3.05 Biosynthesis of Glycosaminoglycans and Proteoglycans

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3.05.1 Introduction

Sulfated glycosaminoglycans (GAGs) are linear polysaccharides consisting of repeating disaccharide units composed of *N*-acetylhexosamine and uronic acid, and exist as proteoglycans (PGs) by attaching to specific serine residues in the core protein.[1–3] PGs are ubiquitously distributed on the cell surface and in the extracellular matrix and play critical roles in a variety of physiological phenomena such as cell–cell and cell–matrix adhesion, cell proliferation, cell division, morphogenesis, and the regulation of signaling molecules via GAG chains (**Figure 1**).[4–10]

Sulfated GAGs are structurally classified into two groups, chondroitin sulfate/dermatan sulfate (CS/DS) and heparan sulfate/heparin (HS/Hep), on the basis of a difference in the repeating disaccharide unit, CS/DS and HS/Hep being composed of -4GlcAβ(IdoAα)1-3GalNAcβ1- and -4GlcAβ(IdoAα)1-4GlcNAcα1-, respectively (**Figure 2**).[1–3] These GAGs are synthesized onto a common GAG-protein linkage tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser, of the core protein (**Figure 3**). After the synthesis of the GAG sugar backbone on this tetrasaccharide, numerous modifications including sulfation, epimerization, and desulfation are performed in a spatiotemporal manner, producing mature and functional GAG chains that exert biological functions dependent on their specific structure.[3]

Recent biochemical and molecular biological advances have led to the cloning of a series of enzymes responsible for the synthesis and modification of GAG chains, and the elucidation of their properties.[3] Furthermore, functional analyses of GAGs using model organisms have revealed unexpected roles of these molecules.[3] Here, we summarize the biosynthetic mechanism and the physiological functions of GAGs.

3.05.2 Biosynthesis of the GAG-Protein Linkage Region Tetrasaccharide and Its Modification

The GAG-protein linkage region tetrasaccharide is sequentially synthesized onto a specific serine residue in the core protein by the corresponding glycosyltransferases (**Figure 3**).[1–3] The molecular cloning of four glycosyltransferases responsible for the biosynthesis of the linkage region tetrasaccharide has been achieved (**Table 1**). First, xylosyltransferase (XylT) transfers a Xyl residue from UDP-Xyl to specific serine residues in core proteins in the endoplasmic reticulum and the *cis*-Golgi compartments (**Figure 4**). Since the biosynthesis of GAGs is initiated by the action of XylT, this enzyme may be critical in the regulation of the expression level of GAGs. Two XylTs, XylT-1 and XylT-2, were cloned, and their amino acid sequences found to be significantly homologous. However, enzymatic activity was

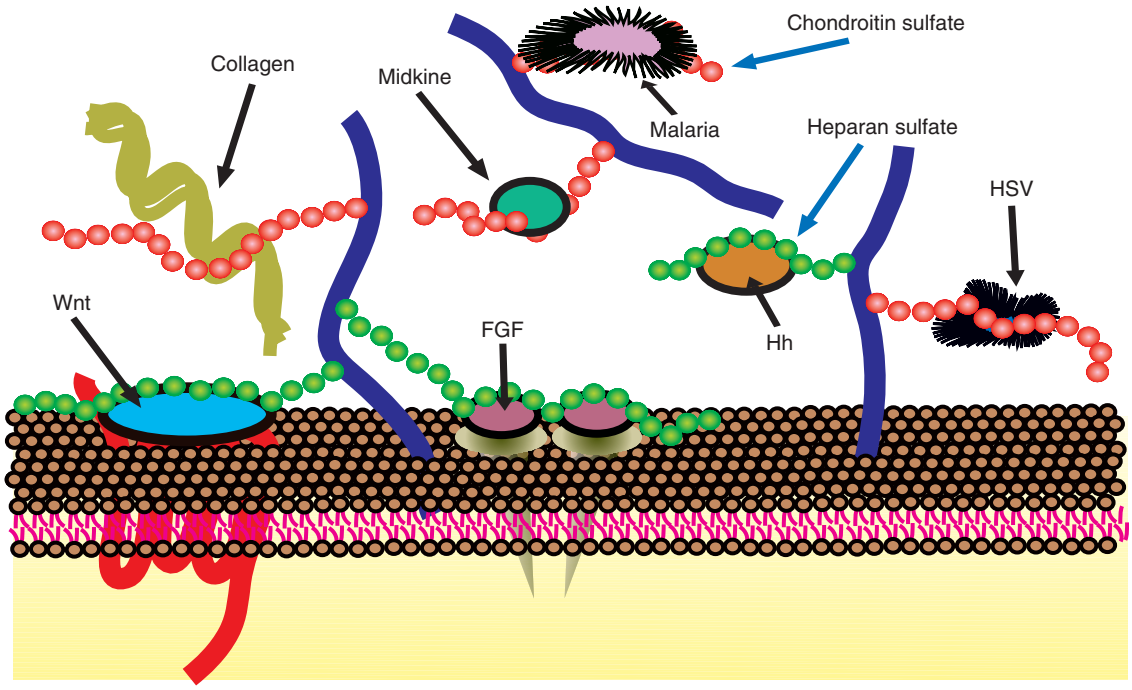

Figure 1 Various functions of GAG chains. GAGs including HS and CS expressed on the cell surface and in the extracellular matrix interact with various proteins such as growth factors, morphogens, and adhesive molecules.

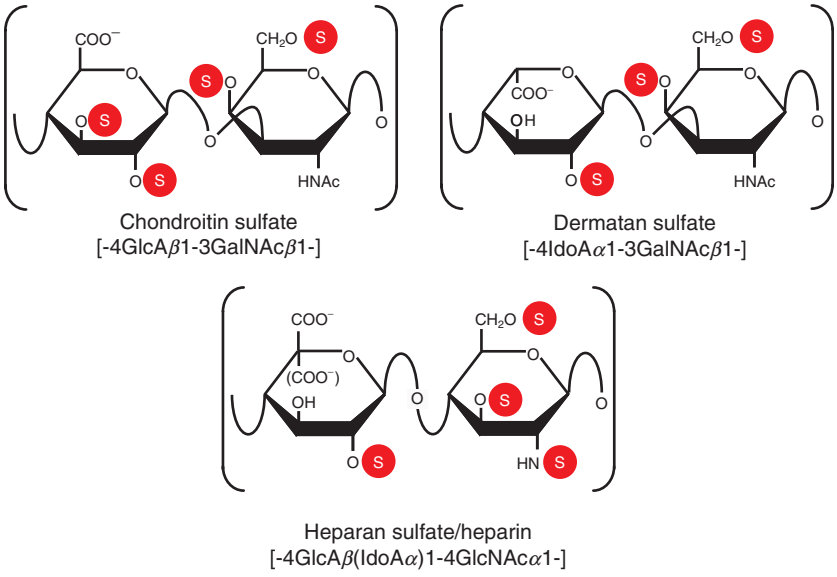

Figure 2 Typical repeating disaccharide units in CS/DS and HS/Hep and their potential sulfation sites. CS and DS are constituted of uronic acid and GalNAc. DS is a stereoisomer of CS, including IdoA instead of or in addition to GlcA. HS and Hep consist of uronic acid and GlcNAc residues with varying proportions of IdoA. These sugar residues can be esterified by sulfate at various positions as indicated by "S."

