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FROM GENE TO STRUCTURE: Formation of Iduronic Acid in Dermatan Sulfate by Two DS-epimerases

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FROM GENE TO STRUCTURE

Formation of Iduronic Acid in Dermatan Sulfate by Two DS-epimerases

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TO MONICA

*“Failure is the condiment that give success its flavor”
Truman Capote*

About the cover:

The picture shows a structural model of DS-epimerase 1 obtained based on homology to the bacterial lyase heparinase II. The active site of the enzyme is located in a groove formed by two distinct domains of the protein and is depicted with a chondroitin sulfate tetrasaccharide.

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LIST OF PAPERS

Paper I

*Tiedemann K., ***Olander B.**, *Eklund E., Todorova L., Bengtsson M., Maccarana M., Westergren-Thorsson G. and Malmström A. (2005)

Regulation of the chondroitin/dermatan fine structure by transforming growth factor- β 1 through effects on polymer-modifying enzymes

Glycobiology, **15**, 1277-1285 *These authors contributed equally to this work

Paper II

Maccarana M., **Olander B.**, Malmström J., Tiedemann K., Aebersold R., Lindahl U., Li JP and Malmström A. (2006)

Biosynthesis of Dermatan Sulfate: Chondroitin-glucuronate C5-epimerase is identical to SART2

Journal of Biological Chemistry, **281**, 11560-11568

Paper III

Pacheco B., Malmström A. and Maccarana M.

Two dermatan sulfate epimerases collaborate to form iduronic acid domains in dermatan sulfate

Manuscript

Paper IV

Pacheco B., Maccarana M., Goodlett D.R., Malmström A. and Malmström L.

Identification of the active site of DS-epimerase 1 and requirement of N-glycosylation for enzyme function

Reviewed by Journal of Biological Chemistry and revised version re-submitted

Paper V

Pacheco B., Maccarana M. and Malmström A.

Dermatan 4-O-sulfotransferase 1 is pivotal in the formation of iduronic acid blocks in dermatan sulfate and these regions are involved in FGF2-signaling

Manuscript

ABBREVIATIONS

CS	chondroitin sulfate
DS	dermatan sulfate
ECM	extracellular matrix
ER	endoplasmatic reticulum
FGF	fibroblast growth factor
GAG	glycosaminoglycan
Gal	galactose
GalNAc	<i>N</i> -acetylgalactosamine
GlcA	<i>D</i> -glucuronic acid
GlcNAc	<i>N</i> -acetylglucosamine
HA	hyaluronic acid
HS	heparan sulfate
IdoA	<i>L</i> -iduronic acid
KS	keratan sulfate
PAP	3'-phosphoadenosine 5'-phosphate
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PGs	proteoglycans
ST	sulfotransferase
UTP	uridine 5'-triphosphate
UDP	uridine 5'-diphosphate

INTRODUCTION

During embryonic development and adult life a myriad of cell behaviors such as differentiation, proliferation and migration are in effect to maintain tissue integrity and function. An integral part of these dynamic processes is the interplay between the cells and their environment, *i.e.* the extracellular space. Complex polysaccharides, such as dermatan sulfate play a key role in these processes. Due to its structure, dermatan sulfate can readily bind a multitude of ligands, and can at the cell surface function both as an adhesion molecule and co-receptor for growth factors. More distal to the cell, the polysaccharide sequesters growth factors that can be released upon injury. This diminishes the need for neosynthesis and allows a fast and efficient response in cell behavior when needed.

Dermatan sulfate is a long linear polysaccharide of an alternating disaccharide unit consisting of N-acetylgalactosamine and glucuronic acid/iduronic acid. Through modifications (*i.e.* sulfation and epimerization) each disaccharide unit can potentially exist in 16 different variations. The variable affinity of dermatan sulfate for its ligands is dependent on the presence of particular modifications. Regulating the structure of dermatan sulfate is therefore a way for the cell to fine-tune growth factor signaling, which could also play an important role during pathology such as tumorigenesis. However, dermatan sulfate biosynthesis, and how it is regulated is still poorly understood.

Accordingly, the aim of this thesis was to gain insight into the biosynthesis, especially in regard to enzymes involved and what influences their activity. In the first part of this thesis a general review of the intricate biosynthesis and biological function of dermatan sulfate is given. In the second part, the identification of two key enzymes (*i.e.* DS-epimerase 1 and 2), their catalytic mechanism and how they collaborate together with a sulfotransferase to generate functional domains in dermatan sulfate is reported. In addition, evidence that common growth factors can regulate the structure by influencing the transcription of biosynthetic enzymes is also presented.

BACKGROUND

Chondroitin/dermatan sulfate (CS/DS) is a complex polysaccharide commonly found in the extracellular matrix (ECM) and at the cell surface of animal cells where it elicits a wide range of functions. The hallmark function of CS/DS is its ability to bind several growth factors, chemokines and morphogens. Thus, by sequestering signaling molecules in the ECM or at the cell surface, it can regulate their availability for receptor binding. Examples of growth factors that have been shown to be influenced by CS/DS are Wnt, fibroblast growth factors (FGFs), vascular endothelial growth factor (VEGF), midkine, pleiotrophin and hepatocyte growth factor (HGF)¹⁻⁴. In addition to its role in growth factor binding, CS/DS has important functions in coagulation, infection, neurogenesis and in the adhesion/migration of cells^{5,6}. CS/DS function is strictly dependant on the structure of the polysaccharide and modulating modifications of the chain offers a way for cells to regulate and fine-tune the processes above. This is of particular importance in tumorigenesis where cancer cells can potentially assemble CS/DS chains with a structure that supports a proliferative microenvironment.

In vivo, CS/DS do not normally exist as free chains but are instead covalently attached to different core proteins forming proteoglycans (PGs). In many tissues the most abundant PGs are versican, biglycan and decorin. Several biological functions of PGs are mediated by a combination of that of the protein core and the CS/DS side chain, making it one functional unit. CS/DS is a member of the glycosaminoglycan (GAG) family, which also includes three other members, *i.e.* heparan sulfate (HS), keratan sulfate (KS) and hyaluronic acid (HA). These molecules are long linear polysaccharides ranging in length from 20 to 25 000 (in the case of HA) of a repeated disaccharide unit consisting of hexuronic acid and N-acetyl D-hexosamine residues. The hexuronic residue can either be β -D-glucuronic acid (GlcA) or α -L-iduronic acid (IdoA). In CS/DS, the amino sugar is a N-acetylgalactosamine (GalNAc), and in the case of HS and HA N-acetylglucosamine (GlcNAc). The exceptions of these rules are KS where the hexuronic residue is replaced by a galactose and HA which does not contain IdoA. Of the GAGs, HA is the simplest in the sense that it is an unmodified polymer, while the other three types can be extensively modified by sulfation and epimerisation. In addition, HA exists as a free polysaccharide while CS/DS, HS and KS are all covalently attached

to various proteins. GAGs can be found throughout the animal kingdom even in the most primitive species including in some bacteria as a part of their cell wall. This family of polymers has also been shown to be of clinical relevance, both as a treatment and cause of several pathologies. Heparin (a highly modified form of HS) is one of the oldest and most widely used drugs on the market, and it is estimated that roughly 50-100 tons is produced every year (Eriksson, P.O., Dilafor AB, *personal communication*).

Core Proteins

As mentioned above, CS/DS does not normally exist as a free polysaccharide chain but is instead assembled on a core protein through a linkage region forming a proteoglycan. The linkage region has the structure $\text{-GlcA}\beta\text{1-3Gal}\beta\text{1-3Gal}\beta\text{1-4Xyl}\beta\text{1-}$ and is also the same in HS PGs. Matrix PGs can be divided into three families based on the domain structure of the proteins; basement membrane PGs, hyalectans and small leucine-rich PGs⁷. Basement membrane and small leucine-rich PGs carry few GAG chains while hyalectans can carry up to a hundred individual GAG chains. Interestingly, the structure of CS/DS chain differs between these groups even when produced from the same cell. The most striking difference is that of the hyalectan versican, which contain very small amounts of IdoA compared to that of the small leucine-rich PG member's decorin and biglycan that carry IdoA rich DS chains. The reason for this is not completely known, but it has been hypothesized that they are directed to different compartments of the Golgi during synthesis of the GAG-chain differing in content of the DS-epimerases⁸. Generally, CS/DS PGs are associated with the ECM while HS PGs are more prominent at the cell surface. The difference in distribution probably reflects functional aspects of CS/DS and HS in biology. Interestingly, variations have been observed in the GAG content of PGs, *e.g.* syndecans – a HS PG – can also carry CS^{9,10}.

Chondroitin/Dermatan Sulfate Structure and Function

CS was discovered over 150 years ago and got its name due to its abundance in cartilage. However, it is not until now, after the identification and cloning of most biosynthetic enzymes that we fully grasp and have the opportunity to attack the complex problem that is the machinery generating this polysaccharide. Altogether, more than twenty enzymes are required to assemble a single CS/DS chain (Table I), many of which that seem to have overlapping or seemingly redundant functions. A further complication that is hampering the study of this polymer is the lack of fast and efficient tools to analyze the structure of the chains. In addition, a CS/DS chain is not homogenous, but rather exist as a hybrid molecule due to the distribution of modifications within the chain. Qualitative tools to study the distribution of these functional domains in the polysaccharide are missing, and as a result our knowledge accumulated by over 150 years of research of CS/DS biosynthesis and its biological function is still fragmentary.

Structure

CS is a long linear acidic polysaccharide consisting of the repeating disaccharide unit $\text{-4GlcA}\beta\text{1-3GalNAc}\beta\text{1-}$ and can vary in size up to a hundred of these units ¹¹. A CS/DS chain of decorin produced by human lung fibroblast (HFL-1) consists of roughly 60-80 repeats (30-40 kDa). Conversion of GlcA into IdoA, through the process of epimerization, generates dermatan sulfate. This modification is highly variable, and can range from one IdoA residue in a chain to almost one hundred percent IdoA. The presence of IdoA gives the CS/DS chain key characteristics due to flexibility of the IdoA residue. GlcA is rigid and adopts a ${}^4\text{C}_1$ chair conformation while IdoA can be found in multiple conformations (such as ${}^1\text{C}_4$, ${}^4\text{C}_1$ and ${}^2\text{S}_0$ (skew-boat)) which are also affected by the environment, *i.e.*, sulfation of neighboring residues (Figure 1) ¹²⁻¹⁴. The inherent flexibility of the IdoA residue is believed to be important in binding to proteins. An example that supports this view is the X-ray structure of a HS hexasaccharide in complex with FGF-2, which also have the ability to bind CS/DS. Two IdoA residues in the binding hexasaccharide obtains two different conformations, ${}^1\text{C}_4$ and ${}^2\text{S}_0$ facilitating interaction with FGF-2 ¹⁵. Overall, data indicate that a chain with the same overall sulfate density but with IdoA instead of GlcA interacts better with its substrates in many cases.

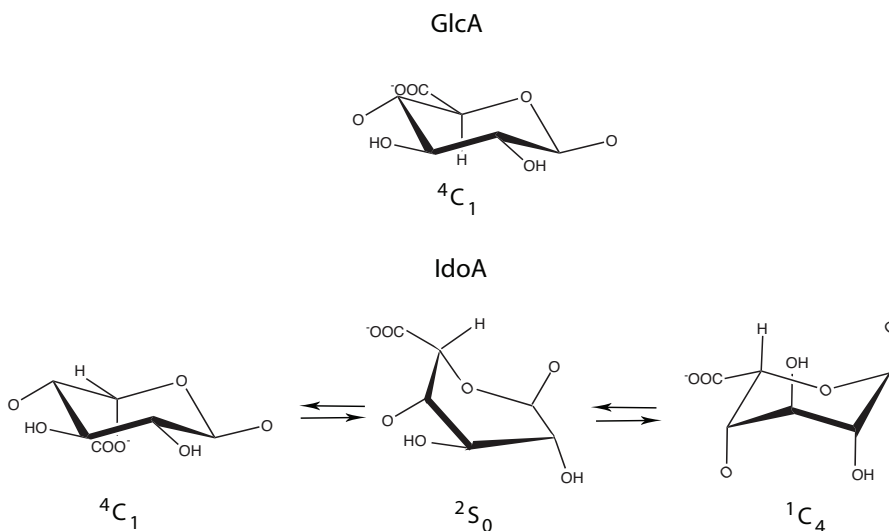


Figure 1. Conformations of glucuronic/iduronic acid (GlcA/IdoA) in dermatan sulfate. Due to the position of the C5-carboxyl in IdoA, this residue can adopt three different conformations that exist in equilibrium, *i.e.* two chair forms (4C_1 , 1C_4) and one skew-boat form (2S_0). The equilibrium is affected by sulfation of IdoA and by the nearby GalNAc. In CS/DS, GlcA can only be found in the 4C_1 -chair conformation.

