Non–pseudogene-derived complex acid β-glucosidase mutations causing mild type 1 and severe type 2 Gaucher disease

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Gaucher disease is an autosomal recessive inborn error of glycosphingolipid metabolism caused by the deficient activity of the lysosomal hydrolase, acid β-glucosidase. Three phenotypically distinct subtypes result from different acid β-glucosidase mutations encoding enzymes with absent or low activity. A severe neonatal type 2 variant who presented with collodion skin, ichthyosis, and a rapid neurodegenerative course had two novel acid β-glucosidase alleles: a complex, maternally derived allele, E326K+L444P, and a paternally inherited nonsense mutation, E233X. Because the only other non–pseudogene-derived complex allele, D140H+E326K, also had the E326K lesion and was reported in a mild type 1 patient with a D140H+E326K/K157Q genotype, these complex alleles and their individual mutations were expressed and characterized. Because the E233X mutation expressed no activity and the K157Q allele had ~1% normal specific activity based on cross-reacting immunologic material (CRIM SA) in the baculovirus system, the residual activity in both patients was primarily from their complex alleles. In the type 1 patient, the D140H+E326K allele was neuroprotective, encoding an enzyme with a catalytic efficiency similar to that of the N370S enzyme. In contrast, the E326K+L444P allele did not have sufficient activity to protect against the neurologic manifestations and, in combination with the inactive E233X lesion, resulted in the severe neonatal type 2 variant. Thus, characterization of these novel genotypes with non–pseudogene-derived complex mutations provided the pathogenic basis for their diverse phenotypes.


Introduction

Gaucher disease (GD) is the most common lysosomal storage disorder and results from the deficient activity of acid β-glucosidase (E.C.3.2.1.45) and the accumulation of its undegraded substrate, glucosylceramide (1, 2). The disease occurs in three distinct phenotypic subtypes that are delineated by the absence (type 1), or presence and severity of neurologic involvement (types 2 and 3). Most patients (~95%) have type 1 GD, the non-neuropathic form characterized by hepatosplenomegaly, secondary hypersplenism, and skeletal involvement (3). In contrast, patients with the neurologic forms, acute infantile type 2 or subacute late-infantile or juvenile type 3, are rare. Recently, severe type 2 neonatal variants with collodion skin or ichthyosis and rapid neuronal degeneration have been described. These variants occasionally present with nonimmune hydrops fetalis (4, 5).

To date, over 100 acid β-glucosidase mutations have been identified in patients with GD (6). Of these, hetero- or homoallelism for the N370S lesion has been found in ~50% of the Ashkenazi Jewish patients with type 1 disease, and the severe L444P mutation has been found as the most frequent lesion in non-Jewish GD patients (6). It is notable that several multiply mutated alleles have been identified that are derived from rearrangements between the structural gene and the 16-kb downstream pseudogene (7), including RecNciI, RecTL, AZRecTL, and Complex C (8–12). Expression of the RecNciI (L444P+A456P+V460V) and RecTL (D409H+L444P+A456P+V460V) alleles (13) and the individual L444P, A456P, and D409H mutations (14) has demonstrated that these complex alleles were severely compromised, having essentially no activity. Prediction of the disease subtype and severity based on analysis of the patient’s genotype has been limited, in part due to the occurrence of numerous family-specific (or private) acid β-glucosidase mutations (6, 15–18). However, some of these mutations have been expressed and characterized, providing correlations between their residual CRIM SAs (specific activity based on cross-reacting immunologic material) and their stability with the disease phenotype (13, 14, 19, 20). For example, the presence of one N370S allele, which encodes a protein with significant CRIM SA, is neuroprotective, precluding the development of neurologic manifestations and resulting in the type 1 phenotype (17, 18). Homoal-
lelism for the L444P mutation, which encodes a very low level of CRIM SA, usually results in neuropathic disease (21–23), while homoollelism for the severe 48GG frameshift allele is a fetal lethal (15).

