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Cathepsin L1, the Major Protease Involved in Liver Fluke (*Fasciola hepatica*) Virulence

PROPEPTIDE CLEAVAGE SITES AND AUTOACTIVATION OF THE ZYMOGEN SECRETED FROM GASTRODERMAL CELLS*

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The secretion and activation of the major cathepsin L1 cysteine protease involved in the virulence of the helminth pathogen *Fasciola hepatica* was investigated. Only the fully processed and active mature enzyme can be detected in medium in which adult *F. hepatica* are cultured. However, immunocytochemical studies revealed that the inactive procathepsin L1 is packaged in secretory vesicles of epithelial cells that line the parasite gut. These observations suggest that processing and activation of procathepsin L1 occurs following secretion from these cells into the acidic gut lumen. Expression of the 37-kDa procathepsin L1 in *Pichia pastoris* showed that an intermolecular processing event within a conserved G*X*N*X*F*X*D motif in the propeptide generates an active 30-kDa intermediate form. Further activation of the enzyme was initiated by decreasing the pH to 5.0 and involved the progressive processing of the 37 and 30-kDa forms to other intermediates and finally to a fully mature 24.5 kDa cathepsin L with an additional 1 or 2 amino acids. An active site mutant procathepsin L, constructed by replacing the Cys^{26} with Gly^{26}, failed to autoprocess. However, [Gly^{26}]procathepsin L was processed by exogenous wild-type cathepsin L to a mature enzyme plus 10 amino acids attached to the N terminus. This exogenous processing occurred without the formation of a 30-kDa intermediate form. The results indicate that activation of procathepsin L1 by removal of the propeptide can occur by different pathways, and that this takes place within the parasite gut where the protease functions in food digestion and from where it is liberated as an active enzyme for additional extracorporeal roles.

Fasciola hepatica is a helminth parasite that causes liver fluke disease in cattle and sheep worldwide and has recently emerged as an important pathogen of humans (1). Cathepsin L1, a major cysteine protease secreted by the parasite plays a pivotal role in various aspects of its pathogenicity. For example, the enzyme takes part in nutrient acquisition by catabolizing host proteins to absorbable peptides (2), facilitates the migration of the parasite through the host intestine and liver by cleaving interstitial matrix proteins such as fibronectin, laminin, and native collagen (3), and is implicated in the inactivation of host immune defenses by cleaving immunoglobulins (4, 5). Further, cathepsin L1 has recently been shown to suppress Th1 immune responses in infected laboratory animals making them susceptible to concurrent bacterial infections (6, 7, 8). Accordingly, the protease has been recognized as an important target at which parasite intervention strategies should be directed (9). In this regard, we have shown that the induction of anti-cathepsin L immune responses prior to a challenge infection of *F. hepatica* larvae elicits high levels of protection in cattle against disease (10, 11, 12).

Phylogenetic studies have shown that the *F. hepatica* cathepsin L1 belongs to an enzyme lineage that eventually gave rise to the mammalian cathepsin Ls, Ks, and Ss (2). The isolation of a cDNA encoding *F. hepatica* cathepsin L revealed that the enzyme, like its mammalian homologs, is synthesized as an inactive preproenzyme consisting of a prepeptide, a propeptide and mature enzyme region (13). By analogy with the mammalian proteases we assume that the prepeptide is removed following translocation into the endoplasmic reticulum and that the N-terminal propeptide extension is involved in several functions including intracellular targeting of the enzyme (14–16), correct folding of the mature enzyme by acting as an intramolecular chaperone (17–19) and prevention of uncontrolled proteolysis by binding to the enzyme substrate cleft in a reverse, non-productive direction (14). As has been demonstrated for the propeptide of human cathepsin L and other papain-like cysteine proteases (14, 17, 19, 20) the free propeptide of the *F. hepatica* procathepsin L is a specific and potent inhibitor of the cognate mature enzyme at neutral pH but does not bind to the enzyme at pH 5.5–3.5 (21).

Human lysosomal procathepsin L is stable at high pH because the propeptide protects the protein from the denaturing effects of the alkali (15). Removal of the propeptide to generate the fully active mature enzyme occurs at the lower lysosomal

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pH of 5.5 (14, 15, 22, 23). It has been widely reported that cleavage of the proregion of cysteine proteases can occur autocatalytically *in vitro* under acidic conditions (15, 24, 25, 26). As the stability of the propeptide-protease complex is dependent on electrostatic interactions, reduction of the environmental pH, weakens the bond between the propeptide and the catalytic site. As a consequence, the proenzyme possibly adopts a looser conformation, in which the propeptide is bound less tightly into the active site making it more susceptible to proteolysis (16, 27).

The precise mechanism of proteolytic conversion from proenzyme to mature enzyme is still actively debated. The three-dimensional structure of procathepsin L (14) and procathepsin B (28, 29, 30) show that the N terminus of the mature enzyme is quite removed from the active site thus making it difficult to visualize an autocatalytic cleavage event. Moreover, circular dichroism studies reveal that activation does not involve significant conformational changes in the structure of procathepsin L (15) or procathepsin B (16). Therefore, the initial event in autocatalysis may involve an active proenzyme, possibly created by the reduced pH, that cleaves another proenzyme in the vicinity of the N terminus and sets off a chain reaction (15, 16). However, more recent studies on procathepsin B and procathepsin S identified autoproteolytic intermediates of processing when cystatin was included in *in vitro* activation reactions, which supported the view that the segment of the propeptide that binds the active site cleft is susceptible to cleavage (31). Accordingly, it was suggested that an initial slow intramolecular cleavage event within this segment of the propeptide triggers a more rapid cascade of intermolecular cleavages at the N terminus (31).

Earlier studies on the processing of yeast-expressed recombinant papain identified a conserved heptapeptide (Gly-Xaa-Asn-Xaa-Phe-Xaa-Asp) motif located between residues −42 and −36 in the propeptide that may be a site of initial cleavage in the pH-dependent autoactivation of the enzyme (32). It was suggested that the lowering of the pH perturbed the negative charge of Asp^{-36} resulting in a conformational change that switched on the processing events by allowing proteolysis to occur at the Ala^{-37}/Asp^{-36} bond. Following this primary cleavage further removal of the remaining amino acids of the propeptide may result from the proteolytic activity of the intermediate species, resulting in fully active mature protease (32).

