

Two New Mild Homozygous Mutations in Gaucher Disease Patients: Clinical Signs and Biochemical Analyses

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Gaucher disease (GD) is a lysosomal storage disorder resulting from impaired activity of lysosomal β -glucocerebrosidase. More than 60 mutations have been described in the GBA gene. They have been classified as lethal, severe, and mild on the basis of the corresponding phenotype. The fact that most GD patients are compound heterozygous and that most type 1 patients bear the N370S allele, which by itself causes a mild phenotype, make it difficult to correlate the clinical signs with the mutations. Besides N370S, about 10 mild mutations have been described, but only one undoubtedly classified as mild was found at homozygosity. Here we report 2 novel mutations, I402T and V375L, at homozygosity in 2 adult Italian type 1 GD patients. Some properties of the I402T fibroblast enzyme have been compared to those of the enzyme from cells of several N370S/N370S patients. Analysis of the catalytic properties and heat stability as well as the response to phosphatidylserine and sphingolipid activator protein indicate a marked similarity between the 2 enzymes. The finding of another, unrelated patient bearing the I402T mutation (in this case as a compound heterozygote with mutation N370S) suggests that this allele might be quite frequent in the area of Sicily from where both patients originated. In conclusion, the phenotypic expression in the 2 homozygous patients presented here and the biochemical data for one of them allowed the classification of these mutations as mild thus extend-

ing the group of mild mutations found at homozygosity. *Am. J. Med. Genet.* 70:437–443, 1997. © 1997 Wiley-Liss, Inc.

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INTRODUCTION

Gaucher disease (GD) is a lysosomal glycolipid storage disorder characterized by the accumulation of glucosylceramide mainly in macrophages. It is common among Ashkenazi Jews [Beutler and Grabowski, 1995]. There are 3 clinical types of GD: type 1, characterized by hepatosplenomegaly, anemia, thrombocytopenia and bone lesions, and by the lack of primary nervous system involvement; type 2, the acute neuronopathic form of the disease, with severe nervous system involvement and death usually within the first 2 years of life; and type 3, the juvenile subacute neuronopathic form, with later onset and a more protracted course than type 2.

Nearly all GD cases are due to mutations in the gene encoding β -glucocerebrosidase (GBA); to date more than 60 mutations have been described [Balicki and Beutler, 1995]. Although genotype-phenotype correlations are difficult to establish, Beutler et al. [1994] classified the mutations on the basis of the severity of the phenotypic expression. Lethal mutations prevent the formation of any enzyme. They have never been found either at homozygosity or as combined heterozygotes with another lethal mutation. Severe mutations include those associated with the neuronopathic forms of the disease. Mild mutations are those that are always associated with type 1 disease, even when they are present in combination with a severe or a lethal mutation.

Severe mutations tend to produce enzymes with decreased stability and severely reduced catalytic activity, while mild mutations (e.g., N370S) lead to mutant GBA with reduced activity but nearly normal stability.

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It is difficult to classify a mutation as mild because most type 1 GD patients bear the common N370S mutation. As the presence of this mutant allele implies by itself a type 1 phenotype, the severity of the accompanying mutation is difficult to ascertain. Moreover, most reported GD genotypes are heterozygous compounds, and so it is difficult to correlate the clinical findings with either of the 2 mutations, particularly in the absence of expression experiments.

Here we report on 3 patients of Italian origin affected with type 1 GD. Two of them were homozygous for 2 different, previously undescribed mutations: I402T and V375L, and the third one was heterozygous for the I402T mutation. The homozygosity for I402T prompted us to compare some properties of the mutant enzyme in cells from this patient to those from patients homozygous for N370S. The lack of available material from the patient homozygous for V375L precluded further enzymatic characterization.

