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Article Aging

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Thyroid functions of mouse cathepsins B, K, and L

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Thyroid function depends on processing of the prohormone thyroglobulin by sequential proteolytic events. From in vitro analysis it is known that cysteine proteinases mediate proteolytic processing of thyroglobulin. Here, we have analyzed mice with deficiencies in cathepsins B, K, L, B and K, or K and L in order to investigate which of the cysteine proteinases is most important for proteolytic processing of thyroglobulin in vivo. Immunolabeling demonstrated a rearrangement of the endocytic system and a redistribution of extracellularly located enzymes in thyroids of cathepsin-deficient mice. Cathepsin L was upregulated in thyroids of cathepsin $K^{-/-}$ or $B^{-/-}/K^{-/-}$ mice, suggesting a compensation of cathepsin L for cathepsin K deficiency. Impaired proteolysis resulted in the persistence of thyroglobulin in the thyroids of mice with deficiencies in cathepsin B or L. The typical multilayered appearance of extracellularly stored thyroglobulin was retained in cathepsin $K^{-/-}$ mice only. These results suggest that cathepsins B and L are involved in the solubilization of thyroglobulin from its covalently cross-linked storage form. Cathepsin $K^{-/-}/L^{-/-}$ mice had significantly reduced levels of free thyroxine, indicating that utilization of luminal thyroglobulin for thyroxine liberation is mediated by a combinatory action of cathepsins K and L.

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Introduction

Thyroid function depends on cycles of synthesis of the prohormone thyroglobulin (Tg) and its proteolytic degradation for the maintenance of constant levels of thyroid hormones in the blood of vertebrates (1, 2). Within thyroid follicles, newly synthesized Tg is transported along the secretory route to the apical plasma membrane of thyroid epithelial cells. After exocytosis, Tg is stored within the extracellular lumen of thyroid follicles in a covalently cross-linked form (3–6). Thyroid hormone liberation begins with the solubilization of Tg from the covalently cross-linked globules (2). Soluble Tg is then subjected to limited proteolysis, leading to the rapid liberation of the thyroid hormone thyroxine (T_4) (7–10). This step of utilization of Tg precedes its endocytosis and its complete degradation within lysosomes of thyroid epithelial cells (2, 7, 10).

In vitro, proteolysis of Tg is achieved by its incubation with cysteine proteinases like cathepsins B and L (7, 11, 12). Recently, we have shown that thyroid epithelial cells additionally express cathepsin K, and that this protease is able to liberate T_4 from Tg by limited and extracellular proteolysis (8). These observations suggested that cathepsin K by itself, or in a combined action with the cysteine proteinases cathepsins B and L, might have an important function in the maintenance of constant levels of thyroid hormones in the blood (2). Here, we have analyzed cathepsin-deficient mice in order to clarify which of the cysteine proteinases are important for solubilization and for utilization of Tg in vivo. So far, thyroid function has not been analyzed in mice with single or double deficiencies in the cathepsins B, K, and/or L.

Although the gross phenotype of cathepsin B–deficient mice is not altered (13), a critical role of cathepsin B was demonstrated for trypsinogen activation in experimental acute pancreatitis (14). Furthermore, cathepsin B–deficient mice are less susceptible to TNF-α–induced hepatocyte apoptosis (15).

Homozygous cathepsin $K^{-/-}$ mice are fertile, and until the age of 10 months no overt phenotypic abnormalities were detected. Radiological examination showed, however, that cathepsin $K^{-/-}$ mice developed an osteopetrosis due to a severely impaired resorption of bone matrix (16). Therefore, cathepsin $K^{-/-}$ mice represent a valuable animal model for the rare bone disorder pyknodysostosis (16) in which the cathepsin K gene is

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Nonstandard abbreviations used: thyroglobulin (Tg); thyroxine (T_4); free T_4 (FT_4); triiodothyronine (T_3); thyroid-stimulating hormone (TSH).

affected by various mutations resulting in the expression of nonfunctional enzyme (17).

Homozygous mice with a deficiency in cathepsin L are fertile but develop periodic hair loss due to alterations of hair follicle morphogenesis and cycling (18, 19). Cathepsin L–deficient mice develop cardiac alterations that closely resemble human dilated cardiomyopathy (20). Furthermore, cathepsin L is essential for degradation of invariant chain during MHC class II–restricted antigen presentation in cortical thymic epithelial cells (21).

Mice with double deficiencies in cathepsins B and L were recently reported to suffer from an early-onset postnatal brain atrophy, and to die during the second to fourth week of life (22). In our hands, mice with double deficiencies in cathepsins B and L were also lethal in a very early postnatal state, whereas those with deficiencies in cathepsins B and K, or K and L survived (our unpublished observations). Cathepsin $B^{-/-}/K^{-/-}$ mice did not show an overt phenotype, whereas cathepsin $K^{-/-}/L^{-/-}$ mice were smaller in size as compared with WT littermates. This growth defect of cathepsin $K^{-/-}/L^{-/-}$ mice might be due in part to the osteopetrotic phenotype caused by the deficiency in cathepsin K. However, thyroid hormones have long been known as important regulators of vertebrate development and growth. Hence, if cathepsin K is indeed important for the liberation of thyroid hormones (2, 8), the smaller stature of cathepsin $K^{-/-}/L^{-/-}$ mice might additionally result from a thyroid phenotype.

To test this hypothesis in vivo, and to determine which cysteine proteinase is important for T_4 liberation from its prohormone Tg, here, we have analyzed thyroid morphology and function in cathepsin-deficient mice; i.e., cathepsin $B^{-/-}$, $K^{-/-}$, or $L^{-/-}$ mice were used. Because it could not be excluded that an overlapping function of the various enzymes is needed for proteolysis of Tg, double-deficient animals, i.e., cathepsin $B^{-/-}/K^{-/-}$ or cathepsin $K^{-/-}/L^{-/-}$ mice, were included in the analysis of the biological significance of cathepsins for thyroid physiology. The results demonstrate that cathepsin $K^{-/-}/L^{-/-}$ mice show a significant reduction of serum T_4 levels, indicating that both cathepsins K and L are necessary for thyroid hormone liberation from Tg in mice. In addition, the typical multilayered appearance of the storage form of the prohormone Tg in the lumen of thyroid follicles was retained in cathepsin $K^{-/-}$ mice only, and increased amounts of Tg persisted in mice with deficiencies in cathepsin B or L. These results suggest that cathepsins B and L are most important for the solubilization step that precedes liberation of T_4 from Tg. Hence, our results support the notion that cathepsins B, K, and L have an important impact on proper function of the mouse thyroid.

Methods

Generation of mice with deficiencies in cysteine proteinases. Cathepsin-deficient mice were generated by targeted disruption of the *ctsb*, *ctsk*, or *ctsl* gene. The detailed procedures have been described for the generation of cathepsin $B^{-/-}$ (13, 14), cathepsin $K^{-/-}$ (16), and cathepsin $L^{-/-}$ mice (18).

For generation of double-deficient mice, null mutant cathepsin B, K, or L mice on a mixed 129Ola:C57BL/6J background were intercrossed. Double-heterozygous mice were identified by PCR and bred to produce double-deficient mice and controls. Double-deficient mice were identified by PCR and Southern and Western blotting as described (13, 16, 18). All genotypes were obtained with the expected mendelian frequencies.

The animals used in all studies were maintained and bred according to institutional guidelines in the animal facilities of the University Medical Center (Freiburg, Germany) and of the University of Göttingen (Göttingen, Germany). All subsequent preparations, including blood sample collection and serum and tissue preparations, were performed in accordance with the same guidelines and after prior approval by the institutional animal care committees at the Universities of Bonn, Freiburg, and Göttingen (Germany).

Blood sample collection and quantitation of serum T_4 levels. For the determination of serum T_4 levels, mice were anesthetized, and blood samples were taken at 10 am by puncturing of the blood lacuna of the orbital sinus or the tail vein, or by heart puncturing. Blood samples were allowed to clot for 30–60 minutes at 37°C, and, after incubation overnight at 4°C, clotted material was carefully removed. Sera were cleared by centrifugation at 10,000 *g* for 10 minutes at 4°C, and stored at –20°C. For the quantitative determination of serum levels of free T_4 (FT_4), serum samples were analyzed in duplicates using an immunoradiometric assay according to the manufacturer's protocol (DYNOtest FT_4; Brahms Diagnostica GmbH, Henningsdorf/Berlin, Germany). Determination of serum FT_4 concentrations used the IRMA program of an LB 2111 gamma counter (Berthold Australia Pty Ltd., Bundoora, Victoria, Australia).

Preparation of thyroid tissue. For analysis of morphological signs of alterations in cathepsin-deficient mice, the following genotypes were used: WT mice (+/+), 12 months old, one female and two males; cathepsin $B^{-/-}$ mice, 8–9 months old, two females and one male; cathepsin $K^{-/-}$ mice, 11–12 months old, one female and two males; cathepsin $L^{-/-}$ mice, 10.5–12 months old, one female and two males; cathepsin $B^{-/-}/K^{-/-}$ mice, 7 months old, one female and one male, and 4 months old, one female; cathepsin $K^{-/-}/L^{-/-}$ mice, 9 months old, three females. Mice were anesthetized and bled by opening of the aorta descendens. Prewarmed PBS supplemented with 10 IU/ml heparin (B. Braun Melsungen AG, Melsungen, Germany) followed by 3% paraformaldehyde in PBS was perfused via the heart. Thyroid glands were dissected and freed of connective tissue. For analysis of protein expression levels, thyroid glands were excised after bleeding and perfusion with heparinized PBS.

