BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 202–207 (1998)
ARTICLE NO. RC989613

Multiple Processing of Procathepsin L to Cathepsin L *in Vivo*

Kazumi Ishidoh,*[,1] Takaomi C. Saido,† Seiichi Kawashima,† Miyoko Hirose,‡ Sumio Watanabe,‡ Nobuhiro Sato,‡ and Eiki Kominami*

**Department of Biochemistry, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan; †Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan; and ‡Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received October 2, 1998

Three amino-terminal-specific peptidic antibodies against cathepsin L were generated. These antibodies recognize *in vitro* processing products of procathepsin L in time-course-dependent fashion. Immunoblot analyses with these antibodies followed by immunoprecipitation with anti-cathepsin L antibody showed that the amino terminal amino acid sequences of intracellular cathepsin L are heterogeneous: the single chain form of cathepsin L starts with either EPLML, LKIPK or IPKSV, and the heavy chain of the two chain form with IPKSV. Percoll density gradient and fluorescence immunohistochemistry suggested that these three species of cathepsin L localize in the lysosomes where procathepsin L processing occurs.

Cathepsin L, a lysosomal cysteine proteinase, plays a major role in the degradation of endocytosed proteins as well as intracellular proteins (1). The results of studies on processing in cultured cells and of amino acid sequence at both the protein level and that deduced from the nucleotide sequence show that cathepsin L is translated as preprocathepsin L, processed to procathepsin L with a molecular mass of 39kDa, and localized in lysosomes as the mature enzyme comprising a single chain form with a molecular mass of 30kDa and a two-chain form with molecular masses of the chains of 25kDa and 5kDa (2–5). Only the two-chain form of cathepsin L has been sequenced at the protein level because purified cathepsin L converts from the single chain form to the two-chain form during storage at −80°C. Recently, cathepsins C and H, other cysteine proteinases belonging to the same papain family, were shown to have heterogeneous amino terminal amino acid sequences (6,7).

Procathepsin L, the same protein as the Major Excreted Protein secreted from both transformed and normal cells treated with growth factors, was purified from the medium of *v-Ha-ras* transformed NIH3T3 cells (8,9). Purified procathepsin L provides a useful material to analyze the mechanism of processing of procathepsin L to the mature form *in vitro* (10,11). In the presence of dextran sulfate under acidic conditions, purified procathepsin L undergoes autocatalytic processing to the mature form in at least two steps: First, a 31kDa form with a 6-amino acid amino terminal extension is generated; then, the 30kDa form with a 2-amino acid amino terminal extension is processed. Although these processing mechanisms work well in the presence of glycosaminoglycans, components of proteoglycans, instead of dextran sulfate, it is unclear that these mechanisms also apply *in vivo*. (12). Intracellular forms of mature cathepsin L may have heterogeneous amino termini similar to those of cathepsins C and H, because *in vitro* processing studies show that processed cathepsin L has heterogeneous amino termini.

Previously, we generated a procathepsin L-specific antibody to identify where procathepsin L is processed *in situ* (13). Although this antibody recognizes only procathepsin L and not mature cathepsin L, we failed to identify the site of procathepsin L processing since little procathepsin L exists in cells and tissues and it is difficult to detect the disappearance of the signal bound to the procathepsin L-specific antibody *in situ*. Thus, the site of procathepsin L processing to the mature enzyme *in vivo* remains unclear.

[1] All correspondence should be addressed to Dr. Kazumi Ishidoh. Fax: +81-3-5802-5889. E-mail: kishidoh@med.Juntendo.ac.jp.

Abbreviations used: E-64-c, N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucine-3-methylbutylamide; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemagglutinin; PAGE, polyacrylamide electrophoresis; PBS (−), Dulbecco's phosphate buffered saline; APMSF, (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride; and SDS, sodium dodecyl sulfate.

0006-291X/98 $25.00

In this study, we generated amino terminal-specific antibodies generated using synthetic peptides including EPLML, corresponding to the 31kDa *in vitro* processing form, LKIPK, corresponding to the 30kDa *in vitro* processing form, and IPKSV, corresponding to the amino terminal of purified cathepsin L, to identify where procathepsin L is converted to cathepsin L. These three antibodies recognize not only the *in vitro* processed forms of procathepsin L, but also various forms of cathepsin L in mouse tissues. Fluorescence immunohistochemistry using anti-EPLML antibody and anti-cathepsin L antibody showed that procathepsin L processing occurs in lysosomes.

