

# Regulation of Cell Substrate Adhesion: Effects of Small Galactosaminoglycan-containing Proteoglycans

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**Abstract.** Cell adhesion is a process which is initiated by the attachment of cells to specific sites in adhesive matrix proteins via cell surface receptors of the integrin family. This is followed by a reorganization of cytoskeletal elements which results in cell spreading and the formation of focal adhesion plaques. We have examined the effects of a class of small galactosaminoglycan-containing proteoglycans on the various stages of cell adhesion to fibronectin-coated substrates. Our results indicate that dermatan sulfate proteoglycans (DSPGs) derived from cartilage, as well as other related small proteoglycans, inhibit the initial attachment of CHO cells and rat embryo fibroblasts to substrates composed of the 105-kD cell-binding fibronectin fragment, but do not affect cell attachment to intact fibronectin. Although this effect involves binding of DSPGs

to the substrate via the protein core, the intact proteoglycan is necessary for the observed activity. Isolated core proteins are inactive. The structural composition of the galactosaminoglycan chain does not appear to be functionally significant since both chondroitin sulfate and various dermatan sulfate proteoglycans of this family inhibit cell attachment to the fibronectin fragment. Neither the percentage of cells spread nor the mean area of spread cells adhering to substrates of intact fibronectin was significantly affected by the DSPGs. However, significantly fewer cells formed focal adhesions in the presence of DSPGs as compared with untreated control cells. These results suggest that the binding of small galactosaminoglycan-containing proteoglycans to a fibronectin substrate may affect several stages in the cell adhesion process.

**A**DHESION of cells to supporting substrates is of fundamental importance for normal cell behavior and has been extensively studied at the molecular level during the past decade. It is now known that attachment of cells to a substrate is specifically promoted by a family of adhesive matrix proteins (including fibronectin, laminin, vitronectin, fibrinogen, and some collagen types). Specific domains in these proteins, which often include the amino acid sequence RGD, are recognized by corresponding cell surface receptors. The different receptors are structurally related and are, therefore, grouped together as a family of receptor proteins referred to as integrins (1, 4, 21, 29).

The initial attachment of a cell to a substrate composed of an adhesive matrix is followed by a reorganization of cytoskeletal components resulting in a flattening and spreading of the cell body. Subsequently, specialized adhesive structures (i.e., focal adhesions) are often formed (for reviews see 5, 37). In vivo, the adhesive process must be carefully regulated, and in certain situations, i.e., during mitosis and migration, the cells must dissolve and reassemble the adhesive structures in an orderly fashion. The molecular mechanisms involved in the later stages of the cell adhesion

process and in regulation of cell adhesion are poorly understood. Previous studies have shown that certain extracellular matrix components such as thrombospondin (25), tenascin (26) and SPARC (26, 30) may regulate cell adhesion by interfering with some of the later stages in the adhesive process.

Some proteoglycans have also been shown to inhibit the early stages of cell adhesion, i.e., cell attachment. For example, a large chondroitin sulfate proteoglycan (CSPG) purified from a rat yolk sac tumor (3) and a mixture of dermatan sulfate proteoglycans (DSPGs)<sup>1</sup> (22) isolated from cartilage were shown to inhibit the attachment of cells to fibronectin-coated substrates. In these cases, the proteoglycans were presumed to exert their inhibitory effects by binding via their polysaccharide chains to glycosaminoglycan binding sites in the fibronectin molecule. It was proposed that the bulky proteoglycans sterically interfered with the interactions between the cell-anchored integrins and the corresponding binding site in the substrate. Furthermore, cartilage was found to contain two species of DSPG (DSPG I or "biglycan" and

1. *Abbreviations used in this paper:* DSPG, dermatan sulfate proteoglycan; REF, rat embryo fibroblast.

DSPG II or "decorin") with similar, but genetically distinct core proteins (28). One of these DSPGs, DSPG II, was shown to bind to fibronectin through protein-protein interactions (32) and also to a cell surface endocytosis receptor (12, 13). These findings prompted us to reexamine the effects of various proteoglycans on the different stages of cell adhesion.

## Materials and Methods

### Materials

Dermatan sulfate proteoglycan I ("biglycan," DSPG I), dermatan sulfate proteoglycan II ("decorin," DSPG II), each containing 40% iduronic acid, and the large CSPG were isolated from bovine articular cartilage (6, 28, 34). PG-S1, containing 100% glucuronic acid, from nasal cartilage (15), PG-S2, containing 73% iduronic acid, from bovine flexor tendon (35), and fibromodulin, a small keratan sulfate proteoglycan, from bovine articular cartilage (16) were generously provided by Dr. Dick Heinegard (Department of Medical and Physiological Chemistry, Lund University, Lund, Sweden). The scleral DSPG II (7) containing 50–60% iduronic acid, was provided by Dr. Anders Malmstrom (Department of Physiological Chemistry, Lund University, Lund, Sweden). Ham's F-12 Growth Medium (F-12), DME, trypsin-EDTA, and glutamine were purchased from Gibco Laboratories (Grand Island, NY). FBS was obtained from Flow Laboratories (McLean, VA) or from Hyclone Laboratories (Logan, UT). Tissue culture flasks were acquired from NUNC, Irvine Scientific (Santa Ana, CA) or from Sumilon, Vanguard International (Neptune, NJ). [<sup>3</sup>H]Thymidine (196 mCi/mg), Na<sup>125</sup>I (16.9 mCi/mg) and aqueous counting scintillant were purchased from Amersham Corporation (Arlington Heights, IL). Glass coverslips, Falcon 3072 microtiter tissue culture plates, Falcon 3047 24-well tissue culture plates, and Tween 20 were obtained from Fisher Scientific Co. (Atlanta, GA). Immulon 2 Removawells were purchased from Dynatech Laboratories Inc. (Chantilly, VA). All other reagents used were procured from Sigma Chemical Co. (St. Louis, MO).

### Cell Culture

CHO cells (CHO-K1 ATCC# CCL 61) were provided by Dr. Jeffrey D. Esko (Department of Biochemistry, University of Alabama at Birmingham) and rat embryo fibroblasts (REF) were provided by Dr. Anne Woods (Department of Cell Biology, University of Alabama at Birmingham). CHO cells were maintained in F-12 growth medium, and REF cells were maintained in DME supplemented with 2 mM glutamine. All growth media were supplemented with 10% FBS (vol/vol) unless otherwise stated. Cells were routinely maintained in monolayer culture and detached with trypsin (CHO cells) or trypsin-EDTA (REF) for sequential passage every 4–7 d.

