Drosophila Heparan Sulfate, a Novel Design*5

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Background: Heparan sulfate (HS) has important roles in cellular signaling that depend on the arrangement of sulfated domains.

Results: Drosophila HS contains an essentially single sulfated domain.

Conclusion: The domain organization of Drosophila HS is novel and differs from previously described HS structures.

Significance: The findings may provide novel insight into structure-function relationships for HS.

Heparan sulfate (HS) proteoglycans play critical roles in a wide variety of biological processes such as growth factor signaling, cell adhesion, wound healing, and tumor metastasis. Functionally important interactions between HS and a variety of proteins depend on specific structural features within the HS chains. The fruit fly (Drosophila melanogaster) is frequently applied as a model organism to study HS function in development. Previous structural studies of Drosophila HS have been restricted to disaccharide composition, without regard to the arrangement of saccharide domains typically found in vertebrate HS. Here, we biochemically characterized Drosophila HS by selective depolymerization with nitric acid. Analysis of the generated saccharide products revealed a novel HS design, involving a peripheral, extended, presumably single, N-sulfated domain linked to an N-acetylated sequence contiguous with the linkage to core protein. The N-sulfated domain may be envisaged as a heparin structure of unusually low O-sulfate content.

Heparan sulfate (HS) glycosaminoglycans constitute a family of polysaccharides structurally related to (but less sulfated than) heparin and characterized by extensive structural heterogeneity and variability. In contrast to heparin, which occurs exclusively in connective tissue-type mast cells, HS is produced by most cells in the body and is expressed, largely in proteoglycan form, at cell surfaces and in the extracellular matrix. HS has been ascribed diverse and fundamental roles in development and homeostasis due to interactions, more or less electrostatic in nature, with a multitude of proteins. The polysaccharide occurs throughout the evolutionary system, from Cnidaria onward (1, 2). Several reviews dealing with the structural, metabolic, and functional aspects of HS proteoglycans have been published (3–8).

Biosynthesis of HS involves polymerization of alternating d-glucuronic acid (GlcUA) and 2-deoxy-2-acetamido-d-glucose (N-acetyl-d-glucosamine; GlcNAc) residues, yielding a (GlcUAβ1,4GlcNacα1,4)n precursor polymer covalently attached to a proteoglycan core protein through a distinct “linkage region” (8–10). The product is modified through a complex series of reactions involving N-deacetylation/N-sulfation of GlcNAc (yielding GlcNS, where NS is an N-sulfate group) residues, C-5 epimerization of GlcUA to iduronic acid (IdoUA) units, and O-sulfation of hexuronic acids (HexUA) at C-2 and Glucosamine residues at C-3 and C-6. Because these reactions generally engage only a fraction of potentially available target residues, the final products have heterogeneous structures that vary with tissue source (11). The bifunctional N-deacetylation/N-sulfation enzymes (N-deacetylase/N-sulfotransferase (NDST); four mammalian isoforms described in mammals) have a key role in the overall polymer modification process because subsequent GlcUA C-5 epimerization and O-sulfation reactions generally depend on the presence of GlcNS residues. Due to selective NDST action, vertebrate HS chains typically display domains of consecutive N-sulfated disaccharide units (NS-domains), alternating N-acetylated and N-sulfated units (NA/NS-domains), and essentially unmodified N-acetylated sequences (NA-domains) (see Fig. 1A). IdoUA and O-sulfate residues are confined to the NS- and NA/NS-domains (12). Heparin may be considered an unusually extended and highly O-sulfated NS-domain.

Available information regarding HS structure derives predominantly from analysis of vertebrate samples. Characterization of HS/heparin from non-vertebrate sources revealed some unusual disaccharide units (1, 13) but, so far, no information regarding domain structure. Manipulation of genes encoding enzymes involved in biosynthesis of Drosophila HS defined roles of HS in various developmental events, again, however, only in terms of overall disaccharide composition (14–19). We therefore decided to approach Drosophila HS from a different angle, concentrating on domain organization as revealed by selective deaminative cleavage. The results point to a glyco-

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¶ The abbreviations used are: HS, heparan sulfate; NDST, N-deacetylase/N-sulfotransferase; aMan, 2,5-anhydro-α-mannitol (formed by reduction of terminal 2,5-anhydromannose with NaBH₄).

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saminoglycan domain of novel design, best envisaged as a low-sulfated heparin.