Presumably, the severe type 2 neonatal form of GD is caused by very severe mutations that render the enzyme essentially nonfunctional and/or unstable (5, 24–33). However, the causative genotype has not been defined in most of these cases (5, 24–27). In fact, the complete genotype of only one patient with documented skin abnormalities has been determined: homozygosity for the complex mutation RecNcil (5, 26). Note that mouse models with totally deficient (knockout) or severely reduced (RecNcil or L444P homozygotes) acid β-glucosidase activity present with features similar to those seen in the neonatal GD type 2 cases, including skin abnormalities, poor skin turgor, and neonatal demise in the first days of life (30, 34, 35). Because the lesions in these type 2 variants result in mutant proteins with little, if any, enzyme activity and/or stability, knowledge of these severe mutations can provide structure/function information, as well as further delineation of genotype/phenotype correlations in GD.

In this study, mutation analysis of a severe GD type 2 patient with colloidon skin abnormalities led to the identification of two novel alleles: E233X and a new complex allele with two mutations, E326K and L444P. Interestingly, one other complex allele containing the E326K lesion has been reported in a mild type 1 GD patient with the genotype K157Q/D140H+E326K (36). To determine if the E326K lesion was a polymorphism and the role of these non–pseudogene-derived complex alleles in the causation of these diverse phenotypes, the individual and complex mutations were expressed and characterized. It is notable that the D140H+E326K was neuroprotective, having sufficient residual activity to prevent the development of neurologic disease, even in combination with the essentially inactive K157Q mutation. In contrast, the compound E326K+L444P allele was neuronopathic, having a very low CRIM SA, which in combination with the inactive E233X allele, produced the neonatal type 2 GD variant with the colloidon skin abnormality.

Methods
Case report. The patient was a 1,965 g newborn female (Fig. 1) delivered by emergency cesarean section due to fetal bradycardia at 32 weeks gestation to a 29-year-old G5P0 mother. The parents were not related; the father had Irish/Polish ancestry and the mother had Mexican ancestry. No pathological details were available on four prior pregnancies that terminated spontaneously during the first trimester. Apgar scores were 0 and 4 at 1 and 5 min, respectively. The pregnancy was complicated by intermittent vaginal bleeding, polyhydramnios that were decompressed by amniocentesis, and premature onset of labor at 31 weeks. At birth, the child had a facial scaling rash and edematous, shiny, and peeling colloidal skin. There were no major dysmorphic features; however, the ears were low-set and dysplastic. The infant had hepatosplenomegaly and was hypotonic. Laboratory studies revealed severe anemia (hemoglobin, 5 g/dl) and thrombocytopenia, normal liver function, normal prothrombin time (PT) and activated prothrombin time (APPT), negative TORCH and hepatitis titers, and a normal female karyotype (46, XX). A bone marrow biopsy showed typical GD cells by light microscopy. There was marked deficiency of acid β-glucosidase activity in leukocytes (0.4 × 10⁻⁴ μmol/min/mg protein; control value ~2.0 × 10⁻⁴ μmol/min/mg protein).

The infant had a fulminant course characterized by severe and progressive thrombocytopenia, necessitating two to three platelet transfusions a day. The initial neurologic symptoms of hypotonia and lethargy evolved to a frank encephalopathy, with associated opisthotonus and flexor hypertonicity of the upper limbs. A computed tomography scan of the brain showed an extensive subdural hematoma, cortical atrophy, ventriculomegaly, and encephalomalacia. The patient had a cardiorespiratory arrest and died at the age of 10 weeks.

Reagents. Triton X-100 and NBD-glucocerebroside were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Ethylenediame was purchased from Fisher Scientific Co. (Pittsburgh, Pennsylvania, USA). 4-methylumbelliferyl-β-D-glucopyranoside (4MU-Glc) was from Genzyme Corp. (Cambridge, Massachusetts, USA). Sodium taurocholate, deoxyxojirimycin, and castanospermine were purchased from Calbiochem-Novabiochem Corp. (La Jolla, California, USA). Dynabeads were obtained from Dynal Inc. (Lake Success, New York, USA). The Sculprior In Vitro Mutagenesis System kit was from Amersham Life Sciences Inc. (Arlington Heights, Illinois, USA). Spodoptera frugiperda (Crl 1711) cells were purchased from American Type Culture Collection (Rockville, Maryland, USA). Baculogold was obtained from PharMingen (San Diego, California, USA).

