A Multienzyme Network Functions in Intestinal Protein Digestion by a Platyhelminth Parasite*

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Proteases frequently function not only as individual enzymes but also in cascades or networks. A notable evolutionary switch occurred in one such protease network that is involved in protein digestion in the intestine. In vertebrates, this is largely the work of trypsin family serine proteases, whereas in invertebrates, cysteine proteases of the papain family and aspartic proteases assume the role. Utilizing a combination of protease class-specific inhibitors and RNA interference, we deconvoluted such a network of major endopeptidases functioning in invertebrate intestinal protein digestion, using the parasitic helminth, Schistosoma mansoni as an experimental model. We show that initial degradation of host blood proteins is ordered, occasionally redundant, and substrate-specific. Although inhibition of parasite cathepsin D had a greater effect on primary cleavage of hemoglobin, inhibition of cathepsin B predominated in albumin degradation. Nevertheless, in both cases, inhibitor combinations were synergistic. An asparaginyl endopeptidase (legumain) also synergized with cathepsin B and L in protein digestion, either by zymogen activation or facilitating substrate cleavage. This protease network operates optimally in acidic pH compartments either in the gut lumen or in vacuoles of the intestinal lining cells. Defining the role of each of these major enzymes now provides a clearer understanding of the function of a complex protease network that is conserved throughout invertebrate evolution. It also provides insights into which of these proteases are logical targets for development of chemotherapy for schistosomiasis, a major global health problem.

Proteolytic enzymes (proteases) are ubiquitous enzymes that operate in virtually every biological phenomenon. They function not only as individual enzymes but often in cascades or networks (1). Digestion of proteins in the intestine is one noteworthy example of the function of multiple proteases of different classes as part of a coordinated physiological process. In vertebrates, protein digestion is largely the work of pancreas-derived serine proteases, primarily members of the trypsin family (clan PA). This group of enzymes is remarkably conserved among vertebrates.

A very different picture emerges from analysis of intestinal protein digestion by invertebrates. Here cysteine proteases of the clan CA (also known as the papain family) and aspartic proteases homologous to cathepsin D (clan AA) have been described in the gut of organisms as diverse as platyhelminths (2), nematodes (3, 4), and arthropods (5, 6). Interestingly, the invertebrate cathepsin B and L proteases have higher pH optima and often function extracellularly (7). The transition from cysteine/aspartic to serine proteases appears to have occurred in arthropods or mollusks.

A proteolytic cascade or network involving aspartic and cysteine proteases has been proposed as catalyzing hemoglobin degradation in the blood-feeding helminths (7, 8). However, several important biochemical questions remain unanswered. Is degradation of host proteins a systematic hierarchical event with individual proteases performing precise cleavage events in sequence? Or alternatively, is protein digestion functionally redundant, irrespective of both substrate and protease? Do certain substrates bias the activities of proteases, whereby they are “preferred” by some proteases but not by others? To begin to address these issues, we have chosen as a model digestive pathway that of the blood fluke, Schistosoma mansoni. This organism utilizes a number of proteases to digest hemoglobin and host serum proteins to maintain successful parasitism of its human host (9).

Schistosomiasis (bilharzia) is a major global health problem affecting over 200 million people (10). It is caused by several species of schistosomes, or blood flukes, of which S. mansoni is a convenient experimental model and a major agent of disease in the Middle East, Africa, and South America. Following the invasion of human skin by aquatic larvae (cercariae), immature parasites (schistosomula) enter the vascular system and in 5–6 weeks mature to adults, which pair and produce eggs. Larval development, adult worm viability, and production of eggs by female worms are all dependent on the acquisition of nutrients from the host bloodstream, including hemoglobin from red blood cells (11) and the abundant serum proteins. Remarkably, no serine proteases have been localized to the gut lumen or gastrodermis of schistosomes, so the proteases involved in digestion are clearly distinct from those key to vertebrate digestion. Three other classes of proteases have been implicated in host protein digestion and localized to the gut of S. mansoni.

*This work was supported by National Institutes of Health Grant AI-053247 and The Sandler Family Supporting Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 51, pp. 39316 – 39329, December 22, 2006 © 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
These include a metallo-aminopeptidase (12) and a cathepsin D-like aspartic protease as well as cysteine proteases, including a cathepsin B, a cathepsin L (also known as cathepsin F), a cathepsin C, and an asparaginyl endopeptidase (reviewed in Refs. 9 and 13). The precise function each of these enzymes plays in degradation of host nutrients remains speculative and occasionally controversial (9, 14, 15).

Identifying the key enzymes that facilitate host nutrient degradation is important not only to understand the biology and pathogenesis of schistosome infection but also to identify those enzymes that might be the most suitable targets for the development of new chemotherapy. Previous studies have suggested that inhibitors of either cysteine proteases (16) or aspartic proteases (18) may block hemoglobin degradation and arrest schistosome development and egg production.

In order to address how such a network or cascade of proteases might function in the schistosome gut, we focused on the endopeptidases, cathepsin B1, D, and L1, and the asparaginyl endopeptidase, which may function to trans-activate the cathepsin B1 zymogen (19). We utilized a combination of class-specific protease inhibitors and transcriptional silencing to deconvolute the specific roles and dynamic interplay of these schistosome gut-derived proteases in host protein degradation.

**EXPERIMENTAL PROCEDURES**

**Parasites—** *S. mansoni* (Puerto Rican strain) was maintained in the laboratory using *Biomphalaria glabrata* snails and golden hamsters (*Mesocricetus auratus*) as intermediate and definitive hosts, respectively. Cercariae harvested from infected *B. glabrata* were used to infect C57BL/6 mice by subcutaneous injection (2,000 cercariae/mouse). Worms were perfused from mice 3 weeks postinfection (20) in Basch *Schistosoma* culture medium 169 (SCM) with 10% fetal bovine serum instead of human serum (21) and complemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Worms were washed thoroughly and cultured in SCM at 37 °C in a 5% CO2 incubator. SCM was changed every 48 h. Adult worms were obtained from hamsters 6 weeks postinfection.

**Protease Inhibitors—** K11777 was synthesized by Dr. James Palmer (Celera Genomics, South San Francisco, CA). EA-1 was a gift of Dr. Jonathan Ellman (University of California, Berkeley, CA). API-1 and API-2 were a gift of Dr. Ben Dunn (University of Florida College of Medicine). Lopinavir was provided by Dr. Sunil Parikh (University of California, San Francisco, CA) through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health). DCG-04 and KMB-09 were provided by Drs. Kelly Sexton and Matthew Bogyo (Stanford University School of Medicine) and were radioiodinated as previously described (22). MG-256 was a gift of Dr. Marion Götz (Elmhurst College, Elmhurst, IL). Pepstatin A and iodoacetamide were purchased from Sigma. E-64, E-64D, CA-074, Z-Phe-Ala-DMK, and Z-Phe-Phe-DMK were purchased from Bachem (Torrance, CA).

**Fluorescent Substrates—** Z-Phe-Arg-AMC, Z-Phe-Phe-AMC, Z-Ala-Ala-Asn-AMC, and Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-Arg were purchased from Bachem. Rhodamine-labeled bovine serum albumin (Rh-BSA) and DQ red BSA were purchased from Molecular Probes (Eugene, OR). Rhodamine-labeled hemoglobin (Rh-Hb) was synthesized as follows. 50 mg of human hemoglobin (Sigma) was incubated with 10 μg of N-hydroxysuccinimide-rhodamine (Pierce) in 500 μl of phosphate-buffered saline for 1 h at room temperature. Unreacted NHS-rhodamine was then blocked with 20 mM Tris–HCl buffer, pH 7.4. Following desalting using a PD-10 column (Amersham Biosciences) and lyophilization, Rh-Hb was resuspended in and dialyzed against phosphate-buffered saline with a 3.5-kDa cut-off Slide-A-Lyser cassette (Pierce) overnight at 4 °C.

