Biosynthetic Processing of Cathepsins and Lysosomal Degradation Are Abolished in Asparaginyl Endopeptidase-Deficient Mice

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Running title: Characterization of AEP-deficient mice
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Summary
Asparaginyl endopeptidase (AEP)/legumain, an asparagine-specific cysteine proteinase in animals, is an ortholog of plant vacuolar processing enzyme which processes the exposed asparagine residues of various vacuolar proteins. In search for its physiological role in mammals, here we generated and characterized AEP-deficient mice. Although their bodyweights were significantly reduced, they were normally born and fertile. In the wild-type kidney, where the expression of AEP was exceedingly high among various organs, the localization of AEP was mainly found in the lamp-2-positive late endosomes in the apical region of the proximal tubule cells. In these cells of AEP-deficient mice, the lamp-2-positive membrane structures were found to be greatly enlarged. These aberrant lysosomes, merged with the late endosomes, accumulated electron-dense and membranous materials. Furthermore, the processing of the lysosomal proteases, cathepsins B, H, and L, from the single-chain forms into the two-chain forms was completely defected in the deficient mice. Thus, the AEP-deficiency caused the accumulation of macromolecules in the lysosomes, highlighting a pivotal role of AEP in the endosomal/lysosomal degradation system.
Introduction

Lysosomes contain a variety of acid hydrolytic enzymes including proteases, glycosidases, and lipases and thereby provide the major site for degradation of macromolecules within a cell (1,2). Two processes are responsible for the uptake of molecules in the lysosomes, autophagy and endocytosis. Cytoplasmic components, including long-lived proteins and organelles, are delivered to the lysosomes via autophagy. Extracellular or cell surface molecules are transported to the lysosomes via endocytic pathways by passing through two sequential intermediates, early and late endosomes. Obstruction of the degradation process in human leads to severe diseases generally called lysosomal storage disease (3,4). Most of these diseases are autosomal recessive inherited disorders caused by a deficiency of a lysosomal enzyme. Thus, lysosomal degradation contributes to maintain homeostasis not only intracellularly, but also throughout the body.

Asparaginyl endopeptidase (AEP, EC 3.4.22.34), also called legumain, belongs to the cysteine peptidase family C13 (5). This family was originally identified in higher plants as vacuolar processing enzyme (VPE), which is the enzyme responsible for the processing and maturation of seed storage proteins in the vacuole (6). AEP shows the strict substrate specificity toward the carbonyl side of exposed asparagine residues on the surface of substrate molecules (7,8). AEP is synthesized as an inactive preproprotein and auto-catalytically processed into the active mature forms. C-terminal and N-terminal propeptides of AEP are sequentially removed during its delivery to the acidic compartment (9,10). C-terminal propeptide acts as an auto-inhibitory domain that masks the catalytic site, therefore the removal of C-terminal propeptide is sufficient for the activation of AEP (11,12).

Although AEP is postulated to have a regulatory role in the biosynthesis of lysosomal enzymes, only a few studies have examined the physiological function of AEP in mammals. The microbial antigen tetanus toxin C fragment is processed into peptides by AEP in the endosomal/lysosomal system of B cells. The generated peptides are then loaded on class II major histocompatibility complex (MHC II) molecules for antigen
presentation (13). Myelin basic protein (MBP) is a potential human autoantigen for MHC II that is implicated in the pathogenesis of multiple sclerosis. A dominant epitope of MBP contains a processing site for AEP. The destruction processing of this epitope by AEP limits its presentation in thymus (14-16). In addition to these functions of AEP, the C-terminal propeptide of AEP was identified as an inhibitor of osteoclast differentiation (17). The C-terminal propeptide was found to act as an inhibitor of AEP (11,12), and so there is a possibility that AEP is involved in the process of osteoclast differentiation.

