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Sulfation pattern in glycosaminoglycan: Does it have a code?

Hiroko Habuchi[1], Osami Habuchi[2] and Koji Kimata[1]

[1] *Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195,* [2] *Department of Chemistry, Aichi University of Education, Igaya-cho, Kariya, Aichi 448-8542, Japan*

Heparan sulfate chains (HS) are initially synthesized on core proteins as linear polysaccharides composed of glucuronic acid—*N*-acetylglucosamine repeating units and subjected to marked structural modification by sulfation (*N*-, 2-*O*-, 6-*O*-, 3-*O*- sulfotransferases) and epimerization (C5-epimerase) at the Golgi lumen and further by desulfation (6-*O*- endosulfatase) at the cell surface, after which divergent fine structures are generated. The expression patterns and specificity of the modifying enzymes are, at least partly, responsible for the elaboration of these fine structures of heparan sulfate. HS interacts with many proteins including growth factors (GF) and morphogens through specific fine structures. Recent biochemical and genetic studies have presented evidence that HS plays important roles in cell behavior and organogenesis. In knock-down experiments of heparan sulfate 6-*O*-sulfotransferase, 6-*O*-sulfated units in HS have been shown to act as a stimulator or suppressor according to individual GF/morphogen signaling systems.
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Introduction

Heparan sulfate (HS) proteoglycans are present ubiquitously on the cell surface and in the extracellular matrix including basement membranes. HS chains are known to interact with divergent bioactive ligands such as growth factors and morphogens (FGFs, Wnts, BMPs, VEGFs, Hh *etc*.) and their receptors (FGFR, *etc*.), extracellular matrix molecules (collagen, fibronectin, laminin, *etc*.), proteases, *etc*. Such interactions may regulate the activity, gradient formation, and stability of ligands. Therefore, HS plays important roles in a variety of developmental, morphogenetic, and pathogenic processes [1–3]. The specificity of the interactions between HS and ligands is thought to be due, at least in part, to the fine structure of HS with specific monosaccharide sequences characterized by the sulfation pattern and the specific location of isomeric hexuronic acid residues [4,5]. The fine and divergent structures of HS are synthesized in the Golgi apparatus by the actions of heparan-sulfate chain modification enzymes, *N*-, 2-*O*-, 6-*O*-, 3-*O*-sulfotransferases, and C5-epimerase [6–8], and further modified at the cell surface by endo 6-*O*-sulfatase (Sulf1) [9] (Figure 1). These enzymes have several isoforms differing in specificity and expression patterns with the exception of C5-epimerase and heparan sulfate 2-*O*-sulfotransferase (HS2ST). Biochemical and cell biological studies have revealed that growth factor (GF) and/or GF-receptor forms complexes with HS and the GF signalings are regulated by the formation of complexes [10,11]. Recent genetic studies on HS modifying enzymes are now uncovering the functions of HS in GF signaling (Table 1) [12–16, see ref. [17] for review]. In this review, we describe recent studies by us and the collaborators on the structure and function of HS, especially the HS synthesized by isoforms of HS 6-*O*-sulfotransferase (HS6ST) and HS2ST.

To whom correspondence should be addressed: Koji Kimata, Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195. Tel.: 81-52-264-4811; Fax: 81-561-63-3532; E-mail: kimata@amugw.aichi-med-u.ac.jp

Substrate preferences and expression patterns of different isoforms of HS6ST and HS2ST

HS6ST catalyzes 6-*O*-sulfation of the *N*-sulfoglucosamine residue of heparan sulfate, and has been shown to comprise three isoforms and one alternatively spliced form [18–21]. All the HS6ST isoforms transferred sulfate to position 6 of not only the internal *N*-sulfoglucosamine residues but also the nonreducing terminal *N*-sulfoglucosamine residue; however, all the isoforms showed little activity toward *N*-acetylglucosamine residues *in vitro*. In contrast, all the HS6ST isoforms are able to transfer sulfate to these residues *in vivo*. Such an apparent discrepancy in the specificity observed *in vitro* and *in vivo* may