shown by only XylT-1, not by XylT-2.[11] After the transfer of a Xyl residue, two Gal residues are transferred to the Xyl residue by two kinds of galactosyltransferases (GalTs), GalT-I and GalT-II, in the *cis*- and *medial*-Golgi compartments (**Figure 4**).[12–14] Interestingly, a defect in the expression of GalT-I leads to the progeroid variant of Ehlers–Danlos syndrome. Although there have been no reports of a deficiency in other glycosyltransferases involved in the

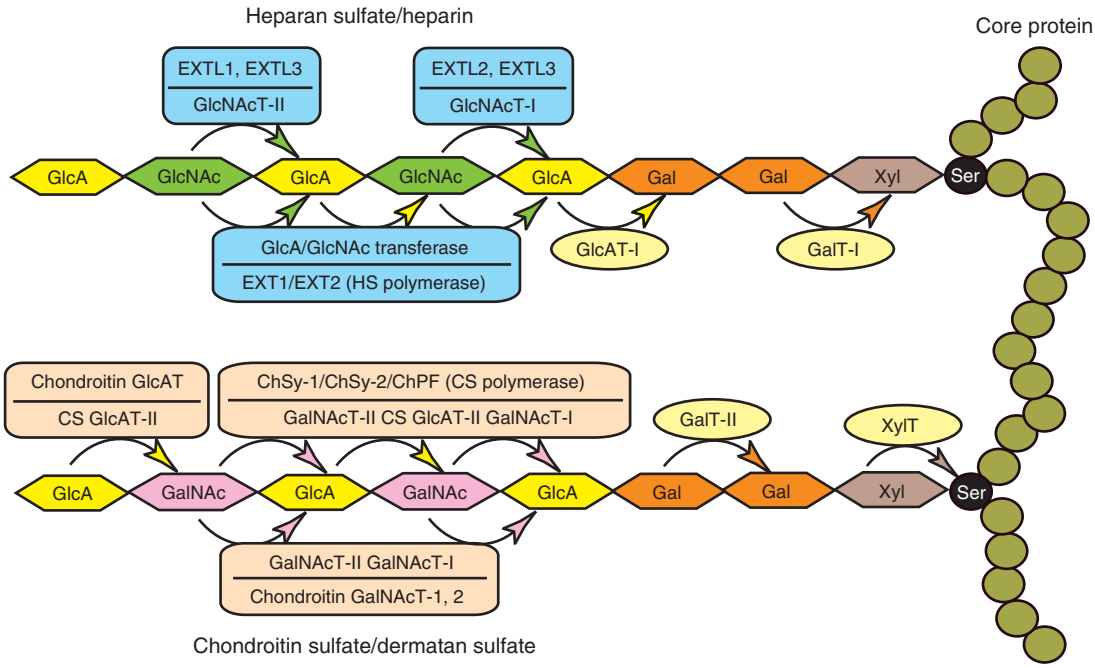

Figure 3 Schema of the biosynthetic assembly of the GAG backbones by various glycosyltransferases. A number of glycosyltransferases are required for the synthesis of the backbones of GAGs. XylT, Xyl transferase; GalT-I, Gal transferase-I; GalT-II, Gal transferase-II, GlcAT-I, GlcA transferase-I; GalNAcT-I, GalNAc transferase-I; CS GlcAT-II, CS GlcA transferase-II; GalNAcT-II, GalNAc transferase-II; CS polymerase, GlcA/GalNAc transferase; GlcNAcT-I, GlcNAc transferase-I; GlcNAcT-II, GlcNAc transferase-II; HS polymerase, GlcA/GlcNAc transferase; ChSy-1, chondroitin synthase-1; ChSy-2, chondroitin synthase-2; and ChPF, chondroitin polymerizing factor.

Table 1 Human glycosyltransferases involved in the synthesis of the linkage region

Name	*Abbreviation*	*Chromosome location*	*Amino acid*	*mRNA expression*	*mRNA accession*
Xyl transferase	XylT	16p13.1	>827	Ubiquitous	AJ295748
Gal transferase-I	GalT-I	5q35.1–q35.3	327	Ubiquitous	AB028600
Gal transferase-II	GalT-II	1p36.3	329	Ubiquitous	AF092050
GlcA transferase-I	GlcAT-I	11q12–q13	335	Ubiquitous	AB009598

biosynthesis of the linkage tetrasaccharide, defects in XylT, GalT-II, and glucuronyltransferase-I (GlcAT-I) may result in the same disease.[15,16] GlcAT-I completes the biosynthesis by transferring a GlcA residue to the linkage trisaccharide, Galβ1-3Galβ1-4Xylβ1-*O*-Ser, in the *medial*- and *trans*-Golgi compartments (**Figure 4**).[17,18] Notably, overexpression of GlcAT-I also resulted in the formation of the human natural killer cell carbohydrate antigen-1 (HNK-1) epitope, (HSO_3)GlcAβ1-3Galβ1-4GlcNAc, on the surface of COS-1 cells, which are deficient in the expression of HNK-1 epitope due to a loss of *GlcAT-P* expression.[19] Normally, the transfer of GlcA involved in the synthesis of the HNK-1 epitope is catalyzed by two enzymes, GlcAT-P and GlcAT-S, and a deficiency of GlcAT-P results in almost a complete loss of HNK-1 expression and impaired neuronal functions.[20–22] In contrast, the introduction of GlcAT-P into CHO cells deficient in GlcAT-I expression partially rescued GAG expression.[18] These results suggested that GlcAT-I and GlcAT-P show functional redundancy in certain cells. In rat articular cartilage explants, the introduction of GlcAT-I enhanced GAG synthesis and deposition, which was attributable to an increase in the abundance rather than length of GAG chains.[23] In contrast, an antisense inhibition of GlcAT-I expression impaired PG synthesis.[23] Thus, the expression level of GlcAT-I correlates well with the amount of GAGs, suggesting that GlcAT-I regulates the expression of GAGs.

