Expression and maturation of human cathepsin D in baby-hamster kidney cells

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In medium and in homogenates from baby-hamster kidney cells (BHK) transfected with human cathepsin D cDNA, an elevated activity of cathepsin D was found as compared to non-transfected cells. The elevated activity was removed by titrating the homogenates with an anti-(human cathepsin D) antibody. Metabolic labelling and immunoprecipitation revealed that, in the transfected cells, human cathepsin D was synthesized as a 53-kDa precursor indistinguishable from that found in human cells. A portion of the precursor was secreted and the remainder was processed to intermediate and mature chains within a few hours of synthesis. The precursor that was released from the transfected cells had a slightly smaller apparent size than that from cultured human fibroblasts. This difference was abrogated when the precursors were treated with glycopeptidase F. In the intracellular small chain a difference was observed in the size of carbohydrate chains that were cleavable with endo- β -*N*-acetylglucosaminidase H. Sequence analysis of the *N*-termini of mature intracellular cathepsin D indicated a *N*-terminal trimming in both large and small chains from both human and transfected hamster cells. The proteolytic maturation of human cathepsin D in BHK cells closely resembles that in human cells, whereas a portion of the carbohydrate side chains is processed differently. The trimming of the *N*-termini in mature cathepsin D is proposed to be a part of the maturation and aging of this protein.

INTRODUCTION

Human cathepsin D is a lysosomal pepstatin-sensitive aspartic proteinase consisting of two polypeptides [1]. It is synthesized as a high-molecular-mass (53 kDa) precursor that is subject to maturation upon segregation from the secretory pathway [2]. In a cell-free system using porcine cathepsin D, cDNA synthesis of a preprocathepsin D with an *N*-terminal signal sequence has been demonstrated [3]. The amino acid sequence of human preprocathepsin D has been deduced from the nucleotide sequence of the corresponding cDNA [4–6]. Its sequence is similar to that of porcine procathepsin D and pepsinogen [7].

In cells, signal sequences initiate translocation of nascent proteins into the lumen of the endoplasmic reticulum. During the translocation of preprocathepsin D the signal sequence is removed and procathepsin D is formed [3]. In procathepsin D, carbohydrate side chains become phosphorylated. This modification facilitates the segregation of procathepsin D into a prelysosomal compartment [8,9]. The efficiency of this transport varies with the cell type [10]. During its subsequent maturation, procathepsin D is subject to several endo- and exo-proteolytic cleavages [11,12]. Firstly, the pro-sequence from the *N*-terminus is removed and secondly, a two-chain mature enzyme with the larger fragment originating from the *C*-terminus is formed [11]. In human cells these two stages in the maturation of cathepsin D have been demonstrated by pulse-chase labelling experiments [2,13,14]. A more precise structural analysis, including the determination of terminal sequences, has been performed on porcine and bovine cathepsin D [7]. Like human cathepsin D, porcine and bovine cathepsin D is fragmented into two chains. Tang and co-workers [11] have pointed out that in the precursors of human, porcine and bovine cathepsin D, the two chains are connected with a proteinase-prone peptide loop, which is missing in the precursors of single-chain cathepsin D and in pepsinogen.

The identity of the proteinases involved in the fragmentation and trimming of the polypeptide chains of cathepsin D is not known.

Currently it is assumed that the segregation of procathepsin D into the prelysosomal compartment is followed by a self-catalysed intramolecular cleavage, which reduces the apparent molecular mass of the precursor by 1–2 kDa [15,16] and/or cleavage of the prosequence, which removes fragment(s) of approx. 3.5 kDa. This cleavage results in the formation of a 48-kDa processing intermediate and is insensitive to several known proteinase inhibitors [14]. The subsequent fragmentation of the single-chain intermediate is inhibited by leupeptin [14]. To study the segregation and the maturation of human cathepsin D we decided to prepare heterologous cells expressing the human enzyme at a high rate. Here we report on the synthesis of human cathepsin D in transfected BHK cells to show that an apparently normal, enzymically active, two-chain human cathepsin D is formed. The *N*-termini of the two chains of human cathepsin D are heterogeneous indicating that fragmentation of procathepsin D is followed by a slow *N*-terminal trimming of the chains.

