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Processing Properties of Recombinant Human Procathepsin L

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Human procathepsin L is highly expressed in mouse myeloma cells and processed into the mature enzyme under the acidic condition below pH 5.5. Different from the mature enzyme, it is stable at a neutral pH. To examine whether or not procathepsin L is autoprocessed intramolecularly, we constructed a mutant procathepsin L cDNA in which the codon for Cys^{138} proposed as the active site was mutated to encode Ser by PCR-mutagenesis. The mutant procathepsin L (C138S) was secreted into the culture medium from mouse myeloma cells expressing this mutant cDNA, but not processed into the mature form under the acidic condition. In addition, the mutant C138S was not processed by the incubation at 37 °C with wild-type procathepsin L or mature cathepsin L under the acidic condition. These findings showed that Cys^{138} is the active site of cathepsin L and that the autocatalytic processing occurs intramolecularly.

Cathepsin L is overexpressed in malignantly transformed cells and in cells treated with growth factors or tumor promoters, and predominantly secreted as a proenzyme instead of being transported to lysosomes (1-5). This phenomenon has been ascribed to its high levels of synthesis and the low affinity of procathepsin L for mannose-6-phosphate receptors (6). Procathepsin L secreted from murine fibroblasts is processed rapidly at pH3.0 (2), or in contact with negatively charged materials such as dextran sulfate at the physiological lysosomal pH5.5 (7). The activation of procathepsin L in guinea pig sperm at pH 3.0 is inhibited by cysteine proteinase inhibitors such as leupeptin, Z-Phe-Ala-CHN_2 and $HgCl_2$, suggesting that it undergoes autoactivation (8). However, whether this autocatalytic activation is caused by intermolecular or intramolecular mechanisms has not been clarified. Here we report some processing properties of procathepsin L, the isolation of mutant procathepsin L and the mechanism of autocatalytic activation.

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The abbreviations used: Z-, benzyloxycarbonyl; -MCA, 4-methyl-7-coumarylamide; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride.

MATERIALS AND METHODS

Enzyme assays. Human procathepsin L was prepared as described previously (9). The enzymatic activity of cathepsin L was assayed using 5 μM Z-Phe-Arg-MCA (Sigma Chemicals, Co., MO, USA) as the substrate, as described by Barrett and Kirschke (10). The fluorescence of free aminomethylcoumarin was determined by excitation at 365nm and emission at 450nm using a Fluorescence Concentration Analyzer (Baxter Healthcare Co., IL, USA).

Processing of recombinant procathepsin L. Procathepsin L was incubated in the absence of thiol activator at 37°C in 100mM buffer at pH values (see below) for 5 min, 30 min and 90 min. Each mixture was then diluted with 200mM sodium acetate buffer (pH5.5) containing 2mM EDTA and the enzymatic activity was assayed as described above. The buffers used for the pH profiles were 100mM sodium formate (pH 3.0 - 4.0), 100mM sodium acetate (pH4.0 - 6.0), 100mM sodium phosphate (pH6.0 - 8.0) and 100mM Tris-HCl (pH8.0 - 8.5).

Construction of mutant procathepsin L (C138S). PCR- mutagenesis was performed by the method described by Ito et al. (11). The *Eco*RI fragment of human cathepsin L cDNA inserted into M13tv19 (Takara Shuzo Co., LTD. Kyoto, Japan) was used as the template. The mutagenic oligonucleotide (5′-CAGTGTGGTTCTTCTTGGGCTTTTAGTGCT-3′) corresponded to the sequence encompassing the wild-type active site Cys at the amino acid position No. 138 (the residue No. 1 is the amino terminus of preprocathepsin L). The underlined nucleotide represents the mutation which resulted in the substitution of Ser for Cys at the position No. 138 in human preprocathepsin L. Mutation was confirmed by DNA sequencing. The mutated cDNA (*Eco*RI fragment) was inserted into pTBN-HCLneo as described previously (9), resulting in the mutant procathepsin L (C138S) expression plasmid pTBN-HCL(C138S)neo. To express C138S, the mouse myeloma cell line Sp2/0-Ag14 (ATCC, MD, USA) was transformed with pTBN-HCL(C138S)neo and the expression clone was selected as described previously (9).

