Specific and flexible roles of heparan sulfate modifications in Drosophila FGF signaling

Keisuke Kamimura, Takashi Koyama, Hiroko Habuchi, Ryu Ueda, Masayuki Masu, Koji Kimata, and Hiroshi Nakato

1Department of Genetics, Cell Biology, and Development, The University of Minnesota, Minneapolis, MN 55455
2Institute for Molecular Science of Medicine, Aichi Medical University, Aichi 480-1195, Japan
3Invertebrate Genetics Laboratory, Genetic Strain Research Center, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan
4Department of Molecular Neurobiology, Institute of Basic Medical Sciences, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Ibaraki 305-8577, Japan

Specific sulfation sequence of heparan sulfate (HS) contributes to the selective interaction between HS and various proteins in vitro. To clarify the in vivo importance of HS fine structures, we characterized the functions of the Drosophila HS 2-O and 6-O sulfotransferase (Hs2st and Hs6st) genes in FGF-mediated tracheal formation. We found that mutations in Hs2st or Hs6st had unexpectedly little effect on tracheal morphogenesis. Structural analysis of mutant HS revealed not only a loss of corresponding sulfation, but also a compensatory increase of sulfation at other positions, which maintains the level of HS total charge. The restricted phenotypes of Hsst mutants are ascribed to this compensation because FGF signaling is strongly disrupted by Hs2st; Hs6st double mutation, or by overexpression of 6-O sulfatase, an extracellular enzyme which removes 6-O sulfate groups without increasing 2-O sulfation. These findings suggest that the overall sulfation level is more important than strictly defined HS fine structures for FGF signaling in some developmental contexts.

Introduction

Secreted signaling proteins, such as BMPs, Wnts, Hedgehog, and FGFs, play key roles in animal development. Although it is established that reception of these molecules on the cell surface is mediated by heparan sulfate proteoglycans (HSPGs), the mechanism producing selective binding of proteins to heparan sulfate (HS) in a growth factor–rich environment remains a fundamental question. HS is synthesized as disaccharide polymers, which then undergoes a series of modification events including N, 2-O, 6-O, and 3-O sulfation. A number of in vitro studies showed that interactions between HS and various growth factors require unique HS structures in which 2-O and 6-O sulfate groups contribute to generate specific sulfation patterns (for reviews see Nakato and Kimata, 2002; Habuchi et al., 2004). Crystallographic studies also supported this biochemical evidence, showing that the 2-O and 6-O sulfate groups form hydrogen bonds with heparin binding residues of FGFs and/or FGF receptors (FGFRs) to induce dimerization of FGFRs (Schlessinger et al., 2000). Thus, in vitro studies showed that specific sulfation patterns on HS have critical roles in its selective binding to ligand proteins. However, the in vivo importance of these sulfation events is poorly understood.

FGF signaling regulates tracheal system formation in Drosophila (Klambt et al., 1992; Sutherland et al., 1996). The tracheal precursor cells express Breathless (Btl), a Drosophila FGF receptor, and migrate toward regions expressing Branchless (Bnl; a Drosophila FGF) to form primary branches in the embryo. FGF also controls the formation of the adult tracheal system, the air sac, which develops from a group of cells called “tracheoblasts” in the wing disc (Sato and Kornberg, 2002). A previous study showed that Btl-dependent activation of MAP kinase relies on sulfatless (sfl), which encodes N-deacetylase/ N-sulfotransferase (NDST), indicating that HS has a crucial role in these processes (Lin et al., 1999). Because the reaction catalyzed by NDST is the first step in HS modification and is critical for subsequent reactions, mutation of sfl results in the production of sugar chains with no sulfation (Toyoda et al., 2000). To determine what structural features of HS are required for regulating FGF signaling, we characterized functions of HS 2-O sulfotransferase (Hs2st) and HS 6-O sulfotransferase (Hs6st) genes during tracheal development.

Correspondence to Hiroshi Nakato: nakato003@umn.edu

Abbreviations used in this paper: bnl, branchless; btl, breathless; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; Hs2st, heparan sulfate 2-O sulfotransferase; Hs6st, heparan sulfate 6-O sulfotransferase; sfl, sulfatless; trh, trachealless.

