Characterization of β -galactosidase mutations Asp³³² → Asn and Arg¹⁴⁸ → Ser, and a polymorphism, Ser⁵³² → Gly, in a case of G_{M1} gangliosidosis

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We have identified and characterized three missense mutations in a patient with type I G_{M1} gangliosidosis, namely a substitution of G for A at nucleotide position 1044 (G1044 → A; in exon 10) on one allele, which converts Asp³³² into asparagine, and both a mutation (C492 → A in exon 4, leading to the amino acid change of Arg¹⁴⁸ → Ser) and a polymorphism (A1644 → G in exon 15, leading to a change of Ser⁵³² → Gly) on the other allele. This patient had less than 1 % residual β -galactosidase activity and minimally detectable levels of immunoreactive β -galactosidase protein in fibroblasts. To account for the above findings, a series of expression and immunolocalization studies were undertaken to assess the impact of each mutation. Transient overexpression in COS-1 cells of cDNAs encoding Asp³³²Asn, Arg¹⁴⁸Ser and Ser⁵³²Gly mutant β -galactosidases produced abundant amounts of precursor β -galactosidase, with activities of 0, 84 and 81 % compared with the cDNA clone for wild-type β -galactosidase (GP8). Since the level of vector-driven expression is much less in Chinese hamster ovary (CHO) cells than in COS-1 cells, and we knew that exogenous β -galactosidase undergoes lysosomal processing when expressed in these cells, transient expression studies were performed of Arg¹⁴⁸Ser and Ser⁵³²Gly, which yielded active forms of the enzyme. In this case, the Arg¹⁴⁸Ser and Ser⁵³²Gly products gave rise to 11 % and 86 % of the control activity respectively. These results were not unexpected, since the Arg¹⁴⁸Ser mutation introduced a major conformational change into the protein, and we anticipated that it would be degraded in the endoplasmic reticulum (ER), whereas the polymorphism was expected to produce near-normal activity. To examine the effect of the Asp³³²Asn mutation on the catalytic activity, we isolated CHO clones permanently transfected with the Asp³³²Asn and Asp³³²Glu constructs, purified the enzymes by substrate-analogue-affinity chromatography, and determined their kinetic

parameters. The $V_{\max}$ values of both mutant recombinant enzymes were markedly reduced (less than 0.9 % of the control), and the K_m values were unchanged compared with the corresponding wild-type enzyme isolated at the same time. Both the Arg¹⁴⁸Ser β -galactosidase in CHO cells and Asp³³²Asn β -galactosidases (in COS-1 and CHO cells) produced abundant immunoreaction in the perinuclear area, consistent with localization in the ER. A low amount was detected in lysosomes. Incubation of patient fibroblasts in the presence of leupeptin, which reduces the rate of degradation of lysosomal β -galactosidase by thiol proteases, had no effect on residual enzyme activity, and immunostaining was again detected largely in the perinuclear area (localized to the ER) with much lower amounts in the lysosomes. In summary, the Arg¹⁴⁸Ser mutation has no effect on catalytic activity, whereas the Asp³³²Asn mutation seriously reduces catalytic activity, suggesting that Asp³³² might play a role in the active site. Immunofluorescence studies indicate the expressed mutant proteins with Arg¹⁴⁸Ser and Asp³³²Asn mutations are held up in the ER, where they are probably degraded, resulting in only minimum amounts of the enzyme becoming localized in the lysosomes. These results are completely consistent with findings in the cultured fibroblasts. Our results imply that most of the missense mutations described in G_{M1} gangliosidosis to date have little effect on catalytic activity, but do affect protein conformation such that the resulting protein cannot be transported out of the ER and fails to arrive in the lysosome. This accounts for the minimal amounts of enzyme protein and activity seen in most G_{M1} gangliosidosis patient fibroblasts.

Key words: active site, gene expression, immunofluorescence, polymorphisms, protein folding.

INTRODUCTION

A deficiency of human lysosomal β -galactosidase activity is the primary defect in patients with the autosomal recessive storage diseases, G_{M1} gangliosidosis, and Morquio disease, type B [1]. In G_{M1} gangliosidosis, G_{M1} ganglioside accumulates in the neurons of the central nervous system. G_{M1} patients have a marked

deficiency (0–3 % of normal) of lysosomal β -galactosidase activity [2], accounting for the neuronal and visceral organ storage of galactose-terminal substances. Morquio disease type B patients are neurologically normal, do not store G_{M1} ganglioside, but display severe skeletal dysostosis multiplex because of an accumulation of keratan sulphate, a glycosaminoglycan, characterized by poly(galactose-*N*-acetylglucosamine)-repeating units

Abbreviations used: CAT, chloramphenicol acetyltransferase; CHO, chinese hamster ovary; COS-1, African green monkey kidney cells; ER, endoplasmic reticulum; GP8, cDNA clone for wild-type β -galactosidase; poly(A)⁺, polyadenylated; SSCP, single-strand conformation polymorphism; SSPE, sodium chloride–sodium phosphate–EDTA.

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with sulphate attached at the 6-position of both sugars. While Morquio type B patients have been reported to have a higher residual activity (activity 5–10 % of normal) of lysosomal β -galactosidase [3,4] than do G_{M1} gangliosidosis patients (0–3 %), why this should lead solely to the storage of keratan sulphate, and not the other natural substrates of the enzyme, is at present poorly understood.

The β -galactosidase gene, residing on chromosome 3p21.33, encodes a 677-amino-acid prepolypeptide, from which a 23-amino-acid N-terminal signal sequence is removed on entry into the ER to generate a polypeptide precursor of 654 residues [5,6]. When N-glycosylated at most, if not all, of its seven candidate sites, the nascent chain migrates as an 84-kDa propolypeptide and, after obtaining its mannose-phosphate lysosomal tag (whereby the mass as determined by SDS/PAGE increases to 88 kDa), is then rapidly transported to the endosomal-lysosomal compartment [7], where it is proteolytically processed to the mature 64-kDa form of the enzyme. We have previously shown that purified lysosomal β -galactosidase undergoes rapid and irreversible inactivation by 2,4-dinitrophenyl-2-fluoro-2-deoxy- β -D-galactopyranoside, a potent mechanism-based suicide substrate [7]. These results indicate that lysosomal β -galactosidase cleaves substrates by a Koshland double-displacement mechanism, involving two acidic residues; one residue, the catalytic nucleophile, forms a galactosylated-enzyme intermediate. The second acidic residue, the acid-base catalyst, donates a proton for cleavage of the galactosyl amino-acid bond, and extracts a proton from water, allowing attack by the hydroxy ion with release of free β -D-galactose and restoring the enzyme to an active state. Using electrospray ionization mass spectrometry after proteolytic digestion and HPLC separation of peptides, we identified the galactosylated nucleophile as being Glu²⁶⁸ [8]. Glu²⁶⁸ is conserved in the mouse, dog and all other members of Family 35 β -galactosidases defined to date, which includes a variety of plant species (tomato, asparagus, cauliflower, apple, *Aspergillus* and others) [8]. The second acidic residue in the mechanism remains to be identified. The majority of the mutations in the β -galactosidase gene are of the missense type, which are spread uniformly throughout the gene and give rise to G_{M1} gangliosidosis [1]. No mutation involving Glu²⁶⁸ has been described to date, but one mutation in particular, Trp²⁷³Leu, located only five residues from Glu²⁶⁸, has been found regularly in patients with Morquio disease type B [1].

