

Biosynthesis and Transport of Cathepsin D in Cultured Human Fibroblasts

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ABSTRACT For study of the time order of glycosylation, formation of complex oligosaccharides and proteolytic maturation as well as the site of proteolytic maturation of cathepsin D, fibroblasts were subjected to pulse-chase labeling, and cathepsin D was isolated from either total cell extracts or subcellular fractions by immune precipitation and analyzed for its molecular forms and sensitivity to endo- β -N-acetylglucosaminidase H. After a 10-min pulse, cathepsin D was detected in its glycosylated precursor form, indicating an early, probably a cotranslational, N-glycosylation of cathepsin D. Conversion of the high-mannose oligosaccharide side chains into forms resistant to endo- β -N-acetylglucosaminidase H started after \sim 40 min, indicating that transport of cathepsin D from the endoplasmic reticulum to the trans-Golgi apparatus requires \sim 40 min. Processing of the 53-kdalton precursor polypeptide of cathepsin D to a 47-kdalton intermediate followed about 20 min after the formation of complex oligosaccharides, and, another 30 min later, 31-kdalton mature forms of cathepsin D were detected. Processing of cathepsin D was first observed in light membranes as a partial conversion of the 53-kdalton precursor into the 47-kdalton intermediate. Both the precursor and the intermediate are transferred into the high density-class lysosomes. After 8 h, the processing to the mature 31-kdalton form of cathepsin D is mostly completed.

It is now well established that cathepsin D is synthesized on membrane-bound ribosomes and cotranslationally translocated into the lumen of the endoplasmic reticulum (1–3). Cathepsin D synthesized in human skin fibroblasts contains two asparagine-linked oligosaccharides per polypeptide chain. During passage through the Golgi apparatus, a portion of these oligosaccharides may become phosphorylated or converted into complex-type oligosaccharides. In the presence of NH_4Cl , \sim 90% of the newly synthesized cathepsin D is secreted. In the NH_4Cl -induced secretions, about half of the cathepsin D molecules have one high-mannose and one complex oligosaccharide. The remaining cathepsin D polypeptides have either two high-mannose or two complex oligosaccharides (4). Of the high-mannose oligosaccharides, about half are phosphorylated (5). Subcellular fractionation experiments (6) and kinetic studies (7) established that phosphorylation represents an early reaction in the Golgi apparatus and precedes the formation of complex oligosaccharides. The segregation of cathepsin D and of other lysosomal enzymes from the secretory pathway is dependent on binding to mannose 6-phosphate-specific receptors and is thought to occur at an

intracellular site (for review, see reference 8). However, the sites of the binding to the receptors and of the segregation from the secretory pathway remain to be determined. After segregation, cathepsin D is subjected to a limited proteolysis, in which the 53-kdalton precursor polypeptide is converted to a 47-kdalton intermediate and the 31-kdalton mature forms (9). This maturation is accompanied by activation of cathepsin D and is thought to be a lysosomal event (10).

In the present study we report on the early stages of cathepsin D biosynthesis with emphasis on the timing and location of the various modification reactions.

MATERIALS AND METHODS

Materials: [^{35}S]Methionine (sp act, 1.2 Ci/nmol) and ^{14}C -methylated standards were obtained from New England Nuclear (Boston, MA). Immuno-precipitin, a 10% *Staphylococcus aureus* cell wall preparation was obtained from Bethesda Research Laboratories (Rockville, MD), and endo- β -N-acetylglucosaminidase H, from Seikagaku (Tokyo, Japan). Rabbit antiserum raised against human placental cathepsin D (11) was purified by affinity chromatography on a cathepsin D-Sepharose 4B column.

Cell Culture: Human diploid fibroblasts were maintained at 37°C under 5% CO_2 in Eagle's minimal essential medium supplemented with anti-

biotics, nonessential amino acids, and 10% fetal calf serum (Boehringer Mannheim Biochemicals, Mannheim, Federal Republic of Germany) as described in reference 12.

