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Characterization of Human Glucocerebrosidase from Different Mutant Alleles*

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Human cDNA was mutagenized to duplicate six naturally occurring mutations in the gene for glucocerebrosidase. The mutant genes were expressed in NIH 3T3 cells. The abnormal human enzymes were purified by immunoaffinity chromatography and characterized. The Asn^{370} → Ser mutant protein differed from normal enzyme in its inhibition by both conduritol B epoxide and glucosphingosine demonstrating that the 370 mutant enzyme has an abnormal catalytic site. In addition, the 370 mutant enzyme is less activated by saposin C, but more stimulated by phosphatidylserine than the wild type enzyme. The Arg^{463} → Cys mutant protein was normal with respect to conduritol B epoxide and glucosphingosine inhibition, but was less activated by both saposin C and phosphatidylserine. The Arg^{120} → Gln mutant protein was catalytically inactive. The Leu^{444} → Pro, the pseudopattern, and the Pro^{415} → Arg mutants appear to have reduced amounts of enzyme protein in cells. The studies demonstrated that mutations in the gene for glucocerebrosidase have different effects on the catalytic activity and stability of the enzyme.

An inherited deficiency of glucocerebrosidase (EC 3.2.1.45) is the basis for the lysosomal storage of glucosylceramide in the heterogeneous group of disorders known collectively as Gaucher disease. Biochemical analyses of the enzyme from tissues and cells have been inconclusive, but have suggested that the clinical phenotype is related to a specific abnormality in either the amount or activity of the mutant enzyme (1–3). In a molecular approach to the study of the mutations responsible for Gaucher disease, the cDNA for glucocerebrosidase (GC)[1] was cloned (5), and its sequence was determined (6, 7). Sequencing of mutant GC genes has been carried out, and six different mutant alleles have been identified: codon Leu^{444} → Pro (8), codon Asn^{370} → Ser (9), codon Arg^{120} → Gln (10), codon Pro^{415} → Arg (11), codon Arg^{463} → Cys (12), and a crossover with the pseudogene (12, 41). Only recently have mutant proteins derived from single alleles been available for study. In this paper, we report properties of mutant glucocerebrosidases produced from six different alleles known to cause Gaucher disease.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonuclease, reagents for DNA sequencing, T4 DNA ligase, T4 DNA polymerase, and M13mp18 were purchased from Bethesda Research Laboratories or New England Biolabs. Mutant *Escherichia coli*, strain CJ 236, and T4 gene 32 protein were obtained from Bio-Rad. Cyanogen bromide-activated Sepharose 4B was from Pharmacia LKB Biotechnology Inc. Cell culture reagents and G418 were from Gibco Laboratories. [α-^{35}S]dATP and [α-^{32}P]dCTP were from Du Pont-New England Nuclear. Conduritol B epoxide (CBE) was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA). 4-Methylumbelliferyl-β-D-glucopyranoside (4MU-glc), phosphatidylserine (PS), sodium taurocholate (TC), and glucosphingosine were obtained from Sigma. Other reagents were of the highest grade available.

Constructions

Human cDNA was mutagenized using two different methods. The first method was site-directed mutagenesis (13). Briefly, the cDNA that encodes normal human glucocerebrosidase was cloned into the *Eco*RI site of M13mp18 in the 3′ → 5′ orientation for the Arg^{120} → Gln mutation and 5′ → 3′ orientation for the Pro^{415} → Arg mutation. Double-stranded cDNA was transformed in CJ 236(dut$^-$,ung$^-$) *E. coli*, to make a uracil-containing single-stranded DNA. Following the isolation of the uracil-containing single-stranded DNA, a mutagenic primer was annealed, and the second strand was synthesized with T4 DNA polymerase and T4 DNA ligase. The oligonucleotides used to modify cDNA were 5′-ACATCATCCAGGTACCCAT-3′ for the Arg^{120} → Gln mutation and 5′-TAGAACATGCGCTGTTTGT-3′ for the Pro^{415} → Arg mutation. These oligonucleotides were synthesized on an Applied Biosystem Model 380B DNA Synthesizer and purified using a 20% polyacrylamide/urea sequencing gel. The double-stranded phage DNA was transformed in JM107 (ung$^+$). In this step, the uracil containing strand (containing normal cDNA) was inactivated, and only the mutagenized cDNA strand was grown. Twelve plaques were picked at random and sequenced by the dideoxynucleotide chain termination method to confirm that only the mutant cDNA was present (14). The other method used to define and construct mutant cDNA for GC in expression vectors employed cDNA amplified by the polymerase chain reaction. The details of these procedures have been reported recently (12). Briefly, total RNA was extracted from Gaucher disease fibroblasts, and the complementary DNA was synthesized by avian myeloblastosis virus reverse transcriptase. The cDNA was amplified by the polymerase chain reaction. Amplified cDNA was ligated into M13mp18 and sequenced (14). Mutant cDNA containing the Arg^{463} → Cys mutation was cut with *Nsi*I/*Bam*HI, and a small fragment which contained the 463 mutation was isolated from low melting agarose. This *Nsi*I/*Bam*HI fragment was cloned into the *Nsi*I/*Bam*HI site of normal cDNA. The mutant cDNA containing the Asn^{370} → Ser mutation was cut with *Sca*I/*Nsi*I, and a small fragment was purified which contained the 370 mutation. This fragment was cloned into the *Sca*I/*Nsi*I site of normal cDNA.

