Maturation and degradation of β-galactosidase in the post-Golgi compartment are regulated by cathepsin B and a non-cysteine protease

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Abstract Lysosomal β-galactosidase precursor is processed to a mature form and associated with protective protein in lysosomes. In this study we used two cysteine protease proinhibitors, E64-d for cathepsins B, S, H, and L, and CA074Me for cathepsin B. They are converted intracellularly to active forms, E-64c and CA074, respectively. Both active compounds inhibited maturation of the exogenous β-galactosidase precursor, but E-64c did not inhibit further degradation to an inactive 50-kDa product. We concluded that cathepsin B participated exclusively in maturation of β-galactosidase, and a non-cysteine protease was involved in further degradation and inactivation of the enzyme molecule.

Key words: β-Galactosidase; Lysosome; Protease inhibitor; Cathepsin B; E-64c; CA074

1. Introduction

Lysosomal β-galactosidase (EC 3.2.1.23) is primarily deficient in β-galactosidosis represented by G_{M1}-gangliosidosis and Morquio B disease [1], and secondarily deficient in galactosialidosis that is caused by the primary defect of protective protein (cathepsin A; EC 3.4.16.1) [2]. β-Galactosidase is synthesized as an 86-kDa precursor that is processed to a 64-kDa mature form by lysosomal proteases. Early in this processing, β-galactosidase forms a stable complex with protective protein, and becomes protected against degrading proteases [3,4]. The level of intracellular β-galactosidase activity is therefore regulated by lysosomal proteases responsible for maturation of the precursor and for ultimate degradation. Details of this molecular regulation are not known at present, but our previous studies indicated that two different proteases were suggested to be involved in processing of the enzyme: one inhibited by leupeptin and the other not inhibited by leupeptin [5,6].

Lysosomes in mammalian cells contain several cysteine proteases; cathepsin B (EC 3.4.22.1), cathepsin H (EC 3.4.22.16), and cathepsin L (EC 3.4.22.15) in most tissues [7], and also cathepsin S (EC 3.4.22.27) in some tissues [8]. Cathepsin B has been shown to be involved in processing of protein precursors [9–11], antigen presentation [12], and tumor metastasis [13]. Cathepsin L is involved in bone resorption [14]. However, little is known about their authentic intracellular substrates. Recently, a proinhibitor, CA074Me [15], has become available for the study of intracellular protein turnover. It is almost inactive itself, but rapidly de-methylated intracellularly to an active form, CA074, a highly specific inhibitor of cathepsin B. It has little effect on cathepsin H, L, S, or *m*-calpain (EC 3.4.22.17) [16,17]. Another cysteine protease proinhibitor, E64-d, after being converted to an active form, E64-c, shows more generalized inhibitory effects [18], including cathepsin B. They should allow us to specify the function of cathepsin B in proteolytic processing of exogenous β-galactosidase precursor in β-galactosidase-deficient fibroblasts, where the precursor enzyme is endocytosed via the mannose 6-phosphate receptor (MPR) and transported to lysosomes in these cells [19–21].

In this report we demonstrated that both E64-d and CA074Me inhibited maturation of the β-galactosidase precursor, but further degradation was not inhibited. This result indicated that cathepsin B is involved in processing of the β-galactosidase precursor and another non-cysteine protease is present for further degradation.

2. Experimental procedures

2.1. Materials

CA074Me and E64-d were obtained from Taisho Pharmaceutical Co. (Tokyo, Japan). The expression vector, pCAGGS-neo, was provided by Dr. J. Miyazaki (University of Tokyo) [22]. Pepstatin A, chymostatin, and Z-Arg-Arg-AMC were purchased from Peptide Institute (Osaka, Japan); 4-methylumbelliferone (4MU) from Nacalai Tesque (Kyoto); restriction enzymes and T4 DNA ligase from Takara Shuzo (Tokyo); Lipofectin, G418, Ham's F-10 medium and fetal bovine serum from Gibco BRL (Grand Island, NY); Enhanced Chemiluminescence (ECL) for Western blotting from Amersham (Buckinghamshire); and mannose-6-phosphate (M6P) and goat anti-rabbit IgG peroxidase conjugate from Sigma (St. Louis, MO).

