Biodistribution, Kinetics, and Efficacy of Highly Phosphorylated and Non-phosphorylated β-Glucuronidase in the Murine Model of Mucopolysaccharidosis VII*

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Mark S. Sands‡§¶, Carole A. Vogler¶, Kevin K. Ohlemiller**, Marie S. Roberts‡, Jeffrey H. Grubb**, Beth Levy*, and William S. Sly‡‡

From the Departments of *Internal Medicine and §Genetics, and the **Research Department, Central Institute for the Deaf, Washington University School of Medicine, St. Louis, Missouri 63110 and the ‡Department of Pathology and the ¶Edward A. Doisy Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104

Enzyme replacement therapy (ERT) has been shown to be effective at reducing the accumulation of undegraded substrates in lysosomal storage diseases. Most ERT studies have been performed with recombinant proteins that are mixtures of phosphorylated and non-phosphorylated enzyme. Because different cell types use different receptors to take up phosphorylated or non-phosphorylated enzyme, it is difficult to determine which form of enzyme contributed to the clinical response. Here we compare the uptake, distribution, and efficacy of highly phosphorylated and non-phosphorylated β-glucuronidase (GUSB) in the MPS VII mouse. Highly phosphorylated murine GUSB was efficiently taken up by a wide range of tissues. In contrast, non-phosphorylated murine GUSB was taken up primarily by tissues of the reticuloendothelial (RE) system. Although the tissue distribution was different, the half-lives of both enzymes in any particular tissue were similar. Both preparations of enzyme were capable of preventing the accumulation of lysosomal storage in cell types they targeted. An important difference in clinical efficacy emerged in that phosphorylated GUSB was more efficient than non-phosphorylated enzyme at preventing the hearing loss associated with this disease. These data suggest that both forms of enzyme contribute to the clinical responses of ERT in MPS VII mice but that enzyme preparations containing phosphorylated GUSB are more broadly effective than non-phosphorylated enzyme.

Lysosomal storage diseases are inherited disorders usually caused by the lack of a single lysosomal enzyme activity (1). These diseases are usually progressive in nature, and children often present with a wide spectrum of clinical symptoms. It has been shown previously that exogenous lysosomal enzymes can be taken up by affected cells and can correct the metabolic defect (2). This process, referred to as “cross-correction,” forms the basis for several therapeutic approaches (2). Uptake of lysosomal enzymes is mediated by either the mannose 6-phosphate or mannose receptors (3, 4). Conceptually, the simplest therapeutic approach involves the systemic injection of a recombinant lysosomal enzyme containing oligosaccharides with exposed mannose or mannose 6-phosphate residues. Macrophage-targeted glucocerebrosidase with exposed mannose residues has been shown to be highly effective for Gaucher’s disease where the pathology is largely restricted to fixed tissue macrophages. It is generally believed that enzymes modified with mannose 6-phosphate would be more efficacious for most other lysosomal storage diseases. This is due to the fact that, unlike the mannose receptor, which is limited to cells of the reticuloendothelial system, the mannose 6-phosphate receptor is present on most cell types (5–7).

Enzyme replacement therapy has proven effective at preventing or reversing lysosomal storage in patients and animal models with lysosomal storage diseases (8–17). We previously showed that enzyme replacement prevents the accumulation of lysosomal storage in the murine model of mucopolysaccharidosis type VII (MPS VII), a lysosomal storage disease caused by a deficiency in β-glucuronidase (GUSB) activity (12, 18, 19). If untreated, the deficiency in GUSB activity leads to the progressive accumulation of partially degraded glycosaminoglycans (GAGS) in many tissues of the body. The resulting clinical symptoms include cognitive deficits, auditory defects, visual impairment, skeletal dysplasia, and shortened life span. A murine model of MPS VII has been described that shares most of these clinical symptoms (20–25). Enzyme replacement initiated at birth in MPS VII mice prevents the accumulation of GAGS in many tissues (12). The reduced lysosomal storage following enzyme replacement therapy correlates with dramatic improvements in bone development, cognitive ability, hearing, immune function, and life span (26–29). Like most other enzyme replacement studies, the recombinant enzyme used in the MPS VII mouse was produced in mammalian cells and was composed of a mixture of phosphorylated and non-phosphorylated enzyme (12, 30). Therefore, it has been difficult to determine which form of the enzyme produced which aspects of the clinical improvements. Here we show that the biodistribution and clinical efficacy of phosphorylated and non-phosphorylated murine GUSB are different following intravenous injection into MPS VII mice.

