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Review

Heparan Sulfate : Structure, Biosynthesis, and Functions

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Abstract : Heparan sulfate shows marked structural diversity and binds various heparin-binding proteins. These interactions in *in vitro* and *in vivo* systems have been shown to be implicated not only in various cell behavior such as cell growth, differentiation, adhesion, and migration, but also in tissue-morphogenesis during development. Furthermore, heparan sulfate is involved in various patho-physiological phenomena such as inflammation, blood coagulation, tumor cell malignancy and microbial infection. These biological functions are achieved mostly through the interaction between heparan sulfate and various growth factors. Heparan sulfate functions as a coreceptor for the high affinity receptors of various growth factors. Heparan sulfate present in the extracellular matrix or on the cell surface regulates the local concentrations of growth factors or morphogens, thereby forming a concentration gradient reviewing heparan sulfate proteoglycans). The divergent structures of heparan sulfate are synthesized by the successive actions of heparan sulfate-modifier enzymes (*N*- and *O*-sulfotransferases and C5-epimerase). The modification of heparan sulfate largely occurs at the Golgi but some reactions appear to occur in the extracellular space after secretion. By now, most of these modifier enzymes have been cloned and characterized. Through functional analyses of these enzymes at the level of the cell, organ and whole animal, the roles of heparan sulfate in various biological systems have been revealed.

Key words : heparan sulfata proteoglycan, sulfotransferase, glycosyltransferase, cell growth factor, morphogenetic factor

Structural Diversity and Interaction with Multiple Binding Proteins of Heparan Sulfate

Heparan sulfate proteoglycans (HSPGs) are glycoconjugates in which sulfated glycosaminoglycans, heparan sulfate, are attached to core proteins[1-10]. HSPGs are present in various tissues as two major forms, basement membrane-type HSPG and cell surface-type HSPG. The basement membrane-type HSPGs are present in the extracellular matrix including the basement membrane, and include perlecan, agrin and type XVIII collagen[11]. The cell surface-type HSPGs are classified into two groups, syndecans and glypicans. The syndecan family (syndecan 1-4)[12),13] is immobilized on the cell surface by intercalating the transmembrane domain of the core protein into the plasma membrane. The glypican family (glypican 1-6) is attached to the plasma membrane through a glycosyl phosphatidyl inositol anchor that is covalently bound to the C-terminal of the core protein[14),15]. In addition to these major types, so-called part-time HSPGs such as betaglycan and CD44 are reported, in which depending on the cellular conditions, HS-free forms also exist.

Heparan sulfate chains are initially synthesized as a copolymer composed of alternating glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc), and subsequently modified by uronosyl C-5 epimerization and sulfation. Because of the partial and regional modification reactions, various disaccharide units (shown in Fig. 1) are generated in a single heparan sulfate chain. Thus a heparan sulfate chain is able to have extremely divergent structures so that, through structurally specific regions, it interacts with various proteins such as heparin-binding growth factors (BMP, CXC chemokine, FGF family, GM-CSF, HBEGF, HGF, IL8, MIP-1β, PDGF, VEGF, Wnt, and Hh), protease inhibitors (antithrombin III, heparin cofactor II, and protease nexin I), proteases, lipoprotein lipases and extracellular matrix components (laminins, collagens, and fibronectin). Functional structural units, so-called domain structures, involved in the binding to several ligand proteins are shown in Table 2. In the FGF family, FGF-1, FGF-2 and FGF-8b bind hexasaccharides containing IdoA(2S)-GlcNS(6S)[16),17], IdoA(2S)-GlcNS[18-21] and IdoA(2S)-GlcNS(6S)[22], respectively. FGF8b requires oligosaccharides longer than tetradecasaccharide for the enhancement of activity, whereas FGF-1 and FGF-2 require octa to decasaccharides for activation. FGF-18 binds oligosaccharides containing IdoA-GlcNS(6S)[23], whereas FGF-4 binds oligosaccharides containing IdoA(2S)-GlcNS or IdoA-GlcNS(6S)[24]. Laminin and fibronectin bind a highly sulfated cluster containing five to six consecutive IdoA-(2S)-GlcNS(6S) that is prominent in heparin[25),26], suggesting that these extracellular matrix proteins bind

