Expression and characterization of 14 *GLB1* mutant alleles found in GM1-gangliosidosis and Morquio B patients

Raül Santamaria,[*,†,§] Amparo Chabás,[†,] John W. Callahan,[††] Daniel Grinberg,[1,*,†,§] and Lluïsa Vilageliu[1,2,*,†,§]**

Departament de Genètica, Universitat de Barcelona, Barcelona, Spain; Centero de Investigación Biomédica en red de Enfermedades Raras, Barcelona, Spain[†]; Institut de Biomedicina de la Universitat de Barcelona, Barcelona, Spain[§]; Institut de Bioquímica Clínica,** Hospital Clínic, Corporació Sanitària Clínic, Barcelona, Spain; and Research Institute,[††] Hospital of Sick Children, Toronto, Canada

Abstract GM1-gangliosidosis and Morquio B disease are lysosomal storage disorders caused by β-galactosidase deficiency attributable to mutations in the *GLB1* gene. On reaching the endosomal-lysosomal compartment, the β-galactosidase protein associates with the protective protein/cathepsin A (PPCA) and neuraminidase proteins to form the lysosomal multienzyme complex (LMC). The correct interaction of these proteins in the complex is essential for their activity. More than 100 mutations have been described in GM1-gangliosidosis and Morquio B patients, but few have been further characterized. We expressed 12 mutations suspected to be pathogenic, one known polymorphic change (p.S532G), and a variant described as either a pathogenic or a polymorphic change (p.R521C). Ten of them had not been expressed before. The expression analysis confirmed the pathogenicity of the 12 mutations, whereas the relatively high activity of p.S532G is consistent with its definition as a polymorphism. The results for p.R521C suggest that this change is a low-penetrant disease-causing allele. Furthermore, the effect of these β-galactosidase changes on the LMC was also studied by coimmunoprecipitations and Western blotting. The alteration of neuraminidase and PPCA patterns in several of the Western blotting analyses performed on patient protein extracts indicated that the LMC is affected in at least some GM1-gangliosidosis and Morquio B patients.— Santamaria, R., A. Chabás, J. W. Callahan, D. Grinberg, and L. Vilageliu. **Expression and characterization of 14 *GLB1* mutant alleles found in GM1-gangliosidosis and Morquio B patients. *J. Lipid Res.* 2007.** 48: **2275–2282.**

Supplementary key words β-galactosidase • heterologous expression • lysosomal multienzyme complex

The deficiency of lysosomal β-galactosidase caused by mutations in the *GLB1* gene is the cause of the rare lysosomal storage disorders GM1-gangliosidosis (Mendelian Inheritance in Man 230500) and Morquio B disease (Mendelian Inheritance in Man 253010). Natural substrates for β-galactosidase are ganglioside GM1, keratan sulfate, and various glycopeptides. The sphingolipid ganglioside GM1 is the main substrate that accumulates in GM1-gangliosidosis patients, and the glycosaminoglycan keratan sulfate is the main substrate that accumulates in Morquio B patients. Three main clinical forms, according to age of onset and severity of symptoms, have been established in GM1-gangliosidosis patients: type I (infantile form), type II (late infantile/juvenile form), and type III (adult form). The different clinical forms in this sphingolipidosis are mainly attributable to different residual activities of the mutant enzymes and, thus, to different levels of substrate accumulation in tissues. Residual β-galactosidase activity in fibroblasts from patients, measured using the artificial 4-methylumbelliferyl β-galactopyranoside substrate, varies from 0.07–1.3% of control values in infantile patients to 0.3–4.8% in the juvenile form and up to 9% in adults. Morquio B patients have no neurological involvement but display severe skeletal dysostosis multiplex as a result of a high accumulation of keratan sulfate (1).

The human *GLB1* gene, localized to chromosome 3 at 3p21.33 (2), contains 16 exons that give rise to two alternatively spliced mRNAs: a major transcript of 2.5 kb that encodes the β-galactosidase and a minor transcript of 2 kb encoding the elastin binding protein (EBP). The β-galactosidase protein is targeted to the lysosome as an 88 kDa polypeptide that is then proteolytically processed to a mature 64 kDa form. EBP is a major component of the nonintegrin cell surface elastin receptor complex, in which EBP binds and protects tropoelastin (3). This complex consists of three units: EBP, neuraminidase, and protective protein/cathepsin A (PPCA). The β-galactosidase

Manuscript received 5 July 2007 and in revised form 27 July 2007.

Published, JLR Papers in Press, July 30, 2007.
DOI 10.1194/jlr.M700308-JLR200

[1] D. Grinberg and L. Vilageliu are co-last authors.
[2] To whom correspondence should be addressed.
e-mail: lvilageliu@ub.edu

protein also forms a complex, the lysosomal multienzyme complex (LMC): on reaching the endosomal-lysosomal compartment, the enzyme associates with the preassociated PPCA and neuraminidase. This complex also includes the *N*-acetylgalactosamine-6-sulfate sulfatase enzyme. The correct interaction of these proteins in the complex is essential for their correct activity (4). Within the LMC, PPCA has not only a protective role for both glycosidases against lysosomal proteolysis but also plays an important role in the correct maturation of β-galactosidase to the 64 kDa form (5–7).

