Secondary Storage of Dermatan Sulfate in Sanfilippo Disease*

William C. Lamanna, Roger Lawrence, Stéphane Sarrazin, and Jeffrey D. Esko

From the Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, California 92039-0687

Mucopolysaccharidoses are a group of genetically inherited disorders that result from the defective activity of lysosomal enzymes involved in glycosaminoglycan catabolism, causing their intralysosomal accumulation. Sanfilippo disease describes a subset of mucopolysaccharidoses resulting from defects in heparan sulfate catabolism. Sanfilippo disorders cause severe neuropathology in affected children. The reason for such extensive central nervous system dysfunction is unresolved, but it may be associated with the secondary accumulation of metabolites such as gangliosides. In this article, we describe the accumulation of dermatan sulfate as a novel secondary metabolite in Sanfilippo. Based on chondroitinase ABC digestion, chondroitin/dermatan sulfate levels in fibroblasts from Sanfilippo patients were elevated 2–5-fold above wild-type dermal fibroblasts. Lysosomal turnover of chondroitin/dermatan sulfate in these cell lines was significantly impaired but could be normalized by reducing heparan sulfate storage using enzyme replacement therapy. Examination of chondroitin/dermatan sulfate catabolic enzymes showed that heparan sulfate and heparin can inhibit iduronate 2-sulfatase. The discovery of a novel storage metabolite in Sanfilippo patients may have important implications for diagnosis and understanding disease pathology.

Heparan sulfate, chondroitin sulfate, and dermatan sulfate are the common sulfated glycosaminoglycans produced by mammalian cells (1). These glycosaminoglycans undergo lysosomal degradation through a multienzyme process, involving an array of sulfatases and glycosidases that act in concert to remove sulfate groups and monosaccharides from the nonreducing end of the polysaccharides (2). Because of the sequential nature of the degradative process, disruption of any one of the many enzymes involved in glycosaminoglycan catabolism results in lysosomal storage, causing a range of diseases known as mucopolysaccharidoses (MPS2 diseases). The Sanfilippo class of MPS diseases results from a deficiency in heparan sulfate catabolism caused by genetic defects in any of four lysosomal enzymes, including sulfamidase (MPSIIIa; OMIM code 252900), α-N-acetyl-glucosaminidase (MPSIIIb; OMIM code 252920), acetyl-CoA: α-glucosaminide N-acetyltransferase (MPSIIIc; OMIM code 252930) and N-acetylgalactosamine-6-sulfatase (MPSIIId; OMIM code 252940) (2). The primary pathological manifestation in Sanfilippo is severe mental retardation, whereas peripheral abnormalities such as defective bone development and enlargement of the spleen and liver remain more mild (2, 3). The greater involvement of the CNS in disease pathology is unique to Sanfilippo and makes this form of MPS particularly difficult to treat (2).

Classically, the pathology of MPS diseases is thought to depend on the degree of enzyme deficiency, the type of glycosaminoglycan that is stored, and the cell types most affected by lysosomal accumulation (2, 3). However, correlations that might explain differences in disease manifestation between different MPS disease types, or in some cases even between patients with the same genetic mutation, are not apparent (3), suggesting the contribution of other secondary metabolites and/or modifying genes to disease pathology. Indeed, for Sanfilippo, the molecular relationship between heparan sulfate storage and extensive neuropathology remains unclear. Some evidence suggests that defects in the CNS of Sanfilippo patients may be associated with the accumulation of secondary metabolites in the brain, such as GM2 and GM3 gangliosides (4). Storage of gangliosides in the CNS has been shown to disrupt Ca2+ uptake by the sarco/endoplasmic reticulum Ca2+-ATPase, a process that is essential for proper neuronal function and survival (5–7). Correlations between ganglioside accumulation and specific pathogenic cascades such as ectopic dendritogenesis have also been documented (8, 9). In addition to gangliosides, sequestration of cholesterol in the cell bodies of neurons and glia has been observed in Sanfilippo, causing aberrant lipid distribution throughout the cell that may disrupt membrane dynamics and intraendosomal transport (4, 10). The cause of these secondary defects remains unresolved but likely results from the disruptive effects of primary storage metabolites on lysosomal hydrolases and cellular trafficking (4, 10–12).

In this study, we describe dermatan sulfate accumulation in Sanfilippo patient cells. Enzyme replacement therapy demon-
strated a direct correlation between heparan sulfate storage and deficient dermatan sulfate turnover in the lysosome. Iduronate 2-sulfatase, a dermatan sulfate and heparan sulfate catabolic enzyme, exhibits exceptional sensitivity to heparan sulfate accumulation, suggesting that its inhibition in lysosomes by stored heparan sulfate may cause secondary storage. The combined accumulation of heparan sulfate with dermatan sulfate in Sanfilippo cells may explain some of the pathological features of Sanfilippo diseases and has important implications for diagnostic approaches that rely on the detection of storage material for the characterization of MPS disease types.