**Preparation of Gastrointestinal Contents (GIC)—** Adult *S. mansoni* worms were washed thoroughly in 37 °C-prewarmed 0.85% saline and transferred to a 10-ml glass beaker. The saline solution was discarded. Worm regurgitation (150–200 worms) was triggered by the addition of distilled water (23) twice into a total volume of 1.5 ml at room temperature for 20 min. The GIC was stored at −80 °C.

**Preparation of Worm Extract—** Three-week-old worms (~100) were washed thoroughly in 37 °C-prewarmed 0.85% saline and homogenized on ice with a pellet pestle motor (Kontes, Vineland, NJ). Extracts were centrifuged at 10,000 × g for 15 min at 4 °C, and the supernatant was collected.

**Determination of Protein Concentration—** Protein concentration was determined by the Bradford assay (24), using reagents obtained from Bio-Rad, on a Spectramax Plus 384 spectrophotometer ( Molecular Devices, Sunnyvale, CA) in triplicate for each sample.

**Cathepsin B and Cathepsin L Activity Assays—** Enzyme activity was monitored at room temperature, in black microtiter plates (Corning Glass), by hydrolysis of the fluorogenic substrates Z-Phe-Arg-AMC for cathepsin L (CatL) and cathepsin B (CatB), and Z-Arg-Arg-AMC for CatB (25). Worm extract (0.2 μg) was preincubated for 10 min at room temperature in 100 μl

**References**

1. Abdulla, M. A., Lim, K. C., Sajid, M., McKerrow, J. H., and Caffrey, C. R. (2006) PLoS Med. in press.
2. The abbreviations and trivial names used are: SCM, *Schistosoma* culture medium; AE, asparaginyl endopeptidase; API-1, lysyl-prolyl-isoleucyl-norleucyl-phenylalanine-(CH_{2}NH)-phenylalanyl-arginyl-leucine; API-2, lysyl-prolyl-phenylalanyl-norleucyl-phenylalanine-(CH_{2}NH)-phenylalanyl-seryl-arginine; BSA, bovine serum albumin; CA-074, N{\text{-}}3-trans-propylcarbamoyloxirane-2-carboxyl-yl-Imid-Pr-OH; CatB, cathepsin B; CatD, cathepsin D; CatL, cathepsin L; DCG-04, N{\text{-}}trans-acetyl-lysyl(biotinylated)-tyrosyl-hexyl-leucyl-epoxysuccinyl-lysyl-leucyl ethyl ester; DQ red BSA, self-quenched red bodipy dye conjugate of bovine serum albumin; dsRNA, double-stranded RNA; DTT dithiothreitol; EA-1, 1-acyclic-piperidine-4-carboxyl acid (2-benzo[1,3]dioxol-5-yl-ethyl)-{3-[2-(3-chloro-phenoxy)acetyl]-lysinyl(biotinylated)-tyrosyl(biotinylated)-hexyl-leucyl-epoxysuccinyl-leucyl ethyl ester; GIC, gastrointestinal content(s); IAA, iodoacetamide; K11777, N-methylpiperazine-urea-phenylalanine-homophenylalanine-vinylsulfone-benzene; KMB-09, acetyl-biotinylated-lysyl-tyrosyl-hexyl-valyl-alanyl-asparyl-acetylomocytylethyl ketone; Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-Arg, (7-methoxyxycoumarin-4-yl)-acyctylglycyl-lysyl-lysyl-isoleucyl-isoleucyl-phenylalanyl-phenylalanyl-arginyl-leucyl-lysyl-2,4-dinitrophenyl-alanyl-Arg-NH_{2}; MG-256, 25(S)-3-(N{\text{-}}(N-benzoylcarbonyl-alanyl-alanyl)-N{\text{-}}carbomethoxymethyleneazidocarbonyl)-oxirane-2-carboxylic acid phen-ethyl ester; PA, pepstatin A; Rh-Hb, rhodamine-conjugated bovine serum albumin; Rh-Hb, rhodamine-conjugated human hemoglobin; RNA, RNA interference; SmAE, S. mansoni asparaginyl endopeptidase (also known as S. mansoni le-gumain, Sm32; SmCB1.1, S. mansoni cathepsin B1 (also known as Sm31) isofrom 1; SmCB1.2, S. mansoni cathepsin B1 isofrom 2; SmCD, S. mansoni cathepsin D; SmCL1, S. mansoni cathepsin L1 (also known as SmCF); Z, benzoylcarbonyl; AMC, 7-amido-4-methylcoumarin; DMK, diazomethyl ketone.
of 100 mM phosphate citrate, 2 mM DTT, pH 5.5. Substrates (stocks of 10 mM in Me$_2$SO) in 100 µl of the same buffer were then added to give a final concentration of 20 µM. Release of the free AMC was measured at excitation and emission wavelengths of 355 and 460 nm, respectively, in a Flexstation spectrophuorometer (Molecular Devices), for 10 min. Assays were performed in duplicate. To confirm that CatL and CatB activity was being measured, worm extract in buffer was preincubated for 10 min prior to substrate addition with E-64, a general clan CA cysteine protease inhibitor (26), or CA-074, a selective inhibitor of CatB (27), both at a final concentration of 20 µM.

**Cathepsin D Activity Assay**—Enzyme activity was monitored by cleavage of the quenched fluorogenic decapeptide substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-Arg (28). Worm extract (2 µg) was preincubated for 10 min in 100 µl of 100 mM phosphate citrate, 1 mM iodoacetamide (IAA), pH 4.0. Substrate (1 mM stock in Me$_2$SO) in 100 µl of the same buffer was added to give a final concentration of 20 µM. Fluorescence from the substrate hydrolysis was measured for 10 min at excitation and emission wavelengths of 330 and 393 nm, respectively. To confirm that cathepsin D (CatD) activity was being measured, worm extract was preincubated for 10 min with a 20 µM concentration of the aspartic protease inhibitor, pepstatin A (PA), prior to the addition of the substrate (29).

**Asparaginyl Endopeptidase Activity Assay**—Enzyme activity was monitored by hydrolysis of the fluorogenic substrate Z-Ala-Ala-Asn-AMC (30). Worm extract (2 µg) was preincubated for 10 min in 100 µl of 100 mM phosphate citrate, 2 mM DTT, 20 µM E-64, pH 6.0. Substrate (10 mM stock in Me$_2$SO) in 100 µl of the same buffer was added to give a final concentration of 20 µM. Fluorescence was measured for 20 min as described above, and assays were performed in duplicate. To confirm that asparaginyl endopeptidase (AE) activity was being measured, worm extract in buffer was preincubated for 10 min prior to substrate addition with the azapeptido epoxide MG-256 (31) at a final concentration of 20 µM.

**Labeling of GIC with the Radiolabeled Cysteine Protease Inhibitors**—125$^n$-DCG-04 and 125$^n$-KMB09—GIC was preincubated for 10 min with 10 µM K11777, E-64, Z-Phe-Ala-DMK, CA-074, MG-256, Z-Phe-Phe-DMK, or IAA (1 mM) at room temperature in 100 mM phosphate citrate, 2 mM DTT, pH 4.0. Subsequently, GIC was incubated in the presence of 125$^n$-DCG-04 (32) or 125$^n$-KMB09 (33) for 1 or 2 h, respectively, at room temperature. To assess the selectivity of CA-074 and Z-Phe-Phe-DMK against CatB and CatL over time, preincubation times with the inhibitors were 10 and 40 min prior to the addition of 125$^n$-DCG-04. Samples were resolved by SDS-PAGE (12.5% Tris-glycine Criterion gels; Bio-Rad) and visualized on a Typhoon Trio 8600 Imager (Amersham Biosciences) with the tetramethylrhodamine filter (532 and 580 nm as excitation and emission wavelengths, respectively).