### Preparation of Substrates

Human fibronectin was purified from outdated plasma (American Red Cross, Birmingham, AL) according to the method of Engvall and Ruoslahti (10) as modified by Miekka et al. (24) or purchased from the New York Blood Center Inc. (New York). The 105-kD fibronectin "cell-binding" peptide, generously provided by Dr. Staffan Johansson (University of Uppsala, Sweden), was generated by chymotrypsin digestion of intact fibronectin and was isolated and characterized as previously described (38). 24-well tissue culture plates (2.0 cm<sup>2</sup> surface area/well) or glass coverslips (1.2 cm<sup>2</sup> surface area/coverslip) were coated with human fibronectin (8 μg per well or 4.52 μg per coverslip) or equimolar amounts of 105-kD fibronectin peptides (3.8 μg per well or 2.16 μg per coverslip). Microtiter wells (0.6 cm<sup>2</sup> surface area per well) were coated with 1.12 μg fibronectin or 0.53 μg of the 105-kD fibronectin fragment per well by incubation at 4°C for 12 h followed by a rinse with PBS. To block unoccupied protein binding sites, the coated wells were incubated with either 2% BSA, heat treated at 56°C for 1 h and filtered, or with PBS containing 1% native BSA for 1 h at 4°C. The substrates were then rinsed with PBS and the appropriate assay buffer.

### Cell Attachment Assay

Cells (10<sup>5</sup>) were seeded in 75-cm<sup>2</sup> tissue culture flasks and incubated for 2–4 d. Cells, still in the log phase of growth, were labeled for 3 h before harvest by adding 2 μCi of [<sup>3</sup>H]thymidine per ml culture medium. In all at-

tachment assays cycloheximide (10 μg/ml) was added to the culture medium 2 h before harvest and included in all subsequent washing and assay buffers. At harvest, radiolabeled cells were detached by trypsin treatment, washed twice with 5 ml of F12 containing 10% PBS (F12/PBS), and resuspended in F12/PBS. Cell numbers were determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL) or a hemocytometer.

Radiolabeled cells suspended in F12/PBS were seeded in substrate-coated 24-well tissue culture plates (60,000 cells per well) or in 96-well microtiter plates (20,000 cells per well). In some experiments, the substrates were preincubated with 100 μg/ml proteoglycans, isolated core proteins, or isolated glycosaminoglycan chains (see figure legends for more details). The cells were incubated at 37°C for the indicated periods of time and the percent of cells attached to the substrate was determined essentially as described by Couchman et al. (8). Briefly, unattached cells were collected, wells were washed three times with PBS, and the wash was combined with the unattached cells. Attached cells were solubilized by incubation with 1% SDS in PBS for 6–12 h at 37°C and collected. The wells were washed three times with PBS and the wash was combined with the solubilized cells. Radioactivity in the two pools was determined and the percentage of cells attached was calculated assuming all the radioactivity was associated with the cells. Each value was the average of at least two determinations.

### DSPG Binding Assays

Proteins were iodinated either using the chloramine-T method of Hunter (20) or using "Enzymobeads" (Bio-Rad Laboratories, Richmond, CA) in a modified lactoperoxidase reaction performed according to manufacturer's instructions. The labeled macromolecules were then separated from free iodine by gel permeation chromatography on a PD-10 column (Pharmacia-LKB Biotechnology, Inc., Piscataway, NJ). Iodinated DSPGs were diluted with PBS and  $5 \times 10^4$  cpm (specific activity =  $\sim 1 \times 10^6$  cpm/μg DSPG) of [<sup>125</sup>I]-DSPG in 50–100 μl were placed in substrate-coated wells and incubated at 37°C (see figure legends for more details). Unbound DSPGs were removed and the wells were rinsed with PBS containing 0.1% Tween 20. The radioactivity bound to substrate containing the adsorbed protein was determined by breaking off the washed individual wells of the Immulon 2 removawell plates and placing them into gamma tubes before counting in an LKB gamma counter.

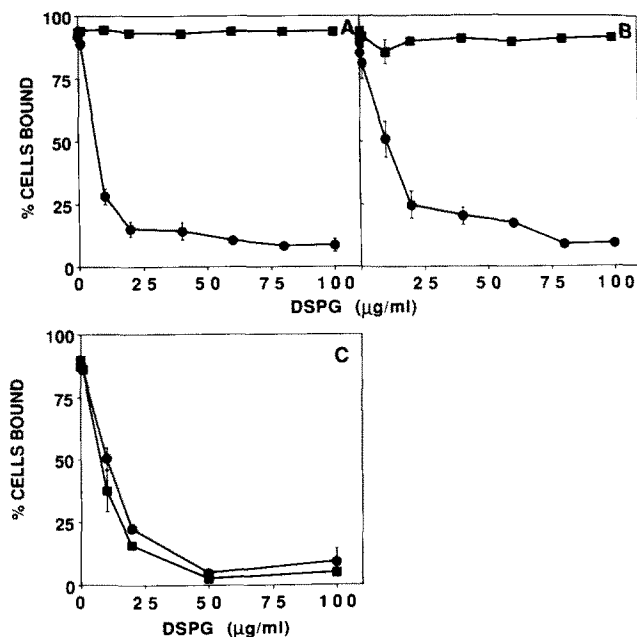
Assays to determine dissociation constants on substrates composed of either fibronectin or the 105-kD fragment of fibronectin were performed using [<sup>125</sup>I]-labeled intact DSPG II or isolated core protein from DSPG II and analyzed as previously described (2).

### Cell Spreading and Focal Adhesion Assays

Cell spreading and focal adhesion formation were assayed as described by Woods et al. (38) and Murphy-Ullrich and Hook (25). Glass coverslips were coated with 4.25 μg human plasma fibronectin or 5 μg BSA in 100 μl PBS and air dried overnight. Substrates were rehydrated, washed, and additional protein binding sites were blocked by incubation with 1% heat-denatured BSA. Coverslips were washed and then incubated for 1 h at room temperature with 0.5 ml DME containing 100 μg/ml DSPG I or DSPG II in PBS. In some experiments, DSPGs were added at the time of seeding cells onto the coverslips. Cells were harvested as described above, pelleted and resuspended in DME.  $\sim 60,000$  cells were added to each coverslip in a 24-well plate. Unless otherwise stated, cycloheximide was maintained at a concentration of 10 μg/ml throughout the experiment. Cells were allowed to attach and spread for 3 h at 37°C, fixed with 3% warmed glutaraldehyde for 30 min, washed, and examined by phase and interference reflection microscopy using a Nikon Optiphot microscope (Nikon Inc., Melville, NY). Spread cell area was measured using a digital planimeter system (Microplan II; Laboratory Computer Systems, Inc., Cambridge, MA). To quantify the percent of cells positive for focal adhesion formation, a minimum of 150 cells per coverslip was examined by interference reflection microscopy. Cells were scored as positive if they had at least three plaques per cell. Spread cells were designated as those which were noncircular in shape and had cytoplasm visible by phase microscopy.

