European Journal of Human Genetics (2000) 8, 95–102

www.nature.com/ejhg

ARTICLE

Gaucher disease: expression and characterization of mild and severe acid β-glucosidase mutations in Portuguese type 1 patients

Olga Amaral[1], Ana Marcão[2], MC Sá Miranda[1], Robert J Desnick[3,4] and Marie E Grace[3]

[1]Instituto de Genética Médica Jacinto Magalhães, Unidade de Enzimologia; [2]Instituto de Biologia Molecular e Celular, Unidade de Neurobiologia Genética, Porto, Portugal; Departments of [3]Human Genetics and [4]Pediatrics, Mount Sinai School of Medicine, New York, USA

Type 1 Gaucher disease (GD), the most prevalent lysosomal storage disease, results from the deficient activity of acid β-glucosidase. Molecular analysis of 12 unrelated Portuguese patients with type 1 GD identified three novel acid β-glucosidase mutations (F109V, W184R and R395P), as well as three previously reported, but uncharacterized, lesions (R359Q, G377S and N396T). The type 1 probands were either heteroallelic for the well-characterized common lesion, N370S, and the F109V, W184R, R359Q or N396T lesions or homoallelic for the G377S or N396T mutations. Expression of the W184R, R359Q, and R395P mutations revealed very low specific activities based on cross-reacting immunologic material (CRIM SAs of 0.0004, 0.016 and 0.045, respectively), consistent with their being found only in type 1 patients who had a neuroprotective N370S allele. In contrast, the F109V, G377S and N396T alleles had significant acid β-glucosidase activity (CRIM specific activities of 0.15, 0.17, 0.14, respectively), in agreement with their being mild type 1 alleles. Thus, these studies identified additional acid β-glucosidase mutations in the Portuguese population and demonstrated that the G377S and N396T mutations were neuroprotective, consistent with the mild clinical phenotypes of the type 1 patients who were homoallelic for the G377S and N396T lesions. *European Journal of Human Genetics* (2000) **8**, 95–102.

Keywords: baculovirus; mutation analysis; Gaucher; neuroprotective allele; phenotype

Introduction

Gaucher disease (GD) is an inherited storage disorder resulting from the deficiency of acid β-glucosidase (E.C.3.2.1.45) and the lysosomal accumulation of its undegraded substrate, glucosylceramide, particularly in the cells of the reticuloendothelial system.[1,2] The majority (about 95%) of GD cases have non-neuronopathic or type 1 disease and present with systemic features including pancytopenia, hepatosplenomegaly and skeletal involvement.[3] Patients with neurologic GD present with either an acute (type 2) or subacute (type 3) course.

Correspondence: Marie E Grace, PhD, Department of Human Genetics, Box 1498, Mount Sinai School of Medicine, Fifth Avenue and 100th Street, New York, NY 10029. Tel: +1 212 659 6778; Fax: +1 212 849 2508; E-mail: grace@msvax.mssm.edu
Received 16 April 1999; revised 17 September 1999; accepted 11 November 1999

The isolation and sequencing of the full-length cDNA and the entire genomic sequence,[4–6] provided the opportunity to identify common disease-causing mutations, determine their frequencies, and identify genotype/phenotype correlations. To date, over 120 mutations have been identified in the acid β-glucosidase gene;[7,8] (online compilation [http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html][9]), including base substitutions causing missense, nonsense and splicing mutations, small and large insertions and deletions, and complex rearrangements with the pseudogene located 16 kb downstream.[6] Of these mutations, only a few have been proven to be common: the N370S lesion in the Ashkenazi Jewish and the Portuguese populations and the panethnic L444P mutation.[10–13]

Prediction of disease type and severity by characterizing a patient's genotype has been difficult since most of the mutations identified are rare or private. In fact, most non-consanguineous GD patients, with the notable exception of

Ashkenazi, Norbottnian, and Portuguese patients, are heteroallelic for a rare or private allele. However, genotype/phenotype correlations have been possible for the most frequently identified mutations, N370S and L444P.[7,14–18] The presence of at least one N370S allele precludes development of neurological manifestations, even if the heteroallele is completely inactive;[19,20] and homoallelism for the L444P mutation is generally predictive of neurological disease.[21–24] Nonetheless, significant phenotypic variability occurs within the subtypes,[25,26] and in particular within type 1 GD, as illustrated by patients homoallelic for the N370S allele, whose condition, although primarily mild, can range from clinically asymptomatic to severely involved with massive hepatosplenomegaly and debilitating bone disease. Of note, type 1 GD patients homoallelic or heteroallelic for the N370S allele are unusually prevalent among Ashkenazi Jewish and non-Jewish Portuguese patients, the carrier frequencies being about 1 in 17.5 and 1 in 118 in these populations, respectively.[10,13]

The identification and expression of acid β-glucosidase mutations is useful for understanding the normal function of this lysosomal hydrolase and for further delineation of genotype/phenotype correlations in GD, especially in non-Jewish populations.[12,15] Screening for four mutations (N370S, L444P, G377S and N396T) in the Portuguese population, where type 1 GD is the most prevalent lysosomal storage disease (being some 25 times more frequent than the neuronopathic forms), detected 85% of mutant or about 15% unknown alleles.[27] In this communication, three new mutations (F109V, W184R, R395P) and three previously reported but uncharacterized lesions (R359Q,[28] G377S,[29] N396T[12]) were detected in 12 Portuguese patients with type 1 GD. Heterologous expression and characterization studies confirmed causality of the mutations for GD, identified three mild alleles (F109V, G377S and N396T) and led to greater insight into the roles of individual residues in maintaining acid β-glucosidase function.

Materials and methods

Patient descriptions

The clinical and laboratory features of the 12 Portuguese type 1 patients studied are summarized in Table 1. The use of human materials was approved by the Institute of Medical Genetics Jacinto de Magalhães.