Studies assessing mutations in the β -galactosidase gene for their impact on the expression of the protein in fibroblasts from G_{M1} gangliosidosis patients have been hampered by the general lack of well-characterized antibodies. Although β -galactosidase exits the ER-Golgi compartment and might be secreted from cells as a fully active precursor [7], its stability upon arrival in the lysosome and its productive processing to the mature enzyme are critically dependent on it forming a complex with two other independently synthesized and targeted hydrolases, namely protective protein/Cathepsin A and neuraminidase [9,10]. This renders studies on the impact of mutations more difficult to assess, because the products must survive both the ER 'quality-control' system and a unique lysosomal-processing system [11,12]. In the present work, we have identified Arg¹⁴⁸Ser and Asp³³²Asn mutations in a patient with type 1 G_{M1} gangliosidosis. The Asp³³²Asn mutation results in a catalytically defective enzyme, as judged from expression studies, where conversion of Asp³³² into Asn³³² or into Glu³³² resulted in markedly reduced catalytic activities in the enzymes, suggesting that Asp³³² serves as an acidic active site residue. In addition, the Asp³³²Asn mutant localizes in the ER, suggesting it is also transport-defective. The Arg¹⁴⁸Ser mutation was found on the same allele as a Ser⁵³²Gly

mutation, which has been shown to be a common polymorphism. Arg¹⁴⁸Ser and Ser⁵³²Gly were transiently expressed in both COS-1 and CHO cells, and show that the enzyme containing the Arg¹⁴⁸Ser mutation, although catalytically active, is localized and degraded in the endoplasmic reticulum (ER), whereas the common Ser⁵³²Gly polymorphism results in an active enzyme. A preliminary report on this work has appeared in abstract form [13].

EXPERIMENTAL

Patient information

The proband, case 1, was born by Caesarean section to non-consanguineous parents after a normal pregnancy. Developmental delay, hypotonia, cherry red spots, kyphoscoliosis and hepatosplenomegaly were noted at 6 months of age. He died at 3 years. The urinary oligosaccharide pattern and the level of residual enzyme activity measured with synthetic substrate were typical for G_{M1} gangliosidosis. Fibroblasts from case 2 (patient 1 of [14]) were referred to us by Dr C. A. Rupa and Dr B. Gordon (Children's Psychiatric Research Institute, London, ON, Canada). Cultured fetal fibroblasts (case 3) were available from a case detected prenatally (courtesy of Dr Don Whelan; McMaster University, Hamilton,). These fibroblast strains were established for diagnostic studies, which were subject to informed consent. Cells from a fourth case [case 4; from NIGMS Human Genetic Mutant Cell Repository (GM05652A)] displayed no detectable acid β -galactosidase activity, and normal levels of β -hexosaminidase, β -glucuronidase and neuraminidase were found. Cells from a case of Morquio disease type B, also from the NIGMS Human Genetic Mutant Cell Repository (GM01602), gave a residual activity < 5 % of control values. In general, skin fibroblasts were grown in α -MEM with penicillin (100 units/ml) and streptomycin (100 mg/ml), supplemented with 10 % (v/v) fetal-calf serum at 37 °C in an air/CO₂ (19:1) atmosphere until confluent. These cell cultures were free from mycoplasma contamination, as analysed by a PCR-based method. Origin-defective simian-virus-40-transformed African green monkey kidney cells (COS-1) and Chinese hamster ovary cells (CHO) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

Northern blot analysis

Extraction of polyadenylated [poly(A)⁺] mRNA from five 100-mm plates of confluent fibroblasts was performed by following the Oligotex™ direct mRNA kit (Qiagen, Chatsworth, CA, U.S.A.)-guanidinium isothiocyanate method [15]. Samples of poly(A)⁺ RNA (30 or 50 μ g) were heat-denatured at 65 °C for 30 min, quickly loaded on to a 1 % (w/v) agarose gel, and after electrophoresis were transferred to a nitrocellulose blot. A *SalI*-digested fragment of the full-length β -galactosidase cDNA (called GP8, described in [16], and kindly supplied by Professor Y. Suzuki, International University of Health and Welfare Kita Kanemaru, Otawara, Japan) was used as the β -galactosidase probe, whereas a Cox6a cDNA fragment (derived from cytochrome oxidase protein, 6a) was used as an internal control. Both were labelled with ³²P using the random-primer-labelling system (Life Technologies, Rockville, MD, U.S.A.). Pre-hybridization of the membrane was performed at 42 °C in 50 % (v/v) formamide, 5 $\times$ sodium chloride-sodium phosphate-EDTA (SSPE), 0.1 % (w/v) SDS, 5 $\times$ Denhardt's solution (where 1 $\times$ Denhardt's solution is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.002 % BSA), 200 mg/ml denatured salmon-sperm DNA and 10 % (w/v) dextran sulphate. The RNA was hybridized

overnight at 42 °C with the labelled probes, and washed twice in $6 \times$ SSPE/0.1 % (w/v) SDS at room temperature for 20 min, followed by two washes in $1 \times$ SSPE/ 0.1 % (w/v) SDS at 55 °C for 30 min. The blot was air-dried, and then exposed overnight at -70 °C to Kodak XAR-5 film for developing.

Isolation of genomic DNA

Genomic DNA was isolated from harvested cultured skin fibroblasts by freeze-thawing (ten times) the cells suspended in lysis buffer (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA, pH 8, 0.5 % SDS, 0.1 mg/ml proteinase K) in a dry ice/ethanol bath [17]. An equal volume of phenol/chloroform was added, and DNA was precipitated by adding 0.5 vol. of 7.5 M ammonium acetate and 2 vols. of 100 % ethanol. The DNA pellet was rinsed with 70 % ethanol, and dissolved in TE buffer (10 mM Tris/HCl, 0.1 mM EDTA, pH 8).

PCR analysis

Exons 2–16 were amplified from 500 ng of patient or normal genomic DNA as described by the procedure by Saiki et al. [18]. The primers used to amplify the appropriate exons of the β -galactosidase gene and those used for site-directed mutagenesis are on the basis of the published intron–exon junction sequences [19]. DNA-amplification reactions were performed in a total volume of 100 μ l, containing 100 ng of each primer, 10 units of *Taq* polymerase (Promega, Madison, WI, U.S.A.) and 0.5 mM each of dCTP, dGTP, dTTP and dATP, in 10 mM Tris/HCl, pH 8.3/50 mM KCl/1.5 mM MgCl₂. The reaction conditions used for PCR were 30 cycles comprising 1 min at 94 °C (denaturation), 70 s at 56 °C (annealing) and 1 min at 72 °C (extension), using the RoboCycler 40 (Stratagene, La Jolla, CA, U.S.A.) thermal cycler. PCR products were run on a 1 % agarose gel to check for insertions or deletions, before single-strand conformation polymorphism (SSCP) analysis was performed.