Given the importance of cathepsin L proteases in the virulence of *F. hepatica* and other helminth pathogens (2) it is important to understand the mechanisms of their synthesis, processing and activation. We have previously shown that cathepsin L1 is secreted by this parasite and therefore functions extracellularly (4, 33) similar to the mammalian cathepsin Ls involved in the processing of the hormone thyroglobulin (34–36). In the present study we have employed confocal laser and electron immunocytochemistry to show that the enzyme is stored in secretory vesicles of the gastrodermal epithelial cells in its inactive proenzyme form. We investigate the autoactivation of *F. hepatica* cathepsin L1 using the wild-type proenzyme expressed in *Pichia pastoris*. Intermolecular processing was studied by generating an inactive procathepsin L mutant, where the catalytic Cys residue at position 26 was substituted with a Gly, which was incapable of autoactivation. Our results show that *P. pastoris*-expressed cathepsin L can autoactivate at low pH to an active intermediate form by an initial cleavage within the conserved heptapeptide (Gly-Xaa-Asn-Xaa-Phe-Xaa-Asp), as previously described for papain (32). Further activation of the enzyme occurs by cleavage in the vicinity of the N terminus. In contrast, intermolecular processing of the mutant inactive procathepsin L to a mature product by exogenous mature cathepsin L did not involve the formation of the above intermediate but was initiated by a direct cleavage close to the N terminus. These data provide a mechanism by which *F. hepatica* synthesizes and secretes a fully activated protease that is essential to its existence as a parasite.

EXPERIMENTAL PROCEDURES

Materials—Z-Phe-Arg-NHMec[1] and Z-Phe-Ala-CHN_2 were obtained from Bachem (St. Helens, UK). DTT and EDTA were obtained from Sigma (Dorset, Poole, UK). Prestained molecular weight markers and the AvrII and SnaBI restriction enzymes were obtained from New England Biolabs (UK) Ltd. (Hitchin, UK). Primers were obtained from Sigma-Genosys (Pampisford, UK). The pPIC9K vector and *Pichia pastoris* strain GS115 were obtained from Invitrogen Corp. (San Diego, CA). Ni-NTA agarose and columns were obtained from Qiagen (Crawley, UK).

In Vitro Cultivation of Parasites—Adult *F. hepatica* were obtained from infected cattle at a local abattoir and cultured *in vitro* in RPMI 1640 containing 30 mM HEPES, 1% glucose and 25 mg/ml gentamycin as described by Dalton and Heffernan (37). The medium was collected after 6 h, cleared by centrifugation at 14,000 × *g* for 30 min at 4 °C and stored at −20 °C.

Preparation of Antipropeptide and Antimature Cathepsin L1 Antisera—Native mature cathepsin L1 (nFheCL1) was purified from excretory-secretory (ES) products of adult *F. hepatica* by gel permeation and ion exchange chromatography and antiserum prepared in rabbits as previously described (4, 38). Recombinant cathepsin L1 propeptide was generated and purified as described by Roche *et al.* (21) and antiserum prepared by immunizing New Zealand White rabbits five times with 20 μg of protein formulated in Freund's Complete and Incomplete Adjuvant.

Immunofluorescence and Immunoelectron Microscopy—Adult *F. hepatica* were recovered from infected cattle at a local abattoir, washed, and transported to the laboratory in mammal saline (0.9% NaCl) at 37 °C. Parasites were rinsed in mammal saline and allowed to regurgitate their gut contents before being flat-fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.2) for 4 h. They were washed in antibody diluent (ABD: PBS with, 0.1% bovine serum albumin; 0.3% Triton X-100; 0.1% sodium azide) for 24 h before being incubated for 48 h at 4 °C in antiserum prepared against purified mature cathepsin L1 (diluted 1:3000) and subsequently washed in ABD (24 h, 4 °C). Swine anti-rabbit tetramethyl rhodamine isothiocyanate (TRITC; 1:100; Dako Ltd.) was used to visualize bound primary antibody before the worms were washed in ABD (24 h, 4 °C) and mounted on glass microscope slides in PBS/glycerol (1:9) containing 2.5% 2,4-diazabicylco 2.2.2 octane. Specimens were viewed using a Leica TCS-NT confocal scanning laser microscope.

For electron microscopy worms were washed in mammal saline and fixed for 1 h in 2% double-distilled glutaraldehyde (GTA) (Agar Scientific) in 0.1 M sodium cacodylate buffer (pH 7.2) containing 3% sucrose at 4 °C. Following thorough washing in buffer, specimens were dehydrated through graded ethanol to propylene oxide, infiltrated and embedded in Agar 100 resin (Agar Scientific). Ultrathin sections (80–90 nm) were cut on a Reichert Ultracut E ultramicrotome, collected on bare 200-mesh nickel grids and dried at room temperature. For immunogold labeling sections were etched with 10% hydrogen peroxide for 5 min and rinsed thoroughly with 20 mM Tris-HCl buffer (pH 8.2) containing 0.1% bovine serum albumin and Tween 20 (1:40 dilution). Grids were incubated in normal goat serum (1:20 dilution) for 30 min and then transferred to primary antibody diluted to 1:20,000 with 0.1% bovine serum albumin/Tris-HCl buffer for 12–18 h. Grids were then washed in bovine serum albumin/Tris-HCl and transferred to a 20 μl drop of 10 nm gold-conjugated goat anti-rabbit IgG (Bio Cell International) for 2 h at room temperature. Following another buffer wash, grids were lightly fixed with 2% double-distilled GTA for 3 min, and finally washed with buffer and rinsed with distilled water. Grids were double stained with uranyl acetate (5 min) and lead citrate (3 min) and examined in a FEI (Philips) CM100 transmission electron microscope, operating at 100 keV.

Controls consisted of (i) incubation of whole-mounts/sections with secondary antibody in the absence of primary antibody and (ii) incubation with preimmune serum followed by the secondary antiserum.

[1] The abbreviations used are: Z-Phe-Arg-NHMec, benzyloxycarbonyl-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide; Z-Phe-Ala-CHN_2, benzyloxycarbonyl-L-phenylalanyl-L-alanine-diazomethylketone; DTT, dithiothreitol; PBS, phosphate-buffered saline; NTA, nitrilotriacetic acid.

Construction of Expression Vectors Encoding cDNA for Wild-type Procathepsin L1 (rFheproCL1) and Gly^{26} Procathepsin L1 (rmutFheproCL1) and Transformation into Pichia pastoris—The *F. hepatica* procathepsin L (FheproCL1) was amplified by PCR from the pAAH5 *Saccharomyces cerevisiae* expression vector into which the full-length cDNA had been previously cloned in our laboratory (13). Primers were used (see primers A and D below) to incorporate a SnaBI restriction site at the 5′-end of the gene and an AvrII restriction site and His_6 tag sequence at the 3′-end. The 980-bp fragment was inserted into the AvrII/SnaB1 site of *P. pastoris* expression vector pPIC9K (Invitrogen).

Mutants were generated from this construct by a PCR-based site-directed mutagenesis method known as gene splicing by overlap extension (SOEing) (39) using the pPIC9K-FheproCL1 DNA as a template. The construction of the inactive FheproCL1 mutant involved changing the active site cysteine (Cys^{26}) residue to a glycine in a two-step PCR process. The primers used were as follows: primer A, 5′-GCGGCTACGTATCGA-ATGATGATTTGTGGCAT-3′; primer B, 5′-GAATGCCCAACCGGAGCC-AC-3′; primer C, 5′-GTGGCTCCGGTTGGGCATTC-3′; primer D, 5′-GC-GCCTAGGTCAGTGGTGGTGGTGGTGGTGGGCCC-3′.

