Physiological Substrates for Human Lysosomal \textit{\beta-}Hexosaminidase S*

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Human lysosomal \textit{\beta-}hexosaminidases remove terminal \textit{\beta-}glycosidically bound \textit{\textit{\textSigma}}-acyethylhexosamine residues from a number of glycoconjugates. Three different isozymes composed of two noncovalently linked subunits \textit{\alpha} and \textit{\beta} exist: Hex \textit{\alpha} (\textit{\alpha}\textit{\textSigma}), Hex \textit{\beta} (\textit{\beta}\textit{\textSigma}), and Hex \textit{\textSigma} (\textit{\textSigma}\textit{\textSigma}). While the role of Hex \textit{\Lambda} and Hex \textit{\textSigma} for the degradation of several anionic and neutral glycoconjugates has been well established, the physiological significance of labile Hex \textit{\textSigma} has remained unclear. However, the striking accumulation of anionic oligosaccharides in double knockout mice totally deficient in hexosaminidase activity but not in mice expressing Hex \textit{S} (Sangio, K., M. P. Crawford, J. N., Mack, M. L., Tiff, C.J., Skop, E., Starr, C. M., Hoffmann, A., Sandhoff, K., Suzuki, K., and Proia, R. L., (1996) Nat. Genet. 14, 348–352) prompted us to re-examine the substrate specificity of Hex \textit{S}. To identify physiological substrates of Hex \textit{S}, anionic and neutral oligosaccharides excreted in the urine of the double knockout mice were isolated and analyzed. Using ESI-MS/MS and glycosidase digestion the anionic glycans were identified as products of incomplete deraminate sulfate degradation whereas the neutral storage oligosaccharides were found to be fragments of \textit{\textSigma}-glycan degradation. In vitro, recombinant Hex \textit{S} was highly active on water-soluble and amphiphilic glycoconjugates including artificial substrates, sulfated GAG fragments, and the sulfated glycosphingolipid SM2. Hydrolysis of membrane-bound SM2 by the recombinant Hex \textit{S} was synergistically stimulated by the \textit{\textSigma}2 activator protein and the lysosomal anionic phosphopholipid bis(\textit{\textSigma}acyethylglycerophosphate).

Lysoosomal hexosaminidases (EC 3.2.1.52) release terminal \textit{\beta-}glycosidically linked \textit{\textSigma}-acyethylhexosamine and \textit{\textSigma}-acyethylgalactosamine residues from a number of glycoconjugates (1). They are composed of two subunits, \textit{\alpha} and \textit{\beta}, derived from homologous genes HEXA and HEXB. Hexosaminidase A (Hex \textit{\alpha}, \textit{\alpha} prefix) and Hex B (\textit{\beta} prefix) were believed to be the major functional isozymes, and Hex S (\textit{\textSigma} prefix) a minor labile form without significant activity (2). Several attempts to isolate pure Hex \textit{S} from human tissues or cell homogenates yielded preparations with poor enzymatic activity (3–5).

Each subunit possesses an active site characterized by its own substrate specificity (5). The active site of the \textit{\beta}-subunit hydrolyzes uncharged substrates, whereas the \textit{\alpha}-subunit, in addition, cleaves negatively charged substrates. Only the \textit{\beta}-heterodimer Hex \textit{A} is able to degrade ganglioside GM2 (Fig. 1B) at significant rates in the presence of the GM2 activator protein (GM2AP).

A group of severe neurodegenerative storage diseases, the GM2 gangliosidoses, results from mutations in any of the genes encoding the two hexosaminidase subunits and GM2AP. Tay-Sachs disease is caused by mutations in the \textit{HEXA} gene resulting in a deficiency in Hex \textit{A} and Hex \textit{S}, whereas in Sandhoff disease the lack of Hex \textit{A} and Hex \textit{B} activity is observed due to mutations in the \textit{HEXB} gene. The GM2 gangliosidoses are characterized by a massive accumulation of ganglioside GM2 and related glycolipids in neuronal lysosomes. Depending on the defect and its severity, other tissues may also be affected by lipid and oligosaccharide accumulation (1). The severity of the clinical phenotype is directly related to the amount of residual enzyme activity (6). In addition, the isozyme that is not affected by mutation is able to compensate in part for the loss of the affected hexosaminidase activity (1).

Mouse models have been generated for Tay-Sachs disease (deficient in the \textit{\alpha}-subunit but expressing Hex \textit{B}), for Sandhoff disease (deficient in the \textit{\beta}-subunit and expressing Hex \textit{S}) as...
well as double knockout mice (totally deficient in hexosaminidase activity) (7, 8) (Table I).

Surprisingly, the double knockout mice show a mucopolysaccharidosis phenotype with an accumulation of anionic oligosaccharides in addition to the pathological and biochemical features of GM2 gangliosidoses (8, 9). In the HexS−/− mice no increased accumulation of glycosaminoglycans (GAGs) was observed indicating that Hex S, which is still expressed in the mutant mice, is involved in GAG catabolism. GAGs contain β(1–4)- and β(1–3)-linked N-acetylgalactosamine residues which become sensitive to hexosaminidases when exposed as terminal sugar residues during their degradation (10).

We have purified and characterized recombinant Hex S (rHexS) after expression in insect cells. To study its substrate specificity the storage glycans were isolated from the urine of the hexosaminidase double knockout mice. In addition, other water-soluble and lipid substrates were evaluated. The results demonstrate that Hex S is highly active on a wide range of substrates and support the contention that the enzyme is physiologically important in vivo.