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[1] The abbreviations used are: GC, glucocerebrosidase; CBE, conduritol B epoxide; 4MU-glc, 4-methylumbelliferyl-β-D-glucopyranoside; PS, phosphatidylserine; TC, sodium taurocholate; SDS, sodium dodecyl sulfate; CRIM, cross-reacting materials; Bes, *N*,*N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid.

For the 444 mutation and the "pseudopattern" mutation, mutant cDNA containing the desired mutation was cut with *Mst*II/*Bam*HI. The fragment was purified and cloned into the *Mst*II/*Bam*HI site of normal cDNA. These mutant cDNAs were confirmed to have the desired mutation by sequencing. The pseudopattern mutation contains single base substitutions in codons 444, 456, and 460 in exon 10 and is probably the result of a gene conversion (12, 15).

Transfection of NIH 3T3 Cells

Mutant and normal cDNA were ligated into the *Eco*RI site of the pCDE vector. Transfection was carried out by the method of Chen and Okayama (16). NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (high glucose)/10% fetal calf serum. For transfection, 5×10^5 cells were plated in 100-mm dishes and incubated overnight at 37 °C under 5% CO_2. Then 20 μg of DNA (construct: pSV_2Neo, 2:1 molecular ratio) was mixed with 0.25 M $CaCl_2$ and 0.5 ml of 2 × BBS (50 mM) Bes (pH 6.95), 280 mM NaCl, 1.5 mM Na_2HPO_4). The mixture was incubated for 10 min at room temperature. Calcium phosphate-DNA solution was added to cells in a dropwise manner. The cells were incubated at 35 °C under 3% CO_2 overnight. The next day, cells were washed with medium four times and incubated overnight at 37 °C under 5% CO_2. After 24 h, the cells were split at 1:10, 1:5, and 1:3 and incubated for another 24 h. Then, G418 was added to culture medium at a final concentration of 400 μg/ml to select stable transfectants. After about 3 weeks, stable transfectants were picked and grown up to 5–20 confluent 150-mm dishes.

Immunoaffinity Purification of Normal and Mutant Glucocerebrosidase

Purification of the enzyme was carried out by the method of Aerts *et al.* (17). This method is specific of human glucocerebrosidase (18). Briefly, cells were harvested with trypsin-EDTA and washed with phosphate-buffered saline two times. Then cells were sonicated 10 s × 2 (40 watts/s) in 4 volumes of 50 mM potassium phosphate buffer (pH 6.5), 0.25% Triton X-100, and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatant was incubated overnight with 50 μl of cyanogen bromide-activated Sepharose 4B coupled to monoclonal antibody (8E4). The affinity resin was prepared according to the manufacturer's instructions. The monoclonal antibody (8E4) is specific for human glucocerebrosidase and does not react with mouse glucocerebrosidase (18). After binding of the enzyme, the resin was washed successively with 0.1 M citrate phosphate buffer (pH 6.0), 0.5 M NaCl, 30% ethylene glycol in 0.1 M citrate phosphate buffer (pH 6.0), 1% Triton X-100 in 0.01/0.02 M citrate phosphate buffer (pH 5.4), and 50% ethylene glycol in 0.1 M citrate phosphate buffer (pH 6.0). The enzyme was eluted with 90% ethylene glycol in 0.1 M citrate phosphate buffer (pH 6.0). After dilution of the solution to 30% ethylene glycol, the immunoaffinity purification was repeated.

Determination of K_m Values for 4MU-glc

Enzymtic activity of glucocerebrosidase was determined using 4MU-glc as a substrate (17). For K_m studies, the reaction mixture (200 μl) contained various concentrations of 4MU-glc (0.1–7.5 mM), 0.1 M citrate phosphate buffer (pH 5.4), 3.5 mM TC, 2.1 mM Triton X-100, 0.1% bovine serum albumin, and a constant amount of enzyme activity. The reaction was incubated at 37 °C for 30 min and terminated by adding 3.8 ml of 0.17 M glycine carbonate buffer (pH 10.4). Enzyme activities were expressed as nanomoles of substrate cleaved per h (nmol/h). The amount of enzyme was adjusted so that less than 5% of the substrate was hydrolyzed. K_m values were determined from Lineweaver-Burk plots.

