In Vitro Studies of the Substrate Specificities of Heparan Sulfate 2-O- and 6-O-sulfotransferases

BY
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Abstract


Heparan sulfate (HS), a linear negatively charged polysaccharide located at the cell surface and in the extracellular matrix, interacts with, and thereby regulates the functions of numerous proteins. HS-protein interactions depend on the fine structure of HS, especially its sulfation pattern. This thesis aimed to understand how differently sulfated domains in HS are generated. Specifically, the substrate specificities of HS hexuronic acid 2-O-sulfotransferase (2OST) and HS glucosaminyl 6-O-sulfotransferases (6OSTs) were investigated.

Three different 6OSTs (6OST1-3) have been cloned and characterized. To study the mechanisms controlling 6-O-sulfation we incubated the recombinant purified 6-OST isoforms with different 6-O-desulfated poly- and oligosaccharide substrates and the active sulfate donor 3'-phosphoadenosine 5'-phospho[35S]sulfate (35S-labeled PAPS). All three enzymes catalyzed 6-O-sulfation of both N-acetylated (GlcNAc) as well as N-sulfated (GlcNS) glucosamines next to a nonreducing iduronic acid (IdoA) or glucuronic acid (GlcA). Similar specificities were demonstrated, although some differences in substrate preferences were noted.

To understand how pre-existing 2-O-sulfates affects 6-O-sulfation, 6OST2 and 6OST3 were incubated with pair-wise mixed octasaccharide substrates with different contents of 2-O-sulfates. The specificities for substrates with two or three 2-O-sulfates were higher compared to octasaccharides with no or one 2-O-sulfate indicating that 2-O-sulfate groups substantially promote the subsequent 6-O-sulfation.

Overexpression of the 6OSTs in a mammalian cell line resulted in increased 6-O-sulfation of -GlcA-GlcNS- and -GlcA-GlcNAc- sequences. The results were not isoform specific, but affected by the overexpression level.

The 2OST catalyzes 2-O-sulfation of both IdoA and GlcA residues, with high preference for IdoA units. To study how 2-O-sulfation of GlcA and IdoA is regulated, we incubated the enzyme with different substrates and 35S-labeled PAPS. Our findings revealed that the 2OST almost exclusively sulfated IdoA also with a ratio of GlcA to IdoA of 99:1, suggesting that 2-O-sulfation of GlcA occurs before IdoA is formed.

Keywords: heparan sulfate, heparin, sulfotransferase, 2-O-sulfotransferase, 6-O-sulfotransferase, polysaccharide biosynthesis, glycosaminoglycan, O-sulfotransferase, proteoglycan

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To all who supported me
"Wisdom is not a product of schooling but of the lifelong attempt to acquire it" – Albert Einstein
List of Papers

This thesis is based on the following papers:


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* Both authors contributed equally to this manuscript.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>aManR</td>
<td>2,5-Anhydromannitol</td>
</tr>
<tr>
<td>AT</td>
<td>Antithrombin</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
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<td>GlcA</td>
<td>β-D-Glucuronic acid</td>
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<td>GlcN</td>
<td>D-Glucosamine</td>
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<td>GlcNAc</td>
<td>N-Acetyl-D-glucosamine</td>
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<td>GlcNS</td>
<td>N-Sulfate-D-glucosamine</td>
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<tr>
<td>HexA</td>
<td>Unspecified hexuronic acid</td>
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<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>IdoA</td>
<td>α-L-Iduronic acid</td>
</tr>
<tr>
<td>NDST</td>
<td>N-Deacetylase/N-sulfotransferase</td>
</tr>
<tr>
<td>OST</td>
<td>O-Sulfotransferase</td>
</tr>
<tr>
<td>PAPS</td>
<td>3'-Phosphoadenosine-5'-phosphosulfate</td>
</tr>
<tr>
<td>PG</td>
<td>Proteoglycan</td>
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<tr>
<td>UDP</td>
<td>Uridine diphosphate</td>
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Introduction

Glycobiology
Glycobiology is the study of the structure, biosynthesis and biology of saccharides that are widely distributed in nature. It is one of the more rapidly growing fields in biomedical sciences, with relevance to basic research, biomedicine, and biotechnology (Varki et al. 1999). Proteins carrying carbohydrate modifications can be divided into two different groups. Glycoproteins are proteins that carry one or more oligosaccharide chains covalently linked to an asparagine, serine or threonine residue. The other group, the proteoglycans, instead has one or more glycosaminoglycan (GAG) chains covalently linked to a serine or in rare cases, a threonine residue. The glycoprotein oligosaccharides are built with different individual monosaccharides and may be branched, whereas GAGs are linear and composed of repeated disaccharide units (Varki et al. 1999).

Proteoglycans
Proteoglycans (PGs) are proteins with one or more covalently attached glycosaminoglycan (GAG) chain. There are two different main types of GAGs found in PGs: the glucosaminoglycan family comprising heparan sulfate (HS) and heparin; and the galactosaminoglycan family containing chondroitin sulfate (CS) and dermanan sulfate (DS) (for review see (Kjellén and Lindahl 1991)). In addition, keratan sulfate (KS) belongs to the glucosaminoglycan family, but will not be discussed further.

Chondroitin sulfate and dermanan sulfate
Chondroitin sulfate and dermanan sulfate are sulfated polysaccharides and despite their wide distribution, CS and DS have until very recently attracted less attention than HS. Growing evidence suggests that CS and DS chains have intriguing functions in the central nervous system (CNS), growth factor signaling, morphogenesis and cell division, in addition to their structural roles (reviewed in (Sugahara et al. 2003)). CS and DS also interact with heparin-binding proteins, and the interactions of DS chains with fibroblast growth factor (FGF)-2 and FGF-7 are implicated in wound repair
Heparan sulfate proteoglycans (HSPGs) are found in vertebrates as well as in invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans* (for review see (Selleck 2001)). HS chains are found covalently linked to core proteins, constituting HSPGs. HSPGs occur on the surface of all nucleated cells and in the ECM (for reviews see (Bernfield et al. 1999; Esko and Lindahl 2001)). HS substitutes a variety of cell surface proteins but is consistently found on members belonging to two major families of membrane-associated core proteins, the syndecans and the glypicans. Syndecans (four members) are membrane spanning, whereas glypicans (six members) are glycosyl phosphatidyl inositol (GPI) anchored to the plasma membrane. In the ECM, the major HS core proteins are perlecan and agrin. Each syndecan and glypican gene product bears HS chains as a constant feature in contrast to the so-called part-time PGs that exist with or without HS. Examples of part-time PGs carrying HS chains are: CD44, agrin and collagen XVIII (Bernfield et al. 1999). The principal differences between the PGs serglycin (the heparin PG), perlecan, syndecan and glypican are shown in Fig. 1.

HS has been known to be ubiquitous at animal cell-surfaces for more than 30 years (Kraemer 1971a; Kraemer 1971b). Although HS is generally more abundant at the cell surface than most receptors, HS has only recently been recognized as a physiologically important cell surface molecule. HSPGs interact with a variety of protein ligands including growth factors, matrix components, proteases, protease inhibitors and microbial proteins (for review see (Bernfield et al. 1999)). It is generally believed that the interaction between proteins and HS depends on the relative positions of sulfate and carboxyl groups in the HS chain. These negatively charged groups are recognized by positively charged amino acids on the protein surfaces. The HS chains of syndecans and glypicans bind a variety of soluble and insoluble extracellular ligands, with relatively high affinity (K_D of 1-100 nM) mediating binding at physiological salt concentrations (Bernfield et al. 1992; Conrad 1998).
Figure 1. Different heparan sulfate proteoglycans. The glypicans and the syndecans are associated with the plasma membrane, whereas perlecan is found in the extracellular matrix. Serglycin, present in the granules of connective tissue mast cells, carries heparin chains. Adopted from (Lindahl et al. 1994).

Heparin

Heparin is a highly sulfated variant of HS that is synthesized by connective tissue-type mast cells. Heparin chains are assembled on the core protein serglycin (Fig. 1) and stored in intracellular granules that are released during certain inflammatory reactions (Kjellén and Lindahl 1991). Although heparin has long been used as an antithrombotic drug, endogenous heparin is not present in the blood, showing that there is no physiological role for heparin in regulating blood coagulation. The role of heparin in mast cells is not clear, but heparin appears to be important for packaging of proteases present in the granules. Mast cells lacking heparin have altered morphology and reduced amounts of histamine and mast cell proteases (Forsberg et al. 1999). Furthermore, the mouse mast cell protease MCP-6 depends on heparin for for-
formation of active tetramers (Hallgren et al. 2000). HS and heparin not only differ in their respective tissue distribution, they also show structural differences. HS polysaccharide chains are generally more variable in structure and less sulfated than heparin chains (Lindahl and Kjellén 1991). However, a particular HS species isolated from oligodendrocyte-type-2 astrocyte precursor cells is structurally similar to heparin (Stringer et al. 1999) and also liver HS is relatively highly sulfated (Lyon et al. 1994). Due to its easy access from the pharmaceutical industry, heparin has commonly been used in experimental systems for determining binding to proteins of interest, although the physiological ligand would be HS. In such studies, numerous proteins have been purified and characterized after procedures including an affinity chromatography step on heparin-Sepharose.