DNA isolation, PCR amplification, and mutation detection. Skin biopsies for fibroblast culture were obtained with informed consent. Genomic DNA was isolated from skin fibroblasts using standard techniques (37). Initial mutation screening for the four common mutations (N370S, L444P, 846G, and IVS2–1) was performed by PCR amplification and restriction digestion as described previously (17, 21, 38, 39). To detect the unknown acid β-glucosidase mutations, the complete coding region and adjacent intron/exon boundaries were amplified from genomic DNA (500 ng) using biotinylated sense (exons 1+2, 3, 4, 6, and 7) and antisense (exons 5, 8+9, and 10+11) primers (17). Each PCR product was denatured with NaOH and the biotinylated single-strand template was subsequently immobilized on streptavidin attached to polystyrene Dynabeads. Solid-phase direct sequencing was performed by the dyeoxy chain termination method (40, 41). The designations for the mutations refer to the position of the amino acid substitution, where amino acid 1 is the NH₂-terminus of the mature protein. The cDNA base numbers refer to the position of the nucleotide in the cdNA (42, 43), where nucleotide 1 is the A in the first ATG. The genomic designations are based on the updated acid β-glucosidase GenBank sequence (7, 44),

Figure 1
The patient at 4 weeks old clearly showing features of colloidal skin — shiny areas over abdomen and extremities.
accession no. J03059. The first nucleotide of exon 1 is at genomic position 1230.

Screening for the E326K mutation. Genomic DNAs from 53 GD patients and 50 unrelated normal individuals were PCR-amplified across exons 8 and 9 to yield 10-kb nucleotide PCR products (encompassing the genomic region 5862–6941) that were then subjected to restriction digestion with BstI (New England Biolabs, Beverly, Massachusetts, USA). The presence of the G→A substitution at genomic position 6195, which creates a BstI restriction site, resulted in two DNA fragments of 340 and 740 bp.

Construction of expression plasmids. Point mutations for K157Q, D140H, E233X, and E326K were introduced into the acid β-glucosidase cDNA by an M13mp19-based, oligonucleotidedirected, site-specific mutagenesis procedure (Sculptor In Vitro Mutagenesis System) employing the phosphorothioate selection method (45, 46) as described previously (14, 19). The double mutants (D140H+E326K and E326K+L444P) were created by a second round of mutagenesis, beginning with a single-strand template carrying the E326K mutation. The complete sequence of each mutated cDNA was determined to confirm that no spurious mutations were incorporated during the mutagenesis procedure. The mutant cDNAs were then cloned into the EcoRI site of the baculovirus expression vector pAc1392. The final acid β-glucosidase cDNA inserts in the expression plasmid was 1.562-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; K157Q, D140H, E233X, E326K, D140H+E326K, and E326K+L444P) were produced in cloned Spodoptera frugiperda (SPF) cells by calcium phosphate–mediated transfection and homologous recombination between the expression plasmid and baculovirus genomic DNA (Baculogold) as described (47). Pure recombinant baculovirus clones containing the normal or mutant cDNA for acid β-glucosidase were isolated by plaque hybridization and then amplified, titrated, and used at a multiplicity of infection >10 to infect Sf9 cells (47). The previously characterized common mutations, N370S and L444P, were introduced into the acid β-glucosidase activity, using the fluorescently labeled natural substrate NBD-glucosylceramide or 12-[N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)]dodecanoyl-sphinogosyl-1-O-β-D-glucopyranoside (NBD-GC) or the synthetic substrate (4-methyl-umbelliferyl-β-D-glucopyranoside; 4MU-Glc). The final reaction mixtures contained either 0.3 mM NBD-GC (reaction volume of 100 or 200 μl) or 4 mM 4MU-Glc (reaction volume of 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 4MU-Glc reaction was terminated by raising the pH with the addition of 2.3 ml of a 0.1 M aqueous solution of ethylenediamine. Fluorescence was determined with a fluorometer (Model 450; Sequoia- Turner, Mountain View, California, USA). The NBD-GC assays were termed and processed as described (53), and the fluorescence of the extracted reaction products were read using a Farrand Optical System 3 spectrophuorometer (Farrand Optical Co., New York, New York, 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). One unit of acid β-glucosidase activity was that amount of enzyme that hydrolyzed one micromole of substrate per min at 37°C.