Metabolic Labeling: Conditions for labeling with [³⁵S]methionine were as described (13). Confluent cultures in 60-mm dishes were incubated for 10 min with 1 ml of medium containing 300 μCi of L-[³⁵S]methionine. The same radioactive medium was used for sequential labeling of up to 6 dishes. Labeling was terminated by either harvesting or feeding the cultures with 2 ml of Eagle's minimal essential medium containing 10% fetal calf serum and 0.25 mg/ml methionine. For harvesting, cultures were washed twice with ice-cold medium and the cells were solubilized in 50 μl of 1% Nonidet P-40 and 1% sodium deoxycholate. The solubilized cells were recovered in a final volume of 35–45 μl. For subcellular fractionation, cultures in 75-cm² flasks were incubated for 20 min with 4 ml of labeling medium containing 0.6 mCi of [³⁵S]methionine. The same medium was used sequentially for labeling of three cultures.

Treatment with Endo-β-N-Acetylglucosaminidase H: The cell extracts were adjusted to 10 mM sodium phosphate, pH 6.0, and 0.1 M NaCl and split in two equal parts. Endo-β-N-acetylglucosaminidase H from a 1-U/ml stock solution was added to reach a final concentration of 50 mU/ml. The same amount of enzyme was added again after 16 h. Samples with and without endo-β-N-acetylglucosaminidase H were incubated for a total of 30 h at 37°C.

Subcellular Fractionation in Percoll Gradients: After labeling, the 75-cm² flasks were washed twice with ice-cold 10 mM sodium phosphate, pH 7.4, in 0.14 M NaCl, and once with 0.25 M sucrose and 1 mM EDTA adjusted to pH 7.4 with NaOH. The cells were detached with a rubber policeman, suspended in 2 ml of 0.25 M sucrose, 3 mM imidazole-HCl, pH 7.4, and broken by 20 strokes in a tight-fitting Dounce homogenizer (Wheaton Scientific, Millville, NJ). Nuclei and unbroken cells were removed by centrifugation for 10 min at 600 g. On average, ~85% of β-hexosaminidase (latency >80%) was recovered in the postnuclear supernate.

Percoll, 25.69 ml, and 10 ml of 2.5 M sucrose were added to 64.31 ml water. In a tube, 30 ml of this gradient medium (ρ = 1.065) was underlaid with 2 ml of 2.5 M sucrose. The postnuclear supernates were applied to the top of the gradient, and the samples were centrifuged at 20,000 rpm, ω²t = 1.38 × 10¹⁰ rad²/s in a VTi 50 rotor (Beckman Instruments, Inc., Fullerton, CA). Fractions of 2 ml were collected and assayed for β-hexosaminidase activity (14), radioactivity, and density (15). Two to three fractions were combined and subjected to centrifugation for 3.5 h at 100,000 g. The membranous layers above the pelleted Percoll were collected and adjusted to 1% Nonidet P-40, 1% sodium deoxycholate, 10 mM sodium phosphate, pH 7.4, and 0.15 M NaCl.

Immune Precipitation of Cathepsin D: The cell extracts and subcellular fractions were adjusted to 10 mM sodium phosphate, pH 7.4, and 0.15 M NaCl, using 10-fold concentrated stock solutions. After freeze-thawing, the assays were kept at 4°C overnight. 0.2 vol of immunoprecipitin was added. After incubation for 30 min at room temperature, the suspensions were centrifuged for 1 h at 45,000 g. The supernates were mixed with 0.8 vol of buffered detergent (1% Nonidet P-40, 1% SDS, 0.5% sodium deoxycholate, 5 mg/ml bovine serum albumin, 0.14 M NaCl in 10 mM sodium phosphate, pH 7.4), and immunopurified rabbit immunoglobulins against placental cathepsin D (30 μg per cell extract of a 60-mm dish) were added. After incubation for 30 min at room temperature and for 16 h at 4°C, immunoprecipitin (2 μl/μg of added immunoglobulins) was added. After 30 min at 4°C, the immunoprecipitin was spun down in an Eppendorf centrifuge (30 s) and washed once with 800 μl of cold buffered detergent and twice with 800 μl of 1 M phosphate-buffered 14 mM NaCl, pH 7.4. The bound material was solubilized by heating the pellet for 5 min at 95°C in 70 μl of a solution of 1% SDS, 10 mM dithiothreitol, 10% glycerol, 125 mM Tris-HCl, pH 6.8. Immunoprecipitin was removed by centrifugation and supernates were subjected to PAGE (16) and fluorography (17) as described (9). Radioactivity in the cathepsin D polypeptides was quantified by densitometry of the fluorograms.