The anticoagulant effect of heparin requires the participation of antithrombin (AT), a plasma protease inhibitor. AT inhibits coagulation by forming stable complexes with proteases (most predominantly thrombin). The reactions between AT and thrombin are slow in the absence of heparin (Björk and Lindahl 1982). However, heparin can accelerate these reactions more than thousand-fold, which effectively inhibits fibrin formation. The AT binding sequence of heparin has been characterized in detail (Lindahl et al. 1979; Lindahl et al. 1984). The minimum requirement for AT binding to heparin is a pentasaccharide sequence with a rare 3-O-sulfated GlcNS residue. The structure of the AT binding region is described in a subsequent section.

Biosynthesis of heparan sulfate

The biosynthesis of HS and heparin is a multistep process, which is carried out by a large number of enzymes (reviewed in (Esko and Lindahl 2001; Lindahl et al. 1998; Lander and Selleck 2000)). Biosynthesis of complex carbohydrates is a template-free process, contrary to the generation of other polymers such as mRNA and proteins. The theoretically possible structural diversity of HS exceeds that of other macromolecules such as nucleic acids and proteins (Sasisekharan and Venkataraman 2000).

The initial event in HS biosynthesis is the addition of xylose (Xyl) from the corresponding UDP sugar to a serine residue in the core protein (Fig. 2). The attachment site in the core protein generally consists of a Ser-Gly dipeptide flanked by one or more acidic residues (Zhang and Esko 1994; Esko and Zhang 1996). Addition of two galactose (Gal) residues and one glucuronic acid (GlcA) unit completes the formation of the core protein linkage tetrasaccharide. Distinct glycosyltransferases catalyze each of these reactions; see Fig. 2. The linkage region can be phosphorylated at C2 on the Xyl but the function of this modification remains unclear (Sugahara and Kitagawa 2000). After synthesis of the linkage region common to HS- and CSPGs, one
or more N-acetylglucosaminyl (GlcNAc) transferases add a single α1,4-linked GlcNAc residue to the chain which thus will be committed to the HS type. CS will be synthesized if instead a β1,4-linked N-acetylgalactosamine (GalNAc) residue is added to the linkage region (Esko and Zhang 1996).

**Figure 2. The biosynthesis of the heparan sulfate backbone.** The numbers indicate the corresponding transferase (T): 1, XylT; 2, GalT-I; 3, GalT-II; 4, GlcAT-I; 5, GlcNAcT-I; and 6, GlcAT-II/GlcNAcT-II. Symbols: Gal, galactose; GlcA, glucuronic acid; GlcNAc, N-acetylated glucosamine; Ser, serine; and Xyl, xylose.

The HS chain is polymerized through the action of GlcA- and GlcNAc-transferases (EXT1/2) to generate a backbone of alternating GlcAβ1,4-linked and GlcNAcα1,4-linked residues from their respective UDP-sugars (Lind et al. 1993; Lind et al. 1998). Concomitant with polymerization the modifica-
tion reactions occur. First, N-acetyl groups are replaced by N-sulfate groups, reactions catalyzed by combined N-deacetylase/N-sulfotransferases (NDSTs) (Orellana et al. 1994; Eriksson et al. 1994; Aikawa and Esko 1999; Aikawa et al. 2001) of which four different isoforms are known to date (for review see (Esko and Lindahl 2001)). Because not all potential targets are modified by the NDSTs, the HS chains contain three principally different blocks: the N-acetylated (NA) regions, the N-sulfated (NS) regions and the NA/NS regions with mixed N-acetylated and N-sulfated glucosamine residues (Turnbull et al. 2001; Gallagher 2001). A small proportion of the glucosamine units is N-unsubstituted (GlcNH$_2$), thus containing a free amino group (van den Born et al. 1995; Norgard-Sumnicht and Varki 1995). How free amino groups are formed is not known, it may be a random NDST catalyzed event of deacetylation, without corresponding N-sulfation, or it may exist hitherto unknown intracellular/extracellular N-deacetylases or N-sulfatases. Recent results indicate that neither NDST1 nor NDST2 is involved in these unsubstituted amino groups (Holmborn et al. 2004).

The N-sulfated regions are further modified by the glucuronyl C5 epimerase (only one form known) that converts some GlcA residues into iduronic acid (IdoA) residues (Li et al. 1997; Li et al. 2003). This reaction can only occur downstream (toward the reducing end (Conrad 1998)) of a GlcNS residue, due to the substrate specificity of the epimerase (Hagner-McWhirter et al. 2000). The HS chain is O-sulfated at C2 of hexuronic acid residues by a single 2-O-sulfotransferase (2OST) (Bai and Esko 1996; Rong et al. 2001; Merry et al. 2001), at C6 of GlcN residues by three 6-O-sulfotransferases (6OSTs) (Habuchi et al. 2000) and, more rarely, also at C3 of GlcN by possibly up to seven 3-O-sulfotransferases (3OSTs) (Kusche et al. 1988; Shworak et al. 1999; Kamimura et al. 2004). Also GlcNAc residues in the NA/NS regions can be 6-O-sulfated and it has been estimated that more than half of the 6-O-sulfates are located outside the N-sulfated block regions (Maccarana et al. 1996). Furthermore, although GlcA residues may become 2-O-sulfated, this is much less common than 2-O-sulfation of IdoA residues (Lindahl et al. 1998). Interestingly, there is a relatively high proportion of GlcA(2S) residues in human cerebral brain (Lindahl et al. 1995). The functional importance of this modification is not known. Similar to the cytosolic sulfotransferases, the HS sulfotransferases use 3'-phosphoadenosine 5'-phosphosulfate (PAPS) as a sulfate donor. The rate of synthesis of heparin (and presumably HS) is remarkably fast. Using a mouse mastocytoma microsomal fraction it has been shown that a heparin chain is formed and fully modified within a minute (Lidholt et al. 1989). For a schematic illustration of the HS biosynthesis see Fig. 3. The biosynthesis of heparin is principally the same as that of HS, but the end product (heparin) is more sulfated. The predominant AT binding region in porcine mucosa heparin is the pentasac-
charide -GlcNAc(6S)-GlcA-GlcNS(3S)-IdoA(2S)-GlcNS(6S)- where the underlined sulfate groups are absolutely necessary for high affinity binding to AT (Kusche et al. 1990). The biosynthesis of the AT binding sequence has been extensively studied and current notion places 3-O-sulfation of GlcNS as the final step in the biosynthesis of the AT binding region as well as of the overall HS chains.

Figure 3. Heparan sulfate modifications. Summary of the modification steps involved in the heparan sulfate biosynthesis: 1, N-deacetylation/N-sulfation; 2, C5-epimerization; 3, 2-O-sulfation; 4, 6-O-sulfation and 5, 3-O-sulfation. All sulfotransferase reactions require PAPS as sulfate donor. In this figure the 2-O-sulfation of GlcA is shown to occur along with 2-O-sulfation of IdoA, but may in fact be an earlier event.
Heparan sulfate modifying enzymes

N-deacetylase/N-sulfotransferases
The first class of sulfotransferases acting on the growing HS polysaccharide is the NDST enzymes. The enzyme has dual catalytical activities; one domain with the N-deacetylase activity and one domain harboring the N-sulfotransferase activity. NDST is a key regulator of HS biosynthesis, since its action will create the different NA, NA/NS and NS domains found in HS. Despite the important function of the NDSTs, it is not known how the NDSTs select their target GlcNAc residues. However, the N-sulfation patterns of the resulting polysaccharides point to non random incorporation of the N-sulfate groups.

C5-epimerase
The C5-epimerase is catalyzing the epimerization of GlcA into IdoA. Due to the substrate specificity of the enzyme that only attacks downstream of GlcNS residues, epimerization closely follows the pattern formed by the NDSTs (Hagner-McWhirter et al. 2000).

Heparan sulfate O-sulfotransferases
The HS O-sulfotransferases (OSTs) can be divided into three different families, the 2OST, the 6OSTs and the 3OSTs. The OSTs catalyze the incorporation of sulfate groups to the oxygen of hydroxyl groups on monosaccharide units within HS.

Heparan sulfate 2-O-sulfotransferase
The 2OST catalyzes the transfer of a sulfate group to C2 of both IdoA and GlcA (Fig. 4). The enzyme was first purified and cloned from hamster and initially believed to only sulfate IdoA residues (Kobayashi et al. 1996; Kobayashi et al. 1997). However, when the mouse 2OST later was cloned and expressed in mammalian cells it was shown that the same enzyme sulfates both GlcA and IdoA residues (Rong et al. 2000). HS from 2OST overexpressing cells had increased GlcA 2-O-sulfation that was accompanied by decreased formation of IdoA residues, but the overall 2-O- and 6-O-sulfation remained largely unaffected. In vitro experiments revealed that sulfation of IdoA residues were strongly favored (Rong et al. 2001). The $K_M$ for exclusively IdoA-containing saccharide substrates is approximately fivefold lower than the $K_M$ for a GlcA-containing substrate and the IdoA $V_{max}$ is about three times higher than GlcA $V_{max}$ giving a total difference factor of about 15-fold.
Interestingly, the 2OST and the epimerase travel from ER to the Golgi as a complex (Pinhal et al. 2001). Possibly, this is how the cell ascertains a correct biosynthesis of HS in terms of epimerization and concomitant 2-O-sulfation.

