Characteristics of the β -galactosidase–carboxypeptidase complex in G_{M1} -gangliosidosis and β -galactosialidosis fibroblasts

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Lysosomal β -galactosidase (β -Gal) occurs either alone in monomeric and dimeric forms, or in a high- M_r complex with at least two additional proteins. One is neuraminidase and the second is the protective protein, which has also been shown to possess carboxypeptidase activity. β -Gal activity is deficient in G_{M1} -gangliosidosis as a primary defect, and is secondarily affected in galactosialidosis (GS), where the primary defect is the absence of protective protein activity. Fibroblasts from three patients with G_{M1} -gangliosidosis, type 1, showed markedly reduced amounts of β -Gal cross-reacting material (CRM), and a fourth appeared to have normal levels. A patient with type 2 G_{M1} -gangliosidosis was also found to be CRM-normal. These findings demonstrate that patients with G_{M1} -gangliosidosis type 1 are heterogeneous with respect to the level of residual β -Gal protein. Fibroblasts from four patients with GS were strongly CRM-positive with an anti- β -Gal antibody, as was a sample of brain from one of these patients, suggesting that the loss of β -Gal activity is linked to a subtler change in the primary structure of the enzyme than has been previously thought. While three GS cell lines displayed reduced carboxypeptidase activity (to 32–42% of the control), one cell line was completely devoid of activity, demonstrating that while carboxypeptidase activity is a property of the protective protein this action is distinct and separate from its protective role. On direct immunoprecipitation with anti- β -Gal antibody, a portion of the total carboxypeptidase activity co-precipitated with β -Gal from extracts of normal and G_{M1} -gangliosidosis cells, consistent with the presence of the complex in these cells. However, no carboxypeptidase activity was precipitable with this antibody from GS fibroblasts, suggesting the absence of complex from these cells. To examine this further, the various forms of β -Gal were resolved by h.p.l.c. molecular-sieve chromatography. Three forms of β -Gal activity were resolved in normal cells: a complex, a dimer and a monomer. Residual β -Gal activity of GS cells resolved into two of these forms, the complex and the monomer. In normal and G_{M1} -gangliosidosis cells a portion of the total carboxypeptidase activity co-chromatographed with the complex while the bulk of the activity occurred in a single 36000- M_r peak. Only the low- M_r carboxypeptidase activity was detected in GS cells. This confirms our results on immunoprecipitation indicating that portions of the β -Gal and the carboxypeptidase activities exist outside the complex in normal, G_{M1} -gangliosidosis and GS cells. In summary, the loss of protective protein function from GS cells results in disproportionate loss of the dimeric and monomeric forms of β -Gal activity, but does not result in the complete degradation of the protein.

INTRODUCTION

Activity of lysosomal β -galactosidase (β -Gal; EC 3.2.1.23) is markedly decreased in G_{M1} -gangliosidosis, a disease characterized by the neuronal accumulation of G_{M1} -ganglioside, and in Morquio disease, type B, characterized by the accumulation of keratan sulphate. G_{M1} -gangliosidosis is clinically heterogeneous and occurs in infantile (type 1), late infantile (type 2) and juvenile (type 3) forms (for a review, see O'Brien, 1989). Early immunological work on G_{M1} -gangliosidosis suggested the presence of normal amounts of β -Gal polypeptide in tissues, but recent reports have suggested that G_{M1} -gangliosidosis fibroblasts synthesize an 85000- M_r β -Gal precursor that is not processed normally to the mature 64000- M_r form (O'Brien, 1989). The defect in Morquio B does not prevent normal synthesis of the 85000- M_r precursor and normal processing to the 64000- M_r polypeptide (Hoogetveen *et al.*, 1984).

Galactosialidosis (GS) also occurs in at least three clinical phenotypes: an early infantile form where abnormalities can be detected *in utero* (Kleijer *et al.*, 1979); a late infantile form characterized by bony abnormalities, macular cherry-red spot and mild mental retardation (Strisciuglio *et al.*, 1988); and a

juvenile–adult form with variable age of onset of bony abnormalities, corneal clouding, macular cherry-red spot, neurological manifestations and mental retardation (Nanba *et al.*, 1987; Chitayat *et al.*, 1988). All forms of GS are characterized by storage and increased urinary excretion of galactoconjugates and sialyloligosaccharides. GS fibroblasts show a marked reduction in β -Gal activity, and virtually no neuraminidase activity. However, obligate heterozygotes appear normal (Tsuiji *et al.*, 1984).

