Fibronectin-mediated Adhesion of Fibroblasts: Inhibition by Dermatan Sulfate Proteoglycan and Evidence for a Cryptic Glycosaminoglycan-binding Domain

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Abstract. Dermatan sulfate proteoglycans (DS-PGs) isolated from bovine articular cartilage have been examined for their effects on the adhesive responses of BALB/c 3T3 cells and bovine dermal fibroblasts on plasma fibronectin (pFN) and/or type I collagen matrices, and compared to the effects of the chondroitin sulfate/keratan sulfate proteoglycan monomers (CS/KS-PGs) from cartilage. DS-PGs inhibited the attachment and spreading of 3T3 cells on pFN-coated tissue culture substrata much more effectively than the cartilage CS/KS-PGs reported previously; in contrast, dermal fibroblasts were much less sensitive to either proteoglycan class unless they were pretreated with cycloheximide. Both cell types failed to adhere to substrata coated only with the proteoglycans; binding of the proteoglycans to various substrata has also been quantitated. While a strong inhibitory effect was obtained with the native intact DS-PGs, little inhibitory effect was obtained with isolated DS chains (liberated by alkaline–borohydride cleavage) or with core protein preparations (liberated by chondroitinase ABC digestion). In marked contrast, DS-PGs did not inhibit attachment or spreading responses of either 3T3 or dermal fibroblasts on type I collagen-coated substrata when the collagen was adsorbed with pFN alone, DS-PGs alone, or the two in combination. These results support evidence for (a) collagen-dependent, fibronectin-independent mechanisms of adhesion of fibroblasts, and (b) different sites on the collagen fibrils where DS-PGs bind and where cell surface "receptors" for collagen bind.

Experiments were developed to determine the mechanism(s) of inhibition. All evidence indicated that the mechanism using the intact pFN molecule involved the binding of the DS-PGs to the glycosaminoglycan (GAG)-binding sites of substratum-bound pFN, thereby inhibiting the interaction of the fibronectin with receptors on the cell surface. This was supported by affinity chromatography studies demonstrating that DS-PGs bind completely and effectively to pFN–Sepharose columns whereas only a subset of the cartilage CS/KS-PG binds weakly to these columns. In contrast, when a 120-kD chymotrypsin-generated cell-binding fragment of pFN (CBF which has no detectable GAG-binding activity as a soluble ligand) was tested in adhesion assays, DS-PGs inhibited 3T3 adherence on CBF more effectively than on intact pFN. A variety of experiments indicated that the mechanism of this inhibition also involved the binding of DS-PGs to only substratum-bound CBF due to the presence of a cryptic GAG-binding domain not observed in the soluble CBF. When a series of complementary cell-binding fragments generated from pFN by thermolysin digestion and subsequent affinity chromatography (Castellani, P., A. Siri, C. Rosellini, E. Infusini, L. Borsi, and L. Zardi, 1986, *J. Cell Biol.*, 103:1671–1677) were tested, a graded response to inhibition by DS-PGs was observed revealing the proximity of the cryptic GAG-binding domain to the cell-binding domain of the fibronectin molecule.

All of these results taken together demonstrate that DS-PGs can have a marked influence on the adhesive responses of fibroblasts to select extracellular matrices, particularly with regard to the conformation of the fibronectin molecule, and suggest experiments for investigating DS-PG functions in vivo.

SMALL interstitial dermatan sulfate proteoglycans (DS-PGs)[1] are widely distributed in the extracellular matrices of skin (22, 23, 25, 43, 44), sclera (11, 12), tendon (63), fetal epiphyseal cartilage (51), mature articular cartilage (50), and bone with chondroitin sulfate (CS) chains substituting for dermatan sulfate chains in this last case (17–19).

The DS-PGs are polydisperse species with molecular masses ranging from 80 to 140 kD and with core proteins generated

1. *Abbreviations used in this paper:* 120K CBF, 120-kD fragment containing RGDS-sensitive cell-binding activity; CS, chondroitin sulfate; DS, dermatan sulfate; pFN, plasma fibronectin; GAG, glycosaminoglycan; HS, heparan sulfate; KS, keratan sulfate; PF-4, platelet factor-4; PG, proteoglycan.

by deglycosylation with chondroitinase ABC of ∼45 kD; they contain a limited number of dermatan sulfate chains covalently bound to serine residues in the protein core (27, 70). Immunohistochemical studies have localized them in the extracellular matrices of connective tissues (40, 41, 46), hence their description as small interstitial proteoglycans by Heinegard (28). Mature bovine articular cartilage (50), fetal epiphyseal cartilage (51), and tendon (62) contain two different species of DS-PGs (referred to as DS-PGI and DS-PGII) with different primary sequences in their core proteins. Also, DS-PGI readily self-associates whereas DS-PGII from fetal epiphyseal and mature articular cartilages does not (50).

Relatively little is known about the biological functions of the DS-PGs. They are distributed at the "d" band on the surfaces of collagen fibrils in vivo (8, 46, 54–56, 66) and inhibit fibrillogenesis in vitro (64). Iduronate-enriched species of DS-PG have also been demonstrated in the collagenous matrices deposited on the substratum by human dermal fibroblasts (20).

The common occurrence of DS-PGs and/or CS-PGs with many cell types in tissues would suggest their importance in basic cell biological functions and some evidence has been garnered with specific proteoglycans for inhibition of adhesion of cells to certain extracellular matrices. Thus, Knox and Wells (32) first demonstrated that the major cartilage CS/keratan sulfate (KS)-PG at high concentrations could inhibit adherence of chick embryonic fibroblasts to serum- or collagen-coated tissue culture substrata. This activity for cartilage CS/KS-PG was also confirmed by Rich et al. (48) using plasma fibronectin–coated surfaces and a variety of cell types. Brennan et al. (5) then showed that a rat yolk sac tumor cell CS-PG could inhibit adherence of these tumor cells to substrata coated with fibronectin (but not its cell-binding fragment) or type I collagen by interacting with the substratum protein itself, rather than with the cell surface. Exogenous addition of multivalent heparin proteoglycans was shown by Klebe and Mock (30) to inhibit attachment of cells to fibronectin substrata much more effectively than single chains of glycosaminoglycan (GAG). Such studies agreed with biochemical analyses of substratum adhesion sites from fibroblasts adhering to serum-coated surfaces—namely, that CS-PGs appear to function in the detachment processes of cells and not directly in their attachment (15, 16). Until now, the effects of DS-PGs on cell adhesion have not been examined.

The studies described above form the impetus for examining the significance of DS-PGs in the adhesion responses of fibroblasts on biochemically defined substrata. The availability of large amounts of DS-PGs from bovine articular cartilage as recently described by Rosenberg et al. (50) have made such studies feasible. Two different tissue culture model systems have been used. BALB/c 3T3 adherence and spreading have been analyzed since these cells do not deposit a layer of collagen at their undersurfaces (13) and interact with plasma fibronectin (pFN) on the substratum through both heparan sulfate (HS)- and cell-binding domains of the molecule (14, 29, 34); preliminary experiments have been described in this regard (49). In addition, bovine dermal fibroblasts have also been used because they reside in tissue that contains this DS-PG and because they deposit a complex collagen extracellular matrix at their undersurfaces when grown on fibronectin-coated substrata.

Materials and Methods

Cell Cultures

Stock cultures of mouse BALB/c 3T3 (clone A31) cells were grown Mycoplasma-free in DME supplemented with 10% neonatal calf serum, 250 U/ml penicillin, and 250 µg/ml streptomycin sulfate at 37°C in a 10% CO_2-humidified air mixture. Bovine dermal fibroblasts were isolated from the dermis of an adult cow at a local slaughterhouse under sterile conditions and grown in DME with 10% FCS. For experiments, subconfluent cells in stock cultures were rinsed twice with PBS, incubated with gentle shaking in 0.5 mM EGTA in PBS at 37°C for 30 min, and gently pipetted in order to detach them. Suspended cells were collected by centrifugation, rinsed twice with serum-free medium containing 0.25 mg/ml heat-treated BSA (adhesion medium), and then resuspended in fresh adhesion medium.

