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Development of a mouse monoclonal antibody against the chondroitin sulfate-protein linkage region derived from shark cartilage

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Abstract Glycosaminoglycans (GAGs) like chondroitin sulfate (CS) and heparan sulfate (HS) are synthesized on the tetrasaccharide linkage region, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser, of proteoglycans. The Xyl can be modified by 2-*O*-phosphate in both CS and HS, whereas the Gal residues can be sulfated at C-4 and/or C-6 in CS but not in HS. To study the roles of these modifications, monoclonal antibodies were developed against linkage glycopeptides of shark cartilage CS proteoglycans, and one was characterized in detail. This antibody bound hexa- and pentasaccharide-peptides more strongly than unsaturated tetrasaccharide-peptides with the unnatural fourth sugar residue (unsaturated hexuronic acid), suggesting the importance of the fifth and/or fourth saccharide residue GalNAc-5 and/or GlcA-4. Its reactivity was not affected by treatment with chondro-4-sulfatase or alkaline phosphatase, suggesting that 4-*O*-sulfate on the Gal residues and 2-*O*-phosphate on the Xyl residue were not recognized. Treatment with weak alkali to cleave the Xyl-Ser linkage completely abolished the binding activity, suggesting the importance of the peptide moiety of the hexasaccharide-peptide for the binding. Based on the amino acid composition and matrix-assisted laser desorption ionization time-of-flight mass spectrometry analyses, it was revealed that the peptide moiety is composed of four amino acids, Ser, Pro, Gly, and Glu. Furthermore, the antibody stained wild-type CHO cells significantly, but much weakly mutant cells deficient in xylosyl- or galactosyltransferase-I required for the biosynthesis of the linkage region. These results suggest that the antibody recognizes the structure GalNAc (±6-*O*-sulfate)-GlcA-Gal-Gal-Xyl-Ser-(Pro, Gly, Glu). The antibody will be a useful tool for investigating the significance of the linkage region in the biosynthesis and/or intracellular transport of different GAG chains especially since such tools to study the linkage region are lacking.

Keywords Proteoglycans · Glycosaminoglycans · Chondroitin sulfate · Heparan sulfate · Dermatan sulfate · Monoclonal antibody

Abbreviations

2AB	2-aminobenzamide
BSA	bovine serum albumin
CS	chondroitin sulfate

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DS	dermatan sulfate
ELISA	enzyme-linked immunosorbent assay
GAG	glycosaminoglycan
Gal	D-galactose
GalNAc	*N*-acetyl-D-galactosamine
GlcA	D-glucuronic acid
GlcN	D-glucosamine
GlcNAc	*N*-acetyl-D-glucosamine
Hep	heparin
ΔHexA	4,5-unsaturated hexuronic acid or 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid
HPLC	high performance liquid chromatography
HS	heparan sulfate
IdoA	L-iduronic acid
PG	proteoglycan
Xyl	D-xylose

Introduction

Proteoglycans (PGs) are macromolecules composed of linear polysaccharide glycosaminoglycan (GAG) side chains, which are covalently attached to specific Ser residues of core proteins. PGs are distributed mainly in extracellular matrices and at cell surfaces, and implicated in many pathophysiological phenomena. Characteristic GAG moieties appear to play important roles as regulators of various biological processes by interacting with functional protein ligands such as growth factors, cytokines, and morphogens [1–4].

GAGs include chondroitin sulfate/dermatan sulfate (CS/DS) and heparan sulfate/heparin (HS/Hep), which are classified as galactosaminoglycans and glucosaminoglycans, respectively. CS/DS and HS/Hep consist of repeating disaccharide units, GlcA/IdoA-GalNAc and GlcA/IdoA-GlcN/GlcNAc, respectively. GlcA, IdoA, GalNAc, GlcN, and GlcNAc represent D-glucuronic acid, L-iduronic acid, *N*-acetyl-D-galactosamine, D-glucosamine, and *N*-acetyl-D-glucosamine, respectively. GAGs are specifically modified most notably by sulfation at various positions, forming a variety of structures and acquiring functions. Although the structure of the repeating disaccharide region differs between CS/DS and HS/Hep, both types of GAGs are covalently bound to core proteins through the common linkage region tetrasaccharide, GlcAβ1-3Galβ1-3Galβ1-4Xyl [5], where Gal and Xyl stand for D-galactose and D-xylose, respectively.

In the biosynthesis of GAGs, monosaccharide residues are transferred stepwise from the corresponding nucleotide sugars to growing GAG chains, being governed largely by the substrate specificity of the glycosyltransferases involved. This process is initiated by the addition of Xyl to specific serine residues in the core protein, followed by the sequential addition of two Gal residues and a GlcA residue to construct the tetrasaccharide linkage structure [1]. The synthesis of CS/DS chains initiates once GalNAc is transferred by the *N*-acetylgalactosaminyltransferase-I (GalNAcT-I) activity of chondroitin synthases to the GlcA of the common linkage region, whereas that of HS/Hep chains initiates if GlcNAc is first added by the *N*-acetylglucosaminyltransferase-I (GlcNAcT-I) activity [1, 4, 6]. Hence, GalNAcT-I and GlcNAcT-I activities are crucial for the determination of the GAG species to be synthesized on the common tetrasaccharide linkage region. However, it is not clear how these different GAGs are selectively assembled on the common structure.

We have carried out a series of structural studies of the GAG-protein linkage region, based on the working hypothesis that there may be differences in the region's structure among GAG chains and such differences may contribute to the determination of the type and/or character of the GAG species to be synthesized [6, 7]. These structural studies have revealed unique modifications, such as 4-*O*-sulfated Gal, 6-*O*-sulfated Gal, and 2-*O*-phosphorylated Xyl. So far, sulfated Gal residues have been demonstrated only in the linkage region of CS/DS, not in HS/Hep, though a 2-*O*-phosphorylated Xyl residue has been found in both CS/DS and HS/Hep [6, 8, 9], suggesting the sulfate groups on the Gal residues to be involved in the selective assembly of different GAG types. In fact, it has been demonstrated that these modifications influence the catalytic activities of galactosyltransferase-I, which transfers Gal-2 (for the numbering of the sugar residues, see Table 1), and glucuronosyltransferase-I (GlcAT-I), which transfers the first GlcA, involved in the construction of the tetrasaccharide linkage region [10, 11]. However, it is not fully understood if these modifications also affect other biosynthetic enzymes or play a biological role as recognition signals for regulatory proteins such as intracellular transporters.