2.2. Cell strains

We used three different fibroblast strains with primary β-galactosidase deficiency (β-galactosidosis) for this study. Cell strain 1, from a patient with infantile G_{M1}-gangliosidosis, was homozygous for dupli-

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Abbreviations: CA074, *N*-(1,3-transpropylcarbamoyloxirane-2-carbonyl)-1-isoleucyl-1-proline; CA074Me, *N*-(1,3-transpropylcarbamoyloxirane-2-carbonyl)-1-isoleucyl-1-proline methyl ester; E64-c, L-3-carboxy-2,3-trans-epoxypropionyl-leucylamide-(3-methyl)butane; E64-d, {*N*-[-(L-3-trans-ethoxycarbonyloxirane-2-carbonyl-L-leucyl]-3-methyl-butylamine}; 4MU, 4-methylumbelliferone; Z-Arg-Arg-AMC, carboxybenzoyl-L-argininyl-L-arginine 4-methyl-coumaryl-7-amide; CHO, Chinese hamster ovary; M6P, mannose 6-phosphate; MPR, mannose 6-phosphate receptor; DMSO, dimethyl sulfoxide

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cation of nucleotides 1103–1226 [23]. β-Galactosidase activity was not detectable. Cell strain 2 was from another patient with infantile G_{M1}-gangliosidosis. Gene mutation has not yet been identified. The residual enzyme activity was 1.7% of the control mean. Cell strain 3, from a patient with Morquio B disease, was homozygous for the W273L mutation. The residual enzyme activity was 4.9% of the control mean. A fibroblast strain from a patient with an unknown neurological disease and negative metabolic screening was used as a control for this study. They were cultured in Ham's F-10 medium supplemented with 10% fetal bovine serum and antibiotics.

2.3. Enzyme assay and protein determination

β-Galactosidase and α-galactosidase were assayed with 4MU-β-D-galactopyranoside and 4MU-α-D-galactopyranoside as substrates, respectively [24]. Cathepsin B was assayed with Z-Arg-Arg-AMC, according to the method of Barrett and Kirschke [7,24]. One unit represents one nmol of 4MU or AMC substrate released per hour. Protein in the soluble fraction of the fibroblast lysate was measured by the method of Bradford [25].

2.4. Preparation of recombinant β-galactosidase precursor

Recombinant β-galactosidase precursor secreted from stably transformed CHO cells was isolated as described previously [19], except that the expression vector pCAGGS-neo was used in this study. The cDNA for human β-galactosidase [26] digested by *SalI* was subcloned into the *XhoI* site of pCAGGS-neo. CHO cells were transfected with the plasmid using Lipofectin [27], and stable transformants were selected by G418. The cells secreting the highest amount of the β-galactosidase precursor into the medium were cloned and cultured at confluence for 6 days at 30°C. The culture medium containing 125 U/ml of newly synthesized β-galactosidase precursor was used for replacement studies (conditioned medium). The presence of the β-galactosidase precursor was confirmed by Western blotting as previously described [19].

2.5. Intracellular processing of the exogenous β-galactosidase precursor

Cysteine protease proinhibitors were solubilized in dimethyl sulfoxide (DMSO), and added to the culture medium, with final concentrations of 1–25 μM inhibitor and 0.5% DMSO. Confluent cells on 60-mm dishes were pre-incubated in the presence of one of the protease proinhibitors for 3 h, then cultured for another 20 h in the presence of both the conditioned medium containing 500 units of the precursor and the protease inhibitor at the same concentration, and harvested by scraping. Cell lysates were subjected to enzyme assays and Western blotting.