**EXPERIMENTAL PROCEDURES**

*Enzymes and Reagents*—Heparinases I, II, and III (*Flavobacterium heparinum*) and chondroitinase ACII were from Seikagaku, and chondroitinase ABC was from Sigma Aldrich. Sulfamidase and iduronate 2-sulfatase (human) were obtained from R&D Systems. GalNac-4-sulfatase was a kind gift from Thomas Dierks (Bielefeld University). Pronase (type XIV protease from *Streptomyces griseus*) was from Sigma Aldrich. PN-Gase F was from Prozyme. 4-Methylumbelliferyl β-D-N-acetylgalcosaminide (4MU-GlcNAc) was from Calbiochem. 4-Methylumbelliferyl β-D-glucuronide (4MU-GlCα) was from Sigma Aldrich. 4-Methylumbelliferyl α-L-iduronide (4MU-Ido) was from Glycosynth. 4-Methylumbelliferyl β-galactose-6-sulfate and 4-methylumbelliferyl α-iduronide-2-sulfate (4MU-IdoA2S) were from Moscerdam Substrates.

*Cell Culture*—Wild-type human foreskin fibroblasts (ATCC; CRL-1634), wild-type human dermal fibroblasts (GM08398, GM05659, and GM15871), MPSIIia fibroblasts (GM00643, GM00934, GM00879, and GM06110), MPSIIib (GM001426), MPSIIic (GM05157), MPSIIId (GM05093), and Neu1 (GM01718) fibroblasts were obtained from the ATCC or Coriell Cell Repositories and grown in DMEM high glucose medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen). Cells were aged for the indicated number of weeks and provided with fresh medium every 2 weeks.

*Turnover of [35S]Glycosaminoglycans*—Wild-type and Sanfilippo fibroblasts were aged 4 weeks at confluence. Radiolabeling of glycosaminoglycans was carried out by incubating cells 48 h in DMEM+F12 (Invitrogen) medium supplemented with 10% dialyzed fetal bovine serum containing 100 μCi/ml Na[35S]O (PerkinElmer Life Science). Dialyzed serum was used to increase the radioisotopic activity of added [35S]O. To monitor turnover of the radiolabeled glycosaminoglycans, cells were rinsed twice with PBS and incubated for 48 h in DMEM+F12 supplemented with 10% fetal bovine serum. Cells were washed twice with PBS and treated for 20 min with 0.05% Trypsin/EDTA (Invitrogen) to lift the cells and degrade any remaining cell surface proteoglycans. Cell pellets were frozen at −20°C for subsequent glycosaminoglycan analysis as described below.

*Glycosaminoglycan Purification and Analysis*—Cell pellets were solubilized by adding 0.5 ml 0.1 M NaOH. The samples were diluted 1:5 with wash buffer (50 mM sodium acetate, 0.2 M NaCl, pH 6.0) and digested overnight at 37°C with 0.1 mg/ml Pronase and 0.1% Triton X-100 prior to glycosaminoglycan purification. Glycosaminoglycans were purified from cell homogenates using DEAE-Sepharose anion exchange chromatography as described previously (13, 14). Briefly, 0.4 ml DEAE resin was washed with 20 column volumes of wash buffer containing 0.1% Triton X-100. After loading samples, the column was washed with 40 column volumes of wash buffer, and glycosaminoglycans were eluted with 5 column volumes of 50 mM sodium acetate buffer, pH 6.0, containing 1 M NaCl. Subsequently, glycosaminoglycans were desalted on PD-10 columns (GE Healthcare, 10% ethanol). To determine the relative amounts of heparan sulfate and chondroitin/dermatan sulfate, a portion of the purified glycosaminoglycans was digested with 1 milliunit each of heparin lyases I, II, and III or chondroitinase ABC to specifically degrade heparan sulfate or chondroitin sulfate/dermatan sulfate, respectively, and resistant material was repurified using DEAE chromatography as described above. To assess whether dermatan sulfate specifically accumulated, a portion of material was treated with chondroitinase ACII, which only degrades regions of the chains containing glucuronic acid and leaves intact regions containing iduronic acid. Radiolabeled samples were quantified by liquid scintillation counting, and the recovered counts were normalized per μg cell protein. Some variation in counts between sets of experiments occurred (e.g. see and compare Figs. 3, 5, and 6), mostly likely due to differences in radioispecific activity of [35S]O, metabolic status of cells, harvesting of cells, recovery of glycosaminoglycans during anion-exchange chromatography, and protein determinations to which the values are normalized. For nonradiolabeled samples, glycosaminoglycans were quantified by digesting with heparin lyases I, II, and III or chondroitinase ABC, respectively. The resulting disaccharides were subsequently derivatized with isotopically labeled aniline and quantified by mass spectrometry as described previously (14).

*Enzyme Activity Measurements*—The activity of lysosomal enzymes β-hexosaminidase, α-iduronidase, β-glucuronidase, Gal/GalNac-6-sulfatase, and iduronate 2-sulfatase were assayed in wild-type cell lysates using the corresponding fluorogenic 4MU glycoside derivatives as substrates, as described previously (15–19). As no fluorogenic substrate is available that is specific for only GalNac-4-sulfatase, the activity of purified enzyme was tested using p-nitroanisole 4-sulfate as described previously (20). In some experiments, heparan sulfate (Neoparin), heparin (Neoparin), dermatan sulfate (Neoparin), or chondroitin sulfate (Sigma Aldrich) were added to reactions at indicated concentrations prior to activity analysis. All glycosaminoglycans were desalted on PD-10 columns (GE Healthcare, 10% ethanol) prior to use. Monosaccharide analysis of dermatan sulfate and chondroitin sulfate was carried out using pulsed amperometric detection as described previously (21). The dermatan sulfate contained ~95% iduronic and 5% glucuronic acid, whereas the chondroitin sulfate contained ~85% glucuronic and 15% iduronic acid.