Lysosomal β -Gal occurs in at least two forms: as a dimeric enzyme (M_r 150000; containing two 64000- M_r monomers) and as a high- M_r form (> 600000). In some tissues a 64000–70000- M_r active monomer has been reported. The high- M_r form of β -Gal is in reality a complex containing at least two additional proteins, one of which is neuraminidase (EC 3.2.1.18) with a polypeptide of M_r 66000 (Van der Horst *et al.*, 1989). The second is protective protein (native M_r 54000; reduced M_r values 32000 and 20000) which gets its name from its ability to 'protect' β -Gal and neuraminidase from lysosomal proteolytic digestion. Galjart *et al.* (1988, 1990) postulated that this protein was in reality a carboxypeptidase-type of serine protease. D'Azzo (1990) showed, by site-directed mutagenesis, that the protective function of the protein was separate from its carboxypeptidolytic action.

Abbreviations used: β -Gal, β -galactosidase; GS, galactosialidosis; CRM, cross-reacting material; MEM, minimum essential medium; FCS, foetal calf serum; PBS, phosphate-buffered saline (10 mM-phosphate–0.15 M-NaCl); 4-Mu-NeuAc; 4-methylumbelliferyl *N*-acetylneuraminate; Cbz, benzyloxycarbonyl; TBS, Tris-buffered saline; HRP, horseradish peroxidase; DTT, dithiothreitol; NP-40, Nonidet P-40.

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It has been proposed that protective protein plays a pivotal role in formation of a complex that affords protection for β -Gal and neuraminidase against intralysosomal proteolysis. In the absence of the functional protein, it has been suggested that both β -Gal and neuraminidase are degraded at an accelerated rate giving the low- β -Gal, low-neuraminidase phenotype.

In the present work we examine the level of β -Gal cross-reacting material (CRM) and the different forms of the enzyme in normal fibroblasts and from patients with G_{M1} -gangliosidosis and GS. We show that a portion of the total carboxypeptidase activity co-precipitates with β -Gal upon direct immunoprecipitation from normal and G_{M1} -gangliosidosis cells, while in GS cells carboxypeptidase activity cannot be detected in β -Gal immunoprecipitates. Molecular-sieve h.p.l.c. resolves three peaks of β -Gal activity and two peaks of carboxypeptidase in normal cells. A high- M_r form of β -Gal activity was also detected at a reduced level in GS cells but, in contrast to normal cells, no carboxypeptidase activity was found in this fraction.

MATERIALS AND METHODS

Cell lines and culture conditions

Cell strains with the type 1 G_{M1} -gangliosidosis phenotype included two local patients, 1331 (patient CL) and 2304 (patient BJ), and patients 1946 (patient BK; Dr. D. Whelan, Hamilton, Ontario, Canada), and 3045 (patient CG; Dr. Derek Applegarth, Vancouver, British Columbia, Canada). Strain 300 was from a type 2 patient (HS; Lowden *et al.*, 1974). GS cell lines included strain nos. 1104 (BK, infantile form; a sample of brain from this patient was also examined), 1749 (JS, infantile form; Dr. D. Whelan), 2870 [AW, juvenile form; Dr. Derek Applegarth, Vancouver, B.C. (Chitayat *et al.*, 1988)] and 3065 [CM, infantile form; Dr. Michel Potier, Montreal, Quebec (Tranchemontagne *et al.*, 1990)]. The designation of these patients was indicated on the basis of clinical history, and the presence in urine of galactose-terminal and sialic acid-terminal oligosaccharides respectively. Cells were propagated in minimum essential medium (α -MEM)/15% foetal calf serum (FCS) at 37 °C and 5% CO₂ until confluent by using standard cell-culture techniques. At confluency, the monolayer was washed twice with 10 ml of 10 mM-phosphate-0.15 M-NaCl (PBS, pH 7.5). Further PBS (2 ml) was then added and the monolayer was scraped using a rubber policeman. The cell suspension, diluted to 15 ml with PBS, was centrifuged at 2000 rev./min (1100 *g*) for 10 min, and the resuspended cell pellet was transferred in 1 ml of PBS to an Eppendorf tube. The cell pellet obtained after centrifugation was either homogenized immediately or frozen at -70 °C. Homogenates were prepared by sonication for 15 s at 20 W in PBS.