Proteins and Proteoglycans

Human pFN was purified by gelatin–Sepharose affinity chromatography as described previously (33). Platelet factor-4 (PF-4), free of any contaminating pFN, was purified from outdated platelet packs provided by the Cleveland Red Cross as also described (33). Bovine articular cartilage DS-PGs (a mixture of DS-PGI and DS-PGII) were purified as described by Rosenberg et al. (50) and stored at −85°C in 4 M GdnHCl in buffer. A variety of assays was used to demonstrate that these DS-PGs are not contaminated with other proteoglycan classes. Cartilage chondroitin sulfate/keratan sulfate proteoglycan monomer (CS/KS-PG) was purified by conventional means (50), stored at −85°C in 4 M GdnHCl in buffer, and shown to be free of any contaminating DS-PG moieties. For adhesion studies, all proteoglycan solutions were dialyzed at 4°C for 48 h against multiple changes of PBS to rid the solutions of GdnHCl; in all cases, these moieties remained in solution at concentrations ranging from 0.3 to 6 mg/ml.

Cell-binding Fragments of Fibronectin

Two different protocols were used to generate cell-binding fragments of human fibronectins which contain the Arg-Gly-Asp-Ser (RGDS) sequence recognized by the fibronectin receptor (6, 9, 21, 47). As developed by Pierschbacher et al. (45), chymotrypsin treatment of pFN liberates a relatively protease-resistant fragment of 120 kD which lacks heparin- and collagen-binding activity as a soluble ligand but which contains RGDS-sensitive cell-binding activity (3, 29, 34). This will be referred to as the 120K CBF.

The second protocol was developed by Borsi et al. (4) and further by Castellani et al. (7) using thermolysin digestion of human plasma or cellular fibronectins and subsequent affinity chromatography of soluble-phase fragments. The following purified fragments are used from this scheme: 110-kD fragment (f110) from pFN which contains the RGDS sequence but no COOH-terminal heparin-binding domain; 145-kD fragment (f145) which contains all of the sequence of f110 as well as the COOH-terminal heparin-binding domain from the α subunit of pFN and possibly some of the IIICS region at its COOH-terminal end; 155-kD fragment (f155) from the β subunit of pFN which contains all of the sequence of f110 as well as an additional type III homology until at its COOH-terminal end and the COOH-terminal heparin-binding domain; and a mixture of 44- and 47-kD fragments from cellular fibronectin (f44+47) which contains the so-called "extra domain" (ED_a) characteristic of cellular fibronectins and the COOH-terminal heparin-binding domain (f44+47 do not contain the RGDS-dependent cell-binding domain). All fragments were shown to be highly purified by PAGE in the presence of SDS and reducing agent.

Adhesion Assays

In experiments where unlabeled cells were to be photographed by phase-contrast microscopy, cells were inoculated into pre-coated 24- or 48-well cluster dishes (Costar, Cambridge, MA). The substratum had been treated by one of two possible protocols (36, 37). In the first protocol, wells were rinsed twice with PBS; then an appropriate volume (500 µl for 24-well dishes; 200 µl for 48-well dishes) of protein solution (20 µg/ml pFN alone or a mixture of pFN plus the indicated concentration of a specific PG; or 250 µg/ml heat-treated BSA as a control substratum [in all cases, this yielded no attachment]) in serum-free DME was added and the wells were incubated for 1 h at 37°C. BSA was then added to wells to final concentration of 250 µg/ml (adhesion medium), followed by inoculation of 10⁵ cells. In the second protocol, wells were first adsorbed with 20 µg/ml pFN for 1 h at 37°C in serum-free DME, rinsed free of pFN, and then post-incubated

with varying amounts of bovine articular cartilage DS-PGs or cartilage CS/KS-PG in serum-free DME for 1 h at 37°C. This was followed by addition of BSA and finally inoculation of 10^5 cells.

Collagen coating was performed as follows: the wells of a 48-well dish were rinsed twice with PBS and 200 μl/well of vitrogen-100 solution in PBS (50 μg/ml type I collagen) was added to each well; the dish was incubated for 15 min at 37°C to promote gelation and then allowed to dry overnight at room temperature. Use of higher concentrations of collagen for coating or longer periods of adsorption yielded the same results as reported here. The wells were rinsed twice with PBS and post-adsorbed with pFN (at 50 μg/ml), DS-PGs (at 100 μg/ml), or CS/KS-PG (at 300 μg/ml) as described below.

Quantitation of Attachment

For radiolabeling of cells to be used in the attachment assays (37), cells were freshly passaged by trypsinization. 24 h later, the medium was replaced with one containing 0.1 μCi/ml [methyl-H^3]thymidine (sp act = 50-90 Ci/mmol). After 24 h or 48 h, depending on whether the attached or the unattached cells were being assayed as below, the medium was replaced with unlabeled medium for an additional 24 h. Either glass coverslips were placed in 100-mm diameter tissue culture dishes, which were subsequently coated with the specific protein to assay attached cells, or alternatively 24-well cluster dishes were used to assay unattached cells. Triplicate glass coverslips were removed at indicated times, rinsed three times with PBS, and placed in scintillation vials to assay radiolabeled attached cells. To quantitate unattached cells in wells of cluster dishes, all medium in each well was transferred to a BSA-coated plastic vial and then triplicate aliquots were taken to assay unattached cells (N) for comparison with a same-size aliquot of the original cell inoculum (N_o). Percent of cells attached in a well was calculated as $C = (1-N/N_o) \times 100\%$, where N represents radiolabeled and unattached cells in the wells. Both attachment assays yielded the same results.

Preparation of Samples for Microscopy

For phase-contrast microscopy, tissue culture wells with attached cells at the indicated times were rinsed twice with PBS, incubated for 30 min at room temperature with 3% glutaraldehyde in PBS, rinsed twice with PBS, and stored in PBS-containing 0.02% sodium azide at 4°C. Phase-contrast micrographs were taken on a Nikon Diaphot inverted phase-contrast microscope. In some cases, glutaraldehyde fixation was performed directly in the adhesion medium of the well before rinsing and storage.

Affinity Chromatography

pFN or PF4 affinity columns were prepared by cross-linking the ligands to CNBr-activated Sepharose 4B beads as described previously (33), taking precautions to avoid inactivation of the critical lysine residues in the binding site of PF4. Excess residues were blocked with 1 M monoethanolamine for 2 h at room temperature and the beads were then washed and suspended in TMC (0.05 M Tris, pH 7.4, 1 mM MgCl$_2$, 1 mM CaCl$_2$) buffer. The saturation limits of 20 ml pFN-Sepharose or PF4-Sepharose columns were determined by chromatography of [^{3}H]heparin (sp act = 0.2-1 mCi/mg) with varying amounts of nonradioactive heparin. After washing the pFN column with 50 ml TMC buffer, a gradient of 0-0.6 M NaCl in TMC was applied (40 ml total), followed by 10 ml 0.6 M NaCl in TMC and finally 10 ml 2 M NaCl in TMC. With PF4 columns, the gradient applied was 0-0.8 M NaCl in TMC. Fractions were collected, radioactivity was measured by scintillation counting, and salt concentration was determined by refractometry. The elution of proteoglycans was monitored by uronate determinations using the carbazole method (50).

