Role of beta-galactosidase and Elastin Binding Protein in Lysosomal and non Lysosomal complexes of GM1- gangliosidosis patients

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RESEARCH ARTICLE

Role of β-Galactosidase and Elastin Binding Protein in Lysosomal and Nonlysosomal Complexes of Patients with G_{M1}-Gangliosidosis

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G_{M1}-gangliosidosis is a lysosomal storage disorder caused by a deficiency of β-galactosidase (GLB1). The *GLB1* gene gives rise to the GLB1 lysosomal enzyme and to the elastin binding protein (EBP), involved in elastic fiber deposition. GLB1 forms a complex with protective protein cathepsin A (PPCA), alpha neuraminidase (NEU1), and galactosamine 6-sulphate sulfatase (GALNS) inside lysosomes, while EBP binds to PPCA and NEU1 on the cell surface. We investigated the function of the GLB1 and EBP mutated proteins by analyzing the clinical, genetic, and cellular data of 11 G_{M1}-gangliosidosis patients. Their molecular analysis, followed by expression studies, lead to the identification of four new and 10 known *GLB1* mutations. Some common amino acid substitutions [c.1445G>A (p.Arg482H), c.622C>T (p.Arg208His), c.175C>T (p.Arg59Cys) and c.176G>A (p.Arg59His)] were present in the GLB1 enzyme of several patients, all of Mediterranean origin, suggesting a common origin. Western blotting analyses against GLB1, EBP, and PPCA proteins showed that the identified mutations affect GLB1 enzyme activity and/or stability. The c.1445G>A (p.Arg482His), c.175C>T (p.Arg59Cys), c.733+2T>C, c.1736G>A (p.Gly579Asp), and c.1051C>T (p.Arg351X) GLB1 mutations, affect the stabilization of PPCA probably because they hamper the interaction between GLB1/EBP and PPCA within the multiprotein complex. The amount of EBP was normal, but the detection of impaired elastogenesis in such patients suggests an alteration in its function. We conclude that the presence of genetic lesions in both GLB1 and EBP coding region does not directly predict impaired elastogenesis and that elastic fiber assembly has to be evaluated specifically in each case. Nevertheless, the degree of EBP involvement may be linked to specific clinical findings. Hum Mutat 25:285–292, 2005. © **2005 Wiley-Liss, Inc.**

KEY WORDS: β-galactosidosis; GLB1; elastin binding protein; lysosomal complex; elastic fiber

DATABASES:
GLB1 – OMIM: 230500; GenBank: NT_022517.16, NM_000404.1

INTRODUCTION

G_{M1}-gangliosidosis is an autosomal recessive lysosomal disorder due to β-galactosidase (GLB1) deficiency (E.C.3.2.1.23; MIM# 230500). In G_{M1}-gangliosidosis, GM1 and GA1 gangliosides accumulate in nerve cells, while glycosaminoglycans and glycopeptides accumulate in visceral organs and other tissues [Suzuki et al., 2001]. G_{M1}-gangliosidosis has been classified into infantile (Type I), late infantile or juvenile (Type II), and adult (Type III) forms according to the age of clinical onset, the GLB1 residual enzymatic activity, and the severity of clinical course. Recently, the late infantile and the juvenile subtypes of GM_1 type II have been clearly defined [Caciotti et al., 2003].

The *GLB1* gene gives rise to two alternatively spliced mRNAs that encode the lysosomal GLB1 enzyme and the elastin binding protein (EBP) [Morreau et al., 1989; Hinek, 1996; Privitera et al., 1998]. The GLB1 precursor (85 kDa) is processed inside lysosomes into a 64-kDa mature enzyme [d'Azzo et al., 1982; Nanba et al., 1988; Van der Spoel et al., 2000]. It has been reported that GLB1 can be present in a 1.27-MDa multienzyme complex with protective protein/cathepsin A (PPCA), neuraminidase (NEU1),

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and N-acetyl galactosamine-6-sulfate sulfatase (GALNS) [Pshezhetsky and Ashmarina, 2001]. A 680-kDa lysosomal complex containing GLB1 and PPCA only has also been described [Pshezhetsky and Ashmarina, 2001]. The latter represents the major form of GLB1 enzyme inside lysosomes, where PPCA fulfils its protective function toward GLB1 [Pshezhetsky and Ashmarina, 2001].

EBP is required in the assembly of tropoelastin monomers into elastic fibers on the cell surface, where it is thought to form a complex with PPCA and NEU1 [Pshezhetsky and Ashmarina, 2001]. In this complex, PPCA seems to act as a protective protein with no enzymatic function, while NEU1 allows the attachment of the complex to the membranes [Pshezhetsky and Ashmarina, 2001].

In order to elucidate the roles of GLB1 and EBP proteins, we studied the clinical, molecular, and cellular characteristics of 11 patients with G_{M1}-gangliosidosis, focusing on the effect of all identified amino acid substitutions on elastic fiber assembly and proteins of the lysosomal and non-lysosomal complexes.

