Iodination of mature cathepsin D in thyrocytes as an indicator for its transport to the cell surface

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Thyrocytes are known for their ability to iodinate thyroglobulin from which the thyroid hormones are generated. In the intact thyroid gland the iodination process is almost exclusively executed at the apical plasma membrane of thyroid epithelial cells. Here, we show that freshly isolated thyrocytes iodinated polypeptides other than thyroglobulin and that one of the major iodinated polypeptides was the mature form of the lysosomal protease cathepsin D (CD). The detection of mature CD as an iodinated polypeptide suggested that a fraction of the lysosomally maturated enzyme was delivered to the apical plasma membrane where it became available for iodination. After labeling of thyrocytes with [35S]methionine/cysteine overnight part of the mature CD was released into the culture medium. This was abolished by inhibiting maturation of CD with NH4Cl, indicating that mature CD appeared in the medium after its proteolytic maturation in an acidic compartment. Besides CD other soluble lysosomal polypeptides like the β-N-acetylhexosaminidase and the sphingolipid-activating protein D (Sap D) were iodinated and partially secreted as mature polypeptides. In contrast, the membrane-associated lysosomal ceramidase was iodinated and partially secreted as immature single-chain enzyme and not as fully maturated two-chain enzyme. These data indicate that a portion of mature CD and other soluble lysosomal enzymes is delivered from lysosomes to the cell surface whereas some membrane-associated enzymes from the terminal lysosomal compartment are efficiently excluded from this process.

Introduction

The main function of the thyroid gland is the synthesis and controlled release of thyroid hormones into the blood stream. The generation of thyroid hormones is initiated by the iodination and coupling of certain tyrosyl residues in the polypeptide chain of thyroglobulin at the apical plasma membrane of thyroid epithelial cells [14, 53]. To efficiently solve this task the apical cell surface is enlarged by numerous microvillar-like protrusions which are studded with the iodinating system consisting of the thyroperoxidase and accessory enzymes. After iodination thyroglobulin is stored extracellularly in the lumen of thyroid follicles at concentrations of at least 100 mg/ml [23, 46]. In some species the follicular content consists of covalently crosslinked thyroglobulin reaching concentrations as high as 700 mg/ml [2, 26]. Upon hormone demand thyroglobulin is reinternalized by thyrocytes which results in the proteolytic degradation of thyroglobulin [50]. Liberation of hormones was shown to start extracellularly and in early endocytic compartments by limited proteolysis of thyroglobulin indicating the presence of extracellularly acting proteases [5, 11, 44]. Thyroid hormone liberation and thyroglobulin degradation is completed in lysosomes.

In thyrocytes CD is considered to be one of the enzymes participating especially in the initial phase of thyroglobulin degradation [12, 13, 55]. CD is a lysosomal endopeptidase with a preference to cleave next to aromatic amino acids. Like most other lysosomal enzymes it is synthesized as an inactive higher molecular mass precursor (48 kDa in porcine cells [16, 25]) which is activated by proteolytic removal of the propeptide in the acidic environment of endosomes generating the single-chain enzyme (46 kDa [16, 40]). Beginning in the late endosome but mainly upon delivery to lysosomes CD is further proteolytically processed, yielding the mature two-chain enzyme, which consists of a light and heavy chain with apparent molecular masses of 15 and 31 kDa [8, 16, 20]. The last maturation step of CD has been shown to depend on acidic pH [19] and the presence of lysosomal cysteine proteases.

We found that besides thyroglobulin other polypeptides were iodinated in cultured thyrocytes [35]. Here, we show that CD is one of the major polypeptides incorporating radioactive


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iodine. Cell-surface biotinylation confirmed that iodination of mature CD occurred at the plasma membrane. Consequently, this unique feature of endogenous iodination was used as an indicator for the intermittent presence of lysosomal enzymes on the cell surface. Other lysosomal enzymes, iodinated by thyrocytes, included β-N-acetylgalactosaminidase, lysosomal ceramidase and Sap D. Interestingly, in most cases the mature forms of lysosomal polypeptides were iodinated, indicating the existence of a retrograde transport pathway from lysosomes to the plasma membrane of thyrocytes.

Materials and methods

Cell culture

Follicle segments were prepared from biopsies of human thyroid glands or from whole porcine thyroid glands as described previously [27]. Follicle segments (5–40 × 10^6 cells) were used for experiments immediately after preparation or were seeded into 25 or 75 cm^2-culture dishes where they formed monolayers. Monolayers were kept in culture for at least two days before starting the experiments. Thyrocytes were maintained at 37°C and 5% CO_2 in Eagle's minimal essential medium supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.2 μg/ml amphotericin B and 10% fetal calf serum (Boehringer Mannheim, FRG) as described for the culture of human skin fibroblasts in [7].