Materials

Cluster dishes and 100-mm petri dishes were obtained from Costar; DME was from Gibco, Grand Island, NY; neonatal calf serum and FCS were from Biologos, Inc., Naperville, IL; No. 1 (11 × 22-mm) micro cover glasses were from Arthur H. Thomas Co., Philadelphia, PA; Thermanox plastic coverslips (13-mm diam) from Miles Scientific, Elkhart, IN; [methyl-H^3]thymidine and [^{3}H]heparin from New England Nuclear, Boston, MA; Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden; vitrogen-100 was from Collagen Corp., Palo Alto, CA; BSA and EGTA were from Sigma Chemical Co., St. Louis, MO; glutaraldehyde and technical pan films from Eastman Kodak Co., Rochester, NY; paraformaldehyde was
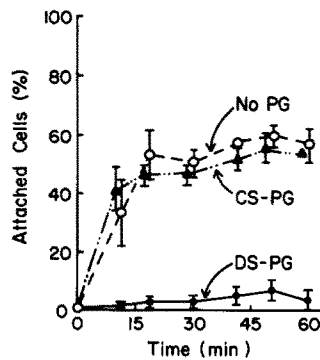

Figure 1. Kinetics of 3T3 attachment: effects of proteoglycan. BALB/c 3T3 cells were radiolabeled by [^{3}H]thymidine incorporation as described in the Materials and Methods. They were then detached from stock cultures by EGTA treatment, rinsed twice with adhesion medium, and then inoculated into dishes containing glass coverslips. These dishes had been previously adsorbed with 20 μg/ml pFN alone in serum-free DME for 1 h at 37°C (no PG; o); or with a mixture of 20 μg/ml pFN plus 300 μg/ml cartilage CS/KS-PG (CS-PG; ▲); or a mixture of 20 μg/ml pFN plus 100 μg/ml articular cartilage DS-PGs (DS-PG; ●). BSA was added to a final concentration of 250 μg/ml and cells were inoculated. At the indicated times, triplicate coverslips were removed from dishes, rinsed twice with PBS, and placed in scintillation vials for determination of the amounts of radiolabeled cells adherent. This number is corrected for the total number of cells inoculated (as the 100% value) as shown on the Y axis. Standard deviations are shown.

Results

Inhibition of pFN-mediated Attachment to DS-PGs

Articular cartilage DS-PGs were tested for possible effects on attachment of BALB/c 3T3 cells on human pFN-coated substrata. For comparison, the inhibitory capacity of cartilage CS/KS-PG monomer from the same mature bovine articular cartilages was also examined. As shown in Fig. 1 using the attached cell assay described in Materials and Methods, a concentration of 300 μg/ml of cartilage CS/KS-PG has little inhibitory effect on the attachment kinetics of 3T3 cells on substrata coated with a mixture of pFN and the PG and when the CS/KS-PG is left in the adhesion medium (however, in agreement with previous studies [32, 48], higher concentrations of this proteoglycan do become inhibitory). In contrast, a concentration of 100 μg/ml of articular cartilage DS-PGs completely abolished attachment (Fig. 1) in the same adhesion assay. Identical results were also obtained when substrata were first coated with 20 μg/ml pFN and then the proteoglycan was added to the adhesion medium at the time of inoculation of cells; also when the unattached cell assay was used.

When bovine dermal fibroblasts were tested in the same assays, their attachment and spreading processes were insensitive to either proteoglycan on pFN substrata, consistent with a multiplicity of mechanisms by which the collagenous matrices (produced constitutively and secreted by these dermal fibroblasts in culture [24a]) provide for their adhesion (see below). When dermal fibroblasts were treated with 2 μg/ml of cycloheximide for 16 h to inhibit synthesis and deposition of their endogenous matrix and then assayed for adhesive responses on pFN, both attachment and spreading

Table I. Quantitation of DS-PG Bound to Various Substrata*

Substratum	DS-PG	OD$_{405}$
	µg/ml	
BSA	0	0.18
DS-PG	5	0.38
	10	0.90
	20	1.3
	100	1.4
DS-PG on pFN	10	0.32
	100	0.49
DS-PG on CBF	10	0.92
	100	1.01

* Wells of a 96-well tissue culture dish were coated at 37°C with BSA only (250 µg/ml), DS-PG only at the indicated concentrations, pFN (20 µg/ml) for 1 h followed by 1 h of DS-PG at the indicated concentrations (along with 250 µg/ml BSA), or CBF (20 µg/ml) for 1 h followed by 1 h of DS-PG at the indicated concentrations (along with 250 µg/ml BSA). All wells were then rinsed, post-adsorbed with heat-treated BSA (250 µg/ml), rinsed again, and bound DS-PG was assayed by ELISA in the linear region of the binding curve using polyclonal rabbit anti–bovine articular cartilage DS-PG and an alkaline phosphatase conjugate. Optical density was measured at 405 nm after 70 min of reaction time and values routinely fell within a range of ±5% of the value given.

were partially inhibited by DS-PG but not by CS/KS-PG (data not shown). These results confirm the complexity and multiplicity of mechanisms that such matrix-producing cells can use in adhesion processes.

Adsorption of DS-PGs or CS/KS-PG to the substratum in the absence of any pFN, followed by inoculation of either 3T3 or bovine dermal fibroblasts, resulted in no detectable attachment. This suggests either that there are no "receptors" on the surfaces of these cells for these PGs (in contrast to other cell populations where proteoglycan receptors have been demonstrated [23a]) or that the binding of PG to the substratum creates steric problems which circumvents the action of possible PG "receptors." To examine the binding of PGs to the substratum, ELISAs were used both with polyclonal anti–DS-PG for direct determination or, alternatively, by indirect detection of the blockage of the substratum by PGs with polyclonal antibody to BSA. As shown in Table I, the ELISA in which we used anti–DS-PG reveals maximal binding of the PG antigen to the substratum between 10 and 20 µg/ml. This level of binding to the naked substratum by DS-PG was approximately threefold higher than the binding of DS-PG to a pFN-coated substratum (Table I). Maximal bind-
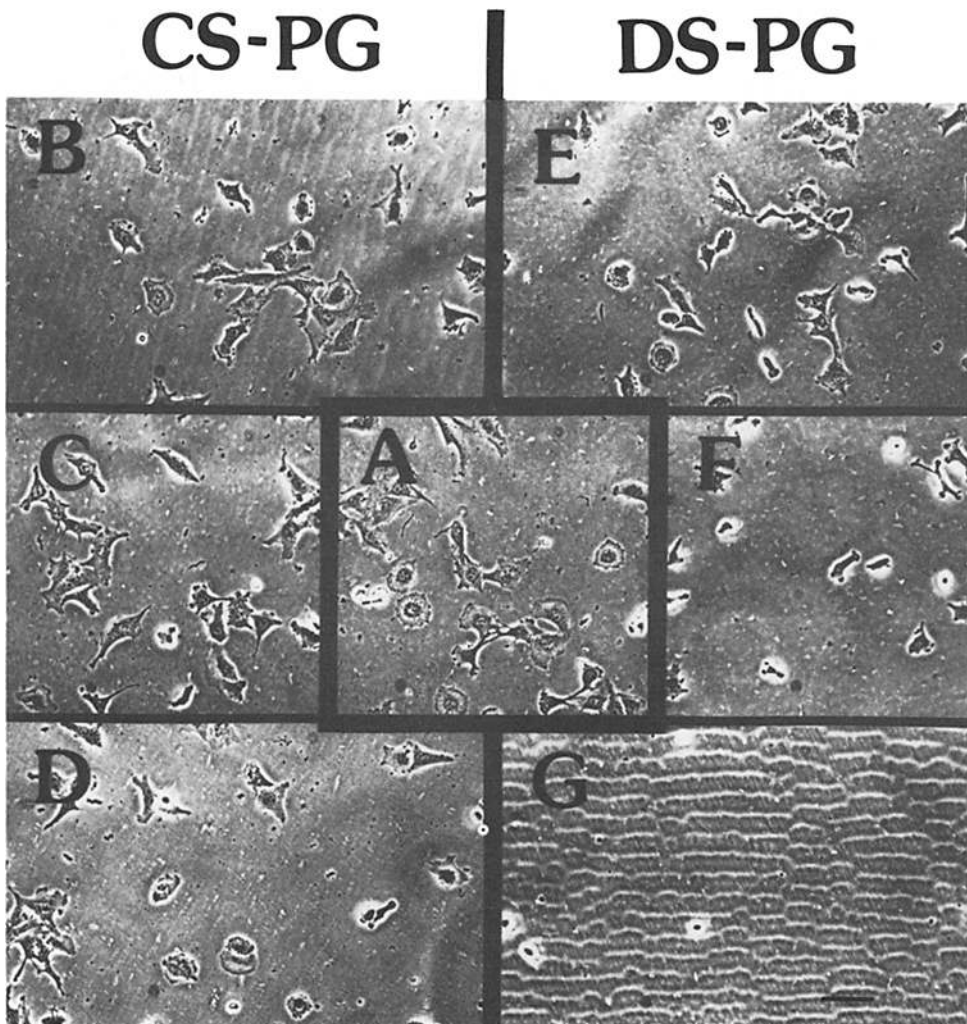

