Coordinate Role for Cell Surface Chondroitin Sulfate Proteoglycan and $\alpha 4\beta 1$ Integrin in Mediating Melanoma Cell Adhesion to Fibronectin

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Abstract. Cellular recognition and adhesion to the extracellular matrix (ECM) has a complex molecular basis, involving both integrins and cell surface proteoglycans (PG). The current studies have used specific inhibitors of chondroitin sulfate proteoglycan (CSPG) synthesis along with anti-$\alpha 4$ integrin subunit monoclonal antibodies to demonstrate that human melanoma cell adhesion to an A-chain derived, 33-kD carboxyl-terminal heparin binding fragment of human plasma fibronectin (FN) involves both cell surface CSPG and $\alpha 4\beta 1$ integrin. A direct role for cell surface CSPG in mediating melanoma cell adhesion to this FN fragment was demonstrated by the identification of a cationic synthetic peptide, termed FN-C/H-III, within the fragment. FN-C/H-III is located close to the amino terminal end of the fragment, representing residues #1721–1736 of intact FN. FN-C/H-III binds CSPG directly, can inhibit CSPG binding to the fragment, and promotes melanoma cell adhesion by a CSPG-dependent, $\alpha 4\beta 1$ integrin-independent mechanism. A scrambled version of FN-C/H-III does not inhibit CSPG binding or cell adhesion to the fragment or to FN-C/H-III, indicating that the primary sequence of FN-C/H-III is important for its biological properties.

Previous studies have identified three other synthetic peptides from within this 33-kD FN fragment that promote cell adhesion by an arginyl-glycyl-aspartic acid (RGD) independent mechanism. Two of these synthetic peptides (FN-C/H-I and FN-C/H-II) bind heparin and promote cell adhesion, implicating cell surface PG in mediating cellular recognition of these two peptides. Additionally, a third synthetic peptide, CS1, is located in close proximity to FN-C/H-I and FN-C/H-II and it promotes cell adhesion by an $\alpha 4\beta 1$ integrin-dependent mechanism. In contrast to FN-C/H-III, cellular recognition of these three peptides involved contributions from both CSPG and $\alpha 4$ integrin subunits. Of particular importance are observations demonstrating that CS1-mediated melanoma cell adhesion could be inhibited by interfering with CSPG synthesis or expression. Since CS1 does not bind CSPG, the results suggest that CSPG may modify the function and/or activity of $\alpha 4\beta 1$ integrin on the surface of human melanoma cells. Together, these results support a model in which the PG and integrin binding sites within the 33-kD fragment may act in concert to focus these two cell adhesion receptors into close proximity on the cell surface, thereby influencing initial cellular recognition events that contribute to melanoma cell adhesion on this fragment.

FIBRONECTIN (FN)[1] has long served as a prototype adhesion molecule with which to understand the molecular basis of cell adhesion. Despite the evidence that has demonstrated a role for integrins in mediating cell adhesion to FN (reviewed in Hynes, 1987; Ruoslahti, 1991; Albelda and Buck, 1990; Humphries, 1990; Springer, 1990), there is also evidence for an important role of cell surface proteoglycans (PG) in mediating cell adhesion to FN (Höök et al., 1984; Couchman and Höök, 1988; Saunders and Bernfield, 1988; Yamagata et al., 1989; Gallagher, 1989). PG are complex macromolecules consisting of a core protein to which highly anionic glycosaminoglycan (GAG) chains are covalently attached by an O-linkage to serine or threonine residues on the core protein. The anionic properties of GAG on cell surface PG are important for the binding of many PG to their respective ligands (Höök et al., 1984; Couchman and Höök, 1988; Gallagher, 1989).

We have previously reported that a 33-kD heparin binding fragment, which originates from the carboxy terminal heparin binding region of FN A-chains, is active at promoting the adhesion and spreading of mouse melanoma cells lines in vitro (McCarthy et al., 1986, 1988*a*, 1990). This fragment, which inhibits lung colonization of tumor cells pretreated ex vivo before tail vein injection (McCarthy et al., 1988*b*), also promotes the adhesion of various other cell types such as

1. *Abbreviations used in this paper*: αDX, *p*-nitrophenyl-α-D-xyloside; βDX, *p*-nitrophenyl-β-D-xyloside; CSPG, chondroitin sulfate proteoglycans; EDC, 1-ethyl-3(3-dimethylaminopropyl)-carbodimide hydrochloride; FN, fibronectin; GAG, glycosaminoglycan; NEM, *N*-ethyl-maleimide; OVA, ovalbumin; PG, proteoglycans.

neurons (Rogers et al., 1987; Wait et al., 1987), endothelial cells (Visser et al., 1989), and lymphocytes (Liao et al., 1989; Wayner et al., 1989). The heparin binding properties of this fragment make it a likely ligand for cell surface GAG and PG (Saunders and Bernfield, 1988).

By the use of synthetic peptides (Humphries et al., 1987; McCarthy et al., 1988*a*, 1990; Haugen et al., 1990), cell adhesion to the 33-kD heparin binding fragment of FN A-chains has been demonstrated to involve several distinct sites. Some of these synthetic peptides bind the GAG heparin, suggesting that they might promote cell adhesion by binding to cell surface GAG and PG. Specifically, two synthetic peptides, termed FN-C/H-I (YEKPGSPPREVVPRPRPGV) and FN-C/H-II (KNNQKSEPLIGRKKT), have previously been shown to bind heparin and promote cell adhesion (McCarthy et al., 1988*a*, 1990; Haugen et al., 1990). Both FN-C/H-I and FN-C/H-II are located within type III repeat #14 of human plasma FN, which is near the carboxy-terminal end of the fragment. Another cell adhesion promoting synthetic peptide from within this fragment (Humphries et al., 1987), termed CS1 (DELPQLVTLPHPNLHGPEILDVPST), is located within the alternatively spliced type IIICS domain (or V-region) of FN that is contiguous with type III repeat #14 on FN A-chains. In contrast to FN-C/H-I and FN-C/H-II, CS1 does not bind heparin (McCarthy et al., 1990) and promotes cell adhesion by binding to the $\alpha 4\beta 1$ integrin receptor (Wayner et al., 1989; Mould et al., 1990; Guan and Hynes, 1990). Therefore, cell adhesion to the 33-kD heparin binding fragment of FN A-chains has a complex molecular basis, likely involving multiple cell adhesion promoting determinants that interact with both cell surface PG/GAG and $\alpha 4\beta 1$ integrin.

In this study, we have further examined the molecular basis for the adhesion of highly metastatic human melanoma cells, A375 SM (Fidler, 1986) to the 33-kD fragment of FN. As with many other human melanoma cell lines previously examined (Bumol and Reisfeld, 1982; Ross et al., 1983; reviewed in Gallagher, 1989), these cells express the overwhelming majority of their cell surface PG as chondroitin sulfate proteoglycan (CSPG). Through the use of specific inhibitors of CSPG function, as well as specific anti-$\alpha 4$ integrin mAbs, we demonstrate that the adhesion of these melanoma cells to the 33-kD fragment involves contributions from both $\alpha 4$-integrin subunits and cell surface CSPG.

Furthermore, we have identified a novel heparin binding, cell adhesion–promoting synthetic peptide from within the 33-kD fragment, termed FN-C/H-III (YRVRVTPKEKTGPMKE). In contrast to the other three previously described cell adhesion promoting synthetic peptides from the 33-kD fragment, FN-C/H-III is located within type III repeat #12, representing residues #1721–1736 near the amino-terminal end of the fragment. Cell adhesion to FN-C/H-III was completely resistant to anti-$\alpha 4$ integrin mAbs, but sensitive to specific inhibitors of CSPG function, indicating that FN-C/H-III supports melanoma cell adhesion by a CSPG-dependent mechanism. In contrast, melanoma cell adhesion to the other three synthetic peptides was sensitive to anti-$\alpha 4$ integrin mAbs, and to a lesser extent, to CSPG inhibitors. The results suggest that these various cell adhesion promoting sites on this fragment act in a coordinated fashion to bind a melanoma cell surface receptor complex, consisting of CSPG and $\alpha 4\beta 1$ integrin, thereby influencing early cellular recognition events that contribute to cell adhesion on the fragment.

Materials and Methods

Cell Culture

Highly metastatic human melanoma cells, A375SM, which were selected by in vivo experimental metastasis assays of parent A375P cells in nude mice (Fidler, 1986) were kindly provided by Dr. I. J. Fidler (M.D. Anderson Hospital Cancer Center, Houston, TX). The cells were maintained in MEM supplemented with 10% FCS, vitamin solution, and 1 mM sodium pyruvate. Cells were routinely used after less than 15 passages from frozen stocks to minimize phenotypic drift.