Materials

14C-methylated molecular mass standards and the luminescence detection kit for Western blots were from Amersham Buchler, Braunschweig, FRG. [251NaI and [35S]-Trans-label, a mixture of L-[35S]methionine and L-[3SS]cysteine, was from ICN, Meckenheim, FRG. Goat anti-rabbit IgGs coupled to 5–4,6-dichlorotriazin-2-ylaminofluorescein hydrochloride (DTAF) were obtained from Dianova, Hamburg, FRG. The polyclonal rabbit antiserum used for immunoprecipitation of CD was a gift from Dr. A. Hasilik (Institute of Physiological Chemistry, University of Marburg, FRG). For immunoprecipitation of CD, a rabbit IgG fraction specific for human CD (IM 16) or non-immune rabbit IgG were purchased from Oncogene Science, Uniondale, NY, USA. The rabbit antisera against human lysosomal ceramidase, Sap D and β-N-acetylgalactosaminidase were kindly provided by Dr. K. Sandhoff (Institute of Biochemistry, University of Bonn, FRG) whereas rabbit antisera against porcine aminopeptidase N or thyroglobulin were raised and described in our own laboratory [34, 35]. Protein A-Sepharose Cl-4B was obtained from Pharmacia, Freiburg, FRG, protein G-agarose from Calbiochem, Bad Soden, FRG, acrylamide (research grade) from Serva, Heidelberg, FRG, and non-sterilized blot membranes from Schleicher and Schuell, Dassel, FRG.

All other reagents, including propylthiouracil, streptavidin conjugated to methacrylate or horseradish peroxidase and biotinamidocaproate N-hydroxysuccinimide ester were of the highest obtainable grade and purchased from Sigma Chemical Company (Munich, FRG).

Biosynthetic labeling with [35S]-Trans-label

If not stated otherwise, all labelings were performed at 37°C. For labeling with [35S]-Trans-label monolayer thyrocytes were kept in methionine/cysteine-deficient Dulbecco's modified minimal essential medium containing 10 mM/L thymotropin and antibiotics (as above) for 6h before the pulse medium was applied, containing 11 MBq [35S]-Trans-label and 10% dialyzed fetal calf serum. In addition, 20 mM NHaCl was or was not contained in the pulse medium. Starvation periods of 1–2h yielded only marginal labeling of newly synthesized proteins, indicating that thyrocytes possess large pools of intracellular methionine or cysteine. These pools were depleted only after prolonged starvation periods.

Biosynthetic labeling with [251]NaI

Follicle segments were labeled for various periods of time with up to 18.5 MBq [251]NaI and up to 50 mU/ml thyrotropin in F-12 medium. Chase periods were initiated by washing cells once with F-12 medium before culturing them in F-12 medium supplemented with 5 mM NaI. Human follicle segments were usually labeled in the presence of 20 mU/ml thyrotropin.

Inhibition of iodination by propylthiouracil

Identical amounts of porcine follicle segments were preincubated for 2h at 22°C in the absence or presence of increasing concentrations of propylthiouracil (10μM–10mM), an inhibitor of the thyroperoxidase [15]. [251]NaI was then added to the culture medium for another 2h at 22°C. CD was immunoprecipitated from aliquots of the cell lysate and analyzed by SDS-PAGE and autoradiography together with aliquots of the total cell lysate. Labeling of cells at 22°C resulted in a selective increase of CD iodination and was chosen to better illustrate the position of CD within the cell lysate.

Biotinylation of cell surface proteins and streptavidin chromatography

In some experiments labeling of cells with [125]I NaI was followed by a cell-surface biotinylation. For this purpose cells were washed three times with ice-cold 145 mM NaCl, 20 mM NaP, pH 7.4 (phosphate-buffered saline, PBS) and biotinylated for 30 min with 0.1 μg/ml biotinamidocaproate N-hydroxysuccinimide ester at 0°C. Biotinylation was stopped by adding one volume of ice-cold F-12 medium. Cells were then washed once with ice-cold F-12 medium before being lysed with lysis buffer containing 0.5% Triton X-100. Cell lysates were split into two identical aliquots, one of which was frozen whereas the other was subjected to the following streptavidin absorption procedure:

