Cathepsin E Deficiency Induces a Novel Form of Lysosomal Storage Disorder Showing the Accumulation of Lysosomal Membrane Sialoglycoproteins and the Elevation of Lysosomal pH in Macrophages*

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Cathepsin E, an endolysosomal aspartic proteinase predominantly expressed in cells of the immune system, has an important role in immune responses. However, little is known about the precise roles of cathepsin E in this system. Here we report that cathepsin E deficiency (CatE−/−) leads to a novel form of lysosome storage disorder in macrophages, exhibiting the accumulation of the two major lysosomal membrane sialoglycoproteins LAMP-1 and LAMP-2 and the elevation of lysosomal pH. These striking features were also found in wild-type macrophages treated with pepstatin A and Ascaris inhibitor. Whereas there were no obvious differences in their expression, biosynthesis, and trafficking between wild-type and CatE−/− macrophages, the degradation rates of these two membrane proteins were apparently decreased as a result of cathepsin E deficiency. Because there was no difference in the vacuolar-type H\(^+/\)ATPase activity in both cell types, the elevated lysosomal pH in CatE−/− macrophages is most likely due to the accumulation of these lysosomal membrane glycoproteins highly modified with acidic monosaccharides, thereby leading to the disruption of non-proton factors controlling lysosomal pH. Furthermore, the selective degradation of LAMP-1 and LAMP-2, as well as LIMP-2, was also observed by treatment of the lysosomal membrane fraction isolated from wild-type macrophages with purified cathepsin E at pH 5. Our results thus suggest that cathepsin E is important for preventing the accumulation of these lysosomal membrane sialoglycoproteins that can induce a new form of lysosomal storage disorder.

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The endolysosome system represents the final destination for many endocytic, autophagic, and secretary molecules targeted for destruction or recycling (15). This system therefore contributes to the maintenance of homeostasis via numerous functions, including the supply of nutrients, the turnover of cellular proteins, the elimination of defective or unfavorable molecules, and the down-regulation of surface receptors (16, 17). These organelles are extremely unique in containing a variety of acidic hydrolases able to degrade or modify the transported macromolecules. Accordingly, the limiting membranes of the endolysosomal organelles most likely not only protect other cellular constituents against the attack by these potent hydrolases but also serve to maintain the acidification of the endolysosomal lumen (16, 18). Intriguingly, the endolysosomal membrane contains several unique integral proteins such as...
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LAMP-1,3 LAMP-2, and LIMP-2/LGP85, whose luminal domains are heavily N-glycosylated with complex poly-N-acetyllactosamines (19, 20). Of further importance, these membrane proteins represent more than 50% of the total membrane proteins of endolysosomes (18, 20) and their glycosylation constitutes about 60% (LAMP-1 and LAMP-2) and 20% (LIMP-2/LGP85) of the total mass of the respective molecules (21), and for the most part are present on the intraluminal side of endosomes and lysosomes, suggesting their significance in protecting the membrane from degradation by lysosomal hydrolases. To better understand the role of cathepsin E in the endolysosomal system, therefore, it was of particular importance for us to investigate the effect of cathepsin E deficiency on expression, trafficking, localization, and turnover of these membrane proteins as well as lysosomal soluble enzymes. We herein report that cathepsin E deficiency leads to a novel form of lysosome storage disorder in macrophages, exhibiting the accumulation of major lysosomal membraneialoglycoproteins, including LAMP-1, LAMP-2, and LIMP-2, and the elevation of lysosomal pH. We also found that the trafficking of soluble lysosomal proteins to lysosomes was partially impaired in CatE-/- macrophages. To address the mechanism underlying these consequences, we determined the synthesis, expression, and turnover of the lysosomal membrane proteins in CatE-/- macrophages in comparison to those of the wild-type cells. In addition, to determine whether cathepsin E is directly involved in the degradation of these lysosomal membrane proteins, the effects of its inhibitors on the cellular levels of lysosomal membrane proteins and the lysosomal pH in wild-type macrophages. Our results indicate that cathepsin E is essential for degradation of these lysosomal membrane proteins, and that its deficiency is implicated in the development of a new form of lysosomal storage disorder associated with the elevation of lysosomal pH.

EXPERIMENTAL PROCEDURES

Materials—Pepstatin A was purchased from Peptide Institute Inc. (Osaka, Japan). Bafilomycin A1 and polyclonal antibodies to V-ATPase (A-subunit) were from Wako Pure Chemicals (Tokyo, Japan). [35S]Methionine was from Amersham Biosciences. Recombinant Ascaris pepsin inhibitor was kindly donated by Dr. Takashi Kagayama, Kyoto University. Antibodies to mouse LAMP-1 and LAMP-2 were from Southern Biotechnology Inc. (Birmingham, AL). Antibodies to rat LIMP-2/LGP85, LAP, and MPR300 were kindly donated by Dr. Masaru Himeno and Dr. Yoshitaka Tanaka, Kyushu University. Antibodies to actin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies to rat cathepsins B and D have been described previously (22).

Animals—Wild-type and CatE-/-mice on C57BL/6 genetic background were used as described previously (14). All animals were maintained according to the guidelines of the Japanese Pharmacological Society. The animals and all experiments were approved by the Animal Research Committee of Graduate School of Dental Science, Kyushu University.

