Major Role of Cathepsin L for Producing the Peptide Hormones ACTH, β-Endorphin, and α-MSH, Illustrated by Protease Gene Knockout and Expression*

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The pituitary hormones adrenocorticotrophic hormone (ACTH), β-endorphin, and α-melanocyte stimulating hormone (α-MSH) are synthesized by proteolytic processing of their common proopiomelanocortin (POMC) precursor. Key findings from this study show that cathepsin L functions as a major proteolytic enzyme for the production of POMC-derived peptide hormones in secretory vesicles. Specifically, cathepsin L knock-out mice showed major decreases in ACTH, β-endorphin, and α-MSH that were reduced to 23, 18, and 7% of wild-type controls (100%) in pituitary. These decreased peptide levels were accompanied by increased levels of POMC consistent with proteolysis of POMC by cathepsin L. Immunofluorescence microscopy showed colocalization of cathepsin L with β-endorphin and α-MSH in the intermediate pituitary and with ACTH in the anterior pituitary. In contrast, cathepsin L was only partially colocalized with the lysosomal marker Lamp-1 in pituitary, consistent with its extralysosomal function in secretory vesicles. Expression of cathepsin L in pituitary AtT-20 cells resulted in increased ACTH and β-endorphin in the regulated secretory pathway. Furthermore, treatment of AtT-20 cells with CLIK-148, a specific inhibitor of cathepsin L, resulted in reduced production of ACTH and accumulation of POMC. These findings demonstrate a prominent role for cathepsin L in the production of ACTH, β-endorphin, and α-MSH peptide hormones in the regulated secretory pathway.

The peptide hormones ACTH‡, β-endorphin, and α-MSH are produced by proteolytic processing of their common proopiomelanocortin (POMC) prohormone precursor (1, 2). The mature peptide hormones are stored in pituitary secretory vesicles for regulated secretion and control of physiological functions in target organs. ACTH regulates the production of glucocorticoids in the adrenal cortex for control of metabolism (3, 4). α-MSH is implicated in the regulation of appetite and the production of melanin (5, 6). β-Endorphin is an endogenous opioid peptide involved in pain regulation (7, 8). The proteolytic mechanisms that generate these functionally distinct peptide hormones from POMC are essential for these hormones to exert their biological functions.

The role of cysteine protease activity for POMC processing has been implicated by several studies (9–11), but the identity of the protease has not yet been achieved. In early studies of POMC processing activity in pituitary secretory vesicles, cysteine protease activity represented a significant portion of POMC cleaving activity, based on its inhibition by the thiol reagent p-chloromercuribenzoate (9). In anterior and intermediate pituitary cells in culture, treatment of cells with the cysteine protease inhibitor E64d reduced cell levels of POMC-derived ACTH, β-endorphin, and α-MSH (10, 11). Thus, it is likely that a cysteine protease participates in POMC processing. Participation of a cysteine protease in POMC processing would represent a new protease pathway, in addition to the subtilisin-like prohormone convertase enzymes (PC2 and PC1/3) that participate in processing POMC (12–15). Therefore, the goal of this study was to identify the cysteine protease that produces ACTH, β-endorphin, and α-MSH peptide hormones derived from POMC.

Herein we provide evidence indicating a key role for the cysteine protease cathepsin L in the biosynthesis of ACTH, β-endorphin, and α-MSH in secretory vesicles. Cathepsin L gene knock-out mice showed major reductions in pituitary tissue levels of ACTH, β-endorphin, and α-MSH. Furthermore, cathepsin L KO mouse pituitaries displayed accumulation of POMC, consistent with proteolysis of POMC by cathepsin L. In vivo cellular localization of cathepsin L in pituitary cells demonstrated a high degree of colocalization with β-endorphin, α-MSH, and ACTH in secretory vesicles. Expression of the cathepsin L cDNA in pituitary AtT-20 cells resulted in green fluorescence protein; CMW, cytomegalovirus; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; KO, knock-out; MCA, methylcoumarinamide.
increased production of ACTH and β-endorphin in the regulated secretory pathway. In addition, CLIK-148, a specific inhibitor of cathepsin L, reduced ACTH production. These gene knock-out and gene expression studies indicate a significant role for cathepsin L in the production of POMC-derived peptide hormones in secretory vesicles.