CS/DS can also be modified by sulfation at various positions. This reaction is catalyzed by a group of enzymes called sulfotransferases that transfer a sulfate group from the universal sulfate donor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to the backbone of the chain in specific positions¹⁶. A GalNAc flanked by GlcA can be sulfated at either position 4 and/or 6, resulting in A and C units respectively (Figure 2). The GlcA residue can also be sulfated at position 2 but has only been found together with a 6-*O*-sulfated GalNAc in mammals forming the D unit. A GalNAc flanked by IdoA however, is mainly 4-*O*-sulfated (iA unit) and has given rise to the hypothesis that 4-*O*-sulfation and epimerization are closely connected processes¹⁷. The IdoA residue in these regions can also be 2-*O*-sulfated (iB unit). Albeit a rare modification, 3-*O*-sulfation of GlcA or IdoA has also been shown to occur in some marine organisms¹⁸⁻²¹. CS containing the latter structure, which contaminated a batch of the commonly used drug heparin, recently got a lot of media attention due to its role in the death of over 80 people in USA, and has led to several law suits²². The over-sulfated CS in the contaminated batches activated the complement system and the kinin-kallikrein pathway in treated patients, resulting in the production of anaphylatoxins and the vasoactive mediator bradykinin, respectively²³. A complete list of disaccharide structures found in

mammals and their nomenclature can be found in figure 2. It is worthwhile mentioning that previously, and also today at some commercial sources, names such as chondroitin sulfate A (4-*O*-sulfated), chondroitin sulfate B (dermatan sulfate) and chondroitin sulfate C (6-*O*-sulfated) were used. However, such macromolecular designations pose a big problem when it comes to the study of CS/DS as we will see below, and proper knowledge is needed to correctly address results obtained using CS from various sources.

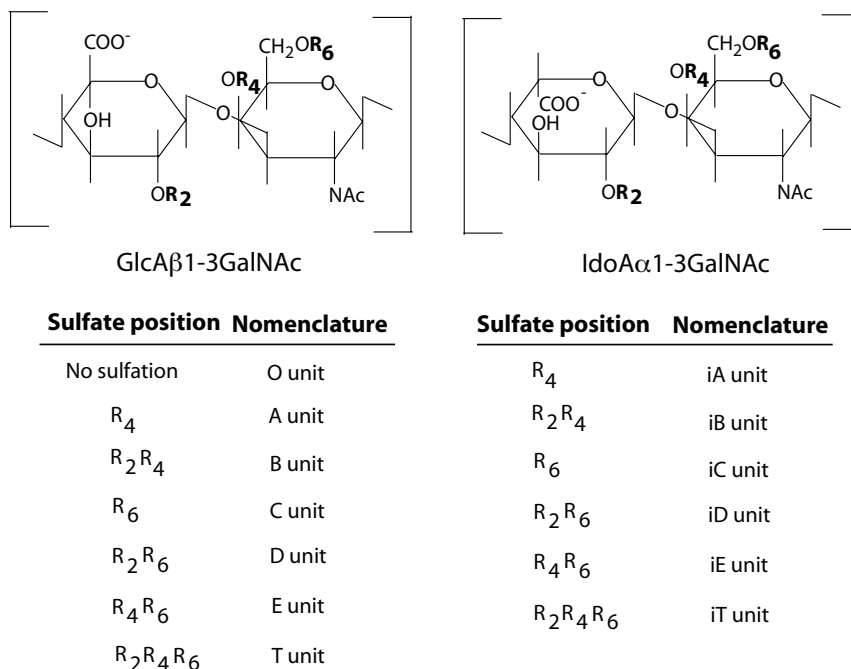


Figure 2. Structural units found in mammalian CS/DS. Sulfation and epimerization generate various chemical forms of the alternating disaccharide unit in CS/DS. Each form is designated with a one-letter code based on the positions of the sulfate groups. If the structure is epimerized, an i (Iduronic acid) is added to the name, *e.g.* iA. Not depicted are the 3-*O*-sulfated forms, which have only been identified in marine organisms.

In the 1960's it became apparent that GlcA and IdoA can exist in the same chain²⁴⁻²⁶. An interesting feature of CS/DS chains is therefore their hybrid structure, *i.e.* the distribution of IdoA and sulfates in the chain. *In vivo*, IdoA is frequently found in blocks (stretch of >4 IdoA residues) separated by blocks of GlcA or regions of alternating IdoA/GlcA residues (Figure 3)^{27,28}. For example, DS from human myometrium contain five distinct domains (2xIdoA blocks and

3xGlcA blocks) with different charge densities, *i.e.* difference in sulfation²⁹. In addition, the amount of IdoA as well as the pattern of these domains within a single chain is also tissue specific, pointing towards a non-random biosynthetic machinery that is highly regulated³⁰. The domains formed in CS/DS reflect functional parts within the chains. For example, alternating IdoA/GlcA structures of two individual DS chains can interact and is thought to be important in collagen fibrillogenesis^{31,32}. IdoA blocks on the other hand have been shown to be important in the binding to several growth factors such as fibroblasts growth factor 2, hepatocyte growth factor and heparin cofactor II^{3,33-35}. In regard to GlcA blocks, an octasaccharide containing a continuous sequence of three GlcA-GalNAc(4S,6S) is required for binding to collagen V³⁶. Both the degree of sulfation, epimerization and the distribution of these modifications varies, not only between tissues but also during development, and could therefore be a way to fine tune processes such as cell signaling and tissue integrity^{37,38}. When studying the fine structure of CS/DS in a biological context it is therefore not only important to determine the degree of modifications, but also their distribution within the chain.

Binding to growth factors

It was early shown that CS/DS had the ability to bind an array of ligands. In general, CS/DS bind their ligands with a much lower affinity compared to the related GAG heparan sulfate. The binding is dependant on the structure of the polysaccharide, *i.e.* particular motifs generated through sulfation and epimerization. However, the existence of specific “sequence codes” for each interaction have at least in the HS system, based on results from knock-out mice, been challenged³⁹. Some interactions do require semi-specific modifications, such as that between heparin cofactor II and DS. The binding of the ligand requires a hexasaccharide with the sequence iB-iB-iB or a 4-*O*-sulfated hexasaccharide rich in IdoA with at least one iE unit^{33,40}. The interaction between these structures and heparin cofactor II is involved in the enzymatic inactivation of thrombin in the coagulation cascade. This binding is physiologically relevant as shown in mice deficient in heparin cofactor II. These mice have a more rapid formation of thrombi upon vascular wall injury than their wild-type counterpart. Injecting deficient mice with wild-type heparin cofactor II, but not a modified form with low-affinity for CS/DS, rescues the phenotype⁴¹. Another example is the binding to the neurotrophic factors midkine and BDNF, which prefer E structures. Changing the position of the sulfates within a synthetic tetrasaccharide – but not the overall charge – was shown to significantly inhibit binding⁴². It has also been shown that domains of 4-*O*-sulfated IdoA blocks in CS/DS can readily bind hepatocyte growth factor (HGF)³.

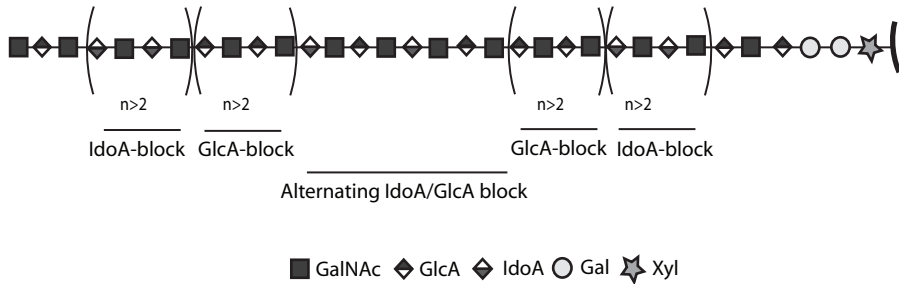


Figure 3. Hybrid structure of CS/DS. A key feature of dermatan sulfate is the distribution of IdoA within the chain. *In vivo*, IdoA is commonly found in clusters (IdoA blocks) separated by clusters of GlcA residues (GlcA blocks) or alternating GlcA/IdoA residues. Together with sulfation these blocks represent functional domains that are binding sites for several growth factors.

The ability of CS/DS to bind various growth factors is an important way of regulating the availability at the cell surface. During inflammation, growth factors sequestered in the extracellular matrix can be released by metalloproteinases that cleave the proteoglycan core protein, thus making them available for signaling. FGF-2 and FGF-7 bind to 4-*O*-sulfated IdoA domains in DS and when released upon injury, the complex promotes FGF signaling^{43,44}. A similar mechanism, mediated by the protease xHtrA1 in the frog *Xenopus leavis*, releases DS/FGF complexes from biglycan which subsequently can engage signaling at a long range⁴⁵.

Neuritogenesis

A very exciting area is the role of CS/DS in brain development where it has been shown to be involved in neural migration, neural plasticity, neural stem cell maintenance and outgrowth of axons/dendrites⁵. During development, the structure of CS/DS is spatio-temporally regulated, both in regard to epimerization and sulfation. Epimerization is more prominent in embryonic than in adult brains, and specific disulfated structures (Figure 2) have a variable expression pattern during development⁴⁶⁻⁴⁸. The structure of these distinct chains correlates with the regulated spatiotemporal expression of the enzymes involved in making them⁴⁸. This suggests an important role of certain structures in development of the brain. Both iB, D/iD and E/iE structures (Figure 2) have been shown to be important in the outgrowth of neurites by mediating binding of the growth factor pleiotrophin to the CS/DS-PG phosphacan and its transmembrane form receptor-type

proteintyrosine phosphatase ζ ⁴⁹⁻⁵¹. In addition, the importance of D/iD and E/iE structures in migration of neural precursor cells during development of the cortex was recently shown by *in vivo* siRNA mediated knockdown of the two sulfotransferases making these type of structures, *i.e.* UST and GalNAc4-6ST (Table I) ⁵².

CS/DS deposition is also induced upon CNS injury, where it is a major component of the scar tissue formed. This has detrimental effects during CNS regeneration, *e.g.* spinal cord injury, where CS/DS is inhibitory ⁵³. Efforts has been made to reverse this process by degrading CS/DS with chondroitinases, with some promising results ^{54,55}.

Infection

CS/DS has also been implied in infectivity of several pathogens. HSV-1 (herpes simplex virus) can utilize CS/DS chains as receptors at the cell surface ⁵⁶. The interaction of the virus with the polysaccharide is mediated via a positively charged region of the viral envelope protein glycoprotein C. Further, this interaction is dependent on E structures (Figure 2), and cells lacking the sulfotransferase C4ST-1 (Table I) are resistant to infection by HSV-1 ^{57,58}. CS/DS has also been proposed to be involved in pregnancy-associated malaria, where it increases the adherence of parasite infected red blood cells to the placental capillary endothelium ⁵⁹. The sequestration of parasite infected red blood cells in the capillaries affects the normal function of the placenta, which can lead to death of both the mother and unborn child. Further, the causative agent of Lyme disease, *Borrelia burgdorferi*, contain two adhesins (decorin-binding proteins A and B) that via CS/DS chains bind to the host cell ⁶⁰. The structural requirements for this binding have not been elucidated in detail but IdoA containing chains are better inhibitors of bacterial binding than those without IdoA ^{60,61}. In addition to its role in adherence, CS/DS chains can be shed from proteoglycans by bacterial proteases which thereby bind and inactivate host antimicrobial defensins ⁶².

Evolution of Chondroitin/Dermatan Sulfate

CS first appeared in the evolutionary tree with the emergence of species with tissues organized into germ layers, *i.e.* the subkingdom of eumetazoa, which includes all animal species except sponges ⁶³. It is worthwhile to mention however, that unsulfated chondroitin and unsulfated heparan can also be found in some bacteria ^{64,65}. These polysaccharides are in contrast to CS and HS found in the

animal kingdom, branched polysaccharides, and not attached to a core-protein. These bacterial strains are today used as a source of chondroitin and heparan for enzymatic studies and has shown to be of great use^{66,67}. The presence of chondroitin and heparan in bacteria has been suggested to be a late event in evolution since these polysaccharides has been implied in pathogenicity of these bacteria⁶³.

One reason for the poor information regarding the biological role of iduronic acid in dermatan sulfate is the lack of this modification in common model organisms. The use of these model organisms has lead to a considerable advancement of the understanding of heparan sulfate in biology^{68,69}. However, DS cannot be found in either the nematode *Ceanorhabditis elegans* or in *Drosophila melanogaster*, and can only be identified in the phylum deuterostomes⁶³. Due to the late emergence, it has been suggested that DS has very specific roles in development and physiology. The presence of iduronic acid blocks, as discussed above, is however not obligatory for life since a recently developed knock-out mouse for DS-epimerases 1 (Paper II) was shown to be viable, albeit with other discrete phenotypes, *Maccarana et. al. manuscript in preparation*. This mouse still contains a small amount of IdoA due to the presence of a second epimerase (Paper III), and it can therefore not be ruled out that a complete loss of iduronic acid is incompatible with proper development.

Sulfation is an earlier event in respect of evolution, and both 4-*O*-sulfation and 6-*O*-sulfation is present in CS chains from both *D.melanogaster* and in snails^{70,71}. The nematode *C.elegans* has a complete lack of sulfation of its chondroitin chains^{70,72}. Based on studies of the fine structure of CS chains in snails, it has been suggested that 6-*O*-sulfation is a more recently acquired modification than 4-*O*-sulfation⁷¹.

