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Effect of proteinase inhibitors on intracellular processing of cathepsin B, H and L in rat macrophages

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The effects of various proteinase inhibitors on the processing of lysosomal cathepsins B, H and L were investigated in cultured rat peritoneal macrophages. The processing of newly synthesized pro-cathepsins B, H and L to the mature single-chain enzymes was sensitive to a metal chelator,1,10-phenanthroline, and a synthetic metalloendopeptidase substrate, Z-Gly-Leu-NH_2, and insensitive to inhibitors of serine proteinases, aspartic proteinases and cysteine proteinases. Inhibitors of cysteine proteinases, E-64-d and leupeptin, inhibited the processing of the single-chain forms of cathepsins B, H and L to the two-chain forms. These results suggest that (a) metal endopeptidase(s) is (are) involved in the propeptide processing of cathepsin B, H and L, and that proteolytic cleavages of the mature single-chain cathepsins are accomplished by cysteine proteinases in lysosomes.

Cathepsin B; Cathepsin H; Cathepsin L; Proteinase inhibitor; Intracellular processing; (Rat macrophage)

1. INTRODUCTION

The lysosomal cysteine proteinases cathepsin B, H and L are synthesized as larger precursors in macrophages [1], rat liver [2,3] and pancreatic islet cells [4], like various other lysosomal enzymes. Pulse-chase experiments showed that procathepsins B (39 kDa), H (41 kDa) and L (39 kDa) were synthesized after a short time of labeling and that these proenzymes were subsequently processed to mature single-chain enzymes (29 kDa for cathepsin B, 28 kDa for cathepsin H and 29 kDa for cathepsin L) by limited proteolysis [1]. The single-chain forms of cathepsins were then processed further to the two-chain forms at different rates [1].

Nishimura and Kato [2,3] recently demonstrated by a radioactive pulse-chase protocol coupled with subcellular fractionation that in rat liver the processing of procathepsins B and H to the mature single-chain enzymes takes place in lysosomes.

However, it is unknown what type of proteinase(s) is involved in processing of procathepsins. In this work, we examined the effects of various proteinase inhibitors on the proteolytic processing of the three cathepsins in rat macrophages to characterize these proteinases. Here we report that the processing of procathepsins B, H and L to the single-chain forms are sensitive to the metal chelator,1,10-phenanthroline and the synthetic metalloendopeptidase substrate, Z-Gly-Leu-NH_2, and that the conversion from the single-chain forms of cathepsins to the two-chain forms is sensitive to E-64-d, a specific cysteine proteinase inhibitor.

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Abbreviation: SDS-PAGEE, SDS-polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

E-64-d (EST, ethyl(2*S*,3*S*)-3-(*s*)-3-methyl-1-(3-methylbutylcarbamoyl)butylcarbamoyl oxirane-2-carbocylaste) was a gift from Dr Hanada (Taisho Pharmaceutical Co.). Phenylmethylsulfonyl fluoride and Z-Phe-Leu-NH_2 were from Sigma. Pepstatin and leupeptin were obtained from the Peptide Center, Osaka, Japan. 1,10-Phenanthroline was from Merck.

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The method for preparation of peritoneal macrophages from rats and the culture conditions were as described [5]. The macrophages were incubated overnight in methionine-free Eagle's MEM containing 10% (v/v) heat inactivated calf serum, and were then labeled with 100 μCi [^{35}S]methionine in 1.0 ml Eagle's MEM for 15 min. Then they were chased in RPMI 1640 containing 2% calf serum. Various proteinase inhibitors were included in the case medium. Immunoprecipitations of cathepsins from cell extracts and media and SDS-PAGE were performed as described in the accompanying paper [1]. Antisera against rat cathepsin B, H [6] and L [7] were prepared as reported.

3. RESULTS AND DISCUSSION

To study the effects of various proteinase inhibitors on the processing of cathepsins B, H and L, the cells were pulse labeled for 15 min and test inhibitors were added to the chase medium.