Analytical assays

β -Gal activity was measured in an assay mixture (0.25 ml) containing 50 mM-citrate-100 mM-phosphate buffer (pH 4.3), 4-methylumbelliferyl β -D-galactopyranoside as substrate (0.5 mM), water and enzyme (Jones *et al.*, 1984). Incubations were for 30 min at 37 °C. For neuraminidase (O'Brien & Warner, 1980) the assay mixture contained 10 μ l of 3.96 mM-4-methylumbelliferyl *N*-acetyl neuramate (4MU-NeuAc), 10 μ l of 0.5 M-acetate, pH 4.3, and 10 μ l of enzyme. Incubations were for 15 min at 37 °C. Hexosaminidase assays (O'Dowd *et al.*, 1986) contained 100 μ l of substrate (3 mM in 0.1 M-citrate-phosphate buffer, pH 4.5), and 100 μ l of enzyme/water were mixed and incubated at 37 °C for 30 min. All the above reactions were stopped by the addition of 2 ml of 0.1 M-2-methyl-2-amino-1-propanol (pH 10.4). Fluorescence was measured with a Turner spectrofluorimeter at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Carboxypeptidase activity was

measured at a final volume of 0.2 ml with 4 mM-*N*-Cbz-Phe-Ala and 20 mM-acetate buffer, pH 5.5. Alanine release was measured by the fluorescamine reaction. Preliminary studies in fibroblast extracts and placental preparations indicated that the reaction was linear with time and protein concentration, and that the enzyme displayed a pH optimum of 5.5 and a K_m value of 2 mM. Protein concentrations were measured as described by Lowry *et al.* (1951), with BSA as standard.

Antisera against β -Gal

Antisera were raised in two New Zealand rabbits. Each was immunized with an initial intramuscular injection with 150 μ g of homogeneous β -Gal coupled to KA1(SO₄)₂ (Williams & Chase, 1967) followed every 2 weeks by injection of 30-50 μ g of homogeneous β -Gal suspended in 100 μ l of Freund's incomplete adjuvant. Blood was allowed to clot at 4 °C and sera were collected and stored at -70 °C until used. The antisera were characterized extensively (Hubbes *et al.*, 1992).

Immunoprecipitation of β -Gal activity

Fibroblast extracts, containing known amounts of β -Gal activity and carboxypeptidase activity, were incubated for 4 h at 4 °C in the presence of antisera and were further incubated in the presence of an excess of protein A-Sepharose for 12 h at 4 °C. The mixtures were centrifuged (12000 *g* for 10 min) and the supernatants and immunoprecipitates were assayed for both β -Gal and carboxypeptidase activity.

PAGE and Western blots

SDS/PAGE was carried out according to the method of Laemmli (1970) by using a 16 cm $\times$ 18 cm $\times$ 1.5 mm slab gel apparatus (Bio-Rad) or a 8 cm $\times$ 10 cm $\times$ 0.3 mm Minigel apparatus (Bio-Rad). Samples were denatured in sample buffer [0.625 M-Tris/HCl, pH 6.8, 20% glycerol, 50 mM-DTT, 3% (w/v) SDS and 0.001% Bromophenol Blue] by heating to 100 °C for 10 min. The following M_r standards were used: phosphorylase *b* (M_r 94000), BSA (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), trypsin inhibitor (M_r 20100), and RNAase (M_r 14400). Gels were stained using either silver stain (Bio-Rad kit) or Coomassie Brilliant Blue R250.