RESULTS AND DISCUSSION

Human skin fibroblasts were labeled for 10 min and subjected to a chase for up to 3 h. As shown in Fig. 1, cathepsin D is synthesized as precursor with a molecular mass of 53 kdaltons. After a chase for 60 min, a 47-kdalton intermediate polypeptide becomes detectable (10% of total). Finally, the proteolytic maturation yields a 31-kdalton polypeptide, which becomes apparent after a chase for 2 h (6% of total). In the medium (not shown), cathepsin D precursor is detectable after 2 h of chase. The chase periods after which the 47-kdalton intermediate and 31-kdalton mature forms accounted for ≥5% of

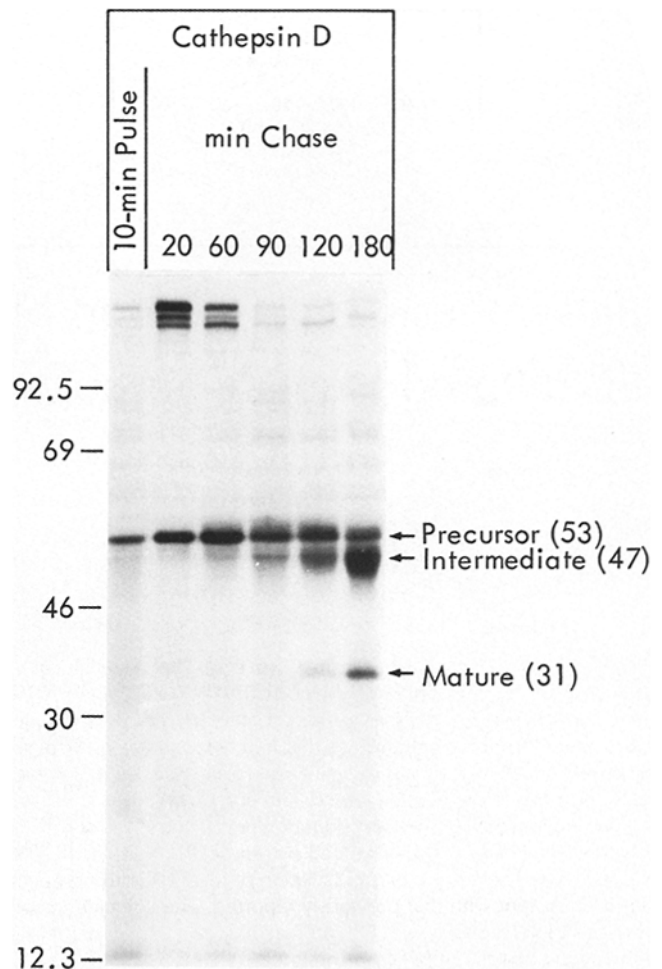


FIGURE 1 Kinetics of maturation of cathepsin D. Fibroblasts were labeled with [³⁵S]methionine for 10 min and subjected to chase periods of up to 180 min. Cathepsin D was immune precipitated and the radioactive polypeptides were separated by PAGE in the presence of SDS and visualized by fluorography. The precursor (53-kdalton), processing intermediate (47-kdalton), and mature form (31-kdalton) of cathepsin D are indicated by arrows. Besides a variety of contaminants present in trace amounts, several major polypeptides of high molecular weights are seen. These polypeptides are not related to cathepsin D. The following ¹⁴C-methylated standards were used: phosphorylase b, 92.5 kdaltons; bovine serum albumin, 69 kdaltons; ovalbumin, 46 kdaltons; carbonic anhydrase, 30 kdaltons; cytochrome c, 12.3 kdaltons.

total cathepsin D varied in five different experiments from 30 to 90 min (mean of 65) and from 60 to 120 min (mean of 90), respectively. The secretions contain (a minor fraction of) lysosomal enzymes in the precursor forms (9). Obviously, the proteolytic processing occurs subsequent to segregation of lysosomal enzymes from the secretory pathway. Our results indicate that cathepsin D is transported from the endoplasmic reticulum to the site of segregation within less than 65 min.

