Cell Surface Phosphatidylinositol-anchored Heparan Sulfate Proteoglycan Initiates Mouse Melanoma Cell Adhesion to a Fibronectin-derived, Heparin-binding Synthetic Peptide

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Abstract. Cell surface heparan sulfate proteoglycan (HSPG) from metastatic mouse melanoma cells initiates cell adhesion to the synthetic peptide FN-C/H II, a heparin-binding peptide from the 33-kD A chain–derived fragment of fibronectin. Mouse melanoma cell adhesion to FN-C/H II was sensitive to soluble heparin and pretreatment of mouse melanoma cells with heparitinase. In contrast, cell adhesion to the fibronectin synthetic peptide CS1 is mediated through an $\alpha 4\beta 1$ integrin and was resistant to heparin or heparitinase treatment.

Mouse melanoma cell HSPG was metabolically labeled with [^{35}S]sulfate and extracted with detergent. After HPLC-DEAE purification, ^{35}S-HSPG eluted from a dissociative CL-4B column with a K_{av} ~0.45, while ^{35}S-heparan sulfate (HS) chains eluted with a K_{av} ~0.62. The HSPG contained a major 63-kD core protein after heparitinase digestion. Polyclonal antibodies generated against HSPG purified from mouse melanoma cells grown in vivo also identified a 63-kD core protein. This HSPG is an integral plasma membrane component by virtue of its binding to Octyl Sepharose affinity columns and that anti-HSPG antibody staining exhibited a cell surface localization. The HSPG is anchored to the cell surface through phosphatidylinositol (PI) linkages, as evidenced in part by the ability of PI-specific phospholipase C to eliminate binding of the detergent-extracted HSPG to Octyl Sepharose. Furthermore, the mouse melanoma HSPG core protein could be metabolically labeled with ^{3}H-ethanolamine.

The involvement of mouse melanoma cell surface HSPG in cell adhesion to fibronectin was also demonstrated by the ability of anti-HSPG antibodies and anti-HSPG IgG Fab monomers to inhibit mouse melanoma cell adhesion to FN-C/H II. ^{35}S-HSPG and ^{35}S-HS bind to FN-C/H II affinity columns and require 0.25 M NaCl for elution. However, heparitinase-treated ^{125}I-labeled HSPG failed to bind FN-C/H II, suggesting that HS, and not HSPG core protein, binds FN-C/H II. These data support the hypothesis that a phosphatidylinositol-anchored HSPG on mouse melanoma cells (MPIHP-63) initiates recognition to FN-C/H II, and implicate PI-associated signal transduction pathways in mediating melanoma cell adhesion to this defined ligand.

TUMOR cell adhesion to components of the extracellular matrix (ECM)[1] and to other cells is critically important in the metastatic process (Liotta et al., 1986; McCarthy et al., 1985). Tumor cell adhesion–promoting fragments or synthetic peptides of ECM proteins, such as fibronectin (FN) and laminin, can inhibit the experimental metastasis of several metastatic tumor cell types (Barsky et al., 1984; McCarthy et al., 1985; Humphries et al., 1986; Saiki et al., 1989). This inhibition is correlated with a corresponding inhibition of the arrest of ex vivo pretreated tumor cells within the pulmonary microcirculation after tail vein injection, suggesting an importance of tumor cell surface receptors for these ligands in tumor cell arrest and/or extravasation. One of these metastasis-inhibiting, cell adhesion–promoting fragments is a 33-kD heparin-binding fragment that is derived from proteolytic digests of human plasma FN A-chains (McCarthy et al., 1988*b*). Previous studies have demonstrated that the 33-kD heparin-binding fragment is active in promoting the RGD-independent adhesion and spreading of mouse melanoma, fibrosarcoma

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; FN, fibronectin; HSPG, heparan sulfate proteoglycan; HS, heparan sulfate; CS, chondroitin sulfate; OA, ovalbumin; PG, proteoglycan; PI, phosphatidylinositol; PLC, phospholipase C.

(McCarthy et al., 1986, 1988*b*), and rat neuroblastoma (Haugen et al., 1990), as well as other cell types (Herbst et al., 1988; Liao et al., 1989; Wayner et al., 1989; Visser et al., 1989). The heparin-binding properties of the 33-kD fragment suggest the importance of cell surface proteoglycans in mediating cell adhesion to this fragment.

Cell surface proteoglycans (PGs) have been implicated as receptors for the ECM, including FN. Focal adhesion formation by fibroblasts on FN-coated substrata requires the coordinated recognition of the heparin-binding and RGD-containing cell binding domains (Laterra et al., 1983; Woods et al., 1986; LeBaron et al., 1988). Analysis of focal adhesion plaques reveal areas enriched in PGs, integrins, and FN, as well as cytoskeletal proteins (Lattera et al., 1983; Lark and Culp, 1984; reviewed in Burridge et al., 1988). Furthermore, cells deficient in heparan sulfate (HS) synthesis exhibit a different adhesion phenotype compared to cells that produce HS (LeBaron et al., 1988). Additionally, syndecan, a cell surface PG of mouse mammary epithelial cells, has been shown to bind the carboxy-terminal, heparin-binding domain of FN and support attachment of epithelial cells (Saunders et al., 1988). These data suggest that cell surface PGs bind to the heparin-binding domain of FN at sites of contact between the cell and the substratum. The structural heterogeneity of core proteins of cell surface PGs suggests several mechanisms by which cell surface PGs may modulate cell adhesion. The core proteins of PGs may be linked to the cell membrane through a transmembrane domain (Saunders et al., 1988; Marynen et al., 1989) or by covalent attachment to phosphatidylinositol (PI) (Yanagishita and McQuillan, 1989; David et al., 1990; Carey and Stahl, 1990), suggesting that there may be specific functional consequences for these two types of plasma membrane linkages in mediating cell recognition of appropriate ligands.

Several melanoma cell adhesion-promoting synthetic peptides have been identified from within the carboxy-terminal heparin binding domain of FN (McCarthy et al., 1988*a*, 1990; Haugen et al., 1990). These synthetic peptides, termed FN-C/H I and FN-C/H II, are relatively hydrophilic, cationic, and bind the glycosaminoglycan (GAG) heparin, implicating a role for cell surface proteoglycans in mediating melanoma recognition of these synthetic peptides. Additionally, a synthetic peptide located within the 33-kD fragment, termed CS1, promotes cell adhesion but does not bind heparin (Humphries et al., 1987; McCarthy et al., 1990). Peptide CS1 interacts with $\alpha4\beta1$ integrin on human lymphocytes and melanoma cells (Wayner et al., 1989; Mould et al., 1990), indicating that $\alpha4\beta1$ integrin is also involved in mediating the adhesion of certain cells to this fragment. The multiple cell adhesion promoting sites identified within the 33-kD fragment indicate that melanoma cell adhesion to this fragment has a complex molecular basis, involving the possible close coordination of cell surface proteoglycans and $\alpha4\beta1$ integrin.

The current studies demonstrate that a cell surface heparan sulfate proteoglycan (HSPG) mediates cell adhesion of K1735 highly metastatic mouse melanoma cells to peptide FN-C/H II (KNNQKSEPLIGRKKT), a heparin-binding synthetic peptide from within the 33-kD heparin-binding fragment that is contiguous with the $\alpha4\beta1$ integrin-binding sequence CS1 (DELPQLVTLPHPNLHGPEILDVPST). Heparitinase, but not chondroitinase ABC, treatment of melanoma cell surfaces completely inhibited cell adhesion to FN-C/H II. Purified cell surface HSPG also bound to FN-C/H II affinity columns and eluted at moderate ionic strength (0.25 M NaCl). Partial characterization of the HSPG core protein demonstrated that it has an apparent molecular mass of 63 kD, and that it is expressed by melanoma cells as an integral plasma membrane protein that is attached by linkage to PI. Polyclonal antibodies generated against the mouse melanoma HSPG protein core stained the surface of mouse melanoma cells and inhibited mouse melanoma cell adhesion to FN-C/H II. In addition to demonstrating a role for cell surface HSPG in the initial recognition of FN-C/H-II, these studies suggest a role for PI-associated signal transduction pathways in mediating melanoma cell adhesion to this defined heparin binding ligand.

Materials and Methods

Cell Culture

A highly metastatic clone (M4) of the K1735 mouse melanoma was generously provided by Dr. I. J. Fidler (M.D. Anderson Hospital Cancer Center, Houston, TX). This tumor cell line was maintained by in vitro culture in DMEM (Sigma Chemical Co., St. Louis, MO) supplemented with 10% calf serum (Sigma Chemical Co.). The number of in vitro passages was limited to eight in order to minimize phenotypic drift.

