Heparan sulfate (HS) regulates the activity of various ligands and is involved in molecular recognition events on the cell surface and in the extracellular matrix. Specific binding of HS to different ligand proteins depends on the sulfation pattern of HS. For example, the interaction between antithrombin and a particular 3-O sulfated HS motif is thought to modulate blood coagulation. However, a recent study of mice defective for this modification suggested that 3-O sulfate groups along the HS chains (for review see Esko and Lindahl et al., 1998) is not essential for binding to FGF, but is critical for activation of the FGF receptor (for review see Nakato and Kimata, 2002). In contrast, binding of platelet-derived growth factor, hepatocyte growth factor, lipoprotein lipase, and herpes simplex virus (HSV) glycoprotein C to HS are all dependent on 6-O sulfation (for review see Lindahl et al., 1998). Thus, the biological functions of HSPGs are controlled by biosynthetic events that define the fine structures of HS.

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Introduction

Heparan sulfate proteoglycans (HSPGs), abundant components of the cell surface and the extracellular matrix, are critically involved in a variety of biological phenomena, including cell adhesion, proliferation, and differentiation. The diverse functions of HSPGs are thought to be mediated by highly specific interactions between various binding proteins and distinct sequence motifs of the heparan sulfate (HS) chains. Such specific structures are predominantly determined by the regulated positioning of N-, 2-, 6-, and 3-O sulfate groups along the HS chains (for review see Esko and Selleck, 2002). For example, FGF-2 requires both N- and 2-O sulfate groups for binding to HS. The 6-O sulfate group is not essential for binding to FGF, but is critical for activation of the FGF receptor (for review see Nakato and Kimata, 2002). In contrast, binding of platelet-derived growth factor, hepatocyte growth factor, lipoprotein lipase, and herpes simplex virus (HSV) glycoprotein C to HS are all dependent on 6-O sulfation (for review see Lindahl et al., 1998). Thus, the biological functions of HSPGs are controlled by biosynthetic events that define the fine structures of HS.

The first and still the most notable example showing the importance of sequence-specific sulfation in HS–protein interactions comes from analyses of 3-O sulfated HS. The 3-O sulfate group is the rarest component in HS, and 3-O sulfation occurs late in the HS biosynthesis pathway (for review

Abbreviations used in this paper: ACV, anterior cross vein; A/P, anterior–posterior; AT, antithrombin; BDGP, Berkeley Drosophila Genome Project; cx, cut; DI, Delta; dpp, decapentaplegic; ds, deltex; D/V, dorsal–ventral; E(spl)C, Enhancer of split Complex; gD, glycoprotein D; HS, heparan sulfate; HS3ST, HS 3-O sulfotransferase; HSPG, HS proteoglycan; HSV, herpes simplex virus; IR, inverted repeat; kuz, kuzbanian; N, Notch; Nic, extracellular domain of N protein; neur, neuralized; N[ICD], intracellular domain of N protein; RNAi, RNA interference; sca, scabreuse; Ser, Serrate; SMC, sensory organ mother cell; ST, sulfotransferase; Su(H), Suppressor of Hairless; UAS, upstream activating sequence; vg, vestigial; wg, wingless.

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Figure 1. Structures and enzymatic activities of Drosophila Hs3sts. (A) Comparison of the amino acid sequences of human and Drosophila HS3STs. The program “Clustal W” was used to align amino acid sequences of human HS3STs and Drosophila Hs3sts. Consensus residues among four HS3STs are colored red. Identical residues between HS3ST-1 and Hs3st-A and between HS3ST-3A and Hs3st-B are marked in blue and green, respectively. The arrowhead indicates the potential N-glycosylation signal conserved in all HS3STs. (B) A dendrogram comparing sulfotransferase (ST) domains from all human and Drosophila isoforms. The dendrogram was generated by calculating the average similarity of amino acid sequences of the ST domain as determined by the University of Wisconsin Genetics Computer Group program “Distances” (Shworak et al., 1999). The preferred ligands of HS treated with HS3ST-1 (Shworak et al., 1997), HS3ST-3A (Shukla et al., 1999), HS3ST-3B (Shukla et al., 1999), HS3ST-2, HS3ST-4 (unpublished data), and HS3ST-5 (Xia et al., 2002; HajMohammadi et al., 2003) are shown. Drosophila Hs3sts are colored red. The HS3ST multigene family is comprised of two structurally distinct groups. The following information for amino acid sequences of human HS3STs was used: GenBank/EMBL/DDBJ accession no. AF019386 (HS3ST-1), AF105374 (HS3ST-2), AF105376 (HS3ST-3A), AF105377 (HS3ST-3B), AF105378 (HS3ST-4), AF50392 (HS3ST-5), and the unpublished sequence for HS3ST-6 (unpublished data). (C) Enzymatic sequence specificity of Hs3st-B. Generation of functional HS10<sup>\text{<sup>10+</sup>}</sup> was assessed by transfecting CHO cells with the indicated plasmids. Transfectants were exposed to various concentrations of HSV-1(KOS)tk12 (Warner et al., 1998), which expresses
see Rosenberg et al., 1997). In humans, there are at least seven HS 3-O sulfotransferases (HS3STs) that produce at least two distinct forms of 3-O sulfated HS (Shworak et al., 1997, 1999; Shukla et al., 1999; Xia et al., 2002). One form of 3-O sulfated HS plays a critical role in the blood coagulation cascade. HS3ST-1 generates HS$^{AT^+}$: HS with a specific 3-O sulfated motif that binds to antithrombin (AT). In therapeutic heparin administration, this interaction leads to inactivation of thrombin, resulting in inhibition of blood coagulation (Rosenberg et al., 1997; Shworak et al., 1999). However, a recent report showed that Hs3st1$^{-/-}$ knockout mice do not exhibit an obvious procoagulant phenotype even though HS$^{AT^+}$ levels in these mice are largely reduced (HajMohammadi et al., 2003). Instead, these mice showed postnatal lethality and intrauterine growth retardation, implying other physiological roles for 3-O sulfation. A different form of 3-O sulfated HS is involved in the entry of HSV-1 into host cells and the fusion of infected cells. HS3ST-3 isoforms generate HS$^{O^-}$: HS with a different 3-O sulfated sequence that mediates the cellular entry of HSV-1. HS$^{O^-}$ on the surface of animal cells interacts with a viral coat protein, glycoprotein D (gD), to promote fusion of the HSV-1 envelope with the cell membrane of host cells (Shukla et al., 1999). Multinucleated giant cells (polykaryocytes), resulting from virus-induced cell fusion, are a hallmark of HSV-1 infection. It has also been demonstrated that CHO cells expressing HS3ST-3 fuse with CHO cells that express viral glycoproteins gB, gD, gH, and gL, suggesting that 3-O sulfated HS has a crucial role both in the entry and spreading of virus. Representative values from a single transfection show the amount of reaction product detected spectrophotometrically (A$^{410}$) as the