Care was exercised not to in- or decrease the volume of the cell lysate. Therefore, a 30 μl-pellet of streptavidin methacrylate was suspended in the cell lysate and incubated for 30 min on an end-over-end mixer at 4°C. After centrifuging the sample for 1 min in an Eppendorf microfuge the supernatant was subjected to two further incubations with streptavidin methacrylate as before. The streptavidin sediments were then washed as described for immunoprecipitates (see below) and eluted by boiling for 5 min in 200 μl of 1.5 × sample buffer under reducing conditions. The eluates were counted in a gamma counter. Usually two rounds of incubation with streptavidin methacrylate were sufficient to obtain more than 95% of the biotinylated, radiolabeled polypeptides. The recovery of radiolabeled polypeptides was in the range of 50% due to losses during the washing procedure and due to incomplete elution from the streptavidin matrix. Because of these losses the degree of biotinylation can best be deduced by comparing cell lysates before and after streptavidin absorption. Incubation of control cell lysates (from cells which were labeled with [251]NaI but not biotinylated) with streptavidin methacrylate showed that binding of radiolabeled polypeptides to streptavidin methacrylate was negligible.

In order to determine the plasma membrane-associated form of CD, freshly prepared follicle sheets were cell surface-biotinylated as described above, lysed and CD was immunoprecipitated from the lysate. After Western blotting of the immunoprecipitate the blot membranes were probed with streptavidin coupled to horseradish peroxidase, and biotinylated CD was visualized by a luminescence detection protocol according to the manufacturer's instructions (Amersham Buchler, Braunschweig, FRG).
Lysis and extraction of cells
Cells were routinely washed 1–2 times before being lysed with 50 mM TrisCl pH 7.4, 145 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, containing 0.5 % Triton X-100 (buffer A) for 30 min at 22°C. Insoluble cellular debris was removed by centrifugation at 15000g for 10 min.

Immunoprecipitation from cell extracts and media
In experiments represented by Fig. 6 secretory proteins were concentrated by (NH₄)₂SO₄-precipitation which was performed according to [24]. Before immunoprecipitating from media of thyrocytes, they were cleared by centrifugation and adjusted to the conditions of buffer A. Up to 10 µl of antiserum were added to aliquots of cell extracts or media and the immunoreaction was allowed to start for 30 min at 22°C and to continue at 4°C overnight. Immunocomplexes were collected with a double excess of the nominal binding capacity of protein A-Sepharose CL-4B or protein G-agarose for one hour at 4°C on a shaker before they were centrifuged for 20s at 15000g and 22°C. Protein A-Sepharose or protein G-agarose sediments were washed twice with 0.8 ml PBS, twice with 0.8 ml PBS containing 1% Triton X-100, 0.5% sodium deoxycholate and 20 mg/ml bovine serum albumine (buffer B), once with 0.8 ml buffer B containing 2 M KCl, once with 0.8 ml of 10 mM TrisCl, pH 8.5, 0.6 M NaCl, 0.1% sodium dodecylsulfate, 0.05% Nonidet P-40 and once with 0.8 ml 10-fold diluted PBS. They were then boiled for 20 µl of 1.5-fold concentrated sample buffer under reducing conditions for 5 min at 100°C. Samples were centrifuged for 2 min and the supernatants were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Western blot analysis
Up to 20 µl of cell extracts were mixed with 5 µl of 5-fold concentrated sample buffer containing 50 mM dithiothreitol and boiled for 5 min at 100°C before being loaded onto a gel. Cell extracts and immunoprecipitates were analyzed on 12.5% or 15% SDS-polyacrylamide gels according to [32] and either followed by autoradiography (for 125I) or fluorography (all other isotopes) of the dried gels [33]. Western blot analysis was performed as described earlier [35] using 100-fold diluted IM 16 rabbit IgGs.

Quantitation of iodination rates
The incorporation of iodine into a specific polypeptide was determined by densitometric analysis of the autoradiographs.

Immunolabeling of thyrocytes for fluorescence microscopy
Porcine thyrocytes were grown on coverglasses, washed in PBS, and fixed with 8% paraformaldehyde in 200 mM Hepes (pH 7.4) for 30 min at 22°C. After washing, some coverglasses were further treated with 0.2% Triton X-100 in Hepes for 5 min. After blockade of nonspecific binding sites, cells were incubated with rabbit anti-human CD antibodies (IM 16 at a final concentration of 10 µg/ml) for 90 min at 37°C and with DTAF-labeled secondary antibodies. For controls either first antibodies were omitted or non-immune rabbit IgG were used instead of specific antibodies. For immunolabeling of cell surface-associated CD, cells were incubated with 10 mM NH₄Cl for 18.5 h at 37°C, and thereafter cooled to 4°C. The following steps were performed at 4°C: after washing and blockade of nonspecific binding sites, cells were incubated with rabbit anti-human CD antibodies (see above) for 120 min and with DTAF-labeled secondary antibodies for 90 min before fixation with 8% paraformaldehyde (for 10 min at 4°C and for 20 min at 22°C). Labeled cells were mounted on microscope slides in a mixture of 33% glycerol and 34% 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, FRG) or a confocal laser scanning microscope (TCS 4D, Leica, Bensheim, FRG). Micrographs were taken on Kodak Tmax films (Eastman Kodak, Rochester, NY, USA).