MATERIALS AND METHODS

Patients

The clinical presentation of the new G_{M1}-gangliosidosis patients is summarized in Table 1. The clinical presentation of the other patients has been previously reported [Morrone et al., 2000]. The numbering of the patients reported in these two articles has been unified.

RNA Isolation, cDNA and Genomic DNA Analyses

All reagents for mRNA extraction from patients' fibroblasts and lymphocytes were purchased from Eppendorf AG (Hamburg, Germany). RNA integrity was verified by 0.8% agarose gel electrophoresis. RT-PCR analysis on *GLB1* cDNA was carried out as previously reported [Morrone et al., 2000]. The RT-PCR products were checked on a 1.5% agarose gel, excised, and purified using the Nucleospin Extract kit (Macherey-Nagel, Düren, Germany). Sequencing reactions were performed using the ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. The nomenclature of the new *GLB1* gene genetic lesions is as designated previously [den Dunnen and Antonarakis, 2000; den Dunnen and Paalman, 2003]. The genomic reference sequence is GenBank NT_022517.16 and cDNA numbering starts from the A of the ATG translation codon in the reference sequence (Genbank: NM_000404.1; GI:10834965). The *GLB1* mutations were confirmed in patients' and their relatives' DNA. Genomic DNA was obtained from patients' lymphocytes and/or fibroblasts using a commercial DNA extraction kit (Qiagen, Hilden, Germany). The oligonucleotides and the PCR amplifying conditions were described previously [Morrone et al., 2000, Caciotti et al., 2003].

Expression Studies

Expression studies were performed on the new and known amino acid substitutions [c.175C>T (p.Arg59Cys), c.176G>A (p.Arg 59His), c.716C>T (p.Thr239Met), c.1561C>T (p.Arg521Cys), c.1736G>A (p.Gly579Asp), c.1772A>G (p.Tyr591Cys), and c.1771T>A (p.Tyr591Asn)] identified in the patients' *GLB1* gene. Site-directed mutagenesis and fragment replacement were used to introduce these genetic lesions in transient *GLB1* expression vectors as described previously [Caciotti et al., 2003]. The oligonucleotide primers for site directed mutagenesis were previously reported [Caciotti et al., 2003] except for the following:

Arg59Cys forward 5′CACTACTCCTGTGTGCCCC 3′ (166/184 nt exon 2)
Arg59Cys reverse 5′GGGGCACACAGGAGTAGTG 3′ (184/166 nt exon 2)
Thr239Met forward 5′CTCTACACCATGGTGGACTTTG 3′ (706/727 nt exon 6)
Thr239Met reverse 5′CAAAGTCCACCATGGTGTAGAG 3′ (727/706 nt exon 6)
Arg521Cys forward 5′GATGCAGTGTGCAGCCACC 3′ (1552/1570 nt exon 15)
Arg521Cys reverse 5′GGTGGCTGCACACTGCATC 3′ (1570/1552 nt exon 15)
Tyr591Asn forward 5′CTTGGCCGCAATTGGCCAGC 3′ (1762/1781 nt exon 16)
Tyr591Asn reverse 5′GCTGGCCAATTGCGGCCAAG 3′ (1781/1762 nt exon 16)
Tyr591Cys forward 5′CTTGGCCGCTGTTGGCCAGC 3′ (1762/1781 nt exon 16)
Tyr591Cys reverse 5′GCTGGCCAACAGCGGCCAAG 3′ (1781/1762 nt exon 16)

The underlined bases correspond to a mispairing with the normal sequence.

The mutated transient expression vectors were used to transform the *E. coli* strain Solo pack gold cells (Stratagene, Amsterdam Zuidoost, The Netherlands), obtaining a great quantity of each recombinant vector. The integrity of the DNA and the presence of only the expected mutations in the GLB1 c-DNA inserts were verified by sequencing both strands.

Transfections into COS-1 Cells

Normal and mutant vectors were transiently over expressed in African green monkey kidney cells (COS-1) as described previously [Caciotti et al., 2003]. In each experiment, the value of GLB1 activity in nontransfected COS-1 cells was used to set up the GLB1 intrinsic activity of the expression system. Standard deviation determination was done using Microsoft Excel 97 SR-2 (www.microsoft.com).

Cell Cultures and Biochemical Enzymatic Assays

COS-1 cells and patients' fibroblasts were cultured in Dulbecco's modified Eagles-Hams F10 medium (1:1 vol/vol) with fetal bovine serum (10%) and antibiotics.

Protein content of transfected COS-1 cells and of patients' fibroblasts was determined in triplicate by the Lowry method [Lowry et al., 1951]. GLB1 enzyme assay on fibroblasts and transfected cells was performed in triplicate by fluorescence measurement using the 4-methylumbelliferyl β-galactopyranoside artificial substrate [Galjaard, 1980].

