Evidence for Extracellularly Acting Cathepsins Mediating Thyroid Hormone Liberation in Thyroid Epithelial Cells*

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ABSTRACT

Thyroglobulin (Tg) is the major secretory product of thyroid epithelial cells and is stored in the lumen of thyroid follicles at high concentrations. Thyroid hormone liberation is assumed to occur separately from this storage compartment within lysosomes. However, for the transfer of Tg to lysosomes, mechanisms to solubilize the luminal content must precede its endocytosis, because part of the luminal Tg occurs in a covalently cross-linked form. Here, by immunoprecipitation and immunoblotting we show that the majority of procathepsin B or L and a fraction of mature cathepsin B are released from porcine thyrocytes in vitro. Released cathepsins were detectable on the cell surface of the thyrocytes by immunocytochemistry and amounted to 27% of the total cathepsin B. Cytochemical studies revealed the proteolytic activity of cathepsin B at neutral pH on the cell surface of thyrocytes. Therefore, the possibility of extracellular proteolysis by cathepsins was investigated by incubating plasma membrane preparations, conditioned media, or lysosomes with Tg. The liberation of thyroid hormones was quantitated by RIA, and the degradation of Tg was determined by SDS-PAGE. Extracellular and plasma membrane-associated proteases rapidly mediated up to 54% of the total Tg liberation by limited proteolysis of Tg at neutral pH under conditions where cysteine proteases were reactivated. We propose that released and proteolytically active cysteine proteases, i.e., cathepsins B and L, provide thyrocytes with a pathway of limited extracellular proteolysis of Tg before endocytosis. (Endocrinology 137: 1963–1974, 1996)
Reactivation of cysteine proteases resulted in enhanced \( \text{I}_2 \) liberation by extracellular means and led us to postulate an involvement of extracellularly active cathepsins B and L in the proteolysis of \( \text{Tg} \).

**Materials and Methods**

**Materials**

Rabbit antiporcine aminopeptidase N (APN) antiserum were produced in our own laboratory (Dippen and Herzog, Institut für Zellbiologie, Munich, Germany), as were rabbit antiporcine \( \text{Tg} \) and rabbit antibovine \( \text{Tg} \) (Herzog, Brix, Summa, Institut für Zellbiologie, Bonn, Germany). Sheep antianthuman \( \text{cathepsin L} \) was obtained from BioAss (Dissen, Germany). Monoclonal mouse antianthuman mature cathepsin B (IM27) and rabbit antianthuman procathepsin L (IM06) were generous gifts from Dr. James R. Zabrecky, Oncogene Science (Uniondale, NY). Affinity-puriﬁed 5-(4,6-dichlorotriazin-2-yl)aminofluorescein hydrochloride or horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Dianova (Hamburg, Germany), and goat antianiimmunoglobulin G (IgG) coupled to 10-nm gold particles (\( \text{Au}_{10} \)) was obtained from Sigma (Deisenhofen, Germany). [\( ^{35} \text{S} \)]Iodine and \( [^{14} \text{C}] \)iodine were purchased from Amersham Buchler (Braunschweig, Germany), protein G-agarose and mowiol-4-88 were obtained from Calbiochem-Novabiochem (Bad Soden, Germany), trans-epoxyuccinyl-l-leucylamido (4-guanidine) butan (E64) and lactoperoxidase (LPO) were obtained from Sigma, and the other protease inhibitors as well as detergents were purchased from Sigma, Serva (Heidelberg, Germany), or Merck (Darmstadt, Germany). N-Benzoyloxycarbonyl-arginyl-arginine-4-mercapto-2-naphthylamine (Z-Arg-Arg-4MPNA) was obtained from Bachem Biochemica (Heidelberg, Germany), 2-hydroxy-5-nitrobenzaldehyde (NSA) was a gift from Dr. E. Spiess (Heidelberg, Germany), and paraoxonase was used as the freshly prepared hexazinium salt (HPR) according to the manufacturer’s protocol (Sigma). Centricon-10 concentrators were obtained from Amicon (Witten, Germany), and RAAs were purchased from Brahms Diagnostica (formerly Henning, Berlin, Germany).

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**Cell culture.** Porcine thyroid glands were obtained from the local slaughterhouse and transported on ice to the laboratory. Thyroid tissue was suspended and cut in Eagle’s Minimum Essential Medium (EMEM) into 0.2-mm fragments using razor blades. After repeated washing, the thyroid fragments were sedimented at 100 x g for 75 sec. Fragments were resuspended in 1 mg/ml collagenase (activity, 215 U/mI) in EMEM and incubated for 30 min at 37 C under gentle agitation (150 rpm). The suspension was then dissociated using siliconized glass pipettes, with diameters from 1.0–0.7 mm, filtered through 250 to 150-Km gaze, and thyroid fragments were sedimented at 100 x 8 for 75 sec. Fragments and incubated for 30 min at 37 C under gentle agitation (150 rpm). The final pellet was resuspended in the above medium containing 10% FCS. Cells were then plated on coverglasses, tissue culture flasks, or dishes and incubated at 37 C in 5% CO\(_2\). For all experiments, cells were grown without any further passage near confluency, which was reached 7–10 days after isolation.