Table 1. Mutants having defective genes for glycosaminoglycan biosynthesis

Species	*Enzyme or gene*	*Enzymatic activity*	*Phenotype, signaling molecule*
HS			
Mouse	NDST-1	*N*-deacetylation & *N*-sulfation of GlcNAc	Neonatal lethal,pulmonary atelectasis, cyanosis
	NDST-2	*N*-deacetylation & *N*-sulfation of GlcNAc	Deficiency in heparin synthesis in mast cell
	C5-epimerase	Conversion of GlcA to IdoA in HS	Neonatal lethal, renal agenesis, lung defects,
	HS2ST	2-*O*-sulfation of glucosamine in HS	Neonatal lethal, renal agenesis
	HS3ST-1	3-*O*-sulfation of glucosamine in HS	Normal coagulation
	EXT-1	HS copolymerase	Embryonic lethal, failure of gastrulation, Ihh
Drosophila	*Sulfateless*	*N*-deacetylation & *N*-sulfation of GlcNAc	Abnormal segmentation, FGF, Wg
	Tout velu	HS copolymerase	Abnormal segmentation, Hh,
C. elegans	*Rib-2*	HS copolymerase	Failure of gastrulation, maternal effect letharity
	Hse-5	Conversion of GlcA to IdoA in HS	Axonal patterning defects
	Hst-2	2-*O*-sulfation of glucosamine in HS	Axonal patterning defects
	Hst-6	6-*O*-sulfation of glucosamine in HS	Axonal patterning defects
CS			
Mouse	C6ST	6-*O*-sulfation of GalNAc in CS	Reduction of native T lymphocytes in spleen
C. eleganse	*Sqv-5*	Chondroitin synthetase	Defect in vulva morphogenesis & cytokinesis

reflect the pathway of heparan sulfate biosynthesis; in which 6-*O*-sulfation of the GlcNAc residue may occur as a well-coupled reaction with the elongation of HS backbone. Although the substrate specificities of these isoforms largely overlapped, the individual isoforms showed characteristic preference for the uronic acid residue neighboring the *N*-sulfoglucosamine. HS6ST-1 predominantly sulfated the IdoA-GlcNSO$_3$ unit. On the other hand, HS6ST-2 transferred sulfate preferentially