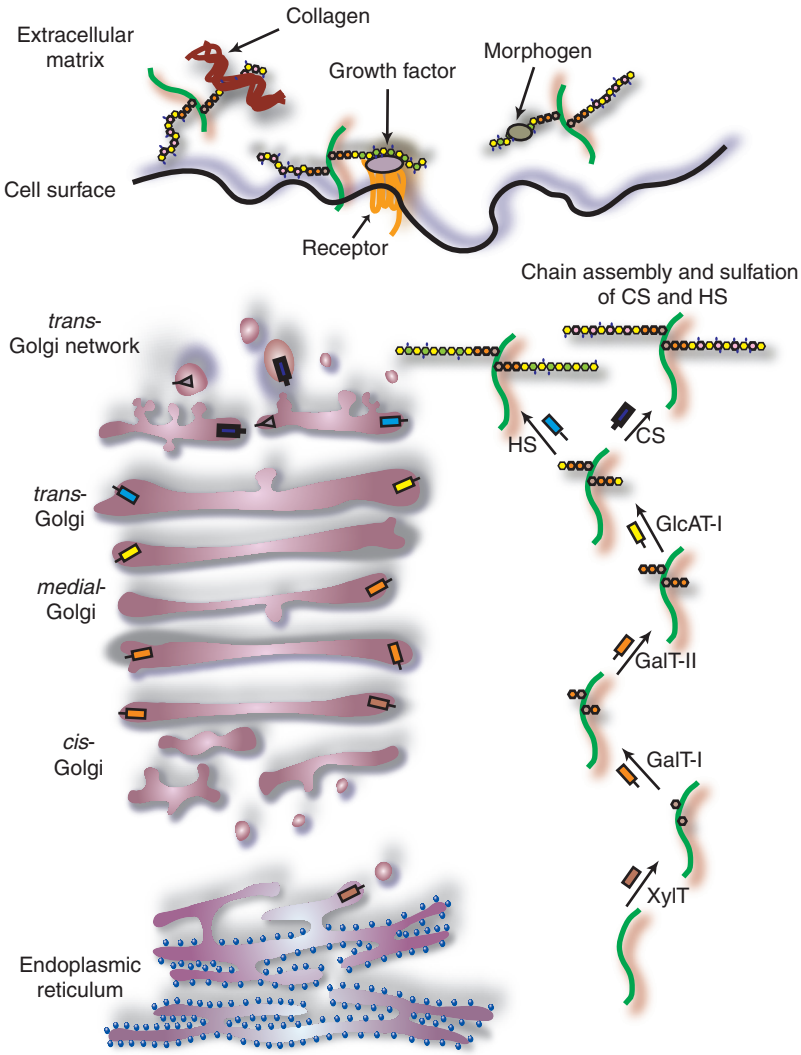

Figure 4 GAG synthesis in intracellular compartments. Following the synthesis of core proteins, GAG chain synthesis is initiated by XylT in the ER. During the translocation of core proteins with immature GAG chains, GAG chains are gradually assembled by various glycosyltransferases and sulfotransferases. Mature proteoglycans composed of a core protein and multiple GAG chains are expressed on the cell surface and in the extracellular matrix, and are involved in various events such as cell adhesion, cell differentiation, and cell division.

Modifications of the linkage region tetrasaccharide have been revealed in several organisms. One of these modifications is phosphorylation of the Xyl residue at position 2.[1–3] This modification occurs in both CS and HS, and appears to affect the transfer of Gal and GlcA residues by GalT-I and GlcAT-I, respectively.[24] *In vitro* experiments using authentic substrates showed that phosphorylation of the Xyl residue prevents the transfer of a Gal residue by GalT-I.[24] In contrast, GlcAT-I could efficiently transfer a GlcA residue to the phosphorylated trisaccharide *in vitro* (Tone *et al.*, unpublished data). These results suggested that the phosphorylation of the Xyl residue occurs after the transfer of the first Gal residue by GalT-I and before the transfer of the GlcA residue by GlcAT-I. Actually, the phosphorylation of the Xyl residue is most prominent after the addition of two Gal residues,[25,26] although the biological role of this modification and the enzyme responsible for the phosphorylation remain to be elucidated. Another modification is sulfation of either one or both Gal residues at position 4 or 6.[27–29] This modification has been shown in only CS and DS, never in HS. Therefore, it is possible that the sulfation of Gal residues is important for the selective assembly of CS and HS onto the linkage region tetrasaccharide, although it has been unclear whether

Table 2 Model organisms with defects in the biosynthesis of GAGs and core proteins