Restriction-Site Analysis

To screen the p.Arg521Cys amino acid substitution, *GLB1* of 100 normal controls was analyzed using the CfoI enzyme (Roche, Mannheim, Germany). The PCR fragments were amplified by the genomic primers as previously reported [Morrone et al., 2000]. A 10-μl aliquot of PCR product was incubated for 1 hr at the temperature recommended by the manufacturer, with a reaction mixture containing 2 μl of 10× reaction buffer and 1 U of the restriction enzyme. The total volume was brought up to 20 μl.

Western Blotting

Patients' fibroblasts and transfected COS-1 cells were harvested by scraping in PBS and sonicated as described previously [Okamura-Oho et al., 1996]. About 20 μg of total fibroblasts and COS-1 cells were used in the blots. Western blots were prepared from 12.5% polyacrylamide gels and probed as previously described [Van Dongen et al., 1985]. Following electrophoresis, proteins were transferred onto nitrocellulose (Bio-Rad, Hercules, CA). Western blotting was carried out with the following antibodies:

- α-85, anti GLB1 antibody [Van der Spoel et al., 2000; Caciotti et al., 2003].
- alf1, anti EBP antibody, conjugated with BSA (bovine serum albumin) and immunized into rabbits, produced by Igtech (Perdifumo, Italy). The identification of EBP antigenic and hydrophilic peptides was used to elucidate the following EBP epitope: NH2-VGSPSAQDEASPLS-COOH (91–104 amino acids).

TABLE 1. Clinical Features of the New GM1-gangliosidosis Patients

Patient	1.1	5.1	6.1	8.1	9.1	10.1	11.1
Ethnic origin	Italy	USA (Dominican Republic origin)	USA	Australia (Maltese origin)	Australia (Maltese origin)	Italy	Italy (Moroccan origin)
Clinical phenotype	Infantile	Infantile	Infantile	Infantile	Infantile	Infantile	Adult
Age of onset	6 months	4 months	2 months	3 months	At birth	3 months	27 years
Presentation	Psychomotor delay, broncho-pneumonia	Psychomotor delay, asthma, macrocrania, flat facies	Lethargy	Psychomotor delay	Edema at birth subsided at 3months hypotonia	Psychomotor delay, facial dysmorphisms	Mild extra pyramidal signs, ataxia
Age of diagnosis	9 months	8 months	5 months	5 months	8 months	6 months	30 years
Skeleton	Kyphosis of the dorsolumbar spine	Kyphosis of the dorsolumbar spine, gibbous	Extreme ankle stiffness	Dysostosis multiplex	Enlarged marrow spaces, decreased tubulation and thinning of cortices	Kyphosis of the dorsolumbar spine, dysostosis multiplex	Spondylo dysplasia
Liver/spleen	Enlarged	Enlarged	Enlarged	Enlarged	Enlarged	Enlarged	Normal
Heart	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Nervous system	Axial hypotonia	Hypotonia, psychomotor delay	Hypotonia, spasticity of lower extremities, psychomotor delay	Hypotonia, seizures	Hypotonia, psychomotor delay	Hypotonia, psychomotor delay	Normal intelligence
Eye	Cherry red spot	Nystagmus	Severe ophtalmological involvement	Cherry red spot	Subtle cherry red spots	Cherry red spot	Normal
Enzymatic assay*	2% of control value in leukocytes	1% of control value in leukocytes	$<1\%$ of control value in leukocytes	$<1\%$ of control value in fibroblasts and leukocytes	$<1\%$ of control value in fibroblasts and leukocytes	$<1\%$ of control value in fibroblasts and leukocytes	30% of normal value in fibroblasts
Course	Alive at 28 months	Alive at 24 months	Death at 20 months	Death at 27 months	Death at 24 months	Death at 25 months	Alive at 37 years

*The results of GLB1 activity are the average of three independent assays performed in triplicate and compared to normal controls.

- a54, anti PPCA antibody [Morreau et al., 1989, Galjart et al., 1991].
- anti actin antibody (SIGMA, Milano, Italy), was used to assay total protein content loaded in the blots.

The results of the blots were visualized by reaction with the secondary antibody anti-rabbit IgG (whole molecule) Alkaline phosphatase conjugate (Sigma, Milano, Italy), revealed by AP Conjugate Substrate kit (Bio-Rad, Hercules, CA).

Immunostaining

The 10-day-old cultures of normal and patients' fibroblasts were immunostained as previously reported [Privitera et al., 1998]. Rabbit polyclonal antibodies against human tropoelastin (Elastin Products Company, Missouri) and fibronectin (Sigma, Milano, Italy), were used as primary antibodies. Direct immunofluorescence was performed by a secondary fluorescein isothiocyanate conjugated (FITC) antibody, purchased from Sigma (Milano, Italy). To stain nucleic acids, fibroblasts were also incubated with propidium iodide (10 μg/ml), as described previously [Malvagia et al., 2004]. Photomicrographs of treated fibroblasts were performed by the BioRad 1024MRCES confocal light microscope, equipped with an Argon-Kripton laser (BioRad). A series of 13 optical sections (512 × 512 pixels), with a thickness of 1.5 μm, was collected for each sample. Single composite images were obtained by superimposition of the optical sections.