Proteins were transferred from the unfixed gel on to the nitrocellulose (Towbin *et al.*, 1979). The unbound sites on the nitrocellulose were blocked with 0.25% gelatin in Tris-buffered saline (TBS) (0.15 M-NaCl, 50 mM-Tris buffer, pH 7.4) for 4 h and then incubated overnight at room temperature with 1/500 diluted antiserum in a solution of blot buffer [TBS containing 0.25% gelatin, 0.05% Nonidet P-40 (NP-40) and 5 mM-EDTA]. The nitrocellulose was washed for 4 h in blot buffer (three changes) and then incubated overnight in a 1:1000 dilution of horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG. The blot was washed with several changes of blot buffer for 4 h and then incubated for 1 h in a 1:100 dilution of peroxidase-anti-peroxidase soluble complex. The blot was then washed twice in blot buffer for 5 min and once briefly in TBS. Substrate, which consisted of two freshly mixed solutions (60 mg of 4-chloro-1-naphthol in 20 ml of ice-cold methanol and 60 μ l of 30% H₂O₂ in 100 ml of TBS) was added, and colour development was stopped by washing with distilled water. Separate lanes containing protein standards were cut into strips, stained with Coomassie Blue, and destained in 50% methanol-5% acetic acid.

H.p.l.c. gel filtration and analysis

Cell supernatants were chromatographed on two TSK 3000 molecular-sieve columns in series (Altex Spherogel, 7.5 mm internal diam. $\times$ 30 cm) equilibrated with 0.1 M-phosphate/0.15 M-

NaCl buffer, at pH 6.0 (Hubbes *et al.*, 1992). All samples were injected using a 250 μ l loop (Beckmann). The columns were eluted at a flow rate of 0.5 ml/min and fractions were collected every 1 min. Aliquots (20 μ l) were assayed for β -Gal and carboxypeptidase activity as described. Hexosaminidase activity served as an internal control. The standards used to calibrate the columns included Blue Dextran, thyroglobulin (M_r 669000), ferritin (M_r 440000), catalase (M_r 232000), aldolase (M_r 158000), BSA (M_r 68000), chymotrypsinogen A (M_r 25000) and vitamin B₁₂. The results were plotted and the area under each activity peak was calculated using the Curves program and a MacIntosh computer.

Fractions containing enzyme activity were pooled (about 1.5 ml per pool) and dialysed overnight against a 1:60 dilution of sample buffer, containing no dithiothreitol (DTT). The sac contents were freeze-dried, dissolved in 0.025 ml of water containing 50 mM-DTT, and subjected to Western blot analysis as indicated above.

RESULTS

Enzymic characterization of G_{M1}-gangliosidosis and GS fibroblasts

G_{M1}-gangliosidosis fibroblasts gave extremely low β -Gal activity in type 1 lines, and approx. 4% of normal residual activity in the type 2 line examined. These lines had normal levels of neuraminidase (Table 1). GS fibroblasts showed the expected 75–90% reduction in β -Gal activity, and no detectable neuraminidase activity (Table 1). Hexosaminidase activity was within normal limits in all cell lines (results not shown). Carboxypeptidase activity was reduced to 32–42% of the normal level in three out of the four GS cell lines, while in one line this enzyme activity was completely absent (Table 2). We verified these cell lines as being different from the G_{M1}-gangliosidosis cell lines by incubating them in the presence of the lysosomotropic agent 10 mM-NH₄Cl. Detectable levels of β -Gal activity were found in the medium above G_{M1}-gangliosidosis type 1 and type 2 cells, while neuraminidase activity was at 158% of the control value. However, medium above the GS cells showed an average β -Gal

activity of 129% and an average neuraminidase activity of 162% of the control values. These data confirm a primary abnormality in β -Gal in the G_{M1} gangliosidosis cells and show the deficiencies in β -Gal and neuraminidase in the GS cells to be secondary to another defect, presumed to be in the protective protein. These data are consistent with those of others (Palmeri *et al.*, 1986; D'Azzo *et al.*, 1982).