Mutants and knockout mouse	*Name*	*Phenotypes*	*Reference*
C. elegans			
sqv-1	UDP-GlcA decarboxylase	Defects in cytokinesis and vulval morphogenesis	30
sqv-2	GalT-II	Defects in cytokinesis and vulval morphogenesis	31
sqv-3	GalT-I	Defects in cytokinesis and vulval morphogenesis	12,32
sqv-4	UDP-Glc dehydrogenase	Defects in cytokinesis and vulval morphogenesis	33
sqv-5	ChSy	Defects in cytokinesis and vulval morphogenesis	34,35
sqv-6	XylT	Defects in cytokinesis and vulval morphogenesis	31
sqv-7	UDP-GlcA, UDP-GalNAc, UDP-Gal transporter	Defects in cytokinesis and vulval morphogenesis	32,36
sqv-8	GlcAT-I	Defects in cytokinesis and vulval morphogenesis	32
pfc-1	ChPF	Defects in cytokinesis and vulval morphogenesis	37
sdn-1	Syndecan	Defects in vulval morphogenesis	38,39
unc-52	Perlecan	Defects in the formation or maintenance of the muscle myofilament lattice, the effects of the regulation of distal tip cell migration	40,41
agrin	Agrin	Embryonic lethality	42
rib-2	EXTL3	Developmental abnormalities in embryonic stage	43
hst-1	NDST	Abnormal	44
hst-2	HS2ST	Abnormal axon guidance	45,46
hst-3	HS3ST	–[a]	47
hst-6	HS6ST	Suppression of kal-1-dependent axon-branching phenotype	48
D. melanogaster			
sugarless	UDP-Glc dehydrogenase	Defects in Wg, FGF signalings	49–52
frc	UDP-sugar transporter	Defects in Wg, Hh, FGF, Notch signalings	53,54
slalom	PAPS transporter	Defects in Wg, Hh signalings	55,56
oxt	XylT		57
beta4GalT7	GalT-I	Abnormal wing and leg morphology similar to flies with defective Hh and Dpp signalings	58,59
ttv	EXT1	Defects in Hh, Wg, Dpp signalings	60–64
sotv	EXT2	Defects in Hh, Wg, Dpp signalings	62–64
botv	EXTL3	Defects in Hh, Wg, Dpp signalings	62–64
sulfateless	NDST	Defects in Wg, FGF signalings	49,65
pipe	HS2ST	Defects in the formation of embryonic dorsal–ventral polarity	66,67
dHS2ST	HS2ST	–	68
HS3ST-B	HS3ST	Defects in Notch signaling	69
dHS6ST	HS6ST	Defects in FGF signaling	70
dally	Glypican	Defects in Wg, Dpp, Hh signalings	65,71–76
dally-like	Glypican	Defects in Wg, Hh signalings	75,77,78
dSyndecan	Syndecan		79–81
trol	Perlecan	Defects in neuroblast proliferation in the CNS	82
Notum	Notum	Defect in Wg signaling	83,84
Zebrafish (Danio rerio)			
jekyll	UDP-Glc dehydrogenase	Defects in cardiac valve formation	85
b3gat3	GlcAT-I	Defective branchial arches and jaw	86
uxs1	UDP-GlcA decarboxylase	Defective cartilage unstained with Alcian blue	86
zHS6ST	HS6ST	Defects in muscle differentiation	87,88
knypek	Glypican	Defects in Wnt signaling	89
GPC3	Glypican-3	Defects in Wnt signaling	90
syndecan-2	Syndecan-2	Defects in angiogenesis	91

(continued)

Table 2 (continued)

Mutants and knockout mouse	*Name*	*Phenotypes*	*Reference*
Mouse			
lzme (lazy mesoderm)	UDP-Glc dehydrogenase	Defects in FGF signaling	92
brachymorphic mouse	PAPS synthase 2	Dome-shaped skull, shortened but not widened limbs, short tail	93
cmd (cartilage matrix deficiency)	Aggrecan	Perinatal lethal dwarfism, craniofacial abnormalities	94
hdf (Versican$^{-/-}$)	Versican	Embryonic lethality with heart defect	95,96
Syndecan-1$^{-/-}$	Syndecan-1	Defects in the repair of skin and corneal wounds, low susceptibility to Wnt-1 signaling	97,98
Syndecan-3$^{-/-}$	Syndecan-3	Reduction of reflex hyperphagia following food deprivation	99,100
Syndecan-4$^{-/-}$	Syndecan-4	Impairment of focal adhesion under restricted conditions	101
Glypican-2$^{-/-}$	Glypican-2	No phenotypes	5
Glypican-3$^{-/-}$	Glypican-3	Developmental overgrowth typical of human Simpson–Golabi–Behmel syndrome	102–104
Agrin$^{-/-}$	Agrin	Perinatal lethality owing to breathing failure, defects of neuromuscular synaptogenesis	105
Perlecan$^{-/-}$	Perlecan	Defective cephalic development	106,107
Decorin$^{-/-}$	Decorin	Abnormal collagen morphology in skin and tendons	108
Biglycan$^{-/-}$	Biglycan	Reduced growth rate and decreased bone mass	109
Neurocan$^{-/-}$	Neurocan	No obvious deficits	110
PTP$^{-/-}$	PTP	Resistance to gastric ulceration caused by VacA of *Helicobacter pylori*	111
Thrombomodulin$^{-/-}$	Thrombomodulin	Embryonic lethality with dysfunctional maternal–embryonic interaction	112
EXT1$^{-/-}$	EXT1	Disruption of gastrulation	113
EXT1$^{-/-}$(generated by gene-trap mutation)	EXT1	An elevated range of Indian hedgehog signaling during embryonic chondrocyte differentiation	114,115
EXT1$^{-/-}$ (specific for brain)	EXT1	Defects in the midbrain–hindbrain region, disturbed Wnt-1 distribution	116
EXT2$^{-/-}$	EXT2	Disruption of gastrulation	117
NDST-1$^{-/-}$	NDST-1	Neonatal lethality due to respiration defects	118–120
NDST-1 (specific for endothelial cells and leukocytes)	NDST-1	Impaired L-selectin- and chemokine-mediated neutrophil trafficking during inflammatory responses	121
NDST-2$^{-/-}$	NDST-2	Loss of Hep, abnormal mast cell	122,123
HS2ST$^{-/-}$	HS2ST	Renal agenesis, defects in the eyes and skeleton	124
HS3ST-1$^{-/-}$	HS3ST-1	Genetic background-specific lethality, intrauterine growth retardation	125
Uronyl C5-epimerase$^{-/-}$ (Hsepi)	Uronyl C5-epimerase	Neonatal lethality with renal agenesis, lung defects, skeletal malformation	126
C6ST-1$^{-/-}$	C6ST-1	Decrease in naive T lymphocytes	127
C4ST-1	C4ST-1	Chondrodysplasia, drastic decrease in the amount of CS	128,129
Human			
Ehlers–Danlos syndrome	GalT-I	Aged appearance, developmental delay, dwarfism, craniofacial disproportion, and generalized osteopenia	15,16
Hereditary multiple exostosis	EXT1 EXT2	An autosomal dominant disorder characterized by the formation of cartilage-capped tumors (exostoses) that develop from the growth plate of endochondral bones, especially of long bones	130,131
Spondyloepiphyseal dysplasia	C6ST-1	Normal length at birth but severely reduced adult height, severe progressive kyphoscoliosis, arthritic changes with joint dislocations, genu valgum, cubitus valgus, mild brachydactyly, camptodactyly, microdontia, and normal intelligence	132

Table 2 (continued)

Mutants and knockout mouse	*Name*	*Phenotypes*	*Reference*
Spondyloepimetaphyseal dysplasia	PAPS synthase 2	Short, bowed lower limbs, enlarged knee joints, kyphoscoliosis, a mild generalized brachydactyly	133
Achondrogenesis type 1B	DTDST (diastrophic dysplasia sulfate transporter)	Autosomal recessive, lethal chondrodysplasia with severe underdevelopment of skeleton, extreme micromelia, and death before or immediately after birth	134
Schwartz–Jampel syndrome, dyssegmental dysplasia Silverman–Handmaker type	Perlecan	Autosomal recessive disorder characterized by permanent myotonia and skeletal dysplasia	135,136
Simpson–Golabi-Behmel syndrome	Glypican-3	X-linked disorder characterized by pre- and postnatal outgrowth	137
Camptodactyly-arthropathy-coxa vara-pericarditis syndrome	CACP	Autosomal recessive disorder characterized by synoviocyte hyperplasia	138