SSCP analysis

SSCP analysis was performed at either room temperature or 4 °C with 6 % (w/v) polyacrylamide gels in $0.5 \times$ TBE running buffer (where $1 \times$ TBE is 45 mM Tris/borate/1 mM EDTA) containing 5 % (v/v) glycerol (40 W), as described in refs. [20] and [21]. The gels were fixed in 50 % (v/v) methanol and 10 % (v/v) acetic acid solution for 1 h, and then were washed three times in distilled water for 15 min, followed by staining in a solution containing equal volumes of 0.5 M sodium carbonate and 10 mM silver nitrate/25 mM ammonium nitrate/3.5 mM tungstosilicic acid/0.8 % (v/v) formalin for 10–30 min. The reaction was stopped by the addition of 10 % acetic acid.

DNA sequencing

PCR products were sequenced directly, or after ligating them into the TOPO TA cloning kit vector (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions. Non-radio-labelled PCR products were run on a 1 % agarose gel, excised, and then purified using the Glassmax DNA isolation system (Gibco BRL, Burlington, ON, Canada). Direct sequencing was performed using 100 ng of purified PCR product, 100 ng of primers, [α -³⁵S]dATP and the Sequenase kit (United States Biochemical, Cleveland, OH, U.S.A.), as recommended by the manufacturer.

Restriction enzyme analyses

DdeI

To examine the C $\rightarrow$ A transversion in codon 148 (Arg¹⁴⁸Ser), a 10- μ l sample containing the 139-bp PCR product from exon 4 (primer sequences are shown in Table 1) was digested with 10 units of *DdeI* for 1 h at 37 °C, run on a 10 % polyacrylamide gel and silver-stained.

BsrGI

For the Asp³³²Asn mutation, PCR reactions included the upstream primer (5'-AGCACAGCCCACCATGTAC-3', where the underlined bases are mismatched), along with the usual downstream primer for exon 10 (Table 1) at 64 °C. In the presence of the G1044 $\rightarrow$ A transition, a *BsrGI* site is created in the amplified product (125 bp), where digestion with the enzyme results in release of 17- and 108-bp fragments.

HpaII

For the Ser⁵³²Gly polymorphic mutation, part of exon 15 was amplified (Table 1) using genomic DNA and a specifically constructed upstream primer (5'-GGGCTGGGGACACCGTG-CC-3') where C corresponds to a mispairing with the normal sequence, whereby, in the presence of the A $\rightarrow$ G transition, a *HpaII* restriction site is created.

Construction in β -galactosidase of missense mutations found in G_{M1} gangliosidosis

Fragment-replacement mutagenesis was used to introduce the following mutations into wild-type β -galactosidase GP8. The Asp³³²Asn, Arg¹⁴⁸Ser and Ser⁵³²Gly mutations were then examined by transient expression in COS-1 and CHO cells. Wild-type β -galactosidase cDNA (GP8-pGEM) was used as the template, and primers are given in Table 1(B).

Arg¹⁴⁸Ser mutation

The 558-bp fragment containing the CGC $\rightarrow$ AGC change, with a 5' *SalI* site and a 3' *PvuII* site, was completely sequenced and then subcloned into these sites in the wild-type GP8 cDNA in pGEM-3Z.

Asp³³²Asn and Asp³³²Glu mutations

The initial 255-bp fragment with a 5' *EcoRI* and a 3' *DraIII* site, containing the GAC $\rightarrow$ AAC or GAC $\rightarrow$ GAG changes leading to the amino acid mutations of Asp³³² $\rightarrow$ Asn or Asp³³² $\rightarrow$ Glu respectively (205 bp after digestion with these enzymes), was purified, sequenced and subcloned into β -galactosidase GP8-pGEM-3Z.

Ser⁵³²Gly mutation

The 505-bp fragment with 5' and 3' *BamHI* sites containing the AGT $\rightarrow$ GGT change was purified, sequenced and subcloned into GP8-pGEM-3Z. For COS-1 cell expression, the wild-type and mutated forms of GP8 were inserted into the *XhoI* site of pSVL vector using T4 DNA ligase (1 unit/ μ g DNA for 12 h at 12 °C). For CHO cell expression, the wild-type and mutated forms of GP8 were inserted into pRc/RSV at the *HindIII* site, as

Table 1 Primers for the amplification of exons 4, 10, and 15 in β -galactosidase (a), and those for site-directed mutagenesis of specific residues in exons 4, 10 and 15 (b)(a) In all cases, the melting temperature (T_m) was 60 °C. (b) The nucleotide changes are underlined.

(a)				
Exon	Upstream (5' → 3')	Downstream (5' → 3')	Product size (bp)	
4	CGGAACAGACATTATAATGGC	GCTTTTATAAATCTTCTCAAGAC	139	
10	TGGCACATTTTGTCTTCTAG	GCACCCACCACAGCTCATA	165	
15	CAGCTCACTGTGCTCTGTTT	GAATTCAAACCTTCCCATGA	315	
(b)				
Location of mutation (codon affected)	cDNA primer position	Oligonucleotides used (5' → 3')	cDNA codon change	Mutation introduced
Exon 4 (Arg ¹⁴⁸)	Upstream	CGTCGACGGGGCGCCGA	CGC → AGC	Arg ¹⁴⁸ Ser
	Middle	CTATTCTTCTCAGCTCCTCCG		
	Downstream	ACTTGTCCACAGCTGCCAGG		
Exon 10 (Asp ³³²)	Upstream	GCAGCTACTTTGCCTGTGATT	GAC → AAC	Asp ³³² Asn
	Middle	GGGGCATCATAGTTGTAGCTG		
	Downstream	AACATATGCTCGATCGTGGAC		
Exon 10 (Asp ³³²)	Upstream	GCAGCTACTTTGCCTGTGATT	GAC → GAG	Asp ³³² Glu
	Middle	GGGGCATCATACTCGTAGCTGG		
	Downstream	AACATATGCTCGATCGTGGAC		
Exon 15(Ser ⁵³²)	Upstream	AATAACCCCTCACTAAAG	AGT → GGT	Ser ⁵³² Gly
	Middle	GACGCTGGCCACCATGATG		
	Downstream	AATACGACTCACTATAG		

described in detail previously [7]. All plasmids were examined for the correct orientation of inserts.

DNA transfection

CHO cells were transfected using the liposome-mediated (Lipofectin™) method, while the COS-1 cells were transfected using the adenovirus-mediated method.