PATIENTS

Patient 1

This patient was from Catania (Sicily, Italy) and his parents were consanguineous. An older sister had hepatosplenomegaly and died at the age of 64 without a specific clinical diagnosis. The patient is now 45 years old and he presented the first symptoms at age 37 years when he complained of asthenia and fatigue. Physical examination at 41 years showed a marked visceral enlargement with liver palpable at 7 cm and spleen at 10 cm below the costal margin. Blood cell counts showed thrombocytopenia (32,000 platelets per microliter). Serum level of acid phosphatase was high (15.2 U/l with normal values < 4.7 U/l). A bone marrow biopsy showed typical Gaucher cells. The patient has no skeletal involvement. He was treated with enzyme replacement therapy, and a gradual improvement of the hematological parameters was observed (platelet count: 105,000 per microliter). Progressive reduction of the visceral enlargement was demonstrated both on clinical and ultrasound examination.

Patient 2

This patient was a 36-year-old man from the same region as patient 1. His parents were not consanguineous. Clinical symptoms began at the age of 33 years with recurrent epistaxis and general weakness. Clinical evaluation at 36 years demonstrated moderate visceral enlargement (liver and spleen 4 cm below the costal margin) and reduced platelet counts (90,000 per microliter). Gaucher cells were detected in a bone marrow biopsy. Skeletal radiographs were normal.

Patient 3

Patient 3 is now 44 years old. He also had an Italian origin but no family data are available. At the time of diagnosis (39 years), he presented with asthenia, stroke-like episodes with paresia of his right arm, and thrombocytopenia. Physical examination demonstrated spleen and liver enlargement and Gaucher cells

were detected in the bone marrow. The patient was splenectomized and the anatomical examination of the spleen confirmed Gaucher disease.

Patients Homozygous for N370S

For comparative enzymatic studies, 6 Spanish type 1 GD patients (N370S/N370S) were analyzed. Four of them have been described previously [I.2, I.7., I.9, and I.12 in Cormand et al., 1995].

MATERIALS AND METHODS

Skin fibroblast cultures were established according to routine procedures in Eagle's minimum essential medium. Leukocytes were prepared according to the method of Skoog and Beck [1956]. Sphingolipid activator protein C (Saposin C, Gaucher activator) was partially purified from a human brain [Chabás et al., 1987].

Biochemical Analyses

Enzymatic activity in fibroblasts and leukocytes was determined in citrate buffer (0.04 M, pH 5.5) using 4MU- β -glucopyranoside as the substrate (MU-Glc, 3 mM) in the presence of sodium taurocholate (T, 0.3% w/v) and Triton X-100 (TX, 0.24% w/v) in a reaction mixture of 0.15 ml.

Km studies. The reaction mixture contained fibroblast extract in a constant amount of enzyme activity and MU-Glc at a concentration range of 0.4–10 mM in citrate buffer (0.04 M, pH 5.5). Km values were determined from Lineweaver-Burk plots.

Heat stability. The fibroblast extracts (20–30 μ g protein) in 0.5 M citrate buffer, pH 5.5, were placed at 50°C and enzyme activity was assayed in samples withdrawn at 0, 5, 10, 20, 30, and 60 minutes as described above.

Effect of natural activator. Stimulation of fibroblast glucocerebrosidase by the combination of Saposin C (SAP, 150 μ g) and phosphatidylserine (PS, 5 μ g), or by PS alone, was carried out using MU-Glc (2.5 mM) in citrate buffer (0.025 M, pH 5.0) in a final volume of 0.2 ml.

Other enzyme assays. Plasma chitotriosidase activity was examined [Hollak et al., 1994]. Activity of β -xylosidase in fibroblasts (50–75 μ g protein) was measured with 4MU- β -xylopyranoside (1.6 mM in 0.04 M citrate buffer, pH 5.0) in the presence of T and TX in a final volume of 0.13 ml.

Molecular Analyses

High molecular weight DNA was prepared from peripheral blood leukocytes using the salting out procedure of Miller et al. [1988]. The A6144G polymorphism, in intron 9 of the GBA gene was studied by *Hha*I digestion of a polymerase chain reaction (PCR)-amplified product. Gene-specific primers (sense: nt 5904–5923, antisense: 6655–6690) were used to amplify a 787 bp

fragment [Sidransky et al., 1992]. The alleles were designated as "+" (*Hha*I site present) or "-" (*Hha*I site absent) according to Beutler et al. [1992].

