Expression of mutated glucocerebrosidase alleles in human cells

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Gaucher disease is a heterogeneous disease characterized by impaired activity of the lysosomal enzyme glucocerebrosidase. This heterogeneity is attributed to a large number of mutations in the corresponding gene. In order to test the biochemical properties of some mutations prevalent among Israeli populations, the normal human glucocerebrosidase cDNA and cDNAs carrying mutations N370S, L444P, D409H, recTL, recNciI, P415R and 84GG were coupled to the T7 RNA polymerase promoter in a vaccinia virus-derived expression vector (pTM-1). Recombinant viruses were produced and used to infect human tissue culture cells. RNA and protein stability, recognition by anti-glucocerebrosidase monoclonal antibodies and intracellular enzymatic activity were measured. The results demonstrated that the D409H allele directed synthesis of cytoplasmic RNA with decreased stability compared with its normal counterpart or other mutated forms. The D409H and L444P mutated proteins had lower stability than that of their normal counterpart, while the recNciI-mutated protein was more stable. Only glucocerebrosidase forms harboring leucine at position 444 were recognized by the anti-glucocerebrosidase monoclonal antibodies used (8E4 and 2C7). Measurements of enzymatic activity of the recombinant proteins in cells loaded with a fluorescent glucosylceramide demonstrated that the N370S mutated enzyme had activity similar to that of the normal enzyme. The other mutated enzymes exhibited varying degrees of activities, generally corresponding to the phenotypes with which they are associated. The results presented demonstrate the use of the vaccinia virus-derived expression system and of loading living cells with fluorescent substrate as efficient tools for studying mutants in Gaucher disease and in other lysosomal diseases.

INTRODUCTION

Gaucher disease, the most prevalent sphingolipid disorder, is characterized by the accumulation of glucosylceramide mainly in cells of the reticuloendothelial system. This accumulation is a consequence of the reduced activity of the lysosomal enzyme glucocerebrosidase (1,2). On the basis of age of onset, clinical signs and involvement of neurological symptoms, the disease has been sub-divided into three clinical categories. Type 1 (adult type, chronic, non-neuronopathic) is the most common form which is characterized by the lack of central nervous system (CNS) involvement. It is very heterogeneous in its clinical features (2,3) and is known as the most prevalent genetic disease among Ashkenazi Jews. Type 2 (infantile, acute neuronopathic) is a rare and lethal form of the disease. It is characterized by the early appearance of visceral signs, CNS involvement and death a few months after birth. Type 3 (juvenile neuronopathic) is characterized by early onset of visceral impairment and a later appearance of CNS symptoms (2,3).

Over 60 mutations identified thus far in the glucocerebrosidase gene have been associated with Gaucher disease (for review, see refs 2–4). Some phenotype–genotype correlations have already been established (5–7). We chose to study the molecular lesions associated with mutations which were identified in Israeli populations. The mutations: N370S (8), 84GG (9), L444P (10), recTL and recNciI (11,12) occur among Jewish patients (6,13). Arab patients have been found to be homozygous for the D409H mutation (14). The P415R mutation was selected as a reference for a severe mutation (15). The N370S mutation had been associated exclusively with type 1 Gaucher disease. Most known patients homozygous for the N370S mutation are mildly affected. However, according to the prevalence of the N370S mutation in the Jewish population (1:15) (16), many more patients than those attending clinics most probably exist. This raises the possibility that in individuals homozygous for the N370S mutation this

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Figure 1. Structure of the pTM-1-derived plasmid containing the normal human glucocerebrosidase cDNA. Initially, an NcoI–SacI fragment of the human glucocerebrosidase cDNA was inserted into the pTM-1 plasmid. Then, a PCR fragment of the glucocerebrosidase cDNA was obtained by amplifying the human glucocerebrosidase cDNA with a sense primer containing the second ATG (underlined below) which was converted to an NcoI site (bold) (5′ AGCCATGGCTGGCAGCCTCA 3′) and an antisense primer downstream of the unique NcoI site in this cDNA. The amplified fragment was digested with NcoI and inserted into the first ligation plasmid. The resulting plasmid (pTM-1-glu3) contained glucocerebrosidase cDNA sequence starting at the second ATG, immediately adjacent to the T7 promoter (pT7) followed by the EMCV-5′-UTR and flanked on either side by part of the vaccinia virus thymidine kinase (tk) gene.

