Biosynthesis of Cathepsin B in Cultured Normal and I-Cell Fibroblasts*

Heike Hanewinkel†, Josef Glossl‡, and Hans Kresse‡

From the †Institut für Physiologische Chemie und Pathobiologie der Westfälischen Wilhelms Universität, D-4400 Münster, Federal Republic of Germany and ‡Zentrum für Angewandte Genetik, Universität für Bodenkultur, A-1180 Wien, Austria

Biosynthesis and processing of cathepsin B in cultured human skin fibroblasts were investigated using immunological procedures. Upon metabolic labeling with [35S]methionine for 10 min, a precursor form with M, 44,500 was identified. During an 80-min chase, about 50% of it was converted to an M, 46,000 form. Further processing yielded mature forms with M, 33,000 and 27,000, in a final quantitative ratio of about 3:1. Processing of cathepsin B was inhibited by leupeptin, which led to an accumulation of the M, 33,000 polypeptide. The M, 33,000 form appeared to be the most active form and showed a half-time of about 12 h. About 5% of newly synthesized enzyme was secreted as precursor, being detectable extracellularly already after 40 min. NH4Cl enhanced the secretion of the precursor about 20-fold. The precursor and the 33-kDa form contained phosphorylated N-linked oligosaccharides. Cleavage by peptide N-glycosidase F or by biosynthesis in the presence of tunicamycin yielded a precursor with M, 39,000. Evidence of a mannose 6-phosphate-dependent transport of cathepsin B in fibroblasts was obtained on the basis of the following results: (i) cathepsin B precursor from NH4Cl-stimulated secretions was internalized in a mannose 6-phosphate detectable manner, and (ii) I-cell fibroblasts secreted more than 95% of newly synthesized cathepsin B precursor. In conclusion, cathepsin B from human skin fibroblasts shows an analogous biosynthetic behavior as other lysosomal enzymes.

Cathepsin B (EC 3.4.22.1) is a well-characterized lysosomal thiol proteinase (for review see Barrett and Kirschke, 1981; Katunuma and Kominami, 1983). Besides its lysosomal role, cathepsin B or cathepsin B-like enzymes have been suggested to be involved in a variety of physiologic and pathological processes, for example in the proteolytic maturation of proinsulin (Docherty et al., 1984; Steiner et al., 1984), or proalbumin (Judah and Quinn, 1978; Matsuda et al., 1986) and of proapolipoprotein A-II (Gordon et al., 1985) to their respective mature forms. They are also thought to play a role in inflammatory and neoplastic disease states (e.g. Mort et al., 1985; Köppel et al., 1984; Sloane et al., 1986a, 1986b). Wide variations in tissue levels of cathepsin B are compatible with specialized functions of this enzyme in distinct tissues (Kominami et al., 1985; San Segundo et al., 1986). Recent studies revealed a growth-regulated expression of thiol-dependent proteinases in different organisms (Williams et al., 1985; Portnoy et al., 1986; Denhardt et al., 1986).

The mature form of cathepsin B is well characterized by amino acid sequencing of the enzyme from rat (Tsakio et al., 1983) and human liver (Ritonja et al., 1985). Analysis of cDNA clones confirmed that cathepsin B of human, rat, and mouse origin is highly conserved at both the protein and nucleotide level and that it is closely related to other cysteine proteinases of animal and plant origin (San Segundo et al., 1985; Fong et al., 1986; Chan et al., 1986).

The lysosomal localization of cathepsin B is well established (Mort et al., 1981). In most instances, lysosomal enzymes are synthesized as proenzyme forms of higher molecular size and they are targeted to lysosomes by the mannose 6-phosphate recognition signal (Hasilik and von Figura, 1984). Mannose 6-phosphate residues are components of high mannose and hybrid oligosaccharides (Goldberg et al., 1984). However, cathepsin B showed only negligible affinity to concanavalin A-Sepharose (Barrett and Kirschke, 1981), and the structure of the carbohydrate moiety of cathepsin B from porcine spleen (Takahashi et al., 1984) and rat liver (Taniuchi et al., 1985) differed drastically from that of other lysosomal enzymes (Goldberg et al., 1984).

In view of the differences in the carbohydrate structure and of the proposed extra- and intralysosomal roles of cathepsin B, the question of its biosynthetic pathway is of high relevance. Nevertheless, detailed information on biosynthesis and intracellular targeting of cathepsin B is not yet available. As cultured human skin fibroblasts are a well-established model for studying lysosomal enzymes (Hasilik and von Figura, 1984), we chose this system to elucidate the biosynthesis and maturation of cathepsin B using immunological procedures. In addition, the behavior of cathepsin B in fibroblasts from patients with I-cell disease was investigated. In I-cell disease, the synthesis of the mannose 6-phosphate recognition signal on lysosomal enzymes is hindered due to a deficient phosphotransferase. This defect leads to a secretion of biosynthetic precursors of most soluble lysosomal enzymes and to an intracellular deficiency of enzyme activity in cultured fibroblasts (for review see Creek and Sly, 1984).

