

Production of glucocerebrosidase with terminal mannose glycans for enzyme replacement therapy of Gaucher's disease using a plant cell system

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Summary

Gaucher's disease, a lysosomal storage disorder caused by mutations in the gene encoding glucocerebrosidase (GCD), is currently treated by enzyme replacement therapy using recombinant GCD (Cerezyme[®]) expressed in Chinese hamster ovary (CHO) cells. As complex glycans in mammalian cells do not terminate in mannose residues, which are essential for the biological uptake of GCD via macrophage mannose receptors in human patients with Gaucher's disease, an *in vitro* glycan modification is required in order to expose the mannose residues on the glycans of Cerezyme[®]. In this report, the production of a recombinant human GCD in a carrot cell suspension culture is described. The recombinant plant-derived GCD (prGCD) is targeted to the storage vacuoles, using a plant-specific C-terminal sorting signal. Notably, the recombinant human GCD expressed in the carrot cells naturally contains terminal mannose residues on its complex glycans, apparently as a result of the activity of a special vacuolar enzyme that modifies complex glycans. Hence, the plant-produced recombinant human GCD does not require exposure of mannose residues *in vitro*, which is a requirement for the production of Cerezyme[®]. prGCD also displays a level of biological activity similar to that of Cerezyme[®] produced in CHO cells, as well as a highly homologous high-resolution three-dimensional structure, determined by X-ray crystallography. A single-dose toxicity study with prGCD in mice demonstrated the absence of treatment-related adverse reactions or clinical findings, indicating the potential safety of prGCD. prGCD is currently undergoing clinical studies, and may offer a new and alternative therapeutic option for Gaucher's disease.

Keywords: cell culture, Gaucher's disease, glucocerebrosidase, mannose, plant.

Introduction

Gaucher's disease is the most prevalent lysosomal storage disorder, and occurs with a particularly high frequency in the Ashkenazi Jewish population (Lee, 1982; Grabowski, 1993; Beutler and Grabowski, 2001). It is caused by mutations in the gene encoding glucocerebrosidase (GCD), a lysosomal enzyme that catalyses the hydrolysis of glucosylceramide (glucocere-

broside, GlcCer), leading to GlcCer accumulation, primarily in the lysosomes of macrophages. The characteristic storage cells, known as Gaucher cells, are found in the liver, spleen and bone marrow. Associated clinical symptoms include hepatosplenomegaly, anaemia, thrombocytopenia and skeletal deterioration (Grabowski and Hopkin, 2003; Jmoudiak and Futerman, 2005).

GCD consists of 497 amino acids, and contains five putative *N*-glycosylation sites, four of which are normally occupied

(Berg-Fussman *et al.*, 1993; Brumshtein *et al.*, 2006). Glycosylation is essential for the production of an active protein, with both high-mannose (Man) and complex oligosaccharide chains found in GCD (Berg-Fussman *et al.*, 1993). As Gaucher's disease is a monogenic disease, it can be treated by replacement of the defective gene product, GCD; hence, the rationale underlying 'enzyme replacement therapy' (ERT) (Barton *et al.*, 1991; Pastores *et al.*, 1993; Grabowski *et al.*, 1995; Weinreb *et al.*, 2002; Grabowski and Hopkin, 2003; Beutler, 2004). An effective clinical outcome is achieved in patients with Gaucher's disease treated with recombinant GCD expressed in mammalian Chinese hamster ovary (CHO) cells: Cerezyme® (Grabowski *et al.*, 1995; Weinreb *et al.*, 2002; Jmoudiak and Futerman, 2005).

In order for GCD to be effective in ERT, terminal Man residues must be present on the glycan chains to permit binding to macrophage Man receptors, and subsequent internalization (Sato and Beutler, 1993; Bijsterbosch *et al.*, 1996). Cerezyme® production involves sequential *in vitro* deglycosylation, using α -neuraminidase, β -galactosidase and β -N-acetylglucosaminidase, to expose terminal Man residues, a procedure which dramatically improves targeting and internalization (Furbish *et al.*, 1981; Doebber *et al.*, 1982; Bijsterbosch *et al.*, 1996; Friedman *et al.*, 1999).

The objective of this study was to provide a novel and alternative plant-based method for the industrial-scale production of GCD for use in ERT. The use of plant cell cultures for the production of human biopharmaceutical proteins has been under evaluation in recent years (Hellwig *et al.*, 2004). Plant cell cultures offer several advantages over both field-grown transgenic plants and mammalian cell cultures. They are cost-effective, do not involve the use of mammalian-derived components in the manufacturing process, have high batch-to-batch reproducibility, allow precise control over the growth process, and enable compliance with current Good Manufacturing Procedures (Hellwig *et al.*, 2004).

In this report, the expression of a recombinant human GCD in a plant cell expression system, based on transgenic carrot cells grown in suspension culture, is described, which is capable of the production of glycoproteins. It is shown that GCD expression in carrot cells (prGCD), together with its targeting to the storage vacuole, generates a protein with terminal Man structures *in vivo*, thus precluding the need for post-production enzymatic modification *in vitro* (Lerouge *et al.*, 1998; Friedman *et al.*, 1999; Gomord and Faye, 2004). It is demonstrated that prGCD is structurally highly homologous to Cerezyme®, and displays comparable enzymatic activity and uptake into macrophages. Furthermore, a single-dose safety study in ICR (CD-1®) mice, with doses up to 18 mg/kg

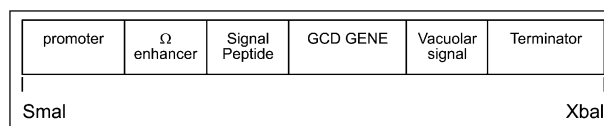


Figure 1 Glucocerebrosidase (GCD) gene used in the plant expression system. The expression cassette consists of the cauliflower mosaic virus (CaMV) 35S promoter, the tobacco mosaic virus (TMV) omega translational enhancer element, a signal peptide, the human GCD sequence, a vacuolar targeting signal and the octopine synthase terminator sequence from *Agrobacterium tumefaciens*.

administered by intravenous infusion, demonstrates that there are no adverse reactions, clinical or pathological findings, indicating the potential safety of the plant cell-expressed GCD.