The underlined nucleotides indicate the replacements introduced. Each reaction used one flanking primer that hybridized at one end of the target sequence (primer A or D) and one overlapping internal primer that hybridizes at the site of the mutation and contains the mismatched base (primer B or C). In the first round of amplification two sections of the cDNA were amplified using primer combinations A+B and C+D. These two PCR products, with an overlap of 21 bp at one end of each fragment, were then combined in a second PCR to amplify the entire rmutFheproCL1 cDNA. Primers for this reaction were the two outside primers used in each of the two first round reactions (primers A and D). For all PCRs, high-fidelity Taq polymerase was used (25 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min). The rmutFheproCL1 cDNA was then inserted into the AvrII/SnaB1 site of expression vector pPIC9K. Both the wild-type (rFheproCL1) and mutant (rmutFheproCL1) plasmid insert were sequenced to verify the presence of correct gene sequence and mutation.

The rFheCLI or rmutFheCL1 plasmids were linearized by digestion with SalI and introduced to *P. pastoris* GSII5 cells by spheroplasting (40). Transformants were selected for their ability to grow on histidine-deficient agar plates and on agar plates containing minimal media and methanol. Insertion of rFheCLI or rmutFheCL1 into *P. pastoris* was confirmed by PCR using primers specific to the yeast genome (41).

Expression and Purification of Procathepsin L1 and Gly^{26} Procathepsin L1—Yeast transformants were cultured in 250 ml of BMGY broth, buffered to pH 6.0, in 1 liter of baffled flasks at 30 °C until an OD_{600} of 2–6 was reached. Cells were harvested by centrifugation at 2000 rpm for 5 min, and protein expression induced by resuspending in 50 ml of BMMY broth, buffered at pH 6.0, 7.0, or 8.0, containing 1% methanol (41). The cultures were grown at 30 °C with shaking at 225 rpm for 3 days, 1 ml samples were removed daily and then filter-sterilized methanol was added to maintain a final concentration of 1%. To assess the effect of the cathepsin L cysteine inhibitor Z-Phe-Ala-diazomethylketone (-CHN_2) on the production of procathepsin L1 by yeast the inhibitor was added at the time of induction to a final concentration of 25 μM and then twice daily at the same concentration over the subsequent 3 days.

Recombinant proteins were purified from yeast medium by affinity chromatography using Ni-NTA-agarose (Qiagen). Briefly, a column prepared with 1 ml of resin was equilibrated by passing through 10 ml of 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole. 10 ml of yeast media supernatant was mixed with 40 ml of the same buffer and applied to the column. The column was washed with 15 ml of 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 20 mM imidazole, and bound protein eluted using 50 mM sodium phosphate buffer, pH 7.0, containing 300 mM NaCl and 250 mM imidazole. Purified recombinant proteases were dialysed against PBS and stored at −20 °C. Samples of yeast medium supernatants (10 μl) and purified proteins (~1–5 μg) were analyzed by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (see below).

Fluorometric Substrate Assay for Cathepsin L1 Activity—Cathepsin L1 activity was determined by the fluorometric substrate assay described by Dowd *et al.* (38). Yeast culture supernatants or purified recombinant protease were assayed in a total volume of 1 ml of substrate/buffer mix (2.5 mM EDTA, 2 mM DTT, 0.1 M sodium phosphate buffer, pH 6.0, and 10 μM Z-Phe-Arg-NHMec). The reaction was incubated at 37 °C and stopped after 30 min by the addition of 200 μl of 10% acetic acid. Fluorescence was recorded at an excitation wavelength of 370 nm and an emission wavelength of 440 nm. The activity of the samples were calculated from a standard curve of NHMec ranging from 0 to 10 μM, and presented as nanomoles of NHMec min^{-1} ml^{-1}.

In Vitro Processing of Procathepsin L1 and Gly^{26} Procathepsin L1—Purified recombinant procathepsin L1 (~5 μg) was incubated in activation buffer (0.1 M sodium citrate buffer, pH 5.0, 1 mM DTT, and 1.25 mM EDTA) at 37 °C. Samples were taken at various time points up to 2 h and the proteolytic cleavage of the propeptide visualized by 12% SDS-PAGE. Aliquots were also assayed for enzyme activity with the specific substrate Z-Phe-Arg-NHMec as described above.

Exogenous processing of Gly^{26} procathepsin L1 was carried out by mixing 5 μg of the purified mutant enzyme with 0.5 μg of wild-type procathepsin L that had been activated as described above. Processing of the mutant protein over a period of 3 h at 37 °C was analyzed by 12% SDS-PAGE. To confirm that [Gly^{26}]procathepsin L1 was expressed as a correctly folded protein a similar experiment was performed in parallel with mutant enzyme preparations that had been unfolded by heating at 95 °C for 3 min.

Identification of Cleavage Products by N-terminal Sequencing—Following 12% SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane using a semidry transfer cell at 15V for 20 min. The membrane was washed with dH_2O and stained with 0.025% Coomassie Brilliant Blue R-250 in 40% methanol (40). Protein bands of interest were subjected to N-terminal sequencing at Genosphere Biotechnologies (Paris, France).

Sequence Analysis—The *F. hepatica* cathepsin L1 protein sequence was aligned with several related cathepsin sequences using ClustalX 1.81. Protein sequences used included *Carica papaya*, *Fasciola hepatica* cathepsin L1, *F. hepatica* cathepsin L2, *F. gigantica* cathepsin L1, *Schistosoma mansoni* cathepsin L2, *Caenorhabditis elegans* CPL-1, mouse cathepsin L, rat cathepsin L, and human cathepsin L.[2] Sequences were numbered according to the papain numbering used by Vernet *et al.* (32) where the propeptide residues are recorded as a negative beginning from the cleavage site between propeptide and mature enzyme.

SDS-PAGE and Immunoblotting—Protein samples were separated by 12% SDS-PAGE and gels stained with a 0.1% w/v solution of Coomassie Brilliant Blue R-250 in 40% methanol/10% acetic acid (42). Immunoblots were prepared by transferring proteins to nitrocellulose membranes, presoaked in a transfer buffer (50 mM Tris, 384 mM glycine, 20% methanol), and then blocking these for 1 h at room temperature with 5% milk in PBS/0.1% Tween 20. The nitrocellulose membranes were probed with polyclonal rabbit antimature cathepsin L1 serum, rabbit anti-recombinant propeptide and preimmunized control rabbit serum diluted 1/1,000 in 1% milk/PBS/0.1% Tween-20 for 45 min at room temperature. They were then washed three times for 5 min each with 1% milk/PBS/0.1% Tween-20, followed by incubation for 45 min at room temperature with a 1/10,000 dilution of secondary antibody (goat anti-rabbit IgG-peroxidase conjugate) prepared in 1% milk/PBS/0.1% Tween-20. Blots were washed as before and bound antibody visualized with 3,3′-diaminobenzidine (DAB, Sigma).