In this report we will show that cathepsin B in cultured fibroblasts is synthesized analogously as other lysosomal enzymes. It carries the mannose 6-phosphate recognition marker, and consequently it is localized inappropriately in I-cell fibroblasts.

* This work was supported in part by Deutsche Forschungsgemeinschaft SFB 310. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Zentrum für Angewandte Genetik, Universität für Bodenkultur, Gregor-Mendel-Str. 33, A-1180 Wien, Austria.

(Received for publication, March 11, 1987)
Human fibroblast cathepsin B is synthesized as an N-glycan-containing precursor with an $M_r$ of about 44,500, a part of which becomes converted to an $M_r$ of 45,000 form. This conversion was not investigated by the authors. Treatment of fibroblasts with an inhibitor of N-glycan synthesis, as well as digestion of the cathepsin B precursor with peptide $N$-glycosidase F led to the formation of an $M_r$ of 39,000 polypeptide. All types of N-linked oligosaccharides are cleaved from glycoproteins by peptide $N$-glycosidase F (Tarentino et al., 1985). Thus, the $M_r$ of 39,000 form represents the cathepsin B precursor free of N-glycosidically linked oligosaccharides. This $M_r$ value is close to the calculated molecular mass of 35,900 for human hepatoma procathepsin B, deduced from the cDNA sequence (Chan et al., 1986). In rat pancreatic islets, a cathepsin B precursor of approximately 44 kDa was found (Steiner et al., 1984), which is in good agreement with the size of the glycosylated precursor from human skin fibroblasts.

In skin fibroblasts, the precursor is processed first to an $M_r$ of 33,000 form and then in part to an $M_r$ of 27,000 polypeptide. The latter proteolytic step was sensitive to leupeptin. Leupeptin treatment of fibroblast cultures led to an increase of specific enzymatic activity of cathepsin B, both to a similar extent. An increased activity of cathepsin B after leupeptin loading is in agreement with observations in galactosialidosis fibroblasts (Kato et al., 1983; Takeda et al., 1986) and in mouse organs (Sutherland and Greenbaum, 1983). Leupeptin is a reversibly acting competitive inhibitor of thiol- and some serine proteases (Knight, 1980; Umezawa, 1976). Therefore, cathepsin B from leupeptin-treated cells might be of normal activity after harvesting the cells in a leupeptin-free buffer in spite of a possible complete inhibition in vivo. Taken together with the results obtained by immunological detection of nonlabeled cathepsin B in fibroblast extracts, it is suggested that the $M_r$ of 33,000 polypeptide is the main form as well as the most active form of cathepsin B in cultivated skin fibroblasts. Similar molecular weights were observed for cathepsin B forms from rat islets of Langerhans (Docherty et al., 1984) and for a cathepsin B-like mouse tumor thiol proteinase (Olstein and Liener, 1983). Additionally, an extracellular form of cathepsin B with an $M_r$ of 40,000, representing tentatively a precursor of cathepsin B, was detected in ascitic fluid of cancer patients (Mort et al., 1983; Mort and Recklies, 1986). This latent form could be rendered active by pepstatin treatment, yielding an $M_r$ of 33,000. A 5-kDa light chain of cathepsin B, representing most likely a product of limited proteolysis in the N-terminal part of the enzyme (Takio et al., 1983; Ritonja et al., 1985) was not detected in skin fibroblasts.

The proposed extratrasosomal functions of cathepsin B (for review see Katunuma and Kominami, 1983; Docherty et al., 1984; Gordon et al., 1986; Slaone et al., 1986a,b) raises the question of its intracellular targeting. Our results demonstrate that at least in skin fibroblasts cathepsin B, as most other lysosomal enzymes, is targeted to the lysosomes via the mannose 6-phosphate recognition marker. This is shown by several lines of evidence. (i) Phosphate label was incorporated into the N-glycan containing cathepsin B precursor, which could be removed by peptide $N$-glycosidase F. (ii) NH$_4$Cl, which inhibits targeting of lysosomal enzymes due to a disturbance of pH-gradients in acidic cell organelles (Hasilik and von Figura, 1984; Creek and Sly, 1984), enhanced secretion of newly synthesized cathepsin B precursor and inhibited proteolytic processing of the precursor. (iii) Cathepsin B precursor from ammonium chloride secretions was internalized by fibroblasts in a mannose 6-phosphate inhibitable manner and further processed intracellularly. (iv) I-Cell fibroblasts secreted most of their newly formed cathepsin B precursor. Accordingly, no incorporation of phosphate was detectable in cathepsin B from I-cell fibroblasts. However, we estimated with almost 15% a relatively high residual enzyme activity of cathepsin B in I-cell fibroblasts. This is not consistent with the observed secretion of about 95% of newly synthesized enzyme. A similar but more extreme situation was observed for acid phosphatase (Lemansky et al., 1985). In I-cell fibroblasts about one-third of the synthesized acid phosphatase polyglyptides remains intracellularly, whereas the intracellular activity of that enzyme is normal.