### Immunofluorescence Microscopy

Immunofluorescence staining of vinculin and F-actin were performed according to procedures described by Murphy-Ullrich and Hook (25). Cells were treated as described above in the cell spreading assays, and then fixed, permeabilized, and stained for vinculin with a mAb to vinculin, (clone VIN-11-5, Sigma Chemical Co.) and for F-actin using NBD-phalloidin (Molecular Probes, Eugene, OR).



**Figure 1.** Inhibition of cell attachment to the 105-kD fibronectin fragment by DSPGs. CHO cells (A and C) or REFs (B) were seeded on substrates composed of intact fibronectin or the 105-kD fibronectin fragment in the presence of increasing concentrations of either a mixture of both DSPG I and DSPG II (A and B) or purified DSPG I and DSPG II (C). Cells were labeled with [<sup>3</sup>H]thymidine and cell attachment assays were performed as indicated in Materials and Methods. The substrates composed of intact fibronectin and the 105-kD fibronectin fragment were preincubated with varying concentrations (0–100 µg/ml) of the DSPG mixture or the individual DSPG species for 1 h before the addition of cells. Cells were allowed to attach for 90 min and then unbound cells were removed, followed by three rinses in PBS. Bound cells were solubilized by incubation with 1% SDS in PBS for 6–12 h and removed. The percentage of cells attached was calculated assuming all radioactivity was associated with the cells. The results are expressed as the mean and ranges of determinations from at least two wells. (A and B) (■) percent cells attached to the intact fibronectin coated substrate; and (●) percent cells attached to the 105-kD fibronectin fragment-coated surface. (C) (■) percent cells attached in the presence of DSPG II; and (●) percent cells attached in the presence of DSPG I.

## Results

### Effects of DSPGs on Cell Adhesion to Fibronectin-Coated Substrates

Attachment of CHO cells to a substrate composed of the 105-kD fibronectin fragment was inhibited by a mixture of DSPG I and DSPG II in a concentration dependent manner (Fig. 1 A). The number of CHO cells attaching to the coated substrate was reduced from 95 to <30% in the presence of 10 µg/ml of proteoglycan. At concentrations of proteoglycan >60 µg/ml, the number of attached cells was comparable with levels seen with a nonadhesive substrate, e.g., BSA (~10–25% cell attachment). In contrast, the presence of DSPG did not affect the attachment of CHO cells to substrates coated with intact fibronectin (Fig. 1 A). Essentially identical results were seen in assays using REFs (Fig. 1 B) except that slightly higher concentrations of DSPG were needed to obtain the same degree of inhibition of attachment on the 105-kD fibronectin fragment-coated surface as that

observed for CHO cells. The effect was not due to nonspecific charge interactions with the glycosaminoglycan side chains, since the presence of heparin at molar concentrations similar to those of the DSPG did not effect the attachment of either cell type to substrates composed of intact fibronectin or its 105-kD fragment (data not shown).

When purified preparations of DSPG I and DSPG II were examined separately for their effects on CHO cell attachment, both proteoglycan species effectively inhibited cell attachment to wells coated with the 105-kD fibronectin fragment (Fig. 1 C). Under the conditions of the experiment, DSPG I seems to be only slightly more effective than DSPG II. In contrast, cell attachment to intact fibronectin was unaffected by the presence of the individual proteoglycan species (data not shown).

Since the DSPGs did not affect cell attachment to substrates composed of intact fibronectin, we examined whether DSPGs affected the later stages in the adhesion process of cells attached to fibronectin. Analysis of cell spreading revealed that neither CHO cells nor REFs seeded onto fibronectin substrates were significantly affected by preincubation of the substrates with either DSPG I or DSPG II. Both the area of spread cells and the percent of cells spread on fibronectin substrates preincubated with either DSPG I or DSPG II were comparable with cells spread on untreated fibronectin (Table I).

In contrast, the ability of cells to form focal adhesion plaques on composite substrates of fibronectin and DSPG was reduced as compared with cells seeded on fibronectin substrates (Table I). When examined by interference reflection microscopy, we observed that 73% of the cells formed focal adhesions on fibronectin-coated substrates, whereas only 51 and 47% of cells formed adhesion plaques on substrates composed of fibronectin preincubated with DSPG I or DSPG II, respectively. Cells spread on substrates containing DSPG often had broad lamellae which lacked focal adhesion plaques (Fig. 2, a and d). These cells had fewer plaque-like clusters of vinculin (Fig. 2, b and e) as compared with control cells spread on fibronectin substrates (Fig. 2 h). In focal adhesion negative cells spread on composite substrates of fibronectin and DSPG II (Fig. 2 f), the density and