Precursor Formation

UDP-sugars

UDP-sugars are the precursors molecules for glycosaminoglycan synthesis and are divided up into two groups, *i.e.* primary and secondary nucleotide sugars⁷³. UDP-Gal and UDP-GlcNAc, both constituents of GAGs, are examples of primary nucleotide sugars, and are formed from UTP and their respective sugar-1-phosphate, *e.g.* GlcNAc-1-phosphate. This reaction is catalyzed by a group of enzymes called pyrophosphorylases. On the contrary, secondary nucleotide sugars are formed by modification of the primary source in processes such as epimerization and decarboxylation.

UDP-GlcA is synthesized from UDP-Glc by an UDP-glucose dehydrogenase. UDP-GlcA is also the precursor for UDP-Xylose, which is the first residue to be added in GAG biosynthesis and is formed by decarboxylation of the GlcA moiety by an UDP-GlcA carboxylase. UDP-GalNAc is formed by epimerization of the primary nucleotide sugar UDP-GlcNAc. The biosynthesis of the various UDP-sugars and their interconnections are summarized in figure 4.

The synthesis of these precursors takes place in the cytoplasm and they must therefore be transported into the ER/Golgi where GAG biosynthesis takes place. To achieve this, non-energy requiring transporters are located in the ER/Golgi membranes. These anti-port transporters are multiple-pass membrane-spanning proteins and exchange the UDP-sugar for a nucleoside monophosphate and are also competitively regulated⁷⁴. These transporters are generally not specific to one UDP-sugar but have the ability to transfer several types across the membrane. An example of this is the human UDP-GlcA/GalNAc transporter. A knock-out of the mouse orthologue of this transporter showed a defect in CS/DS biosynthesis and resulted in skeletal dysplasia⁷⁵. A mutation in the gene coding for this transporter was in the latter study also shown to be the cause of Schneckbecken dysplasia, which is a severe skeletal dysplasia in humans.

PAPS

3'-phosphoadenosine 5'-phosphosulfate (PAPS) is the sulfate donor required for all sulfation reactions within the cell. The synthesis of PAPS takes place in the cytoplasm by the action of two enzyme activities, ATP sulfurylase and APS kinase, which are located in two distinct domains of the protein PAPS synthetase⁷⁶. In the first reaction, two phosphates are removed and a sulfate group added by the ATP sulfurylase to form adenosine 5'-phosphosulfate (APS). Subsequently, APS is phosphorylated by the APS kinase to generate PAPS. Due to the location of many STs to the Golgi, PAPS needs to be transferred across the membrane. This is performed by the PAPS translocase, an antiport transporter which was partially purified and reported to be a 230kDa integral membrane protein in 1996^{77,78}. However, the molecular identification of this transporter was not achieved until 2003 when two independent groups identified the human (PAPST1) and drosophila (*slalom*) PAPS translocases^{79,80}. Later, a second PAPS transporter (PAPST2) has also been identified^{81,82}.

In a sulfotransferase reaction, the sulfate group of PAPS is transferred to the acceptor resulting in the formation of PAP. Since PAP is a competitive inhibitor of the sulfotransferase reaction, proper clearance is needed to maintain sulfation. In the Golgi, PAP is converted into AMP by the resident PAP-phosphatase JAWS^{83,84}. Knock-out mice for this protein die perinatally and present severe skeletal

abnormalities. CS/DS is drastically undersulfated in these mice showing the importance of PAP clearance^{83,84}.

CS/DS Chain Initiation and Elongation

Synthesis of the linkage region

Both CS/DS and HS are assembled to a core protein via a common linkage region, a tetrasaccharide with the structure -GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-. The same enzymes that synthesize the linkage region in CS/DS are also responsible for the same process in HS. This fact was elucidated through loss of function mutants that showed a lack of both types of polysaccharides⁸⁵⁻⁸⁷. The attachment of xylose to a serine residue is the initial step in the synthesis of this linkage region, and is the rate-limiting step of glycosaminoglycan biosynthesis. No true consensus sequence appears to exist for xylosylation. However, the serine is often followed by a glycine with acidic amino acids on one or both sides⁸⁸. Xylosylation of the core-protein is catalyzed by two xylosyltransferases (XylT-I and XylT-II) using the precursor UDP-xylose⁸⁹⁻⁹². It was long believed that this initial step in the biosynthesis took place in the ER. Recently however, evidence suggest that XylT-I and XylT-II reside in the early compartment of the Golgi apparatus⁹³.

The attachment of a xylose is followed by stepwise addition of two galactose residues. This process is catalyzed by two separate Galactosyl transferases, *i.e.* Gal I transferase and Gal II transferase^{94,95}. Gal I transferase has been shown to interact with XylT-I, but no interaction has been reported for Gal II with Gal I or XylT-1/XylT-II⁹⁶. Gal I and Gal II transferase are believed to be localized to the early Golgi compartment since they overlap with the cis and medial Golgi markers CALNUC and α -mannosidase II, respectively^{95,97}. The final step in the synthesis of the linkage region is the addition of glucuronic acid by GlcA I transferase⁹⁸. Loss of function mutants have been observed for all enzymes involved in synthesis of the linker region in the nematode *C. elegans*^{99,100}. The mutants share a similar phenotype with defects in vulva morphogenesis and cytokinesis. Consistent with previous findings presented above, neither HS nor CS is assembled in these mutants. In addition, point mutations in the gene coding for Gal I transferase, causing a reduced enzyme function, is involved in the development of a progeroid form of Ehlers-Danlos syndrome in humans¹⁰¹⁻¹⁰³.

The linkage region has also been shown to be prone for modification, both by phosphorylation and sulfation. Phosphorylation can occur in position 2 of the xylose residue, albeit the kinase responsible for this modification is yet to be identified^{9,104-107}. While phosphorylation of xylose can be found in both CS/DS

and HS, sulfation of the galactose residues has only been identified in the former. Sulfation can occur in position 6 of the first Gal residue and in position 4 and/or 6 of the second Gal^{9,107-109}. 6-*O*-sulfation of both Gal residues of the linkage region has recently been attributed to the sulfotransferase C6ST-1 (Table I)¹¹⁰.

The biological function of these modifications is not known, but has been suggested to play a role in subsequent elongation, and determination if the GAG chain will be HS or CS/DS. Experiments has for example shown that a phosphorylated and/or sulfated linkage region substrate increases the addition of GlcA by recombinant GlcA I transferase¹¹¹. Completion of the linkage region, and initial elongation of a CS/DS chain on decorin, can at least in COS cells be achieved without sulfation¹⁰⁵. It is therefore doubtful that sulfation of the linkage region Gals are a true determinant for CS/DS chain initiation. Further research is needed to elucidate the exact role of the modifications above in CS/DS biosynthesis.

Polymerisation

Polymerisation is initiated in the medial Golgi compartment by the addition of a GalNAc to the common linkage region followed by subsequent addition of alternating GlcA and GalNAc. Considerable progress has been made in the understanding of the polymerization reaction during the last eight years, with the identification and cloning of the enzymes involved in this process.

To date two ubiquitously expressed enzymes, N-acetylgalactosaminyltransferase I (CSGalNAcT-1) and N-acetylgalactosaminyltransferase II (CSGalNAcT-2), have been identified and shown to transfer a GalNAc to the linkage region, which is also a determinant for CS/DS chain elongation¹¹²⁻¹¹⁵. CSGalNAcT-1 is abundant in cartilage, and its expression is affected by the amount of the proteoglycan aggrecan¹¹⁶. Further, in the latter study it was shown that over-expression of CSGalNAcT-1 in cartilage, but not that of the CS synthases discussed below, resulted in an increase of CS content of aggrecan without affecting the chain length or the amount of this proteoglycan. Thus, the number of chains of aggrecan increases upon over-expression of CS-GalNAcT-1, which clearly shows the importance of this enzyme in CS/DS chain initiation. Notably, these enzymes also have GalNAcT-II activity, (*i.e.*, they can add a GalNAc to GlcA in the polymer) suggesting that they have a role in elongation.

Once the first GalNAc is added to the linkage region, and the chain is destined to become chondroitin, elongation starts. In addition to the two CS-GalNAc transferases discussed above, four enzymes with both GalNAcT-II and GlcAT-II activity but no GlcAT-I and GalNAcT-I activity (*i.e.*, they cannot add a GlcA or GalNAc in the linkage region) have been identified (Table I)¹¹⁷⁻¹²¹. An impressive biosynthetic machinery of a total of 11 enzymes is therefore potentially

involved in initiation and elongation of a single CS/DS chain. These enzymes have a type II transmembrane topology with a short cytoplasmic N-terminal region followed by a transmembrane domain, stem region and a catalytical domain. Interestingly, although all chondroitin synthases identified show both GalNAcT-II and GlcAT-II activity, no polymerization occurred when incubating soluble recombinant enzymes with both UDP-GalNAc and UDP-GlcA. However, co-expression of any two of the chondroitin synthases in cells not only increased GalNAcT-II and GlcAT-II activity, but also resulted in polymerization in an *in vitro* assay^{121,122}. Moreover, it was also shown that the enzymes in all combinations tested physically interact. This could not be achieved by simple mixing of separately expressed and purified enzymes but required co-expression. An interesting feature of these different complexes is that they synthesized chains with different lengths¹²¹. If this is an *in vitro* artifact or a true inherent feature of the various complexes within a cell, with a function to regulate chain length remains to be seen. The role of the two GalNAc transferases (CS-GalNAcT-1, CS-GalNAcT-2) has not been investigated in this regard, and their contribution to polymerization remains to be elucidated.

The biological role and activity of these enzymes has also been studied in *C.elegans*. This organism has orthologues to the human enzymes Chondroitin Synthase I (ChSy1) and Chondroitin Polymerizing factor (ChPf), and share 37 and 27 percent sequence identity to their human counterparts, respectively. Using an RNA interference approach, it was shown that knockdown of either ChSy or ChPf resulted in loss of CS, confirming the requirement of both enzymes for proper elongation. In addition, an aberrant cell division and defects in gonadal distal tip cell migration was observed after knockdown^{100,123-125}. These defects were also shown to be more severe when weak alleles (low expression) for the two genes were combined. The expression pattern of these enzymes overlapped, except in distal tip cells where only ChPf expression was observed. However, over-expressing ChSy specifically in distal tip cells in ChSy deficient worms rescued the migration of these cells indicating that the failure to observe ChSy expression was simply due to a low expression level¹²⁵. Altogether the collaborative action of several enzymes is therefore required for CS elongation also *in vivo*. No loss of function models has been reported for the mammalian enzymes.

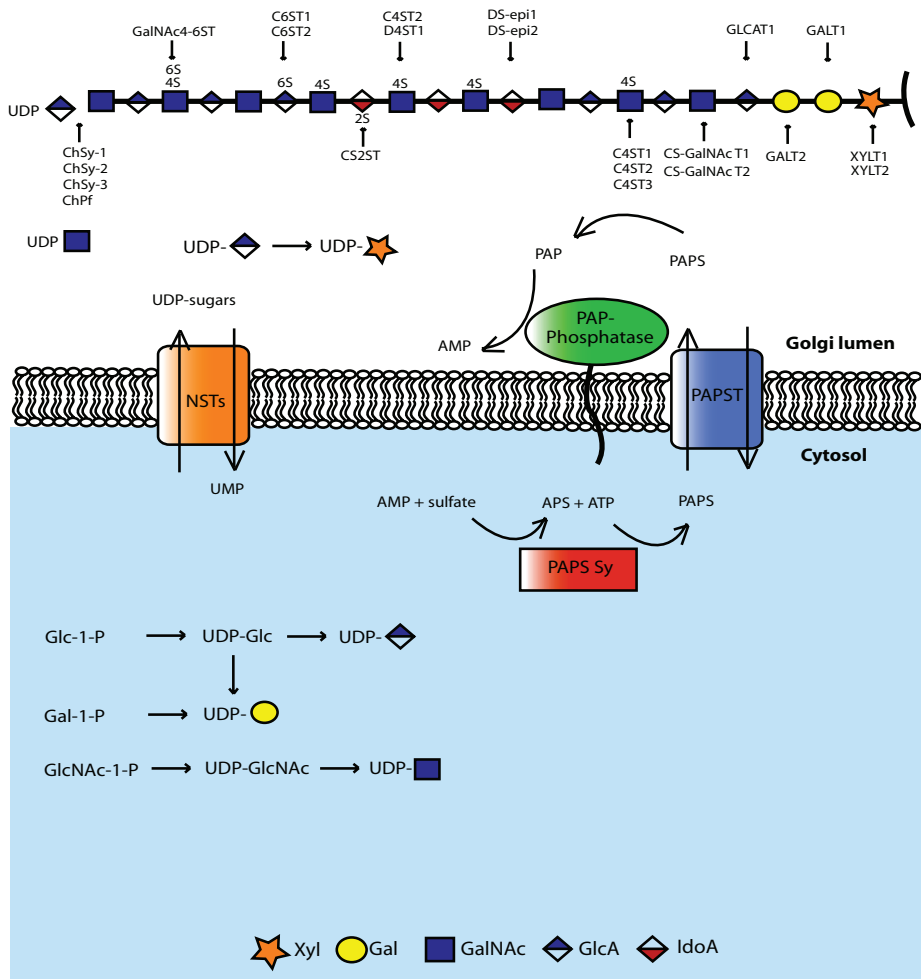


Figure 4. Biosynthesis of CS/DS. The precursors for CS/DS biosynthesis are formed in the cytoplasm. PAPS (synthesized by the PAPS synthetase, PAPS Sy) and nucleotide-sugars are thereafter transported into the Golgi lumen by PAPS transporters (PAPST) and nucleotide-sugar transporters (NSTs), respectively. In Golgi, a linkage region is formed on a core-protein through the sequential addition of UDP-Xyl, UDP-Gal and UDP-GlcA by the glycosyltransferases XylT1/T2, GalT1/T2 and GlcAT1. On this linkage region, chondroitin is assembled through alternating addition of UDP-GalNAc and UDP-GlcA by 6 glycosyltransferases (CS-GalNAc T1/T2, ChSy1-3 and ChPF), creating a chain of between 20-100 disaccharide-repeats in length. During assembly, the chain is modified by epimerization by two DS-epimerases (DS-epi 1 and DS-epi 2, see Paper II and Paper III) and sulfation in various positions by a group of specific sulfotransferases (C4ST-1-3, D4ST-1, C6ST-1/2, CS2ST/UST and GalNAc4-6ST) generating a highly modified dermatan sulfate chain. PAP formed from the sulfate donor PAPS in the sulfotransferase reaction is a competitive inhibitor of sulfation and are cleared by a Golgi-resident PAP-phosphatase.

