A Liquid Chromatography-Mass Spectrometry-based Approach to Characterize the Substrate Specificity of Mammalian Heparanase*

Received for publication, June 27, 2014, and in revised form, October 18, 2014. Published, JBC Papers in Press, October 21, 2014, DOI 10.1074/jbc.M114.589630

Yang Mao,†1 Yu Huang,†1, Jo Ann Buczek-Thomas, Cheryl M. Ethen, Matthew A. Nugent, Zhengliang L. Wu, and Joseph Zaia‡1,2

From the †Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118 and ‡R&D Systems, Minneapolis, Minnesota 55413

Background: Heparanase remodels ECM and is associated with cancer metastasis and angiogenesis.

Results: An LC-MS-based approach was developed to profile the structures of the heparanase cleavage sites in heterogeneous HS chains.

Conclusion: Heparanase cleaves at the non-reducing side of highly sulfated HS domains.

Significance: The results suggest a mechanism for heparanase to activate nascent growth factor binding domains within HS.

Extracellular heparanase activity releases growth factors and angiogenic factors from heparan sulfate (HS) storage sites and alters the integrity of the extracellular matrix. These activities lead to a loss of normal cell matrix adherent junctions and correlate with invasive cellular phenotypes. Elevated expression of heparanase is associated with several human cancers and with vascular remodeling. Heparanase cleaves only a limited fraction of glucuronic linkages in HS. There have been few investigations of the functional consequences of heparanase activity, largely due to the heterogeneity and complexity of HS. Here, we report a liquid chromatography-mass spectrometry (LC-MS)-based approach to profile the terminal structures created by heparanase digestion and reconstruct the heparanase cleavage sites from the products. Using this method, we demonstrate that heparanase cleaves at the non-reducing side of highly sulfated HS domains, exposing cryptic growth factor binding sites. This cleavage pattern is observed in HS from several tissue sources, regardless of overall sulfation degree, indicating a common recognition pattern. We further demonstrate that heparanase cleavage of HS chains leads to increased ability to support FGF2-dependent cell proliferation. These results suggest a new mechanism to explain how heparanase might potentiate the uncontrolled cell proliferation associated with cancer through its ability to activate nascent growth factor-promoting domains within HS.

Heparan sulfate (HS)4 glycosaminoglycans are unbranched polysaccharides found in intracellular granules, on cell surfaces, and in extracellular matrices (ECM) covalently linked to proteoglycan core proteins. HS chains are composed of disaccharide repeating units of uronic acid and glucosamine, which may be modified to contain sulfate groups, including 2-O-sulfation on the uronic acid and 3-O-sulfation, 6-O-sulfation, and N-sulfation on the glucosamine. These sulfate groups are clustered within HS chains into heparin-like regions called NS domains, alternating with NA domains that have N-acetylated glucosamine residues. In addition, the uronic acid in the repeating units can be either glucuronic acid or iduronic acid. These highly variable modifications, introduced during the biosynthesis of HS, define the extremely complex and heterogeneous nature of HS and pose significant analytical challenges (1, 2). Meanwhile, the sulfate groups confer to HS chains a strong negatively charged character under physiological conditions, and provide docking sites for numerous protein ligands involved in diverse biological processes (3). It has long been demonstrated that HS sequesters growth factors, chemokines, and morphogens in ECM, creating a low affinity storage depot that can modulate extracellular growth factor movement and distribution (4, 5). In addition, interactions with HS fragments modulate the activities of various growth factors and enzymes (4).

Mammalian heparanase (HPSE) is an endo-β-D-glucuronidase that catalyzes the partial depolymerization of HS chains and plays a central role in the intracellular degradation of HS in lysosomes (6, 7). Importantly, heparanase is also released into ECM, where it contributes to the remodeling of the HS-containing ECM and basement membranes (8–10). As the ECM provides physical barriers between cells and tissues, extracellular heparanase facilitates cell invasion and is involved in several normal and pathological processes, including, most notably,
Substrate Specificity of Heparanase by LC-MS

cancer metastasis (11–13) and angiogenesis (12, 14). High heparanase expression and activity correlate with aggressive tumor phenotypes. Furthermore, degrading HS chains by heparanase releases HS-bound angiogenic growth factors from ECM, such as FGF2 and VEGF, promoting an indirect angiogenic response (15–18).

Despite the importance of heparanase, however, there have been relatively few studies on its substrate specificity. This is probably because many functions of heparanase can be explained by its ability to degrade HS chains, irrespective of the sulfation pattern of the HS substrate. Further, the heterogeneity and complexity of HS also makes characterizing the substrate specificity of heparanase challenging because it is extremely difficult to obtain structurally diversified yet pure HS substrates. As a result, previous research has taken two primary approaches: 1) testing the enzyme activity of heparanase on structurally defined HS oligosaccharides (19, 20) and 2) using chemo-enzymatically synthesized HS polysaccharides that are homogenously modified (19, 21, 22). These studies identified the favored substrate of heparanase among the defined HS structures tested. However, they were limited, first, because neither the oligosaccharides nor the chemo-enzymatically synthesized HS polysaccharides have enough structural diversity for the conclusion to be applicable to heterogeneous HS substrates. Second, both methods force heparanase to act on unnatural substrates that differ from those found in physiological conditions, which skew the substrate specificity and sometimes lead to conflicting results. For example, using different HS oligosaccharides as substrates, Pikas et al. (19) and Okada et al. (20) reached opposite conclusions regarding the requirement of 2-O-sulfation. Finally, these approaches cannot be used to reveal the extent to which heparanase recognizes common cleavage sites from heterogeneous HS substrates isolated from different sources.

