INTRACELLULAR PROCESSING AND ACTIVATION OF LYSOSOMAL CATHEPSINS

YUKIO NISHIMURA, TAKAHIRO KAWABATA, SHINJI YANO, AND KEITARO KATO

Department of Physiological Chemistry Faculty of Pharmaceutical Sciences, Kyushu University, Higashi-Ku, Fukuoka 812

Precursor forms of lysosomal cathepsins B, H and L in the hepatic endoplasmic lumen were identified as having a molecular weight of 39-, 41-, and 39-kDa, respectively, by immunoblotting analysis. The proenzymes were then concentrated by applying the microsomal contents to a concanavalin A-Sepharose chromatography. The concanavalin A-adsorbed fractions containing the proenzymes showed no appreciable activities of cathepsins B, H and L. When those fractions were incubated at pH 3.0, the enzymatic activities markedly increased. Immunoblotting analysis showed that after 36 hr incubation the proenzymes disappeared and the mature enzymes increased. Thus the proenzymes were processed to the mature enzymes under acidic conditions of pH 3.0. The marked increased of enzymatic activities and the conversion of the proenzymes to the mature forms were completely blocked with pepstatin which is a potent inhibitor of aspartic proteinases. The results strongly suggested that a processing proteinase for procathepsins B, H, and L might be cathepsin D, a major lysosomal aspartic proteinase. Indeed, lysosomal cathepsin D could convert the immunoaffinity-purified microsomal procathepsin B to the mature enzyme in vitro. Therefore, procathepsins B, H, and L seem to be firstly synthesized as the enzymatically inactive forms in endoplasmic reticulum and may successively be converted into the active forms by cathepsin D in lysosomal compartments.

Cathepsins B, H, and L are well-known lysosomal cysteine proteinases which are considered to cause the degradation of tissue proteins in lysosomes (3). Like most lysosomal acid hydrolases, cathepsins B, H, and L are synthesized on membrane-bound polysomes of endoplasmic reticulum as glycosylated precursors which are larger than the mature enzymes (11-13, 16, 17). During the synthesis and the following intracellular translocation to lysosomal compartments, lysosomal cathepsins B, H, L, and D are considered to undergo not only a cleavage of NH2-terminal prepeptide co-translationally but also a proteolytic processing of transient propeptide post-translationally (5, 11, 12, 16, 17, 21).

Mailing Address: Yukio Nishimura, Ph. D., Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan.

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2 To whom correspondence should be addressed.

3 Abbreviations: Z, benzoyloxy carbonyl; MCA, 4-methylcoumaryl-7-amide; DOC, sodium deoxycholate; Con A, concanavalin A; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
The propeptide of lysosomal cathepsin D seems to have similar functions to those of other proteases such as pepsin (22). These enzymes are synthesized as larger molecules namedzymogens, which release NH₂-terminal prosegments by limited proteolysis and reveal the enzymatic activities. Latent cathepsinogen D, an acid protease, is presumably activated by brief acid treatment, and the activation is accompanied by the release of an activation peptide (6, 13, 19). However, it has not been made clear where the processing for procathepsins begin in the biosynthetic pathway and it remains to be determined what enzyme or factors in the acidic compartments or lysosomes carry out the complete proteolytic maturation of procathepsins. Therefore, the present investigation was undertaken to characterize an activation mechanism for the latent precursors of microsomal cathepsins B, H, and L and to identify a propeptide-processing protease which causes the cleavage of propeptides in lysosomes. Our data carried out with the in vitro activation experiments indicate that lysosomal cathepsin D is the propeptide-processing protease responsible for cleaving the propeptides of microsomal procathepsins B, H, and L. In addition, our results with assays of enzymatic activities suggest that this processing step, presumably proceeding in lysosomes in vitro, is accompanied by the activation of the enzymes.

