Interaction of Small Dermatan Sulfate Proteoglycan from Fibroblasts with Fibronectin

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Abstract. Immunogold labeling was used to localize the core protein of small dermatan sulfate proteoglycan (DS-PG) on the surface of cultured human fibroblasts. At 4°C, DS-PG core protein was uniformly distributed over the cell surface. At 37°C, gold particles either became rearranged in form of clusters or remained associated with fibrils. Double-label immunocytochemistry indicated the co-distribution of DS-PG core protein and fibronectin in the fibrils. In an enzyme-linked immunosorbent assay, binding of DS-PG from fibroblast secretions and of its core protein to fibronectin occurred at pH 7.4 and at physiological ionic strength. Larger amounts of core protein than of intact proteoglycan could be bound. Fibronectin peptides containing either the heparin-binding domain near the COOH-terminal end or the heparin-binding NH_2 terminus were the only fragments interacting with DS-PG and core protein. Competition and replacement experiments with heparin and dermatan sulfate suggested the existence of adjacent binding sites for heparin and DS-PG core protein. It is hypothesized that heparan sulfate proteoglycans and DS-PG may competitively interact with fibronectin.

THE small dermatan sulfate proteoglycan (DS-PG)[1] from human fibroblast secretions is a typical member of a possibly ubiquitous family of interstitial proteoglycans that are characterized by the linkage of only a few galactosaminoglycan chains or of even only one chain to a protein core with an M_r of ~40,000–50,000 (21). The galactosaminoglycan chains may exhibit great differences in length and chemical composition. In cartilage, the chains are exclusively (22) or predominantly (41) of chondroitin sulfate-type but they contain a high proportion of L-iduronic acid in skin and sclera (8, 10). The small proteoglycan secreted by cultured human skin fibroblasts is a dermatan sulfate-type proteoglycan, too, since ~80% of its hexuronic acids are L-iduronic acid (23). The glycosaminoglycan chains exhibited a mean M_r of 37,000. From cloned cDNA a molecular weight of 36,319 for the mature core protein was calculated. The sequence includes three Ser-Gly dipeptides (26). In bovine DS-PG the single glycosaminoglycan chain is linked to the serine residue at position 4 (7). The two other Ser-Gly sequences may become substituted by a second chain in some of the molecules (2, 26). Additionally, either two or three asparagine-bound oligosaccharides are present (15). There exists a second, less well characterized species of small DS-PG, the core protein of which is unrelated to that of fibroblast DS-PG (41).

By ultrastructural studies DS-PGs have been shown to be located at the "d" band of type I collagen fibrils in unmineralized connective tissues (47, 48, 64). Tendon DS-PG and its core protein but not similar proteoglycans from cartilage or aorta inhibited fibrillogenesis of both type I and type II collagen (57). DS-PG produced by human skin fibroblasts bound specifically to collagenous fibers of the extracellular matrix of the cells (14). It is therefore apparent that DS-PG exerts one of its biological functions in the extracellular matrix by interacting with collagen fibers.

Indirect evidence raises the possibility that DS-PG is also associated with fibronectin. Immunofluorescence studies indicated that chondroitin sulfate and/or dermatan sulfate proteoglycans are components of the fibronectin-containing pericellular matrix fibers of human fibroblasts (20). Fibronectin added to the medium of cultured hamster fibroblasts could be chemically cross-linked to proteoglycans at the cell surface that contained galactosaminoglycan chains (35). Vice versa, gold-decorated proteoglycans were bound to surface-associated fibronectin fibrils of cultured arterial smooth muscle cells (59). In contrast, ferritin particles bound to chondroitin ABC lyase-degraded proteoglycan exhibited a distribution on cell surfaces quite different from that of fibronectin (19).

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1. *Abbreviation used in this paper*: DS-PG, small dermatan sulfate proteoglycan from human skin fibroblasts.

Fibronectin represents a group of high molecular glycoproteins present in extracellular matrices, basement membranes, and plasma. It consists of two disulfide-bonded polypeptides, the primary structure of which varies in interstitial and plasma fibronectins due to differential splicing of fibronectin pre-mRNA (25, 46). All fibronectin species exhibit pleiotropic functions, which are explained by the existence of several functional domains (see references 24, 56, 61–63 for review) and can be isolated by limited proteolysis. Two domains with high affinity and additional domains with lower affinity for heparin have been characterized (4, 17, 34, 38, 50–53). The high affinity domains are located at the NH_2 terminal and between the cell-binding and the COOH-terminal fibrin-binding domains. Presently it is controversial whether or not heparin and heparan sulfate are bound at physiological pH and at physiological ionic strength (28, 50, 53, 55). It has also been shown that isolated heparin-binding domains may interact with dermatan sulfate, chondroitin sulfate, and hyaluronate at low ionic strength (50). Other investigations, however, observed interactions in vitro between intact fibronectin and hyaluronate (29, 43, 63) or a chondroitin sulfate proteoglycan (33) under simulated physiological conditions.

Here a co-distribution of fibronectin and DS-PG core protein has been found in cultured fibroblasts by electron microscopic immunolocalization. Using an enzyme-linked immunosorbent assay, the interaction of DS-PG and of its core protein with fibronectin and fibronectin peptides was investigated.

