Binding of a Large Chondroitin Sulfate/Dermatan Sulfate Proteoglycan, Versican, to L-selectin, P-selectin, and CD44*

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Here we show that a large chondroitin sulfate proteoglycan, versican, derived from a renal adenocarcinoma cell line ACHN, binds L-selectin, P-selectin, and CD44. The binding was mediated by the interaction of the chondroitin sulfate (CS) chain of versican with the carbohydrate-binding domain of L- and P-selectin and CD44. The binding of versican to L- and P-selectin was inhibited by CS B, CS E, and heparan sulfate (HS) but not by any other glycosaminoglycans tested. On the other hand, the binding to CD44 was inhibited by hyaluronic acid, chondroitin (CH), CS A, CS B, CS C, CS D, and CS E but not by HS or keratan sulfate. A cross-blocking study indicated that L- and P-selectin recognize close or overlapping sites on versican, whereas CD44 recognizes separate sites. We also show that soluble L- and P-selectin directly bind to immobilized CS B, CS E, and HS and that soluble CD44 directly binds to immobilized hyaluronic acid, CH, and all the CS chains examined. Consistent with these results, structural analysis showed that versican is modified with at least CS B and CS C. Thus, proteoglycans sufficiently modified with the appropriate glycosaminoglycans should be able to bind L-selectin, P-selectin, and/or CD44.

Proteoglycans are a heterogeneous family of macromolecules found in all tissues. They provide structural integrity to tissues and mediate cell proliferation, differentiation, and migration. Proteoglycans consist of a core protein to which one or more glycosaminoglycan (GAG) side chains are covalently attached. There are several types of GAGs, which include heparin or heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (CS B), and keratan sulfate (KS). Several of the biological activities of proteoglycans are attributable to the GAGs. For example, heparin binds anti-thrombin III (1), and heparin and HS bind fibroblast growth factor (2) and various chemokines (3–5). Furthermore, CS binds a subset of chemokines, such as RANTES (regulated on activation normal T cell expressed and secreted) (6) and PF-4 (platelet factor-4) (7).

Versican, also known as PG-M, is a large CS proteoglycan that is expressed in cultured fibroblasts (8), proliferating keratinocytes (9), and arterial smooth muscle cells (10), and is found in the kidney, skin, brain, and other tissues (11). Versican has a hyaluronic acid (HA)-binding domain at its amino terminus and a set of epidermal growth factor (EGF)-like, lectin-like, and complement regulatory protein-like domains at its carboxyl terminus (8). It also has a CS-bearing domain in its middle portion that contains two alternatively spliced domains called GAG-α and GAG-β (12). As a result of alternative splicing, versican has four distinct isoforms that contain different numbers of CS chains (13). The amino-terminal domain of versican binds HA with high affinity (14). On the other hand, the carboxyl-terminal lectin-like domain binds simple sugars such as fucose and GlcNAc (15) and sulfated glycolipids (16). The lectin-like domain also binds extracellular matrix proteins such as tenasin-R (17) and fibulin-1 (18), apparently through protein-protein interactions. The EGF-like domain of versican enhances cell proliferation, at least in part through binding to the EGF receptor (19). Until now, little has been known about the counter receptors that interact with the CS chain of versican in vivo.

We have previously shown that versican, derived from a renal adenocarcinoma cell line, ACHN, interacts with a leukocyte adhesion molecule, L-selectin, through versican’s CS chains (20). L-selectin is a member of the selectin family, which is characterized by an amino-terminal lectin-like domain followed by an EGF-like domain and complement regulatory protein-like domains. Studies of the carbohydrate-based ligands for L-selectin initially identified two sialomucins, GlyCAM-1 (21) and CD44 (22), which are expressed on the high endothelial venules of lymph nodes. Subsequent studies revealed that these molecules interact with L-selectin via a sialyl Lewis X (sLeX)-like carbohydrate structure, 6-sulfo-sLeX (23). Other sialomucins, such as podocalyxin-like protein (24), sulfated glycoprotein (Sgp) (25), and PSGL-1 (26) also bind to L-selectin in a carbohydrate-dependent manner. PSGL-1 also binds to the endothelial P- and E-selectin (26, 27).