Reagents

BSA (fraction V, fatty acid free) was purchased from Miles Laboratory (Elkhart, IN). Heparitinase, chondroitinase ABC (protease free), and chondroitinase ACII were purchased from Seikagaku America Inc. (Bethesda, MD). *p*-Nitrophenyl-α-D-xylopyranoside (αDX) was purchased from Koch-Light Ltd. (Suffole, England). 1-ethyl-3{3-dimenthylaminopropyl)-carbodimide hydrochloride (EDC), 3-{3-Cholamidopropyl-dimethylammonio]l-propanesulfate (CHAPS), p-nitrophenyl-β-D-xylopyranoside (βDX), heparin (porcine intestinal mucosa, grade I, 15 kD), Sepharose CL-4B, Sepharose CL-6B, DEAE-Sephacel, CNBr-activated Sepharose CL-4B, *N*-ethyl-maleimide (NEM), PMSF, and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). FITC-goat anti-mouse IgG was purchased from Organon Teknika Corp. (Durham, NC).

mAbs

mAbs against specific integrin subunits have been previously described (Wayner et al., 1988, 1989; Wayner and Kovach, 1992). These mAbs include: P4C2 and P4G9 (anti-α4-integrin), PlD6 (anti-α5 integrin), and P4C10 (anti-β1 integrin).

Protein Isolation

Human plasma FN was purified as a by-product of factor VIII production by sequential ion exchange and gelatin affinity chromatography as described (McCarthy et al., 1986, 1988*a*). The tryptic/catheptic 33-kD heparin binding fragment of FN A-chains was purified according to methods previously reported (McCarthy et al., 1988a). The 75-kD fragment was prepared by extensive trypsinization of FN followed by purification over sequential mAb 180-8 affinity and Spherogel TSK-3,000 columns as previously described (McCarthy et al., 1986). Purity of FN, the 33-kD fragment, and the 75-kD fragment was verified by SDS-PAGE and Coomassie brilliant blue staining.

Peptide Synthesis and Characterization

Peptides from FN were synthesized at the Microchemical Facility of the University of Minnesota (Minneapolis, MN) using a peptide synthesizer (system 990; Beckman Instruments, Inc., Palo Alto, CA). 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 110°C (McCarthy et al., 1990; Haugen et al., 1990; Skubitz et al., 1990). These peptides included FN-C/H-I (YEKPGSPPREVVPRPRPGV; representing residues #1906–1924 of FN A-chains), FN-C/H-II (KNNQKSEPLIGRKKT; residues #1946–1960), CS-1 (DELPQLVTLPHPNLHGPEILDVPST; residues #1961–1985), FN-C/H-III (YRVRVTPKEKTGPMKE; residues #1721–1736), and scrambled FN-C/H-III (MKGKVTREVRYPTKPE). All peptides were synthesized with a tyrosine residue at the carboxy-terminal end to facilitate radioiodination of the peptides for certain analyses.

Conjugation of the Peptides to Ovalbumin

Synthetic peptides were chemically conjugated to ovalbumin (OVA) for all

experiments using EDC, since previous studies have shown that the coupling of synthetic peptides to large carrier proteins results in enhanced heparin binding activity and cell adhesion activities (Humphries et al., 1987; McCarthy et al., 1990; Haugen et al., 1990). Briefly, equal amounts (by weight) of the peptides and OVA were solubilized in water and mixed with a 10-fold excess (by weight) of EDC dissolved in water. The sample was then mixed overnight at 4°C on a circular rotator. The coupled peptides were then dialyzed extensively against PBS to remove the excess EDC and uncoupled peptide (Spectrapore 6, 10-kD exclusion; Spectrum Medical Industries, Los Angeles, CA). The molar ratio of each of the peptides to OVA was 5:1, as determined by using a trace amount of radioactive peptides in the coupling reactions. The conjugates were stored at −20°C until use.

Determination of Peptide Binding to Plastic Wells

Each peptide was radiolabeled with ^{125}Iodine (New England Nuclear, Boston, MA) using an aqueous chloramine T reaction as described by Haugen et al. (1990). Plates were coated with the radiolabeled peptides, which had been coupled to OVA as described above, for 24 h at 37°C. Plates were blocked with PBS/BSA (PBS containing 2 mg/ml BSA) for 3 h and then rinsed three times with the same buffer. Peptide remaining bound was solubilized with 0.5 N NaOH and 1% SDS, and bound radioactivity was quantitated in a Gamma Trac Gamma counter (TM Analytic, Elkgrove Village, IN). The amount of peptide bound was calculated based on the amount of radioactivity recovered and the specific activity of the peptide/OVA conjugates.

Extraction of Cell Surface–associated Proteoglycans

Cell surface PG were extracted from cells radiolabeled with $^{35}SO_4$ according to the methods described by Ross et al. (1988). Briefly, subconfluent cells were radiolabeled overnight with carrier-free $Na_2{}^{35}SO_4$ (sp ac 0.72 Ci/mg; New England Nuclear) at a final concentration of 59 μCi/ml. Monolayer cells were washed with cold serum-free MEM two times, and then cells were incubated for 30 min at 18°C with MEM containing 50 μg/ml heparin to remove weakly associated GAG on the cell surface (Yanagishita et al., 1987). Cells were harvested with 1 mM EDTA and washed three times with cold PBS. Cells (5×10^7 total) were incubated for 60 min at 4°C with 50 ml of extraction buffer that contained 10 mM Tris-HCl (pH 7.1), 0.9% (vol/vol) NP-40, 2 mM EDTA, 0.25 mM DTT, 1 mM NEM, and 1 mM PMSF with gentle shaking. The suspension was centrifuged at 2,000 rpm for 10 min to remove the insoluble cell debris. The supernatant was collected and then centrifuged at 10,000 rpm for 30 min. One-fifth volume of RNase (Sigma Chemical Co.) was added to the supernatant and then incubated for 60 min at 37°C. Crude PG were extensively dialyzed against "urea buffer" containing 6 M urea, 50 mM Tris-HCl (pH 7.0), 10 mM EDTA, 0.1 mM PMSF, 1 mM NEM, and 10 mM 6-aminocaproic acid, and then separated on DEAE-Sephacel chromatography as described below.

Anion-Exchange Column Chromatography of PG

DEAE-Sephacel beads (20-ml bed volume) were packed in a glass column equilibrated with the urea buffer. The crude PG were applied to the column and washed with three column volumes of the urea buffer to remove unbound material. The bound material was eluted with a linear gradient (0 to 1 M) of NaCl in urea buffer, and 1-ml fractions were collected. The radioactive fractions were pooled, extensively dialyzed against water, and then lyophilized. Recovery of [^{35}S]sulfate-labeled macromolecules from this column was routinely >90%.

Size Exclusion Column Chromatography

Hydrodynamic properties of [^{35}S]sulfate-labeled molecules were evaluated by size exclusion chromatography on 100 × 1 cm columns of Sepharose CL-4B equilibrated with 1% Triton X-100, 4 M guanidine-hydrochloride, and 50 mM sodium acetate (pH 5.8). The V_o and V_t of this column were determined as described previously (Klein et al., 1986). Separation was carried out at room temperature at a flow rate of 3 ml/h, and recovery from this column was >95%.

Generation of Glycosaminoglycan Chains

GAG were removed from the core protein by alkaline β-elimination (Klein et al., 1986). Briefly, purified lyophilized PG were dissolved in water and incubated with 2 M $NaBH_4$ and 0.05 M NaOH (final concentrations) for 24 h at 45°C. The reaction was stopped by adding glacial acetic acid to a pH of 5 to 6. The reaction mixture was applied to a Sephadex G-50 column, equilibrated with 0.13 M formic acid and 0.073 M NH_4OH to desalt the reaction and to ensure that the GAG were not non-specifically degraded by the treatment. Under these conditions, over 99% of applied GAG were recovered from the V_o fractions. The V_o fractions were pooled, lyophilized, and further characterized for GAG type and hydrodynamic properties.

Characterization of GAG Chain

To purify and evaluate the hydrodynamic properties of the alkaline borohydride released GAG, the samples were applied to a Sepharose CL-6B column as described by Klein et al. (1986). Nitrous acid deaminative cleavage was performed by first solubilizing GAG in 100 μl of water, 500 μl of 1 N HCl, and 500 μl of *N*-butyl nitrate (Sigma Chemical Co.). The reaction was carried out for 2 h at room temperature and was stopped by adding 500 μl of 1 N NaOH. The samples were frozen, lyophilized and applied to Sephadex G-50 columns, equilibrated with 0.13 M formic acid, and 0.073 M NH_4OH. Nitrous acid–resistant GAG, eluting in the V_o of the Sephadex G-50 columns, were then treated with 1 U of chondroitinase ABC or chondroitinase ACII in 500 μl of 0.1 M Tris-HCl (pH 8.0) containing 0.03 M sodium acetate, 0.01 M EDTA, 0.01 M NEM, 0.01 M PMSF, and 0.36 mM pepstatin for 3 h at 37°C as described previously (Klein et al., 1986). After the enzyme treatment, samples were reapplied to a Sephadex G-50 column and analyzed for relative sensitivity to chondroitinase digestion. Percent recovery from these columns was routinely ≥95%.

