Structural Analysis of Glycosaminoglycans in Animals Bearing Mutations in sugarless, sulfateless, and tout-velu

DROSOPHILA HOMOLOGUES OF VERTEBRATE GENES ENCODING GLYCOSAMINOGLYCAN BIOSYNTHETIC ENZYMES*

Received for publication, April 25, 2000
Published, JBC Papers in Press, May 9, 2000, DOI 10.1074/jbc.M003540200

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Mutations that disrupt developmental patterning in Drosophila have provided considerable information about growth factor signaling mechanisms. Three genes recently demonstrated to affect signaling by members of the Wnt, transforming growth factor-β, Hedgehog, and fibroblast growth factor families in Drosophila encode proteins with homology to vertebrate enzymes involved in glycosaminoglycan synthesis. We report here the biochemical characterization of glycosaminoglycans in Drosophila bearing mutations in sugarless, sulfateless, and tout-velu. We find that mutations in sugarless, which encodes a protein with homology to UDP-glucose dehydrogenase, compromise the synthesis of both chondroitin and heparan sulfate, as would be predicted from a defect in UDP-glucuronate production. Defects in sulfateless, a gene encoding a protein with similarity to vertebrate N-deacetylase/N-sulfotransferases, do not affect chondroitin sulfate levels or composition but dramatically alter the composition of heparin lyase-released disaccharides. N-6-O- and 2-O-sulfated disaccharides are absent and replaced entirely with an unsulfated disaccharide. A mutation in tout-velu, a gene related to the vertebrate Exostoses 1 heparan sulfate co-polymerase, likewise does not affect chondroitin sulfate synthesis but reduces all forms of heparan sulfate to below the limit of detection. These findings show that sugarless, sulfateless, and tout-velu affect glycosaminoglycan biosynthesis and demonstrate the utility of Drosophila as a model organism for studying the function and biosynthesis of glycosaminoglycans in vivo.

Until recently, the analysis of glycosaminoglycan biosynthesis and structure has focused on vertebrate tissues and cultured cells. These studies have produced a wealth of information about the enzymes required for glycosaminoglycan synthesis and the varied structures of these polymers in different cell types and tissues (1). However, glycosaminoglycans are also found in invertebrates, including the fruit fly, Drosophila melanogaster, and the nematode Caenorhabditis elegans. Recent studies have shown that both of these genetically tractable organisms make chondroitin and heparan sulfate (2, 3). Drosophila is notable for a structurally complex heparan sulfate, containing the principal sulfated species represented in vertebrates (2). Both Drosophila and C. elegans therefore provide the means to study the genes required for glycosaminoglycan synthesis in vivo, and the role of these polymers in developmental patterning.

The abbreviations used are: EXT1, Exostoses 1; sgl, sugarless; sfl, sulfateless; ttv, tout-velu; daily, division abnormally delayed; Wg, wingless; Dpp, decapentaplegic; FGFR, fibroblast growth factor receptor; Hh, hedgehog; HPLC, high performance liquid chromatography; GFP, green fluorescent protein; PAPS, adenosine 3'-phosphosulfate; ADP-5-phosphosulfate.
found in mice bearing a gene trap mutation in heparan sulfate 2-O- sulfotransferase argues that distinct modifications of heparan sulfate are indeed crucial for particular patterning events (17). One way to directly investigate the biological functions of specific glycosaminoglycan structures in vivo is to examine patterning in animals bearing mutations in genes affecting distinct biosynthetic activities. A critical step in this approach is to perform structural analysis of glycosaminoglycans from mutants defective for particular genes to establish the molecular changes resulting from the loss of this biosynthetic activity. In earlier work we reported methods for structural analysis of glycosaminoglycans in Drosophila and C. elegans using HPLC separation of disaccharides generated by lyase digestion (2). We have now applied these procedures to the detailed analysis of glycosaminoglycans isolated from animals bearing mutations in three genes proposed to serve roles in glycosaminoglycan biosynthesis on account of their homology to vertebrate genes. Based upon the molecular phenotype of these mutants, we can draw conclusions as to the signaling activities of different classes of glycosaminoglycans during developmental patterning.