Figure 2. Dose dependence of proteoglycans and cell morphology. Cluster dishes (24 well) were coated with 20 µg/ml pFN alone (A) or with a mixture of pFN and a specific PG (cartilage CS/KS-PG in B–D or cartilage DS-PGs in E–G) as described in Materials and Methods and the legend to Fig. 1. EGTA-detached and rinsed 3T3 cells were then inoculated. After 4 h of cell attachment and spreading, the wells were rinsed well, fixed with glutaraldehyde, and photographed for phase-contrast microscopy. The following concentrations of PG were used: (B and E) 10 µg/ml; (C and F) 100 µg/ml; and (D and G) 300 µg/ml. Bar, 20 µm.

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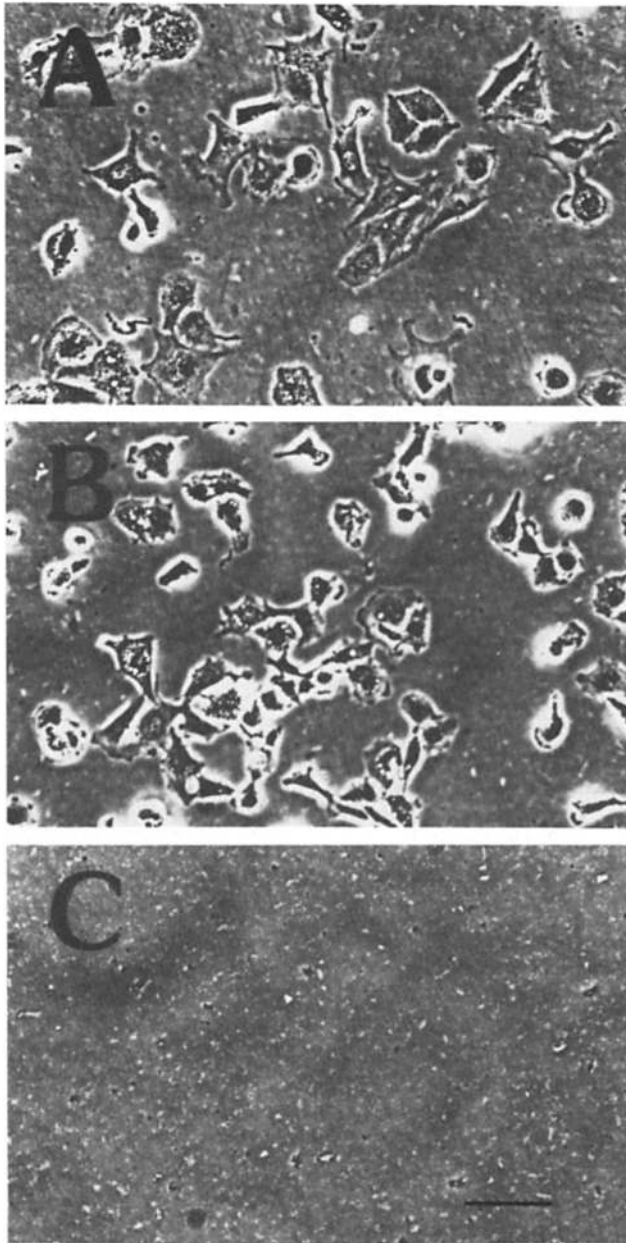*Figure 3.* Post-adsorption of the substratum with DS-PGs. Cluster dishes were coated with 20 μg/ml pFN, rinsed well, and post-adsorbed with (*A*) nothing, (*B*) 10 μg/ml cartilage DS-PGs in PBS, or (*C*) 100 μg/ml DS-PGs for an additional hour at 37°C. The wells were then rinsed twice with adhesion medium and refed with fresh adhesion medium without any additions. Cells were then inoculated for a 4-h incubation and fixed with glutaraldehyde for phase-contrast microscopy. Bar, 20 μm.

ing of CS/KS-PG to the substratum was measured based on blockage of binding of BSA using anti–BSA and was observed at 30–50 μg/ml; furthermore, even at this maximal level of CS/KS-PG binding, ~15% of the substratum sites were still available to BSA binding whereas 20 μg/ml DS-PG completely blocked BSA binding (data not shown).

Dose responses for inhibition of 3T3 attachment and spreading are shown in Fig. 2. At 4 h with adsorption to the substratum with pFN alone or pFN mixed with the particular proteoglycan, cartilage CS/KS-PG at 10 (Fig. 2 *B*) or 100 μg/ml (Fig. 2 *C*) displayed no inhibition of attachment or spreading when compared to the untreated control (Fig. 2 *A*). Even at 300 μg/ml (Fig. 2 *D*), there was a limited effect on the spreading of the cells. In marked contrast, cartilage DS-PGs at 10 μg/ml (Fig. 2 *E*) generated more bipolar cells and at 100 μg/ml (Fig. 2 *F*) prevented any extensive spreading and left cells attached in a fragile manner, requiring very careful rinsing of wells to detect any adherent cells. At 300 μg/ml (Fig. 2 *G*), attachment was completely inhibited. Identical effects were noted when the substratum was first adsorbed with pFN and then PG was added at the time of inoculation of cells.

Several experiments were undertaken to determine whether the effects of the DS-PGs were on the cell surface receptors recognizing pFN or on the substratum-bound pFN itself. When platelet factor-4, which binds effectively to several different proteoglycans including CS-PGs or DS-PGs (34, 67; see below), was tested as the adhesion-promoting ligand on the substratum (36, 37), cartilage CS/KS-PG was just as effective at inhibiting attachment as cartilage DS-PGs, demonstrating differences in specificity between pFN and PF4 substrata (see affinity fractionations below). An experiment was also performed in which pFN-coated wells were post-adsorbed with the DS-PGs for a time period, the excess pFN and PG were rinsed off the substratum, and then 3T3 cells were added to evaluate attachment and spreading. As shown in Fig. 3 *B*, post-adsorption of pFN substrata with 10 μg/ml DS-PGs inhibited the spreading of cells when compared to untreated controls (Fig. 3 *A*) in the absence of any proteoglycan in the adhesion medium. At 100 μg/ml DS-PG post-adsorption (Fig. 3 *C*), the substratum was saturated and inhibition was maximal. When EGTA-detached 3T3 cells were treated in suspension with high concentrations of DS-PGs for 1 h and then rinsed free of excess proteoglycan before inoculation into pFN-coated wells, there was no inhibition of attachment or spreading (data not shown). All of these data taken together indicate that the mechanism of inhibition involves the binding of the DS-PG to the GAG-binding domains of pFN on the substratum, thereby inhibiting the binding of cell surface receptors to the complex on the substratum.

The core proteins of the DS-PGs were maximally stripped of most of their dermatan sulfate chains by chondroitinase ABC digestion under conditions where the integrity of the core protein is conserved, as described previously (50), in order to test for any inhibitory activity by this moiety. DS chains were also prepared by alkaline-borohydride elimination to test their possible inhibitory activities separately. At concentrations as high as 250 μg/ml, neither the core protein nor the DS chains displayed any inhibition of attachment or spreading of 3T3 cells (data not shown). This is consistent with the weaker and less sterically restrictive binding of the "univalent" DS chains to the substratum-bound pFN and the much more effective inhibition by the multivalent proteoglycan as found in previous studies (30). Of equal importance, this demonstrates that the inhibition cannot be explained by interaction of the DS-PG core protein with the pFN on the substratum.