The use of antibodies has become a standard approach in many fields of biochemical and biomedical research. To clarify the biological significance of the modifications in the GAG-protein linkage region, antibodies which recognize specific patterns of modification in the region may be useful. Monoclonal antibodies (mAbs) that recognize specific features of the repeating disaccharide region of CS chains have been generated, and epitopes of some of them have been characterized [12–16]. Studies using anti-CS mAbs have revealed restricted spatiotemporal patterns of the expression of specific CS structures in various tissues during growth and development, and in pathological conditions [16–19]. However, so far, no antibodies have been reported, which recognize the GAG-protein linkage region. In the present study, a mAb against the CS-protein

Table 1 Structure of the major components in the linkage hexasaccharide fractions purified from CS of shark cartilage and whale cartilage

ΔHexAα1-3GalNAcβ1-4GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser

6	5	4	3	2	1	
Modifications[a]						
CS from shark cartilage[b]						Proportion[c] (mol%)
—	—	—	—	—	—	13.1
—	6S	—	—	—	—	8.3
—	4S	—	—	—	—	2.1
—	—	—	—	—	2P	6.7
—	6S	—	—	—	2P	3.4
—	—	—	—	6S	—	5.3
—	6S	—	—	6S	—	4.8
—	4S	—	—	6S	—	1.5
—	—	—	6S	6S	—	5.5
—	6S	—	6S	6S	—	37.4
—	4S	—	6S	6S	—	9.1
—	6S	—	4S	6S	—	1.1
—	4S	—	4S	6S	—	1.6
CS from whale cartilage[d]						
—	—	—	—	—	—	21
—	6S	—	—	—	—	16
—	4S	—	—	—	—	36
—	4S	—	4S	—	—	27

[a] The modifications in each monosaccharide residue are shown in the table. The numbers refer to the corresponding residues in the linkage structures shown above. 4S, 6S, and 2P stand for 4-*O*-sulfate, 6-*O*-sulfate, and 2-*O*-phosphate, respectively

[b] Data are from Sugahara *et al.* 1992 [22] and de Waard *et al.* [27]

[c] The proportion of each structure is expressed as a molar percentage of the total linkage hexasaccharide fraction

[d] Data are from Sugahara *et al.* 1991 [21]

linkage region from shark cartilage was developed and its epitope was characterized.

Materials and methods

Materials PGs from salmon nasal cartilage, chicken cartilage, and whale cartilage were provided by Biomatec Japan Inc. (Kushiro, Japan). PG from rayfish cartilage was obtained from Marukyou Biofoods Co., Ltd. (Kushiro, Japan). PG from birds' nests made with dried saliva of male *Collocalia* swiftlets was provided by Y. T. Li, Tulane University [20]. PG from bovine nasal cartilage was a gift from the late Dr. Albert Dorfman, University of Chicago. A whale cartilage CS-peptide fraction, which contained Ser, Asp, Thr, Glu, Pro, Gly, Ala, Val, Lys and Arg in a molar ratio of 1.00 : 1.74 : 0.42 : 1.99 : 1.53 : 4.22 : 1.00 : 0.52 : 0.63 : 0.65, was prepared as reported [21]. A CS-peptide fraction from salmon PG was prepared by the same protocol as described previously [22].

The following enzymes were purchased from Seikagaku Corp. (Tokyo, Japan): chondroitinase (CSase) ABC (EC 4.2.2.20) from *Proteus vulgaris*, CSase AC-I (EC 4.2.2.5) from *Fravobacterium heparinum*, CSase AC-II (EC 4.2.2.5) from *Artherobacter aurescens*, and chondro-4-sulfatase (EC 3.1.6.9) from *P. vulgaris*. Calf intestinal alkaline phosphatase (EC 3.1.3.1) of special quality for molecular biology was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Anti-HS antibody F58-10E4 and Alexa Fluor 488®-labeled goat anti-mouse IgM antibody were purchased from Seikagaku Corp. (Tokyo, Japan) and Molecular Probes (Eugene, OR), respectively.

Wild-type Chinese hamster ovary (CHO) cells (CHO-K1), xylosyltransferase-deficient CHO cells (pgsA-745) [23], and galactosyltransferase-I-deficient CHO cells (pgsB-618) [24] were purchased from American Type Culture Collection (Manassas, VA). These cell lines were maintained in Ham's F12K medium (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

The structurally defined linkage hexasaccharide ΔHexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl was isolated from whale cartilage CS-PG [21]. ΔHexA stands for 4,5-unsaturated hexuronic acid. A 2-aminobenzamide (2AB)-derivative of the linkage hexasaccharide ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-2AB, was prepared as described previously [25]. The tetrasaccharide peptide GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-Ser-Gly was chemically synthesized [26].

Preparation of the oligosaccharide-peptides from the CS-core protein linkage region The CS-peptide fraction (1.0 g) was prepared from shark cartilage [22, 27] and exhaustively digested with 1.2 IU of CSase ABC in a total volume of 12.5 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 60 mM sodium acetate and 0.1% bovine serum albumin (BSA) for 25 h at 37°C. An additional 0.2 IU of the enzyme was added after 23 h to complete the digestion, and the reaction was terminated by heating at 100°C for 5 min. The digest was fractionated by gel-filtration using a column (2.0×149 cm) of Sephadex G-15 (GE Healthcare, Buckinghamshire, UK) with 0.25 M NH_4HCO_3/7% 1-propanol as the eluent. The isolated linkage hexasaccharide-peptide fraction was digested with CSase ABC again as described above to complete the digestion, and this process was repeated three times until no disaccharide was produced as judged by gel-filtration chromatography. This fraction

contained several amino acids including Ser, Glu, Pro, Gly, Val, and Lys in a molar ratio of 1.00, 1.16, 1.41, 1.23, 0.46, and 0.29, respectively [22].

To eliminate the ΔHexA residue, which has strong immunogenic activity, from the nonreducing end of the linkage hexasccharide-peptides, the glycopeptides were treated with mercuric acetate as described previously [28, 29]. Namely, the linkage hexasaccharide-peptide fraction (1.5 μmol as linkage hexasaccharide) was treated with 0.2 ml of 10 mM mercuric acetate in 130 mM sodium acetate buffer, pH 5.0, overnight at room temperature. After the addition of 2 ml of 5 M NaCl, the reaction mixture was desalted by successive gel-filtration using a Sephadex G-25 (fine) column (1.5×47 cm) equilibrated with 1 M NaCl and then with H_2O.

The linkage tetrasaccharide-peptides were prepared by treatment of the linkage hexasaccharide-peptides (120 nmol as ΔHexA) with 10 mIU of CSase AC-I [30] in a total volume of 30 μl of 50 mM of Tris/HCl buffer, pH 7.3, at 37°C for 20 h. The enzymatic reaction was terminated by heating at 95°C for 1 min.

The linkage oligosaccharide-peptide fraction was treated with LiOH as described below to release the oligosaccharide from the core peptides, labeled with 2AB and analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column (4.6×250 mm, YMC Co., Kyoto, Japan) to confirm the presence of the linkage oligosaccharide components predicted. The molar concentration of the linkage oligosaccharide-peptides was determined by measuring absorbance at 232 nm based on an average millimolar absorption coefficient of 5.5 for the double bond of ΔHexA [31] or by the carbazole reaction to measure uronic acids (GlcA and ΔHexA) colorimetrically [32].

