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Title	Development of a mouse monoclonal antibody against the chondroitin sulfate-protein linkage region derived from shark cartilage
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1 **Development of a mouse monoclonal antibody against the chondroitin**
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4 **sulfate-protein linkage region derived from shark cartilage**
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10 **Chizuru Akatsu, Duriya Fongmoon, Shuji Mizumoto, Jean-Claude Jacquinet, Prachya**
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13 **Kongtawelert, Shuhei Yamada, and Kazuyuki Sugahara**
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19

20 **Abstract**
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23 Glycosaminoglycans (GAGs) like chondroitin sulfate (CS) and heparan sulfate (HS) are
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25 synthesized on the tetrasaccharide linkage region, GlcA β 1-3Gal β 1-3Gal β 1-4Xyl β 1-*O*-Ser, of
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27 proteoglycans. The Xyl can be modified by 2-*O*-phosphate in both CS and HS, whereas the Gal
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29 residues can be sulfated at C-4 and/or C-6 in CS but not in HS. To study the roles of these
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31 modifications, monoclonal antibodies were developed against linkage glycopeptides of shark
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33 cartilage CS proteoglycans, and one was characterized in detail. This antibody bound hexa- and
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35 pentasaccharide-peptides more strongly than tetrasaccharide-peptides, suggesting the importance of
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37 GalNAc. It did not react to the CS linkage region modified by 4-*O*-sulfation. Its reactivity was not
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39 affected by treatment with chondro-4-sulfatase or alkaline phosphatase. The results of an ELISA
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41 using various proteoglycans and glycopeptides with different modifications suggested the
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43 recognition of 6-*O*-sulfate on the GalNAc and/or Gal residues. Treatments with exopeptidases did
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45 not affect the reactivity of the hexasaccharide-peptide fraction, whereas weak alkali to cleave the
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1 Xyl-Ser linkage completely abolished the binding activity, suggesting the importance of the Xy-Ser
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4 linkage for the binding. Furthermore, the antibody stained wild-type CHO cells, but not mutant cells
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7 deficient in xylosyltransferase required for the synthesis of the linkage region. These results suggest
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10 that the antibody recognizes the structure GalNAc-GlcA-Gal-Gal-Xyl-Ser that is modified by
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13 6-*O*-sulfation on GalNAc and/or Gal. The antibody will be a useful tool for investigating the
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16 significance of the linkage region in the biosynthesis and/or intracellular transport of different GAG
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19 chains.
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26 **Keywords** Proteoglycans, Glycosaminoglycans, Chondroitin sulfate, Heparan sulfate, Dermatan
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29 sulfate, Monoclonal antibody
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36 **Abbreviations**

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39 2AB, 2-aminobenzamide; BSA, bovine serum albumin; CS, chondroitin sulfate; DS, dermatan
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42 sulfate; ELISA, enzyme-linked immunosorbent assay; GAG, glycosaminoglycan; Gal, D-galactose;
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45 GalNAc, *N*-acetyl-D-galactosamine; GlcA, D-glucuronic acid; GlcN, D-glucosamine; GlcNAc,
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48 *N*-acetyl-D-glucosamine; Hep, Heparin; Δ HexA, 4,5-unsaturated hexuronic acid or
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51 4-deoxy- α -L-*threo*-hex-4-enopyranosyluronic acid; HPLC, high performance liquid chromatography;
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55 HS, heparan sulfate; IdoA, L-iduronic acid; PG, proteoglycan; Xyl, D-xylose.
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1 Introduction

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4 Proteoglycans (PGs) are macromolecules composed of linear polysaccharide
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7 glycosaminoglycan (GAG) side chains, which are covalently attached to specific Ser residues of core
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10 proteins. PGs are distributed mainly in extracellular matrices and at cell surfaces, and implicated in
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13 many pathophysiological phenomena. Characteristic GAG moieties appear to play important roles as
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16 regulators of various biological processes by interacting with functional protein ligands such as
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19 growth factors, cytokines, and morphogens [1-4].
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23 GAGs include chondroitin sulfate/dermatan sulfate (CS/DS) and heparan sulfate/heparin
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26 (HS/Hep), which are classified as galactosaminoglycans and glucosaminoglycans, respectively.
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29 CS/DS and HS/Hep consist of repeating disaccharide units, GlcA/IdoA-GalNAc and
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32 GlcA/IdoA-GlcN/GlcNAc, respectively. GlcA, IdoA, GalNAc, GlcN, and GlcNAc represent
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35 D-glucuronic acid, L-iduronic acid, *N*-acetyl-D-galactosamine, D-glucosamine, and
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38 *N*-acetyl-D-glucosamine, respectively. GAGs are specifically modified most notably by sulfation at
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41 various positions, forming a variety of structures and acquiring functions. Although the structure of
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43
44 the repeating disaccharide region differs between CS/DS and HS/Hep, both types of GAGs are
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47 covalently bound to core proteins through the common linkage region tetrasaccharide,
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In the biosynthesis of GAGs, monosaccharide residues are transferred stepwise from the

1 corresponding nucleotide sugars to growing GAG chains, being governed largely by the substrate
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4 specificity of the glycosyltransferases involved. This process is initiated by the addition of Xyl to
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7 specific serine residues in the core protein, followed by the sequential addition of two Gal residues
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10 and a GlcA residue to construct the tetrasaccharide linkage structure [1]. The synthesis of CS/DS
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13 chains initiates once GalNAc is transferred by the *N*-acetylgalactosaminyltransferase-I (GalNAcT-I)
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16 activity of chondroitin synthases to the GlcA of the common linkage region, whereas that of HS/Hep
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19 chains initiates if GlcNAc is first added by the *N*-acetylglucosaminyltransferase-I (GlcNAcT-I)
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22 activity [1, 4, 6]. Hence, GalNAcT-I and GlcNAcT-I activities are crucial for the determination of
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25 the GAG species to be synthesized on the common tetrasaccharide linkage region. However, it is not
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28 clear how these different GAGs are selectively assembled on the common structure.
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32 We have carried out a series of structural studies of the GAG-protein linkage region, based
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35 on the working hypothesis that there may be differences in the region's structure among GAG chains
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38 and such differences may contribute to the determination of the type and/or character of the GAG
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41 species to be synthesized [6, 7]. These structural studies have revealed unique modifications, such as
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44 4-*O*-sulfated Gal, 6-*O*-sulfated Gal, and 2-*O*-phosphorylated Xyl. So far, sulfated Gal residues have
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47 been demonstrated only in the linkage region of CS/DS, not in HS/Hep, though a
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50 2-*O*-phosphorylated Xyl residue has been found in both CS/DS and HS/Hep [6, 8, 9], suggesting the
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53 sulfate groups on the Gal residues to be involved in the selective assembly of different GAG types.
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56 In fact, it has been demonstrated that these modifications influence the catalytic activities of
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1 galactosyltransferase-I, which transfers Gal-2 (for the numbering of the sugar residues, see Table 1),
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4 and glucuronyltransferase-I (GlcAT-I), which transfers the first GlcA, involved in the construction of
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7 the tetrasaccharide linkage region [10, 11]. However, it is not fully understood if these modifications
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10 also affect on other biosynthetic enzymes or play a biological role as recognition signals for
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13 regulatory proteins such as intracellular transporters.
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17 The use of antibodies has become a standard approach in many fields of biochemical and
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20 biomedical research. To clarify the biological significance of the modifications in the GAG-protein
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23 linkage region, antibodies which recognize specific patterns of modification in the region may be
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26 useful. Monoclonal antibodies (mAbs) that recognize specific features of the repeating disaccharide
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29 region of CS chains have been generated, and epitopes of some of them have been characterized
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32 [12-16]. Studies using anti-CS mAbs have revealed restricted spatiotemporal patterns of the
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35 expression of specific CS structures in various tissues during growth and development, and in
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38 pathological conditions [16-19]. However, so far, no antibodies have been reported which recognize
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41 the GAG-protein linkage region. In the present study, a mAb against the CS-protein linkage region
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44 from shark cartilage was developed and its epitope was characterized.
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51 **Materials and methods**