Collagen-dependent Adhesion Processes

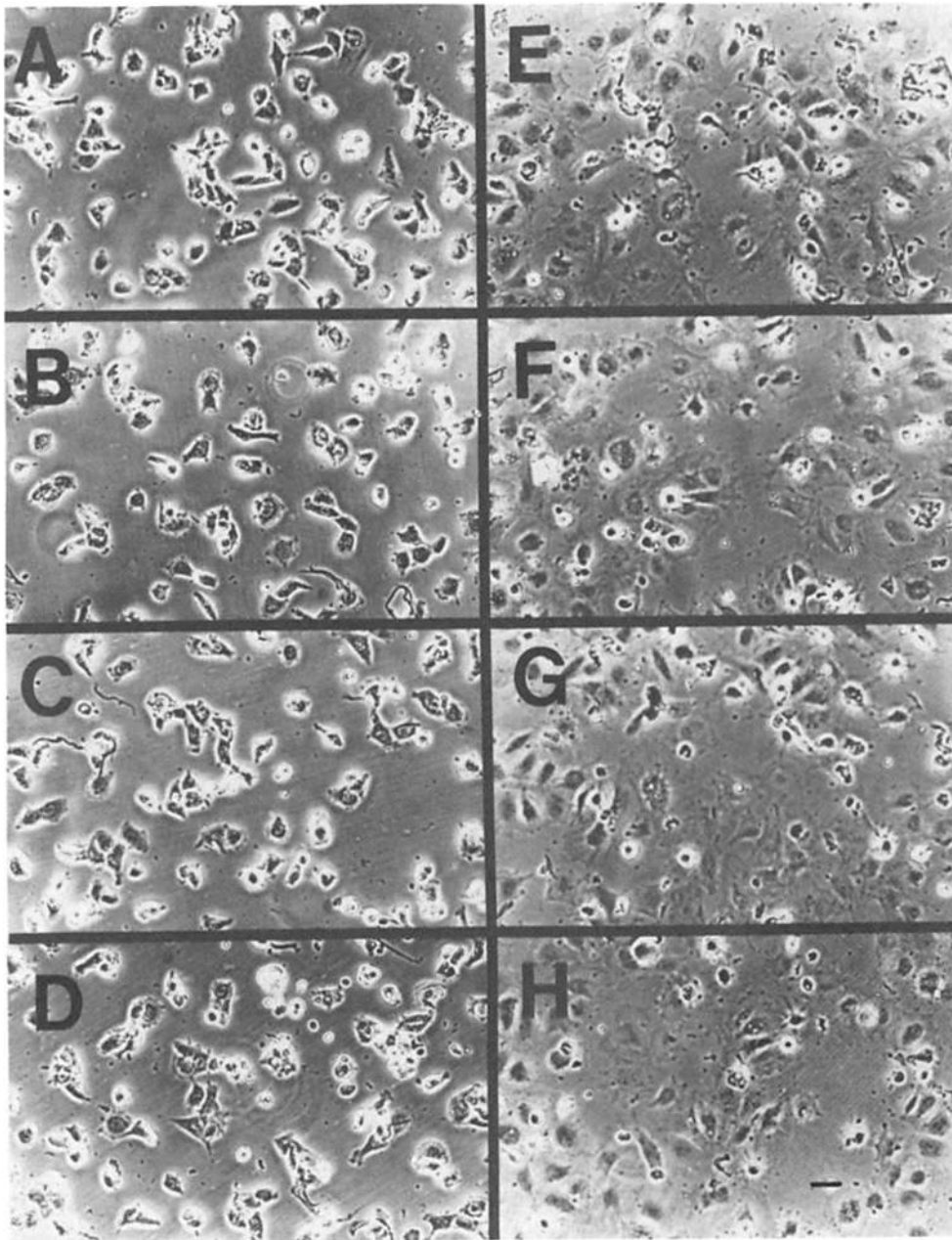A series of experiments were then designed to test whether

Figure 4. Collagen-coated substrata and effects of proteoglycan. Cluster dishes were coated with type I collagen as described in the Materials and Methods. In wells *A–C* and *E–G*, pFN (50 µg/ml) was post-adsorbed for 1 h at 37°C in serum-free DME; in wells *D* and *H*, DS-PGs (100 µg/ml) were post-adsorbed in the absence of any exogenous pFN. After rinsing the wells, BSA was added to 250 µg/ml to all wells; DS-PGs (100 µg/ml) were added to wells *B* and *F*; or CS/KS-PG (300 µg/ml) to wells *C* and *G*. Finally, EGTA-detached 3T3 cells were inoculated into wells *A–D* and EGTA-detached bovine dermal fibroblasts into wells *E–H*. After 4 h of incubation, all wells were rinsed twice with PBS and adherent cells fixed with glutaraldehyde as described in Materials and Methods for phase-contrast microscopy. Bar, 20 µm.

the PGs can inhibit attachment and spreading of either 3T3 cells (Fig. 4, *A–D*) or bovine dermal fibroblasts (Fig. 4, *E–H*) on substrata coated with type I collagen. At 4 h, both 3T3 cells (Fig. 4 *A*) and dermal fibroblasts (Fig. 4 *E*) attach and spread effectively on type I collagen post-adsorbed with pFN. Neither 100 µg/ml DS-PG (Fig. 4, *B* and *F*) nor 300 µg/ml CS/KS-PG (Fig. 4, *C* and *G*) had any inhibitory influence on adhesion processes by either cell type. Similarly, in the absence of any exogenous pFN, adsorption of DS-PGs to the collagen layer had no adverse effect on attachment or spreading by either cell type (Fig. 4, *D* and *H*). These results were also observed at shorter or longer time points, as well as higher concentrations of PG adsorbed over longer periods of time. These data are consistent with collagen-dependent and fibronectin-independent mechanisms of adhesion of these cells, as found previously (26, 31,

39), that are insensitive to the inhibitory influence of these proteoglycans. Therefore, the biochemical complexity of the extracellular matrix is a critical determinant in providing sensitivity or resistance of fibroblasts to the adhesion-modulating effects of proteoglycans.