EXPERIMENTAL PROCEDURES

Commercial Products

Phosphatidylcholine (egg yolk) (PC), cholesterol (Chol), the maltose oligosaccharide standard, concanavalin A-Sepharose, chondroitin 6-sulfate (type IV-S), β-glucuronidase from bovine liver (type B-10), and hyaluronidase (bovine testes) were purchased from Sigma, Deisenhofen, Germany. BMP was purchased from Avanti Polar Lipids. 2,3-Dihydroxybenzoic acid was supplied by ICN Biochemicals. POROS 20 HQ and HS ion exchange resins were purchased from Roche Molecular Biochemicals, Mannheim, Germany. DEAE-Sephacel and Sephadex G-25 fine were purchased from Amersham Biosciences, Inc., Munich, Germany. TSK-Gel HPLC columns of the anionic subunit (13).

Characterization of Post-translational Modifications of rHexS

After proteolytic cleavage and reverse phase separation of the resulting peptides the post-translational modifications of rHexS were analyzed by mass spectrometry: N-glycosylation of rHexS glycopeptides was demonstrated by treatment with N-glycosidase F (15) and by ESI-MS/MS fragment analysis as described below. Disulfide linkages were analyzed by comparing the masses of the proteolytic cleavage products of unmodified active rHexS with those of active rHexS alkylated by iodoaceticamide, and those of reduced and alkylated rHexS as established in our laboratory for the analysis of the hexosaminidase β-subunit (15). In addition, chymotryptic cleavage of rHexS was used under the conditions described for trypsin degradation (15) and proteolytic digestion products were also analyzed by ESI-MS/MS as described below, in addition to MALDI-MS.

Activator Preparation

Recombinant GM2AP was expressed in insect cells using the baculovirus expression system and purified as described previously (16).

Determination of Km and Vmax

The Km and Vmax values for recombinant β-hexosaminidase S were determined by the method of Lineweaver and Burk (17). Enzyme activities were measured as described (5) using various concentrations of the synthetic substrates MUG (final concentrations of 0.25–2.5 mM citrate buffer, pH 4.4) and MUGS (0.1–1.0 mM in 50 mM citrate buffer, pH 4.0), respectively. Enzyme kinetics with these substrates followed Michaelis-Menten theory.

Determination of pH Optimum with the Artificial Substrates MUG and MUGS

Enzyme activities were measured as described (5) using MUG (final concentrations of 1 mM in 50 mM citrate buffer) and MUGS (0.5 mM in 50 mM citrate buffer) with various pH values between 4.0 and 4.9. The pH optimum for both substrates was determined to be pH 4.4.
25 mM citrate buffer), respectively, and varying the pH of the solution in the range from 3.0 to 6.0.

**Presentation of Data**

The data obtained with the artificial substrates are means of at least duplicate determinations. Deviations did not exceed ± 5% of the mean.

**Animals**

Generation of hexosaminidase-deficient mice through targeted gene disruption and cross-breding of the knockout animals to obtain the double knockout mice has been described previously (7, 8). The urine and kidneys of knockout mice and wild type littermates of 10–20 weeks of age were analyzed.

**Isolation of the Sulfoglycolipids SM2 and SB2 from Rat Kidney**

SM2 and SB2 were isolated according to Jennemann et al. (18) with some modifications. Kidneys were homogenized with an Ultra-Turrax, freeze-dried, and extracted twice with aceton using an ultrasonic bath. The residual pellet then was extracted for GSLs two times with chloroform/methanol/water (10/10/1, v/v/v), once with chloroform/methanol/water (30/60/8, v/v/v) using an ultrasonic bath, and the combined chloroform/methanol/water extracts were dried in a rotary evaporator.

To remove most phospholipids the chloroform/methanol/water extract was treated with 0.1 M methanolic KOH for 2 h at 37 °C and neutralized with acetic acid. To remove salts the extract was dialyzed 5 times against water and subsequently freeze dried. Neutral and acidic GSLs were separated on a DEAE column and acidic GSLs were split into fractions by eluting with a stepwise gradient of 20, 80, 200, 500, and 1000 mM potassium acetate in methanol. Again salts were removed by dialyzing 5 times against water and subsequently freeze drying the micellar lipid solution. SM2 then was isolated from the 80 mM potassium acetate fraction and SB2 from the 500 mM potassium acetate fraction. Each fraction was further purified by silica gel flash chromatography using a stepwise gradient of propanol-2/methanol:water extracts were dried in a rotary evaporator. To remove salts the extract was dialyzed 5 times against water and subsequently freeze dried.

**Vesicle Preparation**

Large unilamellar vesicles (LUVs) were prepared by the following procedure: PC (50 mM, toluol/ethanol, 2/1, v/v), BMP (5 mM, chloroform/methanol, 2/1, v/v), Chol (25.6 mM, chloroform/methanol, 2/1, v/v), and SM2 (740 μM, chloroform/methanol/water, 60/35/8, v/v/v). The lipid mixture was hydrated to a total lipid concentration of 2 mM in Tris/HCl buffer (1 mM, pH 7.4) and mixed and dried under nitrogen. The lipid mixture was hydrated to a 3.2.1.31) in 100 mM sodium citrate buffer, pH 5.2, for 48 h at 37 °C. To remove salts the extract was dialyzed 5 times against water and subsequently freeze dried.