[a]Not reported.

all of the linkage region tetrasaccharide of CS is sulfated. Recently, it has been demonstrated that 6-O-sulfation of the first Gal residue markedly enhances GlcAT-I activity, and other sulfations, 4-O-sulfation of the first Gal residue and 4- or 6-O-sulfation of the second Gal residue, inhibit the transfer of a GlcA residue by GlcAT-I.[24] Thus, the sulfation of specific positions of the two Gal residues may regulate the expression level of GAGs by affecting the activity of GlcAT-I, although further experiments including the cloning of sulfotransferases responsible for the sulfation of Gal residues are required for the elucidation of the physiological roles of the sulfated linkage region tetrasaccharide.

Several model organisms deficient in the expression of glycosyltransferases responsible for the biosynthesis of the linkage region tetrasaccharide have been reported (**Table 2**). *Caenorhabditis elegans* (*C. elegans*) is one of the tractable model organisms and synthesizes both HS and Chn.[139,140] Genetic analyses of *C. elegans* revealed a link between GAG and morphogenesis.[6] In the screening of a mutant defective in the formation of the vulva, Horvitz and colleagues isolated eight kinds of mutants, designated *squashed vulva* (*sqv*).[141,142] These mutants showed not only perturbation of vulval invagination but also a defect in cytokinesis in fertilized eggs, resulting in oscillation between cell division and cell fusion.[6] Identification of the genes mutated in *sqv* mutants showed that all of the mutants possess deficiencies in enzymes or a nucleotide-sugar transporter involved in the biosynthesis of GAGs (**Figure 5**).[12,30–36,141,142] Among *sqv* mutants, *sqv-2*, *sqv-3*, *sqv-6*, and *sqv-8* encoded GalT-II, GalT-I, XylT, and GlcAT-I, respectively, and showed the same phenotypes (**Table 2**).[12,31,32] As a defect in the glycosyltransferases involved in the synthesis of the linkage region tetrasaccharide eliminates the expression of both HS and Chn, GAGs play a pivotal role in morphogenesis in *C. elegans*, especially in vulval formation. In *Drosophila melanogaster* (*D. melanogaster*), the inhibition of GalT-I by RNA interference (RNAi) resulted in reduced staining with antibodies recognizing CS and HS, and caused an abnormal wing and leg morphology similar to that of flies with defective Hedgehog (Hh) and Decpentaplegic (Dpp) signalings.[58] These signaling molecules are required for morphogenesis and organogenesis early in development, and aberrations of these molecules lead to the abnormal formation of a series of organs and tissues. Thus, GAGs are apparently required for the regulation of signaling molecules responsible for morphogenesis and organogenesis.[143]

3.05.3 Biosynthesis of CS/DS

Growing evidence has indicated that CSPGs play critical roles in various physiological events such as the regulation of signaling molecules, cell–cell and cell–matrix interactions, neurite extension, and cell division and differentiation.[6,144–146] CS is synthesized onto the GAG-protein linkage region tetrasaccharide shared by another type of GAG, HS (**Figure 3**).[2,3,147] Following the completion of the synthesis of the linkage region tetrasaccharide, the first GalNAc residue is transferred to the GlcA residue in the linkage region tetrasaccharide by GalNAcT-I activity, triggering the synthesis of CS.[2,3] If a GlcNAc residue is added instead of the GalNAc residue, an HS chain is built up onto the linkage tetrasaccharide.[2,3,148] Therefore, this step is critical for the selective assembly of CS and HS. Next, GlcA and GalNAc residues are alternately transferred onto growing CS chains by the actions of GlcAT-II and GalNAcT-II, respectively, resulting in the formation of the polymer sugar backbone of CS. Although the identity of the glycosyltransferases responsible for the biosynthesis and polymerization of the CS backbone had long been an enigma, recent molecular biological techniques in conjunction with the use of databases have revealed that Chn