β -Gal immunoreactivity in G_{M1}-gangliosidosis and GS fibroblasts

With the anti- β -Gal antiserum and Western blot analysis, normal fibroblasts displayed two closely migrating bands, in the range 64000–67000- M_r . The occurrence of two components, by analogy to the findings with hexosaminidase B (Mahuran *et al.*, 1988), were presumed to reflect altered amounts of *N*-linked oligosaccharides attached to the mature form of β -Gal. Fibroblasts from three patients with type 1 G_{M1}-gangliosidosis showed significant reductions in both the 64000- and 67000- M_r proteins (Figs. 1a and 1b), while in a fourth type 1 cell line and the line from the type 2 G_{M1}-gangliosidosis patient (Figs. 1a and 1b), both components were present in near-normal amounts. Two patients with the adult form of G_{M1}-gangliosidosis also showed normal levels of the two immunoreactive components seen in normal cells (J. W. Callahan & H. Taylor, unpublished work). Western blot analysis of four GS cell lines consistently gave levels of the two β -Gal components similar to that seen in the normal and type 2-gangliosidosis G_{M1} lines (Fig. 1c). A brain extract from one of these GS patients gave a similar result (Fig. 1b). In another experiment where only two of the lines were examined, additional immunoreactive bands of slightly lower M_r (59000–64000) were noticed in the GS lines but not in the normal control, suggestive of partial breakdown of the two β -Gal proteins. Western blot analysis of different subcultures of these GS cells consistently failed to show a marked diminution in the amount of β -Gal polypeptides. These data are inconsistent with the results of pulse-chase experiments (D'Azzo *et al.*, 1982; Palmeri *et al.*, 1986).

Multiple forms of β -Gal and carboxypeptidase in G_{M1}-gangliosidosis and GS fibroblasts

Since there were near-normal amounts of β -Gal CRM in all of the GS fibroblasts we examined, it was of interest to determine what proportion of the enzyme occurred in the high- M_r form, the presumptive complex with neuraminidase and protective protein. This was examined in two ways, by direct immunoprecipitation and by molecular-sieve chromatography. Extracts from normal, G_{M1}-gangliosidosis and GS cells were incubated with antiserum and immune complexes precipitated with Protein A-Sepharose beads were assayed directly for both β -Gal and carboxypeptidase activities (Table 2). In the G_{M1}-gangliosidosis and GS cells all the residual β -Gal activity was precipitated. However, whereas immunoprecipitation of β -Gal activity was accompanied by carboxypeptidase activity in normal and G_{M1}-gangliosidosis cell extracts, no carboxypeptidase activity could be detected in the immunoprecipitates from the GS cells (Table 2). All the carboxypeptidase activity detected remained in the supernatant.

These forms of β -Gal activity, the complex (M_r 780000), dimeric β -Gal (M_r 134000) and monomeric β -Gal (M_r 56000), were resolved in the relative proportions 12:42:44 by molecular-sieve h.p.l.c. of normal fibroblast supernatants. About 20% of the carboxypeptidase activity chromatographed with the high- M_r form in control cells, while the bulk of the carboxypeptidase activity eluted later than the β -Gal monomer, with a calculated M_r of 36000. Comparable analyses of one type 1 G_{M1}-gangliosidosis and two GS fibroblast stains were performed to determine the number and size of β -Gal species (Fig. 2). The G_{M1}

Table 1. Enzyme activities in fibroblasts from patients with G_{M1}-gangliosidosis and GS

Cells were homogenized as described in the Materials and methods section. All values represent the average of assays run in triplicate. ND, not detectable.

Cell line	Specific activity (nmol/h per mg)	
	β -Gal	Neuraminidase
Control 1	300	16
2	260	14
G _{M1} , type 1		
Line 1331	ND	12
Line 1946	ND	15
Line 2304	ND	18
Line 3045	1.2	16
G _{M1} , type 2		
Line 300	10	15
GS		
Line 1104	28	ND
Line 1749	55	ND
Line 2870	35	ND
Line 3065	65	ND

Table 2. Immunoprecipitation of β -Gal and carboxypeptidase

Supernatants from cell homogenates containing measured amounts of β -Gal and carboxypeptidase activity were incubated with immune serum for 4 h, and immune complexes were precipitated with Protein A-Sepharose. The supernatant and precipitated enzyme activities were then determined. The specific activities for β -Gal and carboxypeptidase (Carb Y) in the control sample were 925 and 5075 nmol/h per mg of protein respectively. ND, not detectable.