protein behaves very similarly to the normal enzyme in degrading most membrane glucosylceramides. The L444P mutation is more severe than the N370S mutation. Patients homozygous for the L444P usually present with type 3 disease (17). Sometimes they present with severe type 1 Gaucher disease at early stages. The 84GG mutation is a frameshift mutation causing premature termination and is severe. The D409H mutation is peculiar since patients homozygous for it present with oculomotor apraxia and severe heart disease but not visceral disease (14). RecTL and recNciI are complex alleles that most probably originated from recombination between the active glucocerebrosidase gene and a glucocerebrosidase pseudogene (11). They have not been identified in homozygous form. The P415R mutation is a severe mutation associated with type 2 Gaucher disease (15).

Several attempts have been made to explain the correlation between the phenotype exhibited by the different mutations and the biochemical changes they cause. Using the baculovirus-derived expression system and in vitro assays, Grabowski et al. tested the stability and activity of 13 different mutated glucocerebrosidases. The results demonstrated a marked decrease in activity of all the mutated enzymes, ranging from a 4-fold reduction in the activity of the N370S enzyme to 22-fold and 480-fold reductions in the activity of the L444P or the D409H mutated enzymes, respectively (18). However, the low residual activity of the N370S mutation does not explain its association with a very mild phenotype, and the very low activity of the L444P and D409H mutated enzymes does not reconcile with survival of patients homozygous for these mutations. Therefore, we chose the T7/encephalomyocarditis (EMC)/vaccinia virus hybrid expression system to express the normal and several mutated human glucocerebrosidase in human cells (19). The glucocerebrosidase cDNAs were introduced into the pTM-1 vector (20–22) and recombinant viruses were produced. The viruses were then used to infect human cells. There was a 10- to 20-fold overexpression of glucocerebrosidase activity in cells infected with a recombinant virus carrying the normal cDNA, compared with uninfected cells, as measured in vitro using 4-methylumbelliferyl glucopyranoside (4-MUG) as a substrate. RNA and protein stability, recognition by anti-glucocerebrosidase monoclonal antibodies and intracellular enzymatic activity were measured.

RESULTS

Construction of recombinant vaccinia virus containing the human glucocerebrosidase gene

To establish an efficient expression system for normal and mutated human glucocerebrosidase that would permit detection of RNA stability, protein stability and enzymatic activity, the normal cDNA was introduced into the T7/EMC/vaccinia virus hybrid expression system in such a way that the NcoI site of the vector polylinker (CCATGG) corresponded to the second glucocerebrosidase ATG (AAACATCATGGCTGGCAG) (23).
All the mutations were introduced into this vector (Fig. 1) followed by generation of recombinant viruses (19).

RNA expression

To test for the steady-state levels of glucocerebrosidase RNAs obtained in cells infected with the different recombinant viruses, HeLa cells were co-infected with vTF7-3 and each of the different viruses containing the normal or mutated glucocerebrosidase cDNAs. Eighteen hours later, RNA was extracted and subjected to Northern analysis using glucocerebrosidase cDNA as a probe. As shown in Figure 2A, a major 1.8 kb RNA species was detected. This RNA was highly overexpressed, as exemplified by the difference between uninfected HeLa or vTF7-3-infected cells, in comparison with cells co-infected with vTF7-3 and each of the recombinant viruses. Under the exposure conditions used in this experiment, the endogenous glucocerebrosidase RNA levels in HeLa cells or in cells infected only with vTF7-3 were below the level of detection. In order to quantitate glucocerebrosidase RNA in the different clones, the filters were rehybridized with a human rRNA cDNA probe (see Fig. 2B). Phosphor-imager quantitation of Northern analysis is summarized in Table 1. The data indicated that the amounts of steady-state glucocerebrosidase RNA originating from the normal and mutated cDNAs, except D409H cDNA, were comparable. The D409H RNA had 59% of the stability of normal RNA. Student’s t-test demonstrated that this difference in RNA stability is significant (P = 0.0071). On the other hand, the 84GG RNA was as stable as its wild-type counterpart (93%; the result of a Student’s t-test was: P = 0.56). Since all RNAs share identical 5’ and 3’ T7-derived promoter and terminator sequences and they are all transcribed in the cytoplasm, the differences in stability reflect an inherent property of the RNA molecules.