Modifications of the Chain

As described in the introduction, a disaccharide unit in CS/DS can exist in 16 different chemical forms. The biological information contained within one chain is therefore greater than both that of DNA and proteins¹²⁶. This vast array of variability is achieved by modifying the GlcA β 1-3GalNAc building block chemically by the addition of sulfate groups in various positions, and/or converting GlcA into IdoA. These modifications are performed through the action of two different classes of enzymes, *i.e.* sulfotransferases and epimerases, respectively. The addition of sulfate groups gives the chain a negative charge and the opportunity to bind to basic sequences in proteins, while converting GlcA into IdoA render the chain flexible, which facilitates the interaction with proteins. An important aspect is that the various modifications of the chain are incomplete, *e.g.* epimerization, resulting in domains with different structural features (Figure 3). With our identification of two DS-epimerases (Paper II and III), members of all classes of enzymes involved in modifying the CS/DS chain have now been identified.

Epimerisation

One of the major modifications of CS/DS is epimerization and is catalyzed by two DS-epimerases, *i.e.* DS-epi 1 and DS-epi 2 (Paper II and III). These enzymes are ubiquitously expressed but with variations in expression levels. The process of epimerization turns GlcA into its stereoisomeric form IdoA, by changing the directionality of the C5 carboxyl group in space (Figure 1 and 2). Malmstrom *et al* could show that this reaction takes place at the polymer level, *i.e.* on a GlcA already incorporated into the GAG chain, and indeed no UDP-IdoA can be found within the cell¹⁷.

Incubations of enzyme fractions with tritiated water and ³H-C5 labeled substrate showed incorporation and release of tritiated water, respectively¹²⁷. Hence, it was concluded that the initial and final step in the epimerization reaction occurs through proton abstraction and proton addition to the C5-carbon, respectively. Further, due to the spatial redistribution of the C5 carboxyl, proton abstraction and re-addition must occur from opposite sides of the sugar plane. The intermediate in this reaction has been proposed to be a carbanion⁶⁶. The epimerase reaction is reminiscent to that of bacterial lyases, which also act at the polymer level. Gacesa proposed in 1987 that lyases and epimerases must therefore share common characteristics¹²⁸. In lyases however, proton abstraction from the C5 carbon of GlcA/IdoA is followed by beta-elimination resulting in cleavage of the chain and formation of a 4,5-unsaturated product. The most challenging part of this reaction mechanism is the abstraction of the C5 proton due to its very high

pK_a . In the bacterial lyases chondroitinase AC-I and heparinase II, crystal structures have shown that reduction of the pK_a is achieved by forming a hydrogen bond to the carboxylate^{129,130}. For the epimerase, it has been hypothesized that epimerisation runs through a two-base mechanism, with the initial proton abstraction performed by a polyprotic amino acid such as a lysine, and proton donation by a monoprotic amino acid, *e.g.* histidine⁶⁶.

The epimerase reaction is freely reversible and generates a polysaccharide product with a GlcA to IdoA ratio of 9:1 at equilibrium^{131,132}. However, *in vivo* a DS chain can consist of almost solely IdoA residues. A clue to this discrepancy was offered by Malmstrom *et al* who could show that addition of the sulfate donor PAPS to the enzymatic reaction increased the IdoA content in the formed chains¹⁷. This observation was further supported by experimental data showing a decreased IdoA content in human skin fibroblasts when the sulfate concentration in the growing medium was reduced¹³³. Indeed, IdoA residues are rarely if not never found with a flanking unsulfated GalNAc. The GalNAc flanking IdoA is often 4-*O*-sulfated and has generated the hypothesis that the DS-epimerases work closely with a 4-*O*-sulfotransferase. In light of the shown substrate specificity of the 4-*O*-sulfotransferases involved in DS biosynthesis, as discussed below, the most likely candidates for working together with the DS-epimerases are C4ST-2 or D4ST-1 (Table I)¹³⁴. The importance of D4ST-1 was confirmed in this thesis by siRNA-mediated knockdown in human lung fibroblasts (Paper V). Since sulfated structures are not a substrate for epimerization, it has been proposed that 4-*O*-sulfation functions as a lock that hinders back-epimerisation of IdoA¹³².

An interesting feature of DS chains is the highly different IdoA content in various proteoglycans produced from the very same cell. Versican for example contain small amounts of IdoA residues situated as alternating IdoA/GlcA residues (Figure 3). In DS chains of biglycan and decorin on the other hand, IdoA residues are abundant and are distributed in blocks. This core protein dependence has not been observed in the HS system. It was therefore postulated that the DS-epimerase/s could have some core protein specificity. By using chimeric forms of decorin and the part-time proteoglycan CSF (containing versican type CS/DS chains), Seidler *et al* could show that the presence of CSF mediated a change in the decorin CS/DS chains to versican type. Interestingly, epimerisation *in vitro* took place both in the wild-type and chimeric proteins equally well⁸. This clearly spoke against a mechanism where the DS-epimerases specifically recognizes the core protein *per se*. The authors instead suggested that a negative signal for epimerization exists within the core protein, possibly directing it to a sub-compartment within the Golgi lacking – or containing less – of the enzymes involved. DS primed on xylosides (*i.e.* priming without a core protein) are similar to those of decorin speaking in favor of such a mechanism¹³⁵. Thus, epimerisation can be considered a default pathway within the cell.

Sulfation

The second major modification of CS/DS is sulfation. This process takes place in late compartments of the Golgi complex by the transfer of a sulfate group from the donor 3'-phosphoadenosine 5'-phosphosulfate to the growing/already formed GAG chain¹³⁶. Sulfation occurs of both GalNAc and GlcA/IdoA, where GalNAc can be substituted on the 4- and 6-hydroxyl, and GlcA/IdoA on the 2-hydroxyl, respectively (Figure 2 and 4). The enzymes responsible for these modifications can be divided up into three families based on their action, *i.e.* 4-*O*-STs, 6-*O*-STs and finally 2-*O*-ST.

The first member of the CS/DS sulfotransferases to be purified was C6ST-1¹³⁷. This was achieved by utilizing the fact that 6-*O*-sulfotransferase activity could be detected in the conditioned medium of chick embryo chondrocytes thereby facilitating its isolation. Further, during purification, 6-*O*-ST and 4-*O*-ST activity could be partially separated, indicating that the two activities were not shared by one sulfotransferase. Cloning and expression of the chick C6ST-1 was achieved in 1995, and followed by its human orthologue by two independent groups three years later¹³⁸⁻¹⁴⁰. Over-expression of the protein in COS cells resulted in a substantial increase in 6-*O*-ST activity, while no or little increase in 4-*O*-ST activity was observed. Interestingly, C6ST-1 also had activity towards keratan, and it was established that this enzyme can act on the Gal residues in keratan sulfate¹⁴¹. C6ST-1 was also the first CS/DS modifying enzyme studied in a loss of function setting. Albeit a major modification in CS/DS, knock-out mice for C6ST-1 develop normally without any apparent phenotype, and were born at the expected Mendelian frequencies¹⁴². The only phenotype observed was a reduced number of naïve T lymphocytes in the spleen, indicating a function for CS/DS in development of the immune system. Surprisingly, a human disease caused by a missense mutations in C6ST-1 has also been identified and is manifested by severe chondrodysplasia, a feature which is not observed in the C6ST-1^{-/-} mouse^{143,144}. Although drastically reduced, low levels of 6-*O*-sulfation can be found in both the C6ST-1^{-/-} mouse and in affected patients. The remaining 6-*O*-ST activity can be explained by the presence of a protein with 24% amino acid sequence identity to C6ST-1 which has been shown to be an active 6-*O*-sulfotransferase and designated C6ST-2¹⁴⁵. However, when independently cloned by a second group, C6ST-2 showed no activity towards the synthetic substrates α - or β -benzyl GalNAc, but had considerable activity towards β -benzyl GlcNAc residues¹⁴⁶. The authors however, did not use C6ST-1 as a positive control of sulfation of α - or β -benzyl GalNAc, and the use of their synthetic substrate to judge CS 6-*O*-ST activity is therefore doubtful. A 6-*O*-ST specifically sulfating GalNAc when surrounded by IdoA residues has also been partially purified from fetal bovine serum but not cloned¹⁴⁷.

Table I Enzymes involved in dermatan sulfate biosynthesis

Enzyme	Reaction	Reference
Xyl transferase I	Addition of a Xyl to the core protein	92
Xyl transferase II	Addition of a Xyl to the core protein	89-91
Gal I transferase	Addition of first Gal residue in linkage region	94
Gal II transferase	Addition of second Gal residue in linkage region	95
GlcA I transferase	Addition of GlcA to linkage region	98
CSGalNAcT-I	Addition of first GalNAc to linkage region ^a	112, 115
CSGalNAcT-II	Addition of first GalNAc to linkage region ^a	113, 114
ChSy1	Polymerisation ^b	117
ChSy2	Polymerisation ^b	120
ChSy3	Polymerisation ^b	121
ChPf	Polymerisation ^b	118, 119
C4ST-1	Sulfates GalNAc in position 4 in the sequence GlcA-GalNAc-GlcA	149, 151
C4ST-2	Sulfates GalNAc in position 4 in the sequence GlcA/IdoA-GalNAc-GlcA/IdoA	151
C4ST-3	Sulfates GalNAc in position 4 in the sequence GlcA-GalNAc-GlcA	153
D4ST-1	Sulfates GalNAc in position 4 in the sequence IdoA-GalNAc-IdoA	152
C6ST-1	Sulfates GalNAc in position 6	139, 140
C6ST-2	Sulfates GalNAc in position 6	145
CS2ST/UST	Sulfates GlcA/IdoA in position 2	158
GalNAc4S-6ST	Sulfates a 4-O-sulfated GalNAc in position 6	157
DS-epi 1	Epimerisation of GlcA to IdoA	Paper II
DS-epi 2	Epimerisation of GlcA to IdoA	Paper III

^aCan also act in chain elongation

^bCannot add a GlcA or GalNAc in the linkage region

A second family of sulfotransferases adds a sulfate group to the 4-hydroxyl of GalNAc. The first member of this family to be identified was chondroitin 4-*O*-sulfotransferase 1 (C4ST-1) by purification from conditioned medium of a rat chondrosarcoma cell line ¹⁴⁸. Cloning and expression of the human and mouse C4ST-1 was subsequently achieved by two independent groups ¹⁴⁹⁻¹⁵¹. When Hiraoka *et al* used the HNK-1 sulfotransferase as a probe to screen EST databases, they discovered, in addition to C4ST-1, a second ST showing 42% sequence identity to that of C4ST-1. This enzyme was therefore named C4ST-2 ¹⁵¹. The purified and recombinant C4ST-1 showed a strong preference for chondroitin as a substrate when assayed *in vitro*, while no or little activity could be detected using 4-

O-sulfated or 6-*O*-sulfated chondroitin. Moreover, desulfated dermatan sulfate served as an acceptor of C4ST-1 action, albeit to a lesser extent than chondroitin. A similar substrate specificity was also obtained for C4ST-2¹⁵¹. By structural analysis of desulfated DS incubated with C4ST-1 and C4ST-2 above, the authors could illustrate that 50 percent of the sulfate groups added to the substrate was recovered as disaccharides after chondroitinase ACI treatment, while the remaining material was contained in larger structures. Since chondroitinase ACI cleaves –GalNAc-GlcA- linkages, the disaccharides obtained represent a GalNAc flanked by two GlcA in the original structure, while that of the resistant material represent –GlcA-GalNAc-(IdoA-GalNAc)_{N≥1}-GlcA. Since no release of 4-*O*-sulfated disaccharides were observed upon chondroitinase B treatment, which specifically cleave –GalNAc-IdoA- linkages, the conclusion was reached that a GalNAc flanked by two IdoA did not serve as an acceptor of C4ST-1¹⁵¹. This indirectly suggested to the authors that 4-*O*-sulfated IdoA domains in DS were generated by sulfation of GalNAc and subsequent epimerization of GlcA. This hypothesis was supported by the fact that reducing sulfation, by lowering the sulfate content in the growth medium of cultured cells, also resulted in less epimerization¹³³. The problem of this view though is the fact that sulfated CS does not serve as a substrate of the DS-epimerase¹³². An explanation for this discrepancy came with the discovery of D4ST-1. The preferred substrate of this enzyme was desulfated dermatan sulfate, and it was shown that a GalNAc flanked by two IdoA residues served as an acceptor strengthening the view that epimerization precedes sulfation¹⁵². A more detailed analysis of the substrate specificity of these three enzymes using partially desulfated dermatan sulfate showed that C4ST-1 and D4ST-1 prefer two flanking GlcA and two flanking IdoA residues respectively, while C4ST-2 can sulfate both types of structures equally well¹³⁴. Later, yet another member of this family were identified based on homology to the HNK-1 ST, *i.e.* C4ST-3 and desulfated chondroitin was shown to serve as the best acceptor¹⁵³. However, the detailed substrate specificity of this enzyme in regard to flanking IdoA residues has not been studied.