**MATERIALS AND METHODS**

*Enzyme assays and purification.*

The cathepsin B activity was determined according to Ref. (3) using Z³-Phe-Ala-MCA or Z-Arg-Arg-MCA as a substrate. Enzyme assay was performed at pH 6.0 as described previously (3). The cathepsin D activity was determined by a modification of the method of Anson (1). Microsomal procathepsin B was purified by the use of Con A-Sepharose, Sepharose-Gly-Phe-Gly-Sc (20), and immunoaffinity chromatography. The procathepsin B fraction eluted from the immunoaffinity column with 0.1 M glycine-HCl buffer (pH 3.0) containing 0.15 M NaCl was concentrated and dialyzed against 50 mM Hepes-NaOH buffer (pH 7.5). Cathepsin D was isolated to homogeneity from rat liver lysosomes essentially as described previously by Yamamoto et al. (26). Tritosomes were prepared by the method of Yamamoto et al. (27).

*Preparation of antisera.*

Rat liver lysosomal cathepsin B, H, and D were purified to homogeneity essentially as described by Towatari et al. (24), Kirschke et al. (9), and Yamamoto et al. (26), respectively. Rat kidney lysosomal cathepsin L was purified as described by Bando et al. (2). Antisera against rat cathepsins were prepared as described previously (16). SDS-PAGE in 15% slab gel was carried out as described (10). After electrophoresis, proteins were transferred electrophoretically to nitrocellulose paper as described (14).

**RESULTS**

*Intracellular processing of lysosomal cathepsins B, H, and L*

The purified forms of lysosomal cathepsins B, H, and L have been shown to contain a mixture of single-chain forms of cathepsins B of 29 kDa, H of 28 kDa, and L of 30 kDa and their processed two-chain forms of cathepsins B of 25 and 4 kDa, H of 22 and 6 kDa, and L of 23 and 7 kDa, respectively (3). We have recently demonstrated
Intracellular Processing and Activation of Lysosomal Cathepsins

from the *in vitro* pulse-chase kinetic analysis in the primary cultures of rat hepatocytes that lysosomal cathepsins B, H, and L are initially synthesized as an N-glycosylated proform enzymes of 39, 41, and 39 kDa, respectively (16, 17). Therefore, these proform enzymes appear to undergo multiple proteolytic processing steps post-translationally, generating processed mature forms. Recent elucidation of the determined primary structure of rat cathepsins B, H, and L predicted from the nucleotide sequences of isolated cDNA clones has confirmed these intracellular processing events.

Fig. 1. Immunoblotting analysis of microsomal procathepsin L. Rat liver microsomal fraction solubilized with 0.26% DOC was subjected to the Con A-Sepharose column which had been equilibrated with 50 mM Hepes-NaOH buffer, pH 7.5. The column was washed with the same buffer extensively. Procathepsin L was eluted with 0.5 M α-methylglucoside solution. Approximately 200 μg of protein in each fraction was subjected to 15% SDS-PAGE followed by immunoblotting with specific IgG. The molecular masses of the different polypeptides are indicated at the left (K=1000).
Identification of the molecular forms of procathepsins B, H, and L derived from rat liver microsomal fraction.

Since latent proform cathepsins are expected to be present in the endoplasmic reticulum, rat liver microsomal fraction was prepared for the in vitro activation experiments. Microsomal fraction was extracted with 0.26% DOC. After the extract was precipitated with ammonium sulfate, it was applied to a Con A-Sepharose column. Procathepsins were eluted with 0.5 M α-methylmannoside in the 50 mM Hepes-NaOH (pH 7.5) buffer. When the eluted fraction containing procathepsin L was analyzed by immunoblotting using anti-rat kidney cathepsin L antibody after SDS-PAGE, procathepsin polypeptide was seen as a single band of 39 kDa (Fig. 1). Proforms of cathepsins B and H were also seen as a single band of 39 kDa and 41 kDa, respectively (14, 15). These fractions were used as the procathepsins fractions.