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‡ To whom correspondence should be addressed: Dept. of Internal Medicine, Washington University School of Medicine, Box 8007, 660 South Euclid Ave., St. Louis, MO 63110. Tel.: 314-362-5494; Fax: 314-362-9333; E-mail: msands@imgate.wustl.edu.

1 The abbreviations used are: MPS VII, mucopolysaccharidosis type VII; GUSB, β-glucuronidase; NP-GUSB, non-phosphorylated GUSB; P-GUSB, phosphorylated GUSB; GAGS, glycosaminoglycans; ABR, auditory-evoked brainstem response; RE, reticuloendothelial.
Enzyme Replacement for Mucopolysaccharidosis Type VII

EXPERIMENTAL PROCEDURES

Recombinant GUSB Production—Non-phosphorylated murine GUSB was produced in insect cells using the baculovirus system and will hereafter be referred to as NP-GUSB. Briefly, the murine GUSB cDNA was subcloned into the baculovirus transfer vector pBACPAK8. SF21 insect cells were cotransfected with the GUSB transfer vector and the packaging vector pBACPAK6 according to the manufacturer’s instructions (CLONTECH). One virus-producing clone was chosen based on the highest level of GUSB production. Passage 3 supernatants were used to infect 0.5- to 1-liter suspension cultures of SF21 cells at a multiplicity of infection of 5. Seven days after infection, cells and cellular debris were removed from the media by centrifugation at 5000 × g for 10 min at 4 °C. Viral particles were removed by centrifugation of the cleared media at 100,000 × g for 1 h at 4 °C.

Non-phosphorylated GUSB was purified by anti-mouse GUSB monoclonal antibody (31). Uptake and inhibition of NP-GUSB and P-GUSB was assayed for GUSB activity using 4-methylumbelliferyl-D-glucuronide as a substrate (31). Uptake and inhibition of NP-GUSB and P-GUSB was assayed for GUSB activity using 4-methylumbelliferyl-D-glucuronide as a substrate (31). Uptake and inhibition of NP-GUSB and P-GUSB was assayed for GUSB activity using 4-methylumbelliferyl-D-glucuronide as a substrate (31). Uptake and inhibition of NP-GUSB and P-GUSB was assayed for GUSB activity using 4-methylumbelliferyl-D-glucuronide as a substrate (31). Uptake and inhibition of NP-GUSB and P-GUSB was assayed for GUSB activity using 4-methylumbelliferyl-D-glucuronide as a substrate (31).

Phosphorylated murine GUSB was produced in mannose 6-phosphate receptor-deficient mouse L-cells as described previously (12, 30) and will hereafter be referred to as P-GUSB. The enzyme was then purified from the conditioned media by anti-mouse GUSB monoclonal antibody affinity chromatography. The enzyme was eluted in 150 mM NaCl, 25 mM Tris (pH 7.5), 1 mM β-glycerophosphate, and 8 mM urea. The urea was removed by passing the enzyme over BioGel P6 desalting resin in the same buffer described above without urea. The concentration of enzyme was adjusted to 2.5 × 10⁶ units/ml (1 unit = 1 nmol of substrate cleaved/h).

