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Enzyme replacement for GM1-gangliosidosis: Uptake, lysosomal activation, and cellular disease correction using a novel β -galactosidase:RTB lectin fusion

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Abstract

New enzyme delivery technologies are required for treatment of lysosomal storage disorders with significant pathologies associated with the so-called “hard-to-treat” tissues and organs. Genetic deficiencies in the *GLB1* gene encoding acid β -galactosidase lead to GM1-gangliosidosis or Morquio B, lysosomal diseases with predominant disease manifestation associated with the central nervous system or skeletal system, respectively. Current lysosomal ERTs are delivered into cells based on receptor-mediated endocytosis and do not effectively address several hard-to-treat organs including those critical for GM1-gangliosidosis patients. Lectins provide alternative cell-uptake mechanisms based on adsorptive-mediated endocytosis and thus may provide unique biodistribution for lysosomal disease therapeutics. In the current study, genetic fusions of the plant galactose/galactosamine-binding lectin, RTB, and the human acid β -galactosidase enzyme were produced using a plant-based bioproduction platform. β -gal:RTB and RTB: β -gal fusion products retained both lectin activity and β -galactosidase activity. Purified proteins representing both fusion orientations were efficiently taken up into GM1 patient fibroblasts and mediated the reduction of GM1 ganglioside substrate with activities matching mammalian cell-derived β -galactosidase. In contrast, plant-derived β -gal alone was enzymatically active but did not mediate uptake or

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Author contributions

C.L.C, D.N.R., J.C. and W.A. participated in project conceptualization and research strategy development. J.C. and V.K designed and produced the gene constructs; J.A. and A.F. expressed, purified, and characterized the plant-produced proteins with assistance in biochemical characterization from J.R. W.A. developed the Dual bioactivity assay, enzyme kinetic studies, and all human cell-based assays with assistance in cell culture and related assays from R.M. C.L.C., W.A., J.C., and J.A. wrote and edited the manuscript with comments from all other authors.

Conflict of interest

C.L.C and D.N.R. are co-founders of biotech start-up BioStrategies LC. The other authors declare no competing financial interests.

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correction indicating the need for either lectin-based (plant product) or mannose-6-phosphate-based (mammalian product) delivery. Native β -galactosidase undergoes catalytic activation (cleavage within the C-terminal region) in lysosomes and is stabilized by association with protective protein/cathepsin A. Enzymatic activity and lysosomal protein processing of the RTB fusions were assessed following internalization into GM1 fibroblasts. Within 1–4 h, both β -gal:RTB and RTB: β -gal were processed to the ~64 kDa “activated” β -gal form; the RTB lectin was cleaved and rapidly degraded. The activated β -gal was still detected at 48 h suggesting interactions with protective protein/cathepsin A. Uptake-saturation analyses indicated that the RTB adsorptive-mediated mechanisms of β -gal:RTB supported significantly greater accumulation of β -galactose activity in fibroblasts compared to the receptor-mediated mechanisms of the mammalian cell-derived β -gal. These data demonstrate that plant-made β -gal:RTB functions as an effective replacement enzyme for GM1-gangliosidosis – delivering enzyme into cells, enabling essential lysosomal processing, and mediating disease substrate clearance at the cellular level. RTB provides novel uptake behaviors and thus may provide new receptor-independent strategies that could broadly impact lysosomal disease treatments.

Keywords

GM1-gangliosidosis; Lysosomal β -galactosidase; Lectin-mediated delivery; Plant-based bioproduction; RTB lectin

1. Introduction

GM1-gangliosidosis is a lysosomal storage disease (LSD) linked to mutations in the *GLB1* gene that encodes lysosomal acid β -D-galactosidase (β -gal; EC 3.2.1.23) [1]. The hallmark of GM1-gangliosidosis (GM1) is the progressive buildup of glycosphingolipid GM1 ganglioside, particularly in neurons, which results in widespread neurodegeneration. Some therapeutic modalities, e.g. substrate reduction therapy, and *ex vivo* and *in vivo* gene therapies, have been implemented in the animal model [2–6]. Whereas enzyme replacement therapies (ERTs) have been efficacious for many LSDs [7–9], an effective ERT has not yet been developed or tested for this disease. Barriers for development of effective ERTs for GM1 and other lysosomal diseases with strong central nervous systems (CNS) involvement remain a) the need to deliver corrective enzyme doses to the brain and b) the continued high cost of developing recombinant protein-drugs and bringing them to patients (reviewed [10, 11]). In this study, we tested a lectin-based “enzyme delivery module” providing receptor-independent routes of cell entry and used a rapid transient plant-based expression platform with the potential to address bioproduction costs.

All currently approved lysosomal disease ERTs exploit either the mannose-6-phosphate receptor (M6PR) or the high-mannose receptor (MMR) to direct endocytosis and lysosomal delivery of the corrective enzyme. While effective in treating visceral disease manifestations, these products do not effectively cross the blood-brain-barrier (BBB) to treat pathologies of the CNS and show limited correction in other so-called “hard-to-treat” tissues such as heart, lung and eye. In contrast to receptor-mediated endo/transcytosis, the plant lectin RTB initiates endocytotic uptake primarily by binding to the abundant galactose-terminated

glycoproteins and glycolipids present on mammalian cell surfaces, exploiting all known endocytic mechanism to access cells [12, 13]. This non-specific adsorptive-mediated endocytosis may provide an alternative enzyme delivery strategy with broader biodistribution and the potential to impact pathologies of these “hard-to-treat” tissues [14]. The lectin RTB (ricin toxin B-subunit) is the non-toxic carbohydrate-binding subunit of ricin that is responsible for mediating transport both into and within target cells. The uptake and trafficking of RTB, a galactose/galactosamine-specific lectin from *Ricinus communis*, has been extensively studied [15–18]. RTB has high affinity for surface glycolipids and glycoproteins providing access to a broad array of cells and enters cells by multiple endocytotic routes (e.g., clathrin-dependent and -independent; dynamin-dependent and -independent; caveolae-dependent and -independent; macropinocytosis; MMR-mediated) [15–18]. Upon endocytosis, RTB traverses preferentially to lysosomes or cycles back to the cell membrane (transcytosis pathway), with less than 5% moving “retrograde” to the endoplasmic reticulum (route for delivery of the RTA ribosome-inactivating toxic subunit) [15, 19, 20]. *In vivo* toxicity studies with the ricin toxin show injected or ingested ricin is quickly mobilized to both blood and lymphatic systems and accumulates in the spleen, liver, kidneys, heart, thymus, brain, and other tissues [21, 22] (reviewed in [23]). Additional studies show that the non-toxic RTB subunit (i.e., without the RTA subunit) mediates uptake into cells and tissue [24–26]. We therefore hypothesize that RTB will facilitate delivery of genetically associated human β -galactosidase to the critical sites of GM1 pathology. As a first step in testing this, we produced fusion proteins of RTB and human β -gal in plants and characterized their activities in plant tissues and in GM1 patient fibroblasts.

