Overexpression of human glucocerebrosidase containing different-sized leaders

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Gaucher disease results from impaired activity of the lysosomal enzyme glucocerebrosidase. Aiming at overexpressing the human glucocerebrosidase and testing the efficiency of the two in-frame ATGs of its gene in directing synthesis of an active enzyme, it was coupled to the T7 RNA polymerase promoter in a vaccinia virus-derived expression vector (pTM-1). cDNAs containing either one or both ATGs of the glucocerebrosidase mRNA were linked to the T7 polymerase promoter. Recombinant viruses were produced and used for infecting human cells in tissue culture. The results demonstrated that both ATGs directed translation of active glucocerebrosidase, resulting in a 10-fold increase in enzymic activity. Most of the protein remained sensitive to endoglycosidase H. The active enzyme represented a small fraction of the expressed glucocerebrosidase. The recombinant enzyme had the same K_m and optimal pH towards the

artificial substrate 4-methylumbelliferyl glucopyranoside as the authentic endogenous human enzyme. Measurements of intracellular enzymic activity directed by the cDNAs with either one or both ATGs in cells loaded with a fluorescent glucosylceramide demonstrated a 30% increase in activity directed by the cDNAs containing the first ATG over that containing the second ATG. This indicates that the protein synthesized from the first ATG, with a 38 amino acid leader, is translocated through the endoplasmic reticulum more readily than its counterpart directed by the second ATG, with a 19 amino acid leader. The elevation in glucocerebrosidase activity and the reproducibility of the data leads us to propose the use of the vaccinia virus-derived expression system as a tool for studying glucocerebrosidase mutants in Gaucher disease.

INTRODUCTION

Gaucher disease, an autosomal recessive disease, is defined by the accumulation of glucosylceramide mainly in cells of the reticulo-endothelial system. This accumulation is a consequence of the decreased 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 subdivided into three clinical categories. Type 1 (adult type, chronic, non-neuronopathic) is the most common form, characterized by the lack of central nervous system involvement. It is very heterogeneous in its clinical features [2,3] and is prevalent among Ashkenazi Jews [4,5]. Type 2 (infantile, acute neuronopathic) is a rare and lethal form of the disease. It is characterized by early appearance of visceral signs and central nervous system involvement. Death occurs a few months after birth. Type 3 (juvenile neuronopathic) is characterized by an early onset of visceral impairment and a later appearance of central nervous system disease [2].

As a lysosomal enzyme, glucocerebrosidase is translated on polyribosomes, starting at one of two in-frame ATGs [6]. Translation starting at the first ATG results in a glucocerebrosidase peptide harbouring a leader of 38 amino acids. This leader contains a highly hydrophilic sequence of 19 amino acids, followed by a hydrophobic sequence of 19 amino acids. Protein synthesis starting at the second ATG results in the production of a glucocerebrosidase peptide with a leader of 19

hydrophobic amino acids [6]. The second ATG is preceded by a Kozak consensus sequence for translation initiation [7,8]. After translocation through the endoplasmic reticulum membrane, accompanied by leader sequence cleavage, the protein is glycosylated on four asparagine residues. The highly mannosylated sugar moieties are modified while moving into the Golgi network, from which they reach the lysosomes [9], most probably through the plasma membrane [10,11].

In experiments aiming at expressing the normal human glucocerebrosidase, various vectors for expression in eukaryotic cells have been used. When a retroviral vector containing the human glucocerebrosidase cDNA was used for stable transfection of NIH 3T3 cells, elevation of enzymic activity was detected concomitantly with appearance of immunoreactive human glucocerebrosidase [12–14]. Infection of COS cells with a chimaeric virus containing human glucocerebrosidase coupled to the SV40 early promoter, or transfection of COS cells with an SV40-derived expression vector containing the glucocerebrosidase cDNA, resulted in mild (3-fold) elevation of enzymic activity and a marked increase in the value or quality of human glucocerebrosidase cross-reactive material (20–50-fold) [15]. When expressed in sf9 cells by using a baculovirus-derived expression system there was a several-thousandfold overexpression of active human glucocerebrosidase tested *in vitro* [16,17]. All mutated proteins demonstrated very low activity *in vitro* [18,19].

In this study we expressed the human glucocerebrosidase in human cells by using the T7 vaccinia hybrid expression system.