**Immunolabelling of thyrocytes.** Cells were grown on coverglasses, washed in PBS, and ﬁxed with 8% paraformaldehyde in 200 mM HEPES (pH 7.4) for 30 min at room temperature. After blockage of nonspeciﬁc binding sites, cells were incubated with speciﬁc antibodies for 90 min at 37 C or overnight at 4 C: rabbit antiporcine APN (0.7 mg/ml), monoclonal mouse antianthuman mature cathepsin B (0.01 mg/ml), or rabbit antianiimmunoprecipitated \( \text{Tg} \) (0.01 mg/ml). After a 60-min incubation with 0.03 mg/ml 5-(4,6-dichlorotriazin-2-yl)aminoﬂuorescein hydrochloride-labeled secondary antibodies, antibodies were bound on microscope slides 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. Immunostained cells were viewed with a conventional ﬂuorescence microscope (Zeiss, Oberkochen, Germany). Micrographs were taken on Kodak TMax ﬁlms (Eastman Kodak, Rochester, NY).

**Immunolabelling of cytoskeleton from thyroid epithelial cells.** Thyroid epithelial cells were ﬁxed with 8% paraformaldehyde in 200 mM HEPES (pH 7.4) and then washed in 0.2% paraformaldehyde in 200 mM HEPES (pH 7.4) and then ﬁxed with sucrose as a cryoprotectant (2.3 M) and frozen in liquid propane. Cytoskeletons of 300 nm were prepared with a cryotome (Reichert-Jung, Wien, Austria) at −110 C and mounted on 300-mesh grids. Bruchmann monoclonal mouse antianiimmunohistochemical labeling of cytoskeletons was performed with 0.05 mg/ml goat antianimmunoglobulin G (IgG) coupled to \( \text{Au}_{10} \) at a dilution of 1:30 each for 60 min. Sections were stained with 0.3% uranyl acetate in 2.7% polyvinyl alcohol (10 min) and with 4% aqueous ethanol. Sections were visualized on a Zeiss EM902 electron microscope (Oberkochen, Germany).

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as the precipitating agent of proteolytically released 4MjNa. The re-
action was allowed to occur for 15-60 min at 37 °C and without oxygen.
Thereafter, cells were washed with PBS, and vital cells were then fixed
with 1% formaldehyde for 20 min at room temperature. After washing in
distilled water, they were mounted on microscope slides and viewed by
conventional fluorescence microscopy (see above) or with a confocal
laser scanning microscope (TCS 4D, Leica, Bensheim, Germany) using an
argon/krypton mixed gas laser with an excitation wavelength of 488
nm. Scans at a resolution of 1024 × 1024 pixels and a pinhole setting of
about 50 were taken in the line-averaging mode. Micrographs were
made on Kodak RMax films using a hardcopy device (see above) for the
documentation of laser scanning micrographs.

For electron detection of cathepsin B activity, cells were cul-
tured in dishes, washed with PBS, and fixed with 0.5% glutaralde-
hyde in PBS for 15 min at room temperature. After washing in reaction
buffer (pH 7.2) at a final concentration of 0.5 mM, and to four parts of this
solution, one part of HPR was added that was titrated to neutral pH with
NaOH. After 60 min incubation at 37 °C, the reaction was stopped by
washing the cells with 50 mM cacyclate buffer and by fixation for 30 min
at room temperature with 2.5% glutaraldehyde in the same buffer.
After osmification with 1% OsO4 and 0.08% K2Fe(CN)6 in cacyclate
buffer at pH 7.2 for 60 min, cells were dehydrated in ethanol and propy-
ylexane and embedded in Epon. Sections were viewed without counterstain-
ing in the electron microscope (CM120; see above). Some sections were rehy-
drated and immunolabeled with monoclonal mouse anti-mature human cathe-
psin B (0.01 mg/ml) and goat antimouse IgG coupled to 100 at a dilution of 1:30 each for 30 min at room temperature.

Isolation, purification, and iodination of Tg. Tg was purified from bovine
thyroid glands by ammonium sulfate precipitation and anion exchange
chromatography, as previously described (18). Nonradioactive Tg was
used for the thyrom hormone liberation assay (see below), whereas the
degradation of Tg (see below) was investigated using radioiodinated Tg.
Tg (1 mg/ml) dissolved in TBS supplemented with 0.00015% H2O2 was
enzymatically iodinated by 0.1 mg/ml LPO (85 U/mg protein) with 1.85
Mg[125I][1][1]NaI for 1 min at room temperature. Because LPO was not
separated from iodinated Tg and, thus, to avoid further iodination
during the incubation with subcellular fractions (see below), sodium
azide was added to the incubation mixtures.