Our group recently described mutations in >40 GM1-gangliosidosis and Morquio B patients (8, 9), most of them missense. Although their absence in 100 control chromosomes strongly suggests that they are disease-causing mutations, the functional analysis presented here confirms their pathogenicity. In vitro expression in COS-7 cells followed by enzymatic activity analyses were performed for 12 mutations, a functional polymorphism (p.S532G), and a change whose pathogenic status was controversial (p.R521C). Ten of these changes had not been expressed before. Although the 12 mutations caused a complete absence or a great reduction of enzyme activity, p.S532G and p.R521C showed only partial decreases in activity. In most cases, close correlations between residual activity and phenotype could be established. In some cases, an effect of β-galactosidase alterations on other members of the LMC was observed.

MATERIALS AND METHODS

Patients

The mutant alleles, samples, and cells used in this study correspond to GM1-gangliosidosis or Morquio B patients already described by our group (8, 9). The genotypes and clinical types of some representative patients are listed in **Table 1**.

Vector construction

The whole coding region of the *GLB1* cDNA was amplified by PCR in two fragments that were ligated and cloned in a pUC18 vector. Mutagenesis was always performed in the *GLB1*-pUC plasmid. For protein expression, the wild-type and mutated cDNAs were subcloned into the pcDNA3.1 expression vector (p.BGALwt and p.BGALmut, respectively). Coding regions of human PPCA and human neuraminidase-1 (or sialidase) were also cloned using the same procedure (p.NEU and p.PPCA, respectively).

Site-directed mutagenesis

All mutations were generated by site-directed mutagenesis using the QuickChange™ Site-Directed Mutagenesis XL kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. In the case of the double mutation p.L162S+p.R521C, the p.R521C mutation was generated in a vector that already bore the p.L162S mutation. All constructs were resequenced to ensure that no spurious mutation had been introduced.

Cell culture and transfection

COS-7 cells were cultured in 100 mm diameter tissue culture dishes with DMEM (GIBCO-BRL, Grand Island, NY), 10% fetal bovine serum (GIBCO-BRL), and antibiotics. For transfection with wild-type and mutant β-galactosidase cDNAs, 30×10^5 cells were plated. Twenty-four hours later (when the cells were at 90% confluence), 2 μg of the corresponding plasmid mixed with 15 μl of Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA) was added. As a negative control, an empty pcDNA3.1 vector and/or a pcDNA vector carrying antisense β-galactosidase cDNA was transfected. Cells were collected at 48 h after transfection by scraping and centrifuging for 5 min at 300 *g*. Cellular pellets were washed twice with PBS. Approximately 10% of the volume was set aside for Western blotting. The remaining cellular pellet was stored at −80°C until the enzymatic analysis was performed.

Patient fibroblasts were cultured as described previously (8), and whole protein extract was obtained by scraping the cells in lysis buffer (1% Triton X-100, 10% glycerol, 20 mM sodium acetate, and 150 mM NaCl) containing the protease inhibitor cocktail, Complete, Mini, EDTA-free (Roche, Basel, Switzerland).

Enzymatic analysis

β-Galactosidase activity was measured with the fluorogenic substrate 4-methylumbelliferyl-β-D- galactopyranoside.

SDS-PAGE and Western blot analysis

Proteins were subjected to SDS-PAGE (12.5% polyacrylamide) and electrophoretically transferred onto nitrocellulose membranes. For transfected cells, ~30 μg of protein extract was loaded, whereas for patient fibroblasts, ~40 μg was loaded.

TABLE 1. Genotype, clinical type, and residual enzyme activity of patients included in this study

Patient[a]	Mutations		Clinical Type	Activity[b]
GM5, 6, 10, 14, 16, 17, 22	p.R59H	p.R59H	I	0.05–3.5%
GM13	p.R59H	p.D441N	I	2.5%
GM9	p.D441N	p.D441N	I	0.7%
GM19	p.D198X	p.T420P	I	0.8%
GM7	p.R590C	p.R590C	I	0.9%
A1	p.[L162S;R521C]	p.[L162S;R521C]	I	3.2–1.2%
GM1	p.R201H	p.[G272D;S532G]	III	1.2%
GM20	p.T420K	p.T420K	III	3.2%
GM4	c.1479+1G>T	c.1479+1G>T	I	4%
GM8	c.245+1G>A	c.1572_1577insG	I	1.2%
MB1, MB5	p.L173P	p.T500A	Morquio B	1.8–5%
MB2	p.R201H	p.R201H	Morquio B	2.4%
MB3	p.Y83C	p.D441N	Morquio B	11.9%
MB4	p.Y444C	p.G494S	Morquio B	3.3%

[a] All patients are described in Ref. 8, except for A1, described in Ref. 9.
[b] Percentage of residual enzyme activity in patient fibroblasts.

Western blot analysis was performed as described elsewhere (10). The generation of the polyclonal anti-human β-galactosidase was described previously (3, 11). Anti-human PPCA and anti-human neuraminidase-1 antibodies were purchased from Rockland (Gilbertsville, PA). Immunoreactive bands were detected by incubating the membrane for 2 min in the following solution: 10 ml of 100 mM Tris-HCl, pH 9, 50 μl of 45 mM *p*-coumaric acid, 50 μl of luminol, and 10 μl of 30% H_2O_2.

