Journal of Cellular Biochemistry 57:208–217 (1995)

Studies on the Turnover of Exogenous Mannose-Terminal Glucocerebrosidase in Rat Liver Lysosomes

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Abstract Mannose-terminal glucocerebrosidase prepared by exoglycosidase digestion of human placental glucocerebrosidase is reported effective in the treatment of patients with type 1 Gaucher's disease [Barton et al. (1991); N Engl J Med 324:1464–1470]. However, the amount of enzyme that is necessary for therapeutic effect is much higher than would be predicted from in vitro activity measurements. We have investigated the fate of infused enzyme following intravenous administration in Sprague-Dawley rats. In this model system, the enzyme is rapidly cleared from the plasma compartment by receptor-mediated endocytosis via the mannose-specific receptor present on reticuloendothelial cells. Enzyme activity measured in rat liver biopsy specimens at various times post-infusion revealed a rapid initial loss of approximately one-half of the maximum delivered enzyme in the first hour followed by a slower decay with a half-life of approximately 6–8 h. The loss in enzyme activity is paralleled by a loss in enzyme protein when analyzed by Western blots. There is no evidence for return of enzyme activity or inactive enzyme protein to the plasma. Incomplete integration into the lysosomal membrane was demonstrated by the use of differential extraction of purified rat liver lysosomes to distinguish between lumenal and membrane bound enzyme. Immunoelectron microscopy of rat liver following infusion of mannose-terminal glucocerebrosidase confirmed localization of the delivered enzyme primarily within the lumen of the lysosomes of Kupffer cells and to a lesser extent associated with the lysosomal membrane. Enzyme activity was stable in isolated rat liver lysosomes preloaded with mannose-terminal glucocerebrosidase and incubated in the absence or presence of ATP. Acidification of the lysosomes to pH 3 results in a rapid loss of enzyme activity and protein; however, the relationship between the in vitro loss and the loss in enzyme activity in intact liver is not clear. We conclude from these studies that rapid intracellular degradation of administered glucocerebrosidase is the prime factor responsible for the high dose required for effective treatment of Gaucher's disease.

Key words: enzymes, replacement therapy, Gaucher's disease, lysosomes

Gaucher's disease is an autosomal recessive disease resulting from a defect in the lysosomal enzyme glucocerebrosidase (glucosylceramidase; β-D-glucosyl-N-acyl-sphingosine glucohydrolase; E.C. 3.2.1.45) [Brady et al., 1965, 1966]. Accumulation of glucosylceramide in resident macrophages of liver, spleen, and bone marrow [Burns et al., 1977] causes an increase in both the size and number of lysosomes and the appearance of characteristic intralysosomal tubular structures made up of twisted bilayers of glucocerebroside [Lee et al., 1973]. Clinical symptoms include severe hepatosplenomegaly, moderate to severe bone crises, and bleeding tendencies. In addition to these systemic disease symptoms, patients with the rarer forms of Gaucher's disease, type 2 and type 3, also show signs of neurologic degeneration [for review see Barranger and Ginns, 1989; Brady et al., 1993]. Multiple mutations in the gene for glucocerebrosidase occur at a variety of loci [reviewed in Brady et al., 1993]. Identification of the primary enzyme defect led to the proposed use of replacement enzyme targeted for the reticuloendothelial cells as therapy for this and other similar metabolic disorders [Brady, 1966]. In order to be effective, catalytically active enzyme must be

Abbreviations used: BSA, bovine serum albumin; CRM, immunologically cross-reactive material; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate.

Received May 24, 1994; accepted June 21, 1994.

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delivered to the sites of glycolipid storage within the lysosome of macrophages. Thus, successful therapy must incorporate several essential features. Reproducible methods developed for production of large quantities of highly purified enzyme [Furbish et al., 1977] along with advances in targeting strategies for delivering active enzyme to a specific tissue or cell type [Furbish et al., 1981; Murray, 1987] have been of equal importance to attaining this goal.

Studies indicate that enzyme replacement with mannose-terminal glucocerebrosidase results in selective enzyme targeting to the macrophage. Mannose-terminal glucocerebrosidase has been shown to be an effective therapy for patients with type 1 Gaucher's disease when 60 IU/kg are administered on a biweekly schedule [Barton et al., 1990, 1991a,b; Fallet et al., 1992]. Recently, an alternative strategy has been advocated [Beutler et al., 1991a,b; Sato and Beutler, 1993] based on observations in a limited number of splenectomized patients and data obtained from equilibrium binding studies. The former study is of limited value due to the absence of a statistically valid sample population in a disease as clinically diverse as Gaucher's disease. Equilibrium binding of ^{125}I-labelled mannose-terminal glucocerebrosidase in isolated peritoneal macrophages reported in the later studies [Sato and Beutler, 1993] failed to demonstrate saturable binding indicating inadequate control of nonspecific binding. The conclusions of the study remain open to further debate. We have addressed these issues more extensively in a separate study of the receptor binding of mannose-terminal glucocerebrosidase in the absence and presence of added biological response modifiers (G. Murray et al., manuscript in preparation).

