Structural Characterization of Heparan Sulfate and Chondroitin Sulfate of Syndecan-1 Purified from Normal Murine Mammary Gland Epithelial Cells

COMMON PHOSPHORYLATION OF XYLOSE AND DIFFERENTIAL SULFATION OF GALACTOSE IN THE PROTEIN LINKAGE REGION TETRASACCHARIDE SEQUENCE

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Syndecan-1, present on the surfaces of normal murine mammary gland epithelial cells, is a transmembrane hybrid proteoglycan, which bears glycosaminoglycan (GAG) side chains of heparan sulfate (HS) and chondroitin sulfate (CS). Purified syndecan-1 ectodomains were analyzed for disaccharide composition and the GAG-protein linkage region after digestion with bacterial lyases. The HS chains contained predominantly a nonsulfated unit with smaller proportions of two monosulfated, two disulfated, and a trisulfated unit, whereas CS chains were demonstrated for the first time to bear GlcUA-GalNAc(4-O-sulfate) as a major component as well as GlcUA-GalNAc, GlcUA-GalNAc(6-O-sulfate), and an E disaccharide unit GlcUA-GalNAc(4,6-O-disulfate) as minor yet appreciable components. Two kinds of linkage region tetrasaccharides, GlcUA-Gal-Xyl and GlcUA-Gal-Xyl(2-O-phosphate), were found for the HS chains in a molar ratio of 55:45. In marked contrast, an additional sulfated tetrasaccharide, GlcUA-Gal(4-O-sulfate)-Gal-Xyl, was demonstrated only for the CS chains, and the unmodified phosphorylated and sulfated components were present at a molar ratio of 55:26:19. The present study thus provided conclusive evidence for the hypothesis that 4-O-sulfation of Gal is peculiar to CS chains in contrast to the phosphorylation of Xyl, which is common to both HS and CS chains. These modifications may be required for biosynthetic maturation of the linkage region tetrasaccharide sequence, which is a prerequisite for creating the repeating disaccharide region of GAG chains and/or biosynthetic selective chain assembly of CS and HS chains.

Proteoglycans (PGs) are macromolecules composed of glycosaminoglycan (GAG) side chains covalently bound to a protein core (1). PGs have been implicated in the regulation and maintenance of cell proliferation, cytodifferentiation, and tissue morphogenesis where the characteristic GAG moieties specifically interact with protein ligands, which include a wide variety of growth and/or differentiation factors, cytokines, and morphogens (2-5). GAGs include chondroitin/dermatan sulfate (CS/DS) and heparan sulfate/heparin (HS/Hep), which are classified as galactosaminoglycans and glucosaminoglycans, respectively. Major components of these linear GAGs, except for branched keratan sulfate, consist of hexosamine (GlcNAc, GlcNAc, or 2-N-sulfated GlcN) and hexuronic acid (GlcUA or IduUA) units, which are arranged in alternating sequences to form the so-called repeating disaccharide region. These repeating units contain a number of sulfate substituents at various positions (1), which create a massive degree of structural and functional diversity. Both types of GAGs are covalently bound to Ser residues in the core proteins through the common GAG-protein linkage structure, GlcUAβ-3Galβ-3Galβ(1–4Xylβ-1–O-Ser) (6). This conformity contrasts sharply with the structural heterogeneity of the repeating disaccharide region. Hence, the question arises how these different GAGs can be synthetized on the same structure, especially because the chain elongation proceeds in a stepwise fashion and is governed largely by the substrate specificity of the glycosyltransferases involved (7, 8).

We have carried out a series of structural studies of the GAG-protein linkage region based on the working hypothesis that possible structural differences in the linkage region may exist among the various GAG chains and determine the type and/or character of the GAG species to be synthesized (7). These and other studies have led to the identification of novel modified structures, such as GlcUAβ/IdoUAα-3Gal(4-O-sulfate)β-3Gal(±6-O-sulfate)β-1–4Xyl, GlcUAβ-3Galβ-3Gal(±6-O-sulfate)β-1–4Xyl, and GlcUAβ-3Galβ-3Galβ(1–4Xylβ-2-O-phosphate), for CS/DS chains (7, 9-19). Interestingly, sulfated Gal residues have been demonstrated so far in the linkage region of CS and DS but not in HS or Hep, nor in the linkage region of the heparin.

