Differential segregation of human and hamster cathepsin D in transfected baby-hamster kidney cells

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The segregation of human cathepsin D, studied in baby-hamster kidney cells (BHK) transfected with human cathepsin D cDNA and compared with that of hamster cathepsin D in the same cells, showed that, in cells that expressed human cathepsin D at a low rate, most of the enzyme remained intracellular. In contrast, when the enzyme was expressed at a high rate, most was secreted. The segregation was examined with an anti-(human cathepsin D) antibody that reacted with the human enzyme exclusively and an anti-(rat cathepsin D) antibody that reacted with both enzymes. In one protocol the cells were metabolically labelled and the two antibodies were used in sequence to precipitate the enzymes from extracts of cells and medium. High expression of the human enzyme did not interfere with the segregation of hamster cathepsin D. In another protocol the activity of cathepsin D in cells and medium was measured before and after titration with anti-(human cathepsin D) antiserum. Human cathepsin D was found predominantly in the medium, and hamster cathepsin D mainly in the cells. In the presence of 10 mM-NH₄Cl the intracellular segregation of hamster cathepsin D was strongly inhibited, while the segregation of human cathepsin D was only slightly diminished. In BHK cells, at least two systems participate in the sorting of the two cathepsins, one of them being rather insensitive to NH₄Cl.

INTRODUCTION

Cathepsin D is a paradigm of a lysosomal enzyme which is synthesized as a higher-*M_r* precursor [1–3]. This is an inactive proenzyme [4–7] that, in prelysosomal and lysosomal organelles, is subject to several proteolytic cleavages [2,3,8–10]. Similar to other soluble lysosomal enzymes, targeting of cathepsin D to lysosomes depends on its phosphorylation [11], on the formation of mannose 6-phosphate residues in its carbohydrate side chains and on the interaction of the phosphorylated oligosaccharides with specific receptors [8,9]. This dependence, however, is not universal. The existence of alternative segregation mechanisms in various vertebrate tissues has been postulated to explain protein targeting to lysosomes in the absence of phosphorylation and in certain other situations (reviewed in [12,13]).

New perspectives for studies on these problems have been opened by the introduction of recombinant genetic techniques. Recently, Faust *et al.* [14] have shown that, in *Xenopus laevis* oocytes, a mannose 6-phosphate system does process and target human cathepsin D to a subcellular organelle, and Conner *et al.* [15] reported that the same protein can be correctly processed and targeted in cell lines from different rodent species. We have obtained the expression of human cathepsin D in cultured hamster cells [16 (the preceding paper)]. Here we examine the efficiency of the segregation of cathepsin D of the two species in the same transfected cells, and report that high expression rates of the human enzyme in hamster cells preclude its efficient targeting, having little if any effect on the segregation of the hamster cathepsin D.

EXPERIMENTAL

Cells and cell culture

Baby-hamster kidney (BHK) cells (BHK-21, A.T.C.C. no. CCL10) were maintained at 37 °C in air/CO₂ (19:1) in Dulbecco

modified Eagle's medium (Gibco BRL, Eggenstein, Germany), which was 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, Mannheim, Germany). Stable transfection with human cathepsin D cDNA [16] and with human myeloperoxidase cDNA [17] has been described previously. After transfection with human cathepsin D cDNA, individual clones with different rates of expression were prepared by limiting dilution.