MATERIALS AND METHODS

E-64-c, a cysteine proteinase inhibitor, was generously provided by Dr. K. Hanada (Taisho Pharmaceutical Co.). Pepstatin A was purchased from Peptide Institute, Protein A-agarose from Santa-Cruz, KLH from Calbiochem, the ECL western blotting detection system and Percoll from Amersham Pharmacia Biotech, and other materials from Nacarai Tesque, Wako, or Sigma.

The purification of procathepsin L from serum-free culture medium of *v-Ha-ras*-transformed NIH3T3 cells and *in vitro* processing of procathepsin L in the presence of dextran sulfate were described previously (11). SDS-PAGE and immunoblot analyses were carried out according to standard protocols (14, 15) and visualized with Konica immunostaining kits or ECL immunoblot detection kits. Three peptides, EPLMLC, LKIPKC, and IPKSVC, corresponding to the 5 amino acids from the amino terminal of the 31kDa form, the 30kDa form, and purified cathepsin L, respectively, with a carboxyl terminal cysteine extension for conjugation with KLH were synthesized in an ACT396 peptide synthesizer (Advanced ChemTec). Antipeptidic antibodies were produced as described previously (16).

A C57 mouse was sacrificed by cervical dislocation and the tissues were washed with PBS (−) and homogenized in 8 volumes of homogenization buffer comprising 0.25M sucrose, 5mM sodium phosphate buffer (pH7.2), 1mM APMSF, 1mM E-64-c, and 1mM Pepstatin A in a Dounce homogenizer with a loose-fitting pestle for liver and kidney, and in a Teflon-glass homogenizer for skin. After centrifugation at 1,700 ×g for 5 min, the supernatants were subjected to further ultracentrifugation at 100,000 ×g for 1 hour. The microsome fractions were dissolved in immunoprecipitation buffer composed of 50mM Tris-HCl (pH7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.05% SDS, 1mM APMSF, 1mM E-64-c, and 1mM Pepstatin A. One milligram samples were mixed with 100μl of Protein A-agarose in 2ml of immunoprecipitation buffer and rotated for 1 hour. After centrifugation at 1,700 ×g for 5 min, the supernatants were added to 5 μg of anti-rat cathepsin L antibody (17). After rotation at 4°C for 16 hours, the samples were mixed with 100 μl of Protein A-agarose. After 1 hour of rotation, the immune-complexes were pelleted by centrifugation at 1,700 ×g for 5 min. After three cycles of suspension in immunoprecipitation buffer and centrifugation, aliquots of the samples were subjected to 15% SDS-PAGE and immunoblot analyses with anti-mouse cathepsin L antibody and the three peptidic antibodies.

Percoll density fractionation analyses were carried out as described with slight modification (18): A C57 mouse was treated with 3mg of E-64-c and leupeptin dissolved in PBS(−) by intraperitoneal injection. After 1 hour, the mouse was sacrificed by cervical dislocation, and the liver was homogenized in 8 volumes of homogenization buffer. The post-nuclear supernatant was obtained by centrifugation at 1,700 × g for 5 min. The microsome fraction containing mitochondria, lysosomes, Golgi apparatus, endosomes, and plasma membrane, was recovered as the pellet following ultracentrifugation at 100,000× g for 1 hour at 4°C. The microsomal fraction was suspended in 500μl of homogenization buffer and fractionated on 8 ml of 40% Percoll in homogenization buffer by centrifugation at 57,400 × g for 45 min at 4°C. Fractions (0.5ml) were collected from the bottom of the tube and subjected to β-hexosaminidase assay, SDS-PAGE, and immunoblot analyses.

Fluorescence immunohistochemistry was carried out as follows: a C57 mouse was sacrificed by cervical dislocation and its liver was washed in PBS (−), immersed at an optimum temperature cutting compound, and then freezed in liquid nitrogen. Serial sections (5μm) were obtained in a cryostat and fixed in cold ethanol for 3 min. After washing with PBS(−), the sections were immersed in 10% goat serum in PBS (−) for 20 min to reduce non-specific antibody binding. The sections were then soaked in the first antibody solution (12.5μg/ml of IgG) at 4°C for 16 hours. After washing with PBS (−), the goat anti-rabbit IgG was conjugated with FITC (Organon Technica, × 200 diluted) at room temperature for 60 min. The samples were then washed with PBS (−), floated on 50% glycerol 20mM potassium phosphate buffer (pH7.4), 150mM NaCl, and diphenylamine (1mg/ml), and photographed through Axiophot (×125).