Abbreviations used: Br-CBE, bromoconduiritol β -epoxide; DMEM, Dulbecco's modified Eagle's medium; EMCV-5'-UTR, encephalomyocarditis virus 5' untranslated region; FCS, fetal calf serum; LR-12-GC, lissamine rhodamine 12-glucosylceramide; MOI, multiplicity of infection; 4-MUG, 4-methylumbelliferyl glucopyranoside; pfu, plaque-forming unit.

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Vaccinia virus, a member of the smallpox virus family, is a cytoplasmic DNA virus, encoding for its own transcription and RNA-modifying enzymes [20]. Vaccinia is widely used as a viral expression vector both because of its wide host range (mammalian and avian cells) and because it leads to high expression of the target gene. The recombinant vaccinia virus vTF7-3 [21] provides expression of the bacteriophage T7 RNA polymerase, a prokaryotic enzyme that does not contain a nuclear localization signal. The prokaryotic RNA polymerase can transcribe any T7 promoter-containing DNA that is introduced into the cytoplasm of the infected cell. The target gene is coupled to the T7 polymerase promoter, followed by the encephalomyocarditis virus 5' untranslated region (EMCV-5'-UTR). In the presence of T7 RNA polymerase, high transcription levels are obtained and the EMCV-5'-UTR ensures highly efficient cap-independent translation from the transcribed mRNA [22]. The vector used for introduction of the target gene, designated pTM-1, contains vaccinia-derived thymidine kinase sequences flanking the transcription unit, which are used for recombination with a wild-type vaccinia virus (v-WR) to produce a recombinant virus. The recombinant virus efficiently infects a wide range of hosts, and shuts off host protein synthesis [20–23].

The human glucocerebrosidase cDNA containing either one or both in-frame ATGs was introduced into the pTM-1 vector [20] and recombinant viruses were produced. The viruses were then used to infect human cells. The results demonstrated a 10-fold overexpression of human glucocerebrosidase activity in cells infected with a glucocerebrosidase-expressing virus in comparison with non-infected cells, with an estimated 100-fold overexpression of human glucocerebrosidase cross-reactive material. Most of the synthesized glucocerebrosidase was endoglycosidase H sensitive, demonstrating that most of the glucocerebrosidase synthesized remained highly mannoseylated and could not reach the lysosomes.

Expression of human glucocerebrosidase from the first ATG resulted in consistently higher enzymic activity than expression from the second ATG. The K_m and pH requirements of the recombinant enzymes were similar to those of the native enzyme, implying the use of the vaccinia virus-derived expression system for identification and evaluation of mutations in Gaucher disease.

EXPERIMENTAL

Cells and viruses

HeLa cells, CV-1 cells and BSC-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS). Human normal foreskin fibroblasts and primary Gaucher fibroblasts (D140H, E326Q/K157Q; D409H homozygous) were grown in DMEM containing 20% FCS. Thymidine kinase-deficient 143B cells (TK⁻ cells) were grown in DMEM supplemented with 10% FCS and 25 µg/ml 5-bromodeoxyuridine. All cells were grown at 37 °C in the presence of CO₂/air (1:19). Wild-type vaccinia virus (v-WR), vTF7-3 (recombinant vaccinia virus expressing the T7 RNA polymerase) and recombinant viruses (harbouring the normal or mutated glucocerebrosidase cDNAs) were propagated in HeLa cells. Titres were determined with BSC-1 cells. Stocks were stored at –80 °C.

Plasmid construction and generation of recombinant viruses

The construction of recombinant vaccinia viruses containing several human glucocerebrosidase cDNAs is depicted in Figure 1. The vaccinia virus-derived pTM-1 vector harbours the bacteriophage T7 RNA polymerase promoter (T_{7p}) and terminator