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\[\text{\textbeta-galactosidase from an insert in the viral genome.} \]
\[\text{\textbeta-Galactosidase activity, a measure of viral entry, was quantified at 6 h after the addition of virus. Representative values from a single transfection show the amount of reaction product detected spectrophotometrically (A}_{\text{\textbeta-gal}} as the mean \pm SD from three wells per virus dose (PFU, plaque forming units). Comparable results were obtained in three independent transfection experiments. 5’PSB, 5’-phosphosulfate binding site; 3’PB, 3’-phosphate binding site; N.D., not determined.} \]
transferase (ST) domain (Shworak et al., 1997, 1999). Homology comparison of the ST domains of human and *Drosophila* HS3STs indicated two major groups of enzymes with a single *Drosophila* isofrom parsing into each group (Fig. 1 B). Hs3st-A was most similar to HS3ST-1 and -5, which both generate HS$^D+$ (Fig. 1 B; Shworak et al., 1997; Xia et al., 2002). Conversely, Hs3st-B was related to the remaining enzymes, many of which are known to preferentially generate HS$^D-$ (Fig. 1 B; Xia et al., 1999; unpublished data). Substrate specificity of the HS3STs is determined by the structure of the ST domain (Yabe et al., 2001); thus, the homology analysis predicts that Hs3st-A and Hs3st-B might produce different 3-O sulfated HS structures for distinct binding proteins. However, structural similarities do not completely predict enzymatic specificities, as HS3ST-5 can also produce HS$^D+$ and HS3ST-3A can make low levels of HS$^S+$ (Yabe et al., 2001; Xia et al., 2002). Consequently, we analyzed enzymatic specificities of Hs3sts.

We subcloned *Drosophila* and representative mammalian HS3ST cDNAs into the pcDNA3.1 expression vector and determined the enzymatic ability to create functional HS$^D+$ by an in vivo assay in which the sequence specific activity of HS3ST-3 isomorphs generates HS$^D+$ and renders CHO cells susceptible to infection by HSV-1 (Shukla et al., 1999). Thus, CHO cells were transfected with HS3ST expression constructs, and then exposed to a recombinant form of HSV-1, and virus entry was monitored (Fig. 1 C). Negligible viral entry was detected in cells expressing Hs3st-A, the empty vector control, or HS3ST-1, which does not produce HS$^D+$ (Shukla et al., 1999). In contrast, expression of Hs3st-B conveyed cellular susceptibility to HSV-1 entry, as did the HS3ST-3A-positive control. Moreover, coexpression of HSV-1 gD with Hs3st-B reduced cellular susceptibility (unpublished data) through the ability of cellular gD to interfere with viral entry via HS$^D+$ (Shukla et al., 1999). Together, these results demonstrate that Hs3st-B, like HS3ST-3A, preferentially generates HS$^D+$ Thus, the two *Drosophila* HS3ST enzymes exhibit distinct yet phylogenetically conserved sequence specificities.

### mRNA expression of Hs3sts

To determine the temporal and spatial expression pattern of the Hs3st mRNAs, we performed RT-PCR and in situ RNA hybridization. RT-PCR revealed that Hs3st-A mRNA is expressed at low levels in all stages examined (Fig. 2 A). Its transcription levels were especially low in larval and pupal stages. In contrast, Hs3st-B mRNA is abundantly expressed throughout development (Fig. 2 A). We next examined the spatial distribution of Hs3st-B in embryos and larval imaginal tissues by in situ hybridization (Fig. 2 B). In these tissues, Hs3st-B was widely but not evenly expressed. Hs3st-B expression was particularly enriched in the salivary glands in embryos, the morphogenetic furrow of eye discs, and the brain lobes of the larval CNS. In the wing disc, Hs3st-B mRNA was detected at high levels in the wing pouch region (Fig. 2 C). We failed to detect any signals from Hs3st-A mRNA (unpublished data), probably due to the low level of expression.