The common N370S mutation was detected by "mismatched PCR" using a 5' primer mismatched at one nucleotide in order to create a *Xho*I restriction site when the mutation was present [Beutler et al., 1990]. Digested PCR products were subjected to electrophoresis on a 4% NuSieve GTG agarose gel (FMC, Rockland, ME).

Mutation L444P was detected by PCR amplification followed by *Nci*I digestion of the product and electrophoresis on a 1.2% agarose gel [Sidransky et al., 1992]. Mutation D409H was detected by allele-specific oligonucleotide (ASO) hybridization as described by Cormand et al. [1995].

PCR amplification and SSCP analysis of 14 DNA fragments covering all 11 exons of the GBA gene and their flanking sequences were performed on genomic DNA from the patients and relatives. All primer pairs were chosen to amplify the GBA gene and not the highly homologous pseudogene. The size of the amplified fragments ranged from 139 to 292 bp. For all fragments, the PCR reaction was performed in a volume of 50 μ l containing 100 ng of genomic DNA, 1 U of Dynazyme DNA polymerase (Finnzymes Oy, Finland), 200 μ M dNTPs, and 20 pmol of each primer, in the buffer recommended by the manufacturer. The PCR program consisted of 35 cycles of denaturation at 94°C for 40 seconds and a single annealing/extension, step at 55°C for 30 seconds.

For the single-strand conformation polymorphism (SSCP) analysis, 1 μ l of the PCR product was mixed with 6 μ l of 95% formamide, 0.05% xylene cyanol, 0.05% bromophenol blue, 20 mM EDTA solution. The samples were then denatured by incubation at 80°C for 3 minutes and placed on ice. Electrophoresis was carried out using a 18 \times 24 cm nondenaturing polyacrylamide gel. Four SSCP conditions were tested in each fragment, combining different polyacrylamide concentrations (8 or 12% acrylamide:bisacrylamide 29:1), different glycerol concentrations in the gel (0% or 5% glycerol), and 2 running conditions (RT at 200 V, or 4°C at 300 V, always 12 hours). Single and double DNA strands were revealed by silver staining as follows: the gel was incubated for 5 minutes with a 10% ethanol solution and for 3 minutes with a 1% HNO₃ solution. After washing in deionized water, it was incubated for 20 minutes with a 12 mM AgNO₃ solution. The gel was washed again and incubated (up to 10 minutes) with a freshly prepared mixture of 280 mM Na₂CO₃ and 0.02% formaldehyde. The gel was finally soaked in 10% acetic acid solution and dried on Whatman 3MM paper.

Fragments that showed aberrant pattern compared to normal in the SSCP test were amplified, purified (Wizard PCR Preps, Promega, Madison, WI), and directly sequenced by fluorescent dideoxy cycle sequencing (ABI 373A Fluorescent DNA Sequencer, Perkin Elmer Cetus, Norwalk, CT). Genomic sequence numbering is according to Horowitz et al. [1989]. cDNA numbers start from the first ATG.

Computational Analysis

The secondary structure for the normal and mutant proteins was predicted by the method of Chou and Fasman [1978] using the GCG package [Devereux et al., 1984].

RESULTS

Biochemical Analysis

In the presence of T and TX, high residual β -glucosidase activity was detected in leukocytes and in cultured fibroblasts from patient 1, close to the lower limit presented by healthy carriers for GD and higher than in type 1 patients bearing the mild mutation N370S at homozygosity. Patient 2 fibroblasts showed an intermediate level of residual enzymatic activity while in patient 3 leukocyte activity was that expected for the disease (Table I).

We analyzed some properties of the fibroblast enzyme and compared them to those in 4 patients bearing genotype N370S/N370S as well as in control cells (Table II). In the presence of the natural activators PS and SAP, β -glucosidase in patient 1 fibroblasts was stimulated about 2-fold (as compared to enzyme activity in the absence of these activators), similarly to the enzyme from control cells. Under these experimental conditions, enzyme activity from patients with the N370S/N370S genotype increased 2.5 to 3.7-fold (mean: 2.9-fold). PS alone also stimulated β -glucosidase activity in cells from patient 1 and from the N370S homozygous patients to the same extent (about 1.6-fold, data not shown).