**EXPERIMENTAL PROCEDURES**

**Analyses of POMC-derived Peptide Hormones in Cathepsin L Knock-out Mice**—Cathepsin L-deficient mice were generated by gene targeting in mouse embryonic stem cells, as described previously (17, 18). Genotyping established cathepsin L gene knock-out (−/−) and wild-type (+/+ ) mice. Cathepsin L knock-out mice and age-matched wild-type control mice were generated in the C57BL/6J mouse strain. The absence of cathepsin L in the knock-out mice has been confirmed by the absence of cathepsin L mRNA (by Northern blots) and absence of cathepsin L in the knock-out mice and age-matched wild-type control mice were generated in the C57BL/6J mouse strain. The absence of cathepsin L in the knock-out mice has been confirmed by the absence of cathepsin L mRNA (by Northern blots) and absence of cathepsin L enzyme protein (by Western blots) (18).

Brain tissues from adult mice (~3 months of age) were collected and homogenized in 1 n acetic acid, heated at 95% for 10 min, and centrifuged (15,000 × g for 15 min), and the supernatant was analyzed for ACTH, β-endorphin, and α-MSH by radioimmunoassays as described previously (12). Protein concentrations in tissue extracts were measured (DC protein assay kit; Bio-Rad). Tissue content of peptide hormones per unit amount of protein was calculated.

Anti-ACTH Western blots were performed for pituitary from cathepsin L gene knock-out mice and wild type animals. Pituitaries were homogenized on ice in radioimmuno precipitation buffer (50 mm Tris, 150 mm sodium chloride, 1.0 mm EDTA, 1% Nonidet P-40, and 0.25% sodium deoxyolate, pH 7.0) supplemented with a complete protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitor mixture 1 (Sigma). After incubation at 4 °C for 1 h, followed by a centrifugation step at 10,000 × g at 4 °C for 5 min, protein concentration was measured (Bio-Rad). The homogenate preparation was subjected to SDS-PAGE, transferred to nitrocellulose, and incubated with 5% nonfat dry milk overnight at 4 °C with mouse anti-ACTH (N-terminal (residues 1–24) reactive; Cymbus Biotechnology, Eastleigh, UK) at a dilution of 1:400. The washed blots were then incubated with secondary antibodies conjugated to horseradish peroxidase (sheep anti-mouse; Amersham Biosciences) at a dilution of 1:10,000 for 1 h and developed with the KPL western luminesence ECL plus (Amersham Biosciences) chemiluminescent system.

**Localization of Cathepsin L with POMC-derived Peptides in Mouse Pituitary by Immunofluorescence Confocal Microscopy**—Tissues in mice (CD1 Swiss male, adult mice; Charles River Laboratories) were perfused with phosphate-buffered saline (PBS) (intracardially) and then with 4% paraformaldehyde. Pituitary tissue was dissected and fixed in 4% paraformaldehyde overnight and cryoprotected with 30% sucrose. Tissue sections (30 μm) were then incubated and permeabilized with PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton for 1 h at room temperature. Double immunostaining of tissue sections with cathepsin L and each POMC-derived peptide hormone was conducted to assess their colocalization. The extent of colocalization of cathepsin L with the lysosomal marker Lamp-1 was also assessed.

Incubation with primary antibodies consisted of anti-cathepsin L-goat (15 μg/ml; R & D Systems), anti-β-endorphin-guinea pig (1:1000; Bachem-Peninsula Laboratories), anti-ACTH-rabbit (1:1000; NIDDK, National Institutes of Health, Bethesda, MD), anti-α-MSH-rabbit (1:500; Phoenix Pharmaceuticals), or monoclonal anti-Lamp-1-rat (1:100; Abcam). Cathepsin L was detected with secondary anti-goat IgG labeled with Alexa Fluor 488 (green fluorescence; 1:200; Molecular Probes, Eugene, OR); β-endorphin, ACTH, α-MSH, or Lamp-1 was detected with antisera (directed to IgGs from guinea pig, rabbit, or rat, respectively) labeled with Alexa Fluor 594 (red fluorescence; 1:200; Molecular Probes). Tissue sections were incubated overnight at 4 °C with primary antibodies, washed with PBS, and incubated with secondary antibodies for 2 h at room temperature. Sections were mounted on glass slides, coverslipped with mounting medium, and examined with spectral deconvolution confocal microscope FV1000 (Olympus). Images were analyzed with the Olympus Fluoview software.

