Heparanases Produce Distinct Populations of Heparan Sulfate Glycosaminoglycans in Chinese Hamster Ovary Cells*

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Once internalized, cell-associated heparan sulfate proteoglycans are degraded to short glycosaminoglycans by the action of endoglycosidases or heparanases. We have begun to address the question of how many heparanases are responsible for this process by analyzing short heparan sulfate chains produced in vivo by Chinese hamster ovary (CHO) cell heparanases. Short heparan sulfate chains were purified from CHO cells and labeled at the reducing end with [3H]NaBH4. Hydrolysis of the chains to monosaccharides and analysis of the [3H]-sugar alcohols indicate that heparanase activities in CHO cells are endo-[b]-glucuronidases. The modification state of the heparanase-derived glycosaminoglycans was examined by treating the [3H]heparan sulfate chains with nitrous acid or bacterial heparin lyases, which cut the chain at specific sequences, and analyzing the products by P2 gel filtration chromatography. Two populations of short chains were identified that differ in the extent of modification on the nonreducing side of the heparanase cleavage site. One class of chains is unmodified for at least 9 residues from the reducing end, while the other group has a modified domain within 3–7 residues from the heparanase cleavage site. Our results suggest a model of heparanase action where the enzymes recognize differences in sulfate content between modified and unmodified regions and bind to sites that encompass both domains. The enzymes then cleave the glycosaminoglycan at junctions between the modified and unmodified sequences to produce the different populations of short heparan sulfate chains.

Heparanases are a family of mammalian endoglycosidases that degrade long heparan sulfate glycosaminoglycans to shorter chains (1–3). Some heparanases are secreted from cells where they degrade heparan sulfate proteoglycans in basement membranes and extracellular matrices (1). These enzymes are thought to play a role in remodeling basement membranes after injury or at inflammation sites (4–6). Other heparanases are intracellular and are important for degrading cell surface heparan sulfate proteoglycans once they have been internalized (2). Inside cells, heparanases cleave long heparan sulfate glycosaminoglycans on core proteins to short chains that have an approximate Mr of 5000 (2, 3, 7–9). One obvious role of the intracellular enzymes is to generate additional nonreducing ends so heparan sulfate glycosaminoglycans can be efficiently degraded by lysosomal exoglycosidases (10). However, these heparanases may have other important functions. Cell surface proteoglycans have been shown to be receptors for growth factors (11–13), enzymes (14), and viruses (15), so intracellular heparanases may act to release bound ligands from their proteoglycan receptors once the complex is internalized. Short heparan sulfate chains generated by heparanases may also regulate the interaction of a ligand with the proteoglycan in the endosome or, if the chains are secreted, at the cell surface. Heparanases could play a role in modulating the modification state of cell surface proteoglycans. Recent evidence suggests that some proteoglycans are internalized and recycled through the Golgi apparatus (16); thus, heparanases could remove a portion of the heparan sulfate in endosomes so that new chain polymerization and modification could occur when the core protein is rerouted to the Golgi. Degradation of proteoglycans by heparanases may also be an important mechanism to prevent long heparan sulfate glycosaminoglycans from associating with molecules that would be deleterious to the cell. For example, heparan sulfate proteoglycans are components of senile plaques in Alzheimer’s disease due to their association with the b-amyloid peptide (17). Heparanases may cleave heparan sulfate chains from the core protein, thus preventing the formation of the b-amyloid-proteoglycan complex deposited in amyloid plaques.

Although heparanase activities have been described in a variety of cells and tissues, not much is known about how the enzymes recognize and cleave the glycosaminoglycan chain. Heparan sulfate glycosaminoglycans are linear chains of alternating uronic acid (GlcUA or IdceA) and GlcN sugars (18). The GlcN residues may be either N-acetylated or N-sulfated, and all four monosaccharide species may be O-sulfated (18). Structural studies of heparan sulfate indicate the N- and O-sulfate groups are clustered in iduronic acid-rich domains (modified domains), which are separated by regions of (GlcNAc1–4GlcUA) disaccharide repeats that contain very little O-sulfate (unmodified domains) (19). It has been postulated that heparanases cleave within the unmodified regions, since, with the exception of the small platelet enzyme that has been reported to be an endoglucomannidase (6), all of the mammalian enzymes examined are endo-[b]-glucuronidases (7, 8, 20, 21). Inhibitor studies suggest overall modification of the polysaccharide is more important for enzyme recognition than a specific glycosaminoglycan sequence, because N-sulfate groups are required for heparanases to cleave the heparan sulfate substrate, but O-sulfate groups have little effect on enzyme activity (22–25). Finally, most heparanases can only act on heparan sulfate chains greater than or equal to 10 kDa (2, 3, 20, 25), indicating that the length of the glycosaminoglycan chain is also important for substrate recognition.