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55 *Materials* PGs from salmon nasal cartilage, chicken cartilage, and whale cartilage were provided by
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58 Biomatec Japan Inc. (Kushiro, Japan). PG from rayfish cartilage was obtained from Marukyou
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1 Biofoods Co., Ltd. (Kushiro, Japan). PG from birds' nests made with dried saliva of male *Collocalia*
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4 swiftlets was provided by Y. T. Li, Tulane University [20]. PG from bovine nasal cartilage was a gift
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6
7 from the late Dr. Albert Dorfman, University of Chicago. A whale cartilage CS-peptide fraction,
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10 which contained Ser, Asp, Thr, Glu, Pro, Gly, Ala, Val, Lys and Arg in a molar ratio of 1.00 : 1.74 :
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13 0.42 : 1.99 : 1.53 : 4.22 : 1.00 : 0.52 : 0.63 : 0.65, was prepared as reported [21].
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17 The following enzymes were purchased from Seikagaku Corp. (Tokyo, Japan):
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20 chondroitinase (CSase) ABC (EC 4.2.2.20) from *Proteus vulgaris*, CSase AC-I (EC 4.2.2.5) from
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22
23 *Fravobacterium heparinum*, CSase AC-II (EC 4.2.2.5) from *Artherobacter aurescens*, and
24
25
26 chondro-4-sulfatase (EC 3.1.6.9) from *P. vulgaris*. Aminopeptidase M (EC 3.4.11.2) from porcine
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29 kidney, carboxypeptidase Y (EC 3.4.16.5) from yeast, and calf intestinal alkaline phosphatase (EC
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32 3.1.3.1) of special quality for molecular biology were obtained from EMD Biosciences, Inc. (San
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35 Diego, CA), Oriental Yeast Co., Ltd. (Tokyo, Japan), and Boehringer Mannheim GmbH (Mannheim,
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37
38 Germany), respectively. Anti-HS antibody F58-10E4 and Alexa Fluor 488[®]-labeled goat anti-mouse
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41 IgM antibody were purchased from Seikagaku Corp. (Tokyo, Japan) and Molecular Probes (Eugene,
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43
44 OR), respectively. Synthetic peptides, Leu-*p*-nitroanilide and benzyloxycarbonylglycyl-Phe, were
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47 purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Peptide Institute, Inc.
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50 (Osaka, Japan), respectively.
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55 Wild-type Chinese hamster ovary (CHO) cells (CHO-K1) and xylosyltransferase-deficient
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58 CHO cells (pgsA-745) [22] were purchased from American Type Culture Collection (Manassas, VA).
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1 These cell lines were maintained in Ham's F12K medium (Wako Pure Chemical Industries, Ltd.,
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4 Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified
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7 atmosphere containing 5% CO₂.

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10 The Structurally defined linkage hexasaccharide
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12 Δ HexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl was isolated from whale cartilage
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14 CS-PG [23]. Δ HexA stands for 4,5-unsaturated hexuronic acid. A 2-aminobenzamide
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17 (2AB)-derivative of the linkage hexasaccharide
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20 Δ HexA-GalNAc-GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-2AB, was prepared as described previously
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23 [23]. The tetrasaccharide peptide GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-Ser-Gly was chemically
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26 synthesized [24].
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36 *Preparation of the oligosaccharide-peptides from the CS-core protein linkage region* The CS-peptide
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39 fraction (1.0 g) was prepared from shark cartilage [25, 26] and exhaustively digested with 1.2 IU of
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42 CSase ABC in a total volume of 12.5 ml of 50 mM Tris/HCl buffer, pH 8.0, containing 60 mM
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45 sodium acetate and 0.1% bovine serum albumin (BSA) for 25 h at 37 °C. An additional 0.2 IU of the
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48 enzyme was added after 23 h to complete the digestion, and the reaction was terminated by heating at
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51 100 °C for 5 min. The digest was fractionated by gel filtration using a column (2.0 X 149 cm) of
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54 Sephadex G-15 (GE Healthcare, Buckinghamshire, UK) with 0.25 M NH₄HCO₃/7% 1-propanol as
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57 the eluent. The isolated linkage hexasaccharide-peptide fraction was digested with CSase ABC again
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1 as described above to complete the digestion, and this process was repeated three times until no
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4 disaccharide was produced as judged by gel filtration chromatography. This fraction contained
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6
7 several amino acids including Ser, Glu, Pro, Gly, Val, and Lys in a molar ratio of 1.00, 1.16, 1.41,
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10 1.23, 0.46, and 0.29, respectively [25].
11

12
13 To eliminate the Δ HexA residue, which has strong immunogenic activity, from the
14
15 nonreducing end of the linkage hexasaccharide-peptides, the glycopeptides were treated with mercuric
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17 acetate as described previously [27, 28]. Namely, the linkage hexasaccharide-peptide fraction (1.5
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19 μ mol as linkage hexasaccharide) was treated with 0.2 ml of 10 mM mercuric acetate in 130 mM
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21 sodium acetate buffer, pH 5.0, overnight at room temperature. After the addition of 2 ml of 5 M NaCl,
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23 the reaction mixture was desalted by successive gel filtration using a Sephadex G-25 (fine) column
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25 (1.5 X 47 cm) equilibrated with 1 M NaCl and then with H₂O.
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36 The linkage tetrasaccharide-peptides were prepared by treatment of the linkage
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38 hexasaccharide-peptides (120 nmol as Δ HexA) with 10 mIU of CSase AC-I [29] in a total volume of
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40 30 μ l of 50 mM of Tris/HCl buffer, pH 7.3, at 37 °C for 20 h. The enzymatic reaction was terminated
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43 by heating at 95 °C for 1 min.
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48 The linkage oligosaccharide-peptide fraction was treated with LiOH as described below to
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50 release the oligosaccharide from the core peptides, labeled with 2AB and analyzed by anion
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52 exchange HPLC on an amine-bound silica PA-03 column (4.6 X 250 mm, YMC Co., Kyoto, Japan)
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55 to confirm the presence of the linkage oligosaccharide components predicted. The molar
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1 concentration of the linkage oligosaccharide-peptides was determined by measuring absorbance at
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4 232 nm based on an average millimolar absorption coefficient of 5.5 for the double bond of Δ HexA
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7 [30] or by the carbazole reaction to measure uronic acids (GlcA and Δ HexA) colorimetrically [31].
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13 *Production of the monoclonal antibody* Monoclonal antibodies were generated by immunizing
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16 BALB/c mice with the linkage pentasaccharide-peptide fraction from shark cartilage CS. The linkage
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19 pentasaccharide-peptide fraction was conjugated with a carrier protein, keyhole limpet hemocyanin
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22 (KLH) (Sigma, St Louis, MO), using glutaraldehyde [32], which cross-links primary amino groups
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25 of peptides as described below: 1 μ mol of the fraction was conjugated with 4 mg of KLH in 2 ml of
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28 phosphate-buffered saline (PBS), pH 7.2, with the addition of glutaraldehyde to a final concentration
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31 of 0.1% at room temperature overnight. The reaction was terminated by addition of 0.25 ml of 1 M
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34 glycine, and then the conjugates were dialyzed overnight against PBS. The KLH-conjugated linkage
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37 pentasaccharide-peptide fraction was injected into mice at 20 μ g/injection every 2 weeks. After the
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40 fourth injection, the serum was screened for reactivity with the linkage hexasaccharide-peptides.
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44 Spleen B lymphocytes of the positive mice were isolated and fused with myeloma cells. The culture
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47 supernatant of the fused hybridoma cells was screened by enzyme-linked immunosorbent assay
48
49
50 (ELISA) using the linkage hexasaccharide-peptides, and three positive clones, 4E1, 3F11, and 1B5,
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52
53
54 were selected. The clone 4E1 with the highest specificity toward the linkage hexasaccharide-peptide
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56
57 fractions was recloned, and eleven secondary clones were further selected. Among the eleven clones,
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60