Table 1. Phosphor-imager analysis of Northern hybridization

<table>
<thead>
<tr>
<th>cDNA</th>
<th>% of normal</th>
<th>n</th>
<th>Stability compared with normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>100</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>N370S</td>
<td>104 ± 5.4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>L444P</td>
<td>108 ± 5.9</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>recNcil</td>
<td>95.2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>D409H</td>
<td>59.1 ± 9.3</td>
<td>6</td>
<td>↓</td>
</tr>
<tr>
<td>recTL</td>
<td>110.3 ± 11.8</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>P415R</td>
<td>105.5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>84GG</td>
<td>93.4 ± 10.4</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

HeLa cells were co-infected with the recombinant viruses as described in Materials and Methods. Northern analysis was performed as described in the legend to Figure 2. Radioactive bands were quantified using phosphor-imager (Fuji xtraspeed 1000 model) and the results were normalized to represent the amount of glucocerebrosidase mRNA relative to the 28S rRNA present in each sample, compared with normal recombinant glucocerebrosidase (100%). n = number of independent experiments performed.

Protein expression

To test whether all glucocerebrosidase forms are expressed as proteins and are recognized by the same monoclonal antibodies, cell lysates were immunoprecipitated either with polyclonal or monoclonal anti-human glucocerebrosidase antibodies. The immunoprecipitates were resolved by SDS–PAGE, and the gels were then fixed, dried and exposed to an X-ray film.

Figure 3. Recognition of the different mutated proteins by anti-glucocerebrosidase antibodies. D409H homozygous cells (5x10⁶) were co-infected (m.o.i. = 10 for each virus) with vTF7-3 and each one of the normal or different mutated recombinant viruses. Twenty hours later, cells were metabolically labeled for 2 h with [35S]methionine. Cell lysates containing 0.5x10⁶ or 1x10⁶ c.p.m. were immunoprecipitated using polyclonal anti-human glucocerebrosidase antibodies, prepared against a recombinant protein expressed in bacteria (A) or 8E4 anti-human glucocerebrosidase monoclonal antibodies (B), respectively. Immunoprecipitates were subjected to 10% SDS–PAGE, and the gels were then fixed, dried and exposed to an X-ray film.

Protein processing

To measure recombinant glucocerebrosidase stability, pulse–chase experiments were performed. Subconfluent monolayers of Gaucher fibroblasts were co-infected with vTF7-3 and each one of the recombinant viruses at an m.o.i of 10 p.f.u./cell. Twenty hours later, the infected cells were labeled with [35S]methionine for 1 h, followed by a chase of 1, 3, 9 or 18 h. Cell lysate samples containing the same c.p.m. were subjected to SDS–PAGE. The overexpression of the recombinant proteins obviated the need for immunoprecipitation. As shown in Figure 4, the primary normal recombinant glucocerebrosidase was a 55 kDa protein detected during the labeling period (denoted ‘a’ in Fig. 4), concomitant with accumulation of a predominant 65 kDa product. A slight reduction in the molecular weight of the protein to 62 kDa (the 62–65 kDa forms are denoted ‘b’ in Fig. 4) was observed during the chase periods. As has already been shown (19), the 55 kDa...
Figure 4. Metabolic labeling of cells. Subconfluent monolayers of Gaucher skin fibroblasts (D140H,E326K/K157Q) were co-infected with vTF7-3 and each of the different mutated recombinant viruses (m.o.i. = 10 for each virus). Twenty hours later, cells were metabolically labeled for 1 h with [35S]methionine followed by 1, 3, 9 or 18 h of chase. Cell lysates were prepared and samples containing 10^5 c.p.m. were subjected to 10% SDS–PAGE, and the gel was then fixed, enhanced, dried and exposed to an X-ray film.