Preparation of cryosections and immunolabeling. Dissected thyroid glands were postfixed with 8% paraformaldehyde in 200 mM HEPES at pH 7.4. After washing, thyroids were infiltrated with polyvinyl-

pyrrolidone-10 in phosphate-buffered sucrose (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) as cryoprotectant, and frozen in liquid propane. Sections were prepared with a cryotome (Reichert-Jung, Wien, Austria) at –60°C, and mounted on microscope slides. Blocking was performed with 3% BSA for 60 minutes at 37°C. Incubation with specific antibodies diluted in 0.1% BSA in calcium- and magnesium-free PBS occurred overnight at 4°C in a moisturized chamber. Specific antibodies were sheep anti–human cathepsin B (RD Laboratorien für Biologische Forschung GmbH, Diessen, Germany), rabbit anti–human cathepsin D (Calbiochem-Novabiochem GmbH, Bad Soden, Germany), rabbit anti–mouse cathepsin K (kindly provided by T. Kamiya, Y. Kobayashi, and H. Sakai, Nagasaki University School of Dentistry, Nagasaki, Japan) (23), rabbit anti–rat cathepsin L (kindly provided by John S. Mort, Shriners Hospital for Children, McGill University, Montreal, Quebec, Canada), rabbit anti-T_4 (ICN Biomedicals GmbH, Eschwege, Germany), rabbit anti-triiodothyronine (anti-T_3; ICN Biomedicals GmbH), or rabbit anti–bovine Tg (24). Secondary antibodies were incubated for 60 minutes at 37°C. As secondary antibodies, carbocyanine (Cy2) or indocarbocyanine (Cy3) coupled to goat anti-rabbit IgG, and TRITC coupled to donkey anti-sheep F(ab)$_2$ fragments (Dianova, Hamburg, Germany) were used. For negative controls, specific antibodies were omitted, or cryosections of thyroids from mice with deficiencies in cathepsins B, K, and/or L were used. Cryosections were mounted in a mixture of 33% glycerol and 14% mowiol in 200 mM Tris (pH 8.5) supplemented with 5% 1,4-diazabicyclo(2.2.2)octan as antifading agent.

Microscopy, documentation, and morphometric analysis of cryosections. Cryosections were viewed with an inverted confocal laser scanning microscope (LSM 510; Carl Zeiss, Oberkochen, Germany) equipped with an argon and a helium/neon mixed-gas laser with excitation wavelengths of 488 or 543 nm. Scans at a resolution of 1024 × 1024 pixels were taken in the line-averaging mode, and at a pinhole setting of one airy unit. Micrographs were stored in LSM or TIFF format (Zeiss LSM Image Browser version 2.30.011; Carl Zeiss Jena GmbH, Jena, Germany). Color coding and image analysis were by Image-Pro Plus 4.0 software (Media Cybernetics Inc., Silver Springs, Maryland, USA). The diameters of cathepsin D–positive vesicles were determined by automatic detection of bright objects from arbitrarily chosen immunofluorescence micrographs. For the determination of epithelial extensions, i.e., the heights of thyroid epithelial cells, micrographs of cryosections of thyroids of WT and of all cathepsin-deficient genotypes were analyzed by the LSM software (LSM 510 software version 2.8 SP1; Carl Zeiss, Oberkochen, Germany). Cross-sectioned follicles were arbitrarily chosen, and the areas and perimeters of each thyroid follicle and of their luminal contents were measured manually. Because most follicles are oval in shape, the value of mean epithelial extension of a given follicle was calculated using Microsoft Excel 2000 software (Microsoft Corp., Redmond, Washington, USA) according to equation 1.

Equation 1

$$\text{Epithelial extension} = \frac{\text{area}_{\text{follicle}} - \text{area}_{\text{lumen}}}{\text{perimeter}_{\text{follicle}} + \text{perimeter}_{\text{lumen}}} \times 0.5$$

Preparation of thyroid lysates, SDS-PAGE, immunoblotting, and densitometry. Dissected thyroids were homogenized and lysed on ice with 0.2% Triton X-100 in PBS supplemented with protease inhibitors [0.2 μg/ml aprotinin, 10 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64), 2 mM EDTA, and 1 μM pepstatin] for 30 minutes. After clearing by centrifugation at 10,000 *g* for 10 minutes at 4°C, the supernatants were used as thyroid lysates. Protein content was determined with Pierce Coomassie Protein Assay (Perbio Science Deutschland GmbH, Bonn, Germany) (25, 26). Lysates were normalized to equal amounts of protein, and boiled in sample buffer consisting of 10 mM Tris-HCl (pH 7.6), 0.5% (wt/vol) SDS, 25 mM DTT, 10% (wt/vol) glycerol, and 25 μg/ml bromophenol blue. As molecular mass markers, prestained precision protein standards were used (Bio-Rad Laboratories Inc., Hercules, California, USA). Proteins were separated by SDS-PAGE (27), blotted onto nitrocellulose, and detected by immunolabeling of the blots after blocking with 6% (wt/vol) casein, 1% (wt/vol) polyvinylpyrrolidone-40, and 10 mM EDTA in PBS plus 0.3% Tween-20. Specific antibodies were as described above for immunolabelings of cryosections, and, in addition, sheep anti–human cathepsin L (RD Laboratorien für Biologische Forschung GmbH) was used. Secondary antibodies were HRP- or peroxidase-conjugated donkey anti-sheep IgG (BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany) or goat anti-rabbit IgG (Dianova). Immunoreactions were visualized by enhanced chemiluminescence onto Hyperfilm MP (Amersham Pharmacia Biotech UK Ltd., Little Chalfont, United Kingdom). Films from three to four different blots for each immunodetection were scanned (Desk Scan II version 2.9; Hewlett-Packard Co., Palo Alto, California, USA) and evaluated densitometrically by TINA version 2.09d (raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Statistic evaluations. Mean values and levels of significance were calculated by one-way ANOVA (Origin 5.0 and 7.0; OriginLab Corp., Northampton, Massachusetts, USA).

Results

In the thyroid, cysteine proteinases like cathepsins B, K, and L are not restricted to lysosomes of epithelial cells; rather, they are also detected at extracellular locations, i.e., they are associated with the apical plasma membrane, and secreted into the lumen of thyroid follicles (7–10). In vitro, cathepsins were shown to cleave Tg at neutral pH conditions, leading to the direct liberation of T_4. We concluded that the cathepsins might be involved

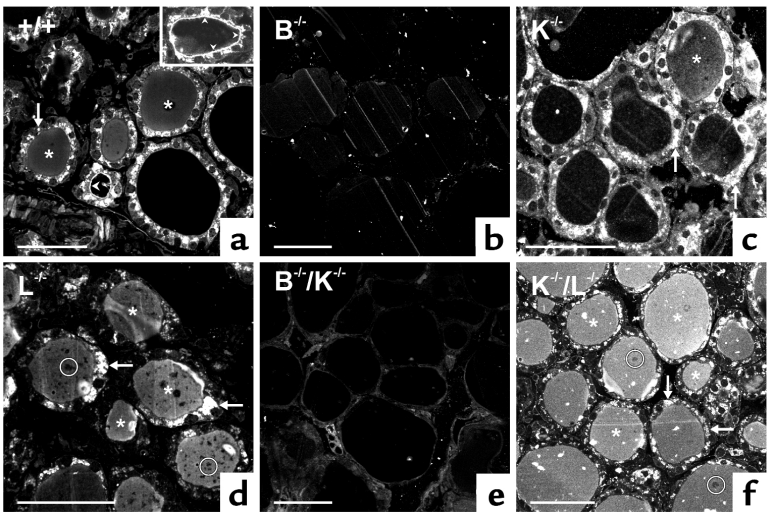

Figure 1
Redistribution of cathepsin B in thyroids of cathepsin $K^{-/-}$, $L^{-/-}$, or $K^{-/-}/L^{-/-}$ mice. Confocal fluorescence micrographs of cryosections of thyroid glands from WT mice (**a**) or cathepsin-deficient mice of the indicated genotypes (**b**–**f**) were immunolabeled with antibodies against cathepsin B. Thyroids from cathepsin B–deficient mice were not stained (**b** and **e**), indicating specificity of antibody labeling. Cathepsin B was detected within endocytic vesicles of thyroid epithelial cells (arrows). In WT mice, cathepsin B was also located at the apical plasma membrane of epithelial cells (arrowheads, inset) and within the lumina of thyroid follicles (asterisks). Deficiencies in cathepsin K and/or cathepsin L resulted in a redistribution of cell surface–associated cathepsin B, since immunolabelings of the apical plasma membranes were no longer observed. Rather, immunofluorescence became detectable over the follicle lumina (asterisks), and it was enhanced when compared with the WTs. Note the presence of non-immunolabeled inclusions within the thyroid follicle lumina of cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice (open circles). Bars: 50 μm.

in thyroid hormone liberation by proteolytic processing of the prohormone Tg (2). To test this hypothesis in vivo, we have analyzed mice with deficiencies in cathepsin B, K, or L, and mice with double deficiencies in cathepsins B and K, or K and L.