Animal and cell culture studies have previously examined the fate of both native placental glucocerebrosidase (unmodified) and macrophage-targeted, mannose-terminal glucocerebrosidase [Furbish et al., 1981; Murray, 1987]. Cell-type selectivity and dependence on uptake via the mannose-specific receptor have been demonstrated for mannose-terminal glucocerebrosidase. A significant increase in enzyme specific activity was observed in rat Kupffer cells compared with unmodified enzyme and could be effectively blocked by coadministration of mannans or other mannose-terminal glycoproteins [Murray, 1987]. Both the total uptake into liver and the preferential association of this enzyme with lysosomal fractions of whole rat liver have been shown to be saturable with half-maximal uptake at approximately 3×10^{-7} M [Furbish et al., 1984].

We report here the rapid turnover of exogenous mannose-terminal glucocerebrosidase in rat liver. By use of whole animals as a model, we have attempted to gain insight into the metabolism of administered enzyme in patients with Gaucher's disease. Definition of the binding, internalization, and subcellular localization of this enzyme in a physiological setting may contribute to the development of more effective enzyme replacement therapy for Gaucher's disease.

EXPERIMENTAL PROCEDURES

Enzyme Administration

Native placental glucocerebrosidase or mannose-terminal glucocerebrosidase (Ceredase®, Genzyme Corp., Cambridge, MA) was diluted in 50 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), containing 1% human serum albumin and administered by intravenous injection to 200–250 g Sprague-Dawley rats (Taconic Laboratories, Germantown, NY). In experiments in which liver biopsy specimens were obtained, a single venous blood sample (0.1 ml) was removed 1 min after injection to determine the amount of enzyme administered. In other experiments, multiple blood samples were taken at various times following infusion from a cannula inserted into the right common carotid artery or from the descending aorta, just above the branch to the femoral artery. Liver biopsies (0.1 g) were removed at various times, weighed, and homogenized. Biopsy tissue specimens for lysosome isolation were homogenized in 0.25 M sucrose, 1 mM EDTA, pH 6.8. For enzyme activity measurements, the biopsy was homogenized in 0.1 M potassium phosphate buffer, pH 5.9, containing 0.15% Triton-X100 and assayed without further processing. All procedures were performed under general anaesthesia induced by intraperitoneal injection of sodium pentobarbital. Bleeding of the cut surface of the liver was controlled by cauterization.

Lysosome Isolation

A lysosome-enriched subcellular fraction was prepared by differential centrifugation, followed by discontinuous gradient centrifugation in Percoll and metrizamide [Madden and Storrie,

1987]. Liver was homogenized in 6 volumes of 0.25 M sucrose, 1 mM EDTA, pH 6.8, and centrifuged at 1,500 rpm (300*g*) for 10 min to obtain a postnuclear supernatant. The pellet was rehomogenized and centrifuged as before, then triplicate aliquots of 4.8 ml of the combined postnuclear supernatants were applied to a gradient consisting of 2 ml of 35% metrizamide, 2 ml of 17% metrizamide, and 5 ml of 6% Percoll. Following centrifugation at 50,000*g* for 30 min, the band at the interface between the 17 and 35% layers was collected, added to 10 ml of homogenization buffer and spun at 50,000*g* for 15 min to pellet the lysosomal fraction.

Carbonate Extractions

In order to differentiate between enzyme protein in the lumen of the lysosome and that bound to the membrane, lysosomal pellets obtained as above were mixed with 1 ml of carbonate buffer (100 mM sodium carbonate, pH 11.5) and incubated on ice for 1 h [Fujiki et al., 1982]. The soluble and particulate fractions were separated by centrifugation at 109,000*g* for 1 h and compared with identical samples incubated for 1 h with homogenization buffer. Each fraction was resuspended in an appropriate volume of buffer and analyzed both by assay of enzyme activity and by Western blotting.

Gels and Blots

Serum and liver samples were solubilized prior to SDS-PAGE by boiling for 3 min in sample buffer containing 2% SDS, 80 mM Tris HCl, 0.01% bromophenol blue, 10% glycerol, and 17 mM dithiothreitol. Electrophoretic separation was effected in a Tris-glycine-SDS buffer (50 mM Tris, 0.38 M glycine, 0.1% SDS) on 1.5 mm thick slab gels containing 10% acrylamide and 0.8% bis-acrylamide [Laemmli, 1970]. For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of glucocerebrosidase in serum, samples were first concentrated by adsorbing to Phenyl-Sepharose® (Pharmacia, Piscataway, NJ) in 0.1 M citrate, pH 5.0. After rotating at 4°C for 15 min to allow binding to occur, samples were centrifuged and resuspended in 25 μl of SDS-PAGE sample buffer. Control experiments showed Phenyl-Sepharose® beads to be effective in removing all glucocerebrosidase activity from the serum.

Proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose sheets (Schleicher and Schuell, Keene, NH) at 0.5 to 1 amp with cooling for 1–3 h [Towbin et al., 1973]. After blotting, protein remaining on the gel was silver stained to confirm transfer efficiency of standard lanes. Silver stain kits (Daiichi) were purchased from Integrated Separation Systems, Hyde Park, MA. Nitrocellulose strips with molecular weight standards were stained with MEMBRAGOLD (Diversified Biotech, Newton Center, MA). After blocking for 1–2 h with 10% (w/v) skim milk powder in phosphate buffered saline (PBS), pH 7.3, containing 0.3% Tween 20, the nitrocellulose sheet was probed with rabbit (polyclonal) antiserum raised to normal placental glucocerebrosidase. 125-I-Protein A, used for developing blots, was obtained from Du Pont-New England Nuclear at an initial specific activity of 86.1 μCi/μg and used within 60 days (one half-life) of receipt.

Immunogold Labeling

Fixation and preparation of tissues for immuno-gold staining were performed essentially as described [Moreira et al., 1989]. Thirty minutes after administration of mannose-terminal glucocerebrosidase, tissues were fixed in situ by cardiac puncture and rapid flushing with 0.1 M sodium cacodylate containing 50% sucrose, pH 7.4, followed by perfusion for 20 min with 1% glutaraldehyde in the same buffer. Tissues were fixed en bloc for 2 h further and then small pieces dehydrated and embedded in epon (Epon 812 kit, Electron Microscopy Sciences, Fort Washington, PA). Post-fixation in osmium tetroxide was omitted in samples for immunocytochemistry. Thin sections were cut on an LKB Ultramicrotome and placed on formvar-coated nickel grids (Electron Microscopy Sciences, Ft. Washington, PA).

Grids were etched by exposure of the tissue face to saturated sodium metaperiodate for 1 h, blocked with 0.5 M glycine for 30 min, then washed with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS), 10 mM sodium phosphate, 0.5 M NaCl, pH 7.4) for 30 min. Grids were incubated for 2 h at room temperature with polyclonal rabbit antiserum against human placental glucocerebrosidase, diluted 1:20, then washed for 1 h with 1% BSA in PBS. Grids were developed by incubation for 30 min with goat anti-rabbit antiserum conjugated with 10-nm gold particles (Ted Pella, Redding, CA) diluted 1:40, then rinsed with PBS, and finally in distilled water before drying and staining with uranyl acetate and lead citrate. Sections

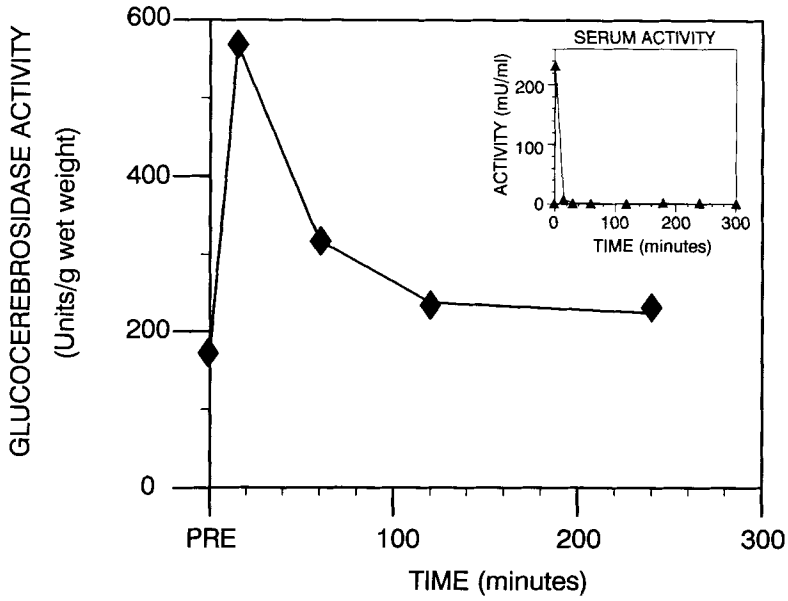

Fig. 1. Liver activity following intravenous infusion of mannose-terminal glucocerebrosidase in rat. Two hundred fifty gram Sprague-Dawley rats were each given 26 units of mannose-terminal glucocerebrosidase per kg by intravenous injection. Immediately prior to injection and at the indicated times, liver biopsies were removed, homogenized, and assayed for glucocerebrosidase activity. **Inset:** Serum activity following intravenous infusion of mannose-terminal glucocerebrosidase in rat. Serum samples removed from rats were assayed for glucocerebrosidase activity.

were viewed by electron microscopy on a JEOL 100-CX at 80 kV.

Enzyme Activity and Protein Measurements

One unit of glucocerebrosidase activity is defined as that quantity of enzyme capable of releasing 1 μmol of [14-C]-glucose from radiolabeled glucocerebroside/min at 37°C under conditions described in Murray et al. [1985]. Proteins were determined by the Lowry method [Lowry et al., 1951].

RESULTS

After intravenous injection of a bolus of mannose-terminal glucocerebrosidase into a rat, peak serum activity declines exponentially with a $t_{1/2}$ of 1–2 min (Fig. 1, inset). Coincident with the fall in serum enzyme activity, activity in homogenates of liver biopsies rises to a maximum value and then declines (Fig. 1). Approximately one-half of the initial liver enzyme activity is lost within the first hour following infusion. Following the initial rapid loss, the remaining enzyme activity decreases more slowly in this experiment and in others over a longer observation period (6 h), the long-lived enzyme activity has a half-life in liver ranging from 6–8 h. There is no measurable return of enzyme activity to the plasma compartment.