EXPERIMENTAL

Cells and cell culture

Baby hamster kidney (BHK) cells (BHK-21, A.T.C.C. CCL10) and human skin fibroblasts were maintained at 37 °C in a mixture of air and CO₂ (19:1) in Dulbecco's modified Eagle medium (Gibco BRL, Eggenstein, Germany) supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), 4.5 mM-NaHCO₃, 10 mM-Hepes/HCl (pH 7.2) and 10% (v/v) foetal-bovine serum (Boehringer Mannheim). The fibroblasts were obtained from Dr. H. Kresse from the Institute of Physiological Chemistry and Pathobiochemistry, University of Münster, Münster, Germany. U937 human promonocytes [17] were grown

Abbreviation used: BHK, baby-hamster kidney.

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in RPMI 1640 (Gibco BRL) medium with 10% (v/v) heat-inactivated foetal-bovine serum and treated with 100 nM-1,25-dihydroxyvitamin D₃ as described previously [18].

Cathepsin D cDNA

Cathepsin D cDNA was isolated from a commercial human placenta cDNA λ gt11 library (Clontech, Palo Alto, CA, U.S.A.). Screening and preparation work were performed according to Maniatis *et al.* [19]. The library was screened with an oligodeoxyribonucleotide corresponding to nucleotide residues 1216–1251 in the sequence of human cathepsin D cDNA of the clone pHKCD45 that had been described by Faust *et al.* [4]; 32 positive clones were found. They were probed with an oligonucleotide representing nucleotide residues 52–69 in cathepsin D cDNA. One clone, CD-1, which contained the entire coding region of human cathepsin D cDNA and corresponded to nucleotide residues 46–2036 of the clone pHKCD45 [4], was selected for further work. In the terminal regions examined, the sequence of clone CD-1 was indistinguishable from that of clone pHKCD45 (nucleotides 46–271 and 1870–2036 in [4]).

Transfection

The insert of clone CD-1 was ligated into the endonuclease-*Eco*RI site downstream of the simian-virus-40 early promoter in the expression vector pBEH [20]. A clone with the proper orientation of the insert was isolated and named pBEH-CD. Stably transformed BHK cells were obtained by transfection using the calcium phosphate co-precipitation method of Wigler *et al.* [21]. Cells grown in 25 cm² tissue-culture flasks were incubated with 0.5 ml suspension of a DNA precipitate in 0.14 M NaCl/0.75 mM-Na₂HPO₄/25 mM-Hepes/HCl, pH 7.1. The precipitate contained 5 μ g of DNA each from pBEH-CD and from mouse L-cells, and 0.5 μ g of DNA from plasmid pSV2-*pac*. This plasmid conferred on the transfected cells resistance to puromycin [22].

Determination of cathepsin D activity

Cells were grown to confluency in 34 mm-diameter Petri dishes, washed twice with phosphate-buffered saline and removed by scraping twice with 0.3 ml of 10 mM sodium phosphate, pH 7.0. The mixture was subjected to freeze-thawing and sonication three times. Aliquots were incubated with 0.1 mg of [¹⁴C]carbamoyl-haemoglobin in 0.1 M sodium acetate, pH 3.65, in a total volume of 0.2 ml in the presence or absence of 2 μ M-pepstatin A for 2 h at 37 °C. The reaction was terminated by adding 0.5 ml of 1% (w/v) casein and 0.5 ml of 25% (w/v) trichloroacetic acid. After 30 min on ice, the samples were centrifuged for 6 min at 12000 *g* and radioactivity was determined in 1 ml aliquots of the supernatant. Carbamoyl-haemoglobin was prepared as described previously [15] and diluted with non-radioactive haemoglobin to provide 25000 c.p.m. per assay. The concentration of protein in the homogenates was determined by the method of Lowry *et al.* [23].