Rearrangement of the endocytic system in cathepsin-deficient mice. Cryosections of thyroids were immunolabeled with antibodies against cathepsin B, K, D, or L as markers of endocytic vesicles in order to analyze their distribution and morphology within thyroid epithelial cells of mice with cathepsin deficiencies.

By immunolabeling, cathepsin B was detected within vesicles of thyroid epithelial cells of WT mice (Figure 1a, arrow). In addition, the apical plasma membrane of thyrocytes (Figure 1a and inset, arrowheads) was immunolabeled, as was the luminal content of follicles of WT mice (asterisks), indicating that cathepsin B is mainly located within lysosomes but also occurred at extracellular locations of WT thyroids. Cryosections of thyroids from mice with deficiencies in cathepsin B or cathepsins B and K were not immunolabeled (Figure 1, b and e, respectively), demonstrating the specificity of cathepsin B immunolabeling. In cathepsin $K^{-/-}$, $L^{-/-}$, or $K^{-/-}/L^{-/-}$ mice, cathepsin B was localized within vesicles of thyroid epithelial cells (Figure 1, c, d, and f, arrows) and within the lumen of follicles (asterisks), whereas a staining of the apical plasma membrane was no longer observed. The results indicated that cathepsin B is associated with the apical plasma membrane in WT thyroids only, suggesting that deficiencies in cathepsins K and/or L led to the redistribution of binding partners of cathepsin B from the apical plasma membrane to other cellular locations.

In thyroids of WT mice, cathepsin K was detected within vesicles of the epithelial cells (Figure 2a, arrows), and within the follicle lumen (asterisks). Specificity of cathepsin K antibodies was proven by the absence of fluorescence signals when cryosections of thyroids from mice with cathepsin K deficiency were immunolabeled (not shown). In thyroids of cathepsin $B^{-/-}$ mice, cathepsin K was no longer detected within the follicle lumen, and cathepsin K–positive vesicles appeared smaller as compared with those of WT thyrocytes (Figure 2b, arrows; compare upper inset in b with inset in a). Furthermore, immunolabeling of cathepsin K at the apical surface of cathepsin B–deficient thyrocytes was observed (Figure 2b, lower inset in b, arrowheads). In contrast to its absence from the luminal content of cathepsin $B^{-/-}$ mice, cathepsin K was detected within the follicle lumen of thyroids from cathepsin $L^{-/-}$ mice (Figure 2c, asterisks), which often contained immunolabeled inclusions (open circles). In comparison with the WT, cathepsin K–labeled vesicles were enlarged in thyroid epithelial cells of cathepsin $L^{-/-}$ mice (Figure 2c, arrows in inset; compare inset in c with inset in a). The results indicated that deficiencies in cathepsin B or L had opposite effects on the sizes of cathepsin K–containing vesicles of thyrocytes.

Cathepsin L was detected by immunolabeling within vesicles of WT thyrocytes (Figure 2d, arrows). In addition, a very faint immunolabeling was observed in association with the apical plasma membrane of WT cells (Figure 2, inset in d, arrowheads). Cell surface association of cathepsin L was not observed in thyroids from cathepsin $B^{-/-}$ (not shown), $K^{-/-}$, or $B^{-/-}/K^{-/-}$ mice, but staining of the follicle lumina occurred (Figure 2, e and f, asterisks). In thyroids from cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice, no immunolabeling with antibodies against cathepsin L was observed (not shown), proving specificity of antibody labeling.

In thyroid epithelial cells from WT mice, cathepsin D–positive vesicles, i.e., lysosomes, were distributed throughout the cells (Figure 3a). Lysosomes of cathepsin $K^{-/-}$ or $B^{-/-}/K^{-/-}$ thyrocytes were comparable in size and distribution to the vesicles of WT cells (Figure 3b). However, immunolabeled vesicles of thyroid epithelial cells from mice with deficiencies in cathepsin B or L

alone, or in both cathepsins K and L, demonstrated a significant enlargement when compared with WT lysosomes (Figure 3b; and compare c–e with a). Furthermore, lysosomes of thyroid epithelial cells of cathepsin $L^{-/-}$ mice often lacked cathepsin D immunolabeling within their lumina, i.e., they appeared as ringlike structures (Figure 3d, arrows), suggesting an association of immunolabeled cathepsin D with vesicular membranes. Similarly, cathepsin B was associated with lysosomal membranes in cathepsin $L^{-/-}$ thyrocytes (Figure 3d, inset, arrows). Accordingly, alterations in the ultrastructure of lysosomes were observed in keratinocytes and cardiomyocytes of cathepsin L–deficient mice (19, 20).

The results of immunolocalization of lysosomal enzymes indicated that deficiencies in cysteine proteinases in thyroid epithelial cells led to an extensive rearrangement of the endocytic system, and to a redistribution of extracellularly located enzymes.

Alterations of cathepsin levels in the thyroid. Because of the altered morphology of the endocytic system of thyroid epithelial cells from cathepsin-deficient mice, we thought to investigate potential compensatory effects on the translational level. For the analysis of the levels of cathepsin expression, lysates of thyroids from mice of the indicated genotypes were normalized to equal amounts of protein, separated on SDS gels, and transferred to nitrocellulose.

Probing of the blots with antibodies against cathepsin B showed, as expected, the lack of its expression in thyroids from cathepsin $B^{-/-}$ or $B^{-/-}/K^{-/-}$ mice (Figure 4a). Levels of cathepsin B expression in thyroids from cathepsin $K^{-/-}$, $L^{-/-}$, or $K^{-/-}/L^{-/-}$ mice were comparable to those in WT thyroids (Figure 4a). The mature forms of cathepsin B were expressed, i.e., single chain and smaller amounts of the heavy chain of two-chain cathepsin B were detected in immunoblots (Figure 4a).

Because it is known that cathepsin D might also be involved in Tg processing (11, 28), and because cathepsin D–positive lysosomes were enlarged in some genotypes (see Figure 3), the expression pattern of this aspartic protease was included in the analysis. In contrast to the principal expression of mature cathepsin B, immunoblotting of cathepsin D demonstrated that the expression of its proform dominated (Figure 4b), indicating that processing of cathepsin D to its fully mature form is negligible in thyroid epithelial cells. Expression of procathepsin D was significantly downregulated in thyroids from cathepsin $B^{-/-}$ mice (Figure 4b). The amounts of procathepsin D were slightly, but not significantly, enhanced in cathepsin $K^{-/-}$ thyroids (Figure 4b). Double deficiency in cathepsins B and K resulted in some reduction of the procathepsin D level, which was, however, not significant when compared with procathepsin D expression of WT thyroids (Figure 4b). In clear contrast, procathepsin D was significantly overexpressed in thyroids of cathepsin $L^{-/-}$ and $K^{-/-}/L^{-/-}$ mice (Figure 4b). Because expression of procathepsin D was only slightly enhanced in thyroids of cathepsin $K^{-/-}$ mice when compared with WT (Figure 4b), the results indicated that it was primarily cathepsin L deficiency that induced overexpression of procathepsin D in the thyroid whereas cathepsin B deficiency resulted in a significant downregulation of procathepsin D levels. The result of cathepsin D overexpression in cathepsin L–deficient mouse thyroids might be indicative of a compensatory function of the aspartic lysosomal enzyme when cathepsin L is lacking. It remains unclear, however, whether cathepsin D compensates for cathepsin L action, because cathepsin D was present mainly in its proform within the thyroid (Figure 4b), hence, in its proteolytically inactive form.