1 six showed high specificity and were injected intraperitoneally into mice to obtain ascitic fluid.

2
3
4 Antibody subclasses were determined using the ImmunoPure[®] Monoclonal Antibody Isotyping Kit
5
6
7 II (Pierce Biotechnology, Rockford, IL). The mAb 4E1/D6 was of particular interest because of its
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9
10 specific activity, and subsequently characterized in detail.
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17 *ELISA* The specificity of the antibody 4E1/D6 in terms of antigen recognition was tested by ELISA.
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19
20 Briefly, various PG preparations or linkage oligosaccharide-peptide fractions (0.5 µg each) were
21
22
23 dissolved in a 0.2 M sodium bicarbonate buffer, pH 9.6 and individually immobilized overnight to a
24
25
26 96-well microtiter plate (Nunc immune plate, MaxiSorp, Nalge Nunc International, Rochester, NY)
27
28
29 at room temperature. The wells were washed once with PBS, pH 7.4, containing 0.05% Tween 20
30
31
32 (PBST), and blocked with 3% (w/v) BSA in PBS for 1 h at 37 °C. The wells were then washed with
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34
35
36 PBST once and incubated with 4E1/D6 for 2 h at 37 °C. After three washes with 25 mM
37
38
39 Tris-buffered saline (TBS) containing 0.05% Tween20 (TBST), the wells were incubated with
40
41
42 alkaline phosphatase-labeled anti-mouse Ig(G+M) (3,000-fold dilution, Chemicon, San Diego, CA).
43
44
45 Enzymatic activity was detected using *p*-nitrophenylphosphate by measuring the absorbance at 415
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47
48 nm.
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50

51
52 For the competitive ELISA, aliquots of the linkage hexasaccharide-peptides from shark
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54
55 cartilage CS, which had been pretreated with peptidase or LiOH, was incubated with 4E1/D6 in a
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57
58 total volume of 50 µL at 37 °C for 1 h, then the mixture was applied to the wells where untreated
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1 linkage hexasaccharide-peptides from shark cartilage CS had been immobilized. The color was
2
3
4 developed as described above.
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10 *Chemical and enzymatic treatments of the linkage hexasaccharide-peptide fraction* To liberate
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12 *O*-linked linkage oligosaccharides from the core peptides, treatment with LiOH was performed as
13
14 described previously [23, 33]. Briefly, the hexasaccharide-peptide fraction (183 nmol) was treated
15
16
17 with 0.5 M LiOH at 4°C for 15 h. The reaction was terminated by neutralization with 2.0 M acetic
18
19
20 acid, and then Li⁺ was removed by cation-exchange chromatography using AG 50W-X2 resin
21
22
23 (Bio-Rad Laboratories, Hercules, CA).
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29
30 Aminopeptidase M or carboxypeptidase Y digestion was carried out using 10 nmol of the
31
32 linkage hexasaccharide-peptide fraction and 3 U of each enzyme in 10 µl of 25 mM imidazole/HCl
33
34
35 buffer, pH 7.6, or 50 mM phosphate buffer, pH 6.0, respectively, for 30 min at 37°C.
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39 To investigate the reactivity of the antibody 4E1/D6 to the 4-*O*-sulfated or
40
41
42 2-*O*-phosphorylated linkage structure, treatment with chondro-4-sulfatase or alkaline phosphatase
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44
45 was conducted. One nmol of the linkage hexasaccharide-peptide fraction or a structurally defined
46
47
48 linkage hexasaccharide, ΔHexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl, was digested
49
50
51 with the indicated enzyme. Digestion with chondro-4-sulfatase was performed with 12 mIU of the
52
53
54 enzyme for 6 h in 3 µL of 50 mM Tris/HCl buffer, pH 7.5, containing 50 mM sodium acetate [34].
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58 Treatment with alkaline phosphatase was carried out with 1 IU of the enzyme in a total volume of 3
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1 μ L of the buffer supplied by the manufacturer at 37 °C for 6 h.
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7 *Immunofluorescence flow cytometry* CHO-K1 and pgsA-745 cells were detached with 2 mM EDTA
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9
10 and suspended in PBS containing 0.1% BSA (PBS-BSA) at a concentration of 10^6 cells/ml. After
11
12
13 three washes with PBS-BSA, the cells were incubated with the antibody 4E1/D6 (400-fold dilution)
14
15
16 or a commercial antibody F58-10E4 against HS (200-fold dilution) at 4°C for 30 min. The cells were
17
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19 washed with PBS-BSA three times and incubated with Alexa Fluor 488[®]-labeled goat anti-mouse
20
21
22 IgM antibody (500-fold dilution). After three washes with PBS-BSA, the cells were analyzed by
23
24
25 immunofluorescence flow cytometry in a BD FACSCanto (BD Biosciences, San Jose, CA). Flow
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27
28 cytometric data were analyzed using Flowjo software (Tree Star, Inc., Ashland, OR).
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36 **Results**

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39 *Production of the monoclonal antibody against the linkage oligosaccharide-peptide fraction of shark*
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41
42 *cartilage CS* The CS-peptide fraction of shark cartilage was exhaustively digested with CSase ABC
43
44
45 to prepare the linkage hexasaccharide-peptide fraction. Approximately 9.2 μ mol of this fraction was
46
47
48 obtained and an aliquot was analyzed by anion-exchange HPLC on an amine-bound silica PA-03
49
50
51 column after cleavage of the bond between the xylose and the serine using LiOH [23, 33] (results not
52
53
54 shown). The fraction contained at least thirteen hexasaccharide compounds as reported (Table 1) [25,
55
56
57 26]. Since the glycopeptides contain a Δ HexA residue at the nonreducing terminus, which is an
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1 unnatural structure causing strong antigenicity, the Δ HexA residue was eliminated by treatment with
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4 mercuric acetate [27, 28]. The pentasaccharide-peptide fraction was conjugated with KLH and used
5
6
7 as an immunogen to develop mAbs against the CS-core protein linkage region.
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9