RESULTS AND DISCUSSION

Secretion of Cathepsin L Proteases by F. hepatica Parasites—When adult *F. hepatica* are maintained *in vitro* they secrete two major proteases that were previously characterized as 27.5 cathepsin L1 and 29 kDa cathepsin L2 in our laboratory (4, 38). N-terminal sequencing revealed a sequence AVPDK for both enzymes that correlated with the N terminus of fully processed mature cathepsin L enzymes. In addition, immunoblotting experiments demonstrated that while they are both reactive with sera prepared against purified mature cathepsin L1, neither reacts with sera prepared specifically against a recombinant propeptide of cathepsin L1 (Fig. 1, see also Fig. 3*B*). Isolation of cDNAs encoding the two enzymes showed that they exhibit a high level of identity (77%) at the amino acid level that explains their cross-reactivity with antiserum prepared against either enzyme (13, 38).

The two proteases are members of a phylogenetic lineage

[2] Protein sequences and accession numbers from GenBank™ are as follows: *Carica papaya* papain (P00784), *F. hepatica* cathepsin L1 (U62288), *F. hepatica* cathepsin L2 (U62289), *F. gigantica* cathepsin L1 (AF112566), *S. mansoni* cathepsin L2 (Z32529), *C. elegans* CPL-1 (NP_507199), mouse cathepsin L (P06797), rat cathepsin L (KHRTL), and human cathepsin L (M20496).

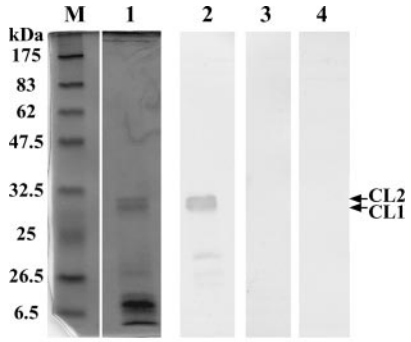

FIG. 1. **SDS-PAGE and immunoblot analysis of *F. hepatica* excretory-secretory (ES) products.** ES products obtained from medium in which *F. hepatica* were cultured was analyzed by SDS-PAGE and immunoblotting. *Lane M*, molecular size markers; *lane 1*, Coomassie Blue-stained 12% SDS-PAGE gel of ES products; *lane 2*, ES products probed with antiserum prepared in rabbits against native mature cathepsin L1; *lane 3*, ES products probed with antiserum prepared in rabbits against recombinant propeptide of procathepsin L1; *lane 4*, ES products probed with normal rabbit serum. *Arrows* indicate position of major secreted and fully processed cathepsin L1 and cathepsin L2.

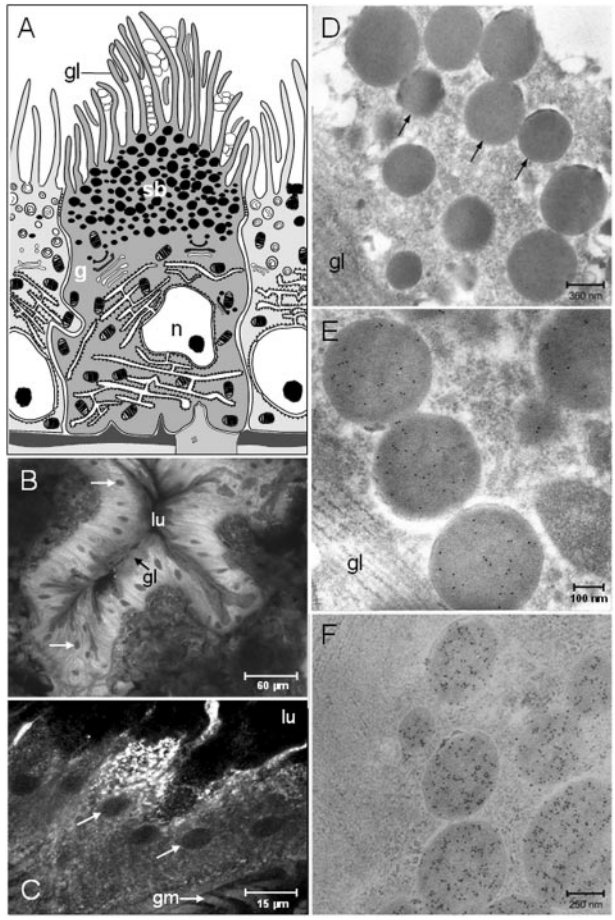

FIG. 2. **Immunolocalization of procathepsin L1 in *F. hepatica* gastrodermal epithelial cells.** *A*, schematic representation of gastrodermal epithelial cells in *F. hepatica* (after Smyth and Halton, Ref. 46). *g*, Golgi; *gl*, gut lamellae; *n*, nuclei; *sb*, secretory bodies. *B*, confocal scanning laser micrograph showing immunoreactivity for mature cathepsin L1 within the gastrodermis of adult *F. hepatica*; the nuclei (*arrows*) of the gastrodermal cells are clearly visible. *gl*, gut lamellae; *lu*, gut lumen. *C*, confocal scanning laser micrograph showing immunoreactivity for mature cathepsin L1 within the gastrodermal cells of adult *F. hepatica*; the nuclei (*arrows*) of the gastrodermal cells are clearly visible. Immunoreactivity appears as a punctuate pattern at the apical end of the cells where the secretory vesicles are located. *gm*, gut muscle; *lu*, gut lumen. *D*, electron micrograph showing non-reactivity of secretory bodies (*arrows*) within the epithelial cells of the gastrodermis with control preimmunized rabbit serum. *gl*, gut lamellae. *E*, electron micrograph showing the localization *F. hepatica* procathepsin L1 to secretory bodies of the gastrodermal epithelial cells with antiserum prepared against the recombinant propeptide of procathepsin L1. *gl*, gut lamellae. *F*, electron micrograph showing the localization of *F. hepatica* cathepsin L1 to secretory bodies of the gastrodermal epithelial cells with antiserum prepared against purified mature portion of cathepsin L1. Note that the labeling is confined to the contents of the secretory bodies.

that includes the mammalian cathepsin Ls and have substrate specificities that are typical of this group, *i.e.* a preference for positively charged residues, such as arginine, in the P1 position and hydrophobic residues, such as phenylalanine and leucine, in the P2 position (14). In contrast to mammalian cathepsin Ls, however, the enzymes exhibit activity over a wide pH range (4.0–8.5) and are remarkably stable at neutral pH; they retain most of their activity following a 24-h incubation at pH 7.0 and 37 °C whereas human cathepsin L is completely inactivated in less than 20 min under the same conditions (43). These enhanced stability properties were suggested to be important in host protein digestion in the parasite gut and in facilitating the migration of the parasite through the host intestine and liver (33).

