Pharmacological chaperones facilitate the post-ER transport of recombinant N370S mutant β -glucocerebrosidase in plant cells: Evidence that N370S is a folding mutant

Gholamreza Babajani^a, Michael B. Tropak^b, Don J. Mahuran^{b,c}, and Allison R. Kermode^{a,*}

^aDepartment of Biological Sciences, Simon Fraser University, 8888 University Dr., Burnaby, BC, Canada V5A 1S6

^bResearch Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8

^cDepartment of Laboratory Medicine and Pathology, University of Toronto, Banting Institute, 100 College Street, Toronto, Ontario, Canada M5G 1L5

Abstract

Gaucher disease is a prevalent lysosomal storage disease in which affected individuals inherit mutations in the gene (*GBA1*) encoding lysosomal acid β -glucosidase (glucocerebrosidase, GCase, EC 3.2.1.45). One of the most prevalent disease-causing mutations in humans is a N370S missense mutation in the GCase protein. As part of a larger endeavor to study the fate of mutant human proteins expressed in plant cells, the N370S mutant protein along with the wild-type-(WT)-GCase, both equipped with a signal peptide, were synthesized in transgenic tobacco BY2 cells, which do not possess lysosomes. The enzymatic activity of plant-recombinant N370S GCase lines was significantly lower (by 81–95%) than that of the WT-GCase lines. In contrast to the WT-GCase protein, which was efficiently secreted from tobacco BY2 cells, and detected in large amounts in the culture medium, only a small proportion of the N370S GCase was secreted. Pharmacological chaperones such as *N*-(*n*-nonyl) deoxynojirimycin and ambroxol increased the steady-state mutant protein levels both inside the plant cells and in the culture medium. These findings contradict the assertion that small molecule chaperones increase N370S GCase activity (as assayed in treated patient cell lysates) by stabilizing the enzyme in the lysosome, and suggest that the mutant protein is impaired in its ability to obtain its functional folded conformation, which is a requirement for exiting the lumen of the ER.

Keywords

β -glucocerebrosidase; N370S mutation; Pharmacological chaperones; Tobacco BY2 cells; Protoplasts; Secretion

*Corresponding author.

1. Introduction

Gaucher disease is a prevalent lysosomal storage disease caused by a deficiency of the lysosomal hydrolase acid β -glucosidase (glucocerebrosidase, GCase, EC 3.2.1.45). This membrane-associated lysosomal enzyme cleaves the β -glucosidic linkage of glucosylceramide; thus the biochemical hallmark of Gaucher disease is the excessive storage of glucosylceramide, primarily in the cells and tissues of the reticuloendothelial system (subtype 1), and additionally in the brain and other nervous tissues, in subtypes 2 and 3 [1]. Almost 300 disease-causing mutations in the *GBA1* gene encoding human GCase have been identified to date. Most cause a deficiency of GCase protein and activity in the lysosome [2,3]. In some GCase variants, the protein is thought to be impaired in its folding in the ER and thus becomes subjected to endoplasmic reticulum-associated degradation (ERAD) involving retrotranslocation of the defective protein to the cytosol and its subsequent disposal by the cytosolic ubiquitin-proteasome machinery. Consequently the cells of an affected patient exhibit a decreased concentration of the hydrolase in the lysosome as a result of an inability of a large percentage of the newly synthesized mutant protein molecules to properly fold in the ER, which in turn prevents post-ER trafficking to the lysosome [4,5].

The N370S missense mutation of the *GBA1* gene is associated with the non-neuropathic subtype (subtype 1), and it is one of the most prevalent mutations causing Gaucher disease, second only to the L444P mutation. For some mutant lysosomal enzymes, including N370S GCase, pharmacological chaperones are being pursued as potential small-molecule ‘*enzyme enhancement*’ therapeutics; some of these active-site-specific inhibitors can ‘rescue’ a proportion of the newly synthesized mutant enzymes when present at sub-inhibitory concentrations, promoting their maturation and lysosomal trafficking. By serving as a folding template in the ER, the pharmacological chaperone is thought to allow a small proportion of the proteins to fold properly and thus pass the protein quality control system of the ER [6–8].