**Degradation of Hemoglobin and Albumin by GIC**—Five µg each of human hemoglobin (α and β chains; Sigma) and mouse albumin (Sigma) were incubated in the presence of GIC (1 µg) in a total volume of 30 µl for 6 h at 37 °C. To monitor the effect of pH on the activity of the GIC, the assays were performed at pH values ranging from 3.5 to 8.0, in 100 mM NaCl, 2 mM DTT. To assess the contribution of the different protease classes to hydrolysis, worm GIC was preincubated for 10 min with cysteine protease inhibitors IAA (1 mM), K11777, CA-074, or KMB-09 with or without the aspartic protease inhibitor PA (all 10 µM). A Me$_2$SO control was also included. Following feeding, worms were washed five times with 0.85% saline, and rhodamine and bodipy fluorescence (excitation/emission: 541/572 and 590/620 nm, respectively) was visualized by microscopy on the LSM 510 PASCAL confocal microscope (Carl Zeiss, Jena, Germany) or on an AXIO Imager.M1 (Carl Zeiss) with the Texas Red filter (excitation/emission: 595/615 nm, respectively). Fig. 1 confirms that when Rh-BSA degradation products are generated by proteinase K (Roche Applied Science) on SDS-PAGE (A), there is a corresponding increase in fluorescence (B), as measured on a Flexstation spectrophotometer (Molecular Devices).
TABLE 1  
PCR and real time PCR primers and GenBank™ accession numbers for genes used in RNA interference studies

| Genes and GenBank™ accession numbers | Forward primers and positions | Reverse primers and positions |
|-------------------------------------|------------------------------|------------------------------|
| SmCB1.1 (A506157)                  | 5'-atgctcaatccctaatggcttggc-3' (1–26) | 5'-caatgaccccaaaagcccttc-3' (502–526) |
| SmCL1 (U07345)                     | 5'-gcccttgactgacgccggctt-3' (33–56) | 5'-cccattttatggtggtgtgac-3' (553–578) |
| SmCD (U60995)                      | 5'-gcttatttgacctctttgtaccc-3' (590–625) | 5'-catctgaagtttgaagaaggcg-3' (1252–1277) |
| SmAE (AA550382)                    | 5'-gatgacctgtagataggccgtaa-3' (76–100) | 5'-gatataaattctacctaggggtaa-3' (472–497) |
| rPhMC1 (GC balanced gene of A555625) | 5'-cacacctagtaagctacttgagac-3' (64–87) | 5'-gacacttgtagatgagatggtttc-3' (556–579) |

| Real time PCR primers |
|-----------------------|
| SmCB1.1 (A506157)    | 5'-actctgggagcgcgccgtctt-3' (849–868) |
| SmCB1.2 (A506158)    | 5'-ggagagaagcctcccacagc-3' (219–238) |
| SmCL1 (U07345)       | 5'-ttctgcaagacactcttgcttg-3' (588–607) |
| SmCD (U60995)        | 5'-ccaggctgctggcattc-3' (801–820) |
| SmCC (Z32531)        | 5'-acacggctgtcaaccaagg-3' (730–749) |
| S. mansoni actin (U9945) | 5'-caacgttccctctccatcgtct-3' (133–152) |
|                      | 5'-gccaggggtgctttctccaga-3' (355–376) |

Gels for albumin. Gels were scanned on a Typhoon 8600 variable mode imager using excitation and emission wavelengths of 633 and 670 nm, respectively. Band intensity was determined with ImageQuant TL software (Amersham Biosciences). Gels were later stained with Coomassie Brilliant Blue R-250 (Bio-Rad). To evaluate the effect of protein conformation on hydrolysis, native or denatured (by boiling for 10 min in the presence of 25 mM DTT) 125I-BSA was incubated for 24 h at 37°C with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol, and the RNA pellet was resuspended in a Typhoon 8600 imager in the phosphorimaging mode.

Degradation of Rh-Hb and DQ Red BSA by S. mansoni Extracts—Worm extracts (2 μg) were incubated at 37°C with 5 μg of Rh-Hb or 1 μg of DQ red BSA in 100 mM phosphate citrate, 2 mM DTT, pH 4.0, or pH 6.5. Proteins were resolved by SDS-PAGE using 10–20% Tris-glycine Criterion gels and visualized using a Typhoon 8600 Imager in the phosphorimaging mode.

Double-stranded RNA (dsRNA) Synthesis—A 400–700-bp fragment was amplified from S. mansoni adult worm cDNA for the target protease genes SmCB1.1, SmCL1, SmCD, and SmAE with gene-specific primers (Integrated DNA Technologies, Coralville, IA; Table 1). Amplicons were cloned into the PCR-II Topo vector (Invitrogen). A resynthesized GC-balanced gene for Plasmodium berghei metacaspase 1 was chosen as a control to monitor any off-target dsRNA effect on the worms. Also, BLAST analysis against the S. mansoni genome data base (available on the World Wide Web at www.genedb.org/genedb/smannsoni/blast.jsp) was used to rule out any sequence identity of 20 nucleotides or more with other Schistosoma genes. A T7 RNA polymerase promoter sequence (underlined) was included at the 5'-end of both the forward and reverse gene-specific primers: 5'-AAG TAA TAG GAC TCA TCA TAG GG-3'. Two separate single promoter transcription reactions were carried out for each cDNA template. dsRNA was synthesized with the T7 RiboMax Express RNAi System (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, in vitro transcription was carried out with 5 μl of unpurified single-T7 promoter PCR product/20-μl reaction (reactions were scaled up to 200 μl). Following a 30-min incubation at 37°C, single-stranded RNAs were pooled, heated at 70°C for 10 min, and cooled down to ambient temperature for 20 min to allow dsRNA annealing. The dsRNA was treated with RNase A and DNase to remove any remaining single-stranded RNA and the DNA template and then purified with the Megaclear kit (Ambion, Austin, TX) to remove salts toxic to the worms and stored at −80°C in the elution solution. dsRNA integrity was verified by non-denaturing 1% agarose gel electrophoresis, and its purity was accessed by the ratio A260/A280 (if the value was equal or superior to 2, the dsRNA sample was considered relatively free of protein). The dsRNA concentration was determined by UV light absorbance at 260 nm (one A260 unit equals 40 μg/ml dsRNA) on an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Typically, the in vitro transcription reaction yielded 4–5 μg/μl dsRNA.

Treatment of S. mansoni with dsRNA—dsRNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 95% ethanol, and the RNA pellet was resuspended in SCM at a concentration of 1 μg/μl. dsRNA (400 μg) was added to 100 3-week-old worms in 1 ml of SCM, and the medium was changed every 48 h. After 6 days in culture, worms were homogenized or frozen at −80°C.

cDNA Synthesis—Total RNA was isolated from homogenized worms using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was resuspended in 30 μl of diethylpyrocarbonate (DEPC)-treated water and incubated at 50°C for 10 min for complete dissolution. The concentration of RNA was determined at 260 nm on an ND-1000 spectrophotometer. For each RNAi-treated sample, 2 μg of total RNA were treated with 2 units of DNase I (Sigma) for 20 min at room temperature. After the addition of the stop solution, RNA mixtures were heated at 70°C for 10 min and then chilled on ice. Total RNA was reverse transcribed using the SuperScript III kit (Invitrogen) according to the manufacturer’s protocol and using random hexamers as primers. For each sample, a reaction was performed omitting reverse transcriptase as a control for

4 M. Sajid, unpublished results.
genomic DNA contamination. Control PCRs were performed on each reverse transcription reaction with SmCD PCR primers.