*Hs3st-B* transgenic RNAi flies show neurogenic phenotypes

To elucidate the function of the Hs3st genes, it is critical to know the loss-of-function phenotypes of animals in which the activities of Hs3sts are compromised. However, there are no mutations identified in these loci. Therefore, we used the transgenic RNAi technique to analyze the in vivo function of Hs3st-A and -B (Kennedell and Carthew, 2000). In this method, a hairpin RNA is expressed from a transgene exhibiting dyad symmetry under the control of GAL4 drivers. Expressed RNAs are double stranded and selectively interfere with expression of the specific target genes. This method has been successfully applied for functional analyses of nu-
numerous genes (Ueda, 2001). We established transgenic fly strains bearing inverted repeats (IRs) of 500 bp DNA sequences corresponding to the NH2 terminus of Hs3st coding regions under the control of upstream activating sequences (UAS) for the GAL4 transcription factor (UAS-IR-Hs3st-A and -B; Brand and Perrimon, 1993). Expression of either IR-Hs3st-A or -B using ubiquitous drivers such as actin-GAL4 caused strong lethality during embryonic and larval stages, showing that Hs3st-A and -B are critically required for viability (unpublished data).

In this work, we focused our analysis on the function of Hs3st-B. To test the effectiveness of Hs3st-B transgenic RNAi, we examined the ability of IR-Hs3st-B to reduce endogenous Hs3st-B mRNA expression. To prepare RNA from actin-GAL4/UAS-IR-Hs3st-B animals, larval stages were reared at 18°C, and then shifted to 25°C for 24 h before RNA preparation. RT-PCR analysis showed that expression of IR-Hs3st-B dramatically reduced the Hs3st-B mRNA level, whereas it had no effect on mRNA levels of Hs3st-A and ribosomal protein 49 (Fig. 3 A), confirming the target specificity of the transgenic RNAi technique.

We found that tissue-specific expression of IR-Hs3st-B using several GAL4 drivers caused a variety of morphological defects. For example, expression of IR-Hs3st-B under the control of GMR-GAL4, an eye-specific driver, caused a severe rough eye phenotype, including an irregular array or fusion of ommatidial bristles and duplication or loss of interommatidial bristles (Fig. 3, C and C). Scanning EM of adult eyes. B' and C' show a high magnification view of adult eye for B and C, respectively. Hs3st-B transgenic RNAi caused necrotic death or fusion of ommatidia and duplication or loss of interommatidial bristles (C and C'). (D and E) Adult nota. (F–M) Adult wings. H and I show a high magnification view of the ACV for F and G, respectively. J and K show a high magnification view of wing vein L3 for F and G, respectively. L and M show the distal region of the wing. (N–Q) Bristles on the sternopleura, a part of the lateral thorax (N and O) and on the leg (P and Q). In the wing, Hs3st-B transgenic RNAi caused reduction of wing size (G), loss of the ACV (I), thickening of wing vein L3 (K), extra or ectopic sensory organs on ACV (I) and L3 vein (K), and notching at the wing margin (M). Extra sensory bristles were also observed on the notum (E), sternopleura (O), and leg (Q).

Expression of IR-Hs3st-B also disrupts the joints between tarsal segments, leading to shortened legs (Q). (R and S) SMCs were labeled by expression of neur-lacZ (red) in dpp-GAL4, UAS-GFP/neur-lacZ (R) and UAS-IR-Hs3st-B/+; dpp-GAL4, UAS-GFP/neur-lacZ wing discs (S). In these figures, anterior of wing disc is up and dorsal is left. The number of SMCs for chemosensory bristles in the A/P boundary marked by GFP were dramatically increased in IR-Hs3st-B animals. We did not observe these defects in any control animals (UAS-IR-Hs3st-B/+; GMR-GAL4, or dpp-GAL4/+).