10 After immunization of BALB/c mice with the immunogen, six positive clones were
11
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13 obtained by screening assays using the CS hexasaccharide-peptide fraction immobilized onto a Nunc
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16 Maxisorp plate. Clone 4E1/D6, with the highest level of activity, was characterized further. This
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19 clone contained an IgM-type immunoglobulin with kappa light chains (data not shown), and the
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21
22 concentration of protein in ascitic fluid was 34.3 mg/ml as determined using the BCA protein assay
23
24
25 kit according to the instructions provided by the manufacturer (Thermo Scientific, Rockford, IL).
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32 *Assessment of the reactivity of the antibody 4E1/D6 toward various PGs* To characterize the
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35 specificity of 4E1/D6, the antibody's reactivity toward various PGs was assessed by ELISA (Fig. 1),
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37
38 where PGs were individually immobilized onto a Nunc Maxisorp plate through their protein moiety.
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41 The linkage hexasaccharide-peptide fraction from shark cartilage CS was also immobilized as a
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44 positive control. Significant binding of 4E1/D6 was observed with the linkage hexasaccharide
45
46
47 fraction and a PG from salmon nasal cartilage, but with no other PGs tested. The reactivity of
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50 4E1/D6 toward this PG was confirmed by a competitive ELISA: a soluble PG from salmon nasal
51
52
53 cartilage inhibited the binding of 4E1/D6 to the immobilized linkage hexasaccharide-peptide fraction
54
55
56 of shark cartilage CS in a dose-dependent manner (data not shown). 4E1/D6 reacted less extensively
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1 to the salmon PG than the linkage hexasaccharide-peptide fraction and not at all to the other PGs
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4 tested. Of course, the possibility exists that the linkage regions of these PGs are masked by CS
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7 polysaccharide side chains, and so are inaccessible to the antibody. However, the structural
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10 difference among these PGs remains investigated.

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16 *Assessment of the reactivity of 4E1/D6 toward the linkage region oligosaccharides* To investigate the
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19 structural features of the linkage pentasaccharide-peptides required for recognition by 4E1/D6, the
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22 reactivity of 4E1/D6 toward linkage hexa-, penta-, and tetrasaccharide-peptide fractions of shark
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24
25 cartilage CS was assessed by ELISA (Fig. 2). The linkage tetrasaccharide-peptide fraction was
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28 prepared by digestion of the linkage hexasaccharide-peptide fraction with CSase AC-I, and the
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31 exhaustive digestion was confirmed by gel filtration chromatography of the digest. The linkage hexa-,
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34 penta-, and tetrasaccharide-peptide fractions (0.5 µg each) were immobilized onto Maxisorp plates.
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36
37 The reactivity was considerably weaker toward the tetrasaccharide-peptides than hexa- or
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39
40 pentasaccharide-peptides of shark cartilage CS (Table 1). These results suggest the fifth saccharide
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43 residue GalNAc-5 in the linkage oligosaccharide-peptide fraction to be important for recognition by
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46 4E1/D6. In strong contrast, the hexasaccharide-peptide fraction of whale cartilage CS, which
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51 contains four major saccharide sequences with different sulfation patterns (Table 1), showed no
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54 reactivity, suggesting that the 4E1/D6 epitope may contain a particular modification in the linkage
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57 region. In addition, 4E1/D6 may distinguish the different amino acid sequences in the peptide moiety
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1 of the linkage oligosaccharide-peptide of shark cartilage CS from those of whale cartilage CS (see
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4 above).

10 *Assessment of the reactivity of 4E1/D6 toward the peptide moiety of the linkage*

13 *hexasaccharide-peptides* To assess whether 4E1/D6 recognizes the peptide moiety of the linkage

16 hexasaccharide-peptides, a competitive ELISA was carried out using the linkage hexasaccharide

19 fraction from shark cartilage CS treated with LiOH, aminopeptidase, or carboxypeptidase as an

22 inhibitor (Fig. 3). Treatment with LiOH liberates hexasaccharides from the core peptide. Upon

25 treatment with aminopeptidase or carboxypeptidase, amino acid residues located on the amino or

28 carboxy terminal side of the Ser residue are trimmed, respectively. The LiOH-treated sample did not

31 inhibit at all the binding of 4E1/D6 to the linkage hexasaccharide-peptides of shark cartilage CS. In

34 contrast, the digest of the linkage hexasaccharide fraction obtained with aminopeptidase or

37 carboxypeptidase inhibited the binding of 4E1/D6 to the un-treated linkage hexasaccharide-peptide

40 to a comparable degree with the linkage hexasaccharide-peptides of shark cartilage CS (Fig. 3). The

43 activities of aminopeptidase and carboxypeptidase were confirmed by colorimetric assays using the

46 artificial substrates Leu-*p*-nitroanilide and benzyloxycarbonylglycyl-Phe, respectively [35, 36]. From

49 these results, 4E1/D6 does not appear to recognize sequences composed of multiple amino acids.

52 Rather, the xylosidic linkage to the Ser residue appears to be recognized by 4E1/D6. The possibility

55 exists that a few amino acids immediately adjacent to the Ser residue are also recognized since in

1 amino acid composition, the hexasaccharide peptide fractions derived from shark and whale cartilage
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4 CS differ considerably (see above).
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10 *Assessment of the reactivity of 4E1/D6 to the sulfatase- or phosphatase-treated linkage*

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12 *hexasaccharide-peptide fraction* Since 4E1/D6 did not react to the hexasaccharide-peptide fraction
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14 of whale cartilage CS (Fig. 2), it seems to recognize a sulfated or phosphorylated component of the
15
16 pentasaccharide-peptide fraction of shark cartilage CS. GalNAc and two Gal residues can often be
17
18 modified by 6-*O*-sulfate, and Xyl can be modified by 2-*O*-phosphate. In addition, GalNAc-5 and
19
20 Gal-3 can be sulfated at position C-4. Therefore, to examine whether 4-*O*-sulfation and
21
22 2-*O*-phosphorylation in the linkage region are required for the epitope's recognition, the reactivity of
23
24 4E1/D6 toward the linkage hexasaccharide-peptide fraction was investigated by ELISA before and
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26 after digestion with chondro-4-sulfatase or alkaline phosphatase (Fig. 4). The reactivity of 4E1/D6
27
28 toward the hexasaccharide-peptide fraction was not diminished after either treatment, suggesting that
29
30 neither the 4-*O*-sulfate nor 2-*O*-phosphate group is recognized by 4E1/D6. The enzymatic activity of
31
32 chondro-4-sulfatase and alkaline phosphatase toward the linkage region was confirmed by digestion
33
34 of the structurally defined linkage hexasaccharides. As shown in Fig. 5, after treatment with
35
36 chondro-4-sulfatase, the position of the disulfated linkage hexasaccharide
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38 Δ HexA-GalNAc(4-*O*-sulfate)-GlcA-Gal(4-*O*-sulfate)-Gal-Xyl (Fig. 5A, closed arrow) shifted to that
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40 position of the nonsulfated linkage hexasaccharide Δ HexA-GalNAc-GlcA-Gal-Gal-Xyl (Fig. 5B,
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1 open arrow). Digestion of the 2AB-derivative of the phosphorylated hexasaccharide,
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4 Δ HexA-GalNAc-GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-2AB, with alkaline phosphatase yielded
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6
7 Δ HexA-GalNAc-GlcA-Gal-Gal-Xyl-2AB as judged by anion-exchange HPLC (data not shown). The
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9
10 recognition of the 2-*O*-phosphorylated linkage region by 4E1/D6 was also examined by competitive
11
12
13 ELISA using a chemically synthesized linkage tetrasaccharide-peptide,
14
15
16 GlcA-Gal-Gal-Xyl(2-*O*-phosphate)-Ser-Gly [24], as an inhibitor. However, it showed no inhibitory
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18
19 activity. No sulfatase which hydrolyzes 6-*O*-sulfate groups in the linkage region is available, and
20
21
22 therefore it is not possible to investigate whether the 6-*O*-sulfate groups are recognized. The
23
24
25 commercial bacterial chondro-6-sulfatase (Seikagaku Corp.) does not act on the 6-*O*-sulfate groups
26
27
28 of the Gal residues in the linkage region [34].
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36 *Analysis of the interactions of 4E1/D6 with CHO cell lines deficient in the biosynthetic enzymes for*
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39 *the linkage region of GAGs* The specificity of 4E1/D6 was investigated further by analyzing its
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41
42 interaction with two CHO cell lines, CHO-K1 and pgsA-745, using immunofluorescence
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44
45 flowcytometry. The pgsA-745 is a mutant cell line deficient in xylosyltransferase, and was
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48 established from CHO-K1 cells (wild type) [22]. In the biosynthesis of CS and HS, a
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50
51 xylosyltransferase initiates the formation of the tetrasaccharide linkage region by transferring a Xyl
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53
54 residue to a specific Ser residue of the core protein. The pgsA-745 cells produce neither CS nor HS
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56
57 polysaccharide chains since they cannot construct the linkage region, which is common to CS and
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59
60