There is some controversy about whether N370S GCase suffers solely from a reduced catalytic activity or whether this mutant hydrolase is also subject to turnover by ERAD. There is considerable evidence that N370S GCase shows reduced catalytic activity, for example when expressed as a recombinant protein in cells of various heterologous hosts — both mammalian (e.g. NIH 3T3 fibroblast) and insect (e.g. *Spodoptera frugiperda*; Sf9) [9,10]. Various studies provide evidence that N370S is an unstable protein, which is destabilized in the environment of the ER (neutral pH) [6,11], and targeted for efficient degradation by ERAD. Sub-inhibitory concentrations of N-(n-nonyl)deoxynojirimycin (NN-DNJ) and the iminosugar isofagomine (IFG) increase the activity of N370S GCase by 2- to 3-fold in cultured N370S-patient fibroblasts; the authors conclude that the chaperones help the mutant protein to fold in the neutral pH of the ER lumen, thus allowing some proportion of the protein to pass ER quality control and undergo transport to the lysosome [6,11]. Similarly others have provided evidence that N370S GCase is impaired in its ability to exit from the ER [12]. Ambroxol (ABX) was uncovered as an enzyme-enhancing therapeutic agent for GCase using a thermal denaturation assay to screen the NINDS, National Institutes of Health, library of 1040 drugs that have been previously used to treat humans. This

chemical stabilizes GCase in a pH-dependent manner and significantly increases the activity and protein levels of N370S GCase in deficient fibroblasts; in ABX-treated Gaucher disease (N370S/N370S) lymphoblasts, the enzyme reaches therapeutically significant levels such that storage of glucosylceramide is reduced [13]. Collectively these data imply that the N370S mutation affects both the catalytic activity, as well as the conformational stability of the enzyme. Yet others still contend that the N370S GCase mutant protein is primarily a catalytically impaired mutant. Based on crystallization of an insect-recombinant human N370S GCase, these authors assert that the mutant protein has an identical folding to that of its normal recombinant counterpart. Interestingly, the authors further speculate that the active site-specific inhibitor NN-DNJ stabilizes and increases the lysosomal levels of N370S GCase by reducing its degradation in the lysosome, rather than in the ER lumen [14].

To resolve this conflicting literature and to determine whether pharmacological chaperones facilitate the post-ER transport of N370S GCase, the recombinant mutant and wild-type (WT) proteins, each equipped with a signal peptide, were produced in transgenic tobacco BY2 cells. The fact that plant cells do not have lysosomes eliminates the possibility of a chaperone-mediated stabilization of N370S GCase in these organelles. As in mammalian cells, the signal peptide effects the co-translational translocation of the recombinant protein into the ER lumen of the plant cell, which similarly provides an oxidizing environment of neutral pH [15,16]. In the absence of additional topogenic information, the protein (if transport-competent) will transit to the plant cell surface by a default mechanism.

We show that in the absence of a pharmacological chaperone, N370S GCase is impaired in its secretion presumably because of its inability to pass the ER protein quality control system. Further, treatment of the transgenic tobacco BY-2 cells with the ABX and NN-DNJ promoted the post-ER transport of N370S GCase, as shown by an increased amount of mutant protein in the media surrounding cultured BY-2 protoplasts. Thus the N370S GCase mutant protein is indeed capable of being 'chaperoned', which further suggest that the mutant protein is somehow impaired in its ability to obtain a functional folded conformation. The steady-state levels of mutant proteins inside the plant cells were also increased in the presence of these chemicals, suggestive of an increase in the concentration of folded mutant GCase in the ER, and/or in transit to the cell surface via the secretory pathway. Similar to the findings of others, there was additionally a significant impairment of the catalytic activity of the recombinant N370S GCase.

2. Materials and methods

2.1. Generation of constructs for production of WT and mutant GCase proteins in tobacco BY-2 cells

The N370S mutation was engineered into the WT cDNA (*GBA1*) clone using the Quick Change site-directed mutagenesis kit (Stratagene, Santa Clara, USA). To allow for secretion of mutant and WT GCase into the culture medium, a sequence encoding the signal peptide of proaleurain (MAHARVLLLALAVLATAAVAVA) was fused to the coding sequences specifying the WT and mutant GCase using the following pair of primers: 5'-GCTCGCCGTCCTGGCCACGGCCGCGTCGCGTCGCC-GCCCGCCCCTGCATCCCTAAAAG-3' (GBA-SPp-F1), 5'-CACCATGGCCCCAC-

CCCCGCGTCCTCCTCCTGGCGCTCGCCGTCCTGGCCACGG-3' (GBA-SPp-F2), and 5'-TCACTGGCGATGCCACAGGTAGG-3' (GBA-SPp-R). Re-amplified cDNA products were cloned into a Gateway entry vector pENTR/D-TOPO (Invitrogen, Burlington, Canada). The nucleotide sequences were then determined using the M13 reverse and forward universal primers. The resulting constructs containing sequences encoding WT and N370S GCase were sub-cloned into a Gateway expression vector (pSITE-0B) [17], which carries the neomycin phosphotransferase II gene (for kanamycin resistance) and the cauliflower mosaic virus (CaMV) 35S promoter (with a duplicated enhancer element; 2X35S) to drive expression of the WT and mutant genes.

