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IDENTIFICATION OF A PRECURSOR FORM OF CATHEPSIN D IN MICROSOMAL LUMEN: CHARACTERIZATION OF ENZYMATIC ACTIVATION AND PROTEOLYTIC PROCESSING IN VITRO

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SUMMARY: A precursor form of cathepsin D with 45 kDa was demonstrated in the rat liver microsomal lumen by immmunoblotting analysis. The microsomal fraction containing procathepsin D which passed through a pepstatin-Sepharose resin showed no appreciable activity of cathepsin D. The *in vitro* incubation of this fraction at pH 3.0 resulted in a gradual increase of proteolytic activity toward hemoglobin as substrate and also, the proteolytic conversion of procathepsin D to the mature form was concomitantly observed. The proteolytic processing step was sensitive to pepstatin. These results suggest that procathepsin D is inactive in the endoplasmic reticulum and may be converted to the active forms by autoproteolytic processing mechanism at acidic pH during biosynthesis.

Cathepsin D is a major lysosomal aspartyl protease with an acidic pH optimum (1). Depending on the species, cathepsin D exists as a single-chain enzyme with 44 kDa or as its processed two-chain enzyme with 30 kDa and 15 kDa in the purified preparations (1,2).

Cathepsin D may be the most extensively studied lysosomal protease for its biogenesis among many lysosomal enzymes. Cathepsin D is synthesized on the membrane-bound polysomes of endoplasmic reticulum as N-glycosylated prepropeptide which is

ABBREVIATIONS: Con A, concanavalin A; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

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larger than the mature enzyme (3-5). During the biosynthesis and the following intracellular translocation to the lysosomes, cathepsin D undergoes not only a cleavage of NH_2-terminal prepeptide co-translationally but also a proteolytic processing of propeptide post-translationally (3-6). Since the partial amino-terminal sequence analysis of the procathepsin D suggested a striking homology to the propeptide of pepsinogen, it was hypothesized that the propeptide of procathepsin D may act as an activation peptide to keep the enzyme inactive until it is delivered to the lysosomes (4), and that procathepsin D may be activated by a similar mechanism to that involved in the activation of pepsinogen (7). Indeed, a precursor form of cathepsin D isolated from the media of fibroblast cells by immunoprecipitation was demonstrated to undergo autocatalytic activation at acidic pH (8).

In this paper, we report evidence showing that a precursor form of cathepsin D is present in the rat liver microsomal lumen as determined by immunoblotting analysis. Our data suggest that procathepsin D is inactive in the microsomal lumen and subsequently undergoes autoproteolytic propeptide-processing step at acidic pH which is accompanied with the activation of enzyme.

MATERIALS AND METHODS

Materials. Con A-Sepharose 4B, AH-Sepharose 4B, and proteins used as molecular weight standards were purchased from Pharmacia Fine Chemicals. Pepstatin and leupeptin were purchased from Peptide Research Foundation, Osaka, Japan. Trypsin inhibitor (soy bean), phenylmethansulfonyl fluoride, aprotinin, bovine hemoglobin, and bovine serum albumin were from Sigma Chemical Co. Acrylamide, N,N'-methylene bisacrylamide, SDS, Triton X-100, 4-chloro-1-naphthol, Hepes and α-methylmannoside were obtained from Nakarai Chemical Co. Horse radish peroxidase labeled goat anti-rabbit Fab was obtained from Medical Biological Laboratory, Japan.

Enzyme assay and purification. Cathepsin D activity was determined by a modification of the method of Anson (9). To the reaction mixture, containing 0.5 ml of 0.1 M sodium acetate buffer, pH 3.8, and 1.0 ml of buffered hemoglobin solution (2.5 %

w/v, in 0.1 M sodium acetate buffer, pH 3.8), 5-100 µl of enzyme solution were added, and the final volume was adjusted to 2.0 ml with distilled water. After 40 min incubation at 37°C, the reaction was stopped by the addition of 2.0 ml of ice-cold 5 % TCA solution. After 10 min at 0°C, the sample was centrifuged; the peptides liberated in the supernatant (1.0 ml) were measured by the Folin-Lowry reaction (10). The enzyme activity was determined with reference to a tyrosine standard curve. Units were expressed as micrograms of tyrosine solubilized per minute (11). Rat liver lysosomal cathepsin D was purified to homogeneity essentially as described by Yamamoto et al. (11). Pepstatin was coupled to AH-Sepharose 4B by the method of Murakami and Inagami (12).