*N-Glycan Quantification*—Wild-type, MPSIIia, and Neu1 (neuraminidase-deficient) fibroblasts were aged at confluence for 4 weeks. Cells were washed with PBS, trypsinized exhaustively, and sedimented by centrifugation. Cell pellets were
resuspended in 2 ml of 20 mM HEPES buffer, pH 8.2, and lysed by brief sonication. Lysates were digested with 1 mg/ml bovine pancreas trypsin (Sigma Aldrich) overnight at 37 °C, and trypsin was subsequently inactivated by heating the samples to 95 °C for 5 min. Precipitate was removed by centrifugation, and glycopeptides were purified from the cell lysate using a Sep-Pak C18 column (Waters) according to the manufacturer’s instructions. Samples were lyophilized, resuspended in 1 ml of 20 mM HEPES buffer, pH 8.2, and N-glycans were released from the glycopeptide fraction by digestion of the samples overnight at 37 °C with PNGase F (15 milliunits/ml). Free N-glycans were separated from the remaining glycopeptides by Sep-Pak C18 chromatography and then depolymerized by treatment with 2 M acetic acid for 3 h at 80 °C. Sialic acid residues were labeled with the fluorophore 1,2-diamino-4,5-methylene dioxybenzene (DMB) as described previously (22). The DMB-labeled N-glycolyneuraminic acid (Neu5Gc) and N-acetyneuraminic acid (Neu5Ac) were separated iso- cratically by HPLC with a C18 reverse phase column (Acclaim TM120) using 11% acetonitrile and 7% methanol in water as a running buffer at a flow rate of 0.9 ml/min. The eluant was monitored for fluorescence as described previously (22, 23), and Neu5Gc and Neu5Ac were characterized by comparing fluorescence and elution position to known labeled standards.

Size Analysis—Wild-type and MPSIIIa fibroblasts were aged 4 weeks at confluence. Cell surface heparan sulfate and chondroitin/dermatan sulfate were liberated by trypsin treatment from cells radiolabeled 48 h with 35SO4. To measure lysosomal glycosaminoglycans, heparan sulfate and chondroitin/dermatan sulfate was purified from cells after a 48-h label/48-h chase with 35SO4. Radiolabeled samples were treated at 4 °C for 24 h with 0.5 M NaOH containing 1 M NaBH4 to residual peptides by β-elimination from the glycosaminoglycan chains and to reduce the terminal sugars to their corresponding alditols. Samples were run on a 0.5 × 75 cm Sepharose CL-6B column (GE Healthcare) in 0.1 M NaCl at 0.1 ml/min collecting 1 ml fractions. The elution position of the radiola- beled glycosaminoglycans was assessed by scintillation counting, and their average Kav values were calculated using the formula (fraction number - Vr/Vt - Vb/Vt) using phenol red and blue dextran as markers of Vr and Vt, respectively. The size of the heparan sulfate and chondroitin/dermatan sulfate chains was estimated by comparing the Kav values of the samples with published values (24).

RESULTS

Secondary Storage of Chondroitin/Dermatan Sulfate in Sanfilippo Fibroblasts—Secondary storage of glycoconjugates such as gangliosides in Sanfilippo occurs, at least in part, due to the inhibition of lysosomal hydrolases by accumulating heparan sulfate (9, 11). To determine whether heparan sulfate storage causes the secondary storage of additional glycoconjugates, we assayed the amount of chondroitin and dermantan sulfate as a mixture and heparan sulfate by mass spectrometry of the enzymatically released disaccharides. This initial approach was reasonable because dermatan sulfate is a hybrid glycosaminoglycan, containing disaccharides comprised of N-acetylgalactosamine and iduronic acid as well disaccharides containing N-acetylgalactosamine and glucuronic acid that typifies chondroitin sulfate. Intracellular glycosaminoglycan levels in confluent Sanfilippo fibroblasts were found to increase over a 4-week period, whereas their levels remained unchanged in confluent wild-type fibroblasts (data not shown). After 4 weeks of confluence, heparan sulfate levels in Sanfilippo disease fibroblasts (MPSIIIa, MPSIIIb, MPSIIIc, and MPSIIId) were 4–15 times higher than levels observed in comparable dermal wild-type control fibroblasts (Fig. 1A). In addition to this expected increase in heparan sulfate, chondroitin/dermatan sulfate levels increased 2–5-fold above the level in wild-type dermal fibroblasts and even greater when compared with human foreskin fibroblasts (Fig. 1B). This accumulation was also noted after only 1 week of confluence, but the effect was not as dramatic (2–10-fold for heparan sulfate across the four Sanfilippo subclasses and 1.5–2-fold for chondroitin/dermatan sulfate). The difference in accumula- tion was not due to variation in pH of the growth medium, which was 7.5 ± 0.05 across all cultures (25).