2.2. Transformation of tobacco BY-2 cells

The expression constructs for production of WT-GCase and N370S-GCase were introduced into *Agrobacterium tumefaciens* (GV 3101) by electroporation. Transformed *Agrobacterium* cells were selected and used to transform tobacco BY-2 cells. Transformed tobacco BY-2 cells were transferred onto solid Murashige and Skoog (MS) media (Sigma, St. Louis, MO) containing kanamycin (100 µg/mL) and cefotaxime (300 µg/mL) and incubated at 28 °C for 3 to 4 weeks until transformed colonies were formed [18,19]. Resistant cell colonies (at least 30 per construct) were maintained on solid MS culture medium and subcultured twice a week.

2.3. Northern blot analyses of transgenic BY-2 lines

Total RNA was extracted (from 13 individual lines per construct) using Trizol reagent following the manufacturer's instructions (Invitrogen). Bulk RNA samples (15 µg) were separated on formaldehyde-denaturing agarose gels (1.2%, w/v) and blotted onto positively charged nylon membranes as described in Sambrook et al. [20]. RNA was fixed to the nylon membranes and the blots were pre-hybridized in 10 mL hybridization solution (100 mM NaH₂PO₄, 50 mM Na₂P₂O₇, 1 mM EDTA and 7% SDS) for 2 h at 65 °C with hybridization proceeding in the presence of a radiolabelled full-length cDNA probe for human *GBA1* at 65 °C overnight. Following hybridization, the blot was washed three times in 2X SSC, 0.1% SDS at room temperature, twice in 1X SSC, 0.1% SDS at 65 °C, and once in 0.5X SSC, 0.1% SDS at 65 °C. The blot was exposed to X-ray film (Kodak, Rochester, USA) and was developed after 1 h.

2.4. Protein isolation from tobacco BY2 cells grown on solid media and from BY2 protoplasts

BY2 cells (100 mg) grown on solid media were ground on ice in a 1.5-mL microcentrifuge tube using glass beads in 100 µL GCase extraction buffer (50 mM sodium phosphate pH 6.0, 50 mM NaCl, 0.1% sodium taurocholic acid, 1 mM EDTA, 0.5 mM PMSF). After centrifugation at 14 000 *g* for 15 min at 4 °C, the supernatant was removed and transferred to a new microcentrifuge tube. To isolate protein from BY2 protoplasts, protoplasts (2×10⁵) were vortexed in the presence of glass beads in 100 µL GCase extraction buffer. Cell debris was removed by centrifugation at 14,000 *g* for 15 min at 4 °C and the supernatant used for western blot analysis. Protein concentrations were determined using the Bio-Rad DC™ (Bio-Rad Laboratories, Mississauga, Canada) protein assay kit and BSA as a standard.

2.5. Immunoprecipitation of GCase and GCase activity assays

GCase proteins (WT and N370S) were immunoprecipitated using a mouse polyclonal anti-GCase antibody (Novus Biologicals, Oakville, Canada) prior to GCase activity assays. Ten micrograms of anti-GCase antibody was incubated with protein G sepharose beads (BioVision, Mountain View, USA) in PBS pH 7.4, containing 0.02% Tween-20 for 1 h at 4 °C with rotation. Beads were plated and incubated with 100 µg of total soluble protein extracts from WT- and N370S-GCase (high expressing lines) for 1 h at 4 °C. Following three washes (5 min each) in wash buffer (PBS pH 7.4 containing 0.5% Tween-20), the beads were re-suspended in 25 µL citrate phosphate buffer (200 mM, pH 5.5). GCase assays were initiated by adding buffer comprised of citrate phosphate (200 mM, pH 5.5), 0.4% taurocholic acid and 10 mM substrate (4-MUGP) to each sample and incubating at 37 °C for 1 h. The assay was terminated by increasing the pH to 10.5 by adding 200 µL of 0.1 M 2-amino-2-methyl-1-propanol (Sigma, Oakville, Canada). GCase activities were determined by the rate of release of the fluorescent product 4-methylumbelliferone (4-MU) monitored by a Spectramax M2e (Molecular Devices Corp, Sunnyvale, USA) fluorometer (excitation, 360 nm; emission, 450 nm). One unit of enzyme activity was defined as 1 nmol of 4-MU produced per minute. To demonstrate the efficiency of the immunocapture step (particularly its equivalence for the WT versus the N370S GCase), protein G beads were boiled for 5 min in 30 µL of SDS sample loading buffer to elute the captured GCase. Similar amounts of eluted proteins (WT and N370S GCase) were fractionated on 12% SDS-PAGE followed by western blot using a mouse monoclonal anti-GCase 2E2 antibody (Novus Biologicals, Oakville, Canada).