1 HS. Although no immunoreactivity with pgsA-745 cells was detected (Fig. 7B, light gray
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3
4 histograms), weak but significant immunoreactivity with CHO-K1 cells was (Fig. 7A, light gray
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6
7 histogram), suggesting the epitope of 4E1/D6 to exist on the surface of wild-type CHO cells but not
8
9
10 the mutant cells. A HS-specific antibody, F58-10E4, was used as a positive control, and as expected,
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13 intensely stained CHO-K1 cells but did not stain pgsA-745 cells (Fig. 7, dark gray histograms).
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20 Discussion

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22
23 Galactosaminoglycans (CS/DS) and glucosaminoglycans (HS/Hep) are synthesized at
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26 specific Ser residues of the core proteins of PGs through the tetrasaccharide linkage region
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28
29 $\text{GlcA}\beta 1\text{-3Gal}\beta 1\text{-3Gal}\beta 1\text{-4Xyl}$ [5]. Although the tetrasaccharide core is common to CS/DS and
30
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32 HS/Hep, the pattern of modification in the linkage region differs between the two types of GAG
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35 chains. Namely, the 4-*O*-sulfation of a Gal-3 residue and 6-*O*-sulfation of both Gal-2 and Gal-3
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38 residues have been found in CS/DS, but not HS/Hep. Interestingly, syndecan-1, a hybrid-type PG
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41 bearing both HS and CS chains, carries a 4-*O*-sulfate on the Gal-3 of only the CS chains [9],
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43
44 supporting the notion that 4-*O*-sulfation is a modification specific to CS chains [7]. In contrast,
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47 phosphorylation occurs on the Xyl of both CS/DS and HS/Hep [9]. Interestingly, these modifications
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49
50 have significant effects on the synthesis of the tetrasaccharide linkage region. The phosphate on Xyl
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53 stimulates the attachment of the first GlcA-4 [10, 11], but then seems to be removed [37]. In contrast,
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56 it inhibits the attachment of Gal-2 [10], suggesting the phosphorylation to take place after the transfer
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1 of Gal-2 but before that of GlcA-4. Intriguingly, the sulfation of Gal-2 promotes the transfer of the
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4 first GlcA [10, 11]. Furthermore, prior 4-*O*-sulfation of Gal-3 stimulates the 6-*O*-sulfation of Gal-2
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6
7 by recombinant human C6ST-1 [38]. Thus, the sulfated linkage region may also contain signals that
8
9
10 influence further sulfation of the linkage region and the repeating disaccharide region as well as
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12
13 chain polymerization. In fact, sulfation in the vicinity of the linkage region has an influence on the
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15
16 β -*N*-acetylgalactosaminyltransferase activity for chain elongation [39, 40]. However, the biological
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19 significance of modifications to the linkage region has not been fully elucidated, and tools such as
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22 antibodies which recognize these modifications are needed.
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26 In this study, a monoclonal antibody, 4E1/D6 (IgM, kappa), which recognizes the
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29 CS-protein linkage region derived from CS of shark cartilage, was developed for the first time. It
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31
32 recognizes the core pentasaccharide GalNAc-GlcA-Gal-Gal-Xyl: the linkage between the Xyl and
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34
35 Ser is also essential. A few other amino acids in the vicinity of the Ser residue may be recognized as
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37
38 well. In addition, the fifth sugar residue (GalNAc-5) from the reducing end is also important. The
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41 2-*O*-phosphate on Xyl is not involved in the recognition. The antibody did not bind the
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44 hexasaccharide glycopeptide fraction
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47 (Δ HexA-GalNAc(\pm 4-*O*-sulfate)-GlcA-Gal(\pm 4-*O*-sulfate)-Gal-Xyl-peptides) prepared from CS of
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49
50 whale cartilage [21], suggesting that it does not recognize the unmodified tetrasaccharide core or
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52
53 4-*O*-sulfate groups. In contrast, the 6-*O*-sulfate groups on the GalNAc and/or Gal residues appear to
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55
56 be recognized. The results altogether indicate that the antibody recognizes at least one of the
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1 following structures isolated from CS of shark cartilage [25, 26]:

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4 GalNAc-GlcA-Gal-Gal(6-*O*-sulfate)-Xyl-Ser,

5
6
7 GalNAc(6-*O*-sulfate)-GlcA-Gal-Gal(6-*O*-sulfate)-Xyl-Ser,

8
9
10 GalNAc-GlcA-Gal(6-*O*-sulfate)-Gal(6-*O*-sulfate)-Xyl-Ser,

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12
13 GalNAc(6-*O*-sulfate)-GlcA-Gal(6-*O*-sulfate)-Gal(6-*O*-sulfate)-Xyl-Ser. Although the precise epitope
14
15
16 remains unidentified due to the inavailability of specific reagents such as a sulfatase to remove
17
18
19 6-*O*-sulfate groups, this antibody will still be useful for investigating the biological significance of
20
21
22 the 6-*O*-sulfation of the GalNAc and/or Gal residues of the core pentasaccharide.
23
24
25

26 The 6-*O*-sulfated Gal residues in the linkage region have been demonstrated in CS chains
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28
29 isolated from shark cartilage [26], as well as bovine and human articular cartilage [8, 41]. The
30
31
32 antibody reacted with the CHO-K1 cells as well, suggesting the presence of the structure in the CS
33
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35 chains on the surface of mammalian cells, although it is extremely difficult to determine using a
36
37
38 chemical method the structure of a minute part of the linkage region of CS/DS or HS/Hep derived
39
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41 from cultured cells, which contain only one mole per single GAG chain. Notably, however, a
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43
44 recombinant human chondroitin 6-*O*-sulfotransferase-1 (C6ST-1), which transfers a sulfate to the
45
46
47 C-6-position of GalNAc in the repeating disaccharide region of CS chains, can also transfer a sulfate
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49
50 group to both the Gal residues in the linkage region [38]. Likewise, a recombinant human
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52
53 chondroitin 4-*O*-sulfotransferase-1 (C4ST-1), which transfers a sulfate to the C-4-position of
54
55
56 GalNAc in the repeating disaccharide region of CS chains, can also transfer a sulfate group to Gal-3
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59