(T_{7t}) regions flanking unique restriction sites for several enzymes, including *Nco*I and *Sac*I, for the insertion of foreign genes. They are all contained within a copy of the vaccinia virus thymidine kinase (*tk*) gene. The T7 polymerase promoter is linked to the EMCV-5'-UTR. At a first stage, a 1420 bp *Nco*I–*Sac*I fragment of the human glucocerebrosidase cDNA was cloned in the pTM-1 vector, digested with the restriction enzymes *Nco*I and *Sac*I, to give pTM-1-glu' (Figure 1A). Then the 5' sequences of the glucocerebrosidase cDNA were amplified by the PCR technique with three different pairs of primers (see Table 1). The first pair was designed to construct the authentic 5' sequence of the glucocerebrosidase cDNA containing the two ATGs. The second pair of primers was similar to the first except for a point mutation at the second ATG, changing it to ATC. The third pair of primers was suitable for creating a cDNA starting from the second ATG. All the 5' sense primers contained an *Nco*I site and the amplified fragments included a unique *Nco*I site existing within the human glucocerebrosidase cDNA (see Figure 1). The amplified fragments were digested with *Nco*I and cloned into the *Nco*I site of pTM-1-glu' (Figure 1B). The plasmids obtained were designated pTM-1-glu1, pTM-1-glu2 and pTM-1-glu3.

Generation of recombinant vaccinia virus

Generation of recombinant vaccinia viruses was essentially as described elsewhere [24]. They were designated vglu1, vglu2 and vglu3.

RNA preparation

Total cellular RNA was isolated by using TRI-REAGENT (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.). Subconfluent HeLa cells (100 mm diam. Petri dishes, 5 × 10⁶ cells per plate) were co-infected with vTF7-3 and one of the viruses harbouring a human glucocerebrosidase cDNA [the authentic cDNA starting at the first ATG, or the cDNA starting at the first ATG, with mutated second ATG (ATG > ATC), or the second ATG; vglu1, vglu2 or vglu3 respectively] at a multiplicity of infection (MOI) of 10 plaque-forming units (pfu) per cell. After 20 h the medium was aspirated and 1 ml of TRI-REAGENT was added to each of the plates. Cells were scraped with a rubber 'policeman' and collected into an Eppendorf tube. After 5 min of incubation at room temperature, RNA was extracted with 0.2 ml of chloroform for 15 min at room temperature. The aqueous phase was precipitated with 0.5 ml of isopropanol for 10 min at room temperature. After centrifugation (12000 g, 15 min, 4 °C), the RNA pellet was washed with 1 ml of 75% (v/v) ethanol and centrifuged. The RNA pellet was air-dried and resuspended in 0.1 ml of distilled water (55 °C, 10 min).

Northern blot analysis

Loading buffer [50% (v/v) formamide, 6% (v/v) formaldehyde, 1 × MEN (20 mM Mops, pH 6.8, 8 mM sodium acetate and 1 mM EDTA)] was added to an equal volume of RNA sample and incubated for 10 min at 65 °C. A 5 µl portion of dye marker [50% (w/v) sucrose, 0.5% Bromophenol Blue, 5 µg of ethidium bromide] was added and the samples were loaded on a 6% (v/v) formaldehyde, 1% (w/v) agarose gel. After electrophoresis (4 h, 50 V), the gel was soaked for 45 min in 10 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate) and blotted overnight in 20 × SSC. The nitrocellulose filter (Hybond N) was baked for 2 h at 80 °C [25]. Hybridization and washes were as described elsewhere [26].

Figure 1 Insertion of glucocerebrosidase cDNA in the pTM-1 plasmid

(A) The *NcoI*–*SacI* fragment of glucocerebrosidase cDNA was purified and inserted into pTM-1 plasmid to yield pTM-1-glu'. (B) PCR fragments of the glucocerebrosidase cDNA were digested with *NcoI* and inserted into pTM-1-glu' plasmid. The resulting plasmids (pTM-1-glu, 1–3) contained glucocerebrosidase cDNA sequences starting at either ATG, immediately adjacent to the T7 promoter (pT7) followed by EMCV-5'-UTR and flanked on either side by part of the vaccinia virus thymidine kinase (*tk*) gene.

Table 1 Primers used for construction of different pTM-1-derived plasmids harbouring human glucocerebrosidase cDNA

The sequence of the different primers used for construction of different pTM-1-derived plasmids harbouring the human glucocerebrosidase cDNAs is shown. Underlined are the ATGs and the mutated ATG. The bold sequences depict the *NcoI* sites. Primer I, sense primer; primer II, antisense primer. Abbreviations: 2nd ATG, glucocerebrosidase cDNA starting at the 2nd ATG (vglu3); 1st ATG, glucocerebrosidase cDNA with a functional 1st ATG and mutated 2nd ATG (vglu2); 1st + 2nd ATG, glucocerebrosidase cDNA with both ATGs (vglu1).

