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Inhibitor studies indicate that active cathepsin L is probably essential to its own processing in cultured fibroblasts

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The lysosomal cysteine proteinase cathepsin L is synthesized in cultured mouse NIH 3T3 cells as a 39 kDa precursor and processed intracellularly into active 29 kDa and 20 kDa + 5 kDa lysosomal forms. Addition to culture media of the peptidyl aldehyde leupeptin, a non-covalent inhibitor of cathepsin L, results in the accumulation of the 20 kDa mature form of the enzyme, resulting in increased activity of cathepsin L as measured in an *in vitro* assay system in the absence of leupeptin. The more potent irreversible cathepsin L inhibitors benzyloxycarbonyl-Phe-Ala-diazomethane and L-*trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane, when added to living cells at low concentrations, result in accumulation of all partially processed forms of cathepsin L, especially the 29 kDa form, suggesting that cathepsin L is responsible for its own processing. Exogenous procathepsin L introduced into CHO cells by endocytosis via the mannose 6-phosphate receptor is processed in a manner similar to endogenous procathepsin L. We conclude that the major intracellular pathway for processing of procathepsin L, either endogenous or exogenous, probably requires active cathepsin L.

INTRODUCTION

Current evidence suggests that lysosomal proteinases have a dual role in lysosomal digestion. In addition to bulk protein breakdown, they are also involved in the post-translational processing of lysosomal proenzymes. Lysosomal enzymes are synthesized as higher-molecular-mass prepropeptides in the rough endoplasmic reticulum (ER). Simultaneous with, or after, cleavage of the signal peptide in the ER, propeptides undergo glycosylation, followed by proteolytic processing and sorting through the Golgi complex to an acid lysosomal compartment [1–3]. Proteolytic processing is already initiated in the pre-lysosomal compartments and completed after arrival of the enzymes to lysosomes [4,5]. The proteolytic processing of lysosomal proteinases, e.g. cathepsin D [6] and cathepsin L [7,8], also results in the activation of a less active or inactive precursor, differing thus from some other lysosomal enzymes [2]. Thus processing of these lysosomal cathepsins may be an important regulatory mechanism for controlling levels of active enzyme in lysosomes.

The cysteine proteinases cathepsin B, H and L, and the aspartic proteinase cathepsin D, are the major proteinases in lysosomes. Cathepsin L is the most active cysteine endoproteinase in lysosomes of several tissues and has activity against a large variety of cellular proteins [7,9,10]. Cathepsin L has also been identified as the major excreted protein (MEP) from Kirsten-sarcoma-virus-infected NIH 3T3 (KNIH 3T3) fibroblasts [11]. The proenzyme of cathepsin L in mouse NIH 3T3 fibroblasts has a molecular mass of 39 kDa [3]. This precursor can be secreted from cells or processed to a 29 kDa intermediate form and finally to a 20 kDa + 5 kDa two-chain mature form in the lysosomal compartment [3]. Similar processing occurs *in vitro* with purified mouse procathepsin L [7] or recombinant human cathepsin L [12]. Thus, although it appears that purified cathepsin L is able to mediate its own processing *in vitro*, it remains to be seen which enzymes are responsible for its intracellular processing. The inhibitor studies described here were undertaken with the goal of determining what enzymes are involved in the processing of procathepsin L in living cells.

There are several types of cysteine-proteinase inhibitors [13]. Most frequently used in studies in living cells are peptidyl aldehydes, e.g., leupeptin, which are of microbial origin [14]. Leupeptin is a transition-state-analogue inhibitor of cysteine proteinases and binds to these enzymes very tightly, but not covalently [13]. Leupeptin also inhibits calpains and serine proteinases. In contrast, peptidyl-diazomethanes e.g. benzyloxycarbonyl (Z)-Phe-Ala-diazomethane (CHN_2) and epoxysuccinyl peptides, e.g. L-*trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane (E-64) and analogues, cause irreversible inhibition by binding covalently to cysteine proteinases [13]. Peptidyl-diazomethanes are the most specific synthetic thiol-proteinase inhibitors known to date and can be used to inhibit cathepsins B and L in cultured cells [15,16]. Cysteine-proteinase inhibitors can penetrate cells easily and are non-toxic at effective inhibitory concentrations. Various experiments have shown 40–60% inhibition in intracellular protein breakdown resulting from treatment with cysteine-proteinase inhibitors [16–18].