Figure 3. Hs3st-B transgenic RNAi produced neurogenic phenotypes. (A) RT-PCR analyses showing expression of Hs3st-A (A), Hs3st-B (B), and ribosomal protein 49 (rp) mRNAs in third instar larvae from wild-type (wt) and Hs3st-B transgenic RNAi (RNAi) animals. Expression of IR-Hs3st-B using actin-GAL4 specifically reduced Hs3st-B mRNA level. (B–S) Phenotypes of control animals (GMR-GAL4/+, B and B'; dpp-GAL4/+; D, F, H, J, L, N, P, and RI and Hs3st-B transgenic RNAi (GMR-GAL4/UAS-IR-Hs3st-B, C and C'; UAS-IR-Hs3st-B/+; dpp-GAL4/+; E, G, J, K, M, O, Q, and S) animals. (B, B', C, and C') Scanning EM of adult eyes. B' and C' show a high magnification view of adult eye for B and C, respectively. Hs3st-B transgenic RNAi caused necrotic death or fusion of ommatidia and duplication or loss of interommatidial bristles (C and C'). (D and E) Adult nota. (F–M) Adult wings. H and I show a high magnification view of the ACV for F and G, respectively. J and K show a high magnification view of wing vein L3 for F and G, respectively. L and M show the distal region of the wing. (N–Q) Bristles on the sternopleura, a part of the lateral thorax (N and O) and on the leg (P and Q). In the wing, Hs3st-B transgenic RNAi caused reduction of wing size (G), loss of the ACV (I), thickening of wing vein L3 (K), extra or ectopic sensory organs on ACV (I) and L3 vein (K), and notching at the wing margin (M). Extra sensory bristles were also observed on the notum (E), sternopleura (O), and leg (Q). Expression of IR-Hs3st-B also disrupts the joints between tarsal segments, leading to shortened legs (Q). (R and S) SMCs were labeled by expression of neur-lacZ (red) in dpp-GAL4, UAS-GFP/neur-lacZ (R) and UAS-IR-Hs3st-B/+; dpp-GAL4, UAS-GFP/neur-lacZ wing discs (S). In these figures, anterior of wing disc is up and dorsal is left. The number of SMCs for chemosensory bristles in the A/P boundary marked by GFP were dramatically increased in IR-Hs3st-B animals. We did not observe these defects in any control animals (UAS-IR-Hs3st-B/+; GMR-GAL4, or dpp-GAL4/+).
of UAS-IR-Hs3st-B driven by decapentaplegic (dpp)-GAL4 produced several characteristic phenotypes in adult tissues, including numerous ectopic sensory bristles (macrochaetae) on the mesothoracic notum (Fig. 3 E). The Hs3st-B RNAi also caused reduction of wing size (Fig. 3 G), loss of the anterior cross vein (ACV; Fig. 3 I), thickening of wing vein L3 (Fig. 3 K), extra or ectopic sensory organs on the ACV and vein L3 (Fig. 3, I and K), and notching at the wing margin (Fig. 3 M). Extra sensory bristles were also observed on sternopleura (Fig. 3 O) and legs (Fig. 3 Q). Expression of IR-Hs3st-B at the anterior–posterior (A/P) boundary using patched-GAL4 produced similar morphological defects, indicating that these phenotypes are not dependent on a specific GAL4 driver. The observed phenotypes are characteristic of a reduced activity of neurogenic genes such as components of the N signaling pathway (Lehmann et al., 1981, 1983). During the formation of sensory organ bristles, an N-mediated process called “lateral inhibition” singles out a sensory organ mother cell (SMC) from the proneural cluster (Simpson, 1990). Anti-β-galactosidase antibody staining for the SMC-specific marker neutralized (neur)-lacZ in the Hs3st-B transgenic RNAi wing discs revealed numerous SMCs from each proneural cluster (Fig. 3 S), suggesting that N-mediated lateral inhibition is disturbed in these animals. Some of the phenotypes that Hs3st-B transgenic RNAi produced are common to those resulting from defective growth factor signaling systems, including Hedgehog, Dpp, and Wingless (Wg). However, several features suggest that Hs3st-B specifically affects N signaling. For example, Hs3st-B transgenic RNAi produces segmental fusions of the leg that are similar to N mutant phenotypes but different from those caused by hedgehog, dpp, and vg mutations (Fig. 3 Q; Goto et al., 2001). In addition, expression of IR-Hs3st-B in the wing discs does not affect expression of Hedgehog target genes, such as patched and dpp (unpublished data). Together, these results raise the possibility that Hs3st-B is involved in N signaling. This finding was further confirmed by analysis of the genetic interactions between Hs3st-B and N signaling components. Hs3st-B showed strong genetic interactions with various N pathway genes such as N, Dl, sca, kuz, and delta (δ), suggesting that Hs3st-B participates in the N pathway (see supplemental data, available at http://www.jcb.org/cgi/content/jcb.200403077/DC1).

**Hs3st-B regulates N target genes during wing margin formation**

During wing development, the N pathway activates a set of genes controlling wing margin formation, such as cut (ct), vg, and vestigial (vg), in a stripe of cells along the dorsal–ventral (D/V) boundary (Fig. 4, A, C, and E; Couso et al., 1995; Kim et al., 1995; Rulifson and Blair, 1995; Michelli et al., 1997). To ask if Hs3st-B regulates N signaling, we examined expression of Ct, Wg, and the vg boundary enhancer, vg<sup>BE</sup>-lacZ, in the Hs3st-B transgenic RNAi animals. Expression of IR-Hs3st-B under the control of dpp-GAL4 completely eliminated expression of these genes in the central region of the disc (Fig. 4, B, D, and F). We also examined expression of E(spl)m8, the m8 gene from E(spl)C, which is induced along the D/V boundary by N signaling, although E(spl)m8 is not required for wing margin formation (Fig. 4 G; de Celis et al., 1996). We found that the Hs3st-B transgenic RNAi also abolished E(spl)m8-lacZ expression (Fig. 4 H). Because transcription of vg and E(spl)m8 along the D/V boundary is induced by direct interaction between their regulatory sequences and Su(H) DNA binding protein (Lecourtouit and Schweisguth, 1995; Kim et al., 1996), our results suggest that Hs3st-B is required for the Su(H)-mediated N pathway. Thus, Hs3st-B regulates N signaling during wing margin formation, which is consistent with its phenotypic effects and genetic interactions.
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Hs3st-B acts downstream of Dl and upstream of N activation