	Primer I	Primer II
1st + 2nd ATG	5'-GGCCATGGAGTTTCAAG-3'	5'-GGAAATCATCAGGGGTGTC-3'
1st ATG	5'- CCATGG AGTTTCAAGTCCTTCCA- GAGAGGAATGTCCCAAGCCTTTGA- GAGGGTAAGCATCTGGCTGGCAG-3'	5'-GGAAATCATCAGGGGTGTC-3'
2nd ATG	5'-AG CCATGG CTGGCAGCCTCA-3'	5'-GGAAATCATCAGGGGTGTC-3'

Metabolic labelling

Cells were infected with the recombinant viruses as described above. After 24 h, medium was removed and methionine-depleted DMEM was added for 30 min. Methionine-depleted medium (0.5 ml), containing 190 mM NaCl [22] and 20 μ Ci of [35 S]methionine (Amersham; 3000 Ci/mmol) was added to 5×10^5 cells. After a 1 h pulse, medium was removed and fresh DMEM containing 10% FCS was added. At the end of the labelling and at different times of chase, medium was removed and lysis was performed by addition of 0.5 ml of PBS containing 1% (v/v) Nonidet P40, 2 mM EGTA, 5 mM MgCl₂ and 1 mM PMSF. The radioactivity was determined by 10% (w/v) trichloroacetic acid precipitation. Equal radioactive counts from different samples were electrophoresed through SDS/poly-

acrylamide [10% (w/v) gel] [27]. Gels were fixed, stained with 50% (v/v) methanol, 10% (v/v) acetic acid, 0.1% Coomassie Brilliant Blue R-250, and dried after 30 min of treatment with Amplify solution (Amersham).

Immunoprecipitation

Samples containing equal radioactive counts were immuno-precipitated with monoclonal antibodies (2C7 or 8E4), with the described method [28]. The precipitates were subjected to electrophoresis through an SDS/10% polyacrylamide gel, which was fixed, dried and exposed to an X-ray film (Fuji, Rx). Phosphor-imaging analysis was performed with an Agfa phosphorimager (Fujix bas 1000).

Endoglycosidase H digestion

Endoglycosidase H digestion was performed on immuno-precipitated samples. The precipitates were boiled for 5 min in denaturing buffer [0.5% SDS, 1% (v/v) 2-mercaptoethanol; New England Biolabs]. After centrifugation, an equal volume of reaction buffer (0.05 M sodium citrate, pH 5.5) was added to the supernatants. Half of the samples were treated with 1000 units of Endoglycosidase H (New England Biolabs) for 20 h at 37 °C. Samples were subjected to electrophoresis through an SDS/10% polyacrylamide gel manipulated as described above.

Enzymic activity

Subconfluent monolayers (5×10^6 cells) were infected with 10 pfu per cell of vTF7-3 and either vglu1, vglu2 or vglu3. After 20 h, 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, as determined

by the Bradford technique [29], were assayed for β -glucocerebrosidase activity by using 4-methylumbelliferyl glucopyranoside (4-MUG) (Genzyme Corp., Boston, MA, U.S.A.) as substrate. The enzymic reaction was performed in 0.2 ml of 100 mM potassium phosphate buffer, pH 5.0, containing 0.15% (w/v) Triton X-100 (Sigma) and 0.125% (v/v) taurocholate (Calbiochem, grade A) in the presence of 3 mM 4-MUG, for 60 min at 37 °C. The reaction was stopped by the addition of 1 ml of 0.1 M glycine, 0.1 M NaOH, pH 10. The amount of 4-methylumbelliferone was recorded with a Perkin Elmer Luminescence Spectrometer, LS 50 (excitation wavelength 340 nm, emission wavelength 448 nm) [30,31].