In this study, we examined the physiological function of AEP in vivo by generating and characterizing the AEP-deficient mice. AEP was abundantly expressed in kidney and localized in the late endosomes of the proximal tubule cells. Disruption of the AEP gene led to the enlargement of lysosomes in these cells in an age-dependent manner, which suggests that materials to be degraded are being accumulated within the lysosomal compartments. Activities of lysosomal proteases, cathepsin B and dipeptidyl peptidase (DPP) II, increased, while the cathepsin H activity slightly decreased. These results suggest that AEP has an important role in the endosomal/lysosomal degradation of kidney proximal tubule cells.
**Experimental Procedures**

**cDNA Cloning and Northern Blot Analysis** - A mouse cDNA encoding AEP was isolated from an 11-days embryonic cDNA library (Clontech). The design of the PCR primers was based on the most conserved region among the plant VPEs, orthologs of AEP, 5’-CA(T/C)CA(G/A)GC(G/A/T/C)GA(T/C)GT(G/A/T/C)TG(T/C)CA(T/C)GC-3’ and 5’-GC(G/A/T)AT(G/A)TC(G/A)TC(G/A)TACAT-3’. The PCR fragment amplified with the primers was used for screening the cDNA library. Northern blot analysis was performed with Mouse RNA Master Blot (Clontech). A probe was prepared with a Megaprime DNA labeling system (Amersham) using an *SphI-HindIII* fragment of AEP cDNA as a template. The signals were detected with BAS-III imaging plate and BAS2000 (Fuji Film). The signal intensities were quantified with the Image Gauge program (Fuji Film).

**Targeted Disruption of the AEP Gene** - A 129/Sv (129) genomic library was donated by Dr. Masahiko Hatano of Chiba University. Genomic clones for AEP were isolated from the library using the AEP cDNA as a probe. Construction of a targeting vector is schematically represented in Fig. 1. A *SacI-BamHI* 2.2-kb fragment including three putative exons, one of which encodes a catalytic center of Cys-191, was flanked by two identically oriented *loxP* recombination sites (floxed). The neomycin resistance gene cassette in a vector pPGK-NeobpA (18) was used as a positive selection marker and the diphtheria toxin A (DT-A) gene cassette in a vector pMC1DTpA (19) was used as a negative selection marker. The targeting vector was linearized with *SpeI* digestion and was electroporated into E14-1 embryonic stem (ES) cells (20) as described previously (21). The homologous recombination was identified by PCR using primers 5’-TCTTGTCTTTTCGAGGGATGGGAGTG-3’ and 5’-TATACGAAATATCTCGAGTCGCTCG-3’. To verify the results of PCR screening, the *BglII* fragment of the genomic DNA extracted from the PCR-positive ES clone was hybridized with the 5’-external probe (data not shown) and the *EcoRI* fragment was hybridized with the 3’-external probe (Fig. 1B, lane 2). Seven ES clones among 578
neomycin-resistant clones revealed the desired homologous recombination. These clones were also subjected to a Southern blot analysis using a neo probe to be tested for a single integration. To obtain a deleted allele, AxCANCre, an adenovirus vector expressing Cre recombinase (22), was used to infect two independent ES clones. Thirty-five clones out of the selected 46 clones had the desired deletion. The \( \text{EcoRI} \) fragment of the genomic DNA was subjected to Southern blot analyses using an external 3’-probe to confirm the deletion (Fig. 1B, lane 3).

Chimeric mice were generated by the sandwich aggregation method (23) using two floxed ES cell clones and two deleted ES cell clones. Germ line transmission was confirmed with every clone. Mice heterozygous for the mutation were obtained by crossbreeding of the chimeras with C57BL/6J (B6) females. The resulting heterozygotes were then inter-crossed to obtain homozygous and wild-type littermates, or further backcrossed with B6 mice. Genotypes were determined by PCR of tail DNA (Fig. 1C). The phenotypes described here were determined with 129/B6 mixed background mice and identical in the two independent lines with the disrupted allele. Mice were kept under specific pathogen-free conditions in an environmentally controlled clean room at the Center for Transgenic Animals and Plants, National Institute for Basic Biology. The experiments were conducted according to the institutional ethical guidelines for animal experiments and the safety guidelines for gene manipulation experiments.

**Antibodies** - Anti-mouse AEP antibodies were prepared by immunizing rabbits with a bacterially expressed 6 x His tagged fusion protein of AEP (from Cys-52 to His-164). We used a monoclonal antibody for lamp-2 (rat clone GL2A7) from the Developmental Studies Hybridoma Bank (University of Iowa), goat polyclonal antibodies for cathepsin D from Santa Cruz Biotechnology, anti-cathepsin B and anti-cathepsin L antibodies from Dr. Kazumi Ishidoh and Dr. Eiki Kominami of Juntendo University, and anti-cathepsin H antibodies from Dr. Yasuo Uchiyama of Osaka University. Cy-3-conjugated secondary
antibodies were purchased from Jackson Immunoresearch Laboratory and Amersham. Alexa Fluor 488-conjugated secondary antibodies were purchased from Molecular Probes.