Phosphorylated murine GUSB was produced in mammalian 6-phosphate receptor-deficient mouse L-cells as described previously (12, 30) and will hereafter be referred to as P-GUSB. The enzyme was then purified from the conditioned media by anti-mouse GUSB monoclonal antibody affinity chromatography. The enzyme was eluted with 8 mM urea and then stored in the same buffer as described above. The enzyme concentration was then adjusted to 2.5 × 10⁶ units/ml and frozen at −70 °C.

The mannose 6-phosphate-containing fraction of P-GUSB was estimated by determining the percentage of the enzyme that is retained on a column of immobilized cation-independent mannose 6-phosphate receptor. The enzyme was applied to the receptor column, which was washed with buffer containing 10 mM glucose 6-phosphate to remove nonspecifically bound enzyme and eluted with 10 mM mannose 6-phosphate. The enzyme activity loaded on the column was quantitatively recovered in the flow-through and eluate. The specifically bound enzyme in the eluate represented 95.4% of the total activity.

In Vitro Uptake and Inhibition Assays—Human primary GUSB-deficient fibroblasts (SB20 cells) were plated in minimal essential medium, 10% FHEPE (pH 7.0), 15% fetal calf serum, and 25 mM tris(hydroxymethyl)aminomethane, and 100 units of penicillin, and 100 μg/ml of streptomycin and were allowed to grow to near confluence. NP-GUSB or P-GUSB was added to the media at a concentration of 4000 units/ml in the presence or absence of 2 mM mannose 6-phosphate (Sigma Chemical Co.). After 2-h incubation at 37 °C, the media was then removed and the cells washed three times with phosphate-buffered saline. The cells were then harvested, resuspended in 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, 0.2% Triton X-100, and lysed by three freeze-thaw cycles. The cellular debris was removed by centrifugation, and the supernatants were assayed for GUSB activity using 4-methylumbelliferyl-β-D-glucuronide as a substrate (31). Uptake and inhibition of NP-GUSB and P-GUSB was also measured in rat alveolar macrophages. Alveolar macrophages were isolated from 6- to 8-week-old rats by bronchoalveolar lavage with phosphate-buffered saline. The cells were washed and resuspended at a concentration of 5 × 10⁷ cells/0.3 ml in the same media as described for the fibroblasts. The cells were exposed to NP-GUSB or P-GUSB at a concentration of 33,000 units/ml in the absence or presence of 5 mM mannose 6-phosphate or 1.7 mg/ml yeast mannan for 2 h at 37 °C. The slides were then extensively washed in 0.05M sodium bicarbonate–acetate buffer (pH 4.5). The slides were then stained with naphthol-AS-BI-phosphate and hexazoxyflavine paraarsonaline for 12-16 h at 37 °C in a humidified incubator. The slides were counterstained with methyl green.

Sections of liver, spleen, kidney, heart, lung, brain, eye, and bone were immersed in ice-cold 2% glutaraldehyde and 4% paraformaldehyde for at least 48 h. The tissue sections were then embedded in Spurr’s resin, and 0.5-μm sections were obtained. Those sections were stained with toluidine blue for examination by light microscopy.

Auditory-evoked Brainstem Response—Auditory-evoked brainstem response (ABR) measurements were obtained as previously described (34). Mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (80/15 mg/kg). Platinum electrodes were placed subdermally at the vertex (reference), below the right ear (active) and on the dorsorsacrum (ground). Auditory stimuli were delivered to speakers located 7 cm directly lateral to the right ear, and tone burst stimuli at each frequency were presented 1000 times at 20/5. The minimum sound pressure level (decibels) required for visual detection of the characteristic ABR waveform was determined at 5, 10, 20, and 40 kHz using a 5-dB minimum step size.

Statistical Analysis—Significance determinations for the biodistribution studies were performed using Student’s t test.