Loading experiments

Loading experiments were performed essentially as described elsewhere [32]. Cells (5×10^5) were supplemented with 5–10 nmol/ml lissamine rhodamine 12-glucosylceramide (LR-12-GC) for 48 h together with 20 μ M bromoconduritol β -epoxide (Br-CBE) (kindly provided by Dr. Legler, University of Cologne, Cologne, Germany). Cells were infected in the same medium with vTF7-3 and each of the recombinant viruses as described above. After 3 h, medium was removed, cells were washed twice with PBS, and DMEM, without Phenol Red and supplemented with 20% FCS, was added to cells. After a further 18 h, medium was collected. Cells were washed with PBS and lysed in 0.5 ml of distilled water. After protein determination, cell lysate and medium were extracted with chloroform/methanol (1:1) and dried. Chloroform/methanol (1:1; 50 μ l) was added and the samples were separated by TLC (Silica gel 60A; Whatman) in chloroform/butanol/ethyl acetate/0.25% KCl/methanol (25:25:9:16, by vol.). The TLC plates were observed with a UV lamp. Glucosylceramide and ceramide were scraped off the plate, eluted with chloroform/methanol (1:1) and their fluorescence measured in a fluorimeter (Perkin Elmer Luminescence Spectrometer, LS 50; excitation wavelength 560 nm, emission wavelength 580 nm) [32].

Protein determination

Protein levels in samples were determined by the Bradford technique [29].

Restriction enzymes

Restriction enzymes were purchased from several companies and employed in accordance with manufacturers' recommendations.

Random-primed labelling

The kit was purchased from Fermentase and labelling was performed in accordance with the manufacturer's recommendations.

RESULTS

Construction of recombinant vaccinia virus containing the human glucocerebrosidase gene

To establish an efficient expression system for human glucocerebrosidase and to follow the efficiency of its two ATGs in-frame in directing the synthesis of active enzyme, its cDNA was introduced into the hybrid T7/EMC/vaccinia virus-derived expression system. Three different human glucocerebrosidase cDNAs were introduced into the pTM-1 plasmid: an authentic cDNA starting from the first ATG, a cDNA starting from the first ATG with mutated second ATG (ATG > ATC),

Figure 2 Northern blot analysis of human glucocerebrosidase RNA

(A) HeLa cells were co-infected with vTF7-3 and the different viruses harbouring the normal glucocerebrosidase cDNA. At 18 h after infection, RNA was extracted and electrophoresed through a formaldehyde/agarose gel, blotted and hybridized to human glucocerebrosidase cDNA. (B) To quantify glucocerebrosidase RNA amounts in the different samples, blots were rehybridized to human rRNA cDNA.

and a cDNA starting from the second initiator methionine. Recombinant viruses were produced and designated vglu1, vglu2 and vglu3 respectively. RNA stability, protein stability and efficiency of the different cDNAs in directing synthesis of active glucocerebrosidase were tested.

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 with each of the vglu1, vglu2 or vglu3 recombinant viruses. After 18 h, RNA was extracted and subjected to Northern blot analysis with glucocerebrosidase cDNA as a probe. As shown in Figure 2(A), a single 1.8 kb RNA species was detected. This RNA was highly over-expressed, as exemplified by the difference between uninfected HeLa or HeLa cells infected only with vTF7-3 (representing endogenous glucocerebrosidase RNA levels) and cells co-infected with vTF7-3 and each of the chimaeric viruses. Under the exposure conditions used in this experiment (3 h), the endogenous glucocerebrosidase RNA levels are below the detection level. To quantify glucocerebrosidase RNA in the different clones, the filter was rehybridized with a human rRNA cDNA probe (see Figure 2B). Phosphorimaging measurements demonstrated that the steady-state amount of glucocerebrosidase RNA originating from the three different clones was comparable as expected, because all three RNAs shared identical 5' and 3' T7-derived promoter and terminator sequences.

Protein expression

Protein processing

To measure recombinant glucocerebrosidase stability, a pulse-chase experiment was performed. Subconfluent monolayers of Gaucher fibroblasts (D140H, E326Q/K157Q) were co-infected with vTF7-3 and each of the vglu1, vglu2 or vglu3 recombinant viruses at an MOI of 10 pfu per cell. After 20 h, the infected cells

Figure 3 Metabolic labelling of cells

Subconfluent monolayers of Gaucher skin fibroblasts (JP) were co-infected (MOI 10) with vTF7-3 and the recombinant viruses containing the different cDNAs. After 20 h, cells were metabolically labelled for 1 h with [35 S]methionine. At the end of the labelling and after 5 or 18 h of chase, cell lysates were prepared. Samples containing the same c.p.m. were immunoprecipitated with the 2C7 anti-(human glucocerebrosidase) monoclonal antibodies. Total samples (A) or the immunoprecipitates (B) were subjected to electrophoresis through an SDS/10% polyacrylamide gel, which was then fixed, dried and exposed to an X-ray film.

