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Cellular uptake and lysosomal delivery of galactocerebroside tagged with the HIV Tat protein transduction domain

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Abstract

A number of studies have shown that a short peptide, the protein transduction domain (PTD) derived from the HIV-1 Tat protein (Tat-PTD) improved cellular uptake *in vitro* and distribution *in vivo* of recombinant proteins bearing such PTDs when administered systemically. To investigate the effects of Tat-PTD addition on the subcellular localization of the lysosomal enzyme galactocerebroside (GALC, EC 3.2.2.46) and with a view towards designing improved therapeutic strategies for Krabbe disease (globoid cell leukodystrophy), mouse GALC was tagged C-terminally with the Tat-PTD. Compared with unmodified GALC, GALC bearing a Tat-PTD, a myc epitope and 6 consecutive His residues [GALC-TMH (Tat-PTD, a myc epitope and 6 consecutive His residues)] was found to be secreted more efficiently. Also, GALC-TMH was found to be taken up by cells both via mannose-6-phosphate receptor (M6PR)-mediated endocytosis as well as by

M6PR-independent mechanisms. GALC-TMH displayed increased M6PR-independent uptake in fibroblasts derived from twitcher mice (a murine model of globoid cell leukodystrophy) and in neurons derived from the mouse brain cortex compared with GALC lacking a Tat-PTD. Immunocytochemical analyses revealed that Tat-modified GALC protein co-localized in part with the lysosome-associated membrane protein-1. Complete correction of galactosylceramide accumulation was achieved in twitcher mouse fibroblasts lacking GALC activity following addition of GALC-TMH. Therefore, GALC-TMH not only maintained the features of the native GALC protein including enzymatic function, intracellular transport and location, but also displayed more efficient cellular uptake.

Keywords: galactocerebroside, globoid cell leukodystrophy, lentiviral vectors, protein transduction, Tat protein transduction domain, twitcher mouse model.

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Galactocerebroside (GALC, EC 3.2.1.46) is a lysosomal hydrolase that degrades galactosylceramide, a lipid component of myelin, and its deficiency results in demyelination. The resulting disease, known as globoid cell leukodystrophy (GLD) or Krabbe disease, affects the CNS and is inherited in an autosomal recessive manner (Wenger *et al.* 2000). Galactosylceramide is found mostly in the brain, particularly in myelin sheaths. Unmetabolized galactosylceramide does accumulate in macrophages, which form characteristic globoid cells. Galactosylceramide is unique among the sphingolipids in its ability to elicit globoid cells (Suzuki 1985). Another substrate of GALC, psychosine (galactosylsphingosine), accumulates in neural tissues. The psychosine metabolism is impaired in patients with Krabbe disease and the brains of patients affected with this disease may contain 10–100 times the normal amount of psychosine (Suzuki 1998). Psychosine is highly cytotoxic primarily affecting oligodendrocytes, the myelin forming cells and causing their apoptotic death (Taniike *et al.* 1999; Zaka and

Wenger 2004). Human GALC has been purified from urine and lymphocytes (Chen and Wenger 1993; Sakai *et al.* 1994a) and its cDNA and genomic sequences have been determined (Chen *et al.* 1993; Sakai *et al.* 1994b; Luzi *et al.* 1995). GALC cDNA sequences from other species including

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Abbreviations used: BSA, bovine serum albumin; CMV, cytomegalovirus; ERT, enzyme replacement therapy; FBs, fibroblasts; GALC, galactocerebroside; GLD, globoid cell leukodystrophy; GUS, glucuronidase; Lamp-1, lysosomal-associated protein-1; HBSS, Hank's balanced salt solution; LSDs, lysosomal storage diseases; M6PR, mannose-6-phosphate receptor; MPS, mucopolysaccharidosis; PBS, phosphate-buffered saline; PBST, phosphate buffered saline with 0.05% Tween 20; PTD, protein transduction domain; TMH, Tat-PTD, a myc epitope and 6 consecutive His residues; WT, wild type.

mice, dogs, and rhesus monkeys have also been characterized (Sakai *et al.* 1996; Luzi *et al.* 1997). The human GALC cDNA codes for 669 amino acids including a 26 amino acid leader peptide. This correlates with an estimated molecular mass of 80 kDa of the purified enzyme (Chen and Wenger 1993). The 80-kDa version of human GALC has been found to undergo proteolysis, resulting in 30- and 50-kDa fragments in the extracellular medium (Nagano *et al.* 1998).

Over 60 disease-causing mutations have been characterized in human patients with Krabbe disease (Wenger *et al.* 2000). A mutation causing GLD in mice has also been identified (Wenger 2000). These mice are referred to as twitcher mice. Twitcher mice are the most widely used animal model of Krabbe disease to date.