EXPERIMENTAL PROCEDURES

Materials—The following standard unsaturated disaccharides from heparan sulfate were purchased from Sigma: 2-acetamido-2-deoxy-4-O-(4-deoxy-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (△UA-GlcNAc), 2-deoxy-2-sulfamido-4-O(4-deoxy-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (△UA-GlcNS), 2-acetamido-2-deoxy-4-O(4-deoxy-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose (△UA-GlcNa6S), 2-acetamido-2-deoxy-4-O(4-deoxy-2-sulfo-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (△UA2S-GlcNAc), 2-deoxy-2-sulfamido-4-O(4-deoxy-2-sulfo-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose (△UA2S-GlcNS), 2-acetamido-2-deoxy-4-O(4-deoxy-2-sulfo-D-threo-hex-4-enopyranosyluronic acid)-D-glucose (△UA2S-GlcNa6S), and 2-deoxy-2-sulfamido-4-O(4-deoxy-2-sulfo-D-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-glucose (△UA2S-GlcNS).

The standard unsaturated disaccharides from chondroitin sulfate and enzymes were purchased from Seikagaku America (Falmouth, MA): 2-acetamido-2-deoxy-3-O-(β-threo-hex-4-enopyranosyluronic acid)-D-galactose (△Di-0S), 2-acetamido-2-deoxy-3-O-(β-threo-hex-4-enopyranosyluronic acid)-D-glucose (△Di-0G), chondroitinase ABC (EC 4.2.2.19), chondroitinase ACII (EC 4.2.2.25), heparinase II (EC 4.2.2.7), heparitinase I (EC 4.2.2.6), and heparitinase II. Ultrafree-MC DEAE and Ultrafree-MC DAEM were obtained from Millipore Corp. (Bedford, MA). All other chemicals used were of analytical reagent grade.

Biomax-5 (5000 NMWL) were obtained from Millipore Corp. (Bedford, MA); 0.1 M sodium acetate buffer (pH 7.0) was used for our analysis, sglP1731 and sgl531. Chromosomes with these mutations were maintained over a Green Fluorescent Protein gene bearing third chromosome balance, TM3 P[w+ Act-GFP], Ser1. Larvae homozygous for sglP1731 and sgl531 were employed for the analysis of sgl function, and maintained over the TM3 balancer as above, tout-velu3h mutants used in this study were maintained as a stock over the second chromosome balance, CyO P[w+, ubq-GFP]. In all cases, homozygous larvae were identified by the lack of GFP fluorescence detectable under UV illumination in a dissecting microscope.

RESULTS

The analysis of glycosaminoglycans in sgl, slf, and ttk larvae consisted of four assays. In the first two, the levels of both chondroitin and 4-O-sulfated chondroitin disaccharides were determined by HPLC separation of chondroitinase-treated material eluted from DEAE membrane under either low (0–0.15 mM NaCl) or high salt (0.15–1.0 mM NaCl) conditions. Similarly, two assays of disaccharides were conducted, one for material retained on DEAE membrane under low salt conditions (0–0.15 mM NaCl), and a second for material that bound to DEAE membrane at salt concentrations between 0.15 and 1.0 M. These experiments therefore provided a profile of chondroitin, low sulfated chondroitin 4-sulfate (Fig. 1, A and B), N-acetyl heparosan (a non-sulfated precursor of heparin), and heparan sulfate (Fig. 2, A and B). The material that elutes prior to the first disaccharide peak for profiles of both chondroitin sulfate and heparan sulfate-derived disaccharides (Figs. 1 and 2) is found enzymatic blank controls, and represents other molecules found in the crude glycosaminoglycan preparation, such as glycoproteins. These molecules react with the fluorophore, 2-cyanoacetamide,
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Fig. 2. Typical chromatograms of unsaturated disaccharides from heparan sulfate in wild-type sgl, sfl, and ttv mutants. A, C, E, and G, 0–0.15 M NaCl fraction; B, D, F, and H, 0.15–1.0 M NaCl fraction. Peaks: 1, ΔUA-GlcNAc; 2, ΔUA-GlcNS; 3, ΔUA-GlcNASeS; 4, ΔUA-GlcNS6S; 5, ΔUA28-GlcNS; 6, ΔUA28-GlcNS6S. sgl compromises the levels of all heparan sulfate-derived disaccharides, whereas sfl mutations block the synthesis of all sulfated disaccharide species without altering appreciably the total amount of heparan sulfate-derived disaccharides (note the increased levels of ΔUA-GlcNAc in the low salt fraction, E, and see Tables II and III). ttv mutations reduce the levels of all heparan sulfate-derived disaccharides to below detectable limits (G and H, see also Tables II and III).