Subcellular fractionation of thyrocytes. Two days before subcellular frac-
tionation, cells were washed and further incubated with scrum-free
medium. Medium, i.e. secretory products of thyrocytes, were col-
lected from the cells, cleared by centrifugation (twice for 10 min, 150 ×
g [4 C], with removal of supernatant between centrifugations) and cato-
lysed three times with ice-cold PBS, then harvested using rubber
polycmen. Cell suspensions were pelleted and washed three times by
centrifugation (10 min, 150 × g) at 4 C. The pellets were resuspended
in 10 mm Sorensen phosphate buffer (KH2PO4 and Na2HPO4, pH 7.2),
supplemented with 0.25 mM sucrose and 5 mM EDTA, and homogenized
on ice using a Dounce homogenizer (Kontes Co., Vineland, NJ). Cellular
debris and nuclei were removed from cell homogenates by centrifuga-
tion (5 min, 900 × g, 4 C). Lysosomes were enriched by centrifugation
at 10,000 × g for 10 min at 4 C. The resulting pellet was resuspended in
Sorensen phosphate buffer (pH 5.0) supplemented with 0.2% Triton
X-100 and further incubated for 30 min on ice. The supernatant of the
final centrifugation step (15 min, 15,000 × g, 4 C) was then used as
isolated lysosomal enzymes for the in vitro incubation assays. Plasma
membrane vesicles were prepared according to the methods of Hubbard
et al. (30) and Barvais et al. (31) with minor modifications. The super-
natant (0.5 ml) of the 10,000 × g centrifugation step was layered on top
of 1 ml 1.2 M sucrose-5 mM EDTA in 0.1 M Sorensen phosphate buffer
(pH 7.2) and 3.5 ml 0.32 M sucrose-5 mM EDTA in 0.1 M Sorensen
phosphate buffer (pH 7.2) and centrifuged for 2 h at 100,000 × g at 4 C.
The resulting band at the interface between 0.32 and 1.2 M sucrose was
carefully removed with a syringe and resuspended in 10 ml PBS. Plasma
membrane vesicles were collected as the pellet of the following centri-
figation (1 h, 100,000 × g, 4 C) and lysed in PBS (pH 7.2), as described
for lysosomes. For the electron microscopic analysis, nonlysed plasma
membrane preparations were fixed with 2% glutaraldehyde and 1% formalde-
hyde in cacyclate buffer and embedded in Epon (see above).

Results

Release of cathepsins B and L and association of the enzymes with the cell surface of thyroid epithelial cells

Immunocytotoxic labeling of thyrocytes with antibodies against cathe-
sins B and L was compared to that obtained with antibodies against the integral membrane ectoprotease
APN. Controls, i.e. reacted with nonspecific or without first antibodies, were negative (not shown). Immunofluorescence
revealed the presence of APN at the plasma membrane of thyrocytes, as expected (Fig. 1a). A punctate fluorescence
staining pattern was observed, and the cell surface recogni-
tion by anti-APN antibodies was most obvious at the borders
between neighboring cells in a monolayer (Fig. 1a, arrow-
heads). In many cells, one ring-like structure was immuno-
labeled with APN antibodies and probably corresponds to
targets where cilia are inserted into thyrocytes (Fig. 1a, dou-
ble arrow). When monolayers were labeled with antibodies
against procathepsin L a punctate staining was observed.
which was prominent at the borders between thyrocytes (Fig.
1b, arrowheads) and thus resembled the cellular location of
APN. In addition, the perinuclear region of epithelial cells
was immunostained (Fig. 1b, arrow). However, when cells
were labeled with monoclonal antibodies against mature
the epithelial cells was identified by their

Thyroid hormone liberation assay. Secretory products of thyrocytes were
released into culture medium. Media, i.e., secretory products of thyrocytes, were col-
llected as the pellet of the following centrifugation step (10 min, 150 × g, 4 C). The pellets were resuspended in
Sorensen phosphate buffer (pH 7.2) at a final concentration of 0.8 mM, and to four parts of this
buffer (see above), Z-Arg-Arg-4MPNA was applied in reaction buffer
(1 mM EDTA, 1 mM E64, 1 mM pepstatin A, 100 μg/ml aprotinin, and 0.06 mM po-
tassium iodide (final concentrations). After an incubation period of 5
min, Tg (7.75 μg) was added to these mixtures and stored at −20°C. For the
30-min incubations, the other aliquots of the subcellular fractions was
supplemented with Tg (7.75 μg) and incubated at 37°C for 30 min before addition of the protease inhibitor cocktail and potassium iodide
(see above). Such preparations were stored at 20°C. For the reactiva-
tion of cysteine proteases, subcellular fractions were preincubated with
2 mM cysteine for 5 min at 40°C before the addition of Tg. For quantification of the amounts of liberated T3 and T4, samples were analyzed by a
commercially available RIA. Zero time controls were subtracted from
values obtained after 30 min of incubation with Tg. They were normal-
ized to equal amounts of cells and are given as the mean ± se (Origin
2.8, MicroCal Software, Northampton, MA).