Materials and Methods

Immunochemical Reagents

Rabbit antibodies against the DS-PG core protein were those used previously (15) after affinity purification (60). They did not react with epitopes shared with other proteoglycans, e.g., with the large glucuronic acid–rich proteoglycan from human fibroblasts. When fibroblasts were incubated for 14 h in the presence of [^{3}H]leucine (15) followed by chondroitinase ABC lyase digestion of the monolayer as described below, exclusively the core protein of the small DS-PG could be visualized upon subsequent immunoprecipitation, SDS PAGE, and fluorography (15). The production of monoclonal antibodies to DS-PG core protein (LN1, LN3, LN4) will be described in detail elsewhere. Briefly, BALB/c mice were immunized with partially purified DS-PG from fibroblast secretions. Lymph node and spleen cells were fused with the myeloma cell line X63-Ag8.653. Hybrid cells responsible for the production of anti–DS-PG antibodies were identified by an ELISA screening as described below and subcloned several times by the method of limiting dilution. Established clones were grown in KC-2000 medium (KC Biological, Raunheim, FRG). Antibodies were purified (6) by chromatography on CM-Trisacryl (LKB Instrument, Gräfelfing, FRG). They could be distinguished by a different size of their heavy chains, but they all belonged to subclass IgG_1. LN1 recognized an epitope near the glycosaminoglycan attachment site, whereas LN3 and LN4 bound to a carbohydrate-free peptide of M_r 17,000. Epitopes were localized by immunochemical staining of peptides obtained by limited digestion of DS-PG and DS-PG core protein, respectively, with staphylococcal serine proteinase V8 (36) followed by SDS PAGE (18, 27) and electrophoretic transfer to nitrocellulose paper (3). Rabbit antiserum to human fibronectin was purchased from Calbiochem (Frankfurt, FRG), goat anti-rabbit IgG from Bio-Rad (Munich, FRG), and rabbit anti-mouse IgG, peroxidase conjugate, from Sigma (Deisenhofen, FRG).

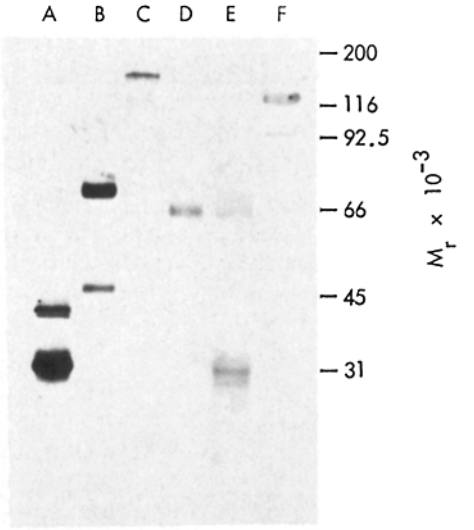


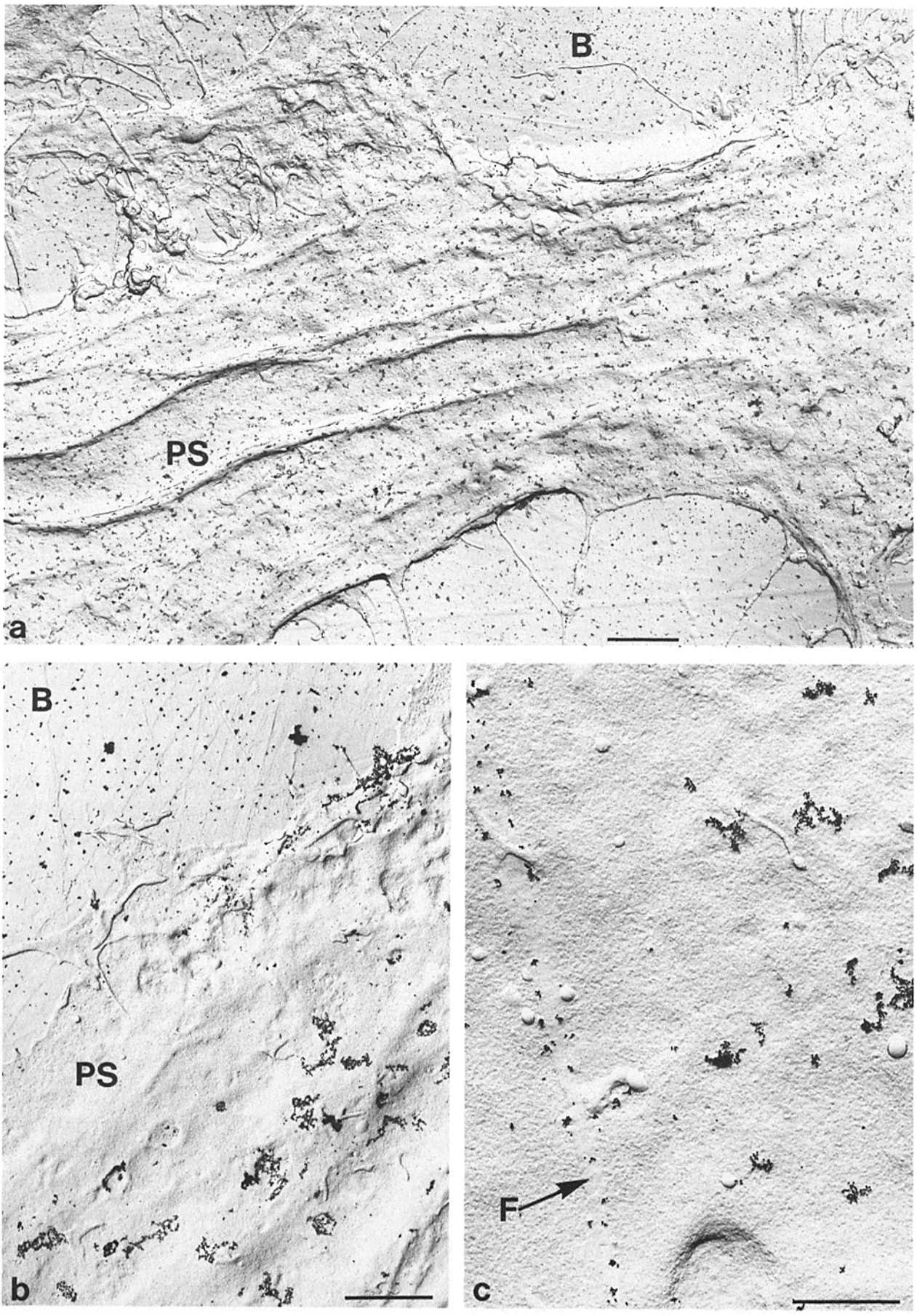
Figure 1. Silver stain of fibronectin peptides after polyacrylamide gel electrophoresis in the presence of SDS (total acrylamide concentration 12.5%). Lane *A*, peptide Tc 31; lane *B*, peptide Ca 70; lane *C*, peptide Ca 140; lane *D*, peptide Tc 66; lane *E*, peptide Tc 33; lane *F*, peptide Cht 125. See text and Fig. 8 for further details.