β -gal functions as an exoglycosidase, removing β -1,4-linked galactose residues from glycoproteins and sphingolipids such as GM1 ganglioside, and keratan sulfate. Depending on the genetic mutation, β -gal deficiencies can lead to the primarily neurodegenerative GM1 gangliosidosis or to Morquio B disease [27, 28]. β -gal can be isolated from mammalian tissues as a monomer, dimer, tetramer and as part of a multi-enzyme complex with N-acetyl- α -neuraminidase (Neu1) and the serine peptidase protective protein/cathepsin A (PPCA) [29–31]. This multi-enzyme complex is critical for enzyme stability and function within lysosomes, as defects in PPCA lead to galactosialidosis disease and deficiencies in both Neu1 and β -gal levels. β -gal is synthesized as an 85 kDa glycoprotein and is proteolytically processed within the lysosome to a 64 kDa “active” form by cleavage of 134 a.a. from the C-terminal end [30]. In PPCA deficient cells, β -gal is synthesized and traffics to lysosomes. However, it is rapidly degraded in lysosomes suggesting that PPCA plays a critical role in stabilizing β -gal in a way that allows the specific catalytic activation events but prevents further degradation [32, 33]. To date, neither plant-based production of functional human β -gal nor production of bioactive β -gal fusions with large heterologous protein domains (mammalian cell-derived HIS-tagged version are available) has been reported.

Plant-based bioproduction provides advantages in safety (no adventitious viral contamination) and cost of manufacture compared to mammalian cell-based technologies [34]. The first commercialized plant-made pharmaceutical protein to receive FDA approval for systemic administration was the glucocerebrosidase ERT for Gaucher disease produced in carrot cells (Protalix’ *Elelyso*, approved in 2012). This product has been used for patient treatment since 2007 [35] with no increase in immunogenicity compared to CHO-cell

derived product [36–38]. In this study, we employ plant-based biomanufacture using transient expression in *Nicotiana benthamiana*. Briefly, gene vectoring components are infiltrated into leaves of intact plants and recombinant proteins are recovered in 3–7 days (e.g. [39–41]). Both monoclonal antibodies (e.g. the ZMapp MAb cocktail for treatment for Ebola) and human virus-like particles (e.g. VLP for influenza A) made with the *N. benthamiana* system are currently in human clinical trials highlighting its potential to make and assemble complex protein products at scale and purity that meet regulatory standards [34, 40–44]. Thus, this production platform may facilitate more cost-effective development of complex biologics for rare diseases.

We recently reported plant-based production of RTB fusions to the human α -L-iduronidase enzyme (ERT for mucopolysaccharidosis I; MPS I) [24]. The fusion product retained both RTB lectin selectivity and iduronidase enzyme activity. The RTB:iduronidase fusion was efficiently taken up into MPS I patient fibroblasts, corrected the lysosomal phenotype, and reduced the glycosaminoglycan disease substrate to “normal” levels by MMR- and M6PR-independent mechanisms [24]. In the present study, β -gal, with and without RTB, was expressed in plants and purified proteins were analyzed for activity, catalytic processing and lysosomal disease correction of GM1 fibroblasts. RTB fusion orientations were developed that supported the complex post-translational processing and multiple protein-protein interaction required for full β -gal functionality within lysosomes and effectively corrected GM1-gangliosidosis at the cell level.

2. Methods

2.1. GLB1 cloning

Native cDNA sequence (*GLB1*, GenBank accession no. NM 000404) and tobacco-codon optimized *GLB1* cDNA were synthesized (GeneArt®, Life Technologies). Synthesized genes included an adapter carrying a hexa-histidine tag (His) and a stop codon at the 3′-end. These constructs, which harbor the endogenous human signal peptide (hSP) were cloned into a pMK-RQ vector. *GLB1* sequences encoding the mature β -gal were also cloned into a pBC-SK(–) vector with an in-house patatin optimized signal peptide (PoSP) using Phusion High-Fidelity DNA polymerase (Finnzymes) and In-Fusion® (Clontech) seamless cloning. PoSP is based on a plant signal peptide derived from potato patatin tuber storage protein [45] with the following modification: Ala-Ser-Ser downstream of the initiation codon [46], a “Kozak” sequence for plants [47], nucleotide modifications to enhance RNA stability, and a cleavage site to yield Val as first amino acid based on the plant N-end rule [48]. Together all these modifications have improved the expression of different recombinant proteins in our system (Condori, unpublished results). Cassettes harboring *GLB1* were subcloned into our plant expression vector [49] derived from pBIB-Kan [50], creating hSP: β -gal^{nat}, hSP: β -gal^{opt}, PoSP: β -gal^{nat} and PoSP: β -gal^{opt} constructs (Fig. 1). In these vectors, expression is driven by the dual 35S promoter [51] and the translational enhancer from tobacco etch virus (TEV) [52]. Constructs were confirmed by DNA sequencing (CRC-DNA Sequencing Facility, Chicago, IL). The vectors were then mobilized into *Agrobacterium tumefaciens* electrocompetent cells LBA4404, using standard procedures [53].

2.2. RTB:GLB1 and GLB1:RTB fusions

For lectin fusion constructs, the B-subunit of ricin (RTB) was modified by removing an internal *KpnI* restriction site (for cloning purposes), replacing GGTACC with GGTACT using a QuikChange II Site-Directed Mutagenesis kit (Agilent), according to manufacturer's instructions. Fusion constructs were carried out using In-Fusion® technology. RTB was cloned at the 5'-end of *GLB1* using PoSP as unique signal peptide for organelle localization inside the plant cell and transferred to our plant expression vector, yielding PoSP:RTB:β-gal^{nat} and PoSP:RTB:β-gal^{opt} constructs (Fig. 1). On the other hand, when RTB was cloned at 3'-end of *GLB1*, two different signal peptides were used (hSP and PoSP), subsequently these constructs were subcloned into the plant expression vectors, creating hSP:β-gal^{nat}:RTB, hSP:β-gal^{opt}:RTB, PoSP:β-gal^{nat}:RTB and PoSP:β-gal^{opt}:RTB (Fig. 1). All constructs were again sequenced and then mobilized into *A. tumefaciens* LBA4404 as described above.