Information for antibody specificity and controls conducted for immunofluorescence staining were utilized to confirm the specificity of immunostaining. With respect to primary antisera, immunostaining of cathepsin L was blocked by preincubation of anti-cathepsin L with cathepsin L enzyme protein. The antisera to ACTH and α-MSH used in our immunochromical studies have been well characterized for specificity in our radioimmunoassays (12). Anti-ACTH (NIDDK, National Institutes of Health) specifically detects ACTH and does not cross-react with α-MSH or β-endorphin. Anti-α-MSH (Phoenix, CA) specifically detects α-MSH and does not cross-react with ACTH, β-endorphin, or (Met)enkephalin. The specificity of anti-β-endorphin has been determined (by Bachem-Peninsula Laboratories), and it detects β-endorphin and does not cross-react with ACTH, α-MSH, or (Met)enkephalin. In addition, the anti-Lamp-1 is a monoclonal antibody that is directed to mouse Lamp-1 (Abcam).

With respect to secondary antisera, specificities have been determined by assessing immunofluorescence with incubation with secondary antiserum alone (i.e. without primary antiserum), which results in lack of immunofluorescence staining. For double immunostaining procedures to detect two antigens (“a” and “b”), the secondary antiserum to antigen “a” has been tested for lack of cross-reactivity to antigen “b”, and the secondary antiserum to antigen “b” has been tested for lack of cross-reactivity to antigen “a”. These controls have been conducted for all colocalization experiments of cathepsin L colocalization with POMC-derived peptide hormones and controls that compared cathepsin L and Lamp-1 localization.

**Evaluation of Cathepsin L Colocalization with ACTH in Mouse Pituitary AtT-20 Cells by Immunofluorescence Confocal Microscopy**—AtT-20 cells were cultured as previously described (19, 20) on polyllysine-coated glass chamber slides (Nalge Nunc International) and fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After permeabilization with Triton X-100 (0.1%), cells were incubated with PBS containing 3% BSA for 30 min at room temperature. Cells were then double-stained for 2 h at room temperature in PBS
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plus 3% BSA with primary antibodies anti-cathepsin L-rabbit (1:50; Athens Research and Technology, Athens, GA) and anti-ACTH-mouse (1:100; Abcam). Primary antibodies were revealed by the secondary antibodies in PBS plus 3% BSA with goat anti-rabbit Alexa Fluor 488 (green fluorescence; 1:200; Molecular Probes) and goat anti-mouse Alexa Fluor 594 (red fluorescence; 1:200; Molecular Probes) for 45 min at room temperature. Slides were mounted with Aqua Poly/Mount (Polysciences) and images were taken with the microscope Delta Vision Spectris Image Deconvolution System on an Olympus IX70 using the software Softworx Explorer from Applied Precision.

Expression of Cathepsin L in AtT-20 Cells and Analyses of ACTH and β-Endorphin Production in the Regulated Secretory Pathway—Cathepsin L cDNA was expressed in AtT-20 cells to assess effects on production of ACTH and β-endorphin in the regulated secretory pathway. Effective expression of transfected cathepsin L in a neuroendocrine cell line has been demonstrated (21).

Cells were plated 1 day before transfection in 6-well plates (Corning Glass) and were at ~70% confluence when transfected with the preprocathepsin L cDNA/cDNA3.1 (bovine) (21) or pcDNA3.1 vector alone (at 2 μg of DNA/well) with transfection reagent FuGene HD (8 μl; Roche Applied Science). Two days after transfection, cells were incubated with BaCl₂ (1 mM) for 2 h to stimulate regulated secretion of ACTH and β-endorphin, measured by radioimmunoassays as previously described (12). Expression conditions were each conducted in replicate wells (six replicates), and experiments were repeated two times. Results are expressed as the mean ± S.E. of ACTH or β-endorphin secreted/well. Cell extracts were also analyzed by anti-ACTH Western blots (anti-ACTH from NIDDK), performed as described previously (12).