**Isolation of Chondroitin 6-Sulfate according to Kresse et al.**

Chondroitin 6-sulfate according to Kresse et al. (21) was isolated from the urine of knockout mice and wild type littermates of 10 weeks of age. The urine was collected in a metabolism cage for mice (Scanbur A/S) in which the mice stayed 8 h per day. Neutral urinary oligosaccharides were prepared by solid phase extraction on graphitized carbon as described by Klein et al. (21). Briefly, the columns (0.5 ml) equilibrated with 3 ml of water were loaded with 150–300 μl of urine sample and washed with 2 ml of water again. Neutral oligosaccharides were eluted with 2 ml of 25% (v/v) acetonitrile in water. Anionic urinary oligosaccharides were eluted according to Lindahl et al. (22) with two pH modifications. A 2.6-ml DEAE-Sephasel column was equilibrated first with 50 mM Tris/HCl, pH 7.2, and then with washing buffer (50 mM sodium acetate buffer, pH 4.0) at a flow-rate of 1.2 ml/min. After loading of 150–300 μl of urine and 15 ml of washing buffer the column was eluted with either 150 mM lithium chloride in 50 mM sodium acetate buffer, pH 4.0, for the isolation of DS-5 (A) or 2 mM lithium chloride in the same buffer for the preparation of GAG OS (B). The eluates A and B were separately desalted by size exclusion chromatography on a TSK-Gel HW40F column (16 × 390 mm) with a flow rate of 1.0 ml/min.

**Enzyme Assays**

Hex S activity was assayed with the artificial substrates MUG or MUGS as described (5). Incubation mixtures using MUG as substrate contained the following components in a final volume of 200 μl: sodium citrate buffer (50 mM, pH 4.4), 20-μl aliquots of enzyme solution, 10 μg of bovine serum albumin, and 1 mM MUG. When using MUGS the final substrate concentration was 0.5 mM and the buffer concentration was 50 mM sodium citrate buffer, pH 4.0. The incubation was stopped after 5 h by adding 1 ml of sodium carbonate/glycine buffer (200 mM each) pH 9.5). Then, the amount of 4-methylumbelliferone released was measured fluorometrically. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of MUG/min at 37 °C.

**Glycolipid Substrates for Human Lysosomal β-Hex S**

The tetrasaccharide was then treated with 0.1 M methanolic KOH for 2 h at 37 °C. The ethanol-soluble degradation products were analyzed by reverse phase high-performance liquid chromatography using a stepwise gradient of methanol:water (0.5 ml) were loaded with sample, washed with water, and eluted with 2 ml of chloroform/methanol 1/1 (v/v). The eluted lipids were applied to a TLC plate.

**Isolation of Urinary Glycans in the Hexosaminidase-deficient Mice**

After incubation stop with 40 μl of methanol the incubation was stopped after 5 h by adding 200 mM sodium citrate buffer, pH 4.3. The micellar assay was also examined in a liposomal assay (B).

**Micellar Assay—Glycolipids**

Glycolipids were incubated with the A, S, and B isozymes under the same conditions as described in the oligosaccharide assay with the following alterations: 8 nmol of glycolipid per assay were used and sodium citrate buffer (5 mM, pH 4.3) replaced the ammonium acetate buffer in a final volume of 20 μl. 1.5 μg of GM2AP was added and incubation times ranged from 15 min to 17 h, as indicated in the legends to the figures. After the incubations were stopped with 20 μl of methanol the assay mixtures were dried in a CVE and dissolved in 40 μl of ammonium acetate buffer (0.3 mM, pH 7.0). Prior to TLC analysis the samples were desalted by reverse phase chromatography, RP 18 columns (0.5 ml) equilibrated with a solution of chloroform, methanol, 0.1 M KCl (3/48/47, v/v/v) were loaded with sample, washed with water, and eluted with 2 ml of chloroform/methanol 1/1 (v/v). The eluted lipids were applied to a TLC plate.

**Liposomal Assay—LUVs containing SM2 as substrate were incubated with rHexS and GM2AP in a liposomal assay. The micellar assay described above was modified as follows. Standard incubation mixtures contained rHexS, GM2AP, 0.1 M sodium citrate buffer, 1.5 μg of GM2AP and 20 μl of protein mixture in citrate buffer, 1 mM, pH 4.3, 4 μg of bovine serum albumin, 1.0 μg of GM2AP, and 300 milliunits of rHexS in a final volume of 40 μl. A total volume of 20 μl of protein mixture in citrate buffer was added to the same volume of unilamellar vesicles dissolved in Tris/Cl buffer (1 mM, pH 7.2). The liposomes (1 mM, amount of total liposomal lipids) were composed of 50 mol % PC, 20 mol % Chol, 20 mol % BMP and 10 mol % SM2 or 70 mol % PC, 20 mol % Chol, and 10 mol % SM2. After incubation stop with 40 μl of methanol the mixtures were con
centrated to dryness by a stream of nitrogen and then subjected to alkaline methanolysis with 0.1 M NaOH in 200 μl of methanol for 4.5 h at 37°C to remove the phospholipids. After neutralization with 1.2 μl of glacial acetic acid, the samples were desalted by reverse phase chromatography on RP18 and applied to HPTLC plates.

Thin-layer Chromatography

Desalted assay samples were applied to thin layer Silica Gel 60 or HPTLC plates (Merck Darmstadt, Germany). The chromatograms were developed with chloroform, methanol, 0.22% (w/v) CaCl2 in water (60/35/8, v/v/v). After development, plates were air-dried, sprayed with 8% (w/v) H2PO4 containing 10% (w/v) CuSO4, and charred at 180°C for 10 min, and lipids were quantitated by photodensitometry (Shimadzu Kyoto, Japan).