Preparation of Peritoneal Macrophages—Thioglycollate-elicited peritoneal macrophages were isolated from mice as described previously (3). Briefly, 8–14-week-old mice were injected peritonally with 4.05% thioglycollate (2 ml/mouse). Three and one-half days later, peritoneal exudate cells were isolated from the peritoneal cavity by washing with phosphate-buffered saline (PBS). The cells were incubated with RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (50 units/ml), and streptomycin (50 µg/ml) at 37 °C with 5% CO₂. After incubation for 2 h, non-adherent cells were removed by washing with Ca²⁺/Mg²⁺-free PBS three times. Peritoneal macrophages isolated as adherent MAC-2-positive cells were obtained at a purity of greater than 95% by this procedure.

Preparation of Media and Cell Lysates—The culture media of macrophages were collected after 24 h and centrifuged at 16,000 × g for 20 min. The supernatant fraction was concentrated at 10-fold using Centriprep-30 and Microcon-30 concentrators (Millipore Co. Bedford, MA). For the preparation of the cell lysates, the cells were washed twice with PBS, removed from the plates with a rubber scraper, and centrifuged at 300 × g for 5 min. The precipitated cells were resuspended in PBS containing 0.1% Triton X-100, and then subjected to sonication for 1 min at 4 °C followed by centrifugation at 100,000 × g for 1 h. The supernatant fraction is referred to as the cell lysate. For SDS-PAGE or immunoblot analyses, the supernatant was precipitated with trichloroacetic acid at a final concentration of 5% and centrifuged at 12,000 × g for 15 min after incubation on ice for 15 min. After washing with ice-cold acetone and evaporating with air, the pellets were suspended in the buffer for SDS-PAGE. The cells were washed twice with PBS, removed from the plates by pipetting, and then subjected to centrifugation at 300 × g for 5 min. The precipitated cells were suspended in PBS containing 0.05% Triton X-100, sonicated 3 times for 5 s at 4 °C, and subjected to centrifugation at 120,000 × g for 30 min at 4 °C, the supernatant fraction was used as the cell lysate.

Preparation of Lysosomal Membranes from Macrophages—The lysosomal membrane fraction was isolated from wild-type macrophages according to the method by Ohsumi et al. (23) with some modifications. Briefly, after suspension in a solution containing 0.25 M sucrose and 0.2 M KCl, wild-type macrophages were homogenized with Potter-Elvehjem type homogenizer. After centrifugation at 650 × g for 10 min, the supernatant was centrifuged at 11,000 × g for 20 min. The pellet was suspended with a dilute solution containing 25 mm sucrose and 20 mm KCl to be lysed, and then further centrifuged at 11,000 × g for 20 min. The supernatant was referred to as the crude lysosome fraction. After addition of CaCl₂ (a final concentration of 10 µm), this fraction was further centrifuged at 1,500 × g for 10 min, and then the supernatant was centrifuged at 5,000 × g for 10 min. Then, the supernatant was centrifuged at 10,000 × g for 30 min and subsequently at 50,000 × g for 30 min. The supernatant was further centrifuged at 105,000 × g for 30 min, the resultant supernatant was referred to as the final
lyosomal membrane fraction, which were almost free from mitochondria, peroxisomes, and endoplasmic reticulum (23).

Degradation of Lysosomal Membrane Proteins by Purified Cathepsin E—The lysosomal membrane fractionation (50 µg) was incubated with purified cathepsin E (0–2 µg) (24) in 0.1 M sodium acetate buffer (pH 5.0) at 25 °C for 12 h. The reaction was stopped by addition of 2.0 M Tris-HCl buffer (pH 9.0) to give a final concentration of 0.2 M. Then, the samples were subjected to SDS-PAGE and Western blot analyses.

Enzyme Assays—Cathepsins B and L were assayed as described previously (22). β-Glucuronidase was assayed by the method of Robins et al. (25). Cathepsin D activity was determined using MOCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-d-Arg-NH₂ (Peptide Institute, Inc., Osaka, Japan) as a substrate according to the method described previously (26). β-Hexosaminidase and α-mannosidase were assayed with 4-methylumbelliferyl-β-d-glucopyranoside and 4-methylumbelliferyl-α-d-mannopyranoside as synthetic substrates essentially according to the method of Robins et al. (25).

Pulse-Chase Analysis—Pulse-chase experiments were performed as described previously (27). Briefly, the cells were preincubated for 1 h at 37 °C in Dulbecco’s modified Eagle’s medium without methionine supplemented with 10% fetal bovine serum and then pulse-labeled for 30 or 60 min with [35S]methionine (100 µCi/ml) and chased in fresh RPMI 1640 medium supplemented with 10% fetal bovine serum (1.5 ml/plate). At the times indicated, the cells were separated from the medium, washed twice with PBS, lysed in PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.02% sodium azide, and a proteinase inhibitor mixture (1 mg/ml of each inhibitor: antipain, chymostatin, leupeptin, pepstatin, phenylmethylsulfonyl fluoride), and then subsequently sonicated for 1 min.

Immunoprecipitation—The cell lysates and media were mixed with 40 µl of Pansorbin for 1 h at 4 °C to prevent nonspecific binding to IgG-protein A beads, and then centrifuged at 6,500 × g for 30 min. For immunoprecipitation of LAMP-1 and LAMP-2, the supernatant fractions were incubated with 10 µl of each monoclonal antibody at 37 °C for 10 min, and then stored at 4 °C for 16 h. The mixtures were further incubated with 20 µg of goat anti-rat antibody at 37 °C for 10 min and stored at 4 °C for 3 h. For the immunoprecipitation of cathepsins B and D, the supernatant fractions were incubated with 10 µl of each polyclonal antibodies at 37 °C for 10 min, and then stored at 4 °C for 16 h. Immune complexes were adsorbed onto protein A-Sepharose beads (50% gel suspension) at 4 °C for 3 h with gentle agitation, followed by three washes with 0.1% SDS, 0.1% Triton X-100, 200 mM EDTA, 10 mM Tris-HCl (pH 7.5). The immune precipitates were washed 3 more times with the same buffer containing 1 M NaCl and 0.1% sodium lauryl sarcosinate and twice with 5 mM Tris-HCl (pH 7.0). The beads were boiled for 5 min at 100 °C with 50 µl of 0.1% SDS, 0.5 mM EDTA, 5% sucrose, 5 mM Tris-HCl (pH 8.0) with 2-mercaptoethanol.