Inhibition of Glucocerebrosidase by Glucosphingosine and CBE

For the glucosphingosine inhibition studies, the activities of normal and mutant enzyme in the incubation were equalized by diluting the enzyme extract with 0.1 M citrate phosphate buffer (pH 5.4), 0.1% bovine serum albumin. Glucosphingosine was dissolved in chloroform:methanol (2:1, v/v), and the solvent was evaporated under a nitrogen stream. Dried glucosphingosine was weighed and dissolved in 0.1 M citrate phosphate buffer (pH 5.4). The solution was sonicated briefly to disperse the lipid homogenously. The reaction mixture (200 μl) contained 7.5 mM 4MU-glc, 0.1 M citrate phosphate buffer (pH 5.4), 3.5 mM TC, 2.1 mM Triton X-100, 0.1% bovine serum albumin/glucosphingosine (0–300 μM), and a constant amount of enzyme activity. The incubation was for 30 min at 37 °C and was terminated by adding 3.8 ml of 0.17 M glycine carbonate buffer (pH 10.4). K_i values were determined from Dixon plots. For CBE inhibition studies, the inhibitor was dissolved in water, and the required amounts (0–300 μM) were added to the reaction mixture. I_{50} values (concentration of CBE required to achieve 50% inhibition of enzyme activity) were determined from semilog plots of the activities.

Activation of Glucocerebrosidase by Synthetic Saposin C and PS

Saposin C was chemically synthesized using peptidylglycine α-amidating monooxygenase resins and an automated solid phase protocol based on the principles outlined by Merrified (19) on an Applied Biosystems model 430 peptide synthesizer. The sequence of the 82-amino acid peptide was as described by O'Brien *et al.* (20). This chemically synthesized saposin C, after refolding, has kinetic properties nearly identical with naturally occurring saposin C. The details of the synthesis, folding, and kinetics of saposin C activation will be described elsewhere.[2] The reaction mixture (200 μl) contained 5 mM 4MU-glc, 0.2 M sodium acetate buffer (pH 5.0), 2.1 mM Triton X-100, 0.1% bovine serum albumin, varying concentrations of saposin C (0–20 μM), and a constant amount of enzyme activity. The reaction was incubated for 30 min at 37 °C and terminated by the addition of glycine buffer. For PS stimulation studies, PS was dissolved in chloroform:methanol (2:1, v/v). A small aliquot of this solution was removed, and the solvent was evaporated under a nitrogen stream. The weight of dried PS was measured and dissolved in distilled water at a final concentration at 1 μg/μl. The solution was sonicated briefly to disperse the lipid homogeneously. Varying amounts of PS (0–80 μg) were added to the reaction mixture described above.

Heat Stability

The normal and mutant enzymes were diluted with 0.1 M citrate phosphate buffer (pH 5.4) and 0.1% bovine serum albumin to equalize the enzyme activity and total protein concentration in all samples. The enzyme solution was placed at 50 °C, and enzyme activity was assayed in samples withdrawn at 0, 2, 5, 30, and 60 min.

Gel Analyses

Western Blot—NIH 3T3 cells transformed by the human genes were harvested with trypsin-EDTA and washed twice with phosphate-buffered saline. The cell pellets were suspended in 50 mM potassium phosphate buffer (pH 6.5), 0.25% Triton X-100 and sonicated twice for 10 s on ice (40 watts/s). The cell homogenate was spun at 14,000 × *g* for 5 min in a Microfuge. An aliquot of the sample was electrophoresed in 7.5% SDS-polyacrylamide gel. The protein in the gel was electroblotted onto nitrocellulose membrane at 100 V for 1 h. The nitrocellulose membrane was reacted with 8E4, and then alkaline phosphatase-conjugated goat anti-mouse IgG was reacted as a second antibody. The alkaline phosphatase reaction was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate to visualize cross-reacting materials (CRIM).

Northern Blot—Analyses were carried out by a standard method as described (22). Briefly, total RNA was extracted from the various transfected cells. Equal amounts of total RNA were applied to a 1.2% agarose/formaldehyde gel and electrophoresed. Then, the RNA was blotted onto nitrocellulose membrane and hybridized overnight at 42 °C in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 50 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, 10% dextran sulfate, and 250 μg/ml salmon sperm DNA containing [α-^{32}P]dCTP-labeled human GC cDNA (5). The membranes were washed with 2.5 × SSC, 0.1% SDS for 4 × 10 min at room temperature and 0.3 × SSC, 0.1% SDS at 55 °C for 20 min. The amount of total RNA (10 μg) applied to each lane was equalized spectrophotometrically. The bands were visualized by autoradiography.

Protein Concentration Determination

The protein concentration was determined using the Quantigold kit obtained from Diversified Biotech (Newton Center, MA). Bovine serum albumin was used as the standard.

[2] S. Weiler, W. D. Carson, T. Ohashi, Y. Kishimoto, S. Morimoto, J. S. O'Brien, J. M. F. G. Aerts, J. M. Tager, J. A. Barranger, and J. M. Tomich, manuscript submitted for publication.

RESULTS

The specific activity of GC in crude extracts of 3T3 cells transformed by either normal or mutant human GC genes is shown in Fig. 1. The amount of additional activity above the endogenous 3T3 activity is that GC activity derived from the human gene. It can be seen in Fig. 1 that transformation of cells with the wild type GC gene results in the greatest increment of activity in the homogenates with the 463 GC having the largest activity among the cells transformed with mutant GC alleles.