The apparent Km value of β -glucosidase for MU-Glc was almost identical in patient 1, the N370S homozygous patients, and controls. Analyses of heat stability of β -glucosidase showed that enzymes from patient 1, the N370S homozygous patients, and controls are similarly stable for 1 hour at 50°C (92–100% of initial activity). Estimations of some secondary biochemical abnormalities such as the plasma chitotriosidase activity in patient 1 demonstrated a 166-fold increase (control mean: 79 nmol/h/ml). Fibroblasts from this patient also showed a high residual β -xylosidase activity (38% of mean control) while in the N370S/N370S patients this activity was only 4% of mean value (control mean: 7.5 nmol/h/mg protein) (data not shown).

TABLE I. Residual β -Glucosidase Activity in Patients Bearing the Novel Mutations I402T and V375L and in Patients Homozygous for N370S

	Genotype	Activity (nmol/h per mg protein) ^a	
		Leukocytes	Fibroblasts
Patient 1	I402T/I402T	4.0	94
Patient 2	N370S/I402T		65
Patient 3	V375L/V375L	1.8	
Type 1 GD ^b	N370S/N370S	1.7 \pm 0.2 (6)	23 \pm 6 (4)
Controls ^b		6.2 \pm 1.3 (42)	261 \pm 102 (56)

^aEnzyme activity measured in the presence of T and TX.

^bEnzyme activity mean \pm SD. Number of cases in parentheses.

TABLE II. Comparative Enzymatic Properties of β -Glucosidase Activity in Fibroblasts From Patient 1 and From Patients Homozygous for the N370S Mutation

	Genotype	Activity (nmol/h/mg) ^a	Activity ratio (-fold) ^b	Km 4MU-Glc (mM)	Heat stability (%) ^c
Patient 1	I402T/I402T	107	2	4.1	92
Type 1 GD (4)	N370S/N370S	103 \pm 44	2.5–3.7	3.8 \pm 1.7	100
Controls		343 \pm 122	2	3.9 \pm 0.3	91–100

^aActivity assayed in the presence of PS and saposin C (SAP). Activity expressed as mean \pm SD.

^bRatio between the activity assayed in the presence of PS and saposin C (SAP) and in the absence of both activators.

^c% initial activity after 1 hour at 50°C.

Mutation Analysis

An initial screening for the previously described N370S, L444P, and D409H mutations in the 3 Italian GD patients showed that patient 2 was heterozygous for the common N370S amino acid substitution. None of these mutations was detected in the other 2 individuals.

In order to identify the other mutations, the 11 exons of the GBA gene were screened by SSCP analysis. The PCR products included all the coding region of the gene, the corresponding exon/intron boundaries, part of the promoter, and the upstream polyadenylation signal. Abnormal SSCP patterns were observed only in

exon 9 in all 3 patients and in some relatives (Figs. 1a, 2a).

Sequence analysis demonstrated that patient 1 was homozygous for a previously undescribed missense mutation due to a T \rightarrow C transition (Fig. 1b) at genomic nucleotide 5937 (cDNA 1322). This mutation leads to an isoleucine to threonine substitution at residue 402 in the mature enzyme. An unaffected brother was found to be a carrier of this mutation.

Patient 2 was a compound heterozygote for the same amino acid substitution (Fig. 1b) together with the N370S mutation. Three unaffected brothers were genotyped as I402T/+, N370S/+, and +/+. Besides, a daugh-