When immunoblots were probed with various antibodies against cathepsin K purified from mouse or

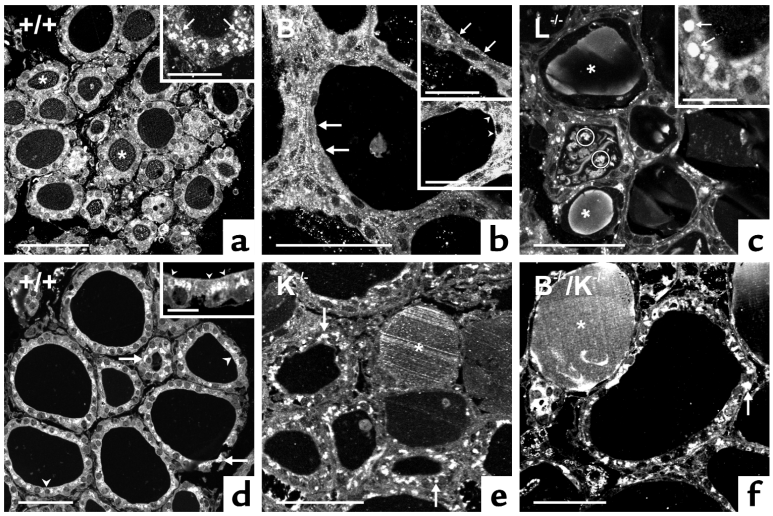

Figure 2
Localization of cathepsins K and L. Confocal fluorescence micrographs of cryosections of thyroid glands from WT mice (+/+) or cathepsin-deficient mice of the indicated genotypes were immunolabeled with antibodies against cathepsin K (**a**–**c**) or cathepsin L (**d**–**f**). Compared with the WT (**a**, inset, arrows), cathepsin K–positive vesicles were smaller in cathepsin $B^{-/-}$ (**b**, top inset, arrows) and larger in cathepsin $L^{-/-}$ thyroid epithelial cells (**c**, inset, arrows). Cathepsin K was absent from thyroid follicle lumina of cathepsin $B^{-/-}$ mice but was detectable within lumina of WT or cathepsin $L^{-/-}$ thyroids (asterisks). Immunolabeling of cathepsin K was also observed at the apical plasma membrane of cathepsin $B^{-/-}$ thyroid epithelial cells (**b**, bottom inset, arrowheads). In addition, cathepsin K–positive inclusions were frequently detected within the luminal content of cathepsin $L^{-/-}$ mice (**c**, open circles). In WT mice, cathepsin L was detected mainly within endocytic vesicles (**d**, arrows) and, in a few follicles, in association with the apical plasma membrane of thyroid epithelial cells (**d** and inset in **d**, arrowheads). Deficiencies in cathepsin B and/or cathepsin K demonstrated a lack of cathepsin L at the apical plasma membrane and resulted in an enhancement of immunolabeling of thyroid follicle lumina (**e** and **f**, asterisks). The redistribution of cathepsin L was less obvious than that of cathepsin B (compare with Figure 1). Bars: 50 μm; in insets: 20 μm.

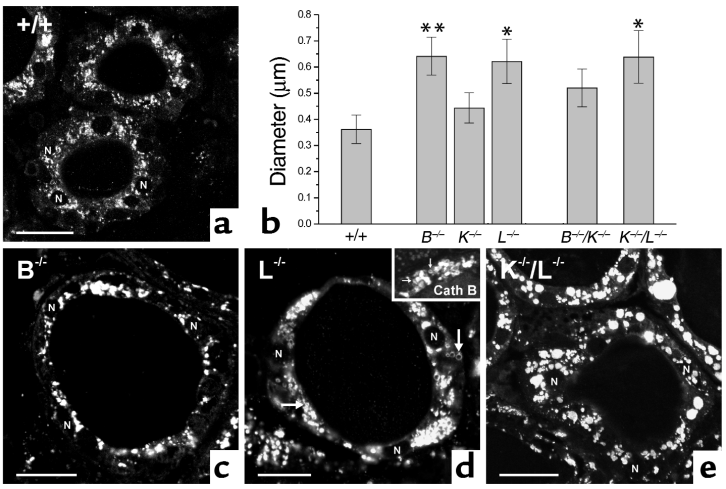

Figure 3
Swelling of cathepsin D–containing lysosomes in thyroids of cathepsin $B^{-/-}$, $L^{-/-}$, or $K^{-/-}/L^{-/-}$ mice. Confocal fluorescence micrographs of cryosections of thyroid glands from WT mice (+/+) or cathepsin-deficient mice of the indicated genotypes were immunolabeled with antibodies against cathepsin D (**a** and **c**–**e**). Diameters of cathepsin D–containing lysosomes were determined morphometrically and are given as means ± SE (**b**). In WT thyroid epithelial cells, cathepsin D–positive vesicles were distributed throughout the cells (**a**). An immunolabeling indicative of cathepsin D at the apical cell surface or over the follicle lumina was not observed in either genotype. The sizes of lysosomes of cathepsin $K^{-/-}$ or $B^{-/-}/K^{-/-}$ thyrocytes were similar to those of WT controls (**b**), whereas those from cathepsin $B^{-/-}$, $L^{-/-}$, or $K^{-/-}/L^{-/-}$ thyroid epithelial cells were significantly enlarged (**c**–**e**). Cathepsin D was absent from the inner portions of enlarged lysosomes of thyroid epithelial cells with a deficiency in cathepsin L (**d**, arrows). Similarly, cathepsin B (Cath B) immunostainings revealed ringlike lysosomes in $L^{-/-}$ thyrocytes (**d**, inset, arrows), indicating a tight association of cathepsins B and D with vesicular membranes in cathepsin L–deficient thyrocytes. N, nuclei. *P < 0.05, **P < 0.01. In **b**, n = 16, 12, 16, 19, 18, and 14, respectively, for sections of the different genotypes indicated. Bars: 20 μm.

human tissues, or against the recombinant enzyme, or against distinct peptide regions of cathepsin K, it became obvious that the antibodies hardly recognized cathepsin K in lysates of mouse thyroids (not shown). However, several of the antibodies tested for immunoblotting indeed cross-reacted with cathepsin K of formaldehyde-fixed mouse thyroid tissue after immunolabeling of cryosections (compare Figure 2, a–c) — and did so in a specific manner, since immunolabeling was not observed in thyroids from cathepsin $K^{-/-}$ or cathepsin $K^{-/-}/L^{-/-}$ mice. From the intensity of immunolabeling it might be concluded that the expression of cathepsin K was unaltered in cathepsin $B^{-/-}$ thyroids (Figure 2, compare b with a), whereas immunolabeling of cryosections from cathepsin $L^{-/-}$ thyroids might suggest a downregulation of cathepsin K (Figure 2, compare c with a). However, further studies are needed to analyze the levels of cathepsin K expression in cathepsin B– or L–deficient mouse thyroids by biochemical means.

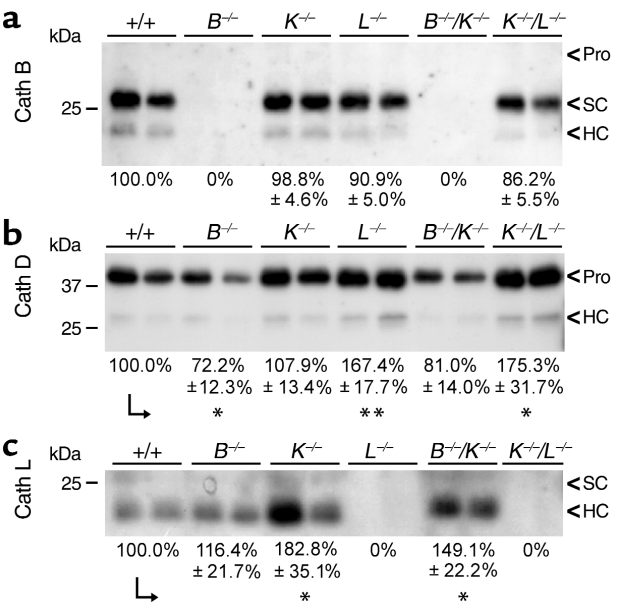

Figure 4
Compensatory effects on the levels of cathepsin expression. Lysates of thyroids from mice of the indicated genotypes were normalized to equal amounts of protein, separated on 15% SDS gels, and transferred to nitrocellulose for subsequent incubation of the blots with antibodies against cathepsin (Cath) B (**a**), D (**b**), or L (**c**). Three to four blots each were used for densitometric evaluation as a measure of cathepsin expression levels. Levels are indicated by numbers below representative immunoblots and are expressed as the mean percentages ± SE of 100% expression in WT controls. Molecular mass markers are indicated in the left margins. Note that cathepsin B expression was absent from cathepsin $B^{-/-}$ and $B^{-/-}/K^{-/-}$ thyroids, as expected, and that it was not altered by cathepsin K, L, or K/L deficiencies (**a**). In contrast, cathepsin D was downregulated in cathepsin $B^{-/-}$ thyroids, but upregulated under conditions of cathepsin L or K/L deficiency, whereas it was not significantly altered in cathepsin $K^{-/-}$ or $B^{-/-}/K^{-/-}$ thyroids (**b**). Cathepsin L was absent from thyroid lysates of cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice, unaltered in cathepsin $B^{-/-}$ thyroids, and significantly upregulated in cathepsin $K^{-/-}$ or $B^{-/-}/K^{-/-}$ mice (**c**). SC, single chain; HC, heavy chain; pro, proform. *P < 0.05, **P < 0.01.

Immunoblotting of cathepsin L proved, as expected, its deficiency in thyroids of cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice (Figure 4c). The major portion of cathepsin L expressed in WT thyroids was processed to its mature form (Figure 4c). Cathepsin B deficiency resulted in a nonsignificant upregulation of cathepsin L expression, whereas a significant cathepsin L overexpression was the result of a deficiency in cathepsin K alone or in cathepsins B and K (Figure 4c). These results indicated that cathepsin L might have a compensatory function when cathepsin K is lacking.