Purification of PG or GAG on Ligand Affinity Columns

Synthetic peptides coupled to OVA, OVA/OVA, or the 33-kD fragment were coupled to CNBr-activated Sepharose 4B according to the methods recommended by the manufacturer. PG or GAG were dissolved in 20 mM Tris-HCl (pH 6.8), 50 mM NaCl, 0.5% CHAPS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, and 1 mM NEM (column buffer), and then applied on OVA/OVA conjugate beads (sham column) to remove PG or GAG that nonspecifically bind to the beads. The flow through fractions (>95% of applied radioactivity) were collected and applied to the FN-C/H-III, CS1, or 33-kD fragment coupled columns. After washing the columns with 30 ml of column buffer, the bound radioactivity was eluted with a linear gradient of NaCl.

In some experiments, the purified PG were preincubated with FN-C/H-III/OVA, scrambled FN-C/H-III/OVA, or OVA/OVA for 2 h at 4°C and then applied to a 33-kD fragment coupled column that had been equilibrated with column buffer containing FN-C/H-III/OVA, scrambled FN-C/H-III/OVA, or OVA/OVA. The bound radioactivity was eluted as described above.

Solid Phase [^{3}H]heparin Binding Assay

Commercial [^{3}H]heparin (0.5 mCi/mg sp ac; ICN Immunobiologicals, Irvine, CA) was purified by charge and size on HPLC-DEAE and Sepharose CL-6B columns as described previously (McCarthy et al., 1990). The ability of various ligands to bind purified [^{3}H]heparin was assessed with a solid-phase binding assay using polystyrene 96-well microtiter plates (Immulon I; Dynatech Laboratories Inc., Chantilly, VA) as a support, as previously described (McCarthy et al., 1988*a*, 1990). The substrata were adsorbed with various ligands as for the cell adhesion assays described below. The plates were then blocked with PBS/BSA, washed, and [^{3}H]heparin was added to the wells in PBS/BSA containing 0.1% CHAPS. The plates were then incubated for 2 h at 37°C, at which time they were washed five times with PBS/BSA/CHAPS to remove the unbound [^{3}H]heparin. The bound [^{3}H]heparin was solubilized with 0.5 N NaOH containing 1% SDS, and quantitated using a liquid scintillation counter (model 3801; Beckman Instruments). Specific binding of [^{3}H]heparin was assessed by performing the binding in the presence of a 100-molar excess of unlabeled heparin.

Generation and Purification of Polyclonal Antibodies against FN-C/H-III

Polyclonal antibodies against FN-C/H-III were generated according to the methods described by McCarthy et al. (1990). Briefly, FN-C/H-III was coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical Co.) using EDC as a coupling reagent as described above for coupling peptides to OVA. The conjugate was mixed with Freund's adjuvant and then used to immunize

New Zealand White Rabbits. IgG was purified from pooled immune sera by precipitation with ammonium sulfate followed by DEAE anion exchange chromatography as described previously (Skubitz et al., 1988). Purity of the IgG was determined by SDS-PAGE and Coomassie brilliant blue staining of the gels. Specificity of the purified IgG was then determined by ELISA as previously described (McCarthy et al., 1990; Haugen et al., 1990; Chelberg et al., 1990).

Cell Adhesion Assays

Cell adhesion assays were performed as described previously with minor modifications (McCarthy et al., 1990; Chelberg et al., 1990; Haugen et al., 1990). Briefly, purified proteins or the peptide–OVA conjugates were diluted to various concentrations in PBS, and 50-μl aliquots were dispensed into Immulon 1 polystyrene microtiter wells. The wells were incubated at 37°C for 4 h, and nonspecific sites on the plastic were then blocked by treating the wells with 200 μl of PBS/BSA. Subconfluent A375SM human melanoma cells that had been radiolabeled overnight with [^{3}H]thymidine (^{3}HTdR, sp ac 6.7 Ci/mmol; New England Nuclear, Boston, MA), were harvested by rinsing with 1 mM EDTA, washed two times with MEM/BSA (MEM containing 2 mg/ml BSA and 0.15 mM Hepes, pH 7.2) and adjusted to a concentration of 10^5 cells/ml in the same medium. Aliquots of 100 μl of the cell suspension were dispensed into the wells and the cells were incubated at 37°C for various lengths of time, as indicated in the Results section. The assays were terminated by aspirating loosely bound and unbound cells from the wells, washing the wells three times, and solubilizing the bound cells in 0.5 N NaOH containing 1% SDS. Bound radioactivity, determined in a liquid scintillation counter (model 3801; Beckman Instruments), was used to calculate the percentage of cells that remained adherent to each substratum relative to the total number of cells added to each well. Unless otherwise indicated, the data represent the means of triplicate determinations.

Inhibition of Cell Adhesion

Plates were coated with the 33-kD fragment or peptide/OVA conjugates at concentrations that promoted half maximal cell adhesion (4.2 pmole of the 33-kD fragment, 20 nmole of FN-C/H-I, 20 nmole of FN-C/H-II, 100 nmole of FN-C/H-III, or 80 nmole of CS1). The plates were then preincubated with various concentrations of polyclonal IgG against FN-C/H-III or purified normal rabbit IgG for 60 min at room temperature. Radiolabeled cells were then incubated in the plates, in the continued presence of the IgG, for 30 min as previously described (McCarthy et al., 1990; Haugen et al., 1990).

Inhibition assays using mAbs against specific integrin subunits were performed as previously described (Wayner et al., 1989; Wayner and Kovach, 1992). Briefly, radiolabeled cells were preincubated with the mAbs for 20 min at room temperature. The cells were then added to wells precoated with half-maximal concentrations of peptides or proteins, and incubated for 30 min at 37°C. The wells were then washed and adherent cells were quantitated as for the cell adhesion assays, as described above.

Chondroitinase ABC or p-Nitrophenyl β-D-xyloside Pretreatments

Treatment of cells with chondroitinase ABC, to remove CS GAG from cell surface CSPGs, was carried out before the cell adhesion assays based on the methods described by Saunders and Bernfield (1988). Briefly, melanoma cells radiolabeled with ^{3}H-TdR were harvested, pelleted, and resuspended in MEM/BSA in the presence or absence of 0.5 U/ml of protease-free chondroitinase ABC or heparitinase. The resuspended cells were then preincubated for 20 min at 37°C prior to dispensing into the microtiter wells. Chondroitinase ABC or heparitinase at 0.5 U/ml was included in the assay to prevent re-expression of intact cell surface CSPGs or HSPGs, respectively, during the course of the 30-min assay.

As a second approach, melanoma cells were pretreated for 24 h with 1 mM *p*-nitrophenyl βDX to inhibit the expression of intact CSPG. αDX, an inactive analogue of βDX, was also used in a similar manner for some experiments as a negative control. βDX has been previously shown to interfere with CSPG synthesis by competing for galactosyl-transferase I, resulting in the uncoupling of CS GAG synthesis from that of CSPG (Schwartz, 1977; Kato et al., 1978; Lohmander et al., 1979). Cells were then harvested as described for the cell adhesion assay and adjusted to the appropriate concentration in MEM/BSA containing 1 mM βDX or αDX to prevent re-expression of the CSPG during the course of the 30-min assay.

Flow Cytometry

Cells were harvested with 1 mM EDTA and washed three times with MEM containing 2 mg/ml BSA and 0.02% NaN_3. Aliquots of 10^6 cells were incubated in 100 μl of the same buffer including primary antibodies (final dilution of 1:100) for 35 min at room temperature with tapping every 5 min. Cells were washed with the buffer three times and incubated in 100 μl of the buffer including FITC-goat anti-mouse IgG (final dilution of 1:250) for 35 min at room temperature with tapping every 5 min. The titers of the primary and secondary antibodies were maximized under these experimental conditions. Cells were washed with the buffer three times, resuspended in 200 μl of the buffer, and then analyzed on a FACS Star (FACS is a registered trademark of Becton-Dickinson & Co., Mountain View, CA).

