Preparation of Lipid-derivatized Glycosaminoglycans to Probe a Regulatory Function of the Carbohydrate Moieties of Proteoglycans in Cell-Matrix Interaction*

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We have shown previously that chondroitin sulfate, but not heparan sulfate/heparin, linked to either natural core proteins or serum albumin interferes with cell-to-substrate adhesion, provided that the external proteoglycans are topologically immobilized on plastic plates. In order to study the roles of glycosaminoglycan chains (GAGs) as recognition structure, a new assay system is now developed which involves the conversion of free GAGs to reactive lactone derivatives selectively modified at the reducing end. The modified GAGs can be coupled to the amino group of phosphatidylethanolamine (PE) for use as probes on either plastic plates or cell surfaces. Incubation of GAG-PE solutions in poly styrene plates results in a time- and dose-dependent increase of the density of the GAG chains noncovalently immobilized onto the plates. No immobilization is detected with any of the GAG-PE samples that have been treated with phospholipase D. A M, 30,000 chondroitin sulfate conjugate to PE (CS-PE), when immobilized onto a fibronectin-coated well for 2 h at an initial concentration of 0.06 μg/100 ul/well, inhibits the adhesion of baby hamster kidney (BHK) cells to the substratum by ~50%, whereas heparin-, heparan sulfate-, hyaluronic acid-, and dermatan sulfate-PE do not. The effect of CS-PE is abolished by treating the CS-PE-coated plates with chondroitinase ABC. A similar level of inhibition by CS-PE is found when the RGD-containing 120-kDa fragment of fibronectin is used in place of fibronectin. CS-PE in soluble form, once exposed to BHK cells in suspension, can be associated with the cell surfaces, thereby exerting some inhibitory effects on cell-to-substrate adhesion. On a per mol basis, however, the activity of cell-associated CS-PE is far lower than that of substrate-associated CS-PE. Together the results indicate that our GAG-PEs offer useful tools for probing regulatory function of the GAG moieties of proteoglycans and further support the hypothesis that the inhibitory regulation of cell-to-matrix adhesion by large chondroitin sulfate proteoglycans is caused by an interaction between the cell surface and the chondroitin sulfate chains topologically immobilized on extracellular matrices.

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Cellular interactions with extracellular matrices are crucial for a number of biological processes that require cell adhesion or migration, including the determination of cell shape and polarity, the control of cell growth and differentiation, the maintenance of tissue integrity, and the invasion and metastasis of malignant cells (for reviews, see Refs. 1–4). Although experimental data show that a large number of adhesive macromolecules such as fibronectin, laminin, and collagen are present in extracellular matrices, it has increasingly become evident that the binding of cells to the matrices is mediated via cell surface receptors (e.g. those termed integrins, syndecans, anchorin CII, laminin-binding protein, and CD44 proteoglycan). In general, however, there is considerably less information about the molecules that regulate cell-matrix adhesion, thereby providing directionality or guiding cells to form cell-to-cell aggregates. Early studies examining the spatial distribution of proteoglycans in cell cultures showed that, although cultured cells of various origins contained dense punctate layers of chondroitin sulfate proteoglycans on both the substrate and the cell surface, the proteoglycan-containing matrix was usually excluded from stable cell-to-substrate focal contacts (5, 6). Complementing these in situ studies, various types of chondroitin sulfate/dermatan sulfate proteoglycan added to cell culture media were shown to inhibit the adhesion of normal and transformed cells to fibronectin (7–11) or a cell-binding fibronectin fragment (12).

Previous studies in our laboratories (13) showed that large chondroitin sulfate proteoglycans from chick embryonic chondrocytes (PG-H) and fibroblasts (PG-M), when immobilized onto fibronectin-coated plates, had a strong inhibitory effect on the adhesion of various types of cells to the substrate and that the immobilized fraction, but not the diffusible counterpart, of external proteoglycan molecules were responsible for this phenomenon. Furthermore, chondroitin sulfate-derivatized serum albumin also displayed a similar inhibitory effect, whereas heparan sulfate/heparin-derivatized serum albumin was far less effective. These findings emphasize the importance of chondroitin sulfate proteoglycans as geometrically controlled inhibitors in the regulation of cell-matrix interaction and raise the interesting possibility that the chondroitin sulfate moieties of the proteoglycans, not the protein cores, are responsible for the inhibitory effect. However, the tests with native proteoglycans or GAG-derivatized serum albumin...
are not suitable for direct quantitative comparisons between the activities of different types of GAG chains, mainly due to the structural ambiguity of the test proteoglycan sample. Let the heterogeneity of the type, number, and distribution pattern of GAG chains attached to the native protein core, and/or the nearly random mode of GAG attachment to the serum albumin core. We have now overcome these problems by preparing novel GAG probes composed of GAG and carrier lipid (GAG-PEs) that can lead their noncovalent binding to plastic culture dishes through the carrier lipid. Here we describe 1) their preparation after selective modification at the reducing terminal saccharide of intact GAG chain to generate a reactive site for the covalent attachment of dipalmitoylphosphatidylethanolamine, 2) their successful applications to the cell-matrix interaction systems, and 3) an exquisite specificity for chondroitin sulfate-PE among various GAG-PEs differing in the type of carbohydrate skeletons.