Coimmunoprecipitation

The interaction between β-galactosidase and neuraminidase-1 was studied by coimmunoprecipitation. COS-7 cells were cotransfected with 2 μg of each plasmid (p.BGALwt/p.BGALmut, p.PPCA, and p.NEU) mixed with 20 μl of Lipofectamine™ 2000 Reagent under the conditions described above. Correct expression of each protein in each sample was checked by Western blotting (data not shown). Coimmunoprecipitation was performed with the Rabbit IgG Trueblot™ Set (eBioscience, Boston, MA) according to the manufacturer's recommendations, except for the following changes. Cell lysate was quantified, and 200 μg of total protein extract was used for each coimmunoprecipitation with 6 μl of commercial anti-neuraminidase antibody. The cell lysis buffer contained 1% Triton X-100, 10% glycerol, 20 mM sodium acetate, and 150 mM NaCl, whereas the washing buffer contained 0.01% Nonidet P-40, 10% glycerol, 20 mM sodium acetate, and 150 mM NaCl. Coimmunoprecipitation was performed at pH 5.5, as described elsewhere (12). Coimmunoprecipitation samples were subjected to Western blot detection with the specific primary antibody and using the Rabbit IgG Trueblot™ as the secondary antibody to avoid detection of the immunoprecipitated antibody, according to the manufacturer's recommendations.

Statistical analysis

For each expression experiment, transfections were performed three times for each mutation as well as for the wild-type construct and for an empty vector used as a negative control. The activity of mutant alleles was expressed as a percentage of the mean of the enzyme activity of the wild-type construct transfected in the same experiment. In both cases, mutant and wild type, the negative control activity was subtracted. For each mutant allele, at least two independent experiments, like those described above, were carried out. The Mann-Whitney *U* test was used to analyze significant differences in enzyme activities between pairs of mutant and/or wild-type enzymes.

RESULTS

Expression and enzyme activity of wild-type and mutant β-galactosidase in COS-7 cells

Different alleles of the *GLB1* gene were expressed in COS-7 cells to establish their residual enzymatic activities. The changes expressed were c.176G>A (p.R59H), c.248A>G (p.Y83C), c.485T>C (p.L162S), c.518T>C (p.L173P), c.602G>A (p.R201H), c.1258A>C (p.T420P), c.1259C>A (p.T420K), c.1321G>A (p.D441N), c.1331A>G (p.Y444C), c.1480G>A (p.G494S), c.1561C>T (p.R521C), c.1594A>G (p.S532G), c.1768C>T (p.R590C), and the double mutant c.[485T>C;1561C>T] (p.[L162S;p.R521C]). The average expressed activity of wild-type β-galactosidase in COS-7 cells was 2,009.6 ± 533.4 nmol/h/mg, whereas the average endogenous activity (COS-7 cells transfected with an empty pcDNA3) was 408 ± 94.7 nmol/h/mg. The residual enzymatic activity for each protein, given as a percentage of wild-type activity, is shown in **Fig. 1**. The following changes resulted in lack of activity: p.R59H, p.L162S, p.L173P, p.T420P, p.D441N, p.Y444C, p.G494S, p.R590C, p.[L162S;R521C]; on the other hand, p.Y83C, p.R201H, p.T420K, p.R521C, and p.S532G produced enzymes with 6.9, 15.9, 10.3, 33.2, and 60.2% of wild-type activity, respectively. **Table 2** shows the statistical analysis of the activity values between all pairs of mutant enzymes with detectable levels of activity and between each of them and the wild-type protein. All changes displayed residual enzyme activities that were statistically different from those of the wild-type enzyme. No significant differences were detected for all pair-wise comparisons for mutations p.Y83C, p.R201H, and p.T420K. The change p.R521C, described previously both as a polymorphism (13) and as a mutation (14), differs statistically from all other mutations but also from the p.S532G polymorphism, which displays the highest residual activity.

Western blot analysis of expressed changes

To confirm that a correct synthesis of the proteins from the transfected construct took place in COS-7 cells, particularly in those cases in which an absence of activity was

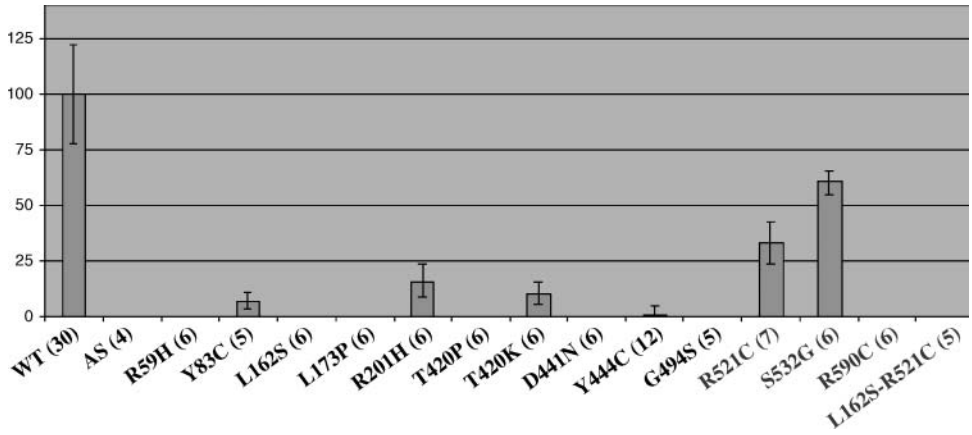

Fig. 1. Residual β-galactosidase enzymatic activity of the proteins from mutant alleles, expressed as the percentage of wild-type (WT) activity. The number of replicates for activity measurements is indicated in parentheses. Error bars correspond to standard deviation.