One approach to address the substrate specificity of intracellular heparanases is to characterize the short heparanase-derived products for the cleaved glycosidic bond and the mod-

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Heparanase-derived Heparan Sulfate Glycosaminoglycans

Heparanase activities from placenta (20), platelets (6, 21), and B16 melanoma cell lines (7), commercially obtained heparan sulfate or heparin was cleaved with partially purified activity, and the newly formed reducing ends were labeled. While these studies yielded useful information about the glycosidic bond cleaved by the partially purified enzymes, they were not ideal for characterizing the sequences at and around the cleavage site for a number of reasons. First, most of the heparan sulfate commercially available is obtained from liver or kidney and may have been partially degraded by intracellular heparanases in the tissue before being purified. These chains may not be the best substrate, since they may no longer have the sequences recognized or cleaved by heparanases, or the chain itself may be too small. Another problem is that in these studies, large quantities of heparan sulfate were incubated with partially purified enzymes in vitro, and the conditions of the reaction may have forced the enzyme to cleave the chain at sequences it would not normally act at in vivo. Finally, it may be important to use heparanase activities and heparan sulfate glycosaminoglycans from the same cell source. Studies have shown the modification state of heparan sulfate glycosaminoglycans is dependent on the cell that synthesizes it (26–28), and it is possible that intracellular heparanase specificities are fine-tuned for the modified glycosaminoglycan it normally encounters. By using a commercial source of glycosaminoglycans, one may be missing the correct sequences for efficient degradation by the heparanase of interest.

We have circumvented these problems by analyzing short heparan sulfate species produced by heparanases in vivo. For these studies we are using Chinese hamster ovary (CHO) cells heparan sulfate glycosaminoglycans. Lyssomes in CHO cells degrade heparan sulfate chains to monosaccharides inefficiently; thus, the short heparanase-derived glycosaminoglycans can be purified from the cells, and the reducing ends can be labeled with [3H]NaBH₄. Analysis of the short [3H]heparan sulfate glycosaminoglycans shows there are distinct populations of heparanase-degraded heparan sulfate chains in CHO cells and suggests that heparanases may recognize and cleave the heparan sulfate chain at junctions between modified and unmodified domains. Our results also indicate that there may be multiple heparanase activities in CHO cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61; Rockville, MD). Cells were maintained in Ham's F-12 medium (Life Technologies, Inc.) from the American Type Culture Collection (CCL-61; Rockville, MD). Cells were grown at 37°C in 100% relative humidity. They were subcultured every 3–4 days with 0.125% trypsin, and after 15–20 passages, fresh cells were revived from frozen stocks stored in liquid nitrogen.

Time Course—Confluent CHO cells were incubated with 50 μCi/ml [3H]NaBH₄ (DuPont NEN) for 7 h at 37°C. Radioactive medium was removed from the plate, the cell layer was washed three times with phosphate-buffered saline, and trypsin was added to release the cells from the culture dish. Trypsinized cells were pelleted, resuspended in F12 media containing 1 mM Na₂SO₄, and replated at approximately 2.5 × 10⁵ cells/60-mm dish. For the zero time point, a comparable aliquot of labeled cells was treated with 0.1 M NaOH (final) for 15 min at room temperature, neutralized with 10 mM acetic acid (27), and stored at −20°C until the end of the chase. Plated cells were allowed to grow for an additional 24, 48, or 72 h to allow for further catabolism of intracellular glycosaminoglycans. At that time labeled heparan sulfate chains were isolated from cells as described (27).