CD44, a distinct class of carbohydrate-binding molecules, is expressed on a variety of cell types, including leucocytes, fibroblasts, endothelial cells, and epithelial cells. Its best characterized ligand is probably HA (28), whereas other types of ligands have also been described. Previous work from our laboratory shows that a CS proteoglycan, serglycin, derived from a T cell line, binds CD44 to stimulate granzyme release from a CD44-
positive cytotoxic cell line (29). The CS form of the invariant chain has also been reported to bind CD44 (30). These carbohydrate-based ligands interact with the link module present on the amino terminus of CD44 (31). The link module appears to have a close structural similarity to the C-type lectin domain (32), suggesting an evolutionary relationship and a possible functional link between CD44 and the selectins.

The present study was performed to determine whether versican binds to other selectin family members, besides L-selectin, and to CD44. Our data show that versican that is derived from ACHN renal adenocarcinoma cells binds L-selectin, P-selectin, and CD44 through its CS chains and that, among the various GAG chains, CS B, CS E, and HS bind L- and P-selectin, whereas HA, CS A, CS B, CS C, CS D, CS E, and chondroitin (CH) bind CD44.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Human L-selectin-Ig and human E-selectin-Ig were provided by Dr. S. R. Watson (Genentech, Inc., South San Francisco, CA). Human P-selectin-Ig was provided by Dr. M. Omata (Suntory Co., Osaka, Japan). Each selectin-Ig chimera consists of the amino-terminal lectin-like domain, the EGF-like domain, the first two complement regulatory protein-like domains, and the C domain from human IgG. Human CD44-Ig was produced as described previously (29). Neuraminidase (Arthrobacter ureafaciens) was purchased from Roche Molecular Biochemicals. CH, CS A (whale cartilage), CS B (pig skin), CS C (shark cartilage), CS D (shark cartilage), CS E (squid cartilage), KS (bovine cornea), HS (bovine kidney), biotinylated sLeX polymeric-probe (sLeX BP-probe), chondroitinase ABC (Proteus vulgaris), chondroitinase AC II (Arthrobacter auruncus), chondroitinase B (Flavobacterium heparitinum), hyaluronidase SD (Streptococcus ducgalactiae), hyaluronidase (Streptomyces hyaluralidicus), anti-sLeX mAb KM-93, anti-versican mAb 2B1, anti-GAG mAbs (CS-56, 2-B-6, 3-B-3, 1-B-6), and biotinylated SLeX-BSA was purchased from Oxford Biotech UK Ltd., Buckinghamshire, UK) according to the instructions provided by the manufacturer.

**Preparation of Lipid-derivatized GAGs**

Lipid-derivatized GAGs were prepared according to the method of Stoll et al. (33) with some modifications. Briefly, 1 mg of each GAG was dissolved in 100 µl of distilled water. To this, 1.9 ml of dipalmityl phosphatidylethanolamine (5 mg/ml) in chloroform/ methanol (1/1, v/v) was added, and the reaction mixture was sonicated for 5 min. After incubation at 60 °C for 2 h, the mixture was dialyzed at 0.5 µl of NaBH₄-CN (10 mg/ml) in methanol was added, and the reaction mixture was incubated at 60 °C for 16 h. The reaction mixture was then lyophilized and dissolved in 2 µl NaCl in 15% ethanol, and the insoluble material was removed by centrifugation. The sample was precipitated with five equivalent volumes of ethanol, and the precipitate was dissolved in 0.2 µl NaCl and applied to a TSKgel Phenyl Toyopearl 650M column (TOSOH, Tokyo, Japan) equilibrated with 0.2 µl NaCl. The lipid-derivatized GAG was eluted with 30% methanol. The eluate was lyophilized and redissolved in distilled water before use.

**Enzyme Treatment**

Chondroitinase ABC or chondroitinase B treatment was performed at 37 °C for 2 h in 50 mM Tris-HCl, 15 mM sodium acetate, pH 8.0. Neutralized antibodies, hyaluronidase (S. dysgalactiae), hyaluronidase (S. hyaluralidicus), or chondroitinase ACII treatment was performed at 37 °C, for 2 h in 50 mM sodium acetate (pH 6.0).