1 in the linkage region (Mizumoto *et al.*, unpublished results). The 4-*O*-sulfated Gal-3 structure has
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3
4 been shown in CS from various mammalian tissues and cells including rat chondrosarcoma [7] and
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6
7 human plasma [42]. The kinase that phosphorylates the Xyl residue has recently been identified as
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10 FAM20B [43]. Thus, the above modifications appear to be widely distributed.
11

12
13 Modifications of the linkage region may also act as a marker for the intracellular transport
14
15 of CS-PGs to the Golgi compartment for biosynthetic processing or elongation and maturation of the
16
17 repeating disaccharide region of CS chains. It is likely that PGs modified with a 4-*O*- or 6-*O*-sulfate
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20 group by the actions of C4ST-1 or C6ST-1 are transported into the suitable Golgi compartment, and
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22
23 consequently sulfated on GalNAc residues for maturation as well. It would be interesting to
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26 investigate putative binding proteins, which recognize the sulfated Gal residues and may help
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29 acceleration of the transport and/or sulfation of the GalNAc residues of the repeating disaccharide
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32 region. Thus, the sulfation of the Gal residues may reflect a possible difference in the Golgi
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35 compartments in which CS and HS chains are synthesized.
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42 It should be noted that C6ST-1 activity is found in medial and trans-Golgi fractions [44].
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44
45 and that GlcAT-I involved in the synthesis of the linkage region is distributed in both medial and
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47
48 trans Golgi/trans Golgi networks. This distribution is similar to that of chondroitin-polymerizing
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51 glucuronyltransferase-II activity [45]. It has also been reported that GlcAT-I has a dual Golgi
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53
54 distribution similar to that of chondroitin polymerizing GlcA transferase-II and distinctly different
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56
57 from the distribution of the two galactosyltransferases found exclusively in cis-Golgi fractions [45].
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1 It has also been reported that xylosyltransferase, galactosyltransferases, and GlcAT-I are distributed
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4 in ER/cis-Golgi, cis-/medial-Golgi, and medial-/trans-Golgi, respectively [46]. These results suggest
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6
7 that nascent PGs are transported from cis- to trans-Golgi compartments during maturation, and that
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10 the sulfation of the linkage region takes place before the transfer of the first *N*-acetylhexosamine
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13 residue to the tetrasaccharide core and could be a signal for the differential assembly of CS and HS
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16 chains as proposed previously [7, 21, 26].
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20 The CHO mutant cells, which are deficient in the glycosyltransferases that synthesize the
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23 linkage region tetrasaccharide core, cannot synthesize either CS/DS or HS/Hep, suggesting the same
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26 glycosyltransferases synthesize the linkage region common to both types of the GAG chains [22, 47,
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29 48]. Therefore, the types of GAG chains to be selectively assembled on the linkage region
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32 tetrasaccharide are determined after or during the construction of the linkage region by these
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35 enzymes. The amino acid sequence around the GAG attachment site (Ser) varies among different
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38 PGs. Therefore, it is unlikely that only the amino acid sequence near the GAG attachment site
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41 determines the type of GAG, although it has some influence [49]. In fact, a PG named serglycine is a
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44 typical example, which can be modified by CS or HS [50].
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48 A number of PGs occur in the form of both a PG and a protein without a GAG chain, and
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51 are called part-time PGs [51]. For example, thrombomodulin, a cell surface glycoprotein, occurs both
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54 as a CS-PG (β -thrombomoduline) and as a protein without a CS chain (α -thrombomodulin), which
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57 has only a tetrasaccharide linkage region, being an immature glycoprotein [52]. The biosynthetic
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1 control mechanism to produce such immature PG forms or to interfere with the glycanation step
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4 remains to be investigated. This antibody may also be useful for investigating the biological
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7 functions and processing of part-time PGs.
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2

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1 **Footnotes**
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3

4 1. Affiliation of the authors
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1 **Figure legends**
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4 **Fig. 1** Reactivity of the antibody 4E1/D6 toward various PG preparations. The reactivity of the
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6 antibody 4E1/D6 with various PGs was analyzed by ELISA. PGs from salmon nasal cartilage,
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8 rayfish cartilage, bird nest, chicken cartilage, bovine nasal cartilage, whale cartilage (10 µg each) and
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10 the linkage hexasaccharide peptide (hexa-pep) fraction derived from shark cartilage CS (0.5 ug) were
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12 individually immobilized to the wells, and processed for incubation with 4E1/D6 followed by
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14 alkaline phosphatase-linked goat anti-mouse Ig(G+M) (diluted 3,000-fold). Bound antibodies were
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16 detected by the addition of *p*-nitrophenylphosphate as a substrate. The reactivity is given relative to
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18 the linkage hexasaccharide-peptide fraction from shark cartilage CS.
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32 **Fig. 2** Reactivity of the antibody 4E1/D6 toward various linkage oligosaccharide-peptide fractions.
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34 The linkage hexa-, penta-, and tetrasaccharide-peptide fractions derived from shark cartilage CS and
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36 the linkage hexasaccharide-peptide fraction from whale cartilage CS (0.5 µg each) were immobilized
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38 to the wells, and the reactivity of 4E1/D6 to the immobilized fractions was analyzed by ELISA as
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40 described in the legend to Figure 1. The assay was performed in duplicate and values represent the
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42 mean ± S.D. The reactivity is given relative to the linkage hexasaccharide-peptide fraction from
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58 **Fig. 3** Reactivity of the antibody 4E1/D6 toward the linkage hexasaccharide-peptide fraction treated
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1 with alkali or peptidases. The linkage hexasaccharide-peptide (hexa-pep) fraction from shark
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4 cartilage CS was treated with alkali, or digested with aminopeptidase or carboxypeptidase. Each
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7 sample was used as an inhibitor (4 nmol each) for the binding of 4E1/D6 to the immobilized
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10 un-treated linkage hexasaccharide-peptide fraction (0.5 μ g) for the competitive ELISA. The
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13 un-treated linkage hexasaccharide-peptide fraction (4 nmol) was also used as a control inhibitor.
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16 Bound antibodies were visualized as described in the legend to Figure 1. This assay was performed
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19 in duplicate, and all values are expressed as a percentage of the reactivity observed without inhibitors
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22 and represent the mean \pm S.D.
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29 **Fig. 4** Reactivity of the antibody 4E1/D6 toward the linkage hexasaccharide-peptide fraction treated
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31 with phosphatase or chondro-4-sulfatase. The linkage hexasaccharide-peptide fraction (500 pmol
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33 each) was treated with alkali phosphatase or chondro-4-sulfatase and each digest was immobilized to
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36 the well. The reactivity of 4E1/D6 toward the digest was analyzed by ELISA. Bound antibodies were
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39 visualized as described in the legend to Figure 1. The reactivity is given relative to the linkage
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42 hexasaccharide-peptide fraction from shark cartilage CS. The assay was performed in duplicate and
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45 values represent the mean \pm S.D.
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55 **Fig. 5** Action of chondro-4-sulfatase on the structurally defined linkage hexasaccharide. The
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57
58 structurally defined linkage hexasaccharide,
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1 by immunofluorescence flow cytometry. A HS-specific monoclonal antibody (F58/10E4) was used as
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4 a control. *Light gray* and *dark gray histograms* represent the 4E1/D6 and F58-10E4-binding,
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7 respectively, as detected with Alexa Fluor 488[®]-conjugated anti-mouse IgM antibody. *Empty*
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10 *histograms* show the background fluorescence.
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Figure1