**EXPERIMENTAL PROCEDURES**

**Materials:** Chondroitin sulfate from shark cartilage (M, 10,000, 30,000); 4-sulfate to 6-sulfate ratio ~1:1); chondroitin sulfate from bovine trachea cartilage (M, 15,000; 4-sulfate to 6-sulfate ratio ~1:1); chondroitin sulfate from whale cartilage (M, 30,000; 4-sulfate to 6-sulfate ratio ~4:1); chondroitin sulfate from bovine serum albumin (M, 18,000 20,000); heparan sulfate from bovine liver (M, 40,000); heparin from porcine intestine (M, 55,000), and heparinase from bovine liver (M, 10,000) were donated by K. Horiie and T. Harada, Seikagaku Co., Tokyo. Although some variations in isolation procedures were used, the procedures used for the GAG samples with M, equal to or lower than 30,000 included, besides the conventional procedure to isolate protein-free GAGs (proteolytic digestion and alkaline β-elimination), either brief acid treatment (0.4 M HCl, 60°C) or testicular hyaluronidase digestion, a known procedure for clearing the hexosaminidic bonds of GAG chains. Chondroitinase ABC, and Δ-unsubstituted disaccharide standards were the products of Seikagaku Co., Tokyo. Fluorescein-labeled glycosaminoglycans and GAG-PEs, in which fluorescein groups were linked to 1–2% of the hexuronic acid carboxyl residues, was prepared by the carbodiimide method using fluoresceinamine (16).

The following materials were obtained from the indicated commercial suppliers: PyA-GlcNAC, PyA-GalNAC, PyA-Gal, and PyA-Xyl was from Organon Teknika Co. (Cappel), Beckman Instruments, Palo Alto, CA; HPTLC Silica gel 60 plates were from Merck, Darmstadt, Germany; Ultrafree C3 (0.22 pm) filter was from Millipore Japan, Tokyo; 96-well polystyrene microtitration plates were from Beckton Dickinson Labware (Falcon), Lincoln Park, NJ.

**Bovine plasma fibronectin** was prepared by the method of Yamada (17). Monoclonal antibody MO-225 directed to chondroitin sulfate was prepared and characterized as described previously (18). The 120-kDa fibronectin fragment that contains the RGD cell binding domain but not any known GAG binding domains (19) was donated by K. Yamakawa, Aichi Medical University, Aichi, Japan. GAG-PEs, prepared by the carbodiimide method (20), polyasaccharides were precipitated with 3 volumes of 1.5 M ammonium acetate (Step 1). The precipitate (Compound II) was dissolved in water, passed through a Dowex 50 x 8 (H+), and then allowed to stand at 4°C for 72 h. The aqueous solution was concentrated at 40°C in vacuo, and the remaining water was replaced by the addition of dimethylformamide followed by evaporation of water at 40°C in vacuo (several cycles) (Step 2). After neutralizing the solution with tertiary butylamine, PE (1-5 mol/mol of Compound III) in chloroform was added, and the mixture was stirred at 60°C (Step 3). The reaction was continued until a maximal level of GAG-PE (Compound IV) was attained (~2 h). The methods for GAG-PE assay as well as those for GAG-PE purification and characterization are described under "Results."

**Cell Adhesion Assay—**Cell-substrate adhesion was assayed by a modification of the methods of Rueslatti et al. (21) and Yamagata et al. (13). Briefly, 96-well polystyrene plates were coated with various adhesive proteins in 0.1 M NaHCO3 (0.5 μg/100 μl/0.32-cm2 well) overnight at 4°C, after which the wells were rinsed three times with Dulbecco’s phosphate-buffered saline. Each well was then filled with 100 μl of Hanks’ salt solution, 20 mm HEPES containing test substances (GAG-PEs and related substances). The stock solutions of GAG-PEs (2–10 mg/ml) had been prepared in Hanks’ solution/HEPES overnight at 4°C, and insoluble materials, if any, removed by filtration on a 0.22-μm Millipore filter. The plates were allowed to stand at room temperature for 2 h unless otherwise indicated and then rinsed three times with Hanks’ solution. Thereafter, an aliquot (1 × 104 cells/100 μl) of single cell suspension in Hanks’ solution was added to each well and the plates were incubated for 1 h at 37°C.

When the effect of heparin was to be tested, heparin (final concentration, 100 μg/100 μl) was added to the cell suspension before seeding. After unattached cells were removed by two washes with Hanks’ solution, attached cells were fixed in 3% (w/v) paraformaldehyde in phosphate-buffered saline at 4°C for 5 min and then stained with hematoxylin reagent. The number of cells in five different randomly chosen areas was determined at 100 × magnification with an Olympus microscope.
transmitted light microscope for the attachment index.

Quantification of the GAG-PEs Immobilized on Plastic Wells—The amounts of GAG-PEs immobilized on plastic wells (see above) were quantitated by taking advantage of the fact that fluorescein-labeled GAG-PEs ranging from 0.1 to 10 μg/ml can be determined by a simple fluorophotometric method. Various concentrations of fluorescein-labeled GAG-PEs (0.1 μg/ml to 100 μg/ml) were mixed with an equal volume of fluorescein-labeled PE (150 μl). After washing, cells were resuspended in Hanks' solution (1.0 mg/ml) and 100-μl aliquots of the suspensions were added to each well. After 1 h at room temperature, the materials remaining in the wells were dissolved with 0.2 M NaOH/0.5% (w/v) Triton X-100 (200 μl/well) for 2 h at room temperature with gentle shaking and measured in an F-3010 fluorescence spectrophotometer (Hitachi Co., Tokyo). The assay protocol was repeated three times and the solution was finally prepared in 3% (w/v) paraformaldehyde. Fixed cells were viewed on a Carl Zeiss confocal laser scan microscope (Carl Zeiss, Oberkochen, Germany).

Comparison of the relative adhesion abilities of CS-PE-bound and unbound BHK cells on fibronectin-coated slides was performed using a Superose 6HR 10/300 column, 10 mm × 30 cm, eluted with phosphate-buffered saline, 0.05% (w/v) Triton X-200 (200 μl/well) for 1 h at room temperature. Insoluble materials were removed by centrifugation (5,600 × g, 30 min) and fluorescence intensity of the resulting supernatant solution was determined fluorophotometrically as described above.

