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Analysis of Where and Which Types of Proteinases Participate in Lysosomal Proteinase Processing Using Bafilomycin A1 and *Helicobacter pylori* Vac A Toxin[1]

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Lysosomal proteinases are translated as preproforms, transported through the Golgi apparatus as proforms, and localized in lysosomes as mature forms. In this study, we analyzed which subclass of proteinases participates in the processing of lysosomal proteinases using Bafilomycin A1, a vacuolar ATPase inhibitor. Bafilomycin A1 raises lysosomal pH resulting in the degradation of lysosomal proteinases such as cathepsins B, D, and L. Twenty-four hours after the withdrawal of Bafilomycin A1, NIH3T3 cells possess these proteinases in amounts and activities similar to those in cells cultured in DMEM and 5% BCS. In the presence of various proteinase inhibitors, procathepsin processing is disturbed by E-64-d, resulting in abnormal processing of cathepsins D and L, but not by APMSF, Pepstatin A, or CA-074. In the presence of *Helicobacter pylori* Vac A toxin, which prevents vesicular transport from late endosomes to lysosomes, the processing of procathepsins B and D occurs, while that of procathepsin L does not. Thus, procathepsins B and D are converted to their mature forms in late endosomes, while procathepsin L is processed to the mature form after its arrival in lysosomes by some cysteine proteinase other than cathepsin B.

Key words: bafilomycin A1, cathepsins, processing, proteinase inhibitors, Vac A toxin.

Lysosomes contain many hydrolases including proteinases, glycosamidases, sulfatases, and phosphatases (*1*). These soluble lysosomal enzymes are translated as preproforms, transported through the Golgi apparatus as proforms, and localized in the lysosomes as the mature enzyme forms *via* endosomes (*2*). These processing mechanisms are quite important for preventing the degradation of cellular components due to inadequate organelles in which these lysosomal hydrolases are inactive. In particular, lysosomal proteinases should be inactive in the Golgi apparatus since inappropriate proteolysis of proteins localized in the Golgi results in cellular dysfunction. Lysosomal proteinases have long propeptides and these proforms show little proteinase activity at neutral pH (*3*). Even at acidic pH, procathepsin L has little proteinase activity against protein substrates or synthetic substrates (*4*). These low proteinase activities cause the autocatalytic processing of recombinant procathepsins B and L (*5, 6*). In contrast, purified procathepsin D does not convert to its mature form even at pH 3.0 (*7*). Previous studies employing a pulse-chase technique in the presence of various proteinase inhibitors produced conflicting results: The processing of lysosomal proteinases was carried out by Pepstatin A-sensitive proteinases such as cathepsin D in cultured hepatocytes, but this processing was inhibited by 1,10-orthophenanthrolin in cultured macrophages (*8, 9*). In yeast, *Saccharomyces cerevisiae, pep4*, a mutant aspartic proteinase failed to be processed to proteinases A and B, resulting in low proteolytic activities in the vacuole, an equivalent organelle to mammalian lysosomes (*10, 11*).

Another unsolved problem is where the precursor forms of lysosomal proteinases are processed *in situ*. If a proteinase is active in the wrong organelle, protein degradation in this organelle may result in cell death. The results of pulse-chase analysis in primary hepatocytes subjected to chloroquine treatment suggested that the processing of procathepsins occurs after their arrival in lysosomes (*8*). Another report describes the participation of cathepsins B and D in the degradation of glucagon in endosomes (*12*). In

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Abbreviations: APMSF, (*p*-amidinophenyl)methanesulfonyl fluoride; BCS, bovine calf serum; CA-074, *N*-(L-3-*trans*-Propylcarbamyloxirane-2-carbonyl)-L-isoleucyl-L-proline; DMEM, Dulbecco's modified Eagle's minimum essential medium; DMSO, dimethyl sulfoxide; E-64-a, *N*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-L-leucine-4-aminobutylamide; E-64-d, *N*-(L-3-*trans*-epoxycarbonyloxirane-2-carbonyl)-L-leucine-3-methylbutylamide; NaPB, sodium phosphate buffer; PAGE, polyacrylamide gel electrophoresis; PBS(−), Dulbecco's phosphate buffered saline.

antigen presenting cells, exogenous proteins are processed by lysosomal proteinases in the MIIC (MHC class II containing compartment) (*13, 14*). This compartment possesses lysosomal integral membrane proteins and proteinases, but not cation-independent mannose 6-phosphate receptor, suggesting this compartment corresponds to some late endosomes. The degradation of the processed invariant chain, p10, that is accomplished in a late endocytic compartment in normal mice, is not observed in thymic epithelial cells in cathepsin L knock-out mice (*15*). Thus, lysosomal proteinases are thought to work in various post-Golgi compartments as well as in lysosomes.

Bafilomycin A1, a macrolide antibiotic isolated from *Streptomyces* sp., inhibits vacuolar ATPase both *in vivo* and *in vitro* (*16*). At low concentration, Bafilomycin A1 causes increases in both lysosomal and intraendosomal pH resulting in the degradation of preexisting lysosomal proteinases as well as alternations in the transport of precursors from cells as proforms. In contrast, this agent does not affect endocytosis or intracellular transport in late endosomes (*17, 18*). Although this antibiotic binds to vacuolar ATPase irreversibly *in vitro*, the activity of vacuolar ATPase is restored in cultured cells after the medium is replaced with fresh medium without Bafilomycin A1 with a slight lag time (*19*). This recovery is presumably due to the *de novo* synthesis of vacuolar ATPase.

Vac A toxin, a 87 kDa protein produced in *Helicobacter pylori*, causes gastric ulcers (*20-22*). When cultured cells are exposed to this toxin, the intracellular vacuoles become enlarged. Vacuolar ATPase participates in the formation of these large vacuoles, since there is a lack of vacuolation in the presence of Bafilomycin A1 (*23, 24*). These large vacuoles are derived from late endosomes since Rabs 7 and 9, markers for late endosomes, are present, while neither Rab 5 or transferin receptor, markers for early endosomes, nor cathepsin D as a marker for lysosomes is observed in enlarged vacuoles (*25*). In addition, Vac A toxin perturbs the vesicular transport from late endosomes to lysosomes, but not from early endosomes to late endosomes (*26*).

In this paper, we analyzed proteinase processing and the sites in organelles that participate in the maturation of lysosomal proteinases in the presence of various proteinase inhibitors or VacA toxin after treatment with Bafilomycin A1 to induce the degradation of lysosomal proteinases. The processing of procathepsins B and D occurs in late endosomes, while that of procathepsin L is completed after arrival in the lysosomes; these processes are carried out by cysteine proteinases other than cathepsin B.