![Figure 4. Enzymatic catalysis of the heparan sulfate HexA 2-O-sulfation.](image)

Heparan sulfate 6-O-sulfotransferases

The 6OSTs transfer a sulfate to the C6 position of GlcN units by the general mechanism indicated in Fig. 5. The first 6OST was purified from serum-free medium of Chinese hamster ovary (CHO) cells (Habuchi et al. 1995), and later cloned from a human fetal brain cDNA library (Habuchi et al. 1998). Three mouse isoforms have been cloned and studies of their mRNA expressions in the adult mouse tissues revealed that 6OST1 (corresponding to the first cloned human enzyme) is strongly expressed in liver, 6OST2 in brain and spleen whereas 6OST3 has a ubiquitous expression (Habuchi et al. 2000). A shorter splice variant of the human 6OST2 has also been reported with expression mainly in ovary and placenta (Habuchi et al. 2003). In naturally occurring heparin and HS, 6-O-sulfate groups are usually found on glucosamine residues on -HexA-GlcNAc-, -HexA±2S-GlcNS- and -HexA±2S-GlcNS3S- sequences. Some information regarding the specificity of the enzymes has been obtained with relatively simple substrates. Recent results have shown that the stem domains of the 6OSTs are required for Golgi localization and enzyme activity (Nagai et al. 2004).
Figure 5. Enzymatic catalysis of the 6-O-sulfotransferases (6OSTs). The hexuronic acid on the target disaccharide can be either glucuronic acid (GlcA) or iduronic acid (IdoA). The target glucosamine is usually either N-sulfated (NS) or N-acetylated (NAc). PAPS indicates the sulfate donor 3′-phosphoadenosine-5′-phosphosulfate, PAP 3′phosphoadenosine-5′-phosphate.

Heparan sulfate 3-O-sulfotransferases
HS 3-O-sulfotransferases transfer sulfate groups to C3 of GlcN residues. The first purified isoform of this enzyme converts non-anticoagulant HS into anticoagulant active HS (Liu et al. 1996), a reaction known to be dependent on 3-O-sulfation of GlcNS (Lindahl et al. 1979; Lindahl et al. 1984). The enzyme was later cloned from both human and murine sources (Shworak et al. 1997). Today, seven different 3OSTs have been cloned (Kamimura et al. 2004), and both 3OST1 and 3OST5 have the capacity to generate anticoagulant active HS (Shworak et al. 1999; Duncan et al. 2004). Several members of the family can catalyze 3-O-sulfation of the rare unsubstituted glucosamine (GlcNH2); 3OST2, 3OST3A, 3OST3B, 3OST4, 3OST5 and 3OST6 (Liu et al. 1999; Shukla et al. 1999; Xia et al. 2002; Kamimura et al. 2004). This reaction generates a high affinity sequence for Herpes simplex adhesion (see “Infectious diseases” paragraph below).

Recent data have indicated that the 3OST2 can be under circadian regulation in the pineal gland, although the physiological importance of this is not fully clear (Kuberan et al. 2004). So far, this is the only HS biosynthetic enzyme known with a preferential daytime expression.

3OST1 was recently crystallized and the structure determined, revealing a cleft containing more positively charged amino acids than the corresponding cleft in NDST1. The observed difference would be expected since the sub-
strate for 3OST1 is more sulfated than that for NDST1 (Edavettal et al. 2004a; Edavettal et al. 2004b). The structure of 3OST3A has also been resolved and was remarkably similar to 3OST1 (Moon et al. 2004), whereas the structures of the other O-sulfotransferases have not yet been elucidated.

**Heparan sulfate endosulfatases**

Recently, a previously unknown type of enzyme was discovered in quail, displaying extracellular sulfatase activity on heparin (Dhoot et al. 2001). The enzyme was named Qsulf1 and was identified in a molecular cloning screen for Sonic hedgehog (Shh) response genes activated during somite formation in quail embryos. Qsulf1 was reported to be a cell surface-associated enzyme sharing extensive sequence homology with human N-acetylglucosamine 6-sulfatase (Dhoot et al. 2001). Qsulf1 was shown to target internal trisulfated IdoA2S-GlcNS6S disaccharide units in HS chains (Ai et al. 2003). Qsulf1 catalyzed 6-O-desulfation reduces Wnt binding to heparin and HS chains. CHO cells with impaired HS biosynthesis are defective in Wnt-dependent Frizzled receptor activation, establishing that HS is required for Frizzled receptor function (Ai et al. 2003). Taken together, these findings suggest a mechanism where Qsulf1 removes 6-O-sulfates from HS chains to promote the formation of low affinity HS-Wnt complexes that can functionally interact with Frizzled receptors to initiate Wnt signal transduction. The extracellular sulfatases have also been cloned in mice and humans (Morimoto-Tomita et al. 2002). Hsulf1 is expressed in testis, stomach, skeletal muscle, lung and kidney, whereas Hsulf2 is expressed in ovary, skeletal muscle, stomach, brain, uterus, heart, kidney and placenta (Morimoto-Tomita et al. 2002). Both enzymes show high activity against trisulfated disaccharide units and have neutral pH optima (Morimoto-Tomita et al. 2002).

**Targeted gene disruption in mice as a tool to study heparan sulfate biosynthesis**

Studies of mice with targeted deletions in HS biosynthetic enzymes have shown that normal HS structure is essential for animal development. Various phenotypic changes have been observed when different genes involved in HSPG biosynthesis have been functionally deleted (for review see (Forsberg and Kjellén 2001)). There are principally two different strategies used to study HS function using gene-targeting. Since HS exists as a HSPG, either the core protein or the HS biosynthetic enzymes can be deleted. When a core protein is deleted, HS is still present on other core proteins, but the mutant should give some information as to whether a certain HSPG species is important for a specific cellular response to e.g. growth factors. The other way
is to target genes for the different biosynthetic enzymes. To date, several mouse strains deficient in HS biosynthetic enzymes have been established using gene-targeting or gene-trapping strategies. These include at least one of the HS polymerases (EXTs) (Lin et al. 2000), two of the NDSTs (Forsberg et al. 1999; Ringvall et al. 2000; Fan et al. 2000; Humphries et al. 1999), the epimerase (Li et al. 2003), the 2OST (Bullock et al. 1998; Merry et al. 2001), and the 3OST1 (Shwora et al. 2002).

Two proteins, EXT1 and EXT2, are responsible for polymerization of HS (Lind et al. 1998). In humans, the disorder hereditary multiple exostoses (HME) is associated with mutations in the EXT1 and EXT2 genes. The disorder has autosomal dominant inheritance and is characterized by exostoses (spikes) on the long bones (reviewed in (Zak et al. 2002)). Malignant transformation of the benign tumor to chondrosarcoma or an osteosarcoma occurs in approximately 1 - 2% of patients. EXT+/− heterozygous mice have reduced HS production, and HS polymerase activity, but do not develop exostoses, although their bone length is diminished by 10% (Lin et al. 2000). Homozygous mice lack HS and die around gastrulation due to lack of organized mesoderm and lack of extraembryonic tissues.

Targeted inactivation of NDST2 had a dramatic effect on the connective tissue mast cells but no apparent effect on HS biosynthesis or the viability of these mice. The mast cells lacked sulfated heparin and had only few granules (Forsberg et al. 1999; Humphries et al. 1999). The effect on connective tissue mast cells may not be so surprising as they express high levels of NDST2 and low levels, if any, of other NDST isoforms. However, it was more unexpected that NDST2 does not seem to be essential for HS biosynthesis although NDST2 mRNA is widely expressed in different tissues.

When instead NDST1 was deleted, the mice synthesize undersulfated HS and die at birth due to lung failure. The type II alveolar cells in the lungs are increased in number but do not secrete sufficient amounts of surfactants (Ringvall et al. 2000; Fan et al. 2000). If surfactant is missing, the surface tension in the lungs will be large and the respiratory muscles not strong enough to expand the lungs. Atelectasia seen in lungs from NDST1−/− pups indicated that their lungs had never been properly inflated. Even though breathing reflexes are present, the possibility cannot be fully excluded that the respiratory failure may be due to disturbances in the brain stem region (Ringvall et al. 2000).

Mice lacking both NDST1 and NDST2 die during gastrulation between E4-E6.5.

1 Katarina Holmborn, personal communication
Targeted deletion of the C5-epimerase is lethal, with renal agenesis, lung defects, and skeletal malformations (Li et al. 2003). Unexpectedly, major organ systems, including the brain, liver, gastrointestinal tract, skin, and heart, appeared normal.

The importance of the fine structure of HS was also illustrated by the phenotype of a mouse lacking the 2OST as a result of a gene-trap mutation (Bullock et al. 1998). The mice die around birth, lack kidneys and have eye and skeletal defects. Kidney development stops at an early stage and may reflect impairment of the signaling by several HS-binding growth factors including members of the Wnt-family, that are known to be involved in kidney morphogenesis. Glial cell derived neurotrophic factor (GDNF) is another possible candidate, since mice lacking GDNF or its receptor (c-ret) show identical kidney phenotypes as mice lacking 2OST (Wilson et al. 2003; Schuchardt et al. 1994).