Lipofectin™ transfection

CHO cells were transfected with the pRc/RSV-GP8-Arg¹⁴⁸Ser, -Asp³³²Asn, -Asp³³²Glu and -Ser⁵³²Gly constructs, and with wild-type GP8. CHO cells were grown until they had reached 80–85 % confluency. Of each construct, 20 µg was transfected separately with the Lipofectin™ reagent (Gibco BRL), as described by Felgner et al. [22]. After a 4-h incubation at 37 °C, medium was added and the cells were incubated for an additional 48–72 h before harvesting. CHO clones permanently transfected with pRc/RSV-GP8-Asp³³²Asn and -Asp³³²Glu were isolated as described previously [7].

Adenovirus-mediated transfection

COS-1 cells were grown to 80 %–85 % confluency for transfection [23]. The growth medium was removed, and the cells were washed twice with 10 ml of PBS. Aliquots (1 ml) of diluted adenovirus-lysed HEK-293 cell media and 8 µg of pSVL-GP8-Arg¹⁴⁸Ser, -Asp³³²Asn and -Ser⁵³²Gly constructs, together with wild-type GP8 and DEAE-dextran (at a final concentration of 80 mg/ml), were added to the diluted adenovirus media. The DNA/adenovirus/DEAE-dextran mixture was added dropwise to the COS-1 cells, and incubated at 37 °C for 2 h. A chloramphenicol acetyltransferase (CAT) construct was included to check for transfection efficiency. After the transfection incubation, the cells were washed once with 10 ml of PBS containing

10 % (v/v) DMSO, twice with 10 ml of PBS, and grown in 10 ml of medium for 2–3 days.

Enzyme purification, protein analysis and enzyme assays

Harvested cultured-skin fibroblasts and transfected cells were resuspended in 0.25 M Tris/HCl, pH 7.8, containing the protease inhibitors PMSF (1 mM) and leupeptin (10 mg/ml), subjected to ten freeze–thaw cycles, and soluble lysates were collected for enzyme and protein analyses. Recombinant Asp³³²Asn and Asp³³²Glu enzymes were purified from the medium above the CHO cells, as described previously [7,24]. To assess the amount of endogenous CHO enzyme in these preparations, we subjected the same volume of medium conditioned with non-transfected CHO cells to this isolation procedure. Protein content was determined either in duplicate or triplicate [25] with BSA (0.5 mg/ml) used as the standard. Enzyme activity was measured in duplicate by adding a 60-µl aliquot of enzyme solution to 190 µl of 0.6 mM artificial substrate, 4-methylumbelliferyl- β -D-galactopyranoside (Sigma, St. Louis, MO, U.S.A.) in 50 mM citrate/100 mM phosphate buffer, pH 4.3 [7,24]. For measurement of the kinetic constants, the volume of substrate used was increased to 240 µl, to which was added a 10-µl aliquot of enzyme diluted into the above buffer containing BSA (0.1 mg/ml). After incubation at 37 °C for 30 min, all reactions were stopped by adding 2 ml of 0.1 M 2-methylpropan-1-ol buffer, pH 10, and the fluorescence was determined with an excitation wavelength of 365 nm and an emission wavelength of 450 nm with a Perkin-Elmer (model 650-40) spectrofluorimeter (Norwalk, CT, U.S.A.). A known amount of 4-methylumbelliferone (Eastman Kodak Company, Rochester, NY, U.S.A.) in 0.1 M 2-methylpropan-1-ol, pH 10, was used as a standard. Transfection efficiency was monitored by the CAT activity using the phase-extraction system [26].

Western blot analysis

Cells were harvested by scraping in PBS, and lysed by sonication, as described previously [9]. Fibroblast protein (30 μ g) and 20 μ g of total COS-1 and CHO cellular protein were analysed using SDS/PAGE. Samples were reduced by boiling for 5 min in 62.5 mM Tris/HCl/3% (w/v) SDS/10 mM dithiothreitol, and then loaded on to an 8% Bio-Rad (Mississauga, ON, Canada) Mini-Gel SDS/PAGE system, run as described by Laemmli [27]. Proteins were transferred overnight to nitrocellulose, and the resultant Western blot was probed using a 1:1500 dilution of our well-characterized rabbit IgG (P-Gal) raised against purified human β -galactosidase precursor, as described previously [7,9]. Immunoblots were developed using the enhanced chemiluminescence system (ECL®) Western Blot (Amersham, Oakville, ON, Canada).

Immunolocalization of β -galactosidase in intracellular compartments

Patient 1 and control fibroblasts were incubated for 48 h in α -MEM medium with 10% (v/v) fetal-bovine serum at 37°C in an air/CO₂ (19:1) mixture, to which 20 μ M leupeptin was added. We have previously shown in normal and galactosialidosis fibroblasts that leupeptin reduces the lysosomal degradation of β -galactosidase, resulting in a prolonged half-life and an increased level of the enzyme activity, without affecting the correct processing of the enzyme to its mature form [9]. Leupeptin-treated fibroblasts were analysed for protein by Western blot analysis, for enzyme activity and for immunolocalization of β -galactosidase. Immunolocalization of β -galactosidase was performed in non-transfected and permanently transfected CHO cells with the wild-type β -galactosidase, Asp³³²Asn and Asp³³²Glu constructs. Cells were grown in α -MEM medium with 10% (v/v) fetal-bovine serum at 37°C in air/CO₂ (19:1) on glass cover slips and, after 48 h, the cells were fixed and gently permeabilized with 100% cold methanol at -20°C for 30 min. The fixed cells were then washed in PBS, blocked with 1% BSA, and incubated for 1 h with rabbit IgG raised against purified human β -galactosidase precursor, as described above (diluted 1:200). The secondary antibody, a green fluorescein-labelled goat-(anti-rabbit) IgG (Fab2), diluted 1:100, was then added for 1 h, either alone or in combination with a 1:10000 dilution of propidium iodide (which, in addition to nuclear DNA, also stains cytoplasmic RNA and RNA bound to the rough ER), thereby marking the position of the ER by the location of red fluorescence. The cells were then washed three times with PBS and mounted with elvanol. In control cultures, the preimmune rabbit IgG was substituted for the primary antibody.