**Table I.** The Effect of DSPGs on Cell Spreading and Focal Adhesion Formation

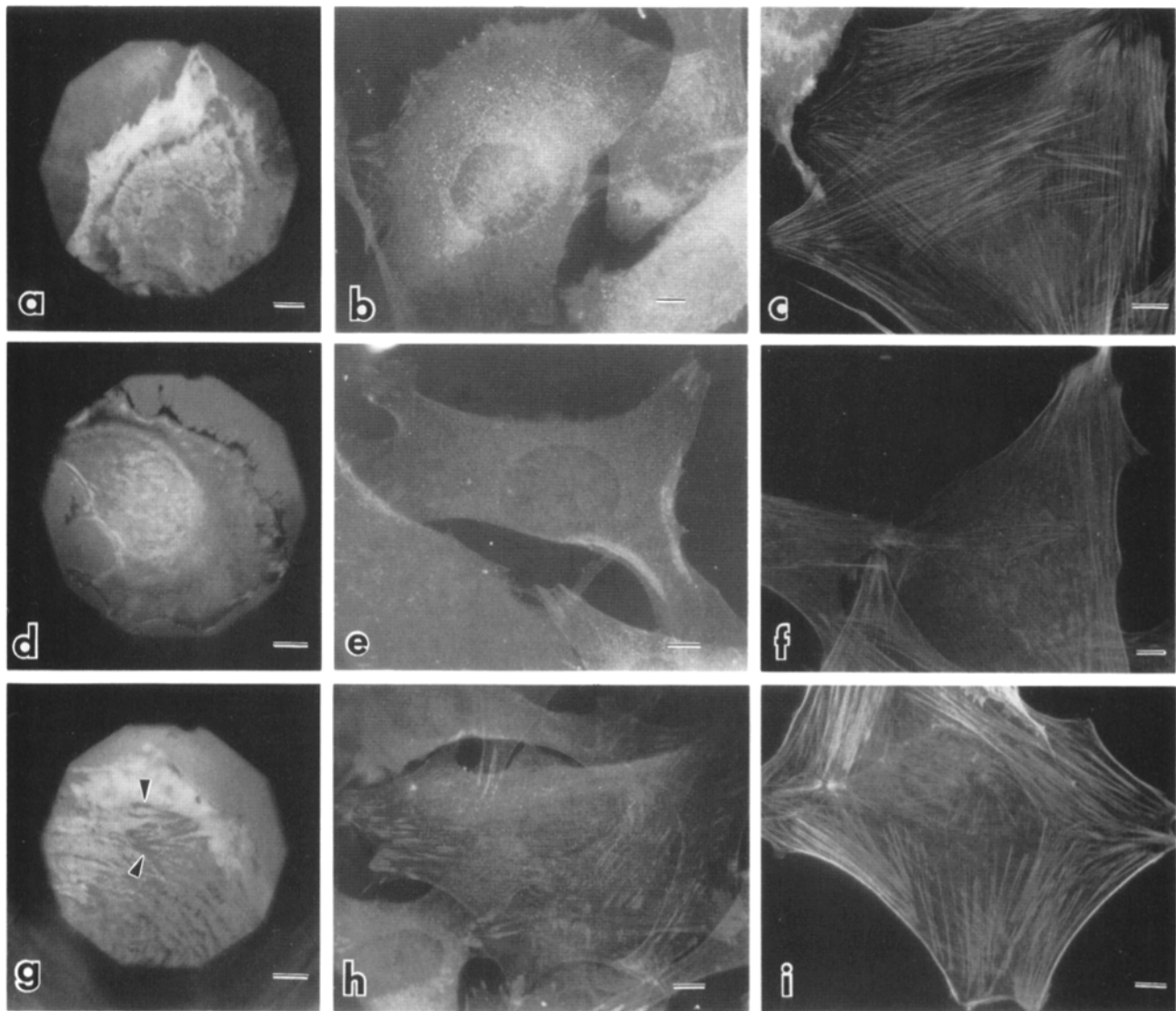
Substrate	Percent REFs positive for focal adhesions*	Cells spread		Spread cell area <sup>§</sup>	
		REF <sup>‡</sup>	REF	CHO-K1	CHO-K1
		%	µm <sup>2</sup>	µm <sup>2</sup>	
BSA	0	0	170 ± 53	47 ± 12	
FN	73 ± 4	94	2092 ± 589	315 ± 100	
FN+DSPG I	51 ± 5	95	2072 ± 579	278 ± 139	
FN+DSPG II	47 ± 6	93	2101 ± 481	215 ± 121	

The effect of DSPG I and DSPG II on cell spreading and focal adhesion formation. Cell spreading and focal adhesion assays were performed as indicated in the Materials and Methods section. Spread cell area was measured using a digital planimeter system and the average spread cell area was calculated. Cells were scored as positive for focal adhesions if they had at least three plaques and spread cells were designated as those which were noncircular in shape and had cytoplasm visible by phase microscopy.

\* These are the results of four experiments in which 150 cells per treatment per experiment were counted.

<sup>‡</sup> n = 100 cells per treatment.

<sup>§</sup> n = 50 cells per treatment.

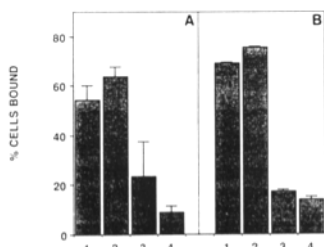


**Figure 2.** The effects of DSPG I and DSPG II on cell spreading and focal adhesion formation on substrates composed of intact fibronectin. REFs were seeded onto fibronectin substrates that had been preincubated with DSPG I (*a*, *b*, and *c*), DSPG II (*d*, *e*, and *f*), or with DME as a control (*g*, *h*, and *i*) and allowed to attach and spread in the continued presence of proteoglycans for 3 h. REFs were examined for focal adhesion formation by interference reflection microscopy (*a*, *d*, and *g*). Cells were also fixed, permeabilized, and stained for the presence of vinculin using the mAb VIN-11-5 (*b*, *e*, and *h*) and F-actin using NBD-phalloidin (*c*, *f*, and *i*). Some cells spread on substrates preincubated with either DSPG I (*a*) or DSPG II (*d*) lack focal adhesion plaques and exhibit ruffling of the membranes. Numerous adhesion plaques are present in cells spread on fibronectin substrates (*arrowheads*, *g*). The clustering of vinculin into adhesion plaques is reduced in cells spread on composite DSPG-fibronectin substrates (*b* and *e*). The density and thickness of the actin-containing stress fibers are reduced in cells on substrates preincubated with DSPG II (*f*) as compared with control cells spread on fibronectin substrates (*i*) or on substrates preincubated with DSPG I (*c*). Bars, 10  $\mu$ m.

thickness of the F-actin-containing microfilaments were markedly reduced as compared with cells spread on substrates of untreated fibronectin (Fig. 2 *i*). The cells spread on substrates of fibronectin and DSPG I (Fig. 2 *c*), which lacked focal adhesions by interference reflection microscopy and had reduced clustering of vinculin, showed organization of F-actin into microfilaments which were only slightly thinner and less dense than in unaffected cells.

To examine if the observed inhibitory effects of cell attachment to the 105-kD fibronectin fragment were a consequence of an interaction between the DSPGs and cells or were a result of a binding of DSPGs to the substrate, the following experiments were performed. Cells (REFs or CHO

cells) were preincubated with DSPG mixtures for 1 h. The cells were washed and subsequently, seeded on substrates composed of the 105-kD fibronectin fragment. Alternatively, the substrates were preincubated with the DSPGs for 1 h, rinsed, and used in cell attachment experiments. The results of these experiments (Fig. 3) showed that the pretreatment of cells with DSPG did not affect their attachment to the substrate. In contrast, preincubation of the substrate with the DSPGs resulted in an inhibition of both CHO (Fig. 3 *A*) and REF (Fig. 3 *B*) cell attachment. The inhibitory effects were almost as pronounced as those observed when the DSPGs were present throughout the incubation (Fig. 3). These data indicate that the inhibitory effect of DSPG on cell