The online version of this article contains supplemental material.
Results and discussion

We generated Hs2st and Hs6st mutations by imprecise P-element excision (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200603129/DC1). The excision alleles, Hs2stD267 and Hs6stD770, delete their respective coding regions, and lethality of Hs2stD267 and Hs6stD770 homozygotes was equivalent to that of their deficiency transheterozygotes, indicating that these mutants are null alleles for each gene. Despite the previous implication of 2-O and 6-O sulfate groups in the binding of HS to many growth factors in vitro (for reviews see Nakato and Kimata, 2002; Habuchi et al., 2004), Hs2st and Hs6st mutants showed only moderate effects on development. Zygotic Hs2st and Hs6st mutants survive to the adult stage without showing obvious morphological defects. We also generated embryos in which both maternal and zygotic Hsst gene activities are eliminated (“Hsst null embryos”). Although such null mutations caused partial lethality during development, significant fractions of these null mutants survive to the adult stage without visible phenotypes. This finding demonstrated that loss of either 2-O or 6-O sulfation does not completely disrupt normal development.

Because 2-O and 6-O sulfations are critical for FGF-HS binding in vitro (for reviews see Nakato and Kimata, 2002; Habuchi et al., 2004), we focused our efforts on the function of Hs2st and Hs6st in btl-mediated tracheal migration. The tracheal system develops from clusters of ectodermal cells that invaginate into the underlying mesoderm and form ten sacs on each side of the embryo. Each sac forms six primary branches by stereotypical cell migration. Some of these branches, such as the dorsal trunk, fuse with corresponding branches in neighboring segments to form a continuous tracheal network (Fig. 1 A).

In btl or sfl mutants, the tracheal cells remain clustered at the site of the tracheal pits without migration (Klambt et al., 1992; Lin et al., 1999). In contrast, we found that maternal and zygotic null mutations of Hs2st or Hs6st had only limited effects on tracheal development (Fig. 1, D–F). Remarkably, only 9% of Hs2st null embryos exhibited a stalled migration of the dorsal branch (Fig. 1 D). A fraction (39%) of Hs6st null embryos exhibited tracheal defects (Fig. 1 E). In these mutant embryos tracheal migration is incomplete, as revealed by the presence of large gaps in the dorsal trunks, as well as stalled tracheal branches. The migration defects in these embryos were observed in all primary branches, but most commonly in the dorsal branch and the dorsal trunk. Surprisingly, however, tracheal morphology was indistinguishable from that of wild-type embryos in the remaining 61% of the embryos (Fig. 1 F).

Next, we examined whether Hs2st and Hs6st mutations affect the formation of the tracheoblast in the wing disc. Normal development of tracheoblasts was observed in all Hs2st and most Hs6st mutants we examined (Fig. 1, I and J), although the tracheoblast was slightly reduced in size in a small fraction (18%) of Hs6st mutant discs (Fig. 1, K and L). The small tracheoblast phenotype of Hs6st mutants was completely rescued by Hs6st expression from a UAS-Hs6st transgene (Fig. 1 M). Thus, null mutations in Hs2st and Hs6st do not completely block btl-mediated tracheal formation, showing that Hs2st and Hs6st mutant animals can produce HS chains that retain a considerable level of activity to mediate FGF signaling.
The modest tracheal phenotypes of *Hs2st* and *Hs6st* null mutants clearly challenge a current view on the role of HS fine structures: numerous biochemical analyses have demonstrated that 2-O and 6-O sulfate groups are critically required for the HS-growth factor interaction (for reviews see Nakato and Kimata, 2002; Habuchi et al., 2004). One possible reason for the restricted phenotypes of these mutants is that the sulfation patterns of mutant HS are altered to restore the growth factor signaling. To examine this possibility, disaccharide profiles of HS from *Hs2st* and *Hs6st* mutant animals were determined using fluorometric post-column HPLC (Toyoda et al., 2000). In wild-type adult flies, the disaccharide composition of HS showed a similar pattern to representative vertebrate tissues (Toyoda et al., 2000). In contrast, HS samples from *Hs2st* or *Hs6st* zygotic mutant adults showed a complete loss of the corresponding disaccharide units, confirming the amorphic nature of these mutant alleles. Significantly, HS disaccharides from *Hs2st* mutants showed not only a loss of 2-O sulfated disaccharide units, but also a remarkable increase of 6-O sulfated disaccharides. Similarly, levels of the 2-O sulfated disaccharides are strikingly elevated in *Hs6st* mutants. As a result, the level of total sulfate groups on HS was not affected in each case, and the total charge of HS in *Hs2st* and *Hs6st* mutants was almost wild type (Fig. 2). These results strongly suggested the existence of a compensation mechanism that adjusts the levels of sulfate groups when a component of the HS-modification machinery is lacking. Importantly, similar compensation of HS sulfation has also been observed in *Hs2st* mutant mice (Merry et al., 2001), implicating this system as a general property of the HS modification machinery that is widely conserved across species. Thus, the unaltered charge levels on HS in the *Hs2st* and *Hs6st* mutants is widely conserved across species. Thus, the unaltered charge levels on HS in the *Hs2st* and *Hs6st* mutants challenges a current view on the role of HS sulfation weakens *Hs2st* and *Hs6st* double-mutant animals. In these animals, the compensation of HS sulfation would not occur due to the absence of both counterparts (*Hs2st* and *Hs6st*) that complement each other in *Hs2st* or *Hs6st* single mutants. In fact, despite the relatively normal development of the single mutants, the *Hs2st*; *Hs6st* zygotic double mutants are completely lethal. In wild-type embryos, tracheal precursor cells invaginate in each hemisegment at stage 11 (Fig. 3 A), and migrate and elongate to form primary branches at stage 12 (Fig. 3 D). One of these branches, the dorsal trunk, fuses with ones in the neighboring segments at stage 14 (Fig. 3 G). Although invagination seems to occur normally in the *Hs2st*; *Hs6st* embryos, they exhibit several characteristic defects in branching morphogenesis. First, mutant tracheal precursor cells failed to migrate to form the primary branches (Fig. 3 H). This defect resembles that of *btl* or *sfl* mutants (Klamt et al., 1992; Lin et al., 1999). Second, clusters of mutant tracheal cells tend to extend dorsally and ventrally, forming long, skinny sacs of tracheal precursor cells of various size (Fig. 3, E and H). Finally, 16% of mutant embryos showed fusion of the tracheal sacs to those in the neighboring segments (Fig. 3, C and I).

