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ER retention and degradation as the molecular basis underlying Gaucher disease heterogeneity

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Gaucher disease (GD), an autosomal recessive disease, is characterized by accumulation of glucosylceramide mainly in cells of the reticuloendothelial system, due to mutations in the acid β-glucocerebrosidase gene. Some of the patients suffer from neurological symptoms (type 2 and type 3 patients), whereas patients with type 1 GD do not present neurological signs. The disease is heterogeneous even among patients with the same genotype, implicating that a mutation in the glucocerebrosidase gene is required to cause GD but other factors play an important role in the manifestation of the disease. Glucocerebrosidase is a lysosomal enzyme, synthesized on endoplasmic reticulum (ER)-bound polyribosomes and translocated into the ER. Following N-linked glycosylations, it is transported to the Golgi apparatus, from where it is trafficked to the lysosomes. In this study, we tested glucocerebrosidase protein levels, *N*-glycans processing and intracellular localization in skin fibroblasts derived from patients with GD. Our results strongly suggest that mutant glucocerebrosidase variants present variable levels of ER retention and undergo ER-associated degradation in the proteasomes. The degree of ER retention and proteasomal degradation is one of the factors that determine GD severity.

INTRODUCTION

Gaucher disease (GD), a sphingolipidosis characterized by impaired activity of the lysosomal enzyme glucocerebrosidase, results from mutations in the glucocerebrosidase gene or, rarely, in the gene encoding the glucocerebrosidase activator (saposin C), designated prosaposin (1,2). Being a very heterogeneous disease, it has been sub-divided into three different clinical types: the adult chronic non-neuronopathic type 1 disease (MIM no. 230800); the infantile, acute neuronopathic type 2 disease (MIM no. 230900); and the juvenile sub-acute neuronopathic type 3 GD (MIM no. 321000). More than 180 Gaucher causing mutations in the glucocerebrosidase gene are known to date. They include point mutations, splice site mutations, deletions and recombinant alleles, resulting from recombination between the glucocerebrosidase gene and a closely related pseudogene, occupying the same locus on chromosome 1q21. The most prevalent among type 1 patients, which has been exclusively associated with this form of the disease, is the N370S missense mutation (3). The most prevalent mutation among non-Jewish patients is the L444P (4). It is a severe mutation, associated, in homozygosity, with type 3 GD (5). It appears in compound heterozygote patients with type 1 or type 2 GD. The D409H (6) is a severe mutation. Patients homozygous for this mutation present occulomotor apraxia and a progressive heart disease with minimal organomegaly (7,8). The P415R is a severe, rare mutation associated with type 2 GD (9). The G202R is a severe mutation, found in homozygosity in patients presenting type 2 GD (10,11), and the V394L (6) is also a severe mutation. The D140H/E326K and the K157Q mutations are rare and were first described in two GD brothers with different manifestation of the disease (12). There is one frameshift mutation, 84GG, which leads to premature termination and, therefore, no expression of protein (13).

There is much diversity in phenotypic expression within all the genotypes, therefore, even siblings with an identical genotype present different disease severities (14), implicating that although mutations in the glucocerebrosidase gene are required to cause GD, other factors play an important role in the manifestations of the disease.

GD is diagnosed by biochemical and molecular means. Biochemically, glucocerebrosidase activity is measured in cell lysates from patients, using fluorescent substrates and following their fluorescent derivatives. Molecular diagnosis, executed by PCR amplification of genomic fragments and detection of specific mutations, provides the definition of the genotype. However, none of the existing methods allows accurate prediction of disease severity from the genotype.

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Being a lysosomal membrane-associated glycoprotein, glucocerebrosidase is translated to a 56 kDa polypeptide by endoplasmic reticulum (ER)-bound polyribosomes. Translocation through the ER membrane is accompanied by leader sequence cleavage and protein glycosylation on four aspargine residues (15). The high mannose sugar moieties are modified while moving through the Golgi apparatus. There, glucocerebrosidase undergoes further modifications in its sugar moieties, finally being transported to the lysosomes as a 59–63 kDa mature protein by a mannose 6 phosphate receptor-independent pathway, since in I cell disease it is found in lysosomes (15,16).

In recent reports, few mutations within the glucocerebrosidase gene were suggested to have a trafficking defect. Thus, it was noted that the G202R mutation results in a glucocerebrosidase variant that does not reach lysosomes (11). Moreover, the addition of sub-inhibitory concentrations of *N*-(*n*-nonyl)deoxynojirimycin (NN-DNJ) to a fibroblast culture medium of N370S homozygous patient led to an increase in the activity of the N370S-glucocerebrosidase variant, suggesting that NN-DNJ led the N370S carrying enzyme to properly fold, thus allowing the stabilized enzyme to transit from the ER to the Golgi, enabling proper trafficking to the lysosomes (17). It was also demonstrated that the carbohydrate mimic *N*-octyl-β-valienamine (NOV), an inhibitor of human glucocerebrosidase (18), could increase the level of the variant enzyme carrying the F213I mutation and up-regulated cellular enzyme activity in F213I homozygous cells. It was therefore suggested that NOV works as a chemical chaperone to accelerate transport and maturation of F213I carrying glucocerebrosidase (18,19).

It is well documented that mutant proteins are identified as misfolded by the ER quality control machinery and retained in the ER. After a certain period of attempts to refold them by the ER chaperones, the misfolded proteins are retro-translocated from the ER back to the cytosol to be eliminated by the ubiquitin–proteasome pathway (20–24). This whole process is known as the ER-associated degradation (ERAD) (22–24).

Recent studies suggested that few mutant variants of lysosomal enzymes are retained within the ER. It was shown that in chronic adult forms of GM2 gangliosidoses, resulting from missense mutations in the β-hexosaminidase A, the mutant variants are retained in the ER resulting in their accelerated degradation (25). In the case of β-galactosidase, whose activity is impaired in GM1 gangliosidosis and Morquio B disease, it was shown that some mutant proteins are unstable in the ER/Golgi apparatus and are rapidly degraded without appropriate molecular folding (26). In the case of Fabry disease, caused by reduced α-galactosidase activity, it was shown that at least in one mutant form (Q279E), the intracellular mutant protein aggregates in the ER and is rapidly degraded (27). Very recently, it has been shown that treatment of Fabry fibroblasts, carrying different mutations, with the competitive α-galactosidase inhibitor 1-deoxygalactonorijimycin (DJG) results in a correction of the lysosomal storage phenotype (28).

In this study, we tested glucocerebrosidase levels, *N*-glycan processing, intracellular localization and proteasomal degradation in GD fibroblasts. Our results strongly indicate that mutant glucocerebrosidase variants exhibit variable degrees of ER retention and proteasomal degradation. The degree of ER retention and proteasomal degradation is one of the factors that determine GD severity.

RESULTS

No correlation between endogenous glucocerebrosidase activity and GD severity

In vitro glucocerebrosidase activity was tested in lysates of primary skin fibroblasts derived from different GD patients, using the artificial substrate 4-methyl-umbeliferyl-glucopyranoside (4-MUG). As shown in Figure 1A, all samples demonstrated low glucocerebrosidase activity of ∼5.2–16.5% of normal. There was no correlation between glucocerebrosidase activity and disease severity (as described in Table 1), indicating that the *in vitro* activity of mutated glucocerebrosidase variants cannot predict GD severity.