We have used both non-covalent and covalent inhibitors of cysteine proteinases to study the mechanism of processing of procathepsin L in living cells. The inhibitors which bind covalently to cathepsin L blocked the processing of procathepsin L irreversibly and at low concentrations, as indicated by the accumulation of intermediate forms of cathepsin L, whereas leupeptin induced the accumulation of the mature 20 kDa form, whose activity in assays *in vitro* increases as protein accumulates. These inhibitors also affected the processing of endocytosed pro-

Abbreviations used: KNIH 3T3, Kirsten-sarcoma-virus-transformed NIH 3T3; CHO, Chinese-hamster ovary; Z, benzyloxycarbonyl; E-64, *trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane; CHN_2, diazomethane; NHMec, 7-(4-methyl)coumarylamide; ER, endoplasmic reticulum; MEP, major excreted protein; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulphoxide.

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cathepsin L in a manner very similar to that of endogenous procathepsin L. These data suggest that cathepsin L mediates its own processing in intact cells.

MATERIALS AND METHODS

Materials

Cysteine-proteinase inhibitors were obtained from Bachem (Z-Phe-Ala-CHN_2 and leupeptin), Sigma Chemical Co. (E-64) and from Enzyme Systems (Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2; see Fig. 6 below). Stock solutions of 50 mM-Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were made in dimethyl sulphoxide (DMSO). In control experiments, the final concentration of 0.2% (v/v) DMSO did not affect cell growth or cathepsin L processing. [^{35}S]Methionine (> 800 Ci/mmol) was from Amersham Corp., Autofluor from National Diagnostics and X-Omat AR film from Kodak. The cathepsin L substrate Z-Phe-Arg-7-(4-methyl)coumarylamide (NHMec) was obtained from Sigma Chemical Co. Protein A–Sepharose CL-4B was purchased from Pharmacia. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were from Gibco Laboratories. α-Modified DMEM was from Advanced Biotechnologies, and calf serum was from Colarado Serum Co.

Methods

Cell culture. NIH 3T3 and KNIH 3T3 fibroblasts were cultured at 37 °C in 5% CO_2 in DMEM supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml) and 10% (v/v) calf serum. Chinese-hamster ovary (CHO) fibroblasts used in the endocytosis experiments were cultured in α-modified DMEM containing 10% fetal bovine serum.

Labelling and pulse–chase analysis. Subconfluent NIH 3T3 and KNIH 3T3 fibroblasts were labelled with [^{35}S]methionine (50–100 μCi/ml) in DMEM (without methionine) for the length of time indicated in the Figure legends. After labelling, cultures were washed twice with DMEM and lysed or processed for chase analysis. During the chase, cells were cultured in normal growth medium with or without cysteine-proteinase inhibitors (see the Figure legends). After the pulse-labelling or chase period, cells were lysed on the dishes with Buffer A as described previously [3] and frozen at −20 °C.

Immunoprecipitation, gel electrophoresis and fluorography. Cell extracts were clarified by centrifugation at 12000 *g* for 1 min. Immunoprecipitation with 3 μl of rabbit anti-(mouse cathepsin L) serum was performed using Protein A–Sepharose binding for 2 h at room temperature [19]. Immunoprecipitates were washed five times, as previously described [19], dissolved in SDS dissociation buffer and boiled for 3 min [3]. Electrophoresis was performed as described by Laemmli [20], using SDS/10% (w/v) polyacrylamide gels. After electrophoresis, gels were treated for 1 h with Autofluor, dried under a vacuum, and exposed to X-Omat AR film at −80 °C for 2–8 days.

Activity measurement of immunoprecipitated cathepsin L. Immunoprecipitation with our previously described rabbit polyclonal antiserum [3] does not affect the activity of cathepsin L (S. Gal & M. M. Gottesman, unpublished work). Cathepsin L bound to Protein A–Sepharose was immunoprecipitated from the supernatant of cell lysates as described above, using 6 μl of antiserum. The enzyme activity of cathepsin L bound to Protein A–Sepharose was assayed by using Z-Phe-Arg-NHMec as substrate, as described in detail previously [21]. Enzyme reactions were carried out in a water bath for 10–30 min at 35 °C with continuous shaking.