Metabolic labelling and isolation of cathepsin D

Cells were grown to confluency in 34 mm-diameter Petri dishes, washed twice with a methionine-free Waymouth medium, incubated for 1 h in this medium and metabolically labelled for 16–20 h with 0.8 ml of medium. For this purpose, the methionine-free medium was supplemented with 4% (v/v) dialysed foetal-bovine serum and 1.6 MBq of [³⁵S]methionine, specific radioactivity 40 TBq/mmol. Cell and medium extracts were prepared as described previously [17]. Cathepsin D was immunoprecipitated using affinity-purified rabbit anti-(human cathepsin D) [18] or rabbit anti-(rat cathepsin D) [19] as the first antibody. The immunocomplexes were collected with goat anti-rabbit immunoglobulin–Eupergit C1Z conjugates. These were prepared at a ratio of 3 µg of antibody/mg of Eupergit C1Z [20] and used at a ratio of 0.3 mg/µg of the first antibody. Eupergit C1Z was purchased from Röhm Pharma, Weiterstadt, Germany. After washing, the complexes were solubilized under reducing conditions [17] and analysed by SDS/PAGE [11,21]. The radioactivity in the gels was revealed by fluorography [22] and quantified by densitometry using Ultrosan densitometer (Pharmacia-LKB, Freiburg i.Br., Germany). As standards the following proteins labelled with [¹⁴C]KCNO [16] were used: phosphorylase *b*, 97.4 kDa; BSA, 69 kDa; carbonic anhydrase, 30 kDa; and cytochrome *c*, 12.3 kDa.

Abbreviation used: BHK, baby-hamster kidney

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Assay and titration of cathepsin D activity

The activity of cathepsin D was determined with [14 C]carbamoyl-haemoglobin [4]. Cell homogenates were prepared, and titration of cathepsin D in the homogenates and in the medium was performed as described in the preceding paper [16]. Aliquots of the samples were incubated without, and with increasing amounts of the anti-(human cathepsin D) antiserum. The immunocomplexes were removed by centrifugation and the residual soluble cathepsin D was determined in duplicate. The activity, which was insensitive to an excess of the antiserum, was referred to as the 'residual' activity. Protein concentration was determined by the method of Lowry *et al.* [23], with BSA as the standard.

RESULTS

High rate of secretion of human cathepsin D in transfected BHK cells

Different clones of BHK cells harbouring human cathepsin D cDNA were examined for the synthesis and secretion of human cathepsin D by metabolic labelling and immunoprecipitation of the antigen (Fig. 1). Precursor, intermediate and large mature cathepsin D chains with nominal M_r values [3] of 53 000, 47 000 and 31 000 respectively were detected in the transfected cells. In SDS/PAGE these chains could not be distinguished from those synthesized in cultured human fibroblasts [16].

As judged from the amount of labelling of cathepsin D in the transfected cells, the rate of synthesis of the human protein varied considerably among the clones. The rate of secretion of cathepsin D varied greatly too. From the distribution of cathepsin D-associated radioactivity in cells and medium it can be judged that, in clones A–E shown in Fig. 1, approx. 13, 44, 6, 48 and 33% of cathepsin D were secreted respectively. Thus cells expressing larger amounts of cathepsin D were targeting a lower proportion of the enzyme to lysosomes.

In transfected cells that synthesized cathepsin D at a high rate, cathepsin D was the major secreted protein as estimated from the metabolic labelling (Fig. 2). In the presence of 10 mM-NH₄Cl secretion of human cathepsin D was little affected, though maturation was inhibited (Fig. 2).

Fig. 1. Biosynthesis and secretion of human cathepsin D in transfected BHK cells

In several experiments, different clones of BHK cells (A–E) transfected with human cathepsin D cDNA were metabolically labelled with [35 S]methionine for 20 h and cathepsin D was immunoprecipitated from extracts of cells (C) and medium (M) with anti-(human cathepsin D) antibody. The radioactive polypeptides were separated by SDS/PAGE and revealed by fluorography. The positions of the precursor (hP), intermediate (hI) and large mature chain (hLM) of human cathepsin D and of molecular-mass standards are indicated.

Fig. 2. Cathepsin D is the major secretory protein in transfected BHK cells and its secretion is little changed in the presence of NH₄Cl

Control and transfected (with human cathepsin D cDNA) BHK cells were subjected to labelling with [35 S]methionine for 16 h in the presence or absence of 10 mM NH₄Cl. Human cathepsin D was precipitated from 25% material in each sample. Total protein was precipitated from 2% aliquots of the medium (Total med.) with 4 vol. of acetone from non-transfected (Co) and transfected (Tf) cells. The positions of the precursor (P), intermediate (I) and large mature (LM) chain of human cathepsin D are indicated. The closed arrow points to a protein whose secretion is enhanced in the presence of NH₄Cl. The open arrow marks an unidentified protein which was secreted more strongly in the transfected cells.