RESULTS

Clinical and Biochemical Presentation

Six of the seven new patients in this study (Patients 1.1, 5.1, 6.1, 8.1, 9.1, 10.1, and 11.1; Tables 1 and 2) presented with the infantile form of the disease. Their clinical findings are summarized in Table 1. Patient 11 was diagnosed as having the adult form of G_{M1}-gangliosidosis and presented with a particularly mild form of the disease. GLB1 deficiency was evident from enzyme assays performed in leukocytes and fibroblasts of all patients (Table 1). Biochemical analysis of fibroblasts and leukocytes from G_{M1}-gangliosidosis Patients 2.1, 3.1, 4.1, and 7.1 was previously reported [Morrone et al., 2000]. NEU1 enzyme activity, assayed in leukocytes, was within the normal range in all patients. The mild clinical presentation of Patient 1.1 correlated with her relatively high GLB1 enzyme activity (Table 1).

Molecular Analysis and Expression Studies

A summary of the four new (p.Arg59Cys, p.Thr239Met amino acid substitutions, c.1309delA small deletion, and c.733+2T<C splicing defect) and the 10 known [p.Arg59His, c.1445G>A (p.Arg482His), c.841C>T (p.His281Tyr), c.602G>A (p.Arg201His), c.1051C>T (p.Arg351X), p.Gly579Asp, p.Tyr591Asn, p.Tyr591Cys, p.Arg521Cys, and c.622C>T (p.Arg208Cys)] amino acid substitutions identified in the GLB1 enzyme in our 11 patients is shown in Table 2. The full length *GLB1* cDNA of the new patients in this cohort (Patients 1.1, 5.1, 6.1, 8.1, 9.1, 10.1, and 11.1) and the exon–intron boundaries of their *GLB1* gene were amplified and directly sequenced on both strands. The mutations detected in the patients' cDNA were confirmed in their genomic DNA and in their parents'. A possible benign polymorphism for each new point mutation was excluded by transient expression studies (Table 2). In order to examine the effect of these missense mutations and that of the p.Arg59His, p.Gly579Asp, p.Tyr591Cys, p.Tyr591Cys, and p.Arg521Cys amino acid substitutions previously described [Silva et al., 1999; Morrone et al., 2000], expression vectors were created by in vitro site direct mutagenesis. The pcD-GLB1 vectors carrying these genetic lesions

TABLE 2. Molecular Characterisation of the Genetic Lesions Identified in GM1-gangliosidosis Patients and GLB1 Activity Resulting from Expression Studies in COS-1 Cells*

Patient	Ethnic origin	Phenotype	Intron/Exon	Nucleotide changes	Amino acid changes	GLB1 activity on COS-1 cells (%) ± SD
1.1	Italy	I	Exon 16	c.1736G>A	p.Gly579Asp	0,16 ± 0.15
			Exon 10	c.1051C>T	p.Arg351X	
2.1	Italy	I+C	Exon 02	c.176G>A	p.Arg59His	0.06 ± 0.25
			Exon 02	c.176G>A	p.Arg59His	
3.1	Italy	I+C	Exon 16	c.1771T>A	p.Tyr591Asn	0.46 ± 0.25
			Exon 16	c.1771T>A	p.Tyr591Asn	
4.1	Italy	I+C	Exon 16	c.1772A>G	p.Tyr591Cys	0.6 ± 0.17
			Exon 16	c.1772A>G	p.Tyr591Cys	
5.1	USA (Dominican Republican origin)	I	Exon 06	c.622C>T	p.Arg208Cys	<1†
			Exon 06	c.716C>T	p.Thr239Met	0.06 ± 0.15
6.1	USA	I	Exon 08	c.841C>T	p.His281Tyr	
			Exon 13	c.1309delA	Frame shift	
7.1	Italy	J	Exon 06	c.602G>A	p.Arg201His	46,5†
			Exon 16	c.1736G>A	p.Gly579Asp	
8.1	Australia (Maltese origin)	I	Exon 14	c.1445G>A	p.Arg482His	0.4†
			Exon 14	c.1445G>A	p.Arg482His	
9.1	Australia (Maltese origin)	I	Exon 14	c.1445G>A	p.Arg482His	0.4†
			?	?	?	
10.1	Italy	I	Exon 02	c.175C>T	p.Arg59Cys	0.23 ± 0.2
			Exon 06	c.733+2T>C	Frame shift	
11.1	Italy (Moroccan origin)	A	Exon 15	c.1561C>T	p.Arg521Cys	24 ± 2
			Exon 15	c.1561C>T	p.Arg521Cys	

*Percentages are referred to GLB1 activity measured in normal controls (COS-1 cells transfected with wild type GLB1 vectors). The values are the average of three independent experiments. SD, Standard deviation. I, Infantile; J, Juvenile; A, adult phenotypes; C, cardiomyopathy. *GLB1* genomic sequence GenBank: NT_022517.16; *GLB1* cDNA sequence GenBank: NM_000404.1. cDNA numbering starts from the A of the ATG translation codon in the reference sequence.