Analysis of Tg degradation by SDS-PAGE. For analysis of Tg degradation, the fractions were each divided into two aliquots to which enzymatically
iodinated Tg was added: one aliquot was incubated without and the other aliquot with the protease inhibitor cocktail (see above) for 30 min
at 37°C. After the incubation, the protease inhibitor cocktail was added to the first aliquot, and both were boiled in sample buffer under reducing
conditions, run on horizontal SDS-gels, and analyzed by autoradiogra-
phy on Kodak X-Omat AR films. The bands corresponding to mono-
clonal Tg or LPO were scanned (see above), and densitometric evaluation
was performed using an automated program (Phoretix International,
Newcastle upon Tyne, UK).

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The extracellular proteolysis of thyroglobulin (Tg) by cathepsins was studied in this research. Cathepsin B was not visible at the plasma membrane when using light microscopical techniques, whereas higher resolving cryoelectron microscopy revealed the presence of the protease at the apical cell surface of thyroid epithelial cells. The numbers of gold particles per μm² were determined to quantitate the relative amounts of cathepsin B immunolabeled at various cellular sites. Cathepsin B labeling within the endosomal/lysosomal system was highest with 68%; however, labeling at the plasma membrane was well above background and amounted to 27% of the total immunolabeled cathepsin B. As cathepsins B and L are soluble proteins without a membrane anchor, an association with the plasma membrane is only possible if the proteins are transported to the cell surface, from where they are partially released. Another portion of the cathepsins remains associated with the plasma membrane.

To further analyze the molecular forms of released cathepsins B and L, lysates and conditioned media of thyrocytes were probed for the presence of the proteases. In the case of cathepsin L, monolayer thyrocytes were biosynthetically labeled with [35S]methionine and [35S]cysteine overnight. The cell lysates and media were subjected to immunoprecipitation with antibodies against cathepsin L, SDS-PAGE, and subsequent fluorography of the immunoprecipitates revealed the presence of the majority of procathepsin L in the conditioned medium of thyrocytes. From cell lysates, the pro form and the mature single and two chain forms of cathepsin L were immunoprecipitated. When comparing the cellular signal of cathepsin L to that observed in the medium, one can deduce that a major portion of newly synthesized procathepsin L is released from thyroid epithelial cells. Whereas immunoprecipitation of porcine cathepsin B was not possible with a variety of antibodies tested, the monoclonal antibody proved to be useful for immunoblotting. Therefore, conditioned media of thyrocytes were concentrated 20-fold by ultrafiltration, and together with cell lysates, these samples were analyzed by SDS-PAGE and Western blotting. Probing the Western blots with monoclonal antibodies against mature cathepsin B revealed the presence of the pro and mature forms of cathepsin B in lysates from thyroid epithelial cells. Most important, the cells released both forms of the cathepsin, as both pro- and mature cathepsin B were recognized in the secretions of the cells. Further evidence for the extracellular presence of mature cathepsin B comes from experiments with human thyrocytes labeled with [125I]NaI in which the mature form of cathepsin B was immunoprecipitated from cell lysates as an iodinated protein. Thyrocytes are able to iodinate proteins that come into contact with neighboring cells (arrowheads in a). In addition, cilia were intensely labeled (double arrow in a). Similarly, antibodies against procathepsin L recognized the borders of neighboring cells (arrowheads in b) and showed an additional perinuclear staining (arrow in b). Cathepsin B was immunolocalized to cytoplasmic vesicles accumulating in the perinuclear region, which most likely represent lysosomes (arrows in c). The results indicated that procathepsin L was not restricted to intracellular pools, whereas mature cathepsin B was mainly localized to lysosomes. N, Nucleus. Bars = 50 μm.
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Fig. 2. Cathepsin B was detected at the cell surface of thyroid epithelial cells by immunoelectron microscopy. Cryosections from 8% formaldehyde-fixed porcine thyroid epithelial cells after labeling with monoclonal antibodies against mature cathepsin B and goat antimouse IgG coupled to Au. The apical plasma membrane was identified by the presence of numerous microvilli in 300-nm thick cryosections (a). Cathepsin B was detected at the plasma membrane, and some gold particles were found in close association with membranous profiles (arrowheads in a and b). Although, by immunofluorescence, cathepsin B seemed to be restricted to lysosomes (compare with Fig. 1c), higher resolving electron microscopy demonstrated the presence of the protease at the apical cell surface of thyroid epithelial cells. In a, bar = 1 μm; in b, bar = 500 nm. C. Quantitation of the amount of immunolabeled cathepsin B by counting the number of gold particles associated with plasma membranous profiles (PM; see arrowheads in a and b) or detected within endosomes and lysosomes (En/Lys). Gold particles over nuclei (N) or on the grids outside of the cells (ex) were evaluated as background staining. The amount of cathepsin B immunolabeled within endosomes and lysosomes was higher (En/Lys; 68%) than the amount of cathepsin B associated with the plasma membrane (PM; 27%), which was well above background staining (ex, 3%; N, 2%).