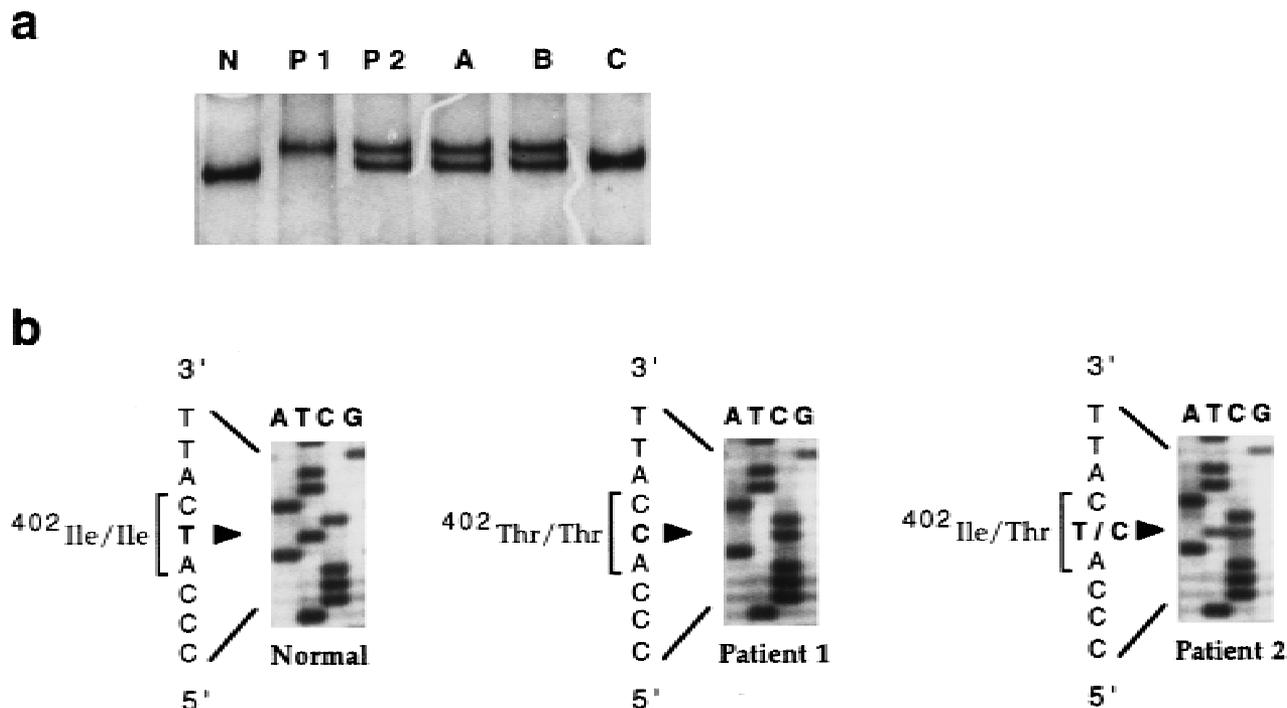


Fig. 1. Identification of mutation I402T by SSCP analysis and sequencing. **a:** SSCP analysis of PCR-amplified DNA fragments containing exon 9 of the GBA gene. Primer sequences (5' \rightarrow 3'): sense: ACTGGAACCTTGCCCTGAAC (nt: 5871–5890); antisense: ATAGGCCTGGTATGGAATGG (6025–6044). The samples were run in a nondenaturing 12% polyacrylamide gel for 12 hours at 300 V, 4°C. Only the single strands are shown. The bands on the gel were revealed by silver staining. **Lane N:** normal control; **lanes P1, P2:** patients 1 and 2, respectively; **lane A:** brother of patient 1; **lanes B, C:** brothers of patient 2. **b:** Direct sequence of exon 9 PCR products from patients 1 and 2 and from a control individual. Patient 1 (central panel) is homozygous for a T-to-C transition in the second position of codon 402, leading to a Thr for Ile amino acid substitution. Patient 2 (right panel) is heterozygous for the same mutation.