Peptide Preparation

Peptides were synthesized at the University of Minnesota Microchemical Facility using a Beckman System 990 peptide synthesizer. The procedures used were based on the Merrifield solid phase system as described previously (Stewart and Young, 1984). Lyophilized crude peptides were purified by preparative reverse-phase HPLC on a C-18 column, using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. The purity and composition of the peptides were verified by HPLC analysis of hydrolysates prepared by treating the peptides under nitrogen in 6 N HCl overnight at 100°C (Skubitz et al., 1988; McCarthy et al., 1990; Haugen et al., 1990). All peptides were synthesized with a tyrosine residue at the carboxy-terminal end to facilitate radioiodination of the peptides. The sequence (minus the tyrosine residue at the carboxy terminal end) and selected properties of the synthetic peptides used in this study are shown in Fig. 1.

Conjugation of Peptides to Ovalbumin

Synthetic peptides were chemically conjugated to ovalbumin (OA) using 1-ethyl-3(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC) (Sigma Chemical Co.) (Humphries et al., 1987; McCarthy et al., 1990; Haugen et al., 1990). Briefly, equal amounts (by weight) of the peptides and OA were solubilized in water and mixed with a 10-fold excess (by weight) of EDC dissolved in water. The sample was mixed overnight at 4°C on a circulator rotator. The coupled peptides were then dialyzed extensively against PBS or water to remove the excess EDC and uncoupled peptides (Spectrapore 6, 10 kD exclusion; Spectrum Medical Industries, Los Angeles, CA). The efficiency of peptide coupling to OA was evaluated using a trace amount of radioactive peptides and was determined to have a stoichiometry of 2.9 mol of FN-C/H II to 1 mol of OA and 6.3 mol of CS1 to 1 mol of OA (Haugen et al. 1990). Peptide-OA conjugates were stored at −20°C until use.

Cell Adhesion Assays

Cell adhesion assays were performed as described previously with minor modifications (Haugen et al., 1990). Briefly, peptide conjugates were diluted to various concentrations in Voller's carbonate buffer and 100-μl aliquots were dispensed in triplicate into Immulon 1 polystyrene microtiter wells. The wells were incubated in a humidified oven at 37°C overnight. Radiolabeled OA-conjugates were used to quantitate the coating of OA-peptides to the wells and shown to be about the same for FN-C/H II-OA and CS1-OA (Haugen et al., 1990). Nonspecific sites were blocked the next day with

5 mg/ml BSA (fatty acid-free; Miles Scientific, Naperville, IL) in Voller's buffer for 2 h. Subconfluent M4 K1735 mouse melanoma cells that had been radiolabeled overnight with ^{3}H-thymidine (^{3}HTdR, specific activity 6.7 Ci/mmol; NEN Research Products, Boston, MA) were harvested by release with trypsin/EDTA, washed, and resuspended to a final concentration of 5×10^4 cells per ml in DMEM supplemented with 20 mM Hepes and 1 mg/ml BSA. 100-μl aliquots of the cell suspension were dispensed into wells and the cells were incubated at 37°C for various lengths of time. The assays were terminated by aspirating nonadherent cells, washing the wells three times, and solubilizing the bound cells in 0.5 N NaOH containing 1% SDS. Radioactivity was determined in a Beckman model 3801 liquid scintillation counter and used to calculate the percentage of cells that remained adherent to each substratum. We have determined through ^{35}S-sulfate labeling experiments of mouse melanoma cells that HSPG is replenished to the cell surface within 15–30 min after trypsinization (not shown).

To evaluate the inhibition of cell adhesion with polyclonal anti-HSPG antibodies, cells were preincubated with the antibody for 15–30 min, then plated directly into the wells precoated with substrata that promoted half-maximal cell adhesion, and allowed to attach for 15–30 min in the continued presence of antibody. For chondroitinase ABC treatment, trypsin/EDTA-released cells were washed in PBS, 0.1% BSA, and 1.25 mM $CaCl_2$ and incubated with or without 0.1 U/ml chondroitinase ABC (Sigma Chemical Co.) for 15 min at 37°C (Saunders and Bernfield, 1988). Cells were then diluted in DMEM, 20 mM Hepes, 0.1% BSA to 5×10^4 cell per ml with a concentration of 0.02 U/ml of enzyme and plated to coated substrata and incubated for 15–30 min. Alternatively, cells were treated with increasing concentrations of heparitinase enzyme then plated in the continued presence of enzyme. Assays were harvested and adherent cells were quantitated as previously described.

Proteoglycan Purification

Subconfluent cultures of K1735 M4 mouse melanoma cells were preferentially labeled with ^{35}S-sulfate for 18 h by replacing the medium with RPMI 1640 containing 0.1 mM Na_2SO_4, 0.36 mM glutamine, and 50 μCi/ml $Na_2{}^{35}SO_4$ (carrier free, specific activity 43 Ci/mg; ICN Biomedicals, Irvine, CA). In other experiments, the cells were doubly labeled for 18 h with ^{35}S-sulfate and ^{3}H-ethanolamine (16 μCi/ml; Amersham International). An extraction protocol utilizing detergent-mediated cell lysis was followed to specifically enrich for intact plasma membrane-associated PGs (Yanagishita and Hascall, 1984). The ^{35}S-labeled cell cultures were replaced with DMEM containing 50 μg/ml heparin for a 30-min incubation at 18°C. The heparin extract was removed and detergent extraction buffer (10 mM Tris-HCl, 0.15 M NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 0.24 mM DTT, 1 mM PMSF, 1% Triton X-100, pH 7.2) was added and incubated at 37°C for 15 min. The detergent extracts were centrifuged at 1,500 rpm for 5 min to remove insoluble material, then dialyzed in small pore dialysis tubing (molecular weight cut off 3,500) against acetate buffer (0.5 M sodium acetate, 0.1 M sodium sulfate, 10 mM EDTA, 0.1 mM PMSF, 10 mM 6-aminohexanoic acid, 1% Triton X-100, pH 6.8) to remove unincorporated ^{35}S-sulfate (Oegema et al., 1979).

^{35}S-PGs were purified by HPLC (Beckman Model 110A) using a 7.5 × 75 mm TSK DEAE 5PW anion exchange column (Bio-Rad Laboratories, Richmond, CA). Extracts were dialyzed against HPLC-DEAE buffer (0.15 M Tris-HCl, 6.0 M Urea, 0.1 M NaCl, 0.01 M EDTA, 0.01 M 6-aminohexanoic acid, 0.2% Triton X-100, or 0.2% CHAPS, 0.1 mM PMSF, pH 7.0) and applied at a flow rate of 1 ml/min, and proteins were eluted with a linear gradient from 0.1 M to 0.8 M NaCl (Klein et al., 1989). 1-ml fractions were collected and sampled for the presence of ^{35}S-sulfate by liquid scintillation (Beckman LS 3801). The salt gradient was monitored by conductivity measurements using a Radiometer Conductivity Meter (model CDM 83). Comparisons were made to standards of known NaCl concentration in DEAE buffer to calculate the actual salt concentrations of the fractions. To insure adequate separation of ^{35}S-proteoglycans, HPLC-DEAE chromatographic peaks were pooled and each rechromatographed on the same column before further characterization. The percent recovery from the HPLC-DEAE columns was 90–95%. Samples were dialyzed into water containing 0.1 mM PMSF and lyophilized. Aliquots were stored at −80°C and used as indicated.

HSPGs were characterized by gel filtration on Sepharose CL-4B (Sigma Chemical Co.) columns (0.9 × 110 cm) equilibrated in 10 mM Tris-HCl, pH 7.2, 4 M guanidine, 1% Triton X-100 at a flow rate of 3 ml/h (Klein et al., 1986). 1-ml fractions were collected with a 85–95% recovery of radioactivity. Rat chondrosarcoma CSPG (Oegema et al., 1975) associated with 4% hyaluronic acid and glucuronolactone were used to mark the column void (V_o) and total (V_t) volumes, respectively. ^{35}S-GAGs were released from proteoglycan protein cores by alkaline β-elimination in 0.05 N NaOH containing 1 M $NaBH_4$ over 24 h at 45°C (Oegema et al., 1979). The reaction mixture was neutralized by the dropwise addition of acetic acid and desalted on Sephadex G-50 (Sigma Chemical) columns. ^{35}S-GAGs were recovered from the column V_o with <5% of alkali-treated material included in the column. The HS and CS content of ^{35}S-GAG samples were determined by sequential nitrous acid deaminative cleavage and chondroitinase ABC treatment by methods modified from Conrad et al. (1977). Detergent-extracted ^{35}S-macromolecules resistant to the effects of nitrous acid and chondroitinase ABC digestion were considered ^{35}S-glycoproteins (Brown et al., 1981) and this population never exceeded 10% of the detergent extract.