**EXPERIMENTAL PROCEDURES**

**Purification of Drosophila HS**—Batches of 15 or 30 g of dechorionated wild-type (Oregon R) Drosophila embryos from different embryonic stages (0–24 h) were digested with Pronase, followed by treatment with NaOH/NaBH₄, essentially as described (1). Briefly, embryos were digested with 100 mg of Pronase E (Merck) in 20 ml of 0.1 M Tris-HCl (pH 8.0) containing 0.002 M CaCl₂ at 55 °C for 24 h. Thereafter, an additional aliquot (50 mg) of enzyme in 2.5 ml of digestion buffer was added, and the digestion was continued for another 24 h. To release O-linked sugar chains (including HS) from the attached peptide generated by the protease digestion and to reduce the liberated O-linked saccharides, 4 M NaOH (final concentration, 0.5 M) and 20 mg of NaBH₄ were added to the digest, and the mixture was kept overnight at 4 °C. After neutralization with 4 M HCl, the mixture was centrifuged at 100,000 × g for 1 h. Supernatants were adjusted to 0.15 M NaCl and pH 8.0 by the addition of Tris-HCl and applied to a 10-ml column of DEAE-Sephacel (GE Healthcare) equilibrated with 0.15 M NaCl and 50 mM Tris-HCl (pH 8.0). The column was first washed with equilibration buffer and then with 50 mM acetate buffer (pH 4.0) containing 0.15 mM NaCl. The pH was increased by washing with 0.15 M NaCl and 50 mM Tris-HCl (pH 8.0), and finally, DEAE-bound material was eluted with 2 M NaCl and 50 mM Tris-HCl (pH 8.0). Two-ml fractions were collected and analyzed for uronic acid content by the carbazole reaction (20). Carbohydrate-positive fractions were pooled and dialyzed against water. The dialyzed material was digested with 125 units of Benzonase (Seikagaku), hence devoid of chondroitin ABC and AC (Seikagaku), and thus converted the original NA-domains to NS-domains to N-sulfated oligosaccharides (see Fig. 1B). Labeled saccharides were separated from unincorporated radioactivity by gel chromatography on a Sephadex G-15 column (1 × 170 cm) in 0.2 M NH₄HCO₃, corresponding to di- and oligosaccharides were pooled and subjected to Protocol A treatment, and the reduced products were analyzed by gel chromatography on a Bio-Gel P-10 column (1.5 × 175 cm, fine grade, Bio-Rad) in 0.5 M NaCl.

**RESULTS**

Frozen mixed-stage Drosophila embryos were digested with Pronase and treated with alkali to isolate free saccharide chains as described under “Experimental Procedures.” Carbohydrate-positive material bound to DEAE and eluted with ≥0.4 M NaCl was considered to represent free glycosaminoglycan chains. Most of the carbohydrate-positive material was eluted with 0.4 and 0.75 M NaCl. Approximately 2–3 μg of uronic acid, corresponding to 5–8 μg of polysaccharide, was obtained per gram of starting material.

The domain organization of Drosophila HS was investigated by selective deaminative cleavage, as outlined in Fig. 1B. Deamination/reduction according to Protocol A will cleave glucosaminidic linkages emanating from N-sulfated GlcNS residues and thus convert NS-domains into hexuronyl-[³H]aMan₉ disaccharides. The mixed NA/NS-domains will yield labeled tetrasaccharides, whereas NA-domains will remain intact. A reference sample of HS isolated from pig intestinal mucosa was subjected to Protocol A treatment, and the reduced products were analyzed by gel chromatography on Bio-Gel P-10 (Fig. 2A). The expected pattern of fragments was observed, with pre-
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FIGURE 2. Gel chromatography of 3H-labeled saccharides obtained after deaminative cleavage of HS. HS samples from Drosophila or pig intestinal mucosa were deaminated and radiolabeled according to Protocol A (A and B) or Protocol B (C and D) (see “Experimental Procedures,” Fig. 1, and supplemental Fig. S1), and the products were analyzed by Bio-Gel P-10 gel chromatography. The numbers above each peak in A indicate oligosaccharide size.

dominant di- and tetrasaccharide peaks along with smaller peaks of larger N-acetylated oligosaccharides. (Note that these oligosaccharides are end-labeled, hence, peak areas need to be multiplied by the number of disaccharide units to reflect relative abundance.) By contrast, Drosophila HS yielded disaccharides as the only product of significance, with only trace amounts of material emerging in the position of tetrasaccharides (Fig. 2B). The generation of disaccharides and the lack of larger oligomers pointed to a parent polysaccharide containing significant extended N-sulfated sequence, uninterrupted by N-acetylated units (Fig. 1). In fact, the degradation pattern of Drosophila HS suggested an N-sulfate content similar to that of heparin.