Western and Northern blots were carried out to estimate the relative amount of RNA and protein produced from each mutant allele compared to that produced by a normal cDNA. Fig. 2 shows the Western blot of crude extracts of 3T3 cells transformed by normal or mutant human GC genes. Fig. 3 shows the Northern blot of total RNA from the same transformed cells probed with a 1700-base pair cDNA for human GC (5). *Panels A* and *B* in each figure represent separate experiments, each with their own controls. These gels show that the amount of cross-reactive protein produced from the 463 GC mutant gene is equal to that produced from the wild type cDNA (Fig. 2*A*, *lane 2*). The amount of RNA produced from the 463 GC allele is about equal to that from the wild type cDNA. In contrast, little or no CRIM for the 444 GC and pseudo-GC proteins could be identified (Fig. 2*A*, *lanes 3* and *4*). The amount of RNA in cells expressing these mutations is slightly greater than normal (Fig. 3*A*, *lanes 3* and *4*). The results suggest that the enzyme protein resulting from these two mutant GC alleles is unstable. Although the possibility exists that 8E4 does not react with 444 GC and pseudo-GC, this conclusion is not consistent with previously reported CRIM and pulse-labeling studies of Type 2 and Type 3 cells which carry the 444 and/or pseudopattern mutations (8, 12, 21, 28, 33, 34, 40). Independently, another group has come to the same conclusion, *i.e.* that 444 GC is an unstable protein (4).

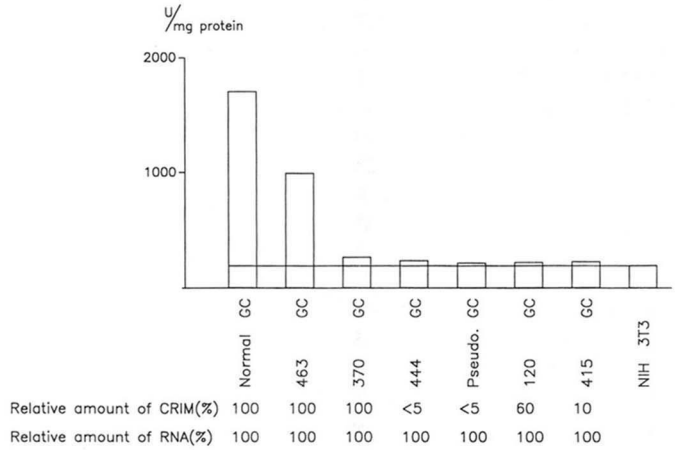

FIG. 1. **The specific activity of glucocerebrosidase from various transfected cells was determined using 4MU-glc as substrate.** The reaction mixture contained 7.5 mM 4MU-glc, 0.1 M citrate phosphate buffer (pH 5.4), 3.5 mM TC, 2.1 mM Triton X-100, and cell extract. Enzyme activity is expressed as nanomoles/h/mg of protein. The relative amounts of CRIM and RNA are also shown. These values are expressed as percent of the CRIM and RNA in cells carrying normal GC cDNA.

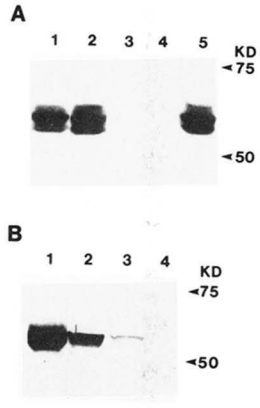

FIG. 2. **Western blot of crude cell extracts of cells transformed with normal and mutant human cDNA.** 100 μg of protein from the crude extracts of cells transformed with the six different human cDNAs was electrophoresed in 7.5% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane. Immunostaining was carried out using 8E4 as described under "Experimental Procedures." *A* and *B* are separate experiments. *A*, NIH 3T3 cells transfected with: *lane 1*, normal cDNA; *lane 2*, $Arg^{463} \rightarrow$ Cys cDNA; *lane 3*, pseudopattern cDNA; *lane 4*, $Leu^{444} \rightarrow$ Pro cDNA; *lane 5*, $Asn^{370} \rightarrow$ Ser cDNA, *B*, NIH 3T3 cells transfected with: *lane 1*, normal cDNA; *lane 2*, $Arg^{120} \rightarrow$ Gln cDNA; *lane 3*, $Pro^{415} \rightarrow$ Arg cDNA; *lane 4*, no cDNA (cell extract of NIH 3T3).