Materials

DS-PG was prepared from spent media of human skin fibroblasts cultured in serum-free media as described previously (15). It was stored frozen in 0.1% (vol/vol) Triton X-100 at concentrations greater than 0.5 mg/ml. Immediately before use core protein was obtained by incubating the stock solution of DS-PG with chondroitin ABC lyase (EC 4.2.2.4; Seikagaku Kogyo, Tokyo, Japan; 1 U/ml) in enriched Tris/acetate buffer (45) for 2 h at 37°C in the presence of 0.1 M 6-aminohexanoic acid, 10 mM EDTA, 5 mM benzamidinium hydrochloride, and 10 mM *N*-ethylmaleimide.

Fibronectin from human serum was obtained from Behring-Werke (Marburg, FRG) and purified to 97% purity by affinity chromatography on a gelatin-Sepharose 4B (Pharmacia, Uppsala, Sweden) column as described (44). Purified material was dialyzed against 50 mM Tris/HCl buffer, pH 7.5, containing 3 mM NaN_3, and stored at 4°C. Fibronectin peptides were prepared according to published procedures (37, 38, 49, 54). Shortly, an NH_2-terminal M_r 70,000 heparin-binding peptide, Ca 70, and an adjacent M_r 40,000 peptide, Ca 140, representing the central part of the fibronectin subunit chains, were isolated from an early digest performed with cathepsin D (EC 3.4.23.5; Sigma). Chromatography on a heparin-Sepharose 4B column yielded a fraction of medium binding strength containing the two fragments, which were separated by gel filtration on Ultrogel AcA 44 (LKB, Stockholm). Ca 70 was further purified on gelatin-Sepharose 4B. The preparation contained additionally a gelatin-binding 42-kD fragment generated by further degradation. A stronger binding fraction from the heparin-Sepharose step contained another M_r 140,000 fragment comprising two disulfide-linked peptide chains from the COOH-terminus of each subunit. It was treated with trypsin (EC 3.4.21.4; Sigma) before rechromatography on heparin-Sepharose 4B. A nonbinding fragment, Tc 31, located close to the COOH-terminus of the longer subunit chain of plasma fibronectin, was collected and the heparin-binding fragments Tc 66 and Tc 33 were eluted and separated from each other by gel filtration on Ultrogel AcA 44. Tc 31

Figure 2. Surface replica of a fibroblast treated with chondroitin ABC lyase before incubation with rabbit anti-human–DS-PG antibodies followed by gold-labeled (12 nm) goat anti-rabbit IgG. (*a*) The gold particles are randomly distributed both on the plasma membrane surface (*PS*) and the bottom of the culture dish (*B*) at 4°C. (*b* and *c*) When chondroitin ABC lyase–treated cells incubated with rabbit anti-human–DS-PG antibodies and gold-labeled (12 nm) anti-rabbit IgG at 4°C were kept at 37°C for 1 h, the gold label on the plasma membrane surface (*PS*) was observed primarily in clusters, whereas gold particles on the bottom (*B*) of the culture dish (*b*) and those associated with fibrils (*F*) on the cell surface did not participate in the clustering process (*c*). Bars: (*a* and *b*) 2 μm; (*c*) 1 μm.


B
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contains minor amounts of a 38-kD fragment most likely produced by an alternative cleavage site. The slightly heterogeneous fragment Tc 33 represents the main heparin-binding domain of the longer subunit chain, whereas Tc 66 contains the heparin-binding and the subsequent nonbinding domain of the shorter subunit chain. Digestion of fibronectin with chymotrypsin (EC 3.4.21.1; Sigma) gave rise to a peptide Cht 125, which differed from Ca 140 by lacking a basic NH_2-terminal domain. It was isolated by chromatography of the digest on heparin-Sepharose 4B followed by Ultrogel AcA 44. The positions of the peptides are shown schematically in Fig. 8. The purity of the fragments is indicated in Fig. 1.