Gel Electrophoresis and Immunoblot Analysis—SDS-PAGE and immunoblotting were performed as described previously (27). The quantification of the immunoreactive bands was analyzed by LAS 1000 and Image Gauge software (Fuji Photo Film Co., Ltd., Tokyo, Japan).

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Two-dimensional Electrophoresis and Proteomic Analyses—The culture supernatant was applied to immobilized pH gradient gel strips (Amersham Biosciences) and then subjected to isoelectric focusing using a Multiphor II (Amersham Biosciences). After isoelectric focusing, the strips were equilibrated for 15 min in 50 mM Tris-HCl (pH 8.8), containing 6 M urea, 30% glycerol, 1% SDS, and 64 mM dithiothreitol and subsequently immersed for 15 min in the same buffer containing 135 mM iodoacetamide instead of dithiothreitol. The strips were then transferred onto 10% SDS-polyacrylamide gels. After electrophoresis, the gels were silver-stained for proteins. Peptide mass mapping was performed by recording the peptide mass fingerprints of typical in-gel digests of the corresponding gel bands using matrix-assisted laser desorption ionization time-of-flight MS (AXIMA-CFR plus, Shimadzu, Tokyo, Japan) and the subsequent use of the mascot search engine (Matrix Science, Tokyo, Japan).

Measurement of Endolysosomal pH—The endolysosomal pH in macrophages was determined in situ by the method of Chen et al. (28) with some modifications. Briefly, after plating on a 96-well plate, macrophages were incubated with 500 µg/ml of an acidotropic probe, Lysosensor yellow/blue dextran (Molecular Probes, Eugene, OR) for 24 h and then washed with PBS. The fluorescence from the acidic compartments in the labeled cells was quantified with a fluorescence microplate reader at an emission wavelength of 430/535 nm with excitation at 340 nm (Wallac 1420 ARVOx, PerkinElmer Inc., Wellesley, MA). To confirm the validity of a pH titration curve, Chinese hamster ovary cells were also incubated with Lysosensor yellow/blue dextran for 24 h and then treated with 10 µM monensin and 0.5 µM bafilomycin A₁. These cells were treated for 20 min with the equilibration buffers consisting of 5 mM NaCl, 115 mM KCl, 1.2 mM MgSO₄, and 25 mM MES varied between pH 4.5 and 7.0. After incubation, the fluorescence intensity was determined.

Measurement of V-ATPase Activity by Immunoprecipitation—Both wild-type and CatE⁻/⁻ macrophages (1 × 10⁶ cells) were lysed with 0.1% Triton X-100 in PBS, and the total ATPase activity was then measured by the method of Ramirez-Monreal and Pearce (29). The cell lysates (50 µg) were mixed with 0–20 µl of anti-V-ATPase A subunit antibody, which was raised using a synthetic peptide corresponding to amino acid sequence 360 AEMPADSGYPAYLGARS 381 of bovine V-ATPase and exhibited cross-reaction with V-ATPase of most organisms such as animals, bacteria, and plant, but not with other ATPases such P-ATPase and F-ATPase, and incubated at 37 °C for 10 min, and stored at 4 °C for 3 h. The immune complexes were adsorbed onto 20 µl of protein A-Sepharose beads (50% gel suspension) at 4 °C for 3 h with gentle agitation. After centrifugation to remove immunoprecipitates, ATPase activity remaining in the supernatant was remeasured. The amount of V-ATPase could be calculated from the amount of ATPase activity remaining in the supernatant. The enzyme unit, U, was defined as micromole of Pi liberated per min per ml.

mRNA Extraction and Quantitative Real-time PCR—The total mRNA of the cells was prepared using a RNasy kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. Complementary DNA was synthesized using 1 µg of total...
RNA incubated with random hexamers, followed by a 50-μl reverse transcription reaction with 6.25 units of Multiscribe reverse transcriptase (Applied Biosystems) at 25 °C for 10 min for binding, at 48 °C for 30 min for reverse transcription, and at 95 °C for 5 min for inactivation, respectively. The cDNA products were amplified using the following oligonucleotide primers for mouse LAMP-1, LAMP-2, and LIMP-2 transcripts. The cDNA samples (1 μl) were added to 12.5 μl of 2X SYBR Green PCR Master Mix, 1 μl of 10 μM for the 5′- and 3′-primers to give a total volume of 25 μl. All reactions were performed in duplicate in the ABI PRISM 7000 Sequence Detector (Applied Biosystems). The thermal cycling was performed at 50 °C for 2 min, at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s plus at 56 °C for 1 min. Real-time PCR data were analyzed for at least three different experiments.