RESULTS

In Vitro Characterization of P-GUSB and NP-GUSB—Insect cells used for the large scale production of recombinant proteins are known to faithfully glycosylate newly synthesized proteins. However, these cells do not have the phosphotransferase activity necessary to confer the mannose 6-phosphate modification of the terminal mannose residues of lysosomal enzymes (35–37). NP-GUSB produced in this way is not taken up by GUSB-deficient fibroblasts that both express the mannose 6-phosphate receptor and actively endocytose P-GUSB (Table 1). In contrast, NP-GUSB is avidly taken up by alveolar macrophages exclusively through a mannose receptor-dependent mechanism, even though they express both mannose and mannose 6-phosphate receptors. Phosphorylated murine GUSB (P-GUSB) is taken up by fibroblasts in a mannose 6-phosphate receptor-dependent manner. However, P-GUSB is also efficiently taken up by alveolar macrophages, both through a mannose 6-phosphate receptor-dependent pathway (45%) and a mannose receptor-dependent pathway (56%). The mannose receptor-dependent uptake reflects the fact that even though 95% of the P-GUSB contains mannose 6-phosphate and is retained on a mannose 6-phosphate receptor column, a significant fraction of the 12-oligosaccharide side chains per tetrasaccharide that are expressed on the surface of the enzyme in adult mice, three 6- to 8-week-old mps/mps mice each received intravenous injections of 2.5 × 10⁶ units of NP-GUSB or P-GUSB, respectively. At 7 and 14 days of age the mice in each group received an additional intraperitoneal injection of the same dose of the respective enzyme. At 21, 28, and 35 days of age, the mice received three injections of the respective enzyme. At 42 days of age three mice from each treatment group were sacrificed and examined histologically for the extent of lysosomal storage. At 9 weeks of age the remaining mice in each group received an intravenous injection of the same dose of the respective enzyme. At 12 weeks of age auditory-evoked brainstem response (ABR) measurements were obtained on six and four P-GUSB- and NP-GUSB-treated mice, respectively.

Histochemical and Histopathologic Analysis—Histochemical detection of GUSB activity in situ was performed essentially as described previously (33). Briefly, portions of tissue were placed in OCT embedding compound (Sakura Finetek, Inc.) and frozen in isopentane at liquid nitrogen temperature. Ten micron-thick sections were obtained and post-fixed in 0.65% chlorohydrylate, 0.6% neutral-buffered formalin, and 70% acetic acid at 4 °C. The slides were then dried at 40 °C. The slides were then stained with naphthol-AS-BI-phosphate and hexazoxyflavine paraarsonaline for 12–16 h at 37 °C in a humidified incubator. The slides were counterstained with methyl green.

Significance determinations for the biodistribution of the two forms of enzyme in adult mice were determined in newborn mps/mps mice. Five mps/mps animals each were identified at birth and injected intravenously through the superficial temporal vein (32) with 2.5 × 10⁶ units of either NP-GUSB or P-GUSB on the day of birth. One animal from each group was sacrificed at 1, 3, 5, 7, and 10 days of age, and the same tissues as described above were removed and assayed for GUSB activity.

To determine the extent of lysosomal storage reduction and the effect on auditory function, groups of nine and seven newborn mps/mps mice each received intravenous injections of 2.5 × 10⁴ units of P-GUSB or NP-GUSB, respectively. At 12 weeks of age the remaining mice in each group received an intravenous injection of the same dose of the respective enzyme. At 42 days of age auditory-evoked brainstem response (ABR) measurements were obtained on six and four P-GUSB- and NP-GUSB-treated mice, respectively.

Animal Injections and Procedures—Homozygous mutant (mps/mps) and phenotypically normal (+/+ or +/−) mice were obtained from the B6.C-H-2−/−ByJir-guspa+/+ colony maintained by one of us (M. S. S.) at the Washington University School of Medicine. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Washington University School of Medicine. To determine the biodistribution of the two forms of enzyme in adult mice, three 6- to 8-week-old mps/mps animals each were injected intravenously through the lateral tail vein with 2.5 × 10⁴ units of either NP-GUSB or P-GUSB. Five hours later the animals were sacrificed and the liver, spleen, kidney, heart, lung, and brain were removed for biochemical and histochemical analysis.