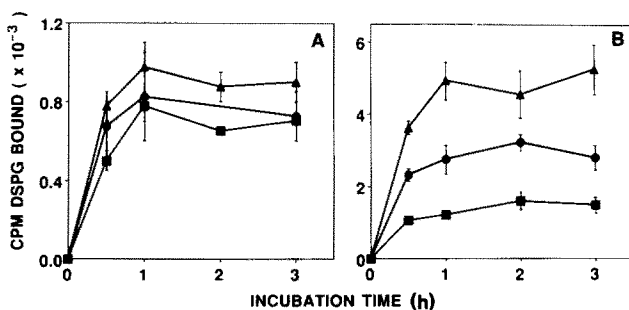
**Figure 3.** The inhibition of cell attachment is due to an interaction between the DSPGs and the fibronectin-coated substrate. Substrates were coated with the 105-kD fibronectin fragment as indicated in Materials and Methods. In treatment 1, CHO cells (A) and REF cells (B) were allowed to

attach to the substrate without previous treatment. In 2, the cells were preincubated with the DSPG mixture (100  $\mu\text{g}/\text{ml}$ ) for 60 min followed by a wash, before seeding on the 105-kD fibronectin fragment substrate. In treatment 3, the substrates were preincubated with the DSPG mixture for 60 min and washed before the seeding of the cells. In 4, the substrates were preincubated with the DSPG mixture for 60 min before addition of cells and the attachment was allowed to proceed in the presence of the DSPGs. The results are expressed as the means and ranges of determinations from at least three wells.

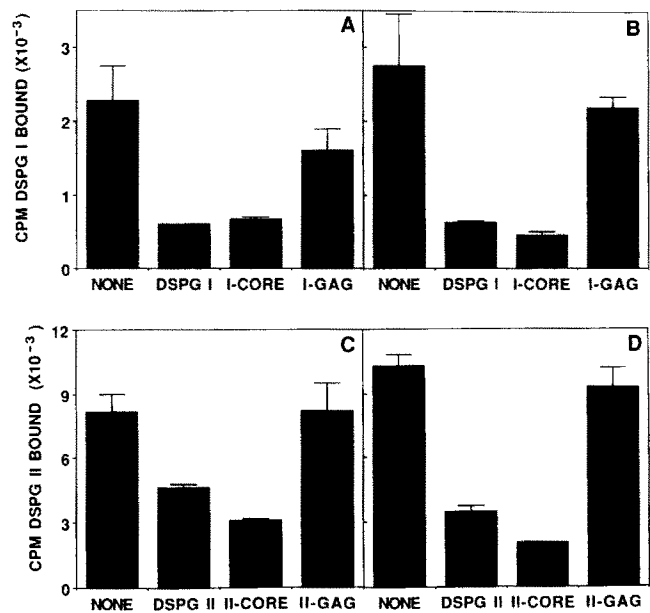
attachment is caused by interaction(s) of DSPG with the substrate and not through DSPG–cell interactions.

#### Binding of DSPG to Intact Fibronectin and the 105-kD Fragment

To examine the possibility that the DSPGs are actually binding to the fibronectin substrate, the proteoglycans were  $^{125}\text{I}$ -labeled and  $\sim 50,000$  cpm of DSPG I or DSPG II were incubated at  $37^\circ\text{C}$  in microtiter wells coated with intact fibronectin, the 105-kD fibronectin fragment or BSA for various time periods. The wells were washed extensively and the amount of bound  $^{125}\text{I}$ -labeled ligand quantified. Both DSPG I and DSPG II bound to all three substrates in a time-dependent manner which reached a plateau at  $\sim 1$  h (Fig. 4). The relative amount of  $^{125}\text{I}$ -labeled DSPG II binding to wells coated with intact fibronectin or the 105-kD fibronectin fragment varied somewhat but was consistently higher than that bound to BSA (Fig. 4 B). When  $^{125}\text{I}$ -labeled



**Figure 4.** Binding of iodinated DSPG I (A) and DSPG II (B) to fibronectin substrates. Fibronectin ( $\bullet$ ), the 105-kD fibronectin fragment ( $\blacktriangle$ ), or BSA-coated substrates ( $\blacksquare$ ) were blocked with BSA for 60 min and incubated with  $50,000$  cpm (approximate specific activity =  $1 \times 10^6$  cpm/ $\mu\text{g}$  DSPG) of  $^{125}\text{I}$ -labeled DSPG I or DSPG II in a volume of  $50$ – $100$   $\mu\text{l}$  PBS at  $37^\circ\text{C}$  for various times before removal of unbound DSPG. The wells were washed with PBS containing  $0.1\%$  Tween 20 and the amount of bound DSPG was determined by breaking off the individual wells of the Immulon 2 removable well plate and counting them in an LKB gamma counter. Results are expressed as the means and ranges of determinations from at least three wells.