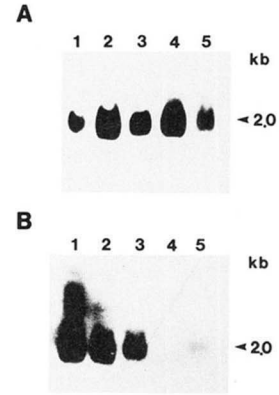

FIG. 3. **Northern blots of various transfected cells.** Total RNA from transfected cells was isolated, and 10 μg was electrophoresed in each lane of a 1.2% agarose formaldehyde gel. The fractionated RNA was blotted onto nitrocellulose membrane and hybridized with a radiolabeled full length cDNA probe from GC. *A* and *B* are separate experiments. *A*, RNA from NIH 3T3 cells transfected with: *lane 1*, normal cDNA; *lane 2*, $Arg^{463} \rightarrow$ Cys cDNA; *lane 3*, pseudopattern cDNA; *lane 4*, $Leu^{444} \rightarrow$ Pro cDNA; *lane 5*, $Asn^{370} \rightarrow$ Ser cDNA. *B*, RNA from NIH 3T3 cells transfected with: *lane 1*, normal cDNA; *lane 2*, $Arg^{120} \rightarrow$ Glu cDNA; *lane 3*, $Pro^{414} \rightarrow$ Arg cDNA; *lane 4*, no cDNA (RNA from NIH 3T3); *lane 5*, RNA from normal human fibroblast.

Cells transformed with the 370 GC mutant allele have an identical amount of CRIM as compared to the normal cDNA (Fig. 2*A*, *lane 5*), and the amount of RNA is approximately equal to normal (Fig. 3*A*, *lane 5*). These results suggested that the 370 GC is stable but catalytically altered. The 120 GC has ~50% CRIM of normal GC (Fig. 2*B*, *lanes 1* and *2*). The amount of RNA for this mutant is slightly lower than normal (~80%) (Fig. 3*B*, *lanes 1* and *2*). Taking into account the amount of RNA, the amount of 120 GC cells could be estimated to be 60–70% of normal GC. It is more likely that reduced catalytic activity, rather than a decrease in enzyme protein, is responsible for the decreased amount of enzymatic activity in cells expressing this mutation. The 415 GC has only ~5% CRIM of normal GC (Fig. 2*B*, *lane 3*). Northern blots show that the RNA for the 415 GC is lower than normal (~60%) (Fig. 3*B*, *lane 3*). Taking these data into account, the 415 GC is probably an unstable protein. These studies of the Western and Northern blots suggest that 370 GC and 463 GC are as stable as normal GC. The 120 GC is somewhat less stable than normal, but that this cannot be causative of the

low activity (<10%) of GC in transfected cells.

Immunoaffinity purification of GC using the monoclonal antibody 8E4 results in a homogeneous preparation of the enzyme from human sources (17, 29).[1] This monoclonal antibody (8E4), which recognizes the N terminus of human GC, does not react with mouse GC either in solution or on Western blot analyses (Fig. 2). The immunoaffinity procedure readily separates the human activity expressed in mouse cells from the endogenous mouse enzyme (18). In the current studies, NIH 3T3 cells served as a control of the immunoaffinity separation of human glucocerebrosidase from the mouse activity. Assays of extracts of nontransfected cells eluted from the 8E4 resin revealed that no measurable GC activity could be recovered. Only those NIH 3T3 cells transfected with normal and mutant human cDNA produced GC that could be harvested by the immunoaffinity procedure. These results demonstrate that only human proteins were isolated using the immunoaffinity method.

Further, this method is useful for the isolation of human glucocerebrosidases from the expression system. Proteins from both the wild type and mutant genes could be isolated. The properties of the enzyme expressed from the normal cDNA were essentially identical with the homogenous enzyme prepared from human placenta (Table I and Figs. 4 through 7). The degree of purification of normal, $Asn^{370} \rightarrow Ser$ mutant (370 GC), and $Arg^{463} \rightarrow Cys$ mutant (463 GC) enzyme was approximately the same being between 2000- and 4000-fold consistently. The method resulted in obtaining enough of two mutant GCs to permit further characterization of their enzymologic properties (see below). The $Arg^{120} \rightarrow Gln$ (120 GC) protein expressed in 3T3 cells cross-reacted with 8E4 (Fig. 2). It could be recovered from the immunoaffinity resin, has the same molecular weight as normal GC (60,000), but had no enzymatic activity. The $Pro^{415} \rightarrow Arg$ (415 GC) reacts with 8E4 and has the same molecular weight as the normal enzyme (Fig. 2). We have previously shown that a cell line (0877) carrying the 444 and pseudopattern mutations (see Ref. 12) produces a protein that cross-reacts with 8E4 (3, 28).[1] However, the signal is consistently weaker. On gels overloaded with protein, these two mutant glucocerebrosidases can be shown to have a molecular weight identical with that of normal GC (data not shown). In standard Western blots of extracts of 3T3 cells expressing these two proteins, the enzyme proteins were at the limit of detection (Fig. 2). These studies demonstrate that the molecular weight of all the mutant proteins does not differ from the wild type. Sufficient amounts of 415 GC, 444 GC, and pseudo-GC were not available to further characterize these mutant proteins. The 463 GC and 370 GC were studied further, and these results are described below.