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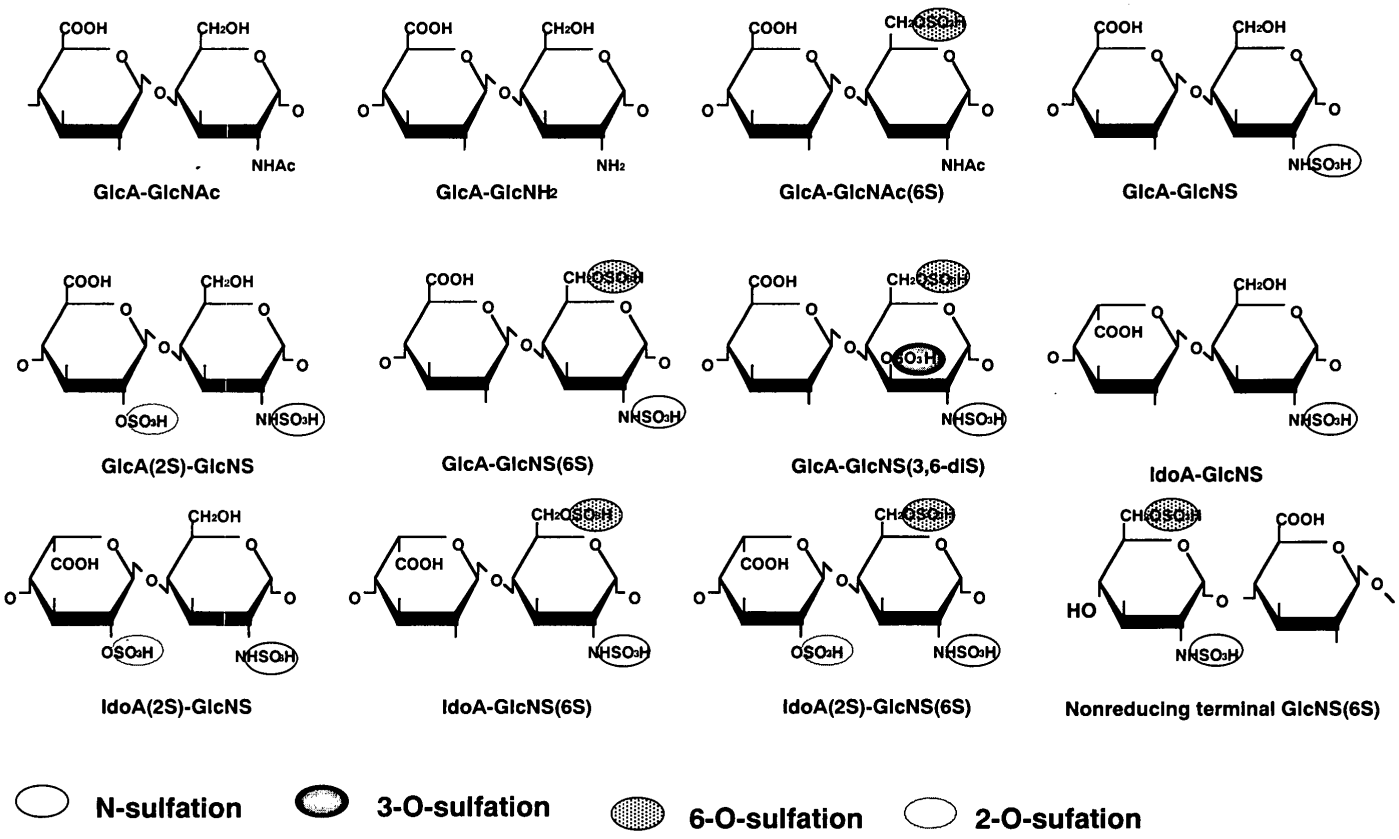

Fig. 1. Main disaccharide structure in heparin/heparan sulfate.
GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GlcNS, *N*-sulfoglucosamine; $GlcNH_2$, glucosamine; IdoA, iduronic acid; 2S, sulfate at position 2 of IdoA or GlcA, 6S, sulfate at position 6 of GlcNS; GlcNAc or $GlcNH_2$; 3S, sulfate at position 3 of GlcNS, GlcNAc or $GlcNH_2$.

Table 1. Heparin • heparan sulfate binding proteins.

Groups	Proteins
Growth factors and cytokines	FGF family (FGF1-23), HGF, HBEGF, VEGF, PDGF, Wnt/Wingless, interferon, interleukin, BMP, MIP-1b
ECM molecules	fibronectin, laminin, thrombospondin, collagen, PGM (versican), vitronectin
Protease inhibitors	Antithrombin III, heparin cofactor II, protease nexin I, plasminogen activator inhibitor, protein C inhibitor
Enzymes	thrombin, lipoprotein lipase,
Cell adhesion molecules	L-selectin, N-CAM, netrin
Viral proteins	Gp120 of HIV, gC and gB and gD of HSV
Nucleus protein	c-fos, c-jun, RNA polymerase, DNA polymerase

Table 2. HS structure required to bind ligands.

Ligands	Binding structures	Reference
FGF-1	hexasaccharides containing $[IdoA(2S)\text{-}GlcNS(6S)]_{1-3}$	16, 17
FGF-2	hexasaccharides containing $[IdoA(2S)\text{-}GlcNS]_{1-3}$	18-21
FGF-4	hexasaccharides containing IdoA(2S)-GlcNS or IdoA-GlcNS(6S) unit	24
FGF-18	octasaccharides containing 2 IdoA-GlcNS(6S) units	23
HGF	octasaccharides containg 2 IdoA(2S)-GlcNS(6S) units	29, 30
Antithrombin III	GlcNAc(6S)-GlcA-GlcNS(3S, ±6S)-IdoA(2S)-GlcNS(6S)	4, 28
HSV gD	oligosaccharides containing IdoA(2S)-$GlcNH_2$(3S, ±6S)	27
Laminine	$[IdoA(2S)\text{-}GlcNS(6S)]_6$	25
Fibronectin	$[IdoA(2S)\text{-}GlcNS(6S)]_6$	26

heparan sulfate *via* mainly electrostatic interaction. Because heparan sulfate generally contains IdoA(2S)-GlcNS(6S) unit as a minor component, the synthesis of the highly sulfated cluster should be achieved by a specific biosynthetic mechanism. Herpes simplex viruses enter infected cells through the binding of the virus glycoprotein gD to cell surface heparan sulfate containing IdoA(2S)-$GlcNH_2$(3S) units[27]. Antithrombin III binds a unique pentasaccharide sequence of heparan sulfate, in which the essential 3-*O*-sulfated *N*-sulfoglucosamine residue is located in the central position of the sequence[4),28].