The slides were analysed, and the proportion of immuno-reactive protein present in the ER or the endosome/lysosome was determined using a fluorescent microscope (Olympus Vanox-AH-3; magnification $\times$ 800) and two narrow-band filters to detect the green and red fluorescence separately. An additional broad-spectrum filter was also used for the simultaneous detection of the green fluorescein-tagged P-gal antibody and the nucleic acids labelled with red propidium iodide fluorescence. In this setting, the overlapping of the red and green fluorescence in the cytoplasm gave rise to a yellow fluorescence, which indicated the co-localization of β -galactosidase with the ER. Multiple images of the same cell obtained with all of the abovementioned filters were captured with a charge-coupled-device (CCD) camera (Optronics, Galeta, CA, U.S.A.), stored in a MacIntosh 9500 computer, and quantitatively analysed using the Image Pro Plus program (Media Cybernetics, Silver Spring, MD, U.S.A.), according to the manufacturer's instructions. In

each of the three experimental groups (wild-type, Asp³³²Asn and Asp³³²Glu), images of 50 cells were analysed, and results were statistically evaluated to give quantitative measurements of the percentage of the area occupied by the anti-(β -galactosidase) reactive ligands that reside in the ER (green and red fluorescence overlapped), and in the endosomal and lysosomal compartments (green fluorescence alone). Cultures of COS-1 cells transiently transfected with wild-type and Asp³³²Asn constructs, maintained in small dishes with coverslips, were fixed as described above. Separate coverslips were incubated for 1 h with PBS-diluted primary antibody (2 μ g/ μ l) followed by a 1-h incubation with the appropriate fluorescein (FITC)-conjugated goat-(anti-rabbit) or goat-(anti-mouse) secondary antibodies. An additional 10-min incubation with propidium iodide (0.1 μ g/ml) ensured the nuclear counterstaining. The coverslips were mounted on glass slides with elvanol, and analysed using an Olympus Vanox AH-3 fluorescence microscope.

RESULTS

Level of β -galactosidase protein

Western blot analysis of cultured skin fibroblasts showed that the patient (Figure 1, lane 3), like most other G_{M1} patients (two examples are shown in Figure 1, lanes 4 and 5) we have examined to date, has barely detectable amounts of the 64 kDa mature form of the enzyme (the normal amount is shown in lane 2). The 84 kDa precursor, which we have previously shown [9] amounts to approx. 20% or less of the total β -galactosidase in fibroblasts, is proportionately lower in abundance in all the G_{M1} cell strains examined compared with normal cells. The Morquio type B strain (lane 6), which has approx. 5% residual β -galactosidase activity, has proportionately decreased amounts of the 84 kDa precursor and the 64 kDa mature form. Northern blot analysis showed that the abundance and size of the mRNAs were normal for case 1, as in all the other cases examined (results not shown).

Identification of the mutations

SSCP analysis was used to screen for mutations in exons 2–16 using intron/exon primers, as described by Morreau et al. [19].

Figure 1 Western blot of fibroblast cell lysates from three G_{M1}-gangliosidosis and one Morquio type B patients

Of total cellular protein, 30 μ g was analysed by SDS/PAGE and probed using an antibody that cross-reacts with the 84 kDa precursor and 64 kDa mature forms of β -galactosidase. Lane 1 (positive control) corresponds to wild-type β -galactosidase transiently expressed in COS-1 cells, and demonstrates the location of the 84 kDa precursor. Lane 2 is from a normal fibroblast control, showing the small amount of precursor and the abundant 64 kDa mature form of β -galactosidase. Lane 3 shows the index case, case 1, where very little precursor and no mature protein is visible. This is comparable with other G_{M1}-gangliosidosis patients, such as cases 3 and 4 (lanes 5 and lane 4 respectively), where no precursor and no mature protein is visible. For comparison, lane 6 corresponds to a fibroblast lysate from Morquio type B (case 5, positive control) where reduced amounts of precursor and mature enzyme are visible.

Figure 2 Sequencing analysis showing three different mutations in the β -galactosidase gene in G_{M1} -gangliosidosis cases

All of the sequencing used PCR-amplified genomic DNA as template. (A) The C492 $\rightarrow$ A transversion mutation is shown, leading to an Arg¹⁴⁸Ser amino acid substitution found in case 1, where the presence of both C and A indicates heterozygosity at this position. (B) The PCR products were subcloned as described. Only the area containing the mutation is shown. The left panel shows the normal sequence found on the one allele in case 1, whereas the right panel shows the G1044 $\rightarrow$ A change in case 1, which converts Asp³³² into Asn. (C) The A1644 $\rightarrow$ G change shown here was from case 4, who was homozygous for this mutation and for Arg³⁵¹ 'Stop' (result not shown). Cases 1 and 3 were heterozygous for the A1644 $\rightarrow$ G mutation.

A 139-bp PCR product, obtained from amplification of exon 4, produced an SSCP-mobility shift in DNA from case 1, but not in cases 2, 3 and 4 (results not shown). Direct sequencing of this product from case 1 identified a C492 $\rightarrow$ A transversion (Figure 2A), which resulted in an Arg¹⁴⁸Ser substitution. This mutation creates a *DdeI* site (generating fragments of 82 bp and 57 bp from the 139-bp product), which was used to analyse all the other G_{M1} cases used in this study. The C492 $\rightarrow$ A transversion mutation was identified on one allele in a second case of infantile G_{M1} gangliosidosis (case 2), described previously [14]. The mutation on the second allele in case 2, G1445 $\rightarrow$ A, converts Arg 482 into His, and has been described previously [16]. The C492 $\rightarrow$ A (Arg¹⁴⁸Ser) mutation was not detected in 50 unrelated normal individuals, indicating that it was unlikely to be a common polymorphism. On SSCP analysis of the proband exon 10 (Table 1), a band shift was noted, and a G1044 $\rightarrow$ A mutation (which converts Asp³³² into Asn) was detected on sequencing both the

Figure 3 Western blot analysis of transfected COS-1 cells

(A) COS-1 cells were transfected with wild-type pSVL-GP8 cDNA (lane 1), pSVL-GP8-Arg¹⁴⁸Ser cDNA (lane 2) and pSVL-GP8-Ser⁵³²Gly cDNA (lane 3), and processed as described in the Materials and methods section. No cross-reacting material (lanes 4 and 5) was detected in the mock-adenovirus-transfected or the untransfected COS-1 cells. (B) COS-1 cells were mock-adenovirus-transfected (lane 1) or transfected with pSVL-GP8-Asp³³²Asn (lane 2) or wild-type pSVL-GP8 cDNA (lane 3). β -Gal, β -galactosidase.

direct and subcloned PCR products (Figure 2B). To examine the occurrence of this mutation in the population, we constructed an oligonucleotide 5'-A₁₀₂₅GCACAGCCCCACCATGTAC₁₀₄₃-3' (where TG are mismatched nucleotides) which, in the presence of the G1044 $\rightarrow$ A mutation, creates a unique *BsrGI* restriction site (*BsrGI* cleavage of the 125-bp product generates 108-bp and 17-bp products). This mutation was not detected in 50 unrelated individuals, indicating it is unlikely to represent a polymorphic change. Upon amplification of exon 15, the 191-bp PCR product undergoes an SSCP shift that was seen in cases 1, 3 and 4. Both the proband and case 3 were heterozygous for this change, whereas case 4 was homozygous. This SSCP shift indicated the presence of a third mutation in case 1, identified as an A1644 $\rightarrow$ G change converting Ser⁵³² into Gly (Figure 2C), and is judged to be a common polymorphism, since it was detected in 5 out of the 20 normal individuals tested. Its occurrence is easily detected using *HpaII* restriction-enzyme digestion, since the 191-bp PCR product is cleaved into 119-bp and a 72-bp fragments when the mutation is absent, and 119-bp and 53-bp products when the A1644 $\rightarrow$ G change is present. The disease-associated C492 $\rightarrow$ A (Arg¹⁴⁸Ser) and the polymorphic A1644 $\rightarrow$ G (Ser⁵³²Gly)

Table 2 β -Galactosidase activity associated with mutations as measured in transiently transfected COS-1 cell lysates

The activities are representative average values from replicate plates, and were corrected for transfection efficiency using the CAT assay. The activity in mock-transfected COS-1 cells was the separately determined baseline value in each experiment, and this value was subtracted from all values in that experiment. This experiment was performed independently three times. —, not determined.