Reagents

Triton X-100 and NBD-glucocerebroside (NBD-Glc) were obtained from Sigma Chemical Co. (St Louis, MO, USA). 4-methylumbellifery 1-β-*D*-glucopyranoside (4MU-β-Glc) was from Genzyme Corp. (Cambridge, MA, USA) and Sigma-Aldrich Quimica (Madrid, Spain). Sodium taurocholate and

Table 1 Clinical and laboratory features of the Portuguese type 1 Gaucher disease patients

Proband/ Gender/ Region of origin	*Age at diagnosis/ Recent evaluation*	*Disease severity for age*	*Leukocyte activity*[a] *(U/mg)*	*Hepato-splenomegaly*[b]	*Bone symptoms*[b]	*Platelet (No/mm^3)*	*Hgb (g/dl)*	*Gentoype*	*Comments*
1/M Azores	28/33	mild–mod	1.5×10^{-5}	splenectomy	–	n.a.[c]	n.a.	F109V/N370S	affected sibs asymptomatic at 31 and 38 years
2/F Azores	35/38	mild	1.6×10^{-5}	+	–	115 000	8.8	N396T/N396T	
3/M Northern	32/36	moderate	2.0×10^{-5}	splenectomy	++	44 500	n.a.	W184R/N370S	
4/F Northern	24/28	mild–mod	3.5×10^{-5}	+	++	n.a.	8.1	R359Q/N370S	
5/F Northern	20/50	mod–sev	4.7×10^{-5}	splenectomy	++	n.a.	n.a.	R359Q/N370S	
6/M Central	39/44	mild–mod	2.3×10^{-5}	splenectomy	+	n.a.	n.a.	G377S/G377S	parents are 1st cousins
7/M Central	9/44	severe	5.2×10^{-5}	splenectomy	++	n.a.	n.a.	G377S/G377S	parents are 1st cousins
8/M Central	48/50	mild–mod	2.8×10^{-5}	+	++	49 000	n.a.	G377S/G377S	parents are 1st cousins; affected father, asymptomatic at 63 years
9/F Central	19/26	mild–mod	2.0×10^{-5}	–	++	50 000	n.a.	N370S/N396T	
10/M Central	13/25	moderate	3.7×10^{-6}	splenectomy	++	51 000	11	N396T/N396T	affected sib, asymptomatic at 35 years
11/M Central	72/72	mild	3.8×10^{-6}	+	+	65 000	10.3	N396T/N396T	parents are 1st cousins
12/F Southern	5/25	severe	3.2×10^{-5}	++	+++	40 000	8.6	N370S/R395P	affected sib, asymptomatic at 21 years

[a]normal range of enzymatic activity, $1.1–2.8\times10^{4}$ U/mg towards 4mMU glucopyranoside. [b]+: mild; ++: moderate; +++: severe; –: minimal to none. [c]n.a.: not available.

deoxynojirimycin were purchased from CalBiochem–Novabiochem (La Jolla, CA, USA). The Sculptor™ *In Vitro* Mutagenesis System kit was from Amersham (Arlington, IL, USA, and Amersham, England). *Spodoptera frugiperda* cells, Baculogold® linearized DNA, and the baculovirus transfer vector, pVL1392, were purchased from PharMingen (San Diego, CA, USA). Restriction enzymes and Taq DNA polymerase were from Boehringer Mannheim (Mannheim GMBH, Germany).

DNA isolation, PCR amplification and mutation detection

Genomic DNA was isolated from skin fibroblasts or peripheral blood using standard techniques.[30] Initial screening for two common (N370S, L444P) and several rare but non-family-specific (84GG, IVS2^{+1}, R463C, R496H, D409H, R120Q, P122S, K157Q, Y212H, F216Y, W312C, G325R, C342G, D409V, P415R, RecTL, and RecNci) mutations[21,31–44] as well as two mutations found in the Portuguese population, G377S[29] and N396T,[12] was performed by polymerase chain reaction (PCR) amplification and restriction digestion or allele specific oligonucleotide hybridization.[31,45,46]

To detect the unknown acid β-glucosidase mutations, the complete coding region and adjacent intron/exon boundaries were amplified directly from genomic DNA (or through nested PCR) and subjected to non-radioactive SSCP analysis.[12] The primers used for the exons in which the novel mutations were found follow: Exon 4, 5'-gggtactgataccctatt-3' and 5'-gggcagagtgagattctgcc-3'; Exon 6 and 8 (nested PCR), 1st PCR: 5'-ctcggactaccatatcttgatca-3' and 5'-gatgggactgtcgacaaagt-3', 2nd PCR (Exon 6) 5'-gtgttccaactctgggtgct-3' and 5'-taaatgggaggccagtcct-3', 2nd PCR (Exon 8) 5'-gatcagttgctcttcctttg-3' and 5'-tttgcaggaagggagactg-3'; Exon 9 (nested PCR), 1st PCR: 5'-aaccatgattccctatcttc-3' and 5'-acgtactctcatctttttgg-3', 2nd PCR: 5'-ccagtgttgagcctttgtct-3' and 5'-gtatggtccggatagtagag-3'.

The relevant PCR products with abnormal electrophoretic mobility were further examined by direct sequencing (Sequenase Version 2.0, USB, Cleveland, Ohio, USA) of the asymmetric PCR generated products. Each mutation identified was confirmed by direct sequencing or restriction analysis of genomic DNA from the proband and affected family members. Genomic DNAs from 50 randomly selected unrelated normal individuals were screened for the F109V, W184R, and R359P mutations by restriction digestion or SSCP analysis, as described above. The designations for the mutations refer to the position of the amino acid substitution, where amino acid one is the *N*-terminus of the mature protein. The cDNA base numbers refer to the position of the nucleotide in the cDNA,[4,47] where nucleotide one is the A in the first ATG. The genomic designations are based on the updated acid β-glucosidase sequence[6,48] available from GenBank [http://www.ncbi.mlm.nih.gov/Entrez/nucleotide.html], accession number J03059 (8/95). The first nucleotide of exon 1 is at genomic position 1230.