The sulfation pattern and the extent of C-5 epimerization thus play important roles in the production of the specific functional domains of heparan sulfate. Recent developments in surface non-covalent association mass spectrometry (SNA-MS) allow the sequencing of ligand-specific oligosaccharides using extremely small amounts of sample (10 to 100 pmol)[31]. The application of such a new technology to the structural analysis of ligand-specific binding domains may accelerate the study of the function of heparan sulfate in the cellular response to alien signal molecules.

Modification Enzymes Responsible for the Production of the Diverse Structure of Heparan Sulfate

Each cell produces a cellular specific heparan sulfate in a temporally and specially regulated manner. The biosynthesis of heparan sulfate is initiated by the addition of linkage tetrasaccharides (GlcA-Gal-Gal-Xyl-) to Ser residues located in the unique sequences of the core proteins. Then, alternating GlcNAc and GlcA are transferred from the respective nucleotide sugars (UDP-GlcA and UDP-GlcNAc) to generate polysaccharides. The polysaccharides are subsequently subjected to *N*-deacetylation/*N*-sulfation, C-5 epimerization and *O*-sulfation at different positions (Fig. 2) (see Ref. 28, 32-36 for a review of the biosynthesis of heparan sulfate). There are significant variations in the order of these biosynthetic steps. The modification enzymes appear to form aggregates and achieve fine cooperation for producing heparan sulfate with defined structures. Recently, an interesting possibility has been shown that heparan sulfate synthesized in the Golgi apparatus may be subjected to further degradation in the extracellular space by unique sulfatases after transport to the cell surface or secretion into the extracellular matrix. The extracellular modification of heparan sulfate may control the activity of the various proteins that bind to heparan sulfate. In the following sections, we have focused on each modification reaction for heparan sulfate.

Modification Enzymes Involved in the Synthesis of Heparan Sulfate

Heparan Sulfate *N*-deacetylase/*N*-sulfotransferase (NDST)

N-Deacetylase/*N*-sulfotransferase (NDST) which catalyzes the initial modification reaction of heparan sulfate has dual functions; *N*-deacetylase activity to remove the *N*-acetyl group of GlcNAc, and *N*-sulfotransferase activity to transfer sulfate to the resulting free amino group of $GlcNH_2$. Four isoforms of NSDT have been reported to date[37-43]. The ratio of *N*-deacetylase activity to *N*-sulfotransferase activity varied from 10.5 to 0.04 among these isoforms. The three-dimensional structure of the *N*-sulfotransferase domain of NDST-1 was determined by X-ray crystallography, and a cleft to which the heparan sulfate chain should be bound was found on the surface of the molecule. When three-