Detergent-extracted ^{35}S-HSPGs, purified by ion exchange chromatography, were resuspended in Octyl Sepharose buffer (20 mM Tris-HCl, 4 M guanidine HCl, pH 6.8). Samples were applied to a 5 ml Octyl Sepharose CL4B (Sigma Chemical Co.) column at a flow rate of 0.5 ml/min. Hydrophobic HSPGs were then eluted with a linear gradient of 0–0.5% Triton X-100 in Octyl Sepharose buffer (Yanagishita et al., 1987). 2-ml fractions were analyzed for ^{35}S-radiolabel by liquid scintillation and percent Triton X-100 was determined by absorbance at 280 nm.

Radioiodination of Heparan Sulfate Proteoglycan

Sepharose CL-4B–purified HSPGs aliquots were radioiodinated using $Na^{125}I$ and iodobead (Pierce Chemical Co., Rockford, IL) in a 50-mM Tris-HCl, pH 7.0, 0.2% CHAPS buffer. After a 15-min incubation, the reaction mixture was applied to a 0.5-ml DEAE-Spetra/Gel column (Spectrum, Los Angeles, CA) equilibrated in DEAE buffer. Free $Na^{125}I$ was removed by washing the column with 0.1 M NaCl DEAE buffer. The ^{125}I-HSPGs were eluted from the column in DEAE buffer containing 1.0 M NaCl. The ^{125}I-labeled HSPGs were dialyzed into deionized water with 0.1 mM PMSF and frozen at −80°C. Radioactivity was quantitated in a gamma counter (TM Analytic, Gamma Trac 1193).

Enzyme Treatment

Aliquots of purified HSPGs were digested with heparitinase or chondroitinase ABC (Sigma Chemical Co. and Seikagaku America Inc., Rockville, MD) in 50 mM Tris, pH 7.0, 10 mM calcium acetate, 1 mM PMSF, 0.036 mM pepstatin, and 10 mM NEM for 4 h at 37°C. For the treatment with PI-specific phospholipase C (PI-PLC) (Boehringer Mannheim), HSPGs were solubilized in 50 mM Tris-HCl, 0.1% Triton X-100, 10 mM EDTA, pH 7.5, and incubated with 50 mU enzyme for 1–2 h at 37°C (David et al, 1990). After the digestion, the treated and control HSPGs were absorbed on DEAE, rinsed to remove Triton X-100, and eluted in 1.0 M NaCl DEAE buffer containing CHAPS. The absence of proteases in the heparitinase and PI-PLC enzymes were verified by a lack of digestion of azocasein after a 60-min incubation with the enzyme at 37°C (Tomarelli et al., 1949).

Peptide Affinity Chromatography

OA-peptide conjugates were coupled to Affigel-10 beads (Bio-Rad Laboratories) following packaging direction (1 mg OA/ml gel). 1–10-ml bed volume columns were made and equilibrated in 50 mM Tris-HCl, pH 7.0, 0.2% CHAPS, 50 mM NaCl, 1 mM PMSF, 0.01 M EDTA, 0.01 M 6-aminohexanoic acid (column buffer). Bound material was eluted with a linear NaCl salt gradient (0.05–0.5 M) in column buffer.

Antibody Preparation

K1735 M4 tumor cells were injected into the leg flanks of C57B16♀ × C3HHeN♂ F1 mice. Tumors were metabolically radiolabeled in vivo by intraperitoneal injection of ^{35}S-sulfate 24 h before tumor excision and extracted overnight in guanidine extraction buffer (4 M guanidine, 2% Triton X-100, 0.1M NaCl, 0.5 mM PMSF, 0.1 M 6-aminohexanoic acid, 10 mM EDTA, 1 mg/ml benzamidine) at 4°C. The extract was centrifuged at 5,500 rpm for 30 min at 4°C and the supernatant was dialyzed into 0.1 M NaCl DEAE buffer containing 0.2% Triton X-100. DEAE-Sephacel was added to form a slurry and incubated overnight at 4°C with rotation. The DEAE-Sephacel slurry was washed with DEAE buffer containing 0.2% CHAPS, then batch eluted in 1.0 M NaCl DEAE buffer. ^{35}S-macromolecules in the eluate were precipitated with 4 vol of ethanol at 4°C overnight, and collected after centrifugation at 5,500 rpm for 30 min. The precipitate was resuspended in guanidine extraction buffer and separated by CsCl density gradient ultracentrifugation (0.44 g of CsCl/g of extract) at 40,000 rpm and 4°C for 60 h using a Ti-70 rotor and a Beckman model L5-65 ultracen-

trifuge. The gradients were cut into a bottom 2/5 (high buoyant density) and a top 3/5 (low buoyant density) fraction (Oegema et al., 1979). The high buoyant density fraction (ρ = 1.44) was dialyzed into DEAE buffer with 0.2% Triton X-100 and purified by HPLC-DEAE ion exchange column chromatography (see above). HSPGs eluted as a single peak (0.32 M NaCl) from HPLC-DEAE columns and were dialyzed against water with 0.5 mM PMSF and lyophilized. Lyophilized tumor-extracted PGs were determined to be HSPG by GAG analysis (see proteoglycan purification). Samples were electrophoresed in a preparative 0.6% agarose–1.8% polyacrylamide gel (Klein et al., 1986), and visualized by staining with 0.2% toluidine blue in 0.1 N acetic acid. A rat chondrosarcoma CSPG (M_r 2.6 × 10^6) and chondroitin 4-sulfate (M_r 20,000) were used as markers. HSPG bands were excised and used to immunize New Zealand White rabbits. Immunization was performed by homogenizing equal volume of excised HSPG gel with complete Freund's adjuvant and injecting mixture into hind legs of rabbits. Subsequent biweekly boost of HSPG and incomplete Freund's adjuvant mixture were injected. Sera were collected 7–10 d after the sixth immunization, and tested by Western blot after transfer of HSPG from agarose-polyacrylamide gel to a nylon membrane. IgG was purified from pooled immune sera as previously described (Skubitz et al., 1988; McCarthy et al., 1990), and adsorbed on FN, type IV collagen or laminin affinity columns to remove any potential cross reacting antibodies to these ECM components.

Fab monomers were generated by digestion of polyclonal anti-HSPG IgG and normal rabbit IgG with papain-agarose (Sigma Chemical Co.) in buffer containing 100 mM sodium acetate, 50 mM cysteine, 1 mM EDTA, pH 5.5, for 6 h at 37°C with gentle agitation. The papain-agarose was removed by centrifugation, and undigested IgG and Fc fragments were removed by protein A–agarose (Pierce Chemical Co.) affinity column chromatography. Digestion and purification was monitored by SDS-PAGE.

Immunofluorescence

K1735 M4 mouse melanoma cells grown in tissue culture were released by treatment with trypsin/EDTA and allowed to adhere on glass coverslips overnight. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Coverslips were blocked in PBS containing 5 mg/ml BSA and 5% normal goat sera for 1–2 h. Anti-HSPG antibodies diluted 1:50 in PBS were added to the coverslips and incubated for 1 h at room temperature, coverslips were washed then incubated for 1 h with rhodamine-conjugated goat anti-rabbit IgG (1:250) (Cooper Biomedical, Malvern, PA). The coverslips were washed and mounted with a polyvinyl alcohol solution to slides. Cells were photographed for 30 s on Tri-X Pan 35-mm film with a Zeiss Model IM microscope using a 63× planapochromat lens.

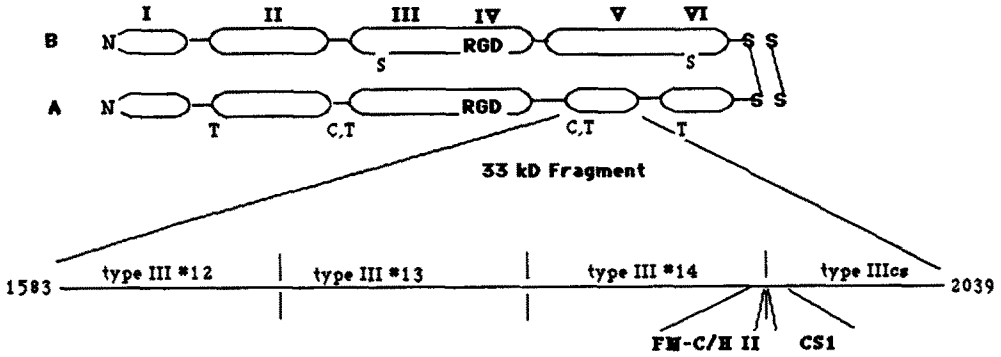
Results

Peptide FN-C/H II Initiates Mouse Melanoma Adhesion by an HS-dependent Mechanism

The location, primary sequences, and selected characteristics of FN-C/H II and CS1 within the 33-kD fragment of FN are shown in Fig. 1. FN-C/H II or CS1 synthetic peptide/OA conjugates (50 μg/ml) promoted the adhesion of virtually 100% of the input cells in a 90-min cell adhesion assay, whereas lower coating concentrations (3–5 μg/ml) promoted melanoma cell adhesion at levels of ~50% of input cells (data not shown).

