Early proteolytic cleavage with loss of a C-terminal fragment underlies altered processing of the β -galactosidase precursor in galactosialidosis

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Processing of human β -galactosidase (β -GAL) was studied in permanently transfected Chinese hamster ovary (CHO) cells and compared with that in normal cells and in cells from subjects with GM1-gangliosidosis, galactosialidosis and I-cell disease. Biosynthesis of β -GAL in CHO cells results in the synthesis of an 88 kDa glycosylated and phosphorylated monomer precursor which is enzymically active and is secreted into the medium. Post-translational processing begins at the C-terminal end of the protein and gives rise to structurally related 67 and 64 kDa mature forms. These are subsequently degraded to give several inactive products, of which a 50 kDa and a 18 kDa species are prominent. In normal fibroblasts only the 84 kDa precursor is readily detected inside cells, while the 88 kDa precursor is the only form secreted from cells in the presence of ammonium chloride. Processing of the precursor in normal fibroblasts results in the appearance of both the 67 and 64 kDa mature forms, which are also degraded to give 50 and 18 kDa products, as in transfected CHO cells. As affected controls, GM1-gangliosidosis cells showed a general loss of all forms of the enzyme, while in I-cell fibroblasts only the 84 kDa precursor and an 18 kDa degradation form were prominent. In galactosialidosis fibroblasts, taken from two different subjects, processing of β -GAL was characterized by the respective appearance of intermediate 80 and 72 kDa enzymically inactive polypeptides, at levels lower than the normal amounts of the 67 and 64 kDa mature forms

and higher than the normal amounts of degradation products, one of which is of 45 kDa and arises by endoproteolytic cleavage of the 80 kDa polypeptide. Incubation for up to 72 h in medium containing leupeptin, a potent inhibitor of thiol-dependent proteases, resulted in a significantly increased level of β -GAL activity to near normal levels in fibroblasts from one galactosialidosis subject. Concordant with this, the abundance of the 84 kDa precursor was increased and the levels of the 80 kDa, 45 kDa and 18 kDa digestion products were diminished. However, in fibroblasts from the second galactosialidosis subject, the amount of the abnormal 72 kDa polypeptide was not influenced by leupeptin treatment. Leupeptin treatment did not increase enzymic activity levels in normal, GM1-gangliosidosis or I-cell disease fibroblasts, despite the fact that the production of the 50 kDa and 18 kDa degradation products was blocked in the presence of leupeptin. We conclude that in galactosialidosis the leupeptin-inhibitable proteolytic cleavage of a small fragment causes a conformational change of the precursor that precludes its further normal processing and results in its enzymic deficiency. This early abnormal trimming of β -GAL is ascribable to a deficiency in the functional protective protein, the function of which is absolutely essential to render β -GAL cryptic from at least two distinct and separate proteolytic attacks that together remove at least 12 kDa from the C-terminal end of the enzyme.

INTRODUCTION

The post-translational processing of β -galactosidase (β -GAL) from its biosynthesis as a glycosylated precursor to its mature lysosomal form is ill defined. What is clear is that the interaction of β -GAL with a protective protein to form a complex is an obligatory step in this process. To begin to dissect these events, we [1] have purified the precursor form of β -GAL secreted from permanently transfected Chinese hamster ovary (CHO) cells and shown it to be a monomer (105 kDa on molecular sieve chromatography and 88 kDa on SDS/PAGE) with a specific activity of 0.7 mmol/h per mg of protein. It hydrolyses synthetic substrates in a double-displacement reaction, with retention of the β -configuration. The purified precursor is readily taken up and corrects the defect in GM1-gangliosidosis cells which are deficient in this enzyme, providing indirect evidence that the precursor is

also phosphorylated. Pulse-chase studies in human fibroblasts have shown that phosphorylated precursor is processed in lysosomes to a 64 kDa mature form [2–4]. Amino acid sequence analysis of the mature protein has revealed an N-terminus identical to that predicted on removal of the signal peptide, suggesting that there is no post-translational processing at the N-terminus of the protein [5], and we have shown that the mature form of the enzyme does not react with a C-terminus-specific anti-peptide antibody [1]. Thus conversion from the precursor to the mature form must involve the loss of about 12 kDa of polypeptide (20 kDa if the possible oligosaccharides are included) from the C-terminal end of the protein [1,5].

The primary defect in galactosialidosis (GS) is dysfunction of the 54 kDa protective protein that associates with β -GAL and neuraminidase to form a complex, thereby controlling turnover of both enzymes [2,6]. This multifunctional protein was deduced

Abbreviations used: α -MEM, Eagle's minimal essential medium; anti-C-Gal IgG, rabbit polyclonal antibody against the β -galactosidase C-terminal sequence; anti-P-Gal IgG, rabbit polyclonal antibody against the β -galactosidase precursor; β -GAL, lysosomal GM1-ganglioside β -galactosidase; CHO, Chinese hamster ovary; GS, galactosialidosis.