were labelled with [35 S]methionine for 1 h, followed by a chase for 5 or 18 h. Cell lysates of samples containing equal radioactivity were separated by SDS/PAGE, before or after immunoprecipitation with 8E4 anti-glucocerebrosidase antibodies. As shown in Figure 3, the vglu3-derived glucocerebrosidase primary protein product is a 55 kDa peptide. During the 1 h labelling period a predominant 65 kDa product accumulated. A slight reduction in the molecular mass of the protein was observed during the chase periods. The same expression pattern was observed for the vglu1- and vglu2-derived recombinant glucocerebrosidase, with the exception that the primary product had a molecular mass of 57 kDa. The recombinant proteins exhibited comparable steady-state levels. The differences in the amounts of glucocerebrosidase immuno-cross-reactivity observed in Figure 3(B), in comparison with the amounts of total recombinant proteins (Figure 3A), might reflect the higher specificity of the monoclonal antibodies used towards the 65 kDa protein form. It is clear from Figure 3 that the use of the T7/vaccinia virus expression system obviates the need for antibodies.

Figure 4 Endoglycosidase H digestion of recombinant glucocerebrosidase

Subconfluent monolayers of HeLa cells were co-infected with vTF7-3 and the virus containing the human glucocerebrosidase cDNA with the second ATG (vglu3), and radioactively labelled as described above. At the end of labelling and after different times of chase, samples containing equal radioactivity were immunoprecipitated (with 8E4 monoclonal antibodies) followed by endoglycosidase H (ENDO H) treatment. Total, immunoprecipitated and endoglycosidase H-treated samples were subjected to electrophoresis through an SDS/10% polyacrylamide gel, which was fixed, dried and exposed to an X-ray film.

Table 2 Glucocerebrosidase activity measured *in vitro* with 4-MUG as substrate

Different cells were infected with vTF7-3 and the different recombinant viruses containing glucocerebrosidase with either one or both ATGs, as described in the Experimental section. After 20 h, cell lysates were prepared and activity was measured in samples containing the same amount of protein, with 4-MUG as substrate. The number of experiments performed on each cell type is shown as *n*. Each experiment was analysed in one to three replicates. Results are percentages of the activity obtained for the protein derived from the cDNA with the 2nd ATG (100%), presented as means $\pm$ S.E.M. See Table 1 for the meanings of 1st ATG, 2nd ATG and 1st + 2nd ATG.

Cell type ...	CV-1 (<i>n</i> = 3)	HeLa (<i>n</i> = 2)	D140H, E326/ K157Q (<i>n</i> = 1)
2nd ATG	100	100	100
1st ATG	123 $\pm$ 9.504	150	130.5
1st + 2nd ATG	136 $\pm$ 8.003	141	127

To test whether any of the recombinant forms seen on the gel is the mature active lysosomal enzyme, HeLa cells were infected and metabolically labelled for 1 h with [35 S]methionine as described above. Samples containing equal radioactivity were immunoprecipitated with 8E4 anti-glucocerebrosidase monoclonal antibodies. Half of the samples were treated with endoglycosidase H, which cleaves sugars with a high mannose content, N-linked to asparagine residues. As seen in Figure 4, most recombinant forms seen after SDS/PAGE were endoglycosidase

Figure 5 Kinetic analysis of recombinant human glucocerebrosidase and the native enzyme

HeLa cells were co-infected with vTF7-3 and the different recombinant viruses. After 24 h, cell lysates were prepared, as explained in the Experimental section, from infected cells as well as from non-infected cells. Samples containing 15 μ g of protein were tested for acid β -glucocerebrosidase activity with increasing concentrations of 4-MUG (0.05–1.5 mM). Velocity (V ; μ M 4-methyl umbelliferone/h per μ g of protein) was plotted against substrate concentration (S ; mM). Kinetic data are shown as Lineweaver–Burk plots.

Figure 6 pH requirements of the normal and recombinant glucocerebrosidase

Cells were infected and enzymic assays were performed as described in the legend to Figure 5, with final substrate concentrations of 1.5 mM, in different pH environments (3–8, 0.5 pH intervals). Units of activity are μ M 4-methylumbelliferone/h per μ g of protein.

H-sensitive, reflecting the fact that they are linked to high-mannose sugar moieties and therefore represent glucocerebrosidase forms that did not reach the lysosome but most probably reside within the endoplasmic reticulum.