While being transported to the site of segregation, cathepsin D acquires mannose 6-phosphate residues and complex oligosaccharides. Because of the location of the enzymes involved, the phosphorylation is assumed to occur in the cis-Golgi apparatus (6, 7). Similarly, the formation of complex oligosaccharides is thought to be accomplished in the trans cisternae of the Golgi apparatus (18, 19). For an estimate of the minimum time required for the transport of cathepsin D into the trans-Golgi compartment, pulse-labeled fibroblasts

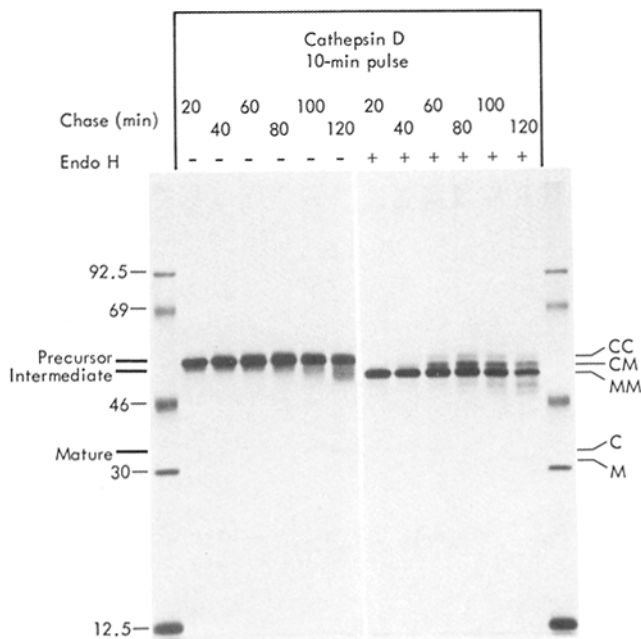


FIGURE 2 Sensitivity of cathepsin D to endo- β -*N*-acetylglucosaminidase H. Fibroblasts were labeled with [35 S]methionine for 10 min and chased for up to 120 min. Cathepsin D was immune precipitated from the cell extracts that had been incubated with or without endo- β -*N*-acetylglucosaminidase H. The three forms of the cathepsin D precursor known to contain either two high-mannose, or one high-mannose and one complex or two complex oligosaccharide side chains (4), are denoted by symbols MM, CM, and CC, respectively. The pattern of glycosylation of the 53-kdalton precursor is consistent with that previously reported. The coincidence of the completely deglycosylated precursor with the resistant 47-kdalton intermediate precludes the interpretation of the glycosylation pattern of the latter. The 31-kdalton mature form contains a single *N*-linked oligosaccharide (4), which is largely sensitive to endo- β -*N*-acetylglucosaminidase H (M-form) and resistant only to a small extent (C-form). For standards, see legend to Fig. 1.

were subjected to chase periods of up to 2 h. Cathepsin D was then isolated from cell extracts, which were incubated with or without endo- β -*N*-acetylglucosaminidase H. As shown in Fig. 2, oligosaccharide side chains in cathepsin D precursor polypeptides were completely sensitive to endo- β -*N*-acetylglucosaminidase H for chase periods of up to 40 min. Resistant (complex) oligosaccharides were detectable after a chase for 60 min. Thus, cathepsin D is transported from the endoplasmic reticulum to trans cisternae of the Golgi apparatus within 40–60 min. Proteolytic maturation of cathepsin D (indicated by the appearance of the 47-kdalton intermediate polypeptide) starts within 20 min after formation of complex oligosaccharides (Fig. 2). The period of 20 min may be the minimum time for export of lysosomal enzyme precursors from the trans cisternae of the Golgi apparatus, for their segregation from the secretory products and the initial proteolytic processing. The site of the segregation, however, has not been determined. Our results do not exclude that the large fraction of cathepsin D molecules, which contain only the high-mannose oligosaccharides (4), may actually bypass the trans cisternae of the Golgi apparatus.

Subcellular Site of the Proteolytic Maturation

Fibroblasts were labeled for 20 min and then subjected to

chase periods of 0.5–8 h. Postnuclear supernates were separated in self-generating density gradients of colloidal silica (Percoll). This procedure separates “heavy” lysosomes from the microsomal membranes. The fractions containing the microsomal membranes are heterogeneous. In these “light” fractions also the marker enzymes of mitochondria and peroxisomes are recovered along with a variable amount of lysosomal enzyme activities (data not shown). Fig. 3A shows a typical profile of β -hexosaminidase activity and radioactivity after centrifugation in the density gradient. In our experiments, the β -hexosaminidase activity (used as a marker for lysosomes) recovered in the lower density region was consistently between 10 and 20% of that recovered in the higher density region. In the original study on the separation of fibroblast lysosomes in Percoll gradients, Rome et al. (20) reported an approximately equal distribution of β -hexosaminidase and other lysosomal markers between the two lysosomal fractions.