Quantification of the Transcript Levels by Real Time PCR—Forward and reverse primers (Table 1) were designed to amplify a 150–300 bp fragment for SmCB1.1, SmCB1.2, SmCC, SmCD, SmCL1, and S. mansoni actin, using the Primer 3 software (available on the World Wide Web at frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Each set of primers was tested on each cDNA sample. Triplicate reactions (20 μl) comprised 1 μl of cDNA, forward and reverse primers (0.1 μl; 2.4 μM each), and 10.5 μl of SYBR-green master mix (Stratagene, La Jolla, CA). Reactions were completed in 96-well plates (Applied Biosystems, Foster City, CA) using a 7300 real time PCR system (Applied Biosystems) with an amplification program of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Trimeta Acid Phosphatase Labeling—Adult S. mansoni were fixed in 3% glutaraldehyde, 1% paraformaldehyde, 0.1 M cacodylacetate, pH 7.4. They were rinsed several times in 0.05 M acetate-veronal, 5% sucrose, pH 5.2, followed by 0.6 mM sodium trimetaphosphate, 4.5 mM acetate, 0.15% lead acetate, 5% sucrose, pH 3.9, for 90 min at 37 °C (35). After incubation, worms were incubated in 2% ammonium sulfide for 10 min at room temperature and washed with the sodium trimetaphosphate buffer until no yellow color was visible in the solution. Worms were then osmicated, in bloc-stained with uranyl acetate, and embedded in Eponate 12 (Ted Pella, Redding, CA). The blocks were sectioned on a Ultracut UCT microtome (Leica, Bannockburn, IL) and examined with a TECNAI10 electron microscope (Philips, Eindhoven, The Netherlands).

RESULTS

Clan CA and Clan CD Cysteine Proteases and a Cathepsin D-like Aspartic Protease Are Detected in Schistosome Gut Contents by Affinity Radiolabeling or Quenched Fluorescent Peptidyl Substrates—Schistosome parasites can be induced to release both luminal contents and GIC by osmotic shock in distilled water (23). Competitive labeling with small molecule inhibitors was used to identify constituent cysteine proteases in the schistosome gut. Labeling of GIC with the clan CD-selective probe 

![Figure 2](https://example.com/figure2.png)

FIGURE 2. Detection of cysteine proteases in S. mansoni GIC and their inhibitor selectivity as documented by competitive labeling with the irreversible radiolabeled cysteine protease probes, DCG-04 and KMB-09. Following labeling of GIC, proteins were resolved by SDS-PAGE and analyzed by phosphorimaging. A. GIC were preincubated 10 min with 10 μM each of K11777, CA-074, MG-256, or both CA-074 and MG-256 before incubation with 125I-KMB-09 or 125I-DCG-04 for 2 or 1 h, respectively, at pH 4.0. Labeling of SmAE with 

![Figure 3](https://example.com/figure3.png)

FIGURE 3. Aspartic protease inhibitors used in this work.
tion with the azapeptide clan CD inhibitor, MG-256 (31), abolished labeling of SmAE by $125$-I-KMB-09 but allowed the resolution of a second protease species of 31 kDa (lane 4), which in turn was inhibited by prior incubation with the cathepsin B-specific inhibitor CA-074 (lane 5). It is therefore concluded that the 31-kDa species is a cathepsin B, most probably S. mansoni cathepsin B1 (SmCB1).

Radiolabeling with the clan CA-selective inhibitor, $125$-I-DCG-04, resolved two molecular species at 31 and 27 kDa (Fig. 2A, lane 6), and this labeling was abolished by prior incubation with K11777 (lane 7). Incubation with CA-074 abolished labeling of the 31-kDa species only (Fig. 2, A (lane 8) and B (lanes 2 and 3), confirming that it is SmCB1, in agreement with a previous report (37). Preincubation of GIC for 15 or 40 min with the cathepsin L preferential inhibitor, Z-Phe-Phe-DMK (38), completely inhibited labeling of the 27-kDa species (Fig. 2B, lanes 4 and 5), suggesting that this is the gut-associated cathepsin L, probably S. mansoni cathepsin L1, SmCL1 (39). Z-Phe-Phe-DMK also inhibited labeling of the 31-kDa cathepsin B to some extent at the longer (40 min) time point (Fig. 2B, lane 5).

Fig. 2C summarizes the selectivity of a battery of protease inhibitors against each of the three major cysteine protease species found in schistosome gut contents. Several of these inhibitors were chosen to evaluate the role of specific clan CA or CD cysteine proteases in parasite digestion of the major host blood proteins hemoglobin and albumin. Although only CA-074 is completely selective, other inhibitors are useful reagents for chemical knock-out when used in a matrix of assays. For example, comparing inhibition by KMB-09 with CA-074 allows the contribution of the SmAE to be sorted from that of SmCB1. KMB-09 was more selective than MG-256, so it was used in subsequent studies. K11777 inhibits both of the major clan CA cysteine proteases, SmCL1 and SmCB1, and was therefore used for comparison with the inhibitors of the aspartic protease SmCD.

Although no active site probes exist for aspartic proteases, a quenched fluorescent substrate assay is selective, and several inhibitors, some of which are cell-permeable, were chosen to identify the role of cathepsin D in schistosome digestion of host proteins (Fig. 2D). PA is a specific non-cell-permeable inhibitor, whereas Lopinavir and EA-1 are cell-permeable inhibitors based on two distinct chemical scaffolds.

**Cell-permeable Aspartic and Cysteine Protease Inhibitors Abolish Fluorescence Released from Fluorescently Labeled Albumin and Hemoglobin in the Schistosome Gut**—To visualize the digestion of host blood proteins in schistosomes in situ, 3-week-old worms were fed 10 µg of DQ red BSA (an internally quenched bodipy-labeled BSA), 50 µg of Rh-BSA, or 50 µg of Rh-Hb for 1 h. After incubation, fluorescence was seen throughout the bifurcated intestine (Fig. 3A). This fluorescent signal was almost completely absent in the presence of a 10 µM concentration of the cell-permeable inhibitors targeting clan CA proteases, K11777 (Fig. 3B) or Z-Phe-Ala-DMK (data not shown). The non-cell-permeable clan CA inhibitor E-64 did not produce any loss of fluorescence, whereas its cell-permeable analog, E-64D, resulted in significant reduction of fluorescence (Fig. 3A). Soluble extracts of worms cultured with the cell-permeable inhibitors, K11777, Z-Phe-Ala-DMK, and E-64D, exhibited less than 5% cathepsin B and L activity compared with control (Fig. 4). Treatment with E-64 did not result in loss of activity, which suggests that E-64, unlike E-64D, did not reach the targeted proteases.

No loss of fluorescence was seen following incubation with a 10 µM concentration of the non-cell-permeable aspartic protease inhibitors PA (Fig. 3B), API-1, and API-2, although these inhibitors inhibited 100, 90, and 90% of the extract aspartic protease activity, respectively (data not shown). Exposure of worms to the cell-permeable aspartic protease inhibitor, Lopinavir, resulted in a marked decrease in fluorescence, comparable with K11777 (Fig. 3B). Another cell-permeable aspartic inhibitor, EA-1 (34), was rapidly lethal to worms, but no gut-specific phenotype was seen. This experiment suggested contributions by both aspartic and cysteine proteases to the digestion of hemoglobin and albumin. However, an unexpected consequence of incubation of worms with cell-permeable aspartic and cysteine inhibitors was an apparent effect on intestinal motility. Although there were no alterations in the appearance of the gut lumen or gastrodermis by ultrastructural analysis following a 3-h incubation with the vinyl sulfone inhibitor K11777, we could not distinguish whether the decreased gut motility was an indirect, downstream effect of gut protease inhibition or an off-target effect. Therefore, we addressed this issue, as described below, by directly analyzing degradation of host blood proteins (hemoglobin and albumin) by the protease activity present in the GIC.

**Protein Degradation in the Gut Takes Place in a Low pH Microenvironment**—Before choosing the conditions for direct assays of protein degradation, we addressed a key question with respect to host protein hydrolysis in the schistosome gut. At what pH values do the GIC proteases optimally operate? Although the pH of the schistosome GIC has been estimated as 6.0–6.4 (23, 37), Fig. 5 shows that efficient degradation of the relevant physiological substrates, hemoglobin and albumin, is optimal at pH 4.0. Indeed, ~95% of both proteins was degraded at pH 4.0 versus 0% of hemoglobin and 20% of albumin at pH 6.5 (Fig. 5, A and B). At pH 6.0, 40% of both proteins was degraded. Denaturation of albumin by boiling in the presence of 25 mM DTT did not facilitate its degradation by worm GIC at pH 6.5 (Fig. 5C), suggesting that pH directly affects protease activity regardless of whether the substrate is in a native or denatured state. These results suggested that proteolysis would be more optimal in luminal or cellular microenvironments that are more acidic. Histochemistry of trimeta phosphatase, which produces an electron dense substrate at pH 3.9, confirmed that fusion of lamellae or “villi” of the gut form sequestered compartments of low pH within the gut lumen (Fig. 6).