Preparation and uptake of radioactive cathepsin L. Cathepsin L labelled with [^{35}S]methionine was produced by KNIH 3T3 fibroblasts, which secrete large amounts of procathepsin L, which is their major excreted protein (MEP) [11]. Subconfluent KNIH 3T3 fibroblasts were labelled with [^{35}S]methionine (250 μCi/ml) for 5 h in DMEM (without methionine). The media were filtered through a 0.22 μm Millipore filter and dialysed against Dulbecco's phosphate-buffered saline, pH 7.2, for 2 days at 4 °C. Dialysis residue (1.0×10^6 c.p.m.) was added to the medium of CHO 10001 fibroblasts (see Fig. 5 below). The uptake of cathepsin L was inhibited by 10 mM-mannose 6-phosphate. At the end of the uptake (30 min or 4 h) or chase period (4 h), cells were washed thoroughly and lysed for immunoprecipitation. Lysates were centrifuged at 12000 *g* for 1 min and the entire supernatant was used for immunoprecipitation, electrophoresis and fluorography as described above.

RESULTS

Effect of cysteine-proteinase inhibitors on cathepsin L activity, synthesis and secretion

Recent studies have shown that prolonged administration of leupeptin paradoxically increased the activities of cathepsin B and L in animal tissues [22–24]. In this study, this effect was verified in cultured NIH 3T3 fibroblasts.

Immunoprecipitable activity of cathepsin L increased 15-fold during 3 days of leupeptin treatment in NIH 3T3 fibroblasts (Fig. 1). By contrast, two other cysteine-proteinase inhibitors, E-64 and Z-Phe-Ala-CHN_2, strongly decreased the total activity of cathepsin L in NIH 3T3 fibroblasts (Fig. 1), as expected, since these are potent covalent inhibitors of this enzyme. Growth curves of NIH 3T3 fibroblasts showed that leupeptin (20 μM) and

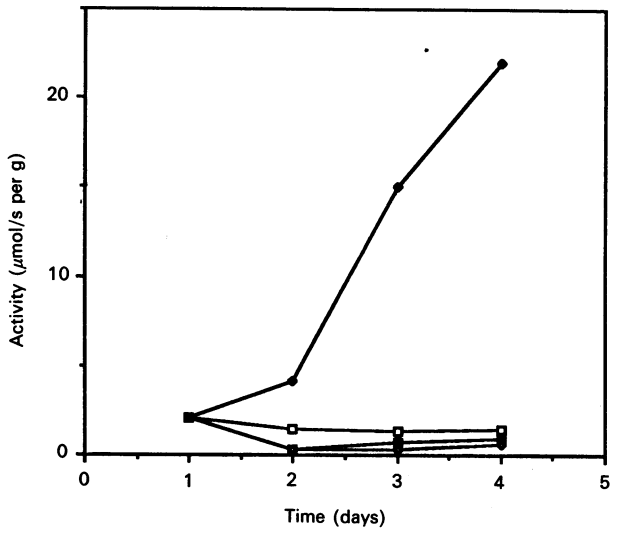

Fig. 1. Effects of prolonged treatment with cysteine-proteinase inhibitors on activities of immunoprecipitated cathepsin L in cultured NIH 3T3 fibroblasts

NIH 3T3 fibroblasts (1.5×10^5 cells in a 100 mm-diameter dish) were plated the day before adding the cysteine-proteinase inhibitors leupeptin, E-64 or Z-Phe-Ala-CHN_2. Drug concentrations were 20 μM for leupeptin and 100 μM for E-64 and Z-Phe-Ala-CHN_2. Media were changed daily. The activities of immunoprecipitated cathepsin L (see the Materials and methods section) are shown as μmol/s per g of protein in the cell supernatant fractions used for immunoprecipitation. Values are means for two different samples. □, Control; ◆, leupeptin; ■, Z-Phe-Ala-CHN_2; ○, E-64.