Production of the monoclonal antibody Monoclonal antibodies were generated by immunizing BALB/c mice with the linkage pentasaccharide-peptide fraction from shark cartilage CS. The linkage pentasaccharide-peptide fraction was conjugated with a carrier protein, keyhole limpet hemocyanin (KLH) (Sigma, St Louis, MO), using glutaraldehyde [33], which cross-links primary amino groups of peptides as described below: 1 μmol of the fraction was conjugated with 4 mg of KLH in 2 ml of phosphate-buffered saline (PBS), pH 7.2, with the addition of glutaraldehyde to a final concentration of 0.1% at room temperature overnight. The reaction was terminated by addition of 0.25 ml of 1 M glycine, and then the conjugates were dialyzed overnight against PBS. The KLH-conjugated linkage pentasaccharide-peptide fraction was injected into mice at 20 μg/injection every 2 weeks. After the fourth injection, the serum was screened for reactivity with the linkage hexasaccharide-peptides. Spleen B lymphocytes of the positive mice were isolated and fused with myeloma cells. The culture supernatant of the fused hybridoma cells was screened by enzyme-linked immunosorbent assay (ELISA) using the linkage hexasaccharide-peptides, and three positive clones, 4E1, 3F11, and 1B5, were selected. The clone 4E1 with the highest specificity toward the linkage hexasaccharide-peptide fractions was recloned, and eleven secondary clones were further selected. Among the eleven clones, six showed high specificity and were injected intraperitoneally into mice to obtain ascitic fluid. Antibody subclasses were determined using the ImmunoPure® Monoclonal Antibody Isotyping Kit II (Pierce Biotechnology, Rockford, IL). The mAb 4E1/D6 was of particular interest because of its specific activity, and subsequently characterized in detail.

ELISA The specificity of the antibody 4E1/D6 in terms of antigen recognition was tested by ELISA. Briefly, various PG preparations (10 μg each) or linkage oligosaccharide-peptide fractions (0.5 μg) were dissolved in a 0.2 M sodium bicarbonate buffer, pH 9.6 and individually immobilized overnight to a 96-well microtiter plate (Nunc immune plate, MaxiSorp, Nalge Nunc International, Rochester, NY) at room temperature. The wells were washed once with PBS, pH 7.4, containing 0.05% Tween 20 (PBST), and blocked with 3% (w/v) BSA in PBS for 1 h at 37°C. The wells were then washed with PBST once and incubated with 4E1/D6 for 2 h at 37°C. After three washes with 25 mM Tris-buffered saline (TBS) containing 0.05% Tween20 (TBST), the wells were incubated with alkaline phosphatase-labeled anti-mouse Ig(G+M) (3,000-fold dilution, Chemicon, San Diego, CA). Enzymatic activity was detected using *p*-nitrophenylphosphate by measuring the absorbance at 415 nm.

For the competitive ELISA, aliquots of the linkage hexasaccharide-peptides from shark cartilage CS, which had been pretreated with LiOH, were incubated with 4E1/D6 in a total volume of 50 μL at 37°C for 1 h, then the mixture was applied to the wells where untreated linkage hexasaccharide-peptides from shark cartilage CS had been immobilized. The color was developed as described above.

Chemical and enzymatic treatments of the linkage hexasaccharide-peptide fraction To liberate *O*-linked linkage oligosaccharides from the core peptides, treatment with LiOH was performed as described previously [25, 34]. Briefly, the hexasaccharide-peptide fraction (183 nmol) was treated with 0.5 M LiOH at 4°C for 15 h. The reaction was terminated by neutralization with 2.0 M acetic acid, and then Li^+ was removed by cation-exchange chromatography using AG 50W-X2 resin (Bio-Rad Laboratories, Hercules, CA).

To investigate the reactivity of the antibody 4E1/D6 to the 4-*O*-sulfated or 2-*O*-phosphorylated linkage structure, treat-

ment with chondro-4-sulfatase or alkaline phosphatase was conducted. One nmol of the linkage hexasaccharide-peptide fraction or a structurally defined linkage hexasaccharide, ΔHexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl, was digested with the indicated enzyme. Digestion with chondro-4-sulfatase was performed with 12 mIU of the enzyme for 6 h in 3 μL of 50 mM Tris/HCl buffer, pH 7.5, containing 50 mM sodium acetate [35]. Treatment with alkaline phosphatase was carried out with 1 IU of the enzyme in a total volume of 3 μL of the buffer supplied by the manufacturer at 37°C for 6 h.

Delayed extraction matrix-assisted laser desorption ionization time-of-flight (DE MALDI-TOF) mass spectrometry (MS) The purified hexasaccharide-peptide fraction was analyzed by DE MALDI-TOF MS in the linear mode by Voyager-DE STR-H (Applied Biosystems, Foster, USA) [25, 36]. Each sample (10 pmol) was mixed with a matrix, 2,5-dihydroxybenzoic acid, on a sample plate well, dried under an air stream, and then analyzed by MS in the positive mode.

Immunofluorescence flow cytometry CHO-K1, pgsA-745, and pgsB-618 cells were detached with 2 mM EDTA and suspended in PBS containing 0.1% BSA (PBS-BSA) at a concentration of 10^6 cells/ml. After three washes with PBS-BSA, the cells were incubated with the antibody 4E1/D6 (400-fold dilution) or a commercial antibody F58-10E4 against HS (200-fold dilution) at 4°C for 30 min. The cells were washed with PBS-BSA three times and incubated with Alexa Fluor 488®-labeled goat anti-mouse IgM antibody (500-fold dilution). After three washes with PBS-BSA, the cells were analyzed by immunofluorescence flow cytometry in a BD FACSCanto (BD Biosciences, San Jose, CA). Flow cytometric data were analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).

Stable overexpression of human chondroitin 6-O-sulfotransferase-1 (C6ST-1) in CHO-K1 cells For construction of the stable overexpression vector of human C6ST-1, the cDNA fragment encoding human *C6ST-1* was amplified by PCR with the pGEM-T Easy-human C6ST-1 as a template [37] using *KOD*-Plus DNA polymerase (Toyobo, Tokyo, Japan). The PCR fragments were subcloned into the *Eco*RV and *Kpn*I sites of the pcDNA3.1/myc-His expression vector (Invitrogen, Carlsbad, CA). The linearized plasmid (6.7 μg) with *Pvu*I was transfected into CHO cells (5×10^6 cells) on 100-mm plates using FuGENE6 (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Transfectants were cultured in the presence of 0.5 mg/ml G418 (Wako Pure Chemical, Osaka, Japan). Then resultant colonies were subcultured on 96-well plates at a low density (1 cell/well), and propagated. To select the clones overexpressing C6ST-1, sulfotransferase activity toward chondroitin was assayed using cell lysates as the enzyme source and [^{35}S]-3′-phosphoadenosine 5′-phosphosulfate as a donor substrate by the method described, previously [37, 38].