To determine the specific step of N signaling that requires 3-O sulfated HS, we analyzed the genetic epistasis of Hs3st-B in the signaling. As mentioned in the previous paragraph, N signaling induces target genes such as wg along the D/V boundary during wing margin formation (Fig. 4 C). Ectopic activation of N results in misexpression of these target genes. For example, when the N ligand Dl is ectopically expressed using dpp-GAL4 (marked by GFP; A', green) induces Wg expression along the A/P boundary in the dorsal compartment. (B and B') Expression of Wg protein (red) and GFP (B', green) is shown for UAS-IR-Hs3st-B+/+; dpp-GAL4 UAS-GFP/ UAS-DI. Coexpression of IR-Hs3st-B with Dl completely suppressed Dl-dependent ectopic expression of Wg in IR-Hs3st-B-expressing cells but not in the adjacent cells (arrows, compare with A'). (C) A dpp-GAL4/UAS-Nact wing disc stained for Wg protein (red). Expression of the activated form of N (Nact) using the same GAL4 driver causes ectopic expression of Wg at the A/P border in both the dorsal and ventral compartment. (D) A UAS-IR-Hs3st-B+/+; dpp-GAL4/UAS-Nact disc stained for Wg protein (red). Ectopic activation of Wg induced by Nact expression was not affected by the Hs3st-B transgenic RNAi (compare with C).

Hs3st-B transgenic RNAi affects N protein levels

The activity of N protein is regulated at multiple steps including proteolytic processing, glycosylation, intracellular transport to the cell surface, and degradation (for review see Baron et al., 2002). To gain insight into the mechanism for control of N function by Hs3st-B, we examined N protein distribution in wing discs from Hs3st-B transgenic RNAi animals using antibodies against the NNECD or NICD. In the third instar larva of wild type, N is evenly expressed throughout the wing disc with the highest protein levels on the apical surface (Fig. 6, A and C; Fehon et al., 1991). We found that expression of IR-Hs3st-B abolished Wg expression only in a cell autonomous fashion and did not affect Wg expression in the neighboring cells (Fig. 5, arrows). This observation shows that (a) Hs3st-B activity is autonomously required only in the signal receiving cells but not in the signal sending cells, and (b) Hs3st-B RNAi does not affect the activity of Dl itself expressed by UAS-Dl transgene.

Hs3st-B acts downstream of Dl and upstream of N.

Figure 5. Hs3st-B acts downstream of Dl and upstream of N. (A and A') Wing disc from a dpp-GAL4 UAS-GFP/UAS-Dl larva stained with anti-Wg antibody (red). Ectopic Dl expression using dpp-GAL4 (marked by GFP; A', green) induces Wg expression along the A/P boundary in the dorsal compartment. (B and B') Expression of Wg protein (red) and GFP (B', green) is shown for UAS-IR-Hs3st-B+/+; dpp-GAL4 UAS-GFP/ UAS-DI. Coexpression of IR-Hs3st-B with Dl completely suppressed Dl-dependent ectopic expression of Wg in IR-Hs3st-B-expressing cells but not in the adjacent cells (arrows, compare with A'). (C) A dpp-GAL4/UAS-Nact wing disc stained for Wg protein (red). Expression of the activated form of N (Nact) using the same GAL4 driver causes ectopic expression of Wg at the A/P border in both the dorsal and ventral compartments. (D) A UAS-IR-Hs3st-B+/+; dpp-GAL4/UAS-Nact disc stained for Wg protein (red). Ectopic activation of Wg induced by Nact expression was not affected by the Hs3st-B transgenic RNAi (compare with C).
Previous papers have shown that high levels of N ligands DI and Ser inhibit N signaling in a cell-autonomous fashion (Michelli et al., 1997). To examine whether or not the reduction of N protein levels in Hs3st-B RNAi animals is caused by high levels of N ligands, we examined expression of DI protein in these animals. In the wild-type wing discs at the late third instar, DI is expressed in the prevein regions, stripes of cells flanking the D/V boundary, and around SMCs (Fig. 6, G–Ĝ; Kooh et al., 1993). We found that IR-Hs3st-B expression did not affect the overall patterns of DI expression, but did increase local levels of DI protein around SMCs (Fig. 6, H–Ĥ). This local up-regulation of DI expression has been previously reported; in N mutant wing discs, high levels of DI protein were observed at regions of proneural clusters (de Celis and Bray, 1997). This finding indicates that Hs3st-B RNAi directly decreases N protein levels and affects DI expression locally as a secondary effect. Together, these findings suggest that Hs3st-B regulates the presentation of N on the cell surface or the stability of N protein.

**Hs3st-B transgenic RNAi affects distribution and morphology of intracellular membrane compartments**

Two lines of evidence prompted us to examine the effects of Hs3st-B RNAi on vesicle trafficking. First, several mutations in genes involved in intracellular trafficking or endocytosis compromise N signaling (for review see Baron et al., 2002). Second, a mammalian homologue of Hs3st-B, HS3ST-3, has a critical role in membrane fusion. 3-O sulfated HS generated by HS3ST-3 mediates fusion of the envelope of HSV-1 and the host cell membrane (Shukla et al., 1999). Furthermore, CHO cells expressing HS3ST-3 fuse with other CHO cells that express viral glycoproteins, resulting in the generation of polykaryocytes (Tiwari et al., 2004).