**Enzyme-linked Immunosorbent Assay**

**Method 1—Recombinant Ig-chimeras, the control human IgG₂, or the mAbs against sLeX or GAGs in PBS (25 µl/well) were added to 96-well flat-bottomed microtiter plates (Costar EIA/RIA plate 3690, Corning Inc., Corning, NY) and kept overnight at 4 °C. The wells were washed with buffer A (0.05% Tween 20, 20 mM HEPES-NaOH, 0.15 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 6.8) and blocked with Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) for 2 h. Biotinylated versican, biotinylated HA, or sLeX BP-probe in buffer A was added to the wells in the presence or absence of mAbs, GAGs, or the soluble Ig-chimeras and incubated for 2 h. After washing the wells with buffer A, alkaline phosphatase-conjugated streptavidin (Promega, Madison, WI), diluted 1:500, was added and incubated for 1 h. To quantify the reaction, Blue Phos substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added, and the optical density was read at 650 nm in a microtiter plate reader (InterMed Co., Tokyo).

**Method 2—Lipid-derivatized GAGs or sLeX-sphingolipid (100 µg/ml, 25 µg/well) were immobilized on 96-well flat-bottomed microtiter plates by drying at 60 °C for 5 h. After blocking with Block Ace, human IgG₂ (5 µg/ml), L-selectin-Ig (5 µg/ml), P-selectin-Ig (3 µg/ml), or CD44-Ig (1 µg/ml) was added to the wells and incubated for 2 h. The binding was detected with alkaline phosphatase-labeled goat anti-human IgG (American Qualex, Santa Clara, CA) diluted 1:1000 and Blue Phos substrate as described above.

**Method 3—Versican (2 or 4 µg/ml, 25 µg/well) or HA (4 µg/ml, 25 µg/well) was added to 96-well flat-bottomed microtiter plates and kept overnight at 4 °C. After blocking with Block Ace, chondroitinase ABC, chondroitinase ACII, chondroitinase B, or the control human IgG (S. hyaluralidicus) was added to the wells and incubated at 37 °C for 2 h. After washing the wells with PBS containing 0.05% Tween 20 and 0.1% BSA, mAb CS-56, 2-B-6, 3-B-3, 1-B-5, or biotinylated HABP (5 µg/ml) was added to the wells and incubated for 1 h. The binding was detected with HRP-labeled goat anti-mouse IgG+M (American Qualex) diluted 1:500 and o-phenylenediamine (0.4 mg/ml) as described previously (20). When biotinylated HABP was used as a primary reagent, the binding was detected with HRP-labeled streptavidin (Zymed Laboratories Inc. Laboratories, San Francisco, CA).

**FACE Analysis**

FACE (fluorophore-assisted carbohydrate electrophoresis) was performed as described previously (34). In brief, 730 ng of purified versican was digested in 50 µl of 0.1 M ammonium acetate, pH 7.3, containing 0.5 unit/ml each of chondroitinase ABC and ACII, for 18 h at 37 °C. The digestion products were separated from enzymes and the deglycosylated core protein by ethanol precipitation, fluorotagged with 2-amino-acridone (Molecular Probes Inc., Eugene, OR), and then analyzed by Western Blotting Analysis

**Binding of Versican to L- and P-selectin and CD44**

**Purification of Versican**

Versican was purified from the conditioned medium of a human renal adenocarcinoma cell line, ACHN, as described previously (20).

**Biotinylation**

Purified versican (50 µg/ml) or HA (1 mg/ml) was dialyzed against 0.1 M NaHCO₃, pH 8.0, containing 0.1 M NaCl, and coupled overnight with 100 µg/ml NHS-LS-biotin (Pierce Chemical Co., Rockford, IL) at room temperature. The reaction was quenched by the addition of a quarter volume of 1× Tris-HCl, pH 7.6. The biotinylated material was dialyzed against phosphate-buffered saline (PBS) and stored at −80 °C until use.

**Western Blotting Analysis**

Versican was subjected to SDS-PAGE or SDS-agarose PAGE (20) and transferred onto an IPVH filter (Millipore Co., Bedford, MA). After blocking with PBS containing 3% BSA and 0.1% NaN₃, the blot was probed with either (i) ABC reagent (Vector Laboratories, Inc., Burlingame, CA), or (ii) anti-versican mAB 2B1 (0.5 µg/ml) and horse-radish peroxidase (HRP)-conjugated goat anti-mouse IgG (American Qualex Co., 1:2000), as described previously (20). After washing with PBS containing 0.1% BSA and 0.05% Tween 20, the blot was developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) according to the instructions provided by the manufacturer.
Cell Binding Assay