**Immunofluorescence Microscopy**—The cells were grown on glass coverslips and briefly washed with PBS and then fixed with 4.0% paraformaldehyde in PBS for 30 min at room temperature. The fixed cells were washed and permeabilized with 50 mM NH4Cl and 0.3% Tween 20 in PBS. The cells were incubated with 1% bovine serum albumin plus 1% normal goat serum for 3 h, and subsequently incubated with the primary antibodies overnight at 4.0 °C followed by fluorescein-labeled secondary antibodies, and then inspected by microscopy using a laser-scanning confocal imaging system (Leica TCS, Leica-Microsystems, Heidelberg, Germany).

**Statistical Analysis**—Quantitative data are presented as mean ± S.D. The statistical significance of differences between mean values was assessed by Student’s t test. p values of <0.05 were considered statistically significant.

**RESULTS**

**Effect of Cathepsin E Deficiency on the Cellular Levels of Major Lysosomal Membrane Glycoproteins**—To determine the effect of cathepsin E deficiency on intracellular levels of lysosomal membrane proteins, we performed SDS-PAGE and immunoblot analysis for the cell lysates of wild-type and CatE−/− macrophages. Two major lysosomal membrane sialoglycoproteins were identified and quantified. 

**FIGURE 1. The effect of cathepsin E deficiency on the intracellular levels of various endolysosomal membrane proteins in macrophages.** A, the cell lysates derived from wild-type (+/+ ) and CatE−/− macrophages (100 μg of protein for each) were subjected to SDS-PAGE followed by immunoblotting with specific antibodies to LAMP-1, LAMP-2, LIMP-2, LAP, V-ATPase (A subunit), MPR 300, and actin. The data indicate the representative immunoblots of five independent experiments. B, a densitometric analysis for the quantification of each protein in the cell lysate of both cell types. The arbitrary density unit was defined as the relative chemiluminescence intensity per mm² measured by LAS1000. The data are the mean ± S.D. of values from five independent experiments. *, p < 0.01 for the indicated comparisons.
proteins, LAMP-1 and LAMP-2, were highly increased in CatE⁻/⁻ macrophages compared with the wild-type cells (Fig. 1). Another major lysosomal membrane sialoglycoprotein LIMP-2 was also increased, but not significantly, in CatE⁻/⁻ macrophages. In contrast, cellular levels of other endolysosomal membrane glycoproteins, including lysosomal acid phosphatase (LAP), which is known to be synthesized and transported to lysosomes as an integral type I membrane protein and slowly released its luminal domain into the lysosomal lumen by proteolytic processing (19), V-ATPase A-subunit, which is an integral endolysosomal membrane protein essential for V-ATPase activity (30, 31), and cation-independent mannose 6-phosphate receptor (MPR300), which is known as a type I integral membrane protein found mainly in the trans-Golgi network and plays a critical role in the intracellular trafficking of soluble lysosomal hydrolases (32), were not different between wild-type and CatE⁻/⁻ macrophages.

Elevation of Lysosomal pH in CatE⁻/⁻ Macrophages—The luminal acidic pH is essential for the normal function of intracellular acidic organelles including endosomes and lysosomes (16). Increasing evidence suggests that the accumulation of undegraded metabolites in lysosomal compartments induces an elevated lysosomal pH, thereby impairing the functions of the endolysosomal system (33). We therefore analyzed the lysosomal pH in CatE⁻/⁻ macrophages using an acidotropic fluorescent probe, namely, Lysosensor yellow/blue dextran. The lysosomal pH of the wild-type cells was estimated to be 5.3 ± 0.4, which closely agreed with the findings previously reported in macrophages (34), whereas that of CatE⁻/⁻ macrophages were 6.4 ± 0.3 (Fig. 2A), indicating the strong induction of the elevated lysosomal pH as a result of cathepsin E deficiency. We further analyzed whether this elevation of lysosomal pH was due to the disruption of V-ATPase, which acts as a major regulating factor for the acidification of the lysosomal lumen (31). Total ATPase activity in CatE⁻/⁻ macrophages was comparable with that of wild-type cells (Fig. 2B). After immunoprecipitation with anti-V-ATPase A subunit antibody, ATPase activity remaining in the supernatant was remeasured in the respective cell lysates. Given no reaction of this antibody with other ATPase types such as P-ATPase and F-ATPase, ATPase activity in the immunoprecipitates calculated from the amount of V-ATPase, which acts as a major regulating factor for the acidification of the lysosomal lumen (31). Total ATPase activity in CatE⁻/⁻ macrophages was comparable with that of wild-type cells (Fig. 2B). After immunoprecipitation with anti-V-ATPase A subunit antibody, ATPase activity remaining in the supernatant was remeasured in the respective cell lysates. Given no reaction of this antibody with other ATPase types such as P-ATPase and F-ATPase, ATPase activity in the immunoprecipitates calculated from the amount of V-ATPase, which acts as a major regulating factor for the acidification of the lysosomal lumen (31).