Affinity Chromatography of Proteoglycans

A series of affinity chromatography experiments were designed to evaluate the binding between these proteoglycans and pFN or PF4 as a biochemical correlation of the adhesion function studies described above. Each proteoglycan applied in milligram quantities (using the carbazole assay to quantitate the uronic acid contents of column fractions) was tested on PF4-Sepharose or pFN-Sepharose under subsaturating conditions. As shown in Fig. 5 *A*, cartilage DS-PGs bind
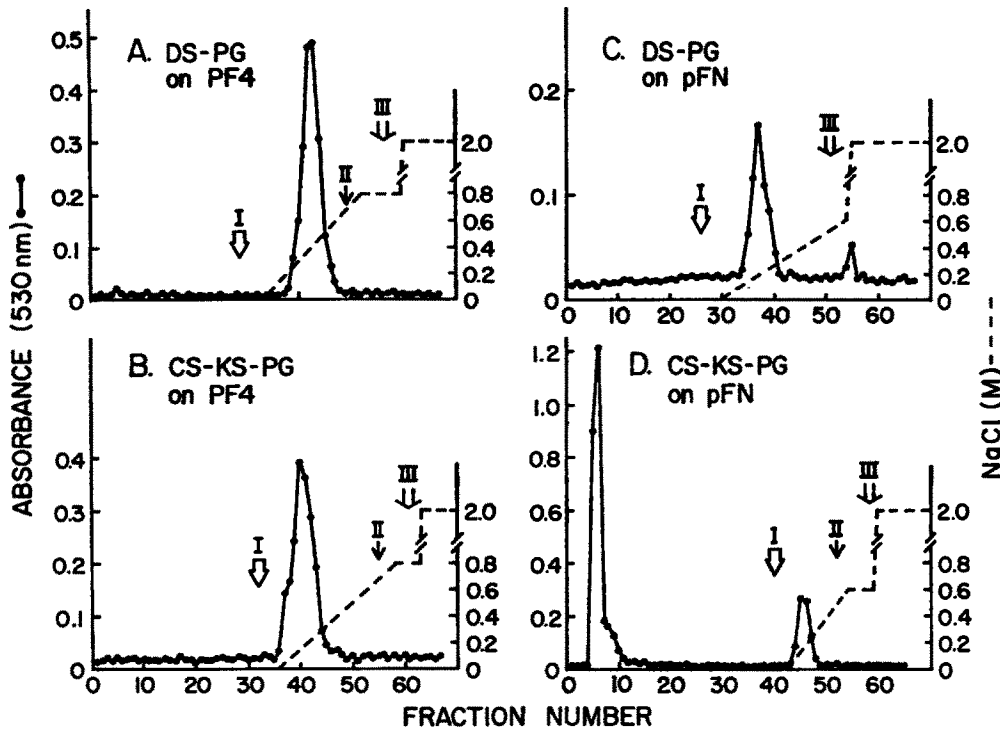
Figure 5. Affinity chromatography of proteoglycans. PF4-(*A* and *B*) or pFN–Sepharose (*C* and *D*) affinity columns were prepared as described in Materials and Methods. Columns were rinsed well with TMC buffer, and 1–2-mg quantities of cartilage DS-PGs (*A* and *C*) or CS/KS-PGs (*B* and *D*) were applied in TMC. At the open arrowhead (*I*), a NaCl gradient in TMC was introduced (0–0.6 M for pFN or 0–0.8 M for PF4). At the single arrow (*II*), a solution of 0.6 or 0.8 M NaCl in TMC was introduced for pFN or PF4, respectively. Finally at the double arrow (*III*), 2 M NaCl in TMC was introduced. Fractions were assayed for uronic acid contents by the carbazole assay at an OD of 530 nm (recoveries approached 82–88% of the material applied). Salt content was determined by refractometry.

completely to PF4 and require relatively high NaCl for elution whereas CS/KS-PG binds completely but requires lower NaCl for elution (Fig. 5 *B*). When DS-PGs are tested on pFN columns (Fig. 5 *C*), binding is complete and a subset of molecules requires very high NaCl for elution. In marked contrast, only a relatively small subset of CS/KS-PG molecules binds to pFN (Fig. 5 *D*) while the vast majority of molecules flow through the column without any retardation; the molecular basis for the binding and nonbinding subsets has not been determined. However, these affinity chromatography studies reveal the basis for the differences in adhesion inhibition on pFN versus PF4 substrata described above—namely, the much more effective and complete binding of DS-PGs to pFN compared with the cartilage CS/KS-PG.

Adhesion on Cell-binding Fragments of Fibronectin

To further evaluate the roles of fibronectin's heparin-binding or cell-binding domains in the inhibitory process, adhesion studies were performed using various fragments of human plasma or cellular fibronectin molecules after their digestion with a specific protease and subsequent purification on affinity columns. 120K CBF purified from chymotrypsin digests of human pFN was used initially, since it lacks collagen- and heparin-binding activities when assayed as a soluble ligand (3, 14, 45). 3T3 cells attach, spread partially, and generate a distinctive pattern of close contacts and microfilament reorganization on this 120K CBF (29, 34).

When 3T3 cells were tested on 120K CBF substratum after post-adsorption with a high concentration of the core protein of DS-PGs (Fig. 6 *A*) or dermatan sulfate chains (Fig. 6 *B*; insufficient amounts of chains restricted the testing of even higher concentrations), attachment and spreading were identical to a CBF-coated well that did not receive chains or core (not shown); leaving the chains or core in the wells through-

out the attachment process also failed to inhibit adhesive responses. However, the intact DS-PGs were highly inhibitory to attachment on CBF, either with re-addition of CBF after the DS-PG treatment period (Fig. 6 *C* and Table II) or with no further addition of CBF (not shown). Also, 100 µg/ml CBF added in the medium along with the DS-PGs (at 5–50 µg/ml) could not "neutralize" the inhibitory activity, indicating that the substratum-bound CBF has a binding activity not displayed by solution-borne CBF; also, pretreatment of cells in suspension with DS-PGs did not alter their responses on CBF (data not shown). When the dose of DS-PGs was varied, concentrations as low as 5 µg/ml were inhibitory (Fig. 6 *D*), demonstrating a far greater sensitivity than that displayed by the intact pFN molecule as described above. In all cases, CS/KS-PG at concentrations as high as 300 µg/ml had no effect on CBF-mediated adhesion (Fig. 6 *E* and Table II), indicating specificity in the process. Treating the CBF substratum for time periods as short as 5 min with the DS-PGs was also inhibitory (Fig. 6 *F*). The effectiveness of binding of DS-PG to a CBF-saturated substratum (such that no BSA binding can be detected in the ELISA using anti-BSA) is demonstrated in Table I. Approximately twice as much DS-PG antigen binds to the CBF coating when compared to the pFN coating; this binding is still well below the level observed for DS-PG binding to naked surfaces in the same assay. This would suggest either that the specific activity for PG binding on CBF is twice as high as that on the intact pFN molecule (consistent with the size difference for the two polypeptides) or that the dissociation process is reduced, thereby permitting detection of higher steady-state levels of binding.

A number of other experiments were used to explore the mechanism of this inhibition. A potential problem would arise if a protease contaminated the DS-PG preparations,
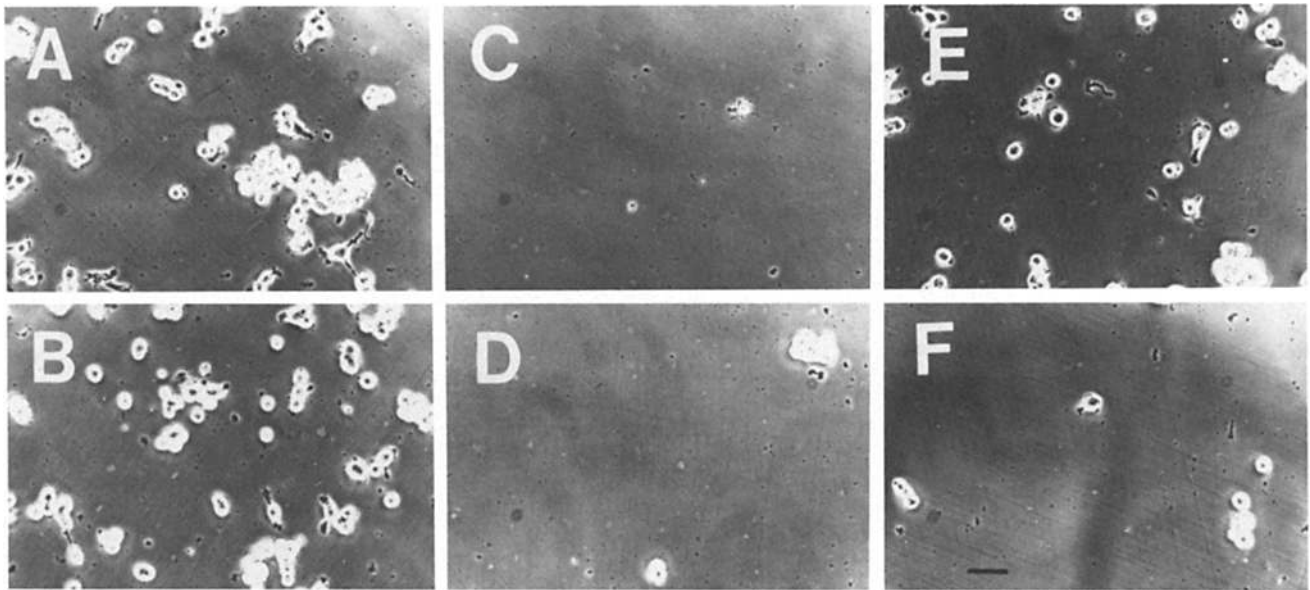
Figure 6. DS-PGs and adherence on 120K CBF of plasma fibronectin. Cluster dish wells were coated for 1 h at 37°C with 20 μg/ml of the 120K CBF generated by chymotrypsin digestion of human plasma fibronectin and purified by affinity chromatography (Materials and Methods). After rinsing the wells of excess CBF, the following solutions were added for a 1-h incubation at 37°C. (*A*) Core protein of DS-PG (250 μg/ml) plus BSA (250 μg/ml), followed by rinsing of the well; (*B*) dermatan sulfate chains of DS-PG (250 μg/ml) plus BSA (250 μg/ml), followed by rinsing of the well; (*C*) DS-PGs (50 μg/ml) plus BSA (250 μg/ml) and CBF (100 μg/ml), followed by rinsing of the well and re-addition of CBF (100 μg/ml); (*D*) DS-PGs (5 μg/ml) plus BSA (250 μg/ml); followed by rinsing of the well; (*E*) CS/KS-PG (300 μg/ml) plus BSA (250 μg/ml) which was left in the well throughout cell attachment period. In the case of *F*, DS-PGs (100 μg/ml) plus BSA (250 μg/ml) were used to treat the substratum for only 5 min before rinsing of the well. 3T3 cells were then inoculated in BSA-containing adhesion medium. After 4 h, adherent cells were fixed with glutaraldehyde and photographed. Bar, 40 μm.