Mouse T lymphomas BW5147 and EL-4 obtained from ATCC (American Type Culture Collection; Rockville, MD) or EL-4 transfected with human L-selectin cDNA (20) were labeled with 5 μM 7-hydroxycoumarin-4-carboxylate-2-carboxyethyl-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes Inc., Eugene, OR) for 30 min at 37 °C. After washing, the cells were suspended in buffer B (20 mM HEPES-NaOH, 0.14 M NaCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 6.8) with or without 20 μg/ml anti-mouse CD44 mAb KM-93 (rat IgG), or 30 μg/ml anti-human L-selectin mAbs TQ-1 or DREG-56 (mouse IgG1). After incubation at 4 °C for 10 min, the cells were directly applied to the wells of a 96-well microtiter plate (5 × 10⁴ cells/well; Costar EIA/RIA plate 3690) that had been coated with 4 μg/ml versican or HA and subsequently blocked with Block Ace. In some experiments, versican- or HA-coated wells were pretreated with 5 milliunits/ml or 100 milliunits/ml of chondroitinase ABC, or 40 TRU/ml of hyaluronidase (from S. hyalurolyticus) at 37 °C for 2 h before applying the cells. The cells were allowed to bind for 40 min at 4 °C. The wells were then filled with buffer B, and the plate was inverted and placed for 30 s. After removal of the unbound cells by gentle aspiration, 1% Nonidet P-40 in PBS was added to each well, and the plate was read at 485 nm of excitation and 538 nm of emission in Labosystems Fluoroskan II (Labosystems Japan, Tokyo).

RESULTS

Versican Binds L-selectin, P-selectin, and CD44—L-selectin-reactive versican was conditioned from the transfected conditioned medium of a renal adenocarcinoma cell line, ACHN, as described previously (20), labeled with NHS-LC-biotin, and subjected to SDS-PAGE (left) or SDS-agarose-PAGE (right), and transferred to a polyvinylidene difluoride membrane. The sample was probed using ABC reagent or anti-versican mAb 2B1 as a primary antibody and HRP-goat anti-mouse IgG as a secondary antibody. Mouse IgG1, (mlpG₁), was used as a control. B and C, binding of biotinylated versican (B) or sLeX BP-probe (C) (0–3 μg/ml) to the wells coated with 5 μg/ml anti-sLeX mAb 2H5 (○), anti-sLeX mAb KM-93 (●), anti-sLeX mAb CSLEX-1 (□), anti-chondroitin sulfate mAb CS-56 (△), or control mouse IgG (●) was determined by ELISA (Method 1) as described under “Experimental Procedures.”

FACe. The molar ratio of Di-0S:Di-6S:Di-4S was assessed by the Eagle Eye documentation system (Stratagene, La Jolla, CA) with illumination at 300 nm.

mAb 2B1. On SDS-agarose-PAGE (20), the purified versican migrated as a broad band of approximately 1600 kDa that was also reactive with mAb 2B1. The purified versican was used throughout this study.

Because the glycoprotein ligands for L-selectin so far identified bear sLeX-like carbohydrates, we examined whether versican also possesses these carbohydrate epitopes. As shown in Fig. 1 (B and C), versican bound minimally, if at all, to immobilized anti-sLeX mAbs (2H5, KM-93, CSLEX-1), whereas the sLeX BP-probe did not. These results indicate that, unlike the other known glycoprotein ligands for L-selectin, versican has CS chains but little or none of the sLeX determinants.

We then explored the possibility that versican binds not only L-selectin but also other selectin family members and CD44 (Fig. 2). As shown in Fig. 2A, biotinylated versican reacted with not only L-selectin-Ig but also P-selectin-Ig and CD44-Ig in a dose-dependent manner, but with neither E-selectin-Ig nor control human IgG. Biotinylated versican did not bind irrelevant Ig chimeras such as CD2-Ig or poliovirus receptor-Ig (data not shown). As expected, sLeX BP-probe bound to the immobilized L-, P-, and E-selectin-Igs (Fig. 2B), consistent with previous reports that sLeX is a common ligand for the selectins (35). Biotinylated HA reacted with CD44-Ig but not with the selectin-Igs (Fig. 2C).