TABLE 2. Significance of the differences in pair-wise comparisons between mutant enzymes with detectable levels of activity and the wild-type enzyme

Y83C	R201H	T420K	R521C	S532G	Alleles	n
$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	Wild type	30
	NS	NS	$P = 0.003$	$P = 0.004$	Y83C	5
		NS	$P = 0.005$	$P = 0.002$	R201H	6
			$P = 0.001$	$P = 0.002$	T420K	6
				$P = 0.001$	R521C	7
					S532G	6

n, number of replicates.

found, immunoblot analysis of the normal and the variant proteins was performed. A unique band of ~84 kDa was detected in all cases using a polyclonal anti-human β-galactosidase antibody. This band corresponds to the precursor protein, indicating a lack of mature protein in COS-7 cells, as suggested previously (15, 16). In any case, this maturation seems not to be necessary to detect the in vitro activity of the enzyme. The 84 kDa band was observed in all of the expressed alleles (**Fig. 2**).

Coimmunoprecipitation

The association of β-galactosidase with other proteins in COS cells has been reported previously (12). Here, we coexpressed the wild-type and mutant β-galactosidase constructs with neuraminidase and PPCA and coimmunoprecipitated β-galactosidase with an anti-neuraminidase antibody to check whether the mutations found in the patients alter the formation of this complex. The results are shown in **Fig. 3**. Controls confirmed that the observed β-galactosidase band came from the specific coimmunoprecipitation, because no band was observed when the anti-neuraminidase antibody was not included (−) or when the pNEU (−NEU) or the pBGALwt (−βGAL) plasmid was not coexpressed. For all mutations, a band of 64 kDa was detected, indicating that under these conditions at least some amount of protein remains associated with neuraminidase in the complex. However, differences in intensity were clearly observed, which suggests that some mutations could affect the interaction ability of the enzyme.

Western blot analysis of patient fibroblasts

To find the effect of mutations on the proteins of the LMC, Western blot analysis was performed using antibodies against human β-galactosidase, neuraminidase, and PPCA on whole protein extracts from fibroblasts of different patients (**Fig. 4**). Western blots were repeated at least twice. Patient characteristics are listed in Table 1.

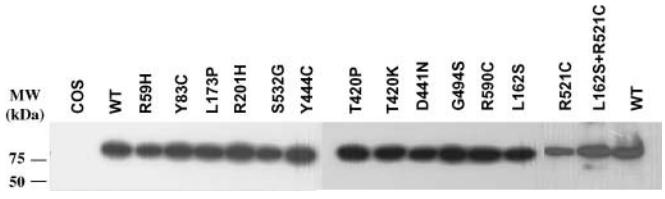

Fig. 2. Western blotting of expressed mutations in COS cells. The COS lane corresponds to cells transfected with an empty pcDNA3.1 plasmid. A total of 30 μg of protein extract was loaded in each lane. MW, molecular mass; WT, wild type.

For β-galactosidase, the wild-type sample gave rise to a main band of 64 kDa corresponding to the mature form of the protein. A faint band, corresponding to the 84 kDa immature protein, was also observed. All patient samples showed the 64 kDa band, although for patient GM4 the 64 kDa band was fainter than for the rest. Most samples showed a weak 84 kDa immature band (except for GM8), and some samples also showed smaller bands, which may correspond to degradation products.

Western blot analysis with anti-neuraminidase antibodies revealed the previously described pattern of two bands caused by different glycosylation states of the enzyme (17, 18). All patients displayed this pattern except for GM4, for whom the neuraminidase bands were nearly undetectable.

Finally, the Western blot for PPCA confirmed the ability of the anti-PPCA antibody to recognize the immature form of the enzyme (54 kDa) as well as the two mature bands (32 and 20 kDa). In most samples, a band of greater molecular mass (>100 kDa) was observed. It could correspond to a not completely denatured multimeric complex. Interestingly, different band patterns were observed for some of the patients. The 32 kDa band was not observed in GM1 and MB3 samples. Patient GM4 seemed to lack the 20 kDa band, whereas patient MB4 showed less 54 kDa immature protein.

DISCUSSION

More than 80 mutations causing GM1-gangliosidosis or Morquio B disease have been described in the *GLB1* gene, but few of them have been expressed to establish their residual enzymatic activities. This needs to be done because residual activities found in patient fibroblasts or leukocytes do not always correlate well with the observed phenotypes (as shown in Table 1). Moreover, most of the patients are compound heterozygotes, making it difficult to know the effect of each mutation individually.

COS cells have been widely and successfully used to express different lysosomal enzymes and, in particular, lysosomal β-galactosidase. For this reason, we decided to use this expression system to study the residual enzyme activities of the various mutant alleles found in our series of patients.