†Reported in Callahan [1999].

were transiently expressed in COS1 cells. All mutations detected in the infantile patients led to no residual enzyme activity, including the p.Gly579Asp amino acid substitution detected both in an infantile and in the juvenile patient (Table 2). In addition, sequence alignments of GLB1 related proteins (glycosyl hydrolase Family 35), indicated that the p.Arg59 amino acid is conserved between species (Fig. 1).

The DNA analysis of Patient 1.1 indicated that she was homozygous for the known p.Arg521Cys amino acid substitution, previously assigned with a benign nature [Silva et al., 1999]. In order to clarify the nature of the p.Arg521Cys mutated protein, we performed expression studies and restriction analysis. We found this amino acid substitution in less than 1% of 200 normal alleles screened, suggesting that it is not a benign substitution. This assumption was confirmed by expression studies that showed a reduction of the activity of the p.Arg521Cys-GLB1 enzyme to about 25% of normal value and that strongly correlate with what we found by in vitro GLB1 assay in the patient's fibroblasts and leukocytes (Table 1).

Western Blotting

Immunoblot analysis using GLB1 antibody resulted in no enzyme detection in the lanes corresponding to COS-1 cells transfected with the p.Thr239Met, p.Gly579Asp, p.Tyr591Cys, and p.Tyr591Asn mutations, respectively (Fig. 2A). Immunoblot analysis was also performed for the detection of other multienzyme complex proteins (EBP and PPCA) in patients' biological materials (from Patients 2.1–5.1, 7.1, 8.1, 10.1, and 11.1). Western blot analysis showed a normal amount of EBP in all lanes corresponding to the patients' fibroblasts, compared both to normal controls and to a normal amount of control protein (β-actin). On the other hand, a reduction of PPCA protein was detected in the lanes corresponding to Patients 1.1, 8.1, and 10.1 (Fig. 2B).

Elastic Fiber Assembly

In order to elucidate the correlation between eventual connective tissue alteration and the clinical/molecular analyses in our patients, experiments on elastic fiber assembly were carried out in patients' fibroblasts by a double immunoassay of tropoelastin and fibronectin. Immunofluorescence studies were performed in those patients presenting with different clinical subtypes of G_{M1}-gangliosidosis: classic infantile phenotype (Patient 10.1), infantile phenotype with cardiomyopathy (Patient 3.1), juvenile (Patient 7.1), and a mild form of the adult phenotype (Patient 11.1). Elastic fiber assembly was markedly reduced in the patient with the infantile form with cardiomyopathy, while a lesser but still marked

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Asparagus                    TYDHKSVIINGQRRILISGSIHYPRSTP-EMWPDLIQKAKDGGLDVIQTY
Malusdomestica               SYDHKAIIINGQKRILISGSIHYPRSTP-EMWPDLIQKAKDGGLDVIQTY
Brassicaoleracea             SHDERAITIDGQRRILLSGSIHYPRSTS-DMWPDLISKAKDGGLDTIETY
BGAL_HUM                     DYSRDSFLKDGQPFRYISGSIHYSRVPR-FYWKDRLLKMKMAGLNAIQTY
BGAL_MOUSE                   DYSRDRFLKDGQPFRYISGSIHYFRIPR-FYWEDRLLKMKMAGLNAIQMY
BacilluscirculansATCC31382   TYD-DSFLLDGKEIRLLSGAMHYFRTVP-EYWEDRLLKLKACGFNTVETY
Xanthomonasmanihotis         QGT--QFVRDGKPYQLLSGAIHFQRIPR-AYWKDRLQKARALGLNTVETY
Arthrobacter                 TIGEQDFLLNDQPHRILAGALHYFRVHP-DQWADRIRKARQMGLNTIETY
Aspergillus                  TWDDKSLFINGERIMIFSGEFHPFRLPVKELQLDIFQKVKALGFNCVSFY
                                   .  :.:    ::* :*  *        * : * :  *:: :. *
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FIGURE 1. **GLB1 sequence alignments between species. Family 35 glycosyl hydrolases and related proteins were aligned in the region surrounding p.Arg59 amino acid of the human GLB1. Both aligned amino acids are indicated by squares. * Total sequence homology;: Very high homology;. High homology.**