In this report, we describe the results of a liquid chromatography-mass spectrometry (LC-MS)-based approach to profile the terminal structures created by heparanase digestion that allowed us to reconstruct the heparanase cleavage sites from the products. The results reveal the common pattern recognized by heparanase as well as substrate-based heterogeneity of the cleavage sites. The newly cleaved HS chains display highly sulfated non-reducing end domains that potentiate FGF2-mediated cellular mitogenesis. Thus, it appears that exposure of cryptic growth factor sites by heparanase may provide a basis for understanding the biological consequences of heparanase with respect to cancer growth in dysregulated ECM.

EXPERIMENTAL PROCEDURES

Material—Heparan sulfate from porcine intestinal mucosa (HSPIM) was purchased from Celsus Laboratories, Inc. (Cincinnati, OH). Heparan sulfate from bovine kidney (HSBK) was purchased from Sigma-Aldrich. Recombinant Human heparanase and syndecan-4 were from R&D Systems (Minneapolis, MN). Recombinant human FGF2 was from Invitrogen.

Preparation of Odd-numbered HS Oligosaccharides—5 µg of HS samples were mixed with 1.25 µg of heparanase in 20 mM Tris/HCl buffer, pH 7.0, or 20 mM NaOAc buffer, pH 5.0, in a total volume of 50 µl. As control experiments, the same amount of HS sample was incubated with the heat-inactivated heparanase under the same conditions. The reactions were incubated for 16 h at 37 °C and then heated at ~100 °C for 5 min to inactivate the enzyme. After centrifugation, the supernatants were mixed with 5 milliunits of Flavobacterium heparinum heparin lyase III in 100 µl of 40 mM Tris/HCl buffer, pH 7.0, containing 4 mM CaCl2 and 0.01% BSA. The reactions were incubated for another 16 h at 37 °C and then terminated as described above. The digests were dried by centrifugal evaporation, reconstituted in 20 µl of water, and purified by size exclusion chromatography (SEC)-HPLC using a 3.2 × 300-mm Superdex Peptide column (GE Healthcare) equilibrated with 100 mM NH4HCO3. The region of the chromatogram corresponding to oligosaccharides (as detected by UV absorbance at 232 nm) was pooled and dried by centrifugal evaporation.

Disaccharide Analysis of HS—The disaccharide compositions of HS samples were analyzed using bacterial polysaccharide lyases and SEC-mass spectrometry as described previously (23). Disaccharide representation codes adopted were defined previously (24).

Amide-HILIC LC-MS Composition Analysis of HS Oligosaccharides—The compositions of HS oligosaccharide samples were profiled using either the chip-based LC-MS method (25, 26) or the capillary column-based LC-MS method, both developed previously in our laboratory (27). The LC-MS raw files from Agilent quadrupole TOF were directly deconvoluted by DeconTools AutoProcessor (Pacific Northwest National Laboratory). The LC-MS raw files from Thermo Fisher LTQ-Orbitrap were converted to mzXML format using MSConvert from ProteoWizard (28) before deconvolution by DeconTools. The composition profiles were generated using GlycReSoft, developed in our laboratory (29), with the output data from DeconTools. HS oligosaccharide compositions are given as [ΔHexA,HexA,GlcNAc,SO3], with a number for each denoting the number of the corresponding residues.

Tandem MS Analysis of HS Oligosaccharides—LC-tandem (CID) was performed on the pulsed chip-based LC-MS system by pulsing sulfolane to the corresponding retention time to enhance the charge state of precursor ions (30). Tandem mass spectra were acquired in targeted MS/MS mode with a list of targeted compositions. The isolation window for the precursor was set to medium (~4 thomsons). The collision energy from 0 to 20 V was ramped throughout the chromatographic peak of the selected composition.

Hydrophobic Trapping Binding Assay—Odd-numbered HS oligosaccharides were prepared from 50 µg of recombinant syndecan-4 (R&D Systems) using the method described above. The purified oligosaccharides were mixed with 20 µg of FGF and incubated for 1 h at room temperature. The mixture was then applied to a C18 reverse phase cartridge (MicroSpin columns, Silica C18 Vydac, 5–200 µl; Harvard Apparatus, Holliston, MA). After washing the hydrophobically trapped complex for three cycles with 70 µl of 200 mM ammonium acetate buffer, the FGF-bound oligosaccharides were eluted with three cycles of 70 µl of 1 M ammonium acetate. Fractions from each step were combined and dried by centrifugal evaporation.

BaF 32 Cell Culture and Proliferation Assays—BaF 32 cells were obtained and maintained as described previously (27). For
the cell proliferation assays, the cells were seeded into 96-well tissue culture plates at a density of 31,250 cells/cm² in the absence and presence of 10 ng/ml FGF2. Native and heparanase-treated HS released from syndecan-4 were added directly to the cells at the indicated final concentrations, and the cells were maintained for 4 days. Changes in cell number were determined using an MTT cell proliferation assay (ATCC, Manassas, VA) according to the manufacturer’s protocol. Changes in relative absorbance were determined at 570 and 670 nm (background) using an Optimax 96-well microplate reader. The data are expressed as the mean, background-corrected absorbance values ± S.D. (n = 3) for each condition.