The half-lives of the two forms of enzyme in different tissues were compared by determining the percentage of the enzyme that is retained on a column of immobilized cation-independent mannose 6-phosphate receptor.
ramer of GUSB does not contain a phosphorylated mannose (38, 39).

In Vivo Biodistribution and Kinetics of P-GUSB and NP-GUSB—Five hours after a single intravenous injection of either P-GUSB or NP-GUSB into 6- to 8-week-old mps/mps mice comparable levels of each enzyme were measured in the liver and spleen (Fig. 1A). The levels of P-GUSB and NP-GUSB activity in the liver represent ~31.2 and 21.7% of normal, respectively. The levels of P-GUSB and NP-GUSB in the spleen represent ~8.1 and 9.2% normal, respectively. In contrast, there was significantly (p < 0.05) more P-GUSB taken up by the kidney, heart, and lung when compared with NP-GUSB (Fig. 1B). Following P-GUSB injection, the activity measured in the kidney, heart, and lung was 2.3, 42.5, and 3.4% normal levels, respectively. Following NP-GUSB injection, GUSB activity in those organs was between 4- and 8-fold less than that observed with P-GUSB. There was very little (<1% normal) of either enzyme activity associated with the brain (Fig. 1B). Although the levels of P-GUSB and NP-GUSB in the liver were comparable, the distribution of the respective enzymes within that organ was different. P-GUSB was uniformly distributed throughout the liver and was present in both hepatocytes and fixed tissue macrophages (Fig. 2A). In contrast, the distribution of NP-GUSB, although uniformly distributed throughout the liver, was restricted to sinus lining cells, which were intensely stained (Fig. 2B). There was little or no GUSB activity associated with the hepatocytes after the injection of NP-GUSB.

The relative tissue distribution of P-GUSB and NP-GUSB in newborn mps/mps mice 24 h after injection (data not shown) was similar to that described above in young adult mice 5 h after injection. Twenty-four hours after an intravenous injection of P-GUSB into newborn mps/mps mice, the levels of GUSB activity in newborn liver, spleen, heart, kidney, and lung were 446.7, 70, 3000, 131, and 185% normal, respectively. This level and distribution of activity was similar to that described in a previous study following an intravenous injection of an enzyme preparation containing a mixture of phosphorylated and non-phosphorylated forms (i.e., a less highly phosphorylated preparation (70%) from over expressing mouse L-cells) (18). The levels of NP-GUSB in the newborn liver and spleen 24 h after injection were 629 and 91% normal, respectively, whereas the levels in the heart, kidney, and lung were 750, 28, and 38% normal, respectively. The half-lives of P-GUSB and NP-GUSB were remarkably similar in various tissues following a single intravenous injection at birth. The half-lives of P-GUSB and NP-GUSB in the liver and spleen range from 1.4 to 1.8 days (Fig. 3). Although the absolute levels of P-GUSB and NP-GUSB taken up by other tissues varied by as much as 8-fold, the half-lives of both forms of enzyme within any tissue were similar and ranged from 1 to 1.5 days (data not shown).

Reduction of Lysosomal Storage following Injection of P-GUSB or NP-GUSB—The two enzyme preparations were compared for efficacy by determining the reduction of lysosomal storage in mice that received multiple injections during the first 6 weeks of life. Newborn mps/mps mice received their first injection on the first or second day of life followed by weekly injections of comparable amounts of either P-GUSB or NP-GUSB. One week after the last of six injections, the mice were sacrificed and examined histologically for the extent of lysosomal storage (Fig. 4). Treatment with P-GUSB reduced storage in many sites, including the meninges, retinal pigment epithelial cells, liver, spleen, kidney, and bone marrow. Storage was only reduced slightly in the osteoblasts of one animal and cortical neurons of two animals in this treatment group. Efficacy in reducing lysosomal storage was much more restricted in NP-GUSB-treated mice. Mice treated with NP-GUSB had reductions in storage in the sinus lining cells of the spleen and liver comparable to those observed in the mice treated with P-GUSB. However, there was much less reduction in storage in the retinal pigment epithelium, spleen trabecular fibroblasts, hepatocytes, renal tubular epithelial cells, and meninges. There was no reduction of neuronal or osteoblast storage fol-