Expression of cathepsin L activity in cell extracts was confirmed by monitoring benzoyloxycarbonyl-Phe-Arg-MCA cleaving activity that is inhibited by the specific cathepsin L inhibitor CLIK-148 (16). Cells were harvested by homogenization in cathepsin L assay buffer (0.1 M sodium acetate, pH 5.5, 1 mM EDTA, and 4 mM dithiothreitol). Homogenate was sonicated and centrifuged (13,000 g) (size of about 1.0 kb) was double-digested with NheI/XhoI and ligated to Nhel/Xhol-digested pEGFP-N1 plasmid expression vector (Clontech). This construct was subjected to DNA sequencing (Davis DNA Sequencing Inc.) to verify the nucleotide sequence and deduced primary amino acid sequence of the bovine preprocathepsin L cDNA.

Preprocathepsin L/EGFP fusion protein plasmid expression vector was transfected in AtT-20 cells with the same conditions as for preprocathepsin L cDNA, described above. Two days after transfection, cells were harvested, and homogenates were prepared for Western blots, as described previously (12). Samples were subjected to electrophoresis on 4–12% BisTris gels (Invitrogen), and proteins were transferred electrophoretically to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked in 3% BSA in TBST buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.05% Tween 20) and were incubated with a mouse anti-EGFP antibody (Abcam) at dilution of 1:500 in TBST overnight at 4 °C. After washing in TBST and incubation with sheep anti-mouse-horseradish peroxidase (GE Healthcare) for 1 h followed by washing, the membrane was developed by ECL plus Western blotting detection systems (GE Healthcare).

Treatment of AtT-20 Cells with CLIK-148, Inhibitor of Cathepsin L—AtT-20 cells were treated with the specific inhibitor of cathepsin L, CLIK-148 (16) (50 μM) (from Enzyme Systems Products, Livermore, CA). Triplicate cell samples of CLIK-148-treated and -untreated conditions were prepared. After treatment, cells were harvested by preparation of acetic acid extracts (as described previously (12)) for measurements of ACTH content by radioimmunoassay. Protein content in extracts was determined (DC protein assay kit; Bio-Rad). The amount of ACTH (pg) per unit amount of protein was calculated. This experiment was repeated three times. Analyses of cells included anti-ACTH Western blots, performed as described previously (12).

Statistical Evaluation of Results—Experiments were conducted with an appropriate number of replicate samples, consisting of 8–10 cathepsin L knock-out mice and 8–10 wild-type age-matched control mice. This experiment was repeated twice. AtT-20 cell experiments were conducted in triplicate samples for each condition, and experiments were repeated two or three times. Differences in peptide hormone levels were evaluated for statistical significance with Student’s t test, with p < 0.05.

RESULTS

Cathepsin L Gene Knock-out Mice Show Substantial Reduction of POMC-derived Peptide Hormones in Pituitary—The peptide hormones ACTH, β-endorphin, and α-MSH are derived from the common POMC prohormone precursor by proteolytic processing. They are especially important as pituitary hormones and are highly expressed in this tissue. To determine the role of cathepsin L in the production of these POMC-derived peptide hormones, cathepsin L gene knock-out (+/−/ genotype) mice were analyzed for pituitary levels of these peptide hormones, with comparison with wild-type controls (+/+ genotype).

Notably, all POMC-derived peptide hormones were substantially reduced in the cathepsin L knock-out mice compared with
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Controls (Fig. 1). ACTH levels in cathepsin L KO mice were reduced to 23% of control levels (100%). β-Endorphin levels in pituitary of cathepsin L KO mice were reduced to 18% of control levels (100%), and α-MSH was reduced in KO mice to 7% of control levels (100%). These data demonstrate a key role for cathepsin L in the production of POMC-derived peptide hormones.

In parallel to the decreased levels of POMC-derived peptide hormones, cathepsin L KO mice showed increased levels of POMC, detected by anti-ACTH Western blots, compared with wild-type controls (Fig. 2). Accumulation of POMC in the cathepsin L knock-out is consistent with its role as a substrate for cathepsin L. The increase in POMC was not due to changes in its mRNA, since POMC mRNA levels in pituitaries of cathepsin L KO mice were compared to wild-type controls were similar. The accumulation of POMC in the cathepsin L KO condition (compared with control wild-type mice) suggests a role for proteolysis of POMC by cathepsin L.