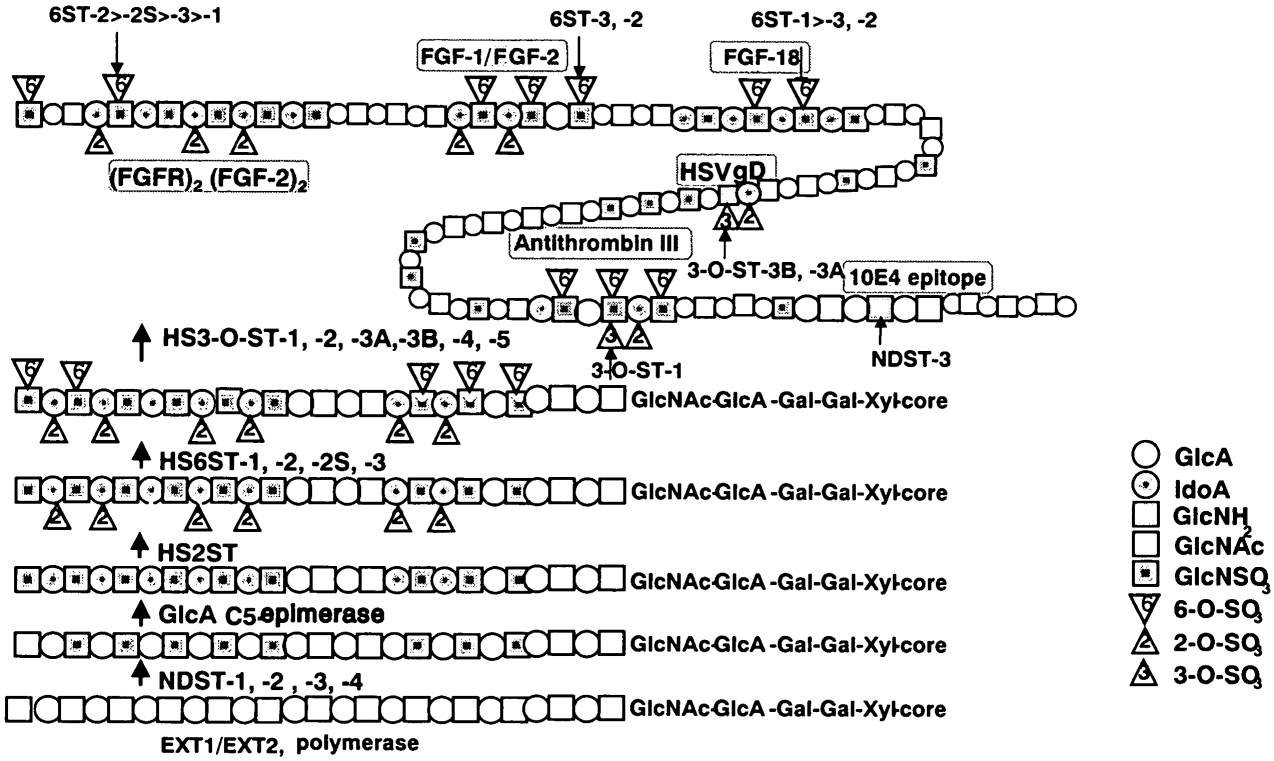

Fig. 2. Scheme for the modification of heparan sulfate biosynthesis.
The symbols used are defined in the figure. Also shown are the binding sites for the specific ligand and sulfotransferase involved in generating these structures. Some sulfotransferases are presumed from the expression tissue and substrate specificities of sulfotransferases.

dimensional models of the three NDST isoforms were constructed on the basis of the three-dimensional structure of the *N*-sulfotransferase domain of NDST-1, several amino acid residues within the cleft were found to differe among these isoforms, suggesting that the difference in substrate specificity among these isoforms is caused by the interactions between heparan sulfate and the amino acid residues located in the cleft. NDST-1 null mice were found to die in the neonatal stage and to be deficient in the synthesis of heparan sulfate, whereas NDST-2 null mice were born normally and fertile, but failed in producing the granules in connective tissue-type mast cells. These phenotypic changes observed in NDST-2 null mice were thought to result from a defect in the synthesis of heparin.

Recently, the epitope for a monoclonal antibody 10E4 that recognizes heparan sulfate was reported to be a unique structure containing -GlcNH$_2$-HexA-[44]. The mAb was found to stain amyloid plaques. Interestingly, at the early stages of infection by prions, the antigen was co-distributed with the infective prion protein in the affected part of the brain. The GlcNH$_2$ residues are thought to be formed by the deacetylation of GlcNAc without successive *N*-sulfation. As described above, the ratio of *N*-deacetylase activity to *N*-sulfotransferase activity was highest for NDST-3 and lowest for NDST-4. Because NDST-3 and NDST-4 were both expressed strongly in the brain, the synthesis of the 10E4 epitope might be regulated by the relative activity of NDST-3 and NDST-4.

Glucuronosyl C-5 epimerase

After the *N*-deacetylation/*N*-sulfation of GlcNAc residues, GlcA residues adjacent to the resulting GlcNS are converted to IdoA by glucuronosyl C-5 epimerase. Only one epimerase has been reported so far and this enzyme is thought to be involved in the biosynthesis of both heparin and heparan sulfate, although proportions of IdoA in these glycosaminoglycans differ markedly[45–47]. The epimerase is thought to work in the Golgi where NDST is present. However, when the *N*-terminal tail of the epimerase was replaced with an ER retention signal derived from the cytoplasmic domain of human invariant chain (p 33), the epimerase became to be relocated in the ER[48]. The relocation of the epimerase to the ER caused a parallel redistribution of HS2ST. Transfected epimerase was also located in the ER in a cell mutant lacking HS2ST, but moved to the Golgi when the cells were transfected with HS2ST cDNA. These observations suggest that the epimerase and HS2ST form a complex in the Golgi and the modification of heparan sulfate is regulated efficiently by the formation of the multi-enzyme complex.

Heparan sulfate 2-sulfotransferase (HS2ST)

Heparan sulfate 2-sulfotransferase (HS2ST) transfers sulfate to position 2 of IdoA residues formed by C-5 epimerization of GlcA, producing IdoA (2S), an essential component of the FGF-2 binding domain. The amount of IdoA (2S) residue in heparan sulfate is generally less than 10 %. However, only one isoform of HS2ST has been reported[49),50]. Because mutant CHO cells having a chemically mutated HS2ST gene and mice in which the HS2ST gene was knocked out by the gene trapping method both synthesized heparan sulfate totally devoid of IdoA (2S), it is unlikely that multiple isoforms of HS2ST would be present[51–53]. HS2ST also appears to be involved in the synthesis of the GlcA (2S) residue, a minor component of heparan sulfate, because the mutant CHO cells mentioned above could not synthesize GlcA (2S)[54),55].

Heparan sulfate 6-sulfotransferase (HS6ST)

HS6ST transfers sulfate to position 6 of GlcNS residues. Three isoforms of HS6ST, HS6ST-1, -2 and -3, have been reported[56–58]. An additional isoform, HS6ST-2S, which was formed from the HS6ST-2 gene by alternative splicing, was recently found (Habuchi et al. unpublished data). The two differently spliced isoforms were expressed in a tissue-specific manner; HS6ST-2 in the brain and HS6ST-2S in the ovary. All HS6ST isoforms are able to synthesize the IdoA (2S)-GlcNS (6S) units that are required for the formation of the ternary complex composed of FGF, FGFR and heparan sulfate; HS6ST-2 showed the highest level of activity. These isoforms are distinguished by their preference for the structure of uronic acid residues adjacent to the targeted GlcNS. In addition to the internal GlcNS residues of heparan sulfate, these HS6ST isoforms were found to transfer sulfate to the nonreducing terminal GlcNS residues and produce GlcNS (6S) residues at the nonreducing terminal. The nonreducing terminal GlcNS (6S) may be one of the substrates for Qsulf1 that is involved in Wnt signaling. The nonreducing terminal structure of heparan sulfate may thus bear important information.