Results

GCD expression in carrot cells

A number of modifications in the native human GCD sequence were made in order to permit its efficient expression and activity. The GCD signal peptide was replaced by that of the *Arabidopsis thaliana* basic endochitinase gene to facilitate co-translational translocation into the endoplasmic reticulum (ER) (Samac *et al.*, 1990), and the storage vacuole targeting signal from tobacco chitinase A, encoding the sequence DLLVDTM (Neuhaus *et al.*, 1991), was fused to the C-terminus to facilitate GCD targeting via the ER to this organelle (Figure 1). Plant storage vacuoles are generally considered to contain relatively low levels of proteases (Neuhaus and Rogers, 1988), and some studies also indicate that plant vacuoles may possess activities that expose terminal Man on complex glycans (Lerouge *et al.*, 1998). A number of antibiotic-resistant transgenic calli were obtained. Many of them expressed a protein band with the expected size of a glycosylated GCD, which cross-reacted in immunoblots with antihuman GCD antibodies. Such a protein band was not detected in control non-transformed calli, verifying its identity as prGCD (data not shown).

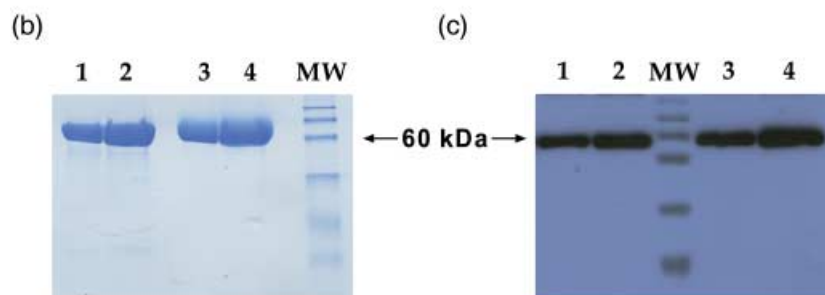
Purification and characterization of prGCD

prGCD was solubilized using Triton X-100 in the presence of an antioxidant, and purified to homogeneity by cation exchange and hydrophobic chromatography (Figure 2b,c). Amino acid sequencing demonstrated that the prGCD sequence corresponds to that of human GCD (Swiss Prot P04062, protein ID AAA35873) (Figure 2a), and includes two additional amino acids (EF) at the N-terminus (designated -2

(a)

	1	9	19	29
-2	EFARPCIPKS	FGYSSVVCVC	NATYCDSFDP	PTFPALGTFS
39	RYESTRSGRR	MELSMGPIQA	NHTGTGLLLT	LQPEQKFQKV
79	KGFGGAMTDA	AALNILALSP	PAQNLLKSY	FSEEGIGYNI
119	IRVPMASCDF	SIRTYTYADT	PDDFQLHNFS	LPEEDTKLKI
159	PLIHRALQLA	QRPVSLASP	WTSPTWLKTN	GAVNGKGLK
199	GQPGDIYHQT	WARYFVKFLD	AYAETHKLQFW	AVTAENEPSA
239	GLLSGYPFQC	LGFTPEHQRD	FIARDLGPTL	ANSTHHNVRL
279	LMLDDQRLLL	PHWAKVVLTD	PEAAKYVHGI	AVHWYLDFLA
299	PAKATLGETH	RLFPNTMLFA	SEACVGSKFW	EQSVRLGSWD
359	RGMQYSHSII	TNLLYHVVGW	TDWNLALNPE	GGPNWVRNFV
399	DSPIIVDITK	DTFYKQPMFY	HLGHFSKFIP	EGSQRVGLVA
439	SQKNLDLAVA	LMHPDGSVV	VVLNRSSKDV	PLTIKDPAVG
479	FLETISPGYS	IHTYLWHRQD	LLVDTM	

Figure 2 Characterization of recombinant plant-derived glucocerebrosidase (prGCD). (a) Amino acid sequence of prGCD. (b) prGCD (lanes 1 and 2; 5 and 10 μ g of protein, respectively) and Cerezyme[®] (lanes 3 and 4; 5 and 10 μ g of protein, respectively) were detected by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. (c) Western blotting using an anti-GCD antibody: prGCD, lanes 1 and 2 (50 and 100 ng of protein, respectively); Cerezyme[®], lanes 3 and 4 (50 and 100 ng of protein, respectively).



and -1 accordingly), derived from the linker used for the fusion of the signal peptide, and an additional seven amino acids at the C-terminus (designated 497–503), derived from the vacuolar targeting signal.

Glycan structures

Glycosylation was analysed (Glycobiology Center of the National Institute for Biotechnology, Ben Gurion University, Beer Sheba, Israel) to determine the glycan structure and glycan quantitative ratio using sequential digestion with various exoglycosidases (see 'Experimental procedures'). It was found that the *N*-linked glycans have a main core of two *N*-acetylglucosamine (GlcNAc) residues and a β 1–4-linked Man, attached to two additional Man residues in α 1–3 and α 1–6 linkages. The additional residues found are shown in Figure 3a, which presents all structures and their relative amounts based on high-performance liquid chromatography (HPLC), enzyme array digests and delayed extraction-matrix-assisted laser desorption ionization-time of flight-mass spectrometry (DE-MALDI-TOF-MS). Figure 3b shows the glycan structure of Cerezyme[®] before and after *in vitro* enzymatic processing. Notably, the analysis of the glycan structures of prGCD revealed that more than 90% of the glycans were Man rich,

bearing terminal Man residues (Figure 3a), whereas, in the case of Cerezyme[®], Man residues were exposed only after a complex *in vitro* procedure (Figure 3b). The dominant glycan in prGCD was the core structure found in most glycoproteins purified from pea, rice, maize and other edible plants (Lerouge *et al.*, 1998; Bardor *et al.*, 2003; Gomord and Faye, 2004). This structure contains a core α -(1,2)-xylose residue as well as a core α -(1,3)-fucose (Figure 3a). DE-MALDI-TOF-MS data contained no signals consistent with typical *O*-linked glycans. Further analysis of the glycan profiles for prGCD obtained from different production batches was performed in order to assess the batch-to-batch reproducibility of prGCD produced in the carrot cell system. As shown in Figure 4, the population of glycans on prGCD was highly reproducible between batches.