resulting in destruction of the CBF on the substratum. This appears not to be the case since BSA added at high concentrations throughout the experiment could not "neutralize" the activity; pretreating the DS-PG with a multitude of protease inhibitors (EDTA, *N*-ethylmaleimide, pepstatin, phenylmethylsulfonyl fluoride, benzamidine hydrochloride, 6-aminocaproic acid) was also without effect. In addition, the inhibition could also be effected by high concentrations of heparin chains (>500 μg/ml), consistent with the much weaker activ-

*Table II. Quantitation of Inhibition of Attachment on CBF by DS-PG**

Substratum	Time point	Unattached cells
	min	*cpm*
CBF	15	330 ± 12
	60	175 ± 8
	120	170 ± 5
CBF + CS/KS-PG	15	490 ± 9
	60	280 ± 8
	120	310 ± 15
CBF + DS-PG	15	850 ± 11
	60	872 ± 7
	120	845 ± 23

* Wells coated with CBF alone (20 μg/ml) or a mixture of CBF (20 μg/ml) and CS/KS-PG (300 μg/ml) or DS-PG (100 μg/ml) as indicated received [³H]thymidine-radiolabeled BALB/c 3T3 cells. At the indicated time points (in minutes), the radioactivity in triplicate wells of the unattached cells was quantitated as described in Materials and Methods: at *t* = 0, this value was 980 cpm ± 16.

ity of univalent binding chains as demonstrated previously (30). PF4 (100 μg/ml) when pre-mixed with DS-PG (50 μg/ml) before treating the CBF substratum completely "neutralized" the inhibitory activity of the proteoglycan (data not shown). All of these data taken together indicate that the 120K CBF contains a cryptic GAG-binding domain that becomes active when the fragment is bound to the substratum and that binding to this domain by DS-PGs (but not CS/KS-PG) interferes with the cell-binding activity of the molecule.

The properties of this cryptic domain were explored further by using a series of fragments of plasma or cellular fibronectin from thermolysin digests as previously purified (4, 7). Using cellular fibronectin as the starting material, fragments of 44 and 47 kD (f44+47) contain the COOH-terminal heparin-binding domain and the so-called "extra domain" (ED$_a$) sequence characteristic of cellular fibronectins but no RGDS-dependent cell-binding domain. As shown in Fig. 7 *A*, 3T3 cells adhere and spread partially on these fragments and these responses are completely inhibited by the DS-PGs as would be expected by their binding to the available heparin-binding domain (Fig. 7 *B*). Of particular interest were 3T3 responses on f110 generated from both the α and β chains of plasma fibronectin, since this fragment contains RGDS-dependent cell-binding activity but no heparin-binding activity when assayed in solution; 3T3 cells attach and spread even more effectively on f110 (Fig. 7 *C*) than on f44+47 (Fig. 7 *A*) and, in agreement with the CBF experiments above, spreading is completely inhibited by DS-PGs and attachment is less than 30% of the control value (Fig. 7 *D*). Similar results were observed on f145 (Fig. 7, *E* and *F*), which contains the RGDS-dependent cell-binding and
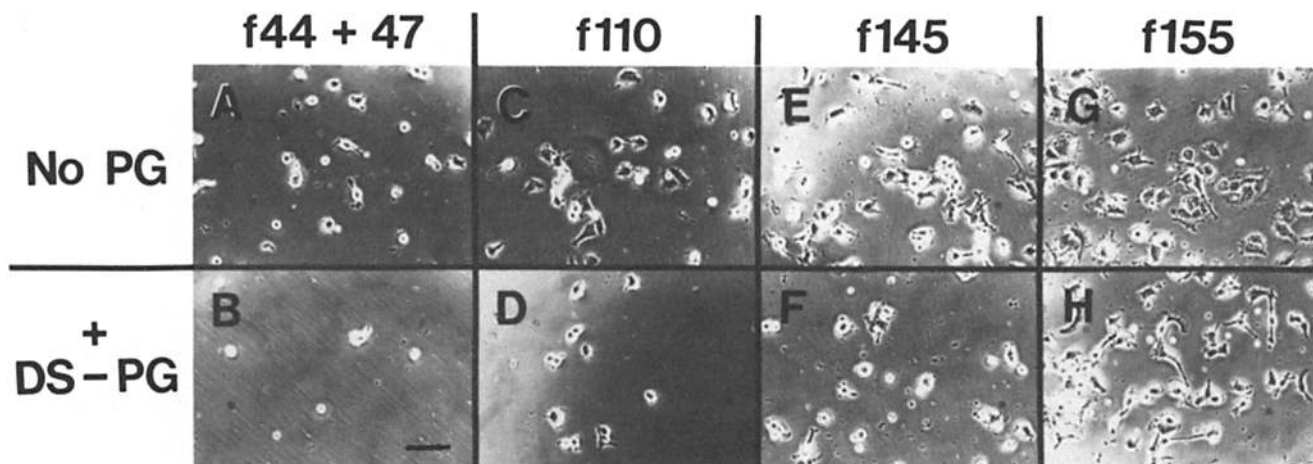
Figure 7. DS-PGs and adherence on various thermolysin-generated fragments of plasma or cellular human fibronectin. Cluster dish wells were coated for 1 h at 37°C with 20 μg/ml of purified fragments generated with thermolysin digestion and subsequent affinity chromatography as described in Materials and Methods and references 4 and 7. (*A* and *B*) f44+47 from cellular fibronectin which contain the COOH-terminal heparin-binding domain but no RGDS-dependent cell-binding domain; (*C* and *D*) f110 from plasma fibronectin which contains the RGDS-dependent cell-binding domain but no heparin-binding domain when assayed as a soluble protein; (*E* and *F*) f145 from the α chain of plasma fibronectin which contains the RGDS-dependent cell-binding domain and the COOH-terminal heparin-binding domain; and (*G* and *H*) f155 from the β chain of plasma fibronectin which contains the RGDS-dependent cell-binding domain and the COOH-terminal heparin-binding domain, as well as an additional type III homology unit. BSA was then added to wells to 250 μg/ml, as well as 100 μg/ml DS-PGs to wells *B, D, F,* and *H* (or No PG in wells *A, C, E,* and *G* as controls) for an additional 1-h incubation. 3T3 cells were inoculated into wells. At 4 h of exposure to the substratum, adherent cells were fixed with glutaraldehyde and photographed. Bar, 60 μm.