Decreased glucocerebrosidase levels in GD fibroblasts

Glucocerebrosidase protein levels in cells of different patients were tested by western blot analysis after peptide:*N*-glycosidase F (PNGase F) digestion. Because PNGase F is an endoglycosidase that removes all aspargine-linked glycans from glycoproteins (29–31), it generates one glucocerebrosidase isoform whose intensity can be readily measured. As presented in Figure 1B–D and Table 1, most patients exhibited decrease in glucocerebrosidase protein levels when compared with normal cells, with some correlation to disease severity. Notably, there was a significant decrease in the amount of glucocerebrosidase in skin fibroblasts derived from patients with type 2 and type 3 GD. To ensure that the decrease in glucocerebrosidase levels did not reflect a general decrease in the amount of lysosomal enzymes, β-hexosaminidase A level was tested and no difference was observed (data not shown), indicating that the decrease is specific to glucocerebrosidase.

Endoglycosidase-H sensitivity of GD glucocerebrosidase

We suspected that the decrease in the glucocerebrosidase protein levels in GD might result from rapid degradation. Therefore, in an effort to follow the intracellular fate of glucocerebrosidase, we monitored the processing of its *N*-glycans in GD fibroblasts. They were subjected to endoglycosidase-H (endo-H) digestion, because endo-H is a specific endoglycosidase that can distinguish between high mannose (more than four mannose residues) and a mature *N*-glycan complex (29,31). It allowed us to follow the traffic of glucocerebrosidase because the removal of two mannose residues to generate the final core of three mannose residues is performed by the Golgi mannosidase II in the mid-Golgi. Therefore, endo-H can distinguish between glycoproteins that have not reached the mid-Golgi and folded, processed, mature glycoproteins. As PNGase F removes all aspargine-linked glycans, its cleavage pattern (as demonstrated in Fig. 1B) was used as a control to confirm that the changes in protein migration on SDS–PAGE originate from protein glycosylations and not from changes in amino acid sequence. The results, presented in Figure 2, showed that in normal cells, ∼90% of

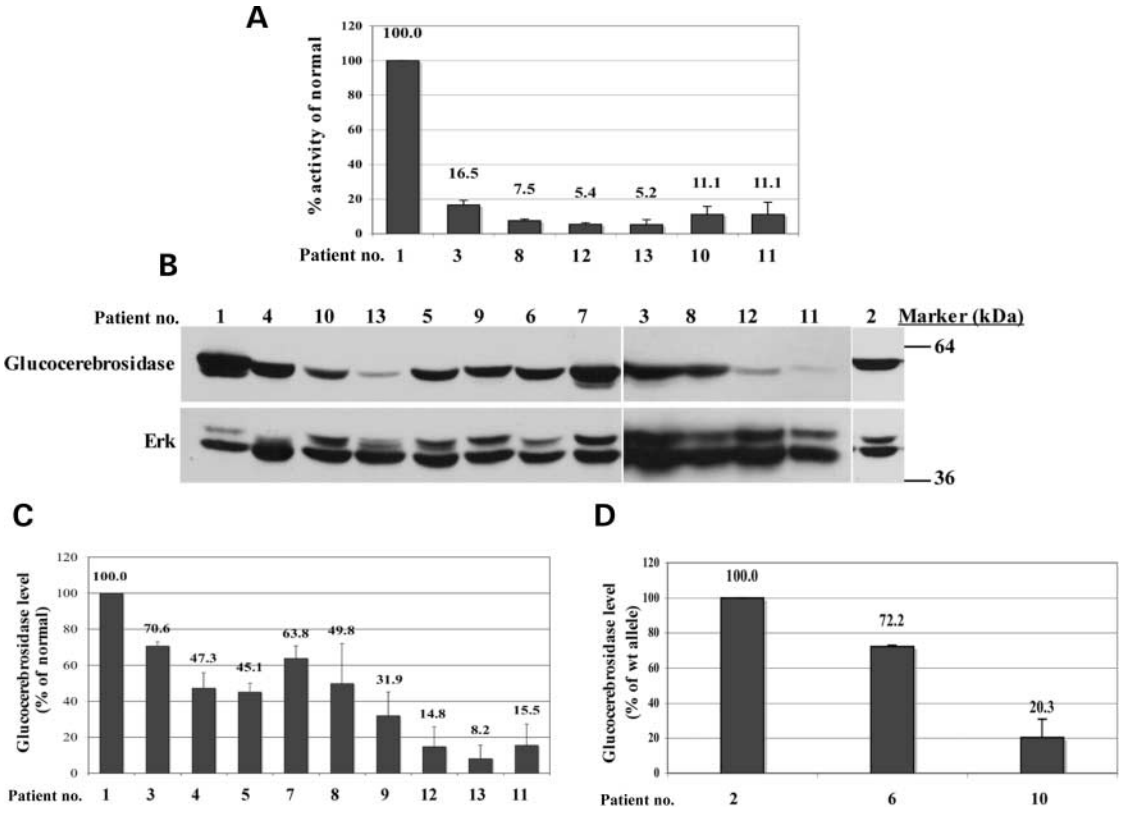

Figure 1. Glucocerebrosidase activity and level in normal and GD cells. (**A**) Samples containing 20 μg of protein were tested for acid β-glucosidase activity using 1.5 mM of the artificial substrate 4-MUG. The results represent the mean ± SEM, of three experiments with two repetition for each one, as percentage of the activity of the normal protein. (**B**) Cell lysates containing the same amount of protein were prepared from skin fibroblasts of GD patients and foreskin fibroblasts (normal) and treated with PNGase F. Following 10% SDS–PAGE, western blot analysis was performed using anti-glucocerebrosidase and anti-erk antibodies. (**C**) To normalize the results, intensity of the glucocerebrosidase band at each lane was divided by the intensity of the erk. The value obtained for the normal glucocerebrosidase was set as 100%. (**D**) Because the anti-human glucocerebrosidase monoclonal antibody used in this study does not recognize the L444P mutant protein (32), glucocerebrosidase levels in L444P containing compound heterozygotes were compared with those of 84GG/+ carriers.

glucocerebrosidase was endo-H resistant, indicating that most of the protein already passed the mid-Golgi (probably mature lysosomal). In contrast, 58–85% of glucocerebrosidase in cells of mildly affected type 1 patients and only 1.9–4% of the enzyme in cells of neuronopathic patients were endo-H resistant, suggesting that a significant fraction of glucocerebrosidase in these cells did not reach the mid-Golgi and therefore was not lysosomal. Furthermore, a difference in endo-H cleavage patterns was observed between patients with the same genotype but with different disease severity. There was a direct correlation between the fraction of endo-H-sensitive glucocerebrosidase and the severity of the disease of the patients. Patients homozygous for the N370S mutation with a type 1 disease (patients 3 and 4) demonstrated 80–85% endo-H resistance, whereas an N370S homozygous patient with severe type 1 disease (patient 8) had only 45% endo-H-resistant glucocerebrosidase. Compound heterozygotes, with the genotype N370S/L444P (patient 6), in whom only the N370S protein could be detected (32), or N370S/84GG (patient 5), in whom one allele is not expressed (13), presented 58–68% of endo-H resistant glucocerebrosidase (Fig. 2B). We included a GD type 1 patient with an unknown genotype (patient 9). This patient presented only 27% endo-H resistant glucocerebrosidase, implying a severe type 1 GD.