Analysis of Glycan Samples by Fluorophore-assisted Carbohydrate Electrophoresis (FACE)

This method is based on the fluorescence labeling of glycans by reductive amination with 8-aminonaphthalene-1,3,6-tris-sulfonic acid (23). 5 μl of a 0.15 M solution of the fluorophore in 15% (v/v) glacial acetic acid and 5 μl of 1.0 M sodium cyanoborohydride in dimethyl sulfoxide were added to the glycan samples which had previously been dried in a CVE. After incubation for 15 h at 37°C the samples were dried in a CVE and dissolved in 8 μl of HPLC water. 4 μl of this solution were mixed with the same volume of 25% (v/v) glycerol in HPLC water and applied to a 32% polyacrylamide gel with a 4% stacking gel. The buffer solutions and gel components were the same as for SDS-PAGE according to Laemmli (24) except that SDS was omitted. The fluorescently labeled glycans resolved in the gel were visualized by illumina-
tion with UV light of 366 nm wavelength. The gel image was photographed by a ccd camera. The migration distance depends on molecular weight and charge, i.e. the m/z values of the labeled glycans. An anionic oligosaccharide carrying intrinsic negative charges has a higher electrophoretic mobility than a neutral oligosaccharide of the same size. Thus, in the FACE gels it is only possible to compare migration distances of identically charged analytes. From our experiments using FACE we conclude for the GAG fragments with high negative net charge that the labeling efficiency decreases with increasing molecular weight of the saccharide. Therefore, band intensities are only comparable between analytes of the same molecular weight and charge. Despite these limitations the FACE display technique is one of the most powerful methods for glycan profiling currently at hand, combining high sensitivity down to the picomole range, high resolution, and relatively high tolerance of buffers and salt concentrations in the samples.

MALDI Mass Spectrometry

MALDI-MS analysis was performed on a ToFSpec E (Micromass, Manchester, UK) mass spectrometer operating at an acceleration voltage of 20 kV with a 337-nm nitrogen laser. Mass spectra were externally calibrated using a maltoligosaccharide standard (4 to 10 glucose units) for glycan samples and commercially available peptides for peptide samples (Sigma). 1 µl of matrix solution were mixed on-target with the same volume of glycan sample. Dihydroxybenzoic acid (10 mg/ml of acetonitrile/ water, 7/3, v/v, freshly prepared) was used as matrix for glycan analysis, α-hydroxycinnamic acid was used for small peptides (10 mg/ml acetonitrile, 0.1% trifluoroacetic acid in water, 6:4, v/v, freshly prepared) and sinapinic acid was used for high molecular weight peptides (10 mg/ml acetonitrile, 0.1% trifluoroacetic acid in water, 1/1, v/v).

ESI Mass Spectrometry

ESI mass spectra were acquired on a Q-Tof2 (Micromass, Manchester, UK) instrument operating at capillary voltages around 1000 V. It uses a quadrupole mass analyzer and an orthogonal acceleration time of flight mass spectrometer with a hexapole collision cell situated between the two mass analyzers. In MS/MS mode, this collision cell was flooded with argon for fragmentation analysis and collision energy was varied in the range from 20 to 60 eV. Peptides were dissolved in acetonitrile/water, 1/1 (v/v) for positive mode analysis. According to Zaia and Costello (25) anionic glycans were dissolved in acetonitrile, 10 mM hydrochloric acid (1/1, v/v) and analyzed in negative mode. In addition, parent and daughter ion scans of DS-5 were performed on a Quattro II

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Fig. 2. Excretion of neutral glycans in the urine of the hexosaminidase deficient mice. The urine of mice deficient in Hex A and S (Hexa −/−) and deficient in Hex A and B (Hexb −/−) as well as the urine of mice totally deficient in hexosaminidase activity (double knockout) and of wild type littermates was collected for 8 h/day in a metabolism cage. The neutral oligosaccharides were prepared by solid phase extraction on Carbograph columns. The neutral glycans were eluted with 25% (v/v) acetonitrile in water. Aliquots of desalted glycan fractions corresponding to equal amounts of urine were evaporated to dryness and, together with a maltose oligosaccharide standard (4 to 10 glucose units), were profiled by FACE. This included fluorescence labeling with 8-aminonaphthalene-1,3,6-tris-sulfonic acid and subsequent electrophoresis on a 32% polyacrylamide gel.

Fig. 3. Enzymic degradation of the neutral glycan H$_3$N$_3$ by rHexS and Hex B. Equal amounts of the neutral glycan H$_3$N$_3$ isolated from urine of Hexb −/− mice corresponding to ~1 to 5 nmol of oligosaccharide were incubated for 17 h at pH 4.5 without enzyme (upper panel) and with rHexS (middle panel), respectively. In the bottom panel the glycan H$_3$N$_3$ was incubated for 15 h with Hex B at pH 5.2. Reaction products were analyzed by MALDI-MS. In the enzyme blank incubated under these conditions no glycan degradation was observed. H, hexose; N, N-acetylhexosamine.

Fig. 4. Preparation of the anionic storage oligosaccharides DS-5 and GAG OS from the hexosaminidase double knockout mice. The urine of mice totally deficient in hexosaminidase activity was applied to an anion exchange column which was eluted by 150 mM LiCl (A) or 2.0 M LiCl (B). The eluates A and B were separately desalted by size exclusion chromatography. The fractions of the desalting step were subjected to FACE analysis.
ES-MS/MS instrument (Micromass, Manchester, UK) similar to the Q-Tof2 except that the masses were detected by a quadrupole analyzer instead of a time-of-flight mass spectrometer.