MATERIALS AND METHODS

Bafilomycin A1 and APMSF were purchased from WAKO Pure Chemicals, Pepstatin A from the Peptide Institute. E-64 derivatives including E-64-a, d, and CA-074 were generous gifts from Dr. K. Hanada (Taisho Pharmaceutical).

Vac A toxin was prepared from *H. pylori* as described previously (*27*). NIH3T3 cells were cultured in DMEM containing 5% BCS, Penicillin G (15 U/ml), Streptomycin (15 μg/ml), and Amphotericin B (37.5 ng/ml), designated as normal culture medium, in 5% CO_2 under a humidified atmosphere. NIH3T3 cells were inoculated in 10 cm dishes at 2×10^6 cells/dish, cultured in normal culture medium for 24 h, and then in medium containing 100 nM Bafilomycin A1. After 24 h, the cells were washed twice with PBS(−), once with normal culture medium, and cultured further in fresh normal culture medium. After 8, 16, and 24 h of culture, cells were harvested, washed twice with PBS(−), and suspended in 500 μl of lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 0.05% SDS, 5 mM EDTA, 1 mM APMSF, 1 mM Pepstatin A, and 1 mM E-64-a. The same samples were mixed vigorously with a vortex mixer for 5 min and subjected to centrifugation at $18,500\times g$ for 15 min at 4°C. Fifty micrograms of supernatant were fractionated on SDS-PAGE and analyzed by immunoblot according to Laemmli and Towbin *et al.* (*28, 29*). The results of immunoblot analysis with anti-LGP-85 and cathepsins B, D, and L antibodies (*30-32*) were visualized with a Konica immunostain kit or an ECL immunoblot detection kit (Amersham). In the case of treatment with proteinase inhibitors or Vac A toxin, 2 h before the end of the Bafilomycin A1 treatment period, a proteinase inhibitor or Vac A toxin was added to the culture medium at a final concentration of 50 μM for proteinase inhibitors or 125 μg/ml for Vac A toxin. After culture for 2 h, the cells were washed twice with PBS(−), once with medium, and placed in fresh normal culture medium in the presence of DMSO, APMSF, Pepstatin A, E-64-d, CA-074, or Vac A toxin and cultured for a further 24 h. Cells treated with each proteinase inhibitor were subjected to SDS-PAGE and immunoblotting as described above. Alternatively, the samples were subjected to immunoprecipitation followed by either immunoblot analysis with anti-peptidic antibodies (*33*) or subjected to amino acid sequence determination (*34*). Proteinase activities were determined by measuring the release of aminomethylcoumarin from 1 mM z-Arg-Arg-MCA for cathepsin B, 1 mM Arg-MCA for cathepsin H, and 1 mM z-Phe-Arg-MCA in the presence of 2 μg of CA-074 for cathepsin L as described previously (*3, 31*). The activity of cathepsin D was measured by determining the amount of amino acids released from denatured hemoglobin (*35*). The activity of β-hexosaminidase was determined by measuring the release of 4-methylumberiferone from 4-methylumberiferyl glucosaminide instead of β-4-methylglucuronide for β-glucuronidase (*32*).

RESULTS

Activities of Lysosomal Proteinases and β-Hexosaminidase in Cells Treated with Bafilomycin A1 and Cultured in Normal Culture Medium—Treatment of cells with Bafilomycin A1, an inhibitor of vacuolar ATPase, causes an elevation in the pH of acidic organelles such as lysosomes and late and early endosomes. Previous reports have described how Bafilomycin A1 causes an alternation in the intracellular trafficking of lysosomal proteinase precursors to secretion, but not lysosomal integral membrane proteins (*17, 18*). This effect of Bafilomycin A1 on cultured cells is reversible (*19*). We explored the effects of Bafilomycin A1 on the activities of lysosomal enzymes in NIH3T3 cells treated with Bafilomycin A1 for 24 h and then cultured in normal culture medium (Table I).

After 24 h in the presence of 100 nM Bafilomycin A1, the cells possessed both β-hexosaminidase and cathepsin H activities similar to those of cells cultured in normal

medium: β-hexosaminidase and cathepsin H activities were 68.5 and 131.6% of those in control cells, respectively. In contrast, the activities of cathepsins B, D, and L were lower than those in normal cells, 8.5, 17.6, and 5.3% of cells cultured under normal conditions, respectively. After the Bafilomycin A1 was removed, intracellular lysosomal enzyme activities recovered gradually with different time courses. The activities of both cathepsins B and D in cells treated with Bafilomycin A1 returned to normal levels within 16 h after the removal of Bafilomycin A1. The activity of cathepsin B was higher after 24 h than in cells cultured continuously in normal medium. In contrast, the activity of cathepsin L in the Bafilomycin A1-treated cells reverted to 70% of that in normal cells after 24 h of culture in normal medium.

Molecular Forms of Lysosomal Proteins in the Bafilomycin A1-Treated Cells Followed by Culture in Normal Culture Medium—Hydrolyzing activities measured using synthetic substrates may include other proteinases such as proteasome, trypsin, and so on. To confirm that the changes in hydrolyzing activities of cells treated with Bafilomycin A1 followed by culture in normal medium correspond to the molecular forms of lysosomal enzymes, we next analyzed the molecular forms of cathepsins B, L, and D by immunoblot analysis with anti-cathepsins B, L, and D antibodies, respectively. In addition, changes in the molecular forms of LGP-85 were explored by immunoblot analysis with anti-LGP-85 antibody, since a previous report showed that lysosomal integral membrane glycoproteins are not affected by Bafilomycin A1 treatment (*18*).

As shown in Fig. 1A, the anti-cathepsin B antibody recognized a 31 kDa protein that corresponded to the single chain form of mature cathepsin B in NIH3T3 cells under normal conditions (lane 1). Bafilomycin A1 treatment caused a loss of the 31-kDa form of mature form of cathepsin B accompanied by an increase in intracellular procathepsin B, a precursor form of cathepsin B with a molecular mass of 41 kDa (lane 2). After removal of the Bafilomycin A1, the amount of the 31 kDa form of mature cathepsin B increased accompanied by a loss of procathepsin B within 8 h. After 16 and 24 h of culture in normal medium, the cells possessed similar amounts of mature cathepsin B to that seen in NIH3T3 cells under normal culture conditions (lanes 4 and 5).