**Both Aspartic and Cysteine Intestinal Proteases Are Required for Degradation of Host Blood Proteins in a Substrate-specific Manner**—To clarify the results from assays with fluorescent proteases and worms in culture, we tested the effect of class-specific protease inhibitors on degradation of hemoglobin and albumin by GIC proteases. Assays were carried out for 6 h, with degradation of hemoglobin and albumin assessed by the appearance of cleavage products after SDS-PAGE. Isolated GIC was preincubated for 10 min with K11777 (10 µM) or IAA (1

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mm) for inhibition of cathepsins B and L, CA-074 (10 μM) for cathepsin B inhibition, PA (10 μM) for aspartic protease inhibition, and KMB-09 for asparaginyl endopeptidase inhibition. These direct assays of GIC activity allowed us to eliminate the variables of cell permeability or off target effects of the live worm experiments.

Fig. 7A, lane 2, shows that 90% of the 16-kDa αβ chain hemoglobin monomer is degraded by GIC within 6 h. Modest but significant inhibition of hemoglobin monomer degradation (10–19%) was seen following preincubation of GIC with PA (lane 3), IAA (lane 4), or K11777 and KMB-09 combined (lane 8). CA-074 (lane 6) alone and KMB-09 (lane 7) alone produced only 2% inhibition of monomer degradation; K11777, which inhibits both cathepsins B and L, produced 8% inhibition (lane 5). All of the cysteine protease inhibitors exhibited the same inhibition profile, preventing further degradation of two major cleavage products of molecular mass higher than 6 kDa. The initial cleavage sites identified by Brindley et al. (15) for S. mansoni cathepsin D give rise to fragments of 11.7 and 11.3 kDa for the α (Phe33-Leu34) and the β (Phe41-Phe42) subunits, respectively. These peptides may correspond to the first cleavage product occurring when only cathepsin D activity is present (lane 8 versus lane 3).

Combinations of class-specific inhibitors were tested next. Combining the aspartic protease inhibitor, PA, with either IAA or K11777 increased inhibition to 56–61% (Fig. 7A, lanes 14 and 15). Inhibiting the aspartic protease activity with PA, combined with KMB-09 to inhibit SmAE (lane 17), did not significantly enhance protection of the 16-kDa hemoglobin monomer relative to PA alone (lane 12). However, a mixture of inhibitors targeting cathepsin D, AE, cathepsin L, and cathepsin B restored native 16-kDa hemoglobin monomer at 89% (lane 16) as well as the hemoglobin tetramer (data not shown). Also of

FIGURE 3. In vivo feeding of 3-week-old S. mansoni worms with Rh-BSA. A, following preincubation with Me2SO (DMSO), 20 μM E-64 or E-64D for 1 h, worms were fed Rh-BSA (50 μg/ml) for 1 h and washed thoroughly in 0.85% saline. Fluorescence was visualized by confocal microscopy. The fluorescent signal outlines the bifurcated gut of the schistosome. Loss of fluorescence occurs only with E-64D, the cell-permeable form of the cysteine protease inhibitor E-64. B, worms were preincubated with Me2SO, 10 μM each of K11777, PA, or Lopinavir for 1 h before exposure to Rh-BSA, as described above. Fluorescence was visualized by light microscopy. Note fluorescence outlining the bifurcated gut. Only the cell-permeable inhibitors K11777 (cysteine proteases) and Lopinavir (aspartic proteases) reduced fluorescence. Scale bar, 0.1 mm.
note is the appearance of the 6 kDa band in lane 15 when preincubating GIC with PA and K11777. The band disappears upon the addition of KMB-09, suggesting that AE may produce, in cleavage of the 16-kDa subunit, a species detected at 6 kDa. Hemoglobin $\alpha$ and $\beta$ chains contain several possible cleavage sites for SmAE. The enzyme potentially cleaves the $\alpha$ chain at Asn$^{69-70}$-Val$^{70}$ and the $\beta$ chain at Asn$^{81-82}$-Leu$^{82}$, yielding 7.9- and 7.2-kDa fragments, respectively. These peptides would correspond to the band detected at 6 kDa when asparaginyl endopeptidase is the only activity present (Fig. 7A, lane 15). The contribution of cathepsin L to hemoglobin cleavage can be estimated when comparing inhibition achieved with PA and CA-074 (lane 18) with inhibition achieved with PA and K11777 (lane 15) (34% versus 61%, respectively).

The effects of the same combinations of inhibitors on serum albumin degradation suggested that the network of proteases was operating differently than seen with the hemoglobin monomer. Fig. 7B shows that 97% of the native 68.7-kDa serum albumin species was degraded within 6 h by GIC (lane 2). Although PA did not protect against the initial cleavage as it had with hemoglobin, it did inhibit degradation of a 50-kDa major degradation product (lane 3). The cysteine protease inhibitors, including CA-074, inhibited degradation of the...
native 68.7 kDa band by 4–16% (lanes 4–6) and generated the same products of 44, 40, and 34 kDa. A combination of PA and K11777 (lane 15) led to 43% inhibition of native 68.7-kDa albumin degradation against 6% for K11777 alone (lane 5). Similarly, PA plus CA-074 (lane 18) gave 17% inhibition of degradation against 4% for CA-074 alone (lane 6). KMB-09 alone produced negligible protection (1%) of serum albumin degradation (lane 7). However, combining this inhibitor with CA-074 and PA resulted in 42% protection of degradation (lane 19) versus 17% without it (lane 18). As was the case with the hemoglobin monomer, a mixture of inhibitors, including PA, KMB-09, and K11777, completely protected serum albumin from protease degradation (lane 16). A contribution of cathepsin L was suggested by comparison of lane 18 with lane 15. The combination of PA with CA-074 (lane 18) led to significantly lower inhibition (17%) than PA with K11777 (43%; lane 15). Fig. 8 shows a possible scenario for albumin degradation fragments generated by SmAE as seen in lane 15.

RNA Interference Confirms the Contribution of both Aspartic and Cysteine Proteases to Host Hemoglobin and Serum Albumin Degradation—In addition to chemical targeting of the GIC proteases, we used protease-specific RNAi to assess the relative contribution of gut-derived proteases to host protein degradation. Three-week-old schistosomes were incubated for 6 days in the presence of 400 μg of dsRNA targeting SmCB1, SmCL1, SmCD, or SmAE. Fig. 9A shows the decrease in transcript levels achieved with RNAi of each protease. Targeting of SmCB1, SmCL1, and SmCD mRNA did not affect the transcription of S. mansoni cathepsin C (SmCC), indicating that dsRNA treatment was gene-specific. Extracts of RNAi-treated worms had decreased protease activity specific to each target with the exception of SmAE RNAi, which also produced a 20% decline in CatB activity (Fig. 9B). Worms soaked in SmCB1 dsRNA lost 85% of CatB activity against Z-Phe-Arg-AMC and Z-Arg-Arg-AMC substrates compared with the rPbMC1 control. SmCL1-RNAi worms lost 98% of CatL-specific activity. This assay was performed in the presence of CA-074 to eliminate CatB activity that accounts for approximately 90% of the activity hydrolyzing Z-Phe-Arg-AMC (41). CatD activity, as measured with the peptidyl substrate Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-Arg, was reduced by 70% in SmCD-targeted worms. The SmAE dsRNA-treated worms lost 98% of their AE activity, as measured with the substrate Z-Ala-Ala-Asn-AMC. Typically, rPbMC1-treated worms exhibited a 10% increase in protease activity compared with nontreated worms (data not shown).