In conclusion, cathepsin B shows a biosynthetic behavior principally similar to that of most other lysosomal enzymes. At least in skin fibroblasts, it is targeted to the lysosomes via the mannose 6-phosphate recognition system and its secretion in I-cell fibroblasts is increased. However, it is likely that in other tissues, due to postulated specialized functions of this enzyme, cathepsin B is sorted and processed in a different way than in fibroblasts.

Acknowledgments—We would like to thank Petra Blumberg and Margit Stichauener for technical assistance and Friederike Sacken for processing the manuscript.

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Supplementary Material to
BIOGENESIS OF CATHEPSIN B IN CULTURED NORMAL AND 1-CELL FIBROBLASTS
Heike Hanewinkel, Josef Gilsz, and Hans Kreuse

EXPERIMENTAL PROCEDURES

Materials - L-[35S]Methionine (specific radioactivity 80 Ci/mol), [-14C]glucosamine (specific radioactivity 40 Ci/mol), [3H]phenol (specific radioactivity 3.0 Ci/mol), HEPES buffer, Tris/HCl buffer, Hanks' balanced salt solution, MEM, RPMI 1640 medium, Eagle's minimal essential medium, dialysis tubing, Ammonium persulfate, Nonidet P-40, sodium dodecyl Sulfate, 1,2-phenanthroline, amino-derivate of bovine casein, actinomycin D, Neuraminidase, Neuraminidase cocktail, Monoblast-Leukemia Cell, pyruvate kinase, NADH, H2O2, NADP, hydrogen peroxide, NADPH, G-6-PDH, bovine serum albumin, trypsin/EDTA, charcoal-stripped serum, fetal bovine serum, antibiotics, penicillin, streptomycin, cycloheximide, ether, acetic acid, DMSO, sodium azide, disodium EDTA, disodium 1,2-phenanthroline dihydrochloride, 1,1-diphosphoglycerate, pH 7.4, 0.1 M sodium phosphate, 0.05 M Tris/HCl, 0.1 M HCl, 0.1 M NaOH, 0.2 M Tris/HCl, 0.1 M KCl, 0.05 M HCl, 0.05 M NaOH, 0.1 M KCl, 0.02 M HCl, 0.02 M NaOH, 0.01 M KCl, 0.01 M HCl, 0.01 M NaOH, 0.005 M KCl, 0.005 M HCl, 0.005 M NaOH, 0.0025 M KCl, 0.0025 M HCl, 0.0025 M NaOH, 0.00125 M KCl, 0.00125 M HCl, 0.00125 M NaOH, 0.000625 M KCl, 0.000625 M HCl, 0.000625 M NaOH, 0.0003125 M KCl, 0.0003125 M HCl, 0.0003125 M NaOH, 0.00015625 M KCl, 0.00015625 M HCl, 0.00015625 M NaOH, 0.000078125 M KCl, 0.000078125 M HCl, 0.000078125 M NaOH, 0.000039062 M KCl, 0.000039062 M HCl, 0.000039062 M NaOH, 0.000019531 M KCl, 0.000019531 M HCl, 0.000019531 M NaOH, 0.000009765 M KCl, 0.000009765 M HCl, 0.000009765 M NaOH, 0.000004882 M KCl, 0.000004882 M HCl, 0.000004882 M NaOH, 0.000002441 M KCl, 0.000002441 M HCl, 0.000002441 M NaOH, 0.00000122M KCl, 0.00000122 M HCl, 0.00000122 M NaOH, 0.00000061M KCl, 0.00000061 M HCl, 0.00000061 M NaOH, 0.00000030 M KCl, 0.00000030 M HCl, 0.00000030 M NaOH, 0.00000015 M KCl, 0.00000015 M HCl, 0.00000015 M NaOH, and 0.000000075 M KCl, 0.000000075 M HCl, 0.000000075 M NaOH.

