Chemically modified β-glucuronidase crosses blood–brain barrier and clears neuronal storage in murine mucopolysaccharidosis VII

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Enzyme replacement therapy has been used successfully in many lysosomal storage diseases. However, correction of brain storage has been limited by the inability of infused enzyme to cross the blood–brain barrier. The newborn mouse is an exception because recombinant enzyme is delivered to neonatal brain after mannose 6-phosphate receptor-mediated transcytosis. Access to this route is very limited after 2 weeks of age. Recently, several studies showed that multiple infusions of high doses of enzyme partially cleared storage in adult brain. These results raised the question of whether correction of brain storage by repeated high doses of enzyme depends on mannose 6-phosphate receptor-mediated uptake or whether enzyme gains access to brain storage by another route when brain capillaries are exposed to prolonged, high levels of circulating enzyme. To address this question, we used an enzyme whose carbohydrate-dependent receptor-mediated uptake was inactivated by chemical modification. Treatment of human β-glucuronidase (GUS) with sodium metaperiodate followed by sodium borohydride reduction (PerT-GUS) eliminated uptake by mannose 6-phosphate and mannose receptors in cultured cells and dramatically slowed its plasma clearance from a $t_{1/2}$ of <10 min to 18 h. Surprisingly, PerT-GUS infused weekly for 12 weeks was more effective in clearing central nervous system storage than native GUS at the same dose. In fact, PerT-GUS resulted in almost complete reversal of storage in neocortical and hippocampal neurons. This enhanced correction of neuronal storage by long-circulating enzyme, which targets no known receptor, suggests a delivery system across the blood–brain barrier that might be exploited therapeutically.

deglycosylation | enzyme replacement therapy | hippocampal neurons | lysosomal storage disease | receptor-mediated endocytosis

The mucopolysaccharidosis (MPS) diseases, including MPS VII, are characterized by widespread storage of glycosaminoglycans in lysosomes in brain and viscera (1). Enzyme replacement therapy (ERT) has resulted in clearance of stored material from visceral organs and in clinical improvement in many patients with lysosomal storage diseases (LSDs) (2–5). Unfortunately, little infused enzyme crosses the blood–brain barrier (BBB), so limited improvement has been achieved in the central nervous system (CNS) (6).

We have focused on improving delivery of enzyme to the brain by using β-glucuronidase (GUS) in the mouse model of MPS VII (Sly syndrome). When enzyme was infused into newborn mice, considerable enzyme was delivered to brain, and CNS storage was reduced (7–9). However, brain storage was resistant to clearance if ERT was begun after 2 weeks of age. Recent studies indicated that this enzyme delivery to the CNS in the newborn period was caused by mannose 6-phosphate receptor (M6PR)-mediated transcytosis (10). Down-regulation of this receptor by age 2 weeks appeared to explain the resistance of brain to ERT in the adult. More recently, however, we observed that multiple infusions of large doses of enzyme partially cleared storage in brain of adult mice (11). Studies from other laboratories in other LSD models also showed evidence of clearance of CNS storage after multiple infusions of large doses of corrective enzyme (12–14). To account for enzyme delivery to adult brain, we speculated that increasing the enzyme dose saturated the clearance receptors and slowed clearance of the enzyme from the circulation (11, 15).

Whether the high circulating levels of enzyme were required for delivery by receptors that were less abundant in adults than neonates or exposure to high circulating levels of enzyme led to delivery by another route is an important question. To address this question, we analyzed ERT in MPS VII mice that were mannose receptor (MR)-deficient (15). When GUS was infused into MR-deficient MPS VII mice, the enzyme clearance was indeed prolonged, although considerably less than expected, because of efficient clearance by hepatic M6PR (11, 15).

Much earlier studies had shown that carbohydrate-mediated clearance, i.e., clearance by the galactose receptor (16), M6PR (17), and MR (18), could be eliminated by chemical modification of the exposed carbohydrates on the glycoproteins. To study the effect of simply prolonging the time the enzyme circulates on the effect of ERT with GUS, we chemically inactivated its terminal sugars by treatment of GUS with sodium metaperiodate followed by borohydride reduction. We confirmed that this treatment produced a ligand (PerT-GUS) that is not susceptible to MR- or M6PR-mediated cellular uptake and plasma clearance (17, 18). We then compared the efficacy of 12 weekly i.v. infusions of 2 and 4 mg/kg PerT-GUS with the same treatment regimen with native GUS in the adult MPS VII mouse.