Table I shows the K_m values of 4MU-glc, K_i values for glucosphingosine specific activities, and molecular weights of GC derived from normal and mutant cDNA compared to purified human placental GC. The K_m and K_i values of GC derived from normal cDNA are almost identical with those of purified human placental enzyme (23, 24). The K_i value of 463 GC is the same as that of normal GC. The 370 GC has a K_i value 15-fold higher than that of normal and the 463 GC (150 μM *versus* 10 μM). Glucosphingosine at 200 μM nearly completely inhibits the activity of normal and 463 GC, but only partially inhibits the 370 GC. The specific activities of normal and mutant GCs are also shown. The specific activity of GC derived from normal cDNA is the same as that of purified human placental enzyme. The specific activity of 370 GC and 463 GC are 5% and 40% of normal GC, respectively.

The inhibition curve for CBE is shown in Fig. 4. CBE putatively reacts in the catalytic site of GC to form a covalent bond with aspartate residues (25). The 370 GC required much more CBE to inhibit the enzyme activity. From the semilog plot of the inhibition curve for CBE, I_{50} values of the 370 GC were 7.8-fold higher than that of normal GC and the 463 GC (585 μM *versus* 75 μM). This result demonstrates that the 370 GC has a catalytic site, which while less efficient, requires more CBE to inhibit it.

Fig. 5 shows the activation profile of normal and mutant GC by saposin C. The 370 GC and 463 GC were both less

TABLE I

Properties of wild type and mutant glucocerebrosidase cDNA expressed in NIH-3T3 cells

Enzymatic properties of normal and mutant glucocerebrosidases K_m values for 4MU-glc were determined by Lineweaver-Burk plots of enzyme activities using different concentrations of 4MU-glc. The reaction mixture contained the required amounts of 4MU-glc (0.1–7.5 mM) 0.1 M citrate phosphate buffer (pH 5.4), 3.5 mM TC, 2.1 mM Triton X-100, 0.1% bovine serum albumin, and a constant amount of enzyme activity. K_i values for glucosphingosine were determined from Dixon plots of enzyme activities using concentrations of glucosphingosine (0–300 μM) and a constant amount of enzyme activity. K_m and K_i for purified human placental enzyme were obtained in three separate experiments. Assays were performed in duplicate. Separate experiments were performed for the enzymes purified from the expression system. For the measurement of K_m, seven concentrations of 4MU-glc were used. For the measurement of K_i, six different concentrations of glucosphingosine were used at three different concentrations of substrate. The assays were performed in duplicate. The specific activities are expressed as nanomoles of 4MU-glc hydrolyzed per h per mg of protein (nmol/h/mg). Molecular weights were determined from Western blots (see Fig. 2).

	Molecular weight ($\times 10^3$)	Specific activity	K_m	K_i	Activation by saposin C	Maximum stimulation by PS	Heat stability
		nmol/h/mg	*mM*	*μM*	*-fold*	*-fold*	
Human placental glucocerebrosidase	65	$1.9 \pm 0.1 \times 10^6$[a]	2.7 ± 0.2[b]	7.5 ± 1.5[b]	4	8	Stable
Wild type cDNA	60–65	2.2×10^6	4.0	10	3	8	Stable
370 Mutant	60–65	1.0×10^5	4.0	150	1.3	30	Stable
463 Mutant	60–65	8.3×10^5	4.0	10	1.5	4	Unstable
120 Mutant	60–65	Inactive					
415 Mutant	60–65	ND[c]					
444 Mutant	60–65	ND					
Pseudopattern mutant	60–65	ND					

[a] Mean ± S.D. of three different preparations.
[b] Mean ± S.D. of three different experiments.
[c] ND, not determined.

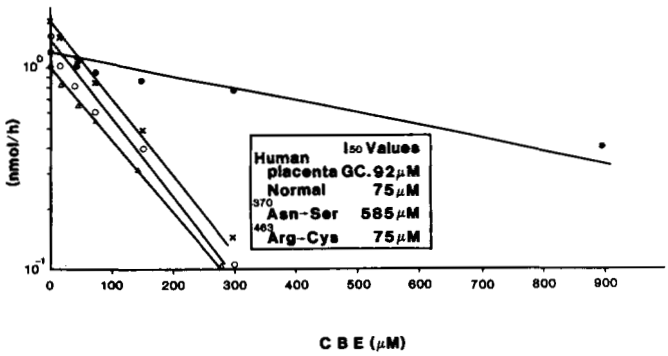

FIG. 4. **Inhibition of GC by CBE.** Normal and mutant GC purified from the expression system were inhibited by various concentrations of CBE. Responses were compared to purified placental GC. The reaction mixture contained 7.5 mM 4MU-glc, 0.1 M citrate phosphate buffer (pH 5.4), 3.5 mM TC, 2.1 mM Triton X-100, 0.1% bovine serum albumin, the required amount of CBE (0–300 μM), and a constant amount of enzyme activity. The amount of enzyme activity in the assay was equalized between samples. I_{50} values were determined as the concentration of CBE required to achieve 50% inhibition of enzyme activity. Δ——Δ, purified placental GC; ○——○, normal GC; ●——●, 370 GC; ×——×, 463 GC.