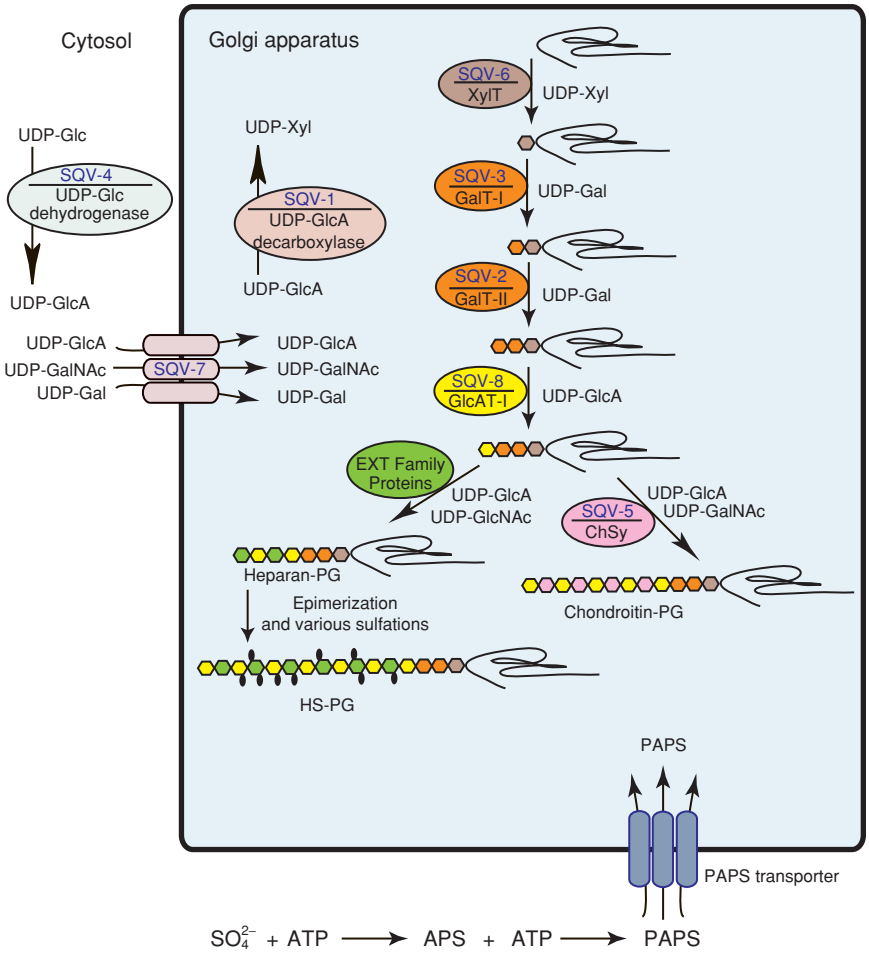

Figure 5 Schematic image of SQV proteins. All *sqv* genes encode proteins involved in GAG biosynthesis, especially chondroitin. SQV-1, UDP-GlcA decarboxylase; SQV-2, Gal transferase-II; SQV-3, Gal transferase-I; SQV-4, UDP-Glc dehydrogenase; SQV-5, Chondroitin synthase; SQV-6, Xyl transferase; SQV-7, UDP-sugar transporter; SQV-8, GlcA transferase-I; EXT family proteins (Rib-1 and Rib-2); and HS polymerase.

polymerization is achieved by an enzyme complex consisting of chondroitin synthase-1 (ChSy-1) and chondroitin polymerizing factor (ChPF) (**Figure 2**).[149,150] ChSy-1 possesses both GalNAcT-II and GlcAT-II activities, whereas ChPF has only marginal GalNAcT-II activity (**Table 3**).[149,150] Although ChSy-1 is a bifunctional glycosyltransferase capable of transferring both GalNAc and GlcA residues, the polymerization of Chn could not be achieved by ChSy-1 alone.[149] However, the interaction of ChSy-1 with ChPF led not only to a dramatic augmentation of the CS-synthesizing activities (GalNAcT-II and GlcAT-II activities) of ChSy-1 but also to the polymerization onto the linkage region tetrasaccharide.[150] Recently, chondroitin synthase-2 (ChSy-2) highly homologous to ChSy-1, also known as chondroitin sulfate synthase-3 (CSS3), was cloned and shown to be a bifunctional enzyme similar to ChSy-1 (Uyama *et al.*, unpublished data) (**Table 3**).[151] Interestingly, the polymerization of Chn could be achieved by enzyme complexes composed not only of ChSy-2 and ChPF but also of ChSy-1 and ChSy-2, and the length of the Chn chains formed by these complexes was different. Although the mechanism by which the interaction of these proteins results in the polymerization activity in addition to the increase in CS-synthesizing activity has been unclear, it is possible that the formation of the enzyme complex leads to an increase in the stability of these proteins, increase in affinity for the acceptor substrate, or structural or conformational alteration of these proteins suitable for the polymerization. Thus, CS polymerization is achieved in multiple combinations among ChSy-1, ChSy-2, and ChPF. In addition to these CS-synthesizing enzymes, chondroitin GalNAcT-1 and -2 have been cloned as enzymes possessing both GalNAcT-I and GalNAcT-II activities,[152–155] and chondroitin GlcAT has been shown to transfer a GlcA residue to CS chains (**Table 3**).[156] Thus, various glycosyltransferases are responsible for the biosynthesis of CS.

Table 3 Human CS glycosyltransferases and CS/DS sulfotransferases

Name	*Abbreviation*	*Chromosome location*	*Amino acid*	*mRNA expression*	*mRNA accession*
Chondroitin synthase-1	ChSy-1 (GalNAcT-II/ GlcAT-II)	15q26.3	802	Ubiquitous	AB071402
Chondroitin synthase-2 (Chondroitin sulfate synthase-3)	ChSy-2 (CSS3) (GalNAcT-II/ GlcAT-II)	5q31.1	882	Ubiquitous	AB175496
Chondroitin GalNAcT-1	ChGn-1 (GalNAcT-I/II)	8p21.3	532	Ubiquitous	AB071403
Chondroitin GalNAcT-2	ChGn-2 (GalNAcT-I/II)	10q11.22	542	Ubiquitous	AB090811
Chondroitin GlcAT-II	GlcAT-II	7q35	772	Ubiquitous	AB037823
Chondroitin polymerizing factor	ChPF	1p11-p12	775	Ubiquitous	AB095813
Chondroitin 4-*O*-sulfotransferase-1	C4ST-1	12q23	352	Ubiquitous	AF239820
Chondroitin 4-*O*-sulfotransferase-2	C4ST-2	7p22	414	Ubiquitous	AF239822
Chondroitin 4-*O*-sulfotransferase-3	C4ST-3	3q21.3	341	Liver	AY120869
Dermatan 4-*O*-sulfotransferase-1	D4ST-1	15q14	376	Ubiquitous	AB066595
Chondroitin 6-*O*-sulfotransferase-1	C6ST-1	10q21.3	479	Ubiquitous	AB017915
Uronyl 2-*O*-sulfotransferase	CS/DS2ST	–[a]	406	Ubiquitous	AB020316
GalNAc 4-sulfate 6-*O*-sulfotransferase	GalNAc4S-6ST	10q26	561	–	AB062423

[a]Not reported.

Although the physiological roles of CS had been obscure due to the lack of cloning of CS-synthesizing enzymes, *sqv* mutants of *C. elegans* provided insights into the functions of Chn in morphogenesis and cell division, especially cytokinesis (**Figure 5**, **Table 2**).[6] In *sqv* mutants, *sqv-5* encodes ChSy responsible for the biosynthesis of Chn,[34,35] whereas other *sqv* genes, except for *sqv-5*, code for the proteins required for the biosynthesis of both Chn and HS. An analysis of worms defective in the expression of *sqv-5* revealed a drastic decrease in the amount of Chn, but not HS. Supporting this result, ChPF in *C. elegans* was cloned and the inhibition of ChPF by RNAi resulted in similar phenotypes to ChSy-depleted worms such as a marked decrease in CS and a defect in cytokinesis in the fertilized eggs.[37] In addition, RNAi treatment of the fertilized eggs of *C. elegans* caused oscillation between cell division and cell fusion, indicating the absolute requirement of Chn in cytokinesis. Taken together, Chn, but not HS, plays critical roles in the cytokinesis of fertilized eggs and morphogenesis such as vulval invagination. However, further experiments are required to elucidate the mechanism by which Chn regulates cytokinesis.