Enzyme-linked Immunosorbent Assay

The interaction of fibronectin and of peptides derived therefrom with DS-PG and DS-PG core protein was studied by a modification of the procedure of Engvall and Perlmann (12). Generally, 50 μl of ligand solutions were allowed to react for 90 min at 37°C. Washing steps were performed at ambient temperature using 200 μl of PBS, pH 7.4, containing 0.1% (vol/vol) of Tween 20 (buffer A). Microtiter plates (Nunc-immuno plates II, Nunc, Wiesbaden, FRG) were coated with fibronectin or fibronectin peptides (10 μg/ml) dissolved in 0.1 M Tris/HCl buffer, pH 7.4, containing 50 mM NaCl (buffer B). After three washing cycles unspecific binding was quenched by incubation with 3% (wt/vol) BSA in buffer A, followed by four washing steps. Increasing concentrations of DS-PG or of its core protein, dissolved in buffer B, were then added. The plates were washed again three times before incubation with one or with an equimolar mixture of the monoclonal antibodies against the DS-PG core protein (125 μg/ml). To reduce unspecific background, washing steps were followed by treatment with 3% BSA in buffer A. The wells were rinsed four times and incubated with second antibody, rabbit anti-mouse IgG, conjugated to peroxidase (dilution 1:1,000 with buffer A). The wells were washed five times and incubated with substrate solution (200 μl of 1 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) and 37 μM H_2O_2 in 50 mM sodium citrate, pH 4.0). Color development was stopped by adding 50 μl 10 mM NaN_3, and the absorbance at 410 nm was measured with a Multiscan photometer (Flow Laboratories, Bonn, FRG). Testing for monoclonal antibodies was performed analogously. The wells were coated with DS-PG core protein (5 μg/ml). Blocking of reacting sites was performed by incubation with 1% (wt/vol) gelatin in 0.1% SDS.

Electron Microscopy and Immunolocalization

Gold particles of ~7-nm diam were prepared by reduction of chloroauric acid with tannic acid as described (32) and saturated with protein A (Sigma) according to Roth et al. (42). Gold particles of 12-nm diam were prepared by reduction of chloroauric acid with sodium citrate (13) and coated with goat anti-rabbit IgG as described (11) or with protein A. The stability of the conjugates was tested by addition of 10% NaCl. All preparations were routinely examined by negative staining before use and discarded if unsatisfactory. Negative staining was performed by placing a Formvar-coated grid for 1–5 min on a drop of a diluted sample of the gold conjugate and then transferring it to a drop of 2% (wt/vol) uranyl acetate, pH 3.5, for 1–5 min.

Indirect immunocytochemistry was used to detect DS-PG and fibronectin on the surface of cultured human fibroblasts. Cells from healthy juvenile donors were maintained in culture as described (5) and used after the fifth to twelfth passage. 1 d after plating the cells in 5.5-cm^2 Falcon plastic dishes, medium was removed, and the cell layer was incubated for 30 min at 37°C with 50 mU chondroitin ABC lyase (Seikagaku Kogyo, Tokyo, Japan) in 0.7 ml of Hanks' balanced salt solution containing 1% BSA. All subsequent steps were performed at 4°C. Nonspecific background labeling was minimized by preincubating the cells in 1% BSA/PBS, pH 7.4, for 15–30 min. Then, rabbit anti-human DS-PG antibodies (1 μg/ml) or rabbit anti-human fibronectin antibodies (22 μg/ml), both in 1% BSA/PBS, were allowed to react for 60 min before the cells were washed three times for 5 min each with 1% BSA/PBS. For visualization of DS-PG core protein, cells were incubated for 30–60 min with gold-labeled goat anti-rabbit IgG (1:30 dilution with 1% BSA/PBS). An analogous incubation with gold-labeled protein A was done for staining of fibronectin. In double-labeling experiments the reactions for immunochemical labeling of DS-PG core protein were performed first. The protein A binding sites of the goat anti-rabbit IgG-gold complexes were then blocked by incubating the cells for 30 min with uncoupled protein A (4 μg/ml PBS) before fibronectin labeling. Labeling was terminated by three 5-min washes with PBS. The cells were then fixed for 30 min in the culture dish with a formaldehyde/glutaraldehyde fixative and subjected to surface replication. In all experimental series, the specificity of the immunostaining was controlled by omitting one or both of the primary antibodies. In case of staining of DS-PG core protein, additional studies were performed by warming up immunogold-labeled cells to 37°C for 15–60 min.

For surface replication, fixed fibroblasts were dehydrated and subjected to critical point or air drying. The bottom of the dish with the attached cells was cut in 1-cm^2 pieces using a soldering iron. Platinum-carbon replicas of the surface of the cell monolayers were made in a Balzers BA 300 freeze-etching apparatus (Balzers, Lichtenstein), equipped with an electron gun evaporator and a quartz crystal thickness monitor. Replicas were obtained by shadowing the cell surface with platinum-carbon at an angle of 38° followed with carbon at 90°.

For post-embedding immunocytochemical labeling of fibronectin, the cells were fixed for 30 min at 4°C in 3% (vol/vol) paraformaldehyde and 3% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The specimens were washed twice with cacodylate buffer, dehydrated in an ascending series of dimethyl formamide, and embedded in Lowicryl K4M as described (58). All subsequent steps were carried out at ambient temperature. The grids were sequentially placed with their section side down on 10-μl droplets placed on wax sheets. Pretreatment with 0.02 M glycine in PBS, pH 8.2, and/or with 1% BSA/PBS, pH 7.4, for 15 min followed by two 5-min washes with PBS, pH 7.4, was performed to block nonspecific binding sites and to quench aldehydes present on the section surface. Reactions with primary antibodies and gold-labeled protein A were performed analogously to surface staining. Labeled grids were finally rinsed with water and stained with aqueous uranyl acetate and lead citrate. All electron microscopic studies were performed with a Philips EM 201 at 60 kV.