From fractions pooled as indicated in Fig. 3, cathepsin D was isolated and analyzed by gel electrophoresis and fluorography (Fig. 3B). After a chase of 30 min, i.e., 30–50 min after biosynthesis, 94% of total cathepsin D was recovered in the light fractions (I and II). However, some material (3% of total) was present also in the fractions containing the higher density lysosomes (V and VI). With prolonged chase periods the radioactivity in cathepsin D in the lower density region decreased and the labeled enzyme accumulated in the higher density lysosomes. After a chase for 8 h, 91% of labeled cathepsin D was found in the higher density fractions.

In each fraction the various molecular forms of cathepsin D were detectable at certain periods of chase. After 30 min of chase, most of the cathepsin D was present in the light fractions as 53-kdalton precursors, though a portion (22% of total) was already processed to the 47-kdalton intermediate. In the light fractions, the ratio between precursor and intermediate remained fairly constant and both disappeared continuously within 3 h of chase. In the dense fractions, the amount was lower than in the light fractions at any time of chase, came to maximum after 60 and 90 min of chase, and disappeared by 3 h of chase. In the dense fractions, the amount of intermediate was higher than in the light fractions after 60 min of chase and, later, came to maximum after 90 and 120 min of chase and was still present after 8 h of chase (4% of total). The mature form of cathepsin D was detected within 1 h of chase (4% of total) in the higher and 30 min later (2% of total) in the lower density fractions. The amount of the mature enzyme finally present in the lower density fractions (4% of total) was far less than that of the 47-kdalton intermediate found therein at various periods of chase (22–8% of total after chase for 30–120 min).

It appears that processing of the 53-kdalton precursor to the 47-kdalton intermediate occurs extensively though not exclusively in the lower density organelles. Most of radioactivity associated with the 47-kdalton intermediate found in the lower density fractions will eventually accumulate in the heavy lysosomes. The conversion of the 47-kdalton intermediate into the mature 31-kdalton form is accomplished predominantly in heavy lysosomes. It is not known whether the appearance of the intermediate in the heavy lysosomes indicates a transport from the light to the dense organelles or a transformation of light into dense organelles. A considerable portion of the precursor cathepsin D is found in the heavy lysosomes. Therefore, proteolysis is neither stringently cou-