To verify that the difference in endo-H cleavage pattern between the different patients is not due to a general defect in sorting or processing of lysosomal proteins, endo-H sensitivity of β-hexosaminidase A was tested. As mature lysosomal β-hexosaminidase A has at least one *N*-glycan complex with more than five mannoses (33), it is endo-H sensitive. The results, shown in Figure 3A, demonstrated no difference in endo-H cleavage pattern of β-hexosaminidase A between normal and GD cells, indicating that the difference in endo-H sensitivity is specific to glucocerebrosidase.

ER retention of glucocerebrosidase in GD cells

Our results strongly suggested that in all tested patients with GD, there was a variable fraction of immature glucocerebrosidase. It is possible that the immature glucocerebrosidase is retained in the ER. To test glucocerebrosidase localization, indirect immunoflourescence was performed. As shown in Figure 3A, in normal cells, glucocerebrosidase accumulated in punctate lysosomal structures, as presented by colocalization with lysotracker (Fig. 3B). Only a negligible fraction of glucocerebrosidase was colocalized with calnexin, an ER marker. In contrast, all mutant glucocerebrosidase variants demonstrated diverse levels of colocalization with calnexin.

Table 1. Correlation between glucocerebrosidase level, glucocerebrosidase endo-H resistance and clinical manifestations

Patient number	Genotype	Disease type	GCase levels (% of normal)	% Endo-H resistant fraction	Correlation: type/ endo-H resistance
1	WT	Normal	100	89.8 ± 4.6	+
2	WT/84GG	Carrier	[a]	91.6	+
3	N370S/N370S	1	70.6 ± 2.3	84.9 ± 4.4	+
4	N370S/N370S	1	47.3 ± 8.5	79.8 ± 4.9	+
5	N370S/84GG	1	45.1 ± 5.0	67.7 ± 4.9	+
6	N370S/L444P	1	72.2 ± 0.7[b]	57.9 ± 5.9	+
7	N370S/V394L	1	63.8 ± 6.9	63.9 ± 6.9	+
8	N370S/N370S	1(severe)	49.8 ± 22.3	45.4 ± 12.5	+
9	R463C/unknown[c]	1	31.9 ± 13.3	27.2 ± 17.4	+
10	P415R/L444P	2	20.3 ± 10.6[b]	1.9 ± 1.9	+
11	Unknown[c]	2	15.5 ± 11.8	4.1 ± 3.6	+
12[d]	Unknown[c]	3	14.8 ± 11.2	34.5 ± 12.4	+
13	D409H/D409H	3	8.2 ± 7.3	3.9 ± 2.8	+

The table summarizes limited data we had on the patients from whom skin fibroblasts had been obtained as well as protein levels and the degree of endo-H resistance in the corresponding cells. The results represent the mean ± SEM, as percentage of four repetitions for each experiment. The correlation between endo-H resistance and the type of GD is also shown. GCase, glucocerebrosidase.
[a]Cells from this individual were used as a control for compound heterozygotes with one undetected allele (84GG).
[b]Results obtained for this individual were compared with those obtained for individual 2.
[c]We excluded the existence of the mutations: N370S, L444P, P415R, 84GG, IVS2+1, D409H, recTL and recNciI in all patients with unknown mutations.
[d]This patient had low glucocerebrosidase level and higher endo-H resistant fraction.

Levels of colocalization with calnexin correlated well with endo-H sensitivity and disease severity. In cells from patients with severe neuronopathic GD, there was almost complete colocalization of glucocerebrosidase with calnexin, indicating that most of the protein was retained in the ER and did not reach the lysosomes. In cells from type 1 patients, part of the protein showed a reticular accumulation in the calnexin positive ER, although it also appeared in punctate lysosomal structures.

To ensure that there is no defect in glucocerebrosidase sorting in GD, cells derived from normal, mildly affected or severely affected patients, transiently expressing myc-tagged wt glucocerebrosidase, were subjected to indirect immunofluorescence. The results demonstrated that in all the tested cells, the wt glucocerebrosidase accumulated in punctate structures and did not colocalize with calnexin in the ER (data not shown). It implies that normal glucocerebrosidase can reach its target lysosomal localization in GD cells, whereas the endogenous enzyme fails to do so, and that the mislocalization of glucocerebrosidase in GD patients is due to the presence of the mutated protein and not to a sorting defect.

Degradation of mutant glucocerebrosidase variants by the proteasomal machinery

Our results strongly indicated that in GD, glucocerebrosidase is retained in the ER and its protein level is decreased. This could result from ERAD, a process in which mutated proteins are identified as misfolded and finally eliminated in the cytosol by the ubiquitin–proteasome pathway (22–24). If this is the case for mutant glucocerebrosidase variants, proteasome inhibitors, such as MG-132 and ALLN, should stabilize this protein and increase its levels. To this end, cells from different patients with GD were grown in the presence of the proteasome inhibitors ALLN and MG-132 and their lysates were subjected to western blot analysis with p53 as a control, because it undergoes proteasomal degradation and can be stabilized by proteasome inhibitors (34). The results, presented in Figure 4A, indicated that although normal glucocerebrosidase was not affected by proteasome inhibitors, mutant glucocerebrosidase variants accumulated in almost all GD patients who were tested, indicating their stabilization in the presence of proteasome inhibitors. There was larger glucocerebrosidase stabilization in patients with neuronopathic GD (from 2.1- to 3.8-fold increase in glucocerebrosidase levels) when compared with patients with type 1 (from 0.9- to 1.7-fold increase in glucocerebrosidase levels) (Fig. 4B). These results indicate that the decrease in glucocerebrosidase protein levels in GD patients is due, at least partly, to proteasomal degradation. Moreover, our results show a correlation between the degree of proteasomal degradation and the severity of the disease. We also compared the effect of ALLN (a non-specific proteasome inhibitor) (35) or MG-132 (a more specific proteasome inhibitor) (36) on the accumulation of glucocerebrosidase variants. The results, presented in Figure 4C, showed that both MG-132 and ALLN had similar effect in stabilizing mutant glucocerebrosidase.