2.6. Western blot analyses

Protein extracts from BY2 transgenic lines (10 µg total soluble protein) were separated by 12% SDS-PAGE and transferred onto Amersham Hybond-P (PVDF) membranes. The blots were blocked overnight at room temperature using 5% (w/v) non-fat dry milk in 10 mM Na₂HPO₄, 150 mM NaCl, and 0.1% (v/v) Tween-20 (PBST) followed by 3 washes (15 min each) with PBST. Blots were incubated with the primary, polyclonal anti-GCase antibodies (1: 5000 dilution) for 3 h at room temperature. After 3 washes with PBST (15 min each) the membrane was incubated with an anti-rabbit HRP (horseradish peroxidase) secondary antibody (1:10,000) for 1 h at room temperature. An enhanced chemiluminescence (ECL) advance western blotting kit (GE Healthcare, Quebec, Canada) was used to detect WT- and N370S-GCase. The same blot was then stripped with guanidine hydrochloride stripping buffer (6 M GnHCl, 0.2% Nonidet (NP-40), 0.1 M β-Mercaptoethanol, 20 mM Tris-HCl, pH 7.5) for 10 min [21] and re-probed with an anti-tubulin or an anti-GAPDH (plant cytosolic NAD-specific glyceraldehyde-3-phosphate dehydrogenase) primary antibody as a loading control.

2.7. Protoplast isolation from tobacco BY2 solid cultures

Tobacco BY2 cells were grown on solid MS medium for 10 d. Ten grams of cells were incubated in 25 mL of enzyme solution (1.2% cellulase “Onozuka RS”, 0.6% Macerozyme R-10 (Yakult Pharmaceutical Ind. Co. Ltd., Tokyo, Japan), 0.5 M mannitol, 10 mM CaCl₂, 12 mM NaOAc) overnight at 28 °C on a rotary shaker at 40 rpm. Protoplasts were filtered

through a 40- μ m mesh (Sigma) and centrifuged in a 50 ml conical tube at 200 *g* for 5 min. The supernatant was discarded and protoplasts were washed with W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.7) at 200 *g* for 5 min. Protoplasts were collected and re-suspended in protoplast culture medium (4.3 g/L MS salts, 100 mg/L myo-inositol, 1 mg/L thiamine, 0.2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), 255 mg/L KH₂PO₄, 30 g/L sucrose, pH 5.7) [22]. The number of protoplasts was estimated by counting with a haemocytometer (Hausser Scientific, Horsham, USA). Viability of protoplasts was determined with 0.02% solution of Evans blue (Sigma) prepared in protoplast culture medium.

2.8. Precipitation of secreted protein from the medium of cultured BY2 protoplasts

For protein precipitation from the cultured media of protoplasts, the culture medium was mixed with chilled acetone in a ratio of 1:5 with gentle shaking and incubated at -20 °C for 1 h, followed by centrifugation at 10,000 *g* for 30 min. The resulting precipitates were re-suspended in GCase extraction buffer and fractionated by SDS-PAGE, followed by western blot analysis as previously described.

2.9. Determination of IC₅₀ values of ambroxol for human WT- and N370S-GCase at pH 5.5 and 7.0

The inhibitory activity of ambroxol (ABX) against WT- and N370S-GCase was evaluated using either purified human GCase (Cerezyme™, Genzyme, USA) or GCase bearing the N370S mutation present in lysates of Gaucher disease patient fibroblasts homozygous for mutation, c.1226A3/c.1226A3G (Hospital for Sick Children, Tissue Culture Facility, Toronto, ON), as described previously [13]. Lysates of Gaucher disease patient fibroblasts from a confluent 175 cm² tissue culture flask were prepared by 10 freeze-thaw cycles in 10 mM sodium phosphate (pH 6.5), 5% glycerol (100 μ L) and then diluted 3-fold in 100 mM citrate phosphate buffer pH 5.5 or 7.0 containing 0.4% taurodeoxycholate. An aliquot (25 μ L) of patient lysate or purified Cerezyme™ containing the same number of units was used to determine the IC₅₀ of ABX in 100 mM citrate-phosphate buffer, 0.2% taurodeoxycholate and 2.5 mM 4-methylumbelliferyl- β -D-glucopyranoside (4-MUGP) according to Maegawa et al. (2009) [13].