Enzymatic activity

The activity of prGCD was compared with that of Cerezyme[®] using a fluorescent GlcCer analogue (Meivar-Levy *et al.*, 1994). Similar specific activities were obtained (Figure 5), with V_{\max} values of $0.47 \pm 0.08 \mu\text{mol } N\text{-}[6\text{-}[(7\text{-nitrobenzo-}2\text{-oxa-}1,3\text{-diazol-}4\text{-yl)amino]hexanoyl]glucosylsphingosine}$ (C6-NBD-GlcCer) formed/min/mg protein for prGCD and

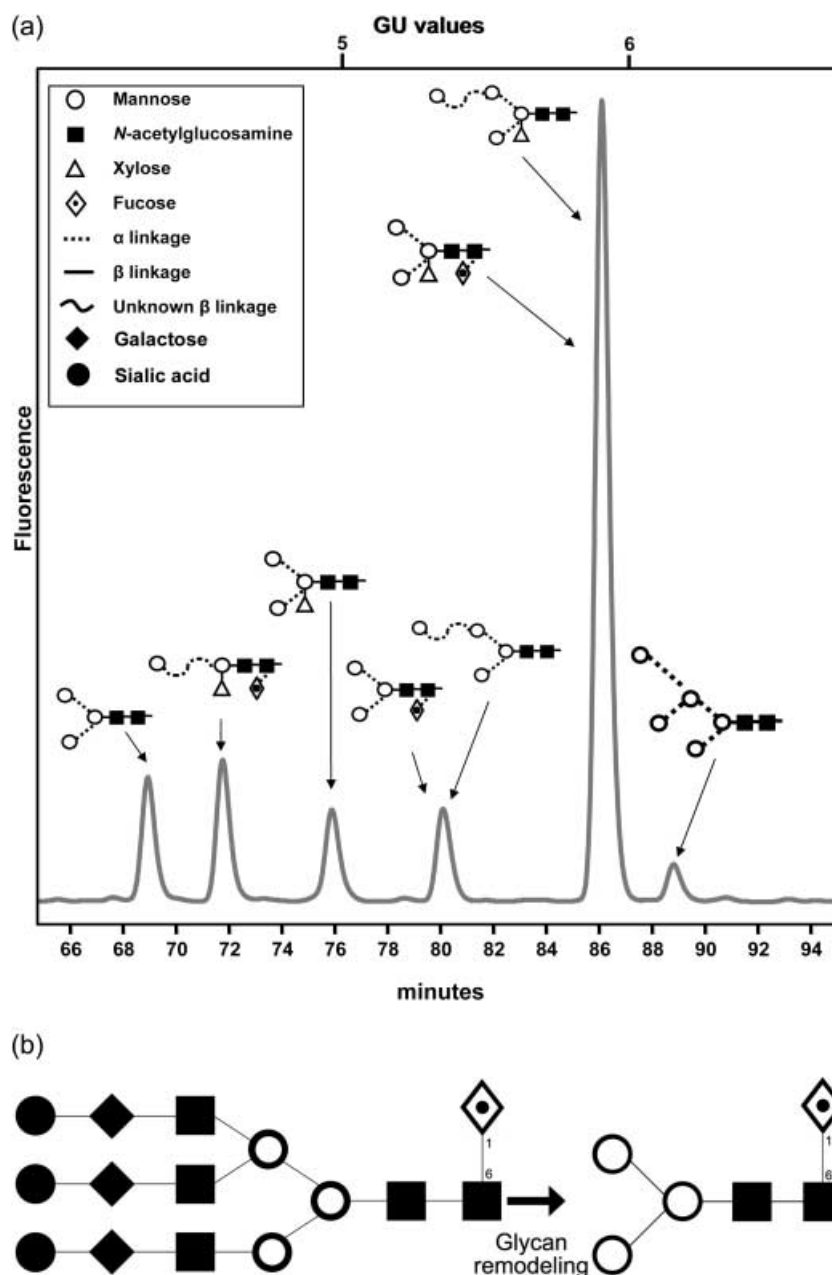


Figure 3 Glycan structures of recombinant glucocerebrosidase (GCD). (a) Major glycan structure analysis of recombinant plant-derived GCD (prGCD). In order to determine the glycan structure and ratios, sequential digestion with various exoglycosidases was performed. The glycans were fluorescently labelled, and sequencing of the glycan pool was achieved by high-performance liquid chromatography (HPLC) analysis. The retention times of individual glycans were compared with those of a standard partial hydrolysis of dextran giving a ladder of glucose units (GU). (b) Major glycan structure of GCD expressed in Chinese hamster ovary (CHO) cells before and after enzymatic deglycosylation.

$0.43 \pm 0.06 \mu\text{mol}$ for Cerezyme[®], and similar K_m values ($20.7 \pm 0.7 \mu\text{M}$ for prGCD and $15.2 \pm 4.8 \mu\text{M}$ for Cerezyme[®]). These kinetic studies show that the activity of prGCD is similar to that of the CHO-expressed enzyme.