In cells under normal culture conditions, the anti-cathepsin L antibody recognized three bands with molecular masses of 39, 31, and 24 kDa corresponding to procathepsin L, the single chain form, and the heavy chain of the two chain form of mature cathepsin L, respectively (Fig. 1B, lane 1). Bands with molecular masses higher than 66 kDa were derived from non-specific binding of the first antibody. After treatment with Bafilomycin A1, the cells contained only the 39 kDa form of procathepsin L, but not mature cathepsin L (lane 2). After the removal of Bafilomycin A1, cells cultured in normal medium for 8 h showed a small amount of the single chain form of mature cathepsin L and a large amount of procathepsin L (lane 3). Further culture for 16 h in normal medium increased the amount of the single chain form of cathepsin L and decreased the amount of procathepsin L (lane 4). After 24 h of culture in normal medium, there was a decrease in both the single chain form of cathepsin L and procathepsin L in these cells (lane 5). Longer exposure of the same filters showed only a small amount of the heavy chain of the two chain form of cathepsin L in cells cultured for 24 h in normal medium after Bafilomycin A1 treatment (data not shown, and see Fig. 3A).

The anti-cathepsin D antibody recognized a 44 kDa protein corresponding to the single chain form of cathepsin D in NIH3T3 cells under normal culture conditions (Fig. 1C, lane 1). Bafilomycin A1 treatment caused an accumulation of procathepsin D with a molecular mass of around 54 kDa and a loss of mature cathepsin D from cells (lane 2). After removal of Bafilomycin A1, the cells cultured in DMEM and 5% BCS showed a small amount of 54 kDa procathepsin D within 8 h (lane 3). After further culture in normal culture medium, only the 44 kDa form of mature cathepsin D was detected by the anti-cathepsin D antibody (lanes 4 and 5).

The anti-LGP-85 antibody recognized an 85 kDa protein corresponding to LGP-85 and a 100 kDa protein that arose presumably due to non-specific binding in NIH3T3 cells grown in normal culture medium (Fig. 1D, lane 1). After Bafilomycin A1 treatment, cells possessed the mature form of LGP-85 with a molecular mass of 85 kDa and its precursor form with a molecular mass of 77 kDa (lane 2). The amount of the intracellular precursor form of LGP-85 with a molecular mass of 77 kDa decreased accompanied by an increase in the mature form of LGP-85 with a molecular mass of 85 kDa when the Bafilomycin A1-treated cells were cultured in normal medium (lanes 3-5).

Immunoblot analysis with anti-cathepsin H antibody revealed that a molecular form of cathepsin H detected as a 30 kDa protein corresponded to the single chain form of mature cathepsin H in NIH3T3 cells. No changes in the amount or molecular form of cathepsin H were observed in either Bafilomycin A1-treated cells or in cells cultured in normal medium following treatment with Bafilomycin A1 (data not shown). Thus, the changes in cathepsin activities in NIH3T3 cells treated with Bafilomycin A1 and then cultured in DMEM and 5% BCS correlate well with their molecular forms.

Changes in the Lysosomal Enzyme Activities in Cells Treated with Bafilomycin A1 Followed by Culture in

TABLE I. **Activities of lysosomal enzymes in NIH3T3 cells.** Representative results are shown. Other cell preparations obtained at different times showed a similar tendency but could not be compared directly because cell conditions have a great effect on enzyme activity.

	β-Hexosaminidase	Cathepsin B	Cathepsin L	Cathepsin H	Cathepsin D
			(nmol/min/μg of protein)		
Normal culture medium	2.115	2.547	2.141	1.189	3.776
Bafilomycin A1-treated	1.440	0.217	0.253	1.565	0.666
Time course after withdrawal of Bafilomycin A1					
8 h	1.311	1.154	0.417	1.117	2.432
16 h	1.551	3.428	1.379	1.519	4.202
24 h	1.851	4.181	1.477	1.583	3.682

Normal Culture Medium in the Presence of Various Proteinase Inhibitors—Previous studies involving pulse-chase analyses in the presence of various proteinase inhibitors to identify groups on proteinases that are involved in the processing of lysosomal proteinases have produced complicated results; aspartic proteinase was found to participate in the processing of procathepsins in primary hepatocytes, while a 1,10-orthophenanthroline-sensitive proteinase was found to be involved in the processing of lysosomal procathepsins in cultured macrophages (*8, 9*). To clarify what types of proteinases are involved in the processing of lysosomal proteinases, we used Bafilomycin A1 treatment followed by culture in normal medium, as described above, in the presence of various proteinase inhibitors.

As shown in Table II, the activity of β-hexosaminidase in cells was slightly increased in the presence of proteinase inhibitors compared with the presence of DMSO, used as the solvent for proteinase inhibitors, when NIH3T3 cells were cultured in normal medium for 24 h after treatment with Bafilomycin A1 for 24 h: 149.1% of control in the presence of APMSF, 165.3% in the presence of Pepstatin A, 134.6% in the presence of E-64-d, and 108.3% in the presence of CA-074.

When the cathepsin B activities in cells cultured with various proteinase inhibitors were compared with the activity in cells cultured with DMSO, the activities were higher in the presence of either APMSF or Pepstatin A, but lower in the presence of either E-64-d or CA-074: 194.0% of control in cells treated with APMSF, 152.3% in cells treated with Pepstatin A, 1.2% in cells treated with E-64-d, and 7.54% in cells treated with CA-074. These results suggest that the degradation of cathepsin B is due in part to both Pepstatin A-sensitive proteinases and APMSF-sensitive proteinases.

TABLE II. **Activities of lysosomal enzymes in cells cultured with various proteinase inhibitors for 24 h after Bafilomycin A1 treatment.** Representative data are shown. Five separate experiments gave similar results but with different magnitudes of enzyme activity.