Versican Interacts with the Lectin Domain of L- and P-selectin. The cells were allowed to bind for 40 min at 4 °C. The wells were then filled with buffer B, and the plate was inverted and placed for 30 s. After removal of the unbound cells by gentle aspiration, 1% Nonidet P-40 in PBS was added to each well, and the plate was read at 485 nm of excitation and 538 nm of emission in Labosystems Fluoroskan II (Labosystems Japan, Tokyo).

RESULTS

Versican Binds L-selectin, P-selectin, and CD44—L-selectin-reactive versican was conditioned from the transfected conditioned medium of a renal adenocarcinoma cell line, ACHN, as described previously (20), labeled with NHS-LC-biotin, and subjected to Western blotting analysis (Fig. LA). When analyzed on SDS-PAGE (Fig. 1A, left), the purified versican was retained at the top of the gel and was specifically reactive with the anti-versican
tin and the Link Module of CD44—To determine which domains of L- and P-selectin and CD44 are involved in binding versican, we examined the effects of various mAbs with known specificities on the binding of versican or known ligands to L- and P-selectin- and CD44-Igs (Fig. 3). As shown in Fig. 3A, although all anti-L-selectin mAbs that block binding to sLeX (MHL-1, MHL-3, TQ1, DREG-56) inhibited the binding between versican and L-selectin-Ig, non-blocking anti-L-selectin mAbs (MHL-2, 4G8) failed to inhibit the binding. EDTA also inhibited the binding of L-selectin-Ig to versican and sLeX. These results suggest that versican interacts with the lectin domain of L-selectin. Similarly, anti-P-selectin mAbs (G1, CLB-Thromb/6, AK4) and EDTA that block binding to sLeX inhibited the interaction between versican and P-selectin-Ig (Fig. 3B). However, mAb 1.2B6, which has dual specificity for E- and P-selectin (36), inhibited the interaction of P-selectin-Ig with the sLeX BP-probe but not with versican. These results suggest that versican binds to the lectin domain of P-selectin as does sLeX, although the binding sites are not identical. Anti-CD44 mAbs BRIC235 and RAMBM 5.5.8, but not F10-44-2 or BU75, inhibited the binding of both versican and HA to CD44-Ig (Fig. 3C), suggesting that versican, like HA, is recognized by the link module of CD44. BRIC235 recognizes epitope 2a in the link module of CD44 as defined in the Vth Human Leukocyte Differentiation Antigen Workshop (37, 38), whereas RAMBM 5.5.8 recognizes a different epitope in the link module of CD44 (39). When these mAbs were added together, the binding of both versican and HA to CD44-Ig was completely inhibited, whereas doubling the concentration of either mAb added alone did not further increase the extent of inhibition (Fig. 3C). These results suggest that at least two distinct epitopes are involved in the binding of CD44 to versican and to HA.

Versican Binds L- and P-selectin and CD44 through Its CS Chains—We next examined whether versican's CS chains are involved in the binding to L- and P-selectin- and CD44-Igs (Fig. 4). The binding of versican to L- and P-selectin- and CD44-Igs was almost completely inhibited by treatment with chondroiti-
nase ABC (Fig. 4, left), whereas the binding of versican to the anti-versican mAb 2-B-1 was not affected (data not shown). As expected, neuraminidase treatment inhibited the binding of the sLeX BP-probe to L- and P-selectin-Igs, whereas hyaluronidase treatment inhibited the binding of HA to CD44-Ig (Fig. 4, right). In contrast, neither of these treatments affected the binding of versican to the L- and P-selectin-Igs. These results indicate that versican binds L- and P-selectin and CD44 through its CS side chains. Although chondroitinase ABC can cleave both CS chains and HA, the processing rate for HA is slower (40); thus, we used a concentration of chondroitinase ABC (5 milliunits/ml) that selectively cleaves CS but not HA in Fig. 4C (also see Fig. 9C).