For localization of fluorescein-labeled CS-PE in BHK cells, live cells were incubated with either fluorescein-labeled CS-PE or fluorescein-labeled chondroitin sulfate (control) on glass coverslips (for conditions, see above). Media were gently aspirated from cultures in the wells after the addition of the phosphate substrate (10 mg/ml of o-phenylenediamine in methanol diluted 1:100 into 0.003% (v/v) H₂O₂) as measured by a MTP-100 microplate reader (Corona Electro Co., Kalsada, Ibaragi, Japan). Measurements were expressed in the amounts (micrograms/well) of immobilized CS-PE using the standard curve prepared with known amounts of fluorescein-labeled CS-PE (see above).

Localization and Quantification of the Fluorescein-labeled CS-PE Bound to Live Cells—Single BHK-21 cell suspensions in Ca²⁺, Mg²⁺-free Hanks' solution (2.5 × 10⁵ cells/ml) were mixed with an equal volume of fluorescein-labeled CS-PE or fluorescein-labeled chondroitin sulfate (negative control) in Ca²⁺-, Mg²⁺-free Hanks' solution. After incubation for 1 h at 37 °C with gentle shaking, the cells were collected by centrifugation (189 × g, 5 min), washed twice with Ca²⁺-, Mg²⁺-free Hanks' solution, and then extracted with 0.5 ml of 0.5 M NaOH, 0.5% (w/v) Triton X-100 for 1 h at room temperature. Insoluble materials were removed by centrifugation (5,600 × g, 30 min) and fluorescence intensity of the resulting supernatant solution was determined fluorophotometrically as described above.

For localization of fluorescein-labeled CS-PE in BHK cells, live cells were incubated with either fluorescein-labeled CS-PE or fluorescein-labeled chondroitin sulfate (control) on glass coverslips (for conditions, see above). Media were gently aspirated from cultures which were then washed with phosphate-buffered saline and lightly fixed in 3% (w/v) paraformaldehyde. Fixed cells were viewed on a Carl Zeiss confocal laser scan microscope (Carl Zeiss, Oberkochen, Germany).

Comparison of the relative adhesion abilities of CS-PE-bound and unbound BHK cells on fibronectin-coated plates was performed with cell suspensions that had been incubated with or without CS-PE (the conditions were the same as above except that unlabeled CS-PE and chondroitin sulfate were used in place of the fluorescein-labeled CS-PE) (150 μl). After washing, cells were resuspended in Hanks' solution (1.0 × 10⁶ cells/ml) and 100-μl aliquots of the suspensions were added to each fibronectin-coated well for adhesion assay (see above).

Enzymatic Digestion of GAG-PEs—Digestion of CS-PE (500 μg) with chondroitinase ABC was carried out in 500 μl of 50 mM Tris-HCl, pH 8.0, 0.01% (w/v) bovine serum albumin, 0.1% (w/v) Triton X-100 containing 1 unit of enzyme at 37 °C for 1 h with shaking. After chondroitinase ABC digestion of the CS-PE immobilized on polyurethane microtitration plates, each well was filled with 100 μl of 50 mM Tris-HCl, pH 8.0, 0.01% (w/v) bovine serum albumin containing 0.002 unit of enzyme and then the plates were incubated at 37 °C for 1 h. After washing with phosphate-buffered saline, the materials remaining in the wells were assayed by ELISA with monoclonal antibody MO-225 directed to chondroitin sulfate (see above). Digestion of PE with phosphatidylethanolamine (100 μg) was carried out in 500 μl of 4 mM CaCl₂, 0.25% (v/v) Triton X-100, 0.01% (w/v) bovine serum albumin, 40 μM Tris-HCl buffer, pH 8.0, containing 2 units of enzyme at 37 °C for 1 h with shaking.

Chromatography—Thin-layer chromatography of CS-PE and related substances was performed on Merck HPTLC silica-gel 60 plates. Chromatograms developed for 40 min with 1-butanol/acetic acid/1 M ammonium hydroxide (2:3:1 by volume) were stained with a 1% (w/v) solution of toluidine blue in 70% (v/v) ethanol, 5% (v/v) acetic acid and dimethylformamide. For chondroitin sulfate detection or with the modified Dittmer-Lester reagent (for detection of phospholipid derivatives) (22).

Hydrophobic chromatography of GAG-PEs and related substances were performed using two different systems. The columns and flow rates used were a TSK-gel Phenyl 5PW column, 7.5 mm × 75 cm, at a flow rate of 1.0 ml/min (column A) and a TSK-gel Phenyl Toso-Tomogel 650 μl column, 5.0 × 20 cm, at a flow rate of 100 ml/h (column B). Each sample (∼0.1 mg for column A and ∼100 mg for column B) dissolved in 50 mM NaCl was applied to a column equilibrated with 50 mM NaCl. Afterwards, Column A (for analytical use) was eluted with a reversed linear gradient of NaCl (0 to 50 μM), Column B (for preparative use) was washed with 5 column volumes of 50 mM NaCl and then eluted with 30% (v/v) methanol. The effluents were analyzed for hexuronic acid (23).

Molecular size separation of GAG-PEs and related substances were performed using a Superose 6HR 10/300 column, 7.5 mm × 30 cm, eluted with phosphate-buffered saline, 0.05% (w/v) Triton X-100. The column was eluted with the same solution, and the effluents were analyzed for hexuronic acid (23).