Results

A375SM Cells Express Predominantly Chondroitin Sulfate Proteoglycans

We first isolated and partially characterized $^{35}SO_4$-PG from human melanoma cells. Others have demonstrated (Bumol and Reisfeld, 1982; Ross et al., 1983; reviewed in Gallagher, 1989) that many human melanoma cell lines almost exclusively express CSPG on their surface. To determine if the A375SM human melanoma cells used in our studies are similar in this regard, $^{35}SO_4$-PG were detergent extracted, partially purified, and characterized as described in Materials and Methods. As an initial purification step, detergent extracted $^{35}SO_4$-labeled macromolecules were purified by anion exchange chromatography, eluting as a broad peak from DEAE columns at 0.45 M NaCl (Fig. 1 *A*). Fractions from the DEAE column containing $^{35}SO_4$-macromolecules were pooled and subjected to dissociative size exclusion chromatography on Sepharose CL-4B columns (Fig. 1 *B*). The $^{35}SO_4$-macromolecules had a large hydrodynamic size, eluting in the V_o of these columns. Alkaline borohydride reduction of the $^{35}SO_4$-macromolecules pooled from the fractions of the Sepharose CL-4B column greatly retarded their elution from size exclusion columns, eluting at K_{av} of 0.6 from Sepharose CL-6B columns (Fig. 1 *C*), which is what would be expected if the radioactive sulfate had been incorporated into O-linked GAG. The $^{35}SO_4$-GAG were 95% resistant to degradation by nitrous acid and 90–95% sensitive to treatment with chondroitinase ABC or chondroitinase AC II (not shown), consistent with their identity as CS.

Human Melanoma Cell Adhesion to the 33-kD Fragment Involves both CSPG and α4β1 Integrin-dependent Mechanisms

We next evaluated the relative contributions of melanoma cell associated PG and the α4β1 integrin receptor in mediating A375SM human melanoma cell adhesion to the 33-kD fragment of FN, since this fragment can act as ligand for both PG/GAG and α4β1 integrin (McCarthy et al., 1988, 1990; Saunders et al., 1988; Wayner et al., 1989; Haugen et al., 1990; Mould et al., 1990). To initially evaluate potential contributions of cell surface PG in mediating melanoma cell adhesion, the cells were pretreated with either chondroitinase ABC or heparitinase to determine if removal of either cell surface CS or HS could interfere with melanoma cell adhesion to this fragment. Chondroitinase ABC pretreatment of the melanoma cells caused a partial (55%) inhibition of melanoma cell adhesion to the 33-kD fragment, whereas pretreatment with heparitinase had no effect on

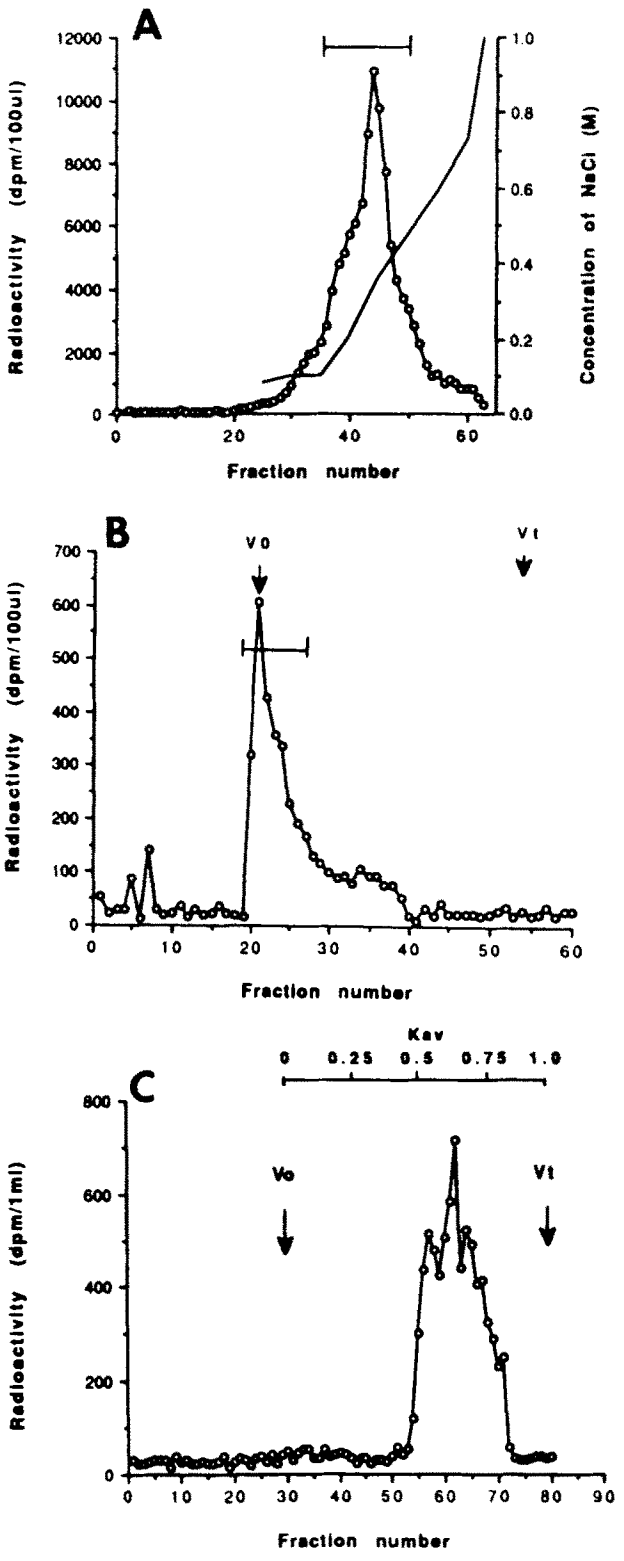


Figure 1. A375 SM human melanoma cells express predominantly CSPG. Cell cultures were metabolically labeled overnight in the presence of 50 μCi/ml of [^{35}S]sulfate and the cell layer was subsequently harvested and dialyzed. (*A*) The dialyzed extract was then applied to a DEAE-Sephacel column and eluted using a linear NaCl gradient from 0.1 to 0.8 M as indicated (————). The radioactive fractions were then pooled (indicated by the bracket), dialyzed, and lyophilized. (*B*) The pooled fractions were applied to a Sepharose CL-4B column under dissociative conditions. The indicated fractions in brackets, including the V_o (⊢————⊣), were collected, dialyzed, and lyophilized. (*C*) The pooled purified PG from the Sepharose CL-4B column were treated with alkaline borohydride and then desalted on a Sephadex G-50 column. The resulting GAG were then applied to a Sepharose CL-6B column.

melanoma cell adhesion. These results support our data that these human melanoma cells express mainly CSPG (Fig. 1) and also indicate that cell surface CS partially mediates melanoma cell adhesion to the 33-kD fragment of FN.

As a further test of the potential involvement of cell surface CS in mediating melanoma cell adhesion, the cells were treated for 24 h with 1 mM βDX, an inhibitor of CSPG synthesis (Schwartz, 1977; Kato et al., 1978; Lohmander 1979; Robinson and Gospodarowicz, 1984). As was observed with chondroitinase ABC pretreatment, the exposure of these cells to 1 mM βDX also caused a significant (~50%), but not complete, inhibition in melanoma cell adhesion to the 33-kD fragment. The inactive analogue of βDX, αDX, had no effect on melanoma cell adhesion to the fragment. The inhibition of cell adhesion produced by these treatments was not due to cytotoxic effects of the chondroitinase ABC as assessed by trypan blue exclusion as well as by incorporation of ^{3}H-TdR or [^{35}S]methionine into cells after treatment with βDX or αDX (not shown).

To ensure that these treatments did not affect the cell surface expression of α4β1 integrin, the treated cells were examined in the presence of specific anti-α4 integrin or anti-β1 mAbs by flow cytometry (Fig. 2). These experiments demonstrated that A375SM cells express both α4 and β1 integrin subunits, consistent with previous reports (Wayner et al., 1989; Mould et al., 1990). Importantly, cells pretreated for 24 h with 1 mM βDX (Fig. 2) or chondroitinase ABC (not shown) exhibit identical levels of α4 and β1 integrin subunits compared to untreated controls. These results indicate that the inhibition of cell adhesion observed in the presence of the CSPG antagonists was not due to removal of α4β1 integrin from the cell surface, thus further supporting the notion that cell surface CSPGs play a direct role in mediating human melanoma cell adhesion to the 33-kD fragment.