Fig.1 shows the effects of inhibitors on the processing of cathepsin L. In this experiment, procathepsin L was found even after 20 h of chase and its processing to the single- and two-chain forms was only partial. However, some inhibitors, such as E-64-d, a specific inhibitor of cysteine protease,

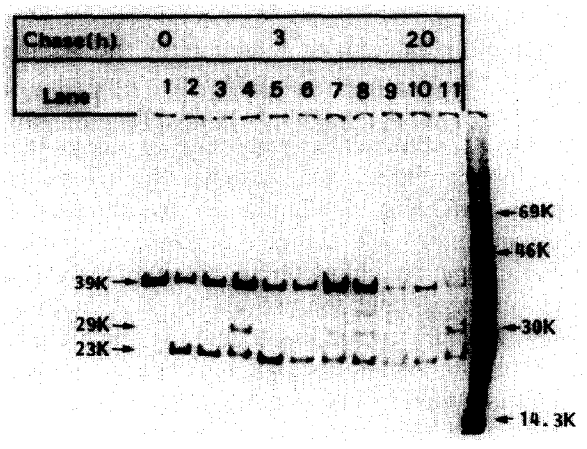

Fig.1. Effects of proteinase inhibitors on intracellular processing of cathepsin L. Rat peritoneal macrophages were pulse chased for 15 min with [^{35}S]methionine and incubated in chase medium with various proteinase inhibitors. At the indicated times, cells were collected in 0.5 ml of phosphate buffered saline containing 0.2% Triton X-100. The mixtures were centrifuged, and the supernatants were incubated with anti-cathepsin L mono-specific antibody. Immunoprecipitates were washed, resuspended in gel sample buffer and subjected to SDS-PAGE followed by fluorography. Lanes: 1, 2 and 9, no addition; 3, leupeptin (40 μg/ml); 4, E-64-d (40 μg/ml); 5, pepstatin (50 μg/ml); 6, phenylmethylsulfonyl fluoride (1 mM); 7, 1,10-phenanthroline (0.4 mM); 8, Z-Gly-Phe-NH_2 (3 mM); 10, pepstatin (50 μg/ml); 11, E-64-d (40 μg/ml). K, kilodalton.

the metal chelator 1,10-phenanthroline and a competitive inhibitor for metalloendopeptidase, Z-Gly-Phe-NH_2, clearly retarded the processing of cathepsin L. E-64-d caused accumulation of the single-chain form of cathepsin L and its effect was seen even in 20 h of chase (lane 11). The two inhibitors of metal protease seemed to inhibit the conversion of procathepsin L to the mature form of the enzyme (lanes 7 and 8). However, two other metal chelators, EDTA and phosphoramidon, had no effect (not shown). Phenylmethylsulfonyl fluoride, a serine proteinase inhibitor, and pepstatin, an aspartic proteinase inhibitor, did not inhibit the processing.

The effects of E-64-d and 1,10-phenanthroline on the processing of cathepsins B, H and L were studied further. High concentrations of Z-Gly-Phe-NH_2 (more than 3 mM) caused rapid detachment of the cells from the culture plates and so its effect was studied no further. When E-64-d was added at 40 μg/ml to the chase medium, it inhibited conversion of the single-chain form of cathepsin L to the two-chain form, resulting in accumulation of the mature single-chain enzyme and procathepsin L (fig.2). Control cells gave only a faint band of the heavy chain after a 21 h chase period, whereas E-64-d-treated cells gave much