Other Methods—Light scattering molecular weights of GAGs and related substances were measured at the concentration range from 0.1 to 10 mg/ml in a LALL-70 light scattering spectrophotometer (Otsuka Electronics Co., Tokyo) as described by Ueno et al. (24). Nuclear magnetic resonance spectra were recorded with a 400-MHz JNM-EX400 nmr spectrometer (JEOL, Tokyo). Exchangeable protons on the polysaccharide-lipid were converted into the deuterated form by dissolving in D₂O and freeze-drying. This cycle was repeated three times, and the solution was finally prepared in D₂O at a concentration of 10 mg/ml. The reducing terminal saccharides of GAGs were analyzed by the fluorescence labeling method of Takemoto et al. (25) as modified by Takagaki et al. (26). Briefly, the reducing-terminal GAGs were reductively aminated with a fluorescent reagent, PyA, by the use of sodium cyanoborohydride. The GAGs thus labeled were purified chromatographically on a Sephacryl S-200 column and then hydrolyzed with 2 M HCl at 100 °C for 6 h under N₂. The resultant PyA-laabeled terminal sugars were N-acetylated and then identified by HPLC on a Ultrasphere-ODS column using PyA-GlcNAc, PyA-GalNAc, PyA-Gal, and PyA-Xyl as standards.

RESULTS

Preparation and Properties of GAG-PEs—An outline of the chemical reactions and steps used to synthesize GAG-PEs is presented in Fig. 1. Before settling on this scheme, a number of coupling trials were performed with periodate-oxidized GAGs and cyanoborohydride to form Schiff's bases with PE. Although the reduction amination procedures have been used with success for the olsigosaccharide-PE conjugation of the olsigosaccharides derived from glycoproteins (27, 28), our trial with GAGs showed very low overall yields (<3%). The small extent of reactions with GAGs could be due to a partial cleavage of internal hexuronic acid residues (which contain periodate-sensitive vicinal hydroxyl groups) during the oxidation step and/or because long GAG chains are far less soluble in the hydrophobic solvents used for the reductive amination with PE. Heterogeneity of the reducing terminal saccharides of GAGs might also have some effects on reactivity with PE. We therefore analyzed the reducing terminal saccharide residues of four kinds of GAG preparations (M., 15,000 and 50,000 chondroitin sulfates from shark cartilage, M., 21,000 dermatan sulfate from porcine skin, and M. 15,000 heparin from porcine intestine) by the use of periodate oxidation methods (26). Acid hydrolysis of PyA-GAGs followed by N-acetylation yielded predominantly (>85%) PyA-GalNAc from the chondroitin sulfate/dermatan sulfate sample or PyA-GlcNAc from the heparin sample, suggesting that the reducing terminal saccharides of these polysaccharides are mostly non-
Glycosaminoglycan Probes in Cell-Matrix Interaction

Sulfated and/or sulfated hexosamines.

In the present approach, intact GAG chains (Fig. 1, Compound I) were modified selectively at the reducing terminal sugar to generate the lactone group (Compound III) which can be exploited to couple amines. The lactone-carrying GAGs can be dissolved in the amphiphilic solvent dimethylformamide for the subsequent N-acylation of PE to form GAG-PEs (Compound IV).

The final reaction mixture was concentrated to dryness in vacuo, redissolved in 50 mM NaCl, and then subjected to hydrophobic chromatography on either a TSK-gel Phenyl 5PW column (for analytical use) or a TSK-gel Phenyl Toyopearl 650 M column (for preparative use). Fig. 2 shows the results of analysis of the Step 3 reaction (Fig. 1) in which M, 30,000 chondroitin sulfate (shark cartilage) was used as a starting material. As Fig. 2A shows, free chondroitin sulfate (Compound I in Fig. 1) appeared exclusively in the flow-through fraction. When a 2-h sample at Step 3 was subjected to the same chromatographic procedure (Fig. 2B), a hexuronate-containing material (which comprised about 25% of the applied hexuronate) emerged during reversed linear gradient elution (50 mM to 0 mM NaCl). Rechromatography of once adsorbed, eluted, and desalted component still yielded a flow-through material but its amount was only ≤7% of the applied hexuronate (Fig. 2C). The results suggest that the bound fraction represents chondroitin sulfate chains with covalently linked PE which can bind through hydrophobic interactions to the phenyl 5PW column. The flow-through fraction in Fig. 2B was not studied further but presumably represents lipids-free chondroitin sulfate chains. Hydrophobic chromatography of PE-derivatized hyaluronic acid, dermatan sulfate, heparan sulfate, and heparin showed elution profiles qualitatively similar to those in Fig. 2, B-C, although there was quantitative variation between preparations, presumably due to differences in the efficiencies of coupling reactions. The material with affinity to the hydrophobic phenyl 5PW column (Fig. 2C) was prepared on a large scale using a TSK-gel Phenyl Toyopearl 650 M column and subjected to analyses by molecular sieving HPLC, light scattering, nmr, and enzymatic fragmentation (see below). Hereafter, the M, 30,000 chondroitin sulfate conjugated to PE is referred to as “CS-PE.”

Fig. 3 shows the elution profiles of CS-PE on a Superose 6HR 10/30 column equilibrated with phosphate-buffered saline, pH 7.4, in the absence (A) and presence (B) of 0.1% Triton X-100. In the absence of Triton X-100, the majority of the material was eluted in the V, region with a small amount of materials retained on the gel (Fig. 3A). The presence of Triton X-100 resulted in a shift of all the material eluting in the V, region of the detergent-free column in an included region (Fig. 3B). The results suggest that amphipathic CS-PE molecules, when added to phosphate-buffered saline, tend to form detergent-sensitive aggregates of variable size. It is noteworthy that all the GAG-PE samples used in the present study are identical in regard to this property (the data with heparin-PE are shown in Fig. 3, C and D). Measurement of the size of the V, material (Fig. 3A) by light scattering indicated the occurrence of large aggregates (M, 450~600 kDa) that are about 15~20 times larger on an average than the free chondroitin sulfate molecule (M, 30,000) used for the synthesis of CS-PE. Likewise, the V, material in Fig. 3C was shown to have an aggregate weight of 300~500 kDa which is 20~30 times larger than the M, of the heparin used.

