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Procathepsin L-specific antibodies that recognize procathepsin L but not cathepsin L

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Procathepsin L was purified to apparent homogeneity from the culture medium of v-Ha-ras transformed NIH3T3 (Ras-NIH) cells in three steps; anion-exchange chromatography, gel filtration, and re-gel filtration. SDS-PAGE analyses revealed that the purified samples contained only the precursor form, procathepsin L, but not the mature enzyme, cathepsin L. Antibodies against purified procathepsin L were raised. These recognized both rat cathepsin L and the purified procathepsin L. To isolate procathepsin L-specific antibodies that did not recognize cathepsin L, sequential affinity chromatography procedures were carried out. Immunoblot analyses showed that the procathepsin L-specific antibodies recognized only procathepsin L, but not cathepsin L.

Procathepsin L; Cathepsin L; Procathepsin L-specific antibody

1. INTRODUCTION

Cathepsin L, a cysteine proteinase, is localized in lysosomes and participates in intracellular protein degradation [1]. The amino acid sequence of this enzyme has been determined at the protein level and deduced from the nucleotide sequence of its cDNA [2,3]. The amino acid sequence of cathepsin L is closely related to those of cathepsins B and H [4]; in contrast, the pro-peptide regions of those cysteine proteinases show less amino acid sequence similarity to one another [5]. A comparison of the amino acid sequence determined at the protein level and that deduced from the nucleotide sequence reveals that the cathepsin L gene is translated as preprocathepsin L, which is then processed to procathepsin L in the rough endoplasmic reticulum. Cathepsin L itself exists in lysosomes. Although these results are consistent with those obtained in pulse-chase analyses [6], the in situ location where procathepsin L is processed to cathepsin L remains unclear.

Recently, procathepsin L was revealed to be identical to a major excreted protein (MEP) from NIH3T3 cells transformed by v-Ki-ras [7], a cyclic protein-2 secreted from sertoli cells [8], and a progesterone-dependent endometrial secretory protein [9]. In addition, the expression levels of the cathepsin L gene correlate closely with the metastatic potential of v-Ha-ras-transformed C3H-10T1/2 cells in vivo [10]. Thus, procathepsin L is secreted both physiologically and pathologically. It is still unclear whether secreted procathepsin L acts as procathepsin L or cathepsin L, to which procathepsin L is processed upon re-uptake into the cells.

Antibodies against cathepsin L react with both cathepsin L and procathepsin L. Although these antibodies were able to reveal that cathepsin L is localized in lysosomes, they could not identify where the processing of procathepsin L occurs in situ. Some evidence suggests that lysosomal cysteine proteinases are processed to the mature enzymes as soon as or before fusion of prelysosomes to endosomes, but it is still not known whether procathepsin L is processed to cathepsin L continuously or during the uptake of extracellular materials.

In this paper, we describe the purification of procathepsin L and the generation of procathepsin L-specific antibodies which do not react with the mature cathepsin L. These antibodies provide useful tools for answering the above questions and were used to establish an ELISA system for measuring the levels of procathepsin L in serum and extracellular fluids.

2. MATERIALS AND METHODS

Materials used in this work were obtained as follows: Sephadex G-75 and CNBr-activated Sepharose 4B from Pharmacia, DE-52 from Whatman, calf serum from United Biotechnologicals, nylon membranes (GV type) for immunoblot from Millipore, molecular weight size markers from Bio-Rad, horseradish peroxidase- and rhodamine-

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Abbreviations: Con A, concanavalin A; CNBr, cyanogen bromide; DMEM, Dulbecco's modified Eagle's minimum essential medium; ELISA, enzyme-linked immunosorbent assay(s); PBS(−), Dulbecco's phosphate buffered saline; Ras-NIH, NIH3T3 cells transformed by v-Ha-ras; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

conjugated anti-rabbit IgG from Cappel, horseradish peroxidase detection kits for immunoblot from Konika, membrane filters (YM-10) for ultrafiltration from Amicon, all others from Nakarai.

Cathepsin L was purified from rat liver, and antibodies against rat cathepsin L were raised according to Bando et al. [11]. Both NIH3T3 and Ras-NIH cells, a generous gift from Dr. Michael Botchan [12], were cultured in DMEM supplemented with 5% bovine calf serum under a humidified 5% CO_2 atmosphere at 37°C. Ras-NIH cells were grown under subconfluent conditions, washed three times with PBS(−), and maintained in DMEM for 24 h. The medium was then concentrated by ultrafiltration using a membrane filter (YM-10) and dialyzed against 10 mM Tris-HCl (pH 7.5). This was then used as the starting material for the purification of procathepsin L.