RESULTS

Structural Analysis of Recombinant Hex S—Recombinant human Hex S was partially purified from the serum-free culture medium of transfected insect cells by affinity chromatography on a concanavalin A-Sepharose column. This preparation contained precursor [H9251]-subunit and the processed [H9251]-subunit in a 1:1 ratio. By Edman sequencing the N terminus of the precursor [H9251]-subunit was identified as L23WPWPQNFQT and that of the polypeptide r[H9251]m in the processed [H9251]-subunit as G85KRHTLKNV which starts five amino acid residues upstream of the N terminus of the native [H9251]m-chain (26, 27). After further purification by cation and anion exchange chromatography the preparation contained 90% processed rHexS according to SDS-PAGE. Rechromatography on a POROS 20 HQ resin yielded small amounts of apparently homogeneous processed rHexS and precursor rHexS. This purified rHexS was 40 times more active against the neutral substrate MUG (52.9 units/mg of protein) than the purified pro-rHexS (1.3 units/mg). Both enzymes, precursor rHexS and rHexS, hydrolyzed the sulfated substrate MUGS at the same rate as the neutral substrate MUG. Since the complete separation of the precursor...
and the processed rHexS by rechromatography caused considerable loss of material and activity we used the purified rHexS preparation still containing up to 10% precursor rHexS for the following studies.

The α-subunit of native Hex S carries three potential N-linked glycosylation sites, Asn-115, -157, and -295, each of which has been found to be modified by an oligosaccharide chain in a previous study (28). We analyzed the N-glycosylation of the rHexS using proteolytic cleavage and mass spectrometric analysis of the glycopeptides separated by reverse phase HPLC. N-Glycans were linked to all three N-glycosylation sites. Asn-115 carried an N-linked GlcNAc2Man4GalNAc5 oligosaccharide as found for the processed α-subunit in Hex A (29), or was not glycosylated; Asn-157 carried either GlcNAc or GlcNAc2Man4 (data not shown); and Asn-295 carried GlcNAc, GlcNAcFuc, or GlcNAcMan4 (data not shown).

To further characterize the structure of the rHexS the disulfide linkage pattern was examined using mass spectrometric analysis of proteolytic peptides as established before for the analysis of Hex B (15). We found that the postulated cystine linkage between the peptides α1 and α2 (27) is formed between Cys-58 and Cys-104 (data not shown). Another disulfide bridge of the α-subunit is formed by Cys-277 and Cys-328 (data not shown) as it was postulated after analysis of the native β-subunit (30). The third disulfide bond was detected between Cys-505 and Cys-522 (data not shown). This disulfide linkage pattern corresponds to that of the homologous β-subunit in Hex B (15). The α-subunit contains an additional cysteine residue in position 125. Both cystein residues, Cys-125 and Cys-458 of the unreduced and active enzyme protein could easily be alkylated by iodoacetamide, indicating that they are not involved in any disulfide bridge (data not shown). Taken together, the glycosylation and disulfide pattern of the recombinant α-subunit in rHexS are in good agreement with data obtained for the hexosaminidase subunits in other studies (15, 27-30).

The rHexS Cleaves the Synthetic Substrates MUG and MUGS at High Rates—The purified rHexS showed higher specific activities toward the artificial substrates MUG and MUGS (Fig. 1A) than described previously for partially purified Hex S preparations obtained from liver and an apparently homogeneous preparation from human fibroblasts (4, 5). As given in Table II the corresponding Vₘₐₓ values were of the same order of magnitude as for Hex A. Interestingly, rHexS catalyzed the hydrolysis of the anionic MUGS substrate even at a higher rate than the other isozymes (Table II). rHexS hydrolyzed the neutral substrate MUG optimally at pH 4.5 (data not shown) as did Hex S and Hex A prepared from human liver (3). The pH optimum of MUGS degradation by rHexS was at pH 3.8 (data not shown).

Neutral Oligosaccharides Are Excreted in the Urine of Hexosaminidase Knockout Mice—Uncharged glycans were found in the urine of both, the Hexb /−/− mice and the double knockout mice (Hexa /−/−; Hexb /−/−) (Fig. 2). The most abundant neutral storage oligosaccharide was isolated and analyzed by ESI-MS/MS (data not shown) and enzymatic degradation by hexosaminidases (Fig. 3). Based on the data obtained from this and additional monosaccharide and methylation analysis (31) we assign the following structure to the major neutral storage glycan: GlcNAcβ1(2–Manα1–3)(GlcNAcβ1–2Manα1–6)Manβ1–4GlcNAc (H₆N₉, Fig. 1D) which appears to be a product of partial N-glycan degradation (data not shown). Other neutral glycans were detected by MALDI-MS that contained 3 hexose (H) and 3–6 N-acetylgalactosaminic residues (N) and 4 hexose and 4–7 N-acetylgalactosaminic residues. In the urine of Hexb /−/− mice, ions that could be assigned to a saccharide exclusively composed of N-acetylgalactosamine and hexose residues were observed up to m/z = 2841.4 and corresponded to the sodium adduct of H₆N₉ (calculated average mass: 2841.6 Da). In the urine of double knockout mice, H₆N₁₅ compounds were detected up to a signal at m/z 3816.4 corresponding to H₆N₁₅ (calculated average mass: 3816.5 Da; data not shown). In the urine of human patients deficient in Hex A and B activity, H₆N₁₅, two structural isomers of H₆N₁₅, and smaller fragments have been identified by NMR spectroscopy (32) carrying only N-acetylgalactosamine residues at their non reducing ends. The structure of H₆N₁₅ obtained from human urine is identical to the N-glycan fragment H₆N₃ that we characterized in the knockout mice (Fig. 1D).