Activation of procathepsins B, H, and L

The microsomal fraction eluted from the Con A-Sepharose column with 0.5 M α-methylmannoside did not show appreciable proteolytic activities of cathepsins B, H,

![Graph](image-url)

**Fig. 2.** Latency in the protease activities of cathepsins B and L. A sample of the microsomal fraction eluted from the Con A-Sepharose column was incubated in 0.1 M sodium formate buffer, pH 3.0. The protease activities of cathepsins B (●) and L (○) were measured. The reaction was allowed to take place at 20°C for various periods and the amounts of the fluorescent product were measured. Values for the determined activity were mean values of at least three separate determinations.
and L. When the procathepsins B and L fraction was incubated with acidic buffer in vitro, a marked increase of protease activity was observed: the optimum pH for the increase was around pH 3.0. As shown in Fig. 2, the activity of cathepsin L increased to 210-fold of the control after 36 hr incubation, and that of cathepsin B to 60-fold of the control (14). The increase in cathepsin H activity was also found previously when the fraction was incubated under acidic conditions (15).

**Characterization of activation process of procathepsins**

The molecular changes of procathepsins B and L during the incubation at pH 3.0 were examined by immunoblotting analysis (Fig. 3). The results clearly indicated that the 39-kDa procathepsin B was gradually converted to the molecular form similar in size to the 29-kDa mature cathepsin B. It has been reported that lysosomal cathepsin B bears an almost deglycosylated form with a unique tetraoligosaccharide structure (23). Actually the immunoblotting experiment showed that the processed cathepsin B revealed a molecular weight slightly higher than the 20-kDa mature enzyme, as would be expected (Fig. 3A, lanes 3–6), because microsomal procathepsin B might not undergo any further deglycosylation process during the incubation at pH 3.0. It is

![Fig. 3. Immunoblotting analysis for the processing of microsomal procathepsins B and L. The microsomal fraction obtained from the Con A-Sepharose column was incubated with 0.1 M sodium formate buffer, pH 3.0, at 20°C as described in Fig. 2. Aliquots of the reaction mixture at different time points were withdrawn for analysis of immunoreactive molecular forms of cathepsins B (Panel A) and L (Panel B) by 15% SDS-PAGE followed by immunoblotting using respective specific IgG. Samples in Panel A were the lysosomal fraction (lane 1); lanes 2–6 were the microsomal fraction incubated for 0 hr (lane 2), for 12 hr (lane 3), for 24 hr (lane 4), for 36 hr (lane 5), and for 48 hr (lane 6). Samples in Panel B were the lysosomal fraction (lane 1); lanes 2–5 were the microsomal fraction incubated for 0 hr (lane 2), for 12 hr (lane 3), for 24 hr (lane 4), for 36 hr (lane 5). The positions of the three cellular forms of the enzymes are indicated.](image-url)
notable that a single-chain form of cathepsin B, 29-kDa, was not converted to a two-chain form, 25-kDa, even after prolonged incubation, suggesting another processing protease for the cleavage of the 29-kDa form.

Procathepsin L, 39-kDa form, was similarly converted to a 30-kDa form corresponding to the single-chain form of cathepsin L, and completely disappeared after 36 hr incubation (Fig. 3B, lanes 2-5). Procathepsin H was also found to be processed to the mature enzyme by the acidic incubation previously (15).

Fig. 4. Effects of inhibitors on the processing of microsomal procathepsin L to mature enzyme. Samples (200 µg) of microsomal fraction eluted from the Con A-Sepharose column were incubated with 0.1 M sodium formate buffer, pH 3.0, for 36 hr at 20°C in the absence or presence of an inhibitor. Samples were then taken for the analysis of the immunoreactive molecular forms of cathepsin B on 15% SDS-PAGE followed by immunoblotting using specific IgG.
Identification of a propeptide-processing proteinase involved in activation step of procathepsins