Released cathepsin B is proteolytically active in the extracellular space and at the plasma membrane of thyrocytes

The proteolytic activity of cathepsin B was analyzed by cytochemical means. Vital or formaldehyde-fixed thyrocytes on coverglasses were incubated at pH 6.2 or pH 7.2 with the cathepsin B-specific substrate Z-Arg-Arg-4MβNA in the presence of NSA as the precipitating agent for proteolytically released 4MβNA. The reaction product was then made vis-
were precipitated from cell lysates (cells). The majority of procathepsin L was detected in the media of thyrocytes (SEC), indicating that the enzyme was released. Western blots from lysates and conditioned media of porcine thyroid epithelial cells after incubation with \([35S]\)methionine and \([35S]\)cysteine at 37°C and immunoprecipitation with antibodies against cathepsin L. The pro and mature forms of cathepsin L were precipitated from cell lysates (cells). The majority of procathepsin L was detected in the media of thyrocytes (SEC), indicating that the enzyme was released. Western blots from lysates and conditioned media of porcine thyroid epithelial cells after incubation with monoclonal antibodies against mature cathepsin L and HRP-coupled secondary antibodies. The pro and mature forms of cathepsin L were detected in both cell lysates (cells) and media (SEC). Note that lane 1 (cells) was overexposed to demonstrate the presence of procathepsin L. A major portion of the procathepsin L and a minor portion of the mature cathepsin L were detected in the media (SEC), indicating that both forms were released by thyrocytes. Fluorography of SDS-gels from lysates of human thyroid epithelial cells after 2-h incubation with \([125I]\)NaI and immunoprecipitation with antibodies against mature cathepsin B. The mature form of the enzyme was detected as an iodinated polypeptide, indicating its appearance at the apical plasma membrane where iodination takes place. Note that this evidence for the cell surface localization of mature cathepsin B was only possible with human thyrocytes, because the antibodies did not immunoprecipitate the porcine antigen.

Fig. 3. Besides the release of procathpsins B and L, a fraction of mature cathepsin B was released by thyroid epithelial cells. a. Fluorography of SDS-gels from lysates and conditioned media of porcine thyroid epithelial cells after overnight incubation with \([35S]\)methionine and \([35S]\)cysteine at 37°C and immunoprecipitation with antibodies against cathepsin L. The pro and mature forms of cathepsin L were precipitated from cell lysates (cells). The majority of procathepsin L was detected in the media of thyrocytes (SEC), indicating that the enzyme was released. Western blots from lysates and conditioned media of porcine thyroid epithelial cells after incubation with monoclonal antibodies against mature cathepsin B and HRP-coupled secondary antibodies. The pro and mature forms of cathepsin B were detected in both cell lysates (cells) and media (SEC). Note that lane 1 (cells) was overexposed to demonstrate the presence of procathepsin B. A major portion of the procathepsin B and a minor portion of the mature cathepsin B were detected in the media (SEC), indicating that both forms were released by thyrocytes. Fluorography of SDS-gels from lysates of human thyroid epithelial cells after 2-h incubation with \([125I]\)NaI and immunoprecipitation with antibodies against mature cathepsin B. The mature form of the enzyme was detected as an iodinated polypeptide, indicating its appearance at the apical plasma membrane where iodination takes place. Note that this evidence for the cell surface localization of mature cathepsin B was only possible with human thyrocytes, because the antibodies did not immunoprecipitate the porcine antigen.

Extracellular proteolysis of Tg leads to the rapid liberation of T₄

As the thyroid hormones T₃ and T₄ are liberated from the prohormone Tg by proteolysis and because Tg is the major secretory product of thyroid epithelial cells, we envisioned a function for the released procathepsin L and the mature cathepsin B in extracellular proteolysis of Tg before endocytosis and delivery of the protein to lysosomes. Therefore, the possibility of extracellular proteolysis was tested by monitoring the liberation of thyroid hormones from Tg and measuring the degradation of Tg polypeptide mediated by secreted or plasma membrane-associated proteases and was compared to lysosomal proteolysis.