The importance of both cysteine and aspartic protease activity to schistosome degradation of host proteins and the substrate specificity suggested by the assays with class-specific inhibitors were in some cases validated or in others clarified by RNAi of these proteases. Fig. 10 shows the effect of RNAi of gut...
FIGURE 9. A, quantitative PCR on cDNA from *S. mansoni* worms exposed to RNAi. mRNA levels of the worms treated with dsRNA targeting rPbMC1 (dsRNA control), SmCB1, SmCL1, and SmCD were determined by real-time PCR. Primers for SmCB1.1, SmCB1.2, SmCL1, SmCD, SmCC, and *S. mansoni* actin were tested on each sample. *S. mansoni* actin was used to standardize the results. SmCB1.1 and SmCB1.2 are isoforms of SmCB1 (37). RNAi targeted both their mRNA sequences. B, CatB, CatL, CatD, and AE activity in extracts of worms exposed to RNAi of SmCB1, SmCL1, SmCD, and SmAE genes, compared with the activity present in worms exposed to rPbMC1 RNAi. The Z-Phe-Arg-AMC substrate measured both CatB and CatL activity. Z-Arg-Arg-AMC substrate measured CatB activity. CatD activity was assessed by the cleavage of Mca-Gly-Lys-Pro-Ile-Leu-Phe-Arg-Leu-Lys-Dnp-Arg in the presence of IAA (1 mM). AE activity was measured by the cleavage of Z-Ala-Ala-Asn-AMC substrate. CA-074 (20 μM) was added to the CatL assay with Z-Phe-Arg-AMC to eliminate overlapping CatB activity. Each value is the mean of triplicate experiments.
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proteases on degradation of fluorescently labeled hemoglobin and albumin substrates by worm extracts. In the case of hemoglobin, effects were minor except for RNAi of cathepsin D. RNAi of SmCB1 alone resulted in a 13% reduction of hemoglobin degradation, SmCL1 11%, SmCD 27%, and SmAE 8%. In the case of albumin, RNAi of SmCB1 alone decreased albumin degradation by 46%, SmCL1 by 15%, SmCD by 50%, and SmAE by 56%. When comparing these data with Fig. 7, A and B, note that Rh-Hb and DQ Red BSA were trichloroacetic acid-precipitated in Fig. 10 so that both the 68.7- and 42-kDa albumin species would be detected as “undegraded” albumin.

DISCUSSION

Degradation of host blood proteins by developing and adult schistosome parasites is a key catabolic process for establishment and maintenance of infection and production of eggs for disease transmission. Male adult S. mansoni worms have been estimated to ingest tens of thousands of erythrocytes/h. Female worms, burdened with the additional nutrient requirements of egg production, have been estimated to ingest hundreds of thousands of erythrocytes/h (42). Lysis of erythrocytes takes place in the esophagus, although the exact mechanism remains unclear. Hemolysin activity has been identified in other helminth parasites (43) and correlates with the presence of an “amoebapore”-like membrane channel. A gene homologous to this protein has been identified in S. japonicum. It is also conceivable that the acid pH of the gut lumen, combined with rapid peristalsis churning the gut contents, may be sufficient to lyse red blood cells or certainly optimize the action of any membrane pore-forming protein.

Previous immunohistochemical studies have localized a number of proteases to the schistosome gut. We confirmed the presence of active proteases by active site labeling (Fig. 2). These include a cathepsin B1 (44, 45), a cathepsin L1 (also known as cathepsin F) (14), a cathepsin D (46), a cathepsin C (47), an asparaginyl endopeptidase (36, 45), and an aminopeptidase (12). To deconvolute the role of the major endopeptidases in primary degradation of hemoglobin and serum proteins, we utilized a combination of RNA interference and class-specific protease inhibitors. Our results suggest that SmCB1, SmCL1, SmAE, and SmCD function in a cooperative network for protein degradation in the schistosome gut and that specific proteases may preferentially initiate degradation of specific host proteins.

For the digestion of albumin, the cysteine proteases initiated substrate cleavage and also protected three major cleavage products (34–44 kDa) from further degradation. Inhibition of both cathepsins B and L is optimal, suggesting some redundancy between these two related proteases. The aspartic protease inhibitor, PA, had a minor effect on initial albumin cleavage but did protect a 42-kDa primary cleavage product from further degradation (Fig. 7B, lane 3). Combining aspartic and clan CA cysteine protease inhibitors was synergistic, restoring 43% of degraded albumin (Fig. 7B, lane 15). However, complete restoration of the 68.7-kDa albumin species required the cooperativity of PA, the clan CA protease inhibitor, K11777, and the clan CD inhibitor, KMB-09 (Fig. 7B, lane 16). The result is consistent with RNAi of individual proteases (Fig. 10) and the effects of protease inhibitors on fluorescently labeled BSA ingested by live worms (Fig. 3).

Cathepsin D plays a greater role in the primary cleavage of hemoglobin (Fig. 7A, lane 3), as was hypothesized by Brindley et al. (15). However, the RNAi data also suggest that the initial cleavage of hemoglobin is more redundant than that of albumin degradation. Although cathepsin D is most effective, even when it is absent some cleavage can still occur (Fig. 10). Initial cleavage by cathepsin D of hemoglobin releases two peptides of molecular mass between 6 and 16 kDa, which are, in turn, degraded by cathepsins B and L. The less abundant cathepsin L provides significant redundancy to cathepsin B in hemoglobin degradation. RNAi of SmCB1 or SmCL1 resulted in the same level of inhibition of hemoglobin degradation (Fig. 10), and inhibition of both of these cysteine proteases produces more profound rescue of hemoglobin than inhibition of CatB alone (Fig. 7A, lanes 15 and 18). Interestingly, other trematode parasites, such as Fasciola hepatica, express abundant cathepsin L rather than cathepsin B in their gut (48, 49). Therefore, the primacy of one cysteine protease versus another may be species-specific.

The clan CD cysteine protease, SmAE (also known as legumin), is a major schistosome gut protein, as demonstrated by both quantitative reverse transcription-PCR analysis of schistosome transcripts and proteome analysis of gut contents. inhibition of this enzyme alone by the clan CD inhibitor KMB-09 had little or no effect on degradation of albumin or hemoglobin (Fig. 7A (lane 7) or 3B (lane 7)). However, although RNAi knock-out of SmAE versus hemoglobin was consistent with the chemical knock-out results, RNAi assays with albumin (Fig. 10) suggested a major role for SmAE. There are two possible explanations for this discordance. Dalton and Brindley (19) proposed that SmAE might activate endoprotease zymogens present in the schistosome gut, including cathepsin B,

5 P. J. Brindley, unpublished results.

6 M. Bahgat, C. R. Caffrey, and M. Delcroix, unpublished data.
Degradation cascades are substrate-specific, with cathepsin D playing a primary role in initiating cleavage but again with some redundancy suggested by inhibitor synergy. Cathepsin D is also known to have exopeptidase activity. A putative role of the AE in cathepsin B activation (19, 37) or in cooperating with cathepsin B and L in direct substrate degradation is indicated. In model A, the primary cleavage of hemoglobin is generally facilitated by cathepsin D, as proposed by Brindley et al. (15) for schistosomes, Williamson et al. (4) for hookworms, and Goldberg et al. (51) for malaria parasites. Therefore, cathepsin D is in boldface type. Nevertheless, some redundancy exists with the cysteine proteases for the initial cleavage. The cysteine proteases also are responsible for cleavage of two species of a molecular mass between 16 and 6 kDa. The albumin degradation pathway corresponds to model B, where cathepsin B now appears to play the primary role in initiating cleavage but again with some redundancy suggested by inhibitor synergy. Cathepsin D is italicized in model B to indicate that combinations of inhibitors are synergistic in preventing the primary cleavage. Cathepsin D also plays a major role in degradation of a 42-kDa species derived from the primary cleavage event by the cysteine proteases (in boldface type). The cysteine proteases play a role in degradation of 34–44-kDa species.

cathepsin L, and cathepsin D. The capacity of SmAE to activate procathepsin B1 was validated experimentally (37). Fig. 9B shows that 20% of cathepsin B protease activity was lost when SmAE production was knocked down with RNAi. This loss may be an underestimate, since in assays performed with the dipeptidyl substrates, both the proenzyme and the mature enzyme can cleave the substrates. Therefore, the effect of SmAE knockdown on cathepsin activation may be more profound than what was apparent. On the other hand, the chemical knock-out data (Fig. 7B) suggested that a synergy exists between SmAE and not only cathepsin B but also cathepsin D (lane 16). Therefore, an alternative explanation for the chemical knock-out/RNAi discrepancy is that the asparagine-specific SmAE produces rare site-specific cleavages in albumin, which do not lead to complete degradation but do facilitate cleavage by cathepsin B and cathepsin D. This may be reflected in the difference in band pattern seen with SmAE activity alone, as seen in lane 15, versus SmCL activity alone in lane 19 (Fig. 7, A and B).