A potential treatment option for Krabbe disease may involve supplying sufficient GALC activity to still functioning oligodendrocytes by enzyme replacement therapy (ERT) or using gene therapy-based approaches to degrade the psychosine that is starting to accumulate (Wenger *et al.* 2000; Lee *et al.* 2005b). A problem for treating GLD, and other lysosomal storage diseases (LSDs), is that virtually all cells in an organism are affected. Clearly, it is not feasible to correct all cells using gene-based approaches. Fortunately, however, this is not necessary when cross-correction can occur. It has been demonstrated that several lysosomal enzymes, including GALC can be secreted by cells producing the enzyme and taken up by other cells via the mannose-6-phosphate receptor (M6PR) (Kornfeld 1987; Nagano *et al.* 1998). For example, GALC released from GALC-overexpressing cells was shown to be taken up by fibroblasts (FBs) derived from Krabbe patients (Rafi *et al.* 1996) or by twitcher mouse-derived glial cells (Luddi *et al.* 2001). In principle, a limited number of overproducing cells should be able to provide sufficient enzyme to the rest of the organism. However, the delivery of therapeutic proteins into tissues and across the blood–brain barrier can be limited due their size and their biochemical properties. Schwarze *et al.* (1999) described the successful uptake of functional *Escherichia coli* β -galactosidase fused to a short cationic peptide derived from the HIV-1 Tat protein through the blood–brain barrier involving a mechanism known as protein transduction (Schwarze and Dowdy 2000). In subsequent studies, Xia *et al.* (2001), Elliger *et al.* (2002), and Orii *et al.* (2005) tested how the Tat protein transduction domain (PTD) affected secretion, uptake and biodistribution of β -glucuronidase (GUS), the enzyme that is defective in mucopolysaccharidosis type VII (MPS VII). The Tat motif allowed for M6PR-independent uptake of the enzyme *in vitro* and significantly increased the distribution of GUS secreted from transduced cells after intravenous or direct brain injection of recombinant adenovirus vectors encoding GUS tagged with the Tat-PTD (Xia *et al.* 2001; Elliger *et al.* 2002). Improved intracellular delivery of glucocerebrosidase (the enzyme that is defective in Gaucher disease) mediated by the HIV-1

Tat-PTD was also observed (Lee *et al.* 2005a). Thus, enzymes modified to contain protein transduction motifs may represent a general strategy for improving the distribution of secreted proteins following *in vivo* gene transfer.

The optimal pH for GALC is around 4.2 (Sakai *et al.* 1994a), a feature that strengthens the feasibility of applying Tat-PTD-modification to GALC as PTD-mediated uptake is into endosomes with reduced pH. At least a portion of Tat-PTD-modified GALC in endosomes is expected to proceed to lysosomes.

With a view towards establishing improved therapeutic strategies for GLD, we analyzed in this report the impacts of a Tat-PTD on enzymatic function, cellular uptake and intracellular location of mouse GALC.

Experimental procedures

Cell culture

Human embryonic kidney 293 (CRL-1573) and 293T (CRL-11268) cells, African green monkey COS-7 cells (CRL-1651), and HeLa cells (CCL-2) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen–Gibco, Gaithersburg, MD, USA) with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT, USA), 2.5 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin (all from Invitrogen–Gibco). Primary cultures of mouse skin FBs were established from 4-week-old B6.CE-GalC^{twi} twitcher mice (The Jackson Laboratory, Bar Harbor, ME, USA) and from wild-type mice. The genotypes of animals were determined by PCR analysis using tail DNA isolated from 1-week-old mice as described (Sakai *et al.* 1996). Skin FBs were maintained in Dulbecco's modified Eagle's medium with 15% heat inactivated fetal bovine serum, 2.5 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin. Mouse brain cortex neurons (M-CX-300; Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA) were plated on plates coated with poly-D-lysine and laminin and maintained in neurobasal medium containing 2% B27 supplement, 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen–Gibco).

GALC expression constructs

The lentiviral plasmid pNL-GALC-IRES-Neo containing a mouse GALC cDNA sequence (Sakai *et al.* 1996), driven by the human cytomegalovirus (CMV)-IE promoter was constructed by inserting a 2.3 kb *SmaI*–*Bam*HI fragment derived from pSVL/MGal (kindly provided by Norio Sakai, Osaka University) into pNL-IRES-Neo cut with *Eco*RI and *Bam*HI. pNL-IRES-Neo was derived from pNL-Neo (Mochizuki *et al.* 1998) by substituting the Neo sequence with an IRES-Neo fragment derived from pIRES1-Neo (Clontech, Palo Alto, CA, USA). To construct pNL-GALC-MH/CMV, a 1.1-kb mouse GALC cDNA fragment was generated by PCR, using a forward primer (5'-GACAGCGGGCCATCTAGAGAA-3') and a reverse primer (5'-TTGTTTGAAGGGCCCGGAGCAGCTTC-CACGCGAAA-3'). This 1.1-kb fragment encodes the C-terminal part of the mouse GALC protein (amino acids 348–668) and also contains a GGG codon replacing the TAG stop codon. The fragment