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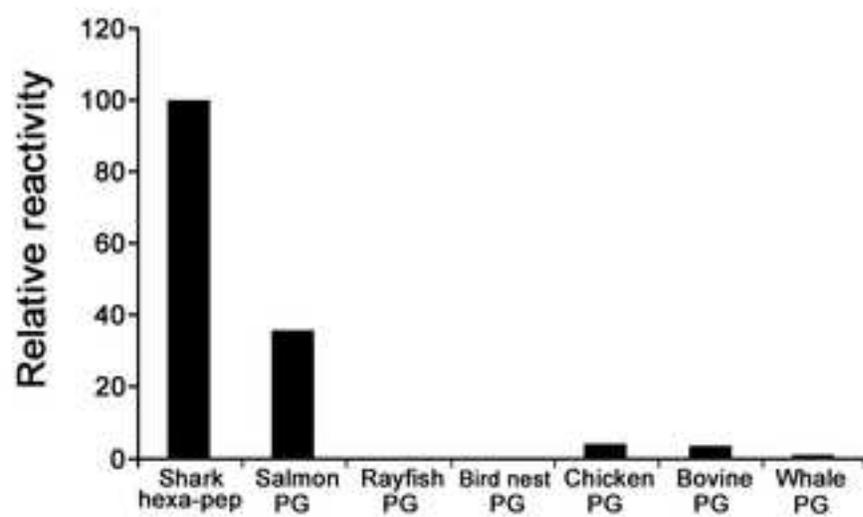


Figure2

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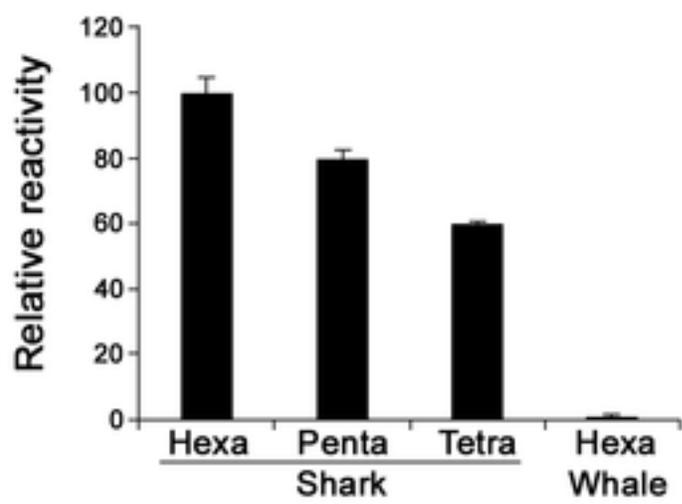


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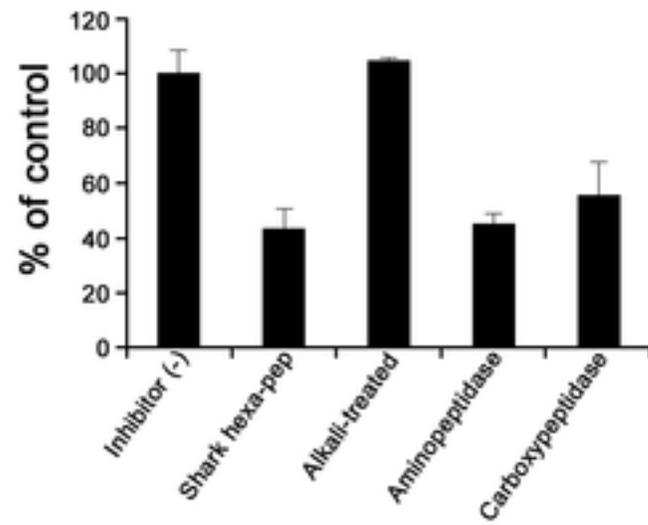


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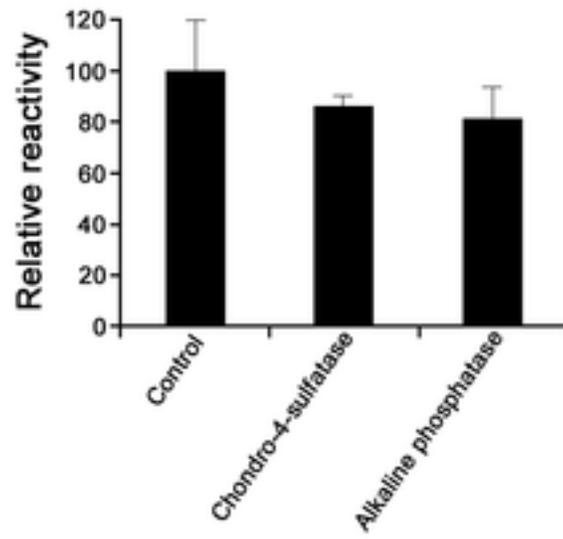