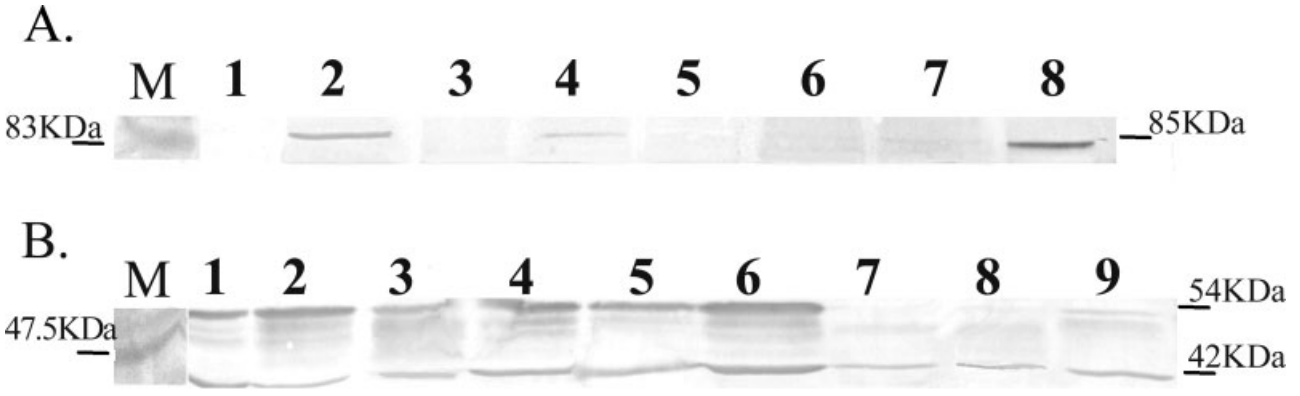

FIGURE 2. **A: Immunoblot analysis of COS-1 cells with α-85 GLB1 antibody. Each lane represents protein lysate (approximately 15μg) of COS-1 cells transfected with either: no vector (1); pcDGLB1-wild type (2); pcDGLB1-Thr239Met (3); pcDGLB1-Arg59His (4); pcDGLB1-Gly579Asp (5); pcDGLB1-Tyr591Asn (6); pcDGLB1-Tyr591Cys (7); or pcDGLB1-Arg521Cys (8); Protein Molecular Weight Marker (M; New England Biolabs). B: Immunoblot analysis of patients' fibroblast with PPCA antibody. About 20 μg of proteins was analyzed by Western blots. Total cellular proteins were probed using a54, which reacts with the PPCA precursor form. Fibroblasts derive from the following: normal control (1); Patient 11.1 (2); Patient 7.1 (3); Patient 3.1 (4); Patient 4.1 (5); Patient 2.1 (6); Patient 10.1 (7); Patient 1.1 (8); Patient 8.1 (9); Protein Molecular Weight Marker (M; New England Biolabs).**

reduction of elastin assembly was detected in the patient with the classical infantile form. A remarkable decrease of elastin assembly compared with normal control was also observed in the juvenile and adult patients' fibroblasts (Fig. 3). In order to evaluate a parameter of normal elastic fiber deposition, fibronectin amount was evaluated. As expected, normal amounts of fibronectin were found in all these patients' fibroblasts (data not shown) as previously described in fibroblasts from patients affected by Morquio B and G_{M1}-gangliosidosis [Hinek et al., 2000].

DISCUSSION

Mutations in the gene encoding the GLB1 enzyme cause the lysosomal disorder G_{M1}-gangliosidosis [Suzuki et al. 2001]. However, the molecular basis of the disease is related to the role of both *GLB1* gene products, GLB1 and EBP, and to the interactions of these proteins within their complexes inside lysosomes and on cell surface, respectively [Pshezhetsky and Ashmarina, 2001]. In addition, the tertiary structure of GLB1 has not been resolved, although the Glu268 and the Asp332 residues, conserved between species, have been proposed to be part of the catalytic sites [McCarter et al., 1997; Callahan, 1999; Pshezhetsky and Ashmarina, 2001]. In order to elucidate the effect of each *GLB1* gene mutation, both common and private, on the GLB1 functional domains and on the stability of the proteins of the complexes, we performed detailed molecular and biological analyses of cell lines from 11 patients with this rare metabolic disease.

Hotspot regions for the GLB1 mutations have been suggested [Silva et al., 1999] and both p.Thr239Met and p.Arg208Cys mutations, mapping to exon 6, confirm this exon to be a hotspot. The importance of the p.Arg59 amino acid to the function of the enzyme has previously been reported in patients of Italian and Brazilian origin [Silva et al., 1999; Morrone et al., 2000]. In addition, the p.Arg482His amino acid substitution has been previously reported at homozygous or heterozygous level in eight unrelated Italian patients [Mosna et al., 1992]. In the current work, the p.Arg482His mutation was identified in two Australian patients of Maltese origin. Moreover, it has been previously suggested that the p.Arg208Cys mutation is of an Hispanic origin with a relatively high frequency in North and South America, via Puerto Rico [Chiu et al., 1996]. This hypothesis is supported by the ethnic background of Patient 5.1 (from the Dominican Republic), who carries the p.Arg208Cys mutation at a heterozygous level. Thus, it can be surmised that the p.Arg59Cys, p.Arg59His, and p.Arg482His, as well as the p.Arg208Cys mutations are of a Mediterranean origin.