was cloned into the *XbaI/ApaI* sites of pcDNA3.1/myc-His (Invitrogen, Carlsbad, CA, USA) resulting in pcDNA-GALC(FC)MH encoding GALC with in-frame myc epitope and His tag sequences. A 1.2-kb *XbaI* fragment containing the 5' part of the GALC cDNA (encoding amino acids 1–347) from pLITMUS-GALC (generated by cloning the GALC cDNA into the *NcoI/BamHI* sites of pLITMUS 29, New England Biolabs, Beverly, MA, USA) was then inserted into the *XbaI* site of pcDNA-GALC(FC)MH to generate pcDNA-GALC-MH. Finally, the *SnaBI/PmeI* fragment derived from pcDNA-GALC-MH was cloned into the *SnaBI/XhoI* sites of pNL-EGFP/CMV (Reiser *et al.* 2000) resulting in pNL-GALC-MH/CMV. To generate pcDNA-GALCΔMH, a 2.1-kb *KpnI/FspI* fragment from pLITMUS-GALC was cloned into the *KpnI/XbaI* sites of pcDNA3.1/myc-His. To construct pNL-GALC-TMH/CMV, the pcDNA3.1/Tat-myc-His plasmid was modified by replacing the pcDNA3.1/myc-His-derived myc-His epitope-encoding sequence with a sequence encoding a HIV-1 Tat-PTD. This sequence consisted of a 104-bp oligonucleotide encoding the HIV-1 TAT-PTD (YGRKKR-RERRRG) (Green and Loewenstein 1988) in frame with a myc-His epitope sequence. The primers used in the overlap extension reaction were: forward, 5'-GAGGGCCCACCATGGGCTATGGCAGGAAGAAGCGGAGACAGCGACGACGAAGAGGCTTCGAACAA-3'; reverse, 5'-ATGACCGGTATGCATATTCAGATCTCTTTCTGAGATGAGTTTTGTTCGAAGCCTCTTCGTCGCTG-3'. This fragment with an *ApaI* site at the 5'-end and an *AgeI* site at 3'-end was cloned into the *ApaI/AgeI* sites of pcDNA3.1/myc-His, resulting in pcDNA3.1/Tat-myc-His. Next, the *XbaI/ApaI* fragment from pcDNA-GALC-MH was cloned into the *XbaI/ApaI* sites of pcDNA3.1/Tat-myc-His resulting in pcDNA-GALC(FC)TMH. Finally, the *SnaBI/PmeI* fragment derived from pcDNA-GALC(FC)TMH was cloned into the *SnaBI/XhoI* sites of pNL-EGFP/CMV, resulting in pNL-GALC-TMH/CMV.

Test of recombinant GALC proteins by transient transfection

293T cells were subjected to transient transfection as described (Reiser *et al.* 1996). Plasmid pNL-LacZ(co) (Kuroda and Reiser, unpublished) encoding *E. coli* β-galactosidase was co-transfected to monitor transfection efficiencies. β-galactosidase activity was determined as described by Bao *et al.* (2004).

Lentiviral vector production and transduction of cells

Lentivirus vector stocks were prepared as described previously (Reiser 2000). Virus titers and vector copies in transduced cells were determined by real-time PCR using primers and probes specific for the HIV-1 group-specific antigen (Gag) coding region (Zhang *et al.* 2004). Target cells were transduced with lentiviral vectors using multiplicities of infection of 30.

Western blot analysis

Cell culture supernatant aliquots (200 μg) from 293T and HeLa cells transduced with GALC-encoding lentiviral vectors were collected by centrifugation and concentrated using StrataClean Resin (Stratagene, La Jolla, CA, USA). Concentrated proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 4–12% NuPAGE Bis–Tris gels (Invitrogen) and then blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 3% non-fat milk in Tris buffered saline with Tween 20, TBST (Sigma-Aldrich, St. Louis,

MO, USA) buffer, and incubated with a monoclonal anti-myc antibody (Invitrogen) followed by alkaline phosphatase conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA). Blots were treated using the Lumi-Phos WB reagent (Pierce Biotechnology Inc., Rockford, IL, USA) and then exposed to X-ray films.

GALC assay

Cultured cells were harvested from tissue culture plates using a cell lifter and washed twice with phosphate-buffered saline (PBS). Cell pellets were re-suspended and homogenized in H₂O containing 1 mmol/L phenylmethylsulfonyl fluoride (Sigma–Aldrich) at 4°C. Cell extracts were centrifuged (22 088 g for 15 min, 4°C). Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce). GALC activity was determined using ³H-galactosylceramide (American Radiolabeled Chemicals, St Louis, MO, USA) as a substrate as described by Raghavan and Krusell (1986). Twenty micrograms of cell extract or 50 μL of cell culture supernatant from lentivirus-transduced cells were used for the assay. GALC units are defined as nmoles of ³H-galactosylceramide hydrolyzed per hour.

Determination of GALC uptake

For GALC production, COS-7 cells were transduced with lentiviral vectors encoding GALC-MH or GALC-TMH for 22 h in the presence of 8 μg/mL polybrene (Sigma–Aldrich). Transduced cells were passaged twice and cell culture supernatants were collected. To determine GALC uptake, 4 mL of conditioned media containing GALC-MH or GALC-TMH (60–120 GALC units) were added to 4 × 10⁵ untransduced cells including 293T cells, COS-7 cells or twitcher mouse FBs (Twi FBs) in the presence or absence of 5 mmol/L M6P (Sigma–Aldrich). For GALC uptake by mouse brain cortex neurons, GALC-MH or GALC-TMH (90 GALC units) were added in the presence or absence of 5 mmol/L M6P. Twenty-four hours later, the cells were washed with PBS and harvested, and cell extracts prepared and GALC activities determined.

Immunofluorescence labeling and microscopy

To assess the subcellular distribution of the GALC-TMH protein, transduced Twi FBs were plated on coverslips in six-well plates, fixed using 3% paraformaldehyde in PBS for 20 min and blocked with PBS containing 10% goat serum and 0.05% saponin (both from Sigma–Aldrich) for 1 h. The coverslips were incubated with mouse monoclonal anti-myc (Invitrogen; 1 : 250 dilution) or rat monoclonal anti-mouse lysosomal-associated protein-1 (Lamp-1) (BD Biosciences, Bedford; 1 : 250), diluted in phosphate buffered saline with 0.05% Tween 20 (PBST) containing 1% bovine serum albumin (BSA) and 0.05% saponin for 2 h at 25°C followed by three 10-min washes with PBST. Alexa 488-conjugated goat anti-mouse-IgG or Alexa 568-conjugated goat anti-rat-IgG (Molecular Probes, Eugene, OR, USA; 1 : 1500 dilution) were then applied and the cells incubated for 1 h at 25°C followed by three 10-min washes with PBST. The coverslips were mounted with ProLong Gold anti-fade reagent with 4'-6-Diamidino-2-phenylindole (Molecular Probes). Images were taken using a Bio-Radiance 2100 scanning confocal microscope (Bio-Rad).