Immunotitration of cathepsin D activity

BHK cells grown to confluency or calcitriol-treated U937 cells [18] were washed three times with 0.14 M-NaCl/10 mM-sodium phosphate, pH 7.4, and taken up in 10 mM-Tris/HCl, pH 7.2. The samples were frozen and thawed three times and sonicated. Aliquots (40 μ l) were incubated for 16 h at 4 °C with 10 μ l of a polyclonal rabbit anti-(human cathepsin D) antiserum that was diluted in 0.14 M-NaCl/10 mM-Tris/HCl, pH 7.4. The mixtures were centrifuged for 30 min at 40000 *g* and the activity of cathepsin D was determined in two 20 μ l aliquots of the supernatant.

Metabolic labelling and immunoprecipitation

Confluent cultures in 34 mm Petri dishes were washed twice with a methionine- or leucine-free Waymouth's medium [24], incubated for 1 h in this medium and then labelled by incubation for a specific period with 0.8 ml of medium. This labelling medium was supplemented with 4% (v/v) dialysed foetal-calf serum and 1.6 MBq of [³⁵S]methionine (specific radioactivity 44 TBq/mmol) or 1.6 MBq of L-[4,5-³H]leucine (specific radioactivity 5 TBq/mmol). Both radioactive amino acids were purchased from Amersham-Buchler, Braunschweig, Germany. After labelling, the medium was removed and 0.2 ml of fivefold concentrated lysis buffer A containing detergents, proteinase inhibitors and DNAase I (20 μ g/ml) was added [25]; the cells were washed twice with 0.14 M-NaCl/10 mM-sodium phosphate, pH 7.0, and harvested by scraping twice with 0.5 ml of lysis buffer A. After freezing and thawing, extracts were prepared by centrifugation for 1 h at 40000 *g*. For immuno-precipitation the extracts were incubated with affinity-purified rabbit anti-(human cathepsin D) antibody [13] and a covalent conjugate (0.3 mg/ μ g of antibody) of affinity-purified goat anti-rabbit immunoglobulin with Eupergit C1Z (Röhm Pharma, Weiterstadt, Germany). The conjugate with Eupergit C1Z was prepared with 3 μ g of anti-antibody/mg of beads as described previously [26]. Cathepsin D was immuno-precipitated from one-quarter aliquots of the extracts from the cultures of BHK cells in 34 mm Petri dishes using 2 μ g of affinity-purified antibody. The immunoprecipitates were washed and then solubilized by heating in the presence of SDS and dithiothreitol as described previously [25]. Digestion with endo- β -N-acetylglucosaminidase H was performed under the conditions previously described [25].

Gel electrophoresis and fluorography

After immunoprecipitation, the radioactive polypeptides were separated by PAGE in the presence of SDS using the method of Laemmli [27] as described by Hasilik & Neufeld [2]. The radioactivity in the gels was detected by fluorography [28].

N-Terminal sequence analysis

Human cathepsin D was isolated by immunoprecipitation from extracts of calcitriol-treated human promonocytes U937 [18] and of transfected BHK cells. Small and large polypeptide chains were separated from each other and from immunoglobulins by denaturation in the presence of SDS and subsequent PAGE [27]. The polypeptides were transferred on to a poly(vinylidene difluoride) membrane (Millipore, Eschborn, Germany) and stained with Coomassie Blue R-250. The stained bands corresponding to cathepsin D polypeptides were excised and the adsorbed protein was subjected to ten cycles of micro-sequencing in model-477A sequencer with on-line model 120 analyser (Applied Biosystems, Weiterstadt, Germany). The results of these analyses were unusual in that the signals of the individual amino acids gradually increased and distributed through at least four consecutive cycles. This indicated the presence of a staggered series of peptides with a trimmed N-terminus. The signals, expressed in pmol, were plotted against the cycle number and corrected for background, which was estimated graphically. Data from four adjacent cycles contributing the largest signals were summed, and the relative distribution of the signal was calculated as a measure of the proportion of the differently trimmed N-termini in the sample. The lag correction was omitted. The average distribution of the N-termini was calculated from the distribution of the signals of three amino acids in each analysis. The large polypeptides from each source were sequenced twice. The results of the two analyses were similar.