Table 3 shows the activities of previously expressed mutations found in the same codons of the mutations that we expressed. The results are also included. Our results are consistent with previous data, in spite of the different

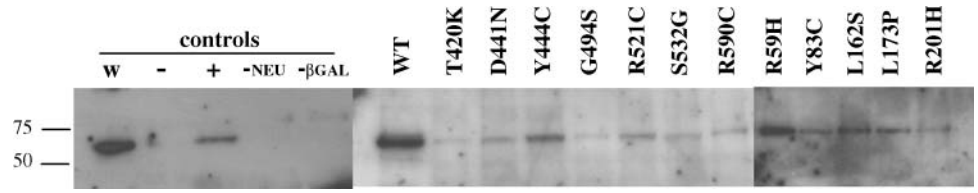

Fig. 3. Coimmunoprecipitation. Shown are Western blots using anti-β-galactosidase antibody. Controls are as follows: W, Western control: whole protein extract of coexpressed β-galactosidase, protective protein/cathepsin A (PPCA), and neuraminidase (30 μg of protein extract); −, coimmunoprecipitation without anti-neuraminidase antibody; +, positive control: coimmunoprecipitation of coexpressed β-galactosidase, PPCA, and neuraminidase using anti-neuraminidase antibody; −NEU, coimmunoprecipitation of coexpressed β-galactosidase and PPCA without expressed NEU; −βGAL, coimmunoprecipitation of coexpressed NEU and PPCA without expressed β-galactosidase. At right are the different mutations coexpressed and coimmunoprecipitated. A total of 200 μg of total protein extract was used for each coimmunoprecipitation. WT, wild type.

expression systems used. It should be noted, however, that few of these studies actually demonstrated the production of expressed protein. The only case for which our results are different from those described previously is that of p.R201H. Studies by two groups reported very different results, and we obtained an intermediate value. This mutation was found in patients with a relatively mild phenotype, which makes the result reported by Kaye et al. (19) difficult to understand. Those authors reported a very low value for the wild-type enzyme, which may explain the null result for the mutant construct. In fact, as they did not demonstrate the production of expressed protein, there is no way to correlate the low level of enzyme activity obtained with the level of protein synthesized. Our results for p.R201H activity and those of Ishii et al. (20) are higher than those described for the p.R201C mutation, consistent with the phenotypes associated with these two genetic changes.

The case of p.R521C change is particularly interesting because it was originally described as a polymorphism and found in 4% of a Brazilian control population (13) and in 1% of Spanish control individuals (8). Recently, Caciotti et al. (14) described a patient homozygous for this change and, after expressing it in COS-1 cells, reported that it was likely to be the disease-causing mutation. In our case, the

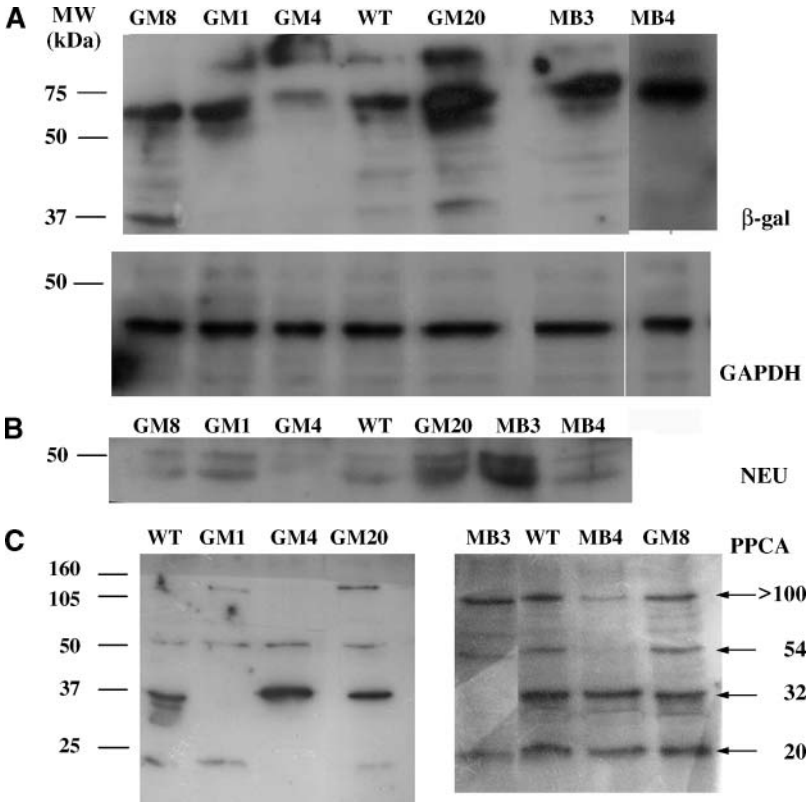

Fig. 4. Western blots of patient fibroblasts using antibodies against β-galactosidase (A), neuraminidase (B), and PPCA (C). At left, molecular masses are displayed; at right, the antibodies used are listed. Arrows indicate expected molecular masses for the observed PPCA bands. Above each lane, the patient sample loaded is shown. A total of 40 μg of protein extract was loaded in each lane.