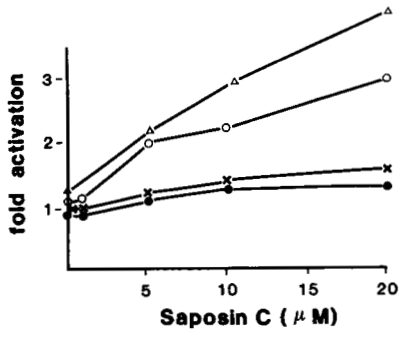

FIG. 5. **Stimulation of normal and mutant GC by synthetic saposin C.** Normal and mutant GC purified from the expression system were stimulated by various concentrations of saposin C and compared to the effects of saposin C on homogeneous placental enzyme. The reaction mixture contained 5 mM 4MU-glc, 0.2 M sodium acetate buffer (pH 5.0), 2.1 mM Triton X-100, 0.1% bovine serum albumin, required concentration of saposin C (0–20 μM), and a constant amount of enzyme activity. The amount of normal and mutant GC added to the reaction was equalized after determining the activity of each in an assay without added saposin C. Each point is an average of duplicate assays. The experiments on purified placental GC were repeated at least three times. Separate experiments were performed for each enzyme purified from the expression system. Δ——Δ, pure placental GC; ○——○, normal GC; ●——●, 370 GC; ×——×, 463 GC.

activated by saposin C compared to normal GC. Normal GC was activated by saposin C 3-fold, but the 463 GC was activated only 1.5-fold by up to a 20 μM concentration of saposin C. The 370 mutant GC was activated only 1.3-fold by saposin C. These results show that both of these mutant proteins are less well activated *in vitro* by saposin C.

The activation profile of normal and mutant GC by PS is shown in Fig. 6. Normal GC is stimulated by PS 8-fold, and the 463 GC was stimulated 4-fold. These results show that these two mutant GCs have very different catalytic properties with respect to stimulation by the acidic lipid PS. The data also show that *in vitro* PS is less stimulatory for the 370 GC at higher concentrations under the conditions used. Other lipids have been shown to have a range of stimulatory concentrations which when exceeded are less effective in stimulating the activity of pure or crude preparations of GC (26, 27, 38). The reasons for this effect are not understood, but

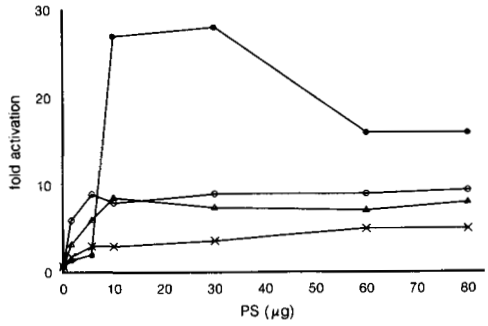

FIG. 6. **Stimulation of normal and mutant GC by PS.** Normal and mutant GC were stimulated by various concentrations of PS. The reaction mixture was as previously described under "Experimental Procedures." The range of PS concentrations was 0 to 80 μg. The amount of normal and mutant enzyme in the reaction was equalized after determining its activity without PS. The data presented are the average of duplicate assays. Δ——Δ, pure placental GC; ○——○, normal GC; ●——●, 370 GC; ×——×, 463 GC.

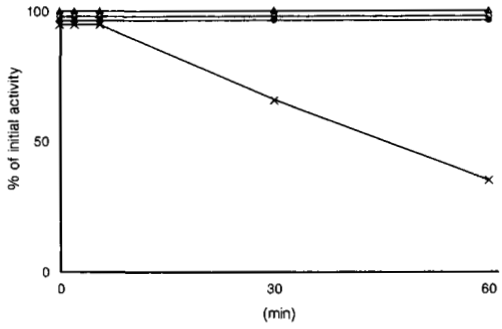

FIG. 7. **Heat stability of normal and mutant GC.** The activity of normal and mutant GC in separate tubes was equalized by adjusting the amount of enzyme added to 0.1 M citrate phosphate buffer (pH 5.4). The total protein concentration was made equal in each sample by adding an appropriate amount of 0.1% bovine serum albumin. The enzyme was placed at 50 °C, and enzyme activity was assayed at 0, 2, 5, 30, and 60 min. Δ——Δ, pure placental GC; ○——○, normal GC; ●——●, 370 GC; ×——×, 463 GC.

probably relate to the interaction and optimal proportions of the multiple components involved in the reaction. In other experiments on extracts of cells from type 1 Gaucher cases, the combination of saposin C and PS *in vitro* results in activity that approximates normal enzyme activity (42). Since cells from Type 1 cases have the highest probability of carrying a 370 mutation (8, 9, 33, 34), the effect of PS on the 370 GC is most likely to be responsible for these observations.