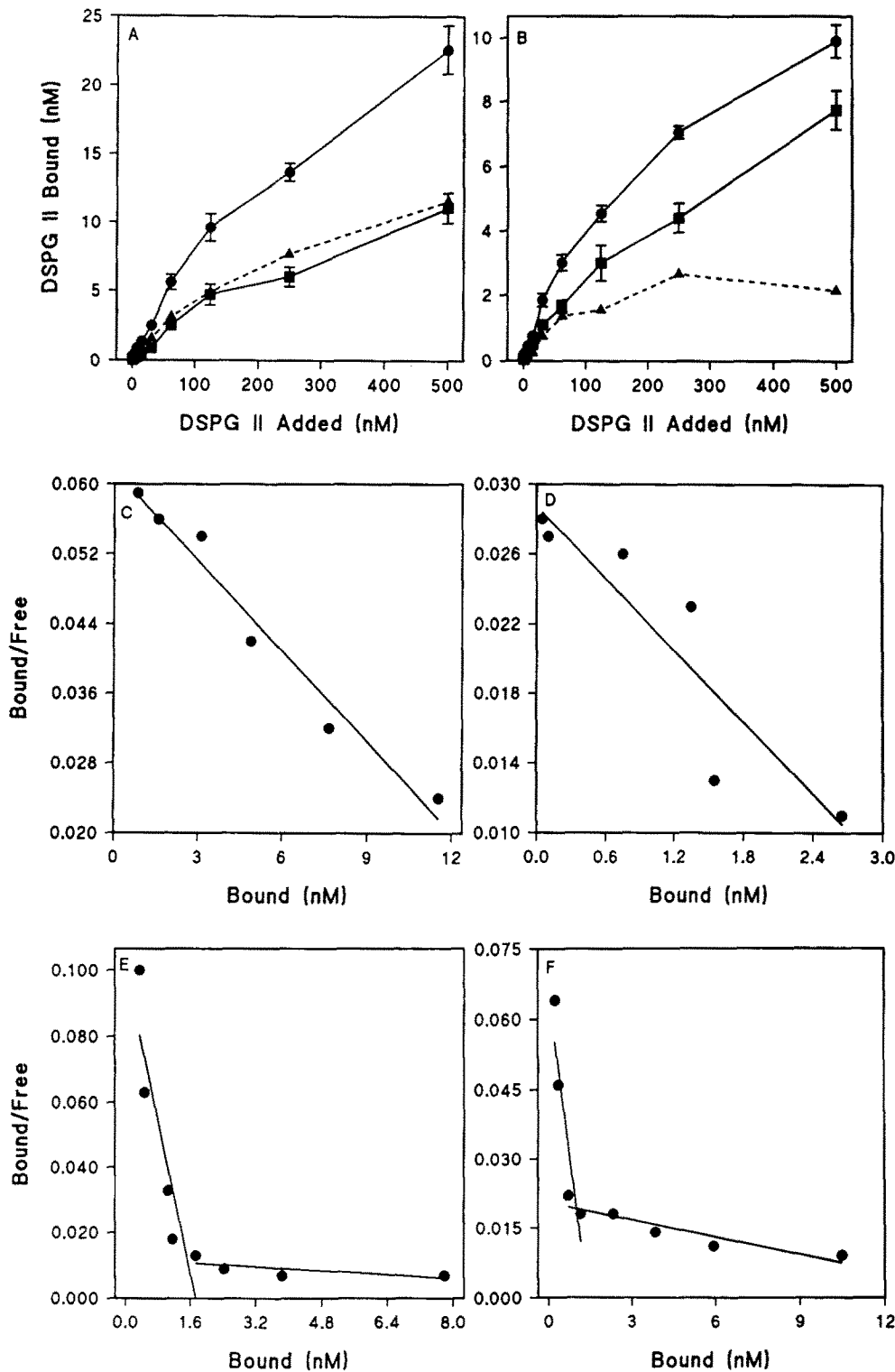


**Figure 5.** Inhibition of  $^{125}\text{I}$ -DSPG I and  $^{125}\text{I}$ -DSPG II binding to intact fibronectin (A and C) or the 105-kD fibronectin fragment (B and D) by unlabeled DSPG or its components. Fibronectin or 105-kD fibronectin fragment-coated substrates were blocked with  $1\%$  BSA, and preincubated with  $50$   $\mu\text{g}/\text{ml}$  of intact DSPG I or DSPG II, isolated protein core from DSPG I or DSPG II, or protein-free dermatan sulfate chains from DSPG I or DSPG II for 60 min.  $50,000$  cpm of iodinated DSPG I or DSPG II was then added and allowed to incubate for 3 h at  $37^\circ\text{C}$ . Unbound DSPG was removed followed by three washes with PBS containing  $0.1\%$  Tween 20. The wells were separated and then counted individually to quantify the bound material. Results are expressed as the means and ranges of determinations from at least three wells.

DSPG I was used as a ligand, only small differences were observed between the amounts bound to wells coated with intact fibronectin, the 105-kD fibronectin fragment or BSA alone (Fig. 4 A).

It is noteworthy that the 105-kD fibronectin fragment is a potent binder of the DSPGs despite the fact that this segment of the fibronectin molecule does not contain a known glycosaminoglycan binding site. This prompted us to locate the fibronectin binding portion of the DSPG molecule. Wells coated with intact fibronectin or the 105-kD fibronectin fragment were incubated with  $^{125}\text{I}$ -DSPG I or  $^{125}\text{I}$ -DSPG II in the presence of unlabeled DSPG, isolated dermatan sulfate chains from DSPG I or DSPG II, or DSPG I or DSPG II core protein depleted of glycosaminoglycan chains. The results of these experiments showed (Fig. 5) that binding of radiolabeled DSPG to intact fibronectin or the 105-kD fibronectin fragment was inhibited by both intact proteoglycan and the isolated protein core. Isolated dermatan sulfate chains only marginally affected the binding of DSPG I or DSPG II to either fibronectin substrate.

These data suggest that the binding of DSPGs to intact fibronectin or the 105-kD fibronectin fragment is primarily mediated by the core protein. In fact, we could demonstrate a direct binding of  $^{125}\text{I}$ -labeled DSPG II core protein to these substrates in a time-dependent and reversible reaction (data not shown). When increasing amounts of  $^{125}\text{I}$ -labeled DSPG II or its isolated core protein were added to wells



**Figure 6.** The saturability of binding of  $^{125}\text{I}$ -DSPG II or its core protein to substrates composed of either fibronectin or the 105-kD fragment of fibronectin. Immulon 2 microtiter removawells, coated with fibronectin (A, ●), the 105-kD fibronectin fragment (B, ●), or 1% BSA (A and B, ■), were blocked with 1% BSA for 60 min and incubated with  $^{125}\text{I}$ -DSPG II (A and B; specific activity = 150,000 cpm/ $\mu\text{g}$ ) or  $^{125}\text{I}$ -DSPG II core protein (data not shown; specific activity = 101,500 cpm/ $\mu\text{g}$ ) at concentrations from 0–10  $\mu\text{g}/\text{well}$  in 0.2 ml of PBS containing 1% BSA. After a 3-h incubation at 37°C, the wells were washed, and the amount of bound radioactivity was determined. Specifically bound DSPG II (A and B, ▲—▲) was calculated as the amount bound to fibronectin or the 105-kD fibronectin fragment minus that which bound to BSA-coated wells. In C–F, the data are plotted according to the methodology of Scatchard (31). (C) DSPG II binding to fibronectin, (D) DSPG II binding to the 105-kD fibronectin fragment; (E) DSPG II core protein binding to fibronectin; and (F) DSPG II core protein binding to the 105-kD fibronectin fragment. The results are expressed as the means of determinations from quadruplicate wells. Bars represent the standard deviation of  $^{125}\text{I}$ -labeled protein bound.

coated with fibronectin or the 105-kD fibronectin fragment, the amount of labeled ligand bound increased (Fig. 6, A and B). By analyzing the amounts of ligand specifically bound to the adsorbed fibronectins, saturable binding could be demonstrated. Assuming molecular masses of 100 kD for DSPG II and 43 kD for the DSPG II core protein, the analyses of these binding data were performed according to the methodology of Scatchard (31) and dissociation constants

were calculated. The binding of intact DSPG II to fibronectin or the 105-kD fibronectin fragment appears to involve one binding site with calculated dissociation constants of  $3 \times 10^{-7}$  M (Fig. 6 C) and  $1.5 \times 10^{-7}$  M (Fig. 6 D), respectively. Analysis of the binding data for the  $^{125}\text{I}$ -labeled core protein revealed two classes of binding sites in wells coated with either fibronectin or the 105-kD fragment of fibronectin with dissociation constants of  $1.3 \times 10^{-6}$  M and  $1.7$