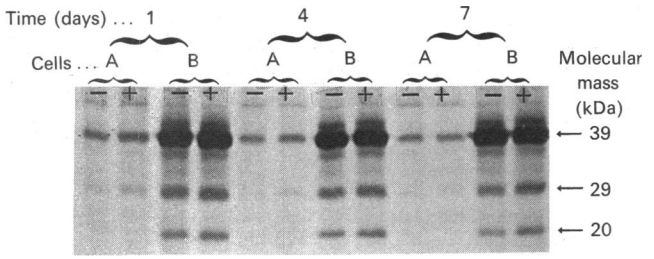

Fig. 2. Leupeptin treatment for 1–7 days does not affect the synthesis rate of cathepsin L in NIH and KNIH 3T3 fibroblasts

NIH 3T3 (1×10^6 cells in a 100 mm-diameter dish) (A) and KNIH 3T3 (2×10^6 cells in 100 mm dish) (B) fibroblasts were precultured for 1, 4 or 7 days without (−) or with (+) leupeptin (20 μM), and then labelled with [^{35}S]methionine (50 μCi/ml) for 3 h. At 7 days, cells were split twice. Leupeptin was in the medium during labelling. Cell extracts were prepared as described in the Materials and methods section, and a volume of extract containing 5×10^5 trichloracetic acid-precipitable c.p.m. was used for immunoprecipiation of cathepsin L.

E-64 (100 μM) did not affect the proliferation of NIH 3T3 cells, but Z-Phe-Ala-CHN_2 (100 μM) did inhibit the growth of the NIH 3T3 cells by approximately 20% during the 3-day experiment (results not shown).

The continuous increase in cathepsin L activity resulting from leupeptin treatment could be due either to increased synthesis, decreased secretion or decreased turnover of active cathepsin L molecules. Fig. 2 shows that leupeptin treatment of KNIH and NIH 3T3 cells for up to 7 days did not affect the synthesis rate of cathepsin L in either cell line. After 3 h of labelling, the 39 kDa procathepsin L was the major form in fibroblasts. As Fig. 2 shows, KNIH cells synthesize much more procathepsin L than do NIH cells, and the 3 h labelling period is long enough to reveal the 29 kDa and 20 kDa processed forms in these cells.

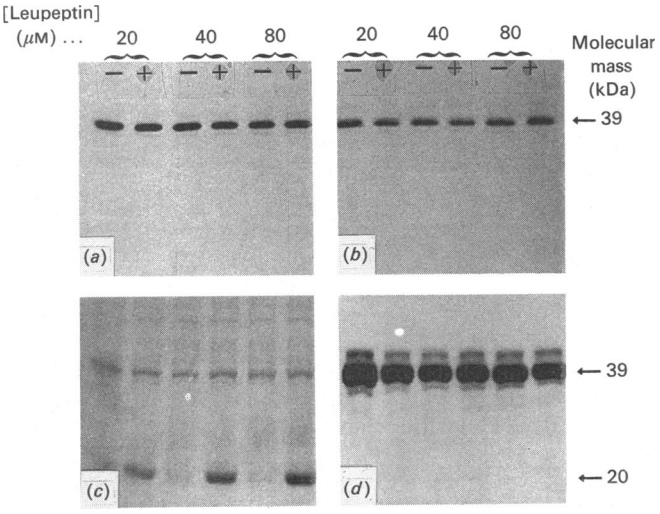

Fig. 3. Effect of increasing concentrations of leupeptin on the synthesis, secretion and processing of cathepsin L in KNIH 3T3 fibroblasts

KNIH 3T3 fibroblasts (1×10^5/60 mm-diameter dish) were precultured for 2 days before labelling for 90 min with [^{35}S]methionine (50 μCi/ml). The leupeptin concentration was 20, 40 or 80 μM in preculture, labelling and chase (8 h) medium. (*a*) and (*b*) show the labelled cathepsin L after a 90 min labelling period, and (*c*) and (*d*) show the same after an 8 h chase period in KNIH 3T3 fibroblasts. (*a*) and (*c*) show labelled cathepsin L in cell lysates, and (*b*) and (*d*) show labelled procathepsin L secreted into the medium.

We found that increasing the concentration of leupeptin up to 4-fold (80 μM) does not affect the synthesis of procathepsin L in KNIH cells (Fig. 3*a*). Since procathepsin L is the MEP of KNIH cells [11], we also examined the effect of leupeptin on procathepsin L secretion. Fig. 3(*b*) (90 min label) and Fig. 3(*d*) (90 min label, followed by an 8 h chase) show that leupeptin did not affect the secretion of procathepsin L. Fig. 3(*c*) shows intracellular cathepsin L, indicating that, after a 90 min labelling period followed by an 8 h chase, leupeptin induced the accumulation of the 20 kDa mature form of cathepsin L in KNIH 3T3 fibroblasts. The accumulation was proportional to the concentration of leupeptin in the culture medium.