Results
Iodination of cathepsin D by porcine thyrocytes
Thyrocytes are polarized epithelial cells and known for the iodination of thyroglobulin. This task is executed by the iodinating system at the apical plasma membrane with the thyroperoxidase as one of the key enzymes. We observed that several polypeptides other than thyroglobulin were iodinated by cultured thyrocytes, one of which was the lysosomal protease CD.

To test, whether CD was iodinated by the apically located thyroperoxidase, porcine follicle segments were preincubated in the absence or presence of increasing concentrations of propylthiouracil, an inhibitor of the thyroperoxidase. Cells were then incubated with [125I]NaI in the continued absence or presence of the drug and CD was immunoprecipitated from the cell lysate and analyzed by SDS-PAGE and autoradiography together with aliquots of the total homogenate. In addition, the steady state distribution of the molecular forms of CD in thyrocytes was visualized by immunoblotting.

The antibodies were able to detect and immunoprecipitate all forms of the enzyme: procathespain D, intermediate CD and mature CD (see Fig. 1, IB and Fig. 2). Although all forms of CD were detectable in the cell lysate by immunoblot analysis (Fig. 1, IB), only the mature form of CD was iodinated (Fig. 1, CD-iptt). The inhibition of CD-iodination was dependent on the concentration of propylthiouracil and paralleled the inhibition pattern of all other iodoproteins, including thyroglobulin (Fig. 1, compare total iodoproteins to CD-iptt). This demonstrates that the thyroperoxidase was responsible for the iodination of all these polypeptides. The propylthiouracil-induced decrease of CD-iodination was observed with immunoprecipitated CD and with CD from total lysates (Fig. 1, mCD).

The iodination of mature CD raised the question whether it was the mature form of CD which was iodinated or whether...
the mature form of CD became visible after iodination of the precursor and its subsequent maturation in the endocytic pathway. To address this question, freshly isolated porcine follicle sheets were labeled with [\(^{125}\)I]NaI for 30 min and chased for up to 6 h. Immunoprecipitation of CD from cell lysates (lanes 2-5) and media (lanes 6-9) revealed that only the mature form of CD was iodinated. Part of the radiiodinated mCD was secreted in a time-dependent fashion. In addition, one batch of cells was precooled for 1 h on ice and then labeled with [\(^{125}\)I]NaI on ice. Upon inhibiting endocytic uptake by low temperature only the mature enzyme was iodinated (lane 10). In order to visualize all forms of CD, thyrocytes were labeled with [\(^{35}\)S]Trans-Iabel overnight and CD was immunoprecipitated from the cell lysate (lane 1).

Upon cell-surface biotinylation of unlabeled cells and Western blotting of immunoprecipitated CD only the mature form of the enzyme was detected by a streptavidin based luminescence detection protocol (not shown) providing further evidence that mature CD was associated with the plasma membrane of thyrocytes.

**Fig. 2.** Cathepsin D is iodinated as mature enzyme. Thyrocytes were labeled for 30 min with [\(^{125}\)I]NaI and chased for up to 6 h. Immunoprecipitation of CD from cell lysates (lanes 2-5) and media (lanes 6-9) revealed that only the mature form of CD was iodinated. Part of the radiiodinated mCD was secreted in a time-dependent fashion. In addition, one batch of cells was precooled for 1 h on ice and then labeled with [\(^{125}\)I]NaI on ice. Upon inhibiting endocytic uptake by low temperature only the mature enzyme was iodinated (lane 10). In order to visualize all forms of CD, thyrocytes were labeled with [\(^{35}\)S]Trans-Iabel overnight and CD was immunoprecipitated from the cell lysate (lane 1).