HS6STs transferred very little sulfate *in vitro* to GlcNAc residues of heparan sulfate, although GlcNAc residues are abundant in heparan sulfate. However, overexpression of HS6ST-1 in CHO cells resulted in an increase of GlcNAc (6S) in the metabolically labeled heparan sulfate; the content of GlcNAc (6S) was nearly proportional to the HS6ST activity in the transfected cells (Habuchi et al. unpublished data). Furthermore, the GlcNAc residue contained in the unique ATIII-binding domain was reported to be sulfated by recombinant HS6ST-1 *in vitro*[59]. Taken together, HS6ST may retain activity toward GlcNAc residues under certain conditions. The sulfation of GlcNAc residues by HS6ST could occur in a specific sugar sequence such as the ATIII-binding domain or take place when the heparan sulfate chain is elongating.

Heparan sulfate 3-sulfotransferase (HS3-OST)

Heparan sulfate 3-sulfotransferase (HS3-OST) transfers sulfate to position 3 of GlcNS residues. At least five isoforms of HS3-OST (HS3-OST-1, HS3-OST-2, HS3-OST-3A, HS3-OST-3B and HS3-OST-4) are known to exist[60–63]. Recently, an additional isoform (HS3-OST-5) was identified[64]. Each of these isoforms is expressed in various tissues differently and shows a distinct substrate

specificity. The 3-O-sulfation of GlcNS residue in the ATIII-binding sequence was carried out by HS3-OST-1[61] and partly by HS3-OST-5[64]. HS3-OST-2 is able to synthesize the IdoA(2S)-GlcNS(3S) unit that is present in the glomerular basement membrane in a large quantity and is thought to be involved in the control of the protein filtration. The IdoA(2S)-GlcNH$_2$(3S) unit identified as binding glycoprotein gD of the herpes simplex virus could be synthesized by HS3-OST-3A and -3B[27], and partly by HS3-OST-5.

Recently, many studies on the role of HSPGs in development, morphogenisis and basic physiological functions have been carried out using invertebral model animals such as *Drosophila* and *C. elegans*. As described above, vertebrates such as humans and mice contain multiple isoforms for the enzymes involved in the synthesis and modification of heparan sulfate chains except for C-5 epimerase and HS2ST, whereas only one gene for each biosynthetic step is known in *Drosophila* and *C. elegans*. The presence of multiple isoforms in vertebrates may be important for the synthesis of the tissue-specific and unique structures of heparan sulfate.

Modification of Heparan Sulfate in the Intracellular Matrix and on the Cell Surface

N-Acetylglucosamine 6-sulfatase (QSulf1) ?

After its secretion into the extracellular space, heparan sulfate may be subjected to structural modification by QSulf1 and thereby regulate Wnt signaling. QSulf1 was identified in a molecular cloning screen for Sonic hedgehog response genes activated during somite formation in quail embryo[65]. QSulf1 was identified as a member of a family of evolutionarily conserved sulfatases related to the lysosomal *N*-acetyl glucosamine 6-sulfatases that catalyze the hydrolysis of 6-*O*-sulfates from *N*-acetylglucosamine at the nonreducing end of heparan sulfate during the degradation of HSPGs[66]. QSulf1 is localized on the cell surface and regulates heparan-dependent Wnt signaling in C2C12 myogenic progenitor cells through a mechanism that requires its catalytic activity, providing evidence that QSulf1 regulates Wnt signaling through desulfation of cell surface HSPGs. However, it remains to be determined whether QSulf1 bears sulfatase activity toward heparan sulfate.

Heparan sulfate deacetylase (Notum/Wingful) ?

The *Notum* gene was identified in a gain-of-function genetic screen that caused the loss of the wings and duplication of the dorsal thorax[67),68]. *Notum* encodes a secreted protein similar to a member of the α/β-hydrolase superfamily including pectin acetylesterase in plants. Notum overexpression phenotypes resemble the defect caused by the *Wg* (Wnt for mammals) mutant, whereas loss of Notum function leads to increased wingless activity by altering the shape of the wingless protein gradient. From these observations, *Notum* acts as a novel secreted antagonist of *Wg*.

Notum is postulated to be a deacetylase that is able to act on GlcNAc residues of heparan sulfate for the following reasons: (1) Notum showed homology to pectin acetylase, (2) Dally, *Drosophila* glypican, and dally-like protein all decreased when Notum was expressed, and (3) the expression of NDST abolished the effects of Notum. The nature of Notum should be clarified further by the biochemical approach.