3. Results

3.1. Steady-state levels of transcripts and protein (WT-GCase and N370S-GCase) in tobacco BY2 cell lines are variable

Tobacco BY2 cells were stably transformed with the GCase gene constructs (Fig. 1) and regenerated lines were screened by PCR. Northern blot analysis of the PCR-positive transgenic cell lines showed that the steady-state level of transcripts corresponding to both WT- and N370S-GCase was variable amongst the population of lines in which abundant transcript was detected (Figs. 2A, B). Of thirty lines screened (for WT-GCase and N370S GCase), all were PCR positive, and about 25% and 30% of the lines expressed abundant transcripts corresponding to N370S and WT GCase, respectively. Western blot analysis of selected high-expressing lines transgenic for WT- and mutant-GCase (6 lines per construct) detected variable levels of an ~60 kDa protein (Figs. 2A, B). In general N370S protein levels

were equivalent or slightly lower than those of WT-GCase in individual transgenic lines, particularly in light of the steady-state levels of transcripts in these lines (Figs. 2A, B).

3.2. Plant recombinant N370S-GCase is catalytically impaired

GCase activities of WT- and N370S-transgenic lines were determined using the same lines selected for western blot analyses (Figs. 2A, B). In the tobacco BY-2 cells there are several endogenous plant β -glucosidases with activities that interfere with detection of that corresponding to the bonafide (i.e., human) GCase. Therefore the WT and N370S GCase proteins were immunoprecipitated with a mouse polyclonal anti-GCase antibody prior to determination of GCase activities. Untransformed tobacco BY2 cells served as the control for immunocaptured GCase activity; these values (0.05 nmol/mg protein/min) were subtracted from the values presented in Fig. 2. Purified WT-GCase immunocaptured from the selected transgenic lines exhibited specific activity levels of 0.51–1.36 units per mg total soluble protein (TSP) (Fig. 2A). The purified N370S GCase enzymes were catalytically active, but exhibited much lower specific activities as compared to the WT-GCase enzymes, which ranged from 0.024 to 0.24 units per mg protein (Fig. 2B). Western blot analyses confirmed that there was equal immunocapture of the WT GCase and the mutant GCase prior to the enzyme activity determinations (Fig. 2C).

Of the various lines expressing GCase, N370S line 21 and GCase line 60 were selected for further analyses to examine the effects of pharmacological chaperones (Sections 3.4 and 3.5). For verification of general trends, the same analyses were conducted on an additional N370S GCase line (line 24).

3.3. Confirmation that ambroxol is an effective active-site directed inhibitor of N370S GCase

As compared to the WT GCase, the N370S mutation has been reported to increase the K_i of some active-site directed inhibitors by ~4-fold [23]. To determine if ambroxol (ABX) behaved in a manner consistent with it being an active-site directed pharmacological chaperone for the N370S GCase, we compared the IC_{50} s of ABX at neutral and acidic pH, against purified commercial GCase (Cerezyme) and the N370S GCase from the lysate of a patient fibroblast cell line homozygous for the mutation (Fig. 3). At the acidic pH of 5.5 the IC_{50} of ABX for the WT GCase was 26 μ M, as compared to 58 μ M for the N370S GCase (~2-fold increase). Shifting to pH 7.0, the neutral pH of the ER, the IC_{50} shift between the two forms of the enzyme was only barely significant, 1.1-fold. However the magnitude of the decrease in IC_{50} s caused by the increased pH was ~5-fold for the WT, 5.7 μ M, and ~9-fold for the N370S, 6.4 μ M (Fig. 3A). Thus, at the neutral pH of the ER, ABX binds with similar strength to either the WT GCase or the N370S mutant. Additionally its pH-dependent advantage as a pharmacological chaperone, i.e., tighter binding at neutral pH than at acidic pH, is even more pronounced with the mutant.

It could be argued that undegraded glucocerebrosides present in the Gaucher disease fibroblast lysates may interfere with the IC_{50} determinations in Fig. 3A, as they could bind to the catalytic site of mutant GCase. To confirm that this was not the case, the lysate from a L444P homozygous fibroblast line, containing ~3% of normal endogenous GCase activity,

was spiked with a small amount of Cerezyme, such that its specific activity resembled that of the N370S homozygous cell lysate (~20% of normal). This was followed by construction of an IC₅₀ curve with ABX; notably the two curves corresponding to the spiked lysate versus pure Cerezyme were superimposable (Fig. 3B). Thus if stored glucocerebrosides exist in the lysate of Gaucher cells, they are insufficient to affect the GCase assay that uses saturating levels of an artificial substrate (Fig. 3B).

3.4. Pharmacological chaperones facilitate the post-ER secretion of N370S GCase into the surrounding media of transgenic protoplasts

To standardize cell numbers and allow for secretion studies, protoplasts (plant cells without their surrounding cell walls) were isolated from the tobacco BY2 transgenic cells. The viability of protoplasts and the secretion of WT GCase into the medium were determined daily from days 1–5. Day 2 and day 3 protoplasts retained reasonable viability (~80%), and further exhibited efficient protein secretion, and so these time points were selected as optimum incubation times for subsequent experiments.