**Immunoblot Analysis** - Mice were deeply anesthetized and perfused with PBS before organs were removed. Organs were immediately frozen in liquid nitrogen, disrupted with Cryo-press (Microtek, Chiba, Japan), and homogenized in suitable buffers with a Polytron. Protein concentrations were determined with BCA Protein Assay Reagent (Pierce) using BSA as a standard. The homogenates were subjected to SDS-PAGE. The separated proteins were electrically transferred to PVDF membranes followed by an immunoblot analysis. The bands were visualized with the ECL detection system (Amersham) and analyzed with LAS1000 plus (Fuji Film).

**Enzyme Assays** - Enzyme activity was measured at 37 °C with the selective fluorescent substrate (final concentration of 1 mM) for each protease. Buffers used were 0.1 M sodium citrate-phosphate (pH 4.5), 1 mM EDTA and 0.1 M dithiothreitol for AEP (5), 75 mM phosphate (pH 6.0), 1 mM EDTA, and 2 mM cysteine for cathepsin B, 0.1 M acetate (pH 5.5), 1 mM EDTA, and 2 mM dithiothreitol for cathepsin B/L, 0.1 M phosphate (pH 6.8), 1 mM EDTA, and 10 mM cysteine for cathepsin H, and 80 mM borate, 20 mM citrate, 0.1 M phosphate (pH 5.3) for DPP II (24). The substrates used were Z-Ala-Ala-Asn-MCA for AEP, Z-Arg-Arg-MCA for cathepsin B, Z-Phe-Arg-MCA for cathepsin B/L, Arg-MCA for cathepsin H, and Lys-Ala-MCA for DPP II. All the substrates were purchased from Peptide Institute (Osaka, Japan). The fluorochromes were quantified using an excitation wavelength of 360 nm and an emission wavelength at 460 nm. One unit of the enzyme activity is the amount of each enzyme that cleaves 1 nmol of substrate per min.

**RNA isolation and RT-PCR** - Total RNA was isolated from kidney. First-strand cDNA was synthesized by incubating 1 µg of the purified RNA with Superscript II reverse
transcriptase (Invitrogen) and oligo-dT primers at 37 °C. The resulting cDNA was then subjected to PCR using primers 5'-ATGACCTGGAGAGTGGCTG-3' and 5'-CGTTGATGTCGTCGGGCA-3' for AEP transcript, and 5'-GTGGGCCGCTCTAGGCACCA-3' and 5'-CGTTGGCCTAGGTTGAGG-3' for β-actin transcript.

Immunofluorescent Microscopy - Mice were deeply anesthetized and fixed by vascular perfusion with 4% paraformaldehyde, 0.2% picric acid, and 0.1 M sodium phosphate (pH 7.4) at a room temperature. The fixed kidneys were cut into small pieces and cryoprotected in PBS containing 20% sucrose overnight at 4 °C. The samples were embedded in the Tissue-Tek OCT compound (Sakura Finetechanical, Tokyo, Japan) and frozen on the surface of liquid nitrogen. Six μm-thick sections were cut on a cryostat (Frigocut 2800E, Reichert-Jung) and collected on coated slides. The sections were washed briefly in PBS and permeabilized in PBS containing 0.5% BSA and 0.3% Triton X-100 to detect both AEP and cathepsin D. In the case of double staining of AEP and lamp-2, sections were permeabilized in PBS containing 0.5% BSA and 0.3% saponin. The sections were incubated with the primary antibodies followed by fluorescence-labeled secondary antibodies and inspected with a laser-scanning confocal imaging system (LSM510, Carl Zeiss).

Electron Microscopy - Mice were fixed as described above except that 2.5% glutaraldehyde was used instead of 4% paraformaldehyde. The removed kidneys were further fixed for 2 h in the same fixative, and subjected to standard electron microscopic techniques as described previously (25). For immunoelectron microscopy, the cryosections, prepared as described above, were reacted with anti-AEP antibodies followed by colloidal gold-conjugated secondary antibodies. The gold labeling was intensified with a silver enhancement kit (Nano Probes) as previously described (26). The cryosections were then dehydrated, embedded in LR-white, and processed further.
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according to standard electron microscopic technique (25). Ultra-thin sections were observed under a Hitachi H700 electron microscopy (Hitachi, Japan).