The lessons that have been learnt from gene-targeted mice are often physiologically significant, such as the NDST2 deficient mice, those lack heparin yet have no obvious coagulation disorders. The viable NDST2 null mice clearly show that heparin has no role in the coagulation homeostasis, but probably in regulating the mast cell proteases (Forsberg et al. 1999). Several modifying enzymes have not yet been gene-targeted in mice. Once these mutated mice have been generated, the resulting information will be important, but very hard to predict.

A number of core protein genes have also been knocked out by gene-targeting. Perlecan-targeted mice show rupture of the basement membrane in the heart (Costell et al. 1999; Arikawa-Hirasawa et al. 1999). Mice lacking z-agrin (a HSPG of the neuromuscular junction) develop normally until the last fetal day or are stillborn (Gautam et al. 1996). Deletions of syndecan-1 and -4 or glypican-2 and -3 result in various phenotypes. Syndecan-1 deficient mice are viable and fertile but have defective wound healing (Alexander et al. 2000), whereas syndecan-4 deficient mice are healthy and fertile (Ishiguro et al. 2000; Echtermeyer et al. 2001), but have a higher mortality if exposed to lipopolysaccharide (Ishiguro et al. 2001). Syndecan-3 targeted mice are resistant to diet-induce obesity (Reizes et al. 2001; Strader et al. 2004). No abnormalities were found in the glypican-2 null mice (Lander and Selleck 2000), but the glypican-3 deficient mice have a severe phenotype (Cano-Gauci et al. 1999) with abnormal lungs and perinatal death.
Mutations in other model organisms

No mice with mutations in any of the 6OSTs have been published yet, but experiments using morpholino-mediated knockdown of the only characterized 6-O-sulfotransferase in zebrafish have shown that the 6OST is essential for muscle development in zebrafish (Bink et al. 2003). In the developing zebrafish, 6OST is expressed in brain, somites and the fins, the same structures that were affected by morpholino-induced depression of transcription. A single gene for 6OST has been identified in Drosophila and whole mount in situ hybridization revealed highly specific expression in embryonic tracheal cells (Kamimura et al. 2001). Interestingly the pattern resembles that of the Drosophila FGF receptor (breathless) mutant. RNA interference experiments demonstrated that reduced 6OST activity caused embryonic lethality and disruption of the primary branching of the tracheal system. The downstream MAPK signaling was also significantly reduced in these embryos.

**In vitro assays to study HS biosynthesis**

The HS biosynthesis has been studied in various ways. HS chains have been purified and characterized from various organs and tissues from different animals. Using cell culture lysates and microsomal fractions incubated with various polysaccharides, the understanding of the HS and heparin biosynthesis has increased. The order and efficiency of the different biosynthetic steps have been defined and assays developed to study the catalytic activities of the biosynthetic enzymes *in vitro*. To further explore the substrate specificities of the biosynthetic enzymes, more elaborate modified heparin derived oligosaccharide substrates have been used. Methods for sequencing HS have been developed, but full-length HS chains are not possible to sequence with today’s technology; besides, it would probably not be meaningful, in view of the predicted sequence variability. HS oligosaccharides can be sequenced but in practice few oligosaccharides longer than a decasaccharide have been resolved. Throughout this thesis, many experiments are performed *in vitro* to study the substrate specificities of the 2OST and the 6OSTs. As mentioned above, one way is to use different polysaccharides with or without different chemical modifications. The most commonly used polysaccharides are derived from heparin, HS or the K5 polysaccharide. K5 polysaccharide is the capsular polysaccharide from the uropathogenic *Escherichia coli* K5 strain and has the same backbone structure as the unmodified HS precursor; -GlcA-GlcNAc-. These polysaccharides can be differentially chemically modified to generate numerous target structures. The disaccharide composition of the chains can be analyzed to determine different substitutions. It is important to keep in mind that the determined composition is the statistical
average and the polysaccharides may be more or less heterogeneous in structure. An oligosaccharide sequence that has been used to study the substrate specificity of biosynthetic enzymes is the antithrombin-binding pentasaccharide sequence, where incorporation of certain N- or O-sulfate groups will determine whether the saccharide will bind AT or not (Fig. 6) (Atha et al. 1987; Kusche et al. 1988). The amounts used are often too small for analysis with colorimetric methods and instead radiochemical methods are used. Many studies are performed using radioactive $^{35}$S-PAPS in mixture with non-radioactive $^{32}$S-PAPS, where the non-radioactive PAPS corresponds to the physiological sulfate donor molecule. The sulfotransferases will incorporate both “cold” (non-radioactive) and “hot” (radioactive) sulfate groups into the HS chain. The $^{35}$S-sulfate groups can then be traced by scintillation counting which is a sensitive method.

Metabolic labeling of HS can be achieved by including radioactive molecules such as $^3$H-glucosamine or $^{35}$S-sulfate in the culture media of cells. The radioactive molecules will be incorporated into the polysaccharides and thus enable structural studies of the newly synthesized HS chains. HS oligosaccharides can be radiolabeled in different ways. One way is to cleave the chains chemically or enzymatically followed by reduction with NaB$_3$H$_4$ that will lead to incorporation of $^3$H-label at the reducing end of the oligosaccharide.

![Figure 6. An antithrombin binding pentasaccharide.](image)

**Figure 6. An antithrombin binding pentasaccharide.** Sulfate groups required for high affinity binding to antithrombin are circled.

### Heparan sulfate and disease

HS has been implicated in a number of diseases, some of which are briefly described below.
Amyloid diseases

Amyloid diseases are characterized by formation of insoluble fibrillar aggregates of misfolded proteins. HS has for a long time been implicated in amyloid diseases, including primary amyloidosis and Alzheimer’s disease (Ohashi 2001). In all amyloid diseases, the aggregates form fibrils that are ordered in a characteristic β-sheet conformation. The polypeptides are often degradation products of some larger protein. To date 22 different polypeptides are known to refold and assemble in highly organized fibrils, which associate with HSPGs to form tissue deposits termed amyloid (Elimova et al. 2004). The mechanisms behind the cytotoxicity of the aggregates are still poorly understood. Recent data demonstrated the effective inhibition of formation of inflammation-associated amyloid A protein (AA)-amyloid by a synthetic peptide corresponding to the HS binding site of serum amyloid A protein (SAA1.1) (Elimova et al. 2004). This peptide blocks amyloid deposition at concentrations several orders of magnitude lower than any other peptide-based inhibitor previously reported. This indicates that SAA1.1:HS interaction is a critical step in AA-amyloidogenesis and suggests a novel treatment strategy also for other amyloidoses. HS is also known to promote fibrillogenesis of both amyloid β peptide (Aβ) (McLaurin et al. 1999) and islet amyloid polypeptide (IAPP) in vitro (Castillo et al. 1998). When added to cell culture, soluble HS has been shown to inhibit Aβ-induced neurotoxicity, possibly by preventing binding of Aβ to the cell surface (Pollack et al. 1995; Woods et al. 1995).

Cancer and metastasis

HS and enzymes acting on HS are of importance for development of cancer, through different mechanisms. Heparanase is an endo-β-D-glucuronidase that can degrade heparin and HS chains into shorter fragments (reviewed in (Vlodavsky et al. 2002)). The first report on the heparanase came in 1971, when heparin-degrading activity was observed in a mouse mastocytoma tissue (Ögren and Lindahl 1971). Later, it was reported that HS degradation correlated with the invasive and metastatic properties of mouse melanoma cells (Nakajima et al. 1983). Heparanase is known to cleave glycosidic bonds via a hydrolase mechanism, and is thus distinct from the bacterial HS-degrading enzymes, heparitinases, that depolymerize HS by eliminative cleavage. Increased expression of heparanase has been observed in many tumors, such as head and neck tumors, pancreatic tumors, hepatocellular carcinoma (Simizu et al. 2004). Therefore, there has been interest into the development of heparanase inhibitors as cancer therapy and one such inhibitor, designated PI-88, is in clinical trials in Australia (Simizu et al. 2004).
The Sulf family of enzymes is associated with the cell surface and modifies HS chains by hydrolysis of 6-O-sulfate groups. The Sulfs have turned out to be of interest in a number of cancers. Loss of human Sulf1 (Hsulf1) up-regulates heparin-binding growth factor signaling in cancer (Lai et al. 2003). The enzyme is readily detectable in normal tissues, but is undetectable or markedly reduced in a number of carcinoma cell lines, including ovary, breast, pancreatic, renal and hepatocellular carcinoma cell lines (Lai et al. 2003). Re-expression of Hsulf-1 in ovarian cancer cell lines resulted in reduced HSPG sulfation and diminished phosphorylation of receptor tyrosine kinases that require sulfated HSPGs as co-receptors for their cognate ligands. Re-expression of the enzyme also resulted in reduced proliferation and increased sensitivity to the chemotherapeutic agent cisplatin (Lai et al. 2003). In a study of hepatocellular cancer, down-regulation of HSulf1 contributed to carcinogenesis by enhancing growth factor signaling and resistance to apoptosis (Lai et al. 2004).