Results

Characterization of Purified Recombinant GUS. The purified recombinant GUS used in these experiments was similar to that described (11, 19). The apparent molecular mass of the enzyme monomer was 75 kDa on reducing SDS/PAGE. The tetrameric enzyme had a molecular mass of ≈300 kDa when analyzed by sizing gel filtration chromatography (data not shown). The specific activity of the purified enzyme was 4.9×10^6 units/mg. The K_{uptake}, where K is the concentration of enzyme giving half-maximal uptake, was 1.25- 2.50 nM, calculated from uptake saturation curves by using human MPS VII fibroblasts in which the uptake is almost entirely M6PR-dependent.

Treatment of Purified GUS with Periodate and Borohydride. To inactivate the mannose and M6P recognition markers that normally mediate clearance of infused GUS, we treated the enzyme sequentially with sodium metaperiodate followed by

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Table 1. Uptake of GUS and PerT-GUS by human fibroblasts

Enzyme	Uptake, units/mg in 1 h		
	−M6P	+2 mM M6P	M6P-specific
GUS	380	3	377
PerT-GUS	1	1	0

sodium borohydride (18). Sodium metaperiodate treatment inactivates the exposed carbohydrate recognition markers on glycoproteins by oxidative degradation (18). Subsequent reduction by sodium borohydride prevents aggregation that results from cross-linking of the reactive aldehydes on periodate-treated enzyme with free amino groups on other enzyme molecules (20). This sequential treatment of GUS resulted in only 14% loss in specific activity (4.9×10^6 units/mg to 4.22×10^6 units/mg).

To determine the impact of this treatment on receptor-mediated uptake, we measured the M6PR-mediated uptake in human fibroblasts (Table 1) and the MR-mediated uptake in mouse J774E macrophages (Table 2). The data presented in Table 1 show that the PerT-GUS had essentially no uptake by fibroblasts in the presence or absence of M6P, confirming that the M6P recognition marker had been completely inactivated. Similarly, Table 2 shows that PerT-GUS had lost its mannan-inhibitable uptake by macrophages. This result indicates that all of the exposed mannose residues on the carbohydrate chains of GUS had been inactivated.

Comparative Stability of GUS and PerT-GUS. The carbohydrates on glycoproteins often confer enhanced thermal stability, and removal of oligosaccharide chains often destabilizes glycoproteins (21). Human GUS has been shown to be relatively stable to thermal inactivation at 65°C (22–26). As shown in Fig. 1, recombinant GUS retained 90% of initial activity after 3 h at 65°C, whereas PerT-GUS retained 40% of its activity under these conditions (Fig. 1).

To compare the stability of GUS and PerT-GUS in lysosomes of living cells at 37°C, we studied their half-life after uptake by MPS VII fibroblasts. The low rate of endocytosis of PerT-GUS by fibroblasts required exposure to 100,000 units/ml PerT-GUS per plate for 48 h to accumulate sufficient enzyme by fluid phase pinocytosis (28 units per plate) to allow measurement of its half-life. By contrast, fibroblasts exposed to 500 units/ml M6P containing native GUS for 48 h contained 228 units per plate. Fig. 2 shows the half-life for the two enzymes in fibroblasts upon subsequent incubation at 37°C. The $t_{1/2}$ of GUS was 18.9 days. The $t_{1/2}$ of PerT-GUS was shorter (12.9 days), but nearly one-third of the initial activity was still present at 21 days.

Clearance of GUS vs. PerT-GUS from the Circulation After Infusion. Plasma clearance was measured in MPS VII mice after infusion of 4 mg/kg of each enzyme (Fig. 3). GUS was cleared with a $t_{1/2}$ of 11.7 min. This rapid clearance has been shown to depend on the MR and the M6PR clearance systems (15). In contrast, the clearance of PerT-GUS was dramatically prolonged to a $t_{1/2}$ of 18.5 h.

Table 2. Uptake of GUS and PerT-GUS by J774E macrophage line

Enzyme	Uptake, units/mg in 1 h		
	−Mannan	+Yeast mannan, 1.69 mg/ml	Mannan-specific
GUS	366	50	316
PerT-GUS prep 1	8	5	3
PerT-GUS prep 2	11	9	2
PerT-GUS prep 3	12	21	0