Results

AEP-Deficient Mice – AEP mRNA expression was by far the highest in kidney (Fig. 1D) as reported previously (5,27,28). The expression levels in other organs such as eye, liver and lung, were less than an eighth of that in kidney. To demonstrate an \textit{in vivo} physiological role of AEP, we generated AEP-deficient (AEP\textsuperscript{−/−}) mice. Neither AEP transcript (Fig. 1E) nor protein (Fig. 3A) was detected in the AEP\textsuperscript{−/−} mice, indicating the disruption of this gene.

Crosses of the AEP\textsuperscript{+/-} mice resulted in progeny with the expected Mendelian frequencies, indicating AEP was not essential for normal development. The AEP\textsuperscript{−/−} mice were fertile and had no gross anatomical and morphological abnormalities, although they were slightly smaller than the wild-type (AEP\textsuperscript{+/+}) and AEP\textsuperscript{+/-} mice (Fig. 2). The increase in bodyweight of the AEP\textsuperscript{−/−} mice was almost the same as that of their AEP\textsuperscript{+/+} and AEP\textsuperscript{+/-} littermates for 20 weeks after weaning. Thereafter the weight increase of the AEP\textsuperscript{−/−} mice began to stagnate. The bodyweights of the AEP\textsuperscript{−/−} mice significantly diverged from the AEP\textsuperscript{+/+} and AEP\textsuperscript{+/-} mice after 40 to 50 weeks (P<0.05 for females and P<0.01 for males). At 50 weeks, the mean weights of AEP\textsuperscript{+/+} and AEP\textsuperscript{+/-} mice were about the same. Their combined mean weights were 38.3 $\pm$ 6.34 g for females and 48.21 $\pm$ 6.91 g for males. At the same stage, the mean weights of female and male AEP\textsuperscript{−/−} mice were 31.1 $\pm$ 4.35 g and 38.7 $\pm$ 5.85 g, respectively. The mean weights of the 50-week-old AEP\textsuperscript{−/−} mice were about 80% of those of the AEP\textsuperscript{+/+} and AEP\textsuperscript{+/-} littermates.

\textit{Processing of Lysosomal Proteases in AEP\textsuperscript{−/−} Mice} – Cathepsins B, H, and L are synthesized as preproproteins and processed into their two-chain forms via single-chain forms (29). To determine the effect of AEP-deficiency on the processing of cathepsins,
the kidney homogenates of 4- and 7-month-old mice were subjected to immunoblot analyses with antibodies against each of the cathepsins. In AEP\textsuperscript{+/+} mice, cathepsins B, H, and L were detected as both the single-chain and two-chain forms (Fig. 3A). In contrast, only the single-chain form of each cathepsin was detected in the AEP\textsuperscript{−/−} mice (Fig. 3A). This result clearly demonstrates that AEP is responsible for processing from the single-chain form into the two-chain form for cathepsins B, H, and L. In addition, it is noteworthy that the amount of cathepsins, especially for cathepsin L, increased in the AEP\textsuperscript{−/−} mice (see discussion below).

Next we determined how the defects in the processing into the two-chain forms influenced the activities of the lysosomal proteases. Protease activities were based on the ability of homogenates of kidneys from 4-month-old mice to cleave selective fluorescent substrates to each enzyme (Fig. 3B). The activity to cleave Z-Ala-Ala-Asn-MCA, a selective substrate for AEP, in the AEP\textsuperscript{−/−} mice was less than 10% of the AEP\textsuperscript{+/+} activity, while the activity in the AEP\textsuperscript{+/−} mice was 65% of the AEP\textsuperscript{+/+} activity. The residual activity found in the AEP\textsuperscript{−/−} mice was inhibited by 10 \( \mu \text{M} \) trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) (Fig. 3B, right panel), but not by 1 mM phenyl- methylsulfonyl fluoride (PMSF) or 10 \( \mu \text{g/ml} \) pepstatin A (data not shown). This result indicates that the E64 sensitive cysteine proteinase(s) may contribute to the remaining activity to cleave the selective substrate for AEP. We did not find notable differences between AEP\textsuperscript{+/+} and AEP\textsuperscript{+/−} mice in the activities of cathepsins B, L, and H and DPP II (Fig. 3B, left panel). Interestingly, cathepsin B and DPP II activities in the AEP\textsuperscript{+/−} mice were almost twice those in their AEP\textsuperscript{+/+} littermates, while the activity toward the cathepsin B/L substrate was only slightly higher. On the other hand, cathepsin H activity in the AEP\textsuperscript{+/−} mice was only about half the activity in AEP\textsuperscript{+/+} mice.