Effect of inhibitors on cathepsin L processing

Mouse cathepsin L (39 kDa) has two processed forms of molecular masses 29 kDa and 20 kDa [3]. We compared the effects of leupeptin (non-covalent binding), and of E-64 and Z-Phe-Ala-CHN_2 (covalent binding), on the processing of cathepsin L in NIH 3T3 fibroblasts. Pulse–chase analysis showed that leupeptin decreased both the processing rate and turnover rate of cathepsin L (Fig. 4*a*). The most prominent form which accumulated after 48 h was the 20 kDa mature cathepsin L, but at intermediate times the 29 kDa form was also prominent. Interestingly, Z-Phe-Ala-CHN_2 and E-64 had a somewhat different effect on the processing of cathepsin L than leupeptin, causing the accumulation of a 29 kDa form of cathepsin L rather than the 20 kDa form (Figs. 4*b* and 4*c*). All of these inhibitors caused the accumulation of the 39 kDa procathepsin L to some extent, seen at the earliest chase times. Furthermore, E-64 and Z-Phe-Ala-CHN_2 treatment, and to a much lesser extent, leupeptin treatment, resulted in the appearance of an intermediate form between 29 kDa and 39 kDa in the first processing step seen after the 2 h and 4 h chases.

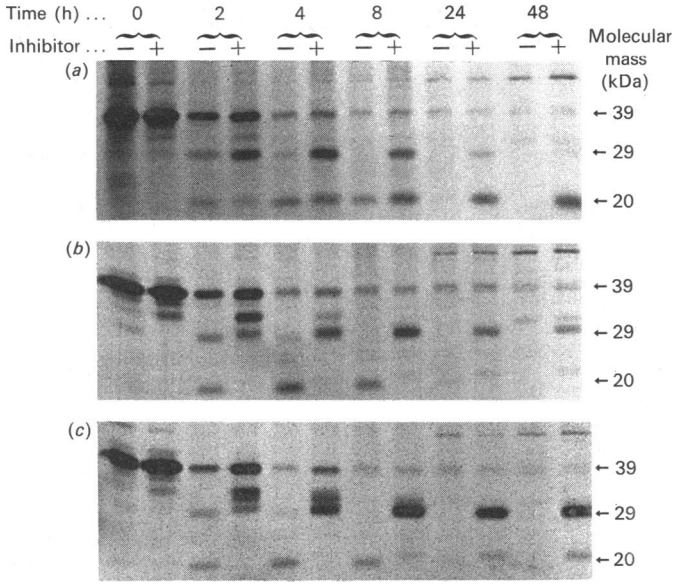

Fig. 4. Effects of treatment with leupeptin, E-64 and Z-Phe-Ala-CHN_2 on the processing of cathepsin L in NIH 3T3 fibroblasts

NIH 3T3 fibroblasts (1.5×10^5 cells/60 mm-diameter dish) were plated the day before labelling for 90 min with [^{35}S]methionine (50 μCi/ml). Cysteine-proteinase inhibitors were included both in preculture (20 h), labelling (90 min) and chase (2–48 h) media. The concentrations of inhibitors in culture media were 20 μM for leupeptin and, for E-64 and Z-Phe-Ala-CHN_2, 100 μM. (*a*) Shows leupeptin-treated, (*b*) E-64-treated and (*c*) Z-Phe-Ala-CHN_2-treated cells. Fibroblasts were cultured either without (−) or with (+) inhibitor.

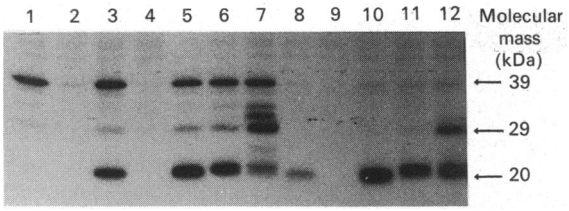

Fig. 5. Effects of cysteine-proteinase inhibitors on the endocytosis and processing of exogenous cathepsin L in CHO fibroblasts