Fig. 1. Titration of cathepsin D activity in homogenates from BHK, transfected BHK and from U937 cells with anti-(human cathepsin D) antiserum

(a) Homogenates were prepared from confluent cultures of control BHK (▲) and transfected BHK-CD (●) cells, and the titration was performed as described in the Experimental section. The activity in the homogenate from BHK-CD cells that was incubated without the antiserum was set 100%. (b) Homogenates were prepared from the transfected BHK-CD cells and from calcitriol-treated U937 cells. The protein concentration of the homogenates was 1.5 mg/ml and 1.9 mg/ml respectively. The details of the titration are described in the Experimental section.

Fig. 2. Specificity of the precipitation of human cathepsin D from transfected BHK-CD cells

Control BHK (Co) and transfected BHK-CD (T) cells were metabolically labelled for 16 h with [35 S]methionine and cathepsin D was precipitated from extracts of cells and medium (Med.). The labelled polypeptides were separated by SDS/PAGE. The positions of precursor (P), intermediate (I) and large mature chain (LM) of cathepsin D and of standards phosphorylase *b* (97.4 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and cytochrome *c* (12.3 kDa) that have been separated in the same gel are indicated.

RESULTS

Expression of human cathepsin D antigen and activity in transfected BHK cells

Crude homogenates were prepared from control and transfected BHK cells and from U937 human promonocytes. In all three homogenates, cathepsin D activity as determined with [14 C]carbamoyl-haemoglobin was > 98% inhibited in the presence of 1 μ M-pepstatin A. In the homogenate from the transfected

BHK cells, the activity was several times higher than in control BHK cells (Fig. 1a). In that from transfected cells, a large proportion of cathepsin D activity was sensitive to anti-(human cathepsin D) antibody (Fig. 1a). The activity remaining soluble after a titration with a surplus of the antibody was similar to that in non-transfected cells. In the latter, cathepsin D was not sensitive to the antibody.

In extracts from metabolically labelled transfected cells, the antibody precipitated three major radioactive polypeptides of 53, 48 and 33 kDa (Fig. 2) that are known components of cathepsin D produced in cultured human cells. Such polypeptides were not precipitated from extracts of control BHK cells.

The relationship between the reactivity with the antibody and enzyme activity of human cathepsin D expressed in the transfected BHK cells and in human cells was examined in a titration experiment. Aliquots of homogenates from the transfected cells and from U937 cells containing similar activity of the enzyme were titrated with increasing amounts of anti-(human cathepsin D) antiserum (Fig. 1b). The parallel course of the two titration curves indicated a great similarity between the two cathepsin D activities.

Maturation of human cathepsin D in BHK cells

The initial experiment on the metabolic labelling of human cathepsin D in transfected BHK cells indicated formation of similar polypeptides as in human cells. In a mixing experiment, human cathepsin D was immuno-precipitated from the transfected BHK cells and from cultured fibroblasts. Aliquots of each sample and their mixture were subjected to PAGE (Fig. 3). While the intracellular forms of cathepsin D could not be distinguished, the enzyme secreted by the transfected cells migrated faster than that from human cells. This difference was lost upon removal of carbohydrate side chains with glycopeptidase F (not shown).

The maturation of human cathepsin D in transfected BHK cells resulted in the conversion of the precursor first into an intermediate of approximately 48 kDa (Fig. 4). Within 4 h a considerable amount of the intermediate was converted into the mature polypeptide. This conversion proceeded somewhat more slowly than that found in an earlier study of human cells [2].