Construction of expression plasmids

Point mutations for F109V, W184R, R359Q, G377S, R395P, and N396T were introduced into the acid β-glucosidase cDNA by an M13mp19-based oligonucleotide-directed site-specific mutagenesis procedure (Sculptor™ *In Vitro* Mutagenesis System) employing the phosphorothioate selection method[49,50] as previously described.[51,52] The complete sequence of each mutagenized cDNA was determined to confirm that no spurious mutations were incorporated during the mutagenesis procedure. The mutant cDNAs were then cloned into the *Eco*RI site of the baculovirus expression vector, pVL1392. The final acid β-glucosidase cDNA inserts in the expression plasmid were 1562 bp fragments beginning 12 bp upstream of the second ATG, ending at the stop codon, and containing the correct amino acid (arginine) at position 495.

Construction and purification of recombinant baculovirus

Recombinant baculovirus containing each of the different acid β-glucosidase cDNAs (normal in the sense [Nl] and antisense [Rev] directions, F109V, W184R, R359Q, G377S, R395P, and N396T) were produced in cloned *Spodoptera frugiperda* (Sf9) cells by calcium phosphate-mediated transfection and homologous recombination between the expression plasmid and baculovirus genomic DNA (Baculogold®) as described.[53] Pure recombinant baculovirus clones containing the normal or mutant cDNA for acid β-glucosidase were isolated by plaque hybridization, amplified, titered, and used at a multiplicity of infection greater than 10 to infect Sf9 cells.[53] The previously characterized common mutations, N370S and L444P,[51,52,54,55] were re-expressed for comparative purposes.

Immunoblotting

Immunoelectroblotting using a polyclonal anti-human acid β-glucosidase antibody was conducted as described.[56] In brief, Sf9 cells infected with pure recombinant virus were harvested 3 days post-infection. The pellets were sonicated in 0.04 M citrate/phosphate buffer, pH 5.5, containing 1 mM EDTA, 4 mM β-mercaptoethanol, 0.1% Triton X-100, and 0.1% sodium taurocholate, using a probe sonicator (Heat Systems-Ultrasonics, Inc, Framingdale, NY, USA). Aliquots of the clarified (875 X g; 20 min) crude sonicates containing determined amounts of protein and enzymatic activity were run on tricine-SDS-polyacrylamide gels[57] and immunoblotted. Specific activities based on the amount of cross-reacting immunological material, CRIM specific activity (CRIM SA), were determined as described.[51,52,54] The relative amounts of CRIM per unit of glucosylceramide-cleaving activity for each mutant allele was determined by computer analysis of the immunoblotting membrane using the NIH Image™ program, version 1.60, and referenced to that of the normal enzyme. To illustrate: computer assisted comparison of the mature about 63 kDa band of the normal and F109V enzyme proteins

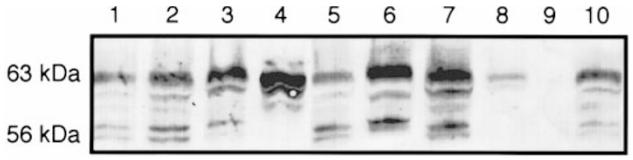

Figure 1 Immunoblot of the normal and mutant acid β-glucosidases expressed in Sf9 cells. Lane 1, F109V (1.0×10^{-4} U, 15 μg); lane 2, W184R (1.0×10^{-6} U, 15 μg); lane 3, R359Q (1.9×10^{-5} U, 15 μg); lane 4, N370S (3.5×10^{-4} U, 15 μg); lane 5, G377S (1.1×10^{-4} U, 15 μg); lane 6, R395P (7.5×10^{-5} U, 15 μg); lane 7, N396T (1.9×10^{-4} U, 15 μg); lane 8, L444P (2.0×10^{-5} U, 15 μg); lane 9, the antisense construct (10 μg); and lane 10, Normal (9.6×10^{-4} U, 10 μg). See text for details.

(Figure 1, lanes 10, and 1, respectively) demonstrated the immunoblot signal for the F109V mutant (1.0×10^{-4} U) was about 0.7 times that for the expressed normal enzyme (9.6×10^{-4} U). The CRIM SA for the F109V mutant enzyme is calculated as the relative units of enzymatic activity divided by the relative CRIM (ie 0.1/0.7 = 0.15). F109V had a CRIM SA that was 15% of that for normal acid β-glucosidase, representing a 6.7-fold reduction in catalytic efficiency or turnover rate.