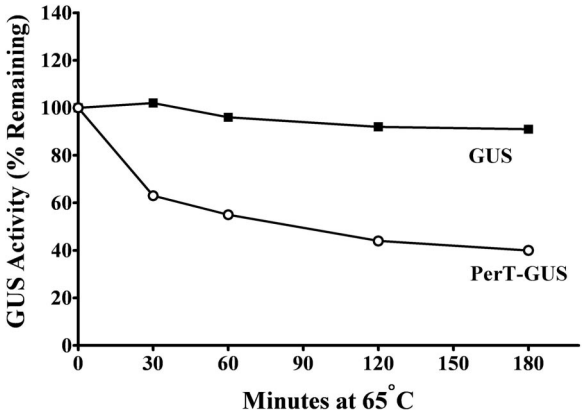

Fig. 1. Stability of native GUS or PerT-GUS at 65°C. GUS or PerT-GUS was incubated at 65°C in heat-inactivation buffer, and aliquots were taken at 0, 30, 60, 120, and 180 min, cooled on ice, and then assayed for GUS activity. GUS (■) retained 90% of the initial activity after 180 min. PerT-GUS (○) was somewhat less stable but retained 40% of the original activity after 180 min.

Tissue Distribution of GUS vs. PerT-GUS 48 h After Infusion. Previously, we observed that treating MPS VII mice with high-dose GUS slowed the plasma clearance of the enzyme and facilitated enzyme delivery to the brain (11). In these experiments, it was not clear whether it was the higher dose of enzyme itself or the delayed plasma clearance of the enzyme that accounted for improved delivery to brain. To address this question, we compared the distribution of GUS and PerT-GUS in brain and other tissues 48 h after infusion into MPS VII mice (Table 3). Mice were perfused with Tris-buffered saline before collection of tissues to ensure that tissue was not contaminated with residual plasma enzyme.

As evident from the data in Table 3, delivery of native GUS to brain at 48 h was minimal. However, native GUS was delivered to other tissues at levels similar those reported. PerT-GUS was delivered to heart, kidney, muscle, lung, and eye at levels higher than those seen with native GUS. The levels in liver and spleen were nearly 4-fold lower after PerT-GUS infusion than after GUS infusion. This result undoubtedly reflects the curtailment

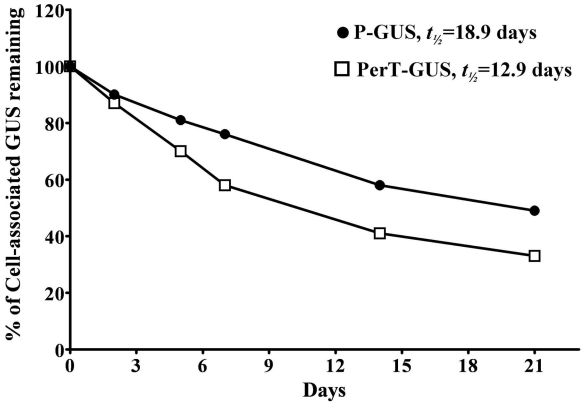

Fig. 2. Stability of native GUS or PerT-GUS in the lysosome after endocytosis by human fibroblasts. Human MPS VII fibroblasts were allowed to endocytose/pinocytose native GUS or PerT-GUS present in the normal growth medium in 35-mm culture dishes for 48 h at 37°C. After the indicated times, the cells were harvested and assayed for GUS activity. In fibroblasts, GUS (●) had a $t_{1/2}$ of 18.9 days, whereas PerT-GUS (□) had a $t_{1/2}$ of 12.9 days.

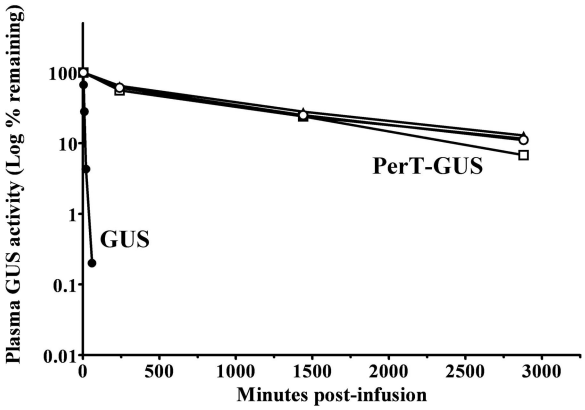

Fig. 3. Clearance of infused GUS or PerT-GUS from the circulation. MPS VII mice were infused with GUS or PerT-GUS at a dose of 4 mg/kg of body weight. Blood samples were taken at the indicated times by supraorbital eye sticks into heparinized capillary tubes. After centrifugation, plasma was collected and assayed for GUS activity. GUS (●) was cleared from the circulation with a $t_{1/2}$ of 11.7 min. Clearance of the PerT-GUS (□, ○, ▲) in three separate experiments was greatly prolonged with a $t_{1/2}$ of 18.5 h.

of receptor-mediated uptake by the MPR and M6PR that are highly expressed in these two tissues. By contrast, brain levels were greatly increased (7.8% of wild type) in PerT-GUS-infused animals. This result suggests that the long circulating PerT-GUS has an advantage in crossing the BBB. Thus, it was of great interest to study its effectiveness in clearing storage from cells in the CNS.