To analyze uptake of GALC-TMH by Twi FBs, 2 mL of conditioned medium containing GALC-TMH (43 GALC units) were added to 2 × 10⁴ Twi FBs plated on coverslips in six-well

plates. Twenty-eight hours later, cells were fixed using 3% paraformaldehyde in PBS for 20 min and blocked with PBS containing 10% goat serum and 0.05% saponin for 1 h. The coverslips were incubated with affinity-purified rabbit anti-GALC polyclonal antibodies (CL1475 1 : 800) (Lee *et al.* 2005b, 2007) or rat monoclonal anti-mouse Lamp-1 (BD Biosciences; 1 : 500), for 2 h at 25°C followed by three washes with PBST. Alexa 488-conjugated goat anti-rabbit-IgG (1 : 500 dilution) or Alexa 568-conjugated goat anti-rat-IgG (1 : 1000 dilution) were then applied and the cells incubated for 2 h at 25°C followed by three 10-min washes with PBST.

Analysis of cells using fluorescent lipids

Twf FBs, wild-type FBs or lentiviral vector NL-GALC-TMH/CMV-transduced FBs plated on Cell-Tak adhesive-treated coverslips (BD Biosciences) were incubated with a solution containing BODIPY-FLC₁₂-galactoceramide (BODIPY-GalCer; Molecular Probes) and BSA (5 μmol/L each in Hanks'-buffered salt solution, containing 10 mmol/L HEPES, pH 7.4; Hanks' balanced salt solution (HBSS)/HEPES) for 45 min at 37°C. Cells were washed twice with HBSS/HEPES and incubated with fresh medium for 1 h at 37°C, followed by a 'back-exchange' with 5% BSA (fatty acid free, Sigma-Aldrich) in HBSS/HEPES at 10°C (Martin and Pagano 1994). The coverslips were rinsed with PBS and mounted with ProLong Gold anti-fade reagent. Images were taken using an Olympus TH 4-100 inverted fluorescence microscope. Similar analyses were conducted using Twf FBs incubated with conditioned medium collected from 293 cells stably expressing GALC-TMH for 24 h before applying BODIPY-GalCer.

Results

C-terminal addition of a Tat-PTD does not impair GALC activity

To distinguish recombinant mouse GALC from endogenous GALC and to assess the effects of the Tat-PTD on the activity of recombinant GALC, a number of different GALC expression constructs were prepared. These constructs encoded 24 additional amino acids at the C-terminus of the GALC protein including a myc epitope sequence and six consecutive His residues. Another construct encoded an eleven amino acid PTD derived from the HIV-1 Tat protein (Green and Loewenstein 1988; Schwarze and Dowdy 2000) in addition to the myc epitope sequence and the six His residues (Fig. 1a). The corresponding GALC proteins are referred to as GALC-MH and GALC-TMH, respectively. The results presented in Fig. 1b show that the specific activity of the GALC-MH enzyme in total cell extracts was similar to that of the unmodified (untagged) recombinant GALC enzyme following transient transfection of 293T cells using GALC and GALC-MH-encoding plasmids. However, the specific activity of GALC fused to the Tat-PTD was reduced by about 37% relative to that of GALC-MH (Fig. 1b). A similar reduction in GALC-TMH activity relative to GALC-MH (about 40%) was observed when GALC levels

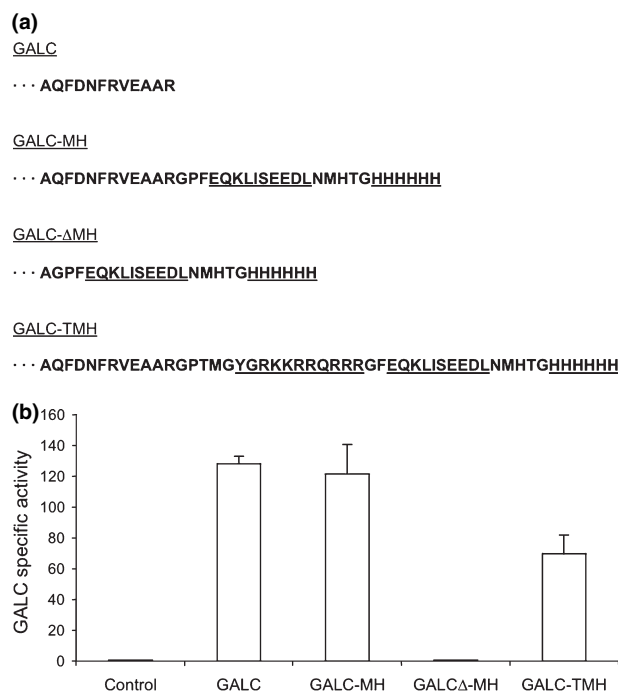


Fig. 1 Design and functional analysis of galactocerebrosidase (GALC) expression constructs. (a) Expression constructs. GALC: unmodified GALC protein; GALC-MH: GALC fusion protein containing a C-terminal myc-tag and six His residues; GALC Δ -MH: GALC fusion protein containing a C-terminal myc-tag (EQKLISEEDL) and six His residues with the last 11 amino acids at the C-terminus of GALC deleted; GALC-TMH: GALC fusion protein containing a C-terminal Tat-PTD (YGRKKRRQRRR), a myc-tag and six His residues. (b) Specific activities of recombinant GALC proteins. 293T cells were transiently transfected with plasmids encoding unmodified GALC, GALC-MH, GALC Δ -MH, or GALC-TMH. Cell extracts were prepared 3 days later and GALC-specific activities (GALC units per mg of cell extract) determined. Control: extracts from untransfected 293T cells. Data shown are from three independent experiments and expressed as mean values \pm SD.