form is the primary peptide that is synthesized on the polyribosomes. The 62–65 kDa proteins are glucocerebrosidase forms accumulating in the endoplasmic reticulum. The same expression pattern was observed for all the recombinant glucocerebrosidase-derived proteins with the exception of 84GG, which showed no detectable product (data not shown). The results of Phosphoimager analysis of 3–5 pulse–chase experiments are summarized in Table 2. The results were analyzed statistically using ANOVA with repeated measures. They indicated that the stability of the D409H and L444P proteins was significantly lower than that of their normal counterpart. The stability of the recTL protein was also lower than that of the normal protein (P = 0.049). P415R and N370S proteins were comparable with the normal recombinant protein while the recNciI protein seemed to be more stable than the normal recombinant protein (P = 0.047).

Enzymatic activity
In order to assess the activity of the different mutated enzymes, measurements were made in vitro and in situ. For in vitro assays, subconfluent monolayers of various cells in tissue culture were co-infected with vTF7-3 and each of the recombinant viruses
Figure 5. Cellular localization of glucocerebrosidase. Foreskin fibroblasts (A) and Gaucher fibroblasts (B) (D409H/D409H) were grown on coverslips. The foreskin fibroblasts were either fixed and stained with anti-human glucocerebrosidase monoclonal antibodies and fluorescein-conjugated goat anti-mouse antibodies (left) or loaded with LR12-GC for 24 h and fixed 24 h after chase (right) as detailed in Materials and Methods. The Gaucher fibroblasts were double stained.

harboring either the normal or the mutated glucocerebrosidase cDNAs. Twenty hours later, cell lysates were prepared and samples containing the same amount of protein were assayed for glucocerebrosidase activity using 4-MUG as a substrate (Table 3). Analysis of variance demonstrated that the activity varied significantly between the different cell types. The results demonstrated that all the mutated recombinant enzymes had low activity toward the artificial substrate, ranging between 0 and 18% of the activity directed by the normal recombinant enzyme. However, there was no correlation between the activity tested in vitro and the severity of the disease with which the mutated enzymes are associated. We decided to use loading of cells with the fluorescent substrate lissamine rhodamine-12-glucosylceramide (LR12-GC) in order to be able to better correlate the severity of a mutation with its intracellular activity.

It was suggested, though never demonstrated directly, that LR12-GC is endocytosed by the cells, reaches the lysosomes and is hydrolyzed there by glucocerebrosidase and saposin C to glucose and LR12-ceramide. The LR12-ceramide leaves the lysosomes and reaches the Golgi network where some of it interacts with phosphorycholine to produce fluorescent sphingomyelin, which is secreted through the plasma membrane into the media (24). To show that the substrate really reaches the lysosomes and is hydrolyzed there by glucocerebrosidase, foreskin fibroblasts and Gaucher skin fibroblasts were loaded for 24 h with the fluorescent substrate LR12-GC, followed by a chase of 24 h. Lysosomes were also stained using anti-human glucocerebrosidase monoclonal antibodies. As shown in Figure 5A, the fluorescein-conjugated antibodies stained the lysosomal glucocerebrosidase in normal foreskin fibroblasts, while the LR12-ceramide accumulated in the Golgi apparatus. Figure 5B indicates that in the Gaucher fibroblasts tested, LR12-GC stained the lysosomes, since these cells contain glucocerebrosidase which was unable to degrade much of the substrate, and therefore most of it remained in the lysosomes.