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to be a serine protease based on its degree of identity with other known enzymes. Subsequent work has proven it to be a catheptic carboxypeptidase A (cathepsin A) with properties similar to those of carboxypeptidase Y of yeast [7]. It also possesses C-terminal deamidase activity [8] and when released by physiological stimuli inactivates tachykinins by both deamidolytic and carboxypeptidolytic reactions [9,10]. Conversion of an essential active-site residue, serine-150, to alanine or a histidine at position 429 to glutamine resulted in a significant decline in its cathepsin A activity, but neither substitution affected the protective function of the protein, indicating that the protease activity and the protective function are separate and distinct properties of the protein [11].

To date there has been no direct evidence indicating the number of steps involved in the maturation of β -GAL, the identity of the proteolytic enzyme(s) responsible for this processing or the identity of the mature C-terminal amino acid. In this work we present evidence from studies on fibroblasts and transfected CHO cells permanently expressing β -GAL that processing of the 88 kDa precursor results in the appearance of both 67 kDa and 64 kDa mature forms of β -GAL, which are subsequently degraded to 50 kDa and 18 kDa polypeptides. This processing begins with removal of the C-terminal end of the protein. In GS fibroblasts, deficient in protective protein, processing of β -GAL is characterized by the separate appearance of two intermediate polypeptides, lower than normal or negligible amounts of the mature enzyme and the appearance of 45 kDa and 18 kDa degradation products. We propose that the correct processing of the β -GAL precursor requires interaction of the protective protein with its C-terminal end to render it cryptic to proteolytic digestion by blocking at least one cathepsin B cleavable site and one non-cathepsin B cleavable site.

A preliminary report of this work has appeared in abstract form [12].

MATERIALS AND METHODS

Materials

Human skin fibroblasts were from a healthy control (HSC # 3858), GM1-gangliosidosis (GM1) infantile phenotype patients cases 1 and 2 (ATCC # GM05652A and HSC # 4992), and late infantile phenotype case 3 (HSC # 3634). The residual β -GAL activities expressed as a percentage of control values were 0%, 1% and 4% respectively. The I-cell strain (HSC # 5569) was from a 34-month-old male patient who displayed the clinical hallmarks and biochemical features (urinary sialyloligosaccharides, high plasma levels of acid hydrolases, low enzyme levels in fibroblasts) associated with the disease. GS cases 1 and 2 were ATCC # GM05076 and # GM00806 respectively, where case 1 was a 7-year-old male homozygous for a single base-pair substitution (T1324G) which resulted in a change from phenylalanine-412 to valine, while case 2 was a 30-year-old female with a Tyr-221 $\rightarrow$ Asn and a second unknown mutation [6,13].

The cells were propagated in Eagle's minimal essential medium (α -MEM) containing 10% (v/v) fetal bovine serum and harvested at confluency by scraping. Cells were lysed by sonication (10 s) in 50 μ l of 0.1 M Tris/HCl, pH 6.7, or 10 mM phosphate, 0.15 M NaCl, pH 7.4 (PBS). Protease inhibitors (0.1 mM PMSF and 20 μ M leupeptin) were not always included in homogenizing solutions. However, where checked, we observed no differences in the protein profiles whether or not they were present. Carbon tetrachloride (50 μ l) was then added to the lysate, the mixture was vortexed and then centrifuged at 10000 *g* at 4 °C for 10 min, and the supernatant (50 μ l) was analysed by Western blotting.

For activity measurements and for immunoprecipitation studies the carbon tetrachloride step was omitted.

Permanently transfected CHO cells

Permanently transfected CHO cells secreting β -GAL precursor into the medium were cultured at 30 °C in CHO-S-SFM (Gibco) [1]. Cells were lysed and processed as above. The human β -GAL precursor was purified from the medium by affinity column chromatography on *p*-aminophenylthiogalactose coupled to CH-Sepharose as previously described [1,14]. The eluted proteins were analysed by direct immunoprecipitation, SDS/PAGE and Western blotting.

Preparation of anti-C-Gal and anti-P-Gal antibodies

The anti-C-Gal antibody was raised in rabbits against a synthetic 14-residue C-terminal amino acid sequence deduced from the β -GAL cDNA. The IgG was purified on Protein A-Sepharose CL-4B (Pharmacia) and affinity purified on an oligopeptide-Sepharose 4B column as described [1,15]. The antibody against the β -GAL precursor (anti-P-Gal) was also raised in rabbits. Briefly, a young male New Zealand rabbit was initially injected with 0.2 mg of purified precursor [suspended in 0.5 ml of a 1:1 (v/v) PBS/complete Freund's adjuvant emulsion]. This was followed at 3-week intervals by two additional 0.2 mg injections as above, except that incomplete Freund's adjuvant was used. The animal was exsanguinated under anaesthesia 3 weeks after the third injection, and serum was collected and stored at -20 °C. IgG was isolated by chromatography on Protein A-Sepharose according to the manufacturer's instructions (Pharmacia).

Enzyme assay and protein determination

Enzyme activity was measured with 4-methylumbelliferyl β -D-galactopyranoside as a substrate, where 1 unit is defined as the release of 1 nmol of 4-methylumbelliferone/h [14]. Protein was determined by the method of Bradford [16] (Bio-Rad).