Certain GAGs Inhibit the Interactions between Versican and L-selectin, P-selectin, and CD44—We next examined the effects of various GAGs on the binding of versican to L- and P-selectin- and CD44-Igs (Fig. 5). Binding of versican to the L- and P-selectin-Igs was strongly inhibited by specific GAGs, i.e. CS B, CS E, and heparan sulfate (HS) but not by any other GAGs examined (Fig. 5, A and B). CS E was particularly potent in that it inhibited the binding of versican to the L- and P-selectin-Igs by about 50% at a concentration as low as 15 ng/ml (approximately 1 nM) (data not shown). Binding of versican to the L- and P-selectin-Igs was also inhibited by sLeX-BSA, in agreement with the notion that versican binds the lectin domain of L- and P-selectin that is similar to sLeX. Conversely, the binding of the sLeX-BP probe to the L- and P-selectin-Igs was inhibited by versican (data not shown). In contrast, the binding of versican to CD44-Ig was inhibited by all of the CS chains examined, as well as by CH and HA, but not by sLeX-
BSA, HS, or keratan sulfate (KS) (Fig. 5C). The binding of the biotinylated HA to the CD44-Ig was inhibited by versican, CH, and all the CS chains examined (data not shown), suggesting that versican, CH, and CS all bind the link module of CD44, similar to HA. HA inhibited the binding of versican to CD44-Ig by 50% at a concentration of 10 ng/ml (data not shown), whereas CH or CS chains inhibited the binding by 50% at about 100 to 3000 times higher concentrations (Fig. 5C), suggesting that HA has substantially higher affinity for CD44 than other GAGs. Inhibitory activity of CH and CS chains on the binding of versican to CD44-Ig appears not due to minor contamination of HA, because CH and CS preparations treated with hyaluronidase (S. hyalurolyticus) retained the same inhibitory activity (data not shown).

L- and P-selectin Bind to the Lipid-derivatized CS B, CS E, and HS, whereas CD44 Binds to the Lipid-derivatized CH, CS A, CS B, CS C, CS D, CS E, and HA—To determine whether L- and P-selectin and CD44 can directly recognize GAGs, we next prepared lipid-derivatized GAGs, immobilized them on ELISA plates, and performed a direct binding assay using soluble L- and P-selectin- and CD44-Igs (Fig. 6). The efficiencies for immobilizing each lipid-derivatized GAG onto the plastic surface were comparable, and ranged from 44% to 68% as assessed by the m-hydroxydiphenyl chromogenic reaction (41) (data not shown). As shown in Fig. 6, L- and P-selectin-Igs bound to the lipid-derivatized CS B, CS E, HS, and sLeX, but not to CH, CS A, CS C, CS D, KS, or HA. On the other hand, CD44-Ig bound to the lipid-derivatized CH, all the CS chains examined, and HA but not to HS, KS, or sLeX. These results are in agreement with those obtained in the preceding section, demonstrating a direct interaction between certain GAG chains with L- and P-selectin and with CD44. The results also indicate that the GAG binding specificities of L- and P-selectin are similar to each other but different from that of CD44.

Close or Overlapping Sites on Versican Are Recognized by L- and P-selectin, whereas Separate Sites Are Recognized by CD44—To compare the binding sites on versican for L- and P-selectin and CD44, we next performed cross-blocking experiments (Fig. 7). Binding of versican to immobilized L-selectin-Ig was inhibited with soluble L- or P-selectin-Ig but not with soluble CD44-Ig. Similarly, binding of versican to immobilized P-selectin-Ig was inhibited with L- or P-selectin-Ig but not with CD44-Ig. In contrast, binding of versican to immobi-
lized CD44-Ig was inhibited with CD44-Ig but not with the selectin-Igs. These results indicate that close or overlapping sites on versican are recognized by L- and P-selectin, whereas separate sites are recognized by CD44.

**Versican Is Modified with At Least CS B and CS C**—We next attempted to characterize the GAGs in the versican molecule by FACE (fluorophore-assisted carbohydrate electrophoresis) analysis (Fig. 8A). After treatment with a mixture of chondroitinase ABC and ACII, the mAb CS-56, 2-B-6, 3-B-3, or 1-B-5 (5 nM each), was digested with a mixture of chondroitinase ABC (Chase ABC), chondroitinase ACII (Chase ACII), or chondroitinase B (Chase B) at 37 °C for 2 h. The binding was determined by ELISA (Method 3) as described under "Experimental Procedures." Each bar represents the mean ± S.D. of triplicate determinations.

**Fig. 8. Analyses of the GAG moieties of versican.** A, FACE analysis. Versican was digested with a mixture of chondroitinase ABC and ACII (0.5 unit/ml each), fluorotagged, and analyzed by FACE. B, mAbs CS-56, 2-B-6, 3-B-3, or 1-B-5 (5 µg/ml) were added to wells coated with versican (2 µg/ml) treated with or without 100 milliunits/ml chondroitinase ABC (Chase ABC), chondroitinase ACII (Chase ACII), or chondroitinase B (Chase B) at 37 °C for 2 h. The binding was determined by ELISA (Method 3) as described under "Experimental Procedures." Each bar represents the mean ± S.D. of triplicate determinations.