To determine whether pharmacological chaperones facilitate the post-ER transport of mutant GCase, we examined the effects of the previously characterized chaperones, NN-DNJ and ABX, on the secretion of GCase into the culture medium surrounding the transgenic protoplasts. In contrast to the WT-GCase protein, which was efficiently secreted from tobacco BY2 cells, and detected in large amounts in the culture medium, only a small proportion of the N370S GCase was secreted in the absence of pharmacological chaperone (Figs. 4A and B; 0 μ M ABX and 0 μ M NN-DNJ). Treatment of N370S-GCase transgenic protoplasts with ABX or NN-DNJ promoted the secretion of N370S mutant protein into the surrounding media, with the maximum amounts of GCase protein present in the culture medium as a result of the 30 μ M ABX (3-d) treatment and the 20 μ M NN-DNJ (2-d) treatment. Interestingly ABX and NN-DNJ also mediated an increase in the secretion of WT GCase into the culture media (e.g. when applied at 40–50 μ M and at 10 μ M, respectively). These results clearly indicate that the pharmacological chaperones enhanced the amount of N370S GCase that is transported beyond the ER along the secretory pathway.

The inclusion of a protease inhibitor cocktail in the protoplast culture medium during incubation had no effect on the stability of the secreted GCase (data not shown).

3.5. Pharmacological chaperones increase the accumulation of N370S GCase protein inside protoplasts

The positive effect of pharmacological chaperones on facilitating the secretion of mutant GCase is presumably mediated through facilitating protein folding in the ER. We further sought to determine whether these compounds stabilize the mutant protein inside the protoplasts. The 3-d ABX-treated and 2-d NN-DNJ-treated protoplasts showed significantly higher levels of N370S GCase protein as compared with untreated controls; a positive and similar effect was noted across the range of concentrations of the two pharmacological chaperones that were tested (Fig. 5A). Interestingly, significant cytotoxicity was observed when the plant cell protoplasts were treated with high concentrations of the chemicals (>30 μ M NN-DNJ and >60 μ M ABX) (data not shown).

Treatment with the chemical chaperones had no significant effect on the steady-state intracellular protein levels of WT GCase in ABX- and NN-DNJ-treated protoplasts (Fig. 5B).

4. Discussion

Gaucher disease is a prevalent lysosomal storage disease that affects 1 in 60,000 people in the general population; its incidence is greater in the Ashkenazi Jewish population, in which it affects 1 in 800 people. One of the most prevalent disease-causing mutations in human *GBA1*, the gene encoding lysosomal GCase, is a missense mutation, N370S. There are two views currently in the literature concerning the function and fate/homeostasis of this mutant enzyme. One view is that the N370S mutant GCase possesses reduced catalytic activity, but is able to fold normally; affected cells exhibit normal steady-state GCase protein levels. This view is partly based on the comparative analysis of the crystal structure of an insect recombinant human N370S GCase as compared to the WT protein. These authors also assert that the major mechanism by which pharmacological chaperones enhance lysosomal levels of N370S GCase is by reducing its degradation within the lysosome rather than by assisting the mutant protein with folding in the ER, a process which facilitates its subsequent trafficking out of this compartment [14]. The other camp contends that the N370S GCase protein is compromised in its folding in the ER, making it subject to ERAD, instead of undergoing proper folding and trafficking to the lysosome [5,7,12,24]. Pharmacologic chaperones, such as NN-DNJ and isofagomine, are thought to be effective as *enzyme enhancement therapies* for this disease-causing mutation because they stabilize the folded and near-folded forms of the N370S enzymes in the ER. This in turn increases the population of folded mutant GCase enzymes that can pass ER quality control and undergo subsequent trafficking to the lysosome. The enzyme becomes active upon its arrival in the lysosome because the small molecule is replaced (out-competed) by the large accumulation of the natural GCase substrate (glucosylceramide); displacement of the pharmacological chaperone may also be facilitated by the low pH of this organelle. For example, ABX loses its ability to inhibit the enzyme below pH 4.7 [13]. In order to confirm that the interaction of ABX with the N370S form of GCase was comparable to that of the WT GCase, we determined the IC₅₀s of each enzyme at pH 5.5 and 7.7. At pH 5.5 the binding of ABX to the WT enzyme was ~2-fold greater than the N370S mutant form. However, at pH 7.0, near the pH of the ER, the IC₅₀s of the two forms of the enzyme were nearly identical.