TABLE 3. Previously reported activities of the changes found in codons expressed in this study and activities of the changes in those codons expressed in this study

Mutation	Activity	Expression System[a]	Reference
p.R59H	0.06%	COS cells, DEAE dextran	14
p.R59H	0%	COS cells, Lipofectamine™	This study
p.R59C	0.23%	COS cells, DEAE dextran	14
p.Y83H	2–5%	$ASVG_{M1}$ cells, calcium phosphate	23
p.Y83C	6.9%	COS cells, Lipofectamine™	This study
p.R201C	8.3%	$ASVG_{M1}$ cells, calcium phosphate	24
p.R201C	3.4%		20
p.R201C	12.9%	COS cells, DEAE dextran	25
p.R201C	8.5%	$ASVG_{M1}$ cells, calcium phosphate	26
p.R201C	0%	COS cells, Lipofectin™	27
p.R201H	46.5%		20
p.R201H	0%	COS cells, Lipofectamine™	28
p.R201H	15.9%	COS cells, Lipofectamine™	This study
p.R521C	24%	COS cells, DEAE dextran	14
p.R521C	33.2%	COS cells, Lipofectamine™	This study
p.S532G	81.0%	COS cells, adenovirus-mediated	22
p.S532G	75–97%	CHO cells, Lipofectin™	22
p.S532G	60.2%	COS cells, Lipofectamine™	This study
p.R590H	<1%	COS cells, Lipofectamine™	29
p.R590C	0%	COS cells, Lipofectamine™	This study

[a] Cell line and transfection system are shown.

p.R521C change resulted in 33.2% of residual activity. This activity is statistically different from that of the other polymorphisms, but it is also different from that of all of the other disease-causing mutations (Table 2). This result could explain the apparent paradox that 4% of the Brazilian population bears the p.R521C mutation but no patients with such a change have been reported to date as the cause of the disease in that population. Individuals bearing p.R521C, an allele showing a relatively high residual activity, could often remain asymptomatic. Only a few patients, like the one reported by Caciotti et al. (14), probably attributable to particular differences in genetic background or environmental factors, would develop the disease. Thus, the p.R521C change could be considered a low-penetrant mutation. A fact that supports this idea is that the p.T420K mutation, which in our experiments showed 10.3% of residual activity, was found in homozygosity in patient GM20, a mildly affected adult patient. This patient had the first symptoms of the disease at age 26 and a mild course of the disease. Accordingly, a mutant allele, such as p.R521C, with 3-fold higher activity, would only be pathogenic in a particular context. In addition, the p.S532G polymorphic change was also expressed. This allele resulted in 60.2% of activity, confirming that this is a nonpathogenic change.

In general, in the present study, good correlations were established between the expressed mutations and the phenotypes of the patients who bore them. The changes p.R59H, p.T420P, p.D441N, p.R590C, and p.[L162S;R521C], found in infantile patients, had null residual activity. The mutations found in adult patients, p.R201H and p.T420K, had 15.9% and 10.3% of residual activity, respectively. Mutations found in Morquio B are expected to have some residual activity, because they can barely cleave the terminal β-galactosyl residue of keratan sulfate but they keep some enzymatic activity against ganglioside GM1 (1). This is true for p.Y83C, which shows 6.9% of activity. The p.L173P mutation, found in two Morquio B patients, had null enzymatic activity, suggesting that the accompanying mutation (p.T500A) should be the Morquio B-causing allele, as reported previously (8).

In contrast, it was difficult to establish any correlation for mutations p.Y444C and p.G494S, found in another Morquio B patient. It was expected that at least one of them would have shown some residual activity. However, p.G494S showed null activity, and although p.Y444C had residual activity different from 0 (0.65%), this was too low to confirm it as the mutation causing Morquio B. Additional cases should be analyzed to establish this correlation.

The differences in residual activity found for all of the expressed amino acid changes cannot be attributed to the lack of correct synthesis or a complete protein degradation, as shown by Western blot analyses (Fig. 2). However, this system does not allow the cause of this absence of activity of the variant proteins to be determined, which could be either a direct effect on the catalytic site or a misfolding and/or erroneous trafficking of the protein. The misfolding/trafficking hypothesis seems to be more likely, because all of the mutations expressed in the present study affect residues that are far from those described as essential for the catalytic process: glutamate 268 (21) and aspartate 332 (22).

The possible effect of the β-galactosidase mutations and polymorphisms on the interactions with the other proteins within the LMC was studied by coimmunoprecipitating β-galactosidase with anti-neuraminidase antibody (Fig. 3). Apparently, all of the changes allow some level of interaction between β-galactosidase and neuraminidase. The differences in intensity suggest that some of the mutations could affect the interaction ability of the enzyme.

A complementary approach to studying the effect of the mutations on the protein interactions within the LMC was the analysis by Western blots of protein extracts from patient fibroblasts (Fig. 4) using antibodies against β-galactosidase, neuraminidase, or PPCA.