Other Methods

Uronic acids (1) and protein (30) were quantitated as described. 1.3 μmol of hexuronic acid is equivalent to 1 mg of DS-PG. Polyacrylamide gel electrophoresis in the presence of SDS followed by silver staining (31) was performed by a modification (18) of the procedure of Laemmli (27).

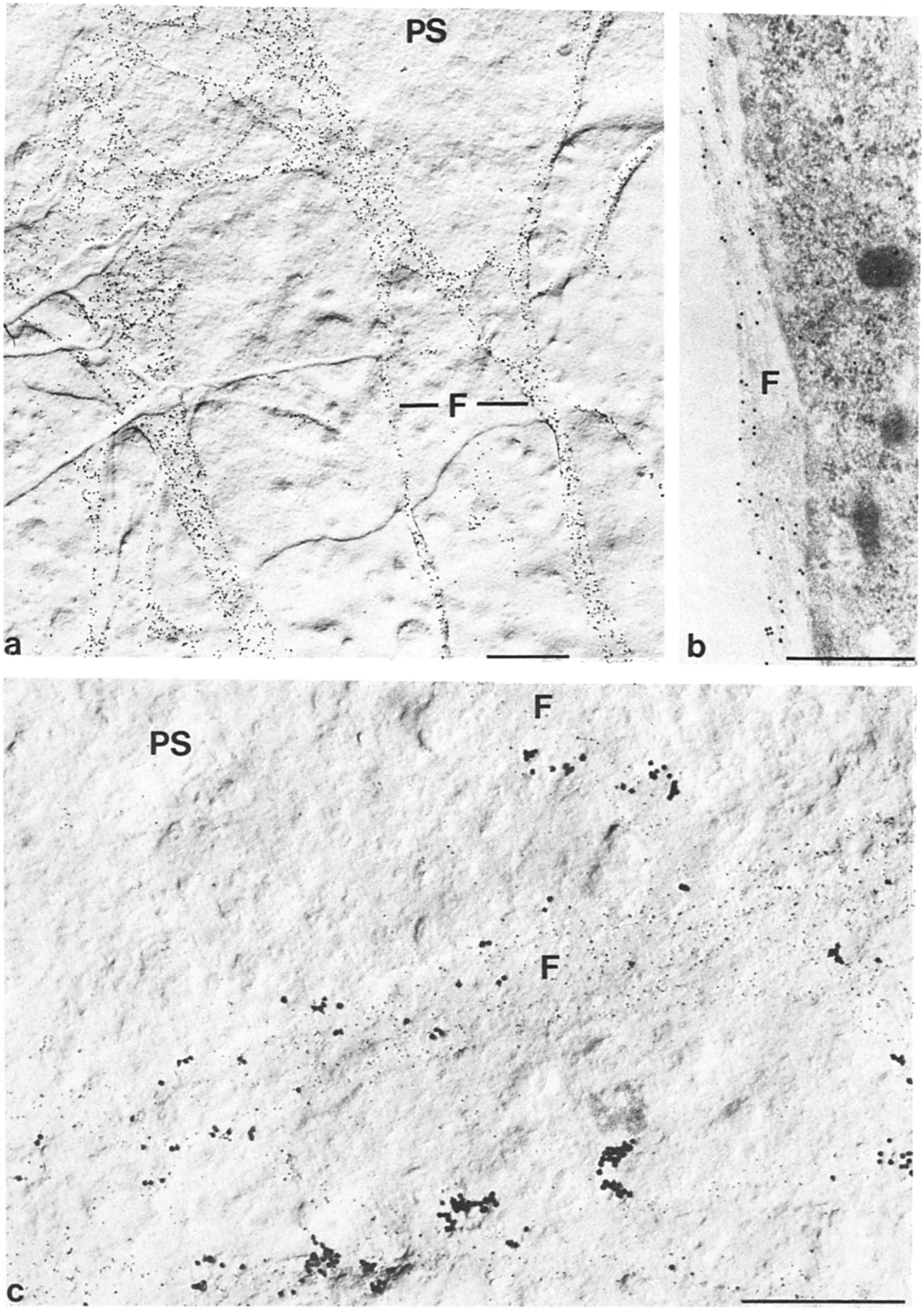
Results

Immunolocalization of DS-PG and Fibronectin

With the aid of platinum-carbon replicas, large continuous areas of the plasma membrane of cultured fibroblasts can be visualized in electron micrographs. The cell surface is smooth, interrupted only by few microvilli or fibrillar structures, whereas numerous peripheral processes of variable length stretch from the cell border.

When intact unfixed cells were treated with chondroitin ABC lyase followed by immunostaining of DS-PG core protein at 4°C, an intense labeling by gold particles could be observed. The gold particles occur singly, form small aggregates, or are aligned in short rows of three to five particles

Figure 3. (*a*) Surface replica of a fibroblast incubated with rabbit anti-human fibronectin antibodies followed by gold-labeled (12 nm) protein A. The gold label is exclusively associated with a fibrillar network (*F*) on the plasma membrane surface (*PS*). (*b*) Thin section of a Lowicryl K4M embedded fibroblast incubated with rabbit anti-human fibronectin antibodies followed by gold-labeled (12 nm) protein A. The gold particles are found over extracellular fibrils (*F*) confirming that fibronectin is a major component of the extracellular matrix. (*c*) Surface replica of a fibroblast sequentially labeled for DS-PG core protein by rabbit anti-human-DS-PG antibodies and gold-labeled goat anti-rabbit antibodies and for fibronectin by rabbit anti-human fibronectin antibodies and gold-labeled protein A. A co-distribution of fibronectin (7-nm-diam gold) with DS-PG core protein (12-nm-diam gold) can be observed. *PS*, plasma membrane surface. *F*, fibril. Bars: (*a*) 1 μm; (*b* and *c*) 0.5 μm.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and are randomly distributed both on the cell surface and on the bottom of the culture dish (Fig. 2 *a*). In controls in which the primary antibodies were omitted, only a negligible amount of label was found.