Since peptide FN-C/H II binds ^{3}[H]heparin, whereas CS1 does not (McCarthy et al., 1988*b*, 1990), we evaluated the ability of soluble heparin to inhibit mouse melanoma cell adhesion to the two synthetic peptides. Cell adhesion to substrata coated with 3 μg/ml of FN-C/H II-OA was significantly inhibited in the presence of nanogram levels of soluble heparin, and inhibition was complete in the presence of 0.5 μg/ml of soluble heparin (Fig. 2 *a*). By contrast, cell adhesion to CS1-OA was totally resistant to the effects of soluble heparin, even at concentrations (5 μg/ml) of heparin that were 10-fold higher than those that maximally inhibited cell adhesion to FN-C/H II.

To further define the nature of the cell surface PG that might mediate mouse melanoma cell adhesion to FN-C/H II, we examined the ability of specific GAG-degrading enzymes to interfere with mouse melanoma cell adhesion. Mouse melanoma cells were pretreated with heparitinase, then added to FN-C/H II-OA– and CS1-OA–coated substrata (Fig. 2 *b*). Heparitinase digestion resulted in a concentration-dependent inhibition of adhesion to FN-C/H II-OA, and inhibition was complete (90%) at 0.10 U of enzyme. Mouse melanoma cell adhesion to the control synthetic peptide, CS1-OA, was unaffected by heparitinase digestion. By con-



Peptide	Sequence[a]	Heparin[b] Binding	Cell Adhesion[c]
FN-C/H II	KNNQKSEPLIGRKKT	+++	+++
CS1	DELPQLVTLPHPNLHGPEILDVPST	---	+++

Figure 1. Location of the 33-kD heparin binding fragment and synthetic peptides, FN-C/H II and CS1, within the intact FN molecule. The IIIcs region is found only in the A isoforms of human plasma FN. The amino and carboxy ends of the 33-kD fragment are based on previous sequence data (McCarthy et al., 1988*b*). Selected biological domains, indicated by roman numerals, are based on the nomenclature of Furcht (1981). I, weak heparin binding; II, collagen binding; III, free sulfhydryl; IV, RGD-mediated cell adhesion; V, carboxy-terminal strong heparin binding and cell adhesion; VI, free sulfhydryl. Approximate locations of tryptic (*T*) and cathepsin D (*C*) sites on intact FN are shown. The peptide sequences (*a*), heparin binding properties (*b*), and cell adhesion properties (*c*) are summarized and based on McCarthy et al. (1988*a*, 1990) and Haugen et al. (1990).

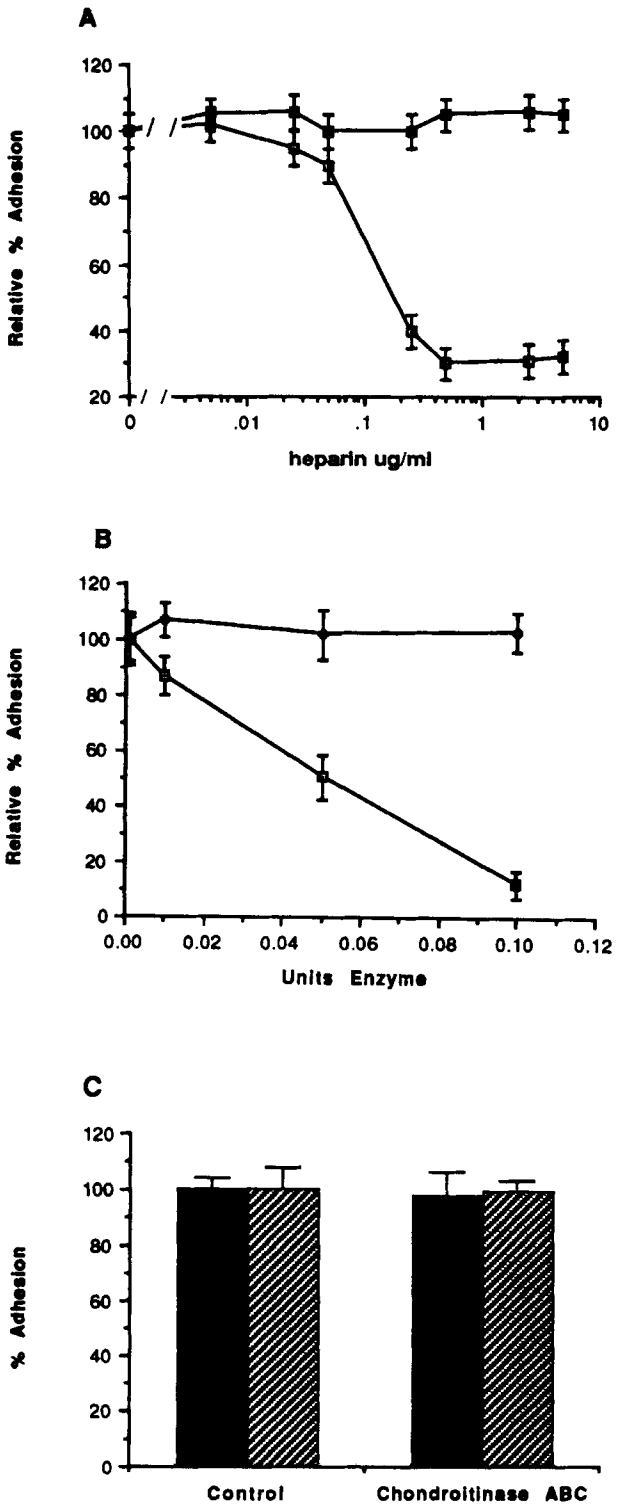


Figure 2. Mouse melanoma cell adhesion to FN-C/H II (but not CS1) is HS-dependent. (*A*) Mouse melanoma cells were added to 96-well plates adsorbed with 5 μg/ml of FN-C/H II-OA (*open squares*) or CS1-OA (*solid squares*) that had been preincubated with increasing concentrations of heparin. (*B*) Mouse melanoma cells were pretreated with increasing concentrations of heparitinase and then added to wells adsorbed with 5 μg/ml of FN-C/H II-OA (*open squares*) or CS1-OA (*solid diamonds*). (*C*) Mouse melanoma cells were pretreated with chondroitinase ABC at 0.1 U/ml then added to wells adsorbed with 5 μg/ml of FN-C/H II-OA (*solid bars*) or CS1-OA (*hatched bars*).

trast, chondroitinase ABC pretreatment of cells had no effect on cell adhesion to FN-C/H II-OA- or CS1-OA-coated substrata (Fig. 2 *c*). Cell viability, as determined by trypan blue dye exclusion, was >95% following all enzyme treatments. These results suggest a primary involvement of cell surface HS, but not CS, in mediating the initial adhesion of mouse melanoma cells to peptide FN-C/H II.