As expected based on previous reports (Wayner et al., 1989; Mould et al., 1990; Wayner and Kovach, 1992), melanoma cell adhesion to the 33-kD fragment was also inhibited in a concentration dependent fashion by the addition of anti-α4 integrin mAb (Fig. 3 *A*). In contrast to the effects of CSPG antagonists on cell adhesion to this fragment, the inhibition observed in the presence of high levels of certain anti-α4 integrin mAb (P4C2) was essentially complete (90%). However, another anti-α4 integrin mAb (P4G9) could only partially (50–60%) inhibit melanoma cell adhesion to the 33-kD fragment, even when high concentrations (1:25 dilution of P4G9 ascites) of the mAb were tested (not shown). Anti-α5 integrin mAb (P1D6), which completely inhibited melanoma cell adhesion to a tryptic 75-kD RGD-containing fragment of FN (not shown), had no effect on melanoma cell adhesion to the 33-kD fragment, even at the highest concentration tested (Fig. 3 *A*). Similarly, anti-β1 integrin mAb (P4C10) could also inhibit melanoma cell adhesion to 33-kD fragment or to the RGD-containing 75-kD fragment (not shown). These results confirm previous reports (Wayner et

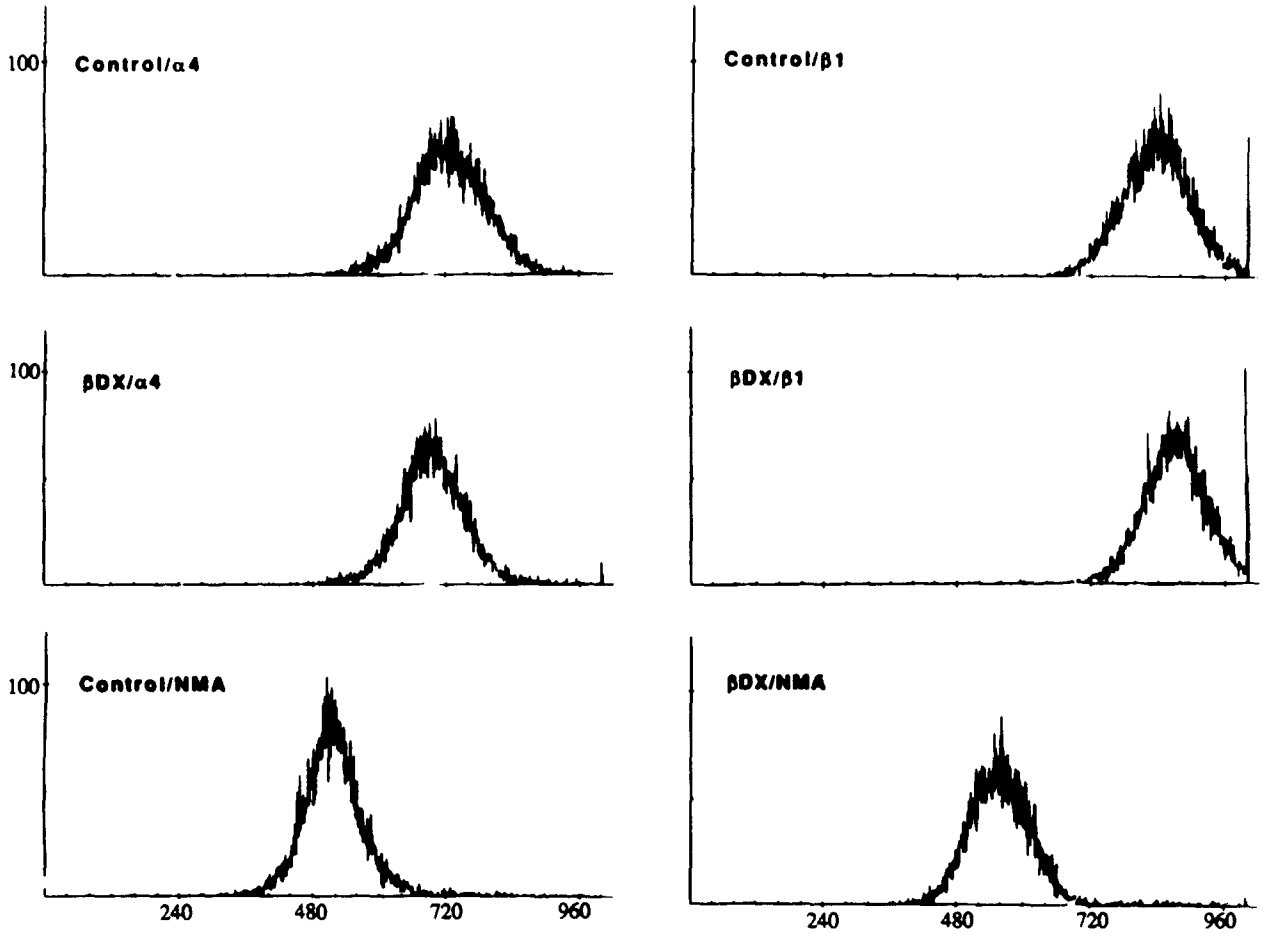


Figure 2. Inhibition of CSPG synthesis by βDX does not affect cell surface expression of α4β1 integrin. Cultured melanoma cells were preincubated (or not) with 1 mM βDX for 24 h as described in Materials and Methods. Cultured cells were then released using EDTA and incubated with anti-α4 integrin mAb (P4C2), anti-β1 integrin mAb (P4C10), or normal mouse ascites (*NMA*) at final dilutions of 1:100 for 30 min at room temperature. Cells were washed three times, incubated with FITC goat anti-mouse IgG at a final dilution of 1:250 for 30 min at room temperature and then analyzed by flow cytometry.

al., 1989; Mould et al., 1990) and illustrate that specific integrin subunits are important for melanoma cell recognition of the 33-kD fragment.

The inhibitory effects of submaximal levels (1:625 dilution) of anti-α4 integrin mAb (P4C2) on melanoma cell adhesion to this fragment could be potentiated by pretreating melanoma cells with chondroitinase ABC (Fig. 3 *B*) or 1 mM βDX (not shown). Similar results were obtained when using suboptimal levels of another anti-α4 integrin mAb (P4G9; not shown). No such potentiation of inhibition was observed in the presence of anti-α5 integrin mAb (Fig. 3 *B*) or normal mouse ascites (not shown), demonstrating that the chondroitinase ABC-mediated potentiation of anti-α4 integrin subunit mAb was specific in nature. These results suggest that α4β1 integrin and cell surface CSPG play a closely coordinated role in mediating melanoma cell adhesion to the 33-kD fragment; consistent with our hypothesis that cell adhesion to this fragment involves both α4β1 integrin-dependent and cell surface PG-dependent mechanisms (Haugen et al., 1990; McCarthy et al., 1990).

Synthetic Peptide FN-C/H-III Binds [³H]heparin and Promotes Cell Adhesion

We have previously used the properties of positive net charge and negative net hydropathy indices as selection criteria for synthesizing FN-C/H-I and FN-C/H-II, two peptides that bind GAG and promote cell adhesion (McCarthy et al., 1988, 1990; Haugen et al., 1990). Using these same criteria, we have synthesized a novel peptide, termed FN-C/H-III, which is in type III repeat #12 of human plasma FN. FN-C/H-III (YRVRVTPKEKTGPMKE) represents FN residues #1,721–1,736 and would be predicted to occur in all isoforms of FN identified to date (Kornblihtt et al., 1985). In contrast to FN-C/H-I, FN-C/H-II, and CS1, peptide FN-C/H-III is located near the amino-terminal end of the 33-kD fragment. FN-C/H-III is similar to FN-C/H-I and FN-C/H-II in that it is also cationic (net charge of +3) and relatively hydrophilic (net hydropathy index of −23.7). FN-C/H-III was coupled to OVA, adsorbed to microtiter wells (Haugen et al., 1990; McCarthy et al., 1990), and evaluated for the ability to bind [³H]heparin or promote melanoma cell adhesion.

Purified [³H]heparin bound to substrata coated with 10 μM FN-C/H-III in a concentration-dependent, specific, and saturable manner, while negligible amounts of [³H]heparin bound to OVA/OVA (not shown). FN-C/H-III also promoted melanoma cell adhesion in a concentration dependent manner (Fig. 4 *A*). Soluble FN-C/H-III inhibited cell adhesion to substrata coated with FN-C/H-III/OVA, whereas OVA/OVA had no inhibitory effect on melanoma cell adhesion