RESULTS AND DISCUSSION

Purification of recombinant human procathepsin L. Recombinant human procathepsin L was highly puri-

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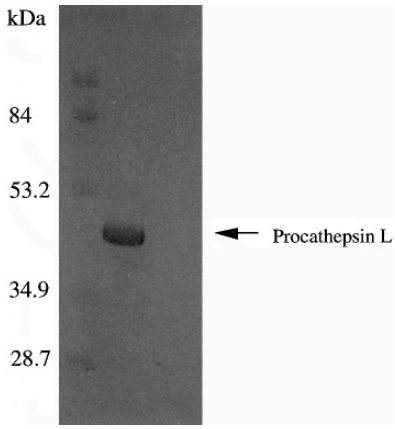

FIG. 1. SDS-polyacrylamide gel electrophoretogram of 10μg of purified recombinant human procathepsin L (CBB staining).

fied from the culture medium of Sp-HCL/HE14 as described previously (9). The purity of procathepsin L was found to be approximately 95%, judging from the densitometric analysis of SDS-polyacrylamide gel stained with Coomassie Brilliant Blue (CBB) (Fig. 1). Protein sequence analysis revealed that the N-terminal residues Nos. 1 to 19 were the same as those encoded by the cDNA sequence (12). The isoelectric point of procathepsin L was shown to be pH5.4 using a chromatofocusing Mono P HR5/20 column (Pharmacia Biotech, Uppsala, Sweden), which was nearly the same as the value calculated from the amino acid composition encoded by the cDNA sequence.

Processing properties of recombinant human procathepsin L. The pH dependent processing of the recombinant human procathepsin L is shown in Fig. 2. Procathepsin L was processed to its mature form by incubation at pH3.0 for 5 min (Fig. 2A) or below pH5.5 for 90 min (Fig. 2C). This processing was not observed above pH6.5 (Fig. 2C), although partial processing occurred at pH6.0 (Fig. 2C). On the other hand, incubation of the precursor in the culture medium above pH5.0 for 24 hours did not result in autocatalytic processing (data not shown), suggesting that this processing was inhibited by some elements in the culture medium.

After the processing at pH3.0 and 4.0 for 30 min, almost complete processing and higher specific activity was observed (Fig. 2B and 3). In contrast, although the processing was apparent after 30 min incubation at pH4.5 - 5.5 (Fig. 2B), the specific activity was low (Fig. 3). This might be due to the formation of processed enzymes having several extra amino acids at the N-terminal of the mature polypeptide. In fact, the incompletely processed procathepsin L with several amino acids added to the N-terminal of mature cathepsin L has been reported to have low proteolytic activity (13). Also, as reported by Mason and Massey (7), procathepsin L might not be activated completely without negatively charged materials such as dextran sulfate at the physiological lysosomal pH5.5.

pH-stability of procathepsin L. After incubation at 37°C for 1, 6 and 24 hours at pH7.0 - 8.5, during which the processing was not observed, procathepsin L was incubated for 5 min at 37°C under the acidic conditions (pH3.0) and the enzymatic activity was measured under the standard assay conditions (Fig. 4). The preincu-