Isolation and Partial Characterization of Mouse Melanoma Cell Surface HSPG

Mouse melanoma cell surface HSPG was purified from detergent extracts of [^{35}S]sulfate-labeled mouse melanoma cells grown in vitro by HPLC-DEAE chromatography. ^{35}S-macromolecules eluted as three peaks from this column (Fig. 3 *a*). The central peak, eluting at 0.32 M NaCl, was pooled

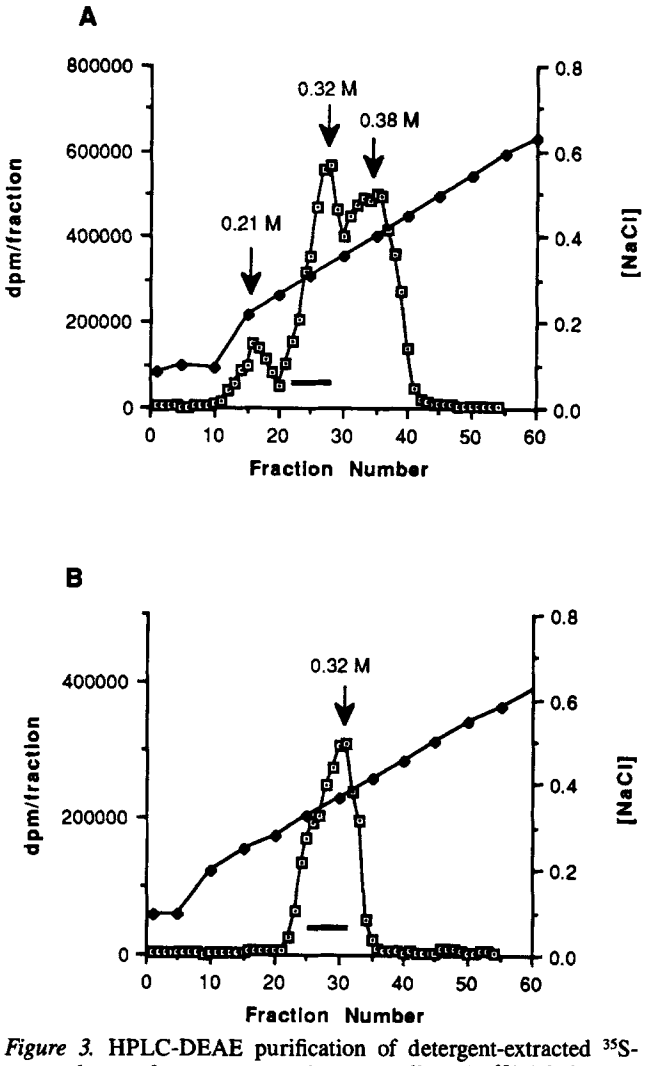


Figure 3. HPLC-DEAE purification of detergent-extracted ^{35}S-proteoglycans from mouse melanoma cells. (*A*) ^{35}S-labeled extracts were dialyzed into DEAE buffer (see Materials and Methods) and applied to a HPLC-DEAE column and eluted with a linear NaCl gradient. Fractions of 1 ml were monitored for radioactivity (*open squares*) and the salt gradient monitored by conductivity measurements (*solid diamonds*). The bar indicates the fractions that were pooled and rechromatographed (*B*).

as indicated, and rechromatographed on HPLC-DEAE (Fig. 3 *b*). Characterization of the ^{35}S-GAGs in this peak indicated that it contained 99% HS, as determined by nitrous acid sensitivity and resistance to chondroitinase ABC. The other two HPLC-DEAE peaks, representing ^{35}S-glycoproteins (0.21 M NaCl) and chondroitinase ABC–sensitive ^{35}S-CSPG (0.38 M NaCl), were not characterized further in these studies. ^{35}S-HSPGs purified by HPLC-DEAE were subjected to chromatography on Sepharose CL-4B in 4 M guanidine and detergent. ^{35}S-HSPG eluted as a single peak at K_{av} ~0.45 (Fig. 4). ^{35}S-HS, released from core proteins by alkaline borohydride, eluted as a single peak from these columns at K_{av} ~0.62. ^{35}S-HSPGs were also analyzed by Sepharose CL-6B chromatography using 0.5 M sodium acetate and 0.2% CHAPS as the buffer. The intact HSPG eluted with a K_{av} ~0.20 and alkaline borohydride released HS eluted with a K_{av} ~0.40 (data not shown).

^{35}S-HSPGs purified by HPLC-DEAE and Sepharose CL-4B were iodinated to identify putative HSPG core proteins. ^{125}I-labeled samples were subjected to separation by SDS-PAGE and visualized by autoradiography (Fig. 5). Digestion of samples with heparitinase resulted in the appearance of a core protein with a M_r of 63 and a minor core protein with a M_r of 32 (Fig. 5, lane 2). Under nonreducing conditions, the major core protein appeared slightly smaller (M_r 57), while the M_r of the minor species remained unchanged (Fig. 5, lane *4*). In subsequent HSPG preparations the M_r 32 band was much reduced or absent. Undigested samples, or samples digested with chondroitinase ABC (Fig. 5, lanes *1* and *3*, respectively), failed to generate protein bands, confirming the specificity of the heparitinase digestion. Samples were also digested with both heparitinase and chondroitinase ABC and yielded a single band at 63 kD, thus, suggesting that the sample did not contain a HS/CS hybrid proteoglycan (data not shown). While iodinated samples appeared to contain contaminating proteins due to the presence of minor bands in all lanes, their appearance was not the result of enzymatic digestion with either chondroitinase ABC or heparitinase.

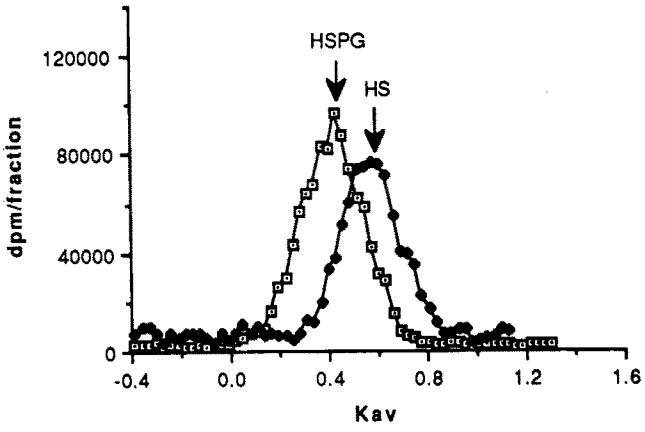


Figure 4. Analysis of HPLC-DEAE–purified ^{35}S-HSPG by Sepharose CL-4B chromatography before (*open squares*) and after (*solid diamonds*) release of ^{35}S-HS by alkaline borohydride. Radioactivity was monitored for each 1-ml fraction.

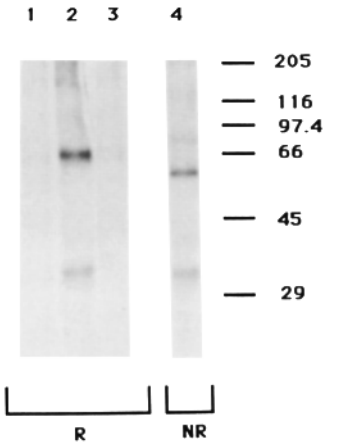


Figure 5. Identification of 63-kD core protein of mouse melanoma HSPG by autoradiography. ^{125}I-labeled HSPGs were digested and analyzed by 12.5% SDS-PAGE under reducing (*R*) and nonreducing (*NR*) conditions. (Lane *1*) Undigested HSPG; (lanes 2 and *4*) heparitinase-digested HSPG; (lane *3*) chondroitinase ABC–digested HSPG. Relative mobilities of the molecular weight standards ($\times 10^3$) are indicated at the right.

Characterization of Anti-mouse Melanoma HSPG Antibodies

HSPG purified from mouse melanoma grown in vivo (see Materials and Methods) were injected into rabbits to generate anti-HSPG antibodies. The anti-HSPG IgG was used to stain mouse melanoma cell cultures by indirect immunofluorescence. As shown in Fig. 6, the antisera stained the cell surface of melanoma cells in a punctate fashion (Fig. 6 *a*). Control cultures incubated with normal rabbit IgG and rhodamine-conjugated goat anti-rabbit secondary antibody were negative (Fig. 6 *b*).

The anti-HSPG IgG fraction was tested for its ability to react with HSPG or HSPG core proteins isolated from mouse melanoma cells grown in vivo or in vitro (Fig. 7). Western blots of intact HSPG, isolated from melanoma grown in vivo and electrophoresed in agarose-polyacrylamide gels, demonstrated that the anti-HSPG IgG reacted with a single, chondroitinase-resistant, heparitinase-sensitive band (Fig. 7 *a*). The anti-HSPG IgG also reacted strongly with the 63-kD core protein produced by heparitinase digestion of HSPG that had been extracted and purified from mouse melanoma cells grown in vitro (Fig. 7 *b*), or in vivo (data not shown). This purified, adsorbed anti-HSPG IgG also cross-reacted with the smaller core protein species at M_r 32. In control experiments, anti-HSPG IgG did not detect proteins from a mouse melanoma cell lysate (Fig. 7 *b*). K1735 mouse melanoma cells also express a CD44-related cell surface CSPG with a core protein of 110 kD (Faassen et al., 1992). Anti-HSPG IgG failed to detect the 110-kD CSPG core protein by Western blot (data not shown).

Since mRNA for the large basement membrane HSPG core protein has previously been detected within these cells (Noonan et al., 1988), we also examined whether or not mouse melanoma HSPG core protein could be detected with a polyclonal antibody to the major EHS basement membrane HSPG, perlecan (Hassell et al., 1980). By immunoblot of heparitinase digests of mouse melanoma HSPG, we determined that the 63- or 32-kD HSPG core proteins from mouse melanoma cells were not detected by anti-perlecan antibodies. In control experiments, anti-perlecan antibodies recognized a 400-kD HSPG core protein isolated from EHS tumor (data not shown).