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whereas a 2-O-phosphorylated Xyl residue has been found in both HS/Hep and CS, indicating that the sulfate groups on the Gal residues may be signals for biosynthetic selective assembly of CS/DS (7, 8), and that the modifying sulfate and/or phosphate groups may be the key elements that control the glycosyltransferases involved in the formation of the linkage region, thereby regulating the maturation of “part-time PGs” (20). Despite the intriguing contrast in the Gal sulfation and the attractive possibilities, it remains to be examined whether such differential and common modifications indeed reflect the indispensable features of the constitutive biosynthetic machinery or cell-, tissue-, or species-dependent modifications.

To clarify these issues and also examine whether the Gal sulfation is indeed characteristic of only CS/DS chains and not of HS chains, we analyzed in this study syndecan-1, a transmembrane hybrid PG that contains both HS and CS side chains on the same core protein. Syndecan-1 is involved in a variety of important biological phenomena through interactions, at least, of its HS chains with specific protein ligands, such as fibroblast growth factors, various morphogens, and microbial pathogens (4, 21–23). The functions of the CS chains have not been investigated as thoroughly. In view of the hybrid structural feature, the biosynthetic sorting of HS and CS is of critical importance to express HS- or CS-specific biological activities. Recently, we developed a sensitive analytical method for GAG–protein linkage region oligosaccharides derived from the extracellular domains (ectodomains) of purified syndecan-1 prepared from the conditioned medium of cultured NMuMG (normal murine mammary gland) epithelial cells. Differential sulfation of one of the galactose residues and common phosphorylation of the xylose residue were demonstrated.

**EXPERIMENTAL PROCEDURES**

**Materials—**Heparitin-sulfate lyase (EC 4.2.2.8) available as heparitinase, heparinase (EC 4.2.2.7), chondroitin ABC lyase (EC 4.2.2.4), chondroitin AC lyase from Arthrobacter aurescens (EC 4.2.2.5), chondro-4-sulfatase (EC 3.1.6.9), chondro-2-sulfatase (EC 3.1.3.1) of special quality for molecular biology was from Roche Molecular Biochemicals (Tokyo, Japan), Ampullaria (freshwater apple shell) heptapancracces β-glucuronidase (EC 3.2.1.31) purified to homogeneity (25) was provided by Tokyo Zouki Chemical Co. (Tokyo, Japan). 2-Aminobenzamide (2AB) was purchased from Nacalai Tesque (Kyoto, Japan). Calf intestine alkaline phosphatase (EC 3.1.3.1) of special quality for molecular biology was from Roche Molecular Biochemicals (Tokyo, Japan), Ampullaria (freshwater apple shell) heptapancracces β-glucuronidase (EC 3.2.1.31) purified to homogeneity (25) was provided by Tokyo Zouki Chemical Co. (Tokyo, Japan). Ampullaria (freshwater apple shell) heptapancracces β-glucuronidase (EC 3.2.1.31) purified to homogeneity (25) was provided by Tokyo Zouki Chemical Co. (Tokyo, Japan).

Preparation of Cell Surface Syndecan-1 Ectodomains—Soluble proteoglycans are shed from the surfaces of subconfluent NMuMG cell cultures. NMuMG cells at relatively early passages (10–14) were cultured to 50% confluence (27), and the conditioned media were used to prepare purified syndecan-1 ectodomains as described previously (28). After anion exchange chromatography, the samples were subjected to CsCl density gradient centrifugation, and the fractions at density higher than 1.35 g/ml were collected for purification of syndecan-1 at higher than 1.35 g/ml were collected for purification of syndecan-1. Syndecan-1 was purified from the extracellular domains (ectodomains) of purified syndecan-1 from the conditioned medium of cultured NMuMG (normal murine mammary gland) epithelial cells. Differential sulfation of one of the galactose residues and common phosphorylation of the xylose residue were demonstrated.