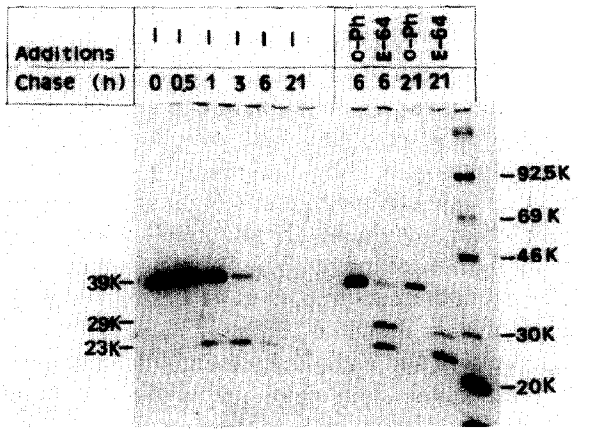

Fig.2. Effects of 1,10-phenanthroline and E-64-d on intracellular processing of cathepsin L. Macrophages were pulse labeled for 15 min with [^{35}S]methionine and incubated in chase medium for the indicated times (left) or in chase medium with 1,10-phenanthroline (1.6 mM) or E-64-d (40 μg/ml) for 6 h or 21 h (right). Immunoprecipitation, SDS-PAGE and fluorography were carried out as described in the legend to fig.1. K, kilodalton.

more intense bands of the single chain and heavy chain. A high concentration of 1,10-phenanthroline (1.6 mM) inhibited processing of procathepsin L almost completely, and so an intense band of procathepsin L was seen even after a chase time of 21 h.

The effects of two inhibitors on the processing of cathepsin B and H are shown in fig.3. Treatment of cells with E-64-d also inhibited the conversions of cathepsin B and H to the two-chain forms and 1,10-phenanthroline inhibited the conversions of procathepsins B and H to the mature enzymes. These two inhibitors did not appreciably affect the releases of procathepsins into the culture medium (not shown).

Since synthetic oligopeptide inhibitors of metalloendopeptidase activity have been reported to block exocytotic secretion from mast cells and chromaffin cells [6] and myoblast fusion [7], metalloendopeptidase activity has been suggested to be involved in the process of membrane contact and fusion. The enzymes that have been investigated as signal peptidases are also metalloproteases [8]. Thus, the processing of procathepsins to the mature enzymes, that is sequential membrane reactions, may be mediated by metalloendopeptidase(s). Nishimura and Kato reported [2,3] that the removal of the propeptides and procathepsins B and H takes place in the lysosomes, so the metalloendopeptidase(s) should be present in the lysosomes.

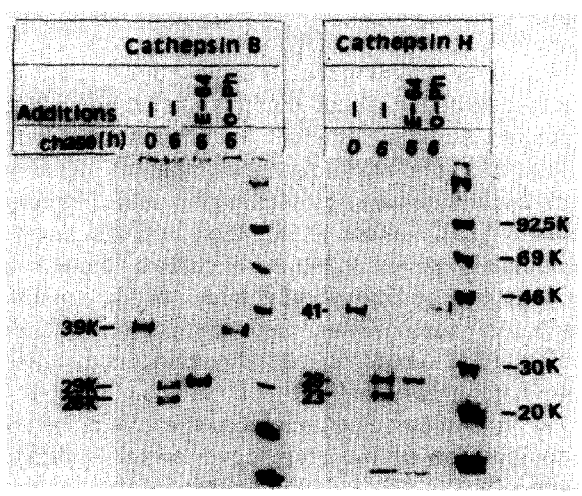

Fig.3. Effects of 1,10-phenanthroline and E-64-d on the intracellular processing of cathepsin B (left) and H (right). Macrophages were pulse labeled for 15 min with [^{35}S]methionine and incubated in chase medium with 1,10-phenanthroline (1.6 mM) or E-64-d (40 μg/ml) for 6 h or 21 h. Cathepsin B and H were sequentially immunoprecipitated from the detergent phase of Triton X-100 lysates. K, kilodalton.

On the other hand, there is accumulating evidence that inhibitors of metalloendopeptidases are general perturbants of many types of membrane-associated biological functions [9]. Thus inhibitors of metallopeptidase may interfere with the transport of procathepsins in various organellae from the endoplasmic reticulum through the golgi apparatus to lysosomes. We are now investigating the locations of procathepsins in 1,10-phenanthroline-treated cells by pulse-chase experiments coupled with subcellular fractionation.

We found [12] that prolonged incubation of macrophages with E-64-d caused marked increase in the contents of cathepsins B, H and L. This finding is consistent with the present observation that E-64-d inhibits the processing of the single-chain forms to the two-chain forms resulting in degradations of the former.

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