RESULTS

Specificities of Anti-peptidic Antibodies

In vitro procathepsin L processing in the presence of dextran sulfate is slower on ice than at 37°C (11). Immunoblot analyses with antibody against the purified cathepsin L show that the processed form, with a molecular mass around 30kDa, appears within 30 min when procathepsin L is incubated with dextran sulfate at pH5.5 on ice (Fig. 1A). This antibody reacts mainly with the two chain form of purified cathepsin L since purified cathepsin L converted from single chain form to two chain form during storage at −80°C (lane 1). Further incubation causes a decrease in the amount of procathepsin L with a molecular mass of 39kDa accompanied by an increase in the amount of the processed forms. After more than 4 hours of incubation on ice, no procathepsin L was detected by the anti-cathepsin L antibody. Although this antibody can not recognize differences in the processed forms with molecular masses between 31kDa and 30kDa, the processed forms remain stable for more than 24 hours. The anti-EPLML antibody recognizes the 31kDa form generated within 30 min from procathepsin L in the presence of dextran sulfate at pH5.5 on ice (Fig. 1B). The amount of this 31kDa processed form increases for up to 2 hours and then decreases. After more than 4 hours incubation, no 31kDa form is detected by the anti-EPLML antibody. No species of purified cathepsin L is detected by the anti-EPLML antibody. The anti-LKIPK antibody detects the 30kDa processed form of procathepsin L obtained by incubation in the presence of dextran sulfate on ice for more than 2 hours (Fig. 1C). Although this 30kDa form recognized by the anti-LKIPK antibody is stable for more than 24 hours, the amount reaches a peak at 8 hours of incubation. This antibody recognizes faint 30kDa bands corresponding to the single chain form of purified cathepsin L (Fig. 1C, lane 1). The anti-IPKSV antibody recognizes the processed form generated from procathepsin L after