![Fig. 2](image-url)
Immunocytochemical detection of cathepsin D

Iodination and cell-surface biotinylation of mature CD strongly suggested transport of the enzyme to the plasma membrane of thyrocytes. To further prove the occurrence of CD at the cell surface immunocytochemistry of monolayer thyrocytes was performed. When formaldehyde-fixed thyrocytes were permeabilized with Triton X-100 before immunolabeling, CD was detectable in cytoplasmic vesicles of various sizes. These vesicles were numerous in the perinuclear region of the cells and thus resembled lysosomes (Fig. 4a, arrows). Immunolabeling of NH₄Cl-treated thyrocytes with anti-CD antibodies at 4°C before fixation revealed a punctate fluorescence staining pattern that was obvious at the borders between neighboring cells in a monolayer (Fig. 4b, arrowheads). This staining pattern was not observed when first antibodies were omitted (not shown) indicating the specificity of CD-labeling at the cell surface of thyrocytes (Fig. 4b, arrowheads). Pretreatment of thyrocytes with NH₄Cl was necessary to detect cell surface-associated CD. The staining pattern at the borders between neighboring cells was below the detection limit when non-treated cells were labeled at 4°C (not shown). In parallel, iodination of mCD was increased by almost one order of magnitude in NH₄Cl-treated thyrocytes (not shown) indicating that NH₄Cl greatly enhances the delivery of lysosomal polypeptides to the cell surface [36]. The immunofluorescence results suggest cell-surface occurrence of CD whereas the iodination data presented in the preceding Figures suggest that at least part of this material consists of mature CD.

Influence of inhibitors on the transport of mature cathepsin D

Having obtained evidence for the transport of mature CD to the plasma membrane of thyrocytes we were interested to know how this transport was brought about and where it originated.

In order to test whether iodination of mature CD relied on the integrity of the cytoskeleton, porcine follicle segments were labeled with [¹²⁵I]NaI for 2 h in the absence and presence of nocodazole, a microtubule-disrupting agent. Thyroglobulin, aminopeptidase N and CD were then immunoprecipitated from the lysates.

In all three cases the yield of iodinated polypeptides was profoundly reduced in noccodazole-treated cells as compared to control cells (Fig. 5, panel A). The relative iodination rates during nocodazole treatment were 0.41, 0.36 and 0.47 for thyroglobulin, aminopeptidase N and CD, respectively (mean of

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**Fig. 4.** Cathepsin D was detected at cell-to-cell borders by immunofluorescence. Immunofluorescence micrograph of formaldehyde-fixed and Triton X-100 permeabilized thyrocytes. Cells were immunolabeled with anti-CD antibodies and DTAF-coupled secondary antibodies. CD was localized in lysosomes of permeabilized cells (arrows) as expected (a). Immunolabeling of CD of NH₄Cl-treated cells at 4°C before fixation revealed a punctate staining pattern that was prominent at the borders between neighboring cells in a monolayer (b, arrowheads). The results indicated that CD was not restricted to intracellular pools such as lysosomes (see Fig. 4a), but was also detectable at the cell surface of thyrocytes (b, arrowheads). A phase-contrast micrograph corresponding to the immunofluorescence micrograph of Fig. 4b is shown in (c). Bars = 100μm.
duplicate determinations, see bottom of Fig. 5, panel A). Similar results were obtained when follicle segments were iodinated in the presence of brefeldin A (not shown), a drug which is known to block transport through the Golgi complex and to induce tubulation of lysosomes thereby blocking vesicular transport out of lysosomes [54].

Thyroperoxidase, as measured by its enzymatic activity, is eliminated from primary thyrocytes with a half-life of approximately 15 h upon culturing [37]. Therefore, it can be ruled out that turnover of thyroperoxidase was responsible for the observed decrease in iodination. The data exclude the appearance of mature CD at the plasma membrane after passive release from lysed cells. They rather imply that mature CD, as well as thyroglobulin and aminopeptidase N, were delivered to the plasma membrane by an active process of vesicular transport.

We then examined whether CD derived from the biosynthetic route or whether it derived from the endosomal/lysosomal compartment for iodination. In the latter case inhibition of protein biosynthesis by cycloheximide should have no effect on the iodination of CD because the lysosomal pool of lysosomal hydrolases is not depleted by short term inhibition of protein biosynthesis. Polypeptides such as thyroglobulin or aminopeptidase N, which are proteins of the secretory pathway and transported to the apical plasma membrane immediately after biosynthesis [17] should exhibit a strong cycloheximide sensitivity of iodination due to a rapid depletion of newly synthesized protein at the plasma membrane.

Identical amounts of porcine follicle segments were preincubated for 1.5, 4.5, and 6 h in the absence and presence of cycloheximide and labeled with \[^{125}\text{I}]\text{NaI}\) for 1.5 h in the continued absence or presence of the drug. Thyroglobulin, aminopeptidase N and CD were immunoprecipitated from aliquots of the cell lysates. Only the immunoprecipitates after 6 h pretreatment with cycloheximide are shown (Fig. 5, panel B). The relative iodination rates after a 6 h cycloheximide pretreatment are given below each immunoprecipitated antigen.