CHO 10001 fibroblasts (5×10^5 cells/60 mm-diameter dish) were precultured overnight before adding [^{35}S]methionine-labelled procathepsin L (see the Materials and methods section). Total radioactivity per dish was 1×10^6 c.p.m. Inhibitors (concentrations as in Fig. 3) were included in the culture media 1 h before endocytosis, during endocytosis (0.5–4 h) and during the chase (4 h). Mannose 6-phosphate, when included, was at a final concentration of 10 mM for the endocytosis and chase period. After the endocytosis period (4 h), cells were carefully washed three times with DMEM, and either were lysed, or chase medium was added. Lanes 1 and 2 show labelled cathepsin L in cells after 30 min uptake (lane 2 with mannose 6-phosphate); lanes 3–7 after 4 h uptake (lane 3 without inhibitors, lane 4 with mannose 6-phosphate, lane 5 with leupeptin, lane 6 with E-64 and lane 7 with Z-Phe-Ala-CHN_2) and lanes 8–12 after 4 h chase (lane 8 without inhibitors, lane 9 with mannose 6-phosphate, lane 10 with leupeptin, lane 11 with E-64 and lane 12 with Z-Phe-Ala-CHN_2).

Inhibitors and processing of endocytosed cathepsin L

Several studies (e.g. [25]) have shown that exogenous lysosomal enzymes taken up by mannose 6-phosphate receptors from extracellular fluid are also processed in cells to mature forms. We studied the effect of cysteine-proteinase inhibitors on the processing of endocytosed, [^{35}S]methionine-labelled, procathepsin L in CHO 10001 fibroblasts. These cells were used for the uptake studies, since the uptake of cathepsin L by CHO cells is more efficient than in NIH 3T3 fibroblasts. Fig. 5 shows that the endocytosis of procathepsin L occurred via mannose 6-phosphate receptors, because 10 mM-mannose 6-phosphate blocked the uptake (lanes 2, 4 and 9). As had been observed for processing of endogenous procathepsin L, leupeptin inhibited the turnover of the 20 kDa mature form (lane 10), whereas Z-Phe-Ala-CHN_2 (lanes 7 and 12) caused the accumulation of the 29 kDa form during the 4 h uptake and processing period. The effect of E-64 (lanes 6 and 11) was very similar to that of leupeptin (lanes 5 and 10) in this experiment. The processing rate seems to be somewhat higher for endocytosed enzymes than for endogenously synthesized enzymes (compare Fig. 4 and 5) and, in addition, Z-Phe-Ala-CHN_2 (lanes 7 and 12) inhibited processing of the 20 kDa form, which accumulates. A higher processing rate for lysosomal enzymes after endocytosis as compared with the endogenous enzymes had also been shown previously [25]. However, two different cell types were used in our experiments (NIH versus CHO), and hence comparing the processing rates is difficult.

Fig. 5 also shows that the 20 kDa form of cathepsin L is slightly larger in E-64- and Z-Phe-Ala-CHN_2-inhibited samples than in those of control and leupeptin-treated fibroblasts. The same phenomenon can be seen in Fig. 4, in both the 29 kDa and 20 kDa forms. This small molecular-mass discrepancy may represent the covalent binding of these inhibitors to cathepsin L molecules or could reflect differences in the sizes of the processed forms. The unprocessed 39 kDa form does not show any shift in molecular mass in samples containing inhibitors.

Comparison of the effects of Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2

Peptidyl-diazomethanes are selective inhibitors of cysteine proteinases [15]. Furthermore, Z-Phe-Phe-CHN_2 is much more effective against cathepsin L than cathepsin B [26]. To address the question of the role of cathepsin B and L in the processing of cathepsin L *in vivo*, we compared the effects of Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 on the processing of cathepsin L with decreasing inhibitor concentrations. A concentration of 3×10^{-6} M caused a prominent inhibition of cathepsin L processing, Z-Phe-Ala-CHN_2 being slightly more effective than Z-Phe-Phe-CHN_2 (Fig. 6). The difference may be due to a low solubility of Z-Phe-Phe-CHN_2 in aqueous tissue-culture medium. Also, in this experiment, the processed forms of cathepsin L were slightly higher in molecular mass in the inhibited samples than in the control samples, suggesting that both inhibitors bind to cathepsin L. Given the extreme sensitivity of the processing to these inhibitors with known relative specificity for cathepsin L, it seems very likely that the processing of cathepsin L in intact cells can be attributed to cathepsin L rather than cathepsin B.