Inhibition of expressed normal and mutant acid β-glucosidases. Aliquots of crude lysates were assayed in the absence or presence of each of the following active site–directed inhibitors: conduritol B epoxide (CBE; 0–600 μM), deoxynojirimycin (DNM; 0–600 μM), and castanospermine (CS; 0–80 μM). Concentrated aqueous stock solutions of the inhibitors were prepared so that aliquots between 0 and 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 concentration of inhibitor resulting in 50% reduction in activity (IC50) for each of the normal and mutant acid β-glucosidases was determined from a plot of activity remaining versus inhibitor concentration.

Results

Mutation analysis. Sequence analysis of the entire coding region and all exon/intron boundaries in the acid β-glucosidase gene was performed on genomic DNA isolated from cultured skin fibroblasts from the proband. As indicated in Table 1, these studies revealed three point substitutions: two missense mutations (E326K and L444P) and a nonsense mutation (E233X). Family studies demonstrated that the patient inherited the E233X mutation from her father and the complex allele with the E326K and L444P lesions from her mother. Sequence analysis indicated that the complex mutation was not derived from a recombinant event as evidenced by sequencing of the downstream pseudogene.

Previously, the E326K mutation was described in another non–pseudogene–derived complex allele (D140H+E326K) in a patient with mild type 1 GD (36). Thus, studies were undertaken to investigate the possibility that the E326K substitution was a benign polymorphism in the acid β-glucosidase gene. Genomic DNA from 50 random normal individuals were PCR-amplified across exons 8 and 9 and tested for the presence of the E326K lesion by restriction digestion with BstI. Additionally, 33 L444P (non-RecNciI) alleles and 30 unknown alleles in 53 GD patients previously screened for the common GD mutations (N370S, L444P, 84GG, and IVS2–1) also were tested for the E326K mutation. None of the 100 normal alleles or 106 GD alleles contained the E326K mutation, a G→A transition at
Table 1
Phenotypes and genotypes of the GD patients carrying the E326K mutation

<table>
<thead>
<tr>
<th>Patient</th>
<th>Phenotype</th>
<th>Ancestry</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Type 2</td>
<td>Irish/Polish/Mexican</td>
<td>E233X/E326K+L444P</td>
</tr>
<tr>
<td>2*</td>
<td>Type 1</td>
<td>Non-Jewish American</td>
<td>K157Q/D140H+E326K</td>
</tr>
</tbody>
</table>

*Mutations reported by Eyal et al. (36). K157Q also reported by Latham et al. (12). GD, Gaucher disease.

Table 2
Acid β-glucosidase mutations expressed and characterized in Sf9 cells using the baculovirus system

<table>
<thead>
<tr>
<th>Designation</th>
<th>cDNA position</th>
<th>Genomic position</th>
<th>Codon change</th>
<th>Exon</th>
<th>Amino acid change</th>
</tr>
</thead>
<tbody>
<tr>
<td>D140H</td>
<td>535</td>
<td>4001</td>
<td>CAT→CAT</td>
<td>5</td>
<td>aspartate→histidine</td>
</tr>
<tr>
<td>K157Q</td>
<td>586</td>
<td>4052</td>
<td>AAG→AAG</td>
<td>5</td>
<td>lysine→glutamine</td>
</tr>
<tr>
<td>E233X</td>
<td>814</td>
<td>5045</td>
<td>GAA→GAA</td>
<td>7</td>
<td>glutamate→termination</td>
</tr>
<tr>
<td>E326K</td>
<td>1093</td>
<td>6195</td>
<td>GAG→GAG</td>
<td>8</td>
<td>glutamate→lysine</td>
</tr>
<tr>
<td>L444P</td>
<td>1448</td>
<td>7139</td>
<td>CTC→CCG</td>
<td>10</td>
<td>leucine→proline</td>
</tr>
</tbody>
</table>

*Expressed as single mutations and as the complex alleles D140H+E326K and E326K+L444P.