For unknown reasons, cycloheximide slightly augmented the general iodination process in freshly isolated follicle segments. As determined from pulse/chase experiments halflives of thyroglobulin, aminopeptidase N and CD were above 16 h ruling out that lack of iodination was caused by high turnover rates (not shown).

Increasing inhibition of iodination with time was observed for thyroglobulin and aminopeptidase N and is shown after 6 h pretreatment (Fig. 5, panel B). In contrast, no inhibition of iodination was detected for CD even after a pretreatment for 6 h with cycloheximide, regardless whether CD was examined as an immunoprecipitate (Fig. 5, panel B) or in total cell lysates (not shown). This finding suggests that opposite to thyroglobulin and aminopeptidase N, CD is delivered from a cycloheximide-insensitive compartment for its transport to the plasma membrane. This compartment is most likely the lysosome.

Many cell types utilize mannose-6-phosphate receptors (MPRs) to target their soluble lysosomal enzymes to lysosomes. \(^{125}\text{I}]\text{Cl}\) treatment of these cells is known to interfere with the acidification of endosomes and lysosomes thus inhibiting MPR-mediated transport and proteolytic maturation of lysosomal enzymes [29]. To test whether mature CD is secreted by thyrocytes and whether secretion of mature CD can be blocked by inhibiting transport of CD to and maturation within lysosomes, porcine monolayers were labeled overnight with \[^{35}\text{S}]\text{Trans-label}\) in the absence or presence of \(^{125}\text{I}]\text{Cl}\). Immunoprecipitation of CD from corresponding amounts of cell lysates or media was followed by gel electrophoretic analysis and fluorography (see Fig. 6).

Release of procathepsin D into the medium was augmented in the presence of \(^{125}\text{I}]\text{Cl}\), indicating that CD is subject to MPR-mediated transport in thyrocytes. As expected, a small fraction of mature CD was secreted into the medium from control cells. In \(^{125}\text{I}]\text{Cl}\)-treated cells maturation of \[^{35}\text{S}]\text{Labeled CD}\) was inhibited and consequently appearance of \[^{35}\text{S}]\text{Labeled mCD}\) in the medium was abolished suggesting that proteolytic

**Fig. 5.** A. Nocodazole inhibits iodination of cathepsin D. After a 10 min pretreatment, follicle sheets were labeled with \(^{125}\text{I}]\text{NaI}\) \(0.26\) in the absence or presence of brefeldin A. Thyroglobulin (TG), aminopeptidase N (APN) and CD were immunoprecipitated from lysates of these cells. The iodination of these polypeptides was uniformly inhibited by brefeldin A. The relative iodination rate of each antigen following nocodazole treatment is given at the bottom of panel A. B. Iodination of cathepsin D is resistant towards cycloheximide. Porcine follicle sheets were preincubated with or without \(0.1 \text{ mg/ml cycloheximide}\) for up to 6 h and then labeled with \(^{125}\text{I}]\text{NaI}\) \(1.34\) in the absence or presence of the drug. Immunoprecipitates of \[^{125}\text{I}]\text{thryroglobulin}, \[^{125}\text{I}]\text{aminopeptidase N (}^{\alpha-}\text{ and }^{\gamma}\text{-chain)}\) and \[^{125}\text{I}]\text{CD}\) after a 6 h pretreatment and a 1.5 h pulse with \(^{125}\text{I}]\text{NaI}\) are shown with the position of the polypeptides indicated at the left margins. Numbers below each pair of autoradiographs give the relative iodination rate of a protein after 6 h of cycloheximide pretreatment. In contrast to the iodination of CD, iodination of aminopeptidase N and thyroglobulin, both polypeptides of the secretory pathway, showed strong inhibition following cycloheximide pretreatment.
The following experiment was performed to elucidate whether thyrocytes with eSSj-Trans-label in the absence or presence of 20 mM NH4Cl overnight. Immunoprecipitation of CD from cells and media (Me) revealed that most lysosomal enzymes were iodinated by NH4Cl. A small fraction of mature 35S-labeled CD was secreted by control thyrocytes which was no longer detectable in the medium of NH4Cl-treated cells.

maturation of CD in an acidic compartment preceded its release into the medium of control cells.

**Most lysosomal enzymes were iodinated as fully maturated polypeptides in human thyrocytes**

The following experiment was performed to elucidate whether other lysosomal enzymes besides CD were iodinated by thyrocytes and whether iodination was restricted to the mature forms of lysosomal polypeptides.