Effect of Proteinase Inhibitors on Cellular Levels of Major Lysosomal Membrane Proteins in Wild-type Macrophages—We next explored whether cathepsin E is directly involved in the degradation of these lysosomal membrane sialoglycoproteins. When wild-type macrophages were cultured for 24 h in the presence of pepstatin A, a potent inhibitor of the aspartic proteinase family, we found the accumulation of LAMP-1, LAMP-2, and LIMP-2, but not LAP, within the cells in a dose-dependent manner (Fig. 3A). In contrast, pepstatin A did not induce an additional increase in the cellular levels of all the lysosomal membrane proteins examined in CatE⁻/⁻ macrophages. Similar results were obtained when the effect of Ascaris pepsin inhibitor (100 μM), a strong inhibitor of cathepsin E but not cathepsin D, was analyzed on wild-type and CatE⁻/⁻ macrophages (Fig. 3B). These results indicate that cathepsin D is not directly involved in the accumulation of these lysosomal membrane proteins in the cells. Furthermore, of interest is the observation that lysosomal pH in wild-type macrophages treated with pepstatin A increased in comparison to that in the

**FIGURE 2. Lysosomal pH and V-ATPase activity in wild-type and CatE⁻/⁻ macrophages.** A, the intralysosomal pH measurements were performed by the addition of Lysosensor yellow/blue dextran to wild-type and CatE⁻/⁻ macrophages followed by incubation for 24 h. After washing with PBS, the fluorescence in each cell type was measured by the emission intensity ratio at 430 and 535 nm using an excitation at 340 nm. *, p < 0.01 versus the wild-type cells. B, the measurement of V-ATPase activity in wild-type and CatE⁻/⁻ macrophages by immunoprecipitation (IP) with anti-V-ATPase A subunit antibody. ATPase activity in cell lysates form both cell types (1 × 10⁵ cells) is expressed as total units (left). The amount of V-ATPase activity calculated from the amount of ATPase activity remaining in the supernatant after immunoprecipitation was expressed as percentages of the total ATPase activity in both cell types (right). The data are the mean ± S.D. of values from five independent experiments.
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A

\[ \text{LAMP-1} \]

\[ \frac{+/+}{-/-} \]

\[ \frac{+}{-} \cdot \star \]

\[ \frac{0}{10} \cdot 100 \]

Arbitrary densitometry units

Peptatin A (μM)

\[ \frac{+}{-} \cdot \star \]

B

\[ \text{LAMP-1} \]

\[ \frac{+/+}{-/-} \]

\[ + \cdot + \cdot + \]

Arbitrary densitometry units

API:

\[ ++ \cdot -/- \]

\[ +/+/+ \cdot -/- \]

\[ +/+-/- \cdot + \]

\[ +/+/+ \cdot -/- \]

C

\[ \frac{+/+}{-/-} \]

\[ \text{Peptatin A} \]

\[ - \cdot + \cdot - \cdot + \]

\[ \text{pH} \]

\[ 4 \cdot 5 \cdot 6 \cdot 7 \]

D

\[ \text{pH} \]

\[ 4 \cdot 5 \cdot 6 \cdot 7 \]

\[ 0 \cdot 6.25 \cdot 12.5 \cdot 25 \cdot 50 \cdot 100 \cdot 200 \cdot 400 \]

Bafilomycin A1 (nM)
non-treated cells (5.8 ± 0.4 versus 5.3 ± 0.4) (Fig. 3C). The lysosomal pH levels in CatE−/− macrophages treated with pepstatin A also increased in comparison to that in non-treated CatE−/− macrophages (7.0 ± 0.3 versus 6.4 ± 0.3), implying an additive effect of cathepsin E deficiency and pepstatin A on the elevation of lysosomal pH. The finding that the extent of the increase in lysosomal pH by pepstatin A was smaller than that by cathepsin E deficiency suggests that this agent may not be distributed into endolysosomes ubiquitously, most probably due to the heterogeneity or complexity of this system. Therefore, our results indicate that the inhibition of aspartic proteinases, especially cathepsin E, leads to the elevation of lysosomal pH in macrophages.

It has previously been demonstrated that procathepsins D and H in primary cultured rat hepatocytes were mostly secreted into the medium by enhanced lysosomal pH upon treatments with bafilomycin A1 (at the order of millimolar), where no mature forms were found within the cells (36). These results were apparently different from our results indicating that cathepsins B and D in CatE−/− macrophages were mostly processed (Fig. 5B). To shed light on why procathepsins B and D were normally processed in CatE−/− macrophages despite the elevated lysosomal pH, we assessed the effect of different concentrations of bafilomycin A1 on lysosomal pH in wild-type macrophages. As shown in Fig. 3D, lysosomal pH was dose-dependently increased by bafilomycin A1 and attained the maximal value (pH 7.5) at 50 nM. Given that the elevated lysosomal pH induced by cathepsin E deficiency was far lower than that by bafilomycin A1 (pH 6.4 versus 7.5), the discrepancy in previous studies and our results is more likely due to the difference in the extent of the elevation of lysosomal pH between bafilomycin A1-treated cells and CatE−/− macrophages. Therefore, our data suggest that normal processing of cathepsins B and D within CatE−/− macrophages may be impaired when the relative increase of lysosomal pH is higher than the value with cathepsin E deficiency.

Expression, Biosynthesis, and Degradation of Major Lysosomal Membrane Glycoproteins—To study the mechanism for the accumulation of the lysosomal membrane sialoglycoproteins induced by cathepsin E deficiency, we analyzed the expression, biosynthesis, localization, and degradation of these proteins. Quantitative real-time PCR revealed that the mRNA levels of LAMP-1 and LAMP-2, like LIMP-2, were identical between wild-type and CatE−/− macrophages (Fig. 4A), implying that their accumulation in CatE−/− macrophages are not due to either an enhanced gene expression or transcriptional stability. When both cell types were pulse-labeled with [35S]methionine for 1 h and then chased up to 12 h, newly synthesized LAMP-1 and LAMP-2 proteins were time dependently decreased in both cell types but these remained more stable in CatE−/− macrophages than the wild-type cells (Fig. 4B). The half-lives of LAMP-1 and LAMP-2 in CatE−/− macrophages
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A

Cathepsin B Activity (U/mg)

\[+/+\] \[+/-\] \[-/-\]

Cathepsin D Activity (U/mg)

\[+/+\] \[+/-\] \[-/-\]

Cathepsin L Activity (U/mg)

\[+/+\] \[+/-\] \[-/-\]

* indicates a significant difference.