Late Endosomal Localization of AEP in the Kidney Proximal Tubule Cells - AEP was abundant in kidney (Fig. 1D). To determine its distribution in kidney, we performed immunofluorescent microscopy with anti-AEP antibodies. Kidneys of 1-, 3- and 12-
month-old-mice were fixed by perfusion and subjected to the analyses. Essentially the same results were obtained from each of 1-, 3- and 12-month-old-mice. AEP was found in a quite restricted region in the cortex (Fig. 4A, right), but not in the medulla including the collecting tubule or the urinary tubule cells (data not shown). In the cortex, prominent AEP signals were detected at the proximal tubules and weak signals were observed at the distal tubules. In contrast, considerable signals were not detected at glomeruli. Pre-immune serum did not make any signals (data not shown).

In the kidney proximal tubule cells, AEP was localized to the punctate structures in the apical region within the cell. To elucidate what the structures were, we performed double-labeling immunofluorescent microscopy with antibodies against cathepsin D, a lysosomal protease (Fig. 4B). The AEP-containing structures were mostly found on the apical side of the cells (Fig. 4B, left), while cathepsin D-positive lysosomes were distributed in the middle region of the cells (Fig. 4B, middle). AEP was scarcely colocalized with cathepsin D (Fig. 4B, right). These results suggest that AEP is localized in a subcellular compartment other than lysosomes.

We next compared the localization of AEP with that of lamp-2, a marker for the late endosomes and lysosomes (30). Most structures stained with anti-AEP antibodies were stained with anti-lamp-2 monoclonal antibody (Fig. 4C, right). This result suggested that AEP was localized in the late endosomes of the kidney proximal tubule cells. We excluded the possibility that AEP was localized to the early endosomes, because almost all AEP signals overlapped with those of lamp-2.

To demonstrate the subcellular localization in greater detail, immunoelectron microscopy was performed with ultra-thin sections prepared from a kidney of a 4-week-old mouse. In the proximal tubule cells, AEP was localized in electron-lucent multivesicular-body-like structures, but not in the electron-dense uniform structures (Fig. 4D). The endocytic apparatuses in the proximal tubule cells are classified as follows; apical clathrin-coated pits, small coated and noncoated early endosomes (< 0.5 μm), large noncoated endosomes (> 0.5 μm), dense apical tubules, and the lysosomes (31). The
electron-dense uniform structures represent dense/mature lysosomes. We did not find any gold particle representing the AEP localization on these structures. This result corresponds with the observation that AEP was not colocalized with cathepsin D. The average diameter of the structures containing gold particles was 0.638 +/- 0.153 µm (n=10). The large noncoated lysosomes can be subdivided into early and late endosomes by the presence of internal coat (31), and the AEP-positive structures did not have internal coat. Taken together, these results show that AEP is concentrated in the late endosomes of the kidney proximal tubule cells.

Enlarged Lysosomes in the Proximal Tubule Cells of AEP<sup>-/-</sup> mice - To clarify the effect of AEP deficiency on the endosomal/lysosomal functions, we examined the distribution of lamp-2 in the kidney of the AEP<sup>-/-</sup> mice. Kidneys were isolated from 4- and 7-month-old mice and subjected to immunofluorescent microscopy. We detected only a small difference between 4-month-old AEP<sup>-/-</sup> and AEP<sup>+/+</sup> mice (data not shown). On the contrary, excessive enlargement of the lamp-2 positive structures was found in the kidney proximal tubule cells of 7-month-old AEP<sup>-/-</sup> mice (Fig. 5B). Lamp-2 signals were observed as ring structures with a maximum diameter of 1 µm. Distribution of lamp-2-containing structures within the proximal tubule cells was also influenced in the AEP<sup>-/-</sup> mice. Lamp-2 positive structures were mainly found in the apical region of the AEP<sup>+/+</sup> kidney proximal tubule cells (Fig. 5A and Fig. 4 C, middle), although they were widely distributed within the cells of the AEP<sup>-/-</sup> mice (Fig. 5B). We did not find these structures in other regions of the kidney such as the distal tubule, glomerulus or collecting tubule (data not shown). Electron microscopic analyses (Fig. 5C, D) show that most lysosomes were large and aberrant in the proximal tubule cells of AEP<sup>-/-</sup> mice. The abnormal lysosomes contained electron-dense and/or membranous materials. These results indicate that AEP<sup>-/-</sup> mice develop lysosomal storage within the kidney proximal tubule cells.
Discussion