To confirm these findings, we applied inverse-mode deaminative cleavage of N-deacetylated material according to Protocol B. This approach is expected to generate variously extended oligomers from NS-domains, tetrasaccharides from NA/NS-domains, and disaccharides from NA-domains (Fig. 1B). In our experience, some depolymerization of polysaccharide during the extended hydrazinolysis is unavoidable, and the fairly modest yield of higher oligosaccharides released from the intact HS standard (Fig. 2C) probably underestimates the actual contribution of N-sulfated structure to the intact polysaccharide. By contrast, the corresponding elution profile relating to Drosophila HS covered the entire separation range of the P-10 column (Fig. 2D), indicating oligosaccharides composed of consecutive N-sulfated disaccharide units. In addition, Protocol B yielded significant amounts of labeled disaccharide (Fig. 2D), presumably derived from NA-domain(s) that escaped detection by Protocol A (Fig. 2B) (see “Discussion”). The size of the NA-domain(s) in the parent HS chain was not defined through this experiment, nor was the precise extension of the N-sulfated sequence(s). Small oligosaccharides (in particular, tetrasaccharides) could be due to incomplete N-deacetylation of N-acetylated structure, but could also reflect partial depolymerization of NS-domains during hydrazinolysis. Nevertheless, the results demonstrate the occurrence of NS-domains in Drosophila HS that exceed the size of the corresponding domains in vertebrate HS. The N-sulfated nature of this material was confirmed by release of reducing terminal labeled disaccharides through deamination at pH 1.5 (see Fig. 4C and supplemental Fig. S2C). (The peak of excluded material in Fig. 2D presumably represents 3H-labeled macromolecules not related to HS judging by its resistance to deamination at pH 1.5 and enzymatic digestion with heparin lyases (data not shown).)

The disaccharides obtained according to the different cleavage procedures were identified by high-voltage paper electrophoresis, paper chromatography, and anion-exchange HPLC (which separated the various O-sulfated but not the non-sulfated species). High-voltage paper electrophoresis of the labeled disaccharides released by deamination according to Protocol A (Fig. 2B) showed major non-sulfated and mono-O-sulfated disaccharides, along with smaller amounts of di-O-sulfated disaccharide (Fig. 3A), compatible with a relatively low-sulfated parent NS-domain. Similar analysis of disaccharides obtained through Protocol B (Fig. 2D) revealed a single non-sulfated component (Fig. 3B), as predicted for a (GlcUA-GlcNAc)n NA-domain; the lack of O-sulfated species was verified also by anion-exchange HPLC (Fig. 4B). Paper chromatography of the non-O-sulfated NS-domain-derived disaccharide fraction indicated GlcUA-aManR and IdoUA-aManR, representing -GlcUA-GlcNS- and -IdoUA-GlcNS-, respectively, in intact HS, at an ~8:2 ratio (supplemental Fig. S2A), whereas the NS-domain-derived disaccharide lacked IdoUA, as expected (supplemental Fig. S2B). Further separation of the O-sulfated disaccharides by anion-exchange HPLC (Fig. 4A) enabled the identification of all NS-domain-derived disaccharide units (Table 1). Results were confirmed by repeated HPLC following digestion with a mixture of β-glucuronidase and α-iduronidase, which selectively eliminated all disaccharides containing nonsulfated HexUA units. For comparison, the results are related to previous compositional analysis of Drosophila HS (based on complete lyase digestion) by Toyoda et al. (18). The composition of the resultant pool of N-sulfated disaccharides showed reasonable agreement with our present results (Table 1). Notably, contrary to our current protocol, their approach enabled assessment also of N-acetylated units that amounted to ~30% of total disaccharides residues. The NS-domain(s) of Drosophila HS, although reminiscent of heparin, contained much less O-sulfate

5 U. Lindahl, unpublished data.
as shown by comparative disaccharide analysis (Table 1). Particularly notable, the trisulfated -IdoUA2S-GlcNS6S- unit (where 2S and 6S are 2-O-sulfate and 6-O-sulfate groups, respectively), most abundant in heparin (Table 1), is a minor component of the fly NS-domain. The labeled disaccharides released by low-pH deamination of oligosaccharides generated through Protocol B represent the transition zone between NS- and NA-domains (Fig. 1 and scheme to the right of Fig. 4C). Due to the influence of GlcN N-substituents on O-sulfotransferase reactions, 2-O-sulfate groups are essentially lacking outside NS-domains in HS, and the trisulfated -IdoUA2S-GlcNS6S- unit is entirely restricted to NS-domains (12, 24). Accordingly, ~85% of the labeled disaccharides released by deamination at pH 1.5 of end-labeled Protocol B oligosaccharides were non-O-sulfated, the remainder being mono-O-sulfated exclusively at the 6-O-position (Fig. 4C). Further separation of the non-O-sulfated disaccharides by paper chromatography (supplementary Fig. S2C) showed a similar ratio of GlcUA-aMan6 to IdoUA-aMan6 as seen after direct low-pH deamination (supplementary Fig. S2A).