Fig. 3. Immunoprecipitation of cathepsin D from control and transfected cells with anti-(rat cathepsin D) antiserum

(a) Cells transfected with human cathepsin D cDNA (CD) and a non-transfected control (O) and (b) cells transfected with human myeloperoxidase cDNA (MPO) were metabolically labelled with [35 S]methionine for 20 h. Cathepsin D was immunoprecipitated with anti-(rat cathepsin D) antiserum, and the radioactive polypeptides were separated by SDS/PAGE and revealed by fluorography. The positions of human (h) and hamster (m) precursor (hP, mP), intermediate (hI, mI) and large mature (hLM, mLM) polypeptides are indicated. The material with $M_r < 30 000$ is probably unrelated to cathepsin D. C, cells; M, medium.

The possibility was considered that an overloading of the segregation system in cells synthesizing a large amount of human cathepsin D could account for its inefficient targeting to lysosomes. Therefore it was of interest to examine the segregation of hamster cathepsin D synthesized in the same cells.

Segregation of metabolically labelled human and hamster cathepsin D

Two anti-(cathepsin D) antibodies with different specificities were available to us. As shown above, anti-(human cathepsin D) reacted with human cathepsin D and did not precipitate hamster cathepsin D (see also [16]). In contrast, anti-(rat cathepsin D) reacted with both human and hamster cathepsin D. In the experiment shown in Fig. 3, the precipitation was performed with the latter antibody and extracts from metabolically labelled control BHK cells and BHK cells that were transfected with cDNA coding for cathepsin D or for an unrelated protein. Two rather broad bands corresponding to approx. 46 and 30 kDa were found in all three cell types and were considered to represent hamster cathepsin D. In cells that were transfected with human cathepsin D cDNA both hamster and human cathepsin D were found (Fig. 3; compare with Fig. 1). A polypeptide of approx. 52 kDa was found in both the transfected and the non-transfected cells and in the medium. In a separate pulse-chase experiment (not shown) this polypeptide was identified as the precursor and the 46 kDa polypeptide as an intermediate form of hamster cathepsin D. The latter was slowly converted into the 30-kDa form. On SDS/PAGE the precursor migrated slightly ahead of its human counterpart. After the processing, the polypeptides of hamster cathepsin D migrated decidedly faster than those of human cathepsin D.

The 46 kDa polypeptide may be referred to as the single-chain hamster cathepsin D [9]. The relative labelling intensity of the intermediate as compared with the large mature chain indicated that maturation proceeded faster in human than in hamster cathepsin D. Probably owing to a weak labelling with

[³⁵S]methionine, the small mature chains of either hamster or human cathepsin D could not be detected.

Because of the similarity in their size, we attempted to separate the two cathepsins by stepwise precipitation with the two antibodies. In the first step, aliquots of extracts from cells and the medium of metabolically labelled transfected BHK cells were titrated with either anti-(human cathepsin D) or anti-(rat cathepsin D) antibody. The immunoprecipitates were collected by centrifugation and the supernatants were incubated with anti-(rat cathepsin D) antibody to obtain a second precipitate. Most of the human cathepsin D in the cell extract could be precipitated with the anti-(human cathepsin D) antibody (compare in Fig. 4, lanes 1–3 with 5–7). The precipitation of human cathepsin D with anti-(rat cathepsin D) antibody was incomplete and continued through the second step (Fig. 4, lanes 4 and 8).