2.3. Plant transient expression

GLB1-containing constructs were expressed in *Nicotiana benthamiana* using an *Agrobacterium*-mediated transient expression system [39]. *A. tumefaciens* cultures containing our gene of interest were inoculated into 5 ml YEP media containing 100 µg/ml kanamycin and 60 µg/ml streptomycin and incubated at 28°C for 24 h with shaking at 250 rpm. After 24 h, 1 ml was transferred into 100 ml of YEP in 500 ml baffled flask containing appropriate antibiotics and incubated for about 7 h at 28°C and 250 rpm. Once the bacterial growth reached log phase, the bacterial pellet was harvested by centrifugation at 5,500xg for 7 min and re-suspended in 10 mM MES hydrate, 10 mM MgCl₂ hexahydrate and 0.5% glucose, pH 5.6. The culture was then induced for 24 h at 28°C by the addition of acetosyringone (final concentration of 200 µM). The final OD₆₀₀ at infiltration was close to 0.2. Bacterial suspension was vacuum-infiltrated in leaves of intact *N. benthamiana* plants at 4–5 weeks of age as described [39] and harvested 48, 72 and 96 h post infiltration (5 plants/time point). For later (and larger scale) production, this protocol was modified to a simpler version in which no acetosyringone was used, eliminating the 24 h induction period and centrifugation steps. For this procedure, the bacterial suspension in YEP was simply diluted into the infiltration media before infiltration. Changes in the bacteria growth conditions did not impact expression levels (data not shown). However, optimal harvest times for recovery of *GLB1*-containing products shifted to five days post-infiltration as compared to days 3–4 for the initial infiltration method.

2.4. β-galactosidase activity assay

β-gal activity in plant crude extracts was determined using the fluorogenic substrate 4-methylumbelliferyl β-D-galactopyranoside (4MUGal, Sigma). Frozen infiltrated *N. benthamiana* leaves were ground in 1:2 w/v of tissue to buffer (100 mM Tris, 150 mM MgCl₂, 10 mM Na₂S₂O₅, pH 7.5 containing 0.1% Tween 20) and centrifuged at 16,000xg for 30 min at 4°C. This crude extract was diluted 1:10 in enzyme buffer (100 mM sodium acetate buffer, pH 4.3). Ten µl of the diluted sample was loaded onto a MicroFluor2 black 96-well plate (Thermo-Fisher) and then mixed with 20 µl/well of substrate solution (1 mM 4MUGal in 100 mM sodium acetate buffer, pH 4.3). The same procedure was done with

crude extracts from plants infiltrated with an “empty vector” (pBIB-Kan) to serve as a blank. After addition of the substrate, the plate was wrapped in aluminum foil and incubated at 37°C for 30 min. The reactions were terminated by the addition of 270 µl of stop solution (0.2 M glycine, 0.125 M sodium carbonate, pH 10.7). Serial dilutions of 4-methylumbelliferone (4MU) (Sigma) from 0.07 to 4.5 nM were used in order to establish a standard curve. Fluorescence was measured on the PolarStar (BMG Labtech) using 350 and 420 nm for excitation and emission, respectively.

2.5. Dual bioactivity assay

The “Dual” assay is a combination of a lectin binding [21] and β -gal activity assays and was developed to directly quantify products having both active RTB and active β -gal in a fusion product. The fusion protein is captured based on the affinity of RTB to bind glycans on the surface of the asialofetuin glycoprotein. First, asialofetuin (Sigma) is bound to the surface of an Immulon 4HBX 96 well plate (Thermo-Fisher) at a concentration of 3 µg/ml. After RTB is bound to asialofetuin, the enzymatic activity of the β -gal is measured as described in the β -gal assay (2.4). The dual bioactivity assay was modified from a previous study [24]. Briefly, 100 µl of crude extract, 1:100 dilution in PBS containing 1% BSA, was loaded onto Immulon 96-well plate. After incubating for 1 h at room temperature, unbound proteins were washed away and 20 µl of substrate solution (1 mM 4MUGal in 100 mM sodium acetate buffer, pH 4.3) was added and incubated at 37°C for 30 min in the dark. The reaction was stopped by adding 280 µl of stop solution (see 2.4). The same procedure was done with crude extract from plants infiltrated with an empty vector to serve as a blank. The standard curve and fluorescence reading were all done as described in section 2.4. For both activity and Dual assay, the activity of the proteins is defined as nmol/min or unit of activity (U), which is equal to nmol/min.

2.6. Western blot analysis

Ten µg of total soluble protein from plant crude extracts and/or 30 ng of purified protein were run on 10% Novex NuPage *bis*-tris gel (Invitrogen) and transblotted onto nitrocellulose membrane (0.2 µm, Bio-Rad). The procedure used 1X PBS with 0.1% Tween 20 and 3% bovine serum albumin (BSA, Sigma) as blocking buffer and 1X PBS with 0.1% Tween 20 (Sigma) for washing the membrane. Sheep anti- β -gal at 1:3,000 dilution (R&D Systems) and rabbit anti-sheep AP-conjugated at 1:30,000 dilution (Invitrogen) were used for detection. Detection of immunoblots was carried out using the CDP-Star (Roche) and Nitroblock Enhancer II system (Applied Biosystems) in accordance with manufacturers' procedures and membrane image was captured on X-ray film (GE Healthcare Life Sciences). Total soluble protein was quantified with Pierce™ Coomassie Plus (Bradford) assay kit (Thermo Scientific) according to manufacturer's procedure.

2.7. Protein purification

Frozen leaf tissue was ground in a Waring commercial blender (Waring) for 5 min in 2X grinding buffer (100 mM Tris, 150 mM MgCl₂, 10 mM N₂S₂O₅, pH 7.5 containing 0.1% Tween-20 and 20 mM D-(+)-galactose). Homogenate was further homogenized at 12,000 rpm/15 min by using a PT-DA 3007/2T Kinematica probe attached to a PT 3100 Polytron® (Brickman Instruments). Heavy matter was removed by centrifugation at 12,000xg for 15

min at 4°C. The supernatant was further filtered through 4 layers of Miracloth (Calbiochem) and the pellet was discarded. Unlike RTB:β-gal and β-gal:RTB, the grinding buffer for pldβ-gal did not contain galactose.