Enzymic activity

Next we aimed at assessing whether synthesis of active enzyme is directed by the various cDNAs that differ in their 5' coding region. To that end, subconfluent monolayers of various cells in tissue culture were co-infected with vTF7-3 and each of the vglu1, vglu2 or vglu3 recombinant viruses. After 20 h, cell lysates were prepared and samples containing the same amount of protein (15 μ g) were assayed for glucocerebrosidase activity with

Figure 7 Activity of recombinant glucocerebrosidase in infected cells

Normal foreskin fibroblasts and Gaucher skin fibroblasts (D409H homozygous) were loaded with 10 nmol of LR-12-GC for 48 h in the presence of 20 μ M Br-CBE. Cells were then infected with vTF7-3 and the different recombinant viruses. After 3 h, cells were washed thoroughly and media replaced. After a further 18 h, media and cells were collected, lipids were extracted, and separated by TLC. The spots were scraped, extracted and counted in a fluorimeter. (A) TLC plate of media; (B) the plate of cells; (C) schematic representation of cellular ceramide (fluorimeter units/ μ g of protein) produced by control cells and the different recombinant viruses. Data are presented as means $\pm$ S.E.M. for experiments in duplicate. Each treatment was repeated two or three times.

4-MUG as a substrate. The results presented in Table 2 demonstrate that the levels of enzyme expressed from the cDNAs containing either the first ATG alone or the first and second ATGs were constantly higher than the levels of enzyme expressed from the cDNA harbouring only the second ATG.

To prove the authenticity of the enzyme produced in all cases, the K_m and pH optimum were measured. HeLa cells were co-infected with vTF7-3 and each of the vglu2 or vglu3 recombinant viruses. After 20 h, cell lysates were prepared and glucocerebrosidase activity was measured in samples containing the same amount of protein and increasing concentrations of substrate. As shown in Figure 5, there was a 10-fold elevation in glucocerebrosidase activity in cells co-infected with vTF7-3 and a recombinant virus harbouring both types of glucocerebrosidase cDNA in comparison with cells infected with vTF7-3 alone or uninfected cells. K_m and V_{max} were determined by Lineweaver–Burk plots. The K_m obtained for the vglu2- and vglu3-derived

enzymes was 0.625 mM, whereas the K_m for the native enzyme, as measured in uninfected HeLa cells was 0.4 mM. The V_{max} for all the recombinant enzymes was 5 μ M/h per μ g of protein whereas that for its native counterpart was 0.67 μ M/h per μ g of protein. These results reflect an increase of an order of magnitude in the amount of active enzyme in cells infected with the recombinant viruses harbouring the glucocerebrosidase cDNA. The pH requirements of the native and recombinant enzymes were also compared. As illustrated in Figure 6, maximal activity for the native as well as the recombinant enzymes was obtained at pH 4.3.

The experiments described above were performed in test tubes. To measure recombinant protein activity within the infected cells, loading experiments were performed. For this purpose, Gaucher skin fibroblasts (derived from a patient with the genotype D409H/D409H) were incubated for 48 h with the fluorescent substrate LR-12-GC and the β -glucocerebrosidase inhibitor Br-CBE [32]. In this system the substrate is endocytosed by the cells, reaches the lysosomes and is hydrolysed there by glucocerebrosidase to glucose and LR-ceramide. The LR-ceramide leaves the lysosomes through vesicles and reaches the Golgi network. Some of it interacts in the Golgi with phosphatidyl choline to produce fluorescent sphingomyelin, which is secreted into the medium (results not shown). After loading of the cells with the fluorescent substrate and inhibiting endogenous glucocerebrosidase activity with Br-CBE, cells were co-infected with vTF7-3 and vglu1, vglu2 or vglu3 recombinant viruses. After 20 h, lipids were extracted from the infected cells and the respective culture media and were separated by TLC, as described in the Experimental section. As shown in Figure 7, the recombinant protein directed by vglu1 was the most efficient in degrading the fluorescent glucosylceramide to glucose and ceramide. Less so was the recombinant glucocerebrosidase derived from vglu2, and the lowest levels of ceramide accumulated in cells that expressed the recombinant glucocerebrosidase deriving from vglu3. The LR-12-GC is hydrolysed only by the lysosomal β -glucocerebrosidase in the presence of saposin C, the β -glucocerebrosidase activator (reviewed in [33]) (M. Horowitz and S. Gatt, unpublished work).