Results

Production of the monoclonal antibody against the linkage oligosaccharide-peptide fraction of shark cartilage CS The CS-peptide fraction of shark cartilage was exhaustively digested with CSase ABC to prepare the linkage hexasaccharide-peptide fraction. Approximately 9.2 μmol of this fraction was obtained and an aliquot was analyzed by anion-exchange HPLC on an amine-bound silica PA-03 column after cleavage of the bond between the xylose and the serine using LiOH [25, 34] (results not shown). The fraction contained at least thirteen hexasaccharide compounds as reported (Table 1) [22, 27]. Since the glycopeptides contain a ΔHexA residue at the nonreducing terminus, which is an unnatural structure causing strong antigenicity, the ΔHexA residue was eliminated by treatment with mercuric acetate [28, 29]. The pentasaccharide-peptide fraction was conjugated with KLH and used as an immunogen to develop mAbs against the CS-core protein linkage region.

After immunization of BALB/c mice with the immunogen, six positive clones were obtained by screening assays using the CS hexasaccharide-peptide fraction immobilized onto a Nunc Maxisorp plate. Clone 4E1/D6, with the highest level of activity, was characterized further. This clone contained an IgM-type immunoglobulin with kappa light chains (data not shown), and the concentration of protein in ascitic fluid was 34.3 mg/ml as determined using the BCA protein assay kit according to the instructions provided by the manufacturer (Thermo Scientific, Rockford, IL).

Assessment of the reactivity of the antibody 4E1/D6 toward various PGs To characterize the specificity of 4E1/D6, the antibody's reactivity toward various PGs was assessed by ELISA (Fig. 1), where PGs were individually immobilized onto a Nunc Maxisorp plate through their protein moiety. The linkage hexasaccharide-peptide fraction from shark cartilage CS was also immobilized as a positive control. Significant binding of 4E1/D6 was observed with the linkage hexasaccharide fraction and a PG from salmon nasal cartilage, but with no other PGs tested. The reactivity of 4E1/D6 toward this PG was confirmed by a competitive ELISA: a soluble PG from salmon nasal cartilage inhibited the binding of 4E1/D6 to the immobilized linkage

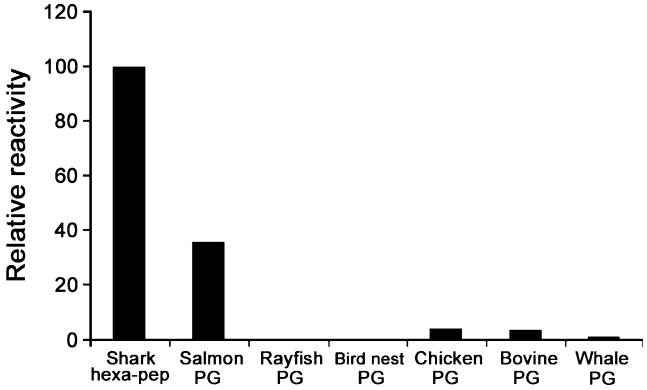

Fig. 1 Reactivity of the antibody 4E1/D6 toward various PG preparations. The reactivity of the antibody 4E1/D6 with various PGs was analyzed by ELISA. PGs from salmon nasal cartilage, rayfish cartilage, bird nest, chicken cartilage, bovine nasal cartilage, whale cartilage (10 μg each) and the linkage hexasaccharide peptide (hexa-pep) fraction derived from shark cartilage CS (0.5 μg) were individually immobilized to the wells, and processed for incubation with 4E1/D6 followed by alkaline phosphatase-linked goat anti-mouse Ig(G+M) (diluted 3,000-fold). Bound antibodies were detected by the addition of *p*-nitrophenylphosphate as a substrate. The reactivity is given relative to that of the linkage hexasaccharide-peptide fraction from shark cartilage CS

hexasaccharide-peptide fraction of shark cartilage CS in a dose-dependent manner (data not shown). 4E1/D6 reacted less extensively to the salmon PG than the linkage hexasaccharide-peptide fraction and not at all to the other PGs tested. The difference in the reactivity among PGs may be due to the difference in the CS modification of the linkage region and/or the structure of the core protein. The structural difference among these PGs remains to be investigated. Although the same amount (10 μg) of PGs was used, the molar amount of the linkage region is most likely different.

Assessment of the reactivity of 4E1/D6 toward the linkage region oligosaccharides To investigate the structural features of the linkage pentasaccharide-peptides required for recognition by 4E1/D6, the reactivity of 4E1/D6 toward linkage hexa-, penta-, and tetrasaccharide-peptide fractions of shark cartilage CS was assessed by ELISA (Fig. 2a). The linkage tetrasaccharide-peptide fraction was prepared by digestion of the linkage hexasaccharide-peptide fraction with CSase AC-I, and the exhaustive digestion was confirmed by gel-filtration chromatography of the digest. Notably, the fourth saccharide residue GlcA-4 in the linkage region should have been converted to an artificial unsaturated hexuronic acid (ΔHexA) due to the eliminative cleavage by digestion with CSase. The linkage hexa-, penta-, and tetrasaccharide-peptide fractions (0.5 μg each) were immobilized onto Maxisorp plates. The reactivity was considerably weaker toward the tetrasaccharide-peptides than hexa- or pentasaccharide-peptides of shark cartilage CS (Table 1). These results suggest the fifth and/or fourth saccharide residue GalNAc-5 and/or GlcA-4 in the linkage oligosaccharide-peptide fraction to be important for recognition by 4E1/D6. In strong contrast, the hexasaccharide-peptide fraction of whale cartilage CS, which contains four major saccharide sequences with different sulfation patterns (Table 1), showed no reactivity, suggesting that the 4E1/D6 epitope may contain a particular modification in the linkage region. In addition, 4E1/D6 may distinguish the different amino acid sequences in the peptide moiety of the linkage oligosaccharide-peptide of shark cartilage CS from those of whale cartilage CS, since their amino acid composition differs considerably (see above).