Fig. 7 is a plot of the heat stability of normal and mutant GC. The normal and the 370 GC are stable for 1 h at 50 °C, but the enzyme activity of the 463 GC was decreased to 37% of the initial activity. This result clearly demonstrates that different mutant glucocerebrosidases have different heat stabilities.

DISCUSSION

Recently, several mutations in the gene for glucocerebrosidase have been described (8–12). However, the relationship between genotype and phenotype remains obscure. For example, the $Leu^{444} \rightarrow Pro$ mutation is found predominantly in severe cases of Gaucher disease (Types 2 and 3), but to a lesser extent occurs in clinically mild cases (Type 1 Gaucher disease) (8, 9, 33, 34). In addition, cells derived from Gaucher patients often have been shown to carry two different mutant alleles, so-called compound heterozygotes (8–12, 30, 34). The enzyme isolated from patient materials therefore is often

composed of two different mutant proteins. In the past, biochemical studies were carried out on residual glucocerebrosidase activities from Gaucher patients using crude or partially purified enzyme preparations. The shortcomings of biochemical studies in assigning a property characteristic of any phenotype are explainable given the molecular genetics cited above. The presence of more than one enzyme protein in samples no doubt is the reason for different biochemical results obtained in different laboratories (1, 31). In this study, we characterized the purified normal and mutant GC enzymes derived from single alleles to define the properties of the mutant proteins. We can conclude that different mutations result in mutant enzymes with different properties. This approach helps clarify previous studies of the enzyme and provides direct evidence for the differences in the properties of glucocerebrosidase that have been observed in patient material. For example, glucocerebrosidase studied in Ashkenazi Jewish patients with Type 1 Gaucher disease has been reported to be either catalytically altered or unstable or both (1, 2, 25, 26, 31, 32, 35).[1] Several mutations occur in the Jewish population heteroallelically most often in combination with the 370 mutation (8, 9, 11, 30, 33, 34). One of these is the codon 444 mutation. Our data show that the 444 GC protein is probably unstable which is consistent with earlier CRIM, pulse-labeling, and immunocytochemical data (2, 3, 21, 28, 40). Thus, reports demonstrating alteration of different properties of GC in the same phenotype are supported by our data, *i.e.* GC in Type 1 patients may be catalytically inefficient, unstable, or both, depending on the mutations present.

The activation of GC by saposins (heat-stable factors or sphingolipid-activating factors) and acidic lipids has been reported to be altered in some studies of GC, but not others (31). Our data show that saposin C poorly stimulates the 370 GC and 463 GC mutant protein as compared to normal. However, the 370 GC is stimulated to a greater extent by PS than either the normal or 463 GC enzyme. Moreover, Type 1 cells which probably carry the 370 codon mutation can be stimulated to near normal activity *in vitro* by the combination of PS and saposins.[4] The studies reported in this paper show that the K_i for glucosphingosine and I_{50} for CBE are identical between 463 GC and normal GC indicating that the active sites of the proteins are very similar. In light of the specific activity of 463 GC being about 40% of normal, these observations and conclusions are not too surprising. However, 463 GC is poorly stimulated by saposin C and PS, implying that the activity of GC in the lysosome may depend on its ability to be activated by *in situ* saposins and endogenous lipids. Indeed, two examples of saposin C deficiency as a cause of Gaucher disease have been reported (36, 37). The 463 GC may be the first example of a refractory response to saposin stimulation as a cause for Gaucher disease. The interaction of saposin and GC is being studied further in our laboratory.

Two proteins and other cellular constituents are important to the activity of glucocerebrosidase. We have shown in this report that mutations in the glucocerebrosidase gene can alter one or more components of the enzymatic catalysis of glucosylceramide. In this regard, Morimoto *et al.* (38) proposed a four-binding site model of glucocerebrosidase: one for the carbohydrate of substrate, one for the aglycon, one for the lipid, and one for the saposins. Other investigators have reported that PS and glucosphingosine bind to the same domain (39). However, in our study, the 463 mutant enzyme had a normal response to glucosphingosine and an abnormal response to PS. This suggests that PS and glucosphingosine may bind to different domains, and that the number of sites interacting in the catalysis may be more than previously predicted.

Finally, previous studies of thermostability of mutant glucocerebrosidases yielded different results in material obtained from patients (26, 31). The present data show that the 370 mutant enzyme is as stable to heating as normal enzyme, but the 463 mutant enzyme is less thermostable. These data show that the apparent inconsistencies of earlier results are probably the consequence of true differences in the products of different mutant genes.

The results reported in this paper describe the different properties of six different mutant glucocerebrosidase enzymes. It is apparent that one or more abnormalities may contribute to low enzymatic activities in patient materials. Our results confirm that Gaucher disease is a genetically heterogeneous disorder caused by different mutations producing gene products with distinctly different properties. Elucidation of the biology of the disorders resulting from the mutations acting either alone or in combination will require considerably further investigation in order to understand the relationship of genotype to phenotype in this group of disorders.

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