When cells incubated with primary antibodies and gold-labeled anti-rabbit IgG at 4°C were warmed up to 37°C, the gold particles were rearranged into clusters within 15 min (Fig. 2 *b*). However, gold particles associated with fibrils on the cell surface (and the particles on the bottom of the culture dish) did not participate in the clustering process (Fig. 2 *c*).

Fibroblasts incubated with anti-fibronectin antibodies followed by gold-labeled protein A exhibited a fibrillar labeling pattern in surface replicas. As shown in Fig. 3 *a*, immunoreactivity was associated exclusively with a fibrillar network. The bottom of the culture dish exhibited only a small amount of label, whereas the peripheral processes traversing the bottom could be intensively stained. Post-embedding immunocytochemistry of thin sections also confirmed the finding that fibronectin is a major component of the extracellular fibrillar matrix (Fig. 3 *b*).

Double-label immunocytochemistry was used to study the distribution of DS-PG core protein in relation to the distribution of fibronectin. A co-distribution of fibronectin with DS-PG core protein was found (Fig. 3 *c*). However, DS-PG of fibrillar localization tended to exhibit a more punctate labeling than that of fibronectin.

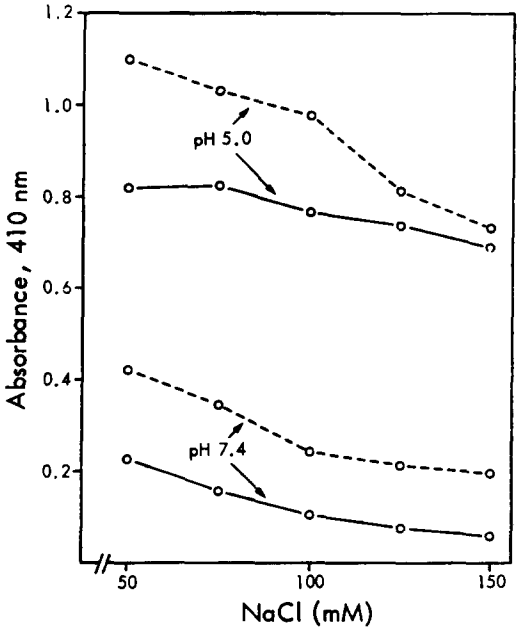

Figure 5. Ionic strength- and pH-dependent interaction between fibronectin and either DS-PG (o——o) or its core protein (o - - - o). The DS-PG concentration was 10 μg/ml.

In Vitro Incubation of Fibronectin with DS-PG and Its Core Protein

Enzyme-linked immunosorbent assays were performed to investigate the interaction of fibronectin with DS-PG in more detail. Advantage was taken by the availability of monoclonal antibodies that specifically recognize epitopes on the core protein of DS-PG. It is shown in Fig. 4 that fibronectin immobilized on the bottom of polystyrene wells can bind DS-PG as well as its core protein in a saturable manner. The enzyme-linked second antibody produced greater absorbance when core protein instead of native DS-PG was used as a ligand. Use of [^{3}H]leucine-labeled DS-PG and core protein, respectively, as a ligand followed by solubilization of bound material with papain (8 mg of enzyme in 200 μl of 0.1 M sodium acetate, pH 6.5, containing 5 mM EDTA and 5 mM cysteine, 2 h at 37°C) revealed that up to 2.2 ng of DS-PG and up to a 2.5-fold equivalent of core protein could be bound per well. Comparing these values with the absorbance obtained in the standard assay it was calculated that in the case of fibronectin-bound ligands the monoclonal antibodies recognized native DS-PG 1.5 times better than the core protein.

The interaction between fibronectin and DS-PG was dependent on pH and ionic strength (Fig. 5). Optimal binding of both proteoglycan and core protein occurred at pH 5.0. At pH 8.0 binding of core protein was almost negligible, whereas DS-PG reacted similarly as at pH 7.4 (result not shown). Binding of DS-PG and of its core protein to fibronectin was facilitated by low ionic strength but still occurred at physiological ionic strength.

As shown above, greater quantities of core protein than of intact DS-PG could be bound per fibronectin-coated well. This could be explained by the existence of additional binding sites for glycosaminoglycan-free core protein. Preincubation of fibronectin with native DS-PG to saturate all DS-

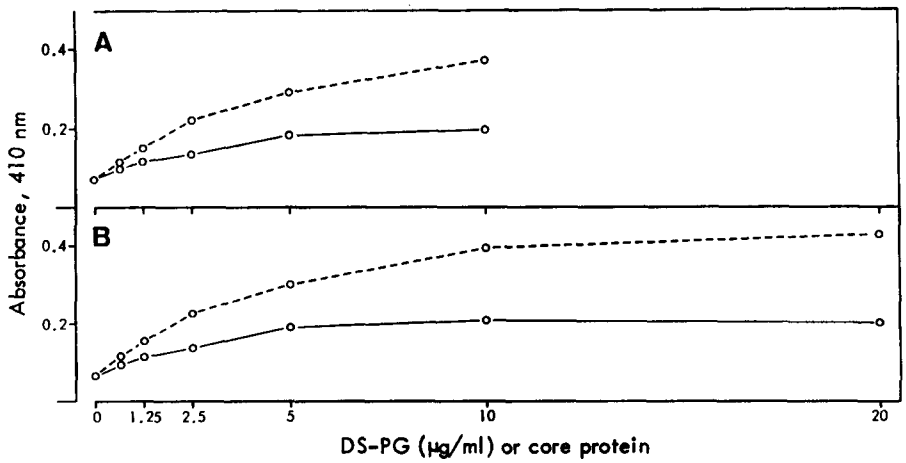

Figure 4. Enzyme-linked immunosorbent assay using fibronectin-coated wells and either DS-PG (o——o) or equivalent amounts of its core protein (o - - - o) as ligands. In *A*, the wells were coated with 10 μg/ml, in *B* with 20 μg/ml of fibronectin.