Expanding our study to other lysosomal enzymes we had to turn to human thyrocytes because antibodies against lysosomal enzymes were only available for the human antigens and showed only little crossreactivity towards the porcine antigens.

Human follicle segments were labeled for 2-3 hours with [125I]NaI, lysed and CD, the β-N-acetylhexosaminidase (β-Hex), sphingolipid-activating protein D (Sap D) and the lysosomal ceramidase (Cer) were immunoprecipitated from corresponding amounts of cell lysates and secretory products. They were then analyzed by SDS-PAGE, autoradiography and densitometry.

The lysosomal ceramidase is a membrane-associated polypeptide and is synthesized as a single-chain precursor with a molecular mass of approximately 55 kDa whereas the mature enzyme within lysosomes consists of a 13 kDa α-chain and a 40 kDa β-chain [28]. In case of the lysosomal ceramidase, it was the immature single-chain enzyme which became iodinated (Fig. 7, Cer). Part of the immature single-chain enzyme was secreted into the medium after a 2 h labeling period (Fig. 7, Cer, open arrowhead). During this period the immature single-chain enzyme was processed to the mature enzyme to a small extent, indicating that, within cells, the lysosomal ceramidase was transported from the cell surface to lysosomes where it was processed to yield the mature enzyme (Fig. 7, Cer).

In contrast to the lysosomal ceramidase, the soluble human lysosomal polypeptides CD, Sap D and the β-N-acetylhexosaminidase were mainly detected as radioiodinated mature polypeptides in the cell homogenate and to a small extent in the medium (Fig. 7, CD, Sap D, β-Hex). Detailed information on the proteolytic processing of these lysosomal enzymes is available from a large body of published reports (CD: [19]; Sap D: [18]; β-N-acetylhexosaminidase: [24, 38]).

The percentage of secretion was 15 %, 11 %, 20 %, and 7 % for CD, Sap D, β-N-acetylhexosaminidase and lysosomal ceramidase, respectively. Although the mechanism of secretion for the immature form of the membrane-associated lysosomal ceramidase is not known it was also observed by others [28].

This experiment demonstrates that the transport of mature CD from the late endosome/lysosome is also operative in human thyrocytes. Immunoprecipitation of CD from [125I]NaI-labeled bovine thyrocytes also showed, that the mature fraction of the enzyme was radiolabeled and partially secreted (not shown), suggesting that the retrograde transport of mature CD from the late endosome/lysosome to the cell surface is a general transport pathway in thyrocytes.

The differential handling of soluble lysosomal polypeptides versus the membrane associated ceramidase points to a selectivity in the transport of lysosomal enzymes from late endosomes/lysosomes to the plasma membrane, in which membrane association apparently plays a crucial role.
**Discussion**

**Iodination of lysosomal enzymes at the plasma membrane of thyrocytes**

Thyrocytes are able to iodinate proteins (for review see [50]) among which thyroglobulin, the macromolecular precursor of thyroid hormones, is the main acceptor of iodine [35]. In the intact gland, the iodination reaction was shown to take place on the apical plasma membrane of thyrocytes [14, 53]. In cultured thyrocytes, however, part of the iodination is executed within intracellular compartments [30, 31]. We therefore employed freshly isolated follicle sheets in all of our iodination experiments to maintain extracellular, apical localization of thyroperoxidase-mediated iodination. Thus, in freshly prepared follicle sheets the iodination of proteins can be regarded as a marker for apical localization. This was proven by combining cell-surface biotinylation and iodination of mature CD in freshly isolated follicle sheets from porcine glands.

The iodination of mature CD was inhibited by noccodazole and brefeldin A, indicating that it was dependent on intact microtubules and on active vesicular traffic. Unlike polypeptides from the secretory pathway, mature CD derived from a cycloheximide-insensitive compartment for its transport to the plasma membrane. Part of the mature form of CD was released into the medium. The appearance of 35S-labeled mature CD in the medium was blocked by treating cells with NH\(_4\)Cl, indicating that proteolytic maturation of CD in an acidic compartment preceded its secretion. Following biosynthesis, complete maturation of lysosomal enzymes is obtained not earlier than in the late endosome but is mainly accomplished in mature lysosomes [8]. Therefore, we conclude that mature CD is transported from the late endosome or lysosome to the plasma membrane and into the medium.