The results for β-galactosidase revealed that mutations in patients GM4 and GM8 allowed the synthesis of a β-galactosidase protein of ~64 kDa. This is surprising because these patients both carried either insertions or mutations affecting position +1 of a donor splice site, which would be expected to give rise to proteins with different molecular masses. In fact, these patients, as indicated in Table 1, also showed some residual enzymatic activity in their fibroblasts, especially GM4. In the case of GM8, both mutations (c.245+1G>A and c.1572_1577insG) were expected to cause nonsense-mediated mRNA decay, but both alleles were amplified by RT-PCR (data not shown). In particular, the c.1572_1577insG allele, if not degraded, was expected to give rise to a truncated protein of ~64 kDa (similar in size to the mature form of the wild-type protein). The truncated protein corresponding to the other allele (with exon 2 skipping) would be very small if synthesized. The exon skipping caused by the mutation present in homozygosity in patient GM4 does not generate a frame shift. However, the absence of exon 14 implies a reduction of ~4.8 kDa in the protein molecular mass, which does not correspond to any of the bands on the Western blot. An altered maturation and/or a modified posttranslational process could be the explanation for the observed bands.

Western blot analysis performed with anti-neuraminidase and anti-PPCA antibodies showed that, in some patients, the band patterns for these two proteins were also altered. Patient GM4 showed a clear reduction of neuraminidase band intensity and a lack of the 20 kDa PPCA band, whereas for patients GM1 and MB3, the 32 kDa PPCA band was not seen. Compared with the rest of the bands, patient MB4 also had great reduction in the intensity of the 54 kDa PPCA band. The altered band patterns observed for the PPCA and neuraminidase assays indicate that some mutations in the β-galactosidase protein affect the stability of the other proteins in the LMC. In fact, most GM1-gangliosidosis patients also show reduced neuraminidase activity (5–18% of the control mean), although higher than in sialidosis or galactosialidosis patients, which is another sign of this LMC alteration.

R.S. is the recipient of a fellowship from the Spanish Ministry of Education. The authors thank M. Cozar and H. Sellés for technical assistance. The authors are also grateful to R. Rycroft for revising the English. Financial support was provided by Centro de Investigación Cientifica y Technológica (Grants SAF2003-00386 and SAF2006-12276), Fondo de Investigación Sanitaria (Redes Temáticas, REDEMETH Grants G03/054, PI051343, and PI051182), and Agència de Gestió d'Ajuts Universitaris i de Recerca (Grant 2005SGR00848).