Comparison of the Effectiveness of GUS and PerT-GUS in Clearing Neuronal Storage. Fig. 4 compares the morphology of neocortical (Fig. 4 *A–C*) and hippocampal (Fig. 4 *D–F*) neurons and meninges (Fig. 4 *G–I*) in untreated (Fig. 4 *A*, *D*, and *G*) and GUS-treated (Fig. 4 *B*, *E*, and *H*) and PerT-GUS-treated (Fig. 4 *C*, *F*, and *I*) MPS VII mice. There was a marked reduction in storage in the neocortical (Fig. 4*C*) and hippocampal (Fig. 4*F*) neurons in the PerT-GUS-treated mice and a slight reduction in storage in neocortical neurons (Fig. 4*B*) with native GUS treatment. Meningeal storage was moderately reduced by both GUS and PerT-GUS treatment (Fig. 4 *H* and *I*).

To obtain a more objective measure of the reduction in neuronal storage in the CNS in response to the two types of GUS, we devised a morphometric approach described in *Materials and Methods*. Table 4 summarizes the results of assessment of storage in neocortical and hippocampal neurons of untreated, GUS-treated, and PerT-GUS-treated MPSVII mice. ERT with GUS over 12 weeks with both 2 mg/kg and 4 mg/kg GUS reduced storage in neocortical neurons compared with untreated MPS VII mice ($P = 0.002$ and $P = 0.003$, respectively), although hippocampal neurons failed to show a morphological response to this therapy. PerT-GUS at 2 mg/kg also reduced neocortical neuronal storage ($P = 0.001$). At 4 mg/kg, the therapeutic effect of PerT-GUS was even more striking ($P = 0.003$ for 2 vs. 4 mg of PerT-GUS and $P < 0.001$ compared with untreated). In addition, there was virtually no storage in the hippocampal neurons in the three PerT-GUS-treated mice available for quantitation (the CA2 region was not present in the section and was therefore not available for quantitation in two of the five PerT-GUS-treated mice). These results indicate that ERT with PerT-GUS is remarkably more effective than traditional GUS at clearing storage in the neocortical and especially hippocampal neurons in the MPS VII mouse. As a group, the PerT-GUS-treated mice also had slightly less storage in glial and perivascular cells than the GUS-treated mice. However, the dose-dependent reduction in storage in meninges, which was moderate at 4 mg/kg, was equivalent in the PerT-GUS- and the GUS-treated animals.

Table 3. Tissue GUS activity (units/mg tissue protein, mean ± SD) 48 h after infusion with GUS or PerT-GUS

Tissue	Wild-type levels* ($n = 4$)	GUS†, 4 mg/kg ($n = 2$)	PerT-GUS, 4 mg/kg ($n = 3$)
Brain	16.7 ± 2	0.23 ± 0.005	1.30 ± 0.28
Liver	185 ± 11.9	892 ± 45.5	230 ± 63
Spleen	301 ± 26.6	558 ± 54	122 ± 51
Heart	20.8 ± 12.5	13.0 ± 1.8	44.1 ± 16.3
Kidney	108 ± 7.5	11.9 ± 0.19	21.7 ± 3.6
Lung	ND‡	5.1 ± 0.4	19.9 ± 6.1
Muscle	4.95 ± 1.80	1.2 ± 0.07	6.3 ± 3.5
Bone + marrow	161 ± 35	75.6 ± 17	59.5 ± 24.8
Eye	4.88 ± 0.68	0.90 ± 0.52	4.9 ± 1.5

Mice were perfused before removal of tissues as described in *Materials and Methods*.
*Prior data by Vogler *et al*. (11).
†Data presented as average ± range.
‡ND, not done.

Clearance of Storage in Nonneuronal Tissues. Clearance of storage was nearly complete in liver and spleen sinusoidal cells and kidney at the 2 mg/kg and 4 mg/kg doses with both GUS and PerT-GUS. In bone, the chondrocytes showed no improvement with either enzyme. Storage was more consistently cleared by PerT-GUS in osteocytes at both doses than by GUS, although 4 mg/kg GUS also produced moderate to marked decrease in storage in most osteocytes.