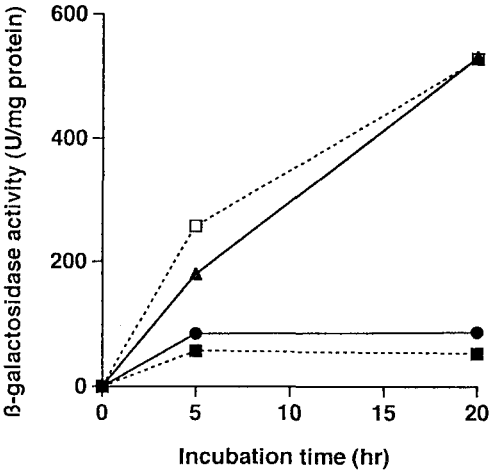

Fig. 1. Effects of protease inhibitors on uptake of exogenous recombinant β-galactosidase precursor by β-galactosidase fibroblasts. Enzyme-deficient fibroblasts were incubated for 3 h in the presence of protease inhibitors, where indicated. Cells were then incubated for up to 20 h in the culture medium containing β-galactosidase precursor only (125 U/ml) (□), precursor and 10 mg/ml M6P (■), precursor and 25 μM CA074Me (●), and precursor and 25 μM E64-d (△). Restoration of activity was inhibited by M6P or by CA074Me, but there was no effect with E64-d. The same result was obtained in two independent experiments.

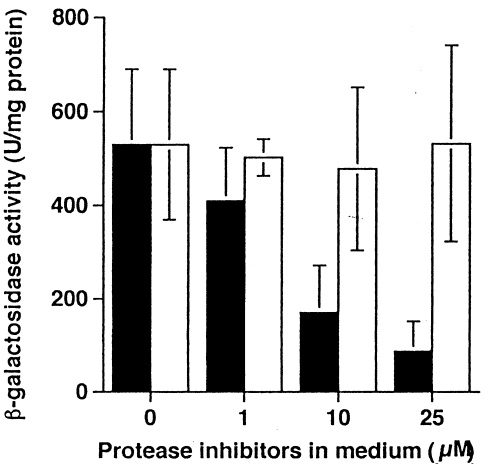

Fig. 2. Dose-dependent effect of protease inhibitors on restoration of enzyme activity in β-galactosidase-deficient fibroblasts by exogenous β-galactosidase precursor. Enzyme-deficient fibroblasts were incubated in the presence of 0.25 μM of CA074Me (■) or E64-d (□) as described in Fig. 1. Restoration was inhibited by CA074Me in a dose-dependent manner, but there was no effect with E64-d. Each value is expressed as mean ± S.E.M. ($n = 3$).

2.6. Western blotting

For electrophoresis 30 μg of protein was applied to each lane of the 8% SDS-polyacrylamide gel. Western blotting was performed with the anti-recombinant precursor β-galactosidase IgG (1:750 dilution) [6] and ECL as described before [6,28].

3. Results

The enzymatically active recombinant precursor [19] was taken up by infantile G_{M1}-gangliosidosis fibroblasts (cell strain 1), and restored the enzyme activity after 20 h of incubation (Fig. 1). This uptake was inhibited in the presence of M6P (Fig. 1). The enzyme activity was not restored in galactosialidosis fibroblasts (data not shown). CA074Me inhibited the restoration of enzyme activity, but E64-d did not (Fig. 1). The effect of CA074Me was dose-dependent (Fig. 2).

The enzyme activity was slightly elevated in the cells (1023 ± 79 nmol/h/mg protein) after incubation for 72 h in the culture medium containing E64-d (10 μg/ml), as compared with the activity in control cells without addition of the inhibitor (656 ± 60 nmol/h/mg protein). Chymostatin and pepstatin A had no activating effect (754 ± 30 and 611 ± 55 nmol/h/mg protein, respectively).

For analysis of intracellular turnover of the exogenous precursor, we used the cell strain 1 that had no detectable residual activity and expressed only a trace of detectable enzyme protein by Western blotting (Fig. 3, lane 1). The endocytosed 86-kDa β-galactosidase precursor was incorporated into the cells and rapidly processed to the 64-kDa mature form (Fig. 3, lane 2). A small portion of the mature enzyme was further degraded to a 50-kDa protein (Fig. 3, lane 2). This 50-kDa protein was also observed in untreated normal fibroblasts (Fig. 3, lane 5).