We also assayed sialylated asparagine-linked glycans in Sanfilippo fibroblasts by quantifying N-glycolyneuraminic acid (Neu5Gc) and N-acetyneuraminic acid (Neu5Ac) associated with purified glycans from wild-type and disease fibroblasts. As shown in Fig. 2, no difference in N-glycan levels was observed between wild-type dermal fibroblasts, wild-type foreskin fibroblasts, and MPSIIIa fibroblasts. As a positive control, fibroblasts from a patient with sialidosis, a lysosomal storage disease in which N-glycans accumulate due to neuraminidase 1 deficiency (Neu1) (26), were also assayed. As expected, Neu5Gc and Neu5Ac levels in Neu1 cells were in- increased at 20-fold above wild-type, respectively (Fig. 2). Overall, these results suggest that chondroitin/dermatan sulfate accumulates as a secondary metabolite in Sanfilippo cells and that the cause of this secondary storage is likely specific to this glycosaminoglycan and not the result of a general storage defect.

Reduced Heparan Sulfate and Chondroitin/Dermatan Sulfate Turnover in Sanfilippo Fibroblasts—To determine whether the cause of increased chondroitin/dermatan sulfate
levels observed in Sanfilippo fibroblasts resulted from altered catabolism, a label chase assay was used (27). Fibroblasts were labeled for 48 h to allow steady state incorporation of $^{35}$SO$_4$ into the glycosaminoglycans and then chased for 48 h in the absence of label. Under these conditions intracellular $^{35}$S heparan sulfate accumulated 5–25-fold in fibroblasts from all four types of Sanfilippo compared with wild-type dermal fibroblast controls, consistent with reduced lysosomal turnover. In addition, $^{35}$S chondroitin/dermatan sulfate levels in Sanfilippo fibroblasts were 1.5–3-fold higher than dermal wild-type fibroblasts after label chase, indicating that, like heparan sulfate, secondary chondroitin/dermatan sulfate degradation was altered (Fig. 3A). Glycosaminoglycan turnover was also characterized in four different MPSIIIa fibroblast isolates to assess patient variation (Fig. 3B). Fibroblasts from all four patients exhibited similar elevations in $^{35}$S heparan sulfate and $^{35}$S chondroitin/dermatan sulfate levels compared with wild-type dermal fibroblasts, substantiating that defects in heparan sulfate and chondroitin/dermatan sulfate turnover can be generalized across the Sanfilippo diseases.

**FIGURE 2. Testing for the storage of N-glycans in wild-type and MPSIIIa fibroblasts.** Wild-type human dermal fibroblasts (DF), wild-type human foreskin fibroblasts (FF), and dermal fibroblasts from MPSIIIa patients were aged 4 weeks at confluence, and N-glycans were purified from cell lysates subsequent to trypsin treatment. The amount of intracellular N-glycans was determined by labeling N-glycan associated Neu5Gc and Neu5Ac with the fluorophore DMB, followed by HPLC separation and quantification relative to cell protein. As a positive control, N-glycan storage was also assessed in neuraminidase-deficient fibroblasts (Neu1, GM01718), which are known to exhibit lysosomal storage of N-glycans. Wild-type dermal fibroblast values represent the average from three different cell lines (GM03898, GM05659, and GM15871). Error bars represent the range of data obtained in two independent experiments.

**FIGURE 3. Analysis of heparan sulfate and chondroitin/dermatan sulfate turnover in wild-type and Sanfilippo fibroblasts.** Wild-type human dermal fibroblasts (DF), wild-type human foreskin fibroblasts (FF), and dermal fibroblasts from MPSIIIa, MPSIIIb, MPSIIIc, and MPSIIId patients (A) or from four different MPSIIIa patients (B) were aged for 4 weeks at confluence prior to 48 h label/48 h chase with $^{35}$SO$_4$. The amount of radiolabeled heparan sulfate (black bars) and chondroitin/dermatan sulfate (gray bars) remaining in the cells after trypsin treatment was quantified relative to cell protein. Wild-type dermal fibroblast values represent the average from three different cell lines (GM03898, GM05659, and GM15871). Error bars represent the range of data obtained in at least three independent experiments.