Recombinant myc-tagged mutated glucocerebrosidase variants are endo-H sensitive and are retained in the ER

It was interesting to test whether recombinant glucocerebrosidase variants behaved similar to their endogenous counterparts. To do that, cell lysates prepared from HeLa cells, transiently expressing normal or mutated myc-tagged glucocerebrosidase variants, were subjected to endo-H treatment and western blot analysis using anti-myc antibody. The results (Fig. 5A) showed that a major fraction of the normal myc-glucocerebrosidase was endo-H resistant. However, all tested mutants were

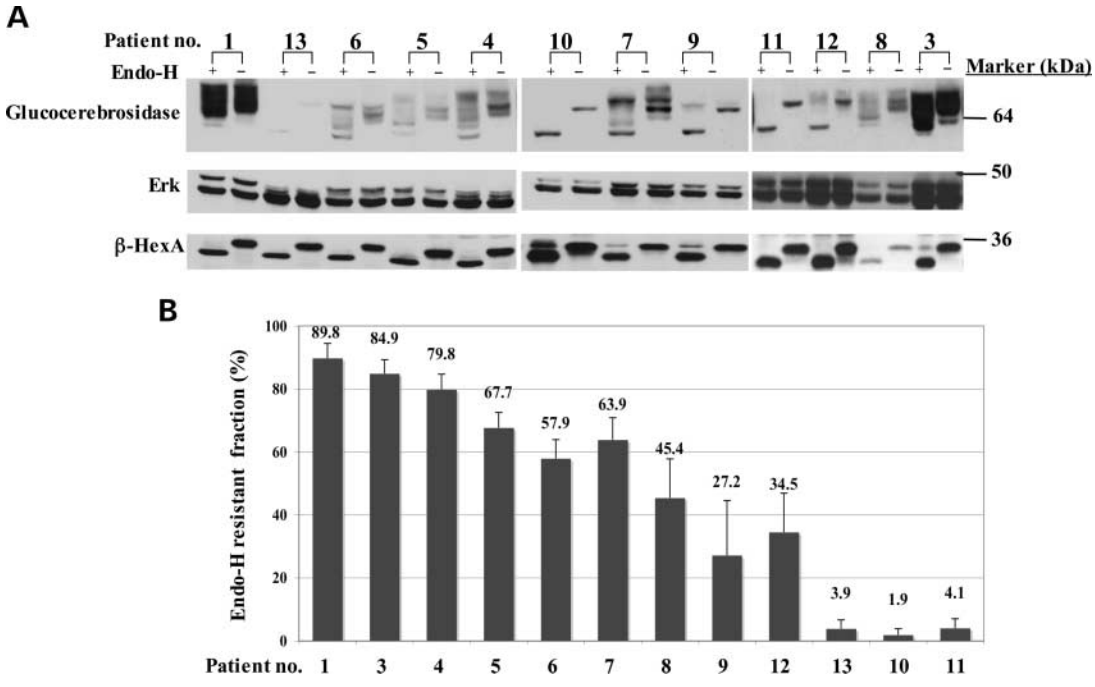

Figure 2. Endo-H resistance of glucocerebrosidase in GD patients. (**A**) Cell lysates containing the same amount of protein prepared from skin fibroblasts of patients with GD and from normal foreskin fibroblasts were subjected to endo-H digestion and western blot analysis with anti-glucocerebrosidase, anti-β-hexosaminidase A and anti-erk antibodies. (**B**) To determine the endo-H resistant fraction, the blots were scanned and the intensity of each band was measured. Glucocerebrosidase resistant fraction was calculated by dividing the intensity of the endo-H sensitive fraction (in the endo-H + lane) by the intensity of the entire amount of the glucocerebrosidase in the same lane. The results represent the mean +SEM, of four independent experiments.

endo-H sensitive. No difference in endo-H sensitivity between the various mutant variants was detected, most probably due to the overexpression of the recombinant proteins, which could have overwhelmed the quality control capacity.

To verify our speculation that all myc-tagged mutated glucocerebrosidase variants were retained in the ER and therefore were endo-H sensitive, their intracellular localization was tested. As presented in Figure 5B, most overexpressed wt glucocerebrosidase localized in punctate lysosomal structures, with no calnexin colocalization. In contrast, all mutants exhibited major colocalization with calnexin, indicating that most of the mutated recombinant proteins were retained in the ER.

Mutated glucocerebrosidase variants interact with calnexin

Because all tested recombinant mutant glucocerebrosidase variants were endo-H sensitive and retained in the ER, we tested their possible association with the ER chaperone calnexin. Calnexin is a type I transmembrane protein localized in the ER, which associates selectively with incompletely folded glycoproteins containing monoglucosylated *N*-glycans (37). It participates in ERAD of several misfolded glycoproteins and was shown to transiently interact with a large number of newly synthesized transmembrane and secretory glycoproteins, from which it dissociates after they attain a native conformation (38–41). Calnexin fails to dissociate or re-associate with misfolded glycoproteins and participates in their targeting to ERAD (41–43). Lysates of cells, transfected with plasmids expressing different mutant myc-tagged glucocerebrosidase variants, were subjected to immunoprecipitation with anti-myc antibody and western blot analysis with anti-calnexin antibodies. The results, presented in Figure 6A, indicated that the wt myc-tagged glucocerebrosidase bound calnexin very poorly, probably reflecting the small fraction of the overexpressed newly synthesized glucocerebrosidase that was still in the ER. However, mutant glucocerebrosidase variants exhibited enhanced binding to calnexin, ranging between 1.7-fold (for the N370S variant) and 15.3-fold (for the K157Q variant) higher than the binding of wt glucocerebrosidase to calnexin, as demonstrated in Figure 6B.

We also tested interaction between endogenous glucocerebrosidase and calnexin. For that purpose, cell lysates of normal, type 3 and type 2 GD patients, treated with MG-132, were immunoprecipitated with anti-calnexin antibodies and subjected to western blot analysis using anti-glucocerebrosidase and anti-calnexin antibodies. The results (Fig. 6C) showed that mutant variants of glucocerebrosidase interacted with calnexin, whereas there was no detectable interaction with the normal protein, although there were high glucocerebrosidase levels in the corresponding lysates (Fig. 6D). The level of calnexin-bound glucocerebrosidase increased in MG-132-treated cells. The fact that wt myc-tagged glucocerebrosidase, overexpressed in cells, interacted with calnexin, whereas endogenous normal glucocerebrosidase did not, implied that there was some retention of the normal overexpressed protein in the ER, as presented by its marginal endo-H sensitivity (Fig. 5A).