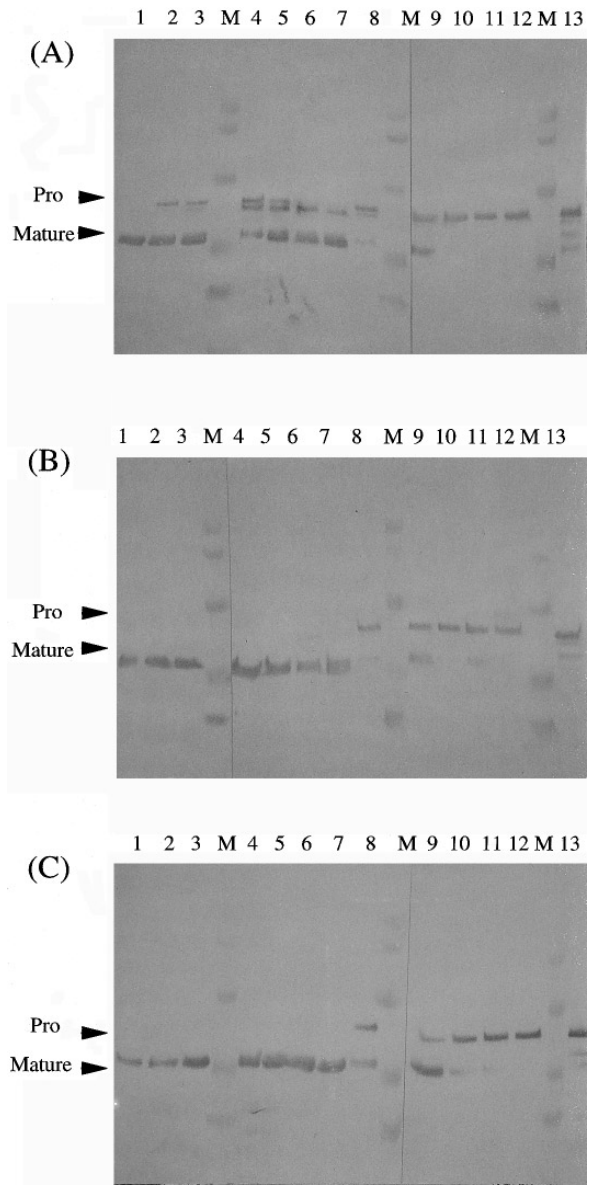

FIG. 2. Western blotting analyses for the effects of pH on the processing of procathepsin L. Human procathepsin L was incubated at 37 °C in buffer indicated below. (A) 5 min of incubation; (B) 30 min of incubation; (C) 90 min of incubation. Lane 1, pH 3.0; lane 2, pH 3.5; lane 3, pH 4.0; lane 4, pH 4.0; lane 5, pH 4.5; lane 6, pH 5.0; lane 7, pH 5.5; lane 8, pH 6.0; lane 9, pH 6.0; lane 10, pH 6.5; lane 11, pH 7.0; lane 12, pH 7.5; lane 13, unincubated precursor. Buffers used were 100mM sodium formate (pH3.0–4.0), 100mM sodium acetate (pH4.0–6.0) and 100mM sodium phosphate (pH6.0–7.5).

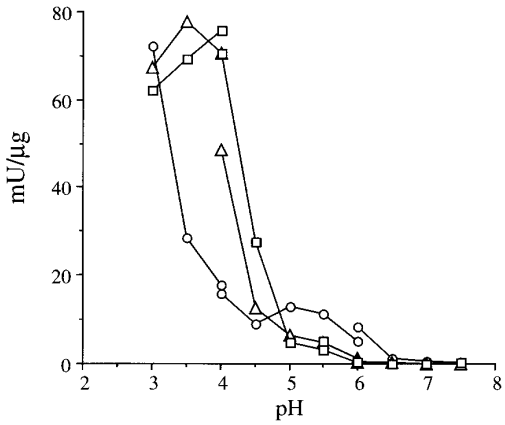

FIG. 3. Effects of pH on the processing of procathepsin L. After human procathepsin L was incubated at 37 °C for various times in 100mM buffer at the indicated pH values, the enzyme activity was measured under the standard assay conditions. Buffers used were 100mM sodium formate (pH3.0–4.0), 100mM sodium acetate (pH4.0–6.0) and 100mM sodium phosphate (pH 6.0–7.5). (○), 5 min of incubation; (△), 30 min of incubation; (□), 90 min of incubation.