To measure recombinant protein activity within the infected cells, loading experiments were performed using LR12-GC (24). Following loading of cells with the fluorescent substrate and inhibition of endogenous glucocerebrosidase activity with bromoconduritol-β-epoxide (Br-CBE) for 48 h, cells were co-infected with vTF7-3 and the normal or mutated recombinant viruses. Twenty hours later, lipids were extracted from the infected cells and separated by TLC. As shown in Figure 6, the recombinant protein carrying the N370S mutation was as efficient as the normal recombinant protein in hydrolyzing the fluorescent glucosylceramide, while the recombinant enzymes carrying the L444P or the D409H mutations were less efficient. Very low activity was found for the recombinant glucocerebrosidase forms recTL, recNciI and the P415R alleles, all of which had activities just above the background level. The 84GG form did not exhibit any activity. Results of Student’s t-test demonstrated that there was no significant difference between the activity of the normal and the N370S enzymes, while the differences between the other mutated enzymes and the normal recombinant enzyme were significant (see Fig. 6).
**DISCUSSION**

In this study, our aim was to evaluate different mutated alleles of the glucocerebrosidase gene for their defective biochemical properties. The hybrid T7/EMC/vaccinia virus expression system was used for this purpose due to several advantages: (i) efficient transcription and translation of the target gene; and (ii) the shut-off of host protein synthesis obviates the need for antibodies to follow expression of the target gene. Since transcription is initiated at the bacteriophage T7 promoter, high RNA levels are obtained from the T7-driven cDNA. It is convenient, therefore, to study the stability of different RNA molecules by following their steady-state levels in infected cells. By doing so, we were able to demonstrate that six recombinant viruses containing the glucocerebrosidase mutations N370S, L444P, recTL, recNciI, P415R and 84GG produced RNA with stability comparable with that of the normal RNA (Table 1). However, the D409H RNA was 2-fold less stable than the normal RNA. Since the same promoter was used in all cases, the changes in the amount of steady-state RNA levels reflect changes in stability of the different RNA molecules.

Translation was efficient due to the encephalomyocarditis virus (EMCV) 5′-untranslated region (UTR). All cDNAs directed the synthesis of a 55 kDa glucocerebrosidase form concomitant with accumulation of 62–65 kDa forms, residing within the endoplasmic reticulum (19,23). These results are in agreement with previously published results (25,26). Only a small fraction, measured by activity but not demonstrated by polyacrylamide gel electrophoresis analysis, reached the lysosomes. The stability of the N370S and the P415R mutated glucocerebrosidase forms was comparable with that of their normal counterpart, as demonstrated by pulse–chase experiments (Fig. 4). The stability of the D409H and L444P proteins was significantly lower, and that of recTL somewhat lower than that of the normal protein, while the recNciI protein was more stable than the normal recombinant protein.

**Table 2. Phosphor-imager results of pulse–chase experiments**

<table>
<thead>
<tr>
<th>Recombinant protein</th>
<th>1 h chase</th>
<th>3 h chase</th>
<th>9 h chase</th>
<th>18 h chase</th>
<th>Significance (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (n = 4)</td>
<td>84 ± 19.6</td>
<td>125 ± 35.0</td>
<td>82 ± 19.4</td>
<td>52± 15.2</td>
<td>–</td>
</tr>
<tr>
<td>N370S (n = 5)</td>
<td>95 ± 14.6</td>
<td>120 ± 19.8</td>
<td>97 ± 16.1</td>
<td>59 ± 7.4</td>
<td>0.691</td>
</tr>
<tr>
<td>L444P (n = 4)</td>
<td>111 ± 21.0</td>
<td>70 ± 4.7</td>
<td>53 ± 6.6</td>
<td>46 ± 15.9</td>
<td>0.016</td>
</tr>
<tr>
<td>D409H (n = 5)</td>
<td>104 ± 4.3</td>
<td>69 ± 5.8</td>
<td>57 ± 9.0</td>
<td>41 ± 5.8</td>
<td>0.001</td>
</tr>
<tr>
<td>P415R (n = 4)</td>
<td>89 ± 6.9</td>
<td>89 ± 6.9</td>
<td>75 ± 11.7</td>
<td>69 ± 9.7</td>
<td>0.394</td>
</tr>
<tr>
<td>recNciI (n = 4)</td>
<td>73 ± 9.8</td>
<td>82 ± 15.3</td>
<td>80 ± 9.7</td>
<td>63 ± 17.5</td>
<td>0.047</td>
</tr>
<tr>
<td>recTL (n = 3)</td>
<td>108</td>
<td>103 ± 14.5</td>
<td>81 ± 5.2</td>
<td>67</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Phosphor-imager analysis was performed on the results of experiments (no. of experiments = n) similar to that presented in Figure 4. The area of the region denoted b in Figure 4 was measured for each time point and calculated in relation to the number obtained for ‘1 h pulse’ which was taken as 100.