_Dermatan Storage in Sanfilippo_

Stored Chondroitin/Dermatan Sulfate Is Not Endolytically Processed in Sanfilippo Fibroblasts—The first step in heparan sulfate degradation is endolytic processing by human heparanase (endogluconidase), which cleaves full-length chains into smaller fragments (28). Similarly, the first step in chondroitin/dermatan sulfate degradation is thought to involve partial endolytic processing by two enzymes with endo-$\beta$-N-acetylhexosaminidase and endohexuronidase activities, respectively (29, 30). Endolytic processing might expedite catabolic turnover by creating more ends on which exolytic hydrolases act, but other studies suggest that the overall rate of catabolism of heparan sulfate may not be affected by reduced endoglycosidic cleavage (31). To examine the size of the chains that accumulate in Sanfilippo cells, intracellular $^{35}$S glycosaminoglycans were purified from trypsin-treated cells after a 48-h label/48-h chase to enrich for radiolabeled lysosomal glycosaminoglycans. Very little radiolabeled material was obtained from the wild type under these conditions, obviating further analysis in the wild type. For comparison, cell surface $^{35}$S glycosaminoglycans were isolated by labeling cells for 48 h with $^{35}$SO$_4$ followed by exhaustive trypsin-treatment and subsequent purification of the labeled glycosaminoglycans from the trypsin solution. Cell surface heparan sulfate generated in this way exhibited an average size of ~45 kDa in wild-type and MPSIIIa cells, which was reduced to fragments of ~10 kDa after the chase in MPSIIIa cells, indicating that stored heparan sulfate had been processed by heparanase (Fig. 4). When chondroitin/dermatan sulfate was analyzed in a similar way, cell surface chondroitin/dermatan sulfate exhibited an average size of ~25 kDa in both wild-type and MPSIIIa cells but did not appear to be processed into shorter chains in MPSIIIa cells (Fig. 4). Overall, these data indicate that chondroitin/dermatan sulfate chains are not cleaved into significantly smaller fragments when they accumulate as secondary storage metabolites in Sanfilippo fibroblasts.

Correction of Heparan Sulfate Storage in MPSIIIa Normalizes Chondroitin/Dermatan Sulfate Turnover—We hypothesized that if heparan sulfate storage in Sanfilippo is the direct cause of secondary chondroitin/dermatan sulfate accumulation then correcting heparan sulfate storage in these cells should normalize chondroitin/dermatan sulfate turnover.
test this hypothesis, glycosaminoglycan turnover was assessed in MPSIIa fibroblasts by label/chase in the presence or absence of recombinant sulfamidase. Addition of exogenous sulfamidase to MPSIIa fibroblasts resulted in an almost complete normalization of heparan sulfate turnover (Fig. 5). The amount of [35S]chondroitin/dermatan sulfate remaining after 48 h of chase was also reduced (~50%), indicating a direct cause and effect relationship between heparan sulfate storage and altered chondroitin/dermatan sulfate turnover (Fig. 5). Addition of sulfamidase only affected intracellular [35S]glycosaminoglycan levels, whereas cell surface and secreted [35S]glycosaminoglycan levels remained unchanged (Fig. 6), substantiating that sulfamidase addition only affected intracellular turnover and not glycosaminoglycan biosynthesis, cell surface processing, or secretion.

Heparan Sulfate Inhibits Iduronate 2-Sulfatase—To determine whether heparan sulfate storage causes secondary chondroitin/dermatan sulfate storage by directly inhibiting lysosomal hydrolases involved in chondroitin/dermatan sulfate catabolism, we tested the sensitivity of the relevant degradative enzymes to heparin, heparan sulfate, and dermatan sulfate. Among the various enzymes tested, only N-acetylgalactosamine-6-sulfatase and iduronate 2-sulfatase activity were found to be sensitive to heparin and heparan sulfate. Iduronate 2-sulfatase was inhibited >95% at glycosaminoglycan concentrations of 100 μg/ml (Fig. 7A) with IC50 values of ~4 and 14 μg/ml, respectively (Fig. 7b). Interestingly, iduronate 2-sulfatase activity was unaffected by dermatan sulfate at a concentration of 100 μg/ml (Fig. 7A) nor was it inhibited by chondroitin sulfate at the same concentration (data not shown). In addition to testing the inhibitory capacity of commercial glycosaminoglycans, we tested the impact of endogenous heparan sulfate purified from the intracellular fraction of MPSIIa cells on iduronate 2-sulfatase activity. At a concentration of 25 μg/ml, endogenous MPSIIa heparan sulfate inhibited iduronate 2-sulfatase activity by >50%, indicating that despite endolytic processing by heparanase, stored MPSIIa heparan sulfate retains most of its inhibitory effect toward this enzyme (Fig. 7B). Degradation of heparin or heparan sulfate to its component disaccharides by treatment with bacterial heparin lyases was found to completely abrogate their inhibitory capacity (data not shown).

The high sensitivity of iduronate 2-sulfatase to heparan sulfate suggested that accumulating heparan sulfate in Sanfilippo lysosomes might be sufficient to inhibit its activity and cause the secondary accumulation of dermatan sulfate, which contains substantial amounts of iduronic acid (32, 33). To examine whether dermatan sulfate accumulates, we isolated 35S-labeled glycosaminoglycans from MPSIIa cells and purified the chondroitin/dermatan sulfate fraction. Digestion of the sample with chondroitinase ABC resulted in 95% degradation (Fig. 8). In contrast, treatment with chondroitinase ACII,

FIGURE 5. Impact of enzyme replacement therapy on primary and secondary storage in MPSIIa fibroblasts. Dermal wild-type and MPSIIa (GM00643) fibroblasts were aged for 4 weeks in cell culture prior to 48-h label/48-h chase with 35SO4. To test the impact of enzyme replacement therapy on storage, 2 μg/ml sulfamidase was added during the chase. Heparan sulfate and chondroitin/dermatan sulfate remaining after the chase were purified from cell lysates and quantified relative to cell protein. Error bars represent the range of data obtained in at least three independent experiments. Data significance between MPSIIa untreated and sulfamidase treated samples was calculated using the two-tailed t test; **, p < 0.01.

