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 NH₂ 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) 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 Mₐ 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 Mₐ 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).
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 NH₂ 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 [³H]leucine (15) followed by chondroitinase ABC 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 elsewhere.

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 benzamidinum 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 column (Pharmacia, Uppsalia, Sweden) as described (44). Purified material was dialyzed against 50 mM Tris/HCl buffer, pH 7.5, containing 3 mM NaN₃, and stored at 4°C. Fibronectin peptides were prepared according to published procedures (37, 38, 49, 54). 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, Gifhöft, FRG). They could be distinguished by a different size of their heavy chains, but they all belonged to subclass IgG1. LN1 recognized an epitope near the glycosaminoglycan attachment site, whereas LN3 and LN4 bound to a carbohydrate-free peptide of Mr 17000. Epitopes were localized by immunochromatographic staining of obtained by limited digestion of DS-PG and DS-PG core protein, respectively, with staphylococcal serine proteinase V8 (36) followed by SDS PAGE (68, 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).
contains minor amounts of a 38-kD fragment most likely produced by an alternative cleavage site. The slightly heterogeneous fragment Te 33 represents the main heparin-binding domain of the longer subunit chain, whereas Te 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% (vol/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 secondary 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-ethylbenzthiazole-sulfonic acid) and 37 μM H\(_2\)O\(_2\) in 50 mM sodium citrate, pH 4.0). Color development was stopped by adding 50 μl 10 mM Na\(_2\)HPO\(_4\), 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 7-9 nm diameter 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 diameter 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 coated particles was performed according to Roth et al. (42). Gold particles of 12-nm diam were prepared 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% (vol/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 secondary 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-ethylbenzthiazole-sulfonic acid) and 37 μM H\(_2\)O\(_2\) in 50 mM sodium citrate, pH 4.0). Color development was stopped by adding 50 μl 10 mM Na\(_2\)HPO\(_4\), 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.

### 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.
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.

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 intact DS-PG was used as a ligand. Use of [3H]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-

[Figures and graphs are not transcribed but are referenced in the text.]
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 NH2-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 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 residue at the NH₂ 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 poly-
saccharide. 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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