### Table I

| Cells | Enzyme | Inhibitor | Units | Inhibition |
|-------|--------|-----------|-------|------------|
| S820  | P-GUSB | None      | 99    | None       |
| S820  | P-GUSB | M6P       | 2     | 98         |
| S820  | P-GUSB | None      | 1     | None       |
| S820  | P-GUSB | M6P       | 0     | 0          |
| MΦ    | P-GUSB | None      | 555   | None       |
| MΦ    | P-GUSB | M6P       | 305   | 45         |
| MΦ    | P-GUSB | Mann      | 242   | 56         |
| MΦ    | NP-GUSB| None      | 1960  | None       |
| MΦ    | NP-GUSB| M6P       | 1778  | 9          |
| MΦ    | NP-GUSB| Mann      | 0     | 100        |

* S820 refers to primary human skin fibroblast derived from an MPS VII patient; MΦ refers to primary rat alveolar macrophages isolated from 6- to 8-week-old animals.

* P-GUSB refers to phosphorylated murine GUSB; NP-GUSB refers to non-phosphorylated murine GUSB.

* M6P refers to mannose 6-phosphate, the concentration in the media of S820 cells and MΦs was 2 and 5 mM, respectively; Mann refers to 1.67 mg/ml yeast mannans in the media.

* Inhibition (%) is calculated as: (units without inhibitor − units with inhibitor)/(units without inhibitor) × 100.

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respectively.

The half-lives of both forms of enzyme were similar and ranged from 1.8 and 1.4 days, respectively. The half-lives of both forms of enzyme in the kidney, heart, and lung were similar.

A separate cohort of newborn mps/mps mice received weekly injections of P-GUSB or NP-GUSB starting at birth until 5 weeks of age and then received an additional injection of enzyme at 9 weeks of age. Both groups of treated mice had elevated ABR thresholds compared with untreated age-matched normal control animals (Fig. 5). However, the average sound intensity required to elicit an ABR was lower at every frequency in mps/mps mice injected with P-GUSB when compared with mice injected with NP-GUSB (Fig. 5). This decreased threshold was significant (p < 0.05) at 5 and 10 kHz.

**DISCUSSION**

Direct enzyme replacement therapy has been proposed as a form of therapy for lysosomal storage diseases. Intravenous injection of glucocerebrosidase, a non-phosphorylated lysosomal enzyme, has been shown to be effective for the treatment of Gaucher’s disease (8, 9). Gaucher’s disease is a lysosomal storage disease that primarily affects cells of the RE system. To produce a therapeautic enzyme, glucocerebrosidase has to be treated with a series of exoglycosidases to expose mannose residues, which target it to the mannose receptor on RE cells (4, 7). However, for the majority of lysosomal storage diseases where both RE and non-RE cells are affected, enzyme will have to be targeted to cells that do not express the mannose receptor (i.e. the majority of non-RE cells). The mannose 6-phosphate receptor may provide access to many of those cells.

We previously showed that multiple injections of murine GUSB, containing ~70% phosphorylated enzyme, prevented the accumulation of lysosomal storage in multiple tissues when initiated in newborn mps/mps mice (12). This reduction of lysosomal storage correlated with improvements in bone length, longevity, hearing, immune function, and cognitive ability (26–29). We also showed that enzyme injections in adult animals reduced established lysosomal storage in many tissues with the exception of the brain (12, 40). This approach has also been shown to be effective in other models of lysosomal storage disease and, more recently, in children with Hurler-Schie and Fabry disease (8–17). However, most of these studies have been performed with enzyme produced in mammalian cells that contains a mixture of phosphorylated and non-phosphorylated enzyme. Therefore, it has been difficult to determine which form of enzyme contributes to which component of the clinical response.