The identity of the protease responsible for the processing of cathepsins into their two-chain forms has been controversial. Chen et al. have suggested that AEP may be responsible to the processing of cathepsins B and H (5). In this study, we show that the processing of cathepsins B, H, and L into the two-chain forms was completely defective in the AEP<sup>−/−</sup> mice (Fig. 3A). This result was consistent with the finding that the processing sites of cathepsin B and cathepsin H are asparagine residue (32), which are preferential sites for AEP activity. The processing of cathepsin L occurs at a site containing an aspartic acid (33,34). A plant AEP ortholog, VPE, has been shown to recognize aspartic acid residues as well as asparagine residues (35). We observed that AEP also cleaves a peptide bond at the carbonyl side of aspartic acid, although the activity toward aspartic acid is quite lower than that toward asparagine<sup>2</sup> (10). The AEP<sup>−/−</sup> mice lacked the activity to cleave the asparaginyl bond (Fig. 3B), showing that AEP is the major protease specific to asparagine under acidic conditions. Thus, AEP must be directly involved in the producing the two-chain forms of cathepsins B, H, and L.

Here we found that the lysosomes of the AEP<sup>−/−</sup> mice became enlarged in the proximal tubule cells (Fig. 5). We did not find the enlarged lysosomes in other regions of kidney. This is in good agreement with the distribution of AEP in kidney (Fig. 4A). The proximal tubule cells, which abundantly express AEP, have excessive apical endocytic compartments that are critical for the reabsorption and degradation of macromolecule (31). These cells are the most active sites for endocytosis and subsequent lysosomal degradation. AEP and lamp-2 were colocalized in the late endosomes of the proximal tubule cells (Fig. 4). In addition, we found that the lysosomes were enlarged in the AEP<sup>−/−</sup> mice; therefore it is reasonable that AEP would function in the degradation of macromolecules internalized via endocytosis. The lysosomes of AEP<sup>−/−</sup> mice contained electron-dense and/or membranous materials (Fig. 5D). These features are analogous to the specific features of lysosomes whose degradation activities are impaired (1). Thus, the AEP deficiency resulted in the development of the lysosomal storage in the kidney.
proximal tubule cells. The species of molecules accumulated in the lysosomes are still to be determined. A preliminary experiment indicated the accumulation of albumin in the homogenates of AEP\(^+\)/ kidneys\(^2\). Yamane et al. reported that AEP cleaved vitamin-D binding protein in a limited proteolytic manner (36). Vitamin-D binding protein and albumin are macromolecules absorbed by proximal tubule cells. AEP\(^+\)/ mice may accumulate these proteins in the lysosomes to produce large and aberrant lysosomes.

In this study, we found that AEP was mainly localized in the late endosomes (Fig. 4C and D). Cathepsins B, D, H, and L were found to be mainly localized in the lysosomes, which have been shown to be electron-dense and uniform structures (37-40). Therefore, AEP was located upstream of the cathepsins in the endocytic pathway. We presume that the endocytosed proteins are digested into peptides by AEP within the late endosomes to accelerate the efficiency of their subsequent lysosomal degradation. Indeed, we found the lysosomes of AEP\(^+\)/ mice became larger with age. We did not detect the large and aberrant lysosomes in 4-month-old AEP\(^+\)/ mice (data not shown), but we did find them in 7-month-old mice (Fig. 5). The amounts of cathepsins had already increased in 4-month-old AEP\(^+\)/ mice (Fig. 3A). The activities of cathepsin B and DPP II in AEP\(^+\)/ mice were almost twice those in AEP\(^+\)/+ mice (Fig. 3B), possibly due to the increased protein levels. These results suggest that lysosomal proteases are induced to compensate for the loss of degradation activity of the AEP\(^+\)/ lysosomes. Lysosomal degradation is managed through the induction of other proteases in younger AEP\(^+\)/ mice, and may finally collapse with aging because of the lower efficiency resulting from the AEP deficiency.