Survival of thyroid epithelial cells depends on expression of cathepsin L. A striking observation was the occurrence of inclusions within the lumina of thyroid follicles from cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice (Figures 1, 2, and 8, open circles; and Figure 5). Luminal inclusions were immunolabeled with antibodies against cathepsin K (Figure 2c, open circles) but appeared black after immunolabeling with antibodies against cathepsin B (Figure 1, d and f, open circles), T_3 (Figure 5, d, e, g, and h), T_4 (not shown), or Tg (Figure 8, d and f, open circles). In phase-contrast micrographs, the inclusions demonstrated a granular structure and were comparable in size to thyroid epithelial cells lining the follicle lumen (Figure 5, f and i), suggesting that luminal inclusions represented remnants of dead thyrocytes. Because such inclusions of remnants of dead cells were not present in the lumina of thyroid follicles of WT or cathepsin $B^{-/-}$, $K^{-/-}$, or $B^{-/-}/K^{-/-}$ mice, the results indicated that cathepsin L is necessary for survival of thyroid epithelial cells. The molecular mechanism by which the survival of thyrocytes is maintained remains, however, unclear.

T_4 liberation is mediated by cathepsins K and L. Because the endocytic system and the levels of protease expression of thyroid epithelial cells showed alterations in cathepsin-deficient mice, we postulated that Tg processing and Tg utilization, i.e., thyroid hormone liberation, might be affected.

Epithelia of thyroids from mice of all cathepsin-deficient genotypes were less extended than those of WTs (Figure 6a), indicating that flattening of thyroid epithelia was a result of cysteine proteinase deficiency. In euthyroid vertebrates, a demand of thyroid hormones is signaled by a rise in thyroid-stimulating hormone (TSH) levels in the blood as a result of negative feedback regulation (for reviews, see refs. 29, 30). Under such conditions of acute TSH stimulation, thyroid epithelial cells change from a cubic to a prismatic appearance, which reflects enhanced activity of thyrocytes, i.e., enhanced Tg turnover. Hence, flattening of epithelia might indicate a reduction in thyroid functional activity.

Because T_4 is the major hormone released from the thyroid (29) and becomes converted into its biologically active form T_3 by deiodinases upon entry of target cells, we considered serum T_4 levels as a relevant systemic marker of thyroid function. In general, T_4 levels were high in young animals and dropped with increasing age of WT or cathepsin $B^{-/-}$ or $K^{-/-}$ mice (Figure 6c, black, red, and green, respectively). An exception from this downregulation of T_4 levels with aging were mice with double deficiencies in cathepsins B and K, in which T_4 levels increased from significantly reduced levels in young mice to slightly elevated levels in mice older than 8 months (Figure 6c, orange). In cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice, the decline of serum T_4 with age was less well pronounced as compared with WTs (Figure 6c, blue and cyan, respectively), indicating that cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice developed reduced T_4 levels at an early

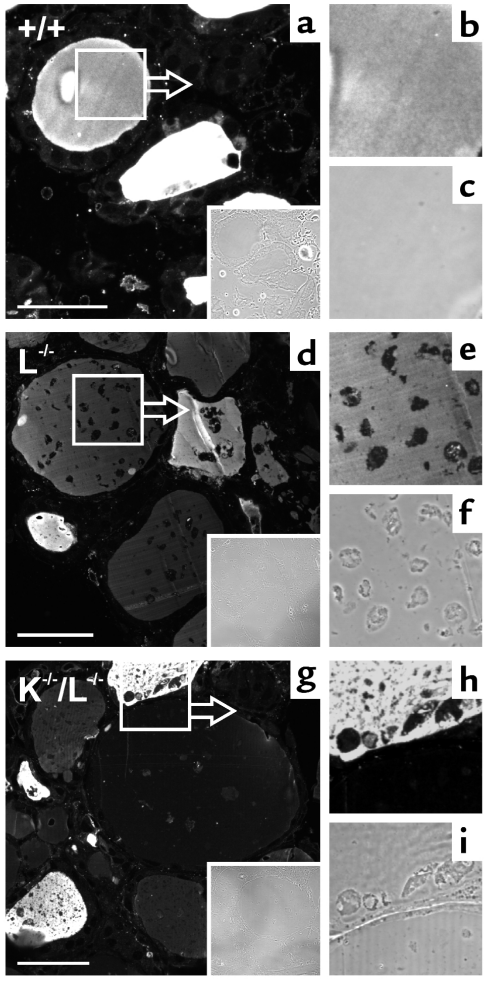

Figure 5
Cathepsin L is essential for survival of thyroid epithelial cells. Confocal fluorescence (**a**, **b**, **d**, **e**, **g**, and **h**) and corresponding phase-contrast micrographs (insets in **a**, **d**, and **g**; and **c**, **f**, and **i**) of cryosections of thyroid glands from WT mice (+/+) or from mice with deficiencies in cathepsin L ($L^{-/-}$, $K^{-/-}/L^{-/-}$) after immunolabeling with anti-T_3 antibodies. Thyroid glands of mice with deficiencies in cathepsin L alone or in cathepsins K and L were characterized by numerous inclusions in the follicle lumina, which were not stained by the antibodies (**d**, **e**, **g**, and **h**). The inclusions were of irregular shapes with a granular appearance in phase-contrast micrographs (**f** and **i**), and their sizes were comparable to those of thyroid epithelial cells lining the follicle lumina, suggesting that luminal inclusions represented remnants of dead cells. Dead cells were not present under conditions of cathepsin B deficiency, i.e., in cathepsin $B^{-/-}$ or $B^{-/-}/K^{-/-}$ mice. Bars: 50 μm; boxes in **a**, **d**, and **g** indicate regions that are shown in higher magnification in **b** and **c**, **e** and **f**, or **h** and **i**, respectively.

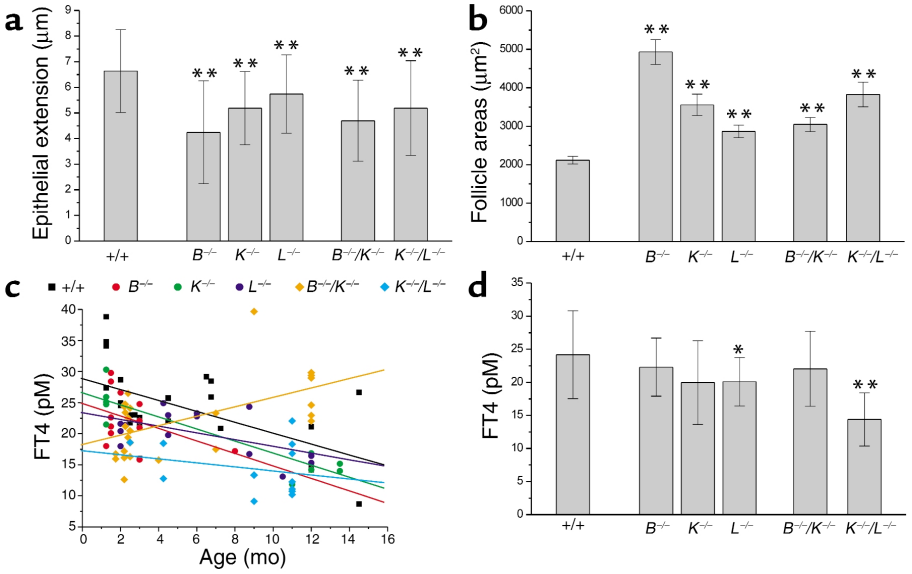

Figure 6
Alterations in thyroid physiological parameters. (**a** and **b**) Morphometric analysis of epithelial extensions, i.e., cell heights (**a**), and follicle dimensions (**b**) of thyroids from WT and cathepsin-deficient mice. Extensions of thyroid epithelia were significantly reduced (**a**) whereas follicle areas were significantly increased (**b**) in mice of all cathepsin-deficient genotypes. (**c** and **d**) To determine whether flattening of epithelia reflects a reduction in the functional activities of thyroid glands, serum T_4 levels were determined by a radiometric immunoassay. (**c**) The scatter graph indicates distribution of serum T_4 levels plotted against mouse age. (**d**) Data from mice of all ages were analyzed. Serum T_4 levels were significantly but slightly reduced in cathepsin $L^{-/-}$ mice (**c** and **d**, blue). A systemic defect in thyroid function was apparent from the very significantly reduced serum T_4 levels in cathepsin $K^{-/-}/L^{-/-}$ mice (**c** and **d**, cyan). Mean values ± SD are given in **a** and **d**, mean values ± SE in **b**. Lines represent linear regressions of the single data plotted against mouse age in **c**. $*P < 0.05$, $**P < 0.01$. In **a** and **b**, arbitrarily chosen follicles were analyzed; n = 162, 70, 71, 114, 150, and 58, respectively. In **c** and **d**, T_4 levels of different animals were determined; n = 25, 12, 10, 14, 28, and 13, respectively.

age. The serum levels of FT4 were significantly reduced ($P < 0.05$) in the sera of mice with cathepsin L deficiency. In mice with double deficiency in cathepsins K and L (Figure 6d), the reduction was even greater ($P < 0.01$). reduced in mice with double deficiency in cathepsins K and L (Figure 6d). The systemic defect in thyroid function, i.e., reduction in serum T_4, was established in both sexes of cathepsin $K^{-/-}/L^{-/-}$ mice.