Enzyme assays

Aliquots of the crude sonicates, prepared as described above, were assayed for acid β-glucosidase activity using the fluorescently-labeled natural substrate NBD-GC (12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]dodecanoyl-sphingosyl-1-O-β-*D*-glucopyranoside or NBD-glucosylceramide), or the synthetic substrate, 4-methylumbelliferyl-β-*D*-glucopyranoside (4MU-Glc). The final reaction mixtures for the natural substrate contained 0.3 mM NBD-GC (reaction volume of 100 or 200 μL) in 0.04 M citrate/phosphate buffer, pH 5.5, 1 mM EDTA, 4 mM β-mercaptoethanol, 0.25% Triton X-100, and 0.25% sodium taurocholate. The W184R mutant also was assayed in the presence of 2.5 mM conduritol B epoxide (CBE). The NBD-GC assays were terminated and processed as described.[58] The fluorescence of the extracted reaction products were read using a Farrand Optical System 3 spectrofluorometer (Optical Technology Devices, Elmsford, NY, USA). Background levels were determined by comparison with results obtained from Sf9 cells infected with recombinant baculovirus containing the cDNA for acid β-glucosidase in the antisense direction (Rev). The contribution to turnover of the synthetic 4MU-Glc substrate in the crude lysates due to the expressed human acid β-glucosidase enzymes was calculated by determining the CBE inhibitable activity.[59] Aliquots of the crude sonicates assayed were first incubated at pH 5.2 for 0.5 h at room temperature in the presence and absence of 1 mM CBE before being assayed for 2 h at 37°C. The final assay mixtures (reaction volume of 60 μL) contained 0.1/0.2 M citrate/phosphate buffer, pH 5.2, 1 mM EDTA, 4 mM β-mercaptoethanol, 0.25% Triton X-100, and 0.25% sodium taurocholate. The 4MU-Glc reaction was terminated by raising the pH with the addition of 1 M glycine. The difference between the activity with and without CBE was determined for each expressed allele. One unit (U) of acid β-glucosidase activity was that amount of enzyme that hydrolyzed one micromole of susbtrate per min at 37°C.

Inhibition of expressed normal and mutant acid β-glucosidases by active site directed inhibitors

Aliquots of crude lysates were assayed in the absence or presence of CBE and deoxynojirimycin (DNM). Concentrated aqueous stock solutions of CBE and DNM were made such that aliquots of 0–50 μL gave the desired final concentrations in the 200 μL reaction mixture. The assay conditions employing the synthetic 4MU-Glc substrate were as described above. The IC_{50} (the concentration of inhibitor resulting in 50% reduction in activity) values for CBE for the normal and mutant acid β-glucosidases were determined from a plot of activity remaining versus inhibitor concentration (0–600 μM) after subtracting the 4MU-Glc activity remaining at 3 mM CBE (which represents the non-CBE inhibitable endogenous Sf9-derived activity). Inhibition by DNM was assessed by determining the percentage of inhibition resulting from a single high concentration of DNM (600 μM).

Results

Identification of acid β-glucosidase mutations

Complete SSCP analysis of the acid β-glucosidase coding region and intron/exon boundaries from non-Jewish Portuguese patients with type 1 GD (Table 1) resulted in the identification of three new (F109V, W184R and R395P) and three rare, but uncharacterized, missense mutations (R359Q,[28] G377S,[29] and N396T[12]). Table 2 summarizes the nucleotide substitutions and the corresponding changes at the protein level for each mutation. The only other allele identified in these patients was the common N370S mutation. None of these mutations were derived from the tightly linked pseudogene.[6] The W184R mutation was previously reported only as part of a complex allele[36] found in an Ashkenazi/Sephardic Jewish type 1 GD patient containing seven missense mutations (Complex C: R120W + W184R + N188K + V191G + S196P + G202R + F213I), of which all but the W184R mutation occurred in the pseudogene sequence.

As indicated in Table 2, five of the six mutations altered a restriction site, facilitating the screening of family members to confirm the lesions. Additionally, to rule out any of the three newly identified mutations being benign polymorphisms, genomic DNA from 50 randomly selected normal Portuguese individuals was screened for the F109V, W184R and R395P mutations by restriction digestion and/or SSCP analysis. None of the 100 alleles screened had any of these new base substitutions.

Table 2 Mutations identified in Portuguese patients with type 1 Gaucher disease

Designation	*Exon*	*cDNA position*[a]	*Genomic position*[a]	*Codon change*	*Amino acid change*	*Restriction site change*
F109V	4	442	2943	TTC → GTC	Phe^{109} → Val	+ Mae II
W184R	6	667	4343	TGG → CGG	Trp^{184} → Arg	—
R359Q	8	1193	6295	CGA → CAA	Arg^{359} → Glu	– Taq I
G377S	9	1246	6748	GGC → AGC	Gly^{377} → Ser	+ Pvu II
R395P	9	1301	6803	CGT → CCT	Arg^{395} → Pro	+ Ban II
N396T	9	1304	6806	AAC → ACC	Asn^{396} → Thr	+ Rsa I

[a]See Methods for definition of cDNA and genomic sequence position.

Table 3 Characterization of normal and mutant acid β-glucosidases expressed in Sf9 cells

Acid β-glucosidase allele	*Specific activity*		*CRIM SA*[a]	*1/CRIM SA*
	Glucosylceramide μmol/min/mg	*4MU-β-glucoside μmol/min/mg*		
Normal	9.6×10^{-2}	3.3×10^{-2}	1.00	1.00
Antisense	0	3.3×10^{-4}	–	–
F109V	6.7×10^{-3}	1.7×10^{-3}	0.15	6.7
W184R	2.4×10^{-5}[b]	3.2×10^{-4}	0.0004	2500
R359Q	1.3×10^{-3}	1.4×10^{-3}	0.016	63
G377S	7.5×10^{-3}	1.5×10^{-3}	0.17	5.9
R395P	5.0×10^{-3}	1.8×10^{-3}	0.045	22
N396T	1.3×10^{-2}	9.8×10^{-4}	0.14	7.2
N370S	2.3×10^{-2}	2.7×10^{-3}	0.18	5.5
L444P	1.3×10^{-3}	8.8×10^{-4}	0.057	17.5

[a]CRIM SA = total units of specific activity per cross-reacting immunologic material (CRIM).
[b]Calculated as CBE inhibitable glucosylceramide-cleaving activity.