All procedures in the purification of procathepsin L were carried out at 4°C. The samples were subjected to anion-exchange chromatography on a DE-52 column equilibrated with 10 mM Tris-HCl (pH 7.5). The column was first washed with 30 column bed vols. of the equilibration buffer, and then fractions containing procathepsin L were eluted with 30 column vols. of 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl. After concentration with a membrane filter (YM-10), the samples were applied to a Sephadex G-75 gel filtration column equilibrated and eluted with 10 mM Tris-HCl (pH 7.5), 150 mM NaCl at a flow rate of 6 ml/h. Aliquots from each fraction were subjected to SDS-PAGE and immunoblot analyses with anti-rat cathepsin L antibodies to identify the fractions containing procathepsin L [13,14]. Positive fractions were concentrated by ultrafiltration using the same membrane filter (YM-10), and the samples were again subjected to gel filtration on Sephadex G-75 under the same conditions as the first gel filtration.

Antibodies against purified procathepsin L were raised in rabbits by injecting the purified protein by emulsifying with an equal vol. of Freund's complete adjuvant three times intracutaneously at 1 week intervals. Bleedings were carried out at 1 week intervals beginning 10 days after the final immunization. The crude antibody fraction was obtained by two cycles of ammonium sulfate precipitation (40% and 35% saturation), dialyzed against 50 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, and applied to cathepsin L–Sepharose prepared by coupling purified rat cathepsin L to CNBr-activated Sepharose 4B in accordance with the manufacturer's protocol. The column was washed with 30 column vols. of 50 mM sodium phosphate buffer (pH 7.0), containing 0.5 M NaCl, and the antibodies bound to cathepsin L–Sepharose were eluted with 3 mM HCl. Immediately, the fractions were neutralized with 2 vols. of 0.5 M sodium phosphate buffer (pH 7.0). Immunoaffinity chromatography was repeated until antibodies against cathepsin L were no longer eluted. After the pass-through fraction adsorbed to the cathepsin L–Sepharose five more times, the remainder was applied to procathepsin L–Sepharose. Antibodies bound to this column were obtained as mentioned above.

Purified rat cathepsin L and mouse procathepsin L were electrophoresed in 10% SDS-gels and followed by immunoblot analyses using the antibodies purified with cathepsin L–Sepharose, or with procathepsin L–Sepharose after cathepsin L–Sepharose treatment. Antibodies bound to cathepsin L or procathepsin L were detected by horseradish peroxidase-conjugated anti-rabbit IgG and visualized using Konica Immunostaining kits.

For fluorescence immunocytochemistry, NIH3T3 cells were fixed with 3% formaldehyde, permeabilized with PBS(−) containing 0.5% Triton X-100, and incubated with either anti-cathepsin L antibodies or anti-procathepsin L-specific antibodies. After incubation with rhodamine-conjugated anti-rabbit IgG, samples were examined using Zeiss Axiophot with a No. 15 filter.

3. RESULTS AND DISCUSSION

3.1. *Purification of procathepsin L*

Cathepsin L has been purified from various tissues and cells, including liver, kidney, brain, primary cultured sertoli cells, and flushings of the uterus [1,2,8,9]. Although the ratio of cathepsin L to procathepsin L varied, these starting materials all contained both cathepsin L and procathepsin L. To purify only procathepsin L, we chose culture medium without serum of Ras-NIH cells as the starting material. Ras-NIH cells secrete large amounts of procathepsin L, but no cathepsin L, and can be maintained in serum-free medium for more than 24 h.

Since procathepsin L is autocatalytically processed to cathepsin L under acidic conditions [15], all procedures in the purification of procathepsin L should be carried out above pH 7.0. The purification procedure we utilized in this study consists of only three steps: anion-exchange chromatography (DE-52), gel filtration (Sephadex G-75) and re-gel filtration (Sephadex G-75). Anion-exchange chromatography effectively removes procathepsin L from other proteins. A representative gel filtration profile from the first Sephadex G-75 column is shown in Fig. 1. The fractions containing procathepsin L (fraction numbers 44–48) were identified by SDS-PAGE and followed by immunoblot analyses with anti-rat cathepsin L antibodies (data not shown). The fractions were combined, concentrated on a membrane filter (YM-10), and subjected to the second gel filtration step. The fractions containing procathepsin L were combined and concentrated by ultrafiltration on a membrane filter (YM-10). Fig. 2 shows that procathepsin L was purified to apparent homogeneity. From the starting material in 30 dishes (10 cm diameter), 200 μg of procathepsin L was isolated. Con A Sepharose, which has been used many times in previous studies, could not be utilized in this study for the following reasons. First, the Con A that co-elutes from Con A–Sepharose could not be separated from procathepsin L under neutral conditions by various chromatographic procedures, including anion-exchange chromatography and gel filtration. Second, the molecular weight of Con A is 29 kDa, close to that of cathepsin L; thus, any autoprocessed cathepsin L present could not be distinguished from Con A. Third, if purified procathepsin L contaminated with even a small amount of Con A was used to immunize rabbits, antibodies against Con A would probably be raised in larger amounts than those against procathepsin L.