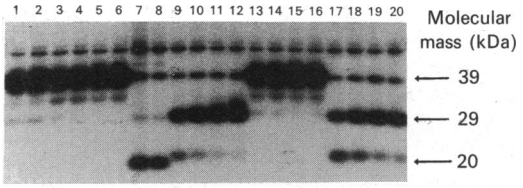

Fig. 6. Effects of Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 on the processing of cathepsin L in NIH 3T3 fibroblasts

NIH 3T3 fibroblasts (2×10^5/60 mm dish) were plated 24 h before labelling with [^{35}S]methionine (100 μCi/ml) for 90 min. Chase time was 6 h. Inhibitors were included in prelabelling (1 h), labelling and chase medium in final concentrations of 3, 10, 30 or 100 μM. Lanes 1–6 show the synthesis of cathepsin L after pulse-labelling (lanes 1–2 without inhibitor and lanes 3–6 with increasing concentrations of Z-Phe-Ala-CHN_2). Lanes 7–12 show labelled cathepsin L after 6 h chase (lanes 7–8 without inhibitor and 9–12 with increasing concentrations of Z-Phe-Ala-CHN_2). Lanes 13–16 show cathepsin L synthesis during the pulse-labelling period (lanes 13–16 with increasing concentrations of Z-Phe-Phe-CHN_2). Lanes 17–20 show the labelled cathepsin L after the 6 h chase period with increasing concentrations of Z-Phe-Phe-CHN_2.

DISCUSSION

The inhibitor studies reported here suggest that in intracellular processing and degradation of procathepsin L to its mature intracellular form is catalysed by cathepsin L itself. This result suggests that the pathways of processing of cathepsin L are similar in intact cells and *in vitro*, since purified cathepsin L has been shown to process itself at low pH [7,12]. Furthermore, secreted procathepsin L is processed rapidly after uptake via mannose 6-phosphate receptors by an autoprocessing mechanism similar to that which occurs after endogenous synthesis.

Experiments using leupeptin as an inhibitor in intact cells have produced seemingly contradictory results. During a brief exposure, activities of cathepsin B and L are inhibited, but later, paradoxically, these activities measured in extracts strongly increase [22,24]. Ultrastructural studies show a prominent accumulation of undigested material in lysosomal vacuoles, suggesting that these enzymes are inhibited in the intact cells [24]. An increase in cysteine-proteinase activities by leupeptin measured in extracts could be due to increased synthesis, decreased turnover or changed balance between endogenous inhibitors and proteinases. The present results both with NIH 3T3 and KNIH 3T3 fibroblasts indicate that the synthesis rate for procathepsin L was not affected, but the processing and turnover rate of cathepsin L were prominently reduced, resulting in the accumulation of active 29 kDa and 20 kDa intracellular forms.

Kominami *et al.* [27] have also shown that the administration of Ep-475, a synthetic analogue of E-64, induces the accumulation of cathepsin B, H and L in rat liver, owing to inhibition of turnover. Previous studies have shown that the administration of E-64 [28] or Z-Phe-Ala-CHN_2 [22] does not increase enzyme activities. The contradictions between these observations most probably reflect the mechanism of enzyme inhibition. A non-covalent inhibition of cysteine proteinases by leupeptin reduces lysosomal protein breakdown, including that of cathepsin L, in lysosomes in intact cells, resulting in the accumulation of 29 and 20 kDa forms of cathepsin L. In enzyme assays, especially in those with immunoprecipitated enzymes, the leupeptin present in cells or tissues is diluted or washed away and increased activities are observed, whereas the covalent inhibitors remain bound to the enzyme.