Ammonium sulfate precipitation was accomplished by a two-step protocol. First, ammonium sulfate was added to 22% saturation for both RTB:β-gal and β-gal:RTB, and 38% for pldβ-gal. After incubation with tumbling at 4°C for 30 min, samples were centrifuged at 16,000xg for 20 min at 4°C. Pellets were discarded and the amount of ammonium sulfate increased to 45% saturation for RTB:β-gal and β-gal:RTB and 52% for pldβ-gal. After incubation and centrifugation the supernatant was discarded and the pellet briefly rinsed without disturbing with 200 mM Tris, 150 mM NaCl, pH 7.8 for RTB:β-gal and β-gal:RTB, and 50 mM NaPO₄, 300 mM NaCl, 10 mM Imidazole, pH 7.5 for pldβ-gal. The pellets were re-suspended in the same buffer as in the rinsing step in a volume equivalent to half the amount of grinding buffer used in the grinding step. Re-suspension of the pellet was facilitated by the addition of 4 mm glass beads, followed by incubation with tumbling at 4°C for 30 min and centrifugation at 12,000xg for 10 min at 4°C. The supernatant was then filtered through a 0.45 μm Tuffryn membrane filter (PALL) before affinity chromatography.

Affinity chromatography was performed in glass column packed with α-lactose agarose gel (Sigma) and size exclusion chromatography (SEC) in HiLoad 26/60 Superdex 200 pg (GE Healthcare Life Sciences). Chromatography was conducted using a Biologic Duoflow FPLC system (Bio-Rad). For purification of RTB:β-gal and β-gal:RTB, α-lactose agarose gel was conditioned with 20 column volumes (CV) of 200 mM Tris, 150 mM NaCl, pH 7.8. Samples were loaded at 3.0 ml/min and unbound protein removed by washing with 4 CV of the above buffer. Bound proteins were eluted with 200 mM Tris, 150 mM NaCl pH 7.8 containing 500 mM of D-(+)-galactose (Sigma). For purification of pldβ-gal, nickel resin (Clontech) was packed into a column and conditioned with 10 CV of 50 mM sodium phosphate, 150 mM NaCl, pH 7.2 buffer. Sample was loaded at 2.0 ml/min and unbound protein removed by washing with 5 CV of the above buffer. Bound protein was eluted with 50 mM sodium phosphate, 150 mM NaCl, 300 mM imidazole buffer (pH 7.5).

Elution fractions from lactose- and nickel-chromatography containing the proteins of interest were concentrated by using a 30K VivaSpin (Sartorius). The SEC column was equilibrated with 2 CV of 50 mM sodium phosphate, 150 mM NaCl pH 7.2 buffer, before injecting the sample. Proteins were eluted with the same buffer at 1.0 ml/min. The peak corresponding to the protein of interest was concentrated using a 30 K VivaSpin column and buffer-exchanged to PBS (Corning cellgro®) by using a 7 MWCO Zeba Spin Desalting Column (Thermo-Fisher). The final product was then filter sterilized by using Ultrafree® – MC 0.22 μm GV Durapore® centrifugal filter (Millipore), quantified by measuring absorbance at A280 using Nanodrop® ND-1000 Spectrophotometer (Thermo-Fisher) and taking into consideration the molar extinction coefficient of 139,930 for pldβ-gal and 203,620 for both RTB:β-gal, and β-gal:RTB. Aliquoted samples were stored at –80°C.

2.8. Proteomic analysis of pldβ-gal, RTB:β-gal and β-gal:RTB by MS/MS

Three μg of purified pldβ-gal, RTB:β-gal, and β-gal:RTB protein were size-separated by SDS-PAGE (10% gel; Life Technologies) under reducing conditions. Proteins were

visualized with SimplyBlue® SafeStain (Life Technologies) following manufacture's procedures. Bands of expected size were excised and submitted for protein identification to the Proteomic Core Facility at the University of Arkansas for Medical Sciences, Little Rock.

2.9. X-Gal staining for β -gal uptake in fibroblasts

Fibroblasts were cultured in MEM (Gibco) supplemented with 15% FBS (Atlanta Biologicals), Glutamax 100x (Gibco) and penicillin/streptomycin 100x (Sigma). Normal human fibroblasts (*GLBI*^{+/+}; Coriell GM-00010) and GM1 fibroblasts (*GLBI*^{-/-}; GM-10919) were grown in 35 mm glass bottom dishes (MatTek) for 24 h. The media was then replaced in the GM1 fibroblasts with serum-free MEM containing 500 ng/ml of β -gal:RTB. The media was also replaced with serum-free MEM in normal and GM1 fibroblasts as controls. Samples were incubated for 2 h at 37°C to allow uptake of β -gal:RTB. Cells were then fixed with 0.1 M phosphate buffer, pH 7.3 supplemented with 5 mM EGTA (Sigma), 2 mM MgCl₂ and 0.2% glutaraldehyde (Sigma) for 15 min at room temperature. Cells were subsequently washed 2X with wash buffer (5 min each; 0.1 M phosphate buffer, 2 mM MgCl₂ pH 7.3). X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) at 1 mg/ml was prepared in staining buffer [0.1 M citrate phosphate buffer, pH 4.6, 5 mM K₄Fe(CN)₆·3H₂O (Sigma) and 5 mM K₃Fe(CN)₆ (Sigma)] and filtered through a 0.2 μ m filter to remove crystals. X-gal solution was added to the fibroblasts and incubated overnight inside a humidified chamber at 37°C. X-gal solution was removed; fibroblasts were washed 3X with wash buffer, and images were acquired using a Nikon TE2000 phase contrast microscope with a 60X objective.

2.10. High throughput imaging assays

A high-through-put high-content imaging assay was developed to quantify the levels of GM1 gangliosides in GM1 fibroblasts to assess GM1 disease and β -gal-mediated disease correction. Details of this assay are described in the associated *data in brief* [54]. Briefly, normal (*GLBI*^{+/+}) and GM1 (*GLBI*^{-/-}) fibroblasts plated in clear-bottom 96-well microtiter plates were treated with 100 μ l fresh serum-free media or serum-free media containing 6 nM of mcd β -gal (R&D Systems), pld β -gal, RTB: β -gal, or β -gal:RTB, incubated for 24 h, and then fixed. To detect GM1 ganglioside, cells were permeabilized, blocked with BSA-glycine, and then incubated with Cholera Toxin B protein (List Biologicals) that had been conjugated to Dylight 594 (CTXB-Dylight⁵⁹⁴). Cells were subsequently counterstained with 600 nM DAPI and images acquired using the BD Pathway 855 High Content Bioimager (BD Biosciences) as described [24]. For each image/repetition the ratio of CTXB total pixels (594 nm) was divided by number of cells (nucleus count). Data was expressed as the average of CTXB-Dylight⁵⁹⁴ pixels per cell ratios in each treatment. Twelve to sixteen images (n=12–16) per treatment were used for quantitation with approximately 150 cells captured per image (each treatment point represents an average of 1800–2400 individual cells).