Identification of Cathepsin B-related protein bands in Fibroblast extracts

To identify cathepsin B-related polypeptides, cell extracts were prepared from exponentially growing human fibroblasts and incubated with 14C-methionine and amino-derivate of bovine casein. The proteins were then precipitated with 10% trichloroacetic acid (TCA) and solubilised in SDS-PAGE sample buffer. After electrophoresis, the gels were silver stained, autoradiographed, and the relative amount of radioactivity in each band was measured by densitometry. The gel was then stained with Coomassie blue and the bands were compared with the autoradiogram. The gels were then stained with Coomassie blue and the bands were compared to the autoradiogram. The gels were then stained with Coomassie blue and the bands were compared to the autoradiogram.
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**FIG. 2.** Crossed rocket immunoelectrophoresis of cell and medium extracts from non-labeled fibroblasts. Cell and medium extracts were prepared as described in "Experimental procedures". 2.5 mg (cellia) and 1.0 mg (medium) of protein were subjected to SDS-gel electrophoresis. The acrylamide concentration of the separation gel was 4%. Appropriate lanes were cut out and transferred as described in "Experimental procedures". (W), indicates the position of the rocket obtained from culture medium.

**FIG. 3.** Synthesis and processing of cathepsin B. Fibroblasts were labeled with [35S]methionine for 10 min (150 uCi/ml) and 4 h (30 uCi/ml), respectively, and chased for the times indicated. Cathepsin B was immune precipitated from cell and medium extracts and subjected to SDS-polyacrylamide gel electrophoresis. After the chase times indicated, the acrylamide concentration of the separation gel was 12.5%. In addition to phosphorylase b, the same Mr standards were used as in Fig. 1. Cathepsin B polypeptides are indicated on the left margin. Additional bands seen in some cell extracts were not observed in other experiments and should therefore represent impurities of the immune precipitate.

**FIG. 4.** Inhibition of processing by leupeptin. Fibroblasts were preincubated for 24 h in the absence or presence of 50 uM leupeptin/ml and labeled under the same conditions for 1 h with 30 uCi [35S]methionine/ml. After the chase times indicated extracts were further treated as given in the legend of Fig. 3. Cathepsin B polypeptides are indicated on the left margin.

**FIG. 5.** Synthesis of cathepsin B in the presence of tunicamycin. Fibroblasts were preincubated for 16 h in the presence of tunicamycin (10 M) or dimethyl sulfoxide (20 M), the solvent of the drug. Medium was then replaced by normal medium (20 M). The experiment was continued for 3 h in the presence of the same tunicamycin concentrations. Cathepsin B was immune precipitated from cell (upper panel) and medium (lower panel) extracts prior to SDS-gel electrophoresis. The cathepsin B forms are indicated on the left margin.

**FIG. 6.** Phosphorylated oligosaccharides of cathepsin B. Fibroblasts were double-labeled with [3H]leucine (40 uCi/ml) and [32P]orthophosphate (80 uCi/ml) (a lane) or with [3H]leucine (40 uCi/ml) and [35S]methionine (150 uCi/ml) (b lane) and then chased for 6 h. Cathepsin B was immune precipitated from cell and medium extracts, subjected directly (a) or after treatment with peptidase-M-glycosidase F (b) or buffer alone (c) to SDS-gel electrophoresis. For Mr standards see Fig. 1. Cathepsin B forms are indicated on the left margin.

**FIG. 7.** Synthesis and secretion of cathepsin B in normal and 1-cell fibroblasts. 1-Cell and normal fibroblasts were labeled for 1 h with 30 uCi [35S]methionine/ml and then chased for 5 h (lanes a and b) and 24 h (lanes c and d). In (c), 10 M mannose was present in the culture medium during the chase period. Cathepsin B was immune precipitated from cell and medium extracts prior to SDS-gel electrophoresis. For Mr standards see Fig. 1. Cathepsin B forms are indicated on the left margin.

**FIG. 8.** Phosphorylation and secretion of cathepsin B in normal and 1-cell fibroblasts. Fibroblasts were double-labeled with 45 uCi each of [3H]leucine and [32P]orthophosphate for 1 h (lanes a and b) and then chased for 20 h (lanes a and c). In (c), 10 M mannose was present in the culture medium during the experiment. Cathepsin B was immune precipitated from medium extracts prior to SDS-gel electrophoresis. [3H] and [32P] were detected from the same gel. For Mr standards see Fig. 1.

**TABLE I**

**Influence of leupeptin treatment of fibroblasts on cathepsin B activity**

| Treatment          | Activity (nM/mg of protein) |
|--------------------|-----------------------------|
| Control            | 160                         |
| Leupeptin 300       | 254                         |
| Leupeptin 3000      | 232                         |

Confluent cultures were grown for 48 h in the absence or presence of 50 uM leupeptin and then harvested for enzyme activity determinations as described in the Experimental section. 0.5 is 1 n mole of substrate trans:aminized.