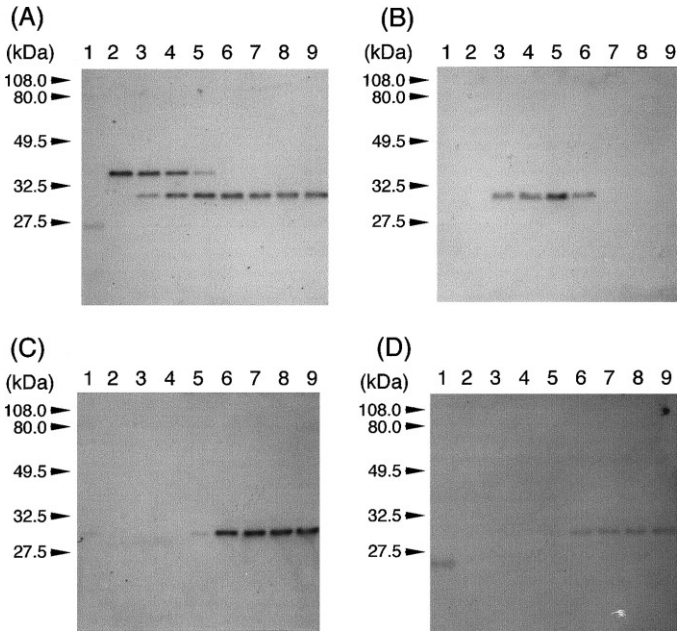

FIG. 1. Specificities of anti-peptidic antibodies. One microgram of purified procathepsin L in 1ml of 100mM sodium acetate buffer (pH5.5) and 1mM dithiothreitol in the presence of dextran sulfate (10μg/ml) were incubated on ice. At each time point, 100 μl of the sample was removed and mixed with E-64-c at a final concentration at 1mM. In (A)–(D), lane 2 is the sample incubated for 0 hour, lane 3 for 0.5 hour, lane 4 for 1 hour, lane 5 for 2 hours, lane 6 for 4 hours, lane 7 for 8 hours, lane 8 for 12 hours, and lane 9 for 24 hours. Mixtures of 50 ng of purified cathepsin L (lane 1) and 25μl of each sample were fractionated by SDS–PAGE in 15% polyacrylamide gels and subjected to immunoblot analyses with (A) anti-mouse cathepsin L (2.5μg/ml), (B) anti-EPLML antibody (2.5 μg/ml), (C) anti-LKIPK antibody (2.5 μg/ml), or (D) anti-IPKSV antibody (2.5 μg/ml). Arrowheads indicate molecular mass markers (Bio-Rad prestained molecular markers): phosphorylase B at 106.0 kDa, bovine serum albumin at 80.0 kDa, ovalbumin at 49.5 kDa, carbonic anhydrase at 32.5 kDa, soybean trypsin inhibitor at 27.5 kDa, and lysozyme at 18.5 kDa.

more than 4 hours of incubation on ice in the presence of dextran sulfate (Fig. 1D). The amount of this form increases after 12 hours of incubation, and is stable for more than 24 hours. This antibody recognizes 25kDa bands corresponding to the heavy chain of the two-chain form of cathepsin L (Fig. 1D, lane 1). Thus, these three anti-peptidic antibodies recognize different species of *in vitro* processed cathepsin L.

Intracellular Forms of Cathepsin L in Vivo

Previous reports have shown that the major form mature cathepsin L differs among various tissues, e.g., the single chain form predominates over the two-chain form in liver, while the two-chain form predominates in kidney; only the single chain form of cathepsin L exists in the skin (17, 19). To evaluate these differences, we analyzed the reactivities of cathepsin L from these tissues with amino terminal amino acid sequence-specific antibodies.

Immunoblot analysis with anti-mouse cathepsin L antibody (Fig. 2A) showed more proteins with molecular masses around 30 kDa than with molecular masses of 25kDa in liver; in contrast, the 25kDa band was stronger than the 30kDa band in kidney. In addition, the predominant band around 30kDa and the faint band around 25kDa were detected in skin. Fifty kilo dalton bands were derived from IgG heavy chain. The anti-EPLML antibody detected the 31kDa form of processed cathepsin L in the tissues that we examined (Fig. 2B). The anti-LKIPK antibody also reacted with the 30 kDa form of cathepsin L (Fig. 2C). Figure 2D shows that both the single chain form and the heavy chain of the two-chain form of cathepsin L were recognized by the anti-IPKSV antibody. A faint band corresponding to the heavy chain of two-chain form of cathepsin L was detected in liver. The 30 kDa band predominated over the 25kDa band in kidney suggesting another species of the two chain form with different amino terminal may exist. Apparently, no 25 kDa form of the heavy chain of the two chain form of cathepsin L was not detected in skin. Long exposure of the same membrane on the film showed that a faint 25 kDa form existed in skin (data not shown). These results suggest that there are at least four forms of processed cathepsin L, i.e., single chain forms with the amino terminal amino acid sequences EPLML, LKIPK, and IPKSV, and a two-chain form that starts with the amino acid sequence IPKSV.

Intracellular Distribution of Various Forms of Cathepsin L

The intracellular processing of cathepsin L *in situ* has been reported to occur in lysosomes, early endosomes, late endosomes, or the prelysosomal compartment (2,3), but the organelle taking part in the processing remain unidentified. To explore where cathepsin L processing occurs, we used immunoblot analyses with our peptidic antibodies of fractions isolated from the organelle of mouse liver by Percoll density gradient centrifugation (18). The activity of β-hexosaminidase, a lysosomal enzyme, was distributed mainly in the heavy fractions corresponding to lanes 1–3 in Fig. 3, but small amounts of activity were also recovered in the light density fractions corresponding to lanes 13–15 (data not shown). Under the conditions of our study, the heavy density fractions contain lysosomes including dense bodies, while the light density fractions are composed of endoplasmic reticulum, Golgi apparatus, endosomes, and some of the lysosomes.