The bodyweights of AEP\(^+\)/ mice were about 80% of those of their AEP\(^+\)/+ and AEP\(^+\)/ littermates. The AEP\(^+\)/ mice grew normally for about 20 weeks, and gradually diverged from their AEP\(^+\)/+ and AEP\(^+\)/ littermates. This divergence of bodyweights and the appearance of lysosomal storage seem to be associated. Macromolecules reabsorbed at the proximal tubule cells are reutilized as nutrients. The small bodyweights of AEP\(^+\)/ mice may be the result of defects in reabsorption by the kidney proximal tubule cells.
It has been shown that some lysosomal storage diseases result in severe defects of the renal functions and become worse with age. Additional aging may cause renal dysfunction such as proteinuria in the AEP−/− mice. We found the AEP−/− lysosomes contained autofluorescent materials as well as electron-dense and/or membranous materials. Accumulation of autofluorescent substances has been reported in the case of neuronal ceroid lipofuscinosis, a lysosomal storage disease (41). Further analysis should establish the AEP−/− mice as a model of lysosomal storage disease of human.
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References


Footnotes

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The abbreviations used are: AEP, asparaginyl endopeptidase; VPE, vacuolar processing enzyme; DPP, dipeptidyl peptidase; AMC, 7-amino-4-methylcoumarin; Z, benzyloxycarbonyl, E64, trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PMSF, phenyl- methylsulfonyl fluoride.

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Figure Legends

**Fig. 1.** Targeted disruption of the *AEP* gene and expression of AEP in wild-type mice.

(A) A targeting vector for disruption of the *AEP* gene and the resulting targeted loci. Solid boxes represent exons. Solid triangles and gray pentagons indicate *LoxP* recombination sites and FRT recombination sites, respectively. A neomycin cassette (Neo) was used as a positive selection marker and a diphtheria toxin A gene cassette (DT-A) was used as a negative selection marker. Abbreviations for restriction enzymes are: RI, *Eco*RI; Bg, *Bgl*II; Sa, *Sac*I; BH, *Bam*HI; Sp, *Spe*I.

(B) Genomic DNAs isolated from the wild-type ES cells (lanes 1 and 4) and the targeted ES cells before (lane 2) and after (lane 3) the treatment with Cre recombinase were digested with *Eco*RI and then were subjected to Southern blot analysis with the 3′-external probe that is shown in A. The 17-kb fragment was derived from the wild-type locus, the 12-kb fragment was from the targeted locus and the 10-kb fragment was from the disrupted locus as indicated by double-headed arrows in A.

(C) PCR was performed with tail DNAs of the AEP*+/+* (+/+), AEP*+/-* (+/-) and AEP*−/−* (−/−) mice. DNA fragments of 150 bp and 290 bp were amplified with the wild-type allele and the mutant allele, respectively.

(D) Mouse RNA Master Blot was used to determine an organ specific expression of the *AEP* mRNA. The signal intensities were quantified with the Image Gauge program.

(E) Total RNAs prepared from kidney of the AEP*+/+* (+/+), AEP*+/-* (+/-) and AEP*−/−* (−/-) mice were subjected to RT-PCR with a specific primer set for *AEP* or *β-actin*.

**Fig. 2.** Bodyweights of AEP*−/−* mice were less than those of AEP*+/+* and AEP*+/-* mice. Bodyweights of the AEP*−/−* mice (n=10) and the wild-type mice (AEP*+/+* and AEP*+/-*, n=10) were monitored for 50 weeks after weaning. Differences were significant at the P<0.05 level for females and at the P<0.01 level for males.
Fig. 3. AEP is responsible for processing of multiple lysosomal proteases.

(A) Kidney homogenates (20 µg proteins) prepared from AEP<sup>+/+</sup> (+/+), AEP<sup>+-</sup> (+/-) and AEP<sup>-/-</sup> (-/-) mice were subjected to immunoblotting with specific antibodies against each of AEP, cathepsin H, cathepsin B and cathepsin L. The AEP deficiency caused an increase in the level of single-chain forms of these proteases. A single-chain form (S) and a two-chain form (T) are indicated on the blot for each enzyme.