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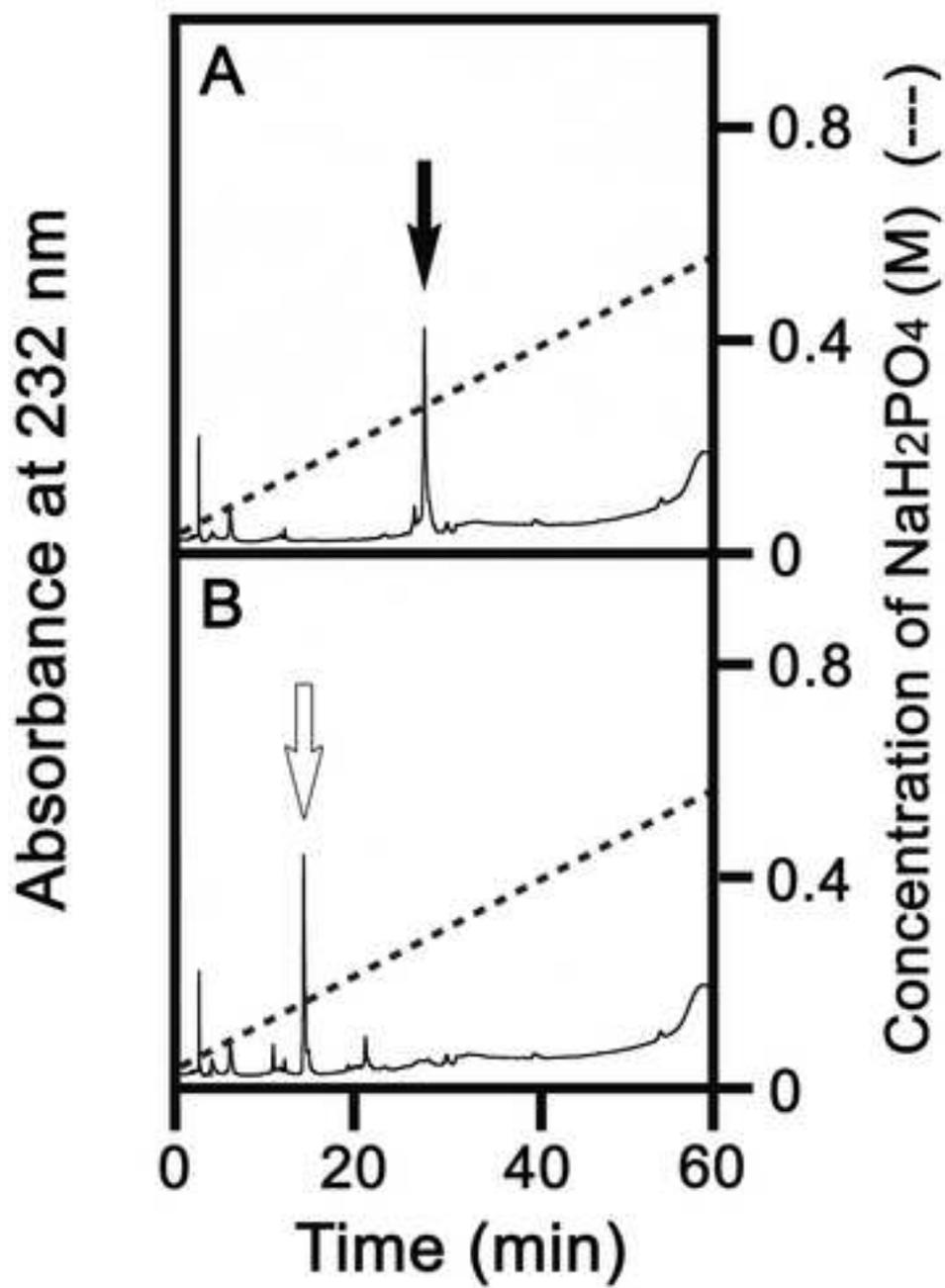


Figure6

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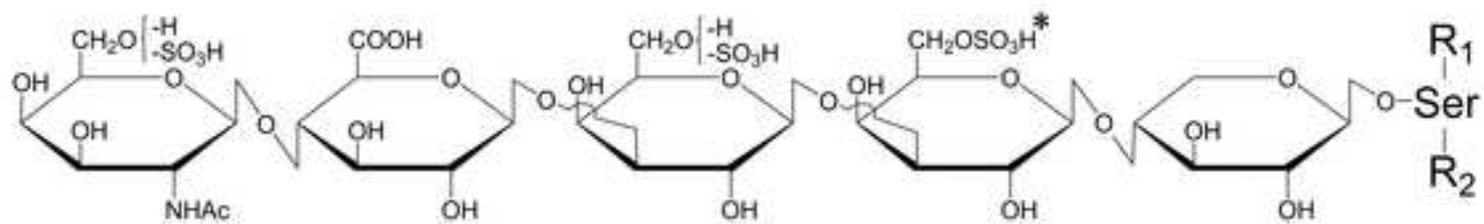


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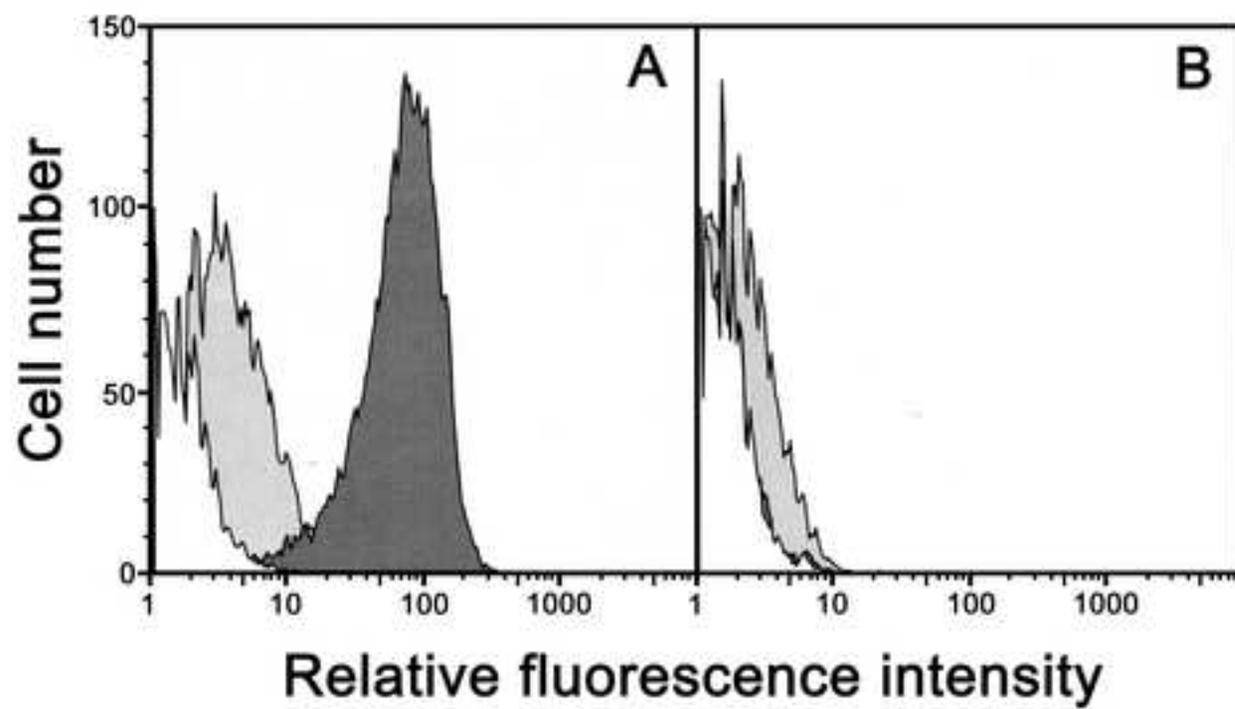


Table 1. Structure of the major components in the linkage hexasaccharide fractions purified from CS of shark cartilage and whale cartilage

$\Delta\text{HexA}\alpha\text{1-3GalNAc}\beta\text{1-4GlcA}\beta\text{1-3Gal}\beta\text{1-3Gal}\beta\text{1-4Xyl}\beta\text{1-O-Ser}$							
<u>6</u> <u>5</u> <u>4</u> <u>3</u> <u>2</u> <u>1</u>							
CS from shark cartilage ^a							
						Proportion ^c	
						(mol%)	
<u>6</u>	<u>5</u>	Modifications ^b				<u>1</u>	
		<u>4</u>	<u>3</u>	<u>2</u>			
—	—	—	—	—	—	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							
						Proportion ^c	
						(mol%)	
<u>6</u>	<u>5</u>	Modifications ^b				<u>1</u>	
		<u>4</u>	<u>3</u>	<u>2</u>			
—	—	—	—	—	—	21	
—	6S	—	—	—	—	16	
—	4S	—	—	—	—	36	
—	4S	—	4S	—	—	27	

^aData are from Sugahara *et al.* 1992 [25] and de Waard *et al.* [26].

^bThe 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.

^cThe proportion of each structure is expressed as a molar percentage of the total linkage hexasaccharide fraction.

^dData are from Sugahara *et al.* 1991 [21].