The purified syndecan-1 ectodomains (3.7 μg) were analyzed by gel filtration chromatography on a PD-10 column. The O-linked saccharides of the purified syndecan-1 ectodomains were liberated from the core protein by treatment with LiOH and derivatized with 2AB as described under “Experimental Procedures.” The 2AB-derivatives were separated from the reagents by paper chromatography. To further purify the 2AB-derivatives, the samples were subjected to gel filtration on a PD-10 column using 50 mM pyridine-acetate buffer, pH 5.0. Eluates were monitored by fluorescence with excitation and emission wavelengths at 330 and 420 nm, respectively. The flow-through fraction containing 2AB-derivatized GAG chains, the right shoulder (Fr. A) of the GAG fraction, and a broad fluorescent peak (Fr. B) eluted afterward, both of which might contain O-linked oligosaccharides, were separately pooled, lyophilized, and analyzed.

Comparison with the average values obtained by automated amino acid and amino sugar analyses (32). Purity was assessed by comparing silver-stained gels from SDS-polyacrylamide gel electrophoresis, which bore various amounts of the immunoadfinity purified GAG-free ectodomain core protein. No contaminating proteins were noted at a level of detection corresponding to one-tenth the amount of ectodomain core protein. This lack of detectable contaminant(s) indicates that at least 90% of the preparation was the ectodomain core protein.

**Preparation of the 2AB-derivatives of the GAG Chains from Syndecan-1 Ectodomains**—The purified syndecan-1 ectodomains (3.7 μg as the core protein) were treated with 0.5 M LiOH at 4 °C for 13 h to liberate O-linked saccharides from the core protein (24, 25). After neutralization with 1.7 M acetic acid, the sample was applied to a column (300 μl) of anion-exchange resin AG 50W-X2 (H+ form, Bio-Rad) equilibrated with H2O. The flow-through fraction containing the liberated O-linked saccharides was neutralized with 1 M NH4HCO3. Derivatization of the saccharides with 2AB and initial purification of the derivatives were carried out by paper chromatography as described previously (24). To further purify the 2AB-derivatives, the samples were subjected to gel filtration on a PD-10 column (Amersham Pharmacia Biotech) using 50 mM pyridine-acetate buffer, pH 5.0, as an eluent (24). Eluates were monitored by fluorescence with excitation and emission wavelengths at 330 and 420 nm, respectively. The flow-through fraction containing 2AB-derivatized GAG chains, the right shoulder (Fr. A) of the GAG fraction and a broad fluorescent peak (Fr. B) eluted afterward, both of which might contain O-linked oligosaccharides, were separately pooled as shown in Fig. 1 and lyophilized. The 2AB-derivatized GAG chains were analyzed as described below. Frs. A and B were analyzed by anion-exchange HPLC on an anion-bound silica PA-03 column or by gel filtration HPLC on a column (7.6 × 500 mm) of Asahipac GS320 as described previously (12, 34) before and after β-glucuronidase digestion. Enzymatic Treatments and HPLC Analysis—Enzymatic treatments for disaccharide composition analysis of GAG chains were carried out as follows. The purified syndecan-1 ectodomains (150 or 38 ng as core protein) were treated with chondroitin ABC lyase (5 mU) in a total volume of 20 μl of 0.05 mM Tris-HCl buffer, pH 8.0, containing 0.06 mM sodium acetate at 37 °C for 10 min (35) or with a mixture of heparinase and heparitin-sulfate lyase (0.5 mU each) in a total volume of 20 μl of acetate-NaCl buffer, pH 7.0, containing 10 mM Ca(OAc)2 at 37 °C for 1 h (36), respectively. The digests were treated with a fluorophore 2AB to label reducing termini of GAG disaccharides and analyzed by HPLC on an anion-bound silica column as described previously (34).

Enzymatic treatment of 2AB-derivatized GAG chains (∼20 pmol) with chondroitin ABC lyase was carried out as reported (34) using 7.5 mU of the enzyme in a total volume of 25 μl for 30 min. The chondroitin...
Figure 2. Disaccharide composition analysis of HS and CS chains of syndecan-1. Syndecan-1 ectodomains (38 or 150 ng as core protein) were digested with a mixture of heparinase and heparitin-sulfate lyase or chondroitin ABC lyase for disaccharide composition analysis of HS (A) or CS (B), respectively. The digests were labeled with a fluorophore 2AB and analyzed by HPLC on an amine-bound silica column as described under “Experimental Procedures”. The elution positions of authentic 2AB-disaccharide standards derived from HS and CS are indicated by numbered arrows in A and B, respectively. 1, ΔDiHS-0S; 2, ΔDiHS-6S; 3, ΔDiHS-NS; 4, ΔDiHS-diS; 5, ΔDiHS-diS; 6, ΔDiHS-triS; 7, ΔDi-0S; 8, Δdi-4S; 9, Δdi-4S; 10, ΔDi-diS; 11, ΔDi-diS; 12, ΔDi-triS.