Reduced levels of thyroid hormones normally result in an increase in TSH levels, which may, after long intervals of several weeks, induce an enlargement of thyroid follicles due to hyperproliferation of thyroid epithelial cells. Therefore, the dimensions of thyroid follicles were analyzed morphometrically in mice with cathepsin deficiencies. In all cathepsin-deficient genotypes, an enlargement of thyroid follicles was observed when compared with the WTs (Figure 6b). The highest values of follicle areas were observed in cathepsin $B^{-/-}$ thyroids (Figure 6b), which were also characterized by extremely flat epithelia (Figure 6a) and by the highest amounts of Tg (see below). In mice with double deficiencies in cathepsins K and L, thyroid follicle areas were enhanced by about 80% as compared with the WTs (Figure 6b). Hence, the systemic defect in serum T_4 in cathepsin $K^{-/-}/L^{-/-}$ mice (Figure 6d, $K^{-/-}/L^{-/-}$) correlated with an enlargement of thyroid follicles (Figure 6b, $K^{-/-}/L^{-/-}$), a phenotype reminiscent of hypothyroidism. Deficiencies in cathepsin B, K, or L alone or in cathepsins B and K were not sufficient to induce this phenotype, indicating that a combinatory action of both cathepsins K and L is necessary for proper hormone liberation from the thyroid. Phenotypic alterations with the development of signs of hypothyroidism were observed in the mice with double deficiencies for cathepsins K and L only at the level of thyroid follicles, whereas the volumes or wet weights of thyroids from cathepsin $K^{-/-}/L^{-/-}$ mice were unaltered compared with the WTs (not shown). This is most probably explained by enhanced cell death induced by cathepsin L deficiency (see above, Figure 5).

The results demonstrate that thyroid function is impaired by a deficiency in cysteine proteinases. An obvious phenotype with significantly reduced serum T_4 levels was observed in cathepsin $K^{-/-}/L^{-/-}$ double-deficient mice, indicating that a combinatory action of cathepsins K and L is necessary for T_4 liberation, i.e., utilization of Tg.

Alterations in luminal Tg depositions are caused by deficiency in cathepsin B or L. Because reduced levels of serum T_4 in cathepsin $K^{-/-}/L^{-/-}$ mice are most probably caused by an impaired Tg degradation, the molecular status of Tg (Figure 7) and its localization (Figure 8) were analyzed next.

Equal amounts of protein of thyroid lysates from mice of the indicated genotypes were loaded onto SDS gels, and blots were probed with antibodies against Tg (Figure 7, a and b). Protein bands representing

monomeric and dimeric Tg were present in all cathepsin-deficient genotypes (Figure 7b, thin and thick lines, respectively). Thyroid lysates from cathepsin $K^{-/-}/L^{-/-}$ mice contained an additional band with higher electrophoretic mobility than monomeric Tg (Figure 7b, $K^{-/-}/L^{-/-}$, broken line). This band represented a high–molecular weight degradation fragment of Tg and indicated an alteration of Tg degradation in cathepsin $K^{-/-}/L^{-/-}$ thyroids as compared with those of the other cathepsin-deficient genotypes. Densitometry of the gels demonstrated that the amounts of total Tg were higher in thyroids from mice of all cathepsin-deficient genotypes when compared with the WTs (Figure 7d). Comparable results were obtained when single bands representing intact Tg or its degradation fragments were analyzed by densitometry (Figure 7c). Five- to sixfold higher amounts of Tg were observed in thyroids from cathepsin $B^{-/-}$, $L^{-/-}$, $B^{-/-}/K^{-/-}$, or $K^{-/-}/L^{-/-}$ mice, whereas cathepsin K deficiency alone resulted in Tg levels that were increased only about twofold over those of the WTs (Figure 7d), indicating that cathepsins B and L are more important than cathepsin K for the gross degradation of Tg.

Because Tg degradation is not restricted to intracellular locations like lysosomes but begins with its extracellular solubilization from the covalently cross-linked storage form, an alteration of Tg degradation might also affect its luminal and/or lysosomal depositions. Therefore, cryosections from thyroids were immunolabeled with antibodies against Tg (Figure 8). Within thyroid epithelial cells of all cathepsin-deficient genotypes, numerous reticular and vesicular structures were immunolabeled with Tg antibodies (Figure 8, insets, arrows). Such structures resemble cisternae of the endoplasmic reticulum as well as exo- and endocytic vesicles, indicating that Tg synthesis, export, and uptake generally occur in thyroid epithelial cells of all cathepsin-deficient genotypes. Additionally, immunofluorescence signals were often observed in association with the basal lamina surrounding individual thyroid follicles (Figure 8, insets, arrowheads), which are most probably representative of Tg reaching the circulation by transcytosis.

Luminal Tg showed the typical multilayered appearance within thyroid follicles of WT controls (Figure 8a). Storage of Tg within follicle lumina occurs in a highly compacted form in which Tg is multimerized and covalently cross-linked. Hence, antibodies are less able to interact with highly compacted Tg in the center of thyroid follicle lumina, resulting in dim fluorescence intensities. In contrast, the luminal portions apposed to the apical plasma membrane are characterized by intense immunofluorescence, i.e., soluble Tg is easily accessible for the antibodies (Figure 8a, broken arrows). Tg depositions within the lumina of thyroid follicles of cathepsin K–deficient mice (Figure 8c, broken arrows) were indistinguishable from those of WTs, whereas thyroids of all other cathepsin-deficient genotypes lacked the multilayered appearance of luminal Tg (Figure 8, b and d–f; compare with a), indicating that a deficiency in either cathepsin B or cathepsin L resulted in the absence of differentially compacted luminal Tg. These results are most probably explained by the notion that cathepsins B and L are involved in the solubilization of Tg from its covalently cross-linked storage form, which is achieved by limited extracellular proteolysis. Further experiments must show whether the extent of covalent cross-linkages in luminal Tg is altered in thyroid follicles of cathepsin B– or L–deficient mice.

The absence of multilayers of luminal Tg might also explain the observation of increased thyroid follicle dimensions in cathepsin $B^{-/-}$ mice, which could not be explained as a result of reduced serum T_4 levels. If the rate of Tg export into the follicle lumen is unaltered, but the rate of removal of luminal Tg is reduced, then,

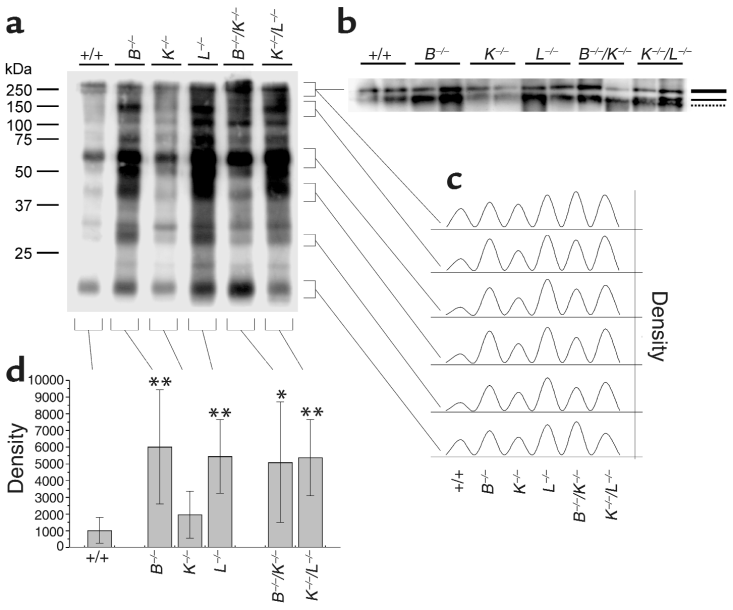

Figure 7
Tg persistence in thyroids of mice with deficiencies in cathepsin B or L. Lysates of thyroids from mice of the indicated genotypes were normalized to equal amounts of protein, separated on 10% SDS gels, and transferred to nitrocellulose for subsequent incubation of the blots with antibodies against Tg. (**a**) A representative immunoblot. (**b**) The uppermost portion of another immunoblot demonstrates the expression of dimeric (thick line) and monomeric Tg (thin line) in WT and all cathepsin-deficient thyroids, as well as the appearance of a high–molecular weight Tg fragment (broken line), which was observed in the lysates of cathepsin $K^{-/-}/L^{-/-}$ thyroids only. Molecular mass markers are indicated in the left margin. (**c**) Densitometric profiles of the indicated bands representing intact Tg and several of its degradation fragments. (**d**) Three different immunoblots with two lanes per genotype were evaluated densitometrically. Mean values ± SD of the densities of the lanes are given. Note that the amounts of immunolabeled Tg were upregulated about five- to sixfold in thyroids of cathepsin B– or L–deficient mice, whereas cathepsin $K^{-/-}$ thyroids contained only about twofold the amounts of Tg present in WTs (**d**).