Heparan Sulfate Glycosaminoglycan Isolation—CHO cells were grown to confluence in 150-mm diameter plates, rinsed three times with cold phosphate-buffered saline, and scraped from the dish with 0.25 mM sucrose, 5 mM HEPES, pH 7.6. Cells were sedimented by centrifuging for 10 min, resuspended in 5 mM HEPES, pH 7.6, and frozen at −20°C. Once cells had been collected from approximately 100 150-mm plates, the suspensions were thawed and treated with 0.1 M NaOH (final) for 15 min at room temperature to break open the cells. After the suspension was neutralized, DNAase (Sigma) and MgCl₂ were added to final concentrations of 50 milliunits/ml and 40 mM respectively, and the mixture was incubated overnight at room temperature to degrade the DNA released from the cells. The next morning, Pronase (Boehringer Mannheim) was added, and the mixture was incubated an additional 24 h at room temperature to degrade cellular proteins. Following protease treatment, the sample was diluted 5-fold with water, insoluble material was pelleted by centrifugation in a clinical centrifuge for 3 min, and the supernatant was applied to a 5-ml DEAE-Sepacel column to isolate the cell-associated glycosaminoglycans. The column was washed with 60 ml of 0.25 mM NaCl, 50 mM sodium acetate, pH 6.0, and glycosaminoglycans were eluted with 25 ml of 1.0 mM NaCl, 50 mM sodium acetate. The glycosaminoglycans in the 1 mM NaCl eluate were concentrated on a new 1-ml DEAE-Sepacel column and then precipitated overnight at 4°C with 95% ethanol. Precipitated glycosaminoglycans were treated with 10 milliunits/ml chondroitinase ABC (ICN Biochemicals, Costa Mesa, CA) for 16 h at 37°C, and the heparan sulfate chains were separated from the newly formed chondroitin sulfate disaccharides by ethanol precipitation. The short intracellular heparan sulfate chains were purified further by a Sepharose CL-6B column (104 × 1 cm), equilibrated in 0.2 mM NH₄HCO₃. The elution position of the short heparan sulfate chains was monitored by applying 5–50 μl of each 1-ml fraction to Immobilon membranes (Millipore Corp., Bedford MA) and staining the bound glycosaminoglycans with 0.2% Alcian blue in 50 mM NaCl and 50 mM MgCl₂ and 50 mM sodium acetate, pH 5.7 (29). Fractions containing short heparan sulfate species (3) were pooled, dialyzed against water, and lyophilized to dryness. For these studies, two separate preparations of short CHO cell heparan sulfate were made and labeled by borohydride reduction (see below).

Heparan Sulfate Glycosaminoglycans with [3H]NaBH₄—Five mCi of [3H]NaBH₄ (American Radiolabeled Chemicals, St. Louis, MO) was resuspended in 80 μl of 50 mM NaOH, and aliquots were added to lyophilized heparan sulfate glycosaminoglycans (10 μg to 1.0 mg). The ratio of [3H]NaBH₄ to heparan sulfate chains was approximately 2.5 mCi for 1.0 μg of glycosaminoglycan (dry weight), and the reaction volume was at least 40 μl. The mixtures were incubated at 4°C for 24 h. The [3H]-labeled glycosaminoglycan was treated overnight at 4°C with 95% ethanol. Precipitated glycosaminoglycans were treated with 10 milliunits/ml chondroitinase ABC (ICN Biochemicals, Costa Mesa, CA) for 16 h at 37°C, and the heparan sulfate chains were separated from the newly formed chondroitin sulfate disaccharides by ethanol precipitation. The short intracellular heparan sulfate chains were purified further by a Sepharose CL-6B column (104 × 1 cm), equilibrated in 0.2 mM NH₄HCO₃. The elution position of the short heparan sulfate chains was monitored by applying 5–50 μl of each 1-ml fraction to Immobilon membranes (Millipore Corp., Bedford MA) and staining the bound glycosaminoglycans with 0.2% Alcian blue in 50 mM NaCl and 50 mM MgCl₂ and 50 mM sodium acetate, pH 5.7 (29). Fractions containing short heparan sulfate species (3) were pooled, dialyzed against water, and lyophilized to dryness. For these studies, two separate preparations of short CHO cell heparan sulfate were made and labeled by borohydride reduction (see below).

Hydrolysis of Heparan Sulfate Chains to Monosaccharides—The 3H-labeled heparan sulfate glycosaminoglycans were hydrolyzed to monosaccharides with 2 mM trifluoroacetic acid and high pH nitrous acid following the procedures of Hook et al. (30). After hydrolysis the mixture was incubated with 1.5 mM NH₄OH (final) for 90 min at room temperature in order to convert any aldonic lactones to acids and then lyophilized and stored at 4°C in the lyophilized form. To separate charge monosaccharide species from uncharged compounds, the base-treated hydrolysis mixture was applied to a 20-ml AGX8 column equilibrated in 0.1 M NaOH (final). Once the sample was applied, the column was washed with 2.5% formic acid to elute any charged species. Fractions containing radioactive material were pooled, and they were lyophilized to remove the formic acid. In some cases, base-treated hydrolysis mixtures were applied to smaller AGX8 columns, equilibrated in 10 mM NH₄OH. Uncharged 3H-species were eluted with 10 mM NH₄OH, while charged 3H-residues were eluted with 5% formic acid.