The media of cultured epithelial cells were concentrated,
and plasma membranes or lysosomes were isolated by sucrose density centrifugation. The purity of the plasma membrane preparation was proven by electron microscopy (Fig. 6) and the presence of APN and its activity (not shown). Lysosomal preparations were analyzed by SDS-PAGE and Western blotting and were shown to contain high amounts of mature cathepsins B and D, as expected (not shown). Such preparations with or without cysteine reactivation (see Materials and Methods) were then incubated with Tg for 30 min at 37 °C. Incubations of secreted or plasma membrane-associated proteases with Tg were performed at pH 7.2, whereas lysosomal proteases were reacted with Tg at pH 5.0. The reaction was stopped by the addition of protease inhibitors and potassium iodide to block deiodination. In 0 min controls, the subcellular fractions were reacted with the protease inhibitors and potassium iodide before the addition of Tg. Values of 0 min controls were subtracted from 30 min values to show the potency of each subcellular fraction to rapidly liberate thyroid hormones.

When cysteine proteases were not reactivated, thyroid hormones were predominantly liberated by lysosomal proteases (Fig. 7, a and c). The contribution of extracellular proteolysis to thyroid hormone liberation ranged from 6–33% (Fig. 7, a and c), indicating that thyroid hormone liberation from Tg is not limited to lysosomal proteolysis. The amounts of liberated thyroid hormones within the 30-min incubation period were enhanced when cysteine proteases were reactivated (Fig. 7, b and d). Most important, the liberation of T₄ by extracellular means increased from 6% to 54% of the total T₄ liberation upon reactivation of cysteine proteases (Fig. 7, a and b). In contrast, lysosomal proteases incubated with Tg at pH 5.0 were most efficient in the liberation of T₃ (Fig. 7, c and d).

The results demonstrated that the potency to liberate thyroid hormones from Tg was not limited to lysosomal proteolysis. Rather, extracellularly acting proteases liberated T₄ as efficiently as lysosomal proteases. The reactivation experiments suggested an involvement of cysteine proteases in this process. As the cysteine proteases cathepsins B and L were detected in the extracellular space of thyrocytes, we propose them to function in extracellular T₄ liberation.

Thyroid hormone liberation occurred by limited proteolysis of Tg.

The degradation status of Tg at steady state was analyzed by SDS-PAGE and immunoblotting with anti-Tg antibodies of the various subcellular fractions, namely secretory products, plasma membranes, and lysosomes. Tg is a glycoprotein with a molecular mass of 330 kDa which migrates as a double band in SDS-gels (Fig. 8a, PBS, Tg monomer). The secretory products of thyrocytes contained Tg in a form indistinguishable from the Tg standard in SDS-PAGE analysis (Fig. 8a, compare SEC with PBS). However, in plasma membrane preparations, monomeric Tg was detected together with high mol wt degradation fragments (Fig. 8a, PM). Within lysosomes, intact monomeric Tg was absent, whereas one degradation fragment of approximately 25 kDa was highly en-
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FIG. 5. Electron microscopic detection of proteolytically active cathepsin B at the plasma membrane of thyroid epithelial cells. Electron micrographs of glutaraldehyde-fixed thyroid epithelial cells after a 60-min reaction with the cathepsin B-specific substrate Z-Arg-Arg-4Mj3NA at pH 7.2 and 37°C in the presence of HPR as the precipitating agent of released 4Mj3NA. Cells were postfixed with 2.5% glutaraldehyde, osmicated, and embedded in Epon. Sections were then viewed in the electron microscope without counterstaining. The section in b was further immunolabeled with monoclonal antibodies against mature cathepsin B and goat antimouse IgG coupled to Au₁₆. The reaction product detectable because of its higher contrast due to osmium fixation was seen within lysosomes (arrow in a) and along the cell surface of thyrocytes (a and b). The reaction product was colocalized to cathepsin B by colocalization with gold particles (b). Immunolabeling was not observed within the cytoplasm or mitochondria; however, ER profiles (arrows) and the cell surface (arrowheads) were recognized by monoclonal antibodies against cathepsin B (b). The close association of some gold particles with plasma membrane profiles. The results showed extracellular cathepsin B activity at neutral pH. N, nucleus; M, mitochondria. In a, bar = 2 μm; in b, bar = 500 nm.


The results indicated that lysosomal proteolysis of the Tg polypeptide exceeded that of extracellular proteolysis under steady state conditions. However, extracellular proteolysis was implicated from the potency of secreted or plasma membrane-associated proteases to liberate thyroid hormones from Tg (cf. Fig. 7).

Therefore, we analyzed the degradation of the Tg polypeptide under conditions that were used for the thyroid hormone liberation analysis. The three subcellular fractions were incubated with enzymatically iodinated Tg for 30 min at 37°C (Fig. 8b, 30 min) and under the corresponding pH conditions, i.e., pH 7.2 for secretory products or plasma membranes and pH 5.0 for lysosomes. Zero time control incubations were reacted with protease inhibitors before the addition of iodinated Tg (Fig. 8b, 0 min). These preparations were then analyzed by autoradiography after SDS-PAGE (Fig. 8b). The amounts of monomeric Tg were densitometrically determined. The results indicated that the polypeptide chain of Tg appeared unchanged in SDS-PAGE after incubation with secretory products (Fig. 8b, SEC) or plasma membrane-associated proteases (Fig. 8b, PM) for 30 min at 37°C. However, 45% of monomeric Tg was degraded by lysosomal proteases during the 30-min incubation period, which resulted in the formation of various degradation fragments (Fig. 8b, LYS, arrowheads). The degradation fragments originated from Tg, as scanning of the LPO band revealed no degradation of this self-iodinated protein. Similar results were obtained when nonenzymatically iodinated Tg or nonradioactive Tg was used (not shown).