The importance of 4-*O*-sulfation of CS/DS became apparent with the creation of a knock-out mouse for C4ST-1^{154,155}. The mice were born at the expected frequencies but died shortly after birth. The authors could show that loss of C4ST-1 resulted in severe chondrodysplasia as a result of an aberrant growth factor signaling (*i.e.* TGF- β and BMP) during development¹⁵⁴. Interestingly, the amount of CS is drastically reduced in these mice and was recently shown to be due to a significant decrease in CS/DS chain length⁵⁸. No loss of function models has been reported for C4ST-2, C4ST-3 or D4ST-1.

Synthesis of oversulfated structures

In addition to the abundant monosulfated structures found in CS/DS, more oversulfated structures frequently occur but to a lesser extent (Figure 2). For example, in cultivated fibroblasts, DS chains derived from decorin/biglycan contain 5 percent iB units, 2 percent D/iD units and 2,5 percent E/iE units as judged after chondroitinase ABC treatment (Paper V). Many of the functions carried by CS/DS are strictly dependant on these types of structures.

When looking at the substrate specificity of C6ST-1/2 and the various 4-OSTs, it is apparent that neither of these proteins have the ability to add a sulfate to an already sulfated GalNAc. The E/iE unit (-GlcA/IdoA-GalNAc-4S6S) is instead synthesized by a separate enzyme called GalNAc4S-6ST, which was originally purified from squid cartilage¹⁵⁶. Using the purified squid enzyme, or that of the recombinant human counterpart, resulted in sulfation of already 4-*O*-sulfated GalNAc^{156,157}. The E/iE units are thus formed by an initial attack by a 4-OST and subsequently by GalNAc4S-6ST. A recent study has suggested that the main 4-OST involved in the formation of the E unit is C4ST-1⁵⁸.

The last type of sulfation occurring in CS/DS is 2-*O*-sulfation and is most often found in the disulfated units D/iD and iB. No 2-*O*-sulfated GlcA/IdoA with a non-sulfated GalNAc on its reducing side have been identified. By using the heparan sulfate 2-*O*-ST sequence to screen databases for homologues, the CS/DS specific enzyme was identified (Uronosyl 2-*O*-sulfotransferase; CS2ST/UST)¹⁵⁸. An interesting question is whether 2-*O*-sulfation by UST happens before sulfation of the GalNAc residue when disulfated units are generated. Two independent groups have shown that both CS/DS rather than their unsulfated counterpart served as good acceptors indicating that sulfation of GalNAc precedes that of the GlcA/IdoA situated at the non-reducing side^{158,159}. In contrast, in a third study, dermatan sulfate served as a poor acceptor¹⁶⁰. The explanation for this discrepancy is not clear, but the authors of the latter study suggested that it could be due to differences in post-translational modifications and/or the effect of host cell-derived factors in the various expression systems. What is clear from these studies is that the activity of UST is increased towards a GlcA with a 6-*O*-sulfated GalNAc on its reducing side and a 4-*O*-sulfate on the non-reducing side^{158,160}. However, UST prefers an IdoA residue with a 4-*O*-sulfated GalNAc on its reducing side. The substrate specificity of UST converges very well with structures found *in vivo*, where D and iB units are more prominent than iD and B units, respectively. B units have only been observed in skin from sharks¹⁶¹. The apparent difference in preference of UST towards GlcA or IdoA is most likely a reflection of the conformation of the uronic acid residue, which influences the interaction of the sulfated GalNAc on the reducing side with the enzyme. This thought is supported

by the fact that IdoA, in contrast to GlcA, can obtain various conformations, which are also sulfate dependant. No crystal structure for UST with either bound CS or DS exists that could unambiguously verify this hypothesis.

The sulfotransferases are all type II transmembrane proteins with a short cytoplasmatic tail, transmembrane segment and a catalytic C-terminal domain. A peculiar characteristic of these enzymes is their secretion into the growth medium as shown by the original purification strategy and over-expression experiments^{137,148,152}. The function of this secretion is unknown but could be a way to regulate the amount of the enzyme in the Golgi. Nagai *et al* showed that by treating cells with a beta-secretase inhibitor, the secretion of HS-6-*O*-sulfotransferase 3 diminished and instead accumulated intracellularly¹⁶². In addition, 6-*O*-sulfation of HS increased during these conditions. Thus, at least in the heparan sulfate system, secretion is a way to regulate the fine structure of the resulting GAG chain.

Structure and Catalytical Mechanism of Sulfotransferases

Several crystal structures have been solved for both cytosolic STs, *e.g.* estrogen sulfotransferase (EST), and Golgi-resident STs, *i.e.* N-deacetylase/N-sulfotransferase NDST-1 (sulfotransferase domain) and several HS-3-*O*-STs¹⁶³⁻¹⁶⁵. Comparison of the structures obtained show that they are very similar, with a single α/β globular domain¹⁶⁶. Structure based sequence alignments have proposed that also the 6-*O*-STs, 2-*O*-STs and 4-*O*-STs acting on CS/DS have the same overall structural fold^{144,159,167}.

The most conserved part of these different STs is the PAPS binding motif, consisting of a strand-loop-helix and a strand-turn-helix structure. The latter part makes up the 3'-phosphate binding site where an arginine and a serine residue form hydrogen bonds to oxygen atoms of the 3'-phosphate¹⁶⁷. Mutation of these two amino acids into alanine was shown to abrogate the binding of PAP to UST¹⁵⁹. These two residues are also conserved among all CS/DS STs identified. The strand-loop-helix motif forms the 5'-phosphosulfate binding site, where in C6ST-1 two serine residues form hydrogen bonds to the 5'-phosphate¹⁶⁷. A lysine residue in the loop of this structure forms a hydrogen bond to the oxygen bridging the 5'-phosphate and the sulfate group. Thus, the lysine has been proposed to weaken the bond between the phosphate and sulfate in PAPS, enabling transfer of the sulfate group to the receiving oxygen in the sugar. This lysine is conserved among 2- and 4-*O*-STs, but is replaced by an arginine in 6-*O*-STs. Mutation of this residue has been shown to result in loss of activity of UST and NDST-1^{159,168}. Studies of the EST have also proposed an interesting interplay between the latter residue and the 3'-phosphate binding serine. When substrate is not present in the active site, the lysine side-chain interacts with the serine, moving it away from the bridging oxygen, and mutation of this residue increases PAPS hydrolysis¹⁶⁹. In addition to

the lysine, the active site also contains a catalytic base that abstracts the proton from the sugar hydroxyl. In several STs, including UST, this is performed by a histidine residue^{159,166}.

In summary, sulfate transfer from PAPS to the sugar proceeds in 4 steps, *i.e.* (1) formation of a ternary complex between PAPS, the sugar substrate and the enzyme, (2) deprotonation of the sugar hydroxyl substrate by a catalytic base such as a histidine, (3) weakening of the bond between the 5'-sulfate and phosphate through the action of the conserved lysine, (4) formation of a sulfated product and PAP.

Chain Termination

An interesting question in CS/DS biosynthesis is how chain length is determined. Variable lengths of CS/DS chains have been observed *in vivo* but very little information exists regarding how it is regulated. Studies trying to elucidate if the non-reducing terminal always contains a certain modification have been unfruitful, and the chain can terminate in GlcA, GalNAc and their sulfated counterparts^{170,171}. The fact that no specific sulfation is observed at the termini, and therefore determining chain length, is supported by a study showing equal size of the chains after chlorate treatment, which inhibits sulfation¹⁷². However, a small percentage of sulfation was still present in this study, and it cannot be ruled out that this is sufficient to support polymerization. In addition, cells deficient in C4ST-1 expression produce CS/DS with a significant reduction in size⁵⁸. These data are consistent with a study showing that addition of GalNAc to the non-reducing end was stimulated when a 4-*O*-sulfated GalNAc was present at the reducing end of a tetrasaccharide substrate¹⁷³. Thus, 4-*O*-sulfation might be needed for elongation but a yet undetermined mechanism must be involved in termination. Of note is that no addition of GlcA occurs when a 4-*O*-GalNAc is present at the non-reducing end¹⁷⁴. Interestingly, different combinations of CS synthases generated a variable chain length *in vitro*^{121,122}.

Degradation

An important aspect of all type of molecules produced in our cells are their turnover. A continuous production without breakdown of old molecules, or an aberrant breakdown without concomitant replacement with new, could easily lead to pathology. A discussion about the breakdown of CS/DS is therefore well motivated since several – very severe – pathological states are due to defects in the breakdown.

Catabolism of CS/DS takes place in the lysosome through the sequential action of endo-acetylhexosaminidases, exo-glycosidases/hexosaminidase and exo-sulfatases¹²⁶. The initial step in this sequence of reactions is the cleavage of the glycosidic linkages at GlcA sites by hyaluronidases, generating shorter CS/DS fragments. Depolymerisation then continues by removal of 4- and 6-*O*-sulfate groups by GalNAc-4-sulfatase and GalNAc-6-sulfatase before removal of the GalNAc and GlcA residues by β -N-acetylhexosaminidase and β -glucuronidase, respectively. 2-*O*-sulfates are removed by iduronate-2-sulfatase before release of the IdoA moiety by α -iduronidase, which like all the other exo-glycosidases only acts on an unsubstituted residue.

Several of the enzymes above are implicated in human pathology. Loss of function leads to accumulation of CS/DS causing a range of disorders designated as mucopolysaccharidoses. These include the Hunter and Hurler syndromes with defects in iduronate-2-sulfatase and α -iduronidase, respectively¹⁷⁵.

PRESENT INVESTIGATION

Aims

Even though the presence of L-iduronic acid in dermatan sulfate has been known for more than 50 years, the enzyme responsible for this modification has never been identified. This missing piece of the puzzle is required to address the biological function of dermatan sulfate as well as elucidating its complex biosynthesis. The objective of this thesis was therefore to isolate and characterize the DS-epimerase, as well as investigating the formation of functional domains within the dermatan sulfate chain brought about due to a possible connection between sulfation and epimerization.

Results

Paper I

The existence of variously sulfated and epimerized forms of DS in different tissues, and during pathological conditions, suggests that the biosynthesis is regulated. It is well established that CS/DS can bind a wide range of growth factors. However, very little is known how growth factors released during normal physiology or inflammation affect CS/DS structure. We have previously shown that TGF- β , EGF and PDGF enhance the production of CS/DS PGs fibroblasts¹⁷⁶. In the first paper of this thesis, we therefore studied the influence of these factors on the structure of CS/DS.

We hypothesized that stimulation of human lung fibroblasts with the growth factors EGF, PDGF and TGF- β would lead to a change in sulfation and

epimerization pattern in CS/DS by affecting the expression of biosynthetic enzymes.

As previously shown, TGF- β induced expression of CS/DS PGs, and was further increased combining TGF- β , EGF and PDGF. Examining the fine structure of CS/DS chains derived from the PGs decorin and biglycan, revealed a reduction in IdoA content going from 70 percent in the unstimulated control to 53 and 36 percent in TGF- β and the combination treatment, respectively. No decrease in IdoA content was observed in versican chains upon treatment. This reduction could be attributed to a decrease in epimerase activity and not simply due to an increased expression of the PG core or an increased chain length. Further, the sulfation pattern in versican-derived CS/DS chains was changed upon treatment, with an increase in 4-*O*-sulfation and a decrease in 6-*O*-sulfation, while no major effects could be detected in decorin/biglycan chains. By measuring sulfotransferase activity using either chondroitin or dermatan, we could conclude that STs preferentially acting towards a GalNAc flanked by two GlcA residues were upregulated. Structural analysis of the product also showed a stronger induction of 4-*O*-sulfation compared to that of 6-*O*-sulfation. This was confirmed with RT-PCR showing a significant induction of C4ST-1 transcription, while the relative mRNA levels for C4ST-2 remained unaffected or slightly decreased. D4ST-1 expression was reduced by 50% after TGF- β treatment. The induced change in 4/6-*O*-sulfation ratio in versican by the growth factors are therefore an effect of a relative higher increase of C4ST-1 compared to that of C6ST-1. The unchanged level of 4-*O*-sulfation in decorin CS/DS can accordingly be attributed to C4ST-1 taking over the role of D4ST-1 when epimerization is reduced.