Liver samples taken 0–4 h after infusion and analyzed by SDS-PAGE and Western blotting show a rapid loss of protein reacting with antibody specific for glucocerebrosidase (Fig. 2). This decrease parallels the loss in activity shown in Figure 1. In an effort to increase signal, plasma samples removed at various times were concentrated using Phenyl-Sepharose® prior to SDS-PAGE and Western blotting. Plasma CRM (Fig. 3) shows an intense initial band, which remains measurable for approximately 30 min after which no positive bands are distinguishable. There is no evidence for any reappearance of enzyme CRM in plasma samples collected during a 4-h observation period.

These results suggest that the enzyme delivered to the liver via receptor-mediated endocytosis is either rapidly degraded to products which no longer cross-react immunologically with antisera raised to the intact enzyme, or that the enzyme is transported out of the liver after only a brief residence. The inability to detect any return of enzyme protein to the plasma compartment may also be due to constant, low level secretion. However, the volumes used for the Western blot assay were calculated to be capable

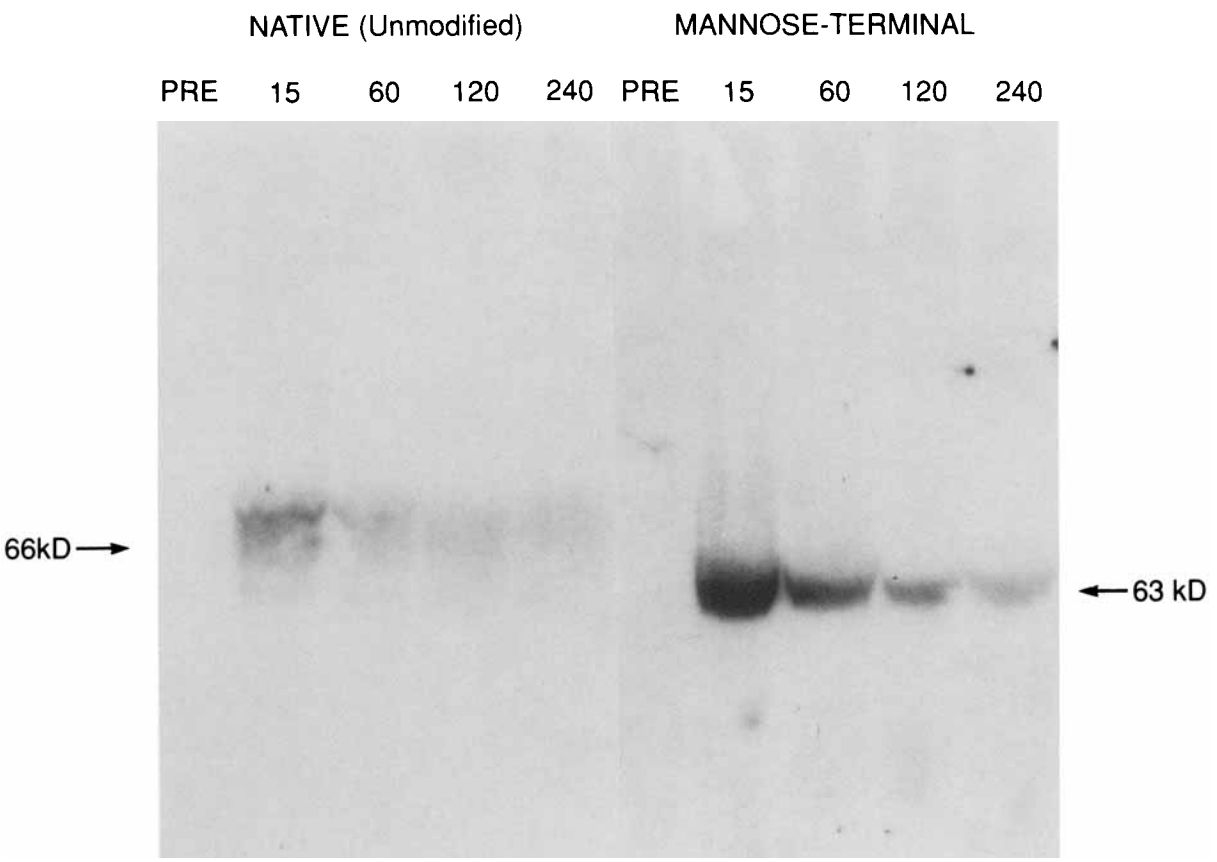

Fig. 2. Liver CRM following intravenous infusion of glucocerebrosidase in rat. Liver biopsies removed in Figure 1 were homogenized in gel solubilizing buffer, run on SDS-PAGE, and Western blotted. Detection of CRM was carried out by reaction with primary rabbit antisera raised against native human placental glucocerebrosidase, followed by 125-I labeled Protein A. A preinfusion biopsy and samples removed at 15, 60, 120, and 240 min were analyzed for both glucocerebrosidase and mannose-terminal glucocerebrosidase injections.

of detecting one-tenth of the delivered enzyme secreted uniformly over a 1-h period. We have also examined the bile produced during the first hour after enzyme administration and found no evidence for excretion via this route (data not shown).