The formation of the large mature subunit (33 kDa) was accompanied by that of a smaller fragment (14 kDa). This fragment was not well visualized in the cells labelled with [35 S]methionine; therefore we documented its presence in cathepsin D in the transfected cells by immunoprecipitation

Fig. 3. Comparison of cathepsin D polypeptides precipitated with anti-(human cathepsin D) antibody from transfected BHK cells and from human fibroblasts

Confluent cultures of BHK cells transfected with human cathepsin D cDNA and of human fibroblasts were metabolically labelled with [35 S]methionine. Extracts from cells and medium were prepared. Aliquots of the extracts corresponding to one-eighth of the transfected BHK-CD cell culture and one-half of the fibroblast culture were subjected to precipitation with anti-(human cathepsin D) antibody. The immunoprecipitates were solubilized and two-thirds aliquots from the samples from transfected BHK cells (T) and fibroblasts (F) and a mixture of one-third aliquots from both (T + F) were analysed by SDS-PAGE and fluorography. The positions of the precursor secreted from fibroblasts (P/F) and from the transfected cells (P/T), of the intermediate (I) and large mature (LM) polypeptides were marked on the margin. The small mature polypeptide of cathepsin D was not identified because of a weak incorporation of the radioactive amino acid.

Fig. 4. Maturation and secretion of human cathepsin D in transfected BHK cells

Confluent cultures of BHK-CD cells were subjected to 1 h pulse-labelling with [35 S]methionine and up to 16 h chase as indicated. Human cathepsin D was immunoprecipitated from extracts of cells and medium and the labelled polypeptides were separated by PAGE. The positions of cathepsin D polypeptides and of standards are marked as in Fig. 2.

Fig. 5. Comparison of large and small fragments of human cathepsin D in U937 and in transfected BHK cells

U937 cells and a confluent culture of BHK cells that were transfected with human cathepsin D cDNA were labelled for 20 h with [3 H]leucine. Cell extracts were prepared and human cathepsin D was isolated by immuno-precipitations. Aliquots of the immuno-precipitates were incubated with or without endo- β -N-acetylglucosaminidase H (Endo H) as indicated. The positions of the main sensitive species among the fragments identifiable as the small subunits and their partially deglycosylated forms are indicated by arrowheads and those of the resistant species by open circles (○). The relationship to cathepsin D of the glycopeptides whose positions are labelled with the closed circles (●) is not clear. Two exposures of the same film, 930 h and 270 h, showing the partial resistance of the larger mature (LM) cathepsin D subunit towards endo- β -N-acetylglucosaminidase are presented. From the comparison of bands with a similar intensity in the shorter exposure of cathepsin D from U937 and in the longer exposure of cathepsin D from BHK-CD cells it followed that the latter contained fewer, if any, resistant side chains. Other abbreviations: I, intermediate; P, precursor; LMd, deglycosylated large mature subunit; SMd, deglycosylated small mature subunit.

from cells that had been metabolically labelled with [3 H]leucine (Fig. 5). In these immuno-precipitates, the majority of cathepsin D could be deglycosylated with endo- β -N-acetylglucosaminidase H, indicating the presence of predominantly high-mannose or hybrid oligosaccharides in both chains of cathepsin D (Fig. 5). The large chain from the transfected BHK cells contained only cleavable oligosaccharides. In this and other experiments we observed that the large chain from U937 cells contained a small amount of non-cleavable oligosaccharides. The small chain of cathepsin D from U937 cells contained both endo- β -N-acetylglucosaminidase H-sensitive and -resistant oligosaccharide side chains. A similar observation has been made previously on the small chain from human fibroblasts [29]. In the small chain from the transfected BHK cells the cleavable oligosaccharides were more preponderant and had apparently different size from those in cathepsin D from U937 cells (Fig. 5). Upon deglycosylation the difference in the apparent size of the small chain was eliminated. These results indicated differences in the carbohydrate moieties in both subunits as expressed in different cells.