Taken together, these results suggest the following scenarios for protease cooperation in invertebrate protein degradation (Fig. 11). Degradation cascades are substrate-specific, with model A best reflecting hemoglobin as a substrate. Cathepsin D most effectively produces the primary cleavage, but the cysteine proteases provide some redundancy as suggested by both inhibitor synergism and the RNAi experiment. Cysteine proteases degrade the fragments produced by primary cleavage of the 16-kDa monomer. The exopeptidase activity of cathepsin B and the exopeptidases cathepsin C (50) and aminopeptidase (12) further degrade peptides released following the action of the endopeptidases B1, L1, and D. For albumin degradation, model B reflects the primary role of the cysteine proteases in producing the cleavage of the 68.7-kDa species with some redundancy by cathepsin D as suggested by synergism of inhibitors. Cathepsin D plays a role in degradation of the 42-kDa species and the cysteine proteases in degrading three species of 34–44 kDa. SmAE may again function to activate cathepsin B1 and/or synergize directly with the cathepsins in protein substrate cleavage.

Where is albumin and hemoglobin degradation taking place? Hemoglobin is only degraded by GIC below pH 6. However, there was significant degradation of albumin (20–40%) at pH 6–6.5. Bogitsh et al. (18, 46) localized cathepsin D to autophagic vacuoles in the gastrodermis, suggesting that the primary site of action of cathepsin D is the gastrodermal lysosome/endoosome. It has been shown that S. mansoni cathepsin D cannot degrade hemoglobin at the estimated pH of the gut lumen (pH 6.0–6.4) (15, 52). Optimal catalysis occurred at pH 3.5–4.0. Since hemoglobin is only degraded in more acidic compartments and not at the estimated pH of the gut lumen, the enhanced effect of cathepsin D in initial hemoglobin cleavage may reflect its cellular location. On the other hand, cysteine proteases exhibit a broader pH range. However, as for the aspartic protease, the cysteine proteases were more efficient at degrading hemoglobin at pH 3.5–4.0 (52). SmCB1 has been immunolocalized to both the lumen and gastrodermis (37). Biologic transformation of adult worms with a green fluorescent protein construct containing a 5′-flanking region of SmCL1 showed expression of reporter gene product in the gut of adult schistosome worms (53), and SmCL1 has been immunolocalized to the gastrodermal cells (14). It is therefore possible that cysteine proteases produce some degradation at a less acidic pH, in the parasite gut lumen at pH 6–6.5. This would correlate with their greater role in initial cleavage of albumin.

Portions of the parasite gut lumen may represent microenvironments of lower pH as suggested by Brindley (15). Ultrastructural analysis demonstrated the presence of a microenvironment formed by the fusion of extremely long villus projections or lamellae from the gastrodermal cells. Although “luminal,” these areas of sequestration have an environment close to pH 4, as suggested by production of electron-dense substrate with the acid phosphatase reaction (Fig. 6B). The only effective inhibitors in the live worm assays were cell-permeable, implying that their target proteases are localized in a compartment that is either within the gastrodermis or in the luminal “pockets” formed by fused gastrodermal lamellae (Fig. 6). Our conclusion is that most of the digestion process occurs at more acidic microenvironments, within the gastrodermis or in the luminal “pockets,” where aspartic and cysteine proteases have been localized and the pH of which would allow both classes of enzymes to efficiently degrade host proteins.

On the one hand, the observation of substrate-specific protease function in schistosome protein digestion suggests that...
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inhibitors of CatB, CatL, AE, or CatD could all be potential leads for antischistosomal therapy. On the other hand, the redundancy observed in key steps of protein digestion suggests that more than one inhibitor might be required. The first assumption is supported by experiments in which two cysteine protease inhibitors that inactivate both CatB and CatL were shown to profoundly affect worm development and female egg production (16). Furthermore, RNAi knockdown of SmCB1 protease inhibitors that inactivate both CatB and CatL were observed differences in the site of action of these major gut proteases. Although it is noteworthy that the differences in albumin and hemoglobin processing by the proteases of the schistosome and the protoscoleces are echoed by degradation of these same substrates in the digestive tract of the cattle tick, Boophilus microplus. In the tick, hemoglobin and albumin are sequestered and degraded independently in different digestive vesicles (17).

To conclude, we propose that a protease gene network comprising cathepsins B, L, and asparaginyl endopeptidase evolved in early metazaoa and remained widespread as a successful digestive network predating the evolution of the pancreas and the subsequent primacy of serine proteases as digestive enzymes in vertebrates.

Acknowledgments—We thank Dr. Fred A. Lewis (The Biomedical Research Institute, Rockville, MD) for providing S. mansoni adult worms and Dr. Stephen J. Davies (Uniformed Services University, Bethesda, MD) for providing S. mansoni GIC.