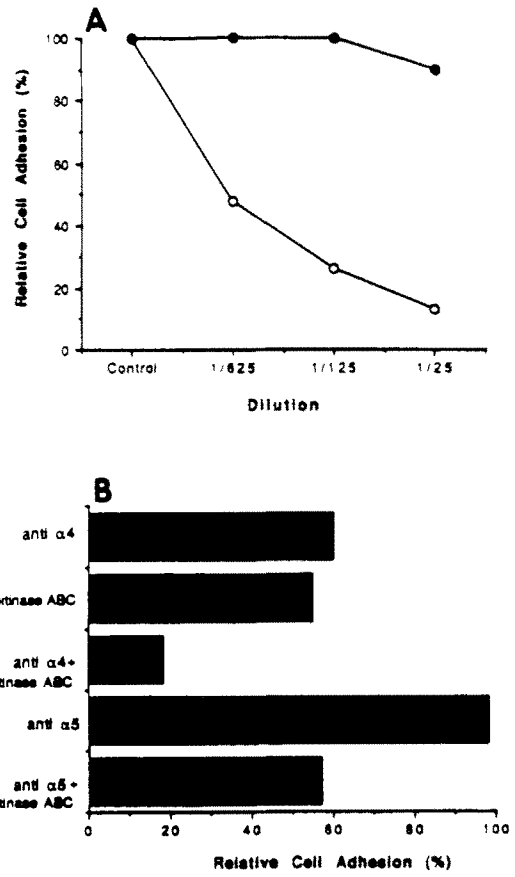
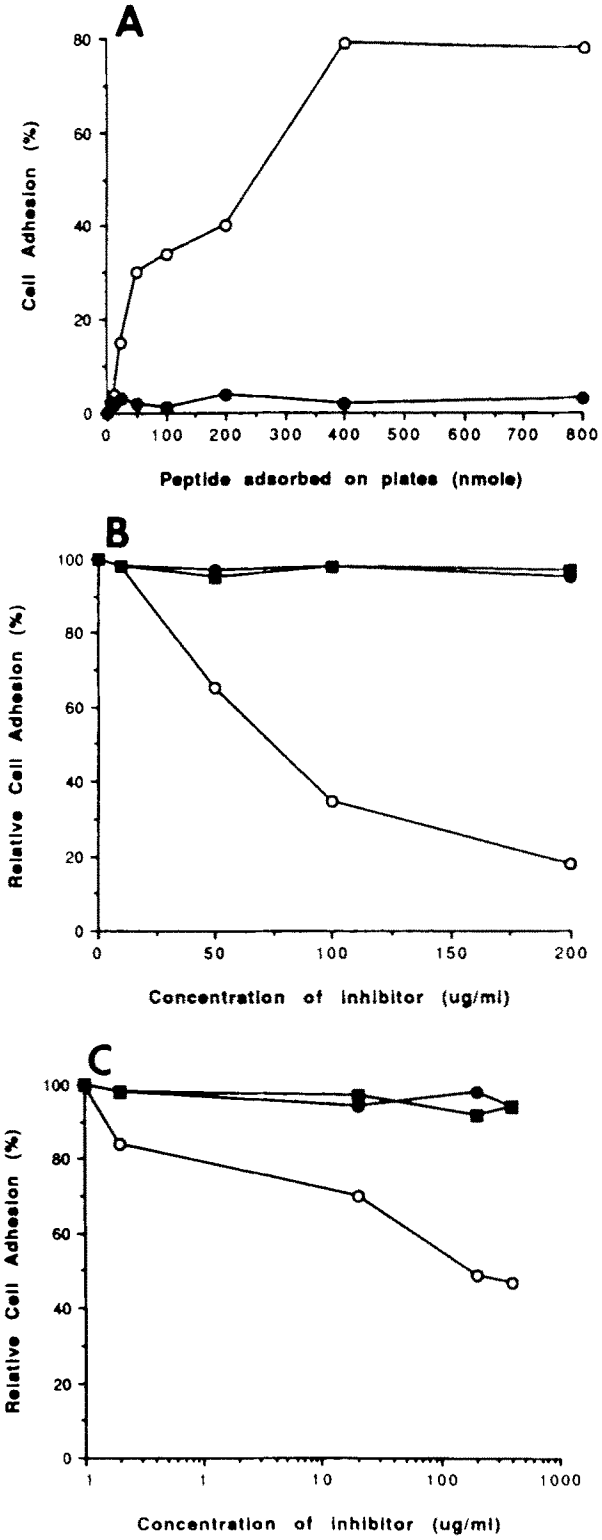


Figure 3. Melanoma cell adhesion to the 33-kD fragment of FN involves both $\alpha 4\beta 1$ integrin and cell surface CSPG. (*A*) Microtiter wells were coated with 4.2 pmoles of the 33-kD fragment, which promoted half-maximal cell adhesion, and then blocked with PBS/BSA as described in Materials and Methods. Cells were preincubated with serial dilutions of anti-$\alpha 4$ integrin mAb (P4C2, ○) or anti-$\alpha 5$ integrin subunit mAb (PlD6, ●) for 30 min at room temperature before the cell adhesion assay. (*B*) Cells were pretreated with chondroitinase ABC and/or a 1:625 dilution of anti-$\alpha 4$ integrin mAb (P4C2) or anti-$\alpha 5$ integrin mAb (PlD6). In each experiment, the cell adhesion assays were carried out for 30 min in the continued presence of anti-integrin subunit mAbs and/or chondroitinase ABC. The standard error of means (SEMs) were <5% of mean values in each case. Data points represent the means of triplicate determinations.

to FN-C/H-III coated substrata (Fig. 4 *B*). In addition, a scrambled version of FN-C/H-III (MKGKVTREVRYPTK-PEY), with the same amino acid composition, net hydropathy index, and net charge as FN-C/H-III, failed to inhibit melanoma cell adhesion to FN-C/H-III coated substrata (Fig. 4 *B*); suggesting that primary sequence, and not just net charge, is important for the cell adhesion promoting activity of FN-C/H-III.

Soluble FN-C/H-III/OVA also inhibited melanoma cell adhesion to the 33-kD fragment in a concentration dependent manner (Fig. 4 *C*). As has been previously observed



III/OVA (○) or OVA/OVA (●) and evaluated for their ability to support melanoma cell adhesion in a 30-min assay as described in Materials and Methods. Alternatively, microtiter wells were coated with 100 nmoles of FN-C/H-III (*B*) or 4.2 pmoles of purified 33-kD fragment (*C*), and evaluated for their ability to support melanoma cell adhesion in the presence of various concentrations of FN-C/H-III/OVA (○), scrambled FN-C/H-III/OVA (■), or OVA/OVA (●) as described in Materials and Methods. SEMs were <10% of mean values in each case, and each experimental point represents the mean of triplicate values.

Figure 4. FN-C/H-III represents a novel cell adhesion promoting synthetic peptide from within the 33-kD fragment of FN. (*A*) Microtiter wells were coated with the indicated amounts of FN-C/H-

Table I. Effects of Polyclonal Anti FN-C/H-III IgG on Melanoma Cell Adhesion to the Various Synthetic Peptides

	Relative Cell Adhesion‡		
	Concentration of IgG (μg/ml)		
Substratum*	4	20	100
FN-C/H-III	78§	72§	25§
FN-C/H-II	110	105	91
FN-C/H-I	93	95	105
CS1	85	95	91

* Plates were coated with the peptides at concentrations that promoted half-maximal cell adhesion as described in Materials and Methods.
‡ IgG was diluted in MEM/BSA to the indicated concentrations and incubated in the wells for 60 min as described in Materials and Methods. Cell adhesion assays were carried out for 30 min as described in Materials and Methods. Data is expressed as a percentage of the cells that adhered in the absence of IgG. Normal rabbit IgG did not affect cell adhesion, even at a concentration of 500 μg/ml (not shown). Data represent the mean of triplicate determinations, and SEMS were <5% of mean values in each case.
§ $P < 0.001$ from values observed in the absence of IgG (by Student's two tailed t test).

for these experiments using FN-C/H-I, FN-C/H-II, or CS1 (Haugen et al., 1990; McCarthy et al., 1990), soluble FN-C/H-III–mediated inhibition of melanoma cell adhesion to the fragment was never complete, reaching levels of ~50–60% at the highest concentrations tested. These results are consistent with the notion that the 33-kD fragment contains multiple distinct cell adhesion promoting sites. Furthermore, OVA/OVA or scrambled FN-C/H-III/OVA were totally ineffective at inhibiting melanoma cell adhesion to the 33-kD fragment, emphasizing the importance of the primary structure of FN-C/H-III in mediating melanoma cell adhesion, either as a synthetic peptide or when present in the context of the larger fragment.

Polyclonal antibodies generated against peptide FN-C/H-III were next evaluated for their ability to inhibit melanoma cell adhesion to FN-C/H-III/OVA or the 33-kD fragment. By ELISA, the purified anti-FN-C/H-III specifically recognized FN-C/H-III and the 33-kD heparin binding fragment of FN, but did not recognize substrata coated with the negative control peptides FN-C/H-I, FN-C/H-II, or CS1 (not shown). The anti-FN-C/H-III IgG specifically inhibited cell adhesion to substrata coated with peptide FN-C/H-III, yet did not inhibit melanoma cell adhesion to substrata coated with the control peptides FN-C/H-I, FN-C/H-II, or CS1 (Table I). Anti-FN-C/H-III IgG also inhibited melanoma cell adhesion to substrata coated with the 33-kD fragment (not shown). However, this effect was only partial (30–35%), which is similar to results we have previously obtained using anti-FN-C/H-I or anti-FN-C/H-II IgG to inhibit cell adhesion to this fragment (McCarthy et al., 1990; Haugen et al., 1990). These results demonstrate that FN-C/H-III is an active cell adhesion promoting site within the 33-kD fragment, and are consistent with the notion that FN-C/H-III represents one of several such active sites within the fragment.