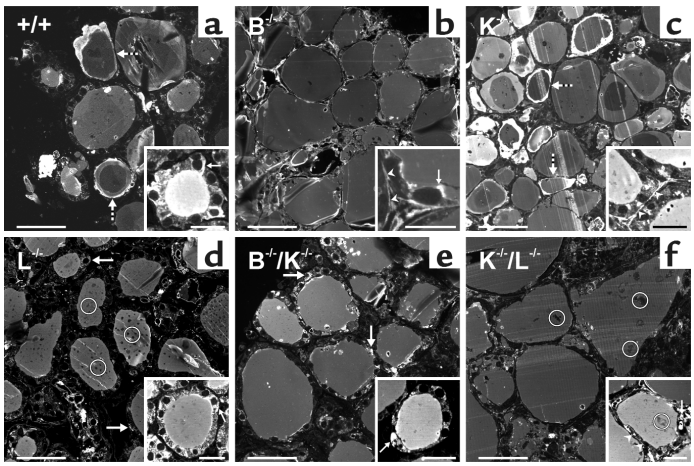

Figure 8
Multilayers of luminal Tg are lacking in thyroid follicles of cathepsin $B^{-/-}$, $L^{-/-}$, $B^{-/-}/K^{-/-}$, and $K^{-/-}/L^{-/-}$ mice. Confocal fluorescence micrographs of cryosections of thyroid glands from WT mice (**a**) or cathepsin-deficient mice of the indicated genotypes (**b**–**f**) were immunolabeled with antibodies against Tg. As expected, Tg was detected within reticular structures and numerous intracellular vesicles (arrows), i.e., within compartments of the biosynthetic and of the endocytic route. Arrowheads indicate immunolabeled Tg in association with the ECM surrounding thyroid follicles. Open circles indicate non-immunolabeled inclusions of dead cells within the follicle lumina of cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice. In WT and cathepsin $K^{-/-}$ mice, immunolabeling revealed the characteristic multilayered appearance of Tg within the lumina of thyroid follicles (broken arrows), representing different states of Tg compaction. In contrast, Tg was homogeneously distributed within the follicle lumina of thyroids of cathepsin $B^{-/-}$, $L^{-/-}$, $B^{-/-}/K^{-/-}$, or $K^{-/-}/L^{-/-}$ mice, indicating that deficiencies in either cathepsin B or cathepsin L resulted in the absence of differentially compacted luminal Tg. Bars: 50 μm; in insets: 20 μm.

as a consequence, the size of the luminal content must increase, resulting in an expansion of thyroid follicles. This is most clearly seen in cathepsin $B^{-/-}$ thyroid follicles (see $B^{-/-}$ in Figures 6, a and b; 7d; and 8b). A similar phenomenon is observed in cathepsin $B^{-/-}/K^{-/-}$ thyroids but might be less pronounced when compared with the cathepsin $B^{-/-}$ genotype, because cathepsin L expression was upregulated in cathepsin $B^{-/-}/K^{-/-}$ mice (Figure 4c) but not in cathepsin $B^{-/-}$ mice. These observations suggest a partial compensation of the double deficiency in cathepsins B and K by cathepsin L overexpression. Because systemic defects, i.e., significantly reduced serum levels of T_4, were observed in cathepsin $K^{-/-}/L^{-/-}$ mice only, the utilization of luminal Tg for T_4 liberation is most probably mediated by a combinatory action of cathepsins K and L. The proposed sequential steps of proteolytic degradation of Tg for its solubilization and utilization are outlined in Figure 9.

Discussion

Our current model of the molecular mechanisms governing thyroid function is mostly derived from in vitro studies of the degradation of the prohormone Tg. Here, we have used mice with deficiencies in the cysteine proteinases cathepsins B, K, and L, which are known to be Tg-degrading enzymes. Cathepsin $K^{-/-}/L^{-/-}$ mice were characterized by significantly reduced serum T_4 levels, clearly indicating that, in vivo, the utilization of Tg, i.e., the liberation of T_4 from its prohormone, is facilitated by the combinatory action of cathepsins K and L. In addition, the absence of multilayers of luminal Tg in cathepsin $B^{-/-}$, $L^{-/-}$, $B^{-/-}/K^{-/-}$, and $K^{-/-}/L^{-/-}$ mice demonstrated that the occurrence of differentially compacted luminal Tg is dependent on the presence of cathepsins B and/or L in mouse thyroid. Hence, our results provide evidence for the notion that the cysteine proteinases cathepsins B, K, and L are important for sequential steps in the complex sequence of proteolytic events leading to the degradation of Tg in vivo (Figure 9).

Solubilization of luminal Tg depends on expression of cathepsins B and L. The maintenance of thyroid function is based on the bidirectional secretion and recapture pathway of Tg (for review, see ref. 1). Newly synthesized Tg is transported along the secretory route to the apical plasma membrane of thyroid epithelial cells, where it becomes iodinated by thyroperoxidase and where thyroid hormone formation by intramolecular coupling of iodinated thyronine residues occurs. After exocytosis, Tg is stored within the extracellular lumen of thyroid follicles in a covalently cross-linked form. The molecular nature of intermolecular covalent cross-linkage of Tg appears to be species-specific. Human Tg globules consist of Tg mainly cross-linked by disulfide bridges (4, 5). In addition to disulfide cross-links, isodipeptide bonds were detected in bovine (3, 6), and dityrosine links in porcine Tg globules (31). Here, we have observed the occurrence of multilayered Tg in mouse thyroids, indicating that covalently cross-linked Tg exists also in the lumen of mouse thyroid follicles (Figure 8). However, no information exists on the nature of cross-linkages in mouse Tg globules. Therefore, further studies are needed to establish an isolation procedure that would then allow, by biochemical and biophysical means (32), a more detailed characterization of mouse Tg globules.

Because globules consisting of covalently cross-linked Tg reach dimensions of up to 120 μm in diameter, proteolysis is a necessary prerequisite for solubilization of Tg from the globules and must precede its endocytosis by thyroid epithelial cells, i.e., the recapturing pathway of Tg. First, extracellularly stored Tg undergoes limited proteolysis, leading to its solubilization from the storage forms and to T_4 liberation; then Tg becomes internalized and reaches endosomes and lysosomes for complete degradation (for review, see ref. 2).

Recently, we proposed that cycles of Tg deposition and Tg proteolysis regulate the size of the luminal content of thyroid follicles and might also explain the multilayered appearance of luminal Tg (2, 8–10). In the present study, we have shown that Tg depositions within the lumina of thyroid follicles of cathepsin $B^{-/-}$, $L^{-/-}$,

$B^{-/-}/K^{-/-}$, or $K^{-/-}/L^{-/-}$ mice lacked the typical multilayered appearance, whereas the luminal content of cathepsin $K^{-/-}$ mice was indistinguishable from that of WT mice. These results support the conclusion that cathepsin K is not involved in the first step of Tg proteolysis, i.e., that it is dispensable for proteolytic solubilization of Tg from covalently cross-linked Tg-globules; whereas both cathepsins B and L need to be present for proper Tg solubilization (Figure 9).

If Tg solubilization were impaired in the absence of cathepsin B or L, and Tg synthesis, secretion, and covalent cross-linking were unaffected, then the amounts of luminal Tg would constantly increase, because Tg could no longer become efficiently removed from the lumen. Indeed, the amounts of Tg were increased within thyroids of cathepsin $B^{-/-}$, $L^{-/-}$, $B^{-/-}/K^{-/-}$, or $K^{-/-}/L^{-/-}$ mice. The most extreme accumulation of Tg was observed in thyroids of cathepsin $B^{-/-}$ mice. These mice were also characterized by the largest follicles. Possibly, the extremely thin epithelia of cathepsin $B^{-/-}$ thyroids were unable to resist the expansion forces of very high amounts of Tg secreted into the lumen. As a result, the volumes of cathepsin $B^{-/-}$ thyroids were twofold higher than those of the WTs (results not shown).

Tg solubilization is, however, not the ultimate prerequisite for efficient T_4 liberation, since serum T_4 levels were reduced in cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice but normal in cathepsin $B^{-/-}$ or $B^{-/-}/K^{-/-}$ mice. Hence, the amount of Tg present under conditions of impaired solubilization from its covalently cross-linked storage form due to cathepsin B deficiency is still sufficient for T_4 liberation, supporting the notion that newly synthesized Tg can become utilized directly, i.e., without being stored in covalently cross-linked Tg globules.

Thyroid function depends on the presence of cathepsins K and L. Because T_4 is the main hormone released from the thyroid gland (29), the proteolytic step leading to the liberation of T_4 from its prohormone Tg is most important for the maintenance of thyroid function. Here, we have observed that serum T_4 levels were significantly reduced in cathepsin $L^{-/-}$ and $K^{-/-}/L^{-/-}$ mice. The normal serum T_4 levels in cathepsin K– and B/K–deficient mice might be explained by the observation that cathepsin L expression was upregulated in thyroid epithelial cells of these mice, supporting the conclusion that cathepsin L is able to compensate for proteolytic activity of cathepsin K. Deficiencies in both proteases, cathepsins K and L, resulted in a systemically established phenotype in that serum T_4 levels were significantly reduced in cathepsin $K^{-/-}/L^{-/-}$ mice when compared with the WTs, suggesting that the utilization of Tg for the liberation of T_4 is mediated by a combinatory action of cathepsins K and L (Figure 9).