The effect of the missense mutations in our patients on the enzyme activity and stability of GLB1 was elucidated by expression studies and Western blot analysis. The severity of the clinical phenotype of the patients seems to depend on whether "severe" GLB1 mutations are present exclusively or in combination with "mild" mutations. The results of expression studies for the p.Arg59His and Arg59Cys mutations correlated with the clinically severe phenotypes of two infantile patients in our cohort; one of whom was homozygous for the p.Arg59His mutation (Patient 2.1;

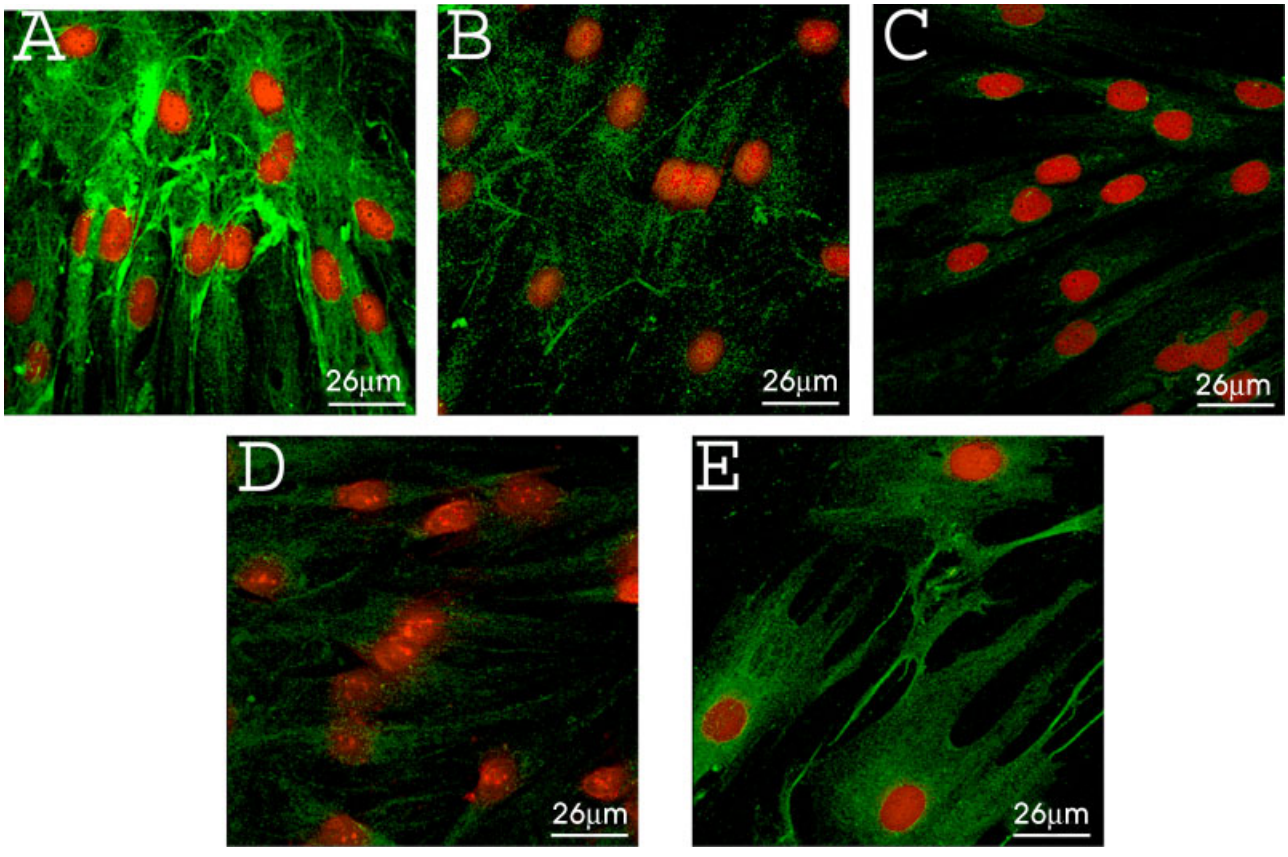

FIGURE 3. **Immunofluorescence studies. Patients' fibroblasts were incubated with polyclonal antibody to tropoelastin. Fibroblasts derive from: normal control (A); Patient 3.1 (B); Patient 10.1 (C); Patient 7.1 (D); and Patient 11.1 (E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

Table 2). These results also confirmed a key role for this amino acid in the function of the enzyme.