FN-C/H-III Promotes Cell Adhesion by a CSPG-dependent Mechanism

To initially examine the potential contribution of cell surface CSPG to cell adhesion on FN-C/H-III, cells were pretreated with chondroitinase ABC, heparitinase, βDX, or αDX before cell adhesion assays. Cell adhesion to FN-C/H-III was inhibited by treatment of the cells with chondroitinase ABC or βDX but not by heparitinase or αDX (Fig. 5 *A*). In contrast, neutralizing anti-α4 integrin subunit mAb (P4C2 or P4G9), which can effectively inhibit melanoma cell adhesion to the 33-kD fragment, did not inhibit cell adhesion to FN-C/H-III coated substrata (Fig. 5 *B*), indicating that melanoma cell adhesion to FN-C/H-III is CS dependent and α4 integrin subunit independent.

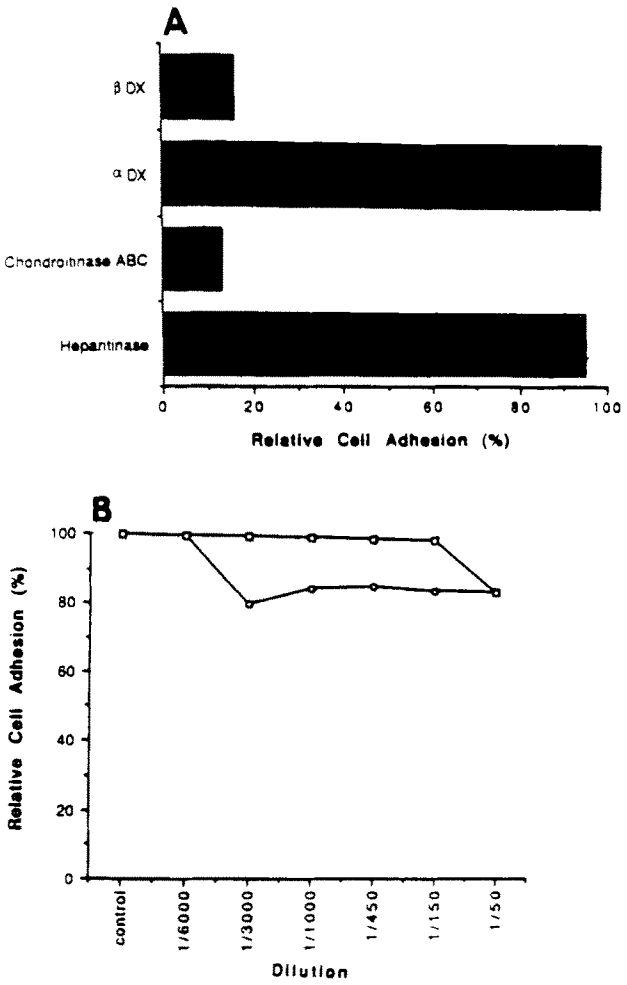


Figure 5. Peptide FN-C/H-III promotes melanoma cell adhesion by a CSPG-dependent, α4 integrin subunit-independent mechanism. (*A*) Microtiter wells were coated with 100 nmoles of FN-C/H-III/OVA and evaluated for the ability to support melanoma cell adhesion following pretreatment of the cells with 1 mM βDX, 1 mM αDX, chondroitinase ABC, or heparitinase. (*B*) Alternatively, microtiter wells adsorbed with 100 nmoles of FN-C/H-III/OVA were evaluated for their ability to support melanoma cell adhesion in the presence of the indicated concentrations of anti-α4 integrin mAb (P4G9; ○), or anti-α5 integrin mAb (P1D6; □). Data points represent the mean of triplicate determinations and SEMs were <10% of mean values in all cases.

CSPG purified by sequential chromatography over DEAE and Sepharose CL-4B columns (Fig. 1 *B*) were evaluated for the ability to bind to FN-C/H-III affinity columns. Purified CSPG bound to FN-C/H-III affinity columns, and eluted at moderately high ionic strength (0.4 M NaCl; Fig. 6 *A*). Alkaline borohydride released CS (Fig. 1 *C*) also bound to the same affinity columns, but CS eluted at a lower ionic strength

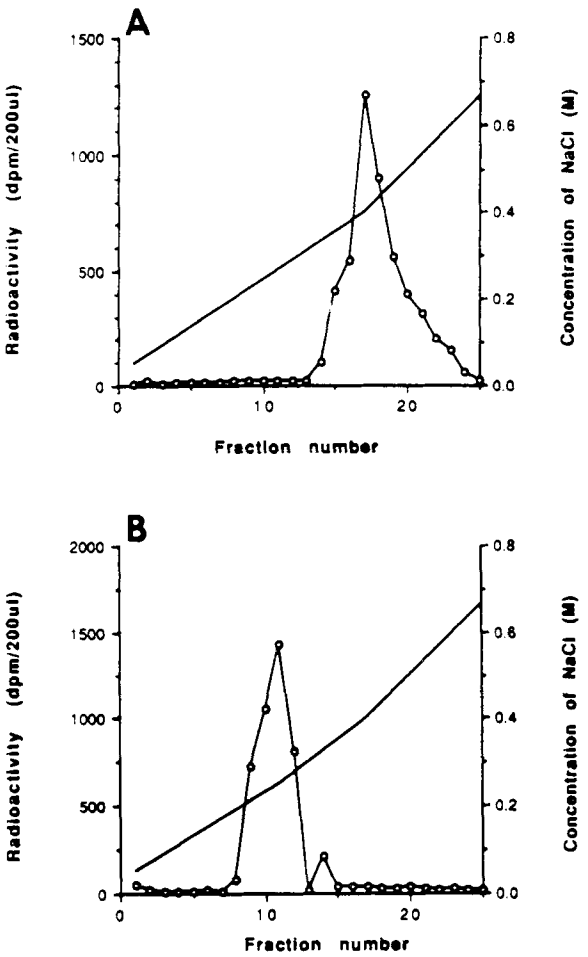


Figure 6. Peptide FN-C/H-III binds purified CSPG and alkaline-borohydride released CS from melanoma cells. (*A*) CSPG purified sequentially over DEAE and Sepharose CL-4B columns were applied to FN-C/H-III/OVA affinity column as described in Materials and Methods, and eluted from the FN-C/H-III/OVA column using a 0.05 to 0.8 M NaCl gradient (————). (*B*) Alkaline borohydride released CS, purified over the Sepharose CL-6B columns, were applied to an FN-C/H-III/OVA affinity column and eluted using the same conditions as in *A*. The recovery for each condition was >95%. Nonspecific binding of the samples, as judged by the ability to interact with OVA/OVA sham affinity columns, was <5% of the total radioactivity applied under these conditions.

(0.25 M NaCl) than the CSPG (Fig. 6 *B*). When purified CSPG was iodinated to radiolabel core proteins, digested with chondroitinase ABC, and then applied to FN-C/H-III affinity column, no radioactivity bound to the columns (not shown). These results indicate that FN-C/H-III binds to CS but not CSPG core proteins. This is consistent with the cell adhesion data in which removal of cell surface CS inhibits melanoma cell adhesion to FN-C/H-III.

We next examined the ability of FN-C/H-III to inhibit the binding of CSPG to affinity columns containing the 33-kD fragment. The purified CSPG was preincubated with FN-C/H-III/OVA, scrambled FN-C/H-III/OVA, or OVA/OVA at final concentrations of 500 μg/ml, and then applied to the 33-kD fragment affinity column as described in Materials and Methods. Under these experimental conditions, FN-C/H-III almost totally prevented the binding of CSPG to the

Table II. Binding of Purified CSPG to a 33-kD Fragment Affinity Column in the Presence of FN-C/H-III or Scrambled FN-C/H-III

Pretreatment*	Flow through‡	Bound‡
	(*dpm*)	(*dpm*)
Buffer	<50	6,982
FN-C/H-III/OVA	6,850	100
Scrambled FN-C/H-III/OVA	150	6,580
OVA/OVA	123	6,745

* The purified CSPG (~7,000 dpm) was preincubated with or without inhibitors (final concentrations of 500 μg/ml) for 2 h at 4°C and then applied to the 33-kD fragment affinity column that had been equilibrated with the buffer containing the corresponding inhibitors at concentrations of 500 μg/ml.
‡ Columns were washed with 6 ml of the buffer (*Flow through*) and then bound material was eluted with 6 ml of buffer containing 0.2 M NaCl (*Bound*).

33-kD fragment affinity column (Table II). In contrast, neither scrambled FN-C/H-III/OVA nor OVA/OVA affected the binding of CSPG to the 33-kD fragment affinity column (Table II). These results indicate that FN-C/H-III serves as a CSPG binding site in the context of the 33-kD fragment, and that the specific binding of FN-C/H-III to CSPG is related to the primary sequence of the synthetic peptide.