N-Terminal sequences in mature cathepsin D

By analogy to porcine cathepsin D it has been proposed that the large polypeptide of the human enzyme commences with Leu-105 of the pro-sequence [4,11]. In the first cycle of the sequencing of the large polypeptide of cathepsin D from either

Table 1. Distribution of selected amino acid signals in adjacent sequencing cycles (pmol) corresponding to various *N*-termini of the large chain of cathepsin D from transfected BHK-CD cells and from human promonocytes U937

The *N*-termini were tentatively assigned to Ala¹⁰⁴–Gly¹⁰⁷. The numbering of the residues is that of Faust *et al.* [4]. The criteria for selecting the amino acids are explained in the Experimental section.

<i>N</i> -Terminus assigned to residue	Amount of amino acid in the allotted cycle (pmol)					
	Lys ¹⁰⁹		Glu ¹¹¹		Gln ¹¹³	
	BHK-CD	U937	BHK-CD	U937	BHK-CD	U937
Ala ¹⁰⁴	8.3	19	10.6	20	3.7	14.6
Leu ¹⁰⁵	16.1	78	19.2	54	26	57
Gly ¹⁰⁶	12.5	41	14.7	31	7.3	30
Gly ¹⁰⁷	2.3	10.2	6.7	16	3.3	19.6

U937 or transfected BHK cells, the strongest signals were those of leucine, glycine and alanine. The last two residues were either contaminants or originated from alternatively processed large chains with a longer *N*-terminus. This latter possibility was supported by the sequencing data from the ten cycles determined. The signals for each amino acid did not increase abruptly. Instead, they increased and decreased gradually through several cycles of sequencing. According to criteria which are explained in the Experimental section, we examined the distribution of lysine, glutamic acid and glutamine through the determined sequence (Table 1). The distribution was similar for all these residues. Apparently, it represented that of four species of the large cathepsin D polypeptide present in the enzyme which was immuno-precipitated from either cell type. The longest detectable species in these preparations started with Ala-104 of the preprocathepsin D sequence [4]; the other three sequences significantly contributing to the signals started with Leu-105, Gly-106 and Gly-107. From the distribution of the signals of the three above-mentioned amino acids through the sequencing cycles (Table 1) we calculated the average approximate proportion of the four *N*-termini in the large polypeptide. In cathepsin D from transfected BHK cells the sequences commencing with Ala-104–Gly-107 were represented in a ratio of approximately 1:2.7:1.5:0.5. In cathepsin D from U937 cells the ratio of the four termini was approximately 1:3.5:1.9:0.7. Although similar to each other, these ratios represent only approximations, because they are based on the minute amounts of cathepsin D that could be isolated from cultured cells.

In preparations of the small polypeptide from cathepsin D from either transfected BHK or U937 cells, four *N*-termini were also detected. The longest of them corresponded to Val⁻³ in the deduced sequence of preprocathepsin D. The sequences started with Val⁻³, Thr⁻², Glu⁻¹ and Gly⁺¹ and these species occurred in a ratio of approximately 1:2:3.3:1.6 in the small chain from the transfected BHK cells and in a ratio of approximately 1:2.2:3.2:1.7 in the small chain from U937 cells. It is unlikely that the occurrence of signals in several consecutive cycles was due to a lag in the Edman degradation, because it was reproduced in another analysis and the broadness of the distribution of the selected signals through consecutive cycles was similar between amino acids that appeared early and late throughout the sequencing (Tables 1 and 2). Therefore it was concluded that the *N*-termini in both chains of human cathepsin D in both autologous and heterologous cells were heterogeneous and started with several consecutive residues as compared with the sequence of the precursor.

Table 2. Distribution of selected amino acid signals in adjacent sequencing cycles corresponding to various *N*-termini of the small chain of cathepsin D from transfected BHK-CD cells and from human promonocytes U937

The *N*-termini were tentatively assigned to Val⁻³–Gly⁺¹. The numbering of the residues is that of Faust *et al.* [4]. The criteria for selecting the amino acids are explained in the Experimental section.