but do not interfere with the analysis of the glycosaminoglycan-derived disaccharides.

Analysis of Wild-type—Separation of glycosaminoglycans released from DEAE membrane with low or high salt washes showed these polymers can be subdivided into distinct classes. The low salt fraction of chondroitinase-sensitive polymers, which represent approximately 20% of the total, was comprised of ΔDi-OS only, whereas the high salt-released fraction showed a mixture of ΔDi-OS and ΔDi-4S (Fig. 1, Table I). Likewise, distinct classes of heparan sulfate polymers were found in the low and high salt DEAE fractions (Fig. 2, Table II). Approximately 10% of the heparan sulfate-derived disaccharide from sgl larvae is nearly the same as that in wild-type, sfl/sgl larvae is nearly the same as that in wild-type, ttv/sgl is not detectable.

sulfataseless—The amino acid sequence of sfl predicts this gene encodes an N-deacetylase/N-sulfotransferase. If sfl does indeed provide this activity in Drosophila, sfl mutants should retain normal levels and composition profiles of chondroitin and chondroitin sulfate, respectively, in sgl mutants (Tables I-III). These findings show that sgl is critical for both chondroitin sulfate and heparan sulfate biosynthesis, and is likely the only gene providing UDP-glucose dehydrogenase activity in Drosophila.

would be expected to be dramatically reduced, but not necessarily eliminated, in sgl mutant second instar larvae (see below). This prediction was borne out; we were unable to detect any chondroitin or chondroitin sulfate in the fraction of glycosaminoglycans from sgl mutants retained on DEAE under high salt conditions (Fig. 1, Tables I and III). A trace amount of ΔDi-OS was detected in chondroitinase-sensitive glycosaminoglycans from sgl larvae that bound to DEAE under low salt conditions (Fig. 1C). sgl mutations also had a profound effect on heparan sulfate biosynthesis. Trace amounts of ΔUA-GlcNAc were found only in the low salt preparation (Fig. 2C, Tables II and III).

Table I

| Unsaturated disaccharide | Total amount in dry tissue | mg/mg |
|--------------------------|---------------------------|-------|
| Wild-type                |                           |       |
| ΔDi-OS                   | 100.0                     | 29.5  |
| ΔDi-4S                   | ND                        |       |
| sgl/sgl                  |                           |       |
| ΔDi-OS                   | 76.6                      | 113.1 |
| ΔDi-4S                   | 23.4                      | 13.2  |
| sfl/sfl                  |                           |       |
| ΔDi-OS                   | 100.0                     | 124.2 |
| ΔDi-4S                   | 19.0                      | 30.2  |
| ttv/ttv                  |                           |       |
| ΔDi-OS                   | 77.5                      | 90.2  |
| ΔDi-4S                   | 22.5                      |       |

* LC, low-charged form.
* ND, not detected.
* HC, high-charged form.