Carbonate extraction of a preparation of lysosomes isolated from rat liver after mannose-terminal glucocerebrosidase infusion was used as a means of discriminating between enzyme associated with the lysosomal membrane and lumenal enzyme (Fig. 4). Although some smearing did result due to the sucrose in samples containing homogenization buffer, we have compared the intensity of bands detected by Western blotting of the particulate (pellet) and supernatant fractions obtained after extraction with 100 mM sodium carbonate or isotonic homogenization medium. For comparison purposes, each sample was adjusted to an appropriate volume such that each subfraction represents an equivalent volume of starting lysosomes. Isolated lysosomes treated with homogenization buffer alone retain most of their enzyme protein in the particulate fraction (Fig. 4., lane 3) and release none to the supernatant (Fig. 4, lane 6). In contrast, a single exposure to carbonate buffer released a significant portion of the enzyme protein as illustrated by both the decrease in signal from the particulate fraction (Fig. 4, lane 4) and increase in the supernatant (Fig. 4, lane 7 or 8). If carbonate treatment is followed by a wash with homogenization buffer, no further decrease in intensity of signal is observed, although some smearing does occur (Fig. 4, lane 5). We conclude that mannose-terminal glucocerebrosidase is present both in a membrane associated state and as a soluble (lumenal) enzyme. It should be emphasized that while an attempt has been made to maintain equivalency between samples in order that comparisons may be made, these data are semiquantitative representations

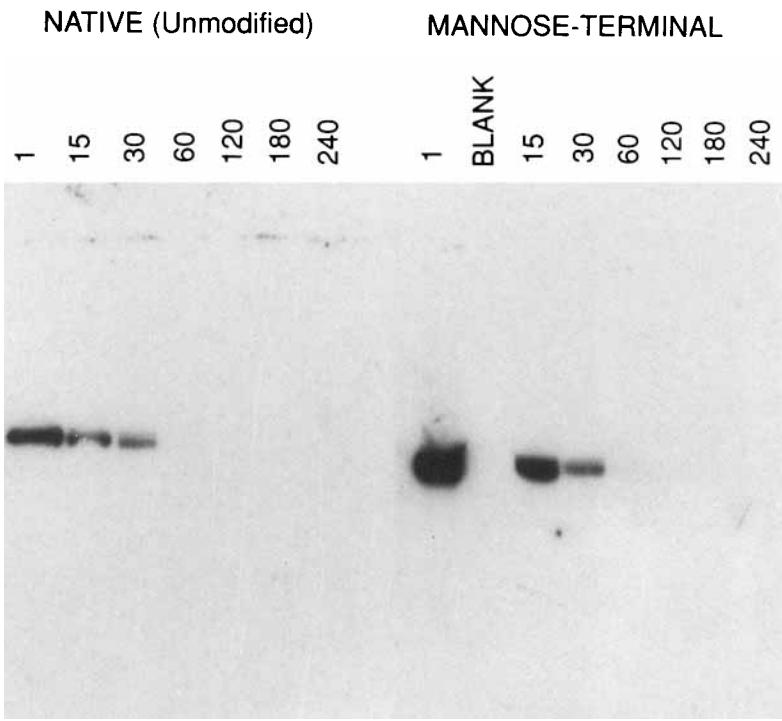

Fig. 3. Serum CRM following intravenous infusion of glucocerebrosidase in rat. Plasma samples obtained following infusion of glucocerebrosidase or mannose-terminal glucocerebrosidase were incubated with Phenyl-Sepharose to concentrate and enrich for hydrophobic components. Proteins were released from the affinity matrix by solubilization in boiling gel preparation buffer containing both DTT and SDS.

of enzyme concentration and do not provide any evidence with respect to the kinetics of association of the enzyme with the membrane.

Post-embedding immunocytochemistry was performed and developed using polyclonal rabbit antiserum against human placental glucocerebrosidase, followed by a secondary goat anti-rabbit antibody-gold conjugate. Figure 5 is a representative section showing a Kupffer cell 30 min after infusion of mannose-terminal glucocerebrosidase. Lysosomes in Kupffer cells from experimental animals (30 IU/kg mannose-terminal glucocerebrosidase) contained 300 ± 160 gold particles/μm^2 compared with background staining of 7–10 particles/μm^2 in other cell-types within the same experimental animal and in all cell-types and organelles from control livers (from saline-infused rats) developed as in the experimental animals, or mannose-terminal glucocerebrosidase-infused rats in which primary antibody was omitted during the development. At the magnification shown, it is possible to observe a relatively small number of gold particles around the periphery of the lysosome compared with the larger number of particles randomly localized over the lumen. A more complete analysis of many immuno-electron micrographs of this type has been completed elsewhere (Gary J. Murray and Fu-Sheng Jin, submitted for publication). In agreement with the differential extraction data reported above, membrane associated particles account for only 18% of the immunogold observed lysosomal compared with 82% over the lumen of the lysosome. Together these observations provide strong evidence that a comparatively small proportion of the administered enzyme is localized to the membrane.