RESULTS

Generating Odd-numbered HS Oligosaccharides Specific to Heparanase Activity—Because of the complexity of HS, one of the standard ways to analyze HS substrates relies on using bacterial heparin lyases to depolymerize the complex HS polysaccharide chains into disaccharide repeating units (ΔHexA-GlcN) (31). In contrast, heparanase is an endo-β-D-glucuronidase that cleaves the glycosidic bonds to the reducing end side of specific glucuronic acid residues. This type of cleavage disrupts the disaccharide repeating units in HS substrates and reduces the abundances of HS disaccharides resulting from analysis of the heparanase-pretreated, polysaccharide lyase-digested HS samples (Fig. 1). Based on the same reasoning, we propose that a partial depolymerization of the heparanase-pretreated HS substrate by heparin lyases would produce a series of odd-numbered HS oligosaccharides (trisaccharide, pentasaccharide, etc.) (Scheme 1). Because each of these oligosaccharides carries half of the heparanase cleavage sites resulting from the heparanase pretreatment, the heparanase cleavage sites can be reconstructed by analysis of the structural features of these odd-numbered HS oligosaccharides. Specifically, our approach takes advantage of our established method using hydrophilic interaction liquid chromatography (HILIC) LC-MS (25, 26, 30) to profile the compositions of the odd-numbered HS oligosaccharides based on their chromatography separation and mass differences.

We first tested our hypothesis using a heterogeneous HS substrate isolated from bovine kidney (HSBK). As expected, compared with the sample without heparanase pretreatment, the oligosaccharide profile of the sample after the serial digestion shows a dramatic increase in the amount of the odd-numbered HS oligosaccharides. Noticeably, there is a distinctive pattern even in the profiles of the trisaccharides (Fig. 2). The profiles unambiguously demonstrate that two groups of trisaccharides are favorably produced by the serial digestion. The first trisaccharide group includes [1,1,1,0,1], [1,1,1,0,2], and [1,1,1,1,1] (HS oligosaccharide compositions are given as [ΔHexA,HexA, GlcN,Ac,SO₃]ο, denoting the number of the corresponding residues and modifications), which contain a Δ-unsaturated hexuronic acid residue generated by the heparin lyase and a saturated uronic acid reducing end presumably released by heparanase (type c products in Scheme 1). The other trisaccharide group includes mainly [0,1,2,0,3] and [0,1,2,0,4] and, to a lesser degree, [0,1,2,1,2] and [0,1,2,1,3], which all contain a glucosamine residue at the non-reducing end. Because it is very unusual for a HS chain to terminate with a glucosamine residue at the non-reducing end (32), those glucosamine residues were also released by the heparanase (type d products in Scheme 1). Together, the data suggest that heparanase recognizes a hex-

![Graphic scheme for generating odd-numbered HS oligosaccharides by the heparin lyases digestion of the heparanase (HPSE)-pretreated HS.](image)

Substrate Specificity of Heparanase by LC-MS

![Substrate Specificity of Heparanase by LC-MS](image)
asaccharide sequence that has a composition of \([1,1,1,0,1]\), \([1,1,1,0,2]\), and \([1,1,1,1,1]\) on the non-reducing end side of the heparanase cleavage site and \([0,1,2,1,2]\), \([0,1,2,1,3]\), \([0,1,2,0,3]\), and \([0,1,2,0,4]\) on the reducing end side of the cleavage site.

**Profiling the Heparanase Cleavage Sites in Different HS Substrates**—To reveal the substrate bias in our analysis, we applied our approach to two additional HS substrates: HSPIM and HS from syndecan-4 (HSSynd4). Although these HS substrates have very different degrees of sulfation (Fig. 3), the trisaccharide profiles produced using the above approach fall into a very consistent pattern. On the non-reducing end side of the cleavage site, we consistently detected \([1,1,1,0,1]\), \([1,1,1,0,2]\), and \([1,1,1,1,1]\) as the major trisaccharide compositions favored by heparanase. Similarly, on the reducing end side of the cleavage site, \([0,1,2,0,3]\) and \([0,1,2,0,4]\) were major HS trisaccharide compositions in all of the different substrates analyzed (Fig. 4). There were small variations among the different HS substrates in terms of the percentage of a particular trisaccharide composition, which reflects the substrate bias. Considering the low degree of overall sulfation in HSSynd4, it is surprising that the highly sulfated trisaccharides \([0,1,2,0,3]\) and \([0,1,2,0,4]\), which contain at least one sulfate per monosaccharide, are overrepresented in the profiling. This may indicate that the limited numbers of sulfates are clustered in NS domains in HSSynd4. These results suggest a consistent pattern recognized by heparanase among substrates from different HS sources.

**Structure Determination of the Heparanase Cleavage Sites**—In order to probe the detailed structural features of the cleavage sites, we subjected the individual HS trisaccharide compositions to tandem MS analysis by collision-induced association (CID-MS2).