![Figure 6](image-url) In intracellular activity of the normal and different mutated recombinant glucocerebrosidase forms in infected cells. Gaucher skin fibroblasts (D409HD409H) were loaded with 10 nmol of LR12-GC for 48 h in the presence of 20 µM Br-CBE, following by co-infection with vTF7-3 and each of the different recombinant viruses at an m.o.i. of 10 p.f.u./cell for each virus. Three hours later, cells were washed thoroughly and the media were replaced. Eighteen hours later, cells were collected and lipids were extracted and separated by TLC. The spots were scraped, extracted and counted in a fluorimeter. Data is presented as percentage ceramide/glucosylceramide/protein/normal recombinant glucocerebrosidase. The data presented is the mean ± SEM of four different experiments, each performed in duplicate. Also shown are the results of a Student’s t-test comparing the activity of the normal recombinant enzyme and the mutated forms (significant p value is ≤0.05). p-p value; Phen/Gen. phenotype–genotype correlation.

**Table 3. In vitro glucocerebrosidase activity measured using 4-MUG as substrate**

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Cell type</th>
<th>HeLa (n = 2)</th>
<th>CV-I (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (vglu3)</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>N370S</td>
<td>12.33</td>
<td>11.0 ± 5.27</td>
<td></td>
</tr>
<tr>
<td>L444P</td>
<td>18.10</td>
<td>7.85 ± 4.70</td>
<td></td>
</tr>
<tr>
<td>recNciI</td>
<td>6.37</td>
<td>2.13 ± 3.20</td>
<td></td>
</tr>
<tr>
<td>recTL</td>
<td>5.90</td>
<td>2.53 ± 2.14</td>
<td></td>
</tr>
<tr>
<td>D409H</td>
<td>12.40</td>
<td>4.47 ± 1.88</td>
<td></td>
</tr>
<tr>
<td>P415R</td>
<td>8.33</td>
<td>3.90 ± 3.09</td>
<td></td>
</tr>
<tr>
<td>84GG</td>
<td>0.70</td>
<td>3.78 ± 3.81</td>
<td></td>
</tr>
</tbody>
</table>

Cells (HeLa or CV-I) were co-infected with vTF7-3 and the normal (vglu3) or each of the recombinant viruses, at 10 p.f.u./cell for each virus, as described in Materials and Methods. Twenty hours later, cell lysates were prepared and activity was measured in samples containing 15 µg of protein using 4-MUG as substrate. n = number of experiments performed in each cell type. Each experiment was repeated 1–3 times. Results are presented as mean ± SEM percentage of the activity obtained for the normal protein (vglu3; 100%).
The anti-human glucocerebrosidase monoclonal antibodies used to immunoprecipitate the recombinant proteins did not recognize those that have proline at position 444 instead of leucine (Fig. 3B). This result explains why the L444P mutated protein was proposed to be an unstable protein (18), though the possibility that the monoclonal antibodies do not recognize the L444P mutated protein has already been suggested (27). It is worth mentioning that the polyclonal antibodies did not recognize the different mutated glucocerebrosidase forms equally well.