3.2. *Generation of procathepsin L-specific antibodies*

Initially, we tried to raise procathepsin L antibodies by immunizing rabbits with synthetic peptides prepared according to the amino acid sequence of the pro-peptide region deduced from its nucleotide sequence. This strategy was unsuccessful, probably because of the poor antigenic properties of the peptides. Therefore, we attempted to purify procathepsin L away from cathepsin L and use it to raise antibodies recognizing procathepsin L but not cathepsin L.

Immunoblot analyses carried out with affinity puri-

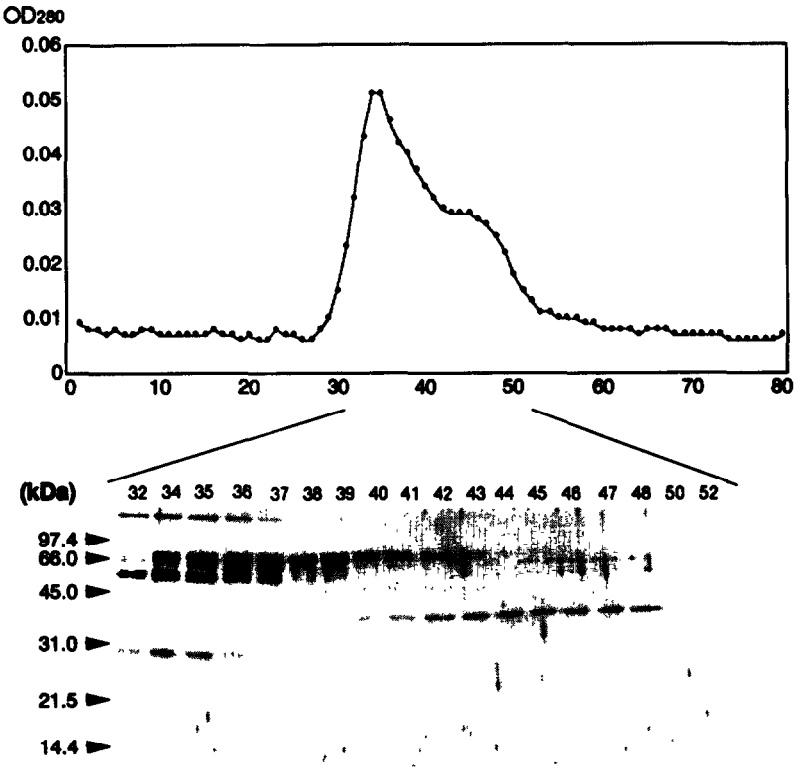

Fig. 1. Gel filtration profiles and SDS-PAGE analyses of procathepsin L purification. The samples eluted from anion-exchange chromatography (DE-52) with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and concentrated by ultrafiltration were subjected to gel filtration on Sephadex G-75. The chromatographic profile is shown at the top with the protein concentration measured at 280 nm plotted on the ordinate vs. fraction number on the abscissa. Aliquots of each fraction were subjected to SDS-PAGE in 12.5% gels. The lower panel shows the SDS-PAGE gel visualized by silver staining. The numbers above the gel indicate the fraction number from the gel filtration column. Arrows to the left of the gel indicate molecular weight markers: phosphorylase B, 97.4 kDa; bovine serum albumin, 66.0 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

fied anti-cathepsin L antibodies and anti-procathepsin L-specific antibodies are shown in Fig. 3. Antibodies purified with cathepsin L–Sepharose recognized both rat cathepsin L and the purified procathepsin L (Fig. 3A and B). In contrast, the procathepsin L-specific antibodies purified with procathepsin L–Sepharose from the flow-through fraction from cathepsin L–Sepharose recognized only the purified procathepsin L, not rat cathepsin L (Fig. 3C and D). Since the amino acid sequence of rat cathepsin L shares 97.7% identity to that of mouse cathepsin L [2,3], we used rat cathepsin L rather than mouse cathepsin L in this study. The procathepsin L-specific antibodies recognized the non-denatured procathepsin L because they bound to procathepsin L–Sepharose. In addition, they also recognized denatured procathepsin L, as is apparent from their use in the immunoblot analyses (Fig. 3C and D) and fluorescence immunocytochemistry (data not shown). Thus, the procathepsin L-specific antibodies probably recognize both the amino acid sequence of the propeptide region and the three-dimensional structure of procathepsin L, although their epitopes have not been identified.