Expression studies. To determine the function of the E326K mutation alone and in combination with the L444P or D140H lesion, each allele was expressed and the mutant proteins were characterized. Constructs were expressed for the E233X mutation, the previously identified but not characterized K157Q lesion (12, 36), and the D140H mutation. For expression, the singly- and doubly-mutated alleles (Table 2) were introduced into Sf9 cells using the baculovirus expression system. The L444P and N370S alleles, previously expressed and characterized (14, 19), were reexpressed for comparative purposes.

As shown in Table 3, the normal acid β-glucosidase allele expressed in Sf9 cells produced ~4.5 × 10⁻² U of activity per milligram of total protein toward the fluorescein-labeled natural substrate, NBD-glucosylceramide. Essentially no activity toward the natural substrate was detected in Sf9 cells infected with the recombinant baculovirus containing the antisense acid β-glucosidase cDNA construct. The four patient-derived alleles had significantly reduced activities. As anticipated, the most severely deficient was the E233X nonsense allele, which had no detectable activity toward NBD-GC. The complex mutant, D140H+E326K, retained ~20% of normal activity in the crude Sf9 cell lysates, while both the K157Q and the complex E326K+L444P alleles had ~3.0% and 3.5% of normal activity, respectively, similar to that of the expressed L444P allele. In contrast, the individual constructs for D140H and E326K alleles expressed mutant enzymes with high (50% and 60% expressed normal activity, respectively) residual activity.

Figure 2 shows the immunoblot analysis of the expressed normal and mutant acid β-glucosidase alleles. The normal allele produced a multiband pattern that reacted strongly with the polyclonal anti-human acid β-glucosidase antibodies. The bands (ranging from 63 kDa to ~56 kDa) represented differentially glycosylated enzyme forms (19). The 56-kDa species, shown previously to be the unglycosylated mature acid β-glucosidase peptide, results from saturation of the glycosylation machinery in the Sf9 cells by the high expression levels achieved with the baculovirus system (19). Similar multiband patterns were detected for all of the enzymes with residual activity encoded by the singly- and doubly-mutated cDNAs. However, no CRIM (i.e., no acid β-glucosidase protein) was detected in the cells expressing the inactive truncation mutant, E233X, or the antisense construct of the normal allele.

The level of catalytic efficiency (kcat) for each mutant enzyme was calculated as the amount of activity per milligram of acid β-glucosidase protein (quantitated as CRIM SA) relative to the expressed CRIM SA for the normal enzyme (Table 3). Because glycosylation is required for acid β-glucosidase activity (54, 55), the CRIM signals for the 56-kDa bands were not included in this analysis. For example, the immunoblot signal for the K157Q enzyme was ~1.3 times that for the normal acid β-glucosidase protein (compare lanes 2 and 4 in Fig. 2). Because the aliquot of the K157Q extract applied to the gel had only 1.4% of the activity applied for the normal enzyme (6.2 × 10⁻³ U vs. 4.5 × 10⁻³ U), the K157Q enzyme had an ~90-fold reduction in kcat, or ~1.1% of the CRIM SA, when compared with the expressed normal enzyme. Table 3 shows the catalytic efficiencies of the individual and double mutants and the finding that the double mutants generally reflected the combined severity of their individual mutations. The combination of the relatively mild mutations, D140H (with its approximately twofold reduction in CRIM SA) and E326K (with its approximately threefold reduction in CRIM SA), resulted in a mutant protein (D140H+E326K) with a kcat of 0.2 (or 20% of the expressed normal enzyme), which was similar to that (0.24) for the common mild allele, N370S (14, 19). Interestingly, while the addition of the very mild E326K mutation (CRIM SA = 0.31) to the deleterious L444P allele (CRIM SA = 0.10) caused a reduction in turnover rate or kcat, the E326K+L444P double mutant enzyme (CRIM SA = 0.045) had a higher kcat than the essentially inactive K157Q enzyme (i.e., CRIM SA ~0.01).