Discussion

Despite the success of ERT in improving many aspects of the pathology in several LSDs for which ERT is available, addressing CNS storage remains a challenge because of inability of enzyme to cross the BBB (6). What seems clear from the studies in the murine MPS VII model presented here is that the long-circulating PerT-GUS was more effective at clearing storage from the CNS than native GUS, which is rapidly cleared from plasma by MR- and M6PR-mediated uptake (15, 18). PerT-GUS also reached other tissues, including heart, kidney, muscle, lung, and eye, better than recombinant native GUS, although PerT-GUS levels were nearly 4-fold lower in liver and spleen, two tissues rich in MR (in Kupffer cells in liver and in macrophages in spleen) and M6PR (in hepatocytes) (15, 18, 23, 27). The lower levels in liver and spleen are likely a direct consequence of the reduced clearance by these abundant carbohydrate recognition receptors in these two tissues (15).

A likely route of entry of PerT-GUS to brain capillary endothelial cells is fluid phase pinocytosis. The uptake of a soluble protein by fluid phase pinocytosis is strictly concentration-dependent, and its rate of uptake is slow compared with the rate of uptake of proteins by receptor-mediated endocytosis. Nonetheless, the very slow clearance of PerT-GUS from the plasma allows some uptake by this route over the entire 12-week interval instead of only during the first few min after each injection, which is the case with rapidly cleared native GUS. Even if PerT-GUS is taken up by endothelial cells by fluid phase pinocytosis, the mechanism by which enzyme crosses the BBB to enter cortical and hippocampal neurons and reduce storage remains to be defined.

Another possibility is that the PerT-GUS is delivered by transcytosis via an uncharacterized receptor that is not observable when enzyme is rapidly cleared or that only recognizes a modification of the enzyme introduced by the PerT treatment.