It is known that insect cells do not contain the phosphotransferase activity required for the modification of terminal mannose residues on lysosomal enzymes, and relatively large quantities of enzymatically active lysosomal hydrolases can be produced in insect cells using the baculovirus system (35–37). Murine GUSB produced in this system (NP-GUSB) has properties that would be expected of a non-phosphorylated enzyme. NP-GUSB is not taken up by human fibroblasts expressing the mannose 6-phosphate receptor but not the mannose receptor. On the other hand, NP-GUSB is taken up by alveolar macrophages, which express the mannose receptor, and this uptake is completely inhibited by yeast mannan.

**FIG. 2.** Non-phosphorylated GUSB is taken up primarily by cells of the reticuloendothelial system in the liver whereas phosphorylated GUSB is taken up by both hepatocytes and Kupffer cells. A, 5 h following an intravenous injection of NP-GUSB, GUSB activity (red) is associated with sinus lining cells (white arrows) between the hepatocytes. B, at the same time after injection of P-GUSB, GUSB activity is associated with both sinus lining cells (white arrows) and hepatocytes (black arrows).

**FIG. 3.** The in vivo half-lives of NP-GUSB and P-GUSB are similar. A, the half-lives of P-GUSB (open squares, broken line) and NP-GUSB (filled circles, solid line) in the liver are ~1.8 and 1.5 days, respectively. B, the half-lives of P-GUSB and NP-GUSB in the spleen are ~1.8 and 1.4 days, respectively. The half-lives of both forms of enzyme in the kidney, heart, and lung were similar and ranged from 1 to 1.5 days (data not shown).

We previously showed that ABR determinations represented one measure of functional improvement in the MPS VII mouse following a therapeutic intervention (23, 26). A separate cohort of newborn mps/mps mice injected with P-GUSB when compared with mice injected with NP-GUSB (Fig. 5). This decreased threshold was significant (p < 0.05) at 5 and 10 kHz.

Enzyme Replacement for Mucopolysaccharidosis Type VII

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Following treatment with NP-GUSB. Neither form of enzyme reduced lysosomal storage in corneal fibroblasts.

**Improvement in Auditory Function (P-GUSB Versus NP-GUSB)—**We previously showed that ABR determinations represent one measure of functional improvement in the MPS VII mouse following a therapeutic intervention (23, 26). A separate cohort of newborn mps/mps mice received weekly injections of P-GUSB or NP-GUSB starting at birth until 5 weeks of age and then received an additional injection of enzyme at 9 weeks of age. Auditory-evoked brainstem responses were measured at 12 weeks of age. Both groups of treated mice had elevated ABR thresholds compared with untreated age-matched normal control animals (Fig. 5). However, the average sound intensity required to elicit an ABR was lower at every frequency in
Highly phosphorylated enzyme produced in mammalian cells (P-GUSB) is efficiently taken up by human fibroblasts exclusively by a mannose 6-phosphate receptor-dependent mechanism. This enzyme is also taken up in vitro by alveolar macrophages but by both the mannose 6-phosphate receptor and mannose receptor systems. Uptake by both non-RE and RE cells is also observed in vivo, because both hepatocytes and Kupffer cells contain enzyme. The macrophages can recognize both the phosphorylated oligosaccharides (via the mannose 6-phosphate receptor) and the non-phosphorylated oligosaccharides on P-GUSB. Interestingly, the half-lives of both forms of enzyme are similar in the tissues examined. This result suggests that the phosphorylation status of GUSB does not affect stability, and once the enzymes localize to the lysosome, they degrade at approximately the same rate in most tissues.