The CID-MS2 spectrum of \([1,1,1,1,1]\) revealed a sulfated \(0,4X\text{GlcNAc}\) fragment, indicating that the GlcNAc residue in the sequence was sulfated at the 6-\(O\) position. This conclusion was further confirmed by cross-ring fragments \(0,2A_2\) and \(Y_2/0,2A_2\). This information, together with the preknowledge that heparanase cleaves after the GlcA residue, determined the structure of \([1,1,1,1,1]\) to be \(\Delta\text{HexA-GlcNAc6S-GlcA}\) (Figs. 5 and 13).

The CID-MS2 spectrum of \([0,1,2,0,3]\) (Figs. 5 and 14) revealed a sulfated cross-ring fragment, \(0,2X_0\), and its complementary fragment, \(0,2A_3\), with two sulfate groups, confirming that the trisaccharide was \(N\)-sulfated at its reducing end. The \(2,4A_3\) cross-ring fragment detected with two sulfate groups indicated that the trisaccharide did not have a 6-\(O\) sulfate group at the reducing end GlcNS. At the same time, the sulfated \(Y_2/0,2A_3\) cross-ring fragment indicated that there had to be a sulfate at either the 2-\(O\) position of the uronic acid or the 6-\(O\) position of the reducing end GlcNS. Therefore, the 2-\(O\) position of the uronic acid in the trisaccharide must be sulfated. Taken together, the structure of \([0,1,2,0,3]\) is determined to be \(\text{GlcNS-HexA2S-GlcNS}\) (Fig. 5).

A similar result was obtained with the HS trisaccharide \([0,1,2,0,4]\) (Figs. 5 and 15). We detected a sulfated \(Y_2/B_2\) frag-
ment, which indicated that the trisaccharide was also sulfated at the 2-O position. In addition, we detected a Y2/0,2A3 cross-ring fragment with two sulfate groups, which not only confirmed the 2-O sulfation but indicated that remaining 6-O sulfate group must be at the reducing end. Therefore, the structure of [0,1,2,0,4] is determined to be GlcNS-HexA2S-GlcNS6S (Fig. 5).

Besides the structural information obtained by tandem MS, the biosynthesis of HS determines that the structure of [1,1,1,0,1] was most likely ΔHexA-GlcNS-GlcA. Combining the structures of the HS trisaccharides from both sides of the heparanase cleavage sites, we were therefore able to reconstruct the most abundant cleavage sites as HexA-GlcNAc6S-GlcA—GlcNS-HexA2S-GlcNS(6S) (Fig. 6).

Profiling the HS Domain Structures in the Vicinity of the Heparanase Cleavage Sites—The trisaccharide analysis above indicates that heparanase cleavage sites appear to have a low to medium degree of sulfation on the non-reducing end side and a high degree of sulfation on the reducing end side. In order to prove that this sulfation discrepancy represents the overall sulfation pattern in the vicinity of the heparanase cleavage sites, we examined the composition profiles of the pentasaccharides and heptasaccharides generated from our methods. As demonstrated in Fig. 7, the composition profiles of those odd-numbered oligosaccharides also fell into a clear pattern, although the absolute signal intensity decreased as the analysis moved to longer HS fragments. On the non-reducing end side of the heparanase cleavage sites, we consistently observed compositions with a low to medium degree of sulfation, such as the pentasaccharides [1,2,2,1,2] and [1,2,2,1,3], and the heptasaccharides [1,3,3,1,3], [1,3,3,2,2], and [1,3,3,2,3] (type c products in Scheme 1). However, on the reducing end side, the highly sulfated pentasaccharide [0,2,3,1,4], [0,2,3,1,5], and [0,2,3,1,6], and heptasaccharide [0,3,4,1,6], and [0,3,4,1,7] stood out in the composition profiles (type d products in Scheme 1). We further quantified the average number of sulfate groups per monosaccharide for all of the major odd-numbered HS oligosaccharides that we observed. The results showed that the averaged sulfation degree on the reducing end side of the heparanase cleavage sites is almost twice as much as the averaged sulfation degree on the non-reducing end side (Fig. 8). Therefore, our data indicate that heparanase cleaves through the boundary of NS and NS/NA domains within an HS chain.

pH Effects—The results described above were generated at acidic pH to mimic the conditions in which heparanase functions in the lysosomes. In order to test the activity and substrate specificity of heparanase on the cell surface, we pretreated HSBK substrate with heparanase at pH 7.0 and repeated the experiment. The analysis shows that heparanase had lower activity at physiological pH, judging by the amount of odd-numbered HS oligosaccharides produced by the serial digestion, which is consistent with previous studies (33). However, heparanase also has more stringent substrate specificity at this condition, demonstrated by the absence of less favored trisaccharide compositions on the reducing end side of the cleavage site in the analysis (Fig. 9). The apparently tightened substrate specificity at pH 7.0 may indicate that the cleavage pattern that we observed is more relevant to the physiological role of the extracellular heparanase than that of the lysosomal heparanase. Therefore, we set up the enzyme reactions at pH 7.0 for the proliferation activity assay described below.

Heparanase-released Non-reducing Ends Bind and Activate FGF—The cleavage of heparanase through the boundary of NS and NS/NA domains would release the formally internal NS domains to the non-reducing ends of the product HS chains. It has been reported previously that HS chains form 2:2:2 complexes with FGFs and FGFRs through their highly sulfated non-reducing end (27, 34). We therefore hypothesized that the
heparanase-released, formally internal NS domain would also bind to FGF and increase the FGF signaling.