	β-Hexosaminidase	Cathepsin B	Cathepsin L	Cathepsin D
	(nmol/min/μg of protein)			
DMSO	1.510	1.737	1.555	4.311
APMSF	2.251	3.370	1.820	4.106
Pepstatin A	2.532	2.646	1.983	1.174
E-64-d	2.032	0.021	0.759	1.521
CA-074	1.636	0.131	1.582	4.412

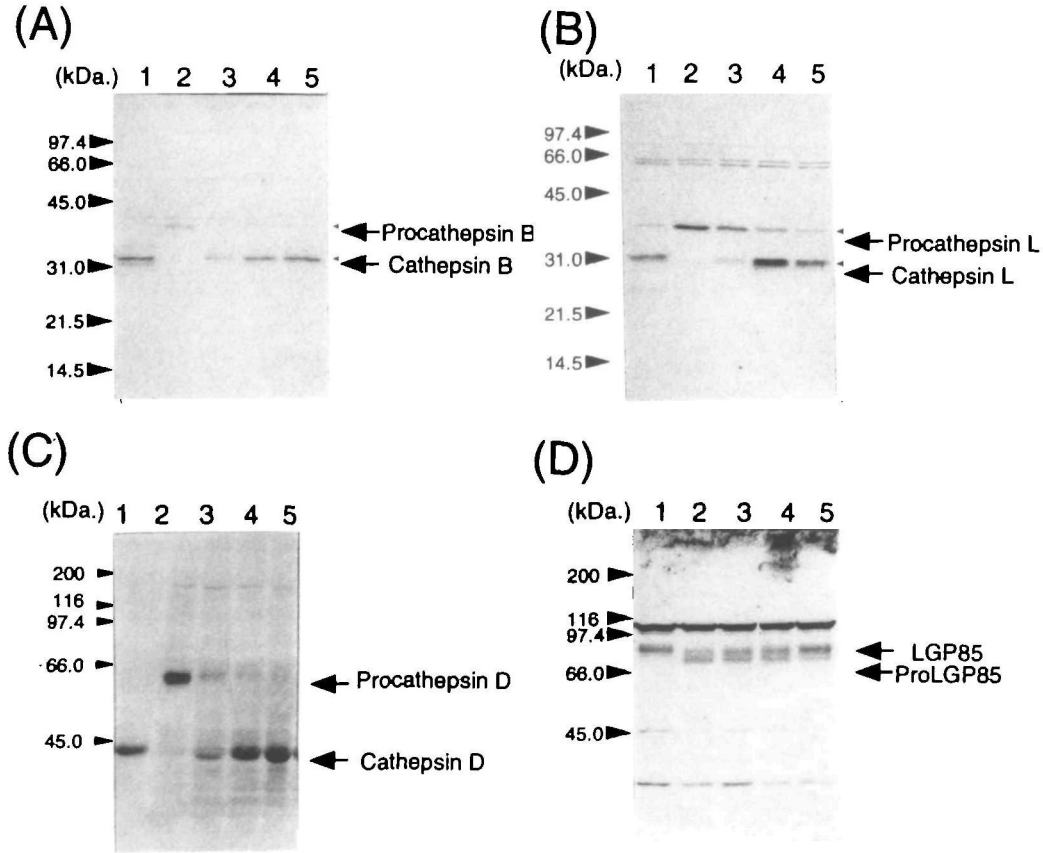

Fig. 1. **Changes in the molecular forms of intracellular lysosomal proteinases and glycoproteins in cells cultured in normal medium after Bafilomycin A1 treatment.** Fifty micrograms of total cell extract was fractionated by SDS-PAGE (13% in A and B, 9% in C and D) and immunoblot analyses with anti-cathepsin B (A), cathepsin L (B), cathepsin D (C), and LGP-85 (D) antibodies were conducted. The cellular extracts were prepared from NIH3T3 cells cultured in DMEM and 5% BCS (lane 1), and treated with 100 nM Bafilomycin A1 for 24 h (lane 2), then cultured in DMEM and 5% BCS for 8 h (lane 3), 16 h (lane 4), or 24 h (lane 5). Arrowheads to the left indicate molecular mass markers: myosin, 200 kDa; β-galactosidase, 116 kDa; 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, 14.5 kDa.

Intracellular cathepsin L activity in the presence of either APMSF or Pepstatin A increased slightly to 117.0 or 127.5% of that in the presence of DMSO. E-64-d treatment caused the lysosomal cathepsin L activity to fall to 48.8% of that in the presence of DMSO. CA-074, an E-64 derivative specific for cathepsin B, did not affect the activity of cathepsin L compared with DMSO.

The cathepsin D activities in cells in the presence of APMSF and CA-074 were comparable to the activity in DMSO. Pepstatin A in the culture medium inhibited cathepsin D activity to 27.2% of that in DMSO. Surprisingly, E-64-d in the medium also affected the intracellular cathepsin D activity; the cathepsin D activity in NIH3T3 cells in the presence of E-64-d was 35.3% that in DMSO. This inhibitory effect of E-64-d on intracellular cathepsin D activity was comparable to that of Pepstatin A.

Effects of Proteinase Inhibitors on the Processing of Lysosomal Proteinases—The enzyme activities of the proteinases were affected by both the presence of inhibitors and by the molecular form, such an inadequately processed form. To clarify which factors affect the proteinase activities in cells cultured in normal medium with proteinase inhibitors after Bafilomycin A1 treatment, we used immunoblot analyses to analyze the molecular forms of the proteinases processed in the presence of various proteinase inhibitors.

As shown in Fig. 2A, cathepsin B from NIH3T3 cells migrated as a 31 kDa band and procathepsin B from Bafilomycin A1-treated NIH3T3 cells migrated as a 41 kDa band (lanes 1 and 2). After 24 h of culture in normal medium following Bafilomycin A1 treatment, both cathepsin B and a faint band corresponding to procathepsin B were detected in cells (lane 3). In the presence of DMSO, the cells possessed similar amounts of procathepsin B as cells cultured in the absence of DMSO, but the level of mature cathepsin B was slightly lower in the presence of DMSO than its absence (lanes 3 and 4). Thus, DMSO has a slight effect on the stability of the mature form of cathepsin B. The intracellular processing of procathepsin B after treatment with Bafilomycin A1 was not affected by APMSF, Pepstatin A, or CA-074 (lanes 5, 6, and 8). When cells were cultured in normal medium with E-64-d after Bafilomycin A1 treatment, forms with molecular masses of around 41 kDa but not 31 kDa were detected by the anti-cathepsin B antibody (lane 7). The cathepsin B activities in cells cultured in either E-64-d or CA-074 were suppressed (Table II). However, the E-64-d treated cells possessed procathepsin B, while CA-074 treated cells contained the mature form of cathepsin B, which was inhibited by CA-074. Thus, the intracellular processing of cathepsin B after Bafilomycin A1 treatment is performed by E-64-d-sensitive and CA-074-insensitive cysteine proteinases.