Cell line	Relative specific activity (% of control)		Units precipitated (nmol/h)	
	β -Gal	Carb Y	β -Gal	Carb Y
Normal (1685)	100	100	42	96
G _{M1} , type 1 (3045)	2.2	28.6	1.6	48
(1946)	ND	58.6	ND	64
G _{M1} , type 2	5.0	94.6	4.1	96
GS				
(1104)	8.4	39.4	6.0	ND
(1749)	9.8	42.9	3.3	ND
(2870)	13.3	31.5	1.3	ND
(3065)	17.6	ND	5.3	ND

Fig. 1. Western blot of β -Gal in G_{M1}-gangliosidosis and GS fibroblasts

(a) Supernatants (containing 200 μ g of protein) of fibroblast homogenates were subjected to SDS/12.5%-PAGE and blotted for Western analysis using the anti- β -Gal antisera. Lanes: 1, G_{M1} (type 1, line 2304); 2, G_{M1} (type 2, line 300); 3, G_{M1} (type 1, line 1331); 4, G_{M1} (type 1, line 1946); 5, control (line 1685). (b) Supernatants (containing 100 μ g of protein) of fibroblast homogenates were subjected to SDS/12.5%-PAGE and blotted for Western analysis using the anti- β -Gal antisera. Lanes: 1, brain from GS patient LK; 2, G_{M1} (type 1, line 3045); 3, G_{M1} (type 1, line 2304); 4, G_{M1} (type 2, line 300); 5, control (line 1685). (c) Supernatants (containing 100 μ g of protein) of fibroblast homogenates were subjected to SDS/12.5%-PAGE and blotted for Western analysis using the anti- β -Gal antisera. Lanes: 1, GS line 1104 (BK); 2, GS line 1749 (JS); 3, ES line 2870 (AW); 4, GS line 3065 (CM). A control was not included on this gel, but the patterns are identical with those of controls and with those obtained in other runs where both controls and GS lines were run simultaneously.

line displayed the same bimodal carboxypeptidase pattern as seen in the control, but little or no β -Gal activity was detectable. Analysis of two GS cell lines revealed two peaks of residual β -Gal activity, one corresponding to the high- M_r form (780 000) and the second eluting in the range of the β -Gal monomer (56 000–60 000). Hexosaminidase activity monitored as an

Fig. 2. H.p.l.c. molecular-sieve analysis of β -Gal and carboxypeptidase activities in fibroblasts

Supernatants (200 μ g of protein) were subjected to h.p.l.c. gel filtration on two TSK 3000 columns as described in the Materials and methods section. Each fraction was assayed for β -Gal activity, while carboxypeptidase activity was assayed on every other fraction. The control (a) was line 1685; the GS lines were 1749 (GS, case 2) (b) and 2870 (GS, case 3) (c), and the G_{M1}-gangliosidosis line (d) was 1331. In (a)–(c) the scale on the left axis describes the activity of β -Gal directly and that of carboxypeptidase divided by a factor of 10 (a, b) or 100 (c). The scale on the left axis in (d) describes carboxypeptidase activity directly, there being little or detectable β -Gal activity. $\square$, β -Gal; $\blacksquare$, carboxypeptidase Y.

internal control eluted at a point intermediate between these two peaks consistent with its M_r (110 000). No evidence for the intermediate, dimeric form of β -Gal (predicted M_r 134 000) was obtained in either cell line. A single peak of carboxypeptidase activity corresponding to the low- M_r form (M_r 36 000) was noted in GS cells, but no high- M_r form of the enzyme was detectable. Supernatants from G_{M1} cells (cell line 1331) and GS cells (line 3065) were mixed, incubated on ice for 30 min, and subjected to h.p.l.c. as described in the Materials and methods section. There were no differences in the profiles compared with the cells alone, and in particular no dimer form of β -Gal was noted. Portions of the h.p.l.c. profile corresponding to the positions expected to contain complex, dimer and monomer forms of β -Gal were subjected to Western blot analysis (Fig. 3). β -Gal CRM was detected in fractions corresponding to all three M_r forms in control cells. In G_{M1}-gangliosidosis cells, CRM was detected, particularly in the complex form, with lower amounts of material seen in the lower- M_r fractions, even though no enzyme activity was detectable anywhere in the gradient. The distribution of CRM in the two GS lines tested was indistinguishable from the control, in spite of the reduced amount of enzyme activity and the apparent absence of β -Gal activity in the M_r range corresponding to the intermediate, dimeric form of the enzyme (Fig. 2).