When the cells were cultured with CA074Me, the β-galactosidase precursor remained unprocessed or was degraded to the 50-kDa protein, and normal processing to the 64-kDa protein was almost completely inhibited (Fig. 3, lane 3). In

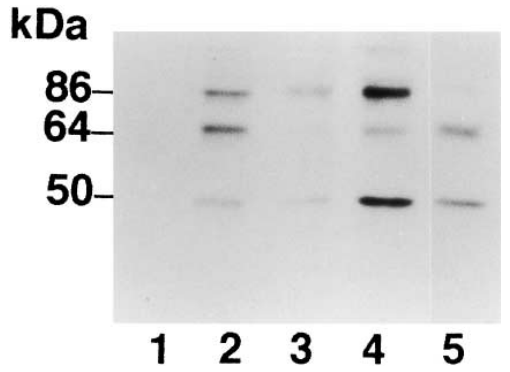

Fig. 3. Proteolytic maturation of the exogenous precursor was altered by cysteine protease inhibitors. Enzyme-deficient fibroblasts were incubated for 20 h with 125 U/ml β-galactosidase precursor (lane 2); precursor and 25 μM CA074Me (lane 3); or precursor and 25 μM E64-d (lane 4) after 3-h preincubation with each inhibitor; or in the absence of precursor or inhibitor (lane 1). Normal fibroblasts also were incubated without protease inhibitor (lane 5). Cell lysates (30 mg of protein) were subjected to SDS-8% polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane as described in the text. Autoradiography: 2 min. No cross-reacting material was detectable in the control (lane 1). The recombinant 86-kDa β-galactosidase precursor was processed to the mature 64-kDa form and degraded to a major product at 50 kDa (lane 2). In the presence of 25 μM CA074Me, the total intracellular enzyme was decreased as compared to that in control cells only for the β-galactosidase precursor, and the degraded 50-kDa form was detectable (lane 3). With 25 μM E64-d, precursor processing was slowed down with little mature form detected. Degradation of the β-galactosidase precursor to the 50-kDa product was not inhibited (lane 4).

the presence of E64-d (Fig. 3, lane 4), most of the endocytosed enzyme was either unprocessed or was degraded to the 50-kDa protein, although the mature enzyme also appeared in relatively small amount.

Cathepsin B activity was completely inhibited after incubation for 23 h with 10 μM CA074Me and almost completely with 10 μM E64-d (Table 1). α-Galactosidase activity was not affected in this experiment (Table 1). This result excluded the possibility of a generalized cytotoxic effect of CA074Me or E64-d in the range of 1–25 μM on cultured fibroblasts, causing a decrease in protease activities and inhibition of normal processing of the enzyme molecule.

4. Discussion

Lysosomal β-galactosidase is synthesized as an 86-kDa precursor, transported to lysosomes, associated with neuraminidase and protective protein, and then processed to the mature form. This maturation is achieved by removal of a 20-kDa peptide from the carboxy-terminal end of the precursor molecule [29]. In galactosialidosis, the complex formation is impaired due to a defect in protective protein expression, and rapid proteolysis of β-galactosidase and neuraminidase ensues. The molecular mechanism is not known for stabilization and activation of β-galactosidase and neuraminidase in the complex [30,31].

Leupeptin protects β-galactosidase against proteolysis at the position 82 residues upstream of the C-terminal. In fact, it restores β-galactosidase activity in galactosialidosis fibroblasts [3,5,6,24,32]. This restoration is also observed in the presence of protective protein. It is not known whether or not protective protein is involved directly in posttranslational processing of β-galactosidase [6,33].

Enzyme loading experiments provide further information about processing of the β-galactosidase protein under physiological conditions in the presence of functionally active protective protein [6,19–21]. Recombinant β-galactosidase precursor in the culture medium is recognized by MPR, endocytosed into the post-Golgi compartment of human β-galactosidosis fibroblasts, and converted to the mature form [21]. On the other hand, transient expression of β-galactosidase cDNA in COS-1 cells produces a large amount of the 86 kDa β-galactosidase precursor, but only a small portion of this precursor is processed to the 64-kDa mature form [26]. Permanently transformed CHO cells also express the β-galactosidase precursor, which is processed to a mature 64-kDa protein but rapidly degraded to an inactive 50-kDa product [6].