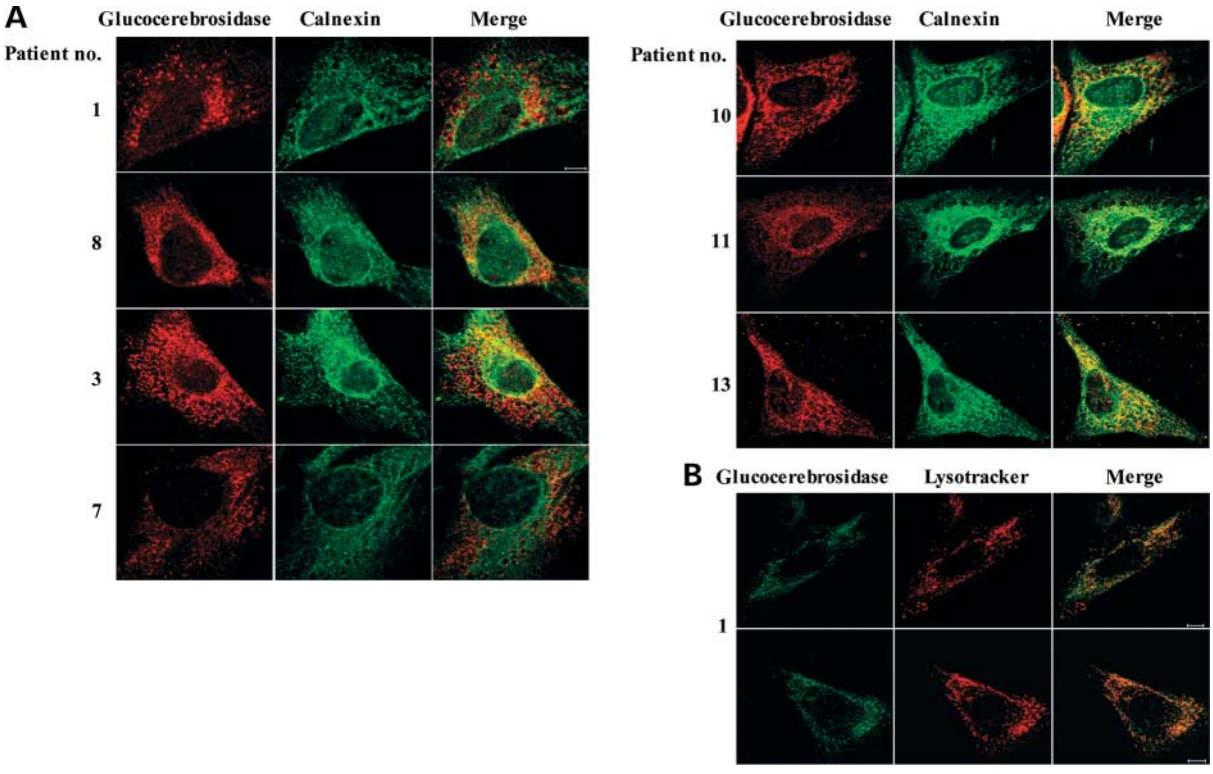

Figure 3. Intracellular localization of glucocerebrosidase in GD patients. (**A**) Cells from normal and GD patients were grown on cover-slips, fixed, permeabilized with 0.1% Triton X-100 and interacted with anti-glucocerebrosidase monoclonal antibody and anti-calnexin polyclonal antibodies. Detection was performed with cy-3-conjugated goat anti-mouse antibodies to demonstrate glucocerebrosidase localization (red) and with cy-2-conjugated goat anti-rabbit antibodies to demonstrate endogenous calnexin (green). Colocalization was illustrated by merging cy-2 and cy-3 images (merge). The results were visualized with a confocal microscope. Scale bar (10 μm) is the same in all pictures. Fibroblasts in the left panel originated from normal foreskin (1) or from patients with type 1 GD, with the following genotypes: 8-N370S/N370S, severe; 3-N370S/N370S, mild; 7-N370S/V394L, mild. Fibroblasts in the right panel originated from patients with neuronopathic forms of GD, with the following genotypes: 10-L444P/P415R, type 2; 11-?/?, type 2; 13-D409H/D409H, type 3. (**B**) To demonstrate lysosmal localization of wt glucocerebrosidase, normal skin fibroblasts, grown on cover-slips were loaded with lysotracker (red), fixed with 4% paraformaldehyde and treated as described earlier. Detection was performed with FITC-conjugated goat anti-mouse antibodies (green). Colocalization was illustrated by merging FITC (green) and lysotracker (red) images (merge). Scale bar: 10 μm.

DISCUSSION

More than 180 mutations in the glucocerebrosidase gene have been associated with GD. In general, they account for the heterogeneity of the disease. However, the heterogeneity among patients with the same genotype could not be explained (44).

In this study, we demonstrate that in patients with GD, there are variable degrees of ER retention and degradation of the mutant glucocerebrosidase protein. Decreased levels of these mutant variants could be partially overcome by proteasome inhibitors, implicating that at least part of this decrease in protein levels is due to proteasomal degradation. We showed preferential binding of the mutant proteins to calnexin, an ER chaperone that associates enduringly with incompletely folded glycoproteins and participates in ERAD of several misfolded glycoproteins (40,41). There was significant correlation between endo-H sensitivity, ER retention and degradation of glucocerebrosidase and disease severity. Endo-H sensitivity of glucocerebrosidase seemed to differentiate not only between patients with neuronopathic and non-neuronopathic disease but also between patients with GD type 1 who presented different clinical disease. We suggest that endo-H sensitivity of glucocerebrosidase is a reliable tool to predict GD severity irrespective of the genotype. It is worth mentioning that our conclusions are based on experiments performed on endogenous, native glucocerebrosidase variants. Although normal recombinant glucocerebrosidase behaved very similar to the endogenous counterpart and only a small fraction of it was endo-H sensitive and bound calnexin, all mutant recombinant variants were retained in the ER. There are documented cases in which maturation of a normal recombinant protein differs from that of the endogenous protein. This could be due to shortage in binding partners, whose association with the expressed protein may be required for proper maturation and/or trafficking (45). Thus, it has been shown that a significant fraction of normal recombinant CFTR is retained in the ER and subjected to ERAD (46–48), whereas endogenous CFTR exits the ER, with no retention (45).

We suggest that the ERAD process of mutant glucocerebrosidase variants plays a significant role in determining the disease heterogeneity. Patients with the same genotype may show different disease severities due to different fidelities of their quality control. The degree of ER retention and concomitant decrease in protein level determine disease severity. To date, there are no direct means to correlate between disease severity and any biochemical/molecular test. We suggest