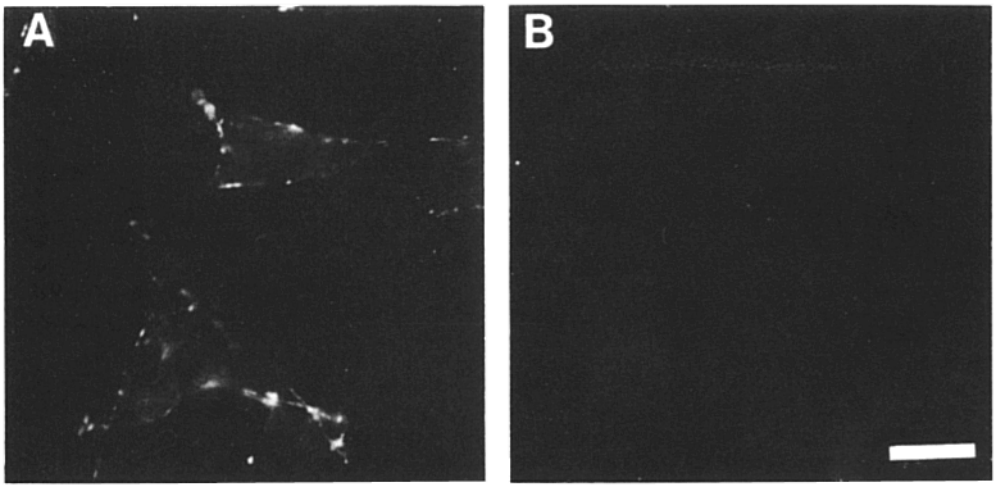


Figure 6. Cell surface staining of cultured mouse melanoma cells with anti-HSPG antibodies. Mouse melanoma cells grown in tissue culture were trypsin released and cultured on glass coverslips overnight. Coverslips were prepared for indirect immunofluorescence (see Materials and Methods) with anti-HSPG IgG (*A*) or normal rabbit IgG (*B*). Bar, ~20 μm.

Mouse Melanoma HSPG Is Anchored through PI

Hydrophobic chromatography has been used by others (Yanagishita et al., 1987) to identify cell surface HSPGs that are potentially integral components of the plasma membrane. One method of anchorage to the plasma membrane occurs through PI (Low and Saltiel, 1988) and can be removed by digestion with PI-PLC. To determine the hydrophobic properties and PI-PLC sensitivity of detergent-extracted, HPLC-DEAE-purified ^{35}S-HSPG, samples were pretreated (or not) with PI-PLC and applied to Octyl

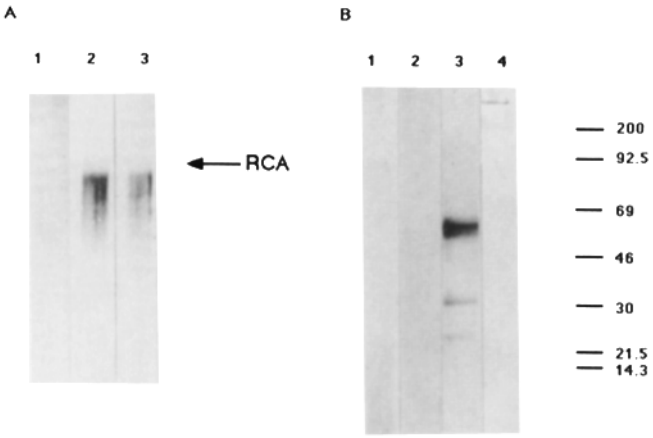


Figure 7. Immunoreactivity of anti-HSPG IgG antibodies by Western blot. (*A*) HSPG purified from mouse melanoma grown in vivo was digested and analyzed by 0.6% agarose-1.8% polyacrylamide gel electrophoresis and electroblotted. (Lane *1*) Heparitinase-digested HSPG; (lane *2*) chondroitinase ABC-digested HSPG; (lane *3*) undigested HSPG. The migration of rat chondrosarcoma CSPG (RCA) (M_r 2.6 × 10^6) is indicated. (*B*) HSPG purified from mouse melanoma grown in vitro was digested and analyzed by 6–15% SDS-PAGE under reducing conditions and electroblotted. (Lane *1*) Total cell extracts from mouse melanoma cells; (lane *2*) heparitinase alone; (lane *3*) heparitinase-digested HSPG; (lane *4*) undigested HSPG. The membranes were probed with anti-HSPG IgG (1:50). Relative mobilities of the molecular weight standards (×10^3) are indicated at the right.

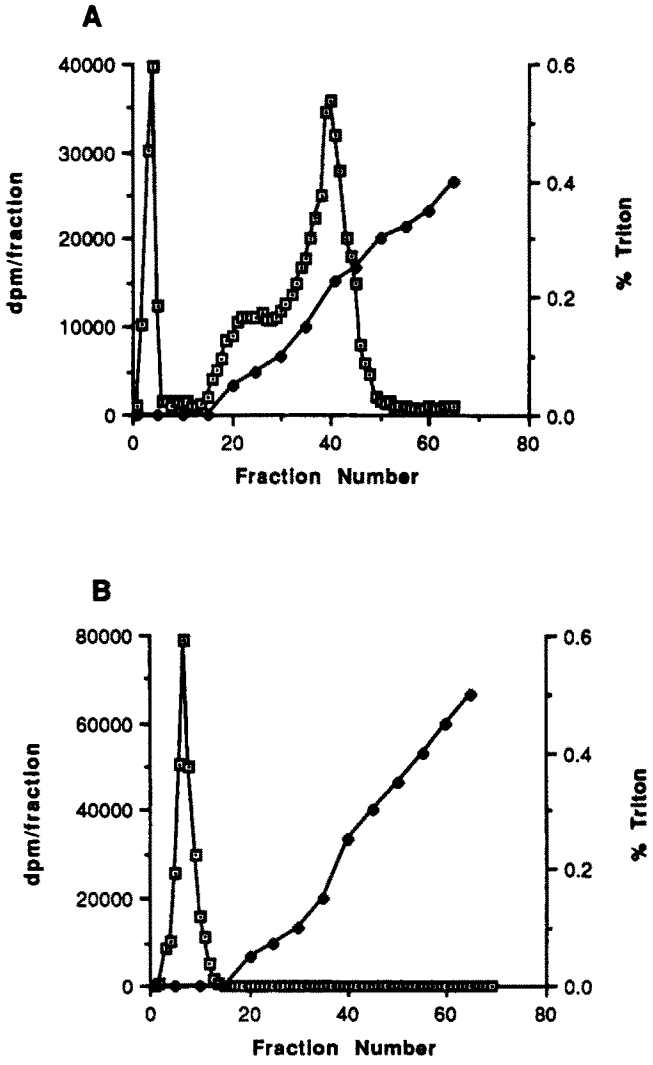


Figure 8. Analysis of ^{35}S-HSPG by Octyl Sepharose CL-4B chromatography before (*A*) and after (*B*) PI-PLC digestion. Fractions were monitored for radioactivity (*open diamonds*) and columns were eluted with a linear gradient of column buffer containing Triton X-100 (*solid diamonds*).

Table I. Metabolic Labeling Data of Mouse Melanoma HSPG with ^{3}H-Ethanolamine and ^{35}S-Sulfate

Mouse melanoma HSPG	^{3}H/^{35}S Ratio*
Intact‡	0.2
Immunoreactive 63-kD core protein§	45

* ^{3}H dpm/^{35}S dpm ratio.
‡ Mouse melanoma cultures were metabolically labeled with ^{3}H-ethanolamine and ^{35}S-sulfate and HSPG was purified from detergent extracts by HPLC-DEAE and dissociative CL-4B chromatography. An aliquot was analyzed for the presence of ^{3}H and ^{35}S by liquid scintillation using a dual label program.
§ Purified, dual labeled HSPG was heparitinase digested and analyzed by SDS-PAGE and Western blot. The immunoreactive 63-kD band was excised and ^{3}H and ^{35}S were determined by liquid scintillation.