Because the thyroid hormones T₃ and T₄ are formed close to the N- and C-terminals of Tg, these results suggested that extracellular liberation of thyroid hormones from Tg occurred by selective, but limited, proteolysis in addition to lysosomal proteolysis, which leads to complete degradation of the Tg polypeptide.
FIG. 7. The potency of thyroid hormone liberation in vitro at distinct cellular sites of thyroid epithelial cells. Evaluation of the amounts of thyroid hormone liberation (T<sub>4</sub> in a and b; T<sub>3</sub> in c and d) after incubation of Tg with secretory products (SEC) or plasma membrane proteases (PM) at neutral pH or with lysosomal proteases (LYS) at acidic pH. The reaction was allowed to proceed for 30 min at 37°C without (a and c) or after reactivation of cysteine proteases (b and d) and was stopped by the addition of protease inhibitors and potassium iodide. The amounts of thyroid hormones were determined by RIA, and the values were normalized to equal amounts of cells and are given as the mean ± SE. The relative contribution of extracellular vs. intracellular proteolysis in the liberation of thyroid hormones is indicated as a percentage. The results demonstrate that thyroid hormone liberation from Tg occurred within the 30-min incubation periods, with higher amounts of liberated T<sub>4</sub> (a and b) than T<sub>3</sub>, (c and d), as expected from the hormonal content of Tg. The amounts of thyroid hormones liberated from Tg were higher when cysteine proteases within the fractions were reactivated (b and d, compare with a and c, respectively). The contribution of extracellular means to the liberation of T<sub>4</sub> increased from 6% to 54% upon reactivation of cysteine proteases (b and d). In contrast, lysosomal proteolysis was most efficient in the liberation of T<sub>3</sub> (c and d). The results demonstrate that extracellular proteolysis mediated by secreted or plasma membrane-associated proteases was as efficient as lysosomal proteolysis in the liberation of T<sub>4</sub> from Tg, whereas lysosomal proteolysis was the most efficient in the liberation of T<sub>3</sub>. The reactivation study (b) showed that extracellular T<sub>4</sub> liberation is dependent on cysteine proteases.

The release of the cathepsins B and L is a unique feature of thyroid epithelial cells

Proteolysis of Tg is the prerequisite for the liberation of thyroid hormones and the complete degradation of the molecule and is considered to be limited to the lysosomes after Tg has followed the secretion, storage, and recapture pathway in thyrocytes. However, proteolysis could, in principle, occur in all compartments along this transport pathway, including the apical cell surface. Here, we report on the release of procathepsins B and L and proteolytically active mature cathepsin B from thyroid epithelial cells in culture. Our results suggest that thyroid hormone liberation is not restricted to lysosomes, but is, as far as the liberation of T<sub>4</sub> is concerned, equally mediated by extracellularly acting proteases. The liberation of T<sub>3</sub> and final degradation of the Tg polypeptide occur mainly intracellularly by lysosomal degradation.

Discussion

Cathepsins B and L (24, 25) belong to the large group of lysosomal enzymes. However, in the past years it became obvious that they are also present in the extracellular environment, i.e. at or near the cell surface, of tumor cells, where they are thought to function in the degradation of extracellular matrix components (29, 33-35). Furthermore, macrophages release cathepsins directly from their lysosomes into the extracellular space during inflammation (36, 37) or bone resorption (38, 39). From cells of the exocrine pancreas, it is known that cathepsin B or L is secreted via the regulated secretory pathway (40). In epithelial cells of the rat thyroid, the presence of cathepsins B and L seemed to be restricted to intracellular vesicles (41). We observed that porcine thyroid epithelial cells have developed a transport pathway that
extracellular liberation of thyroid hormones occurred by limited proteolysis, which was not visible by SDS-PAGE. In contrast, lysosomal
membranous fraction was partially degraded (PM). Intact Tg was absent from lysosomal preparations; however, a 25-kDa degradation fragment of Tg was highly enriched. a, SDS-PAGE and autoradiography of enzymatically radioiodinated Tg after 30-min incubation (30 min) with conditioned medium (SEC at pH 7.2), plasma membrane (PM at pH 7.2), or lysosomal preparations (LYS at pH 5.0) from porcine thyroid epithelial cells. To parallel the thyroid hormone liberation assay (compare with Fig. 7), 0 min controls were produced by incubation of the indicated fractions with protease inhibitors before the addition of radioiodinated Tg. Lane 1 (PBS) shows radioiodinated Tg dissolved in PBS as a standard for densitometric analysis) within the 30-min incubation period and resulted in the accumulation of several Tg degradation fragments (arrowheads). Taken together with the results of the thyroid hormone liberation assay (Fig. 7), these results indicated that proteases efficiently degrade the Tg polypeptide.