Regulated Expression of HS-Modifier Enzymes and Its Physiological Significance

Many FGFs have critical roles during the formation of the central nervous system[69]. FGF-2 and FGF-8 are particularly important in the early phases of patterning, proliferation and neurogenesis. Different FGFs appear to require different structural domains in heparan sulfate to transduce their signals through specific FGFRs. Also, HSPGs are likely to modulate FGF signaling in the developing brain. The structures of HS purified from neuroepithelial cells at different developmental stages were compared. When HSs were isolated from a primary culture of neural precursor cells at middle of the proliferative phase, at embryonic day 10 (E10), and at the stage where neural differentiation begins (E12), the HS from E12 cells had longer chains with more extensively sulfated domains, a higher level of 2-*O*-sulfation and altered patterns of 6-*O*-sulfation and *N*-sulfation compared with the HS obtained from the E10 cells[70]. These stage-specific heparan sulfates showed a distinct ability to enhance the signal transduction through FGF-FGFR complexes, and are implicated in brain development; HS from E10 preferentially activated FGF-8 which functions only in the early stages (E8-E11). HS from stages E10-E13 similarly activated FGF-2 which functions at the same stages, and was a more potent activator for FGFR1c than for the other receptors (Fig. 3)[71]. These structural changes of HS correlated with the expression profiles of HS-ST that varied markedly in both *in vivo* and *in vitro* systems (Fig. 4).

Developing axons are guided to their appropriate targets by a succession of guidance molecules distributed along the pathway, which act as local cues to attract or repel the growth cones of elongating axons[72]. These guidance molecules such as netrin-1 and netrin-2 bind to heparin. Furthermore, HS affects the outgrowth of neurites from neurons *in vitro*. Some HSs, when added exogenously to the developing *Xenopus* optic pathway, cause a severe misrouting of retinal axons at the tectal border. The removal of native HSs by heparitinase digestion at the beginning of optic tract formation retards retinal axon elongation[73]. The structural requirement for HS determined using chemically modified heparins indicated that the 2-*O*- and 6-*O*-sulfate groups had a potent bypass-inducing activity. In situ hybridization in the embryonic brain revealed that HS 6ST is expressed region-specifically along the border of the dorsal optic tract whereas HS2ST is expressed broadly. Taken together, 6-*O*-sulfated HS may play a repulsive role in steering axons across the diencephalic/midbrain border

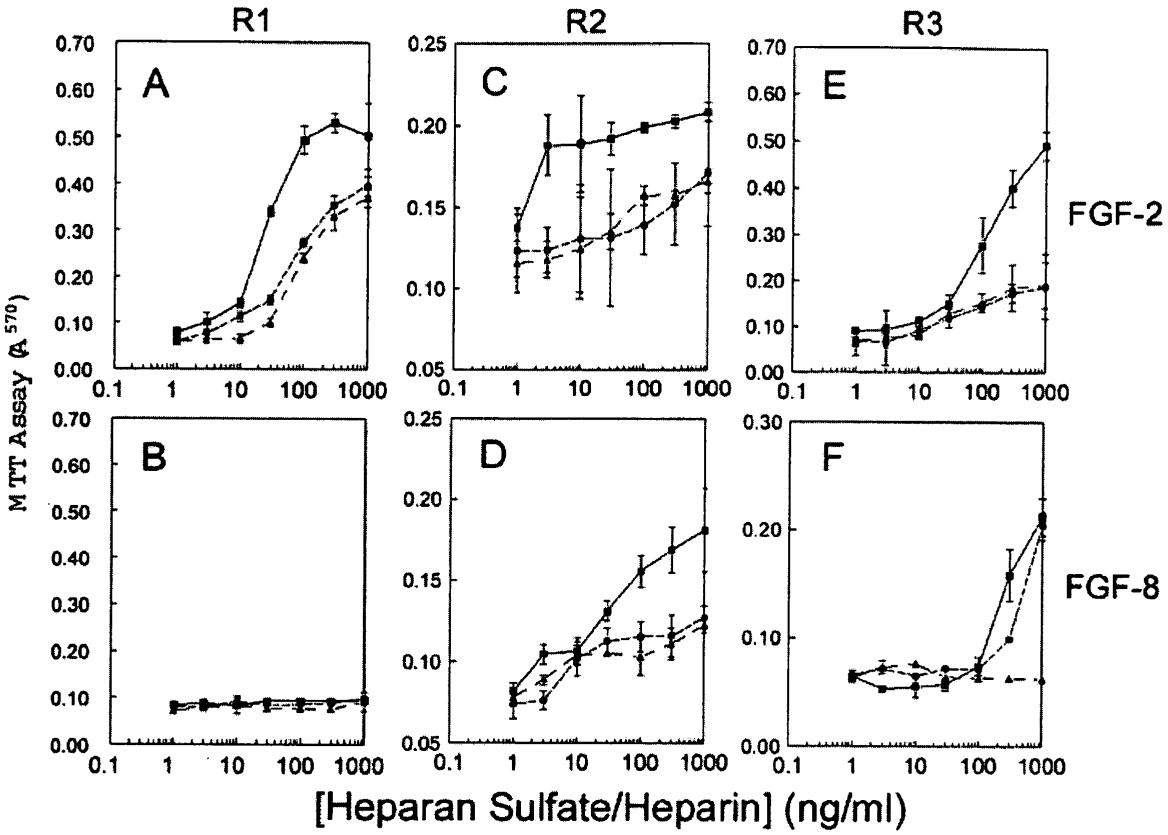

Fig. 3. HSs from different stages of neuroepithelial development have different abilities to activate specific FGF-FGFR signaling complexes (cited with permission from Ref. 71).
HS was purified from E10 or E12 mouse neuroepithelia. BaF3 cells expressing either FGFR1c (A and B), FGFR2c (C and D) or FGFR3c (E and F) were incubated with FGF2 (A, C and E) or FGF8 (B, D and F) and different concentrations of either heparin (squares), E10 HS (circles), or E12 HS (triangles). An MTT assay was used to measure resulting cell numbers.