DISCUSSION

In this study we aimed at an efficient expression of human glucocerebrosidase as a first step in evaluating different mutated alleles for their defective biochemical properties. The hybrid vaccinia/T7 virus expression system was used for this purpose because it offered several advantages: (1) a wide range of hosts can be very efficiently infected with the chimaeric viruses; (2) there is efficient transcription and translation of the target gene; and (3) there is a shut-off of host protein synthesis, obviating the need for antibodies in order to follow the expression of the target gene. Because transcription is initiated at the T7 RNA polymerase promoter by binding of the enzyme to its recognition site, high RNA levels are obtained from any introduced cDNA. It is therefore convenient to study the stability of different RNAs by following their steady-state levels in infected cells. In doing so, we could demonstrate that all three recombinant viruses prepared by us directed synthesis of RNA with comparable stability. Translation in the T7/EMCV/vaccinia system is directed by the internal ribosomal entry site conferred by the EMCV-5'-UTR. Therefore the ATG that is located at the most 3' end of the EMCV segment, and is part of the *NcoI* site, is employed as the initiator ATG for translation [22]. Therefore the vglu1-derived glucocerebrosidase was initiated at the first glucocerebrosidase ATG (Figure 1). All three cDNAs directed synthesis of

enzymically active proteins. The 55 kDa form (starting at the second ATG; vglu3) or the 57 kDa forms (starting at the first ATG; vglu1 and vglu2) were translated on the polyribosomes with identical efficiencies owing to the EMCV-5'-UTR and were translocated and glycosylated with high-mannose side chains, which indicates a residence in the endoplasmic reticulum (63–67 kDa forms). These results are in agreement with previous results [9]. There was a 10-fold increase in enzymic activity. We did not demonstrate directly which cellular fraction of the glucocerebrosidase is inactive or active. However, fluorescence microscopy demonstrated that the substrate (LR-12-GC) accumulates in the lysosomes. When recombinant enzyme for which the synthesis is directed by the chimaeric viral DNA is present in the lysosomes, it hydrolyses the substrate to fluorescent ceramide. The fluorescent ceramide leaves the lysosomes and accumulates in the Golgi apparatus (results not shown).

The stability of the highly mannosylated glucocerebrosidase forms was the same in all cases. Because vaccinia infection (at an MOI of 10) results in cell death after 48 h, there is no way of measuring stability after more than 20–22 h of chase. It seems that the monoclonal antibodies used in our study have a higher affinity for some forms, so there are differences in the amount of glucocerebrosidase seen on gels without immunoprecipitation, in comparison with immunoprecipitated material. The results of loading experiments demonstrated that the recombinant protein translated from the first ATG directed substantial levels of hydrolytic activity in comparison with recombinant protein with the 19 amino acid leader translated from the second ATG. Because in all three cases the mature lysosomal protein obtained after cleavage of the leader is the same, we propose that the efficiency of translocation of the primary recombinant proteins through the endoplasmic reticulum membrane is different and the differences in activity reflect the different rates of accumulation of the recombinant protein in the lysosome. Sorge et al. [6], demonstrating that either ATG can function independently to produce active glucocerebrosidase, noted that the upstream ATG produced two to three times as much protein as did the downstream ATG. Measurements of glucocerebrosidase activity *in vitro*, using 4-MUG as a substrate, demonstrated the same phenomenon (see Table 2). As mentioned above, the lysosomal fraction of glucocerebrosidase from the total recombinant glucocerebrosidase was barely detectable as measured by endoglycosidase H sensitivity. These results demonstrate that most recombinant glucocerebrosidase produced in the cells does not reach the lysosomes, either because of a lack of sufficient amounts of carrier or receptor or alternatively because of a lack of proteins that are responsible for the processing of the enzyme. It is possible that the actual intralysosomal fraction is higher than that presented by the endoglycosidase H resistance [34]. Immunohistochemical experiments would be helpful in confirming the actual amounts of the intralysosomal glucocerebrosidase. The T7/EMCV/vaccinia hybrid expression system provided a 10-fold increase in the activity of lysosomal recombinant human glucocerebrosidase, making it a convenient system for studying the changes in biochemical properties of mutated glucocerebrosidase proteins.

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