To rule out the possibility of chaperone-mediated lysosomal stabilization of mutant GCase, we generated the N370S GCase and its WT counterpart in transgenic tobacco BY2 cells, eukaryotic cells in which lysosomes do not exist. To allow for efficient secretion of the WT and mutant recombinant human proteins in the plant host [25,26], the constructs utilized sequences specifying the signal peptide of the barley cysteine protease proaleurain [27] in place of those encoding the native signal peptide. As no additional targeting information was added, the recombinant proteins (if transport-competent) are expected to transit along the secretory pathway to the plant cell surface by a default mechanism.

We were most interested in the N370S GCase transgenic lines that exhibited robust levels of steady-state GCase transcripts; for several of the lines the transcript levels approximated those of the WT-GCase lines. Western blot detection of GCase protein in total soluble

protein (TSP) extracts from these lines (10 μ g TSP) indicated that GCase protein levels associated with the mutant GCase lines were generally equivalent or slightly lower than those of the WT lines, but to a surprisingly little extent. Because we did not examine the rate of turnover/degradation of the two proteins we cannot state that the N370S-GCase and normal GCase protein are equally stable. Certainly there was considerable variability across all lines (regardless of the GCase construct) in terms of the transcript to protein ratios. A comparative analysis of the activities of the recombinant N370S versus WT GCase (in GCase-immunopurified extracts of BY-2 cells), revealed that the N370S GCase is catalytically impaired, with the specific activities associated with the N370S transgenic lines significantly lower than those of the WT-GCase lines. In transfected NIH 3T3 (mouse embryonic fibroblast) cells, transcript and protein levels corresponding to the N370S GCase versus the WT-GCase are comparable [28], although this work presented only a single transfected 3T3 cell line. The N370S GCase of 3T3 cells exhibits reduced catalytic activity [28] similar to the mutant protein generated in Sf9 insect cells, with the specific activities in both hosts being ~10–20% of those associated with the recombinant WT-GCase [9,10]. Thus in many ways our findings parallel those of others and further support the contention that catalytic deficiency (impaired function) is likely a contributing factor underlying Gaucher disease pathophysiology in N370S/N370S patients.

Our findings of the effects of two pharmacological chaperones, NN-DNJ and ABX, show that both of these small molecules facilitate the post-ER trafficking of N370S GCase, resulting in enhanced secretion of the mutant protein into the culture medium of the transgenic protoplasts. This effect likely occurs through the pharmacological chaperone increasing the proportion of stabilized (folded and near-folded) forms of the N370S enzymes in the ER; a greater proportion of the synthesized enzymes are able to avoid ERAD and to pass the quality control systems of the ER. Thus these transport-competent enzymes undergo subsequent trafficking along the endomembrane system, which in the plant heterologous host cells leads to secretion. In the absence of pharmacological chaperone, only a small proportion of the mutant protein was secreted by the cultured protoplasts, in contrast to the WT-GCase, which was efficiently secreted. Impaired lysosomal trafficking and slower transport of mutant GCase as compared to the WT-GCase is evident in cultured fibroblast cells from two patients homozygous for the N370S mutation [5]. More compelling evidence comes from subcellular localization studies of GCase in N370S/N370S fibroblasts, in which the mutant GCase co-localizes with protein disulfide isomerase, a marker of the ER lumen. The pharmacological chaperone ABX (in which cells are treated with 40 μ M ABX for 5 d) leads to co-localization of the N370S GCase with a lysosomal marker protein, and a corresponding reduction in its ER localization, indicative of transport of the mutant GCase from the ER to lysosomes [13].

ABX enhances the GCase activity of deficient (patient) fibroblasts when applied at 5–60 μ M [13]. The concentrations effective in promoting secretion of N370S GCase from the plant protoplasts were 10–60 μ M, with 30 μ M showing the greatest effect. NN-DNJ is most effective in enhancing the GCase activity of mutant fibroblasts when applied at concentrations below 30 μ M; a treatment of 10 μ M for 9 d leads to a 2-fold increase in the GCase activity in these cells [6]. Similarly, in the present study, enhanced secretion of the

N370S GCase from cultured protoplasts occurred as a result of treatments with 10–30 μ M NN-DNJ, with 20 μ M appearing to be the most effective concentration.

ABX and NN-DNJ not only facilitated secretion of N370S GCase from protoplasts, but further increased the steady-state levels of intracellular GCase in these cells. This could be the result of a stabilization of N370S GCase through direct interactions, since these pharmacological chaperones are active site-specific inhibitors. These direct interactions likely stabilize the folded and partly folded mutant GCase enzymes in the ER, allowing a greater proportion of the synthesized protein to escape ERAD [3,11,12]. Upon passing the ER quality control mechanisms the proteins are able to traffic out of the ER to the cell surface via transit through the secretory pathway.

5. Conclusions

Our present work provides evidence that N370S GCase is a folding mutant and is impaired in its ability to exit the lumen of the ER. Further, N370S GCase is capable of being ‘chaperoned’.