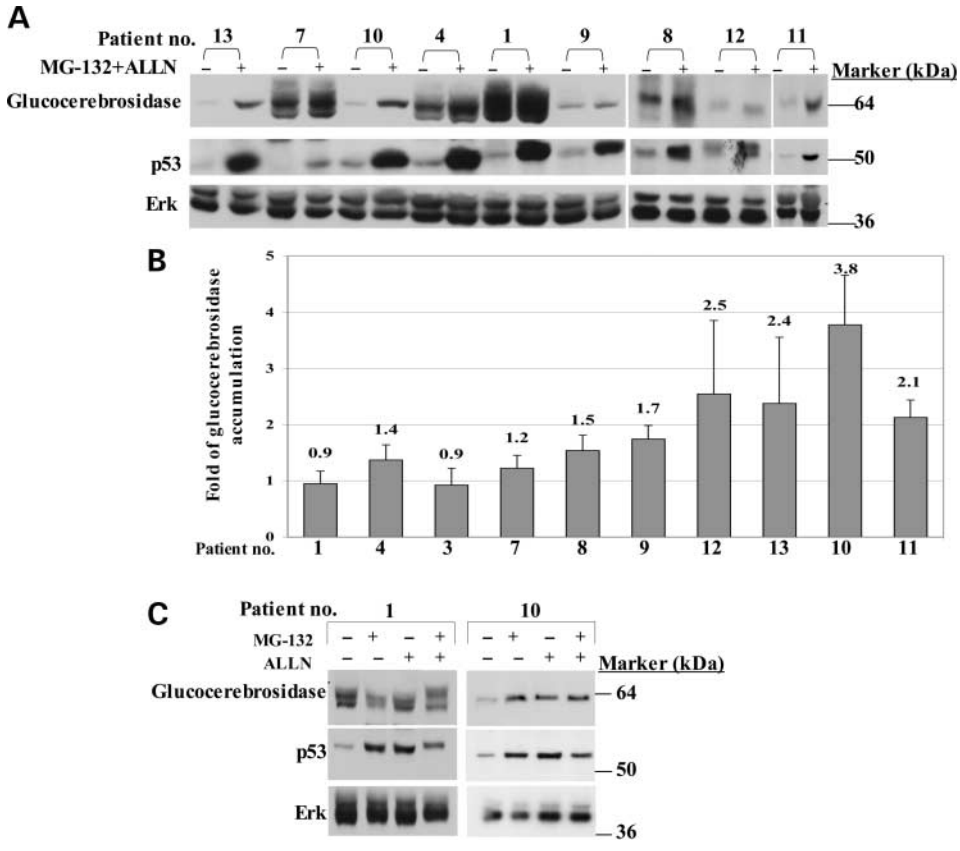

Figure 4 Stabilization of glucocerebrosidase in GD patients with proteasome inhibitors. (**A**) Lysates from GD patients were treated with 25 μM ALLN and 15 μM MG-132 for 20 h and subjected to western blot analysis. The blots were probed with anti-glucocerebrosidase, anti-erk and anti-p53 antibodies. (**B**) To normalize the results, the blots were scanned and glucocerebrosidase intensity at each lane was divided by the intensity of erk and the protein ratio between treated and untreated cells was calculated. The results represent the mean + SEM, of three to six independent experiments, as percentage of the fold increase in protein level due to the treatment of each variant. (**C**) Lysates from GD fibroblasts that were treated for 20 h with either ALLN or MG-132 alone or together in combination. Samples containing equal amounts of protein were subjected to western blot analysis with anti-glucocerebrosidase, anti-erk and anti-p53 antibodies.

using degrees of immature glucocerebrosidase in GD patients, namely, endo-H sensitivity, as a tool to predict disease severity.

The importance of ER quality control, in general, and the ERAD process, in particular, has been indicated in a large spectrum of diseases (21,49,50). The cystic fibrosis ΔF508-CFTR mutated protein does not reach its plasma membrane localization in lung epithelial cells because of its slow or inefficient folding in the ER and excessive degradation (51–53). The intracellular aggregation of the misfolded mutant rhodopsin leads to retinitis pigmentosa. This aggregation results from the retrotranslocation of the misfolded protein by the ERAD machinery, but there is inefficient degradation of this misfolded protein by the proteolytic machinery (54).

It seems that the ERAD process is the mechanism underlying disorders associated with mutant proteins that are processed in the ER, including lysosomal disorders. The possibility that mutant lysosomal enzymes are retained in the ER and undergo ERAD has been suggested for β-hexosaminidase A in chronic adult GM2 gangliosidoses (25), β-galctosidase, mutated in GM1 gangliosidoses and Morquio B disease (26), as well as α-galactosidase, whose impaired activity causes Fabry disease (27).

Studying the ERAD machinery is beginning to provide significant medical insights. Understanding the involvement of this process in pathogenesis opens a novel approach for pharmacological intervention (55,56). Two main strategies are being pursued to obtain functional rescue: the first involves the development of substances that favor correct folding of mutant proteins and, consequently, allow them to pass the quality control machinery. The second strategy involves release of fractions of these misfolded proteins from the ER by preventing their interactions with the quality control components. In both cases, the released mutant proteins, which may have residual activity, reach their normal destination. It has already been shown that low temperature or non-specific chemical chaperones (such as glycerol) release a fraction of ΔF508-CFTR protein to the plasma membrane, where it is active (51,55–57).

Recent studies are focused on using more specific agonists as pharmacological chaperones to rescue proteins with medical relevance from their retention compartments (55,56,58). Thus, it has been shown that in the case of ΔF508-CFTR mutant, A_1 adenosine receptor antagonist 8-cyclopentyl-1, 3-dipropylxanthine (CPX) as well as the benzo(*c*)quinolizinium drugs MBP-07 and MBP-91 leads to restoration of the

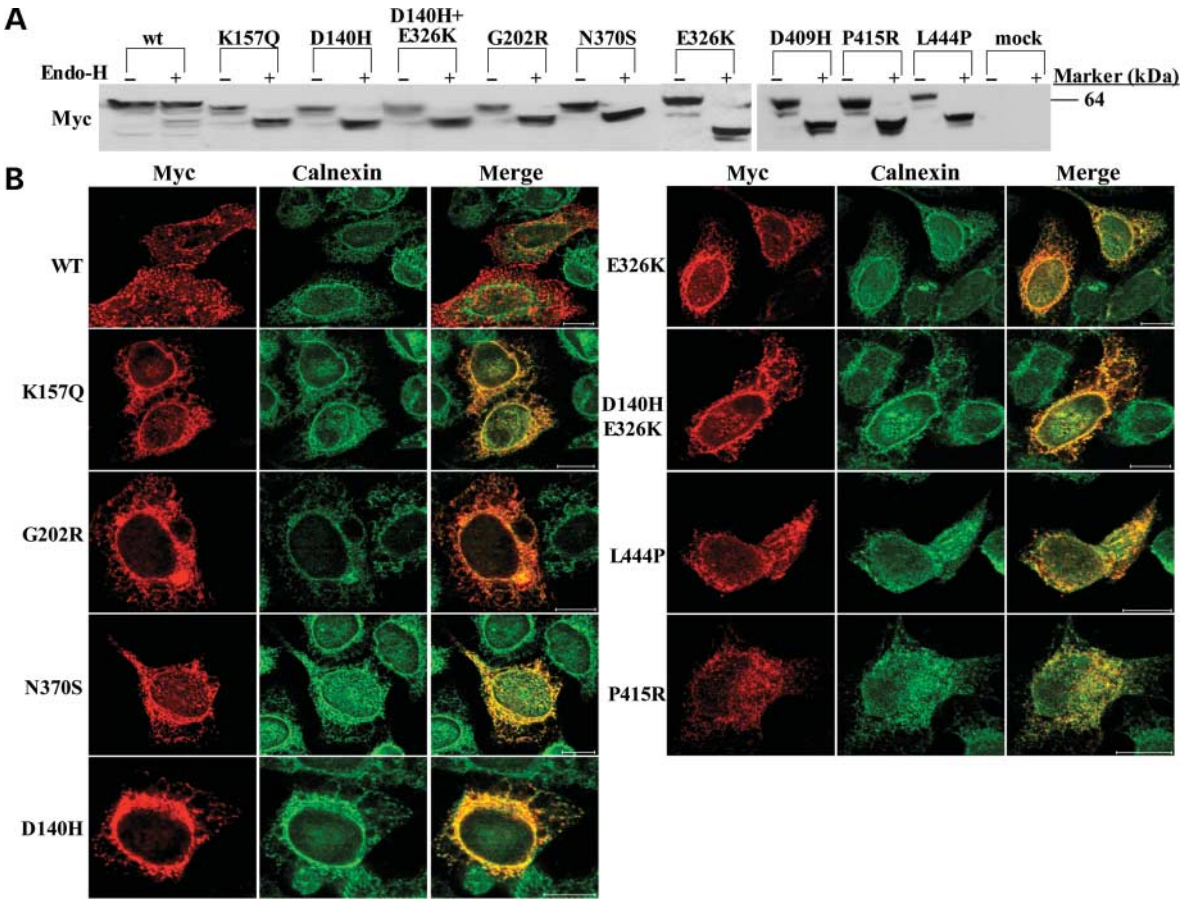

Figure 5. Localization and endo-H sensitivity of recombinant myc-tagged glucocerebrosidase variants. (**A**) Twenty-four hours after transfection of HeLa cells with normal or mutated myc-tagged glucocerebrosidase variants, cell lysates were prepared and subjected to endo-H treatment. Lysates were subjected to 10% SDS–PAGE and blotted. Recombinant glucocerebrosidase was detected by probing the blot with anti-myc antibody. (**B**) HeLa cells, grown on cover-slips, were transfected with normal glucocerebrosidase or its mutated forms. Twenty-four hours after transfection, cells were fixed and permeabilized with 0.1% Triton X-100. Cells were incubated with mouse anti-myc and rabbit anti-calnexin antibodies. Detection was performed with cy-3-conjugated goat anti-mouse antibodies to demonstrate myc-glucocerebrosidase localization (red), and with cy-2-conjugated goat anti-rabbit antibodies to demonstrate endogenous calnexin (green). Colocalization was illustrated by merging cy-2 and cy-3 images (merge). The results were visualized with a confocal microscope. Scale bar: 10 μm.