We studied the effects of Hs3st-B RNAi on the morphology of endosomal/lysosomal compartments. Wing discs from Hs3st-B RNAi animals (UAS-IR-Hs3st-B+/; dpp-GAL4, UAS-GFP+) were cultured in medium containing an endocytosis marker (Texas red dextran) or a lysosomal marker (LysoTracker). We found that IR-Hs3st-B expression strikingly increased the number and size of both dextran and LysoTracker-uptaking vesicular puncta (Fig. 6, I–K). Such enlarged endosomal/lysosomal compartments may lead to a higher rate of protein degradation. We tested whether or not impairment of N signaling causes the same changes of the endosomal/lysosomal compartments and found that expression of either a dominant-negative form of N or an N transgenic RNAi construct driven by dpp-GAL4 does not induce such phenomena (unpublished data). Thus, the changes in number and size of the endosomal/lysosomal compartments are caused by high levels of N ligands, as shown in Fig. 3 (H, blue). (I and J) Wing discs from UAS-IR-Hs3st-B+/; dpp-GAL4, UAS-GFP+/ larvae showing late endosomes visualized by Texas red dextran internalization (red). Expression of IR-Hs3st-B increased the number and size of late endosomes. J shows a high magnification view of a different wing disc with the same genotype as I. (K) Lysosomes are labeled in a different wing disc using LysoTracker (red). Hs3st-B RNAi causes increased and enlarged lysosomes. Expression patterns of dpp-GAL4 are marked by GFP (B’, D’, F’, G’, H’, I, J, and K, green).

Figure 6. **Hs3st-B transgenic RNAi affects N protein levels.** Wing discs from wild-type (A and C) and UAS-IR-Hs3st-B+/; dpp-GAL4, UAS-GFP+/ larvae (B, B’, D, and D’) stained with anti-N(NCD) (A–B’, red) or anti-N(N̅CD) (C–D’, red). Cross sections near the D/V boundary are shown at the bottom of each panel (apical side is up). Hs3st-B transgenic RNAi markedly reduced signals for epitopes in both N(NCD) and N(N̅CD) (E–F’). Wing discs from N(NCD)-lacZ+/ (E) and N(N̅CD)-lacZ+/; UAS-IR-Hs3st-B+/; dpp-GAL4, UAS-GFP+ (F and F’) larvae stained with anti-β-galactosidase antibody (red). Expression of N(NCD)-lacZ was detected strongly at the A/P and D/V compartment boundary (E). This pattern was not affected by the Hs3st-B RNAi (F and F’). (G–H’) Wing discs from dpp-GAL4, UAS-GFP/neur-lacZ (G–G’) and UAS-IR-Hs3st-B+/; dpp-GAL4, UAS-GFP/neur-lacZ larvae (H–H’) stained with anti-DI antibody (red). In wild-type background, DI protein is detectable in prevein regions, stripes of cells flanking the D/V boundary, and around SMCs (G, blue). Hs3st-B RNAi did not affect gross patterns of DI expression, but increased local levels of DI protein around SMCs (H, brackets). Note that the number of SMCs is increased as shown in Fig. 3 (H’, blue). (I and J) Wing discs from UAS-IR-Hs3st-B+/; dpp-GAL4, UAS-GFP+/ larvae showing late endosomes visualized by Texas red dextran internalization (red). Expression of IR-Hs3st-B increased the number and size of late endosomes. J shows a high magnification view of a different wing disc with the same genotype as I. (K) Lysosomes are labeled in a different wing disc using LysoTracker (red). Hs3st-B RNAi causes increased and enlarged lysosomes. Expression patterns of dpp-GAL4 are marked by GFP (B’, D’, F’, G’, H’, I, J, and K, green).
seem to be directly induced by the Hs3st-B RNAi and not through a reduction of N signaling. In summary, these results suggested one possible mechanism in which reduction of N protein levels in Hs3st-B RNAi is caused by defects in trafficking to the cell surface and/or to the endosomal/lysosomal compartments.

Discussion

Function of 3-O sulfated HS in vivo

Several biochemical studies suggest that sulfation at specific positions of HS regulates intercellular signaling. However, the physiological significance of these sulfation events is still poorly understood. 3-O sulfation is one of the best studied examples showing the importance of specific fine structures in controlling the binding affinity of HS to selective ligands. 3-O sulfated HS produced by HS3ST-1 binds to AT (Shworak et al., 1997, 1999), and this interaction has been thought to be a key step in inhibiting blood coagulation. However, several lines of evidence suggest that 3-O sulfated HS also plays roles in evolutionarily fundamental systems such as developmental signaling. First, Hs3st-1−/− mice showed postnatal lethality and intrauterine growth retardation without obvious effects on the blood coagulation (Haj-Mohammadi et al., 2003), suggesting that 3-O sulfated HS may serve other biological roles. Second, recent completion of genomic sequencing in model organisms has revealed the existence of HS3ST genes in invertebrate species including C. elegans and Drosophila, which differ greatly from mammals in their blood coagulation systems. Finally, 3-O sulfation of HS is suggested to be involved in the proliferation of various cancer cells (Miyamoto et al., 2003). These findings prompted us to reassess the roles of 3-O sulfated HS using in vivo systems. This approach was validated by our finding that the enzymatic sequence specificities of HS3STs are phylogenetically conserved. Most importantly, our work showed that reduction of Hs3st-B function using transgenic RNAi caused various morphological defects by compromising N signaling. This result demonstrated for the first time that 3-O sulfated HS controls a particular developmental pathway required for morphogenesis.