In an effort to ascertain whether the decrease in enzyme activity and CRM were related to an intra-lysosomal event or whether the observed losses were due to some form of exocytosis, we have isolated glucocerebrosidase-loaded lysosomes for examination of the susceptibility of mannose-terminal glucocerebrosidase to degradation by lysosomal proteases. By allowing time for endosome-lysosome fusion, mannose-terminal glucocerebrosidase-loaded lysosomes were purified from rat liver and incubated at 37°C under various conditions. Enzyme activity remained constant over a period of 4 h in the presence or absence of added ATP (ADP added as control). At pH 3.0 or lower, both enzyme

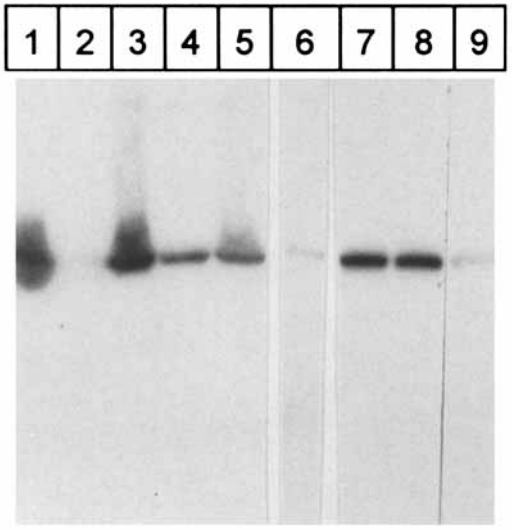

Fig. 4. Differential extraction of lumenal and membrane bound lysosomal glucocerebrosidase. Following intravenous injection of mannose-terminal glucocerebrosidase as in Figure 1, lysosomes were isolated by discontinuous density gradient centrifugation, extracted with carbonate buffers or homogenization buffer, then centrifuged at 109,000*g* to differentials into particulate (lanes 3, 4, and 5) and soluble fractions (lanes 6–10). Lane 1: Post-nuclear supernatant (enzyme infused rat). Lane 2: Post-nuclear supernatant (saline infused rat). Lane 3: Pellet: lysosomes extracted with homogenization media alone. Lane 4: Pellet: lysosomes extracted with carbonate alone (not washed). Lane 5: Pellet: lysosomes extracted with carbonate, centrifuged, then washed with homogenization medium. Lane 6: Supernatant: Homogenization buffer corresponding to lane 3. Lane 7: Supernatant: Carbonate buffer extraction corresponding to lane 4. Lane 8: Supernatant: Carbonate buffer extraction corresponding to lane 5. Lane 9: Standard mannose-terminal glucocerebrosidase.

activity and CRM are lost from rat liver lysosomes with a half-time of 1 h (data not shown). We have incubated mannose-terminal glucocerebrosidase at low pH in the absence of any lysosomal factors and see not only a decrease in enzyme activity, but also the appearance of several new bands on a silver stained gel. These new species, with apparent molecular weights of 26,000, 33,000, and 34,000, suggest an acid-sensitive site on the enzyme. The relationship between this observation at low pH and the loss in enzyme activity and CRM in intact liver is not yet clear.

DISCUSSION

In these experiments we have combined biochemical and cell biologic strategies to follow the delivery and residence time of glucocerebrosidase in liver. Because a significant proportion of enzyme is inactivated during iodination, leading to uncertainties arising from such unpredicted changes in the enzyme molecule, we have chosen an alternative approach in the present study. By measurement of enzyme activity, coupled with use of an immunochemical probe for enzyme protein, we have been able to track both active and inactive enzyme in liver samples. This technique closely approximates physiologic interactions that may occur in patients being treated with exogenous glucocerebrosidase. Although relatively minor changes in structure may be sufficient to remove the immunologically reactive epitope from the enzyme or to inactivate the active site of the enzyme, the combination of two techniques and agreement with previous studies using iodinated probes provides additional validation of the results.

Glucocerebrosidase is normally a membrane bound lysosomal enzyme, purification of which requires a combination of detergent and organic solvent extraction in order to release the active enzyme and strip it of membrane lipids [Furbish et al., 1977]. Once solubilized, however, the enzyme remains in aqueous solution but requires hydrophobic stabilizers such as ethylene glycol or glycerol and a relatively high protein concentration for maximum stability. In vitro, full catalytic activity of glucocerebrosidase can be attained by use of a variety of activators which provide one or more of the following: i) a hydrophobic environment, ii) an anionic activator such as a detergent or phospholipid, and iii) a peptide activator such as saposin A or C. In vivo conditions for maximizing glucocerebrosidase activity cannot be unequivocally defined. The hydrophobic environment may be provided by the phospholipids contained in the lysosomal membrane alone or in combination with activator proteins. These protein factors are known to be present in all tissues and are presumed to play a physiologic role [Morimoto et al., 1990].