The reactivity of the antibody toward the tetrasaccharide linkage region of salmon PG was also examined. The digest of salmon PG with CSase ABC or a mixture of CSases ABC and AC-II, which most likely contains hexasaccharide

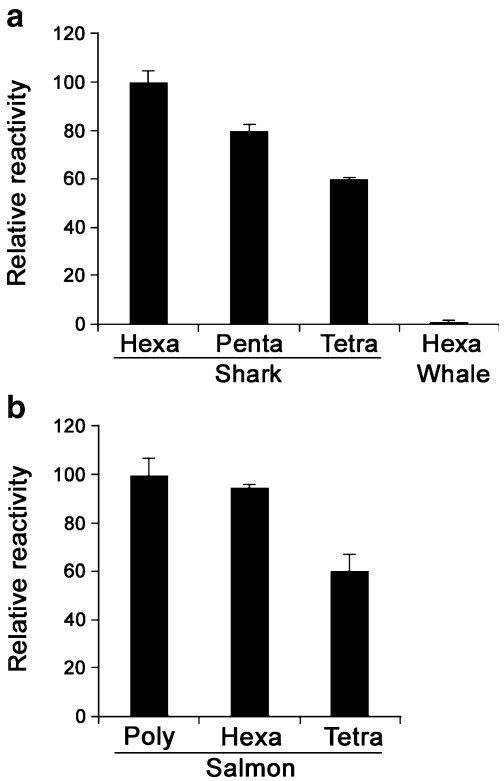

Fig. 2 Reactivity of the antibody 4E1/D6 toward the linkage region. (**a**) The linkage hexa-, penta-, and tetrasaccharide-peptide fractions derived from shark cartilage CS and the linkage hexasaccharide-peptide fraction from whale cartilage CS (0.5 μg each) were immobilized to the wells. (**b**) The digest of salmon PG (10 μg) with CSase ABC (Hexa) or a mixture of CSases ABC and AC-II (Tetra), which most likely contains a hexasaccharide or tetrasaccharide linkage region, respectively, was immobilized to the wells. The reactivity of 4E1/D6 to the immobilized fractions was analyzed by ELISA as described in the legend to Fig. 1. The assay was performed in duplicate and values represent the mean ± S.D. The reactivity is given relative to that of the linkage hexasaccharide-peptide fraction from shark cartilage CS (**a**) or the intact salmon PG (Poly) (**b**)

or tetrasaccharide linkage region, respectively, were immobilized, and their reactivity was analyzed by ELISA (Fig. 2b). 4E1/D6 recognized the digest with a mixture of CSases ABC and AC-II only weaker than the intact salmon PG and its digest with CSase ABC, suggesting the importance of the fifth and/or fourth saccharide residue GalNAc-5 and/or GlcA-4 for recognition by the antibody. Since 4E1/D6 showed the similar reactivity toward the intact salmon PG and its digest with CSase ABC, the accessibility of the antibody to the linkage region does not seem to be inhibited by the CS polysaccharide side chains.

Assessment of the reactivity of 4E1/D6 toward the peptide moiety of the linkage hexasaccharide-peptides To assess whether 4E1/D6 recognizes the peptide moiety of the linkage hexasaccharide-peptides, a competitive ELISA was carried out using the linkage hexasaccharide fraction from shark cartilage CS treated with LiOH as an inhibitor (Fig. 3a). Treatment with LiOH liberates hexasaccharides from the core peptide. Although the hexsaccharide-peptide fraction strongly inhibited the antibody binding, the LiOH-treated sample did not inhibit at all the binding of 4E1/D6 to the linkage hexasaccharide-peptides of shark cartilage CS, suggesting that the xylosidic linkage to the Ser residue and/or a few amino acid residues immediately adjacent to the Ser residue also seem to be involved in the recognition by 4E1/D6.

Analysis of the peptide moiety of the linkage hexasaccharide-peptide from shark cartilage CS The peptide sequence of the major component in the linkage hexasaccharide-peptide fraction was analyzed by automated Edman degradation. However, it failed, probably because the glycopeptide is too small in size and too hydrophilic due to the sulfated saccharide moiety. Thus, the sample was subjected to a DE MALDI-TOF MS analysis in the positive ion mode, and it defined the molecular mass of the major component, from which the amino acid composition was inferred. The molecular ion signals were observed as $[M + 2Na - H]^+$ and $[M + 2Na - SO_3H]^+$ at m/z 1505 and 1425, respectively (Fig. 4), suggesting the molecular mass of the peptide moiety of the linkage hexasaccharide-peptide to be 370 Da. Based on the amino acid composition analysis [22], the major amino acid residues in this fraction are Ser, Pro, Gly, and Glu, and the molecular mass of a tetrapeptide consisting of these 4 amino acids was calculated to be 388. Since the Ser residue is dehydrated in the glycopeptide due to the formation of a glycosidic bond with the Xyl residue, the calculated value was completely consistent with the molecular mass of the peptide obtained by DE MALDI-TOF MS analysis. Therefore, it was concluded that the major component in the linkage hexasaccharide-peptide fraction from shark cartilage contains a tetrapeptide consisting of Ser, Pro, Gly, and Glu residues, although its sequence remains to be established.

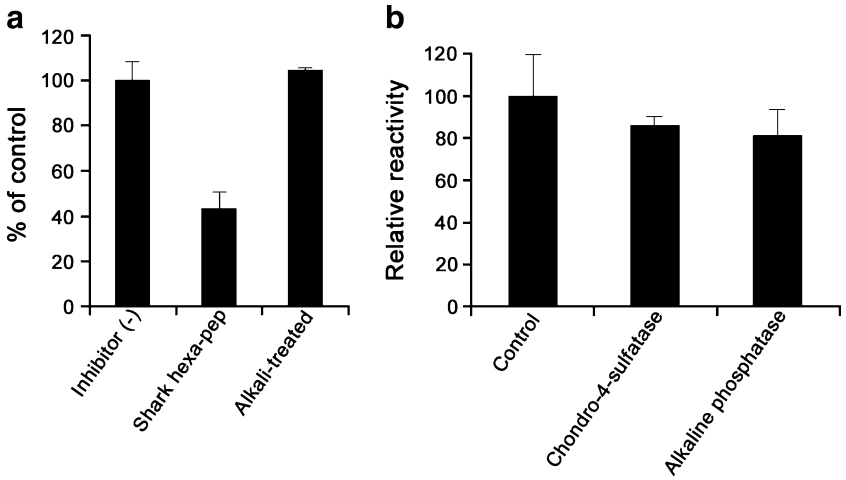

Fig. 3 Reactivity of the antibody 4E1/D6 toward the linkage hexasaccharide-peptide fraction treated with alkali (LiOH), phosphatase, or chondro-4-sulfatase. **a** The linkage hexasaccharide-peptide (hexa-pep) fraction from shark cartilage CS was treated with alkali, and used as an inhibitor (4 nmol) for the binding of 4E1/D6 to the immobilized un-treated linkage hexasaccharide-peptide fraction (0.5 μg) for the competitive ELISA. The un-treated linkage hexasaccharide-peptide fraction (4 nmol) was also used as a control inhibitor. **b** The linkage hexasaccharide-peptide fraction (500 pmol each) was treated with alkaline phosphatase or chondro-4-sulfatase, and each digest was immobilized to the well. The reactivity of 4E1/D6 toward the digest was analyzed by ELISA. The reactivity to the linkage hexasaccharide-peptide fraction from shark cartilage CS was used as a control. Bound antibodies were visualized as described in the legend to Fig. 1. This assay was performed in duplicate, and all values are expressed as a percentage of the reactivity observed without inhibitors and represent the mean ± S.D