Uptake and activity of prGCD by macrophages

The targeting and uptake of GCD by macrophages is mediated by the Man/GlcNAc receptor, and can be determined using murine thioglycolate-elicited peritoneal macrophages (Stahl and Gordon, 1982). Our results demonstrated that

prGCD, expressed and purified from transformed carrot cells, was taken up and targeted macrophage cells specifically through Man/GlcNAc receptors without any need for remodelling *in vitro*. Furthermore, the uptake and cellular activity of prGCD was higher than that of Cerezyme[®] at higher concentrations (Figure 6a). Similar results were obtained using cells isolated from patients with Gaucher's disease (data not shown). The addition of mannan inhibits the specific uptake and internalization of both prGCD (Figure 6b) and Cerezyme[®] (Figure 6c), confirming that prGCD uptake is mediated via Man receptors.

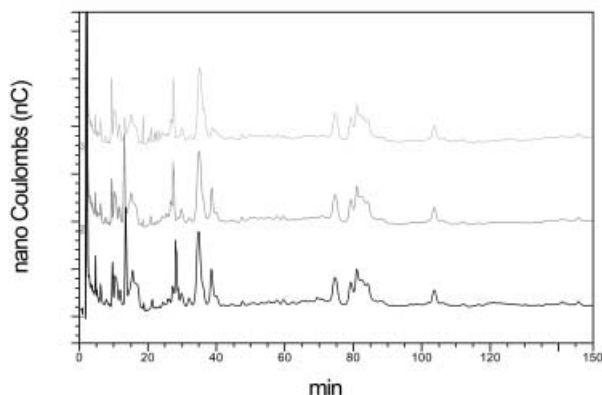


Figure 4 Batch-to-batch reproducibility of the glycan profile of recombinant plant-derived glucocerebrosidase (prGCD). The glycoforms of prGCD (*N*-glycans) present in three independent batches were analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), following digestion with trypsin and peptide *N*-glycosidase A (PNGase A).

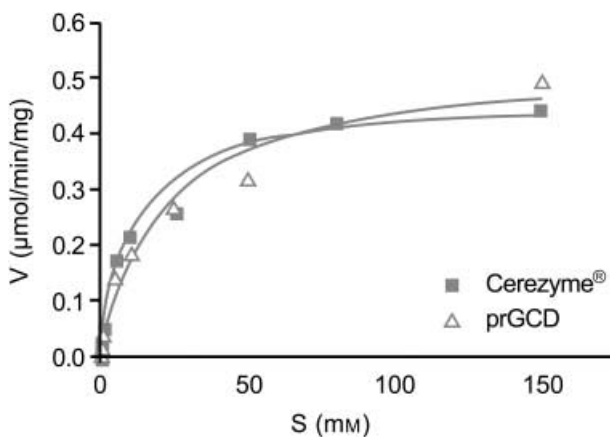


Figure 5 Kinetic analysis of glucocerebrosidase (GCD). Recombinant plant-derived GCD (prGCD) and Cerezyme® (0.2 μg) were assayed using *N*-[6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]-glucosylsphingosine (C6-NBD-GlcCer) (5 min, 37 °C) in 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (50 mM, pH 5.5). The reaction was stopped by the addition of chloroform–methanol, prior to the extraction and analysis of fluorescent lipids. Michaelis–Menten kinetics were analysed using GraphPad Prism software. The data are the means of two independent experiments.

X-Ray structure

The crystal structure of prGCD was determined by X-ray crystallography. prGCD crystallized in a P21 space group with two molecules in the asymmetric unit (Table S1, see 'Supplementary material'). It comprises three domains (Figure 7a). Domain I (residues 1–27 and 384–414) consists of a three-stranded anti-parallel β -sheet flanked by a perpendicular amino-terminal strand. Domain II (residues 30–75 and 431–497) consists of two β -sheets. Domain III (residues 76–

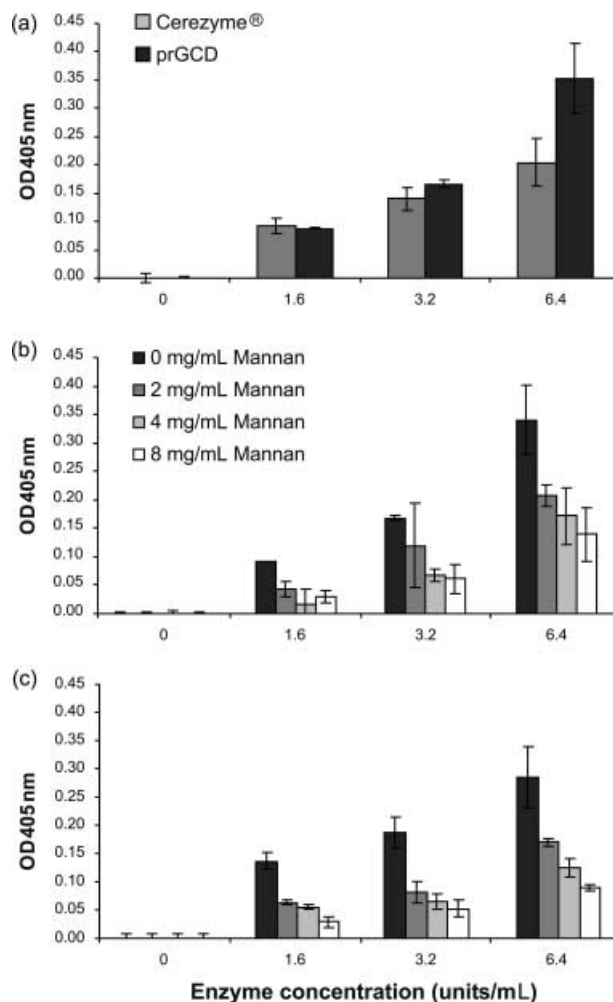


Figure 6 Uptake of recombinant plant-derived glucocerebrosidase (prGCD) by peritoneal macrophages. (a) Mouse macrophages were incubated with prGCD or Cerezyme® at increasing concentrations, and GCD uptake and activity were measured *in vitro* with *p*-nitrophenyl- β -D-glucopyranoside, and presented as the absorbance at 405 nm (OD, optical density). The effect of mannan on the specific uptake of prGCD (b) and Cerezyme® (c) was tested, confirming that prGCD uptake is mediated via mannose receptors. Data are the means \pm standard deviation of three independent experiments.