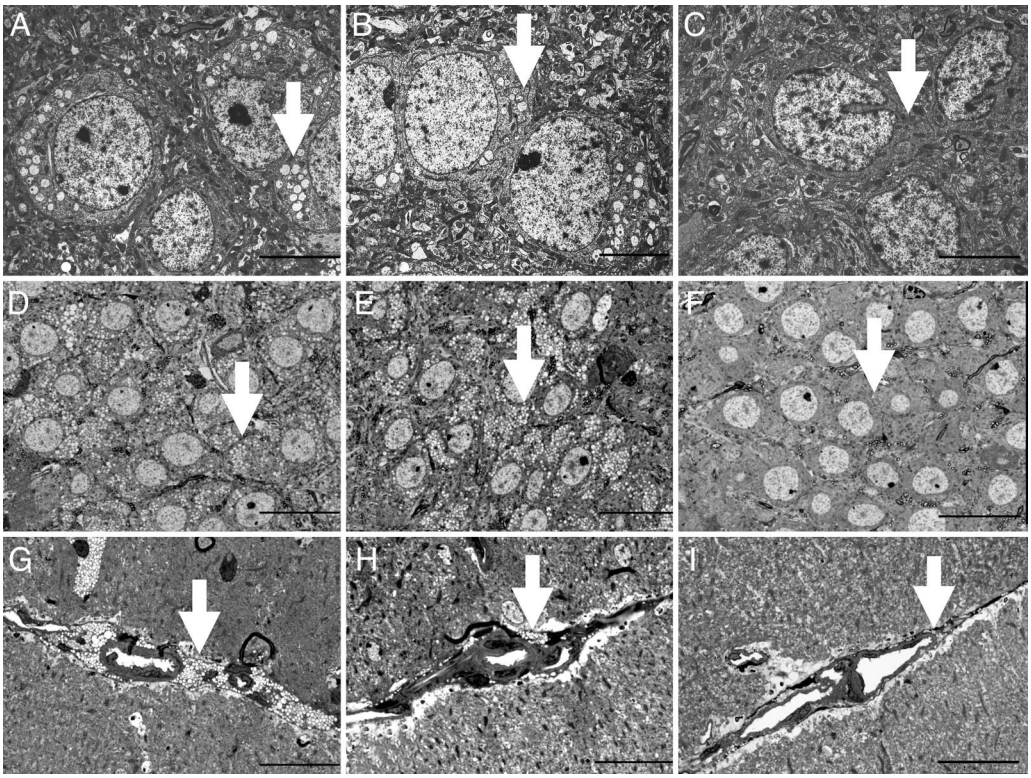

Fig. 4. Reduction in neuronal and meningeal storage with ERT with GUS and PerT-GUS. (*A*) Neocortical neurons from an untreated MPS VII mouse have abundant lysosomal storage in the cytoplasm (arrow). (*B*) After treatment with 4 mg/kg GUS, there is still a moderate amount of cytoplasmic storage (arrow) despite the therapy. (*C*) After 4 mg/kg PerT-GUS, there is a marked reduction in the amount of storage in the neocortical neurons (arrow). (*D*) The CA2 sector hippocampal neurons have abundant storage (arrow) in untreated MPS VII mice. (*E*) After treatment with GUS, the amount of storage in neurons (arrow) the same area of the hippocampus is similar to that of the untreated mouse. (*F*) After treatment with PerT-GUS, there is a remarkable reduction in the amount of storage in neurons (arrow) in the CA2 sector of the hippocampus. (*G*) The meninges of an untreated MPS VII mouse has abundant storage in fibroblasts around vessels (arrow). (*H*) Storage (arrow) is moderately decreased after treatment with GUS. (*I*) Treatment with PerT-GUS also produces moderate reduction in storage (arrow) in the meninges. (Scale bars: *A–C*, uranyl acetate–lead citrate, 10 μm; *D–I*, toluidine blue, 30 μm.)

A third possibility is that PerT-GUS crosses the BBB through the so-called "extracellular route," that allows trace amounts of molecules as large as albumin to enter the CNS (28). Even though the cerebrospinal fluid to serum albumin ratio is 1:200, there is evidence that exclusion of albumin by the BBB is not absolute (28). Furthermore, this extracellular pathway is thought to be the route of delivery of small amounts of erythropoietin, which has a neuroprotective effect in stroke. Also, antibodies to amyloid-β protein, which reverse cognitive impairment in the transgenic mouse model of Alzheimer's disease (28), are possibly transported by this route.

Table 4. Quantitation of lysosomal storage in neurons in control and treated MPS VII mice

	Vacuoles per 500 cells	
Treatment group	Neocortical neurons	Hippocampal neurons
Control MPS VII	1,956	692
	1,685	694
	1,927	
GUS 2 mg/kg	728	641
	744	674
	1,088	
GUS 4 mg/kg	1,274	642
	1,213	
PerT-GUS 2 mg	403	2
	439	
PerT-GUS 4 mg	73	0
	148	5
	72	

One factor that favors the extracellular mechanism for delivery is that it is nonsaturable, and the most important determinant for delivery via this pathway is the $t_{1/2}$ of the protein in the circulation. The very long $t_{1/2}$ of PerT-GUS would make it a candidate for delivery by this route. We suggested previously that high-dose ERT, which favors delivery to the CNS, may gain access to the CNS by this route because it saturates receptors that normally mediate the clearance of the enzyme at lower doses and prolongs the $t_{1/2}$ of the enzyme in the circulation (11).

In addition to the mechanism of delivery of PerT-GUS to the CNS, which remains to be identified, other questions raised by this work are: (*i*) Will treatment of MPS VII mice from birth with PerT-GUS prevent the development of dysostosis multiplex (bone abnormalities) as effectively as native GUS? (*ii*) Will treatment with PerT-GUS from birth be as effective as native GUS in preventing the hearing deficit and cognitive defects? From the morphological data presented here, one might expect that PerT-GUS might even be superior. (*iii*) Will these findings be generalizable to other disease models of lysosomal enzyme deficiencies? The favorable results reported here for MPS VII warrant further studies of this approach to address these questions.

Materials and Methods

Generation of Stable Cell Lines Secreting GUS. Using DNA-cloning techniques, we subcloned the cDNA sequence encoding the full-length cDNA for

human GUS (GenBank accession no. NM_000181) into the mammalian expression vector pCXN (29). This expression vector contains an expression cassette consisting of the chicken β-actin promoter coupled to the CMV intermediate–early enhancer. pCXN also contains a selectable marker for G418 allowing selection of stably expressing mammalian cells.

This plasmid was introduced into the Chinese hamster ovary cell line CHO-K1 by electroporation (30). After selection in growth medium consisting of minimal essential medium + 35 μg/ml proline + 15% FBS + 400 μg of G418, colonies were picked and grown to confluence in 48-well plates. High-level expressing clones were identified by measuring GUS activity secreted into the conditioned medium from these clones (19). The highest-producing clone was scaled up, and secreted enzyme was collected in protein-free collection medium, PF-CHO. Conditioned medium collected in this way was pooled and centrifuged at 5,000 × *g* for 20 min, and the supernatant was collected and frozen at −20°C until sufficient quantities were accumulated for purification.