Mechanisms by which 3-O sulfated HS regulates Drosophila N signaling

What is the mechanism for control of the N signaling system by HS 3-O sulfation? Although our work implicated Hs3st-B in N signaling, there is no direct evidence reported to date of a requirement of HS in this pathway. However, there are several heparin-binding proteins that have been shown to regulate N signaling. Therefore, Hs3st-B may affect N activity through these molecules. First, Kuz, a Drosophila member of the ADAM family, has a domain that is highly conserved among members of the family. Because one of the mammalian homologues, ADAM12, was shown to interact with HS through this domain (Iba et al., 2000), Kuz could also potentially associate with HSPGs. Kuz cleaves the NOD, and this cleavage is required for activation of the N receptor (Lieber et al., 2002). Therefore, it is possible that 3-O sulfated HS interacts with Kuz and regulates the processing of N. The second candidate whose activity may be regulated by 3-O sulfated HS is Sca. Sca is a secreted fibrinogen-related protein that binds to heparin in vitro (Lee et al., 1996). Previous papers showed that Sca has an ability to directly bind and stabilize N protein on the cell surface (Powell et al., 2001). Finally, it is also possible that 3-O sulfated HS affects the N pathway through the activity of Wg, a known heparin-binding factor. It has been suggested that Wg binds to the specific EGF repeats of the NCD (Wesley, 1999) and that this binding inhibits interactions between N and its ligands, DI and Ser, during development (Brennan et al., 1999). Therefore, 3-O sulfation of HS might control N signaling by modulating the N–Wg interaction.

Another model that explains how Hs3st-B regulates N protein levels was suggested by our experiments showing that Hs3st-B RNAi causes aberrant intracellular trafficking events. Previous work has shown that the activity of N signaling is very sensitive to alterations in membrane trafficking (for review see Baron et al., 2002). For example, phosphocholine cytidylyltransferase 1 is involved in the formation of phospholipid membranes and positively regulates N signaling (Weber et al., 2003). Phosphocholine cytidylyltransferase 1 mutant cells show an increase in endocytic activity and have enlarged endosomes, resulting in abnormal subcellular localization of N protein. In addition, mutations in warthog, which encodes a Drosophila homologue of rab6 and regulates trafficking from the Golgi to the trans-Golgi network, result in defective N signaling (Purcell and Artavanis-Tsakonas, 1999). Thus, some genes that are involved in controlling membrane trafficking are known to affect N signal transduction. Together with our observation, these findings suggest that a reduction of N protein by the Hs3st-B RNAi may be caused by abnormal sorting of N protein or enhanced activities of endocytosis/protein degradation. Interestingly, mammalian HSST-3 mediates the fusion of the viral envelope with the host cell membranes (Shukla et al., 1999), as well as the fusion between host cell membranes (Tiwari et al., 2004), suggesting a possible role of 3-O sulfated HS in membrane fusion during trafficking events. It is also possible that the Hs3st-B RNAi independently affects N signaling and intracellular trafficking.

Given that the role of HS in N signaling has not been reported, it is possible that the substrate of Hs3st-B is not HS, but other glycoconjugates such as sugar chains of N protein itself. The O-linked sugar chains on the EGF repeats of N protein play a key role in its activity (for review see Haines and Irvine, 2003), and further modification of this sugar chain (i.e., sulfation) may give a potential regulatory mechanism. It has been shown that fringe (N-acetylglucosaminyltransferase), O-fucosyltransferase 1, and fringe connection (UDP-sugar transporter) are involved in glycosylation of N and affect N signal transduction (for review see Haines and Irvine, 2003). Therefore, it is important to know the effects of Hs3st-B RNAi on modification of glycoconjugates other than HS. Also, we cannot rule out the possibility that Hs3st-B RNAi induces a phenomenon that does not reflect functions of endogenous HS chains. For example, inappropriately modified HS may exert an inhibitory effect on N signaling, even though HSPGs do not normally have any role in this pathway. Further studies on the function of
other HS biosynthetic enzymes and HSPG core protein genes in N signaling and membrane trafficking will reveal the molecular basis underlying the control of these events by 3-O-sulfated HS. In particular, identification of the ligand of 3-O sulfated HS will be important to understand the in vivo functions of this class of enzymes.

Materials and methods

Fly stocks

The following mutant strains were used: N\superscript{H11034} and N\superscript{H11032}, a null and a temperature-sensitive allele of N, respectively; D\superscript{P1360}, a null allele of Dl; kuz\superscript{H11034}, a null allele of kuz; and d\superscript{1}, a temperature-sensitive allele of d. The lacZ reporter lines used were as follows: vg\superscript{H1}-lacZ, vg boundary enhancer; neur-lacZ; E(spl)\superscript{H1}-lacZ, m8 gene from E(spl)C; N\superscript{H11032}. The transgenic genomic used were as follows: UAS-sca; UAS-DI; UAS-N\superscript{H11003}, a constitutively active form lacking the N\superscript{H1}C domain; UAS-GEF; actin-GA4; CMR-GA4; patched-GA4; dpp-GA4. All aforementioned alleles and inserts are described in FlyBase (http://flybase.bio.indiana.edu). The transgenic RNAi animals used were as follows: UAS-sca-AWRI\superscript{H11034}, UAS-IR-Hs3st-B\superscript{H1}, and UAS-IR-Hs3st-B\superscript{H11032} (see also Construction of Hs3st transgenic RNAi flies). We used UAS-IR-Hs3st-B\superscript{H11034} to inhibit Hs3st-B function in all experiments unless stated otherwise in the text.