Expression and characterization of the missense mutations

The F109V, W184R, R359Q, G377S, R359P and N396T missense mutations were expressed in the baculovirus system and characterized (Table 3). For comparison, the previously studied N370S and L444P alleles were re-expressed.[51,52,54] Using the fluorescently-labeled natural substrate, NBD-GC, the expressed normal acid β-glucosidase allele had approximately 9.6×10^{-2} μmol/min/mg (U/mg) of glucosylceramide-cleaving activity in the cell lysates. No activity towards NBD-GC was detected in Sf9 cells infected with the recombinant virus containing the antisense acid β-glucosidase construct. All the expressed mutants had significantly reduced activities, but were relatively stable (ie there was no change in the specific activity of each mutant over a 2 h assay period). Of the six new mutants being characterized, the N396T allele had the highest residual activity (14% of normal). The F109V, G377S and R395P alleles expressed significant activity with 7, 8 and 5% of expressed normal activity, respectively. The R359Q crude lysates had slightly less (1.4% of normal), but easily detectable, residual activity. In contrast, the W184R allele had very low, but detectable activity levels (2.4×10^{-5} U/mg), which was inhibitable by CBE, an active site-directed substrate analogue specific for lysosomal acid β-glucosidase.

Immunoblotting studies were carried out to characterize the expressed normal and mutant acid β-glucosidase and to determine if the mutations altered stability and/or catalytic efficiency. As shown in Figure 1, lane 10, the normal human acid β-glucosidase cDNA produced a multi-band pattern that strongly reacted with polyclonal anti-human acid β-glucosidase antibodies. Consistent with earlier studies,[52] treatment with N-Glycanase™ demonstrated that the multiple bands with molecular weights ranging from 63 kDa to about 56 kDa represented differentially glycosylated forms of the enzyme (data not shown). Similar multi-band patterns were seen for all of the expressed mutant cDNAs. No CRIM was detected in the cells expressing the antisense acid β-glucosidase construct (Figure 1, lane 9).

To assess the level of catalytic efficiency (k_{cat}) for the mutant alleles, the specific activities were normalized for the relative amount of enzyme protein, ie cross-reacting immunologic material (CRIM). The CRIM SA was calculated (see Methods section) for each, relative to the expressed normal acid β-glucosidase (Table 3). Of note, the G377S, N396T and F109V mutant proteins (with CRIM SAs of 0.17, 0.14, and 0.15, respectively) had turnover rates similar to that (0.18) of the mild N370S allele. The R395P mutant protein, with its 22-fold reduced CRIM SA, had a catalytic efficiency (0.045) equivalent to that (0.057) of the L444P mutant enzyme. The R359Q allele was even more severely compromised, having only 1.6% of normal CRIM SA. Of the expressed alleles, the W184R mutant protein was essentially inactive having a 2500-fold reduction in turnover rate, and was not characterized further.

To evaluate active site function and to probe the effect of the various amino acid substitutions on the structural integrity of the active site of each expressed mutant enzyme, the affinities of two active site-directed inhibitors, CBE and DNM, were assessed. Due to its severely reduced activity, the

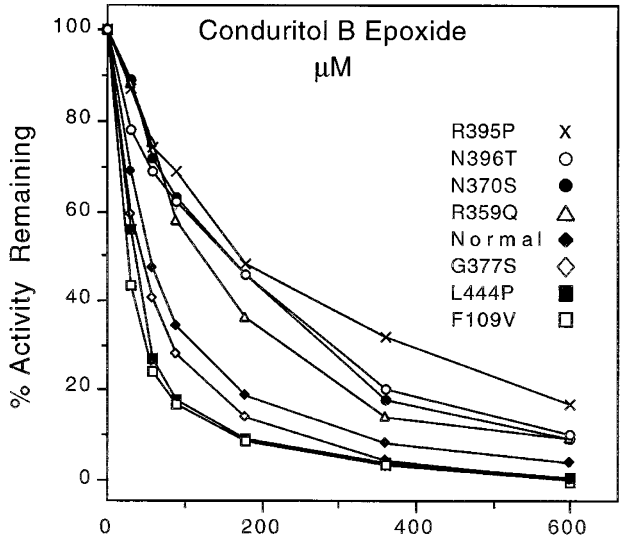

Figure 2 Inhibition of expressed normal and mutant acid β-glucosidases by the active site inhibitor, CBE (0–600 μM). The curve for each mutation is identified by the relative position of its final point from highest to lowest percentage of activity remaining.

W184R mutant was excluded from these studies. For comparative purposes, the previously characterized N370S and L444P alleles were reanalyzed.[51,52,60] The concentration of CBE required to achieve 50% inhibition of the initial activity (IC_{50}) was determined. As shown in Figure 2, two of the mutant proteins (F109V and G377S) interacted normally with this inhibitor, as did the L444P enzyme, and had IC_{50} curves that overlapped that of the expressed normal enzyme. The findings classified these mutant proteins as typical 'group A' enzymes.[61,62] The R359Q, R395P, N396T, and N370S mutant enzymes each had significantly reduced affinity (ie higher IC_{50} values) towards CBE, categorizing them as 'group B' enzymes. These results were confirmed by determining the sensitivity of these mutant proteins to a single high concentration of DNM. Assaying the normal enzyme in the presence of 600 μM DMN resulted in an approximately 80% loss of activity towards 4MU-Glc. The 'group A' enzymes, F109V, G377S, and L444P were similarly affected, with 11, 21 and 11% activity remaining, respectively. However, the 'group B' enzymes encoded by R359Q, R395P, N396T, and N370S were inhibited less, retaining 54, 72, 63 and 76% initial activity, respectively.