Fig. 3. Western blot of multiple forms of β -Gal separated by h.p.l.c.

Fractions corresponding to the monomer, dimer and complex forms of β -Gal from Fig. 2 were pooled separately, and processed for Western blotting analysis as described. (a) Lanes 1–3 represent fractions taken from the complex, dimer and monomer areas respectively from control line 1685; lanes 4–6 represent fractions taken from the complex, dimer and monomer areas respectively from G_{M1} -type 1, line 1331. (b) Lanes 1–3 represent fractions taken from the complex, dimer and monomer areas respectively from GS line 2870; lanes 4–6 represent fractions taken from the complex, dimer and monomer areas respectively from GS line 1749. The M_r values of standard marker proteins are indicated by arrowheads.

DISCUSSION

We have demonstrated a reduction in the amount of mature β -Gal protein in fibroblasts from three type 1 G_{M1} -gangliosidosis patients, and near-normal levels of the protein in the cells of a fourth type 1 patient and of a type 2 patient. Some workers (for a review, see O'Brien, 1989) reported apparently normal amounts of β -Gal CRM in G_{M1} -gangliosidosis cells and tissues. It is possible that patients were distinct from those studied here, and direct analysis of the specific mutations should resolve this question. A recent report by Yoshida *et al.* (1991) documents the heterogeneity in this disease and predicts the occurrence of both CRM-normal and CRM-deficient patients. Other, better characterized, lysosomal-storage diseases have also been shown to be caused by multiple allelic mutations (Neufeld, 1991).

Normal levels of β -Gal CRM were found in fibroblasts from four GS patients, three with early onset of the disease, and one with the juvenile/adult phenotype (Gravel *et al.*, 1979; Chitayat *et al.*, 1988). These were from definite cases of GS as indicated by clinical histories, the presence of urinary sialyloligosaccharides, the level of residual β -Gal and neuraminidase activities in the fibroblasts, and the elevation in these enzymes in media above cells incubated with the lysosomotropic agent NH_4Cl (Chitayat *et al.*, 1988; Tranchemontagne *et al.*, 1990). Previous workers (Hoogveen *et al.*, 1983, 1984, 1986; Palmeri *et al.*, 1986) have presented evidence for a reduction in β -Gal immunoreactivity in GS fibroblasts by using pulse-chase labelling techniques, but the antiserum used in these studies was not monospecific for β -Gal, since several non- β -Gal proteins could be seen on autoradiographs. To our knowledge there have been no reports on steady-state levels of the enzyme in GS cells using direct immunoprecipitation or Western blots. It is possible that the inactivation of β -Gal in GS cells may involve the loss of a primary antigenic site from the native folded β -Gal protein. Thus immunoprecipitation of the native enzyme in pulse-chase experiments may have been incomplete. On the other hand, Western blotting involves antibody interactions with denatured proteins where antigenic determinants tend to be linear sequences of the protein's primary structure.

We were surprised to note no detectable carboxypeptidase activity in one GS fibroblast cell line while the other three strains showed levels of activity intermediate between this cell line and the control value. Tranchemontagne *et al.* (1990) reported complete deficiency of carboxypeptidase activity in GS cell lines, but they used a different substrate, *N*-Cbz-Phe-Leu. The one utilized here, *N*-Cbz-Phe-Ala, was chosen based on studies of yeast carboxypeptidase Y which has a range of activities towards a variety of synthetic substrates (Hayashi & Hata, 1972; Fischer

& Holzer, 1980). The complete absence of carboxypeptidase activity in one GS cell line and the high level of this activity in the other GS lines, albeit lower than in the control, are consistent with the report of D'Azzo (1990) who suggested, on using site-directed mutagenesis techniques, that a functional separation exists in the protective protein between its protease action and its 'protective' functions.