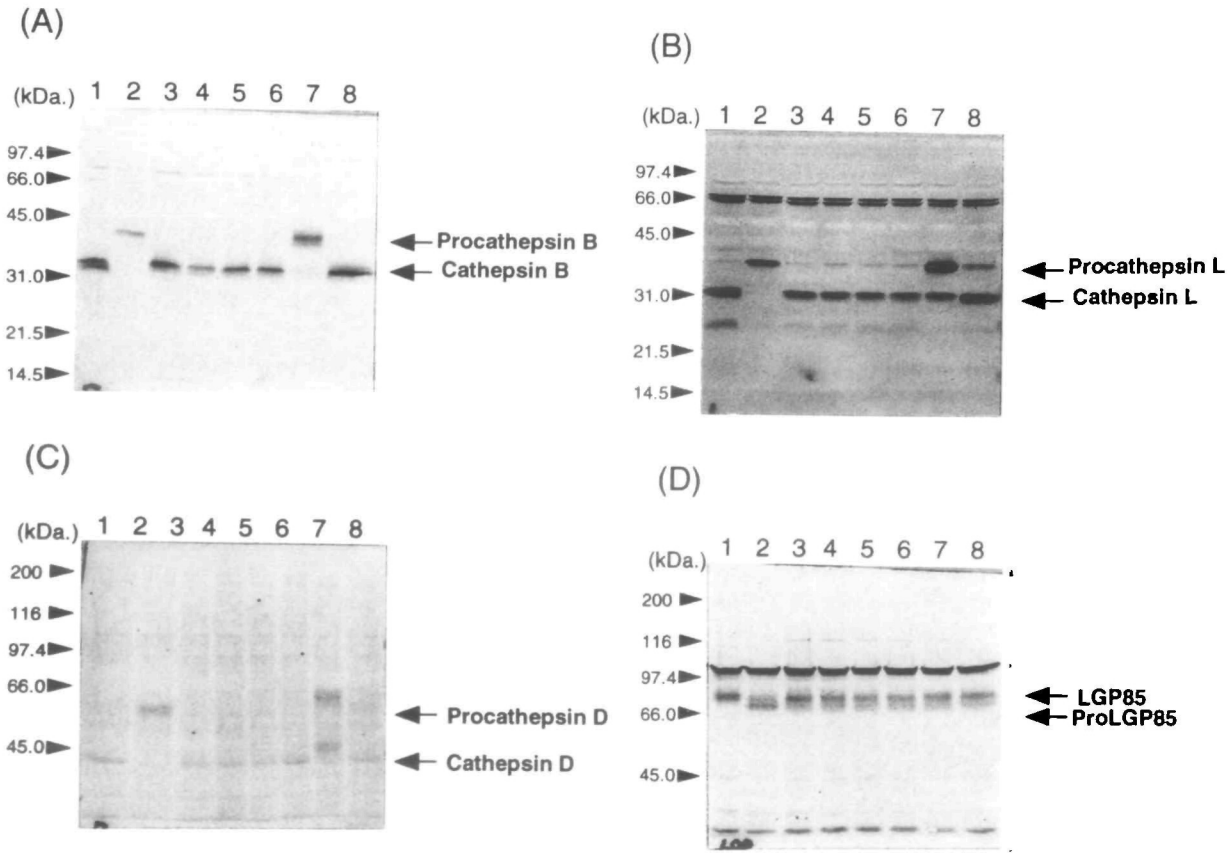

Fig. 2. **Effects of various proteinase inhibitors on the intracellular processing of lysosomal proteins following Bafilomycin A1 treatment.** Fifty micrograms of cell extracts prepared from NIH3T3 cells cultured in DMEM+5% BCS (lane 1), treated with 100 nM Bafilomycin A1 for 24 h (lane 2), and then cultured in DMEM+5% BCS for 24 h in the absence (lane 3) or presence of DMSO (lane 4), 50 μM APMSF (lane 5), 50 μM Pepstatin A (lane 6), 50 μM E-64-d (lane 7), or 50 μM CA-074 (lane 8) were fractionated by 13% (A and B) and 9% (C and D) SDS-PAGE and analyzed by immunoblot analyses with anti-cathepsin B (A), cathepsin L (B), cathepsin D (C), and LGP-85 (D) antibodies. Molecular mass markers as described in the legend to Fig. 1 are indicated on the left.

In the case of procathepsin L processing, the single chain and two chain forms of cathepsin L and procathepsin L were detected by anti-cathepsin L antibody in NIH3T3 cells cultured in normal medium (Fig. 2B, lane 1). Bafilomycin A1-treated cells possessed only procathepsin L (lane 2). After 24 h of culture in normal medium following Bafilomycin A1 treatment, three forms of cathepsin L in the cells were detected (lane 3). Neither DMSO, APMSF, nor Pepstatin A in the culture medium affected the intracellular processing of procathepsin L (lanes 2-4). Cells cultured with E-64-d contained large amounts of procathepsin L and the 31 kDa form compared with cells cultured with DMSO (lanes 4 and 7). CA-074 treated cells possessed large amounts of 31 kDa cathepsin L and slightly elevated amounts of procathepsin L compared with cells cultured in the presence of DMSO (lanes 4 and 8). These results suggest that E-64-d affects the processing of procathepsin L to the 31 kDa form and that CA-074 affects the degradation of the 31 kDa processed form. Thus, the mature forms of cathepsin L were, in part, degraded by cathepsin B. Cathepsin L activity in the cells cultured in the presence of CA-074 did not rise (Table II), since vast amounts of CA-074 slightly inhibit both cathepsin L and cathepsin B *in vitro*.

The anti-cathepsin D antibody recognized both cathepsin D in NIH3T3 cells and procathepsin D in cells treated with Bafilomycin A1 (Fig. 2C, lanes 1 and 2). After 24 h of culture in normal medium following Bafilomycin A1 treatment, proteins with a molecular mass of 44 kDa, corresponding to cathepsin D, were detected (lane 3). Cells treated with DMSO, APMSF, Pepstatin A, or CA-074 all possessed similar amounts of cathepsin D compared with cells cultured in the absence of these agents (lanes 3-6 and 8). When E-64-d was present in the culture medium, two major proteins with molecular masses of about 64 and 46 kDa were observed (lane 7). Judging from their molecular masses, these molecules may possess unusual modifications in their glycosyl side chains and/or might result from the processing of procathepsin D. To clarify these possibilities, we subjected anti-cathepsin D antibody immunoprecipitants from cells treated with E-64-d following Bafilomycin A1 treatment to SDS-PAGE and either amino acid sequencing or lectin blot analysis. Amino terminal amino acid sequencing of the immunoprecipitants revealed that the 64 and 46 kDa bands began with IIRIP and TXEPV, respectively. According to the DDBJ DNA data bank, the deduced amino acid sequences (X52886) of mouse procathepsin D and the mature form begin with IIRIP and EPVSE, respectively. Thus, the amino terminal amino acid sequences of the 64 kDa bands are identical to that of procathepsin D while the terminal sequence of the 46 kDa bands possess a two-amino acid extension compared with mature cathepsin D.