B

Protein level (ng) per 10^6 cells

**cell**

**medium**

**pro**

**mature**

CATHEPSIN B

**cell**

**medium**

Protein level (ng) per 10^6 cells

**pro**

**mature**

**cell**

**medium**

CATHEPSIN D

Protein level (ng) per 10^6 cells

**pro**

**mature**

* indicates a significant difference.

C

Chase (h)

kDa

Cathepsin B

**+/+**

**+/-**

**-/-**

Cell

Medium

0 1 3 6

1 3 6

CATHEPSIN B

**+/+**

**+/-**

**-/-**

CATHEPSIN D

**+/+**

**+/-**

**-/-**

D

**+/+**

**-/-**

LAMP1

Cath.B

Merge

LAMP1

Cath.B

Merge

Cath.B

LAP

Merge

Cath.B

LAP

Merge

LAMP1

LAP

Merge

LAMP1

LAP

Merge
were more than 2-fold those in the wild-type cells. Our results thus indicate that the accumulation of these two lysosomal membrane proteins in CatE⁻/⁻ macrophages is most likely due to their reduced turnover rates.

**Effect of Cathepsin E Deficiency on the Biosynthesis and Traffic of Soluble Lysosomal Proteins**—A number of cell biological studies have demonstrated that the intracellular trafficking of soluble lysosomal enzymes is mediated mainly by mannose 6-phosphate receptors (16, 32), whereas lysosomal membrane glycoproteins are transported from the trans-Golgi network or the plasma membrane by different mechanisms involving the interaction of polypeptide motifs in their cytoplasmic domains with cystolic receptors (19). Given the importance of the acidification of endolysosomal compartments for the normal processing and targeting of soluble lysosomal enzymes (16), we assumed that the elevated lysosomal pH in CatE⁻/⁻ macrophages might perturb the biosynthesis and intracellular trafficking of soluble lysosomal enzymes. We therefore determined the protein and activity levels of various soluble lysosomal enzymes in CatE⁻/⁻ macrophages. The activity levels of cathepsins B, D, and L in CatE⁻/⁻ macrophages were significantly lower than those in the wild-type cells (Fig. 5A). Consistently, the protein levels of these enzymes significantly decreased in CatE⁻/⁻ macrophages in comparison to the wild-type cells, as judged from Western blotting (Fig. 5B). It is important to note, however, that the majority of these cathepsins retained in the cells were normally processed to yield the respective mature forms, as determined by pulse-chase experiments with [³⁵S]methionine (Fig. 5C). Newly synthesized cathepsins B and D as proenzymes of 38 and 46 kDa were normally processed to 32- and 42-kDa single chain forms, respectively, within a chase for 3 h in both cell types. These processed forms remained unchanged even after a 6-h chase. The two-chain forms of cathepsins B and D were not detected under our experimental conditions. A confocal microscopic study also revealed that cathepsin B was mostly found in LAMP-1-positive organelles in both cell types (Fig. 5D). Cathepsin B, likewise LAMP-1, was also found to be colocalized with LAP, which was not affected by cathepsin E deficiency. These results indicate that the localization of cathepsin B within the cells is not affected by cathepsin E deficiency. In this connection, the expression (Fig. 1A) and localization of MPR300 was not significantly changed by a cathepsin E deficiency (data not shown). These results thus suggest that CatE⁻/⁻ macrophages retain the normal biosynthetic and processing machinery for soluble lysosomal enzymes.

We next examined whether a significant decrease in intracellular levels of soluble lysosomal enzymes in CatE⁻/⁻ macrophages was linked to their enhanced secretion. To identify proteins that had been increasingly secreted by cathepsin E deficiency, we used high resolution two-dimensional gel electrophoresis within the range of pl 4.5–7 and 15–200 kDa and compared the proteomic profiles of the culture supernatants from both wild-type and CatE⁻/⁻ macrophages. After silver staining, more than 300 spots were resolved in each gel. After the normalization of the spot volumes, we identified two series of protein spots having apparent molecular masses of 45 kDa (pl 5–6) (a) and 38 kDa (pl 4.5–5.5) (b) that were significantly increased in CatE⁻/⁻ macrophages in comparison to the wild-type cells (Fig. 6A). These spots exhibited a high reproducibility and more than 2-fold changes in abundance between the two cell types, and therefore were excised from two-dimensional gels, digested with trypsin, and then analyzed by mass spectrometry. Of the protein spots identified, the 5 main spots seen in region a were found to be identical with procathepsin D, whereas the 5 spots in region b were all identified as procathepsin B. The presence of such multiple spots for procathepsins D and B are most likely due to the heterogeneity of their carbohydrate moieties. These results indicate that cathepsin E deficiency significantly induces the enhanced secretion of these cathepsins as proenzymes. We additionally analyzed the extracellular levels of soluble lysosomal glucosidases, such as β-glucuronidase, β-hexosaminidase, and α-mannosidase in both cell types. The activity levels of these enzymes in the culture supernatant of CatE⁻/⁻ macrophages were also more than 2-fold those in the wild-type cells (Fig. 6B).