COOH-terminal heparin-binding activities of the α subunit of pFN (and some IIICS sequence at the COOH-terminus of this fragment). However, a similar fragment from the β subunit of pFN (f155 in Fig. 7, *G* and *H*) with an additional type III homology unit is much more resistant to the effects of the DS-PG. Also, PF4 could "neutralize" the inhibitory activity of DS-PGs on these fragments and inhibition was also effected by heparin at concentrations >500 μg/ml (data not shown). These results reveal that the cryptic GAG-binding domain must be located close to the cell-binding domain and is extremely sensitive to the conformation of the protein sequence that lies between the cell-binding and COOH-terminal heparin-binding domains of the molecule. Identical results were obtained when glass substrata were used in place of plastic substrata; however, cell spreading on 120K CBF, f110, and f145 on glass was more pronounced than on plastic.

Discussion

These experiments demonstrate that bovine articular cartilage DS-PGs are effective inhibitors of attachment and spreading of BALB/c 3T3 cells on a plasma fibronectin–coated matrix. By comparison with previous studies (32, 48) and the results of this study using bovine cartilage CS/KS-PG, the DS-PGs are much more potent inhibitors of these processes. This probably reflects the more effective and complete binding of the DS-PGs to pFN, as demonstrated in affinity chromatography experiments of soluble-state molecules or in evaluation of PG binding to pFN on the substratum by ELISA methodologies, than the binding of CS/KS-PG. The inhibition by these DS-PGs is comparable to that observed for a rat yolk sac CS-PG with the use of L2 tumor cells adhering to fibronectin or collagen matrices, as reported by Brennan et al. (5).

A sizable body of evidence indicates that the mechanism of inhibition of adhesion by the DS-PGs requires its binding to the GAG-binding domains on substratum-bound pFN, thereby interfering with pFN's binding to its multiple cell surface determinants (36, 37, 68). This evidence would include (*a*) comparable inhibition by both proteoglycans on PF4 substrata in contrast to the greater specificity for DS-PGs using pFN substrata; (*b*) persistent inhibition when the pFN substratum is post-adsorbed with DS-PGs and rinsed free of excess PG; (*c*) correlation of the affinity chromatography studies with the potency of inhibition by the two PG classes; (*d*) quantitation of PG binding to pFN on the substratum by ELISA; and (*e*) the absence of inhibition when cells are treated in suspension with high concentrations of DS-PGs. The question then arises as to how the binding of DS-PGs to the GAG-binding domains of pFN, binding processes which would be expected to directly interfere with binding to HSPGs on the surface of the cell (14, 30, 33, 36, 37, 68), can additionally interfere with the binding of pFN to the 140-kD glycoprotein receptor (6, 9, 21, 47) or to a possible second receptor for fibronectin (1, 39, 61, 65). As discussed by Brennan et al. (5), there are probably two possible explanations—(*a*) the binding by DS-PGs sterically interferes with the RGDS sequence of pFN's binding to the 140-kD glycoprotein (as well as the second receptor), or (*b*) the binding of the PGs changes the conformation of the protein such that its association constant for receptor decreases markedly. Evidence for a conformational change upon heparan sulfate binding has been recently reported for human pFN (42, 60). Many additional experiments will be required to resolve these possibilities.

In light of the increasing evidence for association of DS-PGs with collagen fibrils and their influence on fibrillogenesis (8, 20, 55, 64), inhibition of cell responses on colla-

gen/pFN matrices was tested with the two PG classes. There was a complete lack of inhibition of attachment and spreading by either cell type on type I collagen matrices, either when the DS-PGs or CS/KS-PG were previously adsorbed to the collagen alone (or to collagen/pFN complexes for short or long periods of time) or when the PG was added to the collagen/pFN matrix at the same time that cells were added. A parallel finding was the relative lack of inhibition by DS-PGs of bovine dermal fibroblast adherence and spreading on pFN-coated substrata; this is consistent with the ability of these cells to make their own endogenous collagen matrix, thereby providing additional mechanisms of adhesion for these cells (1, 24a, 39, 61, 65). That cycloheximide pretreatment makes these dermal cells more sensitive to PG inhibition supports this argument. These results stand in contrast to the common inhibition of rat yolk sac tumor cell adherence to either FN or collagen matrices using the rat yolk sac CS-PG as the inhibitory ligand (5). This would suggest that the mechanisms of association of CS-PGs or DS-PGs with collagen matrices are significantly different and that there are differing influences on cell responses. Since fibronectin complexed to collagen has been shown previously to bind GAGs with the same specificity (5, 52, 69), it is unlikely that these results could be explained by alteration of binding of the proteoglycans once the fibronectin has complexed with the collagen. However, these results suggest that (a) there are fibronectin-independent mechanisms by which DS-PGs bind to the collagen lattice and (b) there are collagen-dependent, fibronectin-independent mechanisms for adherence of these cells as shown by previous studies (26, 31, 38, 39). Furthermore, these processes apply to 3T3 cells, which fail to make a collagen matrix at their undersurfaces, as well as to bovine dermal fibroblasts which do make such a matrix. All of these results suggest that inhibition of cell attachment and/or spreading responses on fibronectin matrices is selectively dependent upon the nature of the matrix in tissues (39, 59). It is known that fibroblast adherence and spreading on collagen matrices results in their eventual contraction in response to cytoskeletal rearrangements (2, 58) and it will be interesting to evaluate the possible modulation by DS-PG binding and function during collagen contraction processes.

Of special interest was the discovery that certain fragments of the fibronectin molecule that contain no apparent GAG-binding activity in solution do expose a cryptic GAG-binding domain when bound to the substratum, thereby becoming highly sensitive to the inhibitory effects of the proteoglycans (or to GAG chains at very high concentrations). The most likely explanation for this finding is a change in conformation of the fragments when they bind to substrata, resulting in accessibility of this binding site to the proteoglycan and subsequent interference with the cell-binding activity by one or both mechanisms summarized above. In fact, DS-PG binds more effectively to CBF on the substratum when compared to pFN as determined by ELISA. Consistent with such a model, binding of human pFN to a number of artificial matrices has been shown to induce significant changes in its conformation (60) and activation of adhesion-promoting activity upon substratum binding has been reported for a chymotrypsin-generated 105-kD fragment of plasma fibronectin via the cell-binding activity of the fragment (53). Brennan et al. (5), using a similar 120-kD cell-binding frag-

ment of pFN, could not detect this inhibitory activity with the rat yolk sac CS-PG and the studies reported here were unable to detect any inhibition with the cartilage CS/KS-PG; these results argue for the specificity of the cryptic site for binding heparan sulfate/dermatan sulfate classes of GAG. Analyses of small fragments from subtilisin digests of human pFN provided initial evidence for a third heparin-binding domain between the collagen- and cell-binding domains of the molecule (24) and binding of thermolysin-generated fragments to heparin can be modulated by pH and Ca^{++} (57).

Of particular note in the studies here are differences in sensitivity of the two large cell-binding fragments purified from thermolysin digests by Zardi and his collaborators from the α and β subunits of pFN (4, 7). F145 from the α subunit was sensitive to inhibition with the DS-PGs while the f155 from the β subunit was relatively resistant. This would indicate that the protein sequence differences of these two fragments between the cell-binding and COOH-terminal heparin-binding domains (or the additional type III homology unit of f155 and small IIICS sequences that persist at the COOH terminus of f145) do not contain this cryptic activity but do regulate accessibility to it. Studies with multiple overlapping combinations of fragments will be required to precisely map this third GAG-binding activity in the fibronectin molecule. These experiments raise the interesting possibility that the ability of certain cells to proteolytically modify the fibronectin at their adhesive contacts (10), thereby generating cell-binding fragments similar to those used here, would become much more susceptible to DS-PGs in their adhesive and migratory activities.

These studies have revealed the versatility in the binding of the dermatan sulfate proteoglycans to the fibronectin molecule and subsequent effects on adhesive functions. The collagenous nature of the substratum, as well as the conformation of the fibronectin molecule itself as it binds to the extracellular matrix, provide critical determinants whether this class of proteoglycan is competent for influencing adhesive responses of many sorts. The availability of large amounts of purified DS-PGs and the construction of more complex, but biochemically defined, matrices now make many studies feasible.

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