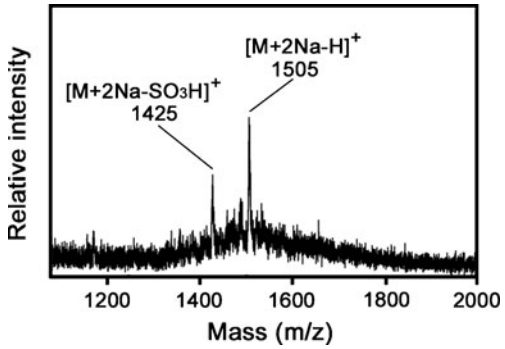

Fig. 4 Identification of the major component in the hexasaccharide-peptide fraction from shark cartilage CS by DE MALDI-TOF MS. DE MALDI-TOF MS was recorded in the positive ion mode with 2,5-dihydroxybenzoic acid as the matrix. Major molecular ion signals were assigned as indicated in the figure

Assessment of the reactivity of 4E1/D6 to the sulfatase- or phosphatase-treated linkage hexasaccharide-peptide fraction Since 4E1/D6 did not react to the hexasaccharide-peptide fraction of whale cartilage CS (Fig. 2), it may recognize a sulfated or phosphorylated component of the pentasaccharide-peptide fraction of shark cartilage CS. GalNAc and two Gal residues can often be modified by 6-*O*-sulfate, and Xyl can be modified by 2-*O*-phosphate. In addition, GalNAc-5 and Gal-3 can be sulfated at position C-4. Therefore, to examine whether 4-*O*-sulfation and 2-*O*-phosphorylation in the linkage region are required for the epitope's recognition, the reactivity of 4E1/D6 toward the linkage hexasaccharide-peptide fraction was investigated by ELISA before and after digestion with chondro-4-sulfatase or alkaline phosphatase (Fig. 3b). The reactivity of 4E1/D6 toward the hexasaccharide-peptide fraction was not diminished after either treatment, suggesting that neither the 4-*O*-sulfate nor 2-*O*-phosphate group is recognized by 4E1/D6. The enzymatic activity of chondro-4-sulfatase and alkaline phosphatase toward the linkage region was confirmed by digestion of the structurally defined linkage hexasaccharides. As shown in Fig. 5, after treatment with chondro-4-sulfatase, the position of the disulfated linkage hexasaccharide ΔHexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl (Fig. 5a, closed arrow) shifted to that position of the nonsulfated linkage hexasaccharide ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl (Fig. 5b, open arrow). Digestion of the 2AB-derivative of the phosphorylated hexasaccharide, ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-2AB, with alkaline phosphatase yielded ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl-2AB as judged by anion-exchange HPLC (data not shown). The recognition of the 2-*O*-phosphorylated linkage region by 4E1/D6 was also examined by competitive ELISA using a chemically synthesized linkage tetrasaccharide-peptide, GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-Ser-Gly [26], as an inhibitor. However, it showed no inhibitory activity. No sulfatase which hydrolyzes 6-*O*-sulfate groups in the linkage region is available, and therefore it is not possible to investigate whether the 6-*O*-sulfate groups are recognized. The commercial bacterial chondro-6-sulfatase (Seikagaku Corp.) does not act on the 6-*O*-sulfate groups of the Gal residues in the linkage region [35].

Analysis of the interactions of 4E1/D6 with CHO cell lines deficient in the biosynthetic enzymes for the linkage region of GAGs The specificity of 4E1/D6 was investigated further by analyzing its interaction with three CHO cell lines, CHO-K1, pgsA-745, and pgsB-618, using immunofluorescence flow cytometry. The pgsA-745 and pgsB-618 are mutant cell lines deficient in xylosyltransferase and galactosyltransferase-I, respectively, and were established from CHO-K1 cells (wild type) [23, 24]. In the biosynthesis of CS and HS, a xylosyltransferase initiates the formation of the tetrasaccharide linkage region by transferring a Xyl residue to a specific Ser residue of the core protein, and then galactosyltransferase-I transfers a Gal residue to the xylosylated Ser residue. The pgsA-745 and pgsB-618 cells produce neither CS nor HS polysaccharide chains, since they cannot construct the linkage region, which is common to CS and HS. A HS-specific antibody, F58-10E4, used as a positive control, intensely stained CHO-K1 cells as expected, but did not stain pgsA-745 cells (Fig. 6, black histograms).

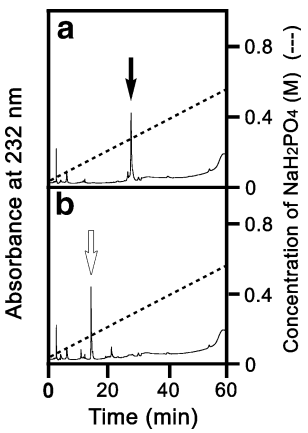

Fig. 5 Action of chondro-4-sulfatase on the structurally defined linkage hexasaccharide. The structurally defined linkage hexasaccharide, ΔHexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl (500 pmol) was analyzed before (**a**) and after (**b**) digestion with chondro-4-sulfatase by anion-exchange HPLC on an amine-bound silica column using a linear gradient of NaH_2PO_4 from 16 mM to 540 mM over 60 min. The peaks were monitored by measuring UV-absorbance at 232 nm. Closed and open arrows indicate the positions of authentic standard linkage hexasaccharides ΔHexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl and ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl, respectively. Treatment with chondro-4-sulfatase caused a shift in the position of the former to that of the latter, indicating the removal of the two sulfate groups

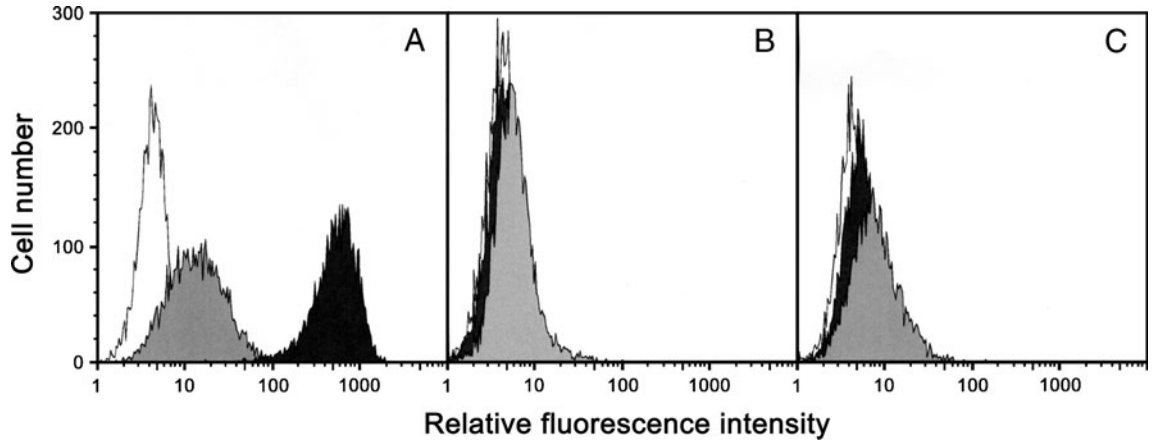

Fig. 6 Interaction of 4E1/D6 with three CHO cell lines. The reactivity of 4E1/D6 toward three CHO cell lines, wild type (CHO-K1) (**a**) xylosyltransferase-deficient (pgsA) (**b**), and galactosyltransferase-I-deficient (pgsB) (**c**) cells, was assessed by immunofluorescence flow cytometry. A HS-specific monoclonal antibody (F58-10E4) was used as a control. *Gray* and *black histograms* represent the 4E1/D6 and F58-10E4-binding, respectively, as detected with Alexa Fluor 488®-conjugated anti-mouse IgM antibody. *White histograms* show the background fluorescence