In conclusion, TGF- β together with EGF and PDGF are thus key players in DS biosynthesis by directly or indirectly regulating transcription of biosynthetic enzymes. This results in CS/DS chains with distinct structural properties, which probably affects the affinity for its ligands, *e.g.* growth factors.

Paper II

For a long time the study of DS has been hampered due to the fact that the key enzyme converting GlcA into IdoA, *i.e.* DS-epimerase, has remained unidentified. The isolation and cloning of the corresponding enzyme involved in biosynthesis of HS, did not reveal a possible candidate for the DS-epimerase, which therefore seem to lack homology¹⁷⁷.

We hypothesized that through a series of chromatography steps would be able to achieve a sufficient purification of the DS-epimerase to identify its amino acid sequence with LC-MS/MS.

To find a suitable enzyme source, several tissues were screened for epimerase activity. The highest expressing tissue was spleen and was subsequently used as a starting point for purification. A seven-step purification scheme was employed, starting from 4kg of bovine spleen, where the DS-epimerase could be purified 43 000-fold with a recovery of 1.4 percent. This proved to be sufficient to obtain a good amount of the protein and a reasonable low-complexity sample to identify a candidate protein with LC-MS/MS. The candidate, SART2, had previously been identified as a squamous cell carcinoma antigen recognized by cytotoxic T-lymphocytes but without a clear described function¹⁷⁸. Over-expression of the candidate protein resulted in a 22-fold increase in epimerase activity and a substantial increase in IdoA content in DS produced by the cell, thus confirming its identity as a DS-epimerase. The human gene for DS-epi 1, consisting of six exons, is located at chromosome 6 and encodes a protein of 110kDa (Figure 5). The protein is predicted to contain an N-terminal signal-peptide and two C-terminal transmembrane domains. This is unlike other enzymes in CS/DS biosynthesis, which are type II transmembrane proteins. Using the protein sequence as a probe to screen for possible homologues we identified a second putative epimerase (see Paper III). In addition, between amino acid 24 and 584, DS-epi 1 shares 21% identity (36% similarity) with a putative bacterial alginate oligo-lyase. This highlights the predicted similarity in reaction mechanisms between the lyase and epimerase reactions and suggests that the DS-epimerase has evolved from bacterial lyases (see Paper IV)¹²⁸. However, over-expressed DS-epi 1 did not show any remnant lyase activity since no depolymerisation of the substrate was observed after incubation with cellular lysates.

In conclusion, we have identified the DS-epimerase and importantly also a second DS-epimerase described in Paper III. Conversion of GlcA into IdoA is an important modification since it is a common constituent of the ligand binding sites in CS/DS. The discovery of these two enzymes therefore offers the opportunity to in detail study the role of this modification in biology.

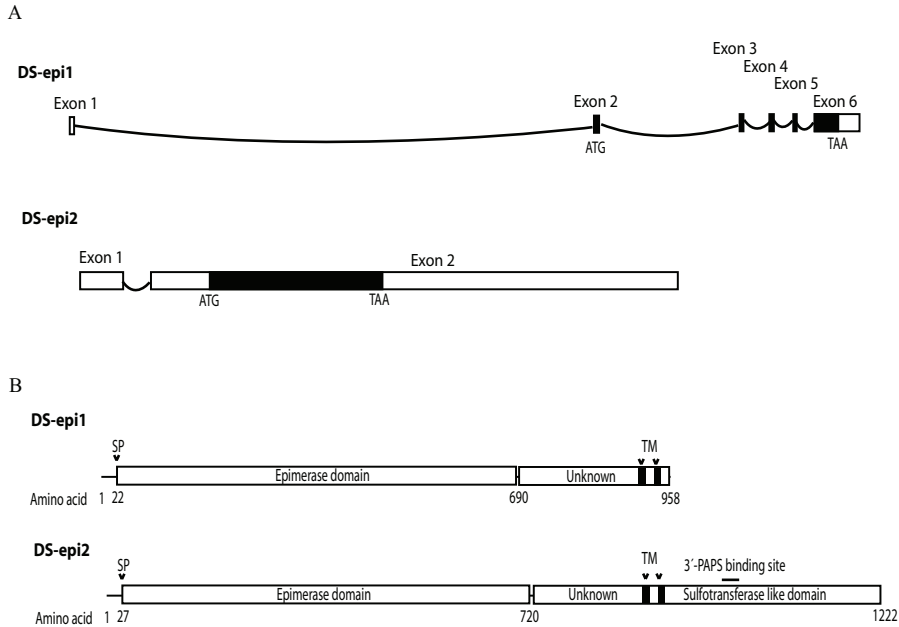


Figure 5. Gene and protein structure of the two DS-epimerases. (A) Schematic view of the gene structure of DS-epi 1 and DS-epi 2. Exons are depicted as boxes while introns are indicated with lines. Protein coding regions are shaded in black. (B) Domain structure of the DS-epimerases showing the common N-terminal epimerase domain and the presence of a C-terminal sulfotransferase-like domain in DS-epimerase 2. Location of signal peptides (SP) and predicted transmembrane regions (TM) are also shown.

Paper III

Using the amino acid sequence of DS-epi 1 to screen the human genome for possible homologues resulted in the identification of a second gene (C18orf4) located at chromosome 18q22. The gene has two exons with a complete open reading frame encoding a protein of 1222 amino acids in exon 2 (Figure 5). The protein, NCAG-1, had previously been identified as a possible player in bipolar disorder II due to the presence of missense single nucleotide polymorphisms overrepresented in patients suffering from the disorder ¹⁷⁹. In addition to the

epimerase domain, which is 51% identical to DS-epi 1(amino acid 62-691), this protein also contains a C-terminal domain with similarities to several sulfotransferases.

We therefore hypothesized that NCAG-1 would potentially be a dual-activity enzyme showing both epimerase and sulfotransferase activity explaining the close connection between sulfation and IdoA content.

Analysis of the gene structure of NCAG-1 had showed that the open reading frame was contained in a single exon¹⁷⁹. Hence, a genomic clone was acquired and the complete ORF was amplified with PCR and subcloned into a pcDNA3.1 vector. For expression studies, a N-terminally FLAG-tagged version was constructed lacking the initial 30 amino acids, which was predicted to contain a signal peptide. Over-expression of the putative enzyme in 293HEK cells resulted in a 21-fold increase in epimerase activity. Interestingly, no sulfotransferase activity could be detected using either desulfated CS or DS or their sulfated counterpart, which was rather surprising due to the similarity to several STs. By creating a structural model of the sulfotransferase domain we could explain our failure to detect sulfotransferase activity, by showing that DS-epi 2 lacks a conserved lysine implicated in catalytic activity of other sulfotransferases (Figure 6).

To further study the role of the two epimerases in DS biosynthesis, DS-epi 1 and DS-epi 2 activity was lowered using a siRNA-mediated approach. Consistent with over-expression data (Paper II), knockdown of DS-epi 1 resulted in loss of IdoA blocks. Surprisingly, IdoA blocks were also significantly reduced after knockdown of DS-epi 2. This suggests that a functional interplay between the two DS-epimerases is required for the formation of IdoA blocks but not for generation of alternating IdoA/GlcA regions. Regulating the availability of the two epimerases in the cell could therefore be a way to generate different IdoA structures.

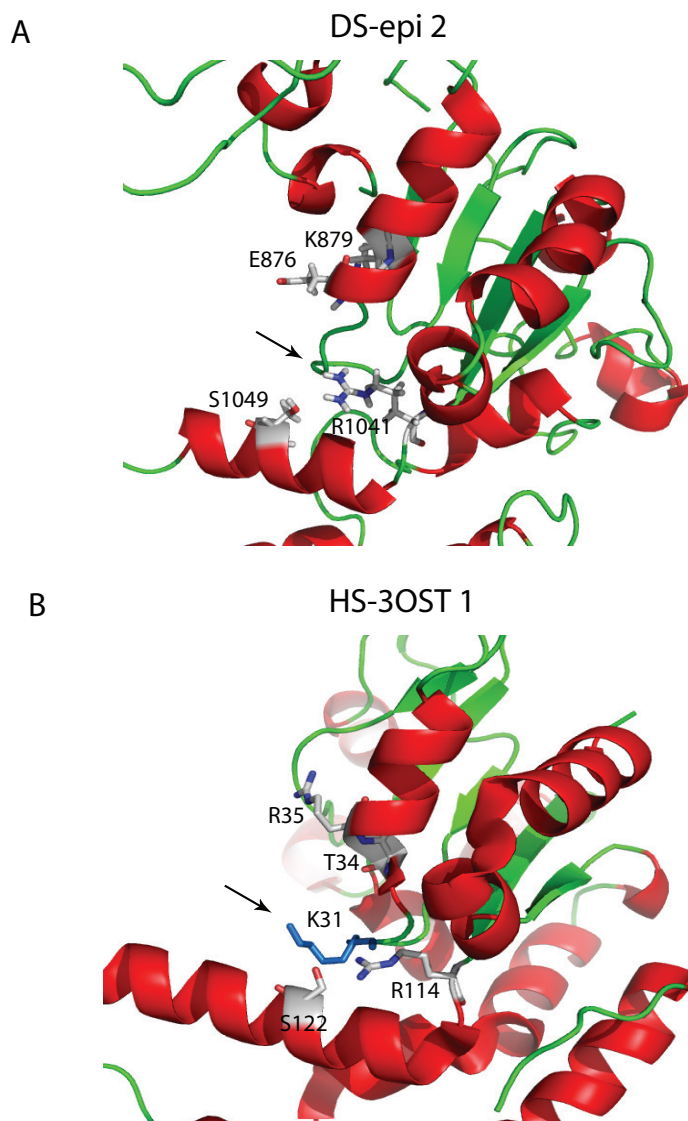


Figure 6. Putative PAPS binding site of DS-epi 2 lacking a catalytic lysine. A homology model of the sulfotransferase-like domain of DS-epi 2 (A) is shown in comparison with the HS-3OST1 crystal structure (1ZRH)(B). Residues involved in binding to the 3'-phosphate of PAPS are conserved in DS-epi 2(R1041 and S1049). In DS-epi 2, a lysine (colored blue in 3OST1) previously shown to be involved in PAPS binding and catalysis in other sulfotransferases is missing (replaced by a serine) and is indicated with an arrow. Residues of 3OST1 involved in binding to the 5'-phosphate of PAPS (T34 and R35) are in DS-epi 2 replaced by a glutamate (E876) and a lysine (K879), respectively.

Paper IV

In paper II and III we reported the identification of two DS-epimerases. Unlike cytosolic epimerases involved in UDP-sugar metabolism, this family does not act on the monomeric sugar. Instead, IdoA is formed by the movement of the C5-carboxyl group of GlcA in an already assembled polysaccharide¹⁷. This family of enzymes also includes the HS-epimerase and alginate epimerases¹⁸⁰. The mechanism of this reaction is poorly known, and no crystal structures for this family of epimerases have been solved. Based on biochemical data, the reaction is thought to involve an initial abstraction of the C5-proton from GlcA by a general base, which is subsequently replaced from the opposite side of the sugar ring by a general acid to achieve the movement of the carboxyl group^{66,127}.

Based on available knowledge we hypothesized that a putative active site would be constructed in a way where the two putative catalytic bases/acids would reside opposite to each other.

Using fold-recognition modeling we identified several bacterial lyases, as distant homologues of the DS-epimerases. This finding is supported by the fact that lyases and epimerases share common characteristics¹²⁸. Both reactions run through an initial proton abstraction from the C5-carbon, but in a lyase reaction a proton is not added back and a 4,5-unsaturated hexuronic acid product is formed. The best match in the homology search was heparinase II¹³⁰, which was subsequently used as a template to build a homology model of DS-epi 1. Based on this homology we could show that DS-epi 1 contains three distinct domains, *i.e.* a N-terminal domain (a.a 1-390) containing 13 α -helices shaped as an incomplete $(\alpha/\alpha)_6$ toroid, a central beta-sheet domain and a C-terminal α -helical domain not present in lyases. The active site is located in a cleft formed by the N-terminal and central domain and show conservation of residues implied in catalysis of heparinase II (Figure 7). Two conserved histidines (H450 and H205), located opposite to each other, fitted the criteria of being the catalytic general base and acid, respectively. Mutations of these amino acids into alanines or asparagines completely abrogated activity confirming their role in catalysis. Interestingly, a conserved tyrosine residue (Y261) was also found, which in lyases, after proton abstraction, function as a general acid in the cleavage of the glycosidic bond. Mutation of this residue into either alanine, or the more conserved phenylalanine, resulted in an inactive DS-epi 1, suggesting that a cleaved intermediate is formed after proton abstraction.