Infectious diseases
HS is involved in a number of infectious diseases; bacterial, parasitic as well as viral infections (reviewed in (Rostand and Esko 1997)) and some examples are described below.

Bacterial infections
When endothelial cells were grown on heparitinase treated ECM, thus lacking HS chains, the cells were more susceptible to infections by *Staphylococcus aureus*. The altered ECM signaled to the uninfected endothelial cells to produce undersulfated cellular HSPGs that increased *Staphylococcus aureus* adherence to endothelial cells (Alston et al. 1997). The intracellular pathogen *Listeria monocytogenes* interacts with the host cell surface, but the mechanisms are largely unknown. Pretreatment of cell culture with heparin or HS, but not other GAGs tested, inhibited attachment and subsequent uptake by murine macrophages and CHO epithelial-like cells (Alvarez-Dominguez et al. 1997). *Pseudomonas aeruginosa* can in vivo activate shedding of syndecan-1 and the resulting syndecan-1 ectodomains enhance bacterial virulence in newborn mice. Newborn mice deficient in syndecan-1 resist *Pseudomonas aeruginosa* lung infection but become susceptible when given purified syndecan-1 ectodomains or heparin (Park et al. 2001).

Viral infections
All sulfated GAGs can modulate HIV-1 infection. HS promotes HIV-1 infection in several cell lines (Patel et al. 1993; Roderiquez et al. 1995). Evidence suggests that HSPGs and CSPGs expressed on the surface of brain microvascular endothelial cells may facilitate HIV-1 transcytosis through the blood-brain barrier (Argyris et al. 2003; Bobardt et al. 2004). Papillomavi-
ruses replicate in the stratified epithelia of both skin and mucosa. Infection with certain human papillomavirus (HPV) types is the main cause of anogenital neoplasia, in particular cervical cancer. The HSPGs mediate initial binding to the cell surface, using syndecan-1 as a primary receptor protein in natural HPV infection of keratinocytes (Shafti-Keramat et al. 2003). The pathogenic dengue viruses cause many clinical manifestations including potentially life-threatening conditions such as hemorrhagic shock syndrome and less frequently acute hepatitis with liver failure and encephalopathy (Malavige et al. 2004). The binding and internalization of dengue virus by hepatocytes have been shown to be mediated primarily by HSPGs (Hilgard and Stockert 2000; Chen et al. 1997; Hung et al. 1999). However, another study showed that enzymatic removal of HS actually increased viral attachment to certain cells (Bielefeldt-Ohmann et al. 2001). Much attention among the HS-mediated viral infections has been on the Herpes simplex viral infections. It has been shown that cell surface HS plays an important role in assisting HSV-1 attachment to host cells (WuDunn and Spear 1989; Trybala et al. 2002), as well as in the viral entry into the target cells (Spear and Longnecker 2003; Shukla and Spear 2001). The role of HS is intriguing since the virus seems to use the rare 3-O-sulfated N-unsubstituted glucosamine unit for increased binding to the HSV-1 envelope D (gD) protein (Liu et al. 2002). Out of seven known 3OST isoforms, two (3OST3 and 3OST5) are able to generate HS structures that increase binding to the viral gD protein (Xia et al. 2002; Xu et al. 2004; Kamimura et al. 2004). HS mediates the initial cellular binding of adenovirus types 2 and 5 (Dechecchi et al. 2001; Dechecchi et al. 2000). Both wild-type TBE virus and TBE virus with a history of passages in cell culture bind HS, although it seems that cell cultured virus acquire increased HS binding (Kroschewski et al. 2003; Mandl et al. 2001).

**Parasite infections**

Treatment of *Trypanosoma cruzi* with soluble HS resulted in significant inhibition of its invasion of cardiomyocytes. Removal of sulfated conjugates from the cardiomyocyte surface using heparitinases demonstrated the specific binding of the parasites to the HSPGs (Calvet et al. 2003). *Plasmodium falciparum*, causing the severe form of malaria, has recently been shown to involve HS in the sequestration of erythrocytes. Unexpectedly, HS present on the cell surface of erythrocytes was found to take part in the sequestration mechanism (Vogt et al. 2003; Vogt et al. 2004).

**Diabetes**

HS has for a long time been suggested to play a role in diabetes pathology. The Steno hypothesis postulates that a genetic defect in the regulation of the production of HS by renal and non-renal cells determines the susceptibility
for the development of proteinuria and macro-angiopathy in patients with diabetic nephropathy. In a recent study, NDST2 mRNA was reduced in diabetic compared with non-diabetic patients. No differences were found in mRNA levels between patients with or without nephropathy, in apparent contradiction to the Steno hypothesis (Yard et al. 2002). On the other hand, hepatocytes isolated from streptozotocin-diabetic rats showed reduced N-deacetylation activity compared to normal rats (Unger et al. 1991).

The HSPG perlecan has been implicated in many complications of diabetes. Decreased levels of perlecan have been observed in the kidney and in other organs, both in patients and in animal models. It is believed that perlecan has an important role in the maintenance of the glomerular filtration barrier and decreased perlecan levels in the glomerular basement membrane has a central role in the development of diabetic albuminuria (reviewed in (Conde-Knape 2001)). The glomerular extracellular matrix HSPGs are lost in association with diabetic nephropathy; this loss results in alteration of the charge-selective properties of glomerular capillaries. This alteration may, in part, be the cause of the proteinuria associated with diabetic nephropathy (Makino et al. 1992).

Lysosomal storage diseases

The degradation of glycans is ordered and often highly specific. Most glycoconjugates are degraded in the lysosomes, and a portion of the monosaccharides is reused for glycoconjugate synthesis. Many of the endo- and exoglycosidases that degrade sugar chains have pH optima between 4.0 and 5.5, but exceptions exist. The exoglycosidases usually do not act unless substituents such as sulfate groups are removed. Lysosomal storage diseases in the degradation of GAGs are referred to as mucopolysaccharidoses (MPS) and can have a variety of clinical symptoms. Lack of a certain degrading enzyme leads to accumulation of non-degradable products in the lysosomes and forms the basis for the pathology. The MPS are rare, the prevalence in Australia has been calculated to be 1 out of 22 500 persons (Meikle et al. 1999) and are inherited in an autosomal-recessive manner, except for MPS II (iduronate-2-sulfatase deficiency), that is X-linked (reviewed in (Muenzer 2004)). Interestingly, many of the MPS give rise to mental retardation. Until the 1980s, only palliative and nonspecific therapies were available for patients with MPS disorders. Prospects changed with bone marrow transplantation, which has proven beneficial, primarily for severe forms of MPS I (iduronidase deficiency) and VI (N-acetylgalactosamine 4-sulfatase or arylsulfatase B deficiency). Recent clinical trials of enzyme replacement therapy have shown promising results in the treatment of MPS I, II and VI (reviewed in (Muenzer 2004)). MPS I patients have been treated with recombinant human iduronidase, MPS II patients with iduronate-2-sulfatase and MPS VI patients
with recombinant human arylsulfatase B. Double-blinded, placebo-controlled phase III enzyme replacement clinical trials for MPS II and MPS VI were both started in September 2003.
Present investigation

Aims of study
The present investigation was undertaken in order to gain specific information regarding the biosynthesis of HS with emphasis on mechanisms controlling 2-O- and 6-O-sulfation. After the purification and cloning of the various biosynthetic enzymes, it has become possible to study the HS biosynthetic machinery using more elaborate methods. The demonstration of three different 6OSTs with differential expression in the mouse, pointed to a possible model whereby differential substrate specificities of the 6OSTs could be responsible for generating different sulfation patterns in different tissues. We therefore studied in more detail the substrate specificities of the murine 6OSTs, (paper I – III) using different approaches. There is only one single 2OST that sulfates both IdoA and GlcA. Previous studies have shown a preference of the enzyme to sulfate IdoA rather than GlcA residues. The regulation of IdoA/GlcA 2-O-sulfation was further explored in paper IV.

The specific aims of this thesis were:
1) To probe the substrate specificities of the three murine 6OSTs (paper I).
2) To investigate how pre-existing 2-O-sulfate groups influence subsequent 6-O-sulfation (paper II).
3) To investigate the effect on HS structure of 6OST overexpression in mammalian cells (paper III).
4) To define the target selection of the 2OST (paper IV)
Results

Substrate specificities of mouse heparan sulfate glucosaminyl 6-O-sulfotransferases. (Paper I)

Three isoforms of the glucosaminyl 6OSTs have been cloned and characterized (Habuchi et al. 2000). In this paper we studied the substrate specificities of the recombinant 6OST enzymes using various O-desulfated poly- and oligosaccharides as substrates and [35S]PAPS as sulfate donor. All three enzymes catalyzed 6-O-sulfation of both -GlcA-GlcNS- and -IdoA-GlcNS- sequences, with preference for IdoA-containing targets, with or without pre-existing 2-O-sulfate groups.