ABC lyase digest was further treated with 7.5 mM of chondroitin AC lyase in a total volume of 50 μl of 0.05 M sodium acetate buffer, pH 6.0, at 37 °C for 30 min. Chondro-4-sulfatase treatment of the chondroitin ABC lyase digests was carried out using 40 μIU of the enzyme in a total volume of 100 μl of 34 mM Tris-HCl buffer, pH 7.5, containing 34 mM sodium acetate and 100 μg/ml bovine serum albumin at 37 °C for 30 min (12). Enzymatic treatment of 2AB-derivatized GAG chains (~20 pmol) with a mixture of heparinase and heparitin-sulfate lyase (3 mg/ml each) was carried out in a total volume of 50 μl for 3 h as reported previously (36). Alkaline phosphatase treatment was carried out using 4 IU of the enzyme in a total volume of 100 μl of 0.08 M glycine/NaOH buffer, pH 9.9, containing 0.5 mM MgCl₂ at 37 °C for 30 min (12). The enzymatic reactions were terminated by heating at 100 °C for 1 min, and each enzyme digest was analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column or by gel filtration HPLC on a column of Asahipac GS-320 as described previously (12, 34). Eluates were monitored by fluorescence intensity with excitation and emission wavelengths of 330 and 420 nm or by absorbance at 232 nm.

RESULTS

Disaccharide Composition Analysis of HS and CS Chains—The purified syndecan-1 ectodomains were digested exhaustively with chondroitin ABC lyase or a mixture of heparinase and heparitin-sulfate lyase, and the resulting disaccharides were labeled with 2AB and analyzed by HPLC on an amine-bound silica column. Each disaccharide peak was identified by comparison of the elution positions with those of the standard 2AB-disaccharides (Fig. 2). The major disaccharide units in HS chains were ΔDiHS-0S ([ΔHexUA-GlcNAc] and ΔDiHS-NS ([ΔHexUA-GlcNAc(2-N-sulfate)]), which accounted for 58 and 23%, respectively (Fig. 2A). The small yet appreciable amounts of other disaccharide units, ΔDiHS-6S ([ΔHexUA-GlcNAc(6-O-sulfate)]), ΔDiHS-diS ([ΔHexUA-GlcNAc(2-N, 6-O-sulfate)]), ΔDiHS-diS ([ΔHexUA-GlcNAc(2-N, 6-O-sulfate)]), and ΔDiHS-triS ([ΔHexUA-GlcNAc(2-N, 6-O-sulfate)]), were also found. These findings are in agreement with previous data (28). Nearly 36% of the disaccharides were N-sulfated, and 11% each of the disaccharides contained hexuronate 2-O-sulfate or glucosamine 6-O-sulfate residue. The 2-N-sulfate content was relatively high compared with that of 2-O-sulfate or 6-O-sulfate. The yield of each disaccharide was calculated based on the fluorescence intensity (34) and the composition of the syndecan-1 HS chains. A summary is given in Fig. 3A.

Chondroitin ABC lyase digestion of the purified syndecan-1 ectodomains yielded ΔDi-0S ([ΔHexUA-GalNAc] and ΔDi-4S ([ΔHexUA-GalNAc(4-O-sulfate)]), ΔDi-6S ([ΔHexUA-GalNAc(6-O-sulfate)]), and ΔDi-diS ([ΔHexUA-GalNAc(4,6-O-disulfate)]) at a molar ratio of 29:59:5:7 (Fig. 2B). Chondroitin AC lyase digestion gave very similar results indicating that most, if not all, of the uronic acid residues were GlcUA. The identity of the latter two minor peaks was confirmed based on the sensitivity to chondro-6-sulfatase. Upon digestion, these peaks were shifted to the positions of ΔDi-0S and ΔDi-4S, respectively (data not shown). The yield of each disaccharide was calculated based on the fluorescence intensity and the composition of the CS chains. The data are summarized in Fig. 3B. The disaccharide compositions obtained using 2AB-derivatization were comparable with those based on the UV absorbance of the underderivatized unsulfated disaccharides (data not shown).