Discussion

Molecular analysis of the acid β-glucosidase gene from 12 unrelated Portuguese type 1 GD patients revealed three novel mutations, and the expression of these, and three other reported, but uncharacterized lesions,[12,28,29] provided insight into the function of these alleles and potential genotype/phenotype correlations. The three novel mutations were missense mutations: F109V, the sterically non-conservative substitution[63,64] of phenylalanine by valine in a weakly conserved (human *vs* murine[65]) region of the acid β-glucosidase protein; W184R, the non-conservative substitution of a tryptophan residue by the positively-charged arginine in the middle of 15 (amino acids 176–189) conserved (in fact, identical) residues; and R395P, the non-conservative substitution of arginine by proline, involving the loss of a positive charge and introduction of a rigid side chain at residue 395, which lies in the longest stretch (amino acids 315–406) of conserved (ie identical except for amino acid 376) residues in the human and murine sequences, suggesting an important functional domain. The three additional alleles characterized also involved residues within this highly conserved 92 amino acid region of acid β-glucosidase which contains the catalytic nucleophile (E340[66]): R359Q, the non-conservative substitution of an arginine by a glutamine (ie resulting in the loss of a positive charge); G377S, the highly favorable substitution of glycine by serine (both having small polar side chains), especially at residues with surface locations; and N396T, the conservative substitution of asparagine by the physio-chemically similar threonine.

Expression studies provided the opportunity to evaluate the function of the new and previously uncharacterized mutations. The expressed recombinant mutant enzyme proteins either had severely compromised function and/or stability (W184R, R359Q and R395P), or had significant residual catalytic activity (F109V, G377S and N396T). Little, if any, functional enzyme was produced from the W184R, R359Q or R395P alleles, consistent with these mutants having severe charge or size changes in highly conserved regions of the coding sequence. Presumably, these lesions must be heteroallelic with a neuroprotective allele, such as N370S, for a type 1 disease phenotype.

Of note, the three mutations with significant residual activity had CRIM SA values (ranging from 0.14 to 0.17) similar to that (0.18) of the well-characterized common N370S allele, the 'neuroprotective' allele in type 1 GD even when heteroallelic with the most severely compromised acid β-glucosidase mutation. The finding that patients homoallelic for either G377S or N396T were essentially asymptomatic or had mild disease (Table 1) suggests that these lesions also are neuroprotective. The identification of type 1 GD patients heteroallelic for either G377S or N396T and a putative or known severe allele would further substantiate the neuroprotective effect of these mutations.

In Portugal, type 1 GD is the most prevalent lysosomal storage disorder. The frequency of the N370S, L444P, G377S and N396T mutations in the Portuguese GD patients was reported as 53.7%, 13%, 7.4% and 5.6%, respectively.[12] Screening for these frequent mutations usually allows the detection of at least one allele in Portuguese patients. To date, the N396T lesion has only been reported in Portuguese type 1 GD patients.[12] In contrast, the G377S allele also has been reported in Spanish type 1 patients[67] and in a Portuguese Sephardic Jewish patient who was homoallelic for G377S,

suggesting that the G377S mutation may have Sephardic ancestry. Of note, the finding of three presumably unrelated patients homoallelic for the G377S allele and four presumably unrelated patients hetero- or homoallelic for the N396T lesion suggests that these lesions have common ancestral founders in the Portuguese population.

Acknowledgements

The authors thank the physicians, patients and their relatives who participated in these studies. The technical assistance of Ms E Pinto is gratefully acknowledged. This work was supported in part by a grant (6-FY96-1062) from the March of Dimes Birth Defects Foundation (MEG) and by grants BD/5547/95 (OA), BIC1542 (AM) and PECS/T/SAU/88/95 from JNICT/FCT (Portugal) (MCSM).