**Table II. DSPG and DSPG Domain Effects on Cell Attachment**

Substrate	Preincubation	Cells bound	Relative cells bound
		%	%
105 kD	—	56.3	100
BSA	—	27.3	0
105 kD	CORE I (CI)	65.4	131.4
	CORE II (CII)	62.0	119.7
	GAG I (GI)	56.3	100
	GAG II (GII)	57.6	104.5
105 kD	DSPG I	32.3	17.2
	CI + DSPG I	53.3	89.7
	CII + DSPG I	35.8	29.3
	GI + DSPG I	19.4	-27.3
	GII + DSPG I	24.3	-10.3
	DSPG II	29.3	6.9
105 kD	CI + DSPG II	58.2	106.5
	CII + DSPG II	51.1	82.1
	GI + DSPG II	27.7	1.4
	GII + DSPG II	25.2	-7.2

The effects of DSPG I, DSPG II, and their domains on cell attachment to substrates composed of the 105-kD fibronectin fragment. Before seeding of the labeled CHO cells, substrates were preincubated with 100  $\mu\text{g/ml}$  of intact proteoglycan, isolated protein core, isolated dermatan sulfate chains, or combinations in the order indicated. The first preincubation was performed with 50  $\mu\text{g/ml}$  of test substance for 1 h and the second with 100  $\mu\text{g/ml}$  for 1 h. Subsequently, cells were seeded, incubated for 90 min, and unbound and bound cells were removed and counted. The number of cells attached to substrates composed of the 105-kD fibronectin fragment in the absence of any preincubation was set at 100%. The number of cells attached to BSA-coated wells was used as a negative control and was set at 0% (the actual percent difference divided by 0.29 = the relative percent difference). For further details see Materials and Methods.

$\times 10^{-8}$  M for wells coated with fibronectin (Fig. 6 E) and  $8 \times 10^{-7}$  M and  $2.1 \times 10^{-8}$  M on substrates composed of the 105-kD fibronectin fragment (Fig. 6 F).

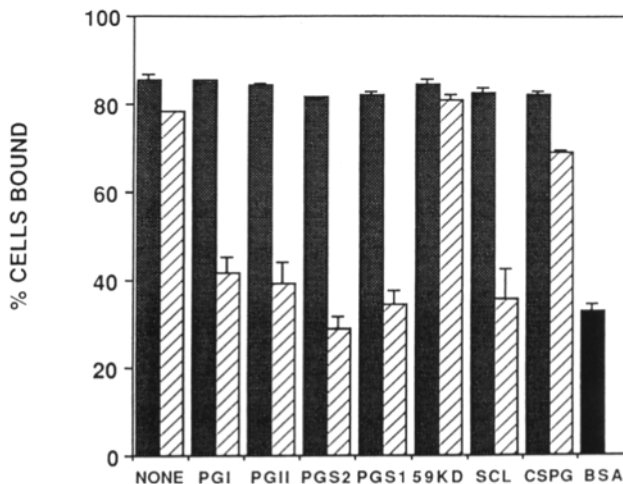
### Intact Proteoglycans Are Required for Biological Activity

In attempts to identify the portion(s) of the DSPG responsible for its ability to inhibit the attachment of CHO cells to substrates composed of the 105-kD fibronectin fragment, cells were seeded on substrates preincubated with intact DSPGs, isolated protein core derived from the DSPGs, or protein free glycosaminoglycan chains. The results of these experiments (Table II) showed that only intact proteoglycans were able to inhibit cell attachment. Preincubation of substrates with isolated protein core or glycosaminoglycan side chains had no inhibitory effect on cell attachment. Hence, the intact form of the DSPG is necessary for inhibition of cell attachment. However, when substrates were preincubated with the corresponding isolated protein core followed by an incubation with intact proteoglycans, the inhibitory activity of DSPG on cell attachment was neutralized. The isolated core protein of DSPG I was effective in neutralizing the inhibitory effects of both DSPG I and DSPG II, whereas the isolated protein core of DSPG II effectively neutralized the effect of DSPG II but only marginally reduced the inhibitory effect of DSPG I. Isolated glycosaminoglycan chains did not affect the biological activity of the intact proteoglycan. Since the core protein, but not the glycosaminoglycan chains, in-

hibited the binding of  $^{125}\text{I}$ -DSPG to fibronectin-coated substrates, our data suggest that inhibition of cell attachment involves the binding of the core proteins to a domain(s) in the 105-kD fibronectin fragment. However, binding of the protein cores to the substrate does not appear to be sufficient to inhibit cell attachment. In addition, glycosaminoglycan chains need to be covalently attached to the core protein, as in the intact proteoglycan, for biological activity to occur.

### Small Glycosaminoglycan Containing Proteoglycans Inhibit Cell Attachment

A number of DSPG-related proteoglycans from different sources were also tested for their ability to inhibit the attachment of CHO cells to fibronectin coated surfaces (Fig. 7). None of the proteoglycans tested affected the attachment of cells to substrates of intact fibronectin. However, the glucuronic acid rich, cartilage PG-S1, the iduronic acid rich, tendon PG-S2, and the scleral DSPG II effectively inhibited the attachment of CHO-K1 cells to substrates composed of the 105-kD fibronectin fragment down to levels obtained with articular cartilage DSPG I and DSPG II. These proteoglycans all have core proteins similar or identical to those of DSPG I or DSPG II but the composition of their glycosaminoglycan side chains varies. PG-S1 contains chondroitin sulfate chains with few if any iduronic acid residues whereas in the tendon PG-S2, >70% of the uronic acid residues are iduronic acid. In contrast to the effects seen with the DSPGs, the large cartilage CSPG and 59-kD fibromodulin proteoglycan were unable to inhibit cell attachment to the 105-kD fibronectin fragment at concentrations tested.



**Figure 7.** The effects of various proteoglycans on cell attachment. Substrates were coated with fibronectin (■) or the 105-kD fibronectin fragment (▨) and the cell attachment assays were performed as previously described. Before the seeding of labeled CHO cells, substrates were incubated with 100  $\mu\text{g/ml}$  of articular DSPG I, articular DSPG II, PG-S1, PG-S2, scleral DSPG, fibromodulin (59-kD), or the large cartilage CSPG for 1 h. The cells were allowed to attach for 90 min. Unbound cells and bound cells were removed and counted as previously described and the percentage of cells bound was determined. (■) A background level of cell binding to a substrate coated with BSA. The results are expressed as the means and ranges of determinations from at least two wells.