381 and 416–430) is a $(\beta/\alpha)_8$ TIM barrel. Structural comparison of prGCD with both Cerezyme® and Cerezyme® covalently modified by an irreversible inhibitor, conduritol-B-epoxide (Dvir *et al.*, 2003; Premkumar *et al.*, 2005) [Protein Data Bank (PDB) ID codes 1OGS and 1Y7V, respectively], revealed highly significant structural identity. A least-squares $C\alpha$ superposition of the crystal structures of prGCD with Cerezyme® and with the Cerezyme®–conduritol-B-epoxide conjugate yielded overall root-mean-square distance (RMSD) values of 0.64 and 0.60 Å, respectively. Furthermore, there was strict conservation of the active site residues, E235 and E340, and of neighbouring residues (Figure 7b).

Table 1 Toxicity study in mice. Six ICR (CD-1[®]) mice per group (three males and three females) were intravenously administered one, five or 10 times the human clinical dose of 60 units/kg. Control animals were administered a solvent control. Animals were monitored for 14 days following treatment. Clinical safety and pathological examinations were performed

Dose group	Clinical observations	Macroscopic examination
1 × clinical dose (1.8 mg/kg)	No obvious treatment-related adverse reactions. Normal body weight gains. No deaths	No gross pathological findings
5 × clinical dose (9 mg/kg)	No obvious treatment-related adverse reactions. Normal body weight gains. No deaths	No gross pathological findings
10 × clinical dose (18 mg/kg)	No obvious treatment-related adverse reactions. Normal body weight gains. No deaths	No gross pathological findings
Solvent control	No obvious treatment-related adverse reactions. Normal body weight gains. No deaths	No gross pathological findings

Toxicity study in mice

A single-dose toxicity study with prGCD was performed at Harlan Biotech (Rehovot, Israel), in which vehicle solution alone, or doses of prGCD in multiples of one, five or 10 times the standard clinical dose (60 units/kg), were given to ICR (CD-1[®]) mice. The animals (six per group, three males and three females) received the drug intravenously in a volume of 10 mL/kg. As shown in Table 1, there were no obvious treatment-related adverse reactions, no gross pathological findings and no mortality incidences observed, even at the highest dose administered. Furthermore, blood samples taken from animals in the high-dose group, which had been administered 10-fold the clinical dose, were tested for haematology and clinical chemistry. All haematology and clinical chemistry values were within normal ranges. In addition, the animals treated with the high dose were subjected to histopathological examination of the liver, spleen and kidney; there were no macro- or micro-histopathological findings.

Discussion

This study demonstrates that carrot cells can produce active human GCD, which has the potential to be used for ERT of Gaucher's disease. Earlier studies demonstrated the importance of GCD glycosylation with terminal Man residues for both its optimal activity and correct targeting to macrophages. Thus, GCD expressed in *Escherichia coli* is inactive, and deglycosylation of GCD extracted from human placenta results in a loss of enzymatic activity (Furbish *et al.*, 1981; Grace and Grabowski, 1990). GCD, after its production in CHO cells, is enzymatically remodelled *in vitro* to expose the terminal Man residues required for uptake by macrophages (Friedman *et al.*, 1999). The fact that prGCD produced in carrot cells naturally possesses terminal Man residues represents an

important advantage of plant cells over mammalian cells for the production of recombinant GCD for ERT. Plant cell cultures have additional advantages over mammalian cells because they do not require the presence of mammalian-derived components in the manufacturing process, making the purified biopharmaceutical products potentially safer and less expensive (Sijmons *et al.*, 1990; Hellwig *et al.*, 2004).

Cerezyme[®] is currently the only recombinant GCD approved for ERT for Gaucher's disease, but its very high cost places a heavy economic burden on healthcare systems worldwide (Futerman *et al.*, 2004). In contrast, prGCD does not require costly enzymatic deglycosylation and provides high batch-to-batch reproducibility of its glycan structures. In addition, kinetic studies show that the activity of prGCD is comparable with that of the CHO-expressed enzyme, Cerezyme[®]. This suggests that large-scale production of prGCD may be more cost-effective than that of Cerezyme[®].

There are conflicting reports regarding the immunogenicity of plant-derived biopharmaceutical glycoproteins (Cabanes-Macheteau *et al.*, 1999; Chargelegue *et al.*, 2000; van Ree *et al.*, 2000). Bardor *et al.* (2003) have shown that 50% of non-allergic blood donors have specific antibodies for core xylose in their sera, and that 25% have specific antibodies to core α -(1,3)-fucose. It remains to be determined whether such antibodies may limit the use of plant-derived biopharmaceutical glycoproteins. However, the current regulatory viewpoint, based on data accumulated in a number of clinical trials involving plant-expressed proteins, is quite promising in this regard (Hellwig *et al.*, 2004). Preclinical data from a single-dose toxicity study with prGCD in mice, in multiples of one, five and 10 times the clinical dose (60 units/kg), demonstrated that there were no obvious treatment-related adverse reactions or clinical findings, indicating the potential safety of plant cell-expressed GCD. Currently, prGCD is undergoing human clinical trials and additional preclinical studies.