References

1 Beutler E, Grabowski GA: Gaucher disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds). *The Metabolic Basis of Inherited Disease*, McGraw Hill: New York, 1994, pp 2641–2670.
2 Desnick RJ, Gatt S, Grabowski GA: *Gaucher Disease: A Century of Delineation and Research*. Alan Liss: New York, 1982.
3 Cox TM, Schofield JP: Gaucher's disease: Clinical features and natural history. *Ballière's Clin Haematol* 1997; **10**: 657–689.
4 Sorge J, West C, Westwood B, Beutler E: Molecular cloning and nucleotide sequence of human glucocerebrosidase cDNA. *Proc Natl Acad Sci* 1985; **82**: 7289–7293.
5 Tsuji S, Choudary PV, Martin BM, Winfeild S, Barranger JA, Ginns EI: Nucleotide sequence of cDNA containing the complete coding sequence for human cerebrosidase. *J Biol Chem* 1986; **261**: 50–53.
6 Horowitz M, Wilder S, Horowitz Z, Reiner O, Gelbart T, Beutler E: The human glucocerebrosidase gene and pseudogene: Structure and evolution. *Genomics* 1989; **4**: 87–96.
7 Grabowski GA, Horowitz M: Gaucher's disease: Molecular, genetic and enzymological aspects. *Baillière's Clin Haematol* 1997; **10**: 635–656.
8 Beutler E, Gelbart T: Hematologically important mutations: Gaucher disease. *Blood Cells Molec Dis* 1998; **24**: 2–8.
9 Krawczak M, Cooper DN: The human gene mutation database. *Trends Genet* 1997; **13**: 121–122.
10 Horowitz M, Pasmanik-Chor M, Borochowitz Z *et al*: Prevalence of glucocerebrosidase mutations in the Israeli Ashenazi Jewish population. *Hum Mutat* 1998; **12**: 240–244.
11 Beutler E, Nguyen N, Henneberg M *et al*: Gaucher disease: gene frequencies in the Ashkenazi Jewish population. *Am J Hum Genet* 1993; **52**: 85–88.
12 Amaral O, Pinto E, Fortuna M, Lacerda L, Sá Miranda MC: Type 1 Gaucher disease: identification of N396T and prevalence of glucocerebrosidase mutations in the Portuguese. *Hum Mutat* 1996; **8**: 280–281.
13 Lacerda L, Amaral O, Pinto R, Oliveira P, Aerts J, Sá Miranda MC: Gaucher disease: N370S glucocerebrosidase gene frequencies in the general population. *Clin Genet* 1994; **45**: 298–300.
14 Beutler E: Gaucher disease: New molecular approaches to diagnosis and treatment. *Science* 1992; **256**: 794–799.
15 Beutler E: Gaucher disease as a paradigm of current issues regarding single gene mutations of humans. *Proc Natl Acad Sci USA* 1993; **90**: 5384–5390.
16 Horowitz M, Zimran A: Mutations causing Gaucher disease. *Hum Mutat* 1994; **3**: 1–11.
17 Erikson A, Bembi B, Schiffman R: Neuronopathic forms of Gaucher disease. *Clinic Haematology* 1997; **10**: 711–723.
18 Levy-Lahad E, Zimran A: Gaucher's disease: Genetic counselling and population screening. *Baillière's Clin Haematol* 1997; **10**: 779–792.
19 Sibille A, Eng C, Kim S-J, Pastores G, Grabowski GA: Phenotype/genotype correlations in Gaucher disease type 1: Clinical and therapeutic implications. *Am J Hum Genet* 1993; **52**: 1094–1101.
20 Zimran A, Sorge J, Gross E, Kubitz M, West C, Beutler E: Prediction of severity of Gaucher's disease by identification of mutations at DNA level. *Lancet* 1989; **2**: 349–352.
21 Tsuji S, Choudary P, Martin B *et al*: A mutation in the human glucocerebrosidase gene in neuronopathic Gaucher's disease. *N Engl J Med* 1987; **316**: 570–575.
22 Eto Y, Kawame H, Hasegawa Y, Ohashi T, Ida H, Tokoro T: Molecular characteristics in Japanese patients with lipidosis: Novel mutations in metachromatic leukodystrophy and Gaucher disease. *Mol Cell Biochem* 1993; **119**: 179–184.
23 Masuno M, Tomatsu S, Sukegawa K, Orii T: Non-existence of a tight association between a ^{444}leucine to proline mutation and phenotypes of Gaucher disease: High frequency of a NciI polymorphism is the non-neuronopathic form. *Hum Genet* 1990; **84**: 203–206.
24 Sá Miranda MC: Phenotypic and genotypic characterization of different Gaucher populations: European/Asian patients with Gaucher Disease. NIH Technology Assessment Conference. *Gaucher Disease* 1995: 61–65.
25 Zimran A, Kay A, Gelbart T *et al*: Gaucher disease. Clinical, laboratory, radiologic, and genetic features of 53 patients. *Medicine* 1992; **71**: 337–353.
26 Tayebi N, Reissner K, Lau E *et al*: Genotypic heterogeneity and phenotypic variation among patients with type 2 Gaucher disease. *Ped Res* 1998; **43**: 571–578.
27 Amaral O, Marcao M, Pinto E, Sá Miranda M: Mutation analyses of the three most common lysosomal storage disorders in Portugal. *Eur J Hum Genet* 1998; **6** (SI): P1.027.
28 Kawame H, Haseqawa Y, Eto Y, Maekawa K: Rapid identification of mutations in the glucocerebrosidase gene of Gaucher disease patients by analysis of single-strand conformation polymorphisms. *Hum Genet* 1992; **90**: 294–296.
29 Laubscher KH, Glew RH, Lee RE, Okinaka RT: Use of denaturing gradient gel electrophoresis to identify mutant sequences in the β-glucocerebrosidase gene. *Hum Mut* 1994; **3**: 411–415.
30 Miller S, Dykes D, Polesky H: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1998; **16**: 1215.
31 Beutler E, Gelbart T, Kuhl W, Zimran A, West C: Mutations in Jewish patients with Gaucher disease. *Blood* 1992; **79**: 1662–1666.
32 Beutler E, Gelbart T, West C: Short communication: identification of six new Gaucher disease mutations. *Genomics* 1993; **15**: 203–205.
33 He G-S, Grabowski GA: Gaucher disease: A G^{+1} A^{+1} IVS2 splice donor site mutation causing exon 2 skipping in the acid β-glucosidase mRNA. *Am J Hum Genet* 1992; **51**: 810–820.
34 Graves PG, Grabowski GA, Eisner RI, Palese P, Smith FI: Gaucher disease: cloning and characterization of a cDNA encoding acid β-glucosidase from an Ashkenazi Jewish patient. *DNA* 1988; **7**: 521–528.
35 Eyal N, Firon N, Wilder S, Kolodny EH, Horowitz M: Three unique base pair changes in a family with Gaucher disease. *Hum Genet* 1991; **87**: 328–332.
36 Latham TE, Theophilus BD, Grabowski GA, Smith FI: Heterogeneity of mutations in the acid β-glucosidase gene of Gaucher disease patients. *DNA Cell Biol* 1991; **10**: 15–21.
37 Beutler E, Gelbart T: Gaucher disease associated with a unique Kpnl restriction site: identification of the amino acid substitution. *Ann Hum Genet* 1990; **54**: 149–153.
38 Eyal N, Wilder S, Horowitz P: Prevalent and rare mutations among Gaucher patients. *Gene* 1990; **96**: 277–283.
39 Tsuji S, Martin BM, Barranger JA, Stubblefield BK, LaMarca ME, Ginns EI: Genetic heterogeneity in type 1 Gaucher's disease: multiple genotypes in Ashkenazic and non-Ashkenazic individuals. *Proc Natl Acad Sci USA* 1988; **85**: 2349–2352.