In addition, increased levels of the POMC-derived 22-kDa ACTH intermediate were observed in pituitaries of cathepsin L KO mice (compared with wild type). Such results implicates cathepsin L in modifying ACTH production from POMC.

In the cathepsin L KO mice, there was no change in levels of PC1/3 or PC2 protein or enzyme activities in pituitaries of cathepsin L KO mice. These controls indicated that there was no detectable compensatory change in PC1/3 or PC2 enzymes. Thus, the significant reduction of ACTH, β-endorphin, and α-MSH is consistent with a role of cathepsin L for production of ACTH, β-endorphin, and α-MSH peptide hormones.

Cathepsin L Colocalizes in Vivo with β-Endorphin, α-MSH, and ACTH in Secretory Vesicles of Mouse Pituitary; Comparison with the Lamp-1 Marker for Lysosomes—A role for cathepsin L in the production of POMC-derived peptide hormones requires localization of cathepsin L with these peptide hormones in secretory vesicles, the primary subcellular site for peptide hormone production, storage, and secretion. Therefore, colocalization studies of cathepsin L with POMC-derived peptide hormones in pituitary (normal wild-type mouse) were conducted by immunofluorescence confocal microscopy.

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In intermediate pituitary, cathepsin L was colocalized with β-endorphin that is present in secretory vesicles (Fig. 3a). Cathepsin L immunofluorescence staining was observed as a punctate pattern of staining in the perinuclear areas of intermediate pituitary cells. The colocalization of cathepsin L with β-endorphin was illustrated by the merged images showing areas of colocalization as yellow fluorescence. All β-endorphin subcellular areas showed the presence of cathepsin L. Controls with only secondary antisera resulted in the absence of immunofluorescence staining (Fig. 3b). These results show selective immunofluorescence detection of cathepsin L and β-endorphin by primary antisera.

Results also showed excellent colocalization of cathepsin L with α-MSH in intermediate pituitary (Fig. 4). Cathepsin L localization (green fluorescence) with α-MSH (red fluorescence) was illustrated by their merged images showing areas of colocalization by yellow fluorescence.

In mouse anterior pituitary, cathepsin L was colocalized with ACTH-containing cells (Fig. 5). The pattern of ACTH immunostaining was consistent with the knowledge that ACTH corticotroph cells comprise 3–5% of cells in anterior pituitary (3, 4). Immunostaining showed that all ACTH-containing cells show colocalization of cathepsin L and ACTH. Furthermore, cathepsin L was present in other cell types of anterior pituitary that are known to produce other anterior pituitary hormones (3, 4). The overlapping localization of ACTH and cathepsin L indicated the presence of cathepsin L in ACTH-containing secretory vesicles.

It was of interest that a large portion of cathepsin L was colocalized with β-endorphin and α-MSH in intermediate pituitary, suggesting perhaps greater localization of cathepsin L with peptide hormone-containing secretory vesicles compared with the presence of cathepsin L in lysosomes. Direct assessment of the localization of cathepsin L with the lysosomal marker Lamp-1 showed that cathepsin L partially colocalized with Lamp-1 in intermediate pituitary (Fig. 6). Similar results for partial colocalization of cathepsin L with Lamp-1 in anterior pituitary were also observed (data not shown). These findings support results of this study showing a high degree of cathepsin L localization in peptide hormone-containing secretory vesicles in pituitary.

Expression of Cathepsin L Increases ACTH and β-Endorphin Production in the Regulated Secretory Pathway of Pituitary AtT-20 Cells—The mouse pituitary AtT-20 cell line produces ACTH and β-endorphin from POMC (19, 20). The homogeneous nature of a clonal cell line allowed further assessment of the colocalization of cathepsin L with ACTH. The punctate immunostaining of cathepsin L was colocalized with ACTH (Fig.7a). In neuritic-like extensions of AtT-20 cells, ACTH-containing secretory vesicles contained cathepsin L (Fig. 7b).