To test this hypothesis, we produced the odd-numbered HS oligosaccharides from syndecan-4, a known FGF2 co-receptor, bound them to FGF2 protein hydrophobically trapped on a C18 column, and subjected the FGF2-sugar complexes to salt wash with different stringencies. As demonstrated in Fig. 10, even after a high stringency salt wash (200 mM), there was still a substantial amount of the highly sulfated pentasaccharides and heptasaccharides, originating from the reducing end side of the heparanase cleavage sites, tightly bound to FGF2. This result is in close agreement with our previous findings of the high affinity binding between FGF2 and the naturally occurring HS non-reducing ends (27), suggesting the heparanase-released non-reducing ends binds to FGF2 in a similar way. We next tested whether the binding between the heparanase-released non-reducing ends to FGF2 would lead to increased FGF2 signaling using a cell proliferation assay. As shown in Figs. 11 and Fig. 12, heparanase treatment greatly enhanced the activity of HSSynd4 on FGF2 signaling. Whereas HSSynd4 was inactive on its own at the concentration tested, after digestion by heparanase, it showed significant activity at stimulating FGF2-mediated mitogenesis. This may be explained by indirect effects of the altered HS chain length. However, taken together with the binding experiment, this result suggests that heparanase cleavage creates new, highly sulfated non-reducing end saccharides that bind FGF2 and facilitate the formation of active FGF

FIGURE 5. CID tandem MS spectra and structure identification of the most abundant trisaccharide generated by the heparin lyase digestion of the heparanase-pretreated HS. a, [1,1,1,1,1]; b, [0,1,2,0,3]; c, [0,1,2,0,4]. The labeled raw mass spectra are shown in Figs. 13–15, respectively. Precursors (represented by blue diamonds in the spectra) with the most charged species are isolated for effective tandem spectra. Major peaks of fragmentation were assigned and labeled, and the corresponding structures were deduced from these fragmentations as shown on the right in each panel. HS oligosaccharide compositions are given as [HexA:HexA:GlcNS:Ac:SO3]. Fragment ions from tandem MS are labeled using the conventional carbohydrate fragmentation nomenclature (43) with HS-specific modifications. Specifically, HS structures without modification of sulfation (S) and/or acetylation (Ac) are named as the backbone, followed by the modifications in parenthesis, such as 0.2X1 (1Ac, 2S) and Y1 (3S). Back slashes are used for alternative assignments.

FIGURE 6. Reconstruction of the most favorable heparanase (HPSE) cleavage. The preferred heparanase cleavage sites were reconstructed from the most abundant trisaccharides observed in our analysis as HexA-GlcNAc6S-GlcNS-HexA2S-GlcNS(6S). Geometric symbols of the glycan structures are the same as defined in Scheme 1.

Substrate Specificity of Heparanase by LC-MS

34146 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 289 • NUMBER 49 • DECEMBER 5, 2014

34146
receptor complexes. We have shown previously that the binding of FGF2 by HS saccharides increases with the degree of sulfation (27). Thus, it appears that heparanase exposes new highly sulfated non-reducing chain saccharides that bind FGF2. The raw tandem mass spectra corresponding to those shown in Fig. 5, including assigned m/z and charge values, are shown in Figs. 13–15.

DISCUSSION

By focusing on the odd-numbered HS oligosaccharides produced by the serial digestion of heparanase and heparin lyases, our LC-MS-based approach details the heparanase cleavage sites within a variety of HS substrates. Compared with previously published methods used in characterizing heparanase activity, which relied on either short HS oligosaccharides or chemo-enzymatically synthesized unnatural HS substrates, our approach was able to analyze the heparanase cleavage sites within the heterogeneous HS polysaccharides, mimicking the physiological substrates and conditions. Therefore, our approach offers a systematic way to evaluate the abundances and characteristics of the heparanase cleavage sites within HS substrates from different sources and/or disease states.

On the trisaccharide level, the results revealed a consistent pattern recognized by heparanase, with the trisaccharide composition \([1,1,1,0,1,0,0,1,2,1,0,2,1,1,1,1,1,0,2,1,3,0,1,2,0,3,0,1,2,0,4,0,1,2,0,4] \) on the non-reducing end side of the cleavage site and \([0,1,2,1,2,0,1,2,1,3,0,1,2,0,3,0,1,2,0,4,0,1,2,0,4] \) on the reducing end side. Our approach reveals more details on the heparanase cleavage sites, particularly on the reducing end side, than previous studies (19–22). This is because previous methods have had limited access to the reducing end side of the heparanase cleavage sites.

On the trisaccharide level, the results revealed a consistent pattern recognized by heparanase, with the trisaccharide composition \([1,1,1,0,1,0,0,1,2,1,0,2,1,1,1,1,1,0,2,1,3,0,1,2,0,3,0,1,2,0,4,0,1,2,0,4] \) on the non-reducing end side of the cleavage site and \([0,1,2,1,2,0,1,2,1,3,0,1,2,0,3,0,1,2,0,4,0,1,2,0,4] \) on the reducing end side. Our approach reveals more details on the heparanase cleavage sites, particularly on the reducing end side, than previous studies (19–22). This is because previous methods have had limited access to the reducing end side of the heparanase cleavage sites.
ends with $[^{3}H]NaBH_4$, which could not be used to specifically label the reducing end side of the cleavage sites.