6OST1 showed relatively higher activity towards -GlcA-GlcNS- target sequences lacking 2-O-sulfates. Sulfation of such non-O-sulfated acceptor sequences was generally favored at low acceptor polysaccharide concentrations. Experiments using a partially O-desulfated antithrombin-binding oligosaccharide as the acceptor revealed 6-O-sulfation of GlcNAc as well as GlcNS3S with each of the three 6OSTs. We conclude that the three 6OSTs have qualitatively similar substrate specificities, with minor differences in target preference.

Oligosaccharide library-based assessment of heparan sulfate 6-O-sulfotransferase substrate specificity (Paper II)

In this project we investigated how pre-existing 2-O-sulfate groups affect subsequent 6-O-sulfation reactions. Purified [3H]-labeled N-sulfated octasaccharides containing different 2-O-sulfated motifs were used as sulfate acceptors, PAPS as sulfate donor, and cell extract from 6OST3-overexpressing 293 cells as enzyme source in the 6OST-catalyzed reactions. The first 6-O-sulfate group was preferentially incorporated at the internal glucosamine unit of the octasaccharide substrate. As the reaction proceeded, the octasaccharides acquired up to three 6-O-sulfate groups. The specificities toward competing differentially 2-O-sulfated octasaccharide substrates were determined for 6OST2 and 6OST3. Both 6OSTs showed a preference for 2-O-sulfated substrates. The specificity toward substrates with two to three 2-O-sulfate groups was three to five times higher as compared with octasaccharides with
no or one 2-O-sulfate group. We predict that oligosaccharide libraries will be useful to determine substrate specificities also of other enzymes involved in glycosaminoglycan metabolism.

Overexpression of heparan sulfate 6-O-sulfotransferases in human embryonic kidney 293 cells results in increased N-acetyl glucosaminy1 6-O-sulfation (Paper III)

To investigate if different isoforms of HS glucosaminy1 6-O-sulfotransferase give rise to differently sulfated domains, we introduced mouse 6OST1, 6OST2 and 6OST3 in human embryonic kidney 293 cells and compared the effects of overexpression on HS structure. Selected stable cell clones were metabolically labeled with $^3$H-GlcN and $^{35}$S-sulfate and labeled glycosaminoglycans isolated. 6OST-expressing cells produced relatively less HS (~50% of total GAGs) than CS compared to control cells (~70%). Overexpression changed the anionic properties of the synthesized HS. HS chains from cells expressing low to medium levels of either of the 6OSTs, synthesized two HS populations, one with similar charge density as the control HS and one less charged population. With increasing level of 6OST expression a highly charged HS appeared. The HS of the control cells synthesized rather homogeneously sized chains. In contrast, HS from transfected cells contained two differently sized populations of chains, one similar to the control HS and one with shorter chains. The shorter chains may reflect a more rapid degradation of HS in the overexpressing cells. High expression of any of the 6OST enzymes resulted in appreciably increased overall sulfation due to increased formation of 6-O-sulfated -GlcA-GlcNS- and -GlcA-GlcNAc-units. The 2-O-sulfation was decreased, whereas N-sulfation remained largely unaffected. The most remarkable effect was the formation of 6-O-sulfated N-acetylated regions. Our results were independent of the particular 6OST-isoform expressed but were influenced by the level of overexpression.

Target selection of heparan sulfate hexuronic acid 2-O-sulfotransferase (Paper IV)

The 2OST catalyzes sulfation of both GlcA and IdoA residues. In the study presented here, we further investigated the substrate specificity of the mouse 2OST. Incubations of 2OST-overexpressing HEK 293 cell lysate with $[^{35}]$PAPS and polysaccharide acceptors with IdoA contents ranging from 12.5 to 50%, resulted in 2-O-sulfation of virtually only IdoA residues. Mixing IdoA-containing polysaccharides with polysaccharides containing only GlcA residues reduced the relative amounts of IdoA to 1-5%. Incubation of such mixtures with 2OST still generated essentially only 2-O-sulfation of IdoA residues. Nevertheless, the 2OST could sulfate N-sulfated K5 decasac-
charides, i.e. oligosaccharides containing only -GlcA-GlcNS- target disaccharides.
The 2OST was also expressed as a fusion protein with a His-FLAG tag (2-OSTHF) and purified from lysate using anti-FLAG agarose beads. When purified 2OSTHF-anti-FLAG beads were incubated with O-desulfated, N-sulfated 3H-end-labeled octasaccharides from heparin, mainly mono- and di-2-O-sulfated products were formed. Results from incubations performed for different periods of time indicated that mono-2-O-sulfated octasaccharides were better substrates than completely O-desulfated octasaccharides. Since the mono-2-O-sulfated product was eluted differently from previously characterized octasaccharide standards with 2-O-sulfates close to the non-reducing end on anion-exchange HPLC, the incorporated 2-O-sulfate group is probably located close to the reducing end. In summary, we conclude that in vitro; (1) the 2OST substrate preference toward IdoA residues is more marked than predicted from the previously determined kinetic parameters (Rong et al. 2001), (2) the 2OST prefers mono-2-O-sulfated before non-O-sulfated octasaccharide substrates.

**Generation of 6OST3 gene-targeted mice (ongoing project)**

Since the cloning of the three murine isoforms of 6OST, attempts have been made to generate mice deficient with regard to each of the isoforms. Our results show that the substrate specificities of the 6OSTs in vitro are qualitatively similar, with only smaller quantitative differences. However, due to the importance of 6-O-sulfation for HS mediated signaling during embryogenesis, small differences in substrate specificity combined with differences in tissue expression of 6OSTs during organogenesis may make a great difference for the developing organism. One way to address the in vivo effects of 6OSTs is by generation of mice lacking 6OST1, -2 or -3. We have chosen to do “classical” targeted deletion of the 6OST3 gene. When the project was started, the genome of the mouse was not publicly available. By screening a genomic BAC library using the mouse 6OST3 cDNA as a probe, several positive clones were obtained. An exon corresponding to half the cDNA sequence including the start codon was identified in a 6 kb XbaI fragment. Later both upstream (7 kb) and downstream (6 kb) SacI fragments were isolated and cloned and a targeting construct was generated, see Fig. 7.
Figure 7. Schematic strategy for a targeted deletion of the 6OST3 gene. Top – schematic structure of the genomic sequence, with the first exon of the 6OST3 gene as a black box. Restriction cleavage sites are as follows: El, EcoRI; E5, EcoRV; E72, Eco72I; S, SacI; Sma, SmaI; X, XbaI and Xho, Xhol. Bottom – targeting construct containing the neomycin resistance cassette (Neo) where 5’ and 3’ indicates the 5’ and 3’ arms of genomic sequences respectively.

Construction of the Targeting Vector

A genomic clone containing the first exon of the 6OST3 gene was isolated from a bacterial artificial chromosome library. The XbaI fragment was subcloned into pAM120 (a derivate of the Bluescript KS- plasmid, given by A. J. MacKrell Jr.). The EcoRI-site was mutated using Klenow enzyme. The resulting plasmid was linearized with NotI/XhoI and purified. The neomycin resistance gene cassette flanked by loxP sites was subsequently cloned into the NotI/XhoI digested pAM120 plasmid. The product was then digested with NotI/EcoRI and the resulting fragment purified. The SacI cleaved upstream fragment (in pBluescript) was reoriented by cleavage with Eco72I/SmaI and ligated into the SmaI site of pBluescript and subsequently cleaved with NotI/EcoRI to generate a 4.8 kb fragment that was then ligated into the NotI/EcoRI sites of the above construct, generating a 11.3 kb construct. The plasmid was linearized with NotI and injected into embryonic stem (ES) cells. ES cells were screened for the presence of neomycin resistance cassette by the addition of G418 to the culture media. Genomic DNA from G418 resistant cells was further screened for homologous recombination by Southern blotting after cleavage with EcoRV.

EcoRV digestion of the wild-type allele gives a restriction fragment of 6 kb, while the mutant allele gives a 5 kb fragment. Alternatively, clones were screened by RT-PCR using a neomycin resistance cassette specific primer.
and another primer located outside the construct. In addition to the first targeting construct, a second (1.2 kb) longer construct was also generated with longer 3’ end genomic sequence.

**Results:**

After screening approximately 400 clones with the shorter construct and 800 clones with the longer construct, no positive clone was detected. The reason for this failure is not known, but could be explained in various ways. The generation of gene-targeted mice deficient in HS biosynthetic enzymes seems to be difficult. Whether this is an effect of positioning of these genes in the chromosomes or due to other reasons is not known. Using genomic databases, 1 kb genomic sequence with high degree of sequence similarity to genomic parts of the targeting construct was found at a different chromosomal location, maybe reducing the frequency of successful recombination.