Figure 3A shows that the anti-cathepsin L antibody recognizes proteins with molecular masses of 25kDa in the heavy density fractions as well as those with masses of 39kDa, 31kDa, and 25kDa in the light density fractions of the Percoll density gradient. These results suggest that heavy lysosomes contain the two-chain form of cathepsin L, while the light fractions

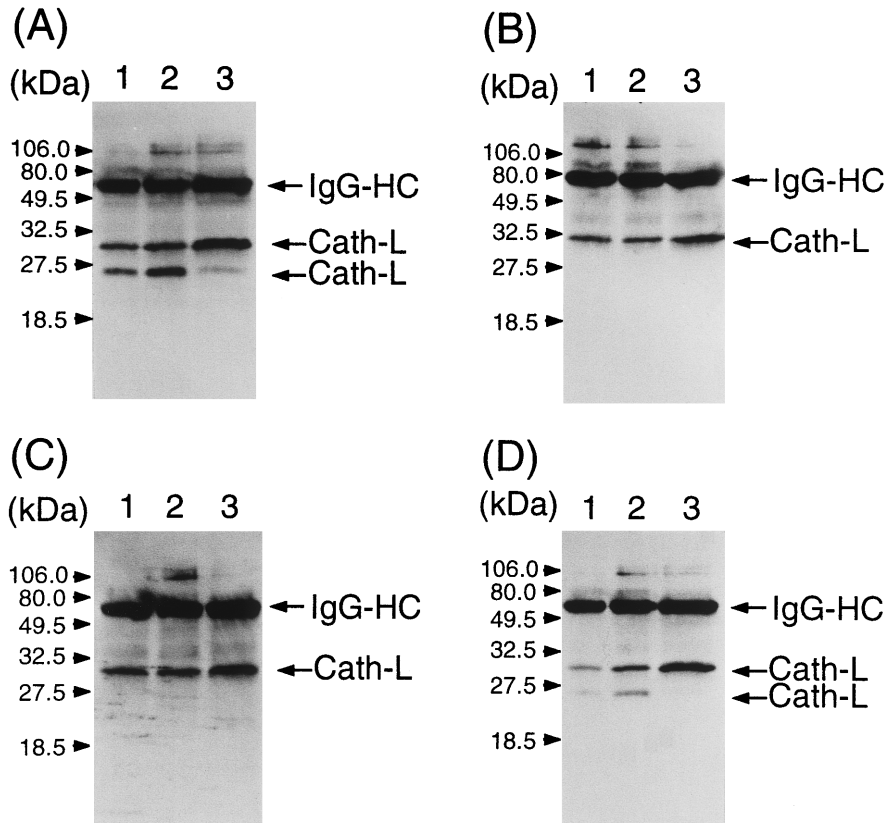

FIG. 2. Intracellular forms of cathepsin L in liver, kidney, and skin. Aliquots of the immunoprecipitants obtained with anti-rat cathepsin L antibody (5μg/samples) from microsomes prepared from mouse liver (lanes 1), kidney (lanes 2), and skin (lanes 3) were fractionated by SDS–PAGE in 15% polyacrylamide gels and immunoblotted with (A) anti-mouse cathepsin L antibody (2.5μg/ml), (B) anti-EPLML antibody (2.5μg/ml), (C) anti-LKIPK antibody (2.5g/ml) or anti-IPKSV antibody (2.5g/ml). Arrowheads to the left indicate molecular masses markers as described in the legend to figure 2. Arrows with IgG-HC and Cath-L indicate immunoglobulin heavy chain and cathepsin L, respectively.

contain procathepsin L, the single chain form of cathepsin L, and part of the two-chain form of cathepsin L. The anti-EPLML antibody bound to 31 kDa proteins in both the light density fractions and the microsomes of mouse liver, but there were no reactive proteins in the heavy density fractions of the Percoll density gradient (Fig. 3B). Figure 3C shows that 30kDa proteins were recognized by the anti-LKIPK antibody in both the light density fractions and the microsome fractions but not in the heavy density fractions. As shown in Fig. 3D, the anti-IPKSV antibody reacted with 25kDa proteins in the heavy fractions and with both 30 kDa and 25 kDa proteins in the light density fractions. These proteins were also recognized by this antibody in the microsome fractions of mouse liver. These results suggest that there is a two-chain form of cathepsin L with an amino terminal sequence of IPKSV in both the heavy and light density fractions; in contrast, the other forms, including procathepsin L and the single chain forms with amino terminal sequences EPLML and LKIPK, only in the light density fractions.