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Amino acid sequence of
  Procathepsin D: IIRIPLRKFTS
  64 kDa band:    IIRIP
  Processing site of mature Cathepsin D: SSPKTT ↓ EPVSELL
  46 kDa band:                             TX  EPV
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Further lectin blot analyses suggested that immunoprecipitated proteins with molecular masses around 64 and 46 kDa are altered in their *N*-linked oligosaccharides, in part by fucose α1-4GlcNAc modification, because these immunoprecipitants reacted with LCA lectin (data, not shown). Thus, suppression of cathepsin D activity in E-64-d treated cells following Bafilomycin A1 treatment (Table II) was presumably due to the failure of procathepsin D undergo appropriate-processing to the mature forms.

The molecular forms of LGP-85 in cells were not affected by culture medium in the presence or absence of proteinase inhibitors (Fig. 2D, lanes 3-8). The precursor form of LGP-85 with a molecular mass of 77 kDa was larger than the mature 85 kDa form in Bafilomycin A1-treated cells; in contrast, cells possessed larger amounts of the mature form than the precursor form when the cells were cultured in normal medium in the presence or absence of any of these agents.

In the case of cathepsin L, we generated anti-peptidic antibodies that recognized three processed forms with amino terminal amino acid sequences of EPLML, LKIPK, and IPKTV (*33*). The 31 kDa form with the amino terminal sequence EPLML showed less proteolytic activity, while the 30 kDa forms with either LKIPK or IPKTV amino termini showed strong activities with the synthetic substrate, z-Phe-Arg-MCA (*36*). When cells were cultured in the presence of E-64-d following Bafilomycin A1 treatment, there was only a small amount of cathepsin L activity. To clarify whether this low hydrolyzing activity against z-Phe-Arg-MCA derives from inadequate intracellular processing of procathepsin L or to complex formation of the processed cathepsin L with E-64-d, we analyzed cell lysates by immunoprecipitation with anti-cathepsin L antibody followed by anti-peptidic antibodies.

As shown in Fig. 3A, only procathepsin L is present in cells after Bafilomycin A1 treatment (lane 1). After 24 h of culture in normal medium in the presence of DMSO following Bafilomycin A1 treatment, the cells possessed procathepsin L and the single chain form and two chain form of mature cathepsin L (lane 2). APMSF had no effect on the processing of procathepsin L to the mature form compared with DMSO (lanes 2 and 3). Treatment with Pepstatin A or CA-074 stabilized the mature forms of cathepsin L including the single chain and two chain forms, but had no effect on procathepsin L (lanes 4 and 6). Both procathepsin L and the single chain processed form, which migrate slightly more slowly in SDS-gels than in the presence of other agents, increased in cells treated with E-64-d (lane 6). In these cells, no two chain form of cathepsin L was detected. No processed form of cathepsin L with the amino terminal amino acid sequence EPLML was detected (Fig. 3B). Small amounts of the processed form with the amino terminal amino acid sequence LKIPK were detected in cells cultured with E-64-d but under other conditions (Fig. 3C). Most of the processed forms in the cells were recognized by the anti-IPKTV antibody, which reacts with both the single chain and the heavy chain of the two chain form of mature cathepsin L, except when the cells were cultured with E-64-d (Fig. 3D). Both anti-LKIPK and anti-IPKTV antibodies failed to recognize most of the processed forms with a molecular mass of 31 kDa (Fig. 3, C and D, lane 5). Further amino terminal amino acid sequence analysis of the 31 kDa form precipitated with anti-cathepsin L antibody from cells treated with E-64-d following Bafilomycin A1 treatment identified the amino terminal sequence RLFQE, which corresponds to a sequence 10 amino acids upstream from the amino terminal of purified cathepsin L (Fig. 3E). These results suggest that the low

activity of cathepsin L in cells treated with E-64-d following Bafilomycin A1 treatment is due mainly to the inadequate processing of procathepsin L.

Processing of Procathepsins B and D Occurs in Late Endosomes, Whereas Procathepsin L Processing Occurs in Lysosomes—The processing of procathepsins B and D occurs faster than that of procathepsin L (Table I and Fig. 1). To determine where these procathepsins are converted to the mature forms, we analyzed processing in cells cultured in the presence of Vac A toxin, a vacuolar toxin from *H. pylori*, following Bafilomycin A1 treatment, since Vac A toxin inhibits vesicular transport from late endosomes to lysosomes and its effect is masked by Bafilomycin A1 treatment (*23-25*).

As shown in Fig. 4A, anti-cathepsin B antibody reacts with the mature form in cells cultured in DMEM and 5% BCS, and procathepsin B in Bafilomycin A1-treated cells (lanes 1 and 2). Procathepsin B is converted to its mature form in the presence or absence of Vac A toxin (lanes 3 and 4). Bands with molecular masses of 42, 60, and 65 kDa are presumably derived from non-specific binding to the anti-cathepsin B antibody.

Anti-cathepsin L antibody recognizes the single chain form and the heavy chain of the two chain form in cells cultured in normal medium, and procathepsin L in Bafilomycin A1 treated cells (Fig. 4B, lanes 1 and 2). After 24 h of culture in DMEM and 5% BCS in the absence of Vac A toxin, a small amount of procathepsin L and a large amount of the single chain form of cathepsin L were detected by the anti-cathepsin L antibody (lane 3). In contrast, in the presence of Vac A toxin, only procathepsin L, but not its processed forms, was detectable in the cells (lane 4). Bands with molecular masses of around 66 kDa were presumably the results of non-specific binding to the first antibody.

In the case of cathepsin D, cathepsin D with a molecular mass of 44 kDa was observed in cells under normal cultured conditions, and procathepsin D with a molecular mass of 54 kDa in cells treated with Bafilomycin A1 (Fig. 4C, lanes 1 and 2). Twenty-four hours after Bafilomycin A1 treatment, both procathepsin D and cathepsin D were detected in cells cultured in the presence of Vac A toxin, similar to the situation in cells cultured in the absence of Vac A toxin (lanes 3 and 4). Bands with molecular masses of 66 kDa were presumably due to non-specific binding to the antibody.