The p.Gly579Asp mutation, present in both an infantile and in the juvenile patient, is a severe disease causing mutation, and the less-severe phenotype of the juvenile patient is due to the p.Arg201His allele. Severe missense mutations (p.Arg59His, p.Arg59Cys, p.Thr239Met, p.Gly579Asp, p.Tyr591Cys, and p.Tyr591Asn) rendered GLB1 enzymatically inactive. Indeed, Western blot analysis showed that the p.Thr239Met, p.Tyr591Cys, and p.Tyr591Asn mutations cause a rapid degradation of the GLB1 precursor. Western blot analysis also revealed that fibroblasts from Patients 1.1 and 10.1 had a markedly reduced amount of PPCA, while no PPCA was detected in fibroblasts from Patient 8.1 (Fig. 2). These findings suggested that some GLB1 regions might be regulation sites in the interaction or stabilization of GLB1 and PPCA in the lysosomal complex. In addition, all mutations detected, except for the p.Arg201His, p.Arg208Cys and the p.Thr239Met mutations, affect both the GLB1 and EBP proteins. Thus, EBP and PPCA are predicted to be involved by the mutations detected in Patient 1.1, 8.1, and 10.1 (Tables 1 and 2). Since these patients carry genetic lesions in more then one specific GLB1 gene region, complicated folding interactions between GLB1 and PPCA in the lysosomal complex or EBP receptor may be suggested.

Expression studies also focused on the effect of the p.Arg521Cys amino acid substitution, previously described as a polymorphism [Silva et al., 1999]. The GLB1 enzyme activity decreased to about 25 to 30% in fibroblasts of Patient 11.1, and in the COS-1 cells transfected with the vector carrying the p.Arg521Cys amino acid substitution, suggesting it to be responsible for the patient's clinical manifestations. However, the patient presents a particularly mild phenotype and the p. Arg521Cys amino acid change has been reported at a high polymorphic frequency (4%) in the Brazilian population [Silva et al., 1999]. Thus, we aimed to exclude other neurodegenerative diseases in this patient, even if we cannot rule out the possibility that such a phenotype arises from the interaction between the partial reduction of GLB1 activity due to the p.Arg 521Cys allele and an unrelated disorder. Causes of ataxia, such as Friedreich ataxia 1, MELAS, and MERRF syndromes (at a molecular level), and an insufficient absorption of vitamins, in particular Vitamins E and B12 (at a biochemical level), were excluded in this patient. The electromyography excluded myopathy, and peripheral neuropathy was excluded by the patient's normal nerve conduction velocity. Other lysosomal disorders have been excluded by biochemical assays. In addition, we underline that the role of polymorphisms as disease causing lesions has been also discussed in previous reports on *GLB1* gene mutations, concerning two inbred strains of mice [Hara et al., 1994], and a G_{M1}-gangliosidosis patient [Caciotti et al., 2003]. The restriction analysis and expression studies in the *GLB1* gene of Patient 11.1 confirmed that the benign nature of a mutation has to be carefully investigated, especially when the affected enzyme is present in a multienzyme complex. In that case, the role of a genetic lesion should be evaluated in the folding process and in the stabilization of all the proteins involved, and a wide range of phenotypes can result from such altered interactions. The p.Arg521Cys mutation introduces a cysteine residue into the GLB1 protein. It can be hypothesized that this amino acid change may interfere with disulfide bridges and may lead to a GLB1 misfolding, that could also affect the lysosomal complex's stabilization.

EBP facilitates the assembly of elastic fibers that have been properly synthesized and secreted in the extracellular matrix [Hinek et al., 2000]. Thus, EBP involvement in G_{M1}-gangliosidosis has been particularly linked to impaired elastogenesis [Hinek et al., 2000] and, consequently, to cardiac involvement [Morrone et al., 2000]. The present study confirms this association. Decreased elastin deposition has been previously found in infantile G_{M1}-gangliosidosis patients, who carried nonsense mutations in the *GLB1* gene [Hinek et al., 2000]. In the present study, elastic fiber assembly was investigated in fibroblasts from patients with different phenotypes of G_{M1}-gangliosidosis. These patients had mutations in the coding region common to GLB1 and EBP proteins, except for the p.Arg201His mutation detected at heterozygous level in the patient with the juvenile form of the disease. In addition, immunofluorescence studies, revealed that elastic fiber assembly is partially altered in fibroblasts from the patient with the classic infantile form of the disease. By contrast, a complete absence of elastic fiber deposition was found in fibroblasts of the patient with cardiomyopathy.

We conclude that the presence of genetic lesions in both GLB1 and EBP coding region does not directly predict impaired elastogenesis, and that elastic fiber deposition has to be evaluated specifically in each case. The degree of EBP involvement may be linked to specific clinical findings, such as the cardiomyopathy in G_{M1}-gangliosidosis. It is predictable that in such patients impaired elastogenesis should be present.

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