Neutral Storage Oligosaccharides Are Preferentially Degraded by Hex B—The N-glycan fragment H₆N₃ (Fig. 1D) was isolated from the urine of Hex B-deficient mice. Long-term incubation with rHexS yielded only minor amounts of degradation products as detected in MALDI-MS (Fig. 3). The bottom panel in Fig. 3 shows that this substrate was most efficiently degraded by Hex B.

The Anionic Urinary Oligosaccharides Are Fragments of Dermatan Sulfate Degradation—The anionic storage glycans were prepared from the urine of the double knockout mice by anion exchange chromatography (Fig. 4). Elution with a buffer containing 150 mM LiCl (A) yielded a single, low molecular weight oligosaccharide termed DS-S in fraction A4 (Fig. 4) whereas elution with 2.0 M LiCl (B) yielded a complex mixture of anionic oligosaccharides including DS-5 in fraction B4 (Fig. 4). This anionic oligosaccharide mixture was named GAG OS. Both, DS-5 and the GAG OS mixture, were analyzed structurally by ESI-MS/MS as well as by enzymatic degradation (Figs. 5-7). Fig. 5A shows a negative mode ESI-TOF-MS spectrum of the GAG OS mixture. Within the series of multiply charged molecular ions the negative charge increases with decreasing m/z values. The masses calculated from these m/z values each differ
by 459 Da, by the expected mass of a disaccharide composed of a sulfated N-acetylhexosamine and a uronic acid residue as it occurs in galactosaminoglycans but not in keratan sulfate. The molecular mass of the free acid DS-5 was determined to be 1139.21 Da, which is identical to the molecular mass of a bis-sulfated GAG pentasaccharide composed of three N-acetylhexosamine and two uronic acid residues (1139.23 Da). Using a triple quadrupole ESI-MS/MS in the negative precursor ion mode, the pure DS-5 sample was scanned for molecular ions that should contain sulfate groups. Only molecular ions were scanned that upon fragmentation in the collision chamber gave rise to the fragment m/z 97, indicating the presence of sulfate or phosphate groups. By this, we detected the singly charged alkali metal ion adducts of DS-5 that corresponded by the loss of one Li\(^+\) to the doubly charged ions found by ESI-TOF-MS previously (Fig. 5B). To confirm that DS-5 contains sulfate but not phosphate we performed an ESI-MS/MS product ion scan of the molecular ion \([M - 4H^+ + Li^+ + Na]^+\) (m/z 582.6, M = DS-5) (Fig. 5C). By increasing the collision energy 4.7-fold (inserted spectrum) a new fragment of m/z 80 (due to [SO\(_3\)]\(^-\)) but not m/z 79 (due to [PO\(_4\)]\(^-\)) appeared (33). Further fragments of this product ion scan confirmed the existence of a sulfated N-acetylhexosamine (HexNAc) residue [HexNAc-sulfate – H\(_2\)O]\(^-\) at m/z 282 and a monosulfated trisaccharide containing one uronic acid (HexUA) and two HexNAc residues in complex with either a Li\(^+\) or Na\(^+\) (m/z 685 or 701: [sulfated (HexNAc)\(_2\)HexUA – 2 H\(^+\) + (Li\(^+\) or Na\(^+\))]\(^-\)) and m/z 667 or 683 due to a further loss of H\(_2\)O (–18 Da), respectively.

To determine whether DS-5 and the GAG OS are derived from either chondroitin or dermatan sulfate the GAG OS were incubated with the bacterial endolysates chondroitinase AC (EC 4.2.2.5) and chondroitinase ABC (EC 4.2.2.4). Whereas chondroitinase AC specifically hydrolyzes chondroitin sulfate and chondroitin sulfate-like units in polysaccharide chains, chondroitinase ABC additionally hydrolyzes dermatan sulfate (34). Both enzymes produce galactosaminoglycan disaccharides unsaturated between C4 and C5 of the hexuronic acid residues when incubated with appropriate substrates (34). By FACE analysis chondroitinase AC activity caused a shift of the complete ladder of oligosaccharides with the formation of low molecular weight products. However, degradation was not complete as it was after incubation with chondroitinase ABC (data not shown). The products of chondroitinase ABC digestion were analyzed by negative mode ESI-MS/MS. The main signal at m/z = 458.07 was identified as a deprotonated monosulfated, unsaturated disaccharide (Fig. 6, calculated molecular mass 458.07 Da). Therefore, the origin of the GAG OS including the pentasaccharide DS-5 is dermatan sulfate.