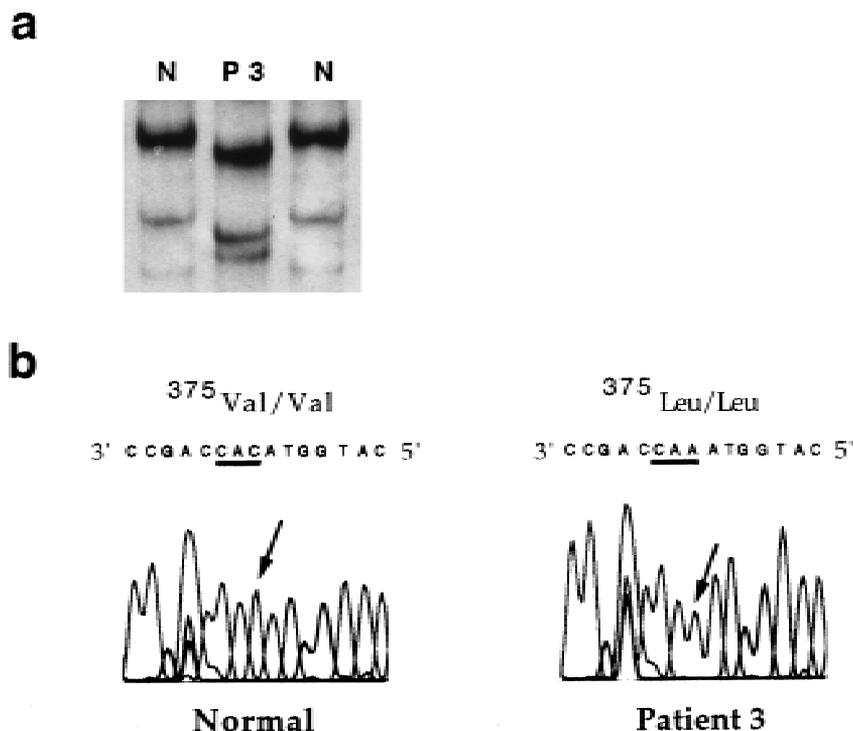


Fig. 2. Identification of mutation V375L by SSCP analysis and sequencing. **a:** SSCP analysis of exon 9 PCR products. Primer sequences (5' - >3'): sense: GTGTTGAGCCTTGTCTCTT (nt: 5800-5819); antisense: GATGGGACTGTGACAAAGT (5919-5938). **Lane N:** Normal individuals; **lane P3:** patient 3. The samples were run in a nondenaturing 12% polyacrylamide gel for 12 hours at 300 V, 4°C. **b:** Reverse sequence of exon 9 PCR products in patient 3 and in a control individual. Patient 3 (right panel) is homozygous for a G - >T transversion in the first position of codon 375, resulting in the substitution of leucine for valine.

ter of the patient was a carrier of the I402T mutation, while a son bore mutation N370S (data not shown).

Patient 3 was homozygous for another novel mutation. This is a G - >T transversion (Fig. 2b) at nucleotide 5855 of the gene (nucleotide no. 1240 of the cDNA, starting from the first ATG) substituting leucine (TTG) 375 of the mature protein for valine (GTG).

The predicted secondary structure for the 2 new mutant proteins did not show any change compared to the normal protein. The nucleotide changes leading to mutations I402T and V375L are not present in the pseudogene sequence. As they do not create or abolish any restriction site, the screening of a number of unaffected individuals was performed by SSCP analysis, comparing the patterns obtained with those from the patient samples. This screening failed to detect any of these changes in 70 normal chromosomes.

The analysis of the *HhaI* intragenic polymorphism showed that the 3 I402T alleles found in patients 1 and 2 are associated with the "-" variant, and the 2 V375L alleles of patient 3 are associated with the "+" variant (data not shown).

DISCUSSION

One important aim of the molecular analysis of the GBA gene is to correlate the nature of the mutations with the clinical manifestations of the disease, in anticipation to prognosis and counseling. However, this is

difficult in a recessive disorder with a well-known allelic heterogeneity such as GD. Only when the mutation under study is present either at homozygosity or compounded with a null or a severe mutation, can conclusions be clearly drawn.