Since some propeptide-processing proteinase for procathepsins B and L seemed to exist in the microsomal fraction eluted from the Con A column, several proteinase inhibitors were examined to identify the processing proteinase in the fraction. When the procathepsins B and L fraction was incubated with 0.1 M sodium formate buffer, pH 3.0, for 36 hr at 20°C in the presence of an inhibitor, very little effect on the enzyme activation was seen with phenylmethansulfonyl fluoride, trypsin inhibitor and aprotinin, however, pepstatin was found to be a potent inhibitor on the activation of procathepsins (Table 1). The enzyme activation was completely blocked in the presence of 5 μM pepstatin. As was expected, the conversion of procathepsins to the corresponding mature enzymes was also completely inhibited by the presence of

![Graph of protease activity](image)

**FIG. 5.** Latency in the protease activity of cathepsin B. A sample of the microsomal procathepsin B (15 μg protein in 100 μl) purified by the immunoaffinity chromatography was incubated with the tritosomal contents (300 μg protein in 100 μl) isolated from rat liver in 0.1 M sodium formate buffer, pH 3.0, at 30°C in the absence or presence of 0.1 mM pepstatin. Aliquots of the reaction mixture at different time points were withdrawn or the analysis of protease activity. The protease activity was measured during activation with Z-Phe-Arg-MCA as described under "Materials and Methods". Values for the determined activity were mean values of at least three separate determinations.
pepstatin (14, 15). Fig. 4 shows the effect of inhibitors on the processing of microsomal procathepsin L to the mature enzyme. Other inhibitors tested such as leupeptin, antipain, phenylmethansulfonyl fluoride, did not show an inhibitory effect on the conversion of the procathepsins B and L (Fig. 4). Since pepstatin is a potent inhibitor of aspartic proteinase, we expected that possible propeptide-processing proteinase might be cathepsin D, a major lysosomal aspartic proteinase.

Fig. 6. Immunoblotting analysis for the processing of microsomal procathepsin B. The purified microsomal procathepsin B was incubated with the tritosomal contents isolated from rat liver in 0.1 M sodium formate buffer, pH 3.0 for 1 hr at 30°C in the absence or presence of an inhibitor. Samples were then taken for the analysis of the immunoreactive molecular forms of cathepsin B on 15% SDS-PAGE followed by immunoblotting using specific IgG. The samples were the lysosomal cathepsin B; and the microsomal procathepsin B, incubated without inhibitor, incubated with 0.1 mM pepstatin.
FIG. 7. Activation of procathepsin B by the treatment with lysosomal cathepsin D. A sample of the purified microsomal procathepsin B (15 µg of protein in 100 µl) was incubated with or without cathepsin D (10 µg of protein in 100 µl) isolated from rat liver lysosomal fraction in 0.1 M sodium formate buffer, pH 3.0, at 30°C for various periods. The protease activity was measured during the activation with Z-Phe-Arg-MCA as in Fig. 2.

Table 1. Effect of inhibitors on the activation of microsomal latent cysteine proteinase activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Activity%</th>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>5 µM</td>
<td>2</td>
</tr>
<tr>
<td>Phenylmethansulfonyl fluoride</td>
<td>1 mM</td>
<td>86</td>
</tr>
<tr>
<td>Trypsin inhibitor (soy bean)</td>
<td>1 mg/ml</td>
<td>90</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>3.8 mTIUb</td>
<td>88</td>
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Note. A sample of the microsomal fraction eluted from the Con A-Sepharose column was incubated with 0.1 M sodium formate buffer, pH 3.0, for 36 hr at 20°C in the presence of an inhibitor. The proteinase activity was measured during activation with Z-Phe-Arg-MCA as described in Materials and Methods.