By analyzing the HS structures on both sides of the cleavage sites in an unbiased way, we were able to identify critical recognition sites that had been missed in previous studies. For example, our results indicate that both of the most abundant trisaccharides on the reducing end side ($[0,1,2,0,3]$ and $[0,1,2,0,4]$) include a 2-O-sulfation, suggesting that 2-O-sulfation is required for heparanase digestion. This result confirms the previous observations made by Bai et al. in vivo (36), whereas it has been ambiguous in previous in vitro studies regarding both the requirement of 2-O-sulfation and its specific location at the cleavage site (19–22). Interestingly, the HS trisaccharide GlcNS-IdoA2S-GlcNS6S was serendipitously discovered as a heparin lyase-resistant sequence after extensive digestion of HSPII with heparin lyases (37). This earlier discovery is in line with our tandem MS analysis of the trisaccharide $[0,1,2,0,4]$, suggesting that the trisaccharide probably resulted from digestion by endogenous heparanase and the subsequent heparin lyase treatment.

Further analysis of the sulfation patterns of pentasaccharides and heptasaccharides from both sides of the heparanase cleavage sites demonstrated that heparanase preferentially targets the boundary of NS and NS/NA domains within an HS chain. To evaluate the biological consequences of this, we measured the FGF2-promoting activity of HS from syndecan 4. Syndecan 4 has been identified previously as a co-receptor for FGF2,
where its HS chains act to enhance FGF2-FGF receptor interactions (38). Our previous work suggests that highly sulfated non-reducing ends of HS chains are able to coordinate with FGF2 and its receptor to produce active signaling complexes, supporting a 2:2:2 stoichiometric model for this process (27). Thus, our observation that heparanase digestion of HSSynd4 was able to enhance FGF2-promoting activity is consistent with its ability to generate new highly sulfated non-reducing ends from HS. Interestingly, it was previously found that the bacterial enzyme, heparin lyase III, also enhances the activities of HS substrates on FGF2 signaling by digesting away lowly sulfated sequences and exposing the highly sulfated NS domains to the non-reducing end (39).

Heparanase has been implicated in cell growth promotion in vivo, with considerable evidence of cancer-promoting activity (40). The cancer-promoting action is believed to result from the ability of heparanase to liberate growth factors such as FGF2 from extracellular matrix storage sites (40) as well as from its

FIGURE 12. Dose response of BAF-32 cell proliferation upon the addition of HSSynd4 (open squares) or heparanase (HPSE)-digested HSSynd4 (black squares). BaF32 cells were grown in the presence of FGF2 (10 ng/ml) with HSSynd4 or heparanase-digested HSSynd4 for 4 days, and cell number was determined by MTT. Error bars, S.D. (n = 3).

FIGURE 13. Raw tandem mass spectrum of trisaccharide [1,1,1,1,1]. Precursor ion is represented by the blue diamond in the spectrum and has an m/z of 316.5429 (1−).

FIGURE 14. Raw tandem mass spectrum of trisaccharide [0,1,2,0,3]. Precursor ion is represented by the blue diamond in the spectrum and has an m/z of 251.0098 (3−).
Substrate Specificity of Heparanase by LC-MS

FIGURE 15. Raw tandem mass spectrum of trisaccharide [0,1,2,0,4]. Precursor ion is represented by the blue diamond in the spectrum and has an m/z of 207.9949 (4−).

ability to destroy the histone acetyltransferase-inhibitory activity of cellular HS (41). Our data suggest an additional role for heparanase whereby it can play an active role in cell growth promotion by activating growth factor-promoting HS domains from within HS chains.

Our results provide a view of the cleavage pattern within heterogeneous HS substrates, from which we conclude that heparanase cleaves near the boundary of NS and NS/NA domains on HS chains. This conclusion is supported by the increased activity of HSSynd4 on FGF2 signaling after heparanase digestion. Because the clustered sulfate groups in the NS domain of HS provide the docking sites for numerous protein ligands and other signal molecules, this type of cleavage near the boundary of the NS domain may serve dual functions in cellular events involving activated heparanase; 1) the targeted cleavage selectively releases growth factors, chemokines, etc. stored in extracellular matrix to cell surfaces, and 2) the removal of the lowly sulfated HS sequences from the non-reducing end of the NS domains would facilitate the interaction between bound protein ligands and their receptors. The ability of heparanase to digest and liberate new activities from within HS suggests that this enzyme and its digestion products play key roles in normal tissue repair and development, where local and controlled activation of growth factor activity is critical. This same activity when inappropriately expressed may also contribute to diseases such as cancer; hence, heparanase may be an important drug target for cancer treatment.

Acknowledgment—We are grateful to Jian Liu who has generously provided recombinant heparin lyases.