Lipophilic pro-inhibitors used in this study, CA074Me and E64-d, have no direct effect on proteases, but they are converted to active forms, CA074 and E64-c, respectively, in cultured fibroblasts [15,18]. E64-c inhibits several cysteine endopeptidases, such as lysosomal cathepsin B, L, H, and cytoplasmic calpains [15,18], but CA074 preferentially inhibits cathepsin B [16,17]. In this study, we demonstrated that CA074Me in the culture medium caused a relatively little accumulation of the exogenous β-galactosidase precursor and inhibited its processing to the mature form in G_{M1}-gangliosidosis fibroblasts. Cathepsin B also may have accelerated endocytosis of the enzyme, and its inactivation may have resulted in low uptake of the precursor protein. E64-d did not affect uptake of the β-galactosidase precursor, and more precursor remained unprocessed although subsequent degradation to the 50-kDa polypeptide was not impaired.

These results indicated that E64-d inhibited processing of

Table 1
Effects of protease inhibitors on lysosomal enzymes

Addition	(μM)	Cathepsin B		α-Galactosidase	
		Mean ± S.E.M.	(%)	Mean ± S.E.M.	(%)
None		1247 ± 85	(100)	125 ± 21	(100)
CA074Me	1	53 ± 6	(4)	137 ± 39	(109)
CA074Me	10	–	(0)	141 ± 33	(113)
CA074Me	25	–	(0)	114 ± 13	(91)
E64-d	1	91 ± 6	(7)	151 ± 20	(121)
E64-d	10	17 ± 2	(1)	203 ± 39	(163)
E64-d	25	–	(0)	121 ± 31	(96)

Fibroblasts with β-galactosidosis (#1–3) were pre-incubated for 3 h in the presence of cysteine protease. This was followed by a 20-h incubation in the conditioned medium containing 125 U/ml of recombinant precursor β-galactosidase, and 0–25 μM of cysteine protease inhibitors (dissolved in 0.5% DMSO). Enzyme activity was expressed as unit/mg protein ($n = 3$). –, not detected.

the β-galactosidase precursor but did not inhibit degradation to the 50-kDa protein. In the presence of CA074Me, little precursor, mature, or degenerative product appeared, suggesting a rapid turnover of β-galactosidase without forming a stable complex with protective protein as observed in galactosialidosis [6]. We concluded that cathepsin B, which is inhibited by CA074 and E64-c, is active in maturation of β-galactosidase, and at least one non-cysteine protease, unaffected by E64-c, degraded the precursor to the 50-kDa protein. Chymostatin (serine protease inhibitor) or pepstatin A (aspartic protease inhibitor) had no effect on processing of the β-galactosidase precursor (data not shown).

Cathepsin B is a major lysosomal protease, whose enzymatic properties have been intensively studied in vitro [7,34], and its crystal structure was determined [35]. It exhibits both endopeptidase and dipeptidyl carboxypeptidase activities in vitro [7,36]. However, its role in intralysosomal maturation of hydrolases has been poorly defined [9,10]. Our results point to an important role of cathepsin B in maturation of lysosomal hydrolases. The candidate cleavage point in conversion of the β-galactosidase precursor to the mature form has been proposed as Arg-530 in a uniquely hydrophilic sequence [29], which is acceptable as a P1 for endopeptidase cathepsin B [7,10,35]. Limited proteolysis of the recombinant β-galactosidase precursor with pepsin or with purified cathepsin B without protective protein could not produce the mature form (Callahan, J.W., unpublished data). To date no one has presented definitive evidence of the true C-terminus of the mature enzyme, and it remains unclear how many steps are involved in normal C-terminal processing. Development of specific inhibitors for proteases will solve this problem. The present study using protease inhibitors suggested the role of specific cathepsins in processing of β-galactosidase, and serves as a model system for the study of posttranslational events and protein-protein interactions in lysosomes, not only for β-galactosidase but also for other lysosomal enzymes.

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