Structural Analysis of the CS-protein Linkage Region—The purified syndecan-1 ectodomains were treated with LiOH to liberate O-glycosylated glycan chains including GAGs from the core protein (24, 33). The liberated saccharides were labeled with a fluorophore 2AB. A mixture of 2AB-labeled GAG chains was recovered in the flow-through fraction on gel filtration using a PD-10 column (Fig. 1). The 2AB-labeled tetrasaccharides derived from the CS-protein linkage region were obtained by digesting the repeating disaccharide region using chondroitin ABC and AC lyases. Depolymerization of CS by chondroitin ABC lyase results in various sulfated disaccharide units and core hexasaccharides that are derived from the linkage region (7). Chondroitin AC lyase degrades a linkage region hexasaccharide into a disaccharide unit and a core tetrasaccharide (11). The resulting 2AB-derivatized linkage region tetrasaccharides were analyzed by HPLC on an amine-bound silica column (Fig. 4). As shown in Fig. 4B, three predominant peaks were observed at the elution positions of the authentic 2AB-tetrasaccharides, HexUA1→3Galβ1→3Galβ1→4Xyl-2AB, HexUA1→3Gal(4-O-sulfate)β1→3Galβ1→4Xyl-2AB, and HexUA1→3Galβ1→3Galβ1→4Xyl(2-O-phosphate)-2AB, in a molar ratio of 55:19:26. When this sample was co-chromatographed with the standard linkage tetrasaccharides, these peaks were co-eluted with the corresponding standards (data not shown), confirming the identity of these peaks.

Upon subsequent chondro-4-sulfatase digestion, the peak eluted at the position of ΔHexUA1→3Gal(4-O-sulfate)β1→3Galβ1→4Xyl-2AB was shifted to the position of the authentic non sulfated 2AB-tetrasaccharide (Fig. 4C). These data indicate that the compound in the peak contained a 4-O-sulfate...
group most probably located on the C4 position of the Gal(II) residue in the linkage tetraraccharide sequence GlcUAβ1-3Gal(II)β1-3Gal(II)β1-4Xyl. Although it was not feasible to distinguish between ΔHexUAα1-3Galβ1-3Galβ1-4Xyl and ΔHexUAα1-3Galβ1-3Galβ1-4Xyl because of the lack of the latter authentic standard, the latter structure has not been reported and is therefore unlikely. Upon subsequent alkaline phosphatase digestion, the peak that eluted at the position of ΔHexUAα1-3Galβ1-3Galβ1-4Xyl(2-O-phosphate)-2AB was shifted by 16 min to the position of the nonsulfated tetraraccharide-2AB in a molar ratio of 55:45. When this sample was co-chromatographed with the standard linkage tetraraccharides, the two peaks were co-eluted with the corresponding standards (data not shown). Upon subsequent alkaline phosphatase digestion, the peak that eluted at the position of ΔHexUAa1-3Gal-Gal-Xyl(2-O-phosphate)-2AB was shifted to the elution position of the nonsulfated tetraraccharide (Fig. 5B), indicating that the compound in the peak contained a phosphate group most probably located on the C2 position of the Xyl residue in the linkage region tetraraccharide sequence as discussed above for the CS linkage region. The broadness of the peak of the dephosphorylated tetraraccharide-2AB in the alkaline phosphate digest was observed as in the case of the HS-derived sample.

Unique modifications of the sequence in the vicinity of the linkage region may render the linkage region resistant to chondroitinases and/or heparinase/heparitin-sulfate lyase. Therefore, the enzymatic susceptibility of the GAG chains was investigated. The 2AB-labeled GAG preparation was analyzed by gel filtration HPLC (see under "Experimental Procedures") before and after the enzymatic digestion to clarify the susceptibility of the linkage region structures to the enzymes. When the enzymatic digest that was prepared by treatment with a mixture of heparinase and heparitin-sulfate lyase and subsequently with chondroitin ABC and AC lyases was analyzed, two major peaks and a minor peak were detected at the elution positions of the authentic linkage 2AB-tetarraccharides, ΔHexUAα1-3Galβ1-3Galβ1-4Xyl(2-O-phosphate)-2AB, and ΔHexUAα1-3Galβ1-3Galβ1-4Xyl(2-O-phosphate)-2AB, respectively (data not shown). The identity of the two major peaks was confirmed by anion-exchange HPLC of the isolated fractions on an amine-bound silica PA-03 column (data not shown). Thus, the peaks detected on the gel filtration HPLC analysis must be derived from the GAG-protein linkage region of syndecan-1. No other peaks were detected on the gel filtration HPLC analysis of the enzymatic digest, indicating that the 2AB-tetraraccharides obtained in this study accounted for all the GAG chains...
of syndecan-1. Taken together, these findings indicate that the GAGs from syndecan-1 are composed of the structures summarized in Fig. 3.