To further characterize the GAG moieties of versican, we used various mAbs and chondroitinas with defined specificities (Fig. 8B). The mAb CS-56, which is specific for intact CS chains (42), reacted with versican; this reaction was abrogated by chondroitinase ABC treatment, as expected. The mAb 2-B-6, which recognizes stubs with the ΔDi-4S terminal structure exposed by chondroitinase digestion (43), reacted with chondroitinase B-treated versican. Because this enzyme selectively cleaves CS B (Ref. 44 and data not shown), this result indicated that versican contains CS B. This mAb also reacted with chondroitinase ACII-treated versican but not with chondroitinase ACII-treated versican. This finding supports the evidence that CD B is present on versican, because chondroitinase ABC cleaves all types of CS chains, whereas chondroitinase ACII cleaves all CS chains except CS B (Ref. 44 and data not shown). The mAb 3-B-3, which recognizes stubs with the ΔDi-6S terminal structure exposed by chondroitinase digestion (45), reacted strongly with chondroitinase ABC-treated versican, indicating that versican contains CS C as well. The mAb 1-B-5, which recognizes stubs with the ΔDi-8S terminal structure (45), reacted with versican; this reaction was abrogated by chondroitinase ABC-treated versican only very weakly, if at all. Together, these results suggest that versican is modified with at least CS B and CS C.

**Lymphoid Cells Can Bind to the CS Chains of Versican in a Manner Dependent on L-selectin or CD44**—We next examined whether the interaction between versican and L-selectin or CD44 can mediate cell adhesion. Mouse lymphoma EL-4 cells expressing an active form of CD44 and no L-selectin bound to versican-coated wells only very weakly, whereas EL-4 cells transfected with human L-selectin bound well to versican-coated wells, as expected. This antibody inhibits the binding of P- and E-selectin to sLe X by recognizing a site outside the sLe X binding site (36). Two anti-CD44 mAbs with different specificities, BRIC235 (37, 38) and RAMBM 5.5.8 (39), inhibited the binding of both versican and HA-coated wells, because it cleaved both CS and HA at this concentration. Hyaluronidase that specifically cleaved HA inhibited the binding of both versican cells to HA- but not to versican-coated wells. Taken together, these results indicate that the interactions between CS chains of versican and L-selectin or CD44 can mediate cell adhesion.

**Fig. 9. Binding of versican to L-selectin.** A, Binding of versican to L-selectin and CD44. After treatment with a lower concentration of chondroitinase ABC (5 milliunits/ml), the binding of BW5147 cells to versican- or HA-coated wells was specifically inhibited (Fig. 9B), indicating that CS chains of versican are involved in cell adhesion. As shown in Fig. 9C, chondroitinase ABC specifically cleaved CS at this concentration. A higher dose of chondroitinase ABC (100 milliunits/ml) inhibited the binding of BW5147 cells to both versican- and HA-coated wells, because it cleaved both CS and HA at this concentration. Hyaluronidase that specifically cleaved HA inhibited the binding of BW5147 cells to HA- but not to versican-coated wells. Taken together, these results indicate that the interactions between CS chains of versican and L-selectin or CD44 can mediate cell adhesion.

**DISCUSSION**

This study shows that versican, derived from a renal adenocarcinoma cell line, ACHN, binds L- and P-selectin and CD44 and this binding is mediated by an interaction between the CS chains of versican and the lectin domain of L- and P-selectin or the link module of CD44. This study also shows that, in the absence of any core protein, a specific subset of GAG chains, including CS B, CS E, and HS, bind L- and P-selectin, whereas a relatively wide range of GAG chains, including CH, CS A, CS B, CS C, CS D, CS E, and HA, bind CD44.