**Fig. 2. Hydrophobic HPLC on TSK-gel phenyl 5PW.** Elution profiles of carbazole reaction-positive materials are shown. The samples applied are: A, shark cartilage chondroitin sulfate (starting material); B, compounds from the 2-h reaction mixture of Step 3 in Fig. 1; and C, materials recovered by ethanol precipitation from the fractions indicated by the bar above curve in B. See “Experimental Procedures” for details.

**Fig. 3. Elution profiles of CS-PE (A, B) and heparin-PE (C, D) on Superose 6HR 10/30 column in phosphate-buffered saline, pH 7.4 (A, C) or in 0.1% (w/v) Triton X-100/phosphate-buffered saline, pH 7.4 (B, D). The arrows denote the positions of M, 10,000 (10K), 30,000 (30K), and 60,000 (60K) chondroitin sulfate (standards). V, void volume. V, total column volume.
The $^1$H nmr spectrum of CS-PE in $^2$H$_2$O (Fig. 4) gave a preeminent peak of the acetamide methyl groups (at 2.10 ppm) of chondroitin sulfate in addition to peaks of the methylene groups (at 1.30 ppm) and terminal methyl groups (at 0.87 ppm) of PE fatty acids. The intensities of these three peaks are consistent with the proposed structure of CS-PE (Compound IV, Fig. 1) where one chondroitin sulfate chain ($M$, 30,000; containing 60 acetamide groups) is linked to one PE molecule ($M$, 692; containing 24 methylene groups and 2 methyl groups).

Analyses with phospholipase D (the enzyme capable of hydrolyzing phosphatidylethanolamine and thereby yielding ethanolamine and phosphatidic acid) provided further evidence to support the proposed structure of CS-PE (Fig. 5). Upon thin-layer chromatography in the solvent 1-butanol/ acetic acid/l ammonia (2:3:1 by volume), intact CS-PE was located at the origin as a band positive to both toluidine blue and molybdate staining (track 1 in A and B). After digestion with phospholipase D, a band ($R_f = 0.80$) staining with both molybdate and toluidine blue reagent was revealed in the position of standard dipalmitoylphosphatidic acid (track 2 in A and B) together with a toluidine blue-positive band left at the origin (track 2 in A). Chondroitinase ABC digestion, on the other hand, resulted in the disappearance of the CS-PE band at the origin with a concomitant appearance of a slowly moving compound ($R_f = 0.21$) that can be visualized with both toluidine blue and molybdate reagent (track 3 in A and B). This band presumably represents a chondroitin sulfate oligosaccharide remnant linked to PE. At present, the limited availability of the material precludes determination of its structure.

Immobilization of GAG-PEs onto Polystyrene Plates—When dilutions of CS-PE were prepared with Hanks' balanced salt solution containing 20 mM HEPES, placed into polystyrene microtiter plates, and allowed to stand at room temperature, CS-PE was immobilized onto the plates in a dose- and time-dependent manner (Fig. 6). In these experiments, the amounts of CS-PE immobilized onto the plates were measured, after washing the plates with phosphate-buffered saline, by immunochemical staining with MO-225, the monoclonal antibody to the GlcA(2-SO$_4$)-GalNAc(6-SO$_4$)$_2$ units of chondroitin sulfate (18). This figure also indicates that immobilization of CS-PE saturated with respect to the concentration of added CS-PE; at 2 h, for example, proportion of immobilized CS-PE decreased from ~20% to ~8% of the added CS-PE, as the concentration (or dose) of CS-PE added to each well increased from 0.1 to 0.5 $\mu$g/100 $\mu$L. When dilutions of hyaluronic acid-PE, dermatan sulfate-PE, heparan sulfate-PE, and heparin-PE were prepared and examined in a similar way, their profiles of time- and dose-dependent immobilization were essentially identical with those in Fig. 6 (data not shown). In view of the amphipathic nature of CS-PE, it is possible that CS-PE molecules may interact preferentially with polystyrene plates to form clusters, in which the molecules are oriented vertically to the plane of the surface, with the hydrophobic PE moiety associating with the solid polystyrene phase and the hydrophilic chondroitin sulfate chains projecting into the

Fig. 4. $^1$H nmr spectrum (400 MHz) of 1% (w/v) of sodium CS-PE in $^2$H$_2$O at 20°C.

Fig. 5. Thin-layer chromatograms developed with 1-butanol/acetic acid/1 M ammonium hydroxide (2:3:1 by volume) and stained with toluidine blue (A) and Dittmer-Lester's molybdate reagent (B). The samples applied are: lanes 1, CS-PE; lanes 2, as in lanes 1 but treated with phospholipase D; lanes 3, as in lanes 1 but treated with chondroitinase ABC; CS, shark cartilage chondroitin sulfate (standard); PA, dipalmitoylphosphatidic acid (standard).

Fig. 6. Time- and dose-dependent immobilization of CS-PE onto polystyrene microtiter plates. Dilutions of CS-PE before (●) or after (○) treatment with phospholipase D were prepared with Hanks' solution, 20 mM HEPES, placed into polystyrene wells, and allowed to stand at room temperature. After 2 or 48 h, the wells were washed with phosphate-buffered saline, and the amounts of chondroitin sulfate moiety immobilized on the plates were measured by immunostaining with MO-225, as detailed under "Experimental Procedures." The numbers on curves indicate incubation time. Error bars, ± SD (n = 5).
aqueous phase. Consistent with this view, the CS-PE molecules immobilized on the plates were sensitive to the chondroitinase ABC added to the aqueous phase, losing their biological activity (see below).