We asked whether FGF signaling is impaired in these animals using an antibody that specifically recognizes the diphosphorylated form of MAP kinase (dpMAPK; Gabay et al., 1997). In wild-type embryos, dpMAPK is detected in the tracheal placodes at stage 10, reflecting activation of DER, a *Drosophila* EGF receptor (Gabay et al., 1997). This dpMAPK signal was not diminished in the *Hs2st*; *Hs6st* embryos, showing that DER signaling is not affected by the double mutations (Fig. 3, K, K'; Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200603129/DC1). At stage 12, wild-type embryos show a strong dpMAPK signal in the migrating tip cells of each primary branch due to activation of FGF signaling (Gabay et al., 1997). In contrast, the *btl*-dependent MAPK activation in the tip cells is disrupted in the *Hs2st*; *Hs6st* embryos (Fig. 3, M, M'; Fig. S2 A). In situ RNA hybridization experiments revealed that *btl* expression is not altered in the double mutant embryos (Fig. S2 C), confirming that the branching defects observed in the

![Figure 2](image-url)  
**Figure 2.** HS disaccharide profiling of *Hs2st* and *Hs6st* mutants. (Left) Representative HPLC chromatograms of wildtype (black), *Hs2st* (pink), and *Hs6st* (green) mutant HS. (Middle) Graphical depiction of disaccharide composition in these mutants, represented as percentage of total HS. (Right) Total levels of sulfate groups in *Hs2st* and *Hs6st* mutants. The value indicates the ratio of total sulfate groups in mutants to that in wild type.
double mutants are caused by disruption of FGF reception but not FGF expression. These results showed that HS with neither 2-O nor 6-O sulfate groups lost the ability to mediate Btl signaling.

Next, we examined whether simultaneous loss of both 2-O and 6-O sulfate groups affects tracheoblast formation in the wing disc. Because Hs2st; Hs6st mutants die during embryogenesis, we analyzed Hs2st homozygous animals bearing a transgene that expresses double-stranded RNA for Hs6st (Hs6st RNAi) under a specific Gal4 driver (Kennerdell and Carthew, 2000). Tracheoblast development was not affected either by homozygosity of the Hs2st null mutation (Fig. 1 I) or by expression of the Hs6st RNAi construct (unpublished data). In contrast, Hs6st RNAi in btl-expressing (tracheal) cells in Hs2st homozygous mutant background completely blocked the formation of the tracheoblast (Fig. 3 O). No such effect was observed, however, when the Hs6st RNAi was induced in bnl-expressing (nontracheal) cells in the same mutant background (Fig. 3 P). Thus, HS requires either 2-O or 6-O sulfate groups for reception of FGF, but these modifications are not essential in the FGF-expressing cells. Collectively, tracheal development could occur in Hs2st or Hs6st single mutants, but not in the double mutants. These findings demonstrated redundant roles of 2-O and 6-O sulfate groups of HS in FGF signaling during tracheal development.