2.11. Internalization capacity of recombinant proteins in GM1 fibroblast

GM1 fibroblasts (*GLBI*^{-/-}) were plated into three 6-well plates (Corning) and grown until confluent. Cells were treated with 6 serial dilutions of mcd β -gal, RTB: β -gal, and β -gal:RTB at protein concentrations ranging from 30 nM to 0.94 nM prepared in 1 ml of MEM. Cells were incubated at 37°C with 5% CO₂ for 30 min to allow surface binding, then washed with

MEM. Fresh MEM medium was added back to each well and incubated for 6 h at 37°C with 5% CO₂. Cells were then washed 3X with PBS containing 100 mM lactose to remove any extracellular lectin-fusion protein bound to the cell membrane, removed by trypsinization, and transferred into a 1.5 ml microcentrifuge tube. After centrifugation at 1000 rpm for 5 min, the pellet was washed by re-suspending in 1 ml PBS. The tubes were centrifuged again (1000 rpm for 5 min) and the supernatant discarded. Cells were lysed by re-suspending the pellet in 60 µl of 1% Triton X-100 and sonicating at 400W for 1 min. The lysate was clarified by centrifugation at 6,000 rpm for 10 min. Supernatant was analyzed for total soluble proteins (TSP by Bradford Assay; Thermo) and β-gal enzyme activity. For enzyme activity determinations, samples (30 µg TSP) were loaded in duplicates and the volume brought to 30 µl using enzyme assay buffer (for buffer compositions see β-gal enzyme assay in section 2.4). Enzymatic reaction was initiated by addition of 30 µl of substrate solution. Plates were then incubated in the dark at 37°C for 6 h. Reaction was terminated by the addition of 240 µl of stop solution. Serial dilutions of 4MU were used to establish the standard curve. Fluorescence was measured on the PolarStar using 350 and 420 nm for excitation and emission, respectively. Best-fit curves were established using GraphPad software. As described in [55, 56], the hyperbolic asymptote value is defined as the maximum uptake capacity. The concentration of protein giving half-maximal uptake is defined as K_{uptake} .

2.12. Enzyme kinetics

Purified extracts were assessed under same assay conditions described in section 2.4. Samples comprising mcdβ-gal, pldβ-gal, RTB:β-gal or β-gal:RTB were diluted to 10 nM in assay buffer. Substrate (4MUGal) was diluted in assay buffer (see section 2.4 for buffer composition) to concentrations ranging from 15.6 to 2000 µM. Equal volumes (25 µl) of protein and substrate solution were combined to initiate the reaction and incubated as described above. Reactions were terminated by the addition of 250 µl of stop solution. Results were plotted as units of β-gal activity per min for each substrate concentration. Data analysis was performed using GraphPad Prism software.

2.13. RTB:β-gal and β-gal:RTB protein processing in fibroblasts

GM1 (*GLBI*^{-/-}) fibroblasts were cultured in 6-well plates (Corning) until confluent. RTB:β-gal and β-gal:RTB were diluted in MEM medium to a final concentration of 10 nM (1.2 µg/ml) and then added to 1 ml/well. After treatment, cells were incubated at 37°C with 5% CO₂ until harvest. After 30 min and 1 h incubation, protein-containing media was removed, cells were pre-washed and harvested as described later. For longer incubations times, protein-containing media was removed after 1 h and cells were washed with 1 ml of MEM media for 5 min and fresh media was added to cells to complete the incubation time. Before harvesting, the cells were washed with PBS containing 100 mM lactose to remove any extracellular bound RTB:β-gal or β-gal:RTB protein still attached to the cell surface. Cells were harvested by trypsinization and transferred into a 1.5 ml microcentrifuge tube. After centrifugation at 1000 rpm for 5 min, the pellet was washed by re-suspending in 1 ml of PBS. The tubes were centrifuged again (1,000 rpm for 5 min) and supernatant discarded. Cells were lysed by re-suspending the pellet in 60 µl of RIPA buffer and cells disrupted by sonicating at 400W for 1 min. The lysates were clarified by centrifugation at 6,000 rpm for

10 min. Supernatant was collected and analyzed for total soluble proteins (Bradford Assay, see section 2.6). Thirty μ g of TSP were loaded in a 10% bis-Tris gel and Western blot analysis was performed as described in section 2.6.

3. Results

3.1. Production of β -gal and β -gal fusions with RTB in *N. benthamiana* leaves

In lysosomes, β -gal activity is based both on interactions with diverse substrates (e.g., sphingolipids, glycoproteins, keratan sulfates) as well as other proteins, including Neu1, PPCA, and the proteinase responsible for catalytic activation. Although the protein structure of β -gal has been determined [57], it is difficult to predict the structural impact of introducing a large fusion partner such as RTB. RTB itself is a 34 kDa glycoprotein with two distinct galactose/galactosamine binding domains that are required for full activity. We therefore developed a series of 10 β -gal constructs (Fig. 1) designed to determine a) if active β -gal could be produced in plants (β -gal with C-terminal His-tag), b) which fusion orientation would support activity of both the lectin and β -gal (β -gal:RTB versus RTB: β -gal), and c) whether plant-based expression could be enhanced by use of a plant-versus human signal peptide, or by codon-optimization based on tobacco codon preferences. These constructs were produced, introduced into *Agrobacterium tumefaciens* (*Agro*; vector for gene delivery into plant cells), and induced *Agro* cultures were vacuum-infiltrated into leaves of intact *N. benthamiana* plants as described in *Methods* (2.3). Plants were harvested 48, 72, and 96 h post-infiltration and leaf extracts were used to assess yield of full-length product, lectin binding, and β -gal activity.

In all cases, abundant product of the expected molecular size (85 kDa for β -gal; 120 kDa for RTB fusions with β -gal) was detected by Western immunoblots using anti- β -gal antibodies (data not shown). Extracts from plants that were infiltrated with *Agro* bearing an “empty vector” control construct showed no endogenous β -galactosidase activity (data not shown). In contrast, enzymatic assays of leaf crude extracts showed that plants expressing β -gal-constructs (with or without RTB) contained β -galactosidase activity (e.g., Fig. 2A). Initial comparisons of β -gal activity levels based on leaf fresh weight showed that constructs using the human signal peptide from *GLB1* showed 55–71% lower yields compared with constructs engineered with our plant optimized signal peptide (PoSP) and these constructs were not analyzed further. However, no consistent differences in yield based on Western analyses and β -gal activity were observed based on “native” versus “tobacco-codon-optimized” versions of the β -gal sequence (data not shown). In order to selectively quantify fusion products possessing both RTB-lectin activity and β -gal enzyme activity, we developed a bi-functional “Dual” assay. In this assay, RTB binds to the galactose-terminated glycoprotein, asialofetuin, which is used to coat 96-well microtiter plates. β -gal activity of the bound product is then directly measured fluorometrically using 4-MUGal (Fig. 2B). Based on these assays, three constructs were selected for subsequent purification and analysis, all of which encompassed the tobacco-codon optimized version of β -gal and our plant-optimized signal peptide. These will be designated **pld β -gal** (His-tagged plant-derived β -gal), **β -gal:RTB**, and **RTB: β -gal** in subsequent analyses, with the β -gal:RTB variant selected as our lead candidate based on “Dual” activity and yield.