DISCUSSION

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*Drosophila* has been increasingly applied as a model organism in studies of developmental processes dependent on HS proteoglycan involvement (25, 26). The diverse functions ascribed to HS include scaffold generation in basement membranes (4), stabilization of morphogen gradients (27, 28), and co-receptor functions in various signaling systems (14, 29, 30). Structural analysis of *Drosophila* HS has been invariably based on exhaustive lyase digestion followed by identification of the generated unsaturated disaccharides. Compositional data seemed in agreement with the current understanding of HS structure, pointing to a polysaccharide with approximately one sulfate residue per disaccharide unit (16, 18). Distinctive features were the relatively low content of N-acetylated glucosamine residues, corresponding to ~30% of the total disaccharide units, and low, close to negligible amounts of 6-O-sulfated GlcNAc residues (16, 18). Notably, analysis of lyase-generated disaccharides containing 4,5-unsaturated HexUA residues did not allow a distinction between GlcUA and IdoUA units. The data provided no information regarding the domain organization of *Drosophila* HS.

The results of this investigation are compatible with the previous compositional data (16, 18), yet indicate a novel HS design. The *Drosophila* HS chains were found to contain extended NS-domains with a relatively low degree of O-sulfation (Fig. 5). The number of such domains per chain is unclear but is presumably low; chains featuring a solitary NS-domain appear entirely plausible. The minor tetrasaccharide peak in Fig. 2B suggests that NS-domains might occasionally be separated by single GlcNAc residues, but could also reflect “anomalous ring contraction” during the deamination reaction (31). More abundant N-acetylated disaccharides, presumably corresponding to those previously observed after lyase digestion,
were revealed through hydrazinolysis followed by deamination at pH 3.9 (Fig. 2D), but were not detected as labeled oligosaccharides after deamination at pH 1.5 and NaBH₄ reduction (Protocol A) (Fig. 2B) and therefore would seem not to be part of peripheral NA-domains. We tentatively propose that the disaccharides released through Protocol B were derived from the (GlcUA-GlcNAc)₅ sequence that occurs attached to the -GlcUA-Gal-Gal-Xyl- linkage region in HS (32, 33) and heparin (34) proteoglycans (Fig. 5). The released fly HS was reduced in the presence of the sulfate donor adenosine 3′-phosphosulfate resulted in formation of extended polysaccharide with either NDST recombinant isoform in vitro in the presence of the sulfate donor adenosine 3′-phosphate 5′-phosphosulfate resulted in formation of extended N-sulfated sequence rather than the interrupted domain structure typical of HS (41). These products thus show clear similarity to the Drosophila HS described here. Drosophila expresses only one NDST gene, sulfataseless. Similarly, mammalian mast cells, which express high levels of NDST2 but no or little NDST1 (42), produce heparin, i.e. an extended NS-domain (43, 44). Conversely, in cells that express more than one NDST isoform, HS domain structure appears to depend on the relative amounts of the enzymes (45, 46). On the other hand, genetic ablation of

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TABLE 1

| Disaccharide composition of NS-domains in Drosophila heparan sulfate |
|-----------------|-----------------|-----------------|-----------------|
|                  | GlcUA-GlcNS     | IdoUA-GlcNS     | GlcUA-GlcNS6S  |
| % of N-sulfated  | 33 ± 2 (<1)     | 8 ± 1.5 (<1)    | 23.6 ± 3.1 (10) |
| Disaccharide     | Total non-O-sulfated | 41.5 ± 7 | 40a |
|                  |                  | ~1b            |                 |
|                  | IdoUA-GlcNS6S   |                  |                  |
|                  | Total mono-O-sulfated | 103 ± 1.2 (3)  | 14.3 ± 3.5 (12) |
|                  |                  | 49.2 ± 3.25     | 8a |
|                  |                  | 52a             | 26b             |
|                  |                  | 73b             |                 |

a Corresponding data relating to previous analysis of Drosophila embryo HS based on complete lyase digestion, yielding ~30% N-acetylated and 70% N-sulfated disaccharides (18).

b Corresponding data relating to mucosal heparin assembled from Ref. 47.

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and demonstration that tout-velu, a Drosophila gene related to EXT tumor suppressors, affects heparan sulfate in vivo. J. Biol. Chem. 275, 2269–2275
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