3.05.4 Modifications of CS

After the synthesis of the sugar backbone of CS, numerous modifications of CS chains are achieved by corresponding enzymes such as GlcA C5-epimerase and a series of sulfotransferases, showing the structural diversity of CS.[1,3,147] GlcA C5-epimerase catalyzes the epimerization of GlcA to IdoA at position 5, converting CS to DS accompanied by an anomeric change of the glycosidic linkage of GlcA from β to α (**Figure 2**). This conversion gives DS the structural flexibility needed to interact with various proteins such as growth factors and cytokines. One famous example is the interaction of Hep cofactor II, which is homologous to antithrombin (AT) III regulating blood coagulation, with DS, but not CS.[157] Thus, although CS and DS are similar in structure except for the existence of IdoA in DS chains, these GAGs are quite different in function. Therefore, the expression of C5-epimerase is critical in the regulation of DS functions. However, GlcA C5-epimerase has not been cloned to date (see 'Note added in proof'). Elucidation of the properties of this enzyme will provide insights into how the synthesis of DS is regulated and what kinds of roles DS plays.

Sulfation of CS at specific positions is catalyzed by a number of sulfotransferases, and the degree of sulfation is regulated in a spatiotemporal manner (**Table 3**).[158,159] Various positions of GalNAc and GlcA in CS have the potential to be sulfated and the resultant sulfated CS shows structural diversity (**Figure 6**). Sulfation of CS occurs predominantly at positions 4 and 6 of GalNAc residues and position 2 of GlcA residues in mammals (**Figures 2** and **6**).[160] Sulfated disaccharides are generally divided into four groups as follows: A unit, GlcA-GalNAc(4S); C unit, GlcA-GalNAc(6S); D unit, GlcA(2S)-GalNAc(6S); and E unit, GlcA-GalNAc(4S,6S) (**Figure 6**).[160] The functions of CS

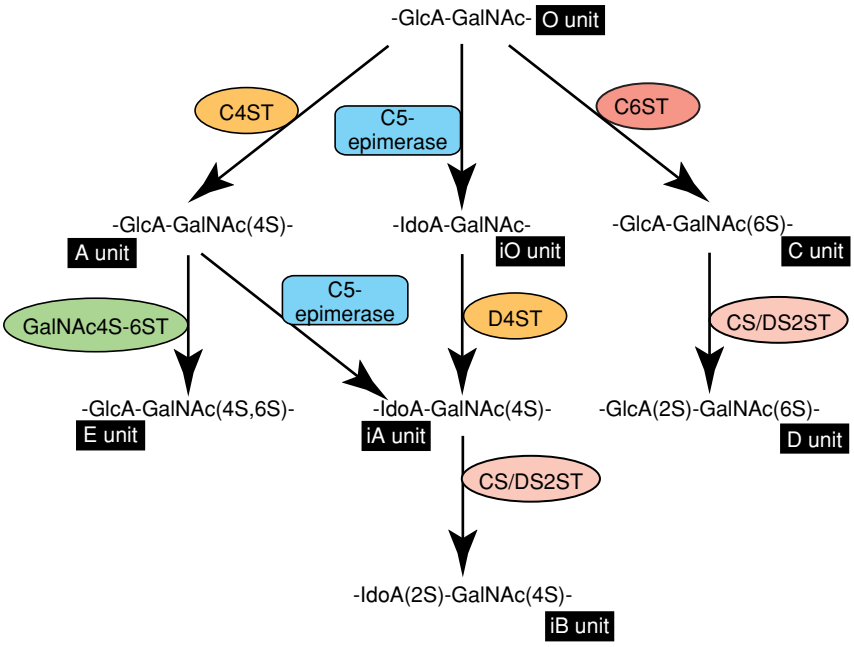

Figure 6 Pathways of biosynthetic modifications of CS and DS chains. C4ST, chondroitin 4-*O*-sulfotransferase; C6ST, chondroitin 6-*O*-sulfotransferase; D4ST, dermatan 4-*O*-sulfotransferase; CS/DS2ST, uronyl 2-*O*-sulfotransferase; GalNAc4S-6ST, GalNAc 4-sulfate 6-*O*-sulfotransferase; O unit, GlcAβ1-3GalNAc; iO unit, IdoAα1-3GalNAc; A unit, GlcAβ1-3GalNAc(4S); iA unit, IdoAα1-3GalNAc(4S); iB unit, IdoA(2S)α1-3GalNAc(4S); C unit, GlcAβ1-3GalNAc(6S); D unit, GlcA(2S)β1-3GalNAc(6S); and E unit, GlcAβ1-3GalNAc(4S,6S).

dependent on sulfation patterns have been elucidated in the brain. In the brain, CS chains containing disulfated disaccharide D and E units (**Figure 6**) appear to play important roles in the regulation of axonal guidance and pathfinding of various neurons.[6] For instance, neurons cultured on substrata coated with CS-D from shark cartilage exhibited a flattened cell soma with multiple neurites, whereas those cultured on substrata coated with CS-E from squid cartilage showed a round-shaped cell soma with a single prominent long neurite.[161,162] The cause of these phenotypic differences was the sulfation patterns of CS. In addition to CS, DS chains purified from various marine organisms exerted marked neurite outgrowth-promoting activity depending on their specific sulfation patterns.[163] CS chains sulfated at specific positions are responsible for the specific binding of growth factors, cytokines, and adhesion molecules.[6] For example, CS/DS hybrid chains purified from embryonic pig brain bind pleiotrophin, an Hep-binding growth factor, to induce neurite outgrowth in mouse hippocampal cells.[164–166] Multiple pleiotrophin-binding sequences derived from CS/DS hybrid chains, which contain at least one D unit, have been demonstrated.

Seven sulfotransferases involved in the sulfation of CS have been cloned to date (**Table 3**).[159] These sulfotransferases catalyze the transfer of a sulfate residue from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) to a specific position of GalNAc, GlcA, or IdoA in CS/DS. For sulfation of position 4 of the GalNAc residue, four sulfotransferases have been cloned. Chondroitin 4-*O*-sulfotransferase-1, -2, and -3 (C4ST-1, -2, and -3) are responsible for the sulfation of position 4 of a GalNAc residue in CS, whereas dermatan 4-*O*-sulfotransferase-1 (D4ST-1) catalyzes the transfer of a sulfate residue to a GalNAc residue next to IdoA in DS.[167–170] Thus, four sulfotransferases are involved in the formation of the A unit. Chondroitin 6-*O*-sulfotransferase-1 (C6ST-1) transfers sulfate to position 6 of the GalNAc residue and is responsible for the formation of C and D units.[171,172] 2-O-Sulfation of GlcA and IdoA is catalyzed by uronyl 2-*O*-sulfotransferase. This enzyme is essential for the formation of the D unit, which is formed by two steps.[173] First, C6ST-1 catalyzes 6-O-sulfation of a GalNAc residue, and then 2-O-sulfation of the adjacent GlcA or IdoA is performed by uronyl 2-*O*-sulfotransferase. GalNAc 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) transfers a sulfate residue to position 6 of GalNAc(4S) formed by C4ST, and is responsible for the formation of the E unit.[174] The sulfation profile of CS is regulated in part by the expression level of corresponding sulfotransferases. In the chick brain at early embryonic stages, the dominant CS structure is 6-O-sulfated CS and the corresponding C6ST activity is quite high.[175] As development proceeds, the amount of 6-O-sulfated CS and level of C6ST activity sharply decrease with a concomitant upregulation of 4-O-sulfated CS and C4ST activity.[175] These findings suggested that the sulfated