Immunolocalization and *in situ* hybridization studies previously carried out by our laboratories had shown that the cathepsin L proteases are synthesized within the gastrodermis that lines the parasite gut (4, 33). The gastrodermis is composed of epithelial cells that are columnar in shape and contain numerous dense vesicles situated at the apical end (see Fig. 2*A*). Although secretory in function, these vesicles have been described as lysosomal-like and for almost thirty years have been suspected to contain proteases (44–47). Confocal laser immunocytochemistry using antiserum prepared against the native mature cathepsin L localized this protease to the gastrodermal epithelial cells (Fig. 2*B*) throughout the gut of adult *F. hepatica*. The pattern of immunoreactive labeling appeared punctate and was more dense toward the apical end of the gastrodermal cells indicating that the cathepsin L protease was associated with the secretory vesicles (Fig. 2*C*). Electron microscopy revealed that the enzyme is indeed stored in secretory vesicles within these cells (Fig. 2, *D–F*). The secretory vesicles were strongly immunoreactive with an antiserum prepared against the recombinant-expressed propeptide (Fig. 2*E*) and to the mature portion of cathepsin L1 (Fig. 2*F*) indicating that the enzymes are present in these vesicles as the inactive proforms, procathepsin L. (The specificity of the sera for the propeptide and mature regions of the procathepsin L1 is demonstrated in Fig. 3*B*.)

The above observations provide important insights into the regulation of protease activity within the gastrodermis of these parasites. First, in agreement with the observation by Halton and co-workers (44, 46, 47) it appears that the function of the vesicles is to package proteolytic enzymes for delivery into the gut lumen. Second, the accumulation of procathepsin L1, the inactive precursor, in the secretory vesicles rather than active mature protease makes good sense given the high abundance of these vesicles in the epithelial cells and hence the possibility of protease leakage into the cytoplasm (see Fig. 2*A*). Third, the data suggest that activation of the procathepsin L1 takes place following secretion of the proteases into the gut lumen. Electron microscopy has shown that the contents of the secretory vesicles are extruded into the gut lumen where they mix with the ingested blood and tissue meal between the long extruding lamellae (Refs. 47 and 48; see Fig. 2*A*). Enzyme activation is likely to take place here as the pH is estimated to be ~5.5 or slightly lower (47, 48). Once activated, the protease would be in direct contact with the bloodmeal and could immediately begin its function in protein catabolism. Since these helminth para-

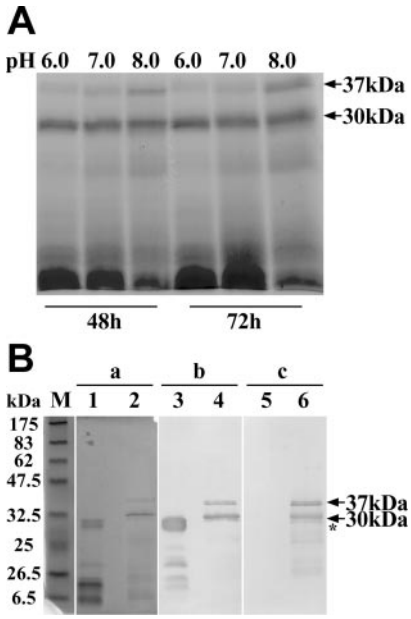

FIG. 3. **SDS-PAGE and immunoblot analysis of *F. hepatica* procathepsin L1 expression from *P. pastoris*.** *A*, cultures of *P. pastoris* transformed with a cDNA encoding procathepsin L1 were induced with 1% methanol. Fermentations were carried out at 30 °C in media buffered to pH 6.0, 7.0, and 8.0. Aliquots (10 μl) removed at 48 and 72 h after induction were analyzed by 12% SDS-PAGE. The 37 and 30 kDa components are indicated. *B*, comparison of *P. pastoris*-expressed procathepsin L with native *F. hepatica*-secreted mature cathepsin L1. Aliquots of adult *F. hepatica* ES products (*lanes 1*, *3*, and *5*) were compared with samples taken from *P. pastoris* medium after induction for 72 h at pH 8.0 (*lanes 2*, *4*, and *6*). *Lane M,* molecular size markers; *panel a,* Coomassie Blue-stained 12% SDS-PAGE gel; *panel b,* samples probed with antiserum prepared in rabbits against mature portion of cathepsin L1; *panel c*, samples probed with antiserum prepared in rabbits against recombinant propeptide portion of procathepsin L1. Note, the antipropeptide serum reacts with the 37 and 30 kDa *P. pastoris*-expressed components (indicated with *arrows*) but not with parasite-produced mature cathepsin L1 or cathepsin L2 (indicated by an *asterisk*). Control preimmunized rabbit serum was not reactive with any proteins (not shown).

sites have a blind-ending gut, removal of undigested products and accumulated hematin is achieved by simply voiding the contents approximately every 3 h (46, 47). In this way active mature cathepsin L would be regularly liberated from the parasite into the surrounding intestinal or liver tissues where it could perform its function in tissue degradation and thus aid the movement of the parasite through these tissues (47, 49).

The secretion of *F. hepatica* procathepsin contrasts with the intracellular trafficking of mammalian procathepsin L through the Golgi apparatus to acidic lysosomes where the enzyme is processed to the mature form by removal and degradation of the propeptide (14, 15). However, a low level of procathepsin L is also secreted from normal cells and is thought to play an extracellular processing function (50), while high levels of secretion has been correlated with the metastatic activity of transformed cells (51, 52). A comparison may also be made with human cathepsin K, which is secreted by osteoclasts and functions in bone resorption (25, 53, 54). Following adherence of osteoclasts to the bone surface an extracellular compartment known as an absorption pit is created between cell and bone into which cathepsin K is secreted. The pH within the pit is slightly acidic and is considered important not only for the dissolution of the bone but also for the activation of procathepsin K to its active proteolytic form (53, 54). Cathepsin B, L, and K have also been shown to be secreted into the extracellular lumen of thyroid follicles where they play a role in the proteolytic processing of covalently cross-linked soluble thyroglobulin to liberate the thyroid hormone thyroxine (T_4) (34–36).

Expression and Processing of Wild-type Procathepsin L1—To study the mechanism by which the *F. hepatica* procathepsin L1 is processed to an active mature enzyme the recombinant proenzyme was expressed in the yeast *P. pastoris*. The transformed yeast cells were induced by 1% methanol in BMMY medium buffered at pH 6.0, 7.0, or 8.0 and then the recombinant protein secreted into the medium analyzed by SDS-PAGE. Two major proteins migrating at 37 and 30 kDa were visualized (Fig. 3*A*). These components migrate higher than the mature cathepsin L1 and cathepsin L2 secreted by the adult *F. hepatica* parasites in 12% SDS-PAGE (Fig. 3*B*). Immunoblotting experiments showed that polyclonal antiserum prepared against the propeptide portion and against the mature portion of procathepsin L both reacted with the *P. pastoris*-expressed 37 and 30 kDa components confirming that these are two forms of the procathepsin L. By contrast, the fully processed enzymes secreted by the parasite are reactive with antiserum prepared against the mature portion of the procathepsin L1 but are not reactive with antiserum prepared against the propeptide portion (Fig. 3*B*).