Expression of cathepsin L in AtT-20 cells was conducted to demonstrate the role of cathepsin L for production of ACTH and β-endorphin. Expression of cathepsin L resulted in significantly increased amounts of ACTH and β-endorphin secreted from the regulated secretory pathway (Fig. 8, a and b). Secretion from the regulated secretory pathway was conducted in the presence of BaCl2, which is known to stimulate regulated secre-
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As a control experiment, cathepsin L expression was demonstrated by transfection of the cathepsin L/EGFP fusion protein (Fig. 9). Western blot detection of the cathepsin L/EGFP fusion protein confirmed expression with the CMV promoter plasmid expression vector. In addition, transfection of cathepsin L cDNA by CMV plasmid expression vector resulted in elevated levels of cathepsin L activity. Overall, expression of cathepsin L resulted in increased production of ACTH and β-endorphin.

Treatment of AtT-20 cells with CLIK-148, inhibitor of cathepsin L, reduced ACTH production. The specific inhibitor of cathepsin L, known as CLIK-148, was utilized to assess its effects on ACTH production in AtT-20 cells. After treatment of cells with CLIK-148, ACTH content in cells was reduced by ~60% compared with untreated controls (Fig. 10a). Furthermore, POMC processing was inhibited by CLIK-148, indicated by accumulation of POMC in cells treated with CLIK-148 (Fig. 10b). Treatment with CLIK-148 also resulted in accumulation of the 22–24-kDa POMC-derived intermediate. These results are consistent with proteolytic processing of POMC by cathepsin L.

DISCUSSION

The biosynthesis of the peptide hormones ACTH, β-endorphin, and α-MSH from their common POMC precursor is critical for targeted regulation of neuroendocrine systems. Herein we provide evidence that the cysteine protease cathepsin L plays a key role in the production of pituitary ACTH, β-endorphin, and α-MSH from the POMC precursor. In vivo localization (22). Analyses of cell extracts with anti-ACTH Western blots showed that expression of cathepsin L resulted in elevated levels of ACTH (Fig. 8c).

FIGURE 7. Cathepsin L colocalization with ACTH in AtT-20 mouse pituitary cells. a, colocalization of cathepsin L with ACTH in the AtT-20 pituitary cell line. Cathepsin L in the AtT-20 mouse pituitary cell line was colocalized with ACTH, demonstrated by dual immunofluorescence confocal microscopy (a and b). Cathepsin L (green fluorescence) showed a punctate pattern of subcellular immunostaining that showed overlapping localization with areas of ACTH immunostaining (red fluorescence), shown by the merged images, with yellow fluorescence indicating colocalization. Controls with only secondary antisera showed the absence of immunofluorescence staining. b, cathepsin L localization with ACTH in neuritic extensions of AtT-20 cells. Examination of the neuritic extensions of AtT-20 cells shows the presence of cathepsin L colocalized with ACTH. Controls with only secondary antisera showed lack of immunofluorescence staining.

FIGURE 8. Expression of cathepsin L increases production of ACTH and β-endorphin in the regulated secretory pathway of AtT-20 cells. a and b, ACTH and β-endorphin production by cathepsin L. Expression of cathepsin L (bovine) in AtT-20 cells by transfection resulted in increased amounts of ACTH (a) and β-endorphin (b) produced in regulated secretory vesicles whose contents were released in the presence of BaCl2. Cells were transfected at 70% confluence with cathepsin L/pcDNA3.1 or pcDNA3.1 vector alone, and 2 days later, cells were incubated in BaCl2 for 2 h, and the medium was collected for radioimmune assay measurements of ACTH and β-endorphin. BaCl2 is a stimulator of regulated secretion (22). Expression of cathepsin L resulted in increased production of ACTH and β-endorphin in regulated secretory vesicles, whose secretion was observed in the presence of BaCl2. Results are shown as the mean ± S.E. (n = 6 for each group, and the experiment was repeated twice) with significant increases of ACTH and β-endorphin produced after expression of cathepsin L with p < 0.05 (by Student’s t test). c, cathepsin L expression and analysis by anti-ACTH Western blots. Western blots with anti-ACTH of AtT-20 cells transfected with the cathepsin L cDNA (lane 2) or with control vector without cDNA (lane 1) showed that cathepsin L-transfected cells displayed increased amounts of ACTH (lane 2) (2 μg of protein/lane).
The proteolytic processing of POMC has been studied as a model prohormone for investigating protease pathways for its conversion into active peptide hormones. The finding of secretory vesicle cathepsin L in this study for the production of POMC-derived peptide hormones indicates cathepsin L as a newly identified protease pathway for POMC processing in addition to the known functions of the subtilisin-like protease convertases PC2 and PC1/3 for POMC processing (12–15).