Preparation of antisera. Male Wistar rats weighing 200-250 g were used. Antisera against rat liver lysosomal cathepsin D was prepared as described previously (5). Antibody was purified by immunoaffinity chromatography using antigen-Sepharose 4B.

SDS-PAGE and immunoblotting analysis. SDS-PAGE in 10 % slab gels was carried out as described (13). After electrophoresis, proteins were transferred electrophoretically to nitrocellulose paper as described by Towbin et al. (14). For immune staining, the nitrocellulose strip was incubated with 50 µg/ml of a rabbit monospecific IgG against lysosomal cathepsin D for 60 min. The strip was then soaked in horse radish peroxidase labeled goat anti-rabbit Fab for 60 min. The peroxidase reaction was initiated by incubating the strip in 0.4 mg/ml 4-chloro-1-naphthol, 0.01 % hydrogen peroxides in 50 mM Tris-Cl, pH 7.0.

RESULTS

Identification of a precursor form of cathepsin D in rat liver microsomal fraction. Rat liver microsomal fraction was solubilized with 0.26 % sodium deoxycholate (15). After the extract was precipitated with ammonium sulfate, it was applied to a Con A-Sepharose column which had been equilibrated with 50 mM Hepes buffer, pH 7.5. The microsomal fraction adsorbed to the Con A-Sepharose column was eluted with 0.5 M α-methylmannoside in the same buffer. The complete amino acid sequence of human preprocathepsin D predicted from the isolated cDNA sequence has shown that two possible N-glycosylation sites were found within the molecule (16). In fact, the isolated single-chain form of cathepsin D was demonstrated to be N-glycosylated with two high-mannose type oligosaccharide chains (17,18). Therefore, the procathepsin D in the microsomal fraction was expected to be

ratained on the Con A-Sepharose column. Thus, the fractions eluted from the Con A column was immediately applied to a pepstatin-Sepharose resin which had been equilibrated with 1 M NaCl/0.5 M sodium acetate buffer(pH 5.0) and the flow-through fractions from the column were pooled, concentrated and used as the microsomal procathepsin D fraction. The procathepsin D fraction showed quite low specific activity with approximately 0.25 units/mg toward hemoglobin. When the procathepsin D fraction was analyzed by immunoblotting using anti-rat liver cathepsin D antibody after SDS-PAGE, procathepsin D certainly revealed as a single band with a molecular weight of 45 kDa, but was heavily contaminated with the lysosomal cathepsin D with 43 kDa (Fig. 1, lane 2). Hence, procathepsin D was not effectively separated from the lysosomal cathepsin D by the pepstatin-Sepharose chromatography.

Activation of procathepsin D in vitro. The effect of pH on the proteolytic activity of procathepsin D was determined by preincubating the procathepsin D fraction in various buffers at 20^{0}C. The appearance of the hemoglobin-hydrolyzing activity became apparent and was time-dependent (Fig. 2). The optimum pH for the increase of proteolytivc activity was observed at pH 3.0. The increase of enzymatic activity was not found when the fraction was incubated at pH 5.0 or 6.0 (Fig. 2). The proteolytic activity of cathepsin D increased to 12-fold of the control after 48 h incubation. This marked increase of proteolytic activity under acidic conditions of pH 3.0 was not inhibited by the presence of phenylmethansulphonyl fluoride, aprotinin, EDTA, or leupeptin.

Immunoblotting analysis for the molecular change of procathepsin D during activation. The molecular change of procathepsin D during incubation at pH 3.0 was investigated by