With anti-(human cathepsin D) antibody the human proenzyme was less efficiently recovered from the medium (Fig. 4, lanes 9–11) than from the cells (lanes 1–3). Nevertheless, after the titration with this antibody most of the human proenzyme was removed from the sample, and the second precipitate contained predominantly the precursor of hamster cathepsin D, as indicated by the shift in the apparent electrophoretic mobility of the precipitated and solubilized protein (Fig. 4, lanes 13–15). The two precursors were not distinguished when the repeated precipitation was performed with the anti-(rat cathepsin D) antibody (Fig. 4, lanes 12 and 16).

A comparison of human cathepsin D in cells and medium revealed that a major fraction of this protein was secreted (Fig. 4, lanes 3 and 11). In contrast, most of the hamster cathepsin D was retained in the cells and processed to either the single- or the two-chain form of the enzyme (Fig. 4, lanes 7 and 15).

Segregation of human and hamster cathepsin D activities

Cathepsin D activity was determined in cell homogenates and medium of transfected and non-transfected cells (Table 1). The cultures, at a comparable cell density, were incubated in fresh

Fig. 4. Sequential precipitation of human and hamster cathepsin D from transfected BHK cells

BHK cells transfected with human cathepsin D cDNA were metabolically labelled with [³⁵S]methionine for 20 h. Aliquots, one-eighth each, of the extracts were incubated with 3 μ g, 6 μ g and 12 μ g of anti-(human cathepsin D) (lanes 1–3 and 9–11) and 6 μ g of anti-(rat cathepsin D) antibody (lanes 4 and 12) to obtain the first precipitates (1st). The supernatants from the first precipitation were incubated with 10 μ g of anti-(rat cathepsin D) and 3 mg of goat anti-rabbit immunoglobulin-Eupergit C12 conjugate (lanes 5–8 and 13–16). The immuno-precipitates were analysed by gel electrophoresis and fluorography. The positions of cathepsin D polypeptides are indicated (see Fig. 3 for symbols). In the first precipitate from the transfected cells (lanes 1–3) the small chain of the mature cathepsin D was also detected. The asterisk marks a presumptive contaminant.

Table 1. Activity of cathepsin D in control and transfected BHK cells and medium

The cells were cultured for 1 day in 34-mm Petri dishes with 1 ml of fresh medium in the presence or absence of 10 mM NH_4Cl . Aliquots of cell homogenates and medium were titrated with dilutions of anti-(human cathepsin D) antiserum. The activity was determined in samples that were incubated without ('Total') and with saturating amounts of the antiserum ('Residual'). The difference between the total and the residual activity represented the activity of human cathepsin D; 90% of the residual activity could be precipitated with anti-(rat cathepsin D) antibody. The activity of cathepsin D in cells and in medium was related to cell protein. The data represent means for two experiments.

Additions in the culture medium	Fraction	Cathepsin D activity (c.p.m./h per μg of cell protein)				
		Transfected BHK cells			Control BHK cells	
		Total	Residual	Difference	Total	Residual
None	Cells	40	12.6	28	10.4	12.6
	Medium	31	1.7	29	1.6	1.8
10 mM- NH_4Cl	Cells	32	10.0	22	8.5	8.0
	Medium	36	6.3	30	5.2	5.1

medium for 24 h in the presence and absence of 10 mM- NH_4Cl . In non-transfected BHK cells, most cathepsin D activity was associated with cells. However, in the presence of NH_4Cl , the activity in the medium was increased severalfold. The activity of hamster cathepsin D was not changed if the samples were pre-incubated with diluted anti-(human cathepsin D) antibody. The intracellular activity of cathepsin D was affected much less than the extracellular, because in fibroblasts NH_4Cl enhances the secretion of the newly synthesized lysosomal enzymes only [3,11].

In cultures of transfected cells, high activity of cathepsin D was found in cells and also in the medium. Most of the activity, particularly in the medium, was precipitated with anti-(human cathepsin D) antibody. After 24 h incubation, 12% of the hamster enzyme was found in the medium of control cells and 39% in that of NH_4Cl -treated cells. By comparison, the corresponding values for the precipitated human enzyme were 49 and 58%. Thus, the segregation of human cathepsin D was rather insensitive to NH_4Cl . This finding is consistent with the results showing the distribution of radioactively labelled cathepsin D (Fig. 2).