	β -Galactosidase activity (nmol/h per mg of protein)	% of wild-type
COS + adenovirus	272	—
Wild-type GP8- β -gal pSVL	720	100
Arg ¹⁴⁸ Ser β -gal pSVL	601	83.5
Ser ⁵³² Gly β -gal pSVL	583	81.0
Asp ³³² Asn β -gal pSVL	256	0

mutations were found in the mother of the proband, and the exon 10 G1044 → A, Asp³³²Asn change was found in the father (results not shown). Interestingly, case 2, the second patient in which we found the C492 → A, Arg¹⁴⁸Ser mutation in exon 4, was also polymorphic for A1644 → G, Ser⁵³²Gly. However, this appears to be a fortuitous association, because the case 4 cell line was found to be homozygous for both a new nonsense mutation and for A1644 → G, Ser⁵³²Gly (S. Zhang, R. Bagshaw and J. W. Callahan, unpublished work).

Expression of mutated β -galactosidase in COS-1 and CHO cells

We examined expression of these mutations in both COS-1 and CHO cells. In general, COS-1 cells amplify the transfected cDNA, and high levels of mRNA encoding the transfected gene are transcribed. In this instance, abundant β -galactosidase protein was produced (Figure 3). Therefore expression in this system allows us to differentiate between mutations that affect the active site, and those that do not. When expressed in COS-1 cells, all of these constructs yielded high levels of the 84 kDa precursor, with small amounts processed to the 64 kDa mature form of the enzyme, as judged by Western blot analysis (Figure 3). Both the Arg¹⁴⁸Ser and the Ser⁵³²Gly β -galactosidases showed substantial levels of enzyme activity, whereas the Asp³³²Asn β -galactosidase gave < 1% residual activity, suggesting that the Asp³³²Asn mutation affected the active site of the enzyme (Table 2). As the COS-1 β -galactosidase is only weakly cross-reactive with our antibody, only small amounts of cross-reacting material (Figure 3) were detected in the untransfected and the mock-

Figure 4 SDS/PAGE of purified Asp³³²Asn- and Asp³³²Glu- β -galactosidase

Wild-type and mutant β -galactosidases were purified from medium conditioned with CHO cells permanently transfected with wild-type β -galactosidase or the respective recombinant β -galactosidases. The enzymes were purified on the substrate analogue-affinity column, as described in the Materials and methods section. Lanes 1–4 respectively were loaded with 5 μ g of the purified wild-type enzyme (lane 1), the purified Asp³³²Glu enzyme (lane 2) and the Asp³³²Asn enzyme (lane 4); lane 3 contains 5 μ g of protein purified from medium above untransfected CHO cells. The molecular-mass standards are indicated. The proteins were detected by Coomassie Blue staining.

adenovirus-transfected host cells. We then performed transient expression in CHO cells where, because only a minor degree of amplification of the cDNA occurs, considerably less mRNA would be transcribed compared with COS-1 cells resulting in less directed β -galactosidase synthesis by these cells. In this case, the ER 'quality-control' system would be expected to function normally, and we would be better able to discriminate between mutations that affected folding and those that did not. In addition, CHO cells process human β -galactosidase to generate both the mature enzyme and its degradation products [7,9]. When the effect of the Arg¹⁴⁸Ser and Ser⁵³²Gly mutations were compared with the GP8 wild-type control using transient transfections in CHO cells, we found markedly reduced levels of activity attributable to the Arg¹⁴⁸Ser mutant enzyme (Table 3), while the levels of activity produced from the polymorphic Ser⁵³²Gly form were virtually indistinguishable from those of the GP8 wild-type control. Taken together, these expression data suggest the Arg¹⁴⁸Ser mutant β -galactosidase, while catalytically active (as judged from the COS-1 expression), is largely degraded (presumably in the ER, as shown in Figure 5A below). This

Table 3 β -Galactosidase activity measured in transfected CHO cell lysates

Enzyme activity was measured in duplicate from each of two separate dishes for each experiment, as described in the Materials and methods section. Corrected specific activities are values obtained after subtraction of the CAT controls. Exper, experiment; —, not applicable.

Transfectant	Specific activity (nmol/h per mg of protein)				% of corrected wild-type control	
	Uncorrected		Corrected			
	Exper 1	Exper 2	Exper 1	Exper 2	Exper 1	Exper 2
Mock	641	548	—	—	—	—
CAT	755	576	—	—	—	—
Wild-type GP8	1122	1165	367	589	100	100
GP8-Arg ¹⁴⁸ Ser	786	658	31	82	8.4	13.9
GP8-Ser ⁵³² Gly	1030	1147	275	571	74.9	96.9

Table 4 Kinetic constants of wild-type and mutant β -galactosidases

The recombinant precursor proteins were purified on the substrate-analogue affinity column and concentrated as described in the Materials and methods section. Total activity represents the units of activity from 600 ml of conditioned medium bound to and eluted from the 1-ml affinity column. Eight substrate concentrations in the range 0.15–0.55 mM were used to derive kinetic constants by curve-fitting to a rectangular hyperbola using the SigmaPlot for Windows program, version 4.0 (Jandell Scientific). All values are the means $\pm$ S.D. for at least three determinations. —, not determined.

Form of the enzyme	Total activity (μ mol/h)	Specific activity (μ mol/h per mg of protein)	K_m (mM)	V_{max} (μ mol/h per mg of protein)
Human placenta, mature*	—	500	0.43	660
Wild type, precursor†	—	827 $\pm$ 338	0.29	989
Wild type, precursor, the present study	1027.0	379 $\pm$ 105	0.36 $\pm$ 0.23	490 $\pm$ 172
Asp ³³² Asn, precursor, the present study	3.53	1.03 $\pm$ 0.61	0.35 $\pm$ 0.02	1.82 $\pm$ 0.05
Asp ³³² Glu, precursor, the present study	5.26	2.94, 3.10	0.32 $\pm$ 0.03	4.44 $\pm$ 0.20
Non-transfected medium, the present study	3.75	1.89	0.15 $\pm$ 0.08	2.26 $\pm$ 0.28

*, Taken from [24]; †, see [7,8].