were normalized based on β -galactosidase expression following co-transfection using the pNL-LacZ(co) plasmid (data not shown). Also, a GALC expression construct with the last 11 amino acid residues of GALC deleted (GALC Δ -MH) lacked enzymatic activity altogether (Fig. 1b). This indicates that the mouse GALC protein can tolerate C-terminal extensions and that a full-length GALC C-terminus is critical for GALC activity.

Impact of the Tat-PTD on the release of GALC into the extracellular medium

To assess the impact of the Tat-PTD on the release of recombinant GALC into the extracellular medium, lentiviral vectors encoding GALC-MH and GALC-TMH were generated. The GALC activity in transduced African green

monkey kidney-derived COS-7 cells is shown in Table 1. A higher proportion of the enzyme activity was found in the extracellular medium of GALC-TMH-transduced cells ($72.5 \pm 3.7\%$) compared with GALC-MH-transduced cells ($58.8 \pm 8.3\%$) (Table 1). These results indicate that fusion of Tat-PTD with the mouse GALC protein facilitated the release of the recombinant enzyme into the extracellular medium.

To further analyze the recombinant GALC enzyme released from the transduced cells into the extracellular medium, a western blot analysis of concentrated cell culture supernatants using a monoclonal antibody directed against the myc epitope was carried out. As shown in Fig. 2, a single band consistent with a molecular weight around 80 kDa was detected in the culture medium harvested from transduced cells including 293T and HeLa cells. The size of the myc-tagged recombinant protein was close to that reported for unmodified human GALC (Chen and Wenger 1993; Nagano *et al.* 1998). Interestingly, the recombinant GALC-TMH protein secreted by 293T and HeLa cells displayed a slightly slower electrophoretic mobility compared with the recombinant GALC-MH protein lacking a Tat-PTD. The same mobility shift was observed with GALC-containing supernatants collected from transduced COS-7 cells (data not shown). The reason for this lower than expected electrophoretic mobility of GALC-TMH relative to GALC-MH is not clear.

Tat-PTD facilitated uptake of recombinant GALC by COS-7 cells, twitcher mouse FBs and by primary mouse neurons

To show that the recombinant GALC enzyme secreted from transduced cells could be taken up by cells through M6PR-mediated endocytosis, enzyme uptake assays were performed. The results presented in Fig. 3 and Table 2 indicate that COS-7 cells, Twi FBs and mouse cortical neurons were

Table 1 Distribution of recombinant galactocerebrosidase (GALC) activity in COS-7 cells

Recombinant enzyme	GALC activity in cell extract (%)	GALC activity released into cell culture medium (%)
GALC-MH	41.2 ± 8.3	58.8 ± 8.3
GALC-TMH	27.5 ± 3.7	72.5 ± 3.7

GALC activities in cell extracts and cell culture supernatants of COS-7 cells expressing GALC-MH or GALC-TMH. Activities were determined 5 days after the cells were plated. The data shown are from four independent experiments and displayed as mean values \pm SD. Endogenous GALC activity in transduced COS-7 cells amounted to 3% of that observed with the recombinant enzyme. GALC activity released is presented as daily GALC activity released into the culture medium.

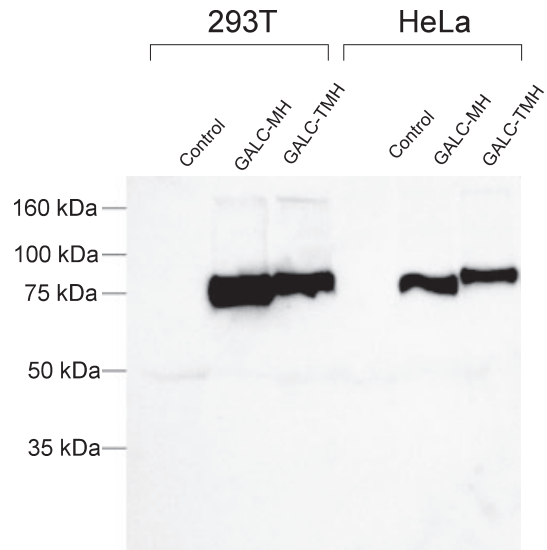


Fig. 2 Western blot analysis of recombinant galactocerebrosidase (GALC) proteins released from lentivirus-transduced 293T and HeLa cells. Cell culture supernatants (200 μ g aliquots) from lentivirus-transduced 293T and HeLa cells were collected and concentrated. Concentrated proteins were analyzed by western blotting using a monoclonal anti-myc antibody. Control: medium from untransduced cells. Protein molecular weight markers are indicated.