In the case of targeted deletion of 6OST3, it cannot be excluded that this isoform plays an essential but unknown role in embryonic stem cells such that the cells die due to loss of the enzyme. On the other hand, a heterozygote would still be expected to have some expression of the enzyme. RT-PCR studies revealed that whereas murine embryonic stem cells express all three isoforms, the main isoform is probably 6OST1, as the levels of the other two enzymes are low (Holmborn et al. 2004). This project will be continued in Prof. Koji Kimata’s group at Aichi Medical University in Japan. To date, no reports on HS 6OST deficient mice have been published.
Discussion

6-O-sulfotransferases in HS biosynthesis

The diversity of HS structure is illustrated by immunohistochemical staining for HS epitopes, using phage display antibodies (van Kuppevelt et al. 1998). Staining of rat kidneys revealed that antibody HS4C3 predominantly stained basement membranes of the glomerulus and of the peritubular capillaries, whereas another antibody, HS4D10, reacted mainly with HS present in basement membranes of tubules and of smooth muscle cells. Antibody HS3G8 on the other hand showed rather promiscuous staining, reacting with most basement membranes in kidney. The differences in staining could be a result of variable HS sulfation patterns that give rise to different protein binding epitopes (Ledin et al. 2004). Maybe the subtle differences in substrate specificities of 6OSTs that we observed in vitro play a crucial role in generating unique protein binding sequences in vivo?

The 6-O-sulfate groups in HS occur in different structural contexts. Approximately half the total 6-O-sulfate in HS substitutes contiguous N-sulfated domains, often in juxtaposition to 2-O-sulfated hexuronic acid units (Maccarana et al. 1996). The remaining portion is found in domains of alternating N-acetylated and N-sulfated disaccharide units, which largely lack 2-O-sulfate groups. It was therefore tempting to speculate that 6-O-sulfation of such widely divergent target sequences would be catalyzed by different 6OST isoforms. In paper I we resolved the substrate specificities of the 6OSTs on the disaccharide acceptor level using oligo- and polysaccharides. Surprisingly, no qualitative differences were found; similar targets were utilized by all three enzymes. The functional role of GlcN 6-O-sulfation was also studied in relation to the AT-binding region in heparin and HS. O-Sulfation of C6 of unit 1 and C3 of unit 3 are essential for high affinity binding (Fig. 6). Recent studies indicated that these sites can be independently sulfated (Zhang et al. 2001). 6-O-Sulfation of the 3-O-sulfated unit 3 is optional and of little importance for AT-binding. While Zhang et al showed that 6OST1 can 6-O-sulfate N-acetylated unit 1, our results demonstrate that 6OST2 and 6OST3 in addition to 6OST1 can sulfate both the N-acetylated unit 1 and N-sulfated unit 3. In our experiments, 6OST2 and 6OST3 show a relative preference for the N-acetylated target compared with 6OST1.
In paper II we examined the role of 2-O-sulfation for the 6-O-sulfation using oligosaccharide substrates. It was established long ago that in HS biosynthesis, 2-O-sulfation precedes 6-O-sulfation (Jacobsson and Lindahl 1980). Our studies showed that preexisting 2-O-sulfate groups and also the presence of a 6-O-sulfate group promoted further 6-O-sulfation. These findings presumably relate to the function of NS-domains, with incorporation of 6-O-sulfate groups preferentially in internal positions of already 2-O-sulfated sequences. They also conform to the lack, by and large, of 6-O-sulfate groups from internal positions of NA-domains. However, we still do not understand by which mechanism about half of the total 6-O-sulfation is directed to the mixed NA/NS-domains that lack 2-O-sulfate groups. In the future, more elaborately designed oligosaccharide substrates with preformed 2-O- and 6-O-sulfate groups may possibly reveal so far undetected differences in 6OST substrate specificity.

We also examined, in a cellular system, how the different 6OST isoforms influence the HS structure (paper III). We overexpressed the murine 6OSTs in HEK 293 cells and examined the structural changes in HS. With the overexpression, the charge density of the HS chains became more varied and at the highest level of overexpression a fraction of the chains became extremely highly charged. Highly overexpressing clones generated a fraction of oligosaccharides, possibly representing N-acetylated fragments with a high degree of 6-O-sulfation. The results were independent of the specific isoform expressed and thus did not give any indication that different 6OSTs generate different HS structures. However, the results indicate that 6-O-sulfation may not be strictly associated with adjacent N-sulfate groups.

Little is known about the interplay of O-sulfotransferases with other HS biosynthetic enzymes, nor how tissue and developmentally specific structures are formed. The general idea is that the biosynthesis of HS starts with the synthesis of the linkage region, followed by polymerization and concomitant modifications of the growing polysaccharide (Lidholt et al. 1989). NDST initiates the modification reactions by N-deacetylation/N-sulfation of some N-acetylated glucosamines. This initial modification sets the limits for further modifications and generates the three different domains that are found in HS. The three domains: NA-, NS- and mixed NA/NS-domains are differently O-sulfated. The NS-domains are rich in IdoA units and highly sulfated, whereas the internal portion of extended N-acetylated NA-domains are devoid of IdoA units and sulfate groups. Mixed regions may still have a significant 6-O-sulfation on both GlcNAc and GlcNS residues, while 2-O-sulfation is low on HexA residues. The notion of strictly regulated modification resulting from distinct substrate specificities of the biosynthetic enzymes should however be somewhat revised. Several pieces of experimental evidence may be noted, especially regarding the regulation of 6-O-sulfation.
In addition to previous results showing that 3-O-sulfation is the final step in HS biosynthesis (Kusche et al. 1988), we show (paper I) that 6-O-sulfation of the antithrombin binding region can occur on 3-O-sulfated GlcNS residues indicating that both 3-O- and 6-O-sulfation can be the final step in HS biosynthesis (Zhang et al. 2001). Furthermore, it was recently shown that HS synthesized by mouse embryonic stem cells deficient in NDST1 and -2 lack N-sulfate groups, but still contain a significant amount of 6-O-sulfate groups (Holmborn et al. 2004). The HS chains synthesized by these cells are essentially composed of long NA regions (with a few N-unsubstituted glucosamines), with considerable 6-O-sulfation. Another piece of evidence against the strictly controlled 6-O-sulfation is our results from 6OST overexpressing HEK 293 cells (paper III). When each of the 6OST isoforms was overexpressed, there was increased 6-O-sulfation in the NS-domains, especially on -GlcA-GlcNS- targets, but also increased sulfation of -GlcA-GlcNAc- targets. These -GlcA-GlcNAc- targets can be found in the mixed NA/NS regions as well as in the NA domains. These results were not merely due to increased 6-O-sulfation in the mixed NA/NS regions since high level of overexpression generated N-acetylated regions with extensive sulfation, presumably 6-O-sulfation. These findings point to some laxity in regulation. On the other hand, one could argue that overexpression of an enzyme might not be physiologically representative as the regulatory mechanisms operating in the unperturbed cell are likely to be more refined. Overexpression of 6OSTs, resulted in decreased C5-epimerization and IdoA 2-O-sulfation, indicating that other modifications were affected. One explanation may be sterical hindrance due to 6OST enzymes occupying the space that the other enzymes normally occupy in the Golgi compartment. A more likely explanation is that once target structures have been 6-O-sulfated, they are no longer substrates for most other biosynthetic enzymes (Jacobsson et al. 1984). Still another option relates to the “gagosome” hypothesis. The gagosome concept visualizes the biosynthetic enzymes as physically interacting in a multimeric complex. Nature shows several examples of complex structures containing many subunits, including the ribosome, responsible for protein synthesis from the mRNA template (reviewed in (Moore and Steitz 2003)) and the oligosaccharyl transferase complex (reviewed in (Dempski and Imperiali 2002)) that transfers a tetradecasaccharide from a dolichol pyrophosphate donor to selected asparagine side chains of nascent polypeptides into the ER. The ribosome consists of many proteins and small RNA molecules. The gagosome would contain many proteins/ enzymes and maybe an overexpression of the 6OSTs would physically compete out some other enzymes from the gagosome (Led 2004). It is also possible that the 6OSTs play a more flexible role in the biosynthesis, i.e. can act on the polysaccharide even outside a gagosome. The gagosome may well contain also non-enzymatic accessory proteins, which may easily escape detection. See Fig. 8 for the differences between the “gagosome” and a “random encounter” model.
The functional significance of the 6OST isoforms remains unclear. Of interest is the level of expression of the various enzymes. In the adult mouse, 6OST1 is mainly expressed in the liver, 6OST2 in brain and spleen, whereas 6OST3 has a diffuse expression in all tissues examined (Habuchi et al. 2000). Variable expression of the 6OSTs may serve to generate HS species with different degrees of 6-O-sulfation in different organs. In a recent study, the levels of 6OST mRNA was investigated in mouse embryos by *in situ* hybridization, showing a dynamic expression of the isoforms in many tissues (Sedita et al. 2004). Analysis of early stages of organogenesis in the embryonic day E8.5-E9.5 mouse, revealed differential expression of 6OST1 and -2 in most structures and no 6OST3 signals. 6OST1 transcripts were localized primarily to endodermal and ectodermal layers, whereas 6OST2 was primarily expressed by mesodermal-derived structures. 6OST3 transcripts were not
detected until E9.5, during formation of the forelimb bud. From around E9.5 and onward, 6OST2 signals were also found in specific regions of the neural tube and brain ectoderm and in the endoderm of the branchial arches. At later stages (E14), expression of 6OST isoforms was also seen in the overlapping domains in regions such as the developing gonads and some substructures of the brain.