Because serum T_4 levels were clearly reduced but T_4 was still present in the blood of mice with deficiencies in cathepsins K and L, it can be concluded that other proteases besides these two are involved in T_4 liberation from Tg. The lysosomal aspartic protease cathepsin D is an enzyme to consider in this respect, because it is known to contribute to Tg degradation (11, 28). Indeed, cathepsin D was upregulated in cathepsin $L^{-/-}$ and $K^{-/-}/L^{-/-}$ thyroids, which might indicate a compensation for cathepsin L function by cathepsin D. However, the proteolytically inactive proform of cathepsin D dominated in the thyroids of all analyzed genotypes. In addition, expression of cathepsin D was strictly lysosomal in mouse thyroid, ruling out its contribution to extracellular T_4 liberation. Furthermore, if cathepsin D were important for T_4 liberation, one would expect a milder phenotype in those mice with cathepsin D overexpression, which is in clear contrast to our observation that cathepsin D–overexpressing cathepsin $L^{-/-}$ and $K^{-/-}/L^{-/-}$ mice suffered from reduced serum T_4 levels. Therefore, and in accordance with results from in vitro degradation studies of Tg (11), it is unlikely that cathepsin D is coresponsible for T_4 liberation in the mouse thyroid.

Besides aminopeptidase N (CD13) and dipeptidylpeptidase IV (CD26), two integral membrane proteins at the apical surface of thyrocytes whose active domains face toward the lumen of thyroid follicles (33, 34), another enzyme that might well contribute to T_4 liberation is cathepsin S. Interestingly, the expression of cathepsin S, a member of the still growing cysteine proteinase family, is upregulated in a TSH-dependent fashion in a thyroid epithelial cell line derived from rats, FRTL-5 cells (35). In addition, cathepsin S cleaves its substrates at neutral to slightly acidic pH conditions, and it is able to degrade Tg while liberating T_4 from its prohormone under conditions simulating the lumen of thyroid follicles (36). Hence, cathepsin S and

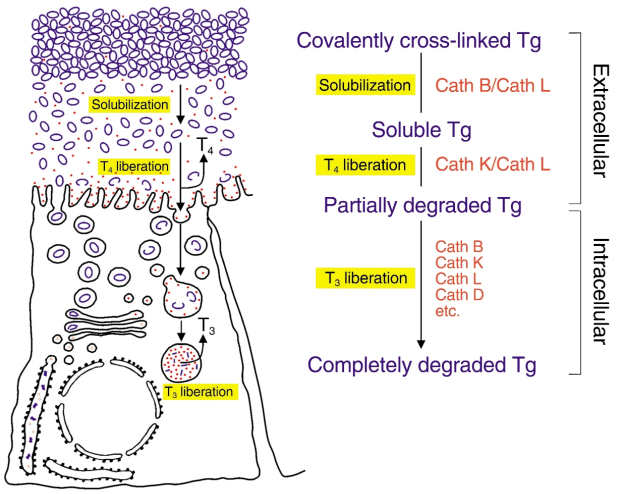

Figure 9
Schematic drawing of the proposed sequence of proteolytic events leading to Tg degradation in mouse thyroid. Solubilization of extracellularly stored Tg from covalently cross-linked globules is mediated by cathepsins B and L. Soluble Tg is then subjected to limited proteolysis mediated by cathepsins K and L for Tg utilization, i.e., extracellular T_4 liberation. Thereafter, partially degraded Tg reenters thyroid epithelial cells by endocytosis and reaches endosomes and lysosomes for its complete degradation by the action of several lysosomal enzymes.

the above-mentioned exopeptidases at the apical plasma membrane of thyroid epithelial cells are promising candidates for further analysis of extracellular proteolysis of Tg leading to T_4 liberation in the thyroid.

From a systemic defect in the body's supply with thyroid hormones, one would expect further alterations to become obvious. Indeed, thyroid follicle dimensions of cathepsin $K^{-/-}/L^{-/-}$ mice were increased by a factor of about two over those of the WT controls. The extension of thyroid follicles of cathepsin $K^{-/-}/L^{-/-}$ mice was more pronounced than that observed in the other genotypes, except for cathepsin $B^{-/-}$ mice, indicating that in addition to an enlargement due to impaired Tg solubilization from its storage form, the reduced serum T_4 levels of cathepsin $K^{-/-}/L^{-/-}$ mice resulted in a further enlargement of thyroid follicles. Reduced serum T_4 levels and extended thyroid follicles of cathepsin $K^{-/-}/L^{-/-}$ mice are reminiscent of phenotypic alterations that indicate hypothyroidism.

Cathepsins and cell death. Despite their enlarged thyroid follicles, cathepsin $K^{-/-}/L^{-/-}$ mice did not develop goiter, since the volumes of their thyroid glands were comparable to those of WT (results not shown). This might be due to the increased number of remnants of dead cells within cathepsin $K^{-/-}/L^{-/-}$ follicles. Similar inclusions of dead cells were abundant in the lumina of thyroid follicles of cathepsin $L^{-/-}$ mice, but generally lacking from thyroids of WT or cathepsin $B^{-/-}$, $K^{-/-}$, or $B^{-/-}/K^{-/-}$ mice, which led us to the conclusion that cathepsin L is needed for survival of thyroid epithelial cells.

A direct contribution of cysteine proteinases to the control of tissue homeostasis has been proven for cathepsin B (15). In TNF-α–treated hepatocytes, cathepsin B was released from the lysosomes into the cytosol, where it enhanced apoptosis by mediating the release of mitochondrial cytochrome *c*, leading to the activation of caspases. Hence, cathepsin B promoted TNF-α–induced apoptosis of hepatocytes. It is unclear whether thyroid epithelial cells of cathepsin $L^{-/-}$ or $K^{-/-}/L^{-/-}$ mice undergo apoptosis or necrosis. Further studies are needed to clarify this issue.

Cathepsin L has been shown to control epidermal cell proliferation (18, 19) in the stratified epithelium of the skin, i.e., epidermis. However, in cathepsin $L^{-/-}$ mice, the increased cell death observed in the thyroid contrasted with the hyperproliferation of basal keratinocytes observed in the epidermis of the same mice. Obviously, cathepsin L deficiency affects proliferation, differentiation, and cell death of epithelial cells of various organs differently. The notion of such diverse effects achieved under conditions of cathepsin L deficiency is further supported by a study in which cathepsin L function was analyzed in *Caenorhabditis elegans* by RNAi technology (37). In *C. elegans*, cathepsin L deficiency resulted in slower rates of cell divisions; hence, proliferation was reduced and led to delayed growth of both larvae and worms.

So far, the precise molecular mechanisms underlying cathepsin L–mediated control of cell proliferation are unknown (18, 37). However, the contrary effects of cathepsin L deficiency on epidermal keratinocytes (18, 19) and on thyrocytes (this study) might indicate that, in mice, growth factors or their receptors are modulated by cathepsin L–mediated proteolytic processing in a cell type–specific manner.

The results of this study support the notion that cathepsin L, together with cathepsin K, mediates thyroid hormone liberation. Hence, cysteine proteinases are not only relevant to the maintenance of tissue homeostasis but are also of significant importance for proper thyroid function.

Possible relevance of cathepsins to thyroid functions in humans. Cathepsins B, K, and L are expressed in the thyroid of all species analyzed so far, including humans (2, 8). Therefore, the question arises as to whether thyroid functions of cathepsins are comparable in mice and humans. Results achieved by using the mouse system might even contribute to a better understanding of molecular mechanisms that lead to the onset of human disorders. In particular, the cathepsin K–deficient mouse has already proved to be a valuable animal model for the human disorder pyknodysostosis, and, hence, the understanding of cathepsin K's function in bone matrix degradation might have important implications for the treatment of osteoporosis (16, 17, 38).

Because the maintenance of constant levels of thyroid hormones is essential for proper development and growth of mice and humans, it is possible that molecular mechanisms leading to Tg degradation and thyroid hormone liberation, similar to those described in this study for mice, also exist in humans. Support for this hypothesis comes from observations of a pathological situation of hyperthyroidism in humans, in which an upregulation of cathepsin B was detected that correlated with its increased occurrence at the apical plasma membrane of thyroid epithelial cells and, interestingly, with enhanced serum levels of thyroid hormones (39). This observation supports our proposal that lysosomal cysteine proteinases of the normal thyroid are involved in extracellular Tg proteolysis at the apical surface of thyroid epithelial cells (Figure 9) (for review, see ref. 2). In addition, the detection of increased amounts of apically located cathepsin B in hyperthyroidism in humans is in agreement with our findings in the mouse system; that is, cathepsin B was detected in association with the apical surface of WT thyrocytes of the mouse, and Tg degradation was clearly impaired when cathepsin B was lacking. Therefore, it can be concluded that at least cathepsin B is important for the degradation of Tg in both humans and mice. Consequently, it might be assumed that the expression and localization of cathepsins K and L are altered during hypo- and/or hyperthyroidism in humans. Hence, future studies must further elucidate the relevance of cathepsins for thyroid functions in humans.

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