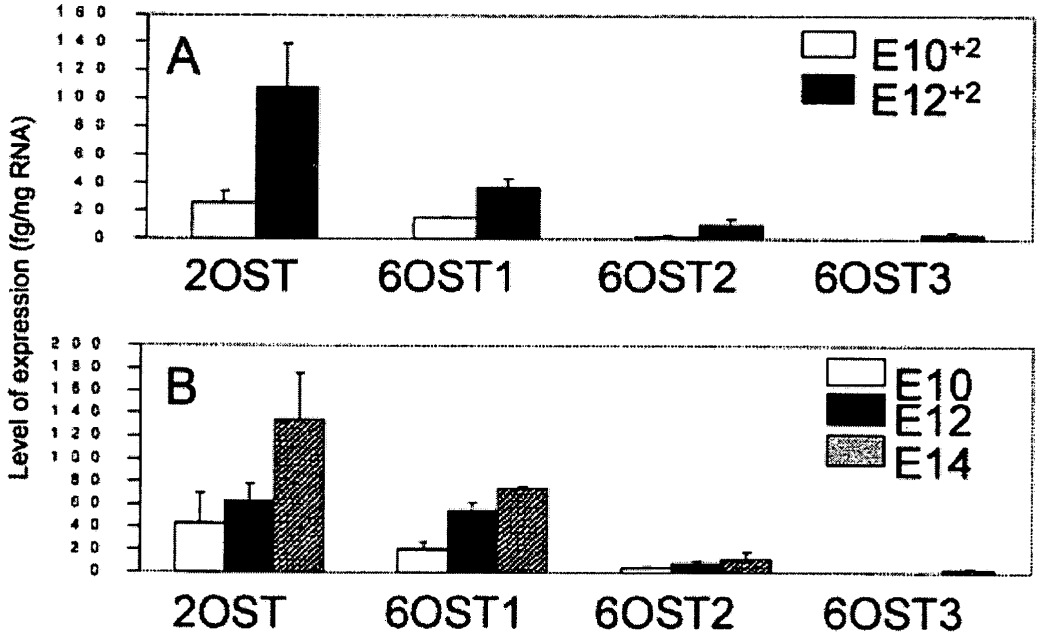

Fig. 4. Quantitative RT-PCR of *O*-sulfotransferase mRNAs in neutral precursor cells (A) and in the developing brain (B) (cited with permission from Ref. 71).
RNAs were isolated from neuroepithelium cells at E10 or E12 (A) and from neuroepithelium tissues at E10, E12 or E14, respectively (B). Expression of HS2ST and HS6ST1-3 was examined by quantitative RT-PCR.

into the tectum[74].

Axon branching and misrouting by the Kallmann syndrome kal-1 gene seems to be induced by a mechanism dependent on HS6ST[75]. Kallmann syndrome is a genetic disease defined as an association of hypogonadotropic hypogonadism and anosmia, *i.e.* the inability to smell. The X-linked form of this disease is caused by mutation in the KAL-1 gene. In those with this disease, the axons of the olfactory receptor neurons still project toward the forebrain, however, they fail to establish connections with their target cells. Moreover, neurons synthesizing gonadotropin-releasing hormone also fail to reach the hypothalamus, thus disrupting the hypothalamic-pituitary hormonal axis and causing hypogonodotropic hypogonadism. To clarify the function of KAL-1 in Kallmann syndrome, Bulow et al. investigated the *C. elegans* ortholog of the human gene[75]. The *C. elegans* homolog of KAL-1 is expressed in selected sensory and interneutron classes, and heterologous *C. elegans* Kal-1 expression causes a highly penetrant axon-misrouting phenotype. On screening for genes capable of modifying the Kal-1 mis/overexpression phenotype, several loci that either suppress or enhance the kal-1-induced axonal defects were identified. One of these modifier genes encoded HS6ST. As KAL-1 protein has the capability to bind various cells and its binding is inhibited by heparin, it is likely that KAL-1 binds by means of a heparan sulfate proteoglycan with a specific HS structure to its cognate receptor or other extracellular cues to induce axonal branching and axon misrouting.

VEGF, FGF-2 and HGF show strong angiogenic activity and have the ability to bind heparin and heparan sulfate. Also, the signal transduction by these growth factors is regulated by heparin. When vascular endothelial cells were incubated under hypoxic conditions to stimulate angiogenesis *in vivo,* the synthesis of heparan sulfate by these cells was promoted and the number of FGF-2-binding sites per cell increased about 2-fold[76]. Similar changes were observed when HIF-1 α, a hypoxia-inducible factor that stimulates the expression of angiogenesis-related genes such as the genes for VEGF and its receptor flt1, was added into the culture medium. When the cells were treated with hypoxia or HIF-1α, the expression level of the mRNA for the enzyme involved in each step of the biosynthesis of HS was altered differently. The mRNA levels of NDST-1, NDST-2 and HS2ST increased markedly, whereas the expression of C5 epimerase was hardly affected at all. A slight decrease (0. 6- to 0. 75-fold) in the mRNA level of HS6ST-2 was observed, while the expression of HS6ST-1 was unchanged. The increase in the proportion of HS in the cell surface glycosaminoglycans and the increase in the number of low affinity binding sites for FGF-2 under the hypoxic conditions may account for the altered sensitivity of the cells to FGF-2. In fact, the cells exposed to the hypoxic conditions demonstrated a greater proliferative response to FGF-2 than the control cells.