## Discussion

In this study, we report that small galactosaminoglycan-containing proteoglycans modulate cell adhesion to fibronectin-coated substrates. In contrast to the results described by Lewandowska et al. (22) and recently by Winnemoller et al. (36), we find that the DSPGs do not interfere with the attachment of cells to substrates composed of intact fibronectin, but that they inhibit the attachment of cells to substrates composed of the 105-kD fibronectin cell-binding fragment. This discrepancy may be due to variations in techniques used and the differences in cell types assayed.

The effects of the small DSPGs on cell adhesion also differ from those of the large, rat yolk sac tumor chondroitin sulfate proteoglycan which inhibit the attachment of rat yolk sac tumor cells to fibronectin coated substrates (3). In this case, the proteoglycan seems to bind to one of the glycosaminoglycan binding sites in the fibronectin molecule, and through its bulkiness, interfere with cell attachment (3). In contrast, in our system, 100  $\mu\text{g/ml}$  of the large chondroitin sulfate proteoglycan from cartilage did not significantly affect CHO cell attachment to either intact fibronectin or the 105-kD fibronectin fragment-coated substrate. In comparison, the small DSPGs showed significant inhibition of CHO cell attachment at concentrations as low as 10  $\mu\text{g/ml}$ .

Yamaguchi and Ruoslahti (39) have reported that CHO cells overexpressing the core protein of DSPG II showed enhanced cell spreading and dramatic changes in growth properties. These data were interpreted to indicate a role for DSPG II in the cell spreading and growth processes. Later results suggested that the observed effects were mediated at least in part by transforming growth factor- $\beta$  (40) and were not a direct effect of a DSPG II interaction with the substrate molecules. In the present study, addition of exogenous DSPG I or DSPG II to a fibronectin substrate did not enhance the number of cells spread or the mean area of spread cells. Instead, the DSPGs were found to impair the ability of cells to form focal adhesions. The extent by which added DSPG reduced focal adhesion formation on fibronectin-coated substrates was similar to that observed in experiments in which thrombospondin was added to endothelial cells attaching to fibronectin substrates (25). However, DSPGs only marginally affected the organization of the actin-containing stress fibers as compared with the extensive F-actin redistribution seen in thrombospondin-sensitive cells. The presence of F-actin microfilaments in the absence of vinculin-containing plaques is consistent with the observation that vinculin does not accumulate until the later stages of adhesion plaque formation (9). In contrast, while the process of focal adhesion formation is apparently sensitive to the presence of DSPGs, the stability of these structures is not affected by DSPGs since the addition of DSPGs to spread endothelial cells grown in serum with existing focal adhesions did not affect the number or distribution of these adhesion structures, whereas thrombospondin caused a partial loss of preformed focal adhesions (25 and data not shown).

The effects of the small DSPGs on cell adhesion appear to require the binding of the proteoglycan to fibronectin molecules or its fragments in the substrate. Preliminary experiments showed that addition of DSPG I or DSPG II did not cause any displacement of either radiolabeled fibronectin or the 105-kD fibronectin fragment from the surface of the tis-

sue culture plastic (data not shown). The interaction between the DSPGs and fibronectin involves the core proteins of the proteoglycans and domain(s) in the central segment of the fibronectin molecule encompassed by the 105-kD fragment. A direct binding of  $^{125}\text{I}$ -labeled DSPGs to fibronectin-coated substrates was demonstrated. This interaction was inhibited by isolated core proteins, but not by glycosaminoglycan chains. However, some apparently unrelated proteins such as casein and nonimmune IgG also inhibited the binding of  $^{125}\text{I}$ -DSPGs to fibronectin whereas other proteins such as ovalbumin, albumin, and fibrinogen were without effect at concentrations of 100  $\mu\text{g/ml}$  (data not shown). Analysis of the binding data suggests one class of binding sites on the fibronectins for intact DSPG II with a moderately high dissociation constant. Binding of isolated protein core to both the 105-kD fibronectin fragment and intact fibronectin demonstrated two classes of binding sites for the protein core ligand, each with markedly different calculated dissociation constants. Schmidt et al. (33) recently reported the presence of two classes of binding sites on intact fibronectin and the 105-kD fibronectin fragment for both the core protein and the intact DSPG II. These differences in the number of binding sites for intact DSPG II may explain some of the differences observed between the two laboratories.

Inhibition of cell adhesion requires a binding of the DSPGs to fibronectin in the substrate. However, addition of isolated core protein to a substrate composed of 105-kD fibronectin fragments is not sufficient to block cell attachment. For this biological activity, an intact proteoglycan is required. Both DSPG I and DSPG II seem to be equally effective. Although, these results demonstrate the importance of the glycosaminoglycan chains for inhibition of cell attachment activity, the structural composition of the galactosaminoglycan chain does not appear to have a determining effect on the activity since DSPG II from tendon with an iduronic-rich glycosaminoglycan chain was as effective in inhibiting cell attachment as the bone-derived PG-S1 with its exclusively glucuronic acid containing glycosaminoglycan chains. It is possible that the role of the glycosaminoglycan chain is to provide enough bulk to sterically interfere with the binding of the appropriate integrin to the corresponding binding site in the fibronectin molecule. When intact fibronectin constitutes the substrate, it is possible that the glycosaminoglycan chains interact with the glycosaminoglycan binding sites of the fibronectin molecules and are effectively folded away from the appropriate binding site. Alternatively, cells may express receptors which interact with domains outside the RGD site in the 105-kD fibronectin fragment. Such additional cell binding sites in fibronectin have been demonstrated (11, 18, 19, 23).

The current study suggests that DSPGs are potential modulators of cell adhesion to substrates composed of fibronectin or certain fragments of fibronectin. It also demonstrates the presence of a possible proteoglycan binding site on the fibronectin molecule and an interaction between the two which is mediated by protein-protein interactions rather than by ionic association with the glycosaminoglycan moiety of the proteoglycan. Previous studies of cells *in vitro* showed that fibronectin may bind to or become associated with a galactosaminoglycan containing molecule (14, 27). In addition, recent studies have shown that a heparan sulfate proteoglycan, isolated from human lung fibroblasts, and related



to the species found in basement membranes, can bind with high affinity to fibronectin via its protein core (17). Obviously, further studies are required to determine the physiological role of the proteoglycan-fibronectin interactions.

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