REFERENCES
1. Kjellen, L., and Lindahl, U. (1991) Proteoglycans: structures and interactions. Annu. Rev. Biochem. 60, 443–475
2. David, G. (1993) Integral membrane heparan sulfate proteoglycans. FASEB J. 7, 1023–1030
3. Sasishekharan, R., Shriner, Z., Venkataraman, G., and Narayanasami, U. (2002) Roles of heparan-sulphate glycosaminoglycans in cancer. Nat. Rev. Cancer 2, 521–528
4. Bernfield, M., Götte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincum, J., and Zako, M. (1999) Functions of cell surface heparan sulfate proteoglycans. Annu. Rev. Biochem. 68, 729–777
5. Dowd, C. J., Cooney, C. L., and Nugent, M. A. (1999) Heparan sulfate mediates bFGF transport through basement membrane by diffusion with rapid reversible binding. J. Biol. Chem. 274, 5236–5244
6. Goldshmidt, O., Nadav, L., Aingorn, H., Irit, C., Feinstein, N., Ilan, N., Zamir, E., Geiger, B., Vlodavsky, I., and Katz, B. Z. (2002) Human heparanase is localized within lysosomes in a stable form. Exp. Cell Res. 281, 50–62
7. Zetser, A., Levy-Adam, F., Kaplan, V., Ginsg-Velitski, S., Bashenok, Y., Schubert, S., Flugelman, M. Y., Vlodavsky, I., and Ilan, N. (2004) Processing and activation of latent heparanase occurs in lysosomes. J. Cell Sci. 117, 2249–2258
8. Vlodavsky, I., Friedmann, Y., Elkin, M., Aingorn, H., Atizmon, R., Ishai-Michaeli, R., Bitan, M., Pappo, O., Peretz, T., Michal, I., Spector, L., and Pecker, I. (1999) Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. Nat. Med. 5, 793–802
9. Hulett, M. D., Freeman, C., Hamdorf, B. J., Baker, R. T., Harris, M. J., and Parish, C. R. (1999) Cloning of mammalian heparanase, an important enzyme in tumor invasion and metastasis. Nat. Med. 5, 803–809
10. Nakajima, M., Irimura, T., Di Ferrante, N., and Nicolson, G. L. (1984) Metastatic melanoma cell heparanase. Characterization of heparan sulfate degradation fragments produced by B16 melanoma endoglucuronidase. J. Biol. Chem. 259, 2283–2290
11. Nakajima, M., Irimura, T., Di Ferrante, N., Di Ferrante, D., and Nicolson, G. L. (1983) Heparan sulfate degradation: relation to tumor invasive and metastatic properties of mouse B16 melanoma sublines. Science 220, 611–613
12. Vlodavsky, I., and Friedmann, Y. (2001) Molecular properties and involvement of heparanase in cancer metastasis and angiogenesis. J. Clin. Invest. 108, 341–347
13. Vlodavsky, I., Elkin, M., Abboud-Jarrous, G., Levi-Adam, F., Fuks, L., Shafat, L., and Ilan, N. (2008) Heparanase: one molecule with multiple functions in cancer progression. Connect. Tissue Res. 49, 207–210
14. Vlodavsky, I., Miao, H. Q., Medalion, B., Danagher, P., and Ron, D. (1996) Involvement of heparan sulfate and related molecules in sequestration and growth promoting activity of fibroblast growth factor from extracellular matrix. Cell Regul. 7, 833–842
16. Vlodavsky, I., Bar-Shavit, R., Ishai-Michaeli, R., Bashkin, P., and Fuks, Z.
(1991) Extracellular sequestration and release of fibroblast growth factor: a regulatory mechanism? Trends Biochem. Sci. 16, 268–271
17. Nadir, Y., Vlodavsky, I., and Brenner, B. (2008) Heparanse, tissue factor, and cancer. Semin. Thromb. Hemost. 34, 187–194
18. Ramani, V. C., Purushothaman, A., Stewart, M. D., Thompson, C. A., Vlodavsky, I., Au, J. L., and Sanderson, R. D. (2013) The heparanase/syndecan-1 axis in cancer: mechanisms and therapies. FEBS J. 280, 2294–2306
19. Piakas, D. S., Li, J.-P., Vlodavsky, I., and Lindahl, U. (1998) Substrate specificity of heparanases from human hepatoma and platelets. J. Biol. Chem. 273, 18770–18777
20. Okada, Y., Yamada, S., Dong, J., Nakajima, M., and Sugahara, K. (2002) Structural recognition by recombinant human heparanase that plays critical roles in tumor metastasis. J. Biol. Chem. 277, 42488–42495
21. Peterson, S. B., and Liu, J. (2010) Unraveling the specificity of heparanase utilizing synthetic substrates. J. Biol. Chem. 285, 14504–14513
22. Peterson, S., and Liu, J. (2012) Deciphering mode of action of heparanse using structurally defined oligosaccharides. J. Biol. Chem. 287, 34836–34843
23. Shi, X., and Zaia, J. (2009) Organ-specific heparan sulfate structural phenotypes. J. Biol. Chem. 284, 11806–11814
24. Lawrence, R., Lu, H., Rosenberg, R. D., Esko, J. D., and Zhang, L. (2008) Disaccharide structure code for the easy representation of constituent oligosaccharides from glycosaminoglycans. Nat. Methods 5, 291–292