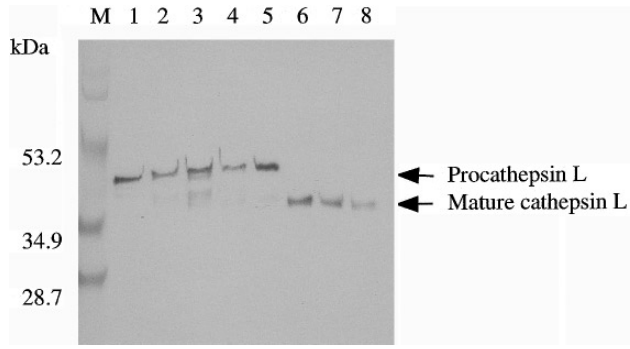

FIG. 5. Western blotting analyses for the processing of the mutant precursor. The mutant C138S (50ng) was incubated in 100mM sodium acetate buffer, pH4.0, with wild-type procathepsin L (12.5ng) or mature cathepsin L (12.5ng). Lane 1, C138S (50ng) unincubated; lane 2, C138S incubated for 30 min at 37°C; lane 3, C138S incubated with procathepsin L for 30 min at 37°C; lane 4, C138S incubated with mature cathepsin L for 30 min at 37 °C; lane 5, wild-type procathepsin L (50ng) unincubated; lane 6, wild-type procathepsin L (50ng) incubated for 30 min at 37 °C; lane 7, mature cathepsin L (50ng) unincubated; lane 8, mature cathepsin L (50ng) incubated for 30 min at 37°C.

bation of procathepsin L at pH7.0 did not effect its auto-processing activity at pH3.0, although procathepsin L lost about 70% of processing activity after the preincubation for 6 hours at a pH above 8.0. The pH stability of procathepsin L was quite different from that of the mature form which rapidly lost activity at a neutral pH (9). This suggest that the propeptide region of cathepsin L is responsible for stabilization at a neutral pH. The propeptide region of cathepsin L has also been reported to be required for proper folding, stability and exit from ER (14, 15, 16). Therefore, the propeptide region might be prerequisite to the biosynthesis and transportation to the lysozomes.

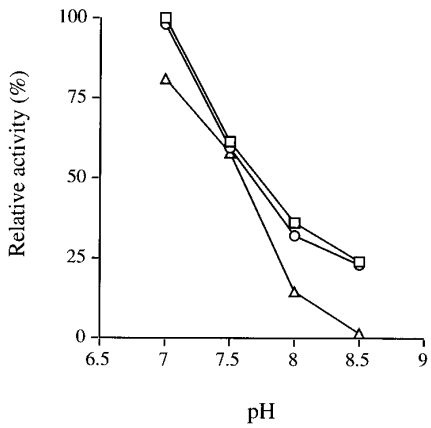

FIG. 4. pH-stability of recombinant human procathepsin L. Procathepsin L was incubated in the absence of a thiol activator at 37°C in 50mM buffer over a range of pH value indicated for 1 hr (□), 6 hr (○) and 24 hr (△). The enzyme activity was measured under the standard assay conditions. 100% of the enzyme activity was derived from the precursor in 50mM Tris-HCl (pH7.0) without preincubation. The buffer used was 50mM Tris–HCl (pH7.0–8.5).

Properties of the mutant procathepsin L C138S. Since the processing of procathepsin L is inhibited by cysteine proteinase inhibitors such as leupeptin, Z-Phe-Ala-CHN_2 and $HgCl_2$, the processing has been suggested to be autocatalytic (8, 13). However, whether the autocatalytic activation is caused by intermolecular or intramolecular mechanisms has not been clarified. To examine whether or not the auto-processing occurs intramolecularly, we have expressed the mutant procathepsin L C138S in mouse myeloma cells. The mutant C138S was purified using the same method by for wild-type procathepsin L (9). Different from the wild-type procathepsin L (Fig. 5, lane 6), the mutant C138S was not processed by the incubation for 30 min at 37°C, pH4.0 (Fig. 5, lane 2). The mutant C138S was not processed by incubation with wild type procathepsin L and mature cathepsin L at pH4.0 (Fig. 5, lanes 3 and 4). These findings suggest that procathepsin L is intramolecularly processed.

The autocleavage scissile bond of procathepsin L has been suggested to be located outside of the active site cleft under the neutral pH, because the crystal structure of recombinant human mature cathepsin L is similar to that of cathepsin B (17, 18, 19). However, the propeptide of procathepsin L would be exposed to the active site and processed under the acidic conditions.

In general, misfolded and unassembled proteins are considered to be specifically and rapidly degraded in the cytosol, mitochondria, chloroplasts and nucleus (20, 21, 22). The mutant precursor C138S was not degraded by the incubation at pH5.5 with mature cathepsin L for 16 hours at 37°C (data not shown), suggesting that the mutant precursor C138S secreted into the cell cul-

ture medium is properly folded and resistant to proteolysis.

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