It was thus concluded that epimerization of GlcA to IdoA possibly proceeds through the following four steps (Figure 7):

- 1 C5-proton abstraction by the general base His450 from GlcA.
- 2 Formation of a cleaved 4,5-unsaturated intermediate by proton donation of Tyr261 to the glycosidic oxygen.
- 3 Proton donation to the C5-carbon by the general acid H205, moving the C5-carboxyl.
- 4 Concomitant recreation of the glycosidic linkage by Tyr261.

It is interesting to note that chondroitinase ACI, which can only attack GlcA, lack the corresponding residue to H205, explaining why it cannot cleave IdoA linkages. Heparinase II and chondroitinase ABC on the other hand, which can cleave both GlcA and IdoA linkages, contain both histidines (Figure 7).

Several studies of biosynthetic enzymes have revealed an important role of N-glycosylation for proper function^{181,182}. We therefore wanted to investigate their role in DS-epi 1 function. This epimerase has four predicted N-glycosylation sites, that we could show were all substituted with N-glycans. Two of these were directly implied in enzyme function since mutation of the asparagine attachment sites to serine resulted in loss of enzymatic activity without affecting production of the enzyme. However, no N-glycans appear to be located close to the active site, which could have an effect on substrate binding. Consistent with this fact, the mutants did not show an apparent higher K_M but significantly lower V_{max} values.

These data do not only provide information regarding the evolutionary origin of the DS-epimerases. Insights into the mechanism of the reaction, and our structural model will allow the design of inhibitors that can potentially be used to study epimerization *in vivo*.

Paper V

In vivo, IdoA is commonly found in blocks where the flanking GalNAc is 4-O-sulfated. These domains are of high interest since they constitute the binding site for several growth factors (e.g. HGF, FGF-2 and FGF-7)^{3,35}. Based on two sets of experiments, it has long been hypothesized that creation of these IdoA-rich domains are dependent on a coupling between the epimerisation and sulfation reactions. (I) The IdoA to GlcA ratio can be increased by adding the sulfate donor PAPS to microsomal incubations, (II) cells grown in sulfate-deprived conditions synthesize DS chains containing less IdoA^{17,133}. In light of the substrate specificity of the identified 4-O-sulfotransferases, the most likely candidates of working together with the two DS-epimerases are D4ST-1 and C4ST-2¹³⁴.

We therefore hypothesized that D4ST-1 would have a pivotal role in epimerization of DS chains in the small PGs biglycan and decorin through a direct physical interaction with the DS-epimerases.

To investigate loss of function of D4ST-1 regarding epimerization, a siRNA-mediated approach was employed. Structural analysis of decorin/biglycan-derived DS-chains after siRNA treatment showed a significant reduction in IdoA content of 70% for the best performing siRNA. Specifically, the prominent IdoA blocks (>4 consecutive IdoA residues) commonly found in the control cells were drastically reduced after knockdown of D4ST-1, while alternating GlcA/IdoA sequences were relatively increased. Interestingly, epimerase activity was not affected indicating that the activity of D4ST-1 *per se* has a pivotal role in formation of IdoA blocks. Surprisingly, we could not detect an interaction between D4ST and either of the two DS-epimerases that could potentially explain the coupling of the reactions.

When D4ST-1 was over-expressed in 293HEK cells, no increase in IdoA was observed. However, 293HEK cells contain very small amounts of epimerase activity and have previously been shown to be a limiting factor when sufficient sulfotransferase activity is present¹⁸³. Our failure to obtain an increase in IdoA could thus be explained by insufficient amounts of the two epimerases.

Since FGF-2 can bind CS/DS via 4-*O*-sulfated IdoA blocks, we investigated if the loss of these structures would affect FGF-2 signaling. Stimulation of control cells with 2.5ng/ml of FGF-2 resulted in an increased phosphorylation of ERK1/2. This increase was also promoted when cells were stimulated in conditioned medium compared to freshly added medium. Notably, siRNA mediated knockdown of D4ST-1 abrogated FGF-2 induced signaling 2.5-fold in conditioned medium but not in freshly added growth medium. Together these data indicate that the presence of CS/DS chains containing the binding site for FGF-2 mediated the increased signaling seen in conditioned medium but not in freshly added medium where no material had been deposited by the cells.

In several studies it has recently been reported that disulfated structures such as iB, D/iD and E/iE (Figure 2) play a major role in CS/DS function. Knockdown of D4ST-1 changed the levels of all these modification with a relative increase in D units and a decrease in B and E units, respectively. D4ST-1 is thus a key enzyme in generating a functional CS/DS chain, and dynamic regulation of its expression by for example TGF- β (Paper I) could be an important way of fine-tuning signaling during processes such as embryogenesis and wound healing.

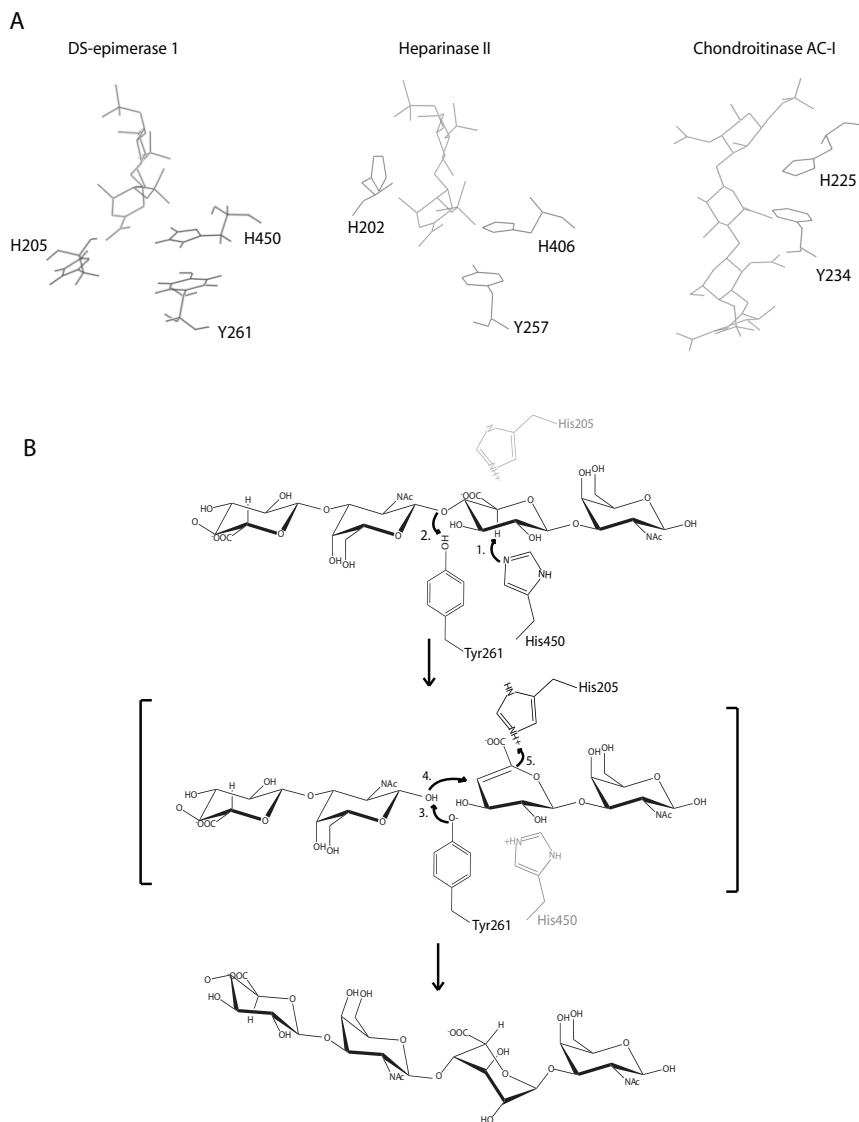


Figure 7. DS-epi 1 active site and catalytic mechanism. (A) Comparison of the active sites of DS-epimerase 1 and the two bacterial lyases heparinase II and chondroitinase AC-I with a bound HS disaccharide product and a CS tetrasaccharide, respectively. The DS-epi 1 active site is depicted with a HS disaccharide product from the heparinase II crystal structure placed by alignment of the two proteins. (B) Proposed catalytic mechanism of epimerization in four steps; (1) C5-proton abstraction from GlcA by the general base His450, (2) formation of a 4,5-unsaturated hexuronic intermediate by proton donation of Tyr261 to the glycosidic oxygen, (3) C5-proton addition by the general acid His205 and (4) concomitant recreation of the glycosidic linkage by Tyr261.

General Discussion

In this thesis, considerable effort has been made to elucidate how IdoA is created and regulated within the cell. We have identified the DS-epimerase (DS-epi 1) and importantly also a second homologous enzyme, which we named DS-epi 2. This is a hallmark discovery since the lack of an identity of these enzymes for many years has been a bottleneck in the study of DS. The implication of this discovery is therefore twofold. Firstly, we have now, through the work in this thesis, started to understand how functional domains (*i.e.* IdoA blocks) in DS are generated. Secondly, it is in ongoing loss of function studies allowing us to address the importance of these domains in development and physiology.

The role of two DS-epimerases and D4ST-1 in CS/DS biosynthesis

Through a classical purification scheme we isolated the DS-epimerase (DS-epi 1) and as a result of that also identified a second epimerase (DS-epi 2) (see Paper II and III). What is then the role of two DS-epimerases in the formation of IdoA? In DS, IdoA can exist in two different types of domains, *i.e.* IdoA blocks and alternating IdoA/GlcA structures (Figure 3). This type of distribution of IdoA in a DS chain, not only differs between tissues but also between different PGs produced by the very same cell. This clearly suggests that a highly regulated biosynthesis is in effect. We have shown that both epimerases are active *in vivo*. Interestingly, siRNA mediated knockdown of both DS-epi 1 and DS-epi 2 specifically diminished the IdoA blocks (Paper III). This suggests that a functional interplay between the two epimerases is pivotal in the formation of these domains. This view is strengthened by that IdoA blocks, but not alternating IdoA/GlcA structures, are missing in DS chains of the DS-epi 1 knock-out mouse, Maccarana *et al.*, *manuscript in preparation*. The longstanding hypothesis that an inherently processive enzyme *in vivo* synthesizes consecutive IdoA residues must therefore be revised. The mechanism for IdoA block formation by two collaborating DS-epimerases is at the present unclear. Esko *et al.* has proposed that the biosynthetic enzymes in the closely related heparan sulfate are organized into Gagosomes¹⁸⁴. Furthermore, it was suggested that the stoichiometry and composition of such enzyme complexes could possibly be involved in determining the structure of the resulting GAG. Several of the enzymes involved in HS biosynthesis have indeed been shown to interact^{97,185,186}. It is thus possible that a Gagosome rich in both DS-epi 1 and DS-epi 2 generates block structures while one with no or less of either epimerase are responsible for alternating IdoA/GlcA structures (Figure 8).

Moreover, we could show that IdoA domains are strictly dependent on the presence of D4ST-1 and were not compensated by other STs such as C4ST-2, which share similar substrate specificity (Paper V). An appealing hypothesis for this close connection between 4-*O*-sulfation and epimerization is a physical interaction between D4ST-1 and one/or both of the epimerases. Such an interaction would allow the 4-*O*-sulfotransferase to act immediately after GlcA has been epimerized into IdoA. However, in our study we could not find an interaction between D4ST-1 and any of the epimerases, but a transient or weak interaction cannot be completely ruled out from these data (Paper V). In addition, hetero-oligomerisation of the enzymes might be induced by the substrate. This idea is supported by experiments *in vitro* showing that binding of heparan sulfate 3-*O*-ST3A to its substrate induces homo-oligomerisation of the enzyme¹⁸⁷. The assembly of a Gagosome, including D4ST-1 and the two epimerases, might therefore be induced by the substrate. If an interaction exists, sulfation would most likely not occur on the immediate flanking GalNAc at the same time as epimerization occurs. The residue undergoing epimerization and the flanking GalNAc, resides within the cleft of the active site of DS-epi 1. Thus, D4ST-1 should therefore not be able to access its substrate. How sulfation of a nearby residue regulates epimerization is unclear. The possibility exists, as previously proposed, that sulfation of a flanking GalNAc locks IdoA from potential back-epimerisation since sulfated structures are not a substrate for the epimerase.

The presence of two epimerases is in contrast to HS biosynthesis, where only one enzyme exists. Moreover, the two DS-epimerases do not share any detectable sequence similarity at the primary level with the HS-epimerase. A fold-recognition modeling procedure for HS-epimerase, as performed for DS-epi 1 (see Paper IV), did not return any significant structural hits, suggesting it has a currently unknown fold. It is thus clear that the two classes of epimerases have evolved through convergent evolution. Epimerization in DS is therefore more reminiscent, at least in part, to that of alginate biosynthesis, where several epimerases carry out the reaction¹⁸⁰. Consistent with previous reports based on structural analysis of CS/DS, the two DS-epimerases can only be found in higher organism and are not found in the genome of *D. melanogaster* and *C. elegans*. It is conceivable that the late acquisition of epimerization in evolution is a direct reflection of the need for more refined structural modifications for binding and fine-tuning of growth factor signaling.