The administration of irreversible inhibitors of cathepsin L, E-64 and Z-Phe-Ala-CHN_2, decreased the activity of immunoprecipitated cathepsin L in NIH 3T3 fibroblasts. Pulse–chase analysis showed that processing from the 39 kDa to the 29 kDa and from the 29 kDa to the 20 kDa mature form of cathepsin L was impeded, and further breakdown was extremely slow in the presence of inhibitors. Interestingly, the 29 kDa and 20 kDa accumulated forms of cathepsin L migrated more slowly in gel electrophoresis than did those in control and leupeptin-treated samples. This is consistent with the view that the inhibitor is irreversibly bound to these processed forms, although it is not possible to rule out small differences in the sizes of the processed forms in the presence of inhibitors. The unprocessed 39 kDa form did not show any molecular-mass shift. Because E-64 and Z-Phe-Ala-CHN_2 are active-site-directed inhibitors of cathepsin L, these observations suggest that unprocessed 39 kDa propeptide is not active in fibroblasts and suggest that the processed intermediate 29 kDa form and the mature 20 kDa form are active and thus exposed to inhibitor binding. These results verify the observations of Mason *et al.* [29]. They used a radiolabelled peptidyl-diazomethane to show that the processed forms of cathepsin L, but not the precursor form, are active in KNIH 3T3 fibroblast.

Several studies have shown that the post-translational processing of lysosomal enzymes, e.g. cathepsin D [30], acid phosphatase [5], α-mannosidase and β-glucosidase [31], can be impeded by cysteine-proteinase inhibitors, suggesting that cathepsin B and/or cathepsin L are involved in proteolytic processing. Our results show that lysosomal cysteine proteinases are also involved in the processing of cathepsin L, especially from the 29 kDa intermediate form to the mature 20 kDa form. In addition, cysteine proteinases most probably also digest the mature 20 kDa enzyme. Nishimura *et al.* [8,32] have shown that the cathepsin D inhibitor pepstatin inhibits the first step in cathepsin L processing in a cell-free system, and that cathepsin D can activate procathepsin L. They conclude from these studies that cathepsin D may be required for the first step in activation of procathepsin L. On the other hand, our previous results [7] demonstrate the autoactivation of the 39 kDa purified procathepsin L to the 29 kDa form at low pH without cathepsin D. Since the cathepsin L inhibitors used in our current study do not completely block conversion of the 39 kDa form into the 29 kDa form, it seems likely that another enzyme, perhaps cathepsin D, may contribute to this initial activation step in intact cells. Subsequent processing steps appear to be mostly cathepsin L-dependent, on the basis of our inhibitor studies.

The present study also suggests that, after cleavage of the signal peptide, there is an intermediate form between the 39 kDa proenzyme and the 29 kDa active form which accumulates as a result of treatment with E-64 and Z-Phe-Ala-CHN_2. A similar intermediate form was also observed during acid-induced autoactivation [7]. These results suggest that the formation of the 29 kDa cathepsin L occurs after one or more intermediate proteolytic clips of the 39 kDa precursor.

The dipeptidyl-diazomethanes, of which Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 are the best studied, are specific inhibitors of thiol proteinases. However, the affinity of these inhibitors, especially that of Z-Phe-Phe-CHN_2, is much higher against cathepsin L than cathepsin B, allowing the selective inhibition of cathepsin L in low inhibitor concentrations [26]. Dipeptidyl-diazomethanes also are relatively ineffective against cathepsin H. In the present study we observed that dipeptidyl-diazomethanes are very effective inhibitors of cathepsin L processing. Even a concentration of 3×10^{-6} M in culture medium caused a significant inhibition in the processing of cathepsin L in fibroblasts. Z-Phe-Ala-CHN_2 was slightly more effective than Z-Phe-Phe-CHN_2, which may be due to the hydrophobicity of Z-Phe-Phe-CHN_2, resulting in solubility and penetration differences. In addition, both inhibitors were observed to bind to cathepsin L. Hence it seems that the effective inhibition of cathepsin L processing in intact cells at very low inhibitor concentrations is due to inhibition of cathepsin L, rather than to inhibition of cathepsin B or H. More complicated scenarios, in which the inhibitors block cathepsin L activation of other cathepsins, which are in turn necessary for activation of cathepsin L, are possible, but seem less likely, given the ability of purified cathepsin L to process itself.

The most coherent model which includes these observations is that the 39 kDa procathepsin L has a small amount of latent activity which is stimulated at low pH or by cleavage with another proteinase such as cathepsin D. The only way to determine the relative role of the cathepsin D, or other cathepsins, in respect of autoprocessing in this initial step, will be to study mutants defective in these activities, since no inhibitor is absolutely specific. Once mature cathepsin L is generated, the procathepsin is rapidly activated [12]. Within the cell, most processing of procathepsin L after the initial step can be attributed to the activity of endogenous cathepsin L already present in the lysosomal and pre-lysosomal compartments.

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