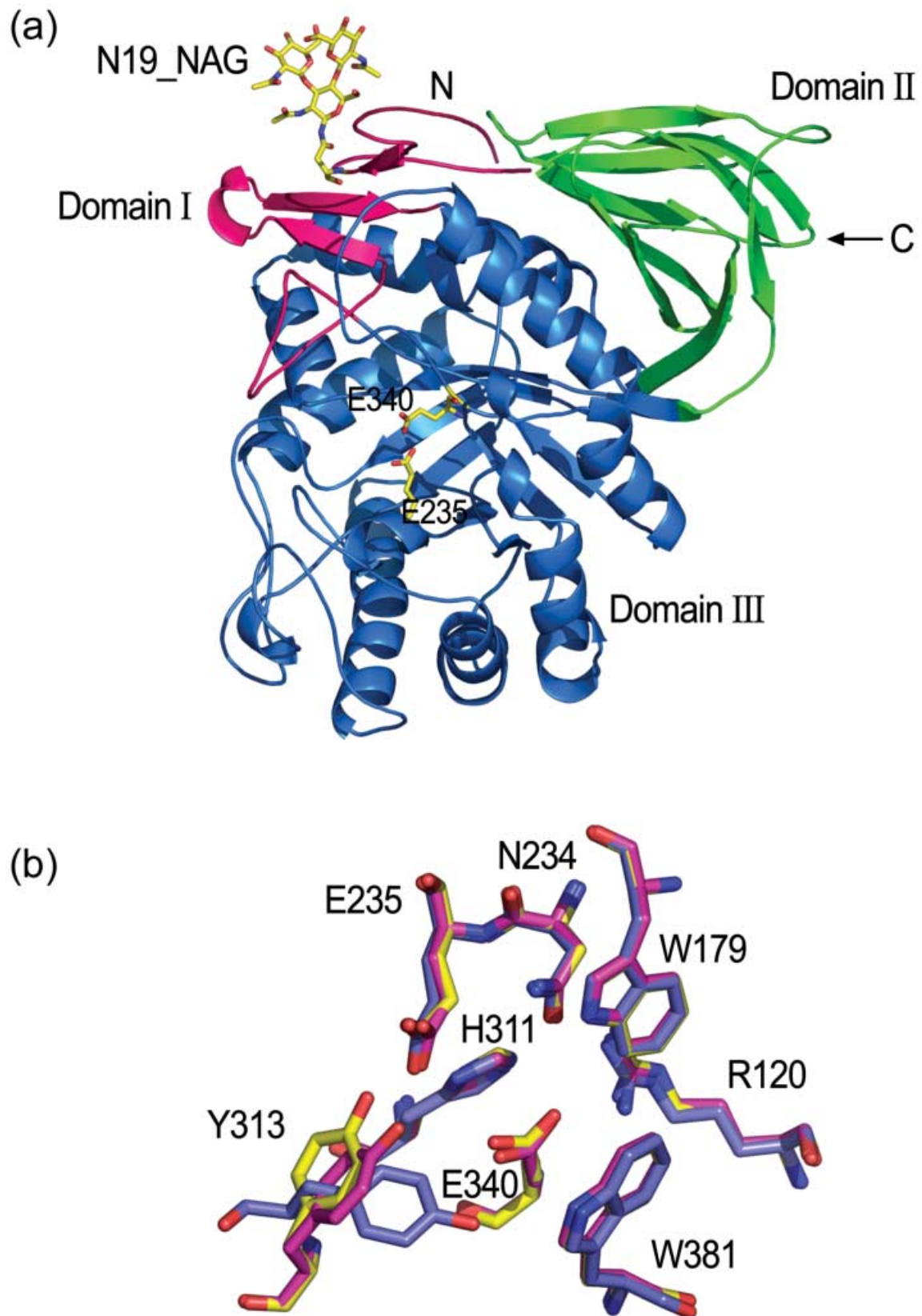


Figure 7 Three-dimensional structure of recombinant plant-derived glucocerebrosidase (prGCD). (a) Domain I is in pink, domain II in green and domain III in cyan. The active site residues E235 and E340 are shown. Glycosylation detected at N19 is also shown. (b) Comparison of the catalytic residues of prGCD (blue), Cerezyme® (pink) and a Cerezyme®-conduritol-B-epoxide conjugate (yellow).

Based on an examination of the entries in the PDB, the structure reported here for prGCD is the first example of the determination of a three-dimensional crystal structure of a human protein produced in a plant recombinant expression system. It is interesting to note the similarity of its three-dimensional structure to that of the same enzyme expressed in CHO cells (Dvir *et al.*, 2003; Premkumar *et al.*, 2005; Liou *et al.*, 2006), showing, for the first time, the extremely high similarity of plant and mammalian cell machineries controlling the folding and assembly of proteins within their endomembrane systems.

In summary, this study describes the expression of recombinant GCD in transgenic plant cells. prGCD is highly homologous to Cerezyme[®], both structurally and biologically. The availability of a high-resolution structure for prGCD will allow future modifications of its structure, perhaps permitting, thereby, improved therapeutic activity. The plant cell system developed here has the potential to be highly advantageous for the production of additional therapeutic recombinant glycoproteins that are not suitable for production in bacterial hosts, and are currently being expressed in costly mammalian expression systems.

Experimental procedures

Construction of the expression plasmid

The cDNA encoding GCD (ATTC clone #65696) was subcloned into a plasmid containing the signal peptide from the *Arabidopsis thaliana* basic endochitinase gene (Samac *et al.*, 1990) and the vacuolar targeting signal from tobacco chitinase A (Neuhaus *et al.*, 1991). At the 5' end of the open reading frame, the plasmid contained the 35S promoter from cauliflower mosaic virus (Odell *et al.*, 1985), followed by the tobacco mosaic virus (TMV) omega translational enhancer element (Gallie and Kado, 1989). At the 3' end, the octopine synthase terminator sequence from *Agrobacterium tumefaciens* (Dhaese *et al.*, 1983) was inserted. The cassette was removed from the intermediate vector and ligated into the binary vector pGREENII (obtained from Dr P. Mullineaux, John Innes Centre, Norwich Research Park, Colney, UK) (Hellens *et al.*, 2000). Kanamycin resistance was conferred by the neomycin phosphotransferase type II (*NPTII*) gene driven by the *nos* promoter.

Transformation and isolation of carrot cells

Carrot calli and cell suspension cultures were produced as described previously (Torres, 1983), and carrot cells were transformed using *Agrobacterium* (Wurtele and Bulka, 1989) with some modifications. Briefly, *Agrobacterium* was transformed with the pGREENII-noskan-GCDvac vector by electroporation (den Dulk-Ras and Hooykaas, 1995), and selected using 30 mg/mL paromomycin. Carrot cells were transformed with *Agrobacterium*, and selected using 60 mg/mL of paromomycin in liquid medium.