a% activity is the per cent of activity in the presence of the inhibitor compared to controls.
b Trypsin-inhibiting units.
To further investigate an activation mechanism for the latent precursor of microsomal cathepsins, the *in vitro* activation experiments have been carried out with the isolated procathepsin B. Procathepsin B was further purified by the Sepharose-Gly-Phe-Gly-Sc column (20) and immunoaffinity chromatography using anti-cathepsin B IgG-Sepharose. When the purified procathepsin B was analyzed by SDS-PAGE, the procathepsin B was seen as a single band of 39 kDa (data not shown). The purified procathepsin B did not show appreciable proteolytic activity toward Z-Arg-Arg-MCA or Z-Phe-Arg-MCA at pH 6.0. However, the treatment of procathepsin B with tritosomal contents at pH 3.0 *in vitro* caused the development of a gradual increase of proteolytic activity. As shown in Fig. 5, the activity of cathepsin B increased to 7-fold that of control after 1 hr incubation. This enzyme activation was completely blocked in the presence of pepstatin (Fig. 5). When the molecular change of procathepsin B during the incubation at pH 3.0 was examined by immunoblotting analysis, the incubation of the procathepsin B with the tritosomal contents for 1 hr caused the disappearance of the 39-kDa polypeptide, concomitantly producing a single-chain form of 25-kDa (Fig. 6). This conversion process was also inhibited in the presence of pepstatin, indicating that the processing proteinase responsible for the activation of procathepsin B would be an aspartic proteinase. Further *in vitro* activation study revealed that the latent procathepsin B was activated when the proenzyme was incubated with the lysosomal aspartic proteinase, cathepsin D, and that the cathepsin B activity increased to 5-fold of the control after 1 hr incubation (Fig. 7). These results suggest that the enzymatically active cathepsins B, H, and L are generated from inactive procathepsins by cathepsin D during the intracellular sorting pathway.

**DISCUSSION**

In an attempt to understand the intracellular processing and activation mechanisms of lysosomal cysteine proteinases of cathepsins B, H, and L, the *in vitro* activation experiments were undertaken employing the rat liver microsomal content fraction.

We have identified a latent form of procathepsins in rat liver microsomal lumen by immunoblot analysis (Fig. 1). The microsomal fraction containing procathepsins
showed no appreciable proteolytic activity, but the in vitro incubation of this fraction under acidic conditions caused a gradual increase in proteolytic activity (Fig. 2). The proteolytic conversion of procathepsin to the mature enzyme was also observed (Fig. 3). Therefore, it seems clear that the activation of procathepsins B, H, and L may be accomplished by limited proteolysis that removes the prosegment extension (14, 15). Since we found clear evidence that the proteolytic conversion of the procathepsins are strongly inhibited by the presence of pepstatin, our data from the in vitro experiments further suggest that aspartic proteinase is involved in the proteolytic processing and activation of procathepsins possibly in the lysosomes (Table 1 and Fig. 4). Pepstatin has also been demonstrated to inhibit the intracellular proteolytic processing event of procathepsins L as revealed by the pulse-chase experiments in vivo in the primary cultures of rat hepatocytes (18). Since pepstatin is a potent inhibitor of aspartic proteinase, we expected that the possible propeptide-processing proteinase might be cathepsin D, a major lysosomal aspartic proteinase. We further carried out the in vitro activation experiments for the purified microsomal procathepsin B. The latent procathepsin B could be activated by treatment with the tritosomal contents or with the lysosomal cathepsin D under the acidic conditions (Figs. 5–7). This rapid activation was found to be closely connected with the conversion of the proform enzyme to the mature enzyme as revealed by the immunoblotting analysis. The present study confirms that cathepsin D is the possible propeptide-processing proteinase which functions to activate other lysosomal cathepsins B, H, and L. We have previously demonstrated the evidence that procathepsin D present in the microsomal fraction was gradually activated by the in vitro incubation at pH 3.0 and that the proteolytic conversion of procathepsin D to the mature form was concomitantly observed (13), suggesting that procathepsin D is inactive in the endoplasmic reticulum and may be converted to the active form by autoprotolytic processing mechanism at an acidic compartment. Taken together, we postulate that lysosomal cathepsin D plays an important role for the intracellular processing and activation of lysosomal procathepsins B, H, and L as shown in Fig. 8.

REFERENCES