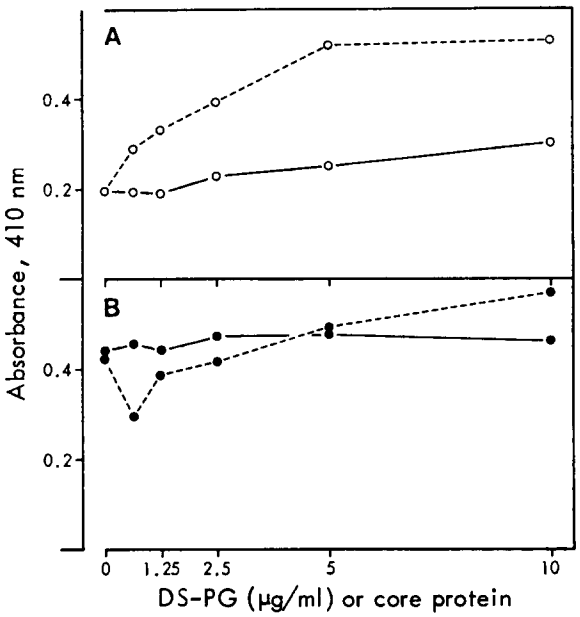

Figure 6. Binding capacity of fibronectin for DS-PG and its core protein. In *A*, fibronectin-coated wells were preincubated with DS-PG (10 μg/ml) before a second incubation with the proteoglycan (o——o) or equivalent amounts of its core protein (o - - - o). In *B*, preincubation was with core protein equivalent to 10 μg/ml of DS-PG followed by incubation with proteoglycan (●——●) or core protein (● - - - ●).

PG binding sites followed by a second incubation with core protein made the binding of additional core protein (Fig. 6) possible. In the converse experiment, preincubation with core protein followed by a second incubation with DS-PG, no additional binding was observed. The interaction between core protein and fibronectin seemed at variance with the assumption of a direct involvement of one of the heparin-binding domains of the fibronectin molecule in core protein binding. Preincubation of fibronectin with either heparin or protein-free dermatan sulfate exhibited no effect on subsequent DS-PG or core protein binding. However, when DS-PG or core protein were incubated together with protein-free dermatan sulfate or heparin, binding of the proteoglycan as well as of the core protein was strongly inhibited, heparin being more inhibitory than dermatan sulfate (Fig. 7). This effect was not due to an unspecific effect of the polyanions since in the presence of chondroitin-4-sulfate only 10% inhibition could be observed. Incubation with the glycosaminoglycans (100 μg/ml) after an interaction between fibronectin and either DS-PG or core protein had been allowed to occur, led to a replacement of maximally 25% of bound DS-PG and 15% of bound core protein. No further desorption was observed when the concentration of heparin or dermatan sulfate was increased to 500 μg/ml.

In Vitro Incubation of Fibronectin Peptides with DS-PG and Its Core Protein

For further investigations of the fibronectin domains that interact with DS-PG and its core protein, various fibronectin peptides were prepared, the binding properties of which have been characterized previously (37, 38, 49, 54). It is shown in Fig. 8 that fibronectin peptides that contain the heparin-binding domain near the COOH-terminal end of the molecule exhibit saturable binding properties for DS-PG and its core protein. Peptides containing the NH_2-terminal heparin-binding domain also interacted with these ligands albeit less efficiently. Almost no interaction was found when peptides containing the cell- and collagen-binding domains were tested.

Discussion

Indirect immunocytochemistry at the electron microscopic level established the dual localization of DS-PG core protein on the surface of cultured human skin fibroblasts. Immune reactive material was either able to form clusters at 37°C or was associated with fibrils and did not participate in the clustering process. At least some of the mobile core protein is considered to be destined for receptor-mediated endocytosis because endocytosis of DS-PG involves recognition of its protein moiety (16). Double-label immunocytochemistry indicated that immobile core protein was associated with fibronectin fibrils. DS-PG core protein, however, was not smoothly distributed along the fibrils but exhibited a punctate staining pattern.