Immunoprecipitation of β -Gal from normal and G_{M1} -gangliosidosis cells resulted in the co-precipitation of 20–25 % of the total carboxypeptidase activity, approximately the same amount as found in the high- M_r complex form by h.p.l.c. analysis by both normal and G_{M1} fibroblasts. This is comparable with the amount of this macromolecular fraction identified in placenta (Hubbes *et al.*, 1992). However, although the anti- β -Gal antibody had the capability of precipitating carboxypeptidase associated with the complex from normal and G_{M1} -gangliosidosis cells, no carboxypeptidase activity was co-precipitated with β -Gal activity in the GS cell lines, indicating lack of functional association of the protective protein with the complex. The presence of a high- M_r form of β -Gal, albeit in lower than normal amounts, in GS cells suggests that protective protein is not solely responsible for the formation or stability of the macromolecular form of β -Gal, and that neuraminidase may play some role in this process. Furthermore, the presence of apparently normal amounts of carboxypeptidase in the high- M_r complex in type 1 G_{M1} gangliosidosis cells, in spite of the reduced amount of β -Gal activity, suggests that active β -Gal is not an absolute requirement for the stability of the complex and that the small amounts of β -Gal protein remaining in the type 1 G_{M1} cells are sufficient to contribute to the complex-formation process. The h.p.l.c. pattern for residual β -Gal activity in GS cells suggests that one of the forms of β -Gal in normal fibroblasts, the dimer, is not active in these cells, but Western blots demonstrated near-normal amounts of CRM throughout the elution profile, consistent with the Western blots of crude cell supernatants.

It is clear from the present data that there is a normal amount of β -Gal protein in GS cells with approximately the same polypeptide M_r as the normal enzyme. It is uncertain if the residual β -Gal activity reflects a small pool of fully active enzyme along with a larger pool of inactive enzyme, or whether the entire pool has undergone a processing event resulting in diminished catalytic capacity. Examination of the h.p.l.c. profiles indicates that there is loss of all forms of β -Gal activity, but particularly the monomer and dimer species, suggesting that it is these forms that are most susceptible to proteolytic degradation. The only known abnormality in GS cells can be ascribed to a protein that both 'protects' β -Gal by binding to it and is capable of hydrolysing the C-terminus of proteins, including β -Gal. How-

ever, since it is necessary for normal trafficking of the enzyme (Lodish, 1988) that it exist as a dimer [as shown in the placenta by Hubbes *et al.* (1992)] when released into the endosome-lysosome compartment, it also follows that the β -Gal dimer would have an unprocessed C-terminal region, evidence for which we have obtained by using an anti-C-terminal antibody (R. M. D'Agrosa & J. W. Callahan: unpublished work). We therefore suggest that the protective protein functions by binding to but not hydrolysing the C-terminus of β -Gal, protecting this area from proteolytic digestion. Our results also demonstrate that this catalytic activity is independent of its protective function. We conclude that the mutation in the protective protein in GS cells results in C-terminal processing of β -Gal, either rendering the enzyme catalytically inactive or changing its substrate-binding capability, but without resulting in its complete proteolysis. The C-terminus of *Escherichia coli* β -Gal is important for catalytic activity, since deletion of the terminal 10 residues results in normal amounts of an inactive and insoluble enzyme (Mandecki *et al.*, 1981; Fowler & Zabin, 1983). The C-terminal domain of human β -Gal is predicted to occur at the surface of the protein, thus making it a likely antigenic site in the native protein. It is also noteworthy that residues 8–11 from the C-terminal Val of human β -Gal show a putative endopeptidase cleavage sequence, Gln-Lys-Asn-Lys, which is remarkably similar to proven endopeptidase cleavage points (Arg-Gln-Asn-Lys in the human and Gln-Lys-Thr-Lys in the mouse) which result in the formation of the β_a and β_b peptides from the β -chain in lysosomal hexosaminidases (Mahuran *et al.*, 1988; Mahuran, 1990). We speculate that loss of β -Gal activity in GS cells is due to cleavage at the Gln-Lys-Asn-Lys sequence, a site normally bound by protective protein and thus rendered cryptic to the action of endopeptidase. Further investigation is required to provide direct evidence for this hypothesis.

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