The anti-LGP85 antibody reacted with a 85 kDa protein

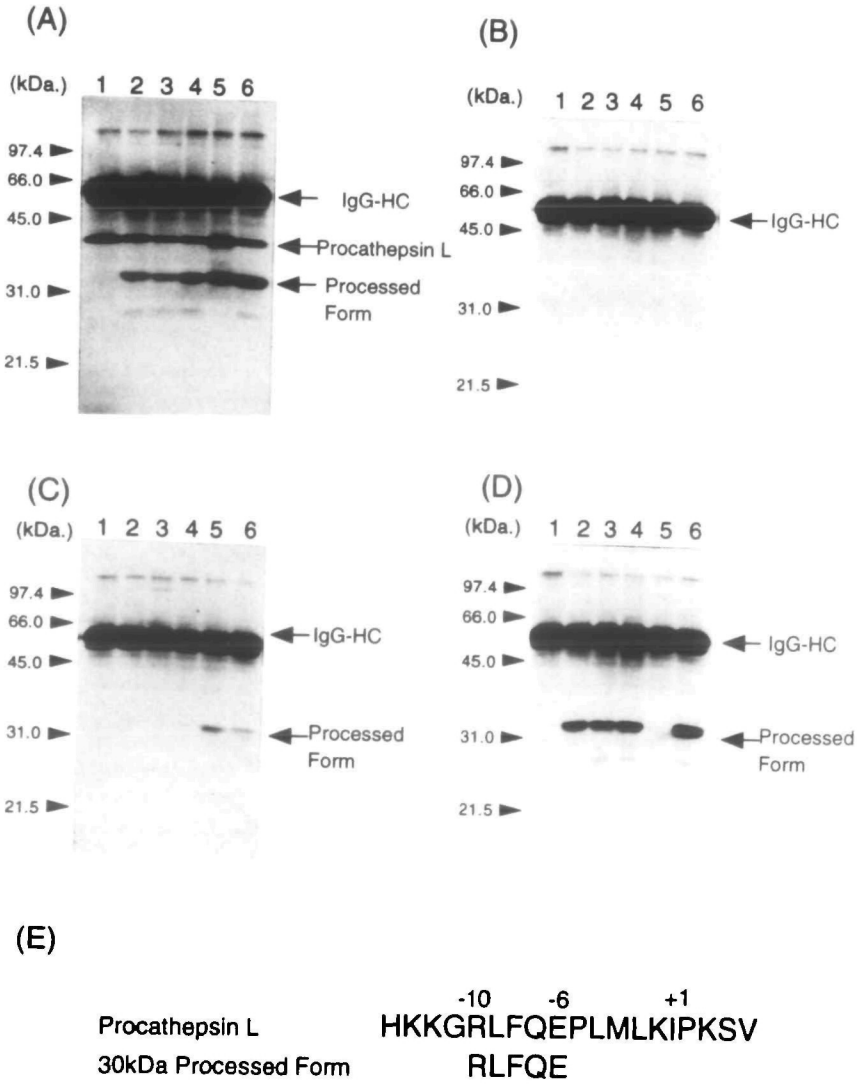

Fig. 3. **Abnormal processing of procathepsin L in the presence of E-64-d.** Cell extracts from NIH3T3 cells were prepared from NIH3T3 cells cultured in 100 nM Bafilomycin A1 for 24 h (lane 1), and then cultured in normal medium in the presence of DMSO (lane 2), APMSF (lane 3), Pepstatin A (lane 4), E-64-d (lane 5), or CA-074 (lane 6). The samples were immunoprecipitated with anti-cathepsin L antibody and fractionated by 12% SDS-PAGE. After transfer to nylon membranes, the immunoprecipitants were analyzed with anti-cathepsin L (A), anti-EPLML (B), anti-LKIPK (C), and anti-IPKTV (D) antibodies. Arrowheads on the left indicate molecular standards as described in the legend to Fig. 1.

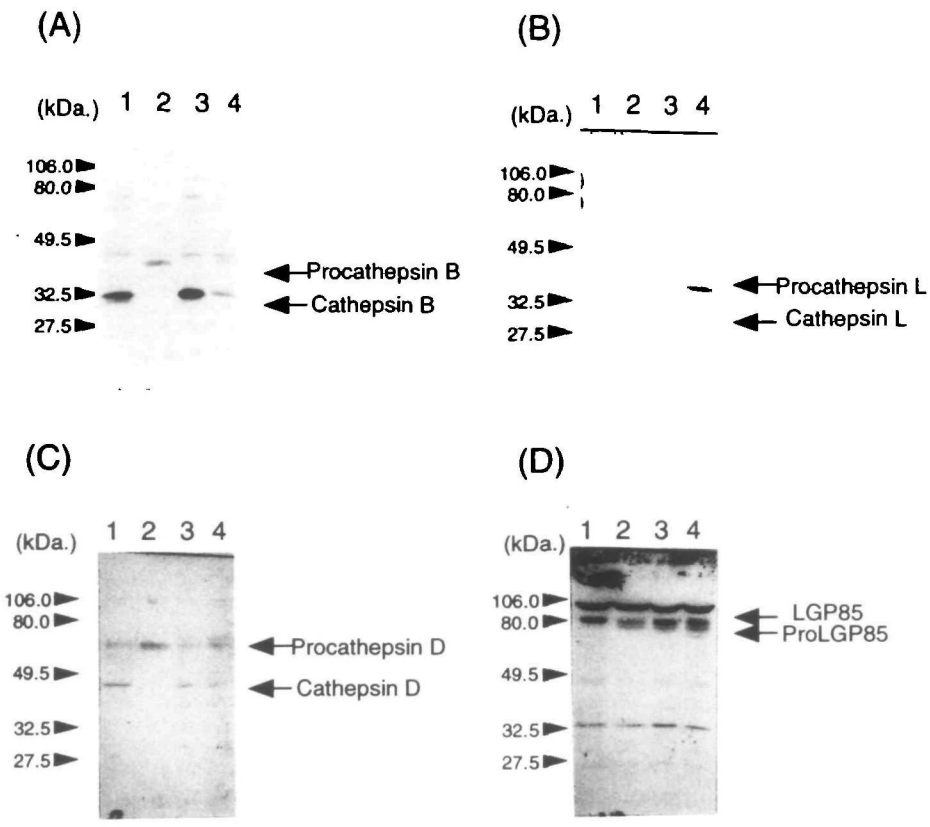

Fig. 4. **Intracellular processings of procathepsins in the presence of Vac A toxin after Bafilomycin A1 treatment.** Fifty micrograms of cellular extracts prepared from cells treated with DMEM and 5% BCS (lane 1), Bafilomycin A1 (lane 2), or normal medium in the absence (lane 3) or presence (lane 4) of Vac A toxin (125 μg/ml) for 24 h after Bafilomycin A1 treatment were subjected to SDS-PAGE (13%) and immunoblot analyses with anti-cathepsin B (A), cathepsin L (B), cathepsin D (C), or LGP-85 (D) antibodies. Arrowheads to the left indicate molecular mass marker: phosphorylase B, 106.0 kDa; bovine serum albumin, 80.0 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa.