When the saturation level of CS-PE immobilization (2 h) onto fibronectin-coated plates was compared with that onto uncoated plates, it was apparent that the former is about 1.5~2 times higher than the latter. The fibronectin-dependent increase of CS-PE immobilization was not affected by excess heparin (1 mg/ml) added to the media, suggesting that this increase is not due to a specific binding of the chondroitin sulfate moiety of CS-PE to GAG-binding sites on fibronectin. In contrast to CS-PE, free chondroitin sulfate chains had no capacity to bind to plates, regardless of whether or not the plates had been coated with fibronectin. The results suggest that the fibronectin-dependent increase of CS-PE immobilization is due to hydrophobic associations between the PE moiety and the coated protein. Although the actual amount of immobilized GAG-PE varies depending on these factors, its range of variation is far smaller than the observed difference among various GAG-PES in the ability to inhibit cell-to-substrate adhesion (see below). In the subsequent studies, therefore, the relative activities of GAG-PES are expressed on a per initial concentration (input dose) basis (micromolars of GAG-PE added/100 µl/well) and, where necessary, the data are translated into “activity/µg of immobilized GAG-PE/well.” It should be stressed that, even in experiments where the activities are expressed on a per dose basis, any GAG-PE molecules that failed to bind to plates were removed prior to cell seeding.

Effects of Immobilized GAG-PES on Cell-to-Substrate Adhesion—After coating with fibronectin, the plastic wells were further incubated with 100 µl of Hanks’ solutions containing 0.01~10 µg/100 µl of CS-PE or other GAG-PES for 2 h at room temperature and subsequently washed with phosphate-buffered saline as described under “Experimental Procedures.” It is noteworthy that the concentration of the fibronectin solution used for the first coating (0.5 µg/100 µl) was chosen to be low enough to leave some unoccupied sites on the plastic substrate (see Ref. 13 for the evidence) which are available for the CS-PE (or other GAG-PES) used for the second coating. Using these plates, a series of BHK cell adhesion experiments were carried out to examine whether CS-PE (or any other GAG-PES) immobilized on the fibronectin-coated plates could act as an inhibitor.

As Fig. 7 shows, fibronectin-mediated cell adhesion was progressively inhibited by increasing the concentration of CS-PE used for immobilization on the plates. The initial CS-PE concentration required for a 50% inhibition (IC50) was 0.06 µg/100 µl/well (note that this effect was actually brought about by 0.024 µg of CS-PE immobilized onto the well). The inhibition by CS-PE is not restricted to the BHK cell-to-fibronectin adhesion. Thus, CS-PE was more or less inhibitory for the adhesion of BHK cells to vitronectin-coated plates, the adhesion of CEF cells to fibronectin- or vitronectin-coated plates, the adhesion of high metastatic B16F10 melanoma cells to fibronectin- or laminin-coated plates, and the adhesion of bovine aorta endothelial cells to fibronectin- or type I collagen-coated plates. At present, however, it is difficult to draw quantitative comparison between the data obtained with different combinations of cell and substrate type. In all these experiments, the ability of CS-PE to inhibit cell-to-substrate adhesion was completely abolished by treatment of the CS-PE-coated plates with chondroitinase ABC prior to the seeding of cells (data not shown). In another control, when the CS-PE was hydrolyzed with phospholipase D before use, all effects on cell-to-substrate adhesion were abolished (Fig. 7), suggesting a requirement for chondroitin sulfate chains immobilized onto the plate through the carrier lipid.

At GAG-PE doses lower than 0.1 µg/100 µl/well, none of the M, 10,000 hyaluronate-, M, 15,000 dermatan sulfate-, M, 40,000 heparan sulfate-, or M, 15,000 heparin-derivatized PE inhibited the adhesion of BHK cells to fibronectin-coated plates (Fig. 7). These compounds had inhibitory effects only at much higher doses (Table I), suggesting that the inhibition is specific with respect to the structure of GAG chain.

Effect of the Chain Size and Sulfation Pattern of the Chondroitin Sulfate Moiety—The data in Table I include differences between five chondroitin sulfate-PE samples with different chondroitin sulfate chain sizes which were tested for their inhibitory effect on cell-to-substrate adhesion. Clearly a decrease of average chain size from M, 60,000 to 30,000 had little effect on the activity, whereas further decrease to M, 10,000 caused a significant reduction of the activity. The 4-sulfate to 6-sulfate ratio of chondroitin sulfate moiety is not critical for the cell adhesion-inhibiting ability, since there was only a slight activity difference between 4-sulfate-rich chondroitin sulfate-PE (whale cartilage) and 6-sulfate-rich chondroitin sulfate-PE (shark cartilage) in the same range of molecular size.

Heparin Insensitive Action of CS-PE on Cell Adhesion—Despite the importance of the RGD sequence to cell adhesion, there is also evidence for the involvement of other biologically active domains in mediating cell adhesion to adhesive proteins, including fibronectin (1-4, 29-32). Consistent with this notion, it has been reported that heparin inhibits a variety of cell adhesive functions, possibly by displacing cell surface proteoglycans from GAG-binding sites on adhesive proteins (for example, see Refs. 31 and 32).
plates may be released from the plate during the 1-h incubation, the CS-PE molecules immobilized on fibronectin-coated with the cell pellets. Chondroitin sulfate is found in the washed cell pellets, about that, under conditions where little or no fluorescein-labeled soluble form, once exposed to intact cell, can be associated with the cell, owing to its hydrophobic long-chain fatty acid. To test this possibility, BHK cells were incubated with fluorescein-labeled CS-PE or fluorescein-labeled chondroitin sulfate (control) in Ca\(^{2+}\),Mg\(^{2+}\)-free Hanks' solution, rinsed with fresh medium, and then subjected separately to confocal laser scanning microscopic examination of fluorescence image and to fluorometric determination of the intensity of cell-associated fluorescence. One hour after their addition, the CS-PE fluorescence, but not the chondroitin sulfate fluorescence, became recognizable on the surface of some cells (data not shown). Determination of the fluorescence intensity (Fig. 8) indicates that, under conditions where little or no fluorescein-labeled chondroitin sulfate is found in the washed cell pellets, about 3-8% of the added fluorescein-labeled CS-PE is associated with the cell pellets.