(B) The homogenates were prepared from kidneys of AEP<sup>+/+</sup> (black column), AEP<sup>+-</sup> (gray column) and AEP<sup>-/-</sup> (open column) mice. Specific activities of proteases in these homogenates were measured with a selective substrate for each of AEP, cathepsin H, cathepsin B, cathepsin B/L and DPPII (left panel). The residual activity to cleave Z-Ala-Ala-Asn-MCA in the AEP<sup>-/-</sup> mice is inhibited by E-64 (right panel). Relative specific activities of the AEP<sup>-/-</sup> and the AEP<sup>+-</sup> mice are indicated by % of the activity of AEP<sup>+/+</sup> mice.

Fig. 4. Subcellular localization of AEP in the late endosomes of the kidney proximal tubule cells.

(A) A frozen section of a mouse kidney was subjected to immunofluorescent microscopy with anti-AEP antibodies and inspected with a laser-scanning confocal microscope (right) or a differential-interference-contrast microscope (left). Asterisks indicate the proximal tubules. gl, glomerulus; d, distal tubule. Bar = 50 µm.

(B) A frozen section of a kidney was subjected to double-immunostaining with either anti-AEP antibodies (left) or anti-cathepsin D antibodies (middle) and was inspected with a laser-scanning confocal microscope. A merged image is also shown (right). Bars = 10 µm.

(C) A frozen section of a kidney was subjected to double-immunostaining with either anti-AEP antibodies (left) or anti-lamp-2 monoclonal antibody (middle) and was inspected with a laser-scanning confocal microscope. A merged image is also shown (right). Bars = 10 µm.
Characterization of AEP-deficient mice

(D) Silver-enhancement immunoelectron microscopic images of the kidney proximal tubule cells that were stained with anti-AEP antibodies. Arrowheads indicate the late endosome in which gold particles are localized. BB, brush border; Ly, lysosome; Mt, mitochondrion; N, Nucleus. Bar = 500 nm.

Fig. 5. Enlargement of lysosomes in the kidney proximal tubule cells of the AEP<sup>−/−</sup> mice. Frozen sections of kidneys isolated from the AEP<sup>+/+</sup> (A) and the AEP<sup>−/−</sup> mice (B) were subjected to immunofluorescent microscopy with anti-lamp-2 antibody. Arrowheads indicate enlarged structures found in the AEP<sup>−/−</sup> mice. Asterisks mark the lumen of proximal tubule. Bars = 5 µm. Electron microscopic images of the proximal tubule cells of AEP<sup>−/−</sup> mice (C, D). Arrowheads indicate the large and aberrant lysosomes containing electron dense and/or membranous materials. BB, brush border; BL, basal lamina; Mt, mitochondrion. Bars = 1 µm.
Figure 1

**A**

Wild-type locus

Targeting vector

Targeted locus

Mutant locus

**B**

(kb) 1 2 (kb) 3 4

17 17

12 10

**C**

(bp) +/- +/- +/- +/

290

150

**D**

Brain

Eye

Liver

Lung

Kidney

Heart

Skeletal muscle

Smooth muscle

Pancreas

Thyroid

Thymus

Submax. gland

Spleen

Testis

Ovary

Prostate

Epididymis

Uterus

7-days embryo

11-days embryo

15-days embryo

17-days embryo

Signal intensity

**E**

AEP

β-actin
### Figure 3

**A**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>4-month-old</th>
<th>7-month-old</th>
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<tbody>
<tr>
<td>AEP</td>
<td>-/-</td>
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</tr>
<tr>
<td>Cathepsin H</td>
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<tr>
<td>Cathepsin B</td>
<td>-/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>-/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

**B**

- **Relative specific activity (%)**
  - AEP: 100, 200
  - Cathepsin H, B, L: 100, 200

- **Specific activity (Units/mg protein)**
  - None: 10 µM E64inhibitor
  - Z-Ala-Ala-Asn-MCA substrate

**Table:**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Specific activity (Units/mg protein)</th>
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<tr>
<td>None</td>
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<td>10 µM E64</td>
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</table>

**Figure 3**
Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice
Kanae Shirahama-Noda, Akitsugu Yamamoto, Kazushi Sugihara, Noriyoshi Hashimoto, Masahide Asano, Mikio Nishimura and Ikuko Hara-Nishimura

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