Sepharose columns (Fig. 8). Approximately 80% of the untreated ^{35}S-HSPG bound to an Octyl Sepharose column (Fig. 8 *a*). The majority (70%) of the bound ^{35}S-HSPG eluted close to the critical micelle concentration of Triton X-100. In contrast, detergent-extracted HSPGs treated with PI-PLC did not bind to Octyl Sepharose and are seen in the unbound fractions (Fig. 8 *b*). In each case, the percent recovery of ^{35}S-HSPG from the Octyl Sepharose columns was >99%. Detergent-extracted, HPLC-DEAE-purified ^{35}S-HSPG digested with low levels of trypsin, or alkaline borohydride-released ^{35}S-HS, also failed to bind this column (data not shown). These data demonstrate that detergent-extracted HSPG can bind to Octyl Sepharose and that this binding is sensitive to the effects of PI-PLC, suggesting that the mouse melanoma HSPG core protein is anchored to the plasma membrane by PI.

To provide additional evidence that the HSPG is linked to the cell surface via a PI-anchor, mouse melanoma cells were metabolically labeled with both ^{3}H-ethanolamine and ^{35}S-sulfate. The cells were detergent extracted as previously described and the HSPG was purified over sequential HPLC-DEAE columns and dissociative Sepharose CL-4B columns. The resulting dual-labeled HSPG preparation was then quantitated for relative amounts of both radioisotopes, yielding a ^{3}H/^{35}S ratio of 0.2 (Table I). This purified HSPG was then

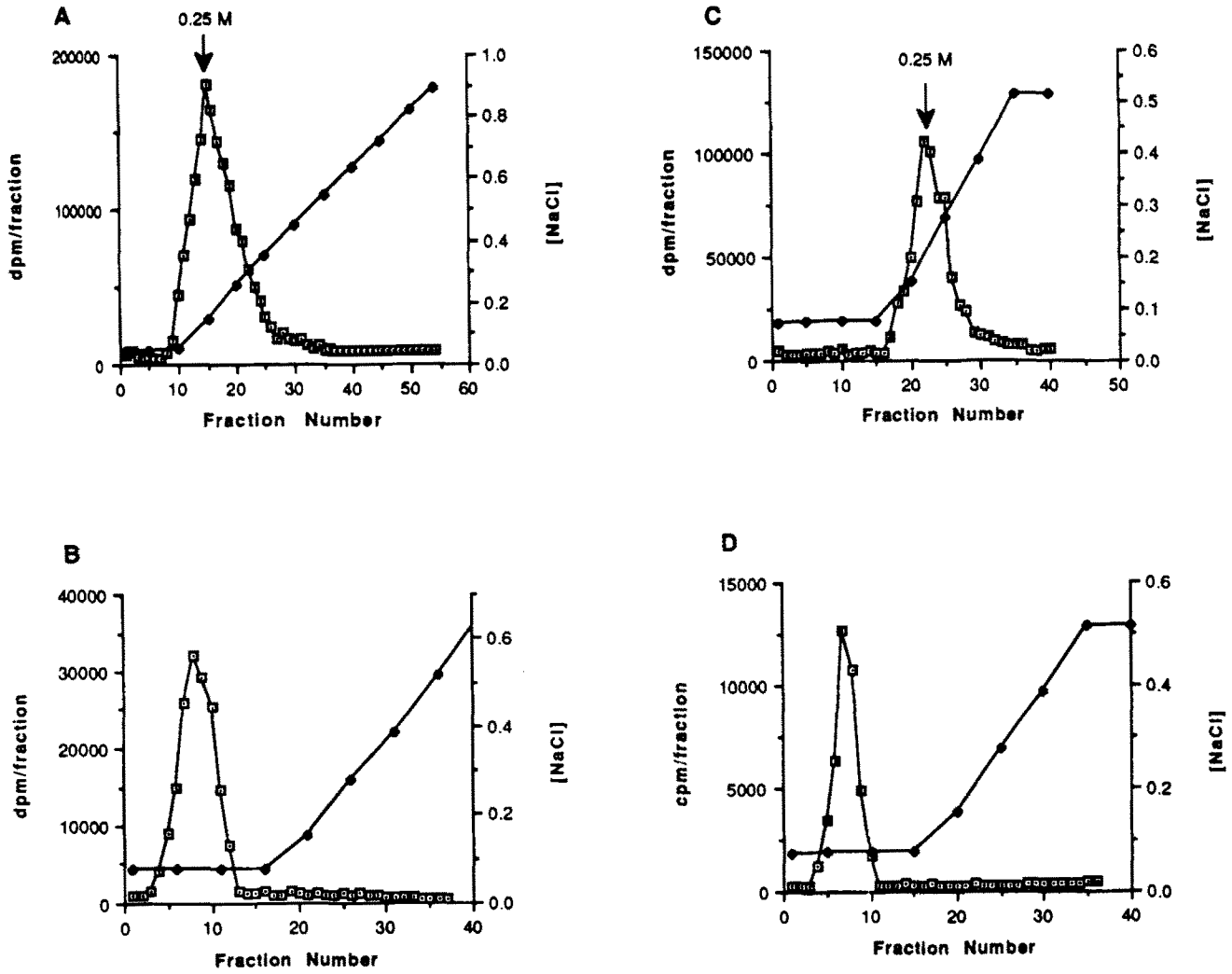


Figure 9. ^{35}S-HSPG and ^{35}S-HS bind to a FN-C/H II-OA affinity column. Detergent-extracted HPLC-DEAE-purified ^{35}S-HSPG was applied to a FN-C/H II-OA (*A*) or CS1-OA (*B*) affinity column in 50 mM Tris, pH 6.8, 50 mM NaCl, 0.2% CHAPS and eluted with a NaCl gradient. Alkaline borohydride-released ^{35}S-HS (*C*) or heparitinase-treated ^{125}I-HSPG (*D*) were applied to a FN-C/H II-OA affinity column. Radioactivity was monitored for each 1-2-ml fraction (*open squares*) and the salt gradient was determined by conductivity measurements (*solid diamonds*).

heparitinase digested, subjected to SDS-PAGE, and electrophoretically transferred to nylon membranes. The immunoreactive 63-kD band was then identified by Western blot, and further analyzed by autoradiography. The results demonstrated that the immunoreactive 63-kD core protein from dual-labeled cultures could also be detected by autoradiography (not shown). The immunoreactive/radioactive 63-kD band was then excised from the nylon membrane, and determined to have a $^{3}H/^{35}S$ ratio of 45 (Table I), demonstrating that the 63-kD heparitinase-digested core protein does label with ^{3}H-ethanolamine. Collectively, these data provide evidence that the 63-kD HSPG core protein of mouse melanoma cells is anchored to the plasma membrane via PI due to its sensitivity to PI-PLC digestion and due to the ability of the HSPG core protein to be metabolically labeled with ^{3}H-ethanolamine.

HSPG Binds FN-C/H II and Initiates Melanoma Cell Adhesion

Virtually 100% of the ^{35}S-HSPG isolated from melanoma cells grown in vitro bound to FN-C/H II-OA affinity columns (Fig. 9 *a*), under conditions for which no binding of ^{35}S-HSPGs occurred to OA-OA columns (not shown). The bound ^{35}S-HSPGs could be eluted from FN-C/H II-OA columns at 0.25 M NaCl using a linear NaCl salt gradient. The ^{35}S-HSPGs did not bind to CS1-OA affinity columns (Fig. 9 *b*). To determine the relative importance of HS versus HSPG core protein in binding to peptide FN-C/H II, ^{35}S-HS GAGs released by alkaline borohydride, or ^{125}I-labeled HSPG core protein generated by heparitinase digestion of ^{125}I-labeled HSPG, were applied to FN C/H-II-OA affinity columns. In the former case, 98% of ^{35}S-HS applied to the FN-C/H II-OA column bound to this column and was eluted at 0.25 M NaCl, using a linear salt gradient (Fig. 9 *c*). The elution profile of ^{35}S-HS was thus essentially identical to the elution profile of the intact ^{35}S-HSPGs. By contrast, ^{125}I-labeled HSPG core protein applied to the column did not bind and was collected in the unbound fractions (Fig. 9 *d*).

Purified anti-HSPG IgG inhibited mouse melanoma cell adhesion to FN-C/H-II-coated substrata (Fig. 10 *a*). This inhibition was concentration-dependent and 80% inhibition was observed in the presence of 500 μg/ml of total IgG purified from immune sera. In contrast, anti-HSPG had no effect on cell adhesion to CS1-OA (Fig. 10 *a*), and normal rabbit IgG had no effect on cell adhesion to either peptide, suggesting the inhibitory effect of anti-HSPG IgG was specific in nature. Anti-HSPG IgG Fab monomers inhibited mouse melanoma cell adhesion to FN-C/H II to approximately the same level (80%) as anti-HSPG IgG (Fig. 10 *b*). Although anti-HSPG Fab monomers appear to be more effective than intact immune IgG, the inhibition is identical if the concentrations of IgG and Fab monomers are compared on a molar basis. Normal rabbit IgG Fab monomers had no effect on cell adhesion to FN-C/H II. Anti-HSPG Fab monomers had no effect on cell adhesion to CS1 (data not shown).