Results of loading experiments indicated that the recombinant protein carrying the N370S mutation had activity comparable with that of its normal counterpart. This result may explain the discrepancy between the data available on the prevalence of the N370S mutation among Ashkenazi Jews (16) and the actual number of registered Ashkenazi patients homozygous for the N370S mutation in Israel. According to the prevalence of this mutation among Ashkenazi Jews (1:15), there should be many more registered patients. The limited number of such patients may be due to the high activity of the N370S mutated enzyme resulting, in most cases, in a normal or very close to normal phenotype.

The L444P mutated enzyme had only 51% of the activity of the normal recombinant enzyme toward the substrate used in this study. This activity was higher than that demonstrated by the D409H enzyme (28%). The reduced activity of the L444P enzyme (51%) can be explained by its reduced stability. The reduced activity of the D409H protein (28% of normal) can be attributed to the reduction in RNA and protein stability. Both mutations are associated with more severe neuropathic forms. However, there are patients homozygous for these two mutations and they may reach adulthood. The other tested mutations that presented very low intracellular activity toward LR12-GC (84GG, recNciI, recTL and P415R) are expected to be associated with a more severe form of the disease. Actually, there are no known patients homozygous for any one of these mutations, and compound heterozygotes with genotypes such as L444P/P415R or L444P/recNciI present with type 2 Gaucher disease (28). The only mutation whose intracellular activity is not in agreement with previous results (29), is the recTL. It showed a much lower intracellular activity than we had expected from our patient data.

It seems that the intercellular activity of a recombinant mutated glucocerebrosidase is a good parameter for determining the severity of a given mutation. Any intracellular activity which is <50% of that of the normal enzyme will be associated with a more severe form of Gaucher disease. However, patients homozygous (or compound heterozygotes) for mutations with intracellular activity which is 25–50% of normal will survive and may even reach adulthood. These include the genotypes N370S/severe mutation; D409H/D409H; D409H/L444P and L444P/L444P.

The results presented indicate that the combination of the T7/EMC/vaccinia virus hybrid expression system with measurement of intracellular activity of introduced substrates is efficient and therefore is a very useful method for analyzing Gaucher disease.

MATERIALS AND METHODS

Cells and viruses

HeLa, CV-1 and BSC-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). Human foreskin fibroblasts (FS11) and primary Gaucher fibroblasts (D140H,E326K/K157Q; D409H homozygous) were grown in DMEM containing 20% (v/v) FCS. Human thymidine kinase-deficient 143B cells were grown in DMEM supplemented with 10% (v/v) FCS and 25 µg/ml (w/v) 5-bromodeoxyuridine. Wild-type vaccinia virus (v-WR), vTF7-3 (expressing the T7 RNA polymerase) and recombinant viruses were propagated in HeLa cells. Titer were determined using BSC-1 cells.

Plasmid construction

Construction of the T7/EMC/vaccinia virus-derived plasmid containing the human glucocerebrosidase cDNA was described elsewhere (19). The construct is depicted in Figure 1. The glucocerebrosidase cDNA was coupled to the T7 polymerase promoter, followed by the ECMV-5’-UTR. The pTM-1 vector used for introduction of the target gene contains vaccinia-derived thymidine kinase sequences flanking the transcription unit, which are used for homologous recombination with a wild-type vaccinia virus (v-WR) to produce a recombinant virus. In the constructed plasmid, pTM-I-glu3, the NcoI site (CCATGG) contains the second ATG (in-frame) of the human glucocerebrosidase cDNA (19,23).

Some mutations were introduced into the normal glucocerebrosidase cDNA using the in vitro mutagenesis technique (N370S, L444P, D409H, 84GG and recTL) according to a published procedure (30). Other mutations (recNciI, P415R) were introduced by subcloning cDNA fragments containing the mutations into pTM1-glu3 (19).

Generation of recombinant vaccinia viruses

Generation of recombinant vaccinia viruses was essentially as described elsewhere (31). The virus expressing the normal glucocerebrosidase was designated vglu3, whereas viruses expressing the mutated glucocerebrosidase were designated vglux (where x denotes the mutation).