Immunoprecipitation of β -GAL activity

Human β -GAL precursor or normal fibroblast lysates containing 400–700 units of β -GAL activity were incubated with anti-P-Gal IgG or anti-C-Gal IgG (0–128 μ g) in 100 μ l of PBS at room temperature for 10 min. This was followed by the addition of 25 μ l of Protein A-Sepharose CL-4B in PBS (100 mg of Protein A-Sepharose CL-4B in 2.25 ml of PBS). The mixture (125 μ l) was incubated at room temperature for another 10 min and was then centrifuged in a Mikroliter centrifuge for 1 min. The residual enzyme activity of the supernatant was measured as described above.

Effects of NH₄Cl, leupeptin and protective protein treatment on fibroblast β -GAL

Fibroblasts were incubated for 3 days in α -MEM containing 0.1% human serum albumin and 10 mM NH₄Cl. The conditioned media were collected and applied to concanavalin A-Sepharose columns (Pharmacia) pre-equilibrated to pH 6.0. The columns were washed with 50 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl. The bound proteins were eluted with 0.5 ml of PBS containing 0.5 M methyl- α -D-mannopyranoside (Sigma), and 100 μ l of the eluate was used for Western blots.

To examine the effect of leupeptin on β -GAL processing, fibroblasts were incubated in a specified medium (α -MEM and 0.1% human serum albumin) containing 20 μ M leupeptin for 0–72 h, and processed as above. I-cell fibroblasts were also incubated in the specified medium containing leupeptin with and without the addition of 1500 units/ml β -GAL precursor [1] and a concentrated solution of protective protein precursor prepared according to Itoh et al. [17].

Western blots

Lysates of cells (30 μ g of protein) and eluates (0.5 μ g of protein) from concanavalin A–Sepharose and *p*-aminophenylthio- β -D-galactopyranoside–Sepharose columns were separated by SDS/PAGE [18], transferred to nitrocellulose membranes, immunoblotted and visualized by the ECL analysis system (Amersham). Anti-P-Gal IgG (20 μ g) or affinity-purified anti-C-Gal IgG (400 ng) was used as a primary antibody. Computer-assisted densitometry of ECL films was performed with a LC 475 colour Onescanner with Ofoto 2.0 software fitted to a Macintosh computer. Densities were compared with protein and immunoblot standard curves.

Immunofluorescent microscopy

For immunofluorescent localization of β -GAL, CHO cells and fibroblasts were grown in Medium 199 with 10% fetal bovine serum for 3 days in 4-well plastic slides. Monolayers were washed in PBS, and either quickly permeabilized in 100% acetone cooled to -20°C and then fixed for 10 min with cold 100% methanol, or left non-permeabilized and lightly fixed in 2% paraformaldehyde in PBS for 5 min. Cells were then washed in PBS, blocked with 1% BSA and 5% normal goat serum in PBS, and incubated with anti-C-Gal or anti-P-Gal IgG, both at a concentration of 10 μ g/ml. The secondary antibody was goat anti-rabbit fluorescein-conjugated immunoglobulin diluted 1:100 [15]. As a control, normal serum was substituted for the primary antibody. Additional controls included the secondary antibody alone.

RESULTS

Direct immunoprecipitation of β -GAL

The polyclonal rabbit IgG raised against the purified β -GAL precursor immunoprecipitated over 90% of the activity secreted into the medium by permanently transfected CHO cells (results not shown), and also immunoprecipitated over 90% of the activity in the lysates of normal fibroblasts (Figure 1). Furthermore the affinity-purified IgG directed against the C-terminus of β -GAL also precipitated 90% of the precursor β -GAL activity either from a purified preparation or from the crude culture medium. However, the same anti-C-Gal IgG preparation immunoprecipitated no more than 20% of the activity in human fibroblast lysates (Figure 1), suggesting that no more than 20% of the total β -GAL activity in these cells contains an intact C-terminal epitope.

Processing of the β -GAL precursor by CHO cells

The purified β -GAL precursor was easily visualized by both anti-P-Gal and anti-C-Gal antibodies (Figure 2A, lane 3, and Figure 2B, lane 1, respectively). In spite of the relative abundance and the unprocessed nature of the β -GAL secreted into the medium, the intracellular specific activity in the transfected CHO cells (1350 nmol/h per mg of protein) was only slightly higher than

Figure 1 Immunoprecipitation using anti-P-Gal IgG and anti-C-Gal IgG

β -GAL from fibroblast lysates (activity 400–700 nmol/h) was subjected to immunoprecipitation by 0–128 μ g of anti-P-Gal IgG (■) or anti-C-Gal IgG (◆). Anti-P-Gal IgG precipitated 90% of the activity in a dose-dependent manner, but anti-C-Gal IgG was unable to precipitate more than 20% of the activity in fibroblasts. Up to 90% of the activity of recombinant β -GAL precursor was precipitated by crude anti-C-Gal IgG (□), indicating that this antibody was also able to precipitate β -GAL precursor in a dose-dependent manner.