Thus, the success of enzyme replacement therapy by administration of a solubilized preparation of purified glucocerebrosidase depends not only on the enzyme being directed to the appropriate target cell via the mannose-terminal glycans, but also on the ability of the enzyme to integrate itself into the lysosomal membrane. One hypothesis is that the hydrophobic environment provided by the membrane is critical for the optimum catalytic activity of glucocerebrosidase and that the residence time in the lysosome will be greatly enhanced by factors which improve the latter characteristic.

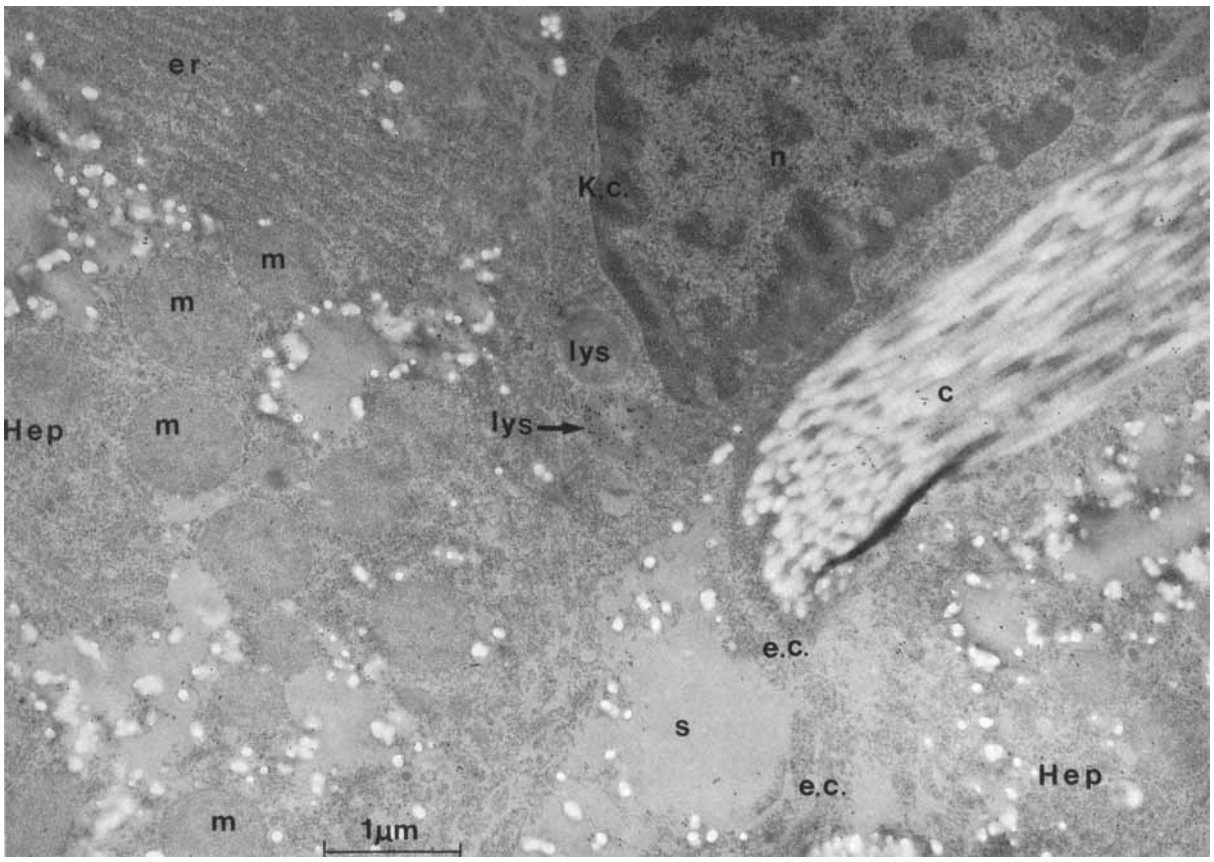

Fig. 5. Electron microscopic immunocytochemical localization of glucocerebrosidase in rat liver Kupffer cells. Thirty minutes following intravenous infusion of 30 IU/kg mannose-terminal glucocerebrosidase into male Sprague-Dawley rats, tissues were fixed with 1% glutaraldehyde by cardiac puncture. Tissue blocks were fixed an additional 2 h en block, then embedded in epon without post-fixation with osmium tetroxide, and stained immunochemically with rabbit antiserum specific for human placental glucocerebrosidase, followed by goat anti-rabbit antibody conjugated with 10μ gold particles. Following the immunolabeling, grids were stained with uranyl acetate and viewed. Original magnification: ×13,000. Bar = 1 μm. K.c., Kupffer cell; Hep, hepatocyte; e.c., endothelial cell; s, sinusoid; lys, lysosome; n, nucleus; c, collagen; m, mitochodria.