Syndecan-1 ectodomains may contain immature truncated small O-linked oligosaccharides, which are structurally related to the GAG-protein linkage region. Therefore, we examined the putative oligosaccharide fractions (Fr. A and B) observed after the 2AB-GAG fraction (Fig. 1) when the 2AB-derivatized saccharide fraction was subjected to gel filtration chromatography on a PD-10 column (see under “Experimental Procedures”). Although both Frs. A and B gave a few major and several very minor fluorescent peaks when analyzed by anion-exchange or gel filtration HPLC (see under “Experimental Procedures”), none of the major peaks was observed at the elution position of the authentic 2AB-tetrasaccharide, GlcUAβ1→3Galβ1→4Xyl-2AB, prepared from α-thrombomodulin (57), or sensitive to the action of β-glucuronidase. These peaks were also resistant to heparitin-sulfate lyase, indicating the absence of the truncated pentasaccharide, GlcNAcα1→4GlcUAβ1→3Galβ1→4Galα1→3Galβ1→4Xyl-2AB. These results were consistent with the data obtained by matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis (not shown), which showed no signals corresponding to the 2AB-derivatives of the linkage pentasaccharides or tetrasaccharides. Hence, the syndecan-1 ectodomains did not contain appreciable amounts of immature linkage region tetrasaccharides or pentasaccharides, although the possibility cannot be excluded because of the limit of the sensitivity of the method that Frs. A or B contained the linkage region-associated disaccharide or trisaccharide stubs.

**DISCUSSION**

In this study, we determined the structure of the GAG-protein linkage region and the disaccharide composition and average size of the CS and HS chains of purified syndecan-1 ectodomains from the surfaces of NMuMG cells. The phosphorylation of the Xyl residue in the linkage region was shown for both HS and CS chains, whereas the 4-O-sulfation of the Gal residue was demonstrated only for the CS but not for HS chains, which was in good agreement with our previous findings (8). In the CS linkage region, the Gal sulfation and Xyl phosphorylation were not found on the same chain, which is also consistent with the previous proposal that they are mutually exclusive (13, 14). These modifications show a rather wide distribution in various tissues of many different vertebrate species. 2-O-Phosphorylation of Xyl has been found for CS from shark cartilage (13), rat (40) and mouse (12) tumors, and for CS/DS from rat fibroblast cell line (41). It was also found for HS/HeP from bovine lung (10, 42). 4-O-Sulfation of Gal has been reported for CS from rat chondrosarcoma (7), whale cartilage (11), bovine cartilage (18, 43), human trypsin inhibitors (16, 17); it was also reported for DS from bovine aorta (15). In addition, Gal 6-O-sulfation has also been revealed for CS from shark cartilage (13, 14) and bovine articular cartilage (19).

The GAG-protein linkage region is formed by the sequential stepwise addition of each monosaccharide residue from the corresponding sugar nucleotide to a specific serine residue in a core protein (1, 8). It is conceivable that the biosynthesis of the linkage region tetrasaccharide is strictly regulated, because it is located at the critical determining point not only for the selective chain assembly of HS/HeP and CS/DS but also for converting proteins into PGs. α-GlcNAc transferase I (44–46) and putative β-GalNAc transferase I (47, 48), which transfer the first GlcNAc and GalNAc residue, respectively, are thought to be the key enzymes that determine the GAG species to be synthesized on the common tetrasaccharide linkage region (8). Hence, an intriguing possibility exists where the Gal 4-O-sulfate structure may be a biosynthetic sorting signal for the selective chain assembly of CS/DS and recognized by putative β-GalNAc transferase I or chondroitin synthase (8), although the amino acid sequence of the core protein also has a profound influence on the type of GAG chains to be synthesized (49–52). The verification of this hypothesis awaits cloning and expression of the transferase gene.