Knockout Mice of HS-Modifier Enzyme Genes

EXT1 knockout mice

The EXT1 enzyme has the activity of both a glucuronyltransferase and a glucoseaminyltransferase and is involved in the polymerization of heparan sulfate chain. On the loss of this gene, homozygous embryos died in the gastrulation period before E 8.5 and lacked an organized mesoderm and extraembryonic tissues. The phenotypes observed in this knockout mouse indicate clearly that heparan sulfate plays pivotal roles in development and differentiation.

NDST-1 knockout mice

NDST is a bifunctional enzyme composed of a single polypeptide that catalyzes the *N*-deacetylation and *N*-sulfation of GlcNAc residue in heparin and heparan sulfate. This modification step is a prerequisite for all other modifications. Since NDST-1 was purified and cloned from rat liver, it is likely that the enzyme is responsible for producing heparan sulfate.

Most NDST-1 deficient embryos survive until birth. However, they turn out to be cyanotic and die neonatally in a condition resembling respiratory distress syndrome[77),78]. Lungs from these newborn NDST-$1^{-/-}$ mice are severely atelectatic. This appears to be due to reduced secretion of Surfactant protein A and B, which are essential for normal respiratory gas exchange, although the number of cells producing these proteins increases and surfactant proteins are present in lungs of NDST-$1^{-/-}$ embryos. In addition, a minor proportion of NDST-1 deficient embryos die during the embryonic period (between E14.5 ane E18.5). The cause of the embryonic lethality is not clear, but incompletely penetrant defects of the skull and the eyes have been observed. NDST-$1^{-/-}$ mouse embryos produce much less *N*-sulfated HS and following a modified reaction, *O*-sulfation and epimerizarion also have been decreased. In cultured fibroblasts, the degree of *N*-sulfation was lowered from $>40\%$ in HS from NDST-$1^{+/+}$ to $<15\%$ in NDST-$1^{-/-}$ HS. As revealed by immunohistochemical staining using antibodies against heparan sulfate, production of heparan sulfate was reduced or ceased in mutant lung, liver and kidney basement membrane. Judging from these observations, NDST-1 is essential for the *N*-sulfation of heparan sulfate in various tissues in contrast to NDST-2 as described below.

NDST-2 knockout mice

Since NDST-2 was purified and cloned from heparin-producing mouse mastocytoma, and its mRNA was expressed strongly in heparin-producing mast cells, it was speculated that this enzyme catalyzes the *N*-sulfation of heparin.

Null mice carrying a targeted disruption of the gene encoding NDST-2 are born normally, are fertile, and show no obvious pathological phenotype[79),80]. However, these null mice show remarkable defects in connective-type mast cells, which are active during inflammatory

conditions and release granular contents, heparin, mast cell specific protease, histamine, and a large number of inflammatory mediators. The synthesis of heparin in NDST-$2^{-/-}$ mice was absent in the peritoneal cells including mast cells, and the connective-type mast cells of these mice showed an altered morphology, contained reduced amounts of histamine, and were lacking mast cell-specific protease (mMCP-4, -5, -6, and mMC-CPA). However, mRNAs for these proteins are expressed at normal levels. Taken together, heparin in mast cells plays a role in storing these materials in secretary granules. Furthermore, NDST-$2^{-/-}$ mice show no obvious signs of thrombosis although heparin has been used clinically as an anticoagulant and antithrombotic agent for over 60 years. Therefore, endogenous heparin is not involved in the regulation of blood clotting.

HS2ST knockout mice

HS2 S T null mice were generated by a random gene trap insertion[52]. In contrast to the early developmental failure of embryos lacking the EXT1 gene, the onset of abnormalities in the mutant mice occurred after midgestation and the mutant mice died in the neonatal period. Homozygous mutants showed bilateral renal agenesis and possessed a blind-ended ureter. However, the remainder of the mutant urogenital system such as the adrenal gland, bladder and ovary was normal. The HS2ST gene was important for mesenchymal condensation around the ureteric bud and the initiation of branching morphogenesis. These null mice exhibited additional defects of the eye and skeleton and some of the homozygous individuals having a cleft palate and polydactyly. 2-O-sulfated groups of hexuronic acid residues were not detected in the mutant HS. Interestingly, compensatory increases in *N*- and 6-*O*-sulfation maintain the overall charge density[53].

The three mutants described above individually generate heparan sulfate having different structures and show different phenotypes and abnormal functions. These observations suggest complicated regulatory mechanisms behind the interaction between heparan sulfate and ligands.

CONCLUDING REMARKS

For determining the functions of the fine structures of heparan sulfate, gene-targeting of the enzymes involved in the biosynthesis of heparan sulfate has been carried out using various animal systems. Phylogenetically lower animals generally having a smaller number of the isoforms, compared with mice or humans, show severe and clear phenotypic changes on the loss of the genes[81]. In mouse systems, however, multiple isoforms exist for a single modification and the activities of these enzymes are regulated in a more complicated manner. The results obtained in the lower animals, therefore, seem to offer genuine clues as to the disease-related genes involved in the synthesis of heparan sulfate. Furthermore, the conditional gene-targeting in mouse systems may allow one to study the functions of heparan sulfate in defined organs.

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