To probe the effects of the various amino acid changes on the structural integrity of the active site of each expressed mutant enzyme, the binding affinities for three active site-directed inhibitors (DNM, CBE, and CS) were assessed by determining for each inhibitor the concentration required to achieve 50% inhibition of the initial activity (IC₅₀). For comparative purposes, the previously characterized L444P and N370S alleles were reanalyzed (14, 19). As shown in Fig. 3, each of the expressed mutant enzymes interacted normally with these inhibitors and had IC₅₀ curves that overlapped those for the expressed normal enzyme, which identified them as group A enzymes (49, 56). None exhibited typical group B kinetics (illustrated by the N370S enzyme; refs. 14, 19), characterized by decreased affinity (i.e., higher IC₅₀ value) toward DNM and CBE.

Discussion

In GD, the occurrence of multiple non–pseudogene-derived point mutations in a single acid β-glucosidase allele was reported only once: a D140H+E326K allele that, together with a K157Q allele, caused type 1 GD (36). Thus, the find-
Figure 2
Composite immunoblots of the normal and mutant acid β-glucosidases expressed in Sf9 cells. Lanes 1–5: D140H+E326K (3.5 × 10⁻⁴ U; 20 µg), K157Q (6.2 × 10⁻⁴ U; 23 µg), D140H (9.9 × 10⁻⁴ U; 24 µg), normal (4.5 × 10⁻⁴ U; 35 µg), and the antisense construct (Rev; 36 µg). Lanes 6 and 7: E326K (7.6 × 10⁻⁴ U; 26 µg) and normal (2.0 × 10⁻⁴ U; 36 µg). Lanes 8 and 9: normal (8.3 × 10⁻⁴ U; 74 µg) and E233X (91 µg). Lanes 10–13: normal (1.1 × 10⁻³ U; 10 µg), L444P (5.1 × 10⁻³ U; 10 µg), E326K+L444P (7.9 × 10⁻³ U; 34 µg), and N370S (4.4 × 10⁻⁴ U; 23 µg). See text for details.

Figure 3
Characterization of acid β-glucosidases expressed in Sf9 cells

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Specific activity¹</th>
<th>CRIM SA³</th>
<th>1/CRIX SA⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4.2–5.0 × 10⁻²</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Antisense</td>
<td>0.0</td>
<td>no CRIM</td>
<td>—</td>
</tr>
<tr>
<td>D140H</td>
<td>2.2–2.5 × 10⁻²</td>
<td>0.55 ± 0.10</td>
<td>1.8</td>
</tr>
<tr>
<td>K157Q</td>
<td>1.4 × 10⁻¹</td>
<td>0.011 ± 0.003</td>
<td>91</td>
</tr>
<tr>
<td>E233X</td>
<td>2.8 × 10⁻²</td>
<td>0.31 ± 0.02</td>
<td>3.2</td>
</tr>
<tr>
<td>N370S</td>
<td>1.1 × 10⁻²</td>
<td>0.24 ± 0.01</td>
<td>4.2</td>
</tr>
<tr>
<td>L444P</td>
<td>1.8 × 10⁻¹</td>
<td>0.10 ± 0.01</td>
<td>10</td>
</tr>
<tr>
<td>D140H+E326K</td>
<td>9.1 × 10⁻¹</td>
<td>0.20 ± 0.03</td>
<td>5</td>
</tr>
<tr>
<td>E326K+L444P</td>
<td>1.6 × 10⁻¹</td>
<td>0.045 ± 0.001</td>
<td>22</td>
</tr>
</tbody>
</table>

¹Using NBD-labeled glucosylceramide as substrate. ³CRIM SA represents total units of specific activity per cross-reacting immunological material based on the glycosylated forms (~63 kDa) only. ⁴To date, found only as part of complex alleles D140H+E326K and E326K+L444P.

detectably different enzyme. That the E326K+L444P/E233X genotype produced a type 2 phenotype indicated that the E326K+L444P allele did not have sufficient residual CRIM SA to prevent the development of the acute neuronopathic variant of GD. This finding was consistent with the double mutant having a CRIM SA less than that of the common neuronopathic L444P allele. Thus, the only acid β-glucosidase activity in the proband was due to the complex allele, producing a severe type 2 phenotype.