that found in the non-transfected CHO cells (1039 nmol/h per mg of protein). Inclusion of protease inhibitors during homogenization raised only slightly (to 1700 nmol/h per mg of protein) the level of intracellular β -GAL activity. The anti-precursor

Figure 2 Immunocomparison of β -GAL polypeptides in permanently transfected CHO cells and those secreted into the medium by CHO cells and by NH_4Cl -treated fibroblasts

Anti-P-Gal IgG (A) and affinity-purified anti-C-Gal IgG (B) were used. (A) Lane 1, 30 μ g of non-transfected CHO cell lysate; lane 2, 30 μ g of transfected CHO cell lysate; lane 3, 0.5 μ g of purified β -GAL precursor. (B) Lane 1, 0.5 μ g of purified β -GAL precursor; lane 2, 0.5 μ g of the concanavalin A–Sepharose column eluate from the medium above normal fibroblasts; lane 3, 0.5 μ g of the concanavalin A–Sepharose column eluate from the medium above GS cells (case 1). The 88 kDa precursor protein, detectable with both IgGs, was the only form of the enzyme secreted from the cells. Values on the left are molecular masses (kDa).

Figure 3 Immunolocalization of β -GAL in CHO cells

Non-transfected CHO cells stained with anti-P-Gal IgG (**A**) and with anti-C-Gal IgG (**C**) showed little immunoreactivity, whereas transfected CHO cells stained with anti-P-Gal IgG showed strong punctate immunofluorescence characteristic of lysosomal localization (**B**). With anti-C-Gal IgG the more diffuse staining was localized to the endoplasmic reticulum area (**D**).

Figure 4 Immunodetection of β -GAL in fibroblasts using anti-P-Gal IgG

N: 30 μ g of normal lysate (lanes 1 and 2 represent two separate strains); GM1: 30 μ g of GM1-gangliosidosis lysate (lane 1–3 are cases 1–3 respectively); GS: 30 μ g of GS lysate (lane 1 and 2 are cases 1 and 2 respectively). Molecular masses were estimated by comparison with protein standards. Normal fibroblast lysates showed 84 kDa, 67 kDa and 64 kDa bands which were decreased in GM1-gangliosidosis lysates. The two GS cell lines showed a clear abnormal band just smaller than the faint 84 kDa precursor, and low amounts of the usual 67 and 64 kDa mature polypeptides were seen clearly only for GS case 1. A 35 kDa band (arrowhead) is a degradation product of β -GAL, as is the 18 kDa band seen in the GS cell lines.

antibody localized strong immunostaining in the lysosome-rich peripheral area, with diffuse immunostaining also noted in the perinuclear areas (Figure 3B). With the affinity-purified anti-C-Gal IgG, diffuse immunostaining was noted almost exclusively in the perinuclear area, with little evidence of the punctate immunostaining associated with the lysosomal compartment (Figure 3D). Western blot analysis of transfected CHO cell lysates clearly showed the presence of the 88 kDa precursor and less abundant amounts of two polypeptides, one at 67 kDa and the second at 64 kDa, which represent the polypeptides of mature β -GAL (Figure 2A, lane 2), while the corresponding areas in the untransfected cells showed a very faint reaction (Figure 2A, lane 1). Of the additional immunoreactive polypeptides identified in the transfected cells with the anti-precursor anti-P-Gal antibody (Figure 2A, lane 2), the most prominent corresponded to a mass of about 50 kDa. Its counterpart was noted in the untransfected

CHO cells. Other degradation products present at approximately equal but lower amounts ranged in molecular mass from about 55 kDa to 18 kDa (Figure 2A, lane 2). Human fibroblasts cultured in the presence of NH_4Cl secrete unprocessed lysosomal hydrolases that are normally targeted to the lysosomal compartment via the mannose 6-phosphate receptor; one of these enzymes is β -GAL. The β -GAL precursor released from both normal and GS fibroblasts under these conditions (Figure 2B, lanes 2 and 3 respectively) displayed the same mass as the precursor secreted from transfected CHO cells (Figure 2A, lane 3, and Figure 2B, lane 1), indicating that the protein has not undergone any C-terminal processing as a result of its secretion from the cells.