A number of PG precursor proteins often lack GAG side chains, thus called part-time PGs (8, 20). However, the biosynthetic molecular mechanism by which a given protein containing the GAG attachment consensus amino acid sequence becomes a PG remains obscure. For investigating this issue, it is essential to clarify the substrate specificities and regulatory mechanisms of the glycosyltransferases involved in the synthesis of the linkage region tetrasaccharide sequence. In this respect, the modification of the linkage region by phosphorylation and sulfation may play important roles in the assembly of GAG chains of PG core proteins. The modification of the linkage region of the CS and HS derived from syndecan-1 purified from the same source was only found on some chains and not on others. The modifications were also heterogeneous as seen for the discrete phosphorylated and sulfated linkage region structures. However, a prominent example of the homogeneous linkage region structure has been revealed for inter-α-trypsin inhibitor (16) and urinary trypsin inhibitor (17), which bear a single CS chain with a uniform linkage region structure, GlcUAβ1→3Gal(4-O-sulfate)β1→3Galβ1→4Xyl. These findings show the wide distribution of modifications including this structure suggest the possibility that the phosphate and/or sulfate groups are added uniformly to the specific positions in the linkage region and removed enzymatically during the early stages in the biosynthesis of the linkage region, thus exhibiting dynamic processing features. Notably, a variety of linkage region structures have been reported for CS chains from shark (13, 14), bovine (43), and human (43) cartilage.

Although the exact subcellular compartments for the Xyl phosphorylation and Gal sulfation remain unknown and the responsible kinase and sulfotransferases have not been identified, they may take place in the endoplasmic reticulum or Golgi. Fransson and his co-workers (41, 53, 54) have reported that the phosphorylation of Xyl is a transient phenomenon and is not inhibited by brefeldin A, which interferes with progression from the endoplasmic reticulum to Golgi. They have also found in the early steps of the CS chain biosynthesis of decorin that the degree of phosphorylation increases to ~90% until the linkage region grows into the Gal-Gal-Xyl trisaccharide, and then dephosphorylation takes places extensively accompanied by glucuronidation. This may indicate that the phosphate group on the Xyl residue plays an important role for the transfer reaction of a GlcUA residue to the trisaccharide. In fact, we have observed that the synthetic phosphorylated trisaccharide, Gal-Gal-Xyl(2-O-phosphate)-Ser, served as a better acceptor substrate than the unmodified counterpart for recombinant human GlcUA transferase. The acceptor recognition by a crystallized form of this GlcUA transferase I has recently been demonstrated by x-ray crystallography (55), and it has further been revealed that the crystallized enzyme specifically recognized the 6-O-sulfate group on the Gal(I) residue adjacent to the Xyl residue, although it remains to be determined whether the phosphate on the Xyl and/or the 4-O-sulfate group on the Gal(I) residue can also be recognized by the crystallized enzyme. These findings suggest that the phosphorylation of the Xyl residue and sulfation of the Gal residues may be required...
for efficient elongation and maturation of the linkage region tetrasaccharide as prerequisites for the assembly of GAG chains.

Syndecan-1 core protein contains three potential HS attachment sites near its N terminus and two possible CS attachment sites in the extracellular domain near its transmembrane region (56). HS and CS of the analyzed core protein (3.7 μg) of 33 kD, which corresponded to 0.11 nmol, yielded 24 and 8 nmol of disaccharides, and 0.23 and 0.17 nmol of linkage oligosaccharides, respectively, indicating that the average sizes of HS and CS chains were ~52 and 23 kDa, respectively. The average size of the HS chains was roughly within the reported range (28), although the average size of the CS chains has not previously been reported. The recoveries of the HS and CS linkage region oligosaccharides were ~70 and 77%, respectively, of the expected values, suggesting that all GAG attachment sites might not be occupied by GAG chains as described previously (56).

We have previously shown that unglycanated α-thrombomodulin (a non-PG form) has the linkage tetrasaccharide at the GAG attachment site (57), suggesting that a critical determinant, modulin (a non-PG form) has the linkage tetrasaccharide at the C4 or C6 position of GalNAc residues but also remains to be clarified what specific functions the CS-E structure of syndecan-1 exhibits in different biological systems.

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