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Abbreviations

ABX	Ambroxol
GCase	β -glucocerebrosidase
NN-DNJ	N-(n-nonyl) deoxynojirimycin

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Fig. 1.

Expression constructs in pSITE-0B vector for generating recombinant WT and N370S GCase in tobacco BY-2 cells. The 5' upstream regulatory sequences included a cauliflower mosaic virus (CaMV) 35S promoter with a duplicated enhancer element, and the translational enhancer element (5' UTR) from the tobacco etch virus (TEV). Both WT and N370S GCase constructs contained in-frame sequences encoding the proaleurain signal peptide, and a 3' end comprised of the CaMV 35S transcription terminator and associated downstream sequences. The *GBA1* coding sequences encoding WT or N370S mutant GCase are also shown.

Fig. 2.

Steady-state GCCase transcripts and protein levels and specific activities associated with independent transgenic BY2 tobacco lines expressing the WT (A) and N370S mutant (B) gene constructs. Northern blot analyses (top panels) were based on total RNA (15 µg) isolated from 10-d-old individual transgenic lines. A radiolabeled full-length cDNA probe was used to detect the GCCase transcripts. Ethidium bromide staining of ribosomal RNA is shown at the bottom. For western blot analysis (middle panels), total soluble protein (10 µg) from 6 selected lines was fractionated by 12% SDS-PAGE, prior to analysis using a polyclonal anti-GCCase antibody. The marker protein GAPDH (NAD-specific glyceraldehyde-3-phosphate dehydrogenase) was used as a control for loading on the same membranes following stripping of membranes and incubation with GAPDH antibodies. Western blots were repeated three times with similar results. For determination of GCCase specific activities (lower panels), GCCase of total soluble extracts from the selected lines was immunocaptured using protein G beads and a polyclonal mouse anti-GCCase antibody. The activity of immobilized GCCase on the protein G beads was determined by GCCase assays; enzyme specific activities are expressed as nmoles/mg/min of total soluble protein. Untransformed tobacco BY2 cells served as the control for immunocaptured GCCase activity; these values (0.05 nmol/mg protein/min) were subtracted from the values presented. Error bars represent the SE derived from three independent experiments. (C) To ensure that there was an equal immunocapture of the WT GCCase and the mutant GCCase, purified proteins from representative lines (N370S line 21 and GCCase line 60) were separately analyzed on a

western blot following the immunocapture step. Cerezyme and untransformed tobacco BY2 cells were used as positive and negative controls, respectively.

Fig. 3.

A. Dose response curves used to calculate IC₅₀ values of ABX for the WT GCase (Cerezyme) and N370S GCase from a homozygous Gaucher patient fibroblast line. Activities, relative to those obtained without ABX (set at 1.0 on the Y-axis) are plotted versus the log of the ABX concentration (X-axis) contained in the assay using 2.5 mM 4-MUGP. Triangles represent the values obtained from the N370S cell lysates and diamonds represent those from the WT GCase. Open triangles and diamonds correspond to data obtained at pH 5.5, solid markers to data obtained at pH 7.0. B. Control experiment to assess potential interference of any stored glucocerebrosides on the IC₅₀ determinations for ABX of Gaucher disease fibroblast lysates. The lysate from a L444P homozygous fibroblast line was spiked with a small amount of Cerezyme, such that its specific activity resembled that of the N370S homozygous cell lysate. IC₅₀ determinations were conducted and the curves of the spiked lysate versus the pure Cerezyme were compared.

Fig. 4.

Effect of ABX and NN-DNJ on N370S GCCase (A) and WT GCCase (B) secretion from transgenic protoplasts. Protoplasts (200,000 per mL) were incubated in culture medium with no pharmacological chaperone or in culture medium containing different concentrations of NN-DNJ or ABX (as indicated) for 2 and 3 days, respectively. Acetone-precipitated proteins from 0.5 mL media were subjected to western blot analyses. The experiment was repeated two times with similar results.

Fig. 5.

The effect of ABX and NN-DNJ on intracellular levels of N370S GCcase (A) and WT GCcase (B). Protoplasts (200,000 per mL) were incubated in culture medium with no pharmacological chaperone or in culture medium containing different concentrations of NN-DNJ or ABX (as indicated) for 2 and 3 days, respectively, prior to western blot analysis. Extracts from the transgenic protoplasts were used to determine the intracellular levels of N370S GCcase and WT GCcase. The marker proteins α -tubulin or GAPDH (NAD-specific glyceraldehyde-3-phosphate dehydrogenase) were used as controls for loading on the same membranes following stripping of membranes and incubation with α -tubulin or GAPDH antibodies. This experiment was repeated two times with similar results.