Paper Chromatography—The 3H-sugar alcohols were applied to cel-
lulose phosphate paper (P-81, Whatman, Hillsboro, OR) and developed in ethyl acetate, pyridine, 5 mM boric acid (3:2:1 by volume) (32). Aldonic and uronic acids move very slowly in this system; therefore, in order to separate the aldonic acid species, the chromatogram was developed for 48 h. Gulo nic acid (Sigma) and idonic acid (graciously provided by Dr. T. Murphy, Pfizer, Inc.) were included in all chromatograms and were identified by a potassium permanganate stain (33). To ensure that the hydrolysis procedure did not affect the migration of the [3H]aldonic acid, both gulo nic acid and idonic acid were hydrolyzed identically to the [3H]-oligosaccharides. All samples were incubated with 1.5 mM NaOH for 90 min at room temperature before being applied to the cellulose phosphate paper to ensure that all of the [3H]-labeled aldonic acids were in the acid form.

**Gel Filtration Chromatography**—Glycosaminoglycan chain size was examined by gel filtration chromatography on a TSK 3000 gel filtration column (7.5 × 30 mm, Tosohaas, Montgomeryville, PA) equilibrated in 0.1 M KH2PO4 (pH 6.0), 0.5 M NaCl, 0.2% Zwittergent (3–12) (3). The column was standardized with heparin, heparan sulfate, and chondroitin sulfate molecules of known molecular weight and with purified, low pH nitrous acid oligosaccharides (hexasaccharide to 14-mer) generated from [3H]glucosamine-labeled CHO heparan sulfate (27).

**Glycosaminoglycan Treatments**—A portion of the [3H]-reduced heparan sulfate (5,000–50,000 cpm) was lyophilized to dryness and treated with nitrous acid or the bacterial polysaccharide lyase, heparanase (EC 4.2.2.7) (Siekagaku America Inc., Ljunsvald MD), to examine the modification of the glycosaminoglycan. Low pH nitrous acid-catalyzed deamination, which cleaves chains at N-sulfated GlcN residues, was performed according to the method of Shively and Conrad (35). High pH nitrous acid-catalyzed deamination, which cleaves chains at unsubstituted GlcN residues, was performed according to the method of Lindahl et al. (36). Heparanase (1.5 milliunits) was added to [3H]-heparan sulfate resuspended in 20 mM sodium acetate, pH 7.0, 1 mM calcium acetate (total volume of 30 μl) and incubated at 37°C. After 16–24 h, the reaction mixture was cooled and the volume was adjusted to 0.5 ml with 0.5 M pyridine acetate, pH 5.0, and then run at a flow rate of 60–100 ml/hr. One-ml fractions were collected. The column was standardized for even numbered oligosaccharides by determining the elution position of the products formed when [3H]glucosamine-labeled heparan sulfate was treated with low pH nitrous acid (27).

**RESULTS**

**CHO Cells Contain Short Heparan Sulfate Species**—In order to isolate chemical quantities of heparanase-derived CHO heparan sulfate glycosaminoglycans, we needed to confirm that there were indeed short chains inside cells. CHO-K1 cells were incubated with [35S]Heparan sulfate chains were isolated separately from the dishes containing chase media. At 0, 24, 48, and 72 h of chase, glycosaminoglycans and the tryptically and replated in fresh media. The columns were developed for 48 h. Gulo nic acid (Sigma) and idonic acid (graciously provided by Dr. T. Murphy, Pfizer, Inc.) were included in all chromatograms and were identified by a potassium permanganate stain (33). To ensure that the hydrolysis procedure did not affect the migration of the [3H]aldonic acid, both gulo nic acid and idonic acid were hydrolyzed identically to the [3H]-oligosaccharides. All samples were incubated with 1.5 mM NaOH for 90 min at room temperature before being applied to the cellulose phosphate paper to ensure that all of the [3H]-labeled aldonic acids were in the acid form.

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**FIG. 1. Size analysis of intracellular heparan sulfate species in CHO cells.** A, CHO-K1 cell-associated [35S]heparan sulfate glycosaminoglycans, purified at 0 (●), 24 (●), 48 (●), and 72 (●) h of chase (see “Experimental Procedures”) were analyzed on a TSK 3000 gel filtration column, equilibrated and eluted with 50 mM KH2PO4, 0.5 M NaCl, 0.2% Zwittergent. The data have been corrected to reflect the amount of [3H]heparan sulfate remaining cell-associated at each chase point. B, short cell-associated heparan sulfate glycosaminoglycans purified from CHO cells were labeled at the reducing end with [3H]NaBH4 (see “Experimental Procedures”), and analyzed on the TSK 3000 gel filtration column.