The data in Fig. 8 raise a question as to whether some of the CS-PE molecules immobilized on fibronectin-coated plates may be released from the plate during the 1-h incubation with cell suspensions, causing a reduced cell-substrate adhesion by binding to the cell surfaces. This possibility was tested by examining whether preincubation of BHK cells on a CS-PE-containing substrate may alter the ability of the cells to adhere to newly prepared fibronectin-coated plates. Thus, BHK cells were preincubated for 1 h on CS-PE-treated (fibronectin-coated) plates, rinsed with assay medium, and then examined for their ability to adhere to a fibronectin substrate with no added CS-PE. This pretreatment of the cells caused no measurable alteration of their adhesion ability (data not shown), indicating that modification of BHK cell adhesion by a released CS-PE is unlikely.

Iida et al. (30) have postulated a model in which chondroitin sulfate proteoglycan located on the surface of melanoma cell may modify the function of \(\alpha 4\beta 1\) integrin on the same surface, thereby influencing initial cellular recognition events that contribute to cell adhesion on the 33-kDa carboxyl-terminal heparin-binding fragment of fibronectin. This prompted us to examine whether CS-PE molecules bound to BHK cell surface may play a regulatory role in the fibronectin-mediated cell adhesion. BHK cells (5 \( \times \) 10\(^5\) cells/ml) were incubated for 1 h directly with a solution of 50 or 500 \( \mu \)g of CS-PE/ml of assay medium, rinsed with assay medium, and then subjected to adhesion assay using newly prepared fibronectin-coated plates with no added CS-PE. This pretreatment caused a significant reduction in the rate of cell adhesion, whereas pretreatment with free chondroitin sulfate (500 \( \mu \)g/ml) had no effect (Fig. 9). The data, coupled with those in Fig. 8, indicate that the amount of cell surface CS-PE required for 40% inhibition is 0.28 \( \mu \)g/10\(^6\) cells. To attain the same degree of inhibition, only 0.014 \( \mu \)g of the CS-PE located on fibronectin-coated plate was enough (Figs. 6 and 7), indicating that specific molar activity of CS-PE varies depending on its topological location; about 20-fold higher on the fibronectin-coated plate than on the cell surface.

### DISCUSSION

We have developed a highly sensitive quantitative assay system that measures the inhibition activity of GAG chains.

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**Table I**

Comparison of the inhibitory effects of GAG-PEs on BHK cell-to-fibronectin adhesion

<table>
<thead>
<tr>
<th>Type and source of GAG moiety</th>
<th>Molecular weight*</th>
<th>IC(_{50})</th>
<th>( \mu )g/100 ( \mu )l/well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin sulfate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shark cartilage</td>
<td>60,000</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Shark cartilage</td>
<td>30,000</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Shark cartilage</td>
<td>10,000*</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Whale cartilage</td>
<td>30,000</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Bovine tracheae</td>
<td>15,000</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>Dermatan sulfate</td>
<td>15,000</td>
<td>0.40</td>
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</tr>
<tr>
<td>Porcine skin</td>
<td>10,000*</td>
<td>2.16</td>
<td></td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mouse EHS tumor</td>
<td>40,000</td>
<td>5.64</td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>15,000</td>
<td>5.86</td>
<td></td>
</tr>
</tbody>
</table>

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* Average molecular weight determined by the light scattering method (24).

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FIG. 8. Incorporation of fluorescein-labeled CS-PE and chondroitin sulfate into the cell surfaces of BHK cells. BHK cells were incubated with fluorescein-labeled CS-PE (\(f\)-CS-PE) or chondroitin sulfate (\(f\)-CS) for 1 h at 37°C. The amounts (micrograms/10\(^6\) cells) of cell-bound fluorescein-labeled materials were assessed from the fluorescence intensities of cell extracts. See "Experimental Procedures" for details.
in cell-to-substrate adhesion and have used this assay to examine the specificity in terms of inhibition abilities of particular GAG chains. The results reveal an exquisite specificity for chondroitin sulfate PE among various GAG-PES differing in the type of carbohydrate skeletons; the amount of Mr, 30,000 CS-PE required to produce a 50% inhibition (IC50) was extremely small, 0.06 µg/100 µl (expressed as the concentration of CS-PE in the solution used for immobilization) or 0.024 µg/0.32-cm² well (expressed as the actual amount of CS-PE immobilized onto the well). Dermatan sulfate PE (Mr of the dermatan sulfate moiety = ~15,000) also showed some activity (Fig. 7), but its IC50 (0.40 µg/100 µl/well, Table I) indicates that the activity is 5-fold lower than the activity of chondroitin sulfate PE in the same molecular weight range. The concentrations of hyaluronic acid PE, heparan sulfate PE, and heparin PE required for 50% inhibition were 30–100 times higher than the IC50 of CS-PE.