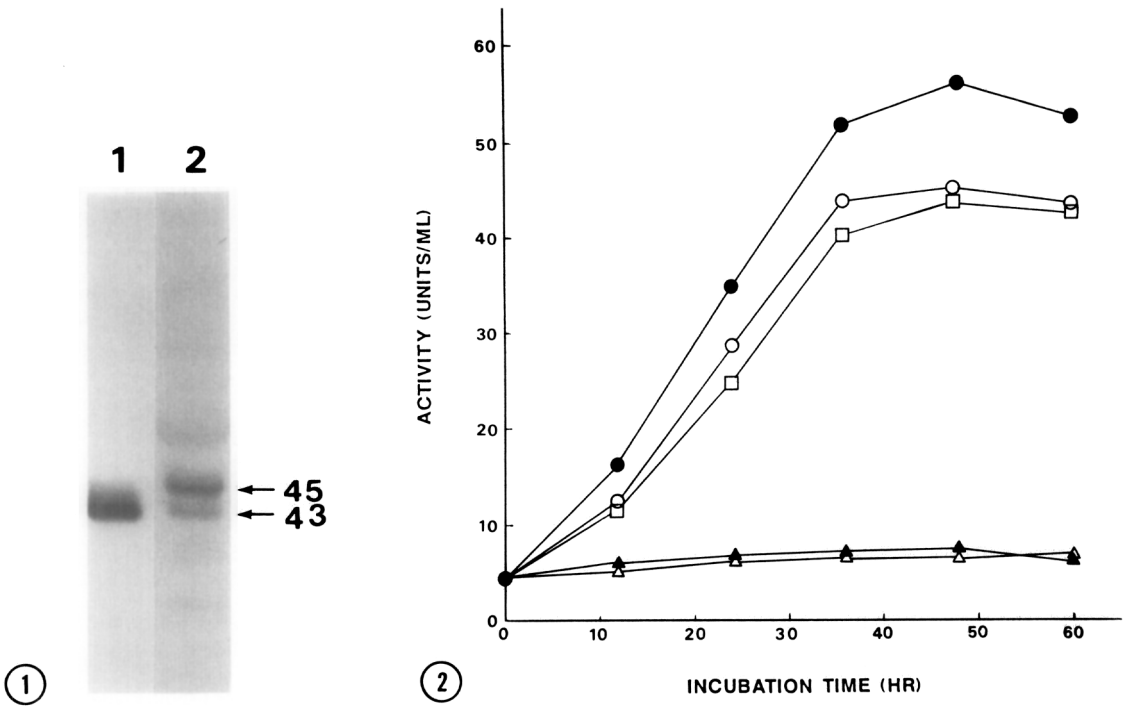

Fig. 1. Immunoblotting analysis of microsomal procathepsin D. Rat liver microsomal fraction solubilized with 0.26 % sodium deoxycholate was subjected to a Con A-Sepharose column which had been equilibrated with 50 mM Hepes buffer, pH 7.5. The fraction adsorbed to the Con A-Sepharose column was eluted with α-methylmannoside in the same buffer and the eluted fractions were subjected to a pepstatin-Sepharose column which had been equilibrated with 1M NaCl/0.5 M sodium acetate buffer, pH 5.0. The flow-through fractions from the column were pooled and used as the microsomal procathepsin D fraction. Approximately 200 μg of protein in the fraction was subjected to SDS-PAGE followed by immunoblotting with specific IgG. Lane 1, the lysosomal fraction; lane 2, the microsomal fraction. The polypeptides are indicated by their apparent molecular weights (in kilodaltons). A two-chain form of enzyme with 30 kDa and 15 kDa was not discernible on the immunoblot.

Fig. 2. Latency in the protease activity of cathepsin D. The microsomal procathepsin D fraction was incubated in 0.1 M sodium formate buffer adjusted to various pH's with NaOH. The reaction was allowed to take place at 20°C for various periods and the protease activity of cathepsin D was measured. Values for the determined activity were mean values of at least three separate determinations. The samples were the fraction incubated at pH 3.0 (●), incubated at pH 3.5 (○), incubated at pH 4.0 (□), incubated at pH 5.0 (▲), and incubated at pH 6.0 (△).

the immunoblotting analysis (Fig. 3). The procathepsin D with 45 kDa disappeared by the incubation at pH 3.0 after 12 h and a 44 kDa form of cathepsin D emerged (Fig. 3, lane 2). This 44 kDa