As another approach to reduce 6-O sulfation without inducing an increase of other sulfation events, we examined the effects of overexpressing Sulf1, a Drosophila extracellular sulfatase (CG6725), on FGF signaling. Vertebrate Sulf genes encode secreted HS 6-O sulfatases, which remove sulfate groups from the HS on the cell surface (Dhooit et al., 2001). Because Sulf1 seems to modify HS fine structure extracellularly, and we hypothesized that compensatory changes in sulfation occur during HS biosynthesis in the Golgi, we expected that the number of sulfate groups on HS in Sulf1-expressing animals would decrease. Indeed, this was the case. Disaccharide profiling of HS from actin-Sulf1 animals showed a significant reduction in the level of 6-O sulfation without the compensatory increase of other sulfate groups (Fig. 4 A). As a result, the total sulfate level is reduced in these animals to 76.3% of the wild-type level. Importantly, overexpression of Sulf1 had stronger effects on viability and FGF-mediated tracheogenesis than Hs6st mutations. actin-Sulf1 animals showed 71% lethality (unpublished data). The tracheoblast was dramatically reduced in size by expression of Sulf1 in btl-expressing (tracheal) cells (Fig. 4 B). The fact that Sulf1-expressing animals show more severe phenotypes than Hs6st null mutants strongly suggests that the compensatory increase of 2-O sulfation in Hs6st mutant HS restores the ability to mediate FGF signaling. From these findings, we conclude that biosynthesis and modification of HS show a striking flexibility. In the absence of a component of the HS-modification machinery, living cells can form HS that lacks normal fine structures but retains normal levels of sulfate groups and a considerable level of activity for growth factor signaling.

Numerous in vitro studies have identified various ligand proteins that bind to specific sulfated HS sequences. Recent studies using animal models have also highlighted the importance of distinct HS sulfation patterns for HSPG functions (Bulow and Hobert, 2004). Thus, it is widely accepted that a specific sequence of sulfation on HS determines a binding site for a ligand, enabling HSPGs to interact selectively with proteins. However, it is not known how strictly ligand binding sites are defined in vivo. Our study demonstrated that living cells show an unexpected level of flexibility in biosynthesis and function of HS. In vivo HS sulfation is flexible in two ways. First, HS modifications can be adjusted in response to a defect in one type of sulfation. Second, mutant HS chains thus synthesized, which do not contain normal sequences of sulfate groups but...
bear normal levels of sulfation, do not completely lose coreceptor activity for growth factor signaling.

We found that *Drosophila Hst* mutations induce compensatory increases in sulfation at other positions, restoring a wild-type net charge on HS. Previously, Merry et al. (2001) showed that HS purified from *Hs2st*−/− embryonic fibroblasts did not have 2-O sulfate groups, but this loss was compensated for by increased N- and 6-O sulfation. This study suggested that a novel structure of HS found in the mutant HS may rescue the wild-type net charge on HS. Previously, Merry et al. (2001) showed that compensatory increases in sulfation at other positions, restoring a wild-type net charge on HS. In particular, sulfation at the 3-O position of various sulfate groups (Kreuger et al., 2005; Jastrebova et al., 2006). Collectively, these observations suggest that some in vivo roles of HS–protein interaction. In particular, sulfation at the 3-O position of various sulfate groups (Kreuger et al., 2005; Jastrebova et al., 2006).

Materials and methods

Fly stocks

The detailed information for fly strains used is described in Flybase (http://flybase.bio.indiana.edu/), except where noted. All flies were maintained at 25°C. The following strains were used: Oregon-R, wild-type strain; P(GSV6)9303 (see the *Drosophila* Gene Search Project web site: http://218.44.182.94/%7Edclust/) and P(AB)A201.1M3, P-element insertion lines for *Hs2st* and *Hs6st*, respectively; *Hs2st*Δ9, and *Hs6st*Δ770, null mutants for *Hs2st* and *Hs6st*, respectively (see below for mutant isolation); *UAS*Δ9, a null allele of *sfl*; Df(2)E55 (breakpoints, 37D02-E01; 37F05-38A01) and Df(3R)94a (breakpoints, 92B02-92C02-03), chromosomal deficiency lines; 1-ve-l, an enhancer trap line for the tracheole (thl) gene. The transgenic animals used were as follows: UAS-GFPnls; UAS-FLP; UAS-Sulf1 (Sulf1 cDNA [SD04414, Berkeley Drosophila Genome Project] was fused to a 344-bp *Sulf1* genomic PCR fragment to complete the coding region and inserted into pUAST vector); UAS-IR-Hs6st (see below for construction of *Hs6st* transgenic RNAi flies); nanos-Gal4; actin-Gal4; and btl-Gal4 (strain number 2211; Drosophila Genetic Resource Center, Kyoto Institute of Technology, Japan).