REFERENCES

1. Barrett, A. J., Rawlings, N. D., and Woessner, J. F. (2004) Handbook of Proteolytic Enzymes, 2nd Ed., Academic Press, London
2. Yamashita, K., Kominami, E., and Aoki, T. (1992) Parasitol. Res. 78, 574–580
3. Brown, A., Burleigh, J. M., Billiet, E. E., and Pritchard, D. I. (1995) Parasitol. 110, 555–563
4. Williamson, A. L., Brindley, P. J., Abbenante, G., Provic, P., Berry, C., Girdwood, K., Pritchard, D. I., Fairlie, D. P., Hotez, P. J., Dalton, J. P., and Loukas, A. (2002) FASEB J. 16, 1458–1460
5. Orr, G. L., Strickland J. A., and Walsh T. A. (1994) J. Insect Physiol. 40, 893–900
6. Boldbaatar, D., Sikalizyo Sikasunge, C., Battsetseg, B., Xuan, X., and Fujisaki, K. (2006) Insect Biochem. Mol. Biol. 36, 25–36
7. Sajid, M., and McKerrow, H. J. (2002) Mol. Biochem. Parasitol. 110, 1–21
8. Williamson, A. L., Brindley, P. J., Knox, D. P., Hotez, P. J., Loukas, A. (2003) Trends Parasitol. 19, 417–423
9. Caffrey, C. R., McKerrow, J. H., Salter, J. P., and Sajid, M. (2004) Trends Parasitol. 20, 241–248
10. Chitsculo, L., Engels, D., Montresor, A., and Savioli, L. (2000) Acta Tropica 77, 41–5124
11. Zussman, R. A., Bauman, P. M., and Petruska, J. C. (1970) J. Parasitol. 56, 75–79
12. McCarthy, E., Stack, C., Donnelly, S. M., Doyle, S., Mann, V. H., Brindley, P. J., Stewart, M., Day, T. A., Maule, A. G., and Dalton, J. P. (2004) Int. J. Parasitol. 34, 703–714
13. Tort, J., Brindley, P. J., Knox, D., Wolfe, K. H., and Dalton, J. P. (1999) Adv. Parasitol. 43, 161–266
14. Brady, C. P., Dowd, A. J., Brindley, P. J., Ryan, T., Day, S. R., and Dalton J. P. (1999) Infect. Immun. 67, 368–374
15. Brindley, P. J., Kalinina, B. H., Wong, J. Y., Bogitsh, B. J., King, L. T., Smyth, D. I., Verity, C. K., Abbenante, G., Brinkworth, R. I., Fairlie, D. P., Smythe, M. L., Milburn, P. J., Bielefeldt-Ohmann, H., Zheng, Y., and McManus, D. P. (2001) Mol. Biochem. Parasitol. 112, 103–112
16. Wasilewski, M. M., Lim, K. C., Phillips, J., and McKerrow, J. H. (1996) Mol. Biochem. Parasitol. 81, 179–189
17. Lara, F. A., Lins, U., Bechara, G. H., and Oliveira, P. L. (2005) J. Exp. Biol. 208, 3093–3101
18. Bogitsh, B. J., Kirschaner, K. F., and Rotmans, J. P. (1992) J. Parasitol. 78, 454–459
19. Dalton, J. P., and Brindley, P. J. (1996) Parasitol. Today 12, 125
20. Smithers, S. R., and Terry, R. J. (1965) Parasitology 55, 695–700
21. Basch, P. F. (1981) J. Parasitol. 67, 179–185
22. Xing, R., Addington, A. K., Mason, R. W. (1998) Biochem. J. 332, 499–505
23. Chappell, C. L., and Dresden, M. H. (1986) J. Parasitol. 72, 161–167
24. Bradford, M. (1976) Anal. Biochem. 72, 248–252
25. Barret, A. J., and Kirschaner (1981) Methods Enzymol. 80, 535–561
26. Barret, A. J., Kembhavi, A. A., Brown, M. A., Kirschaner, H., Knight, C. G., and Hamada, K. (1982) Biochem. J. 201, 189–198
27. Murata, M., Miyashita, S., Yokoo, C., Tamai, M., Hamada, K., Hayataya, K., Towatari, T., Nikawa, T., and Katunuma, N. (1991) FEBS Lett. 25, 307–310
28. Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y., and Yamamoto, K. (1999) J. Biochem. (Tokyo) 125, 1137–1143
29. Umezawa, H., Aoyagi, T., Morishima, H., Matsuzaiki, M., Hamada, M., and Takeuchi, T. (1970) J. Antibiotics 23, 259–262
30. Kembhavi, A. A., Buttle, D. J., Knight, C. D., and Barrett, A. J. (1993) Arch. Biochem. Biophys. 303, 208–213
31. Asgian, L. J., James, K. E., Li, Z. Z., Carter, W., Barrett, A. J., Mikolajczyk, J., Salvesen, G. S., and Powers, J. C. (2002) J. Med. Chem. 45, 4986–4960
32. Greenbaum, D., Medzihrea, C., Burlingame A., and Boggyo, M. (2000) Chem. Biol. 7, 569–581
33. Kato, D., Boatright, K. M., Berger, A. B., Nazif, T., Blum, G., Ryan, C., Chehade, K. A. H., Salvesen, G. S., and Boggyo, M. (2005) Nat. Chem. Biol. 1, 32–38
34. Bi, X., Hase, T., Zhou, J., Sillman, A., Lin, B., Lee, C. E., Kurtz, I. D., Ellman, J. A., and Lynch, G. (2001) J. Neurochem. 74, 1469–1477
35. Doty, S. B., Smith, C. E., Hand, A. R., and Oliver, C. (1977) J. Histochem. Cytochem. 25, 1381–1384
36. Caffrey, C. R., Mathieu, M. A., Gaffney, A. M., Salter, J. P., Sajid, M., Lucas, K. D., Franklin, C. B., Boggyo, M., and McKerrow J. H. (2000) FEBS Lett. 466, 244–248
37. Sajid, M., McKerrow J. H., Hansell, E., Mathieu, M. A., Lucas, K. D., Hsieh, I., Greenbaum, D., Boggyo, M., Salter, J. P., Lim, K. C., Franklin, C., Kim, J., and Caffrey C. R. (2003) Mol. Biochem. Parasitol. 131, 65–75
38. Green, G. D., and Shaw, E. (1981) J. Biol. Chem. 256, 1923–1928
39. Dalton, J. P., Clough, K. A., Jones, M. K., and Brindley, P. J. (1996) Infect. Immun. 64, 1328–1334
40. Bogitsh, B. J. (1978) Exp. Parasitol. 45, 247–254
41. Caffrey, C. R., and Ruppel, A. J. (1997) Parasitol. Res. 83, 632–635
42. Lawrence, J. D. (1973) J. Parasitol. 59, 60–63
43. Don, T. A., Jones, M. K., Smyth, D., O’Donoghue, P., Hotez, P., and Loukas, A. (2004) Int. J. Parasitol. 34, 1029–1035
44. Ruppel, A., Shi, Y. E., Wei, D. X., and Diesfeld, H. J. (1987) Clin. Exp. Immunol. 69, 291–298
45. Klinkert, M. Q., Felleisen, R., Link, G., Ruppel, A., and Beck, E. (1989) Mol. Biochem. Parasitol. 33, 113–122
46. Bogitsh, B. J., and Krischner, K. F. (1986) Exp. Parasitol. 62, 211–215
47. Hola-Jamriska, L., Tort, J. F., Dalton, J. P., Day, S. R., Fan, J., Aaskov, J., (1998) Eur. J. Biochem. 255, 527–534
48. Dalton, J. P., Neill, S. O., Stack, C., Collins, P., Walsh, A., Sekiya, M., Doyle, S., Mulcahy, G., Hoyle, D., Khaznadji, E., Moore, N., Brennan, G., Mousley, A., Kreshchenko, N., Maule, A. G., and Donnelly, S. M. (2003) Int. J. Parasitol. 33, 1173–1181
49. Collins, P. R., Stack, C. M., O’Neill, S. M., Doyle, S., Ryan, T., Brennan, G. P., Mousley, A., Stewart, M., Maule, A. G., Dalton, J. P., and Donnelly, S. (2004) J. Biol. Chem. 279, 17038–17046
50. Butler, R., Michel, A., Kunz, W., and Klinkert, M.-Q. (1995) Protein Pept. Lett. 2, 313–320
51. Goldberg, D. E., Slater, A. F., Cerami, A., and Henderson, G. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2931–2935
52. Caffrey, C. R., Engel, A., Gsell, C., Gohring, K., and Ruppel A. (1998) Parasitol. Int. 47, 11–19
53. Wippersteg, V., Sajid, M., Walsh, D., Khiem, D., Salter, J. P., McKeerlow, J. H., Grevelding, C. G., and Caffrey, C. R. (2005) Int. J. Parasitol. 35, 583–589
54. Correnti, J. M., Brindley, P. J., and Pearce, E. J. (2005) Mol. Biochem. Parasitol. 143, 209–215
55. Semenov, A., Olson, J. E., and Rosenthal, P. J. (1998) Antimicrob. Agents Chemother. 42, 2254–2258
56. Rosenthal, P. J., Lee, G. K., and Smith, R. E. (1993) J. Clin. Invest. 91, 1052–1056
57. Wilson, L. R., Good, R. T., Panaccio, M., Wijffels, G. L., Sandeman, R. M., and Spithill, T. W. (1998) Exp. Parasitol. 88, 85–94
58. Song, C. Y., and Kim, T. S. (1994) Korean J. Parasitol. 32, 231–241
59. Yamakami, K., Hamajima, F., Akae, S., and Tadakuma, T. (1995) Eur. J. Biochem. 233, 490–497
60. Maki, J., Furuhashi, A., and Yanagisawa, T. (1982) Parasitology 84, 137–147
61. Maki, J., and Yanagisawa, T. (1986) J. Helminthol. 60, 31–37
62. Longbottom, D., Redmond, D. L., Russell, M., Liddell, S., Smith, W. D., and Knox, D. P. (1997) Mol. Biochem. Parasitol. 88, 63–72
63. Pratt, D., Cox, G. N., Milhausen, M. J., and Boisvenue, R. J. (1990) Mol. Biochem. Parasitol. 43, 181–191
64. Yatsuda, A. P., Bakker, N., Krijgsfeld, J., Knox, D. P., Heck, A. J., and de Vries, E. (2006) Infect. Immun. 74, 1989–1993