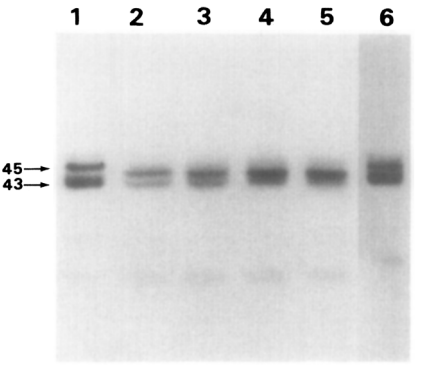

Fig. 3. Immunoblotting analysis for the processing of microsomal procathepsin D. The microsomal procathepsin D fraction was incubated with 0.1 M sodium formate buffer, pH 3.0, at 20°C. Aliquots of the reaction mixture at different time points were withdrawn for analysis of immunoreactive molecular forms of cathepsin D by SDS-PAGE followed by immunoblotting using specific IgG. Samples were the microsomal fraction incubated for 0 h (lane 1), for 12 h (lane 2), for 24 h (lane 3), for 36 h (lane 4), for 48 h (lane 5), and for 48 h in the presence of 5 μM pepstatin (lane 6). The polypeptides are indicated by their apparent molecular weights (in kilodaltons).

form was gradually converted to the molecular form similar in size to the 43 kDa mature cathepsin D after 48 h incubation (Fig. 3, lane 5). The proteolytic conversion of procathepsin D to the mature form of cathepsin D (Fig. 3) seemed to be correlated well with the appearance of proteolytic activity during the enzymatic activation at pH 3.0 (Fig. 2).

The proteolytic conversion of procathepsin D to the mature enzyme was inhibited in the presence of 5 μM pepstatin (Fig. 3, lane 6). On the other hand, no effect on the proteolytic processing was seen with phenylmethansulfonyl fluoride, aprotinin, or leupeptin (data not shown). These results, taken together, support the hypothesis that procathepsin D is latent in the microsomal fraction and the proenzyme undergoes autocatalytic activation process at acidic pH during biosynthesis (4,8,19).

DISCUSSION

It is clear from the biosynthesis study using *in vivo* pulse-chase analysis with [^{35}S]methionine in a culture system (3-6) or

in vitro translation system (3-5) that cathepsin D is initially synthesized as a N-glycosylated preproenzyme. During biosynthesis, the propeptide portion of proenzyme undergoes post-translational proteolytic processing , yielding a mature enzyme. Because the procathepsin D form would have a very short biosynthetic half-life (4,20), it seemed difficult to identify the proenzyme form in preparations isolated from whole tissues. Therefore, the procathepsin D form has not been demonstrated in the rat liver microsomal fraction .

In this report, we have demonstrated evidence that a precursor form of 45 kDa procathepsin D is present in the rat liver microsomal lumen as determined by SDS-PAGE followed by immunoblotting analysis (Fig. 1). The microsomal fraction containing procathepsin D which passed through the pepstatin-Sepharose column showed no appreciable activity of cathepsin D, but the *in vitro* incubation of this fraction with pH 3.0 at 20^{0}C developed a gradual increase of the proteolytic activity toward hemoglobin (Fig. 2). The proteolytic conversion of procathepsin D with 45 kDa to the mature form with 43 kDa was concomitantly observed at pH 3.0 (Fig. 3). This proteolytic conversion of procathepsin D was blocked by the presence of pepstatin (Fig. 3, lane 6). These results suggest that procathepsin D is latent in the microsomal fraction and subsequently undergoes autoproteolytic propeptide-processing which is accompanied with the activation of the enzyme. Since the procathepsin D form with 45 kDa was first converted to the 44 kDa form which was slightly larger than the mature enzyme by 1 kDa during the *in vitro* activation (Fig. 3, lane 2), it is not clear at present whether the same cleavage sites are processed during the *in vitro* processing mechanisms and *in vivo*. Furthermore, we can't rule out the possibility that procathepsin D is processed and

activated by a contamination in the microsomal procathepsin D fraction.

Several lines of evidence have suggested that the propeptide portion of procathepsin D may act as an activation peptide analogous to the case of pepsinogen (4). The precursor form of cathepsin D isolated by immunoprecipitation from the media of fibroblasts was shown to be inactive and undergo autocatalytic activation at acidic pH (8). Moreover, a high molecular weight catalytically inactive cathepsinogen D was isolated from bovine spleen (19); this protein was rapidly activated if incubated at a low pH for a short period. Our results reported here are consistent with those on the activation of procathepsin D reported previously (4,8,19).

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