Most of the residual activity that was not precipitated with anti-(human cathepsin D) antibody was sensitive to anti-(rat cathepsin D) antibody (results not shown). Its distribution between cells and medium resembled that in the non-transfected cells. The determination of the activity of hamster cathepsin D in samples in which human cathepsin D was removed indicated that the rate of secretion of the autologous cathepsin D was much lower than that of the human enzyme. In transfected cells, the secretion of hamster cathepsin D was as stimulated by NH_4Cl as in non-transfected cells. Thus, in transfected cells, which synthesize a large amount of human cathepsin D and are unable to target most of it to lysosomes, the acidification-dependent targeting of hamster cathepsin D is performed with a high efficiency.

DISCUSSION

The results obtained with different clones of BHK cells expressing human cathepsin D show that the efficiency of the lysosomal targeting of the heterologous protein is low when the rate of its synthesis is high. While this work was in progress, it was reported that, in various cells of rodent origin expressing human cathepsin D [15], and in monkey COS (simian-virus-transformed african-green-monkey kidney) cells expressing rat β -glucuronidase [24], the efficiency of the lysosomal targeting of the heterologous proteins was rather low. In contrast, a high

expression rate of human arylsulphatase A in monkey COS and in BHK cells did not preclude an efficient lysosomal targeting of this enzyme [25]. The targeting of human β -glucuronidase in monkey COS cells seems to proceed with an intermediary efficiency [26]. Thus the efficiency of the targeting appears to vary depending on the enzyme expressed, on the rate of its synthesis and on the properties of the host cells. Our contribution is probably the first detailed report on the segregation of the autologous and heterologous enzymes after synthesis in the same cells.

The mannose 6-phosphate-dependent pathway, and possibly other types of the segregation of lysosomal enzymes, rely on two recognition events at least. One of them is required for the phosphorylation, and another for the binding of the phosphorylated enzymes to receptors. An inefficient segregation may be the consequence of a weak interaction with, and oversaturation of, or a low concentration of, any of the components participating in the process. For example, we may refer to the reports by Mueller *et al.* [27] on a defective *N*-acetylglucosaminyl 1-phosphotransferase which does not recognize lysosomal enzymes, and by Kyle *et al.* [28] on an enhancement of the segregation efficiency of at least two lysosomal enzymes upon expression of the human mannose 6-phosphate/insulin-like growth factor II receptor in receptor-deficient mouse cells. Our results on the sorting of human and hamster cathepsin D in transfected BHK cells show that, at least at one stage of the process, different systems recognizing different signals are involved. One of the systems preferentially sorts hamster cathepsin D and may have a low affinity for human cathepsin D, so that the latter does not interfere with the sorting of hamster cathepsin D. This system is sensitive to NH_4Cl . Another system seems to participate in a low-efficiency sorting of human cathepsin D which is rather insensitive to NH_4Cl .

Recently, it has been shown that mouse cathepsin L, which contains mannose 6-phosphate residues and is excessively secreted in transformed mouse fibroblasts, has a low intrinsic affinity for the mannose 6-phosphate/insulin-like growth factor II receptor [29]. In the transformed cells, many other lysosomal enzymes are retained. Thus it appears that cells possess a means to target a major portion of a particular enzyme to lysosomes if it is synthesized slowly and, if synthesized at a high rate, to secrete it into the medium without concomitantly compromising the targeting of other lysosomal enzymes. Human cathepsin D, mouse cathepsin L [29] and proliferin [30] may be representatives of the group of ligands that are predominantly secreted if expressed at a high rate. The low efficiency of the lysosomal

targeting of ligands containing mannose 6-phosphate residues indicates the importance of the whole molecular structure for interactions with the segregation system. Cathepsin D should be a useful model for studies on these interactions, also in view of the biological significance of its secretion in human tumour cells [31].

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