β -GAL in normal, GM1-gangliosidosis, GS and I-cell fibroblasts

Unlike in CHO cells, where the 88 kDa precursor was easily seen, a 84 kDa β -GAL precursor, which we presume to be the glycosylated but non-phosphorylated form of the precursor, was most abundant in human fibroblast lysates (Figure 4). Anti-P-Gal IgG detected both a 67 kDa and a 64 kDa processed form of β -GAL in normal fibroblasts (Figure 4, N, lanes 1 and 2), consistent with the results obtained with the transfected CHO cells. Densitometric scanning analyses of the blots showed that the ratio of the 84 kDa precursor to the 67 kDa and 64 kDa processed polypeptides was in accordance with the immunoprecipitation data (Figure 1). In GM1-gangliosidosis cells from three separate cases, all forms of β -GAL were markedly reduced in abundance (Figure 4, GM1). Both GS cell lines (Figure 4, GS, lanes 1 and 2) showed a faint 84 kDa precursor band and a more prominent lower-molecular-mass band near 80 kDa. In GS case 1, where the residual β -GAL activity was 20% of the normal (Table 1), detectable amounts of the 67 kDa and 64 kDa forms of β -GAL were noted (Figure 4, GS, lane 1), and this correlates with the level of residual enzyme activity. In the second GS case, with only 6% of the normal level of β -GAL activity, only trace amounts of the 67 kDa and 64 kDa polypeptides were noted (Figure 4, GS, lane 2). A prominent degradation product (near 18 kDa) was detected in both the GS and I-cell disease lines, with a barely detectable corresponding reaction seen in controls and in the GM1-gangliosidosis strains. As expected for the I-cell disease line (Table 1), the 84 kDa β -GAL precursor was readily detected in these cells. Variable amounts of other degradation products similar in size and abundance to those in the transfected

Table 1 β -GAL activity in GS fibroblasts is partially restored in the presence of leupeptin

Fibroblasts were incubated in α -MEM containing 0.1% human serum albumin and 20 μ M leupeptin for the times indicated. Medium was removed, monolayers were rinsed with PBS several times and cells were recovered by scraping and homogenized as described. Total β -GAL activity was assayed as described.

Fibroblasts	Incubation time (h)...	β -GAL specific activity (nmol/h per mg of protein)			
		0	5	24	72
Normal		1447	1148	2776	1958
GM1-gangliosidosis		4	< 2	< 2	< 2
GS					
Case 1		291	310	1002	1167
Case 2		90	111	209	297
I-cell disease		11	7	6.5	6.4

Figure 5 Immunolocalization of β -GAL in fibroblasts

Normal (a), GM1-gangliosidosis (b) and GS (c) fibroblasts were stained by anti-P-Gal IgG. The reaction with anti-P-Gal IgG was distinctly punctate and occurred throughout the cytoplasm in the normal cells, with lower levels of reaction in (b) and (c). To assist visualization of the cells, propidium iodide was used to stain the nuclei in (b) and (c).

CHO cells were also seen (Figure 4), but the proteolytic steps giving rise to them remain to be defined. The affinity-purified anti-C-Gal IgG also detected both the 84 and 88 kDa precursors in normal cells, and confirmed the lower precursor levels seen in GM1-gangliosidosis and GS cells (results not shown).

Figure 6 Effect of leupeptin on the processing of β -GAL in normal and GS fibroblasts

Cells were incubated for 0, 5, 24 and 72 h (lanes 1–4 respectively) in medium containing 20 μ M leupeptin. Lysates (30 μ g of protein) prepared from normal (A), GS case 1 (B), GS case 2 (C) and I-cell disease (D) fibroblasts were subjected to Western blots with anti-P-Gal IgG. The signal intensities are not quantitatively comparable, as (A) and (B) were developed for 5 s in the ECL system whereas (C) was developed for 30 s. Processing to the mature 67 and 64 kDa polypeptides in normal cells occurred independently of the presence of leupeptin. Leupeptin blocked the appearance of the 50 and 18 kDa degradation forms in normal cells and of the 45 and 18 kDa products in the GS cell lines. Leupeptin also inhibited the processing of the precursor to an 80 kDa abnormal protein in both GS strains. The occurrence of the intermediate product at 72 kDa seen only in GS case 2 cells was not affected by leupeptin, nor was the appearance of the lower band near 35 kDa. However, the presence of leupeptin was insufficient to restore the 67 kDa and 64 kDa forms in GS cells. The increase in enzyme activity in the presence of leupeptin (see Table 1) appears to result solely from an increase in the level of the 84 kDa precursor.

Immunofluorescent detection of the enzyme with anti-P-Gal IgG in normal fibroblasts localized the immunoreactive material to lysosomes and to the perinuclear areas of the cell (Figure 5a), as seen in CHO cells. An obvious decrease in the number of immunoreactive vesicles was seen in both GM1-gangliosidosis and GS cells (Figures 5b and 5c respectively).

β -GAL processing and degradation

The occurrence of the 80 kDa polypeptide in GS cells suggested to us that altered C-terminal degradation of the β -GAL precursor may be responsible for the deficiency of the enzyme in these cells. To examine this further, normal, GM1-gangliosidosis, GS and I-cell fibroblasts were incubated for up to 72 h in the presence of leupeptin, a potent inhibitor of thiol-dependent proteases. Leupeptin restored β -GAL activity in case 1 GS fibroblasts, and partially restored it in case 2 cells (Table 1). There was a small but demonstrable rise in activity in normal cells, but no change was noted in the activity in GM1 or I-cell fibroblasts. In normal cells, within 5 h of incubation with leupeptin, the 50 kDa and 18 kDa degradation products had disappeared (Figure 6A), and

Figure 7 Effect of conditioned medium and leupeptin on the processing of β -GAL precursor in I-cell disease fibroblasts