The different tissue distributions of P-GUSB and NP-GUSB also result in different degrees of lysosomal storage reduction. The reduction of lysosomal storage in the liver mirrors the distribution of the different enzymes. For example, NP-GUSB is localized almost exclusively to RE cells of the liver, and those are the only cells that have reduced storage; hepatocytes still

![Image](https://via.placeholder.com/150)

**FIG. 4.** Phosphorylated GUSB reduces lysosomal storage in a wider range of cells than does non-phosphorylated GUSB. A, the liver from a 12-week-old MPS VII mouse has extensive lysosomal storage in Kupffer cells and storage in small pericanalicular lysosomes (white arrows) in hepatocytes. B, the liver from an animal treated with P-GUSB has a marked reduction in lysosomal storage in both Kupffer cells and hepatocytes. C, after treatment with NP-GUSB, the Kupffer cells have reduced storage, but hepatocytes still have storage in pericanalicular lysosomes. D, meningeal cells (white arrows) lining the cerebellar folia in untreated MPS VII mice have marked lysosomal storage. E, the same cells (white arrow) have no apparent storage following treatment with P-GUSB. F, storage persists in meningeal cells of MPS VII mice following treatment with NP-GUSB. G, retinal pigment epithelial cells from an untreated MPS VII mouse have marked lysosomal distention (white arrows) that displaces the pigment granules to the periphery of the cytoplasm. H, there is a marked reduction of storage in RPE cells following treatment with P-GUSB. I, there is little or no reduction of storage in RPE cells following treatment with NP-GUSB. J, glomerular epithelial cells (white arrows) and renal tubular cells (black arrows) have distended lysosomes in an untreated MPS VII mouse. K, there is less storage in both glomerular epithelial and renal tubular cells following treatment with P-GUSB. L, there was a reduction in glomerular epithelial cell storage but little reduction in renal tubule cells following treatment with NP-GUSB. M, osteoblasts (white arrow) and bone marrow sinus lining cells (black arrow) from the rib of an untreated MPS VII mouse have lysosomal distention. N, after treatment with P-GUSB, the sinus lining cells (black arrow) have a marked reduction of lysosomal storage, and the osteoblasts (white arrow) have slightly reduced storage. O, treatment with NP-GUSB reduced storage in bone marrow sinus lining cells (black arrow) but had no effect on the storage in osteoblasts (white arrow).

**FIG. 5.** The hearing loss associated with murine MPS VII is more effectively prevented by P-GUSB as compared with NP-GUSB. The average noise intensity (decibels) required to elicit the characteristic ABR waveform is lower at every frequency (kHz) in mps/mps mice treated with P-GUSB (open squares) as compared with NP-GUSB (filled circles). This difference is statistically significant ($p < 0.05$) at 5 and 10 kHz. However, both groups of treated mps/mps mice have significantly elevated ABR thresholds when compared with untreated age-matched normal animals (asterisks).
contain distended lysosomes. In contrast, P-GUSB localizes to both Kupffer cells and hepatocytes, and both cell types are cleared of storage. P-GUSB also localizes in other tissues such as meninges and reduces lysosomal storage there. These data indicate that both phosphorylated and non-phosphorylated enzymes correct lysosomal storage in the cells they target. These data also suggest that, once NP-GUSB is taken up by cells of the RE system in the liver, the enzyme is not transferred to hepatocytes. In addition, because hepatocytes still have extensive lysosomal storage after multiple injections of NP-GUSB, the transfer of partially degraded GAGS from hepatocytes to Kupffer cells for further degradation does not represent a significant mechanism for GAG clearance.

Improvements in the auditory function of treated mps/mps mice were more complete in P-GUSB-treated than in NP-GUSB-treated mice. This may be due in part to improvements in the conductive component of hearing caused by more complete correction of the bony defects by P-GUSB. However, the hearing improvements observed with P-GUSB in this study are less impressive than those reported previously following treatment with an enzyme also produced in mouse L-cells but that was less highly phosphorylated (70%) (26, 30). In addition, the reduction in lysosomal storage by P-GUSB observed in neurons and osteoblasts in the current study was less complete when compared with previous studies (12, 27). One hypothesis that could account for this difference is that the most complete clinical response than that seen using completely non-phosphorylated enzyme produced in insect cells.

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