FIGURE 6. Impact of enzyme replacement therapy on intracellular, cell surface, and secreted glycosaminoglycan fractions. MPSIIa (GM00643) fibroblasts were aged for 4 weeks in cell culture prior to 48-h label/48-h chase with 35SO4. To test the impact of enzyme replacement therapy on intracellular, cell surface, and secreted fractions, cells were chased in the absence (black bars) or with 2 μg/ml sulfamidase added to the growth medium (gray bars). The remaining [35S]glycosaminoglycans (a mixture of heparan sulfate and chondroitin/dermatan sulfate) were purified from the medium (secreted fraction), from the fraction released after trypsin treatment (cell surface fraction), and from the remaining cells (intracellular fraction) and quantified relative to cell protein. Error bars represent the range of data obtained in at least three independent experiments. Data significance between MPSIIa untreated and sulfamidase treated samples was calculated using the two-tailed t test; **, p < 0.01.
Dermatan Storage in Sanfilippo

FIGURE 7. Testing the inhibition of dermatan sulfate catabolic enzymes by glycosaminoglycans. The activity of lysosomal enzymes involved in dermatan sulfate catabolism was assayed using fluorogenic substrates. Wild-type fibroblast cell lysates or purified GalNAc-4-sulfatase were used as an enzyme source. Untreated samples (white bars) were compared with the activity of cell lysates or purified enzyme in the presence of 100 μg/ml heparin (black bars), heparan sulfate (gray bars), or dermatan sulfate (striped bars). Error bars represent the S.D. of at least three independent experiments. Data significance between untreated samples and those in which glycosaminoglycans were added prior to analysis was calculated using the two-tailed t test; **, p < 0.01 (A). The sensitivity of iduronate 2-sulfatase to heparan sulfate and heparin was tested over a range of concentrations to allow the determination of the IC50 values for these glycosaminoglycans. Nonlinear regression curves were fit to the values for the inhibition of iduronate 2-sulfatase with heparin (solid line) or heparan sulfate (dashed line) with R2 values ≥ 0.96. Additionally, the inhibitory capacity of endogenous MPSIIa heparan sulfate toward iduronate 2-sulfatase was tested at a concentration of 25 μg/ml (B). Error bars represent the range of data obtained in at least three independent experiments.

which does not cleave at disaccharides containing iduronic acid, only resulted in a 25% degradation. Thus, the material that accumulates in the MPSIIIa cells is highly enriched in iduronic acid, consistent with its identification as dermatan sulfate. As a control, the heparan sulfate that accumulates was 95% sensitive to heparin lyase treatment. The demonstration that dermatan sulfates accumulates is consistent with inhibition of iduronate 2-sulfatase.

DISCUSSION

Sanfilippo is characterized by extensive central nervous system degeneration with mild somatic defects, making it one of the most severe and difficult to treat forms of MPS (2). Although enzymatic deficiencies associated with Sanfilippo are known to result in the primary accumulation of heparan sulfate, it is unclear why heparan sulfate storage causes such dramatic neuropathology. This disconnect between primary storage defect and pathology has led some researchers to speculate that the accumulation of secondary metabolites may in fact be influencing disease manifestation (4, 9).

In this study, we demonstrate the secondary accumulation of dermatan sulfate in Sanfilippo patients fibroblasts. Screening the inhibitory capacity of different glycosaminoglycans toward a complete panel of dermatan sulfate catabolic enzymes indicated that the cause of this secondary storage may be the inhibition of the lysosomal hydrolase, iduronate 2-sulfatase, by elevated heparan sulfate levels in diseased cells. The IC50 of heparan sulfate toward iduronate 2-sulfatase was ~14 μg/ml. Based on results from this study, the average amount of heparan sulfate in Sanfilippo fibroblasts at 4 weeks of confluence was ~5 μg/mg cell protein (or 5 μg/107 cells). We estimate that at least 80% of this material is lysosomal based on cell fractionation studies (data not shown). If we assume an average cell diameter of 20 μm and that lysosomes in diseased cells make up ~10% of the cell volume, we calculate the amount of heparan sulfate per lysosome to be ~1 mg/ml. Although this calculation is based on a number of assumptions, it demonstrates that the heparan sulfate concentration in Sanfilippo lysosomes is well within range to cause significant inhibition of iduronate 2-sulfatase and subsequent secondary storage.

A major question arising from this study is how the secondary accumulation of dermatan sulfate might affect disease pathology in Sanfilippo. Although further experimentation will be necessary to answer this question, our current understanding of different MPS conditions provides some insight as to the impact of different glycosaminoglycans on disease etiology. For MPS diseases caused explicitly by chondroitin and dermatan sulfate storage (MPS type IVa and VI) and those caused by low level storage of both chondroitin/dermatan sulfate and heparan sulfate storage (Scheie and Hurler-Scheie type MPSI), disease pathology is primarily somatic, affecting bones and organ function, whereas neurological symptoms...
Dermatan Storage in Sanfilippo

In summary, this study describes the secondary storage of dermatan sulfate in Sanfilippo disease fibroblasts and provides strong evidence that the cause of this secondary storage is the result of iduronate 2-sulfatase inhibition by elevated heparan sulfate levels in disease cells. Assuming that this general mechanism of secondary storage results in similar or even greater degrees of dermatan sulfate accumulation in other cell types more relevant to disease pathology, we predict that this secondary storage in combination with heparan sulfate storage could have a significant impact on Sanfilippo pathology. Future experiments will focus on elucidating which cell types exhibit the highest degree of secondary storage and how this might influence disease progression. If accumulation of dermatan sulfate contributes significantly to the pathology of the disease, enzyme supplementation to reduce secondary storage might increase the efficacy of Sanfilippo therapy.