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These results suggest that the short heparanase-derived heparan sulfate chains remaining in the cell are being degraded by the exoglycosidases in the lysosome, albeit not very efficiently. However, since lysosomal enzymes act at the non-reducing end of the polysaccharide (10), the intracellular heparan sulfate species should still have the reducing end that was generated by heparanases. Therefore, we isolated short heparan sulfate from CHO cells and labeled the reducing end with [3H]NaBH4. As expected, the [3H]-reduced heparan sulfate were shorter than the chains initially produced by heparanase (Fig. 1B) but were similar to the size of the [3H]glycosaminoglycans found in the cell after a 24–72 h chase (Fig. 1A). These short [3H]heparan sulfate chains (Fig. 1B) were analyzed to examine the CHO heparanase cleavage sites.

**Heparanase Activities in CHO Cells Are Endoglucuronidases**—Because of the repeating disaccharide structure of heparan sulfate, heparanases will cleave the glycosaminoglycan chain so that either a uronic acid or a glucosamine moiety is at the newly formed reducing end. Since the short heparan sulfate chains were reduced in order to label the end sugar, the [3H]-residues are actually the corresponding sugar alcohol. When reduced, GlcUA and IdceA are converted to gulonic acid or idonic acid, respectively, while GlcNAc and GlcNOSO3 residues are converted to glucosaminitol. To determine the kinds of endoglycosidases present in CHO cells, the short [3H]-reduced chains were hydrolyzed to monosaccharides by a combination of trifluoroacetic acid and high pH nitrous acid treatments. The hydrolysis mixture was treated with base to convert any aldonic lactones to the acid form and then applied to an AGX18 column to separate the neutral and acidic species. Over 80% of

2 S. Tumova and K. J. Bame, manuscript in preparation.
Heparanase-derived Heparan Sulfate Glycosaminoglycans

The migration position of gulonic acid. The acidic fractions from the AGX18 column were pooled and analyzed for the type of 3H-sugar alcohol by ascending cellulose phosphate paper chromatography. The paper was developed in ethyl acetate, pyridine, 5 mM boric acid (3:2:1) to separate the aldonic acids. Once dry, the paper was cut into 0.5-cm fractions and analyzed for radioactivity. The shaded ellipse shows the migration position of idonic acid, and the open ellipse shows the migration position of gulonic acid. A, 3H-sugar alcohols derived from the total reduced chain population; B, 3H-sugar alcohols derived from the nitrous acid oligosaccharides that eluted near the void volume of the P2 gel filtration column (Fig. 3).

Characterization of Chain Modification at the Nonreducing Side of the Cleavage Site—Besides the glycosidic bond that is hydrolyzed, heparanases may be specific for the type of modified residues adjacent to the cleavage site. The modification state of a heparan sulfate chain can be examined by treating the glycosaminoglycan with various reagents that cleave the glycosaminoglycan at unsubstiuted GlcN. To explore this possibility, the 3H-reduced heparan sulfate chains were treated with low pH nitrous acid, and the reaction products were analyzed on a Bio-Gel P2 gel filtration column. The percentage of 3H-chains eluting as different oligosaccharides was calculated for each sample, and results from both preparations were averaged.

### Table I

| 3H-Oligosaccharide | Low pH nitrous acid | Heparinase |
|--------------------|---------------------|-----------|
| V<sub>o</sub>      | 47.1 ± 8.1          | 63.1 ± 18.0 |
| Nonasaccharide     | 7.0 ± 1.6           |           |
| Heptasaccharide    | 8.8 ± 0.4           | 8.7 ± 4.7 |
| Pentasaccharide    | 10.1 ± 1.0          | 15.1 ± 8.7 |
| Trisaccharide      | 26.1 ± 4.5          | 3.0 ± 1.3 |
| Disaccharide       | 5.7 ± 2.3           | 2.2 ± 0.4 |
| Monosaccharide     | 2.3 ± 0.1           | 1.0 ± 1.3 |

The applied radioactivity required a decreased pH to elute from the AGX18 resin (data not shown), indicating that the 3H-sugar alcohols contained a negatively charged functional group and thus were reduced uronic acids. The acidic fractions from the AGX18 column were pooled and analyzed by ascending paper chromatography on cellulose phosphate paper to identify the type of aldonic acid (Fig. 2). Except for a small amount of radioactivity that remained at the origin, all of the 3H-labeled aldonic acids migrated similarly to the gulonic acid standard, suggesting that they were originally glucuronic acid residues (Fig. 2A). These results indicate that the major heparanase activities in CHO cells are endo-β-glucuronidases.

Low pH nitrous acid cleaves heparan sulfate between GlcNSO₃ and uronic acid (fractions 32–40) on a P10 gel filtration column shows that they range from 9 to 13 residues (data not shown). Because nitrous acid cleaves heparan sulfate between GlcNSO₃ and uronic acid residues, the odd numbered oligosaccharides confirm that a uronic acid is at the reducing end of the heparanase-derived chains. The low pH nitrous acid results also suggest that along with endoglucuronidase activity, CHO cells may have a minor endoglucosaminidase activity, since a small amount of 3H-labeled material elutes as disaccharides.