plasma membrane localization of the ΔF508-CFTR, *in vitro*, most likely due to stabilizing correct folding of the mutant protein by involving specific binding sites (59–61). Similarly, the rhodopsin P23H mutant could be rescued by the retinal derivate 11-*cis*-7-ring retinal (62). This approach has been applied already in lysosomal enzymes. A recent study has demonstrated that sub-inhibitory doses of the competitive inhibitor of the α-galactosidase A, DJG (28), release Fabry mutants from the ER chaperone BiP, allowing them to be transported to the lysosomes and leading to clearance of the lysosomal storage. In the case of GD, it has been shown that lysosomal levels and activity of the F213I and the cellular activity of the N370S glucocerebrosidase mutants are increased by treatment with the glucocerebrosidase inhibitors NOV and NN-DNJ, respectively (17,18).

Because only ∼1–5% of normal glucocerebrosidase intracellular activity is required to correct the metabolic defect in GD cells (63), specific small-molecule ligands that act as pharmacological chaperones and enhance the misfolded mutant glucocerebrosidase variants trafficking to the lysosomes may improve their residual activity and serve as a basis for therapy in the treatment of this disease.

To summarize, our results strongly indicate a direct correlation between GD severity and decreased glucocerebrosidase protein levels, endo-H sensitivity, ER localization, binding to calnexin and proteasomal degradation. This is also true for patients with the same genotype who present with different disease severity. Therefore, we suggest using levels of immature glucocerebrosidase in GD patients as a tool to implicate disease severity.

MATERIALS AND METHODS

Materials

The following antibodies were used in this study: mouse monoclonal anti-glucocerebrosidase 2C7 (64) (kindly provided by Professor H. Aerts, E.C. Slater Institute for Biochemical Research, University of Amsterdam, The Netherlands), rabbit polyclonal anti-calnexin (SPA-860; Stressgen Biotechnologies, Victoria, BC, Canada), mouse monoclonal anti-p53 A (DO1, kindly provided by Dr D. Lane, Department of Surgery and Molecular Oncology, University of Dundee, Dundee, UK),

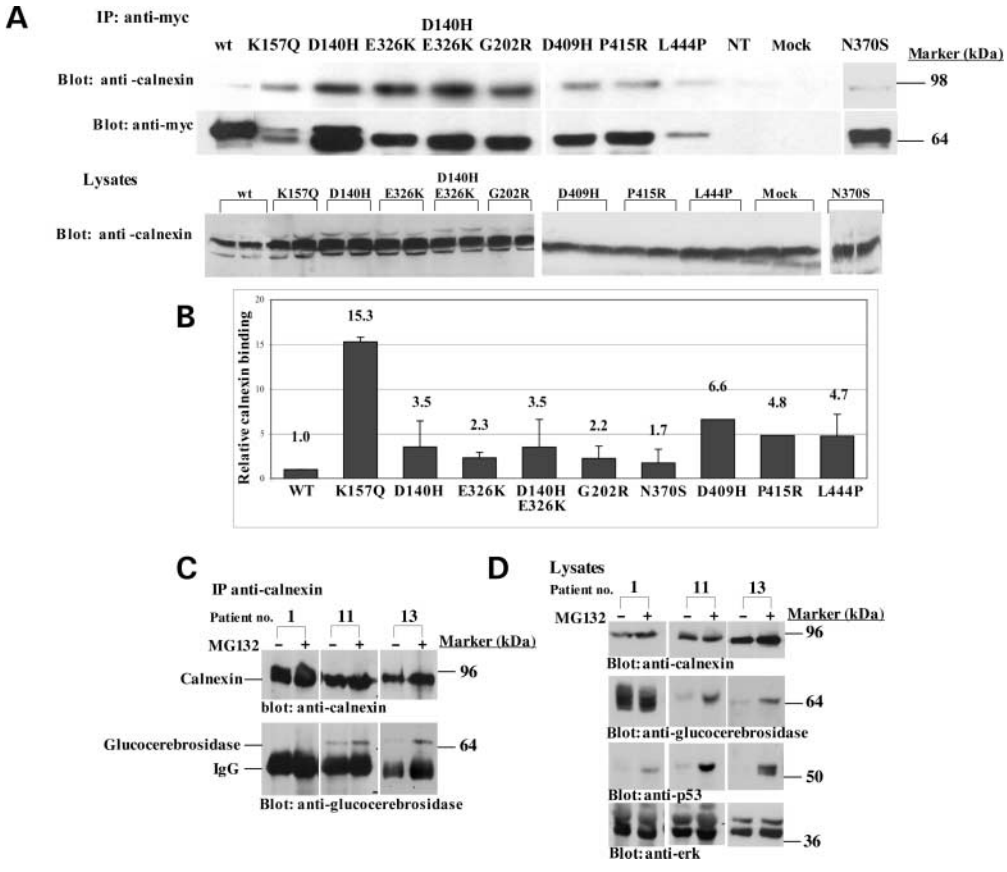

Figure 6. Interaction of calnexin with recombinant and endogenous glucocerebrosidase. (**A**) Lysates of HEK293 cells, transiently expressing wt or mutated myc-tagged glucocerebrosidase variants were immunopercipitated with anti-myc antibody. The precipitates were subjected to 10% SDS–PAGE and blotted, and the blot was probed with anti-myc antibody and re-probed with anti-calnexin antibodies. In parallel, duplicate samples of each lysate were separated through 10% SDS–PAGE and blotted, and the blot was probed with anti-calnexin antibodies. (**B**) To evaluate the amounts of binding to calnexin of each variant, the blots were scanned and the intensity of the calnexin band at each lane was divided by the intensity of glucocerebrosidase. The value obtained for normal glucocerebrosidase was set as 1. The results represent the mean + SEM of one to three independent experiments. (**C** and **D**) Normal, type 3 and type 2 GD patient cells were incubated for 20 h with MG-132 and their lysates were either subjected to SDS–PAGE (lysates) (D) or immunoprecipitated (C) with anti-calnexin antibodies. Following 10% SDS–PAGE, the blots were probed with anti-myc and anti-calnexin antibodies (C and D) as well as with anti-p53 antibody, as a positive control for proteasomal inhibition and anti-erk antibodies, as a control for amount of loaded protein in the different samples (D).

rabbit anti-erk (C16 Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-hexosaminidase A (kindly provided by Dr R. Gravel, Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada) and mouse monoclonal anti-myc (9B11 Cell Signaling Technology, Beverly, MA, USA); secondary antibodies: FITC-conjugated goat anti-mouse, cy-3-conjugated goat anti-mouse and cy-2-conjugated goat anti-rabbit, horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit were purchased from Jackson Immuno Research Laboratories, West Grove, PA, USA.

Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG-132) and *N*-acetyl-L-leucyl-L-leucyl-L-norleucinalAc (ALLN) were purchased from Calbiochem (San Diego, CA, USA). Endo-H and PNGase F were purchased from New England Biolabs (Beverly, MA, USA). Restriction enzymes were purchased from several companies and employed according to manufacturers' recommendations.

4-MUG was purchased from Genzyme Corp. (Boston, MA, USA). NP-40 was purchased from Roche Diagnostic, Mannheim, Germany. Leupeptin was purchased from Sigma-Aldrich, Israel.

Cell lines

HeLa and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). All cells were grown at 37°C in the presence of 5% CO_2. Human primary skin fibroblasts and foreskin fibroblasts, detailed in Table 1, were grown in DMEM supplemented with 20% FCS.

Plasmid construction

Glucocerebrosidase, containing its 38 amino acid residues leader, was cloned into the *Eco*RI and *Xho*I sites of pcDNA4 myc-his-plasmid (Invitrogen Life technologies Co., Carlsbad, CA, USA). *In vitro* site-directed mutagenesis was performed using the Quick Change site-directed mutagenesis kit (Stratagene Life Technologies Co., Austin, TA, USA), to

create variant forms with the mutations: D140H, K157Q, E326K, D140H + E326K, G202R, N370S, D409H, P415R and L444P. Amplified products were digested with *Dpn*I to remove contaminating parental plasmid DNA and subsequently transformed into DH5-α competent *Escherichia coli* cells. Mutations were confirmed by DNA sequencing.

Endo-H and PNGase F treatment

Samples of cell lysates, containing 70 μg of total protein, were subjected to an overnight incubation with endo-H or PNGase F according to the manufacturer's instructions.

Proteasome inhibition

Sub-confluent human skin fibroblasts, grown on 9 mm plates, were treated in the presence or absence of 25 μM ALLN and 15 μM MG-132. Twenty hours later, protein lysates were prepared, and samples containing the same amount of protein, as determined by the Bradford technique (65), were subjected to western blot analysis.

SDS–PAGE and western blotting

Cell monolayers were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed at 4°C in 500 μl of lysis buffer (10 mM HEPES pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$ and 1% Triton X-100) containing 10 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml leupeptin (Sigma-Aldrich). Lysates were incubated on ice for 30 min and centrifuged at 10 000*g* for 15 min at 4°C. Samples containing the same amount of protein were electrophoresed through 10% SDS–PAGE and electroblotted onto a nitrocellulose membrane (Schleicher and Schuell BioScience, Keene, NH, USA). Membranes were blocked with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline (TBS) for 2 h at room temperature (RT) and incubated with the primary antibody for 1 h at RT. The membranes were then washed three times in 0.1% Tween-20 in TBS and incubated with the appropriate secondary antibody for 1 h at RT. After washing, membranes were reacted with ECL detection reagents (Santa Cruz Biotechnology, Inc.) and analyzed by luminescent image analyzer (Kodak X-OMAT 2000 Processor Kodak, Rochester, NY, USA).

Transfections

Transfection was performed using Fugene transfection reagent (Roche Diagnostic) according to the manufacturer's instructions.

Immunoprecipitation

HeLa and HEK293 cells were transiently transfected with wt or mutated myc-tagged glucocerebrosidase. Forty-eight hours after transfection, the cells were washed three times with ice-cold PBS and then lysed at 4°C in 1 ml of lysis buffer (10 mM HEPES pH 8, 100 mM NaCl, 1 mM $MgCl_2$ and 0.5% NP-40) containing 10 μg/ml aprotinin, 0.1 mM PMSF, 10 μg/ml leupeptin, 20 mM *n*-ethyl-maleamide and 10 mM iodoacetamide (Sigma-Aldrich). Following incubation on ice for 30 min and centrifugation at 10 000 *g* for 15 min at 4°C, the supernatants were pre-cleared for 2 h at 4°C with protein A-agarose (Roche Diagnostic). Samples were centrifuged at 15 000 *g* for 1 min at 4°C, and the supernatants were incubated overnight at 4°C with monoclonal anti-myc antibody or polyclonal anti-calnexin antibodies immobilized on protein A–Sepharose (Sigma-Aldrich). Following four washes with 1 ml of lysis buffer containing protease inhibitors, proteins were eluted for 10 min at 100°C with five times loading buffer, electrophoresed through 10% SDS–PAGE and blotted. The corresponding blot was interacted with the appropriate antibodies.

Quantitation

The blots were scanned using Image Scan scanner (Amersham Pharmacia Biotech) and the intensity of each band was measured by the Image Master 1DPrime densitometer (Amersham Pharmacia Biotech).

Immunocytochemical analysis and confocal laser scanning microscopy

Sub-confluent cells, grown on cover slips, were washed twice with PBS, fixed for 5 min at 4°C in methanol, followed by 5 min at 4°C in methanol–acetone (1:1). Following washes, cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min at RT. Cells were then washed three times with PBS, blocked by incubating with PBS containing 1% bovine serum albumin (BSA) and 20% normal goat serum for 30 min at RT and then incubated for 1 h with the corresponding primary antibody (1:100 dilution for 2C7, 1:200 for rabbit anti-calnexin and 1:5000 for anti-myc) in 1% BSA/PBS at RT. Cells were washed three times with PBS and then immunostained with rabbit-cy-2 or mouse-cy-3 conjugated secondary antibodies (1:200 dilution) in 1% BSA/PBS for 45 min at RT. Following three washes with PBS, the cover slips were mounted with galvanol. For lysostracker colocalization, cells, loaded for 1 h with 25 nM; lysotracker (Lysotracker Red DND-99 Molecular probes, Eugene, OR, USA) at 37°C, were fixed 15 min in 4% paraformaldehyde, treated as described earlier and immunostained with mouse-FITC conjugated secondary antibodies. Cells were observed and analyzed with a LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany).

Enzymatic activity

Confluent primary skin fibroblasts were washed twice with PBS, collected with a rubber policeman in 1 ml sterile water and frozen in aliquots at −80°C. About 20 μg of total cell lysates were assayed for acid β-glucosidase activity in 0.2 ml of 100 mM potassium phosphate buffer, pH 4.5, containing 0.15% Triton X-100 (v/v, Sigma) and 0.125% taurocholate (w/v, Calbiochem) in the presence of 1.5 mM 4-MUG for 1 h at 37°C. The reaction was stopped by the addition of 1 ml 0.1 M glycine, 0.1 M NaOH, pH 10. The amount of 4-methyl-umbeliferone was quantified using

Perkin Elmer Luminescence Spectrometer LS 50 (excitation length: 340 nm; emission: 448 nm).

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Conflict of Interest statement. None declared.

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