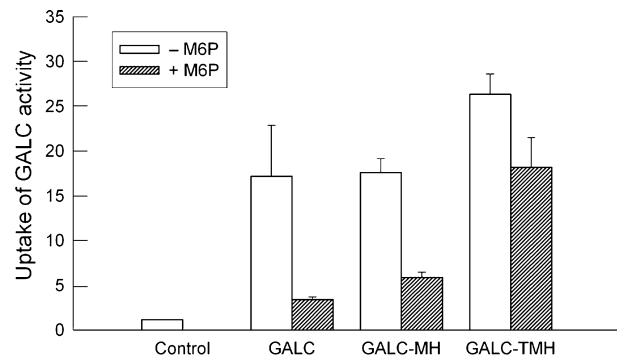


Fig. 3 Uptake of recombinant galactocerebrosidase (GALC) bearing a Tat-protein transduction domain (PTD) in the absence and presence of mannose-6-phosphate (M6P). Conditioned media from COS-7 cells expressing GALC-MH or GALC-TMH were added to COS-7 cells. Media were left on cells for 24 h in the presence or absence of 5 mmol/L M6P as indicated. The cells were analyzed for intracellular GALC activity. The values shown represent GALC units per mg of cell extract. They were obtained from triplicate samples and are expressed as mean values \pm SD.

capable of taking up GALC from the conditioned medium of transduced COS-7 cells expressing GALC-MH or GALC-TMH. Fig. 3 shows that GALC-MH and GALC-TMH in the absence of M6P were taken up as efficiently as unmodified GALC, indicating that modification of the C-terminus of GALC did not affect the enzymes' uptake. As shown in

Recombinant enzyme	Cells	GALC uptake in absence of M6P	GALC uptake in presence of M6P	% Inhibition
GALC-MH	COS-7	17.62 ± 1.53	5.9 ± 0.59	66.97 ± 5.67
GALC-TMH	COS-7	26.35 ± 2.37	18.19 ± 3.34	34.60 ± 11.82
GALC-MH	Twi FBs	11.44 ± 2.14	2.61 ± 1.18	77.19 ± 4.51
GALC-TMH	Twi FBs	12.55 ± 1.22	8.22 ± 2.30	35.03 ± 12.91
GALC-MH	Mouse neurons	43.60	14.1	67.64
GALC-MH	Mouse neurons	203.17	76.22	62.49
GALC-TMH	Mouse neurons	21.40	17.87	16.50
GALC-TMH	Mouse neurons	132.88	111.54	16.06

Table 2 Cellular uptake of recombinant galactocerebrosidase (GALC)

Conditioned media from COS-7 cells expressing GALC-MH or GALC-TMH were added to cells. Media were left on cells for 24 h in the presence or absence of 5 mmol/L mannose-6-phosphate (M6P) as indicated. The cells were analyzed for intracellular GALC activity. The values for COS-7 cells and Twi FBs were obtained from triplicate samples and are expressed as mean values ± SD. For mouse neurons, two independent sets of experiments are shown. The percentage of inhibition of GALC uptake was calculated by comparing the uptake of GALC activity from the conditioned medium in the presence of M6P to that in the absence of M6P. GALC activities are displayed as GALC units per mg of protein.

Fig. 3 and Table 2, uptake of recombinant GALC-MH was largely blocked (67% for COS-7 cells, 77% for Twi FBs, and up to 68% for primary neurons) in the presence of an excess of M6P indicating that uptake was mostly mediated by M6PR-mediated internalization. Uptake of GALC bearing a Tat-PTD (GALC-TMH) was only partially inhibited by M6P (35% in COS-7 cells and in Twi FBs and up to 17% in mouse neurons). Taken together, these results provide evidence that the recombinant GALC enzyme maintained the features of the native GALC enzyme and that the Tat-PTD motif facilitated M6PR-independent GALC uptake.

Localization of Tat-PTD-modified GALC protein and Lamp-1 in twitcher mouse FBs

To assess the effects of the myc epitope and the Tat-PTD on the subcellular distribution of GALC, the intracellular location of the recombinant GALC-TMH protein in transduced cells was analyzed by antibody staining using anti-myc antibody and an antibody directed against the Lamp-1. The confocal images shown in Fig. 4 indicate that the punctate staining observed after incubation with monoclonal anti-myc antibody followed by Alexa 488-labeled secondary antibody overlapped in part with that of Lamp-1 indicating that at least some of the Tat-PTD-modified GALC enzyme was associated with lysosomes. To better resolve the subcellular proximity and potential cross-over of myc-tagged GALC and Lamp-1, a deconvolution analysis was performed. Careful examination of optical sectioning throughout the z-axis revealed that even though the majority of the myc label would apparently yield a negative Pearson's correlation with respect to Lamp-1, a generous amount of signal was in close proximity to the lysosomal compartments (data not shown).

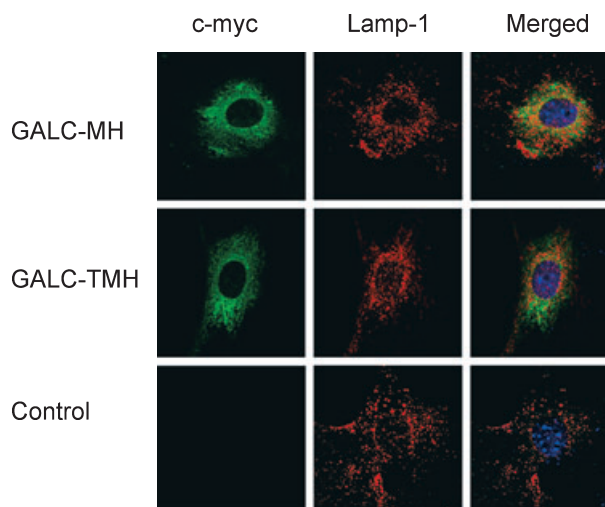


Fig. 4 Confocal analysis of galactocerebrosidase (GALC) and lysosomal-associated protein-1 (Lamp-1) in Twi fibroblasts (FBs) expressing GALC-MH or GALC-TMH. GALC-MH and GALC-TMH-transduced Twi FBs (upper and center panels) and untransduced (control) Twi FBs (lower panel) were stained using anti-myc (green) and anti-Lamp-1 (red) antibody as described in Experimental procedures. Cell nuclei were stained using DAPI.