Compared to F58-10E4, weak but significant immunoreactivity of 4E1/D6 with CHO-K1 cells was detected (Fig. 6a, gray histogram), indicating that the epitope of 4E1/D6 exists on the surface of the wild-type CHO cells. However, the immunoreactivity of 4E1/D6 with pgsA-745 (Fig. 6b, gray histograms) and pgsB-618 (Fig. 6c, gray histogram) cells was very weak, suggesting the lack of the epitope structure of 4E1/D6 on the surface of the mutant cells. Mean fluorescent intensity for the 4E1/D6-binding to CHO-K1, pgsA-745, and pgsB-618 was 685, 184, and 251 in arbitrary values, respectively, with the background value of 134 (Fig. 6, white histograms). These results may reflect that the antibody weakly recognizes the core peptide moiety on the cell surface proteoglycans and that the xylosylated core peptide structure on the pgsB-618 cells is slightly more reactive than the non-glycosylated core peptide structure of proteoglycans on the pgsA-745 cells.

Assessment of the requirement of 6-O-sulfation in the linkage region for recognition by 4E1/D6 To elucidate the requirement of 6-*O*-sulfate groups for recognition by the 4E1/D6 antibody, the structure of the CS-core protein linkage region of salmon PG was analyzed. Salmon PG was treated with LiOH to liberate CS chains from the core protein, the liberated saccharides were labeled with a fluorophore 2AB, and the excess 2AB reagent was removed by paper chromatography. The sample was digested with CSase ABC, and the digest was analyzed by anion-exchange HPLC. Major peaks observed were identified by comparing their elution positions with those of the authentic 2AB-labeled hexasaccharide standards [25]. As summarized in Table 2, besides the nonsulfated hexasaccharide, ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl-2AB, as the major component (65%), monosulfated and monophosphorylated hexasaccharides were also detected. However, no hexasaccharides containing Gal(6-*O*-sulfate) were detected. Even if any, it accounted for less than 6%, indicating that the Gal(6-*O*-sulfate) structure is not a part of the epitope.

The requirement of 6-*O*-sulfation in the linkage region was further investigated by transfection of C6ST-1 to CHO-K1 cells. Recently, Kitagawa *et al.* has demonstrated that human C6ST-1 is a responsible enzyme for the sulfation of Gal residues in the linkage region [39]. Thus, we have transfected this gene to CHO-K1 cells, and its reactivity with 4E1/D6 was analyzed using immunofluorescence flow cytometry. No increase in the reactivity was observed by the transfection with C6ST-1 compared to the mock transfectant (data not shown). When the disaccharide composition of the CS chains from these CHO cells was compared, the proportion of GalNAc(6-*O*-sulfate)-containing disaccharide unit increased from 3 to 59% after transfection with C6ST-1. However, it is extremely difficult to analyze the structure of the linkage region from the CHO cells, because a single CS chain contains only one mole of the linkage region per mole. Thus, it was not possible to

Table 2 Structures of the major hexasaccharides detected in the CS-protein linkage region of PG from salmon nasal cartilage

Structure	Proportion (mol%)
ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl	65.4
ΔHexA-GalNAc(6S)-GlcA-Gal-Gal-Xyl	6.5
ΔHexA-GalNAc(4S)-GlcA-Gal-Gal-Xyl	11.3
ΔHexA-GalNAc-GlcA-Gal-Gal-Xyl(2P)	10.7
Unidentified components	6.1

draw a clear conclusion on the requirement of the 6-*O*-sulfate structure in the linkage pentasaccharide for the recognition by 4E1/D6. The epitope of the 4E1/D6 antibody was presumed to be a hexasaccharide-tetrapeptide structure shown in Fig. 7. The peptide sequence and the requirement of the 6-*O*-sulfation on the GalNAc residue remain to be elucidated.

Discussion

Galactosaminoglycans (CS/DS) and glucosaminoglycans (HS/Hep) are synthesized at specific Ser residues of the core proteins of PGs through the tetrasaccharide linkage region GlcAβ1-3Galβ1-3Galβ1-4Xyl [5]. Although the tetrasaccharide core is common to CS/DS and HS/Hep, the pattern of modification in the linkage region differs between the two types of GAG chains. Namely, the 4-*O*-sulfation of a Gal-3 residue and 6-*O*-sulfation of both Gal-2 and Gal-3 residues have been found in CS/DS, but not HS/Hep. Interestingly, syndecan-1, a hybrid-type PG bearing both HS and CS chains, carries a 4-*O*-sulfate on the Gal-3 of only the CS chains [9], supporting the notion that 4-*O*-sulfation is a modification specific to CS chains [7]. In contrast, phosphorylation occurs on the Xyl of both CS/DS and HS/Hep [9]. These modifications have significant effects on the synthesis of the tetrasaccharide linkage region. The phosphate on Xyl stimulates the attachment of the first GlcA-4 [10, 11], but then seems to be removed [40]. In contrast, it inhibits the attachment of Gal-2 [10], suggesting the phosphorylation to take place after the transfer of Gal-2 but before that of GlcA-4. Intriguingly, the sulfation of Gal-2 promotes the transfer of the first GlcA [10, 11]. Furthermore, prior 4-*O*-sulfation of Gal-3 stimulates the 6-*O*-sulfation of Gal-2 by recombinant human C6ST-1 [39]. Thus, the sulfated linkage region may also contain signals that influence further sulfation of the linkage region and the repeating disaccharide region as well as chain polymerization. In fact, sulfation in the vicinity of the linkage region has an influence on the *N*-acetylgalactosaminyltransferase activity for chain elongation [41, 42]. However, the biological significance of modifications to the linkage region has not been fully elucidated, and the structural analysis of the linkage region is of importance. However, it is extremely difficult to analyze the structure of a minute portion of the linkage region of CS/DS or HS/Hep chains derived from cultured cells, which contain only one mole of the linkage region per single GAG chain. Therefore, tools such as antibodies, which recognize the linkage region, are essential for studying the biological significance of the linkage region.