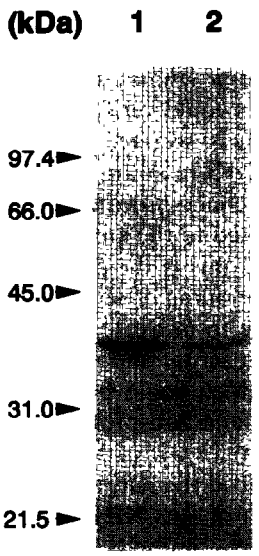

Fig. 2. SDS-PAGE analyses of the purified procathepsin L. Either 1 μg (lane 1) or 0.2 μg (lane 2) of the purified procathepsin L was analyzed by 10.0% SDS-PAGE and the gels were stained with Coomassie brilliant blue R-250. Arrowheads on the left indicate the molecular weight markers described in the legend to Fig. 1. Purified procathepsin L migrated as 35 kDa.

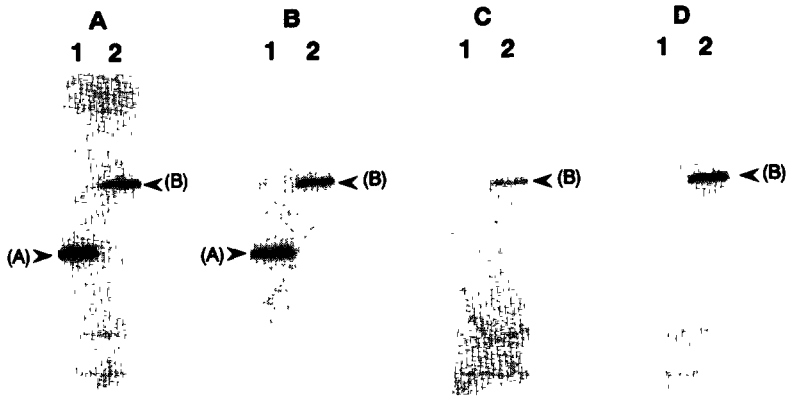

Fig. 3. Immunoblot analyses with antibodies purified with cathepsin L–Sepharose or procathepsin L-specific antibodies. Rat cathepsin L (lanes 1) and purified procathepsin L (lanes 2) were subjected to 10% SDS-PAGE and the proteins were transferred to the nylon membranes. The membranes were blocked with 5% non-fat dried milk in TBS (w/v), and immersed with 0.5 μg/ml (A) or 2.5 μg/ml (B) of the antibodies purified with cathepsin L–Sepharose or with 0.5 μg/ml (C) or 2.5 μg/ml (D) of the procathepsin L-specific antibodies (see text for details). Bound antibodies were detected with horseradish peroxidase-conjugated anti-rabbit IgG, and visualized using Konica immunostaining kits. Arrowheads (A) and (B) indicate cathepsin L and the purified procathepsin L, respectively.

Primary structure and pulse-chase studies have revealed that cathepsin L is translateabd as preprocathepsin L and processed into cathepsin L via procathepsin L [2–4]. However, the in situ location where procathepsin L is processed to cathepsin L has not been identified. Additionally, in vitro investigation has revealed that procathepsin L is autocatalytically processed to cathepsin L at pH's below 5.0, but there are no intracellular compartments this acidic [15]. Recently, it was found that when procathepsin L was incubated with dextran, it was processed into cathepsin L at a higher pH than when dextran was omitted [16]. If the processing of procathepsin L requires that materials be endocytosed, then the processing of the procathepsin L might occur after the fusion of the prelysosome to the endosome. Electron microscopic studies using our procathepsin L-specific antibodies could verify the above hypothesis.

Procathepsin L is secreted from endometrial cells, sertoli cells, and v-Ki-ras-transformed NIH3T3 cells [7–9]. The roles of secreted procathepsin L are unclear, but are probably related to the amount present under various conditions. If this is the case, then it is important to measure the amounts of secreted procathepsin L. It would be possible to measure the amounts of secreted procathepsin L in serum and extracellular fluids by an ELISA system which could be established using both anti-cathepsin L- and anti-procathepsin L-specific antibodies.

We have described the purification of procathepsin L from the medium of Ras-NIH cells, and isolated procathepsin L-specific antibodies from anti-sera against procathepsin L by sequential immunoaffinity purification techniques.

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