The Repeating Disaccharide Unit in the Urinary GAG OS Is Sulfated in Position 4 of the GalNAc Residue—In ESI-MS/MS analysis the unsaturated disaccharide formed by chondroitinase ABC digestion of the GAG OS showed the same fragmentation pattern (Fig. 6D) as the unsulfated standard disaccharide ΔDi-4-sulfate which is sulfated in position 4 of the GalNAc residue (Fig. 6B). We conclude that the dermatan sulfate fragments GAG OS and, thus DS-5, are sulfated in position 4 of the GalNAc residues.

rHexS and Hex A Degrade the Dermatan Sulfate Fragment Mixture GAG OS and the Dermatan Sulfate Pentasaccharide DS-5—The anionic GAG OS fragments isolated from the urine...
of the hexosaminidase double knockout mice were degraded in vitro by rHexS and Hex A (Fig. 7A, lanes 2 and 3), but not by Hex B (Fig. 7A, lane 1). Incubation of the GAG OS or DS-5 with rHexS and Hex A, respectively, gave rise to two products (Fig. 7A, lanes 2 and 3; B, lanes 3 and 4), one of them co-migrating with GalNAc. The second degradation product, derived from the pentasaccharide DS-5 by loss of one monosaccharide unit is termed DS-4. As indicated by the FACE gel in Fig. 7C, N-acetylgalactosamine (lane 3) but not -glucosamine (lane 2) or -galactosamine 6-sulfate (lane 4) was the monosaccharide released from DS-5 (lane 1). This indicates that an exo-sulfatase acted on the nonreducing sugar residue in vivo before DS-5 was excreted with the urine and confirms that the pentasaccharide DS-5 is derived from a galactosaminoglycan. Together with the structural information obtained from mass spectrometry and chondroitinase digestion this suggests the structure of the dermatan sulfate pentasaccharide DS-5 suggested in Fig. 1C.

rHexS and Hex A Degrade the Anionic Trisaccharide C6S-3—To study the degradation of chondroitin 6-sulfate by hexosaminidases A, B, and S. 2 nmol of C6S-3 dissolved in water were incubated for 5 h at pH 4.0 with Hex B (lane 2), rHexS (lane 3), and Hex A (lane 4), respectively, as described under “Experimental Procedures.” Blanks devoid of substrate (lanes 6 and 7) and devoid of enzyme (lane 8) were run under identical conditions. After incubation, the assay samples were subjected to FACE analysis. GalNAc-6-sulfate (lane 5) and the commercially available chondroitin 6-sulfate disaccharide bearing a glucuronic acid residue at the nonreducing terminus (lanes 1 and 9) served as standards for the digestion products.

Fig. 9. Hydrolysis of the sulfated glycosphingolipids SM2 and SB2 by hexosaminidases in the presence/absence of the GM2AP. A, SM2 micelles were incubated for 15 min with the three hexosaminidase isoenzymes Hex A, rHexS, and Hex B, respectively, in the presence and absence of GM2AP. The assays were performed as described under “Experimental Procedures.” B, the same degradation experiment was performed using SB2 instead of SM2 and incubating for 17 h instead of 15 min.

Fig. 10. Hydrolysis of liposomal SM2 by rHexS. LUVs containing 10 mol % SM2, 20 mol % BMP, 20 mol % Chol, and 50 mol % PC were incubated with rHexS in the presence/absence of GM2AP. The assays were performed as described under “Experimental Procedures.” After mild alkaline methanolysis the samples were desalted by reverse phase chromatography and applied to a HPTLC plate. The resolved bands were stained with cuprous sulfate reagent in phosphoric acid. The relative intensities of the product band, corresponding to SM3 and of the substrate band corresponding to SM2 were determined densitometrically. Blanks containing denatured enzyme were run for each assay under identical conditions and subtracted from the experimental values. A, time dependence. LUVs containing SM2 and BMP were incubated with rHexS in the presence of GM2AP in 10 mM citrate buffer (●) and in 50 mM citrate buffer (○). LUVs composed of 10 mol % SM2, 20 mol % Chol, and 70 mol % PC but devoid of BMP were incubated with rHexS in 50 mM citrate buffer in the presence of GM2AP (△). LUVs containing both SM2 and BMP were incubated with rHexS in 50 mM citrate buffer in the absence of GM2AP (□). B, pH dependence. LUVs containing both SM2 and BMP were incubated for 30 min with rHexS in the presence (●) or absence (□) of GM2AP. The pH ranged from 3.6 to 5.7.
osaminidases we prepared the bis-sulfated trisaccharide C6S-3 (Fig. 1C) from commercial chondroitin 6-sulfate using testicular hyaluronidase and β-glucuronidase. After incubation of C6S-3 with the hexosaminidase isozymes the reaction products were separated and visualized by FACE profiling as shown in Fig. 8. In the presence of either rHexS or Hex A two new product bands appeared (lanes 3 and 4) which comigrated with a GalNAc-6-sulfate standard (lane 5) and a commercially available saturated chondroitin 6-sulfate disaccharide standard (C6S-2, lanes 1 and 9), respectively. Hex B exhibited no detectable hydrolyzing activity on C6S-3 (Fig. 8, lane 2).

*rHexS and Hex A Degrade the Sulfated Glycolipid SM2 but Not SB2*—We examined the enzymatic degradation of the monosulfated glycolipid SM2 and the bis-sulfated glycolipid SB2 by hexosaminidases. SM2 is an analogue of ganglioside GM2, and the glycolipid SB2 carries an additional sulfatated ester bound to position 3 of the terminal GalNAc residue. The purified sulfated GSLs were incubated with the different hexosaminidase isozymes in a micellar assay (Fig. 9). rHexS was more active than Hex A and Hex B and catalyzed the degradation of SM2 to SM3 at significant rates even in the absence of GM2AP. Addition of GM2AP stimulated the SM2 hydrolysis by both, rHexS and Hex A. Hex B showed no significant activity in hydrolyzing SM2 in the presence or absence of GM2AP (Fig. 9A). None of the three hexosaminidases showed significant activity in degrading SB2 (Fig. 9B).