Acknowledgment—Technical support was provided by the Glycotechnology core facility at the University of California, San Diego.

REFERENCES

1. Esko, J. D., Kimata, K., and Lindahl, U. (2009) in Essentials of Glycobiology (Varki, A., Cummings, R., Esko, J. D., Freeze, H., Stanley, P., Bertozzi, C. R., Hart, G. W., and Etzler, M. E., eds) 2nd Ed., pp. 229–248, Cold Spring Harbor Laboratory Press, New York
2. Neufeld, E. F., and Muenzer, J. (2001) in Metabolic and Molecular Basis of Inherited Disease, 8th Ed., pp. 3421–3452, McGraw-Hill, New York
3. Futerman, A. H., and van Meer, G. (2004) Nat. Rev. Mol. Cell Biol. 5, 554–565
4. Walkley, S. U., and Vanier, M. T. (2009) Biochim. Biophys. Acta 1793, 726–736
5. Pellet, D., Lloyd-Evans, E., Riebeling, C., Jeyakumar, M., Platt, F. M., and Futerman, A. H. (2003) J. Biol. Chem. 278, 29496–29501
6. Nguyen, H. N., Wang, C., and Perry, D. C. (2002) Brain Res. 924, 159–166
7. Wei, H., Wei, W., Bredesen, D. E., and Perry, D. C. (1998) J. Neurochem. 70, 2305–2314
8. Siegel, D. A., and Walkley, S. U. (1994) J. Neurochem. 62, 1852–1862
9. Walkley, S. U. (2004) Semin. Cell Dev. Biol. 15, 433–444
10. McGlynn, R., Dobrenis, K., and Walkley, S. U. (2004) J. Comp. Neurol. 480, 415–426
11. Baumkötter, J., and Cantz, M. (1983) Biochim. Biophys. Acta 761, 163–170
12. Liour, S. S., Jones, M. Z., Suzuki, M., Bieberich, E., and Yu, R. K. (2001) Mol. Genet. Metab. 72, 239–247
13. Esko, J. D. (2001) Curr. Protoc. Mol. Biol. (Albright, L. M., Coen, D. M., and Varki, A., eds) Vol. 3, pp. 17.21–17.29, John Wiley and Sons, Inc., New York
14. Lawrence, R., Olson, S. K., Steele, R. E., Wang, L., Warrior, R., Cummings, R. D., and Esko, J. D. (2008) J. Biol. Chem. 283, 33674–33684
15. Vouy, Y. V., Keulemans, J. L., and van Diggelen, O. P. (2001) J. Inherit. Metab. Dis. 24, 675–680
16. Leaback, D. H., and Walker, P. G. (1961) Biochem. J. 78, 151–156
17. van Diggelen, O. P., Zhao, H., Kleijer, W. J., Janse, H. C., Poorthuis, R. J., van Pelt, J., Kamerling, J. P., and Galjaard, H. (1990) Clin. Chim. Acta 187, 131–139
18. Hopwood, J. I., Muller, V., Smithson, A., and Baggett, N. (1979) Clin. Chem. Acta 92, 257–265
19. Rome, L. H., Garvin, A. J., Allietta, M. M., and Neufeld, E. F. (1979) Cell 17, 143–153
20. Peters, C., Rommerskirch, W., Modaressi, S., and von Figura, K. (1991) Biochem. J. 276, 499–504
21. Hardy, M. R. (1989) Methods Enzymol. 179, 76–82

remain mild to nonexistent (2). Alternatively, MPS diseases caused by higher levels of heparan sulfate and chondroitin/dermatan sulfate storage (Hurler type MPSI and Hunter type MPSII) are much more severe and exhibit extensive neuropathology in addition to peripheral abnormalities (2). Thus, secondary storage of dermatan sulfate in Sanfilippo cells is consistent with the idea that the combination of heparan sulfate and dermatan sulfate storage might contribute to pathology. Notably, although secondary accumulation of dermatan sulfate was shown to cause only a modest increase in overall glycosaminoglycan levels in Sanfilippo fibroblasts (~10–20%), the contribution of secondary dermatan sulfate to total storage levels may be much greater in tissues that produce high amounts of this glycosaminoglycan such as the brain (34, 35). Future analysis of secondary dermatan sulfate storage in other cell types, particularly in the brain, will be necessary to substantiate the biological significance of our findings.