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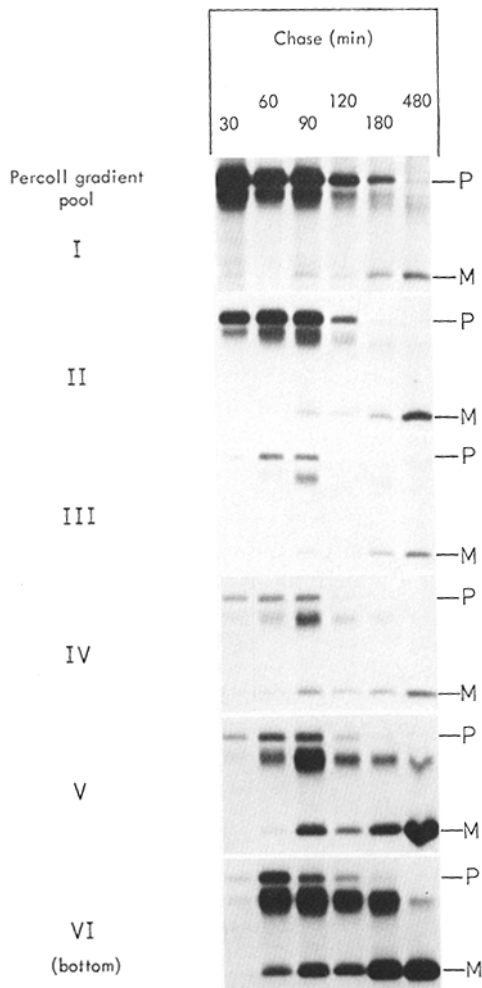
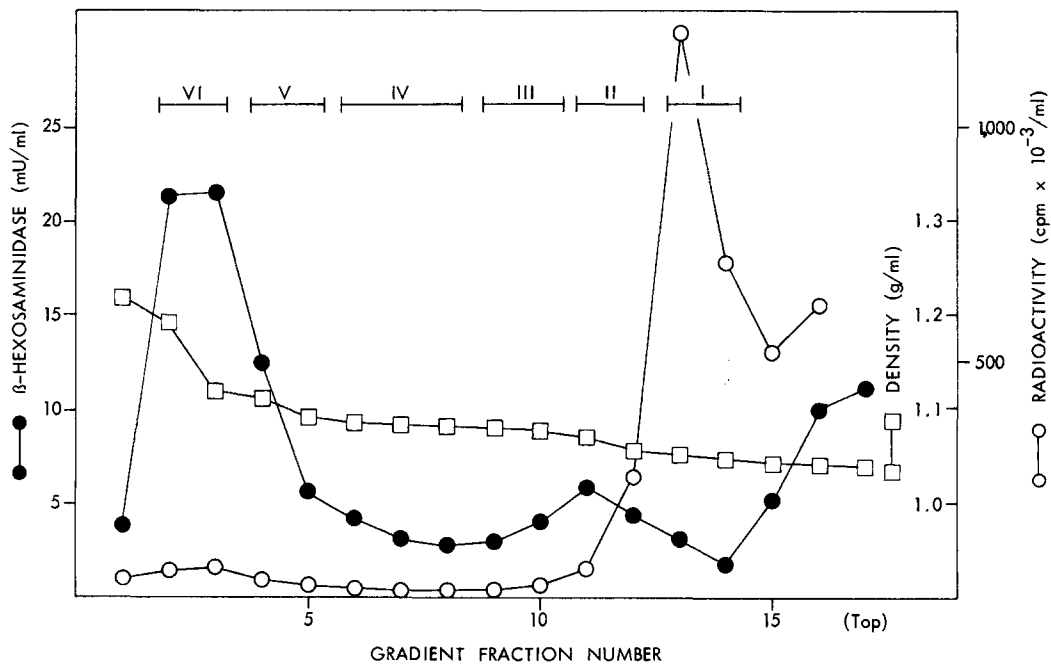


FIGURE 3 Kinetics of maturation of cathepsin D in subcellular fractions. Fibroblasts were labeled with [³⁵S]methionine for 20 min and chased for 30–480 min. Postnuclear supernates were prepared and subjected to centrifugation in a gradient of colloidal silica gel. (A) 18 fractions were collected and assayed for density (□), β-hexosaminidase activity (●), and radioactivity (○). Fractions 1–5 contain the heavy lysosomes; fractions 10–14 contain the microsomes and mitochondria and light lysosomal organelles. Fraction 16 contains nonlatent β-hexosaminidase released from lysosomes. The patterns shown are representative of the other gradients. (B) Fractions of the silica gel gradient were pooled as indicated in A to give pools I–VI. The pools were centrifuged at 100,000 g for 3.5 h to pellet the silica. About 80% of the β-hexosaminidase activity was recovered with the membranous layer assembling above the silica pellet. The fractions were solubilized and cathepsin D was isolated by immune precipitation. The kinetics of the conversion of the 53-kdalton precursor (P) via the 47-kdalton intermediate to the 31-kdalton mature form (M) of cathepsin D in pools I–VI in the order of increasing density can be seen.

pled to nor a prerequisite for the transfer of cathepsin D into the heavy lysosomes.

Worthwhile commenting upon is the presence of mature cathepsin D in the lower density fractions. It has previously

been suggested that the lysosomal enzyme present in these fractions stems primarily from the Golgi endoplasmic reticulum lysosome (GERL) network that is abundant in fibroblasts (20). In our light fractions, the mature cathepsin D bands at

a slightly higher density than the precursor. Pool I contains after 30 and 60 min of chase 2.5–6.5 times more cathepsin D precursor (and intermediate) than pool II, whereas pool II contains two times more mature cathepsin D after 8 h of chase (see Fig. 3). These two forms of cathepsin D in the light fractions probably are contained in different organelles. The mature cathepsin D may originate from the GERL network or other low density lysosomes, and immature enzyme may come from prelysosomal compartments.

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This paper is dedicated to Professor Dr. E. Buddecke on the occasion of his 60th birthday.

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