in cells cultured in normal medium and both 85 and 77 kDa proteins in Bafilomycin A1-treated cells (Fig. 4D, lanes 1 and 2). After 24 h of culture in DMEM and 5% BCS in the absence or presence of Vac A toxin, the amount of mature LGP-85 increased with a reciprocal decrease in the precursor forms (lanes 3 and 4). These results demonstrate that the processing of procathepsins B and D occurs in the late endosome compartment in contrast to procathepsin L processing which takes place in lysosomes.

DISCUSSION

The intracellular processing of lysosomal enzymes, especially proteinases, is one of the most stringently regulated processes, since most lysosomal enzymes that localize in inadequate organelles or the cytosol are toxic and cause in cell death. Lysosomal proteinases translocate through the Golgi apparatus as proenzymes with less activity than mature enzymes at pH 6.5 or higher (*4*). The results of previous studies are ambiguous with respect to where and by which types of proteinases lysosomal proteinases are processed. In this study, we demonstrate that cysteine proteinases other than cathepsin B participate in the processing of procathepsins B, D, and L.

Previous pulse-chase studies using Bafilomycin A1 showed that lysosomal proteinases are not stored in intracellular compartments, but are secreted continuously from treated cells as proforms (*17*). In addition, *in vitro* studies showed cathepsins to be less stable at neutral pH than at acidic pH except for cathepsin H (*37*). Since Bafilomycin A1 inhibits vacuolar type ATPase, cells treated with this agent show suppressed degradation of the extracellular proteins taken up in lysosomes, even though the efficiency of endocytosis is not altered (*19*). This phenomenon is possibly due to a short-term increase in lysosomal pH and to the long-term deprivation of lysosomal proteinases. As shown in Table I, the enzyme activities of the lysosomal proteinases decreased in cells cultured in the presence of Bafilomycin A1 for 24 h. After washing to remove Bafilomycin A1, the enzyme activities of cathepsins B and D increase within 16 h to the levels in normal cultured NIH3T3 cells. In contrast, the activity of cathepsin L does not return to the level in non-treated cells even after 24 h in normal culture medium. Changes in the enzyme activities correspond well to the appearance of the mature enzyme forms. The recovery of enzyme activities suppressed in the presence of various proteinase inhibitors: the activity of cathepsin B was suppressed by both E-64-d and CA-074, cathepsin D by both Pepstatin A and E-64-d, and cathepsin L by E-64-d. The molecular forms of the proteinases suggest that the suppression of the enzyme activities of these proteinases can be divided into two categories, inhibition of the mature enzyme and prevention of the processing of proenzymes into mature enzymes. The former is observed in the case of the inhibition of cathepsin B by CA-074 and cathepsin D by Pepstatin A, the latter case is observed for the inhibition of cathepsins B and D by E-64-d. The inhibition of cathepsin L by E-64-d is due to a mixture of both mechanisms (Fig. 3). These results conflict with those of previous studies involving *in vitro* processing and a pulse-chase technique (*5-9*). These discrepancies derive partially from the experimental conditions: In our study, the proforms of cathepsins are presumably restored at the *trans*-Golgi network and cotransported to late Golgi com-

partments including early and late endosomes and lysosomes where there are no powerful endopeptidases under our conditions. We did not use metallopeptidase inhibitors, since these agents affect trafficking from the *trans*-Golgi network to lysosomes (*38*). There are four candidates for the processing proteinases, the first of which is cathepsin H. Cathepsin H is sensitive to E-64-d but not to CA-074, is active as an endopeptidase at neutral pH, is expressed ubiquitously, and is localized in early endosomes in J774 macrophages (*39*). The second candidate is cathepsin S, which is also sensitive to E-64-d but not to CA-074, works in the invariant chain degradation in endosomes, although the expression of cathepsin S is restricted to the spleen, macrophages, and dendritic cells (*40, 41*). The third candidate is cathepsin C, which is also sensitive to E-64-d but not to CA-074, and whose mRNA is expressed ubiquitously; it shows endopeptidase activity even at neutral pH (*42, 43*). The last candidate is procathepsin L itself. Previously, we reported that procathepsin L itself can degrade extracellular matrix proteins on surface materials (*4*). In late endosomes and lysosomes, many integral glycoproteins possess sialic acid at the ends of their sugar side chains, which might be a substitute for surface materials in a certain compartment (*44*). Further, other tissue specific cysteine proteinases, such as cathepsin W, might participate in the tissue-specific processing of procathepsins (*45*). These hypotheses can be clarified by further analyses using specific inhibitors and pulse-chase analyses in fibroblasts isolated from knock out mice (*15, 46, 47*).

VacA toxin causes homotypic fusion of the intracellular Rab7-positive organelles and the accumulation of enlarged intracellular vacuoles (*25, 26*). Rab 7 localizes on the cytosolic side of late endosomal membranes (*48-50*). Since the *de novo* synthesized precursor forms of lysosomal proteins are transported from the *trans*-Golgi network to lysosomes through early and late endosomes dependent on mannose 6-phosphate receptor, procathepsins B, D, and L are transferred to lysosomes *via* early and late endosomes (*51, 52*). In the presence of cells treated with VacA after Bafilomycin A1 treatment, both procathepsins B and D are converted to their mature forms; in contrast, procathepsin L retains its precursor forms. These results suggest that the processing of both procathepsins B and D occurs in late endosomes, while that of procathepsin L occurs after its arrival in lysosomes. These observations correspond with previous reports: both cathepsins B and D are localized in endosomes of both hepatocytes and the antigen presenting cells (*12, 53*).

In this paper, we show that the processing of procathepsins B and D occurs in the endosomal compartment while that of procathepsin L occurs in lysosomes. These processing events are promoted by cysteine proteinases other than cathepsin B. Further analyses involving enzyme-specific inhibitors are necessary to identify the processing proteinase. To confirm the processing site *in situ*, we plan to analyze the processing of procathepsins in Rab mutant cells after treatment with Bafilomycin A1.

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