Figure 5 Immunofluorescence detection of β -galactosidase

(A) COS-1 cells were transiently transfected with vector alone (left panel), with wild-type pSVL-GP8- β -galactosidase (centre panel) or with pSVL-GP8-Asp³³²Asn (right panel). Nuclei were stained with propidium iodide. Very little β -galactosidase is detected with the P-Gal antibody in the mock-transfected cell (left panel). With wild-type pSVL-GP8 β -galactosidase (centre panel) immunostaining is detected in punctate organelles distributed throughout the cytoplasm, whereas with pSVL-GP8-Asp³³²Asn β -galactosidase (right panel), immunostaining is localized primarily to the cisternae of the ER, with a small but discernible reaction detected in lysosomes. (B) When CHO cells were transiently transfected with the mutant construct, pRc/RSV-GP8-Arg¹⁴⁸Ser β -galactosidase (right panel), strong immunostaining is found in cisternae and in the perinuclear area suggesting localization of the enzyme primarily in the ER, whereas with vector pRc/RSV alone (left panel) a lower level of immunostaining was seen and, as expected, the endogenous host cell enzyme was localized primarily in the lysosomes (left panel). (C) shows the immunolocalization of β -galactosidase in permanently transfected CHO cells by graphical representation of the percentage of the various β -galactosidases [wild-type GP-8 (WT), Asp³³²Asn (332N) or Asp³³²Glu (332E)] localized in the endosome–lysosome compartment or the ER. The bars represent the S.D. of each value obtained from the analyses of 50 cells per experimental group.

would then account in part for the lack of detectable β -galactosidase, cross-reacting material in fibroblasts from this patient.

Since the naturally occurring Asp³³²Asn mutation, when expressed in COS-1, resulted in ample amounts of protein, but negligible catalytic activity, it was of interest to examine in

Figure 6 Immunofluorescence detection of β -galactosidase in fibroblasts incubated in the presence of leupeptin

Control (A and B) and patient's (C and D) fibroblasts were incubated for 48 h in the absence (A and C) or presence (B and D) of leupeptin (20 μ M). As expected, the specific activity of β -galactosidase increased in the control cells (from 681 nmol/h per mg of protein to 994 nmol/h per mg of protein), and the enzyme was immunolocalized almost exclusively in lysosomes. In the patient's fibroblasts there was no change in the enzyme activity (undetectable in the presence or absence of leupeptin), the level of immunodetectable enzyme remained much lower than in normal cells, and was found primarily in the perinuclear area and the cisternae of the ER (C and D), with some found additionally in lysosomes (with leupeptin; D).

greater detail a possible role for Asp³³² in the active site. To do this we converted Asp³³² into both Asn³³² and Glu³³², and isolated permanently transfected CHO cell clones expressing high levels of these enzymes. We have used a similar approach to successfully purify and characterize the normal β -galactosidase precursor [7,8]. Mutant Asp³³²Asn, Asp³³²Glu, and wild-type β -galactosidases were purified to apparent homogeneity (Figure 4, lanes 1, 2 and 4) in a single step from the CHO cell medium on the phenylthio- β -D-galactoside-Sepharose substrate-analogue affinity column, under the standard conditions described previously [8,24]. As a control, a corresponding amount of medium conditioned with non-transfected CHO cells was run to account for any host contribution to the protein (Figure 4, lane 3) and activity (Table 4). We then determined kinetic constants for the mutant enzymes (Table 4). The ' V measured against S ' plots followed Michaelis-Menten kinetics at two enzyme concentrations up to the highest limit of substrate solubility, and the calculated K_m values were the same as those obtained for the wild-type control. Although the V_{max} values alone were substantially lowered, the value for Asp³³²Glu β -galactosidase was twice that of the Asp³³²Asn β -galactosidase, a result that reinforces a putative role for Asp³³² in the active site of the enzyme.

Immunofluorescence localization of mutant β -galactosidases

On the basis of the above results, the residual activity in the patient's cells should be no less than 50% (activity from Arg¹⁴⁸Ser) if all the enzyme synthesized were to arrive in the lysosome, with subsequent stabilization there. However, as shown in Figure 1, the patient's fibroblasts consistently displayed negligible amounts of β -galactosidase activity or cross-reacting material, suggesting that the bulk of the synthesized enzyme either does not reach this compartment or is rapidly degraded upon its arrival. The Arg¹⁴⁸Ser mutation replaces a bulky, positively charged residue with a smaller and less polar one, and a Garnier plot predicts a change in the conformation of the protein, i.e. the resulting protein is at least partially misfolded and, on the basis of studies with other lysosomal hydrolases [28], might be degraded in the ER. However, the Asp³³²Asn mutation does not alter the Garnier plot, suggesting only a minor effect on folding. To address these questions, we used immunofluorescence to examine the localization of the individual mutant enzymes. For the Arg¹⁴⁸Ser mutant, the bulk of the protein was found in the perinuclear network (Figure 5B), consistent with its localization in the ER, with a barely discernible reaction in lysosomes. The Asp³³²Asn β -galactosidase was also found prin-

cipally in the perinuclear area in the transiently transfected COS-1 cells. Lysosome localization was more discernible, although still significantly lower than normal (Figure 5A). The bulk of the wild-type control enzyme was found in the endosome-lysosome compartment, as expected (Figure 5A and 5C). In the permanently transfected CHO cells (Figure 5C), the bulk (approx. 70 %) of the wild-type immunoreaction was localized to the endosome-lysosome compartment, with 30 % detected in the ER (Figure 5C). In contrast, about 65 % of the Asp³³²Asn β -galactosidase and 50 % of the Asp³³²Glu β -galactosidase were found in the ER. Since it could be argued that stability of mutant human β -galactosidase in these hosts might be compromised by the absence of protective protein/Cathepsin A and neuraminidase, key members of a complex found in lysosomes that affect the stability and processing of β -galactosidase, we examined the immunolocalization of the enzyme in the patient's fibroblasts in the presence and absence of leupeptin. This potent inhibitor of lysosomal thiol proteases has no effect on conversion of β -galactosidase from the precursor into the mature form or its ability to form the complex, but does slow its rate of degradation [9]. Furthermore, since the Arg¹⁴⁸Ser mutation results in an active enzyme, we postulated that slowing its degradation might foster its stabilization via complex formation and result in a significant increase in activity in the cells. However, in the presence of leupeptin, the level of β -galactosidase activity remained negligible in the patient's cells, and only a small but detectable increase was noted in the amount of precursor on the basis of the result of a Western blot (results not shown). Although the intensity of immunostaining in the patient's cells (Figures 6C and 6D) was significantly less than in the control (Figures 6A and 6B), an increased cell immunoreactivity of the patient was detectable in the presence of leupeptin, and was localized to both the ER and the endosomal-lysosomal compartment (Figure 6D). These findings are in complete accordance with our immunolocalization (Figure 5), and other expression, studies (Tables 2 and 3).