In order to facilitate further biochemical and *in vitro* disease correction analyses, the selected plant-made β -gal products were purified using a combination of ammonium sulfate precipitation, affinity chromatography, and size exclusion chromatography. The resulting pld β -gal, RTB: β -gal, and β -gal:RTB products showed greater than 98% purity by Coomassie staining (Fig. 3A), cross-reacted with anti-GLB1 antibodies (Fig. 3B) and retained RTB and β -gal activity (data not shown). Based on multiple purification runs for β -gal:RTB, average yields were around 6.4 mg/kg of leaf fresh weight with 15–25% recovery from the crude extract. Plant-made β -gal and β -gal:RTB fusions were recovered as homodimers based on size exclusion chromatography and comparisons of molecular sizes on native versus reducing gel electrophoresis pld β -gal (Fig. 3A–C). The identity of β -gal:RTB product was confirmed by N-terminal sequencing yielding N-term- *VDLRNA* (identical to the N-terminal sequence of mature human β -gal sequence). All three proteins were analyzed by mass spectrometry of tryptic digests. The MS analyses provided 47%, 46% and 33%, coverage for pld β -gal, RTB: β -gal and β -gal:RTB, respectively and identified both protein partners in fusion products.

3.2. Cell uptake and disease correction in GM1 patient fibroblasts

GM1 patient fibroblasts were used to determine a) whether the RTB lectin would effectively mediate cell uptake and lysosomal delivery and b) whether the plant-made β -gal was capable of degrading the GM1 disease substrate, GM1 ganglioside. As a first test to determine if β -gal:RTB was taken up into cells, we used the classic X-gal colorimetric staining to visualize β -gal activity. Whereas untreated GM1 fibroblasts (β -gal^{-/-}) showed no β -gal activity, β -gal activity was detected in GM1 fibroblasts following 2 h treatment with β -gal:RTB (Fig. 4). A high-through-put high-content imaging assay was subsequently developed to assess disease correction. In this assay, cells were grown in 96-well microtiter plates and treated with 6 nM concentrations of β -gal (510 ng β -gal/ml) or β -gal:RTB fusions (720 ng fusion protein/ml; equivalent to 510 ng β -gal/ml). After 24 h incubation, GM1 ganglioside levels were detected based on GM1 ganglioside interactions with Dylight⁵⁹⁴-conjugated cholera toxin B. As shown in Fig. 5, this assay clearly delineates fibroblasts from normal individuals and GM1 patients. Further details on the assay parameters and associated statistical analyses are described in *data in brief* (reviewed in [54]). Significant reduction in GM1 ganglioside levels was observed in GM1 cells after treatment with mammalian cell-derived β -gal (mcd β -gal) as well as both fusion proteins, β -gal:RTB and RTB: β -gal. In contrast, treatment with pld β -gal showed no reduction of GM1 ganglioside levels indicating the critical role of RTB (or mannose-6-P glycans in the case of mcd β -gal) in directing cell uptake and lysosomal delivery. The data provides compelling evidence that RTB can facilitate delivery of active human β -gal into cells and into the key subcellular sites of GM1 disease substrate accumulation.

Whereas all currently approved ERTs for lysosomal diseases use either the mannose-6-phosphate receptor or high-mannose receptor to direct endocytosis into mammalian cells, RTB-mediated endocytosis uses predominantly adsorptive-mediated endocytosis rather than receptor-mediated mechanisms. RTB was previously shown to efficiently delivery a lysosomal enzyme “payload” in the presence of inhibitory levels of M6P and mannan (MMR inhibitor) [24]. A hallmark of receptor-mediated uptake is saturability – uptake levels

depend on the number of receptors present on the cell surface. In contrast, adsorptive-mediated mechanisms are considered “less saturable”. We therefore compared that levels of intracellular β -gal activity in GLB^{-/-} cells treated with various concentrations of either mcd β -gal or RTB fused β -gal. As shown in Fig. 6, the ‘maximum’ amount of mcd β -gal protein that can be internalized (maximum uptake capacity) is 4.5X lower than the value of the lectin-fusion proteins. In other words, the mcd β -gal protein concentration needed to reach the half-maximum protein internalization (12nM), can be achieved using 1/8 of the concentration of the lectin-fused proteins (1.6nM) (Fig. 6, dashed lines). The concentration of protein at which Half-Maximum uptake was achieved (K_{UPTAKE} constant), was the same in all tested recombinant proteins (12 nM). Regardless of the lectin fusion orientation, both proteins showed identical intracellular activity after treatment with the different doses.

3.3. Activity and processing of plant-made RTB: β -gal in mammalian cells

The data described above clearly indicated that our β -gal:RTB fusions are enzymatically active and capable of degrading accumulated GM ganglioside substrate. However, in mammalian lysosomes, β -gal undergoes multiple interactions including catalytic activation (~85 kDa $\rightarrow$ ~64/22 kDa) and association with PPCA which prevents further degradation [30]. Because β -gal shows activity in its full-length form as well as its “activated” form, it is possible that the GM1 ganglioside reduction shown in the experiment above was due to the basal (pre-activation) activity of β -gal. We therefore designed a set of experiments to compare a) β -gal enzyme activities and b) the molecular sizes of RTB- and β -gal-containing polypeptides, before cell uptake and after uptake/lysosomal exposure in GM1 fibroblasts.