The above data agree in qualitative terms with our previous results which showed that both chondroitin sulfate proteoglycans (PG-M from chick embryo fibroblasts or PG-H from chick embryo cartilages) and chondroitin sulfate/dermatan sulfate-derivatized serum albumin (GAG-albumins) in immobilized form inhibited cell-to-substrate adhesion, whereas heparan sulfate proteoglycan (from EHS tumor) and heparan sulfate/heparin-derivatized serum albumin had little or no inhibitory effect (13). In quantitative terms, however, there are some discrepancies between GAG-albumins and GAG-PES in their relative effectiveness; whereas dermatan sulfate-derivatized serum albumin has been reported to be as active as chondroitin sulfate-derivatized serum albumin in inhibiting the adhesion of BHK cells to fibronectin-coated plates, the adhesion-inhibiting activity of dermatan sulfate species is much lower than that of chondroitin sulfate species when applied in the lipid-derivatized form. Also to be added is the fact that, on a per dose basis, the inhibition activities of both chondroitin sulfate- and dermatan sulfate-derivatized serum albumin were ~800-fold lower than the activities of the corresponding GAG-PES used in the present assay. These differences between GAG-albumins and GAG-PES in the apparent inhibition activities could be due, at least in part, to relatively low contents and random distribution of the GAG chains bound by chemical synthesis to serum albumin via their hexuronic carboxyl groups. The GAG-PE preparations used here are free of such structural ambiguities, thus allowing more quantitative evaluation of GAG functions.

The precise mechanism operative for the inhibition of cell-to-substrate adhesion by CS-PE is not entirely clear at present. Since CS-PE has been shown to bind more efficiently to fibronectin-coated plastic plates than to uncoated plates, it may be that some CS-PE molecules are eventually bound to cell adhesion-promoting sites within the precoated fibronectin, making those sites inaccessible to cell surface receptors by steric hindrance or electrostatic repulsion or both. This mechanism, however, could not account for the following observations. 1) In regard to the GAG-PE-mediated inhibition of cell-to-substrate adhesion, the cells were less sensitive to the molecular size of the GAG moieties than to the nature of the GAG carbohydrate backbone, e.g. within a Mr, range from 30,000 to 60,000, the size of chondroitin sulfate moiety had little or no effect on the inhibition of BHK cell adhesion, whereas a marked difference in adhesion-inhibiting activity was seen between the chondroitin sulfate/dermatan sulfate-type chains and the heparan sulfate/heparin-type chains in GAG-PES (Fig. 7 and Table I). It is also noteworthy that, despite the marked difference in their inhibitory activities, heparin-PE resembles CS-PE in its ability to form large aggregates (Fig. 3) with an average light scattering aggregate weight almost equal to that of CS-PE aggregates. 2) Among the GAG-PES used for assay, heparin-PE is highest in net negative charge, but it was lowest in the ability to inhibit cell-to-substrate adhesion (Table I). 3) As shown in our previous studies (13), the inhibitory effect of chondroitin sulfate proteoglycan (PG-M) was abolished when the immobilization of added PG-M onto plastic surfaces of fibronectin-coated plates was blocked by pretreating the plates with serum albumin. Apparently, the chondroitin sulfate chains immobilized onto plastic surfaces, rather than their counterpart bound directly to the fibronectin, are predominant in inhibiting cell-to-substrate adhesion. Although preliminary, our current studies using CS-PE (12) suggested that the inhibition of fibronectin-mediated cell adhesion was caused not only by the CS-PE bound directly to fibronectin but also by the counterpart immobilized onto available plastic surfaces of the fibronectin-coated plates. In these experiments, a solution of fibronectin was first incubated with increasing doses of CS-PE, and the resultant complexes with increasing amounts of CS-PE bound to fibronectin were isolated by gel chromatography on a Superose 6HR 10/30 column equilibrated with phosphate-buffered saline, pH 7.4. Using these as coat solutions, it was shown that the adhesion-promoting properties of fibronectin became progressively lowered with increasing amounts of CS-PE located on the coat of fibronectin complex (CS-PE on the coat was measured by ELISA using monoclonal antibody MO-225 to the chondroitin sulfate moiety). This inhibition, however, could account for only 15% of the inhibition caused by an equal amount of CS-PE immobilized onto a fibronectin precoated plate (i.e. a plate on which both fibronectin coat and uncoated plastic surfaces are available for CS-PE immobilization).

Although more direct evidence is needed to explain the topological effects precisely, one possibility is that the inhibition of fibronectin-mediated cell adhesion may have been caused, at least in part, by an interaction of CS-PE molecules on a solid phase with a cell membrane factor capable of

\[ N. Sugiura, K. Sakurai, Y. Hori, K. Karasawa, S. Suzuki, and Koji Kimata, unpublished data. \]
modulating the properties of receptors (e.g. integrins) required for cell adhesion. As has been found for various synthetic neoglycolipids on liposomes or on silica/plastic plates (27, 28, 33, 34), carbohydrate conjugates to lipids confer certain properties such as ability to bind to natural or synthetic matrices or to impart multivalency of the carbohydrate chains for more efficient interaction with carbohydrate-binding proteins (e.g. receptors, antibodies, and lectins). This carbohydrate-clustering effect may be an important factor in considering the inhibitory effect of CS-PE on cell adhesion.

Regardless of the remaining uncertainties as to the cellular and molecular mechanism, the relatively specific and sensitive mode of action of GAG-PEs suggest that these drugs can be exploited to ascertain whether proteoglycan-induced changes in biological processes (e.g. cell-cell adhesion, cell migration, cell proliferation, cell differentiation, tissue morphogenesis, wound healing, and metastasis; see Refs. 7–11) result specifically from the function of the GAG chains attached. Recently, in a brief report (35), we have demonstrated that CS-PE, but not free chondroitin sulfate, inhibits the invasion of pannus in a brief report, which is consistent with the in vitro observations that rheumatoid synovium extended more preferentially over an eroded fibro-nectin-rich surface of the cartilage than onto an intact surface of the cartilage (36).

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REFERENCES

Glycosaminoglycan Probes in Cell-Matrix Interaction