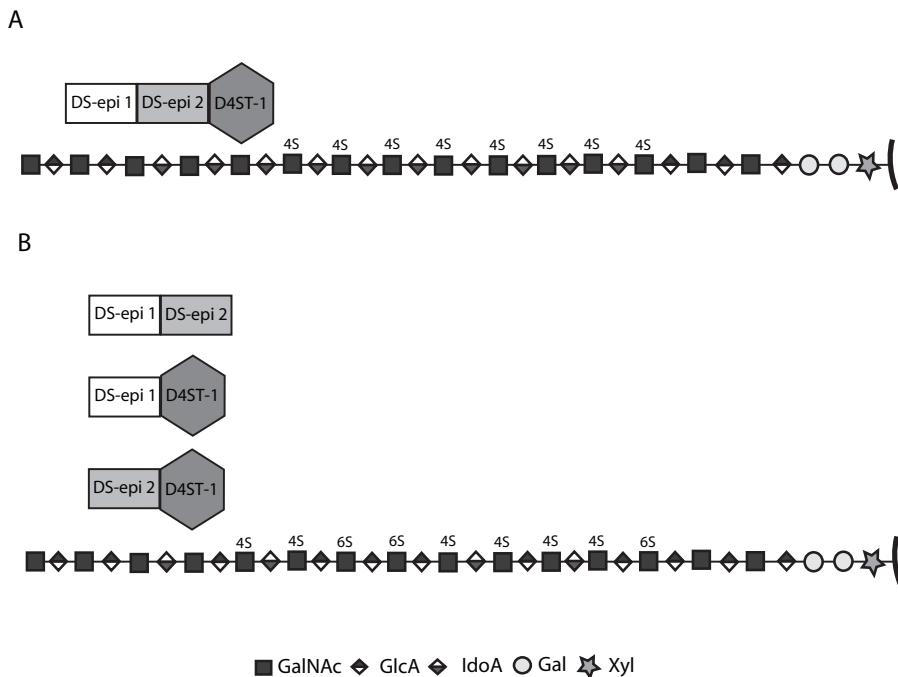


Figure 8. Role of two DS-epimerases and D4ST-1 in IdoA block formation. (A) A functional interaction between DS-epi 1, DS-epi 2 and D4ST-1 generates long stretches of consecutive IdoA residues. (B) When only one epimerase is present, or D4ST-1 is missing, only alternating IdoA/GlcA regions are formed.

Regulation of dermatan sulfate structure and its biological implications

The difference in IdoA content and distribution within the CS/DS chains can potentially be explained as a consequence of regulating the levels of the two epimerases and D4ST-1 within the cell. Several growth factors such as TGF- β , EGF and PDGF have the ability to affect both epimerization and sulfation (see Paper I). TGF- β is a major cytokine involved in normal tissue homeostasis, the immune system, tumorigenesis and wound healing. In addition to its role in inducing the expression of hundreds of genes, or as a result thereof, influences on CS/DS structure will invariably also regulate the availability of other signaling factors at the cell surface. One such example is FGF-2. Binding of FGF-2 to 4-*O*-sulfated IdoA blocks in CS/DS sequesters the growth factor in the extracellular matrix³⁵. Modulating the amount of binding sites for FGF-2 in the matrix by affecting CS/DS biosynthesis could be a key function in wound repair and regeneration where FGF2 plays a key role as a mitogen. In addition, we have obtained evidence that DS might also be implied in direct FGF-2 signaling since

cells treated with siRNA against D4ST-1 showed a reduced phosphorylation of ERK1/2 upon FGF-2 stimulation (see Paper V). This is consistent with data showing that FGF-2-induced proliferation can be maintained by CS/DS chains released upon injury⁴³. The presence of 4-*O*-sulfated IdoA blocks is therefore important for both growth factor availability, and direct signaling. FGFs are known to be important culprits in carcinogenesis. Cancer cell mediated changes of the stromal space could be an efficient way of keeping the mitogen available. Indeed, studies have pointed to differences in CS/DS structures in tumors both in epimerization and sulfation^{188,189}. Of note is the high expression of DS-epi 1 in all cancers tested¹⁷⁸. In summary, functional domains in CS/DS are thus not randomly made, but arise through a complex interplay between several enzymes, which are regulated at the transcriptional level. As a consequence, distinct CS/DS chains are generated with the potential to elicit different biological outcomes.

A multitude of reports have suggested an important role for CS/DS in brain development. In processes such as neural plasticity, neural migration, neural stem cell maintenance, dendritic and axonal outgrowth CS/DS is a key player⁵. The brain contains considerable less IdoA than many other tissues and the IdoA amount is spatio-temporally regulated. Moreover, clusters of IdoA residues are a minor component in the brain⁴⁷. The high expression of DS-epi 2 in the brain compared to DS-epi 1 is therefore of particular interest in this regard since the binding motif for the growth factor pleiotrophin in CS/DS contain IdoA residues⁴⁹. Interestingly, missense single nucleotide polymorphisms in the DS-epi 2 gene are overrepresented in patients suffering from brain polar disease type II¹⁷⁹. These mutations do not occur in the putative DS-epi 2 active site (similar to the DS-epi 1 active site described in paper IV), but in the C-terminal part of the protein containing the sulfotransferase domain. Since DS-epi 2 seem to lack ST activity, the roles of these mutations in catalytic function are unclear and needs to be further investigated. The possibility exists that the sulfotransferase-like domain of DS-epi 2 has a regulatory function over the epimerase domain. Binding of the GAG chain to the ST domain might induce a conformational change, as shown for the heparan sulfate 3-*O*-ST1¹⁹⁰, thereby activating the epimerase. This thought is consistent with that a recombinant DS-epi 2 lacking the ST domain was inactive (data not shown). If the SNPs are indeed associated with an impaired catalytic function of DS-epi 2 it is plausible that particular changes in CS/DS structure would lead to an aberrant binding of several growth factors, *e.g.* pleiotrophin to its proteoglycan receptor, *i.e.* receptor-type proteintyrosine phosphatase ζ . As a consequence, dysregulation of growth factor signaling and/or neuronal pathfinding might occur leading to development of a pathological state.

DS-epimerase structure and clinical implications

In Paper IV we reported the 3D-structure of the epimerase catalytical domain based on homology modeling to the bacterial lyase heparinase II. Our model and description of the DS-epimerase(s) active sites could potentially be used for rational design of inhibitors specific for the epimerases. In addition, we have proposed that epimerization passes through a 4,5-unsaturated hexuronic intermediate, a feature not shared by other GAG biosynthetic enzymes (Paper IV). Thus, compounds mimicking the intermediate might be a way to get around the problem of inhibitor specificity. Such inhibitors could be useful to study the biosynthesis of CS/DS, both *in vitro* and *in vivo*. Interestingly, DS-epi 1 expression is drastically increased in all cancers tested¹⁷⁸. In ongoing studies we have shown that this over-expression is an inherent property of the cancer cell and not primarily of the stromal cells. In addition, tumors show very specific changes in gene expression patterns for several biosynthetic enzymes and proteoglycans. If such changes are indeed involved in creating a proliferative/pro-metastatic microenvironment, inhibiting DS-epi 1 function might be useful as a therapeutic agent.

FUTURE PERSPECTIVE

During the last decade considerable advances have been made in the understanding of CS/DS biosynthesis and the biological function of this type of glycosaminoglycan. This has mostly come about through the identification and cloning of the enzymes involved, as well as generation of loss of function models. However, the organization within the Golgi is still poorly understood, and several major questions such as what determines whether a chain becomes CS or DS or how the biosynthetic enzymes are organized and regulated remains to be answered.

The identification of the two DS-epimerases in this thesis opens up an array of potential opportunities. Studies are now ongoing in our lab to determine the role of IdoA in vertebrate development and physiology, by using loss of function models. This will allow pinpointing processes dependant upon the presence of IdoA and the discovery of new ligands for the DS chain both involved in normal maintenance of the tissue and pathology. The description of the active site of the two DS-epimerases in this thesis offers an opportunity to develop inhibitors towards the enzymes. These could potentially be used to change CS/DS structure in certain settings *in vivo* to further study and manipulate its function, which could also have potential clinical implications.

Further, to fully understand the structural requirements for the interaction of CS/DS with their ligands, chemically defined poly/oligosaccharides need to be obtained. This is challenging due to the complexity of the molecule but promising new approaches has been reported. Using synthetic “clean” molecules of various structures in co-cryztallisation experiments with different ligands (*e.g.* HGF and pleitrophin) would allow mapping of the detailed interactions involved. I am convinced that such an approach would reveal the sequence code of CS/DS, which as previously proposed would be situated in conformational space rather than in the sequence of the chain.

Although important, studies on already known enzymes will only get you so far and the real paradigm shifts come from discovery of new and unexpected links. An interesting approach, which recently resulted in the discovery of a Golgi

resident PAP-phosphatase, is therefore the use of gene-trap technology⁸⁴. Using such a strategy to screen for structural phenotypes in the CS/DS chain could potentially identify novel regulators and organizers necessary for CS/DS biosynthesis. However, this type of technology requires an efficient way to address the CS/DS structures when thousands of cell clones must be screened to observe a desired phenotype. Today, methods to analyze the CS/DS chains are time consuming and unsuitable for such an approach. Considerable effort must therefore be put to develop technologies such as sequencing to correctly analyze the distribution of functional domains within the CS/DS chains fast and efficiently.

Populärvetenskaplig sammanfattning

Kommunikation mellan celler och dess omgivning är en av hörnstenarna inom biologin och resulterar i att ett embryo utvecklas korrekt, att en vävnad fungerar normalt samt att bakterier och virus bekämpas. Tro det eller inte, men sockerbiten du precis lade i kaffet, kommer när du är färdig med denna sammanfattning att i din kropp vara på god väg att vara en del av det informationsflöde som styr processer som ovan.

Alla celler i vår kropp är omgivna av ett komplext nätverk av proteiner och sockerkedjor kallat extracellulär matrix. Förutom att bibehålla strukturen och avgränsa vävnader, reglerar matrixen även tillgängligheten av de signaleringsfaktorer som skickas mellan celler genom att binda och hålla kvar dessa. En molekyl som kan binda dessa signaleringsfaktorer är dermatan sulfat. Denna komplexa polysackarid består av upp till 50 upprepningar av två olika alternerande byggstenar (hexosamin och hexuronsyra) som bildas från glukos (sockret i kaffet). Komplexiteten uppkommer genom att speciella proteiner, s.k. enzymer kemiskt modifierar sockerkedjan på specifika plaster.

I denna avhandling beskrivs identifieringen av två enzymer som är kritiska i syntesen av dermatan sulfat. De två enzymerna, DS-epimeras 1 och 2, utför en tillsynes relativt liten förändring i startmolekylen kondroitin sulfat. En kemisk grupp (karboxyl-grupp) får efter en attack av dessa två enzymer en förändrad position i rymden. Genom denna "enkla" reaktion, som kallas epimerisering, skapas alltså dermatan sulfat. Genom att ta fram en tredimensionell modell av ett av proteinernas struktur (se bild på framsida) har vi också i denna avhandling föreslagit hur enzymerna rent kemiskt kan flytta karboxyl gruppen från en position till en annan. Förutom epimerisering så modifieras polysackariden genom att det sätts på negativt laddade kemiska grupper (sulfat) i olika positioner längs kedjan. Effekterna av dessa förändringar är långtgående i avseende på polysackaridens biologiska funktion. Genom epimerisering och sulfatering skapas inbindningsplatser för signalmolekyler. Vid t.ex. sår läkning kan dermatan sulfat och dess inbundna tillväxtfaktorer frigöras från matrixen och därigenom binda in till

cellytan för att inducera cellerna att dela och därmed hjälpa till att läka såret. Av kuriosa kan nämnas att namnet dermatan sulfat kommer från latinets *dermis* vilket betyder hud, där denna polysackarid finns i rikliga mängder.

Genom att förändra strukturen av dermatan sulfat kan cellen potentiellt kontrollera vilka signaler som når fram. Vi har visat att flera faktorer utsöndrade vid t.ex. sårläkning kan mediera denna förändring genom att direkt påverka mängden av de enzym som är involverade i att göra dermatan sulfat. Resultatet blir en polysackarid som inte längre har samma kapacitet att binda en viss typ av signalmolekyler och cellerna kommer att påverkas annorlunda.

Kan då kunskap om hur strukturen av dermatan sulfat uppkommer och regleras användas även inom medicinen? Mutationer (förändringar i DNA sekvensen som kan leda till förlust av funktion) i DS-epimeras 2 är överrepresenterade i patienter som lider av manodepressivitet. Specifika strukturer i dermatan sulfat som bildas genom en attack av detta enzym har också tidigare visats vara involverade i utvecklingen av hjärnan. Om mutationerna i enzymet *de facto* leder till förlust av funktion av enzymet kan patienterna som lider av sjukdomen teoretiskt behandlas genom att återinföra DS-epimeras 2 med genterapi.

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