REFERENCES

1. Okumiya, T., H. Sakuraba, R. Kase, and T. Sugiura. 2003. Imbalanced substrate specificity of mutant beta-galactosidase in patients with Morquio B disease. *Mol. Genet. Metab.* **78:** 51–58.
2. Takano, T., and Y. Yamanouchi. 1993. Assignment of human beta-galactosidase-A gene to 3p21.33 by fluorescence in situ hybridization. *Hum. Genet.* **92:** 403–404.
3. Privitera, S., C. A. Prody, J. W. Callahan, and A. Hinek. 1998. The 67-kDa enzymatically inactive alternatively spliced variant of beta-galactosidase is identical to the elastin/laminin-binding protein. *J. Biol. Chem.* **273:** 6319–6326.
4. Pshezhetsky, A. V., and M. Ashmarina. 2001. Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Prog. Nucleic Acid Res. Mol. Biol.* **69:** 81–114.
5. Rudenko, G., E. Bonten, A. d'Azzo, and W. G. Hol. 1995. Three-dimensional structure of the human 'protective protein': structure of the precursor form suggests a complex activation mechanism. *Structure.* **3:** 1249–1259.
6. Bonten, E. J., and A. d'Azzo. 2000. Lysosomal neuraminidase. Catalytic activation in insect cells is controlled by the protective protein/cathepsin A. *J. Biol. Chem.* **275:** 37657–37663.
7. Morreau, H., N. J. Galjart, R. Willemsen, N. Gillemans, X. Y. Zhou, and A. d'Azzo. 1992. Human lysosomal protective protein. Glycosylation, intracellular transport, and association with beta-galactosidase in the endoplasmic reticulum. *J. Biol. Chem.* **267:** 17949–17956.
8. Santamaria, R., A. Chabás, M. J. Coll, C. S. Miranda, L. Vilageliu, and D. Grinberg. 2006. Twenty-one novel mutations in the GLB1 gene identified in a large group of GM1-gangliosidosis and Morquio B patients: possible common origin for the prevalent p.R59H mutation among gypsies. *Hum. Mutat.* **27:** 1060.
9. Santamaria, R., M. Blanco, A. Chabás, D. Grinberg, and L. Vilageliu. 2007. Identification of 14 novel GLB1 mutations, including five deletions, in 19 patients with GM1 gangliosidosis from South America. *Clin. Genet.* **71:** 273–279.
10. Montfort, M., A. Chabás, L. Vilageliu, and D. Grinberg. 2004. Functional analysis of 13 GBA mutant alleles identified in Gaucher disease patients: pathogenic changes and "modifier" polymorphisms. *Hum. Mutat.* **23:** 567–575.
11. Okamura-Oho, Y., S. Zhang, W. Hilson, A. Hinek, and J. W. Callahan. 1996. Early proteolytic cleavage with loss of a C-terminal fragment underlies altered processing of the beta-galactosidase precursor in galactosialidosis. *Biochem. J.* **313:** 787–794.
12. van der Spoel, A., E. Bonten, and A. d'Azzo. 1998. Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. *EMBO J.* **17:** 1588–1597.
13. Silva, C. M., M. H. Severini, A. Sopelsa, J. C. Coelho, A. Zaha, A. d'Azzo, and R. Giugliani. 1999. Six novel beta-galactosidase gene mutations in Brazilian patients with GM1-gangliosidosis. *Hum. Mutat.* **13:** 401–409.
14. Caciotti, A., M. A. Donati, A. Boneh, A. d'Azzo, A. Federico, R. Parini, D. Antuzzi, T. Bardelli, D. Nosi, V. Kimonis, et al. 2005. Role of beta-galactosidase and elastin binding protein in lysosomal and nonlysosomal complexes of patients with GM1-gangliosidosis. *Hum. Mutat.* **25:** 285–292.
15. Oshima, A., A. Tsuji, Y. Nagao, H. Sakuraba, and Y. Suzuki. 1988. Cloning, sequencing, and expression of cDNA for human beta-galactosidase. *Biochem. Biophys. Res. Commun.* **157:** 238–244.
16. Morreau, H., N. J. Galjart, N. Gillemans, R. Willemsen, G. T. van der Horst, and A. d'Azzo. 1989. Alternative splicing of beta-galactosidase mRNA generates the classic lysosomal enzyme and a beta-galactosidase-related protein. *J. Biol. Chem.* **264:** 20655–20663.
17. Bonten, E., A. van der Spoel, M. Fornerod, G. Grosveld, and A. d'Azzo. 1996. Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes Dev.* **10:** 3156–3169.
18. Milner, C. M., S. V. Smith, M. B. Carrillo, G. L. Taylor, M. Hollinshead, and R. D. Campbell. 1997. Identification of a sialidase encoded in the human major histocompatibility complex. *J. Biol. Chem.* **272:** 4549–4558.
19. Kaye, E. M., C. Shalish, J. Livermore, H. A. Taylor, R. E. Stevenson, and X. O. Breakefield. 1997. β-Galactosidase gene mutations in patients with slowly progressive GM1 gangliosidosis. *J. Child Neurol.* **12:** 242–247.
20. Ishii, N., A. Oshima, H. Sakuraba, M. Osawa, and Y. Suzuki. 1995. Beta-galactosidosis (genetic beta-galactosidase deficiency): clinical and genetic heterogeneity of the skeletal form. *Dev. Brain Dysfunct.* **8:** 40–50.
21. McCarter, J. D., D. L. Burgoyne, S. Miao, S. Zhang, J. W. Callahan, and S. G. Withers. 1997. Identification of Glu-268 as the catalytic nucleophile of human lysosomal beta-galactosidase precursor by mass spectrometry. *J. Biol. Chem.* **272:** 396–400.
22. Zhang, S., R. Bagshaw, W. Hilson, Y. Oho, A. Hinek, J. T. Clarke, and J. W. Callahan. 2000. Characterization of beta-galactosidase mutations Asp332→Asn and Arg148→Ser, and a polymorphism, Ser532→Gly, in a case of GM1 gangliosidosis. *Biochem. J.* **348:** 621–632.

23. Ishii, N., T. Oohira, A. Oshima, H. Sakuraba, F. Endo, I. Matsuda, K. Sukegawa, T. Orii, and Y. Suzuki. 1995. Clinical and molecular analysis of a Japanese boy with Morquio B disease. *Clin. Genet.* **48:** 103–108.
24. Yoshida, K., A. Oshima, M. Shimmoto, Y. Fukuhara, H. Sakuraba, N. Yanagisawa, and Y. Suzuki. 1991. Human beta-galactosidase gene mutations in GM1-gangliosidosis: a common mutation among Japanese adult/chronic cases. *Am. J. Hum. Genet.* **49:** 435–442.
25. Caciotti, A., T. Bardelli, J. Cunningham, A. D'Azzo, E. Zammarchi, and A. Morrone. 2003. Modulating action of the new polymorphism L436F detected in the GLB1 gene of a type-II GM1 gangliosidosis patient. *Hum. Genet.* **113:** 44–50.
26. Oshima, A., K. Yoshida, K. Itoh, R. Kase, H. Sakuraba, and Y. Suzuki. 1994. Intracellular processing and maturation of mutant gene products in hereditary beta-galactosidase deficiency (beta-galactosidosis). *Hum. Genet.* **93:** 109–114.
27. Nishimoto, J., E. Nanba, K. Inui, S. Okada, and K. Suzuki. 1991. GM1-gangliosidosis (genetic beta-galactosidase deficiency): identification of four mutations in different clinical phenotypes among Japanese patients. *Am. J. Hum. Genet.* **49:** 566–574.
28. Kaye, E. M., C. Shalish, J. Livermore, H. A. Taylor, R. E. Stevenson, and X. O. Breakefield. 1997. β-Galactosidase gene mutations in patients with slowly progressive GM1 gangliosidosis. *J. Child Neurol.* **12:** 242–247.
29. Boustany, R. M., W. H. Qian, and K. Suzuki. 1993. Mutations in acid beta-galactosidase cause GM1-gangliosidosis in American patients. *Am. J. Hum. Genet.* **53:** 881–888.