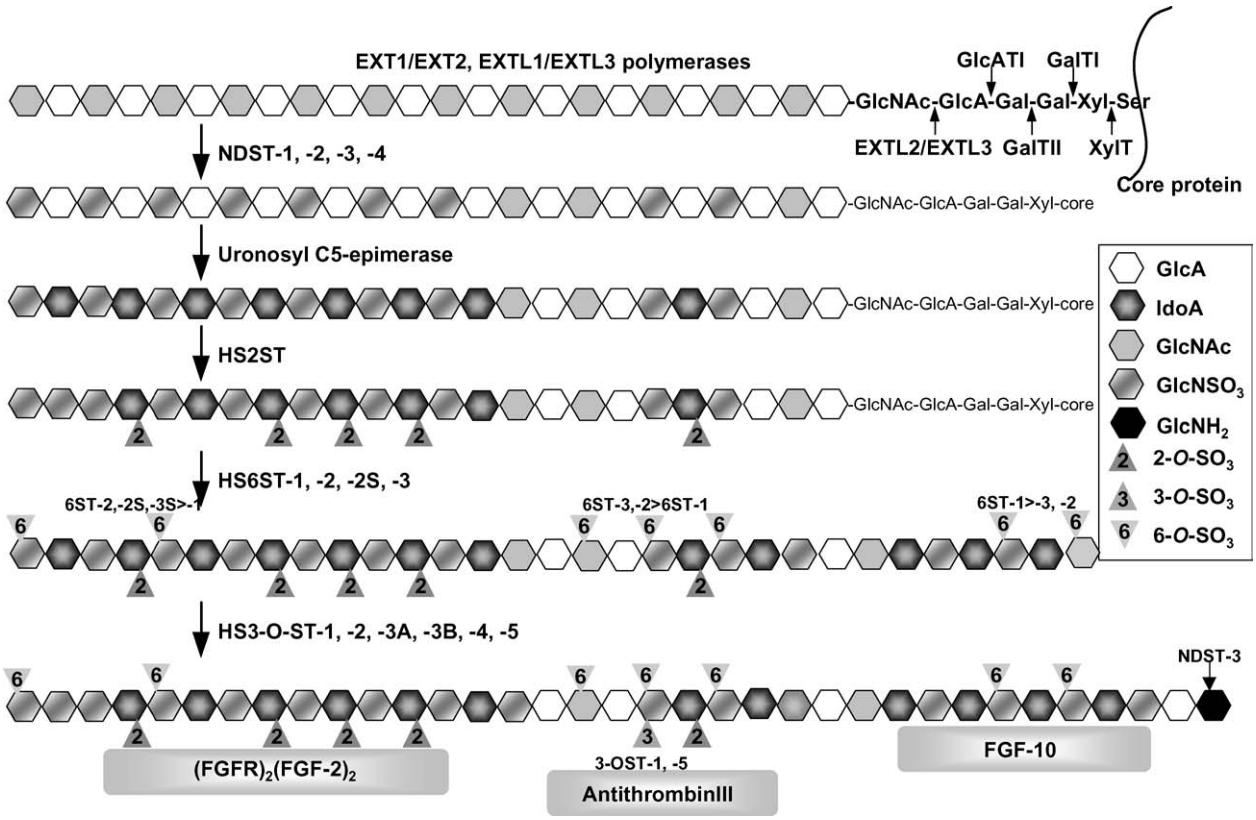

Figure 1. Schema for the modification pathway of heparan sulfate biosynthesis. The symbols used are defined in the figure. Also shown are the binding sites for the specific ligands and sulfotransferases involved in generating these structures. Some of the sulfotransferases are presumed to be involved based on tissue and substrate specificities of sulfotransferases.

to the GlcA-$GlcNSO_3$ unit and the $IdoA(2SO_4)$-$GlcNSO_3$ unit to generate trisulfated disaccharide units in HS. Heparan sulfate 2-*O*-sulfotransferase, HS2ST, transfers sulfate to position 2 of the iduronic acid residues in HS but not to the iduronic acid residues neighboring $GlcNSO_3$ ($6SO_4$) residues [22,23]. Furthermore, HS2ST showed weak activity toward the glucuronic acid residues.

Each HS6ST isoform varied in expression pattern not only in different tissues but also at different developmental stages, whereas HS2ST was expressed rather ubiquitously. Thus the tissue-specific fine structure of HS may largely be accounted for by the occurrence of multiple isoforms of HS6ST.

Fine structure of HS in chick limb buds defined by the distinctive expression patterns of HS-*O*-sulfotransferases

Many heparin-binding growth factors (HBGFs) and morphogens (FGFs, VEGFs, HGF, Wnts, BMPs and SHH) are expressed in a spatiotemporally specific manner in developing limb buds, which has been considered to be essential for limb bud formation and the subsequent patterning of skeletal tissues. The process of limb bud formation in the chick is a good model for examining how the fine structure of heparan sulfate regulates HBGF signaling and the concentration of morphogens. Although there are three isoforms of HS6ST, HS6ST-1, -2, and -3, and one splicing form of HS6S-2, HS6ST-2S, in mice and humans as described above, chickens appear to have only two isoforms of HS6ST, HS6ST-1 and HS6ST-2S [24]. The expression patterns of these genes during chick limb bud development were spatiotemporally specific. The transcripts of HS6ST-1 and HS6ST-2 were preferentially expressed in the anterior proximal region and posterior proximal region, respectively, of the limb bud, during at least stage 21 to stage 24. In contrast, the HS2ST transcript was distributed uniformly throughout the bud. A structural analysis of HS from different regions of the wing bud indicated that 6-*O*-sulfated *N*-sulfoglucosamine/*N*-acetylglucosamine residues are more abundant in the proximal than distal region, and that iduronic acid 6-*O*-sulfated *N*-sulfoglucosamine units tend to be rich in the anterior proximal region while glucronic acid 6-*O*-sulfated *N*-sulfoglucosamine units are abundant in the posterior proximal region. These observations suggest that HS with different sulfation patterns generated by the multiple sulfotransferases could provide the GFs and morphogens with appropriate extracellular environments for their signalings. For example, we have recently found that FGF-10, a mesenchymal FGF essential for limb induction and apical ectodermal ridge (AER) maintenance through activation of the FGFR-2b isoform at the AER, requires a 6-*O*-sulfated structure to bind HS [25]. It is possible that FGF-10 is released as a diffusible form from the distal mesenchyme where low 6-*O*-sulfated HS is predominant, and then reaches the AER, resulting in outgrowth of the AER through the activation of FGFR-2c.