<i>N</i> -Terminus assigned to residue	Amino acid amount in the allotted cycle (pmol)					
	Ile ⁺³		Val ⁺⁶		Leu ⁺⁷	
	BHK-CD	U937	BHK-CD	U937	BHK-CD	U937
Val ⁻³	2.6	5.4	2.6	7.6	2.4	6.0
Thr ⁻²	5.5	15.7	4.3	12.4	5.7	14.2
Glu ⁻¹	10.4	25	7.0	16.3	7.9	19.6
Gly ⁺¹	5.6	16.7	3.1	7.0	3.9	9.6

We cannot exclude the possibility that the heterogeneity was created during the isolation of the polypeptides. However, using immuno-isolation in the presence of several proteinase inhibitors we could rapidly isolate the protein and minimize its degradation. Indeed, it should be noted that the *N*-termini of the small chain described in our work extend further upstream than predicted [4].

With the small amount of cathepsin D isolated from cultured cells we could not examine the *C*-termini in cathepsin D subunits. However, from the comparison of the size of the partially deglycosylated subunits from BHK and u937 cells (Fig. 5) and from the similarity in the *N*-termini, it follows that the *C*-termini may also be alike.

DISCUSSION

We report on the isolation of human cathepsin D cDNA and its expression in transfected BHK cells. Our data extend previous reports on the formation of a partially processed human cathepsin D in *Xenopus laevis* oocytes micro-injected with human cathepsin D mRNA [30] and of a two-chain human cathepsin D in various heterologous cells transfected with human cathepsin D cDNA [31]. We have shown that the enzyme expressed in BHK cells is enzymically active and that its enzyme activity and antibody reactivity are similar to those of cathepsin D produced in human cells.

We have also confirmed the report that the polypeptide pattern of human cathepsin D is similar in human and in transfected heterologous cells [31]. Further, we have shown that the polypeptide backbones of the two subunits of mature cathepsin D obtained by partial deglycosylation are of a very similar size in the two cell types. We have found a microheterogeneity in cathepsin D from both cell types in both subunits at the *N*-termini.

From the analysis of the mature enzyme [1] and from the biosynthetic pattern observed in pulse-chase experiments [2], human cathepsin D has been concluded to comprise of a precursor of approximately 53 kDa, an intermediate of 48 kDa and two mature polypeptides of approximately 33 kDa and 14 kDa. The amino acid sequence of human cathepsin D precursor has been deduced from the nucleotide sequence of the corresponding cDNA and the cleavage pattern has been likened, by analogy, to that of porcine cathepsin D [7].

The *N*-terminus of the small chain of cathepsin D is created by removal of up to 44 residues from the precursor (proenzyme). The precursor itself is also a product of proteolysis. The signal

sequence of human preprocathepsin D is likely to be cleaved in the endoplasmic reticulum at the C-terminal side of Ala⁻⁴⁵ [4]. The next 44 residues are cleaved probably in several steps. Like pepsinogen, procathepsin D is capable of autoactivation through a cleavage of a smaller N-terminal fragment of 1–2 kDa from the 53 kDa precursor [15]. In cultured cells the immediate product of such autoactivation has not been demonstrated. In pulse-chase experiments, a processing intermediate of approx. 48 kDa has been observed, instead, which migrated as a broad band in PAGE. These observations have suggested that the pro-segment is removed in a series of endo- and amino-peptidase reactions. The participation of an aminopeptidase activity in the processing has been suggested earlier [7] and is compatible with our data, which demonstrate a heterogeneity in the N-terminal sequence of the small fragment of human cathepsin D. The occurrence of a gradual digestion of the prosegment is further indicated by a shift in the apparent size of the processing intermediate that can be demonstrated in metabolically labelled cells in the presence of a weak base (S. Grässel, unpublished work).