In this study, a monoclonal antibody, 4E1/D6 (IgM, kappa), which recognizes the CS-protein linkage region derived from CS of shark cartilage, was developed for the first time. It recognizes the pentasaccharide-tetrapeptide GalNAc(±6-*O*-sulfate)-GlcA-Gal-Gal-Xyl-Ser-(Pro, Gly, Glu). Since this antibody bound hexa- and pentasaccharide-peptides more strongly than unsaturated tetrasaccharide-peptides, the fifth and/or fourth sugar residue (GalNAc-5 and/or GlcA-4) from the reducing end is important. An attempt was made to prepare the saturated tetrasaccharide-peptide by digestion of the pentasaccharide-peptide fraction with commercial β-*N*-acetylhexosaminidases from jack bean, *Penicillium oxalicum*, and *Turbo cornutus* (Seikagaku Corp.). However, it was not possible to release the GalNAc-5 due to the resistant nature of the sulfated GalNAc residue to the enzymes (data not shown). Thus, the importance of GalNAc-5 is still unsettled. Neither 2-*O*-phosphate on Xyl nor 4-*O*-sulfate on Gal or GalNAc are involved in the recognition, because digestion with alkaline phosphatase as well as chondro-4-sulfatase did not affect the reactivity. Based on the structural analysis of the linkage region of salmon CS-PG, which is also recognized by the antibody, no or little, if any, 6-*O*-sulfate groups on the Gal residues were detected, suggesting that Gal(6-*O*-sulfate) does not appear to be involved in the recognition. Since transfection of C6ST-1 into CHO-K1 cells resulted in no significant increase in the reactivity of 4E1/D6 to the transfected cells, 6-*O*-sulfate groups do not seem to influence the reactivity of the antibody. However, due to the unavailability of a specific sulfatase to remove 6-*O*-sulfate groups, the requirement of the GalNAc(6-*O*-sulfate) structure remains to be elucidated.

Although the amino acid sequence of the linkage region could not be determined, the peptide moiety is essential for

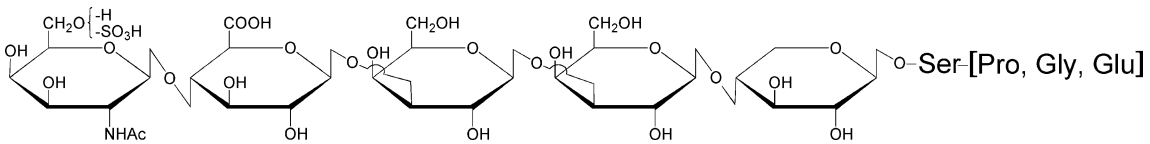

Fig. 7 The predicted structure required for the recognition by the antibody 4E1/D6. The epitope structure recognized by 4E1/D6 was predicted based on the data obtained in this study. The pentasaccharide-tetrapeptide seems to be necessary for recognition by 4E1/D6. The requirement of 6-*O*-sulfation on the nonreducing terminal GalNAc residue has not been confirmed. The amino acid sequence in the tetrapeptide remains to be determined

the recognition by 4E1/D6. The failure of the amino acid sequencing by MS or the automated Edman degradation was probably because the peptide was too short and its modification with the sulfated oligosaccharide gave it strongly hydrophilic properties. Twenty four possible tetrapeptide sequences composed of Ser (S), Pro (P), Gly (G), and Glu (E) were searched in the amino acid sequence of human aggrecan, since the gene of shark aggrecan has not been cloned. One SEPG, one SPGE, one GESP, and 26 PSGE sequences were found, but no other combination sequences existed in human aggrecan. Since the consensus sequence for the attachment of CS has been proposed to be SGXG or (D,E)XSG [43, 44], the Ser residue in the PSGE sequence appears to be substituted with a CS chain. When searched in the amino acid sequence of bovine aggrecan, 20 PSGE, one ESGP, and one EGPS sequences were detected. In mouse, pig, and monkey aggrecans, only the PSGE sequence, but no other tetrapeptide sequences, which meet the above conditions, was present. Therefore, the antibody 4E1/D6 generated against the CS-core protein linkage region in this study, most likely recognizes the PSGE sequence as the peptide moiety of the epitope.

Modifications of the linkage region may also act as a marker for the intracellular transport of CS-PGs to the Golgi compartment for biosynthetic processing or elongation and maturation of the repeating disaccharide region of CS chains. The activity of C6ST-1, which transfers a sulfate to the C-6-position of GalNAc in the repeating disaccharide region as well as the Gal residues in the linkage region [39], is found in medial and trans-Golgi fractions [45]. GlcAT-I involved in the synthesis of the linkage region is distributed in both medial-Golgi and trans-Golgi/trans-Golgi network. This distribution is similar to that of chondroitin-polymerizing glucuronyltransferase-II activity, but distinctly different from the distribution of the two galactosyltransferases found exclusively in cis-Golgi fractions [46]. It has also been reported that xylosyltransferase, galactosyltransferases, and GlcAT-I are distributed in ER/cis-Golgi, cis-/medial-Golgi, and medial-/trans-Golgi, respectively [47]. These results suggest that nascent PGs are transported from cis- to trans-Golgi compartments during maturation, and that the sulfation of the linkage region takes place before the transfer of the first *N*-acetylhexosamine residue to the tetrasaccharide core and could be a signal for the differential assembly of CS and HS chains as proposed previously [7, 21, 27]. The antibody 4E1/D6 will be useful for investigation of the biosynthetic processes of CS in the Golgi compartments by monitoring the linkage region, which is formed at the initial stage of CS biosynthesis.

The CHO mutant cells, which are deficient in the glycosyltransferases that synthesize the linkage region tetrasaccharide core, cannot synthesize either CS/DS or HS/Hep, suggesting the same glycosyltransferases synthesize the linkage region common to both types of the GAG chains [23, 24, 48]. Therefore, the types of GAG chains to be selectively assembled on the linkage region tetrasaccharide are determined after or during the construction of the linkage region by these enzymes. The amino acid sequence around the GAG attachment site (Ser) varies among different PGs. Therefore, it is unlikely that only the amino acid sequence near the GAG attachment site determines the type of GAG, although it has some influence [49]. In fact, a PG named serglycine is a typical example, which can be modified by CS or HS [50].

A number of PGs occur in the form of both a PG and a protein without a GAG chain, and are called part-time PGs [51]. For example, thrombomodulin, a cell surface glycoprotein, occurs both as a CS-PG (β-thrombomodulin) and as a protein without a CS chain (α-thrombomodulin), which has only a tetrasaccharide linkage region, being an immature glycoprotein [52]. The biosynthetic control mechanism to produce such immature PG forms or to interfere with the glycanation step remains to be investigated. This antibody may also be useful for investigating the biological functions and processing of part-time PGs.

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