Gel electrophoresis and Coomassie staining

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under standard conditions, using a Criterion™ cell vertical electrophoresis apparatus (Bio-Rad, Hercules, CA, USA) with premixed tris(hydroxymethyl)aminomethane (Tris)-glycine-SDS electrophoresis running buffer (Bio-Rad); 15% acrylamide gels were prepared using premixed solutions of 40% acrylamide/Bis, resolving gel buffer, stacking gel buffer (Bio-Rad) and 10% aqueous SDS. Commercial molecular weight markers were used to assess the molecular weight of prGCD. Sample buffer (Laemmli, 1970) was used to treat each sample, and the mixture was boiled for 3 min before loading on to the gel. The gels were stained with Bio-Safe™ Coomassie Stain (Bio-Rad), as directed by the manufacturer.

Antibody production and Western blotting

Cerezyme[®] (75 µg) was suspended in 3 mL complete Freund's adjuvant and injected into New Zealand rabbits. A booster injection was given after 2 weeks and the rabbits were bled 10 days later and at 1-week intervals until the antibody titre began to drop. Serum was stored at -20 °C. For Western blotting, protein extracts were separated by SDS-PAGE (Laemmli, 1970) as described above, transferred to a nitrocellulose membrane (Amersham Life Science, Amersham, Buckinghamshire, UK), and GCD was detected using anti-GCD antibody (diluted 1 : 6500) and a peroxidase-conjugated goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (diluted 1 : 15 000) (Sigma, Rehovot, Israel).

prGCD purification

Cells were thawed overnight at 4 °C, and prGCD was extracted by homogenization in 20 mM sodium phosphate, pH 7.2, containing 0.1 mM phenylmethanesulphonyl fluoride, 20 mM ascorbic acid, 0.1 mM DL-dithiothreitol, 1% Triton X-100 and 20 mM ethylenediaminetetraacetic acid (EDTA). Following centrifugation (17 000 *g*, 20 min), the supernatant was applied to a strong cation exchange resin (Macro-Prep high-S support, Bio-Rad) that had been pre-equilibrated with 25 mM sodium citrate, pH 5.5. The prGCD fraction was subsequently applied to a hydrophobic interaction resin (TSK gel, Toyopearl Phenyl-650C, Tosoh Corp, Tokyo, Japan), pooled and further purified using a strong cation exchange column (Macro-Prep high-S support, Bio-Rad). The protein concentration was determined using either the Bradford method (Bradford, 1976), or from the absorbance (optical density, OD) at 280 nm (1 mg/mL = 1.53OD₂₈₀).

Sequence determination

Liquid chromatography-mass spectrometry/mass-spectrometry (LC-MS/MS) and Edman degradation were performed at the Smoler Proteomic Center, Technion, Haifa, Israel. Purified prGCD was separated by SDS-PAGE, followed by tryptic digestion, removal of the sugars using peptide *N*-glycosidase A (PNGase A) and LC-MS/MS. The trypsinized peptides were separated by reverse-phase chromatography on 0.1 × 300-mm fused silica capillaries (J&W, 100 µm inside diameter, Wilmington, DE, USA), and eluted using a linear gradient of 5%–95% acetonitrile containing 0.1% formic acid. Elution fractions were electrosprayed on to an ion-trap mass spectrometer

(DecaXP, Finnigan, San Jose, CA, USA). MS was performed in the positive ion mode using a repetitively full MS scan, followed by collision-induced dissociation (CID) of the most dominant ion selected from the first MS scan. The MS data were compared with simulated proteolysis and collision-induced fragmentation of the proteins, using the NR-NCBI database and Sequest software (J. Eng and J. Yates, University of Washington, Seattle, WA, USA and Finnigan) with unified Score 2400 and Pep-Miner software (IBM Haifa Research Laboratory, Haifa, Israel).

Glycosylation analysis

Glycosylation patterns were analysed by the Glycobiology Center, National Institute for Biotechnology, Ben Gurion University, Beer Sheba, Israel to determine the glycan structure and relative amounts using sequential digestion with various exoglycosidases. The prGCD samples were run on SDS-PAGE, and a 61-kDa band was cut out and incubated with either PNGase A or trypsin followed by PNGase A to release the *N*-linked glycans. The glycans were fluorescently labelled with anthranilamide (2AB) and run on normal phase HPLC.

Sequencing of the labelled glycan pool was achieved by sequential digestion with various exoglycosidases, followed by HPLC analysis. The retention times of the individual glycans were compared with those of a standard partial hydrolysate of dextran giving a ladder of glucose units (GU). Unlabelled glycans were further purified and analysed by DE-MALDI-TOF-MS.

The exoglycosidases used were as follows: bovine kidney α -fucosidase (digests α 1–6 and α 1–3 core fucose; Prozyme, San Leandro, CA, USA); jack bean mannosidase (removes α 1–2, 6 > 3 Man; Prozyme); *Xanthomonas* β -1,2-xylosidase (removes β 1–2 xylose only after removal of α -linked Man; Calbiochem, San Diego, CA, USA); bovine testes β -galactosidase (hydrolyses non-reducing terminal galactose β 1–3 and β 1–4 linkages; Prozyme); *Streptococcus pneumoniae* hexosaminidase [digests β 1–2,3,4,6 *N*-acetyl-D-galactosamine (GalNAc) and GlcNAc; Prozyme].