None of the 6OSTs have yet been crystallized. When the solved 3OST1 structure was compared to the sulfotransferase domain of NDST1 it was obvious that 3OST1 contained more positively charged amino acids. This is what would be predicted from the different substrates for the enzymes: the 3OST substrate is far more negatively charged than the NDST substrate. The NDST substrate is not sulfated, but contains carboxyl groups and maybe some free amino groups. It will be of great interest to see the solved structures of the 6OSTs and compare the number of positively charged amino acids in the binding pockets. It is likely that the 3OST1 and the 6OSTs may be quite similar in this regard. Comparing the crystal structures of the 6OSTs to each other may provide insights into differential substrate specificities.

2-O-sulfotransferase in HS biosynthesis

In contrast to the 6OSTs which contain three isoforms with apparently the same substrate specificities, there is only one 2OST enzyme with two catalytic activities (Rong et al. 2000). The enzyme sulfates both GlcA and IdoA with a preference for IdoA units (Rong et al. 2001). In paper IV we set up a series of experiments to investigate in more detail the substrate recognition properties of the 2OST. Our results show that the preference for IdoA 2-O-sulfation is far greater than would be predicted by the established kinetic parameters of the enzyme. Even at a relative IdoA abundance of only ~1% virtually only IdoA residues were 2-O-sulfated. This result is surprising and the reasons are not understood. However, when either NSK5 (containing only -GlcA-GlcNS-) or de-O-sulfated heparin (essentially composed of -IdoA-GlcNS-) were used separately as substrates for the 2OST, the products were GlcA(2S) and IdoA(2S) respectively. Interestingly, in contrast to previous results using crude cell lysate (Rong et al. 2001), our results with purified recombinant 2OST resulted in similar levels of 2-O-sulfates into each substrate. Thus, the enzyme has the capacity to sulfate both substrates efficiently, however trace amounts of IdoA residues apparently effectively “inhibit” GlcA 2-O-sulfation. In our assays, the radiochemical incorporation into the substrates is low compared to all possible acceptor sites. Even at 1% IdoA concentration not all IdoA targets will become sulfated. However, interactions between enzyme and substrates in solutions are random events and therefore sulfation of two different substrates in solution should reflect the
substrate specificity of the enzyme. It therefore seems likely, that the presence of IdoA or IdoA(2S) residues in the mixture somehow “inhibits” GlcA 2-O-sulfation. The mechanism for this possible “inhibition” is not clear, but will be most interesting to define.

We also noticed that 2-O-sulfation appears to stimulate further 2-O-sulfation. This fact together with our results from paper II, point to an interesting biosynthetic effect: the HS biosynthetic machinery is designed to ensure domains of almost no sulfation and domains with a very high degree of sulfation. Apparently, the 2OST and the 6OSTs are directed to generate a high degree of O-sulfation in NS domains, since both classes of enzymes work better on targets with pre-existing O-sulfate groups. The HS chains grow at the non-reducing end. Since modifications of HS chains most probably take place concomitantly with chain elongation, it would be time-effective to modify the HS chains in the same direction. It is possible that 2-O-sulfation of GlcA residues is catalyzed early in the Golgi, essentially before the epimerase has acted on the polysaccharide. The 2-O-sulfation of GlcA and IdoA residues should maybe be regarded as two distinctly separate events although catalyzed by the same enzyme and in the same organelle.
Future perspectives

Understanding of the HS biosynthesis is not yet accomplished. There are still many aspects where we lack knowledge. We know that the biosynthesis takes place in the Golgi, but many factors involved are not known, including the concentrations of polysaccharide, PAPS and enzymes, nor the topographical positioning of the biosynthetic enzymes. One area of research that might generate interesting results is ongoing experiments to demonstrate the existence of a gagosome. Such investigations could possibly reveal interactions between the biosynthetic enzymes and various regulatory proteins within the Golgi.

The purification and subsequent cloning of the biosynthetic enzymes have given valuable information on substrate specificities in vitro and model organisms mutated in the corresponding genes have provided in vivo data on the biological importance. More data from gene-targeted model organisms could shed light on the complex HS-mediated signaling in vivo and reveal previously unknown interactions involving HS. The structures of some of the biosynthetic enzymes have been resolved, generating increased knowledge of the catalytic properties. Techniques have been developed to synthesize HS oligosaccharides by both enzymatic and chemical routes. These oligosaccharides can possibly be used to inhibit or promote different HS-dependent signaling pathways involved in various disorders. The progress in sequencing HS oligosaccharides may also provide more insights into both HS-dependent signaling and into the mechanisms regulating the biosynthesis machinery.

The more sensitive method for compositional analysis of HS, reversed phase ion pairing (RPIP)-HPLC, may provide detailed information regarding HS structure in both embryonic and adult tissues and organs. Since the RPIP-HPLC method requires only 1 μg saccharide per sample and has shown very reproducible results, this method may significantly improve structural analyses of small amounts of HS from various sources. RNA interference (RNAi) has been performed in Drosophila melanogaster showing lethal phenotype when 6OST levels decreased (Kamimura et al. 2001). Similar procedure applied to each HS biosynthetic enzyme, could generate new knowledge on the biosynthesis. It is likely that the growth factor signaling pathways where HS is involved will be defined in more detail and the understanding of the role of HS will be improved.

2 Dorothe Spillmann, personal communication
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Populärvetenskaplig sammanfattning


I den här avhandlingen har jag studerat hur en annan typ av biologisk polymer, polysackariden heparansulfat, modifieras inuti cellen.


Heparansulfat är mycket negativt laddat. Detta beror till stor del på sulfatjoner som återfinns utmed polysackaridkedjan. Heparansulfatet sulfateras av sulfotransferasen (enzymer som fäster sulfatjoner på andra molekyler).
Den här avhandlingen visar in vitro, det vill säga i provrör, och i cellkulter, hur olika sulfotransferaser arbetar vid bildandet av heparansulfat. Inriktningen har varit på tre 6-O-sulfotransferaser samt ett 2-O-sulfotransferas. 6-O-Sulfotransferasen kan sätta på en sulfatgrupp på syret (bokstaven O) vid kolatom 6 på sockerenheten, medan 2-O-sulfotransferaset kan göra motsvarande på kolatom 2.

Vi bestämde vilka strukturer i heparansulfatet som de tre 6-O-sulfotransferaserna föredrog att sulfatera. Tidigare forskning hade visat att det fanns skillnader mellan de tre typerna (isoformerna) av 6-O-sulfotransferaser. Vi visade i detalj vilka strukturer som sulfaterades av de tre enzymerna. De tre 6-O-sulfotransferaserna kan sulfatera samma typ av strukturer, men med vissa kvantitativa skillnader.

I ett annat projekt som redogörs för i den här avhandlingen, använde vi en uppsättning oligosackarider (i det här fallet åtta sockerheter som kemiskt sitter ihop) med olika antal och positioner av 2-O-sulfatgrupper. Genom att parvis blanda de olika oligosackariderna med ett av 6-O-sulfotransferaserna kunde vi se vilken oligosackarid enzymet föredrog att sulfatera. Oligosackarider med två eller tre sulfatgrupper sulfaterades tre till fem gånger så ofta som oligosackarider med ingen eller en sulfatgrupp. Däremot blev inte oligosackarider med fyra sulfatgrupper snabbare sulfaterade än oligosackarider med tre sulfatgrupper. Vi kunde även visa att 6-O-sulfotransferaset sulfaterar i mitten av oligosackariden först.

Vi överuttryckte 6-O-sulfotransferaserna i cellkulturer, dvs cellerna tvingades att tillverka stora mängder 6-O-sulfotransferaser. Genom att studera heparansulfatets struktur kunde vi visa att strukturen påverkades av överuttrycket. Vid extremt höga enzymnivåer bildades en fraktion av mycket starkt negativt laddade polysackarider i en region av polysackaridkedjan som vanligtvis inte är sulfaterad. Effekten på heparansulfatets struktur var oberoende av vilket av 6-O-sulfotransferaserna som överuttrycktes.

Tillsammans tyder resultaten på att in vitro beter sig 6-O-sulfotransferaserna likartat och att mängden enzym påverkar heparansulfatstrukturen. Eftersom mängden av respektive 6-O-sulfotransferas varierar i olika vävnader, kanske mängdförhållandet är den regulatoriska faktor som bestämmer heparansulfatets struktur och funktion i den levande organismen.

Vi studerade även 2-O-sulfotransferaset som kan sulfatera två olika strukturer i heparansulfatkedjan, glukuronsyra och iduronsyra. Tidigare studier hade visat att om båda strukturerna finns tillgängliga sulfaterar enzym framför allt iduronsyra. Våra resultat visar att sulfatering av iduronsyra var mycket större än vad man skulle ha kunnat förutsäga från de
tidigare studierna. Det visade sig att även mycket små mängder iduronsyra effektivt hämmade sulfateringen av glukuronsyran. En möjlig förklaring till resultaten är att sulfateringen av glukuronsyran måste ske tidigt under heparansulfatets bildande innan iduronsyran har bildats. Genom att blanda 2-O-sulfotransferasenzymet med oligosackarider kunde vi även visa att enzymet föredrog att sulfatera oligosackarider med en 2-O-sulfatgrupp hellre än oligosackarider utan sulfatgrupper.
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