RNA preparation and analysis

Total cellular RNA was isolated using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH). Subconfluent HeLa cells (100 mm Petri dishes, 5×10^6 cells/plate) were co-infected with vTF7-3 and one of the viruses harboring either the normal or the mutated human glucocerebrosidase cDNAs at an m.o.i. of 10 p.f.u./cell. Twenty hours later, RNA was extracted and precipitated according to the manufacturer’s recommendations. The RNA pellet was air dried and resuspended in 0.1 ml of distilled water (55°C, 10 min). Electrophoresis, blotting and hybridization were as described elsewhere (33).

Metabolic labeling

Cells were infected with the recombinant viruses as described above. Twenty four hours later, medium was replaced with methionine-depleted DMEM for 30 min, followed by addition of 0.5 ml of methionine-depleted medium containing 190 mM NaCl (2.1) and 20 µCi of [35S]methionine (Amersham, 3000 Ci/mmol) for 5×10^6 cells. After 2 h pulse, the medium was removed and fresh DMEM containing 10% FCS was added for different chase periods. Cell lysis was performed by addition of 0.5 ml of phosphate-buffered saline (PBS) containing 1% Nonidet P-40, 2 mM EGTA, 5 mM MgCl_2 and 1 mM phenylmethylsulfonyl
fluoride (PMSF). The c.p.m. were determined by trichloroacetic acid precipitation. Samples containing the same c.p.m. were electrophoresed through a 10% SDS–polyacrylamide gel (34). Gels were fixed, and dried following treatment with Amplify solution (Amersham) for 30 min.

**Immunoprecipitation**

D409H homozygous cells (5×10⁶) were infected and treated as described above, and pulse was performed for 2 h. Samples containing the same c.p.m. were immunoprecipitated with anti-glucocerebrosidase antibodies using a described method (35). The precipitates were electrophoresed through a 10% polyacrylamide–SDS gel, which was fixed, dried and exposed to an X-ray film (Fuji, Rx).

**Enzymatic activity**

Subconfluent monolayers (5×10⁶ cells) were infected with 10 p.f.u./cell of YFP7-3 and any one of the recombinant viruses. Twenty hours later, cells were washed twice with PBS, collected with a rubber policeman in 1 ml of sterile water and frozen at –80°C. Samples containing the same amount of protein, determined by the Bradford technique (36), were assayed for β-glucocerebrosidase activity using 4-MUG (Genzyme Corp., Boston, MA) as described (19).

**Loading experiments**

Loading experiments were performed as described elsewhere (24,19).

**Fluorescent staining of cells**

**Fluorescent substrate.** A total of 1×10⁴ cells were grown on coverslips, loaded with 10 nmol of LR12-GC for 24 h and chase was performed for 24 h. At the end of the chase, the coverslips were fixed in formaldehyde (3.7%) for 30 min at room temperature. Following washes in PBS, the slides were glued with Galvanol mounting solution.

**Immunostaining.** A total of 1×10⁴ cells were grown on coverslips and fixed in formaldehyde (3.7%) for 30 min at room temperature, followed by incubation in 3.7% formaldehyde: acetone (1:1) for 5 min at –20°C. The cells were washed in PBS containing 2% bovine serum albumin (BSA) and incubated with normal goat IgG at room temperature for 30 min. Ten μl of anti-human glucocerebrosidase monoclonal antibodies (SE4, 100 μg/ml) were applied for 60 min at room temperature. After extensive washing with PBS containing 2% BSA, 10 μl of fluorescein-conjugated goat anti-mouse antibodies (10μg/ml, Jackson Labs.) were added for 60 min at room temperature, followed by three washes of 10 min each with PBS. Slides were glued with Galvanol mounting solution and visualized (fluorescence microscope, Zeiss). For double staining, loading was performed as described above. Following fixation with formaldehyde and permeabilization of cells in 3.7% formaldehyde:acetone (1:1) for 5 min (–20°C), immunostaining was executed.

**Random-primed labeling**

A kit was purchased from Fermentase and labeling was performed according to manufacturer’s recommendations.

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