I-cell fibroblasts were incubated for 48 h in regular medium (lane 1; intracellular specific activity 8 nmol/h per mg of protein), in medium containing 1500 units/ml β -GAL precursor (lane 2; intracellular specific activity 1345 nmol/h per mg), in medium containing β -GAL precursor plus conditioned medium (derived from GM1-gangliosidosis case 1 cells, a putative source of lysosomal proteins including protective protein) (lane 3; intracellular specific activity 849 nmol/h per mg), or in medium containing β -GAL precursor plus 20 μ M leupeptin (lane 4; intracellular specific activity 2072 nmol/h per mg). Lane 5 contains normal control cells (intracellular specific activity 1769 nmol/h per mg of protein). Lysates (30 μ g of protein) were subjected to Western blots with anti-P-Gal IgG. Values on the left are molecular masses (kDa).

over 72 h the amounts of the 64 kDa, 67 kDa and 84 kDa polypeptides increased slightly, consistent with the rise in enzyme activity (Table 1). It is noteworthy that leupeptin-inhibitable proteases are not involved in the normal maturation of the precursor to the mature forms of the enzyme. In case 1 GS fibroblasts the abnormal 80 kDa intermediate and the 45 and 18 kDa degradation products had disappeared by 5 h of leupeptin treatment (Figure 6B). In GS case 2 cells (Figure 6C) a second abnormal protein, about 72 kDa in size, was also seen. The abundance of this polypeptide was not changed over the course of 72 h in the presence of leupeptin, although there was a notable increase in the amount of the 84 kDa precursor in these cells over the same period. The amounts of the 67 kDa and 64 kDa forms remained negligible in spite of the presence of leupeptin. In I-cell fibroblasts (Figure 6D) both the 84 kDa precursor and the 18 kDa polypeptide bands were detected; neither of these showed any discernible change over the course of 72 h in the presence of leupeptin, nor was there any enhancement in β -GAL activity. As a check on the identity of the 84 kDa precursor in I-cell fibroblasts, these cells were incubated with exogenous β -GAL precursor in the presence and absence of leupeptin (Figure 7). In this instance both exogenously added 88 kDa polypeptide and the 84 kDa polypeptide derived from the host cell were visualized, indicating that the 84 kDa band seen in I-cell disease fibroblasts was indeed the non-phosphorylated form of the β -GAL precursor.

DISCUSSION

The β -GAL precursor secreted from permanently transfected CHO cells migrates as a 88 kDa phosphorylated/glycosylated polypeptide on SDS/PAGE gels. In its native form it is an enzymically active monomer [1] and displays the usual K_m and pH optimum of the fully mature enzyme. It cleaves substrates in a stereospecific manner, with retention of the β -configuration [1]. β -GAL is localized to the lysosomal compartment in CHO cells (as shown by immunofluorescence) and when fully processed both the mature 67 kDa and 64 kDa β -GAL polypeptides are

seen. The amounts of the 67 kDa and 64 kDa forms are approximately the same. The difference between the two forms is unclear, but a 67 kDa intermediate form was seen in pulse-chase studies [2]. Yamamoto et al. [5] predicted, but did not prove, that the C-terminal amino acid in the mature, processed enzyme is arginine-530. This means that the terminal 147 residues must be cleaved off during the maturation process. The deduced sequence downstream from arginine-530 in the region of residues 542–547 includes two side-by-side putative glycosylation sites, only one of which is likely to be glycosylated [1]. The present results suggest that, if this site is not glycosylated, processing probably proceeds to the 64 kDa mature form (i.e. to give Arg-530 as the C-terminal residue), whereas if the site is glycosylated then processing stops C-terminal to it to give rise to the 67 kDa polypeptide. These events have not been proven as yet. In general accord with these results, β -GAL when purified from conventional sources such as placenta shows a broad protein band in the 64–67 kDa range [14], illustrating both protein and oligosaccharide chain polydispersity in the processed enzyme from conventional sources.

Although active β -GAL is readily secreted into the medium by transfected CHO cells, the specific activity of the cell-associated enzyme is only marginally higher than in the non-transfected cells. Although the precursor is processed to the mature enzyme, major digestion products of β -GAL are seen, including 50 and 18 kDa polypeptides (Scheme 1). The lack of enhanced specific activity and the detection of digestion products suggests that in CHO cells, as in human cells, normal processing and stability of β -GAL requires the action of a protective protein which is estimated to be about 6-fold more abundant, at least in human fibroblasts, than β -GAL [19]. To explain this, we assume that a form of the β -GAL/neuraminidase-protective-protein complex exists in CHO cells, as in human fibroblasts, as a non-random association of these proteins [20]. Since human β -GAL is unlikely to enter into a stable association with the host β -GAL/neuraminidase-protective-protein complex [21,22], the requirement for β -GAL processing in these cells would probably exceed the stabilization, protection and processing capability afforded by the host complex. Preferential overproduction of one component such as human β -GAL in the transfected CHO cells would be expected to result in the maturation of a portion of the overproduced β -GAL (hence the 67 kDa and 64 kDa polypeptides), while the rest would be degraded at an accelerated rate as indicated by the appearance of the 50 kDa and 18 kDa degradation products.