The 37 and 30 kDa components were purified from the pH 8.0 yeast medium by Ni-NTA agarose affinity chromatography and subjected to N-terminal sequence analysis. This showed that the 37 kDa protein represented the procathepsin L (plus 5 amino acids of the yeast α-factor secretory signal at the N terminus) while the 30-kDa component represented an intermediate processed form beginning at residue Leu^{-41} (papain numbering) within the propeptide (see Fig. 5).

The amount of the recombinant proteins in the yeast medium increased over the 3 days following induction with methanol; this increase was observed at pH 6.0, 7.0, and 8.0 (Fig. 3). However, the relative ratio of the 37 and 30 kDa proteins was dependent on the buffering pH of the medium, with the proportion of 37 kDa increasing with increasing pH. A pH profile of activity for the recombinant *F. hepatica* cathepsin L showed that it was similar to that of the native mature enzyme (38, 43), *i.e.* the recombinant enzyme was active over the pH range 4.0 to 8.5 but was most active at pH 6.0.[3] Therefore, by increasing the pH of the yeast medium above the pH optimum for activity of the *F. hepatica* cathepsin L the level of processing of the 37-kDa form to the 30-kDa intermediate form was reduced.

To address this issue further, yeast cells were cultured at pH 8.0 in the presence and absence of the cathepsin L-specific inhibitor Z-Phe-Ala-CHN_2 (Fig. 4, *A* and *B*). Although the inhibitor was added twice daily at a concentration of 25 μM, which is 250-fold greater than its K_i for cathepsin L (38, 40), samples of medium taken daily showed that cathepsin L activity was not completely inhibited (it is possible that the inhibitor is not stable in the medium) (see Fig. 4*B*). Nevertheless, the proportion of 37-kDa component in culture supernatants containing inhibitor was greater than that in cultures without inhibitor, although the 30-kDa protein was still evident. These data support the idea that procathepsin L1 secreted from *P. pastoris* autoprocesses to an intermediate active form and that this autoprocessing is prevented by the cathepsin L protease inhibitor Z-Phe-Ala-CHN_2. Estimations of yeast cell viability showed that at the concentrations used the inhibitor did not affect the growth of the yeast cells.[3]

To examine whether further intermolecular processing could take place at a pH lower than 6.0, the 37 kDa procathepsin L1 and 30-kDa intermediate forms were first purified from pH 8.0 yeast culture supernatant by affinity chromatography on Ni-NTA agarose and dialyzed against PBS (pH 7.3). The pH of

[3] P. R. Collins and J. P. Dalton, unpublished data.

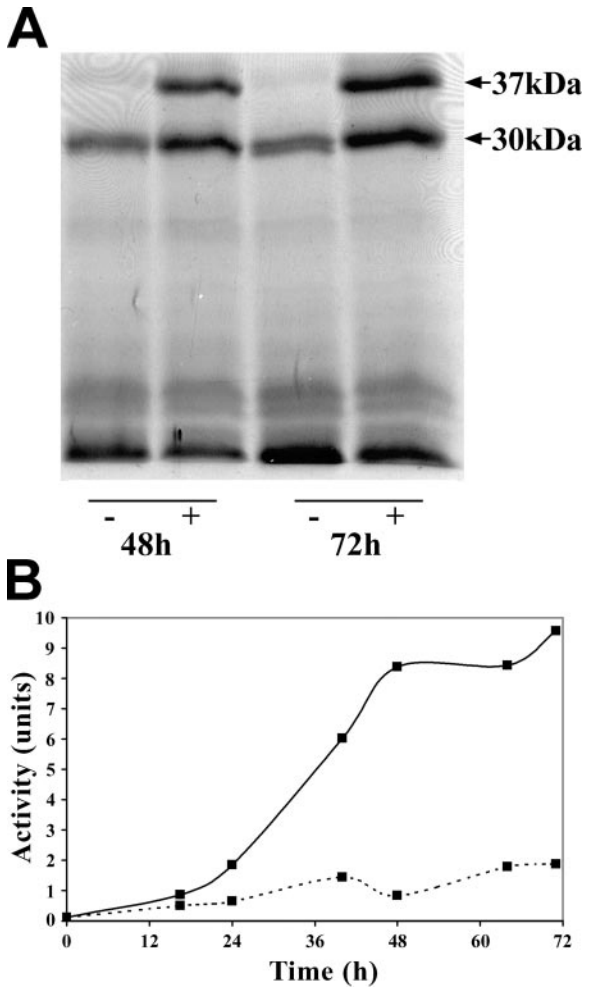

FIG. 4. **Production of procathepsin L1 by transformed *P. pastoris* in the presence and absence of Z-Phe-Ala-diazomethylketone.** *A*, transformed *P. pastoris* were cultured at 30 °C and pH 8.0 in the presence (+) or absence (−) of the cysteine protease inhibitor Z-Phe-Ala-CHN_2 (25 μM). Aliquots of culture supernatant, removed after 48 and 72 h, were analyzed by SDS-PAGE. The 37 and 30 kDa bands are indicated. *B*, recombinant yeast cultured in the presence (*dotted line*) and absence (*solid line*) of 25 μM Z-Phe-Ala-CHN_2. Cathepsin L activity in the culture supernatant was measured with the fluorogenic substrate Z-Phe-Arg-NHMec. Activity units are presented as nmol of NHMec released min^{-1} ml^{-1}.

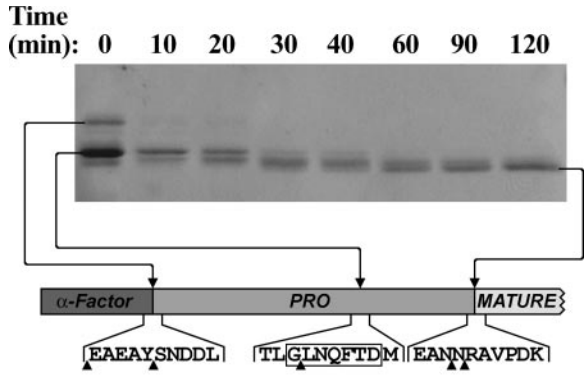

FIG. 5. **Autocatalytic processing of procathepsin L1 at pH 5.0.** Purified procathepsin L1 (5.0 μg) was incubated at 37 °C for 2 h in 0.1 M sodium citrate buffer, pH 5.0, containing 2 mM DTT and 2.5 mM EDTA. Samples removed at various time points were analyzed by SDS-PAGE. The predominant proteins of 37, 30, and 24.5 kDa were subjected to N-terminal sequencing. The schematic indicates the amino acid sequences surrounding the intermolecular processing sites (*arrowed*) that generated these components and their location within the propeptide of procathepsin L1. The *box* indicates the conserved motif G*X*N*X*F*X*D.

samples taken from this preparation were then reduced to 5.0 by the addition of 0.1 M sodium citrate containing 1 mM DTT and 1.25 mM EDTA, and then incubated at 37 °C (Fig. 5). Analysis of samples removed at various time points over a period of 2 h revealed that the 37 kDa procathepsin L is processed to the 30-kDa intermediate form and then through various intermediates that eventually give rise to a single peptide migrating at 25.4 kDa. Corresponding with this processing was the gradual 5-fold increase in the activity of cathepsin L by the end of the 2-h incubation period (data not shown). N-terminal sequence analysis of the 25.4 kDa product revealed two sequences, NRAVP and RAVPD, which are just 2 and 1 amino acids from the mature protein processing site, respectively (see Fig. 5). This intermolecular processing was completely inhibited by adding 10 μM Z-Phe-Ala-CHN_2 to the starting mix and therefore can be attributed to the action of cathepsin L (data not shown).