To reproduce the lysosomal conditions as closely as possible, additional degradation experiments were performed using a liposomal assay system. In this system, the lipid substrate was presented as a component of a unilamellar lipid bilayer to the water-soluble enzyme. Standard liposomes contained phosphatidylcholine and cholesterol as carrier lipids, and BMP, which is a characteristic component of lysosomal and intradosomal membranes (35, 36). Under these conditions rHexS degraded membrane-bound SM2. Significant degradation rates were achieved only in the presence of both, BMP and the GM2AP (Fig. 10A). The optimum for the reaction was pH 4.3 (Fig. 10B).

**DISCUSSION**

Hexosaminidase S (Hex S, αα) is a labile lysosomal enzyme that occurs in much smaller quantities in nonhuman tissues than the major isoenzymes Hex A (αβ) and Hex B (ββ). Since rather low specific activities have been reported for purified Hex S (3–5) its physiological significance has been questioned (2). The striking accumulation of anionic oligosaccharides in the urine of mice deficient in all hexosaminidases compared with those still expressing Hex S (Hexb/−/−) (8) prompted us to reinvestigate the substrate specificity of Hex S.

For these studies we expressed human Hex S cDNA in cultured insect cells and purified the recombinant Hex S from the secretions. All three N-glycosylation sites were utilized in the recombinant α-subunit consistent with previous studies on the enzyme (28). Its disulfide linkage pattern, as analyzed by mass spectrometry after proteolytic cleavage, corresponded to that of the homologous hexosaminidase β-subunit (15). rHexS hydrolyzed the synthetic anionic substrate MUGS at even higher rates than the other isozymes and was as active as Hex A on the neutral substrate MUG (Table II). Interestingly, the precursor form of rHexS showed only 2–3% of the specific molecular activity of processed, mature rHexS.

To identify physiological substrates of Hex S anionic and neutral glycanas were isolated from the hexosaminidase double knockout mice (Hexa −/−, Hexb −/−) and characterized. Analysis by ESI-MS/MS combined with glycosidase digestion indicated that the isolated GAGs were derived from dermatan sulfate. In addition, elevated levels of neutral N-glycan fragments were found in the urine of the double knockout mice. This was expected from their occurrence in the urine of Hexb−/− mice. An anionic (DS-5) and a neutral oligosaccharide (H2N3) were isolated from the urine of the double knockout mice, and used as physiological substrates for rHexS. As an additional putative substrate the sulfated sphingolipid SM2 was isolated from rat kidney.

rHexS was as active as Hex A on the anionic bis-sulfated glycanas, the trisaccharide C6S-3, and the pentasaccharide DS-5, and the sulfated glycolipid SM2. Each of these substrates was refractory to the action of Hex B (Table III). From these anionic substrates rHexS released N-acetylgalactosamine from the nonreducing end and, in the case of C6S-3, N-acetylgalactosamine 6-sulfate, thereby bypassing the exo-sulfatase step. The release of GalNAc-6-sulfate from keratan sulfate oligosaccharides by Hex A has been reported (37) and it was postulated that hexosaminidases are able to release sulfated hexosamines in vivo from the observation that 6- and 4-sulfated hexosamines are excreted with the urine of patients deficient in GAG specific sulfatase activity (38). However, if the terminal N-acetylgalactosamine residue is sulfated in the 3-position, as in the glycolipid SB2, it is resistant against the action of all three hexosaminidases A, B, and S. As with the degradation of other anionic and neutral sphingolipids (39–43), the hydrolysis of the membrane-bound sulfated glycosphingolipid SM2 by rHexS is strongly dependent on both, an activator protein, in this case GM2AP, and the presence of an additional anionic phospholipid such as BMP in the substrate carrying membrane.

As observed by quantitative ESI-MS/MS analysis elevated levels of SM2 were found in the kidney tissues of both Hexa−/− mice and double knockout mice, both lacking Hex S and Hex A activities.2 This suggests an important role of Hex S in the catabolism of SM2, a potentially important glycosphingolipid. High levels of SM2 have been observed in the human renal cancer cell line SMKT-R3 (44) as well as in a uterine endometrial adenocarcinoma (45). Moreover, SM2 and the bis-
Physiological Substrates for Human Lysosomal β-Hex S

sulfated SB2 have been identified as the most potent glycosphingolipid ligands for NKR-P1, a membrane protein on natural killer cells that contains an extracellular C type lectin domain, and their contribution to the activation of natural killer cells has been proposed (46).

Comparing the hexosaminidase concentrations and incubation times used in vitro, anionic glycolipids were degraded much faster than anionic oligosaccharides. Rough calculations suggest that the sulfated GSL SM2 is degraded 500 times faster than the sulfated pentasaccharide DS-5 (Fig. 7B and 9A). Slow degradation rates have also been described for digestion of oligosaccharides by other glycosidasases, e.g. hyaluronidase and β-glucuronidase (34). Presumably, the suitable substrate conformation and the accessibility of the sensitive linkages for the enzyme is less often achieved in a freely movable, water-soluble oligosaccharide than in a glycolipid which may be conformatively fixed in an activator-lipid complex.

The results obtained with Hex A and rHexS expand the array of physiological glycoconjugate substrates acted upon by the lysosomal hexosaminidases. They also add support to the concept that Hex S catalyzes an important set of degradative reactions in vivo. Finally, the unique substrate specificity of Hex S makes the enzyme an interesting and valuable research tool in glycobiology.

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