Correction of galactosylceramide accumulation in twitcher mouse FBs after transduction using lentiviral vectors encoding GALC-TMH or following GALC-TMH uptake

To show that GALC-TMH retained its enzymatic function *in vivo*, the degradation of galactosylceramide in Twi FBs following transduction using lentiviral vectors encoding GALC-TMH was analyzed. FBs were loaded with BO-DIPY-GalCer, a fluorescent analog of galactosylceramide

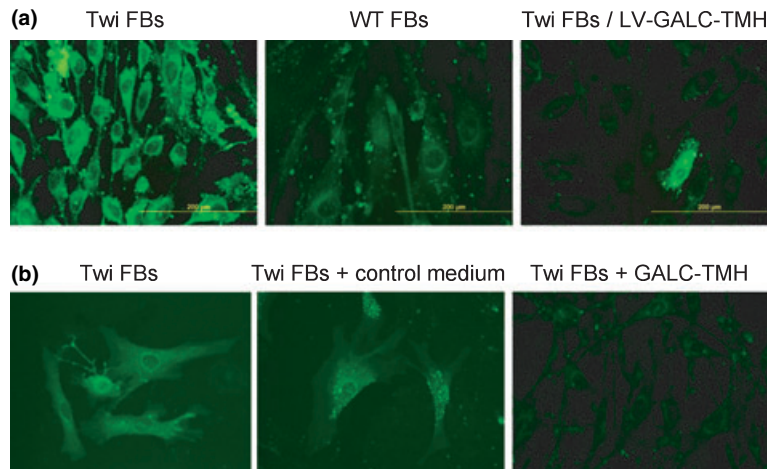


Fig. 5 Hydrolysis of BODIPY-GalCer in Twi fibroblasts (FBs) over-expressing galactocerebrosidase (GALC)-TMH or following uptake of GALC-TMH. (a) Mouse FBs were incubated with 5 $\mu\text{mol/L}$ BODIPY-GalCer. After back-exchange with buffer containing fatty acid-free bovine serum albumin (BSA), images were taken using a fluorescence microscope. Left panel, Twi FBs; Center panel, FBs derived from normal mice; Right panel, Twi FBs over-expressing GALC-TMH. The GALC-specific activities measured in these cells were 0.49, 3.06, and 33.20 GALC units per mg of protein, respectively. (b) Twi FBs were

incubated with conditioned medium for 24 h. Cells were then washed and incubated with BODIPY-GalCer. Left panel, untreated Twi FBs; Center panel, FBs incubated with conditioned medium from control 293 cells; Right panel, Twi FBs exposed to conditioned medium from 293 cells over-expressing GALC-TMH. The GALC-specific activities used for uptake were as follows: Left panel: GALC activity undetectable; Middle panel: 0.3 GALC units per mL; Right panel: 20.2 GALC units per mL.

(Marks and Pagano 2002). The distribution and accumulation of BODIPY-GalCer in FBs from affected mice (Twi FBs), wild type mice (WT FBs) and FBs from affected mice transduced with lentiviral vectors encoding GALC-TMH (Twi FBs + GALC-TMH) were compared (Fig. 5a). In WT FBs, BODIPY-GalCer was cleared, mediated by the endogenous GALC enzyme. However, residual fluorescence was detected in perinuclear structures. In Twi FBs, there was heavy accumulation of unhydrolyzed BODIPY-GalCer all over the cells, presumably because of the lack of GALC activity. The distribution of BODIPY-GalCer in Twi FBs expressing GALC-TMH was similar to that seen in WT FBs, indicating that Twi FBs expressing GALC-TMH rapidly cleared BODIPY-GalCer (Fig. 5a). When Twi FBs were incubated with conditioned medium containing GALC-TMH, Twi FBs were also able to clear BODIPY-GalCer (Fig. 5b, right panel), while Twi FBs incubated with control medium lacking GALC did not (Fig. 5b, middle panel), indicating that clearing was mediated by the exogenously added GALC. To provide more direct evidence for GALC uptake, Twi FBs were incubated with conditioned medium containing GALC-TMH and then stained using polyclonal anti-GALC antibodies (Lee *et al.* 2005b, 2007). The results presented in Fig. 6 show that the staining pattern following incubation with the anti-GALC antibody overlapped at least in part with that obtained with the anti-Lamp-1 antibody. This indicates that following uptake, recombinant GALC was associated at least in part with lysosomes.

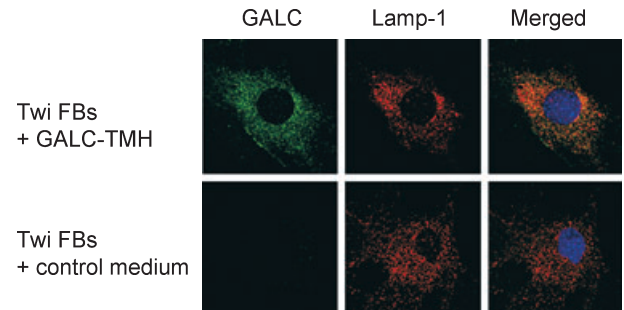


Fig. 6 Confocal analysis of galactocerebrosidase (GALC) and lysosomal-associated protein-1 (Lamp-1) in Twi fibroblasts (FBs) following uptake of GALC-TMH. Twi FBs were exposed to conditioned medium from 293 cells over-expressing GALC-TMH (top panel). The bottom panel shows Twi FBs incubated with conditioned medium from control 293 cells. Twenty-eight hours later, cells were stained with anti-GALC (green) and anti-Lamp-1 (red) antibody as described in Experimental procedures. Cell nuclei were stained using DAPI.