We used an immunofluorescence histochemical technique to analyze the intracellular localization of the single chain form with an amino terminal sequence of EPLML. Fluorescence immunohistochemistry with anti-cathepsin L antibody showed the signal to be localized in the perinuclear area of mouse liver parenchymal cells (Fig. 4A). Since immunohistochemical studies showed cathepsin L to be detectable mainly in lysosomes, this perinuclear profile represents the lysosomal localization of cathepsin L. As shown in figure 4B, the anti-EPLML fluorescence signals were also localized in the perinuclear area in mouse liver. With unknown reason, neither the anti-LKIPK antibody nor the anti-IPKSV antibody gave a fluorescence signal in these studies (data not shown). These results suggest that the 31kDa form of cathepsin L with the amino terminal amino acid sequence EPLML is localized in lysosomes.

DISCUSSION

We generated three species of peptidic antibodies: anti-EPLML, LKIPK, and IPKSV antibodies. These antibodies bound specifically to the three forms of cathepsin L with molecular masses around 30 kDa. Previously we reported that an antibody raised by immunizing rabbit with purified cathepsin L reacted with

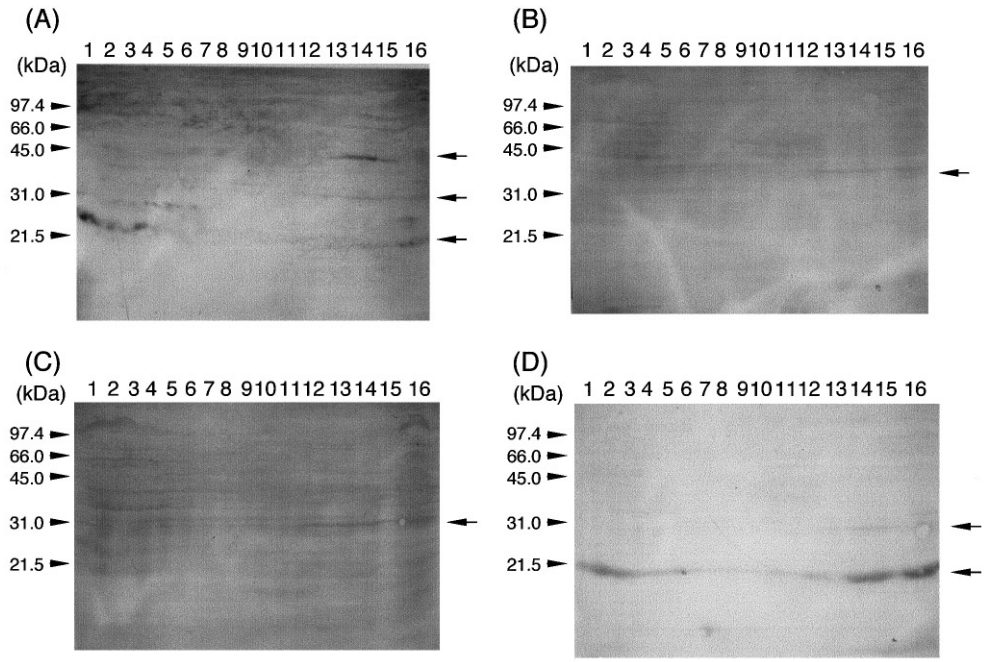

FIG. 3. Immunoblot analyses in Percoll density fractionated organelles from mouse liver. Microsome fractions prepared from mouse liver were fractionated in 40% Percoll density gradients at 57,400 ×g for 45 min. The fractions from bottom to top were numbered from 1 to 15 and they and the microsome fraction (lane 16) were subjected to SDS-PAGE and analyzed with anti-mouse cathepsin L (A), anti-EPLML (B), anti-LKIPK (C), or anti-IPKSV (D) antibodies. The arrows to the right side indicate procathepsin L, 31kDa and 30kDa processed forms, and the heavy chain of the two chain form of cathepsin L, respectively. The arrowheads to the left indicate molecular mass markers: phosphorylase B at 97.4 kDa, bovine serum albumin at 66.0 kDa, ovalbumin at 45.0 kDa, carbonic anhydrase at 31.0 kDa, and soybean trypsin inhibitor at 21.5 kDa.

procathepsin L, and with the single chain form and two-chain form of the mature enzyme (17). The results of pulse-chase analyses in hepatocytes, macrophages, and NIH3T3 cells showed that the intracellular processing of procathepsin L to the mature forms occurs within 1 hour (2,3,20). Nishimura *et al.* suggested that the proteolytic processing of procathepsin L is accomplished after arrival in the lysosomes because of the chloroquine sensitivity. Chloroquine affects not only the pH of lysosomes but also that of transport vesicles