structure of CS is well correlated with the corresponding sulfotransferase activities. Therefore, a better understanding of the expression patterns of sulfotransferases may lead to elucidation of the spatiotemporally specific sulfation of CS and its function *in vivo*.

The importance of the sulfation of CS has been clarified by the analysis of mice and humans with a defect in the expression of sulfotransferases (**Table 2**). In mice defective in the expression of C6ST-1, the level of 6-O-sulfated disaccharides, C and D units, became almost undetectable.[127] Although these mice were healthy and fertile, and reproduced normally, the number of naive T lymphocytes was significantly decreased in spleen, where C6ST-1 is expressed abundantly.[127] This finding suggested that 6-O-sulfated CS formed by C6ST-1 plays a role in the maintenance of naive T lymphocytes in the spleen. In contrast to the mild phenotypes shown in mice deficient in C6ST-1 expression, a loss of function of C6ST-1 in humans causes spondyloepiphyseal dysplasia.[132] In such patients, a missense mutation in the open reading frame of C6ST-1 changes arginine to glutamine (R304Q) at the well-conserved PAPS-binding site.[132] This point mutation completely abolished C6ST activity.[132] The CS disaccharide analysis showed that both C and D units are significantly reduced in the fibroblasts and urine, which was similar to the composition of CS disaccharides in C6ST-1-deficient mice.[127,132] Although why a phenotypic difference is observed between human and mouse is unclear, 6-O-sulfated CS plays critical roles in skeletal development and maintenance in humans. The functions of C4ST-1 have been revealed using mice defective in C4ST-1 expression due to the insertion of a reporter gene by the screening of the gene trap mutation.[128,129] A defect in C4ST-1 expression causes severe chondrodysplasia characterized by a disorganized cartilage growth plate as well as specific alterations in the orientation of chondrocyte columns.[129] Surprisingly, the elimination of C4ST-1 expression led to a drastic decrease in the amount of CS.[129] In addition, the amount of A unit, which is formed by C4ST/D4ST, was reduced by 90% compared to littermates, indicating that C4ST-1 is a major sulfotransferase catalyzing 4-O-sulfation of CS.[129] This result suggests that C4ST-1 regulates not only 4-O-sulfation of CS but also the amount of CS, and other C4ST/D4ST family members including C4ST-2, C4ST-3, and D4ST-1 could not compensate for the loss of C4ST-1. Regarding the roles of sulfated CS, other experiments have shown that CS chains containing E-units are involved in the binding and infection of herpes simplex virus type 1 (HSV-1).[176] CS-E derived from squid cartilage, but not other CS isoforms, more efficiently inhibited the binding to and infection of cells susceptible to HSV-1 than did Hep, which is a highly sulfated form of HS and an inhibitor of HSV-1 infection.[176] Recently, we showed that HSV-1 could not infect and bind sog9 cells, which are defective in the expression of E units due to a defect in *C4ST-1* expression, whereas parental gro2C cells, which express a significant amount of the E unit, were infected by HSV-1 (Uyama *et al.*, unpublished data). These and other results have suggested that CS containing E units is a cell surface receptor for HSV-1 and HSV-2.[6] Thus, it is obvious that CS with specific sulfation is critical for various biological events.

3.05.5 Biosynthesis of HS/Hep

A series of experiments have revealed that HS plays pivotal roles in a number of biological phenomena such as morphogenesis, organogenesis, and neuronal formation including axonal guidance.[4,5,10,143] One well-characterized function of HS is as a co-receptor for growth factors such as fibroblast growth factor (FGF), where HS binds FGF and presents it to the FGF receptor, forming a ternary complex potentiating signal transduction inside cells.

The sugar backbone of HS is synthesized by three enzymes: GlcNAc transferase-I (GlcNAcT-I), GlcNAc transferase-II (GlcNAcT-II), and HS GlcAT-II (**Figure 3**, **Table 4**).[2,3,148] GlcNAcT-I and GlcNAcT-II transfer a GlcNAc residue to the linkage region tetrasaccharide and growing HS chains, respectively, whereas GlcA in HS chains is transferred by HS GlcAT-II activity. Two independent experiments revealed that HS is synthesized by bifunctional enzymes harboring GlcNAcT-II and HS GlcAT-II activities responsible for the elongation of HS chains.[177,178] First, in the screening of cells resistant to HSV-1 infection, sog9 cells were isolated as a GAG-deficient cell line.[179] Then, expression cloning using a Hela-cell cDNA library showed that sog9 cells are defective in *EXT1* expression, resulting in a loss of HS synthesis, and the introduction of *EXT1* into sog9 cells restored the susceptibility to HSV-1.[177,180] *EXT1* was originally cloned as a tumor suppressor gene, the loss of expression of which leads to hereditary multiple exostosis (HME).[181] Characterization of *EXT1* led to the functional elucidation of EXT1, which can transfer GlcNAc and GlcA residues to growing HS chains and is localized in the Golgi apparatus similar to other GAG-synthesizing enzymes.[180,182] Second, another HS-synthesizing enzyme, EXT2, was cloned by the amino acid sequencing of the enzyme purified from bovine serum.[178] Recombinant EXT2 exhibited GlcNAcT-II and HS GlcAT-II activities capable of building up HS chains.[178] Positional cloning of the loci responsible for HME also identified *EXT2* as a tumor suppressor gene.[183] Although neither EXT1 nor EXT2 by itself could polymerize HS chains onto the linkage region tetrasaccharide, an enzyme complex consisting of EXT1 and EXT2 showed significant polymerization activity (**Figure 3**) onto the authentic substrate analogous to the linkage region tetrasaccharide, where EXT2 appears to