Isolation of *Hs2st* and *Hs6st* mutants

To generate *Hs2st*Δ9 and *Hs6st*Δ770 mutants, P(GSV6)9303 and P(AB)A201.1M3 were exposed to P element transposase from *P(y′, δ2-3)* (P{ry}). Their progeny were screened for loss of marker gene expression. Excision chromosomes were analyzed by PCR using flanking primers to find deletions, and the extent of each deletion was determined by sequencing PCR products that spanned the junction (see the legend to Fig. S1 for details). Lethality of *Hs2st*Δ9 and *Hs6st*Δ770 homozygotes [3.8% and 43%, respectively] was equivalent to that of their deficiency transheterozygotes [Df(2)E55/*Hs2st*Δ9; Df(3R)94a/*Hs6st*Δ770, respectively], indicating that these mutants are null alleles for each gene.

Generation of embryos lacking maternal and zygotic function of *Hs2st* and *Hs6st*

Embryos lacking maternal and zygotic activity of *Hs2st* were obtained by crossing *Hs2st*Δ9 homozygous females with *Hs2st*Δ9/CyO wg-lacZ males. To obtain *Hs6st* maternal and zygotic mutant embryos, germ line clones were generated using the autosomal FLP-DHS technique (Chou et al., 1993). Females carrying nanos-Gal4 UAS-FLP/+; FRT82B *Hs6st*Δ770/FRT82B ovoD1 were mated with *Hs6st*Δ770/TM3 Sb ftz-lacZ. The resultant maternal and zygotic mutant embryos were identified with marked balancer.

Construction of *Hs6st* transgenic RNAi flies

Transgenic RNAi flies of *Hs6st* were obtained as described previously (Kamimura et al., 2004). A 500-bp-long cDNA fragment from the first
methionine was amplified by PCR and inserted as an inverted repeat (IR) into a modified pBluescript vector, pSC1, which possesses an IR formation site. IR-containing fragments were subcloned into pUAST, a transformation vector, and transformation of Drosophila embryos was performed using w1118 as a recipient strain.

Immunostaining and in situ RNA hybridization
Antibody staining was performed as described previously (Kamimura et al., 2004) using rabbit anti-β-galactosidase (1:500; Cappell) and mouse anti-diphosphorylated MAP kinase (1:200; Sigma-Aldrich). The primary antibodies were detected with Alexa Fluor-conjugated secondary antibodies (1:500; Molecular Probes). For quantitative analysis of MAPK activation, the percentage of segments that show normal dpMAPK staining in tracheal precursor cells (stage 10 wild type, n = 12; stage 10 Hs2st; Hs6st, n = 18; stage 12 wild type, n = 27; and stage 12 Hs2st; Hs6st, n = 21) was calculated. In situ RNA hybridization was performed as described previously (Kamimura et al., 2004). Light microscopy images were taken using a microscope (model BX50; Olympus) with a 40×/0.75 UPlanFl objective by a CCD camera (DP-50; Olympus) controlled by Studio Lite software. Confocal imaging was performed using a microscope (Axiovert 200M; Carl Zeiss MicroImaging, Inc.) with a 40×/0.75 Plan-Neofluar objective equipped with a confocal microscope system and a software [LCM5 PASCAL; Carl Zeiss MicroImaging, Inc.]. Images were processed using Photoshop 7.0 (Adobe).

Preparation and HPLC analysis of HS disaccharides
HS disaccharide was analyzed by fluorometric post-column HPLC as described previously (Toyoda et al., 2000). Approximately 50 mg of lyophilized adult flies was used to isolate HS. The HS sample was digested with a heparitinase mixture (Seikagaku) and subjected to a reversed-phase C18 column. HS disaccharides were eluted using a gradient of water and methanol, and their retention times were determined with a fluorescence detector. The eluted HS disaccharides were analyzed by reversed-phase HPLC as described previously (Toyoda et al., 2000). Approximately 50 mg of lyophilized adult flies was used to isolate HS. The HS sample was digested with a heparitinase mixture (Seikagaku) and subjected to a reversed-phase C18 column. HS disaccharides were eluted using a gradient of water and methanol, and their retention times were determined with a fluorescence detector. The eluted HS disaccharides were analyzed by reversed-phase HPLC as described previously (Toyoda et al., 2000).

Online supplemental material
Fig. S1 shows the molecular characterization of Hs2st and Hs6st mutants. Fig. S2 shows the quantitative analysis of MAPK activation and in situ RNA hybridization of bln mRNA in wild-type and Hs2st; Hs6st embryos. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200603129/DC1.

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