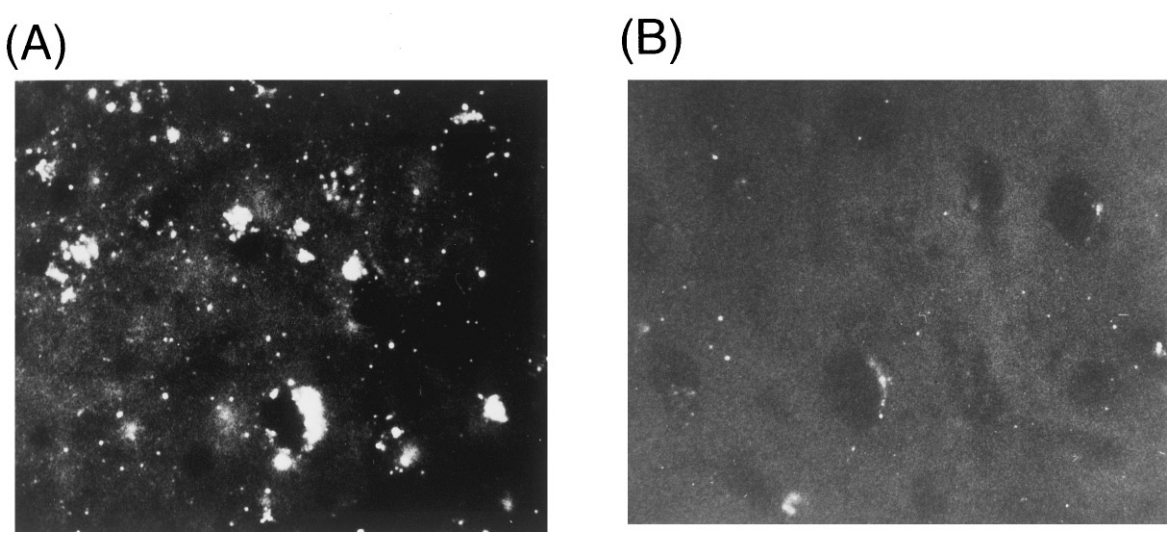

FIG. 4. Fluorescence immunohistochemistry by anti-cathepsin L antibody (A) and anti-EPLML antibody (B) in mouse liver. The liver from a C57 mouse was fixed and immunostained with 12.5μg/ml of anti-mouse cathepsin L antibody (A) or 12.5μg/ml of anti-EPLML antibody (B).

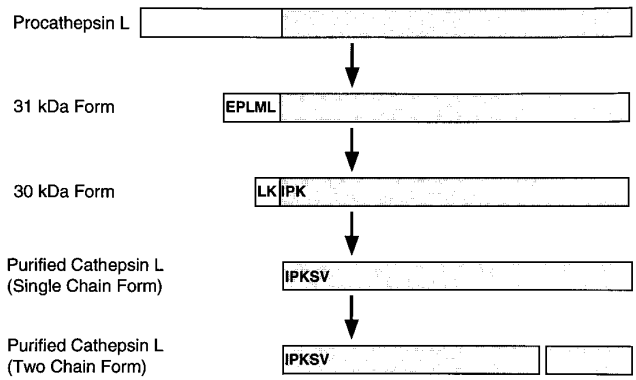

FIG. 5. Schematic illustration for intracellular processing of procathepsin L. Present results *in vitro* and *in vivo* suggest that newly synthesized procathepsin L is processed into the mature single chain form through the intermediates (31 kDa form and 30 kDa form). The mature single chain form is processed further into the two chain form.