Over 90% of the 3H-oligosaccharides generated by low pH nitrous acid are trisaccharides or longer, suggesting that the residue adjacent to the [3H]glucuronic acid must be GlcNAc. However, structural studies of heparan sulfate from endothelial cells have shown that on the chain there is a low level of unsubstituted GlcN residues (37) that are also resistant to cleavage by low pH nitrous acid (35). Therefore, it is possible that the residue next to the reducing end could be an unsubstituted GlcN. To explore this possibility, the 3H-reduced heparan sulfate glycosaminoglycans were treated with high pH nitrous acid, which cleaves the glycosaminoglycan at unsubstituted GlcN but not GlcNAc or GlcNSO₃ residues (35, 36). Acid treatment did not change the elution position of the [3H]glycosaminoglycans on the TSK 3000 gel filtration column (data not shown), indicating that the glucosamine residues on the short CHO heparan sulfate chains are substituted with either acetyl or sulfate groups. These results confirm that CHO cell heparanase activities cleave the glycosaminoglycan so that, in nearly
all instances, the disaccharide at the newly formed reducing end is (GlcNAc1–4GlcUA).

Nearly half of the heparanase-derived chains in CHO cells have a long unmodified sequence on the nonreducing side of the cleaved glycosidic bond, since the first GlcNSO3 is, on average, 10–14 residues from the cleavage site (Fig. 3). One explanation for this result is that these chains were originally attached to core proteins. Studies in fibroblasts and endothelial cells have shown that the glycosaminoglycan chain directly upstream from the linkage tetrasaccharide is unmodified (19), so it would be less sensitive to low pH nitrous acid than heparan sulfate derived from the middle or end of the glycosaminoglycan. However, heparan sulfate glycosaminoglycans are attached to core proteins through a xylose residue (18), so the sugar alcohol at the reducing end of short glycosaminoglycans derived from these sequences would be [3H]xyitol rather than the [3H]gulonic acid found at the reducing end of heparanase-derived heparan sulfates (Fig. 2A). Since over 80% of the short, [3H]-reduced heparan sulfate glycosaminoglycans had gulonic acid at the reducing end (Fig. 2A), it is unlikely that the majority of the voided nitrous acid oligosaccharides came from the end of the chain attached to the protein core. In fact, 80% of the [3H]-sugar alcohols generated from hydrolysis of the long oligosaccharides (Fig. 3, fractions 32–40) bound to the AGX18 resin (data not shown) and co-migrated with the gulonic acid standard on cellulose phosphate paper (Fig. 2B), confirming that nearly all of the nitrous acid oligosaccharides remaining at the void volume of the P2 column (Fig. 3) were derived from internal chain sequences.

The modification state of the glycosaminoglycans on the non-reducing side of the cleavage site was further characterized by incubating the [3H]-reduced chains with the bacterial enzyme, heparinase, and analyzing the reaction products on the P2 column (Fig. 4A, Table I). Heparinase cleaves heparan sulfate glycosaminoglycans between GlcNSO3 and IdceA2S residues (38, 39), which are typically found in modified domains (19). Therefore, the pattern of [3H]oligosaccharides resulting from heparinase treatment will indicate the distance of this modified sequence from the reducing end of the short heparan sulfates. Over half of the [3H]-reduced chains remain at the void volume of the P2 column after incubation with heparinase (Fig. 4A, Table I), indicating that they do not contain the (GlcNSO3–1–4IdceA2S) sequence cleaved by the bacterial enzyme. The remaining [3H]-chains are converted by heparinase to nona-, hepta-, and pentasaccharides (Fig. 4A, Table I). Interestingly, incubation with the bacterial enzyme did not generate any [3H]-trisaccharides. This result suggests that none of the CHO heparanase-derived chains have IdceA2S as the third residue from the reducing end; however, a heparinase-susceptible bond directly upstream of the reducing end may be a poor substrate, due to the proximity of the reduced gulonic acid.