Measurement of GUS Activity and Protein. GUS activity was measured by using 10 mM 4-methyl-umbelliferyl β-D-glucuronide as substrate in 0.1 M sodium acetate buffer (pH 4.8), 1 mg/ml crystalline BSA as described in ref. 31. Protein levels were assayed by the method of Lowry (32).

Purification of GUS. GUS was purified by a multistep procedure with conventional column chromatography. Medium collected from stable cell lines was thawed and filtered through a 0.2-μm microculture capsule filter (Pall Corporation). Filtrate was concentrated over an Amicon YM-100 membrane to minimize volume, then diafiltrated with 2 × 2.25-liter 20 mM $NaPO_4$ + 150 mM NaCl + 0.025% NaN_3 (pH 5.5). Diafiltrated concentrate was then passed over a Blue Sepharose FF (GE Healthcare) column preequilibrated with 20 mM $NaPO_4$ (pH 5.5). After washing with 10 column volumes of 20 mM $NaPO_4$ + 150 mM NaCl (pH 5.5), the column was eluted with 10 mM $NaPO_4$ + 800 mM NaCl (pH 7.5). The fractions containing GUS activity were pooled and applied to a phenyl-Sepharose high-Sub FF column preequilibrated with 10 mM $NaPO_4$ + 1,000 mM NaCl (pH 8.0). After washing with 10 column volumes of 10 mM $NaPO_4$ + 1,000 mM NaCl (pH 8.0), the column was eluted with 10 mM Tris + 1 mM sodium-β-glycerophosphate (pH 8.0). Fractions containing GUS activity were pooled and dialyzed with three changes of 10 mM Tris + 1 mM Na-β-glycerophosphate (pH 8.0). The dialyzed phenyl Sepharose elute was loaded onto a DEAE-Sephacel (Sigma–Aldrich) column preequilibrated with 10 mM Tris + 1 mM Na-β-glycerophosphate (pH 8.0). After washing with 10 column volumes of 10 mM Tris + 1 mM Na-β-glycerophosphate (pH 8.0), the column was eluted with a 0–0.4 M NaCl gradient in the same buffer. The GUS peak was pooled and then dialyzed against three changes of 25 mM sodium acetate + 1 mM Na-β-glycerophosphate + 0.025% NaN_3 (pH 5.5). The dialyzed DEAE eluate was applied to a CM-Sepharose (Sigma–Aldrich) column preequilibrated with 25 mM sodium acetate + 1 mM Na-β-glycerophosphate + 0.025% NaN_3 (pH 5.5). After washing with 10 column volumes of the same buffer, the column was eluted with a 0–0.3 M gradient of NaCl in the equilibration buffer. Fractions containing the peak GUS activity were pooled and dialyzed against three changes of 25 mM Tris (pH 7.5), 1 mM β-glycerophosphate, 150 mM NaCl, 0.25% sodium azide, and then frozen at −80°C where it was stable indefinitely.

Treatment of GUS with Periodate and Borohydride. The mannose and M6P recognition sites on GUS are located in the carbohydrate portion of the enzyme. To inactivate the exposed carbohydrates, the enzyme was treated with sodium metaperiodate followed by sodium borohydride (17, 18). Approximately 10 mg of purified GUS was treated with a final concentration of 20 mM sodium metaperiodate in 20 mM sodium phosphate, 100 mM NaCl (pH 6.0) for 6.5 h on ice, in the dark. The reaction was quenched by the addition of 200 mM final concentration ethylene glycol and incubated for an additional 15 min on ice, in the dark. Afterward, this mixture was dialyzed against two changes of 20 mM sodium phosphate, 100 mM NaCl (pH 6.0) at 4°C. Sodium borohydride was added to the dialyzed enzyme to a final concentration of 100 mM, and the enzyme was held on ice overnight in the dark to reduce reactive aldehyde groups. The enzyme was then dialyzed against two changes of 20 mM sodium phosphate, 100 mM NaCl (pH 7.5) at 4°C and was stable to storage in this buffer at 4°C.

GUS Uptake by Human Primary Fibroblasts and Mouse J774E Macrophages. M6PR-mediated uptake was determined by adding 4,000 units of GUS or PerT-GUS ± 2 mM M6P in 1 ml of growth medium to 35-mm dishes of confluent GM-2784 GUS-deficient fibroblasts. After incubation at 37°C and 5% CO_2 for 2 h, the cells were cooled on ice, washed five times with cold PBS, then solubilized in 0.5 ml of 1% sodium desoxycholate. Extracts were assayed for GUS activity and protein, and values were expressed as units of enzyme taken up per mg of cell protein per hour of uptake.