Zebrafish *HS6ST*, a gene that may participate in neural plate regionalization

Zebrafish *HS6ST (zHS6ST)* was originally found as one of the genes that participate in neural plate regionalization [26]. The amino acid sequences of zHS6ST and all three mouse isoforms were nearly the same. However, the substrate specificity of zHS6ST was most similar to that of HS6ST-1, zHS6ST preferring completely desulfated, *N*-sulfated (CDSNS)-heparin to *N*-sulfated heparosan as an acceptor substrate. The spatiotemporal pattern of *zHS6ST* expression during development was analyzed by whole mount *in situ* hybridization. At the onset of gastrulation, *HS6ST* was ubiquitously expressed in the blastoderm. However, at 24 h post fertilization (hpf), intense staining was detected only in the brain and eye of the embryo. The expression was also restricted to the somite boundaries as well as to the ventral part of the tail. At 48 hpf, the fin bud also expressed a high level of the HS6ST transcript.

To elucidate the functional role of zHS6ST, a loss-of-function analysis was performed by injecting morpholino antisense oligonucleotides into eggs of the zebrafish. The structures of heparan sulfate prepared from the wild type and morphant embryos at 48 h after fertilization were compared. The proportion of 6-*O*-sulfated disaccharides, especially hexuronic acid *N*-sulfoglucosamine 6-sulfate, was markedly decreased in the morphants, suggesting that the morpholino antisense oligonucleotides inhibited 6-*O*-sulfation significantly and specifically. The morpholino-mediated knockdown of HS6ST activity resulted in drastic phenotypic abnormalities, reminiscent of *knypec/glypican* zebrafish mutants. The tail was often curled. The size of the head was reduced in morphants, and the size of the white matter composed of nerve tracts and of the gray matter composed of somata were both significantly reduced. A tiny fin was also often observed at 96 h after fertilization. The most obvious defect in HS6ST knockdown embryos was the abnormal muscle development. In the morphants, muscle cells in the somites had an irregular shape, and in more severe cases, the horizontal myoseptum was not clear or absent. To know the mechanism of zHS6ST's action on muscle development, the expression of marker genes involved in muscle differentiation, *myoD*, *eng2*, *shh*, and *α-tropomyosin*, was examined by *in situ* hybridization. *MyoD* and *eng 2* induced by Wnt were highly expressed in morphant embryos compared to wild-type embryos, suggesting that HS6ST regulates Wnt signaling. As described below, it is suggested that HS6ST in *Drosophila* plays crucial role in the formation of the trachea through the regulation of FGF signaling [27]. At present, however, it remains to be determined whether there is genetic interaction between zHS6ST and FGF signaling. It is also likely that another isoform of zHS6ST is involved in FGF signaling. Further studies are necessary to clarify the signaling molecules involved in abnormalities other than those of muscle differentiation in HS6ST morphants.

Involvement of *Drosophila* HS6ST (dHS6ST) in tracheal formation

Drosophila has only one HS6ST. dHS6ST transferred sulfate to position 6 of the $GlcNSO_3$ residues in CDSNS-heparin and *N*-sulfated heparosan [27]. The ratio of activity toward CDSNS-heparin to that toward *N*-sulfated heparosan was 0.36, suggesting that dHS6ST was similar to mouse HS6ST-3, which has properties intermediate between those of mouse HS6ST-1 and mouse HS6ST-2. During embryogenesis, dHS6ST mRNA was expressed highly specifically in tracheal precursor cells of the stage 10 embryo, and its expression was maintained through stage 12. By stage 16, *dHS6ST* had become uniformly expressed in various tissues. The spatial and temporal expression pattern of *dHS6ST* was similar to that of the *Drosophila* FGF receptor, *breathless* (*btl*), suggesting that dHS6ST functions in tracheal development. As expected, RNAi experiments indicated that the normal formation of the trachea was disrupted by the injection of dsRNA of dHS6ST into the embryo. These observations also supported important functions of 6-*O*-sulfated heparan sulfate in the FGF signaling and the guidance of tracheal cell migration. Vertebral angiogenesis has many similarities to insect tracheal branching. The zHS6ST-knock-down zebrafish described above**,** however, did not show any vascular abnormalities, suggesting that the other isoform of zHS6ST functions in the vascular system.