To look more closely at the properties of the chain populations that differ in their susceptibility to heparinase, both the [3H]oligosaccharides at the void volume (fractions 30–36) and the included [H]-species (fractions 37–53) were pooled and examined for their sensitivity to low pH nitrous acid (Fig. 4, B and C). The nitrous acid profile of the heparinase-voided chains is similar to the profile of the [3H]heparan sulfate treated with nitrous acid alone, except that the proportions of tri-, penta-, and heptasaccharides are reduced compared with the untreated glycosaminoglycans (compare Figs. 3 and 4F). This result shows there is a population of [3H]-reduced heparan sulfate chains that have GlcNSO3 sugars close to the cleavage site, but they lack IdceA2S residues in the N-sulfated region. On the other hand, when the heparinase-susceptible [3H]-chains are treated with nitrous acid, most of them are converted to trisaccharides (Fig. 4C, Table II), indicating that the nona-, hepta-, and pentasaccharides have at least one GlcNSO3 residue on the reducing side of the susceptible heparinase bond. This is not surprising, since IdceA2S sugars are normally present in regions of high N-sulfation (19). Thus, the combined use of heparinase and nitrous acid indicates that there is a population of heparanase-derived chains that have a modified sequence directly upstream of the cleaved glycosidic bond. Most of these chains have the sequence (GlcNSO3–1–4IdceA2S–1–4GlcNSO3–1–4HexA1–4GlcNAc1–4GlcUA).

**Discussion**

Our studies show that there are two populations of short, heparanase-derived heparan sulfate glycosaminoglycans in CHO cells that differ in the distance of the first modified domain from the glycosidic bond cleaved by heparanases (Fig. 5). One population, class I, has relatively unmodified sequences for the first 9–13 residues from the heparanase cleavage site and remain as long oligosaccharides that elute at the void volume of a P2 gel filtration column after either low pH nitrous acid treatment (Fig. 3) or digestion with heparinase (Fig. 4). The other population of short glycosaminoglycans, class II, has modified sequences that begin with 3–7 residues from where heparanases cleaves the chain. Class II chains elute as shorter oligosaccharides after low pH nitrous acid treatment (Fig. 3) and, based on their susceptibility to heparinase, may or may not contain IdceA2S residues within the modified domain (Fig. 4). Both chain populations are produced by endo-ß-glucuronidase.
Heparanase-derived Heparan Sulfate Glycosaminoglycans

Two separate preparations of \(^{3}H\)-reduced chains were incubated with heparinase, and the products were separated on a Bio-Gel P2 gel filtration column. The heparanase-susceptible oligosaccharides from each run were pooled, treated with nitrous acid, and reapplied to the P2 gel filtration column. The percentage of \(^{3}H\)-chains in each oligosaccharide were calculated, and results from both preparations were averaged.

| Oligosaccharide | Before | After |
|-----------------|--------|-------|
| Nonasaccharide  | 24.4 ± 6.8 | 5.4 ± 2.2 |
| Heptasaccharide | 27.9 ± 1.7 | 11.4 ± 3.4 |
| Pentasaccharide | 47.7 ± 5.1 | 22.0 ± 1.6 |
| Trisaccharide   | 54.1 ± 4.1 | 6.2 ± 2.3 |
| Disaccharide    | 6.2 ± 2.3 | 1.0 ± 0.7 |

Fig. 5. Proposed model for heparanase action and the classes of heparanase-derived heparan sulfate glycosaminoglycans in CHO cells. Open blocks represent unmodified domains, and shaded blocks represent modified domains. The reducing end of the glycosaminoglycan chain is indicated by an asterisk.

A model where heparanases bind to both modified and unmodified regions is consistent with what is known about the substrate specificity of the enzymes. Modified domains are defined by the presence of \(N\)-sulfate groups, which have been shown to be essential for heparanases to recognize and cleave a heparan sulfate chain (22–25). Specific \(O\)-sulfate groups, found primarily in the modified regions, are not necessary for enzyme activity (22–25). This is not surprising if, as our model predicts, the enzyme only recognizes the difference in sulfate content between modified and unmodified domains. \(O\)-sulfation should promote the interaction of heparanase with the substrate, since it would increase the sulfate content of the modified domain, but binding should not be dependent on the type or arrangement of \(O\)-sulfate groups. In fact, our model would predict that the spacing and length of the modified domain within the substrate are more important for enzyme recognition than the actual sequence of uronic acid epimers and \(O\)-sulfate groups. The degree of IdceA residues in the modified domain may be important for enzyme recognition as well. Heparan sulfate chains are proposed to form a helix with 2-fold symmetry (34). Spectroscopic and molecular modeling studies indicate that IdceA residues may exist in two conformations that will determine how charged functional groups are oriented to the helix (34). The IdceA in the modified domains may orient the glycosaminoglycan so that the increased sulfate content of the region is emphasized and thus facilitate the binding of heparanase to the heparan sulfate substrate. Our model would also explain the size dependence of heparanase substrates. Studies \textit{in vivo} and \textit{in vitro} suggest that heparan sulfate chains must be at least 10 kDa in order to be cleaved by heparanases (2, 20, 25), which would be the minimum size of a heparan sulfate chain to trisaccharides and oligosaccharides that eluted at the void volume of Sephadex G-25 gel filtration columns (8, 20). Although the sizes of the voided oligosaccharides were not identified in either study, it is likely that they are comparable with the CHO class I products, while the trisaccharide species are comparable with the class II products.