In previous studies using mannose-terminal glucocerebrosidase, the maximum recovery of active enzyme or iodinated enzyme from liver organelles or from isolated cell populations from liver was 30%. The low recovery may be due to a number of factors acting individually or in combination including i) a change in the structure of the protein as a result of oxidation of a critical site during iodination; ii) losses due to artefacts of the cell isolation procedure such as digestion and loss of surface bound enzyme by collagenase; iii) normal lysosomal degradation of an exogenously supplied protein; or iv) exocytosis of the enzyme after a short lysosomal residence. It should also be noted that because of limitations of the techniques used, i.e., cell separations, subcellular organelle preparations, at least 1 h had elapsed before measurement of recovered enzyme. We have demonstrated here that the process of loss of enzyme activity begins relatively rapidly after the attainment of maximum liver level. This observation leads to the conclusion that the low recovery in the previous studies may be less of an artefact of the tissue preparation and more likely due to physiologic losses of the infused protein.

We have tested the extent of enzyme insertion into the lysosomal membrane by differential extraction of isolated rat liver lysosomes after infusion of native or mannose-terminal glucocerebrosidase. Sodium carbonate extraction has been previously reported to be capable of rupturing lysosomal membranes without completely solubilizing the membrane [Madden and Storrie, 1987]. Release of the contents of the lumen permits the discrimination between membrane-

associated and free glucocerebrosidase within the lysosome. We have demonstrated that following infusion, exogenous macrophage-targeted glucocerebrosidase partitions between the membrane and the lumen of the lysosome. The release of a significant proportion of the administered enzyme by use of a high pH carbonate buffer and the morphometric analysis of immuno-electron micrographs of liver cells following infusion provide semi-quantitative proof for the greater localization within the lumen of the lysosome with a smaller fraction of the enzyme associated with the lysosomal membrane.

The incomplete insertion into the lysosomal membrane, and the coincidence of the loss in enzyme activity with the disappearance of CRM, led us initially to conclude that the loss in glucocerebrosidase activity from rat liver after infusion might be due to exocytosis and excretion from the liver, possibly in bile. Losses of non-membrane bound enzyme might be expected to occur relatively rapidly in contrast to enzyme protected within a stable membrane matrix. However, in vitro experiments with isolated liver lysosomes make it clear that the inactivation and loss of CRM take place within the lysosome itself and does not represent a loss due to trafficking of enzyme out of the lysosome. The simultaneous loss of both enzyme activity and protein might be due to either proteolysis or inhibition by an indeterminate process. However, the complete loss of glucocerebrosidase protein (CRM), and the failure to detect any smaller fragments of Western blots, lead to the conclusion that proteolytic inactivation has occurred. The absence of any effect of ATP is remarkable in that the observed losses appear to be unrelated to ATP-dependent lysosomal acidification. This process has been suggested to be required for the lysosomal degradation of mannosylated-bovine serum albumin [Casciola-Rosen and Hubbard, 1991].

The release of much of the glucocerebrosidase protein by the addition of sodium carbonate suggests that a significant proportion of the delivered enzyme is not inserted into the lysosomal membrane in a stable manner. This distribution pattern has been confirmed by direct visualization of immunogold particles by electron microscopy. Thus, although we have no evidence at this time to support increased susceptibility of the lumenal enzyme to proteolysis, it is possible that the initial rapid loss of the infused enzyme from the liver may be at least partially due to the inappropriate membrane localization. Characterization of the "long-lived" enzyme ($t_{1/2}$ = 6–8 h) with respect to its localization within the lysosome may be useful in the design of improved enzyme for replacement therapy. Further immunocytochemical studies at various times post-infusion may be particularly useful in this regard. Extraction studies such as those performed on isolated liver lysosomes may be less useful, however, due to the inability to stop intralysosomal processes during the long sample preparation time.

Increase in enzyme specific activity within a target organ or cell type reflects the attainable enzyme concentration, and as such, is one indicator of therapeutic potency. However, when determining effective therapeutic strategies, the half-life of the enzyme at the target site may be as important as the total amount of enzyme activity delivered. Modification of native glucocerebrosidase bioengineered to direct active enzyme to the sites of lipid storage may be considered the first version of "improved" glucocerebrosidase. Improved target selection has made possible the present effective treatment for Gaucher's disease. In order to further improve the effectiveness of glucocerebrosidase for enzyme replacement therapy of Gaucher's disease it is necessary to examine the problem from a more global perspective and to determine the effects of any modification not only on the cell-specific targeting but also on the "membrane-targeting" addressed in this paper. Increasing the percentage of enzyme inserted into the lysosomal membrane may provide a more economical treatment for Gaucher's disease by prolonging lysosomal residence time. By gaining an understanding of the factors influencing localization of this enzyme to the lysosomal membrane of reticuloendothelial cells, further advances in the bioengineering of this enzyme may be possible.

ACKNOWLEDGMENTS

The authors thank Dr. Norman W. Barton for helpful discussions during the course of these investigations. We also thank Dr. Susan Cheng and the NINDS EM User Facility for training and assistance in electron microscopic techniques. The expert advice of Dr. Jorge Moreira in the design and execution of immuno-gold labeling protocols is gratefully acknowledged. The authors thank Ms. Virginia Tanner and Mr.

Charles Sartor for excellent technical assistance.

This work was supported in part by a grant from the National Gaucher Foundation, Washington, DC.

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