Enzyme-linked immunosorbent assays supported the suggestion of an interaction of fibronectin with intact DS-PG as well as its glycosaminoglycan-free core protein. Larger quantities of core protein than of DS-PG could be bound to a given amount of fibronectin. However, none of the fibronectin peptides tested exhibited binding properties exclusively for core protein. This might indicate that more core

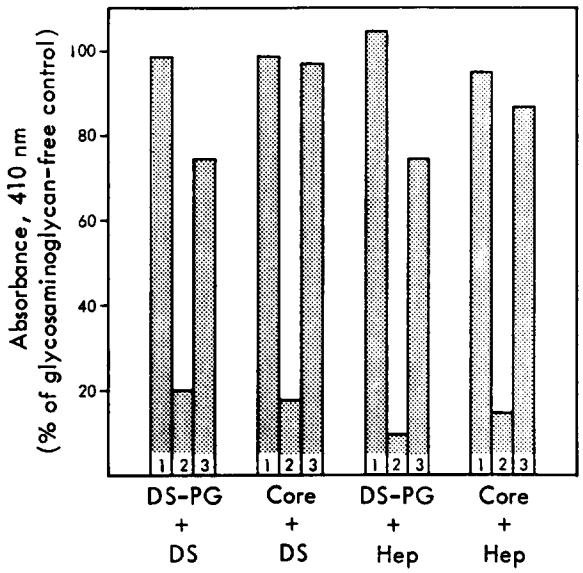

Figure 7. Influence of heparin and dermatan sulfate on binding of DS-PG or its core protein to fibronectin. All values are referred to the difference in absorbance at 410 nm between incubations with DS-PG or core protein without any additions of free glycosaminoglycans and the blank value. In assay 1, fibronectin-coated wells were incubated with protein-free glycosaminoglycans (100 μg/ml) for 90 min at 37°C before incubation with DS-PG (10 μg/ml) or an equivalent amount of its core protein. Assay 2 was performed as assay 1 except that glycosaminoglycans were also present during incubation with DS-PG or core protein. In assay 3, incubation with glycosaminoglycans was performed under the conditions of assay 1 but after preincubation with DS-PG or core protein.

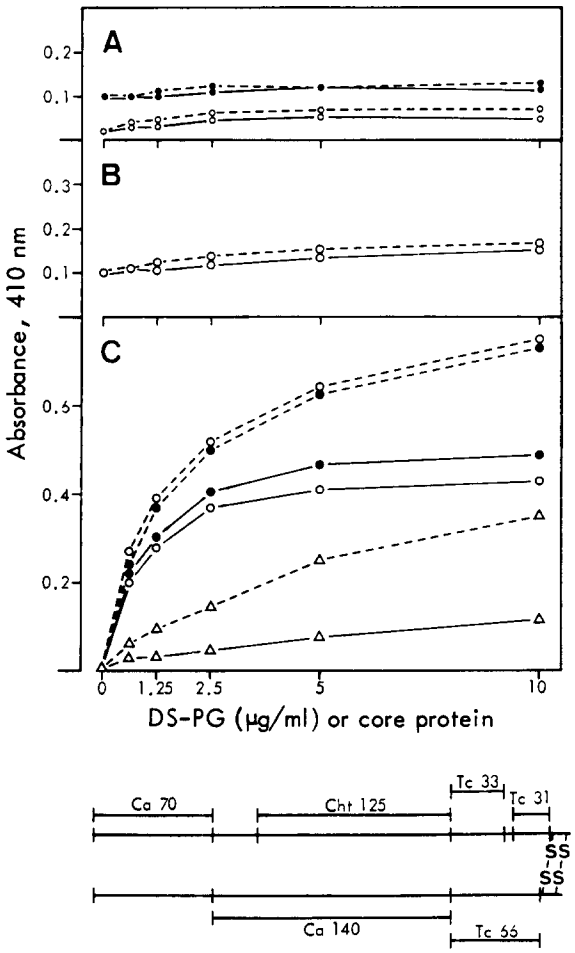

Figure 8. Binding of DS-PG (*solid lines*) and its core protein (*broken lines*) to fibronectin peptides. 10 μg per ml of the respective peptides were used for coating. (*A*) Peptides Cht 125 (●) and Tc 31 (○); (*B*) peptide Ca 140; (*C*) peptides Ca 70 (△), Tc 33 (●), and Tc 66 (○). The position of the peptides is shown schematically at the bottom of the figure.

protein than DS-PG molecules can interact with the respective domains on the fibronectin molecule. Alternatively, some of the binding sites could be accessible only for the small core protein after immobilization of fibronectin to the plastic dish.

The influence of ionic strength and pH on binding of DS-PG and its core protein to fibronectin implies that the interaction is electrostatic and suggests that histidine residues of the fibronectin molecule might be involved in binding. Considering the observation that exclusively heparin-binding fibronectin peptides participated in this interaction, one could postulate that heparin- and core protein-binding sites are identical. However, unsubstituted core protein is very basic (pH 9.8), and it does not appear very likely that the single acidic region at the NH_2 terminus (26) could create a structure that mimics heparin. The results of co-incubation with and replacement by heparin and protein-free dermatan sulfate (Fig. 7) would rather suggest the existence of adjacent binding sites for heparin and DS-PG core protein. Steric hindrance of DS-PG core protein binding would be possible under co-incubation conditions, whereas only minor amounts of bound core protein could be replaced by an excess of polysaccharide. It is obvious that additional assay systems resembling possible in vivo situations are required to study the interactions between fibronectin and DS-PG core protein in greater detail.

It had been shown recently that DS-PG bound completely to fibronectin-Sepharose columns and inhibited the capacity of fibroblasts to adhere to a fibronectin substratum (40). Chondroitin sulfate proteoglycans from cartilage exhibited only a marginal effect in the latter assay.

On the contrary, heparan sulfate proteoglycans play a direct role in forming adhesive bonds on fibronectin (39). In fibroblasts, heparan sulfate binding of fibronectin acted in a cooperative manner with the cell-binding domain of fibronectin (9). Thus, if heparan sulfate proteoglycans and DS-PGs interact with adjacent binding sites on the fibronectin molecule and if the binding of one proteoglycan species influences the binding of the other one, both proteoglycans could participate in the regulation of cellular functions in a competitive manner. Further studies are required to test this hypothesis.

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