The cleavage site between Gly^{-42}-Leu^{-41} that generated the 30 kDa intermediate form observed by SDS-PAGE analysis (29.6 kDa from amino acid sequence) was of particular interest as it occurred within the Gly^{-42}-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} motif that was previously identified as pivotal in the intermolecular processing of papain expressed in the yeast *S. cerevisiae*. By random mutagenesis studies, and by sequence comparisons of various papain-like proteases, Vernet *et al.* (32) showed that the Gly^{-42} and Asp^{-36} residues within this motif were the most constrained and proposed that two cleavage sites, Gly^{-42}-Leu^{-41} and Ala^{-37}-Asp^{-36} were important for proenzyme processing. The latter was considered more significant because cleavage at this site was predicted to perturb the negative charge of the Asp^{-36} residue (32). The Asp^{-36} residue is absolutely conserved in non-cathepsin B-like cysteine proteases of helminth parasites, plants and mammals (including cathepsin L, cathepsin S, and cathepsin K) and crystallographic studies on human cathepsin L showed that it participates in an important salt bridge between propeptide and mature enzyme (14).

On inspection of the *F. hepatica* procathepsin L propeptide sequence we found that the Gly^{-42}-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} motif is completely conserved in this parasitic helminth and in others including *F. gigantica* and *S. mansoni*, and in the free-living helminth *C. elegans* (Fig. 6). Moreover, the Leu^{-41} residue is also conserved in all of these sequences thus preserving the Gly^{-42}-Leu^{-41} bond and implicating its importance in propeptide function in these helminths. However, these two residues are not conserved in the human, mouse, and rat cathepsin L sequences where Gly^{-42} is replaced by Glu or Ala and Leu^{-41} is replaced by Met (Fig. 6). While it is tempting to speculate that the changes at these positions explains why an intermediate 30-kDa form was not observed in studies on the intermolecular processing of human procathepsin L (15), McQueney *et al.* (25) found that the intermolecular processing of an inactive mutant form of procathepsin K by activated cathepsin K did involve a cleavage between Ala^{-42} and Met^{-41}. As pointed out by Coulombe *et al.* (14), while there may be a direct involvement of the Asp^{-36} in the Gly^{-42}-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} motif in the pH-dependant processing of proforms of cysteine proteases, cleavage at other peptide bonds within this motif could influence the local charge state that then may trigger processing.

Expression and Exogenous Processing of Gly^{26} Procathepsin L1—To further study the intermolecular processing of *F. hepatica* procathepsin L1 we expressed an inactive form of the protein by replacing the active site Cys^{26} with Gly^{26}. Expression of this recombinant mutant in medium buffered at pH 6.0

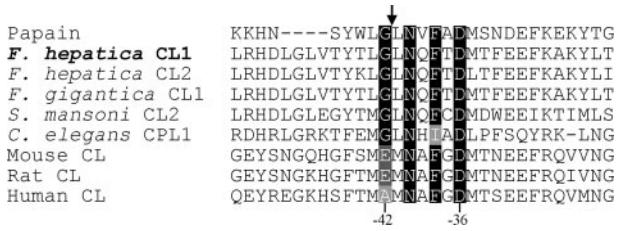

FIG. 6. **Comparison of the G*X*N*X*F*X*D motif between papain and cathepsin L proteases of helminths and mammals.** The G*X*N*X*F*X*D motif sequence of papain is identical to that of *F. hepatica* cathepsin L1 and cathepsin L2 and to the cathepsin Ls of related the trematodes *F. gigantica* and *S. mansoni* (identical residues highlighted in *black*). The *arrow* indicates the position of the intermolecular cleavage site, between the Gly^{-42}-Leu^{-41} bond (papain numbering), that generates the intermediate 30 kDa component of the *F. hepatica* procathepsin L when it is expressed in *P. pastoris.* The Gly^{-42} is substituted for Glu in the rat and mouse cathepsin L sequences, and for Ala in the human cathepsin L sequence (highlighted in *gray*, papain numbering).

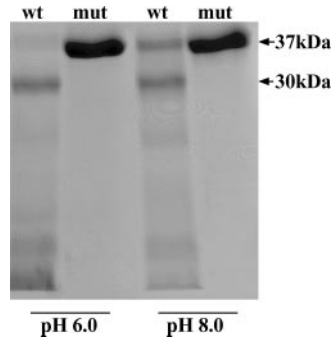

FIG. 7. **Secretion of the mutant procathepsin L1 from *P. pastoris*.** *P. pastoris* cells transformed with wild-type procathepsin L1 (*wt*) or the procathepsin L1 that had the active site Cys^{26} replaced by Gly (*mut*) were cultured at 30 °C in media buffered to pH 6.0 and 8.0. After 48 h of induction with 1% (v/v) methanol samples of culture supernatant were analyzed by SDS-PAGE. The 30-kDa intermediate form of procathepsin L was observed only in the wild-type cultures.

and 8.0 showed that it migrates as a single band at 37 kDa in SDS-PAGE (Fig. 7). N-terminal sequencing confirmed that the 37 kDa protein represented the complete procathepsin L1, and enzyme assays showed that it lacks activity against the substrate Z-Phe-Arg-NHMec.[3] The production of the mutant procathepsin L1 is almost 4-fold greater than that of the combined 37 and 30 kDa components observed in parallel cultures of wild-type procathepsin L1 (see Fig. 7). Furthermore, the mutant enzyme did not process to lower molecular size forms when incubated at pH 5.0.[3]

Addition of recombinant wild-type cathepsin L1, which was activated as described above, to the mutant procathepsin L, at pH 5.0, resulted in the progressive appearance of a minor band at ~35 kDa over an incubation period of 3 h and a second major band at 24.5 kDa that co-migrates with fully active cathepsin L (the intensity of the 24.5-band increases over the reaction period, Fig. 8). N-terminal sequencing attempts were unsuccessful for the 35 kDa protein but a sequence of HGVPY (with less than 10% GVPYE) was obtained for the 24.5 kDa protein; the cleavage site is therefore located 10 amino acids prior to the N terminus of mature cathepsin L1. Experiments in which activated wild-type cathepsin L1 was added to heat-denatured mutant procathepsin L showed that the latter was sensitive to complete degradation by active cathepsin L1 and confirms that the expressed mutant is correctly folded (intensity of the 24.5 does not increase, compare Fig. 8, *A* and *B*).