Structural analysis of heparan sulfate shows that the glycosaminoglycan is composed of alternating modified and unmodified domains (19). Modified regions, made up of 3–7 disaccharides, are rich in GlcNSO\(_3\) and IdceA residues and are the primary sites for \(O\)-sulfation. They are separated by sequences of 14–25 (GlcNAc\(_1\)--GlcUA) disaccharides that contain few \(O\)-sulfate groups (19). Our results suggest that heparanases may recognize and cleave the heparan sulfate chain at the junctions between these domains (Fig. 5). Heparanases may recognize differences in the sulfate content between the modified and unmodified regions and bind to sites on the chain that encompass both domains. The enzymes then cleave the glycosaminoglycan in a relatively unmodified sequence to generate shorter chain products. If heparanases cleave the glycosaminoglycan on the nonreducing side of the modified domain, class I chains will be generated, while if the enzymes act on the reducing side of the modified domain, the products will be class II chains (Fig. 5). In either case, the heparan sulfate substrate may sit loosely in the catalytic site, since both class I and class II products have slight variations in the distance of the first GlcNSO\(_3\) from the cleaved glycosidic bond (Figs. 3 and 4). Model of the heparanase action is similar to the model proposed by Schmidtchen and Fransson (40). They analyzed the structure of heparan sulfate oligosaccharides from fibroblasts and found the chains to be rich in GlcUA residues, with IdceA28 sugars at the periphery of the molecules. From these findings, they proposed that the short glycosaminoglycans had been produced by heparanases that cleaved the chain at or near modified sequences (40).

| Table II: Distribution of heparinase-susceptible \(^{3}H\)-oligosaccharides before and after low pH nitrous acid treatment |
|---------------------------------------------------------------|
| Oligosaccharide | Before | After |
|-----------------|--------|-------|
| Nonasaccharide  | 24.4 ± 6.8 | 5.4 ± 2.2 |
| Heptasaccharide | 27.9 ± 1.7 | 11.4 ± 3.4 |
| Pentasaccharide | 47.7 ± 5.1 | 22.0 ± 1.6 |
| Trisaccharide   | 54.1 ± 4.1 | 6.2 ± 2.3 |
| Disaccharide    | 6.2 ± 2.3 | 1.0 ± 0.7 |

The degree of IdceA residues in the modified domain may be important for enzyme recognition as well. Heparan sulfate chains are proposed to form a helix with 2-fold symmetry (34). Spectroscopic and molecular modeling studies indicate that IdceA residues may exist in two conformations that will determine how charged functional groups are oriented to the helix (34). The IdceA in the modified domains may orient the glycosaminoglycan so that the increased sulfate content of the region is emphasized and thus facilitate the binding of heparanase to the heparan sulfate substrate. Our model would also explain the size dependence of heparanase substrates. Studies \textit{in vivo} and \textit{in vitro} suggest that heparan sulfate chains must be at least 10 kDa in order to be cleaved by heparanases (2, 20, 25), which would be the minimum size of a heparan sulfate chain to trisaccharides and oligosaccharides that eluted at the void volume of Sephadex G-25 gel filtration columns (8, 20). Although the sizes of the voided oligosaccharides were not identified in either study, it is likely that they are comparable with the CHO class I products, while the trisaccharide species are comparable with the class II products.
chain that is composed of one modified domain and one unmodified domain of average sizes.3

How many heparanases are required to generate class I and class II chains? Since our studies characterized short heparan sulfate chains formed in vivo, it is possible they were produced by multiple enzymes. Indeed, the simplest explanation for the formation of class I and class II products is that they were generated by separate heparanase activities. Each enzyme would recognize the substrate by differences in sulfate content between modified and unmodified regions but would bind the chain so that modified domains are oriented differently at the catalytic site. At the present time we do not know how many heparanases exist in CHO cells. We have preliminary evidence for two separate CHO-heparanase activities;4 however, further work is necessary to establish whether each activity catalyzes the formation of one or both classes of heparanase products.

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3 The molecular weight of a chain consisting of one modified and one unmodified domain was calculated by assuming an average molecular weight of 500 g/mol for a modified disaccharide, and an average molecular weight of 400 g/mol for an unmodified disaccharide. A chain with an average modified domain of 5 disaccharides and an average unmodified domain of 20 disaccharides would have an estimated molecular mass of 10.5 kDa (2.5 kDa of modified domain plus 8 kDa of unmodified domain).

4 A. Hassell, C. Sanderson and K.J. Bame, unpublished results.

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