It has been previously demonstrated that glycosaminoglycan storage abrogates autophagy (36, 37), an essential cellular process that, when disrupted in the brain, causes severe neuropathology (38, 39). Thus, one explanation for the severe neuropathology in some MPS disorders may be that overall lysosomal storage breaches a critical threshold where cellular processes such as autophagy are more dramatically affected. It is also important to note that, like heparan sulfate, dermatan sulfate is a bioactive polymer that can inhibit lysosomal enzyme activities (40), modulate enzyme activities in the serum such as serpin-protease complex formation (41, 42), and regulate growth factor signaling and inflammatory response (43). Thus, in addition to contributing to the overall lysosomal load, storage of dermatan sulfate in Sanfilippo could affect biological processes that influence pathology. The fact that dermatan sulfate accumulates as a high molecular weight polymer, at least in some cell types, may accentuate some of its downstream effects.

The discovery of secondary dermatan sulfate storage in Sanfilippo cells also has important implications for MPS disease diagnostics. For example, the dermatan sulfate sensitivity of the heparin cofactor II-thrombin complex formation has been used to detect MPS disorders where this glycosaminoglycan is stored (41, 42, 44). Initial testing of this diagnostic in MPS patients demonstrated the robustness of the assay for detecting MPS disorders where dermatan sulfate catabolism is known to be deficient. However, significant elevations of the heparin cofactor II-thrombin complex were also detected in MPSIIIA patients, which remained unexplained (42). These findings now make sense in light of the data reported here, suggesting significant secondary dermatan sulfate storage in Sanfilippo cells. Our findings also clarify results from a recently reported diagnostic approach where unexplained elevations of chondroitin/dermatan sulfate disaccharides were detected in Sanfilippo patient serum and urine after enzymatic digests of purified glycosaminoglycans (45). Our findings also suggest that diagnostic approaches that are able to detect dermatan sulfate levels as well as heparan sulfate in Sanfilippo may provide a more accurate index for cellular stress and offer a superior indication of disease progression and prognosis.

ACKNOWLEDGMENT—Technical support was provided by the Glycotechnology core facility at the University of California, San Diego.
Dermatan Storage in Sanfilippo

22. Hara, S., Yamaguchi, M., Takemori, Y., Furuhata, K., Ogura, H., and Nakamura, M. (1989) *Anal. Biochem.* **179**, 162–166
23. Klein, A., Diaz, S., Ferreira, I., Lamblin, G., Roussel, P., and Manzi, A. E. (1997) *Glycobiology* **7**, 421–432
24. Wasteson, A. (1971) *J. Chromatogr.* **59**, 87–97
25. Lie, S. O., McKusick, V. A., and Neufeld, E. F. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2361–2363
26. Thomas, G. H. (2001) in *Metabolic and Molecular Basis of Inherited Disease*, 8 Ed., pp. 3507–3534, McGraw Hill, New York
27. Fratantoni, J. C., Hall, C. W., and Neufeld, E. F. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **60**, 699–706
28. Vreys, V., and David, G. (2007) *J. Cell Mol. Med.* **11**, 427–452
29. Nielsen, T. C., Meikle, P. J., Hopwood, J. J., and Fuller, M. (2008) *Glycobiology* **18**, 1119–1128
30. Settembre, C., Fraldi, A., Jahreiss, L., Spamanenato, C., Venturi, C., Medina, D., de Pablo, R., Tacchetti, C., Rubinsztein, D. C., and Ballabio, A. (2008) *Hum. Mol. Genet.* **17**, 119–129
31. Settembre, C., Fraldi, A., Rubinsztein, D. C., and Ballabio, A. (2008) *Autophagy* **4**, 113–114
32. Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006) *Nature* **441**, 880–884
33. Hara, T., Nakamura, K., Matsu, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishina, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006) *Nature* **441**, 885–889
34. Trowbridge, J. M., and Gallo, R. L. (2002) *Glycobiology* **12**, 117R–125R
35. Mitsunaga, C., Mikami, T., Mizumoto, S., Fukuda, J., and Sugahara, K. (2006) *J. Biol. Chem.* **281**, 18942–18953
36. Atsumi, C., Mizumoto, S., Kaneiwa, T., Maccaranà, M., Malmström, A., Yamada, S., and Sugahara, K. (2010) *Glycobiology*, in press
37. Settembre, C., Fraldi, A., Jahreiss, L., Spampanato, C., Venturi, C., Medina, D., de Pablo, R., Tacchetti, C., Rubinsztein, D. C., and Ballabio, A. (2008) *Hum. Mol. Genet.* **17**, 119–129
38. Settembre, C., Fraldi, A., Rubinsztein, D. C., and Ballabio, A. (2008) *Autophagy* **4**, 113–114
39. Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006) *Nature* **441**, 880–884
40. Settembre, C., Fraldi, A., Rubinsztein, D. C., and Ballabio, A. (2008) *Autophagy* **4**, 113–114
41. Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006) *Nature* **441**, 880–884
42. Sumato, M., Matsu, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishina, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006) *Nature* **441**, 885–889
43. Trowbridge, J. M., and Gallo, R. L. (2002) *Glycobiology* **12**, 117R–125R
44. Mitsunaga, C., Mikami, T., Mizumoto, S., Fukuda, J., and Sugahara, K. (2006) *J. Biol. Chem.* **281**, 18942–18953
45. Atsumi, C., Mizumoto, S., Kaneiwa, T., Maccaranà, M., Malmström, A., Yamada, S., and Sugahara, K. (2010) *Glycobiology*, in press