An overview of the cleavage sites observed by autoactivation by the wild-type enzyme and by intermolecular processing of mutant enzyme by exogenously added wild-type cathepsin L1 is shown in Fig. 9. The cleavage sites determined for the native mature cathepsin secreted by *F. hepatica* parasites *in vitro* are also indicated. It is clear that autoactivation and intermolecular activation occur by cleavages at different sites and thus processing of procathepsin L1 to an active enzyme can be achieved by more than one pathway. Of particular note is the absence of a cleavage site within the Gly^{-42}-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} motif that would generate the 30-kDa intermediate component by exogenous activation. The dominance of this cleavage site in yeast-expressed wild-type cathepsin L and the strict conservation of the four relevant residues suggests an important role for this motif in the processing of *F. hepatica* procathepsin L1. The importance of this motif in the processing of papain was demonstrated by Vernet *et al.* (32) using various mutants in Gly^{-42}-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} sequence; certain residue replacements gave rise to some non-functional incorrectly folded enzymes, while others cause the accumulation of inactive or partly active precursors.

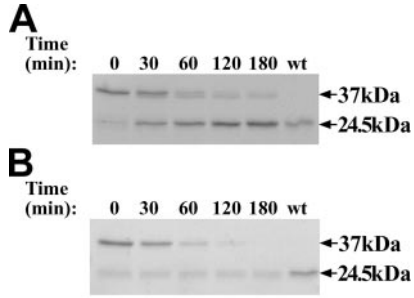

FIG. 8. **Intermolecular processing of procathepsin L1 Cys^{26}-Gly^{26} mutant by exogenously added active cathepsin L1.** *A*, purified procathepsin Cys^{26}-Gly^{26} mutant (5.0 μg) was incubated for up to 3 h in 0.1 M sodium citrate buffer, pH 5.0, containing 1 mM DTT and 1.25 mM EDTA at 37 °C in the presence of 0.5 μg of wild-type cathepsin L1 that was activated according to procedures shown in Fig. 5. The position of the mature wild-type cathepsin L at 24.5 kDa (2.5 μg, loading) is indicated in the lane labeled *wt* with the *arrow*. *B*, parallel experiments were carried as above but in this case purified procathepsin Cys^{26}-Gly^{26} mutant (5.0 μg) was unfolded by heating at 95 °C for 3 min prior to the addition of activated wild-type cathepsin L1.

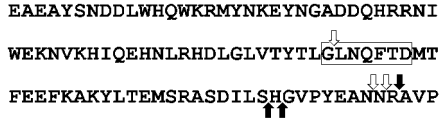

FIG. 9. **Amino acid sequence of the propeptide of *F. hepatica* cathepsin L1 with cleavage sites that lead to activation indicated.** The *downward solid arrow* indicates the cleavage site observed when native mature cathepsin L1 is secreted *in vitro* by adult *F. hepatica.* The *first open arrow* indicates the site of cleavage within the G*X*N*X*F*X*D motif (*boxed*) that gives rise to the 30 kDa intermediate component when procathepsin L1 is expressed in *P. pastoris,* and the second and third *open arrows* show the sites of cleavage when this is further activated *in vitro* at pH 5.0 (see Fig. 5). The upward *solid arrows* show the sites of cleavage of procathepsin Cys^{26}-Gly^{26} mutant by exogenously added mature cathepsin L1.

Further intermolecular processing and activation of the 30-kDa intermediate component and direct activation of procathepsin L1 by exogenous enzyme took place in the C-terminal portion of the propeptide. Elucidation of the three-dimensional structure of human procathepsin L (14) and cathepsin B (29, 30) revealed that this part of the propeptide is less structured and is readily accessible to proteases. Testament to this fact is the collective data showing that papain (32) cathepsin L (14), cathepsin B (29, 30), cathepsin S (31), and cathepsin K (25) can all be activated to mature enzymes either by *cis-* or *trans*-cleavage at various bonds within this segment. Cathepsin B of the helminth parasite *S. mansoni* can also be activated by

trans-cleavage at this segment (55, 56). However, the sites of cleavage of the wild-type and mutant procathepsin L1 differed suggesting that prior cleavage within the Gly^{-42}-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} motif of the wild-type enzyme may have altered the accessibility of the C terminus to protease action. It is also evident that both processing pathways leave additional amino acids at the N terminus of the mature enzyme, whereas cathepsin L1 recovered from the medium in which parasites were cultured was fully processed to the characteristic N-terminal residue. This suggests that additional protease action, possibly by an exopeptidase, may be required to remove these additional amino acids.

In the present study we show that the secretion and activation of *F. hepatica* cathepsin L1 has similarities with that of their mammalian counterparts. Most importantly, we have elucidated that cathepsin Ls are secreted into the parasite lumen from vesicles synthesized within the gastrodermal epithelial cells and that it is possible for these to become active by autocatalysis within the slightly acidic environment of the gut. The cathepsin L proteases are synthesized in copious amounts by *F. hepatica* parasites (0.5–1.0 μg per adult parasite per hour) and early autoradiographic studies by Hanna (57) estimated that the turnover rate from the synthesis of the contents of the secretory vesicles to their liberation into the gut lumen was rapid (~1 h). The means by which these enzymes are trafficked to the secretory vesicles and the possible involvement of the Gly^{-42}-Xaa-Asn-Xaa-Phe-Xaa-Asp^{-36} motif (32) or a pH-dependent interaction with a membrane-bound receptor (58) requires investigation. A role for carbohydrate moieties may be excluded since the *F. hepatica* procathepsin L studied here contains no potential *N*-glycosylation sites, and studies have indicated that the native enzyme is not glycosylated (38, 42), although homologs from other helminths, such a *S. mansoni*, are glycosylated (59).

Mammalian cathepsins play a number of important biological functions such as protein turnover, antigen processing and tissue remodeling (50). However, they have also been implicated in various pathological conditions including tumor invasion and metastasis (50, 60, 61), osteoporosis (53, 54, 62), and chronic inflammatory disease (63, 64). In parasites, cysteine proteases perform a number of pivotal functions such as feeding, tissue penetration and immunomodulation that allow them to infect and establish disease in the host (2, 56). A number of studies have demonstrated that these enzymes represent targets at which new chemotherapeutic and immunoprophylactic strategies can be directed (2, 12, 56, 65). While the goal of improving our knowledge on the activation mechanisms of mammalian cathepsin protease is to provide new ways of treating the diseases they cause, similar studies on the parasite homologs would allow comparative analyses for the purpose of designing parasite-specific treatments. The feasibility of this approach was demonstrated in our laboratory by showing that propeptides of *F. hepatica* procathepsin Ls are potent inhibitors of their cognate enzymes but exhibit little or no activity against their mammalian homologs.

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