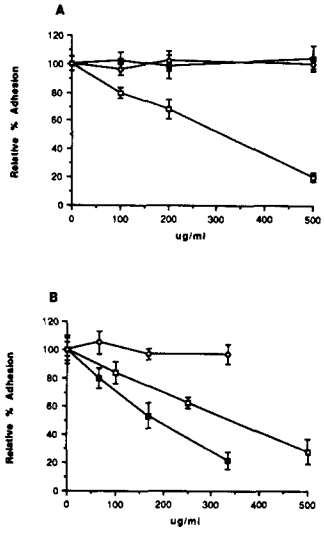


Figure 10. Anti-HSPG inhibits mouse melanoma cell adhesion to FN-C/H II. (*A*) Mouse melanoma cells were pretreated for 15 min with increasing concentrations of anti-HSPG IgG and then added to wells adsorbed with 5 μg/ml FN-C/H II-OA (*open squares*) or CS1-OA (*solid squares*). Additionally, cells were pretreated with normal rabbit IgG and added to wells adsorbed with FN-C/H II-OA (*open circles*). (*B*) Mouse melanoma cells were pretreated with anti-HSPG IgG Fab monomers (*solid squares*), anti-HSPG IgG (*open squares*), or normal rabbit IgG Fab monomers (*open circles*), then added to wells adsorbed with 5 μg/ml FN-C/H II-OA.

Discussion

The current studies demonstrate that cell surface HSPG of mouse melanoma cells initiates cellular recognition and adhesion to a synthetic heparin-binding peptide FN-C/H II, derived from the 33-kD carboxy-terminal fragment of FN A-chains. Initial mouse melanoma cell adhesion to FN-C/H II is largely HS-dependent, since cell adhesion to this peptide is sensitive to heparitinase but not chondroitinase ABC digestion. The mouse melanoma HSPG identified has properties of an integral cell membrane molecule (Hook et al., 1984; Yanagishita et al., 1987) and is anchored through PI, since purified HSPG bound an Octyl Sepharose column, and upon digestion with PI-PLC, the HSPG binding was inhibited. This was confirmed by the metabolic labeling of the HSPG core protein with ^{3}H-ethanolamine, a method used to label the PI-anchor moiety (David et al., 1990; Brunner et al., 1991). Furthermore, neutralizing anti-HSPG antibodies stain the surface of adherent melanoma cells in a punctate fashion. These results strongly support a role for a PI-anchored HSPG in the initial recognition and adhesion of highly metastatic mouse melanoma cells to peptide FN-C/H II.

Iodination of the HSPG followed by heparitinase digestion and SDS-PAGE analysis demonstrated that the isolated HSPG contains a major 63-kD core protein and a much less prevalent 32-kD peptide. Both core proteins were recognized by the polyclonal anti-HSPG antibody used in these studies. While it is possible that the two core proteins represent two distinct gene products, the appearance of the smaller band was variable from preparation to preparation, suggesting that it may represent a proteolytic fragment of the larger 63-kD protein core. Comparison of the Sepharose CL-6B $K_{av's}$ for the intact HSPG or for alkaline borohydride released HS with previously established standards (Wasteson, 1971; Heingard and Hascall, 1974) indicates that the HSPG has an estimated molecular mass of 150–175 kD with HS chains of ~20–25 kD. Considering the estimated size of the HS core protein, we would predict that the melanoma HSPG identified in these studies has two to four HS/core protein, which is similar to what has been observed for other previously described cell surface HSPGs (Gallagher, 1989).

While the exact identity of the core protein(s) identified in the current study has not yet been established, there are biochemical and immunological data that may be helpful to

determine the potential relationship to other previously identified core proteins. Analysis of the major melanoma HSPG core protein by reducing and nonreducing SDS-PAGE indicates that it contains several intrachain disulfide bonds and is PI-PLC sensitive, thereby differentiating it from syndecan, which contains only a single cysteine residue and contains a transmembrane domain (Saunders et al., 1989). Additionally, because these cells express mRNA for the large basement membrane HSPG core protein, we examined the immunological cross-reactivity of mouse melanoma HSPG core protein(s) with a polyclonal antibody to the major EHS basement membrane HSPG, perlecan (Hassell et al., 1980). Mouse melanoma 63- and 32-kD HSPG core proteins did not react with anti-perlecan antibodies, although we can not rule out the possibility that the mouse melanoma HSPG may represent a processed form of the large basement membrane HSPG. The 63-kD mouse melanoma cell surface HSPG does, however, share a number of properties with glypican, a cell surface HSPG described in human lung fibroblasts. Glypican has an estimated core protein molecular mass of 64 kD after heparitinase digestion which is similar to the estimated size of the melanoma HSPG, and upon reduction it exhibits a similar shift in apparent molecular mass. Glypican has also been shown to be linked via phosphatidylinositol (David et al., 1990), as has another HSPG of Schwann cells (Carey and Stahl, 1990). Whether or not these PGs are structurally related to the melanoma cell surface HSPG will be determined in future studies.

Our data support a model in which cell surface HS initiates mouse melanoma cell adhesion to FN-C/H II. This is consistent with previous studies which identify the glycosaminoglycan portion of several proteoglycans as important binding sites for ECM-related and other ligands (Hook et al., 1984; Ruoslahti, 1988; Gallagher, 1989). Using FN-C/H II-OA affinity column chromatography we demonstrated that mouse melanoma HSPG and alkaline borohydride–released HS bind FN-C/H II with the same relative affinity, while the core protein does not bind to FN-C/H-II under the same conditions. Moreover, pretreatment of mouse melanoma cells with heparitinase completely inhibited cell adhesion to FN-C/H II-OA–coated substrata. The specificity of the action of heparitinase on FN-C/H-II–mediated cell adhesion was demonstrated by the failure of the enzyme to inhibit cell adhesion to the control peptide CS1, as well as by the failure of chondroitinase ABC to inhibit melanoma cell adhesion to FN-C/H II. Importantly, these data distinguish these cells from A375 SM human melanoma cells, which express little, if any, cell surface HSPGs (Iida et al., 1992). As might be expected, human melanoma cell adhesion to FN-C/H-II is completely heparitinase-resistant, but is partially sensitive to the effect of chondroitinase ABC (Iida et al., 1992). Collectively, the results from both studies suggest that FN-C/H-II may mediate the adhesion of different cell types in a cell type–specific fashion.

We have shown that mouse melanoma cell adhesion to FN-C/H II is initiated via a HS-dependent mechanism. However, anti-HSPG core protein antibodies, which we have determined to lack HS reactivity (data not shown), can also inhibit cell adhesion to substrata coated with this synthetic peptide. Because anti-HSPG IgG Fab monomers inhibit cell adhesion to FN-C/H II to the same degree, inhibition of cell adhesion to FN-C/H II is not likely to be a result of cell surface HSPG redistribution or endocytosis induced by the anti-HSPG antibodies. Moreover, we have not been able to disrupt binding of purified HSPG to FN-C/H II affinity columns using the anti-HSPG antibodies, suggesting that the inhibitory effects of anti-HSPG on cell adhesion are most likely not due to the ability of the anti-HSPG to disrupt HSPG/FN–C/H II interactions.

While the mechanism(s) of inhibition of the anti-HSPG IgG on melanoma cell adhesion to FN-C/H II remains undefined, it would seem that the core protein plays an important role in mediating cell adhesion that is independent of direct HSPG binding to FN-C/H II. One possibility is that the PI-anchored HSPG core protein could laterally associate with other cell surface adhesion molecules following initial binding to FN-C/H II, and possibly stabilize cell adhesion to FN-C/H II. In support of this hypothesis are studies which demonstrate that PI-anchored proteins have a tenfold greater lateral mobility in the plasma membrane than other integral membrane proteins (reviewed in Cross, 1990). Alternatively, it is possible that FN-C/H-II–mediated ligation of the HSPG can somehow activate specific signal transduction pathways associated with PI turnover. Although it has been suggested that PI-anchored proteins such as Thy-1 (a PI-anchored glycoprotein on neurons and thymocytes) can play a role in signal transduction (Kroczek et al., 1986), the mechanism by which such proteins can transmit signals is not yet clear. Further characterization of this melanoma-associated HSPG (designated as MPIHP-63 for melanoma-associated phosphatidylinositol-anchored HSPG) will yield new insights into the molecular basis for proteoglycan-mediated melanoma cell recognition of the ECM, and could contribute important new concepts for understanding the biology of tumor cell adhesion, invasion, and metastasis.

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