To support these analyses, we initially determined the enzyme kinetic parameters (K_m and V_{max}) for both mammalian- and plant-derived β -gal and for the β -gal:RTB fusions (Fig. 7). Whereas the enzyme parameters for mcd β -gal, pld β -gal, and RTB: β -gal were very similar (V_{max} = 203 $\pm$ 5, 195 $\pm$ 7, and 219 $\pm$ 11 U/nmol, respectively), our “lead” candidate β -gal:RTB (which was selected based on the “dual” activity assay) had a lower specific activity (V_{max} = 133 $\pm$ 5 U/nmol). This data suggest the RTB placement at the C-terminal may interfere with β -gal activity. This was surprising as our data from GM1 fibroblast, treated with the same protein concentrations of RTB: β -gal versus β -gal:RTB, indicated nearly equivalent efficacy in disease correction based on GM1-ganglioside levels (Fig. 5) and identical β -gal enzymatic activity once internalized (Fig. 6).

To further analyze β -gal:RTB proteolytic processing within fibroblasts, protein was extracted from fibroblasts at various times after addition of β -gal:RTB and RTB: β -gal and analyzed by Western immunoblots probed either with anti- β -gal or anti-RTB antibodies. The expected products of β -gal processing (the 85 kDa $\rightarrow$ ~64/22 kDa activation event for native β -gal) are shown in Fig. 8D. As shown in Fig. 8A and B, within 4 to 6 h, β -gal from both β -gal:RTB and RTB: β -gal treated cells is detected only as the processed ~64 kDa form. In contrast, when probed with anti-RTB antibodies analogous full-length product is detected but RTB breakdown products are not detected suggesting degradation (data not shown). Interestingly, processing of the RTB: β -gal product (Fig. 8A) only showed the 64 kDa product and not the expected 99 kDa product indicating removal of the RTB domain as part of lysosomal processing. The “pulse-chase” experimental design (cells were washed of

external β -gal:RTB or RTB: β -gal following 1 h of uptake) provides some insight as to the kinetics of endosomal trafficking to the lysosomes although impacts of transcytosis or protein degradation were not measured. For both fusion orientations, β -gal fusion product (120 kDa) present in endosomes at 1 h was fully processed to lysosome-activated 64 kDa β -gal within 3 h. As shown in Fig. 8C, the 64 kDa “activated” β -gal products is still present at 24 and 48 h incubation suggesting significant stability of the processed form. Overall, these data suggest that although placement of the RTB fusion partner at the C-terminus of β -gal negatively impacts *in vitro* enzymatic activity, both forms are effectively processed within mammalian cells to yield equivalent functional β -gal enzyme capable of mediating disease substrate clearance.

4. Discussion

ERTs have revolutionized patient care and greatly enhanced patient quality of life for a number of lysosomal storage diseases. However, the inability of current ERT approaches to address neurological pathologies limits application to GM1-gangliosidosis, which presents with severe CNS involvement. The plant lectin RTB directs uptake primarily by adsorptive-mediated endocytosis, providing the potential to mediate ERT delivery into tissues and cell types with fundamentally different biodistribution and pharmacokinetics compared to current ERTs based on receptor-mediated endocytosis. The goal of this study was to produce and characterized bi-functional fusions of RTB and β -gal to determine if the fusion products support GM1 disease correction at the cellular level – a critical prerequisite for *in vivo* testing in β -gal^{-/-} mice. Our results demonstrate that plants effectively produce human β -gal in its full-length 85 kDa form. The plant-derived β -gal was enzymatically active but was not taken up into mammalian cells and did not mediate disease correction in GM1 fibroblasts. Although human glycoproteins produced in plants have N-linked glycans at the appropriate sites, plants lack the machinery for M6P modifications [58]. Thus, the RTB lectin provides critical potential cell uptake mechanisms for plant-made β -gal.

In contrast to plant-made β -gal, the β -gal fusions with RTB were efficiently endocytosed into GM1 fibroblasts and mediated degradation of accumulated GM1 ganglioside disease substrate. Although the addition of RTB to the C-terminus of β -gal reduced the specific activity of β -gal compared to mcd β -gal, pld β -gal, and β -gal:RTB (see section 3.3), once internalized into fibroblasts (and trafficked to lysosomes), RTB: β -gal and β -gal:RTB showed no significant difference in enzymatic activity or kinetics of GM1 ganglioside degradation (Fig. 5). This observation is potentially explained by our data on protein processing of the internalized β -gal fusions (Fig. 8). The experiment was set up as a “pulse-chase” to monitor processing kinetics of the internalized product. Our results indicate that, in addition to the “activation” cleavage event within the C-terminal region of β -gal, RTB is cleaved from β -gal and appears to be rapidly degraded within the lysosomes. The removal and degradation of RTB was independent of orientation with its β -gal partner. This has implications with respect to the many lysosomal protein-protein and protein-substrate interactions required for full β -gal functionality. It appears that RTB effectively delivers enzyme into cells and lysosomes, but is then rapidly removed, thus minimizing any potential detrimental impacts on β -gal function. In contrast to RTB, the ~64 kDa β -gal product appears quite stable in fibroblasts with significant product remaining 48 h after uptake (Fig. 8C). Previous reports

indicate that β -gal is rapidly degraded in lysosomes in the absence of PPCA (within 4 h of delivery to *PPCA*^{-/-} cells) [33]. The stability of the activated β -gal product in β -gal:RTB-treated GM1 fibroblasts suggests that the plant-made β -gal successfully interacts with endogenous lysosomal PPCA (either before or after RTB removal). Overall, our results demonstrate that plant-made β -gal:RTB functions as an effective ERT in cells supporting further development and *in vivo* analyses in β -gal^{-/-} mice, the mouse models model for GM1-gangliosidosis.

Delivery of replacement enzymes to “hard-to-treat” tissues, and to the brain in particular, remains a significant barrier to development of new ERTs [11, 59]. The failure of currently approved ERT drugs to treat CNS pathologies indicates that delivery based on high-mannose or mannose-6-phosphate receptors is insufficient for attaining corrective doses in the brain. New strategies involving antibody-fusions and “molecular Trojan horse” approaches have been shown to facilitate delivery of fused enzymes across the BBB [60–63]. These approaches also exploit receptor-mediated endocytotic mechanisms, targeting insulin or transferrin receptors known to be present in brain capillary endothelial cells. In contrast, lectins provide receptor-independent mechanisms for cell uptake and thus may support very different ERT biodistribution and kinetics. Lectins have been shown to facilitate drug delivery across the BBB and into other “hard-to-treat” organs (e.g., lung, eye) [14, 64–66] (reviewed in [67]). Galactose-binding lectin activity was identified as the critical mechanism whereby adeno-associated virus 9 (AAV9; used for brain delivery of gene therapy), but not other AAVs, is able to mobilize across the BBB [68, 69]. The reduced “saturability” of adsorptive-mediated transcytosis, as well as the prevalence of cell surface glycans on brain endothelial cells, suggest that lectins such as RTB may provide CNS access [14]. Highlighting the differences between receptor- and adsorptive-mediated uptake, our data showed that RTB enabled delivery of five-times more β -gal into fibroblasts than mammalian cell-derived β -gal using the M6P receptor (Fig. 6). Based on the promising results described here, we are planning *in vivo* analyses of β -gal:RTB in the GM1-gangliosidosis mouse model to assess biodistribution and GM1 ganglioside reduction in visceral and CNS tissues.