25. Staples, G. O., Bowman, M. J., Costello, C. E., Hitchcock, A. M., Lau, J. M., Leymarie, N., Miller, C., Naimy, H., Shi, X., and Zaia, J. (2009) A chip-based amide-HILIC LC/MS platform for glycosaminoglycan glycomics profiling. Proteomics 9, 686–695
26. Staples, G. O., Naimy, H., Yin, H., Kileen, K., Kraiczek, K., Costello, C. E., and Zaia, J. (2010) Improved hydrophilic interaction chromatography LC/MS of heparinin using a chip with postcolumn makeup flow. Anal. Chem. 82, 516–522
27. Naimy, H., Buszek-Thomas, J. A., Nugent, M. A., Leymarie, N., and Zaia, J. (2011) Highly sulfated nonreducing end-derived heparan sulfate domains bind fibroblast growth factor-2 with high affinity and are enriched in biologically active fractions. J. Biol. Chem. 286, 19311–19319
28. Chambers, M. C., Maclean, B., Burke, R., Amodei, D., Ruderman, D. I., Neumann, S., Gatto, L., Fischer, B., Pratt, B., Egerton, J., Hoff, K., Kessner, D., Tasman, N., Shulman, N., Frewen, B., Baker, T. A., Brunski, M. Y., Paulse, C., Creasy, D., Flashner, L., Kani, K., Moulding, C., Seymour, S. L., Nuwaysir, L. M., Lefebvre, B., Kuhlmann, F., Roark, J., Rainer, P., Detlev, S., Hemenway, T., Huhmer, A., Deutsch, E. W., Moritz, R. L., Katz, J. E., Agus, D. B., MacCoss, M., Tabb, D. L., and Mallick, P. (2012) A cross-platform toolkit for mass spectrometry and proteomics. Nat. Biotechnol. 30, 918–920
29. Maxwell, E., Tan, Y., Tan, Y., Hu, H., Benson, G., Aizikov, K., Conley, S., Staples, G. O., Slys, G. W., Smith, R. D., and Zaia, J. (2012) GlycReSoft: a software package for automated recognition of glycans from LC/MS data. PloS One 7, e45474
30. Huang, Y., Shi, X., Yu, X., Leymarie, N., Staples, G. O., Yin, H., Killeen, K., and Zaia, J. (2011) Improved liquid chromatography-MS/MS of heparan sulfate oligosaccharides via chip-based pulsed makeup flow. Anal. Chem. 83, 8222–8229
31. Ernst, S., Langer, R., Cooney, C. L., and Sasisekharan, R. (1995) Enzymatic degradation of glycosaminoglycans. Crit. Rev. Biochem. Mol. Biol. 30, 387–444
32. Wu, Z. L., and Lech, M. (2005) Characterizing the non-reducing end structure of heparan sulfate. J. Biol. Chem. 280, 33749–33755
33. Gilat, D., Hershkoviz, R., Goldkorn, L., Calahon, L., Korner, G., Vlodavsky, I., and Lider, O. (1995) Molecular behavior adapts to context: heparanase functions as an extracellular matrix-degrading enzyme or as a T cell adhesion molecule, depending on the local pH. J. Exp. Med. 181, 1929–1934
34. Schlessinger, J., Plotnikov, A. N., Ibrahimi, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., Linhardt, R. J., and Mohammadi, M. (2000) Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparan in FGFR binding and dimerization. Mol. Cell 6, 743–750
35. Podyima-Inoue, K. A., Yokote, H., Sakaguchi, K., Ikuta, M., and Yanagishita, M. (2002) Characterization of heparanse from a rat parathyroid cell line. J. Biol. Chem. 277, 32459–32465
36. Bai, X., Bame, K. J., Habuchi, H., Kintara, K., and Esco, J. D. (1997) Turnover of heparan sulfate depends on 2-O-sulfation of uronic acids. J. Biol. Chem. 272, 23172–23179
37. Yamada, S., Sakamoto, K., Tsuda, H., Yoshida, K., Sugahara, K., Kho, K.-H., Morris, H. R., and Dell, A. (1994) Structural studies on the tri- and tetrasaccharides isolated from porcine intestinal heparan and characterization of heparinase/heparitinases using them as substrates. Glycobiology 4, 69–78
38. Horowitz, A., Tkachenko, E., and Simons, M. (2002) Fibroblast growth factor-specific modulation of cellular response by syndecan-4. J. Cell Biol. 157, 715–725
39. Zhang, Z., Coomans, C., and David, G. (2001) Membrane heparan sulfate proteoglycan-supported FGF2-FGFR1 signaling: evidence in support of the “cooperative end structures” model. J. Biol. Chem. 276, 41921–41929
40. Vlodavsky, I., Beckhove, P., Lerner, I., Pisano, C., Meinovitz, A., Ilan, N., and Elkin, M. (2012) Significance of heparanse in cancer and inflammation. Cancer Microenviro. 5, 115–132
41. Purushothaman, A., Hurst, D. R., Pisano, C., Mizumoto, S., Sugahara, K., and Sanderson, R. D. (2011) Heparanse-mediated loss of nuclear syndecan-1 enhances histone acetyltransferase (HAT) activity to promote expression of genes that drive an aggressive tumor phenotype. J. Biol. Chem. 286, 30377–30383
42. Varki, A., Cummings, R. D., Esko, J. D., Freeze, H. H., Stanley, P., Marth, J. D., Bertozzi, C. R., Hart, G. W., and Varki, A. (2009) Symbol nomenclature for glycan representation. Proteomics 9, 5398–5399
43. Domon, B., and Costello, C. E. (1988) A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. Glycoconjugate J. 5, 397–409