Discussion

Enzyme replacement therapy was first proposed as a therapeutic strategy for LSDs soon after the enzymatic defects were elucidated in the human sphingolipid storage disorders Gaucher disease and Niemann-Pick disease (Brady 1966). Currently, ERT is a treatment option for Gaucher disease type I, Fabry disease, and MPS I, and clinical

investigations are underway for Pompe disease, MPS II, and MPS VI (Desnick and Schuchman 2002). This approach is based on the fact that lysosomal enzymes contain oligosaccharide residues (e.g. mannose and/or M6P) that bind to cellular receptors (e.g. mannose or M6PR), leading to internalization of the enzymes by clathrin-mediated endocytosis and subsequent trafficking to lysosomes (Desnick and Schuchman 2002). Inadequate glycosylation of recombinant enzymes and/or lack of appropriate receptors in LSD target cells are obstacles that may affect enzyme delivery and the efficacy of ERT.

Alternative strategies aimed at improving enzyme uptake were described recently. These focus on chimeric enzymes containing alternative targeting moieties including a peptide derived from insulin-like growth factor II (LeBowitz *et al.* 2004), the receptor-associated protein targeting the low-density lipoprotein receptor (Prince *et al.* 2004), or the tetanus toxin C fragment (Jiang *et al.* 2005). However, chimeric enzymes often exhibit diminished activity. Also, the M6PR and low-density lipoprotein receptor family receptors are internalized via clathrin-mediated pathways, which are defective in some lysosomal enzyme-deficient cells (Dhami and Schuchman 2004). Other strategies have focused on chimeric enzymes involving PTDs including the HIV Tat-PTD (Xia *et al.* 2001; Elliger *et al.* 2002; Lee *et al.* 2005a).

Schwarze *et al.* (1999) were the first to show that denatured proteins bearing the Tat-PTD, as large as *E. coli* β -galactosidase, could translocate across membranes and even enter the brain following intraperitoneal injection involving a mechanism known as protein transduction (Schwarze and Dowdy 2000). However, these results are difficult to apply to mammalian lysosomal enzymes in general, in part because hydrophilic oligosaccharide side chains on the glycosylated acid hydrolases prevent proper refolding and assembly of multimeric enzymes following denaturation. Thus, their translocation across cell membranes may be impeded (Orii *et al.* 2005). Results reported by Xia *et al.* (2001), Elliger *et al.* (2002), and Orii *et al.* (2005) showed that the Tat-PTD affected secretion, uptake and biodistribution of native GUS. Furthermore, the Tat motif significantly increased the distribution of GUS secreted from transduced cells after intravenous or direct brain injection in mice with recombinant adenovirus vectors (Xia *et al.* 2001; Elliger *et al.* 2002; Alisky *et al.* 2006).

It is evident from our studies that mouse GALC can also be modified to bear a Tat-PTD without diminishing its function. Our results indicate that the recombinant GALC protein containing a Tat-PTD domain not only maintained the features of the native GALC protein including enzymatic function *in vitro* and *in situ* and intracellular transport, but it also displayed more efficient intercellular uptake via a M6PR-independent pathway.

It was previously shown that BODIPY-lactosylceramide and BODIPY-GalCer are taken up by cells and transferred to

the Golgi compartment (Chen *et al.* 1999). Interestingly, in several LSDs including Krabbe disease, these fluorescent lipids were found to accumulate in late endosomes/lysosomes (Chen *et al.* 1999; Puri *et al.* 1999). Our results show that clearance of accumulated BODIPY-GalCer in Twi FBs did occur following expression (Fig. 5a) or uptake (Fig. 5b) of GALC-TMH by such cells. The residual perinuclear fluorescence seen in WT FBs (Fig. 5a, middle panel) may indicate non-hydrolyzed BODIPY-GalCer residing in a vesicular compartment to which endogenous GALC does not have access. Interestingly, in Twi FBs expressing GALC, this perinuclear fluorescence was not as pronounced possibly because GALC levels in such cells were at least 10 times higher compared with those in WT FBs.

The mechanism(s) affecting Tat-PTD-mediated uptake and distribution of recombinant proteins need to be clarified. Several lines of evidence suggest that the PTDs only mediate cell surface adherence, a property shared with many other positively charged macromolecules. The cell surface adherence results in endocytosis and accumulation of proteins in endosomes. As eight of the 11 amino acid residues of the Tat-PTD are positively charged, Tat may bind negatively charged phospholipids of cell membranes and glycosaminoglycans on the cell surface which may mediate adsorptive endocytosis of Tat-modified proteins (Lundberg *et al.* 2003). This view is supported by recent findings obtained with GUS-Tat fusion proteins (Orii *et al.* 2005). Preliminary studies indicate that the uptake of GALC-TMH was blocked in the presence of heparin (Zhang, unpublished). This supports the view that GALC-TMH may also be taken up by adsorptive endocytosis.

In conclusion, in this study we show that Tat-modified GALC retains enzymatic activity, is efficiently secreted from cells, and is taken up by other cells via M6PR-dependent and independent mechanisms. We expect these findings to ultimately lead to improved therapies for GLD.

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