to the endosomes (21). Thus, the processing site for procathepsin L *in situ* has remained ambiguous. Immunohistochemical analyses with anti-cathepsin L antibody showed that cathepsin L is localized in lysosomes (22, 23). These reports suggest that intracellular procathepsin L transferred to the Golgi apparatus can not be detected by the immunogold technique in part because of the small amount of procathepsin L in the Golgi area. Our previous study with an anti-procathepsin L-specific antibody that recognized only procathepsin L and not the mature enzyme forms showed that procathepsin L colocalizes with the cation-independent mannose 6-phosphate receptor, a marker protein for late endosomes in NIH3T3 cells, but failed to identify the exact processing site *in situ* since the disappearance of procathepsin L *in situ* has not been identified (13). In addition, in fluorescence cytochemical analyses, the three peptidic antibodies failed to bind to the corresponding forms of cathepsin L in NIH3T3 cells (data not shown). In this study, we show that the 31kDa form of cathepsin L with the amino terminal amino acid sequence EPLML, which appears first after the processing of procathepsin L, is colocalized with the signal for the anti-cathepsin L antibody in mouse liver. Thus, the site of procathepsin L processing *in situ* was identified as the lysosome itself. The data presented in this report are summarized in Fig. 5 as a scheme. Further analyses are necessary to identify lysosomal components that participate in the processing of procathepsin L by far western blot analyses with procathepsin L and its processed forms.

ACKNOWLEDGMENTS

We thank the Division of Biochemical Analyses, Central Laboratory of Medical Science for synthesizing the peptides. This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas, (Intracellular Proteolysis, 08278103) and on Basic Areas (10670145) from the Ministry of Education, Science, Sports, and Culture of Japan.

REFERENCES

1. Kominami, E., Ueno, T., Muno, D., and Katunuma, N. (1991) *FEBS Lett.* **287,** 189–192.
2. Kominami, E., Tsukahara, T., Hara, K., and Katunuma, N. (1988) *FEBS Lett.* **231,** 225–228.
3. Nishimura, Y., Furuno, K., and Kato, K. (1988) *Arch. Biochem. Biophys.* **263,** 107–116.
4. Towatari, T., and Katunuma, N. (1988) *FEBS Lett.* **236,** 57–61.
5. Ishidoh, K., Towatari, T., Imajoh, S., Kawasaki, H., Kominami, E., Katunuma, N., and Suzuki, K. (1987) *FEBS Lett.* **223,** 69–73.
6. Ishidoh, K., Muno, D., Sato, N., and Kominami, E. (1991) *J. Biol. Chem.* **266,** 16312–16317.
7. Ritonja, A., Popovic, T., Kotnik, M., Machleidt, W., and Turk, V. (1988) *FEBS Lett.* **228,** 341–345.
8. Kane, S. E., and Gottesman, M. M. (1990) *Seminar in Cancer Biology,* **1,** 127–136.
9. Prene, E. M., Dong, J., and Sahagian, G. G. (1990) *J. Cell Biol.* **110,** 319–326.
10. Mason, R. W., and Massey, S. D. (1992) *Biochem. Biophys. Res. Commun.* **189,** 11659–1666.
11. Ishidoh, K., and Kominami, E. (1994) *FEBS Lett.* **352,** 281–284.
12. Ishidoh, K., and Kominami, E. (1995) *Biochem. Biophys. Res. Commun.* **217,** 624–631.
13. Ishidoh, K., Takeda-Ezaki, M., and Kominami, E. (1993) *FEBS Lett.* **322,** 79–82.
14. Laemmli, U. K. (1970) *Nature,* **227,** 680–685.
15. Towbin, H., Staehlin, T., and Gordon, J. (1982) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
16. Saido, T. C., Nagao, S., Shiramine, M., Tsukaguchi, M., Sorimachi, H., Murofushi, H., Tsuchiya, T., Ito, H., and Suzuki, K. (1992) *J. Biochem.* **111,** 81–86.
17. Bando, Y., Kominami, E., and Katunuma, N. (1986) *J. Biochem.* **100,** 35–42.
18. Ohshita, T., Kominami, E., Ii, K., and Katunuma, N. (1986) *J. Biochem.* **100,** 623–632.
19. Kawada, A., Hara, K., Kominami, E., Hiruma, M., Noguchi, H., and Ishihara, A. (1997) *Arch. Dermatol. Res.* **289,** 87–93.
20. Dong, J., Prence, E. M., Sahagian, G. G. (1989) *J. Biol. Chem.* **264,** 7377–7387.
21. Maxfield, F. R. (1982) *J. Cell Biol.* **95,** 676–681.
22. Yokota, S., Nishimura, Y., and Kato, K. (1988) *Histochemistry,* **90,** 277–283.
23. Watanabe, T., Waguri, S., Watanabe, M., Ishii, Y., Kominami, E., and Uchiyama, Y. (1989) *J. Histochem. Cytochem.* **37,** 1899–1911.