Homozygous mutations are extremely rare in GD patients with the sole exception of N370S, L444P and, to a lesser extent, D409H. Patients with the N370S/N370S genotype have a very mild form of the disease without neuropathic involvement. Among the neuropathic types of the disease, the most frequent homozygote genotype is L444P/L444P. Although this genotype produces neurologic disease in most cases, the phenotypic expression is heterogeneous including both type 2 and 3 patients. At younger ages, it may be associated with aggressive manifestations reported as type 1 GD since neurological signs may appear later [Horowitz and Zimran, 1994a]. Recently, some particular phenotypic manifestations, such as cardiovascular calcifications and supranuclear ophthalmoplegia, have been reported in patients homozygous for D409H [Chabás et al., 1995; Abrahamov et al., 1995; Beutler et al., 1995]. Three reportedly mild mutations, R496C [Kawame et al., 1992], P122S [Beutler et al., 1993], and N188S [Kim et al., 1996] were found at homozygosity in 3 type 1 patients. Regarding the R496C/R496C case, a Japanese patient, no clinical data were reported except for his assignment to type 1. The P122S/P122S geno-

type produces severe visceral disease, and the lack of neuronopathic involvement could be due to the age of the patient (3 years at diagnosis), making it difficult to classify this allele as mild. The N188S/N188S patient was diagnosed at age 17 years with asymptomatic hepatosplenomegaly and no bone disease. From these data, N188S is the only mutation of these 3 that can be undoubtedly classified as a mild mutation.

To date, about 10 GD mutations in addition to the common N370S have been classified as mild [Balicki and Beutler, 1995], in most cases because they were found in compound heterozygosity with a known severe or lethal mutation in type 1 patients. Nevertheless, most type 1 GD patients have at least one allele with the frequent N370S mutation, in both Ashkenazi Jewish [Horowitz and Zimran, 1994b], and non-Jewish populations [Beutler and Gelbart, 1993]. As the presence of N370S is always associated with nonneuronopathic disease, it is impossible to assess the severity of the second mutation in a compound heterozygote bearing N370S.

Mutations I402T and V375L described here, in addition to N370S and N188S, are the only clearly mild mutations that have been analyzed in a homozygous state. Two facts strongly suggest that the 2 amino acid substitutions identified are indeed disease-causing mutations: 1) the changes were found in GD patients and their family members but not in unrelated healthy individuals and 2) the examination of the entire coding region, part of the putative promoter [Doll et al., 1995], all the splice sites, and the first polyadenylation signal of the gene did not show any other mutations.

Our results indicate a marked similarity in the catalytic properties and heat stability of the I402T and N370S mutant fibroblast enzymes. Stimulation of both mutant enzymes by either the physiological activators PS and SAP (activity ratio in Table II) or PS alone (1.6-fold) is also similar. The 2-fold activation observed with the I402T mutant β -glucosidase (in patient 1) by the combination of PS and SAP would then indicate a rather moderate stimulatory effect of SAP. In this regard, a very poor stimulatory response to SAP with the N370S mutant enzyme had been demonstrated in expression studies of β -glucosidase cDNA encoding this mutation [Ohashi et al., 1991; Grace et al., 1994]. These authors state that the responses to PS and SAP were very similar when the overexpressed mutagenized or the natural mutant fibroblast enzymes for N370S were assayed. These findings would validate the analysis of the natural mutant enzyme when a homozygous patient is identified.

However, some differences between mutations N370S and I402T have been observed. The presence of the I402T allele in cells in patients 1 and 2 results in a higher residual activity when T is used as the enzyme activator.

The presence of mutation I402T in 2 unrelated patients from the same geographic region in Sicily suggests that this GD allele might be quite frequent in this small area. The relative isolation of this population, both by historical and geographical reasons [Guglielmino et al., 1991; Rodriguez-Larralde et al., 1994], could explain the expansion of some mutations due to

founder effect and genetic drift. Nevertheless, more cases are needed to support this hypothesis. The analysis of the intragenic *Hha*I polymorphism shows that this mutation is associated with the common “-” variant in patients 1 and 2. In patient 3, mutation V375L is associated with the uncommon “+” variant in both alleles, but no familial information is available. It is tempting to speculate about a common origin for the I402T alleles in the unrelated patients 1 and 2 and for the 2 V375L alleles in patient 3, although further data should be required.

The analysis of additional GBA mutations at homozygosity and the study of their phenotypic effects in the patients may provide more insight into the genotype/phenotype correlation for GD.

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