The 88 kDa β -GAL precursor that is overproduced by transfected CHO cells was detected with the anti-P-Gal and anti-C-Gal IgGs, while in normal fibroblasts the abundance of the 88 kDa precursor was at the lower limit of immunodetectability. However, it was easily detected in medium above fibroblasts incubated with ammonium chloride. In addition the 88 kDa precursor incorporated into I-cell fibroblasts was clearly discernable from the 84 kDa form (Figure 7). Therefore the non-phosphorylated 84 kDa form of the precursor, which we found to represent about 20% of the total β -GAL activity in fibroblasts, probably accounts for the bulk of the total β -GAL activity immunoprecipitable by the anti-C-Gal IgG. These data are in accordance with those of Takiyama et al. [23], who showed that the precursor constitutes up to 20% of the total cell enzyme activity.

In normal fibroblasts, initial processing of the enzyme occurs exclusively at the C-terminal end of the protein (as judged by the loss of reaction with anti-C-Gal IgG) and follows the same route as in the transfected CHO cells; i.e. the appearance of the 67 kDa and 64 kDa polypeptides (Scheme 1). This is followed by the

Scheme 1 Processing of β -GAL

See the text for details.

appearance of the degradation products (50 kDa and 18 kDa). In both cases of GS, abnormal processing is highlighted by: (i) the early appearance of an 80 kDa species that does not react with the anti-C-Gal IgG (results not shown); (ii) lower than normal amounts of the mature 67 and 64 kDa polypeptides; and (iii) replacement of the 50 kDa form in normal cells by a 45 kDa band. The 18 kDa band is the same as is found in the normal fibroblasts, in I-cell fibroblasts and in transfected CHO cells. In case 2 GS a second intermediate at 72 kDa was prominent. The occurrence of the 80 kDa and 72 kDa polypeptides in GS directly reflects rapid partial cleavage at the C-terminal end of β -GAL which appears to be due to the deficiency in the protective protein. The size difference between the 88 kDa precursor and the 80 kDa species (about 6–8 kDa) is comparable with the size difference between the 50 kDa and 45 kDa forms, indicating that they differ at the C-terminal end, while the constant 18 kDa product in CHO cells, normal and GS fibroblasts must arise from an endoproteolytic cleavage at a site about 18 kDa from the unprocessed N-terminal end of the protein.

We can explain these results by the use of the model shown in Scheme 1. The C-terminal 30 residues in β -GAL constitute the most hydrophilic surface sequence of the entire protein, as judged from computer-derived hydrophilicity predictions, and the specificity and potency of our anti-peptide antibody indicates its accessibility at the enzyme surface. A putative cathepsin B cleavage site occurs at Arg-595, about 82 amino acids upstream from the C-terminal end of the protein, again in a region predicted to occur at or near the surface of the protein. Endoproteolytic cleavage at this site would generate a truncated

precursor of about 80 kDa. Leupeptin, a strong inhibitor of thiol-dependent proteases such as lysosomal cathepsins B, H and S [24], inhibited both the abnormal removal of this C-terminal peptide and the endoproteolytic step leading to the appearance of the 45 kDa and 18 kDa products in GS cells and of the 50 kDa and 18 kDa degradation products in normal cells. In addition, leupeptin normalized the β -GAL activity in case 1 GS cells and substantially improved the total activity in case 2 GS cells. However, leupeptin did not prevent the normal processing of β -GAL into the 67 kDa and 64 kDa mature forms of the enzyme in normal cells, nor did it block the appearance of the 72 kDa intermediate in case 2 GS cells. These results indicate that the protective protein renders cryptic two separate and independent cathepsin cleavage sites in β -GAL. The non-leupeptin-inhibitable conversion to the 72 kDa form in case 2 GS cells and its absence in case 1 GS cells illustrates that different mutations in the protective protein can influence in different ways the extent of β -GAL processing, and reflects the genetic heterogeneity described in the protective protein gene.

Whereas the intact 88 kDa precursor and the mature processed 64 kDa enzyme are fully active [1], it is clear that the 80 kDa and 72 kDa intermediate forms seen in GS cells are catalytically inactive. This suggests that the conformational state of the intermediates is so altered as to cause a disassembly of their active sites and to then render them very sensitive to rapid proteolytic digestion by the resident cathepsins. To circumvent the initial deleterious proteolytic event (i.e. cleavage of the C-terminal 8 kDa), the precursor β -GAL, on acidification of the endosomal compartment, immediately associates with homo-

dimeric protective protein precursor to form the complex [25] leading to productive β -GAL maturation, dimerization and oligomerization [19,20,26]. The role played by neuraminidase in this complex is completely unknown [27]. Specific details of complex-formation remain to be revealed, but it is noteworthy that the C-terminal region of β -GAL comprising residues 653–666 is relatively proline-rich and satisfies many of the criteria of an SH3 domain [28], which serves as a module to facilitate protein–protein interactions. Finally, several missense mutations giving rise to infantile and juvenile GM1-gangliosidosis have been identified in the same C-terminal portion of β -GAL that is lost during post-translational processing, further indicating the pivotal role of this portion in the stable conformation and productive processing of the enzyme [29].