Possible involvement of cathepsins B and L in extracellular proteolysis of Tg

The prerequisites for involvement of cathepsins in extracellular proteolysis of Tg, which results in the rapid liberation of T₄, are 1) that they are proteolytically active at neutral pH and 2) that they are able to cleave Tg.

In vitro studies have shown previously that thyroid hormone liberation from Tg is a complex process, which requires synergism among various proteases (22, 23). It became clear that cysteine proteases such as cathepsins B and L (21, 42-44) and the aspartate protease cathepsin D (43, 45-47) are involved in Tg degradation. From studies on the cleavage sites for human cathepsins B, D, and L in rabbit Tg, it was concluded that cathepsins B and L are more important than cathepsin D in the degradation of Tg (21-23). In vitro, cathepsin L cleaves Tg as an endopeptidase near the homologous sites at the N- and C-terminals of Tg, thereby producing smaller peptides (21-23), which are then accessible for exopeptidases such as cathepsin B or APN, the apical ectoprotease of thyrocytes (48). However, extracellular proteolysis at the cell surface is only possible if the involved proteases are proteolytically active at neutral pH. The pH optimum for cathepsin L is approximately 5.0-6.5. However, cathepsin L has a residual proteolytic activity of 30-40% at neutral pH (24, 25). Furthermore, procathepsin L has been shown to be a proteolytically active form of the enzyme stabilized by the propeptide at neutral pH in some cellular systems (49, 50). Thus, an involvement of procathepsin L in extracellular Tg degradation was envisioned; however, direct evidence for the proteolytic activity of procathepsin L in the extracellular environment of thyrocytes is lacking. Cathepsin B has a pH optimum of approximately 6.0 for most substrates (24, 25). It is believed that cathepsin B is irreversibly inactivated above pH 7.0 (25), with a half-life of about 7 min at pH 7.5 (51). In contrast, it was shown recently that cathepsin B from human liver cleaves collagen IV, laminin, and fibronectin at pH 7.4 (35). In addition, when using Z-Arg-Arg-paranitroanilide as a substrate for bovine kidney cathepsin B₁, a residual activity of 46% was determined at pH 7.0, which declined to 21% at pH 7.4 compared to 100% at pH 6.0 (Brix, K., unpublished observations). Here, we have demonstrated that a fraction of mature cathepsin B is released from porcine thyrocytes. Most important, cathepsin B was shown to be proteolytically active at the cell surface of porcine thyrocytes at neutral pH. The proteolytic activity of extracellular ca-
thepsin B was completely abolished by E64, whereas the liberation of T4 from Tg by extracellular means was greatly enhanced upon reactivation of cysteine proteases. Therefore, we assume an involvement of mature cathepsin B in extracellular proteolysis of Tg at neutral pH, resulting in the rapid liberation of T4.

Thyroid hormone liberation by extracellular proteolysis of Tg

We have previously shown that thyroid hormone liberation precedes proteolysis of the protein backbone of Tg in the macrophage cell line J774 (18), and essentially the same mechanism was postulated for Tg degradation in thyrocytes (10, 13). Most of our present knowledge derives from in vitro experiments studying the degradation of radiolabeled Tg (11–13, 19–23, 52–56). Many of these studies analyzed the release of iodocompounds from radiolabeled Tg after long term in vitro incubations, although thyroid hormone liberation from Tg occurs within minutes (7, 13, 18). Therefore, we have reinvestigated the cellular sites of Tg degradation by an in vitro assay system that allowed us to analyze the degradation of unlabeled native Tg within short time intervals. From our finding that thyroid hormone liberation was not restricted to lysosomal proteolysis, we conclude that extracellular proteolysis might be an additional means of T4 liberation. For the liberation of T4 extracellular proteolysis seemed to be as important as lysosomal proteolysis.

Our findings suggest that in the thyroid gland Tg could, in principle, undergo limited extracellular proteolysis. As only a minor portion of luminal Tg is internalized by non-stimulated thyrocytes, it is difficult to speculate on the relative importance of the various cellular sites contributing to thyroid hormone liberation in situ. Studies are currently under way to examine the regulation of Tg degradation by TSH in the different cellular locations of thyroid epithelial cells, e.g., when the rate of Tg endocytosis increases. However, the need of the thyroid for luminal, i.e., extracellular, proteolysis of Tg becomes more obvious for the solubilization of covalently cross-linked Tg (14). We suggest and will analyze in the future whether extracellularly occurring cathepsins might be involved in this process.

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