**Degradation of Lysosomal Membrane Proteins by Cathepsin E**—Finally, we performed experiments showing the selective degradation of LAMP-1 and LAMP-2 by cathepsin E by using the lysosomal membrane fraction isolated from wild-type macrophages. As shown in Fig. 7, LAMP-1 and LAMP-2, as well as LIMP-2, were dose-dependently degraded by cathepsin E upon incubation of the lysosomal membrane fraction (50 μg) with cathepsin E (0–2 μg) at pH 5.0 and 25 °C for 12 h. In contrast, LAP was not significantly degraded under the same conditions.

**DISCUSSION**

In this study, we examined the effect of cathepsin E deficiency on the structural and functional integrity in the endolysosomal system of macrophages. This deficiency was first characterized by the specific accumulation of the two major lysosomal membrane sialoglycoproteins LAMP-1 and LAMP-2 in the cells (Fig. 1). Another major lysosomal membrane sialoglycoprotein LIMP-2 also tended to increase in CatE⁻/⁻ macrophages compared with the wild-type cells. However, we did not detect an increase in other membrane proteins including LAP, V-ATPase, and MPR 300. Whereas there were no significant dif-

![Figure 5](image-url) **Effect of cathepsin E deficiency on the intracellular levels of various soluble cathepsins in macrophages.** A, activity levels of cathepsins B, D, and L in the cell lysates of wild-type and CatE⁻/⁻ macrophages. The data are shown as the mean ± S.D. of the values from five independent experiments. *, p < 0.05 versus the wild-type cells. B, densimetric analyses of immunoblots for quantification of each protein in the cell lysates and media of both cell types. The amounts of cathepsins B and D were determined by LAS1000 from the intensity of known amounts of the respective purified enzymes as standards. The data are the mean ± S.D. of values from four independent experiments. *, p < 0.01 for the indicated controls. Closed and open columns indicate wild-type and CatE⁻/⁻ macrophages, respectively, C, both macrophages were metabolically labeled with [³⁵S]methionine for 30 min and then chased for the times indicated. The cell lysate and culture medium from each cell type were immunoprecipitated with antibodies specific for cathepsins B or D. The immunoprecipitates were then analyzed by SDS-PAGE and fluorography. *, p < 0.01 versus the wild-type cells, D, immunofluorescence microscopy of cathepsin B, LAP, and MPR 300 in wild-type, and CatE⁻/⁻ macrophages. The cells on glass coverslips were fixed, permeabilized with 0.3% Tween 20 in PBS, and then allowed to react with antibodies to each protein. After being washed, the cells were incubated for 3 h with isothiocyanate-conjugated secondary antibodies or tetramethylrhodamine isothiocyanate-conjugated secondary antibodies, and then visualized by confocal laser microscopy.
ferences in the mRNA levels of LAMP-1 and LAMP-2, as well as LIMP-2, and their biosynthesis processes between wild-type and CatE/H11546/H11546 macrophages, we found the marked decrease in the turnover rates of LAMP-1 and LAMP-2 in CatE/H11546/H11546 macrophages compared with wild-type cells (Fig. 4), implying that the accumulation of these membrane proteins is most probably due to the reduced turnover rates by cathepsin E deficiency. In the present study, we also addressed the question of whether cathepsin E was directly involved in the degradation of LAMP-1 and LAMP-2. Our results indicate that LAMP-1 and LAMP-2, as well as LIMP-2, but not LAP, also accumulated in wild-type macrophages upon treatment with pepstatin A and Ascaris pepsin inhibitor (Fig. 3, A and B). In contrast to wild-type cells, CatE/H11546/H11546 macrophages showed no additional increase in the cellular levels of these proteins by treatment with these inhibitors. Taken together with the recent knowledge that congenital ovine neuronal ceroid lipofuscinoses, which is caused by a mutation in the cathepsin-D gene (37), and a novel type of neuronal ceroid lipofuscinoses observed with cathepsin D-deficient mice (38, 39) showed no significant accumulation of these lysosomal membrane proteins, our results suggest that cathepsin E is directly involved in the degradation of these lysosomal membrane proteins in macrophages.

To better understand the pathological consequence of the accumulation of these lysosomal membrane sialoglycoproteins, we determined lysosomal pH, because a significant elevation of lysosomal pH has been reported in a number of lysosomal storage disorders caused by the inactivity of one or several lysosomal proteins (31). Recent evidence has implicated the accumulation of lysosomal membrane proteins involved in the elevation of lysosomal pH. Fibroblasts derived from patients with neuronal ceroid lipofuscinoses having mutations in the integral lysosomal membrane proteins CLN3 and CLN5 showed their accumulation and the concomitant elevation of lysosomal pH (40). Salla disease with mutation in the lysosomal membrane sialic acid transporter sialin, which is known as inherited sialic acid storage disease, also displayed the elevation of lysosomal pH levels (41). Mucolipidosis type IV with elevated lysosomal pH also exhibits an accumulation of a broad spectrum of substances such as mucopolysaccharides and phospholipids (42). In addition, the plasma from patients with various lysosomal storage disorders has been shown to accumulate LAMP-1 and LAMP-2 (43, 44). In this study, we determined lysosomal pH in wild-type and CatE/H11546/H11546 macrophages by using a fluorescent probe Lysosensor yellow/blue dextran, which is known to selectively accumulate into acidic organelles and

FIGURE 6. Two-dimensional gel maps of the culture media of wild-type and CatE/H11546/H11546 macrophages and the activity levels of lysosomal glycosidases in the media. A, comparison of two-dimensional gel maps of the cultured media of wild-type and CatE/H11546/H11546 macrophages. Areas containing the spots with significant abundance changes (as highlighted by squares) were compared in more detail in the magnified figures (lower panels). A proteome analysis revealed that almost all the spots located in areas a and b were identified to be procathepsin D and procathepsin B, respectively. These data indicate the representative two-dimensional gel maps of three independent experiments. B, the activity levels of β-glucuronidase, β-hexosaminidase, and α-mannosidase in the culture media of wild-type and CatE/H11546/H11546 macrophages. The data are the mean ± S.D. of values from five independent experiments. *, p < 0.05 versus the wild-type cells.