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TABLE II
Comparison of heparan sulfates from wild-type and mutant Drosophila larvae

| Unsaturated disaccharide | Wild-type | sfl/sfl | ttv/ttv |
|--------------------------|----------|---------|---------|
| % UA-GlcNAc | % UA-GlcNS | % UA-GlcNa6S | % UA-GlcNS6S | % UA2S-GlcNAc | % UA2S-GlcNS | Total amount in dry tissue |
| LC | 100.0 | ND | ND | ND | ND | ND | 1.2 |
| HC | 32.6 | 23.9 | 2.1 | 19.2 | 16.3 | 5.9 | 12.4 |

a LC, low-charged form.
b ND, not detected.
c HC, high-charged form.

TABLE III
Summary of glycosaminoglycan levels and disaccharide forms represented in wild-type and mutant larvae

| Genotype | Chondroitin sulfate-derived disaccharides | Heparan sulfate-derived disaccharides |
|----------|----------------------------------------|-------------------------------------|
|          | Total amount | Disaccharides* | Total amount | Disaccharides* |
|          | dng/mg dry tissue | | dng/mg dry tissue | | |
| Wild-type | 142.6 | ΔDi-OS (77%) | 13.9 | ΔUA-GlcNAc (33%), ΔUA-GlcNS (24%), ΔUA-GlcNa6S (2%), ΔUA-GlcNS6S (6%) |
|          | 34-fold reduction | Trace amount of ΔDi-OS | 12-fold reduction | Trace amounts of ΔUA-GlcNAc |
| sgl/sgl (UDP-glucose dehydrogenase) | 4.2 | ΔDi-OS (81%) | 12.3 | ΔUA-GlcNAc (100%) |
|          | 12-fold reduction | ΔDi-4S (19%) | polymer of nonsulfated repeats of uronic acid and GlcNAc, called N-acetyl heparosan, all in low salt fraction |
|          | 79% of total in high salt fraction | Composition unchanged | | |
| ttv/ttv (HS copolymerase/EXT1) | 120.4 | ΔDi-OS (78%) | ND | ND |
|          | 13-fold reduction | ΔDi-4S (22%) | At least 15-fold reduction | Composition unchanged |

a Compositions for each disaccharide are those obtained for material in high salt fraction.
b ND, not detected.

as wild-type (12.3 versus 13.9 ng/mg for sfl and wild-type, respectively), indicating that differences in recovery do not account for the changes in composition we observe. These findings show that sfl: 1) affects heparan sulfate synthesis and not chondroitin or chondroitin sulfate generation, 2) is required for the sulfation at the N-position of GlcNAc, the 2-O-position of either glucuronic or iduronic acid, and the 6-O-position of GlcNAc and GlcNS; and 3) results in the accumulation of N-acetyl heparosan, a nonsulfated precursor of heparin, at the expense of sulfated forms of disaccharides.

tout-velu—Earlier studies showed that ttv, a gene related to vertebrate EXT1, affects the levels of heparan sulfate in vivo (2, 9). These finding were consistent with ttv encoding a heparan sulfate co-polymerase. We have extended these experiments to determine precisely what glycosaminoglycans remain in ttv mutant larvae. Chondroitin sulfate-derived disaccharides are largely unchanged, with a modest reduction in levels while retaining a wild-type composition in both the low and high salt fractions (Fig. 1 and Table I). All heparan sulfate-derived disaccharides, however, showed a marked reduction, essentially below our detectable limits of 1.5 ng (Fig. 2, G and H, and Tables II and III). The selective and substantive effect of ttv on heparan sulfate levels in vivo indicates that ttv is critical for the bulk of heparan sulfate biosynthesis.

DISCUSSION

Roles of sgl, sfl, and ttv in Glycosaminoglycan Biosynthesis—We have examined the structures of glycosaminoglycans in animals bearing mutations in genes encoding proteins with homology to three known glycosaminoglycan biosynthetic enzymes. In all three cases, the changes in whole animal glycosaminoglycan profiles are consistent with the Drosophila gene providing the enzymatic activity predicted from the relatedness to the vertebrate genes (Table III, Fig. 3).