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Abbreviations

GM1	GM1-gangliosidosis
β-gal	acid β -galactosidase
LSD	lysosomal storage disease
ERT	enzyme replacement therapy
RTB	B-subunit of ricin

BBB	blood brain barrier
CNS	central nervous system
PPCA	protective protein/cathepsin A
Neu1	neuraminidase

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Highlights

- The RTB plant lectin is proposed as a carrier for lysosomal replacement enzymes.
- Plant-made β -gal:RTB fusions retain galactosidase and lectin-binding activities.
- β -gal:RTB reduces lysosomal GM1 ganglioside levels in GM1 patient fibroblasts.
- β -gal:RTB undergoes lysosomal β -gal proteolytic activation in GM1 fibroblasts.
- Dynamics of GM1 correction of β -gal:RTB differs from mammalian cell-derived β -gal.

Figure 1.

DNA constructs harboring *GLB1*. Ten gene constructs were designed to compare human *GLB1* endogenous signal peptide (hSP) vs. our plant signal peptide (PoSP) optimized from the potato patatin SP; native human DNA sequence (NAT) vs. tobacco codon optimized (OPT) variant; and fusion orientation with the plant lectin RTB.

Figure 2. Relative β -gal activity in plant crude extracts. (A) β -galactosidase enzymatic activity was measured in crude extracts using the fluorometric assay described in methods section 2.4. (B) Quantification of lectin active/enzyme active fusion protein was measured by the Dual assay as described in methods 2.5. Crude leaf extracts were generated from plants harvested 4 days after *Agro* infiltration with the various constructs (construct designations are described in Fig. 1). Values are the average of five biological replicates. Error bars represent standard error.

Figure 3.

Purity and molecular size of plant-derived β -gal and β -gal:RTB fusions. (A) To assess purity, β -gal-containing proteins were size-separated by gel electrophoresis under reducing conditions (2.0 μ g protein/lane) and protein was detected by SimplyBlue® SafeStain (Coomassie stain). (B) Western immunoblots of purified protein (30 ng/lane) utilized the same electrophoresis condition and were detected using anti β -gal antibody as described in the methods section 2.6. (C) Calibration curve for the determination of the molecular weights of native pld β -gal (165 kDa, ▲) and β -gal:RTB (236 kDa, ■) by size exclusion chromatography (HiLoad 26/60 Superdex 200 pg). The standard curve was established by running individual proteins from the Gel Filtration Markers Kit for Protein Molecular Weights 29–700 kDa (Sigma) under the same conditions as test samples (see methods 2.7). The elution volume (V_e) for each protein-standard was divided by the void volume (V_o) of the column. **Insert:** Molecular size of pld β -gal (1) and β -gal:RTB (2) was confirmed by size-separating both proteins in 8% native Tris-glycine gels by electrophoresis.

Figure 4. Detection of β -gal activity in normal fibroblasts (GLB1^{+/+}) and GM1 fibroblasts (GLB1^{-/-}) with and without addition of β -gal:RTB. X-Gal staining was used as a detection method of intracellular β -gal activity in fibroblasts (methods section 2.9). β -gal activity was detected in normal fibroblast (A) but not in GM1 fibroblasts (B). X-Gal staining was also observed in GM1 fibroblasts treated with β -gal:RTB for 2 h prior to fixation and staining, suggesting internalization and activity of the recombinant protein (C).

Figure 5.

GM1 ganglioside levels in treated and untreated GM1 (GLB1^{-/-}) fibroblasts. Dylight⁵⁹⁴ - conjugated Cholera Toxin B was used as a detection method of GM1 ganglioside accumulation in culture fibroblasts (A). Levels of substrate accumulation were quantified using high throughput imaging system. Normal and GM1 fibroblast were cultured in a 96-well plate and incubated for 24 h untreated or treated with 6 nM of the different recombinant proteins as indicated (12–16 wells per treatment; n=12 or 16). Cells were fixed, stained, and images acquired using a High-Content Bioimager as described in methods section 2.10. Data shows difference in GM1 ganglioside accumulation between cell lines and when treated with the different recombinant proteins (B). Statistical significance versus untreated (****) was assessed by 1-way ANOVA, Tukey's multiple comparison test among treatments. ns: no significant difference.

Figure 6.

Maximum uptake capacity of recombinant proteins in GM1 fibroblast. Confluent fibroblast cultures were treated with different protein concentrations as described in methods section 2.11. After incubation time to ensure quantification of only intracellular proteins, cells were washed with 100mM lactose to remove any lectin-fusion still bound to the cell surface. Cells were harvested by trypsinization and washed with PBS. Cell lysates were analyzed for β-gal enzymatic activity and graphically analyzed as described (section 2.11). Dashed lines represents half-maximum uptake capacity of mcdβ-gal.

Figure 7. Michaelis-Menten kinetics of mcd β -gal, pld β -gal and β -gal fusions with RTB. Enzyme kinetics of the different constructs were measured using purified extracts (>98% purity) quantified as described in methods (2.12).

Figure 8.

Intracellular processing of RTB-β-gal fusions in GM1 fibroblasts. GLB^{-/-} fibroblast were incubated with either RTB:β-gal (A) or β-gal:RTB (B) for the times indicated. For the 1/2 h and 1 h time points, treated cells were harvested directly; for longer time points, media was replaced after 1 h of incubation (see methods section 2.13 for full description). At harvest, cells were washed with 100 mM lactose to remove any residual lectin-protein on the cell surface and harvested by trypsinization. A set of untreated cells were incubated for the same time periods as cross-reactive protein controls. Cells lysates were analyzed by Western blot detected with anti-β-gal antibodies. Recombinant mcdβ-gal (20 ng/well) was loaded in gels as positive control. Full length fusion protein (120kDa; (blue dotted box in A,B,C) was the prominent intracellular product up to 2 hours of incubation; after that, this band was reduced suggesting processing to “activated” β-gal form (65kDa; pink dotted box in A,B,C), detected after 1 hour of incubation. Presence of “activated” β-gal product remained in cells even after 24h and 48h (C). Diagram of fusions showing expected sizes of RTB and β-gal products after lysosomal processing of β-gal C-terminal peptide (D).