DISCUSSION

Although our studies focus on the mutations and their impact on the fate of the resulting protein from case 1, G_{M1} gangliosidosis cases 2 and 3, used as positive controls in this study, and indeed in all of the cases we have studied to date (at least 10) where mutations are of the missense type, also have little or no precursor or mature β -galactosidase protein. The reasons for this are readily apparent from the expression and immunofluorescence studies included in the present report, and are able to be generalized, at least in part. Transient expression of the Arg¹⁴⁸Ser and the Asp³³²Asn mutations in COS-1 and CHO cells results in proteins that are largely confined to the perinuclear area of the cell, specifically the ER, presumably because they are not folded to the lowest energy state compatible with its complex quality-control system, and are subsequently degraded there. A small fraction of the enzyme survives, and reaches the lysosome. This alone might account for the low levels of enzyme protein and minimal enzyme activity in the fibroblasts. Of the approximately 29 missense mutations in the β -galactosidase gene that have been reported, few have been studied by expression in host cells where assays such as Western-blotting or immunofluorescence were included (reviewed in [10]). Consequently, details on the impact of many of these mutations on the active site or intra-organellar transit of β -galactosidase must be inferred with little information known directly about how these mutations affect folding of the enzyme in the ER. However, recently Bradford et al. [29] expressed a Cys⁹¹Thr mutation in the gene for *N*-acetyl-

galactosamine 4-sulphatase, the enzyme deficient in Maroteaux-Lamy disease (MPS VI), and found that, although the enzyme was synthesized normally and was active, it was nonetheless rapidly degraded in an early-ER compartment. Their data are in agreement with the present findings.

Transient overexpression in COS-1 cells allowed us to readily discriminate mutations affecting catalytic activity from those that did not, because in both instances the high levels of protein produced forced the folding of the enzyme generating substantial amounts of active (as in the case of Arg¹⁴⁸Ser) or inactive (as with Asp³³²Asn) enzyme. However, in the CHO cells, the vector-directed rate of synthesis of the recombinant enzymes is much lower than in COS-1 cells, and does not exceed the quality-control system of the ER in this cell type [30–32]. Consequently, because of the predicted alteration in the folding arising from the Arg¹⁴⁸Ser mutation, and consistent with our fibroblast results, total activity resulting from this construct was much lower in CHO cells (11 % of normal) compared with COS-1 cells. In the case of the Asp³³²Asn mutation, although the Garnier plot does not predict a major alteration in conformation, a significant proportion of the enzyme nevertheless was localized to the ER, reinforcing the view that the ER quality-control system is exquisitely sensitive, even to slightly misfolded proteins. Taken together, these results indicate that both the Asp³³²Asn and the Arg¹⁴⁸Ser mutations alter the conformation sufficiently such that the newly synthesized enzyme precursor cannot readily exit the ER, and a substantial proportion is degraded there. We have previously shown in both normal and galactosialidosis-affected fibroblasts that treatment with leupeptin results in increased levels of precursor and mature lysosomal forms of β -galactosidase by decreasing their rate of degradation [9]. Since there was no measurable enhancement of β -galactosidase activity in fibroblasts from the proband under these conditions, this suggests that little of the enzyme synthesized actually reaches this organelle, thus supporting the conclusion that the mutations in this patient result in degradation of the enzyme in a pre-lysosomal compartment, i.e. the ER. Earlier studies predicted that secretory and plasma membrane proteins that were not folded absolutely correctly were retained and degraded in the ER [33,34]. Another example is provided by Tay-Sachs disease, caused by mutations affecting the α -subunit of hexosaminidase A, where of the 87 missense mutations described to date only α -Arg¹⁷⁸His results in near-normal amounts of cross-reacting material in lysosomes of fibroblasts [35].

Our identification of Glu²⁶⁸ as the catalytic nucleophile in human β -galactosidase [8], confirming the catalytic mechanism as a Koshland double-displacement reaction [7], means that substrate hydrolysis occurs via a nucleophilic attack, with formation of an α -linked galactosyl-Glu²⁶⁸ intermediate. In this mechanism, which is comparable with that established for *Escherichia coli* β -galactosidase [36], the degalactosylation reaction requires a second acidic amino acid, the acid/base catalyst, for the release of the galactosyl moiety, accompanied by the addition of water and retention of the β -configuration [7,8]. Purified Asp³³²Asn β -galactosidase and Asp³³²Glu β -galactosidase mutant enzymes display at least a 270-fold and 110-fold lower level of catalytic activity, respectively, compared with the wild-type precursor. The fact that both the mutant enzymes result in major decremental effects on the $V_{\max}$ parameter, but not on the K_m value, in the presence of a significant amount of expressed protein provide suggestive evidence that Asp³³² has a role in the active site. Because the host CHO enzyme cannot be easily separated from the expressed human enzymes under the affinity-column conditions, at least a portion of the residual activity must be attributed to the CHO enzyme. Whether Asp³³²

might serve as the second residue in the catalytic mechanism cannot be ascribed with certainty until the contaminating host cell enzyme is decreased to a minimum and the roles of the only other strictly conserved acidic residues (such as Glu¹²⁹, Glu¹⁸⁶, Glu¹⁸⁸ and Glu³³⁹) have been defined. We now have preliminary evidence that conversion of Glu³³⁹ has no effect on enzyme activity, and we have found that introducing a hexahistidine epitope tag at the C-terminal end of the protein has no effect on enzyme activity (S. Zhang and J. W. Callahan, unpublished observations), thus offering a solution to these problems. It is noteworthy that Asp³³² resides in an 11-amino-acid motif (-Thr³²⁹ to Glu³³⁹-), where the five-member sequence containing Asp³³² (-Thr³²⁹Ser³³⁰Tyr³³¹Asp³³²Tyr³³³-) and Glu³³⁹ are absolutely conserved in all species in the family (Family 35, as defined by Henrissat [37–40]), which also includes mouse [41], dog [42] and a series of plant β -galactosidases (asparagus, apple, *Aspergillus* and other plants) [8]. Of the many missense mutations defined to date (reviewed in [10]), only Asp⁴⁹¹Asn, recently identified by Giuliani and co-workers [43], and Glu⁶³²Gly, identified by Boustany et al. [44], involve acidic residues. Asp⁴⁹¹ is not conserved in these β -galactosidases, and resides outside the major conserved domains in the protein, while Glu⁶³²Gly occurs in the C-terminal region of the protein, a part of the enzyme that is removed during intra-lysosomal processing to the mature enzyme and is therefore unlikely to play any role in the catalytic mechanism. In summary, we have defined new mutations in the β -galactosidase gene in a single case of GM1 gangliosidosis, and have shown that, by expressing them in different host cells, it is possible to discriminate those that have effects primarily on the catalytic activity from those that have an effect on enzyme folding in the ER. We also show in the case of Asp³³²Asn that a single mutation can affect both the enzyme's ability to be transported out of the ER and its catalytic activity.

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