Blocking studies using various mAbs showed that versican is recognized by the lectin domain of L- and P-selectin and the link module of CD44 (Fig. 3), both of which mediate the binding of these adhesion molecules to known ligands. The inability of mAb 1.2B6 to inhibit the binding of versican to P-selectin, however, suggests that versican binds a site in the lectin domain of P-selectin that is different from that used for sLe X binding. This antibody inhibits the binding of P- and E-selectin to sLe X by recognizing a site outside the sLe X binding site within the lectin domain, and presumably inducing a conformational change in the sLe X binding site (36). Two anti-CD44 mAbs with different specificities, BRIC235 (37, 38) and RAMBM 5.5.8 (39), inhibited the binding of both versican and HA to CD44 completely when used in combination, although they could not do so on their own (Fig. 3). HA is thought to interact with multiple residues in the link module of CD44, such as Arg-41, Tyr-42, Arg-78, and Tyr-79 (31). Our result suggests that versican also interacts with multiple sites in the link module of CD44.

Our results demonstrate that versican binds L- and P- but not E-selectin (Fig. 2), although the three selectins share considerable structural similarity and all bind sLe X. This finding is reminiscent of previous reports showing that sulfation plays
procedures.

Binding of Versican to L- and P-selectin and CD44

The GAG structure of versican remains to be fully characterized. However, FACE analysis and studies with anti-CS mAbs in combination with chondroitinases with defined specificities indicated that versican is modified with at least CS B and CS E (Fig. 8). Given that both L- and P-selectin bind CS B and CS E avidly (Fig. 6), we speculate that at least CS B or CS E-related structures on versican may serve as binding sites for L- and P-selectin. Due to the absence of an available CS E-specific mAb, we have been unable to verify whether or not CS E is also present in the GAG moiety of versican, although our preliminary analysis of the disaccharide composition of versican after chondroitinase ABC treatment using HPLC failed to detect CS E (data not shown). It has been reported that chondroitin ABC digestion of CS chains containing GlcA(3-sulfate) residues specifically destroys the disaccharide units containing these sugar residues, and thus they cannot be detected by HPLC (54). Thus, it remains possible that disaccharide units containing GlcA(3-sulfate), such as GlcA(3-O-sulfate)β1–3GalNAc(4,6-O-disulfate), which are known to be present in the squid cartilage CS chains (55), remained undetected in our FACE and HPLC analyses. Further study is needed to determine the exact carbohydrate structures of versican that are recognized by L- and P-selectin and CD44.

Previously, our laboratory (29) and others (56, 57) reported that CD44 binds HA but not CS GAGs, which may probably be due to the differential affinity of HA and CS for CD44 (Fig. 5). However, the lipid-derivatized CS GAGs we used in this study allowed us to detect the binding of CD44, probably because of their effective immobilization and/or clustering on the plastic surface. The binding of L- and P-selectin and CD44 to certain lipid-derivatized GAGs in the absence of any core protein (Fig. 6) suggests that proteoglycans, other than versican, that are sufficiently modified with the appropriate GAG chains may also bind these adhesion molecules. We suggest that proteoglycans sufficiently modified with CS B and/or CS E should bind L- and P-selectin and CD44 and that proteoglycans sufficiently modified with HS but not other GAGs should bind L- and P-selectin, but not CD44. Similarly, proteoglycans sufficiently modified with CH, CS A, or CS C, or CS D but not other GAGs may bind CD44 but not selectins. In support of this hypothesis, serglycin from a hematopoietic cell line (29, 53) and the invariant chains on antigen-presenting cells (30) bind CD44 through their CS chains, HA is not a sulfated GAG. Our present result showing that CH, a non-sulfated GAG, can interact with CD44 (Fig. 6) also supports the notion that sulfation is not required for recognition by CD44.

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L-selectin-reactive heparan sulfate proteoglycan, which we have recently described (58), binds L- and P-selectin but not CD44.2

The interaction of L- and P-selectin and CD44 with versican that has been modified with the appropriate GAG chains may have several functional consequences. First, it may promote the binding of leukocytes expressing L-selectin or CD44 to the extracellular matrix, resulting in enhanced cell migration. The fact that the interaction between L-selectin or CD44 and ACHN tumor-derived versican can mediate cell adhesion (Fig. 9) raises the possibility that this interaction plays a role in leukocyte infiltration into the extracellular matrix of the tumor. Second, the interaction of these adhesion molecules with versican may trigger signal transduction, because L-selectin or CD44 and extracellular matrix, resulting in enhanced cell migration. The interaction of L- and P-selectin and CD44 with versican may trigger signal transduction, because L-selectin or CD44 and versican can bind a certain type of che-

mokines.3 Experimental verification is now required to assess the in vivo role of versican under physiological as well as pathological conditions.

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