Glycosylation was further analysed by M-Scan (Wokingham, Berkshire, UK) using gas chromatography-mass spectrometry (GC-MS), fast atom bombardment-mass spectrometry (FAB-MS) and DE-MALDI-TOF-MS. For oligosaccharide determination, the *N*-glycan population was analysed by FAB-MS and DE-MALDI-TOF-MS, following digestion of samples with trypsin and PNGase A, and permethylation of the glycans. *O*-Glycans were analysed following reductive elimination of the tryptic and PNGase A-treated glycopeptides, desalting and permethylation. The similarity of the *N*-glycans in different batches of prGCD was analysed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD, a Dionex method), following digestion with trypsin and PNGase A, to obtain chromatographic profiles for the oligosaccharides released from glycoproteins for the purpose of demonstrating consistency from batch to batch of prGCD. This procedure permits chromatographic comparison of oligosaccharide patterns in a qualitative and quantitative manner.

In vitro enzymatic assay

prGCD was routinely assayed using *p*-nitrophenyl- β -D-glucopyranoside. The assay buffer contained 4 mM β -mercaptoethanol, 1.3 mM EDTA, 0.15% Triton X-100, 0.125% sodium taurocholate and 60 mM

phosphate-citrate buffer, pH 6.0. After 60 min at 37 °C, the reaction was terminated using 5 M NaOH, and the reaction product (*p*-nitrophenol) was determined by its absorbance at 405 nm (Friedman *et al.*, 1999). For kinetic studies, GCD activity was assayed as described by Meivar-Levy *et al.* (1994), with some modifications, using a fluorescent short-acyl-chain analogue of GlcCer, *N*-[6-[(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]hexanoyl]-D-*erythro*-glucosylsphingosine (C6-NBD-D-*erythro*-GlcCer). C6-NBD-GlcCer was synthesized by *N*-acylation of glucosylsphingosine using succinimidyl-6-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)aminohexanoate, as described by Schwarzmann and Sandhoff (1987). The assay was performed using 0.2 μ g of either Cerezyme[®] or prGCD in a final volume of 200 μ L 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (50 mM, pH 5.5). Concentrations of C6-NBD-GlcCer ranged from 0.25 to 100 μ M. Reactions were allowed to proceed for 5 min at 37 °C, and were stopped by the addition of 1.5 mL of chloroform-methanol (1 : 2, v/v) prior to extraction and analysis of the fluorescent lipids.

prGCD uptake by macrophages

Thioglycolate-elicited peritoneal macrophages were obtained from mice (Stahl and Gordon, 1982). Briefly, cells were collected by centrifugation (1200 *g*, 10 min), resuspended in Dulbecco's modified Eagle's medium (DMEM) (Beit Haemek, Israel) containing 10% fetal calf serum, plated at $(1-2) \times 10^5$ cells/well in 96-well plates, and incubated at 37 °C for 90 min prior to the removal of non-adherent cells. Adherent cells were further incubated for 90 min at 37 °C in culture medium containing prGCD or Cerezyme[®], with or without mannan (Sigma). Medium was subsequently removed, the cells were washed three times with phosphate-buffered saline (PBS), followed by lysis using 10 mM Tris, pH 7.3 containing 0.5% Nonidet P40 (NP-40), 1 mM MgCl₂ and a protease inhibitor cocktail (Sigma). The activity of prGCD taken up by the cells was determined by enzymatic activity assay.

X-Ray crystallography

Single crystals of prGCD were obtained by the microbatch method under oil, using an Oryx6 robot (Douglas Instruments Ltd., East Garston, Berkshire, UK). Crystals were grown at 20 °C from a precipitating solution of 25% w/v polyethylene glycol 3350 in 10 mM hexamine cobalt(III) chloride/0.2 M (NH₄)₂SO₄/100 mM bis-Tris, pH 6.5. Crystals were cryoprotected with 25% glycerol. A complete data set was collected from a single crystal on beamline ID14-4 at the European Synchrotron Radiation Facility (Grenoble, France) at 100 K. Diffraction data were integrated, scaled and reduced using the HKL program package (Otwinowski and Minor, 1997). The prGCD structure was solved by molecular replacement using the program PHASER (Storoni *et al.*, 2004), taking the 2 Å refined structure of human GCD (PDB ID code 1OGS) as the starting model (Dvir *et al.*, 2003; Premkumar *et al.*, 2005). Atomic refinement was carried out with the program CNS (Brunger *et al.*, 1998). After one round of simulated annealing, Fo-Fc and 3Fo-2Fc maps were used to fit the prGCD molecule, three sugar moieties, bis-Tris and nine sulphate molecules. Two-fold non-crystallographic restraints were applied in all the refinement cycles. Map display and model rebuilding were performed using the program o (Jones and Kjeldgaard, 1997), and the quality of the model was checked using PROCHECK (Laskowski *et al.*, 1993). Refinement and

model statistics are presented in Table S2 (see 'Supplementary material') Coordinates and structure factors have been deposited in PDB.

Toxicity study in mice

A single-dose toxicity study with prGCD was performed at Harlan Biotech (Rehovot, Israel) by intravenous injection in ICR (CD-1[®]) mice to provide information for the basis for the selection of appropriate dose levels in further repeated dosing trials. In view of the lack of information on the potential acute intravenous toxicity of prGCD in preclinical studies, an initial target dose of 1.8 mg/kg (60 units/kg – the clinical dose level), followed by additional dose levels of 9 and 18 mg/kg, was selected (one, five or 10 times the human clinical dose). prGCD-treated groups comprised six ICR (CD-1[®]) mice (three males and three females) aged 5 weeks at study initiation, and a single group of equal size treated with solvent only (25 mM citrate buffer, 150 mM NaCl, 0.01% Tween 80 and 5% ethanol) served as the control group. In all instances, prGCD-treated animals and controls were administered at a uniform volume dosage of 10 mL/kg. Clinical signs, behaviour and body weight gain/loss were monitored for 14 days following dose administration. Gross pathological examination was performed for all animals. Animals receiving the highest dose tested were subjected to haematology and clinical chemistry evaluation, and to histopathological examination of the liver, spleen and kidney.

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Supplementary material

The following supplementary material is available for this article:

Table S1 Data collection statistics

Table S2 Refinement and model statistics

This material is available as part of the online article from:

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