FIGURE 7. The degradation of major lysosomal membrane proteins by cathepsin E. The lysosomal membrane fraction (50 μg) isolated from wild-type macrophages was incubated with purified cathepsin E (0–2 μg) at pH 5.0 and 25 °C for 12 h. After termination of the reaction, the samples were subjected to SDS-PAGE and Western blot analysis. The data are the mean ± S.D. of values from four independent experiments. *, p < 0.05; **, p < 0.01 versus the controls without cathepsin E.
enable a rapid and reproducible quantitation of a large number of acidic organelles (40). As would be expected, a significant elevation of lysosomal pH was observed in CatE\(^{-/-}\) macrophages (Fig. 2A). It is generally accepted that the luminal pH in the acidic compartments is regulated by at least two major factors, the proton V-ATPase and the non-proton factor luminal polyelectrolyte matrix (possibly Donnan’s membrane equilibrium) (45, 46). We thus measured the activity levels of V-ATPase in CatE\(^{-/-}\) macrophages in the presence of bafilomycin A\(_1\). Importantly, there was no significant difference in the V-ATPase activity between the wild-type and CatE\(^{-/-}\) macrophages (Fig. 2B). Therefore, the elevation of lysosomal pH in CatE\(^{-/-}\) macrophages appeared to be induced by the disorder of the non-proton factor luminal polyelectrolyte matrix, most probably due to the accumulation of the major lysosomal membrane sialoglycoproteins LAPM-1 and LAMP-2 highly modified with acidic monosaccharides, thereby leading to the perturbation of the proton gradients in the lumen of the endolysosomal organelles. Besides the significant accumulation of these lysosomal membrane sialoglycoproteins, we found the elevation of lysosomal pH in wild-type macrophages treated with the inhibitors for cathepsin E, although the extent of the increase in lysosomal pH was smaller than that by cathepsin E deficiency (Fig. 3C), probably due to the heterogeneity or complexity of the endolysosomal system containing the inhibitors. Based on these observations, cathepsin E deficiency was found to induce the accumulation of LAMP-1 and LAMP-2 and the elevation of lysosomal pH in macrophages.

Because pH is essential for the normal properties of endolysosomal compartments and the normal processing and targeting events of lysosomal proteins (16), and because the elevated lysosomal pH has been shown to interfere with the maturation and fusion events of the organelles involved, the elevated lysosomal pH in CatE\(^{-/-}\) macrophages appeared to impair the structural and functional integrity of these cells. Indeed, we found the enhanced secretion of soluble lysosomal enzymes into the culture medium of CatE\(^{-/-}\) macrophages (Fig. 6) and the decreased expression of LAMP-1, LAMP-2, and LIMP-2 on the cell surface as analyzed by flow cytometry (data not shown), thus indicating that the normal processing and targeting events for lysosomal proteins are partially impaired by the elevation of lysosomal pH in these cells. However, it should be noted that large amounts of soluble lysosomal enzymes, including cathepsins B, D, and L, were still retained within CatE\(^{-/-}\) macrophages, and were normally processed and targeted to lysosomal compartments. Literally, pulse-chase analysis revealed that the initially synthesized cathepsins B and D in CatE\(^{-/-}\) macrophages, likewise the wild-type cells, were normally processed to yield the respective mature enzymes (Fig. 5). Previous studies demonstrated that procathepsin D is associated with MPR300 at pH 6.4–6.5 in the Golgi complex and subsequently dissociated in more acidic environments such as the trans-Golgi network or the endosomes (47, 48). The elevation of lysosomal pH upon treatment with lysomotrophic reagents such as chloroquine and ammonium chloride (13) or bafilomycin A\(_1\) (36) causes the enhanced secretion of procathepsin D in the extracellular space. In addition, most of these studies indicated that the intracellular cathepsin D was mainly present in the proform (13, 36). However, in CatE\(^{-/-}\) macrophages, cathepsins B and D within the cells were normally processed to the respective mature forms, despite the elevated lysosomal pH. This discrepancy in the maturation of this enzyme between our and other studies is probably due to the difference in the extent of the increase in lysosomal pH, because the elevation of lysosomal pH by cathepsin E deficiency was much smaller than that by bafilomycin A\(_1\) at concentrations of 50 nM or above (Fig. 3D). Therefore, the enhanced lysosomal pH in CatE\(^{-/-}\) macrophages presumably reflects subtle changes in the transport and processing of soluble lysosomal proteins. The enhanced secretion of soluble lysosomal enzymes in CatE\(^{-/-}\) macrophages may also be induced by such a partial defect in maintaining the endolysosomal pH. Our present data therefore indicate that the mechanism for the elevation of lysosomal pH induced by cathepsin E deficiency as well as pepstatin A is clearly different from that upon treatment with bafilomycin A\(_1\). In conclusion, cathepsin E deficiency induces a new form of lysosomal storage disorder manifesting the accumulation of the major lysosomal membrane sialoglycoproteins LAMP-1 and LAMP-2, probably LIMP-2, a disruption of the acidification capacity of endolysosomal compartments, and a partial traffic defect in the soluble lysosomal enzymes.

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