sgl mutants show dramatic reductions in both chondroitin and heparan sulfate levels, consistent with a defect in UDP-glucuronate generation catalyzed by a UDP-glucose dehydrogenase. These findings support earlier studies showing that modification of Dally (14) and Syndecan (4) is altered in sgl mutants. The residual levels of chondroitin and heparan sulfate-derived disaccharides remaining in sgl mutants most likely results from maternal contributions of wild-type sgl mRNA to the embryo, which provides sufficient activity for normal embryonic development. The trace amounts we detect indicate that zygotic sgl function is required for continued glycosaminoglycan biosynthesis once maternal stores are exhausted.

sfl, a Drosophila gene encoding a protein related to verte-
The analysis of glycosaminoglycans in sgl, sfl, and ttv mutants has shown that the glycosaminoglycan biosynthetic apparatus is largely conserved between Drosophila and vertebrates. The structural forms of chondroitin and heparan sulfate represented in Drosophila are also those found in vertebrate species. In addition, the core proteins for several families of proteoglycans are found in Drosophila, including glypican (13) and syndecan (21). These findings demonstrate the utility of Drosophila as a model organism for studying the biosynthesis and function of glycosaminoglycans and proteoglycans in vivo.

Glycosaminoglycans in Developmental Patterning and Growth Factor Signaling—Our analysis of sgl, sfl, and ttv allows us to make some conclusions concerning the functions of various glycosaminoglycan forms in growth factor signaling and morphogenesis. Of these three mutations, sgl and sfl would appear to have the broadest effects on signaling, compromising both Wg and FGFR-directed events. Furthermore, there is good evidence that sgl is required for dpp signaling in imaginal tissues (4, 22). sfl apparently is also required for normal Hh signaling in the developing wing. While it is not known if sgl and sfl influence Wg, Dpp, FGFR, and Hh signaling equally, their comparable participation in Wg and FGFR signaling suggests that loss of both chondroitin and heparan sulfate in sgl mutants produces no more deleterious effect than a failure to add sulfate modifications to heparan sulfate. It would seem therefore, that at least for FGFR and Wg signaling, heparan sulfate is the critical glycosaminoglycan in vivo.

In contrast to the multiple growth factor pathways affected by sgl and sfl, ttv selectively influences Hh signaling, leaving Wg and FGFR-mediated patterning untouched in both the embryo and in the imaginal discs (8, 22). The large reductions in heparan sulfate levels that accompany loss of ttv function might lead one to predict that ttv would seriously compromise signaling mediated by several growth factors known to require sulfated heparan sulfate, Wg and FGFR in particular. Yet this is not the case. In embryos lacking both maternal and embryonically derived Ttv activity, FGFR and Wg signaling remain (9).

Several explanations for the selective participation of ttv in Hh signaling are possible in light of these findings. First, we have examined glycosaminoglycans in larvae, and the studies where ttv function has been completely removed were primarily performed using embryos. It is possible that there are higher levels of heparan sulfate in ttv mutant embryos compared with larvae, presumably derived from other EXT-related genes that could provide co-polymerase activity. Second, the experiments looking at ttv function in larvae made use of methods to induce recombination events in ttv/+ animals midway through development that produce clones of ttv/+ cells. Sufficient Ttv may remain in these clones to provide significant levels of heparan sulfate co-polymerase activity, levels sufficient for Wg and FGFR signaling, but not for Hh. ttv mutants may also contain small amounts of specific proteoglycans that are heparan sulfate-modified normally and permit normal FGFR and Wg signaling (9). This explanation would require Ttv to have core protein selectivity, placing heparan sulfate chains on a distinct set of proteoglycans. Finally, it is possible that in the absence of any appreciable heparan sulfate co-polymerase activity, proteoglycan core proteins are modified with chondroitin sulfate, and that this sugar polymer is capable of supporting Wg and FGFR signaling, but not Hh signaling or transport. In this scenario, sfl mutations have such a broad affect because N-acetyl heparan sulfate chains are added to their normal protein cores, but do not function because of their lack of sulfation. The presence of heparan chains would preclude their substitution with chondroitin sulfate, preventing rescue of proteoglycan function in Wg and FGFR signaling by chondroitin sulfate modification.
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