For comparison, expression studies were performed to characterize the other E326K complex allele and the genotype, D140H+E326K/K157Q, that caused type 1 disease (36). The surprisingly high residual CRIM SA of the D140H mutation (0.55) suggested that residue 140 was near the surface of the protein where the nonconservative substitution of the negatively charged aspartate by the larger, positively charged histidine would be less disruptive (62). The equivalent position in the murine sequence is an asparagine (61), which is isosteric to aspartate and is a very favorable substitution for aspartate at sites exposed on the exterior of a protein (63). This variation between the human and murine sequences at residue 140 demonstrates that there is no requirement for a positive charge in this position for catalytic competency and that maintenance of the overall structure of the region is important.

The D140H+E326K complex allele also reflected direct, but independent, interaction of its two mutations: the CRIM SA of D140H (0.55) multiplied by that for E326K (0.31) equals 0.17, compared with that of D140H+E326K (0.20). The dependence of the combined mutations was consistent with the kinetic and mutational data, suggesting surface locations for residues 140 and 326 and no gross alteration in active-site structure in the individual or combined mutations, as indicated by their normal interaction with active site–directed inhibitors. Based on the expressed CRIM SA, the K157Q protein was stable, but its enzymatic activity was greatly compromised. Apparently, the substitution of the positively charged lysine residue by the polar, uncharged glutamine was, as expected, highly deleterious (62, 63), suggesting the position was buried in the protein core where the charge change adversely affected protein folding. However, the normal stability and interaction of the K157Q mutant enzyme with active site–directed inhibitors (Fig. 3) argued against gross structural distortion of the expressed protein or direct contact with the bound substrate or inhibitors. Position 157 is located in a stretch of 17 amino acids (residues 146–162) that is invariant between the human and murine sequences (61), suggesting an important functional region that may be involved in the creation or maintenance of an active conformation as
opposed to a direct role in the catalytic mechanism or substrate binding (14, 19, 20, 48). Thus, the complex mutation D140H+E326K was a neuroprotective allele (19, 20). With a CRIM SA equivalent to that of N370S, the D140H+E326K mutant protein had sufficient residual activity to prevent the development of neurologic manifestations, even when associated with the severely compromised K157Q allele. These findings were consistent with the fact that the K157Q/L444P genotype causes type 2 GD (12), confirming that K157Q was not a protective allele. Although these in vitro expression studies characterized the relative turnover rates of the mature partially active mutant enzymes, their potentially altered stability and/or lysosomal transport in vivo may further contribute to their differential phenotypes.

Only recently has it become appreciated that the marked reduction of acid β-glucosidase leads to a severe neonatal form of type 2 GD in which infants present with ichthyosis or collodion skin at birth (5, 24–27, 30, 64). Studies have shown that cutaneous abnormalities result from a deficiency in acid β-glucosidase, which is normally required in the skin to convert glucosylceramide to ceramide, a sphingolipid necessary to maintain the epidermal permeability barrier in the stratum corneum in land-based mammals (64–67). It is notable that the acid β-glucosidase knockout mice and hairless mice whose skin was treated with the specific acid β-glucosidase inhibitor bromo-conduritol B epoxide (Br-CBE) failed to form mature extracellular lamellae and had markedly elevated transepidermal water loss (~40- and 10-fold increases for the knockout and Br-CBE–treated mice, respectively) (64, 66). Thus, the severe deficiency of acid β-glucosidase activity causes the cutaneous manifestations in severe neonatal type 2 GD (4, 5, 30, 64). Interestingly, some patients with this severe subtype did not have clinically apparent colloidion/ichthyotic changes, but had the ultrastructural epidermal abnormalities (5, 25).

In summary, the two non–pseudogene-derived complex mutations that caused GD were characterized by expression and characterization of their respective mutant enzymes. It is notable that both complex alleles had an E326K missense mutation that was shown to produce an altered, partially active enzyme when individually expressed. In combination with either the D140H or L444P allele, the resulting mutant proteins had different residual enzymatic activities, the D140H+E326K protein having sufficient activity to produce a type 1 phenotype, even when the other mutant allele was the severely compromised (essentially inactive) K157Q allele. In contrast, the complex allele E326K+L444P produced a protein that had little, if any, residual acid β-glucosidase activity and together with the nonsense allele, E233X, resulted in a severe type 2 phenotype with the collodion/ichthyosis skin manifestations.

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