Sd and *pipe*, two candidate genes for *Drosophila* orthologs of HS2ST—Sd but not pipe has HS2ST activity

Two genes in drosophila have been identified as orthologs of HS2ST through database analyses. *Sd* is localized to an intron of the *Sd* gene locus, and was once thought to be responsible for a meiotic drive system in *Drosophila* (*the Segregation distorter gene*) hence it was named *Sd* [28]. *Pipe* is one of 11 dorsal group genes and is required for establishing the dorsal-ventral polarity in the embryo [29]. However, since only one HS2ST gene has been detected in any of the vertebrates examined to date, one of the above two gene products was expected to have the HS2ST activity. *Sd* protein showed 53% identity to mHS2ST and 31% identity to human dermatan/chondroitin sulfate hexuronic acid-2-*O*-sulfotransferase (hDS2ST) (Table 2). In contrast, *Pipe* protein showed 26% identity to mHS2ST and 29% identity to hDS2ST. The recombinant proteins encoded by these cDNAs were expressed as fusion proteins with a FLAG peptide in COS-7 cells, and sulfotransferase activity levels of the purified recombinant enzymes were determined using dermatan sulfate and heparan sulfate as acceptors (Habuchi H, Sen J, Stein D, Kitagawa Y, Nakato H, Esko J, Kimata K. unpublished observation). The *Sd* recombinant protein transferred sulfate to position 2 of the hexuronic acid residues in heparan sulfate; the iduronic acid residue was a better acceptor than the glucuronic acid residue. The protein did not sulfate dermatan sulfate. The *pipe* recombinant protein showed neither HS2ST activity nor DS2ST activity, and no sulfotransferase activity was detected toward any other glycosaminoglycans examined. Furthermore, the transfection with *Sd* cDNA of HS2ST-deficient cells, CHO-pgs F17 cells, generated 2-*O*-sulfated hexuronic acid residues in heparan sulfate, but, when *pipe* cDNA was introduced into CHO-pgs F17 cells as well as the cells that had been transfected with *windbeutel* cDNA, no 2-*O*-sulfated hexuronic acid residues were produced. Considering the results, *Sd* not *pipe* is likely the *Drosophila* ortholog of hHS2ST. *Pipe* does not appears to be the gene responsible for 2-*O*-sulfation of DS and HS. Nevertheless, the possibility could not be excluded that pipe may be an enzyme for the sulfation of other glycoconjugates, because the *pipe*-encoded protein contains two PAPS-binding motifs.

Table 2. Homology of Sd and Pipe with HS2ST and DS2ST, and sulfotransferase activity

	Identity (%)			
Protein	*mHS2ST*	*hDS2ST*	*HS2ST activity*	*DS2ST activity*
Sd	53	31	+	–
Pipe	26	29	–	–

Perspectives

Most of the glycosaminoglycan sulfotransferases have now been molecularly cloned and characterized, and shown to be involved in the synthesis of heparan sulfate, chondroitin/dermantan sulfate [30–32], and keratan sulfate. The first evidence of the critical role of glycosaminoglycan sulfotransferase was obtained from HS2ST-knockout mice established by the gene-trap method [33]. These mice showed a characteristic renal agenesis, clearly indicating the importance of the fine structure of HS to the morphogenesis of animals. To date, several sulfotransferase-deficient mutants have been produced (see Table 1). These mutants showed unique phenotypes characteristic of a deficiency of sulfotransferase. The gene knockout approach will provide evidence about the individual roles of multiple isoforms. Inhibition of the expression of a particular sulfotransferase using RNAi or morpholino nucleotides also will be useful for elucidation of the biological functions of sulfotransferases.

Further study of the function of the domain structure of glycosaminoglycan is required. We have recently found a unique and highly sulfated structure (GalNAc4,6 diS –GlcA2S-GalNAc6S) at the nonreducing terminal of chondroitin sulfate [34]. Searching ligands for unique nonreducing terminal structures may help to elucidate the function of the domain structure of glycosaminoglycan. Enzymatically synthesized oligosaccharides with a defined structure will be extremely useful for the quantitative analysis of binding to ligands [25,35,36]. Much of the mechanism by which the functional domain structures are synthesized in the cell remains unresolved, including the

relation between elongation of the sugar chain and sulfation, how the activity of the individual sulfotransferases is regulated, and the order in which each sulfotransferase works [37]. A multi-enzyme complex in the Golgi, which possibly contains not only enzymes but also regulatory proteins, should be present as part of the regulatory mechanism.

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