Cloning and enzymatic assay of Hs3sts

Two genomic loci for putative Drosophila Hs3sts (Hs3st-A: CG33147, and B: CG7890) were identified in the Drosophila genome database using the tblastn program of the BDGP. An Hs3st-A cDNA clone (GH20068) containing the entire protein coding region was obtained from the BDGP. To isolate an Hs3st-B cDNA clone, ~4 X 10\superscript{6} colonies from a Drosophila embryonic cDNA library were screened using Hs3st-B genomic DNA as a probe (Brown and Kafatos, 1988). Hybridization was performed as described by Sambrook et al. (1989). One positive clone was isolated, and the inserted cDNA sequence was determined using a Big Dye\reg Thermsequencing Ready Reaction Kit and the ABI PRISM 310 Analyzer.

To characterize the enzymatic sequence specificities of Hs3sts, expression constructs were generated by inserting an EcorV-Xhol fragment of Hs3st-A and a HindIII-EcoRI fragment of Hs3st-B cDNA into the polycloner of pcDNA3.1 (Life Technologies). pcDNA3.1 expression based plasmids containing mouse Hs3ST-1 or human Hs3ST-3A were described by Sambrook et al. (1989). One positive clone was isolated, and the insert was sequenced using the T7 sequencing primer. Determination of CHO cell susceptibility to HSV-1 entry was performed as previously described (Montgomery et al., 1996; Shukla et al., 1999).

RT-PCR analyses

Total RNA was isolated from wild-type and actin-GA4/UAS-IR-Hs3st-B larvae using the FastRNA kit (Bio101) and FastPrep FP120 (Bio101). Isolated RNA was reverse-transcribed using SuperScript\superscript{reg} First-Strand Synthesis System for RT-PCR (Life Technologies). PCR experiments were performed using the synthesized cDNA as a template and the following primers: Hs3st-AF (5'-CTTGCCGATGACCAAGCACA-3') and Hs3st-ARV (5'-TACATCCGGATCTCAAGCCTG3) for Hs3st-A detection; Hs3st-AR (5'-CTATCGGATCTCAAGCCTG3) and Hs3st-BRV (5'-GCATAAGCCGGATCCCTC-3') for Hs3st-B detection, and m49FW (5'-ATGACCACGCCAGGATTAG3) and m49RV (5'-TGTCTTCTTGAAGCAGGGCCAG3) for ribosomal protein 49 detection.

Construction of Hs3st transgenic RNAi flies

Transgenic RNAi flies of Hs3st-A and -B were obtained using the inducible RNAi method (Kennerdell and Carthew, 2000). A 500-bp-long cDNA fragment from the first methionine was amplified by PCR and inserted as an IR into a modified pBlastn vector, p5C1, which possesses an IR formation site consisting of paired CpGol and StI restriction sites. In all cases, IRs were constructed in a head-to-head orientation. IR-containing fragments were cut with NotI and were subcloned into pUAST, a transformation vector. Transformation of Drosophila embryos was performed using w\superscript{1118} as a recipient strain.

Scanning EM

Adult flies were sequentially dehydrated in 25, 50, 75, and 100% ethanol, for 2 h each. The 100% dehydration was repeated three times. The preparation was subjected to critical point drying before being mounted on stubs and viewed on an S-4700 field emission scanning electron microscope (Hitachi).

In situ hybridization and immunostaining

In situ RNA hybridization was performed as described previously (Kamimura et al., 2001). Antibody staining was performed as described previously (Fujise et al., 2001) using rabbit anti-β-galactosidase (1:500; Cappel), mouse anti-Wg (1:200; Developmental Studies Hybridoma Bank [DSHB]), mouse anti-CT (210; 1:200; DSBH), mouse anti-NECD (C458.2H; 1:20; DSBH), mouse anti-N\superscript{ECD} (C79.9C6; 1:10; DSBH), and mouse anti-DI (C594.9B; 1:200). The primary antibodies were detected with Alexa Fluor conjugated secondary antibodies (1:500; Molecular Probes).

Labeling the endosomal and lysosomal compartments

To visualize late endosomes, wing discs from third instar larvae were incubated in 0.5 mM of Texas red dextran (lysin fixable, MW3100; Molecular Probes) for 5 min in M medium at 25°C and then washed five times for 2 min with ice-cold M medium. Afterwards, these discs were incubated in M medium for 60 min at 25°C and fixed in PFA for 20 min at RT before mounting in VECTASHIELD Mounting Medium (Vector Laboratories). To visualize lysosomes, wing discs were incubated in 100 nM Lysotracker red (Molecular Probes) at 25°C for 2 min in PBS. Subsequently, the discs were washed two times with PBS and incubated in PBS at 25°C for 20 min. These discs were mounted in PBS for observation.

Online supplemental material

Fig. S1 shows the genetic interactions between Hs3st-B and various N signaling components during formation of the wing and notum. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200403077/DC1.

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