Besides CD, several other soluble lysosomal polypeptides (i.e. β-N-acetylgalactosaminidase, Sap D and cathepsin B [5]) were iodinated as mature polypeptides by human thyroid epithelial cells. Interestingly, the lysosomal ceramidase which is tightly associated with the lysosomal membrane [9, 28], was iodinated as newly synthesized immature polypeptide indicating that the soluble content of a lysosome and not the entire lysosome is subject to a transfer to the apical plasma membrane. Our biochemical data are in accordance with ultrastructural data [45] showing that lysosomes move towards the apical plasma membrane of thyrotrop-stimulated thyrocytes but do not fuse with the apical plasma membrane. Rather, the existence of transport vesicles must be postulated which derive from the lysosomal compartment and facilitate transport of mature lysosomal enzymes to the plasma membrane. In fact, a new type of clathrin-coated vesicle was recently discovered, budding from endosomes [47] and, most importantly, from lysosomes [49] thus mediating a retrograde transport from all compartments of the endocytic pathway including the mature lysosome. We postulate that a fraction of soluble, mature lysosomal enzymes (e.g. CD, β-N-acetylgalactosaminidase etc.) is entrapped in these lysosomal recycling vesicles and delivered to the plasma membrane where it becomes available for iodination by thyrocytes. In contrast, membrane-associated/integrated polypeptides can be sorted via cytosolic domains which will direct polypeptides destined for recycling into the newly forming coated pits (e.g. LEP 100 [36], lamp-2 and newly synthesized lysosomal acid phosphatase [1, 3]), whereas other membrane constituents (e.g. the lysosomal ceramidase) are left within the lysosomal membrane. Recycling of some lysosomal membrane proteins is only transient and is abolished upon proteolytic removal of the cytosolic tail determinant interacting with adaptors of the clathrin coat of the recycling vesicles [22]. These proteins are thus rendered to constituents of the terminal lysosomal compartment with some delay.

Recycling of membrane-bounded vesicles from endosomal/lysosomal organelles to the plasma membrane relieves the burden of de novo synthesis of polypeptides as well as membrane lipids in thyrocytes which, at times, are highly active in the endocytic uptake of lumenal thyroglobulin.

Although 125I-labeled mature CD was completely extracted by the membrane perforating detergent saponin [41, 52] from porcine thyrocytes (not shown), there were nevertheless indications that mature CD was membrane associated: (i) It was biotinylated on the cell surface after washing cells repeatedly with PBS (see Fig. 3). (ii) Although much more procathepsin D is secreted by thyrocytes (see Fig. 6), mature CD was preferentially iodinated, suggesting its close contact with the iodinating system on the plasma membrane. However, the nature of this association is unknown at present. Reversible association of mature CD with membranes has also been observed, e.g. with endosomal membranes in rabbit macrophages [10].

**Secretion of mature lysosomal enzymes from other cell types**

The observed retrograde transport in thyrocytes (this study) is different from the fusion of whole lysosomes with the plasma membrane upon parasitic infection [43] or the secretion of entire lysosomes as seen with cells of the haemopoietic lineage, e.g. macrophages, cytotoxic T lymphocytes etc., reviewed in [21]. It also contrasts to the selective diversion of newly synthesized cathepsins B and L into secretion granules of secretory cells, wherein they are processed to the active enzymes which are released upon secretagogue addition [48, 51]. In some breast cancer cell lines synthesis of CD is stimulated by estrogens resulting in hypersecretion of procathepsin D and possible extracellular autoactivation. The release of mature, active cathepsins from cancer cells has been linked to their invasiveness and malignancy (reviewed in [42]). In the latter cases, however, the cathepsins derive directly from the biosynthetic route and not from the lysosomal compartment for secretion.

**The possible function of extracellular mature lysosomal enzymes in thyrocytes**

Thyroid hormones are positioned at the extreme ends of the thyroglobulin polypeptide chain [39] and are highly susceptible to proteolytic attack. In addition, thyroglobulin degradation has been observed to be executed in two steps consisting of a fast proteolytic liberation of hormones extracellularly and in the early endocytic pathway followed by a slow process leading to complete degradation of thyroglobulin within lysosomes [4, 5, 11, 44]. Although it has been known for some time that hormone liberation is mediated by the soluble lysosomal proteases cathepsin B, D and L [12, 13], we only recently could show that this process is executed to a large extent extracellularly and on the plasma membrane by the cathepsins B and L, including the mature two-chain form of cathepsin B [5]. It is noteworthy that proteolytic activity of mature cathepsin B at neutral pH was also observed by others [6]. The data...
presented here may explain how fractions of soluble mature lysosomal enzymes such as the cathepsins are transported to the cell surface where they may fulfill biologically relevant functions, e.g. contribute to the extracellular liberation of thyroid hormones.

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