MR-mediated uptake was measured by adding 10,000 units of GUS or PerT-GUS ± 1.7 mg/ml yeast mannan (Sigma–Aldrich) in 1 ml of growth medium to 35-mm dishes of confluent J774E mouse macrophages (33). After incubation at 37°C and 5% CO_2 for 4 h, the cells were washed as above and then solubilized in 1 ml of 1% sodium desoxycholate and assayed for GUS activity.

Stability of GUS and PerT-GUS at 65°C. Purified GUS or PerT-GUS were diluted in equal volumes of heat inactivation buffer [40 mM Tris·HCl (pH 7.5), 150 mM NaCl, 10 mg/ml BSA], and aliquots were incubated for 0, 0.5, 1, 2, or 3 h at 65°C. After treatment, aliquots were cooled on ice and then assayed for GUS activity. Results were expressed as the percentage of original units of GUS activity remaining at the indicated times.

Stability of GUS vs. PerT-GUS After Uptake by Human MPS VII Fibroblasts. Tissue culture dishes (35 mm) of confluent GM-2784 GUS-deficient fibroblasts were incubated with 500 units of GUS or 100,000 units of PerT-GUS in 1 ml of growth medium at 37°C and 5% CO_2 for 48 h under sterile conditions. The plates were washed twice with sterile growth medium and then fed with 2 ml of the same. Duplicate plates were taken off at 0, 2, 5, 7, 14, and 21 days, washed five times with PBS and frozen at −20°C. Remaining plates were fed twice weekly with 2 ml of growth medium. After all plates had been collected, the cells were solubilized in 0.5 ml of 1% desoxycholate and assayed for GUS activity. Values were expressed as percentage of zero time cell-associated GUS activity remaining at the indicated time points.

Clearance of GUS vs. PerT-GUS from the Circulation After Infusion into Mice. MPS VII mice were infused via tail vein with GUS or PerT-GUS at a dose of 4 mg/kg in a total volume of 125 μl of PBS. After infusion, blood samples were taken by supraorbital puncture at 2, 5, 10, 20, 60, 90, and 120 min for GUS and 4, 240, 1,440, and 2,880 min for PerT-GUS into heparinized capillary tubes. Plasma was collected after centrifugation and assayed for GUS activity. Values were expressed as a percentage of GUS activity remaining compared with the first time point.

Tissue Distribution of GUS vs. PerT-GUS 48 h After Infusion. MPS VII mice were infused via tail vein with GUS or PerT-GUS at a dose of 4 mg/kg in a total volume of 125 μl of PBS. At 48 h after infusion, the mice were perfused with 30 ml of 25 mM Tris (pH 7.2), 140 mM NaCl. Perfused tissues were collected and flash frozen in liquid nitrogen until further processing. Tissues were thawed, weighed, and homogenized for 30 s with a Polytron homogenizer in 10–20 volumes of 25 mM Tris (pH 7.2), 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride. Total homogenates were frozen at −80°C, thawed, and then sonicated for 20 s to produce a homogeneous extract. Extracts were assayed for GUS activity and protein, and the results were expressed as units/milligrams of tissue protein.

Treatment of MPS VII Mice with GUS vs. PerT-GUS at Doses of 2 and 4 mg/kg. MPS VII mice were treated with 12 weekly infusions of GUS or PerT-GUS at doses of 2 or 4 mg/kg body weight. One week after the last infusion, tissues from untreated MPS VII ($n = 3$), 2 mg/kg ($n = 3$) or 4 mg/kg GUS ($n = 2$), or 2 mg/kg ($n = 2$) or 4 mg/kg PerT-GUS ($n = 3$) were obtained at necropsy after normal saline perfusion, fixed in 2% paraformaldehyde and 4% glutaraldehyde, postfixed in osmium tetroxide, and embedded in Spurr resin. For evaluation of lysosomal storage by light microscopy, toluidine blue-stained 0.5-μm-thick sections of liver, spleen, kidney, brain, heart, rib, and bone marrow were assessed without knowledge of treatment group. To evaluate storage in cortical neurons, 500 contiguous parietal neocortical neurons were scored for the number of lucent cytoplasmic vacuoles, indicating lysosomal storage. A maximum of seven vacuoles were counted per cell, and results were evaluated by ANOVA or Student's *t* test. We also evaluated hippocampal neurons by counting the number of vacuoles in 100 neurons in CA2 sector. Other tissues were examined by using a semiquantitative scale, as described in ref. 11.

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