The N-terminus of the small polypeptide originates from the N-terminal region of the processing intermediate [11,12]. This region is well conserved in cathepsin D from several species. Therefore, by analogy with porcine cathepsin D, it has been proposed that the N-terminus of the human small fragment starts with Gly-1 corresponding to the 65th residue of the preproenzyme [4]. Our data show that a significant portion of the small fragments in cathepsin D start up to three residues further upstream from this residue, i.e. at Glu⁻¹, Thr⁻² or Val⁻³. In an independent study, N-terminal microheterogeneity of the small chain has been observed in cathepsin D from human placenta (G. E. Conner, personal communication).

The processing intermediate of human cathepsin D is subject to fragmentation, in which the larger fragment emerges from the C-terminal portion of the intermediate [3]. This fragmentation defines a group of two-chain cathepsins D, which is represented by the bovine, porcine and human enzymes [7]. In these enzymes, between the residues that correspond to Val-91 and Gly-92 of porcine pepsin, several additional residues are localized which are believed to form a hairpin loop that is prone to proteolysis [11]. In porcine cathepsin D this loop has a sequence Pro-Cys-Asn-Ser-Ala-Lys-Ser-Gly-Val- [7] and in the course of the fragmentation the five residues italicized are removed, as determined by the C-terminal and N-terminal analysis of the small and large chains respectively [32]. Similarly, in bovine cathepsin D the C-terminus of the small chain and the N-terminus of the large chain are created by the removal of two serine residues (italicized) from the loop sequence Pro-Cys-Asn-Pro-Ser-Ser-Ser-Ser-Pro [11]. Thus the N-termini of the large fragments in porcine and bovine cathepsin D are of different lengths and begin with Val-Gly-Gly and Ser-Pro-Gly-Gly respectively. In human cathepsin D the connecting loop has the sequence Pro-Cys-Gln-Ser-Ala-Ser-Ser-Ala-Ser-Ala-Leu starting with Pro-95. According to our results the large fragment of the mature human cathepsin D is a mixture of polypeptides starting with Ala-104 (italicized in the preceding sequence), Leu-105, Gly-106 and Gly-107. Thus two or more residues can be removed from the processed loop region in either porcine, bovine or human cathepsin D. Recently, we have sequenced the large chain of cathepsin D that was isolated from human placenta [2]. Similar to cathepsin D from cultured cells, this large chain contained at the N-terminus residues Val-104–Gly-105. The four species were represented in a ratio of 1:5:2.1:0.9.

Previously, among the N-terminal sequences of cathepsin D no microheterogeneity has been described. The absence of such findings may be explained by a difference between cathepsin D preparations from different sources, by a selection for certain

forms of the enzyme during its isolation and by difficulties in interpreting minor sequencing signals. Nevertheless, aminopeptidases have been implicated in the processing of cathepsin D polypeptides [7]. A slow action of the aminopeptidases may be one of several possible causes of the observed microheterogeneity. It appears that at least in the case of human cathepsin D, the processing of its N-termini proceeds slowly, allowing for an accumulation of several polypeptide species with variably trimmed N-termini.

We find sets of similarly trimmed N-termini in cathepsin D in U937 and BHK cells. This may reflect a similarity in the activity of the proteolytic apparatuses in these cell types. We assume that the trimming of the N-termini is physiologically relevant, and propose a testable hypothesis, according to which the N-terminal trimming embodies ageing of lysosomal enzymes. Lysosomal amino- and carboxy-peptidases may gradually trim the termini in various lysosomal enzymes and thus destabilize them. We propose that the aged molecules, such as cathepsin D with more extensively trimmed N-termini, are prone to proteolysis and preferentially catabolised. In cultured fibroblasts the half-life of human cathepsin D has been estimated to be approx. 2 weeks [33]. It should be of interest to see if the aged cathepsin D molecules are enriched in those with more extensively trimmed N-termini.

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