Notably, cathepsin L knock-out mice displayed greater reduction in ACTH and β-endorphin compared with that in PC2 or PC1/3 knock-out mice (12, 13, 15, 23). The cathepsin L knock-out and the PC2 knock-out mice (12, 13, 24) both show substantial reductions in α-MSH. Clearly, cathepsin L plays a major role in the production of all three peptide hormones derived from POMC.

One of the notable findings from this study is the demonstration of intermediate and anterior pituitary showed that cathepsin L possesses a high degree of colocalization with these POMC-derived peptide hormones that are produced and stored within secretory vesicles. In fact, a higher portion of cathepsin L in these regions of pituitary was localized with peptide hormone-containing secretory vesicles, compared with lysosomes. On a functional level, cathepsin L gene knock-out mice showed substantial reduction of POMC-derived peptide hormones in pituitary that were reduced to 23, 18, and 7% of control levels (100%) for ACTH, β-endorphin, and α-MSH, respectively. The increase in POMC and changes in the 22-kDa ACTH-containing intermediate are consistent with a role for cathepsin L in proteolysis of POMC to generate peptide hormones. Expression of cathepsin L in the mouse corticotroph pituitary cell line AtT-20 resulted in increased production of ACTH and β-endorphin in the regulated secretory pathway. Furthermore, inhibition of cathepsin L by the specific inhibitor CLIK-148 (16) resulted in decreased ACTH and accumulation of POMC in AtT-20 cells. These novel findings demonstrate that cathepsin L functions as a significant cysteine protease pathway for production of ACTH, β-endorphin, and α-MSH from their common POMC precursor.
(Met)enkephalin by 50%. These results implicate dual roles for both cathepsin L and PC2 in enkephalin peptide neurotransmitter production. However, little change in enkephalin levels has been observed in brains of PC1/3 knock-out mice. These findings indicate that selected protease components of the dual cathepsin L and PC protease pathways may be utilized for proteolytic processing of proenkephalin or other proneuropeptides and prohormones.

Cathepsin L possesses specificity for processing paired basic residue-processing sites of prohormones and proneuropeptides. Notably, cleavage site studies with peptide-MCA substrates containing dibasic residue-processing sites indicated that cathepsin L prefers to cleave at the N-terminal side of dibasic residues as well as between the dibasic residues (28). In contrast, similar evaluation of PC1/3 and PC2 with dipeptide-MCA substrates showed that these PC proteases prefer to cleave at the C-terminal side of dibasic residue-processing sites (29). These data indicate that, following cathepsin L cleavage, removal of the remaining basic residue extensions at the N terminus of peptide products can be accomplished by Arg/Lys aminopeptidase activity. Arg/Lys aminopeptidase activity is present in pituitary secretory vesicles (30), and this activity has been identified as aminopeptidase B in neuroendocrine-containing secretory vesicles of neuroendocrine chromaffin cells (31).

In contrast to cathepsin L, evaluation of PC1/3 and PC2 with dipeptide-MCA substrates showed that these PC proteases prefer to cleave at the C-terminal side of dibasic residue-processing sites (29). Thus, processing of prohormones by PC2 or PC1/3 results in peptide intermediates with basic residue extensions at the C terminus, which are then removed by the neuroendocrine-specific carboxypeptidase E/H enzyme (32–35). The different exopeptidase steps following prohormone processing by cathepsin L or the PC enzymes implicate extended diversity in proteolytic pathways that may allow several steps for protease regulation in peptide hormone production.

In summary, this study demonstrates that cathepsin L in secretory vesicles functions as a key protease for the production of POMC-derived peptide hormones ACTH, β-endorphin, and α-MSH. These findings indicate the distinct function of cathepsin L in secretory vesicles for production of active peptide hormones, which contrasts with its presence in lysosomes for protein degradation. Significantly, results from this study identify cathepsin L as a novel cysteine protease pathway in secretory vesicles for production of peptide hormones derived from POMC.

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REFERENCES
1. Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N., and Numa, S. (1979) Nature 282, 423–424

4 V. Hook, and T. Toneff, unpublished observations.