40 Theophilus BD, Latham T, Grabowski GA, Smith FI: Comparison of RNase A, a chemical cleavage and GC-clamped denaturing gradient gel electrophoresis for the detection of mutations in exon 9 of the human acid β-glucosidase gene. *Nucleic Acids Res* 1989; **17**: 7707–7722.
41 Wigderson M, Firon N, Horowitz Z *et al*: Characterization of mutations in Gaucher disease patients by cDNA cloning. *Am J Hum Genet* 1989; **44**: 365–377.
42 Hong CM, Ohashi T, Yu X, Weiler S, Barranger JA: Sequence of two alleles responsible for Gaucher disease. *DNA Cell Biol* 1990; **9**: 233–241.
43 Beutler E, Gelbart T, Kuhl W, Sorge J, West C: Identification of the second common Jewish Gaucher disease mutation makes possible population based screening for the heterozygote state. *Proc Natl Acad Sci* 1991; **88**: 10544–10547.
44 Beutler E, Gelbart T, West C: The facile detection of the nt 1226 mutation of glucocerebrosidase by 'mismatched' PCR. *Clin Chim Acta* 1990; **194**: 161–166.
45 Amaral O, Fortuna AM, Lacerda L, Pinto R, Sá Miranda MC: Molecular characterization of type 1 Gaucher disease families and patients: Intrafamilial heterogeneity at the clinical level. *J Med Genet* 1994; **31**: 401–404.
46 Amaral O, Lacerda L, Santos R, Pinto RA, Aerts H, Sá Miranda MC: Type 1 Gaucher disease: Molecular, biochemical, and clinical characterization of patients from Northern Portugal. *Biochem Med Metab Biol* 1993; **49**: 97–107.
47 Sorge J, West C, Westwood B, Beutler E: Correction. *Proc Natl Acad Sci USA*, 1985, **19** 82: 7289–7293. *Proc Natl Acad Sci USA* 1986; **83**: 3567.
48 Beutler E, West C, Gelbart T: Polymorphisms in the human glucocerebrosidase gene. *Genomics* 1992; **12**: 795–800.
49 Taylor JW, Ott J, Eckstein F: The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. *Nucleic Acids Res* 1985; **13**: 8765–8785.
50 Taylor JW, Schmidt W, Cosstick R, Okruszek A, Eckstein F: The use of phosphorothioate-modified DNA in restriction enzyme reactions to prepare nicked DNA. *Nucleic Acids Res* 1985; **13**: 8749–8764.
51 Grace ME, Newman KM, Scheinker V, Berg-Fussman A, Grabowski GA: Analysis of human acid β-glucosidase by site-directed mutagenesis and heterologous expression. *J Biol Chem* 1994; **269**: 2283–2291.
52 Grace ME, Desnick RJ, Patores GM: Identification and expression of acid β-glucosidase mutations causing severe type 1 and neurologic type 2 Gaucher disease in non-Jewish patients. *J Clin Invest* 1997; **99**: 2530–2537.
53 O'Reilly D, Miller L, Luckow V: *Baculovirus Expression Vectors: A Laboratory Manual*, Oxford University Press: New York, 1994.
54 Grace M, Graves P, Smith F, Grabowski G: Analyses of catalytic activity and inhibitor binding of human acid β-glucosidase by site-directed mutagenesis. *J Biol Chem* 1990; **265**: 6827–6835.
55 Grace M, Berg A, He G, Goldberg L, Horowitz M, Grabowski G: Gaucher disease: Heterologous expression of two alleles associated with neuronopathic phenotypes. *Am J Hum Genet* 1991; **46**: 646–655.
56 Aerts J, Donker-Koopman W, Brul S *et al*: Comparative study on glucocerebrosidase in spleens from patients with Gaucher disease. *Biochem J* 1990; **269**: 93–100.
57 Schagger H, von Jagow G: Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987; **166**: 368–379.
58 Dinur T, Grabowski GA, Desnick RJ, Gatt S: Synthesis of a fluorescent derivative of glucosyl ceramide for the sensitive determination of glucocerebrosidase activity. *Anal Biochem* 1984; **136**: 223–234.
59 Sá Miranda MC, Aerts J, Pinto R *et al*: Activity of glucocerebrosidase in extracts of different cell types from type 1 Gaucher disease patients. *Clin Genet* 1990; **38**: 218–227.
60 Grace M, Smith F, Latham T, Horowitz M, Berg A, Grabowski GA: Gaucher disease: Heterologous expression of two alleles associated with neuronopathic phenotypes. *Am J Hum Genet* 1990; **47**: A156.
61 Grabowski GA, Goldblatt J, Dinur T *et al*: Genetic heterogeneity in Gaucher disease: physicokinetic and immunological studies of the residual enzyme in cultured fibroblasts from non-neuronopathic and neuronopathic patients. *Am J Med Genet* 1985; **21**: 529–549.
62 Grabowski GA, Dinur T, Osiecki KM, Kruse JR, Legler G, Gatt S: Gaucher disease types 1,2, and 3: Differential mutations of the acid β-glucosidase active site identified with conduritol B epoxide derivatives and sphingosine. *Am J Hum Genet* 1985; **37**: 499–510.
63 Bordos D, Argos P: Suggestions for 'safe' residue substitutions in site-directed mutagenesis. *J Mol Biol* 1991; **217**: 721–729.
64 Hutchinson C, Swanstrom R, Loebe D: Complete mutagenesis of protein coding domains. *Meth Enzymol* 1991; **202**: 356–390.
65 O'Neill RR, Tokoro T, Kozak CA, Brady RO: Comparison of the chromosomal localization of murine and human glucocerebrosidase genes and of the induced amino acid sequences. *Proc Natl Acad Sci USA* 1989; **86**: 5049–5053.
66 Miao S, McCarter JD, Grace ME, Grabowski GA, Aebersold R, Withers SG: Identification of Glu^{340} as the active-site nucleophile in human glucocerebrosidase by use of electrospray tandem mass spectrometry. *J Biol Chem* 1994; **269**: 10975–10978.
67 Pocovi M, Cenarro A, Civeira F *et al*: Beta-glucocerebrosidase gene locus as a link for Gaucher's disease and familial hypo-alpha-lipoproteinaemia. *Lancet* 1998; **351**: 1919–1923.