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REFERENCES

- Zhang, S., McCarter, J. D., Okamura-Oho, Y., Yaghi, F., Hinek, A., Withers, S. G. and Callahan, J. W. (1994) *Biochem. J.* **304**, 281–288
- D'Azzo, A., Hoogveen, A., Reuser, A. J. J., Robinson, D. and Galjaard, H. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4535–4539
- Hoogveen, A. T., Reuser, A. J., Kroos, M. and Galjaard, H. (1986) *J. Biol. Chem.* **261**, 5702–5704
- Nanba, E., Tsuji, A., Omura, K. and Suzuki, Y. (1988) *Biochem. Biophys. Res. Commun.* **152**, 794–800
- Yamamoto, Y., Hake, C. A., Martin, B. M., Kretz, K. A., Ahern-Rindell, A. J., Naylor, S. L., Mudd, M. and O'Brien, J. S. (1990) *DNA Cell. Biol.* **9**, 119–127
- D'Azzo, A., Andria, G., Strisciuglio, P. and Galjaard, H. (1995) in *Metabolic Basis of Inherited Disease*, vol. 2 (Scriver, C. R., Beaudet, A., Sly, W. S. and Valle, D., eds.), pp. 2825–2837, McGraw-Hill, New York
- Galjart, N. J., Gillemans, N., Harris, A., Van Der Horst, G. T. J., Verheijen, F. W., Galjaard, H. and D'Azzo, A. (1988) *Cell* **54**, 755–764
- Kase, R., Itoh, K., Takiyama, N., Oshima, A., Sakuraba, H. and Suzuki, Y. (1990) *Biochem. Biophys. Res. Commun.* **172**, 1175–1179
- Jackman, H. L., Tan, F., Tamei, H., Beurling-Harbury, C., Li, X.-Y., Skidgel, R. A. and Erdős, E. G. (1990) *J. Biol. Chem.* **265**, 11265–11272
- Jackman, H. L., Morris, P. W., Deddish, P. A., Skidgel, R. A. and Erdős, E. G. (1992) *J. Biol. Chem.* **267**, 2872–2875
- Galjart, N. J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E. J. and d'Azzo, A. (1991) *J. Biol. Chem.* **266**, 14754–14762
- Callahan, J. W., Okamura-Oho, Y. and Zhang, S. (1994) *Proc. Can. Fed. Biol. Soc.* **37**, 89
- Zhou, X. Y., Galjart, N. J., Willemsen, R., Gillemans, N., Galjaard, H. and D'Azzo, A. (1991) *EMBO J.* **10**, 4041–4048
- Hubbes, M., D'Agrosa, R. M. and Callahan, J. W. (1992) *Biochem. J.* **285**, 827–831
- Hinek, A., Rabinovitch, M., Keeley, F., Okamura-Oho, Y. and Callahan, J. W. (1993) *J. Clin. Invest.* **91**, 1198–1205
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Itoh, K., Takiyama, N., Kase, R., Kondoh, K., Sano, A., Oshima, A., Sakuraba, H. and Suzuki, Y. (1993) *J. Biol. Chem.* **268**, 1180–1186
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Pshezhetsky, A. V. and Potier, M. (1994) *Arch. Biochem. Biophys.* **313**, 64–70
- Pshezhetsky, A. V. and Potier, M. (1993) *Biochem. Biophys. Res. Commun.* **195**, 354–362
- Lodish, H. F. (1988) *J. Biol. Chem.* **263**, 2107–2110
- Hurtley, S. M. and Helenius, A. (1989) *Annu. Rev. Cell Biol.* **5**, 277–307
- Takiyama, N., Itoh, K., Oshima, A., Sakuraba, H. and Suzuki, Y. (1993) *Biochem. Biophys. Res. Commun.* **193**, 526–531
- Dawson, R. M. C., Elliott, D. C., Elliott, W. H. and Jones, K. M. (1986), *Data for Biochemical Research*, pp. 328–329, Oxford Scientific Publications, Oxford
- Pshezhetsky, A. V., Elsliger, M.-A., Vinogradova, M. V. and Potier, M. (1995) *Biochemistry* **34**, 2431–2440
- Pshezhetsky, A. V., Levashov, A. V. and Wiederschain, G. Y. (1992) *Biochim. Biophys. Acta* **1122**, 154–160
- Okamura-Oho, Y., Zhang, S. and Callahan, J. W. (1994) *Biochim. Biophys. Acta* **1225**, 244–254
- Ren, R., Mayer, B. J., Cicchetti, P. and Baltimore, D. (1993) *Science* **259**, 1157–1161
- Suzuki, Y., Sakuraba, H. and Oshima, A. (1995) in *Metabolic Basis of Inherited Disease*, vol. 2 (Scriver, C. R., Beaudet, A., Sly, W. S. and Valle, D., eds.), pp. 2785–2823, McGraw-Hill, New York