Melanoma Cell Adhesion to FN-C/H-I, FN-C/H-II, and CS1 Differs from FN-C/H-III in Sensitivity to CSPG Antagonists and Neutralizing Anti-α4 Integrin mAb

As a comparison to the FN-C/H-III cell adhesion data, we also evaluated the ability of the three previously described synthetic peptides, FN-C/H-I, FN-C/H-II, and CS1 to bind CSPG or to promote melanoma cell adhesion in the presence of CSPG antagonists and anti-α4 integrin mAb. As might be expected from their cationic properties, affinity columns containing FN-C/H-I and FN-C/H-II bound purified CSPG (Fig. 7, *A* and *B*), however the concentration of NaCl required to elute the CSPG from these columns (~0.2–0.25 M) was approximately half that required to elute the CSPG from the FN-C/H-III affinity column. In sharp contrast, affinity columns containing CS1 failed to bind the CSPG (Fig. 7 *C*).

Melanoma cell adhesion to FN-C/H-I, FN-C/H-II, and CS1 was also evaluated following treatment of the cells with chondroitinase ABC or βDX (Table III). In contrast to FN-C/H-III, cell adhesion to FN-C/H-II-coated substrata was only partially (~50%) inhibited by chondroitinase ABC or βDX, and cell adhesion to FN-C/H-I was even less affected (~15% inhibition). Of particular interest was the observation that these treatments also significantly (~50%) inhibited melanoma cell adhesion to the CSPG non-binding, α4β1 integrin-binding synthetic peptide CS1. As was observed for the 33-kD fragment, cell adhesion to all three synthetic peptides was resistant to treatment with heparitinase (Table III), or αDX (not shown).

We also tested the ability of anti-α4 integrin mAb to inhibit melanoma cell adhesion to substrata coated with the three synthetic peptides (Fig. 8). As expected (Wayner et al., 1989), melanoma cell adhesion to CS1-coated substrata could be completely inhibited by the addition of P4G9 anti-α4 integrin mAb (Fig. 8 *A*). Importantly, melanoma cell adhesion to FN-C/H-I or FN-C/H-II coated substrata could also be inhibited by pretreatment of these cells with P4G9

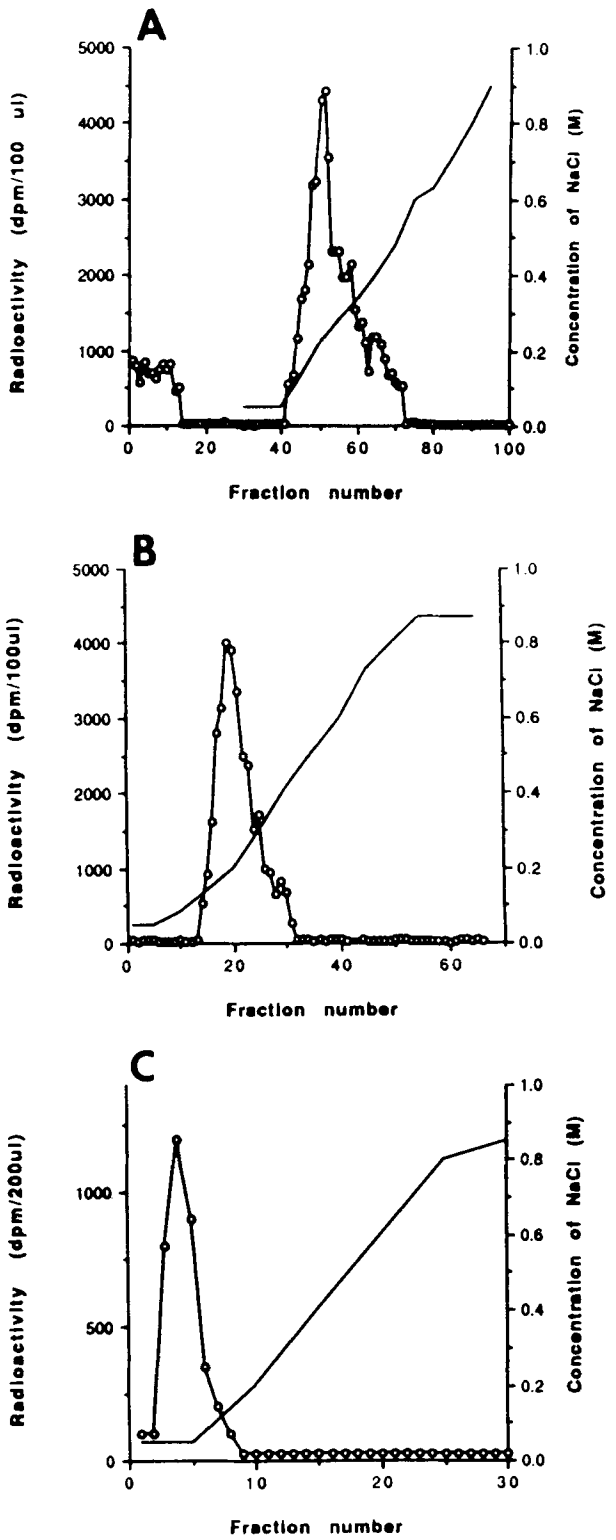


Figure 7. Peptides FN-C/H-I and FN-C/H-II, but not CS1, bind purified melanoma cell CSPG. Affinity columns containing (*A*) FN-C/H-I/OVA, (*B*) FN-C/H-II/OVA, or (*C*) CS1/OVA were evaluated for their ability to bind purified CSPG from melanoma cells. The purified CSPG was applied to these affinity columns in buffer containing 0.05 M NaCl as described in Materials and Methods. The columns were then eluted with a 0.05 to 0.8 M gradient of NaCl (————). The recovery from each affinity column was 95%. Nonspecific binding of the CSPG samples, as judged by their ability to bind to OVA/OVA sham affinity columns, was less than 5% of the total radioactivity applied under these conditions.

Table III. Effect of Removal of Cell Surface CS Glycosaminoglycans on Cell Adhesion to the Various Synthetic Peptides

	Relative cell adhesion in the presence of inhibitors‡		
Substratum*	βDX	Chondroitinase ABC	Heparitinase
		(%)	
FN-C/H-II	45§	43§	96
FN-C/H-I	86	85	98
CS1	50§	53§	97

* Plates were coated with the various ligands at concentrations which promoted half-maximal cell adhesion.
‡ Cells were pretreated with 1 mM βDX overnight, or with chondroitinase ABC or heparitinase in PBS containing 1.25 mM $CaCl_2$ for 20 min. Cell adhesion assays were carried out in the continued presence of the various inhibitors. Percent of inhibition was calculated compared to non-treated group. Data were calculated from the means of triplicate determinations and SEMs were <10% of mean values in each case.
§ $P < 0.001$ from values observed in the absence of the inhibitors (by Student's two tailed t test).

anti-α4 integrin mAb (Fig. 8, *B* and *C*). P4C2, another anti-α4 integrin mAb, could also inhibit melanoma cell adhesion to these three synthetic peptides, although the level of inhibition was somewhat less (50–60%) than that observed with P4G9 (not shown). As controls, anti-α5 integrin mAb (Fig. 8) or normal mouse ascites (not shown) did not inhibit melanoma cell adhesion to these synthetic peptides, demonstrating that the inhibitory effects of anti-α4 integrin mAb on cell adhesion to these three synthetic peptides was specific in nature.

Discussion

Recent studies suggest that cellular recognition of the ECM may involve the coordinated action of distinct receptors on cell surfaces that interact with an array of closely spaced recognition sites on insoluble ECM components. Early work on FN demonstrated that both RGD-containing domains and heparin binding domains within intact FN are required for focal contact and focal adhesion formation in certain cell types (Lark et al., 1985; Woods et al., 1986). More recently, it has been reported that intracellular phosphorylation events, which immediately follow initial cell recognition of FN, appear to involve both GAG-binding and integrin-binding domains (Guan et al., 1991). These reports, as well as others (Kornberg et al., 1991) suggest that the clustering of ECM cell surface receptors may be responsible, in part, for transmitting distinct signals to the interior of the cells, similar to the way in which lymphocytes use receptor clusters to recognize and respond to various target cells (reviewed in Springer, 1990). The current studies, which use synthetic peptides from a FN fragment that has both integrin and PG-binding properties, add further support to this generalized mechanism for cellular recognition of the ECM.

By using specific inhibitors of CSPG or α4β1 integrin subunit function, human melanoma cell adhesion to the 33-kD fragment of FN was shown to involve the activities of both types of cell surface adhesion receptors. As expected, cell adhesion to the 33-kD fragment was inhibited in the presence of anti-α4 integrin or anti-β1 integrin mAbs, but not in the presence of anti-α5 integrin mAbs. Pretreatment of melanoma cells with either proteinase-free chondroitinase ABC (to enzymatically remove cell surface CS) or βDX (to

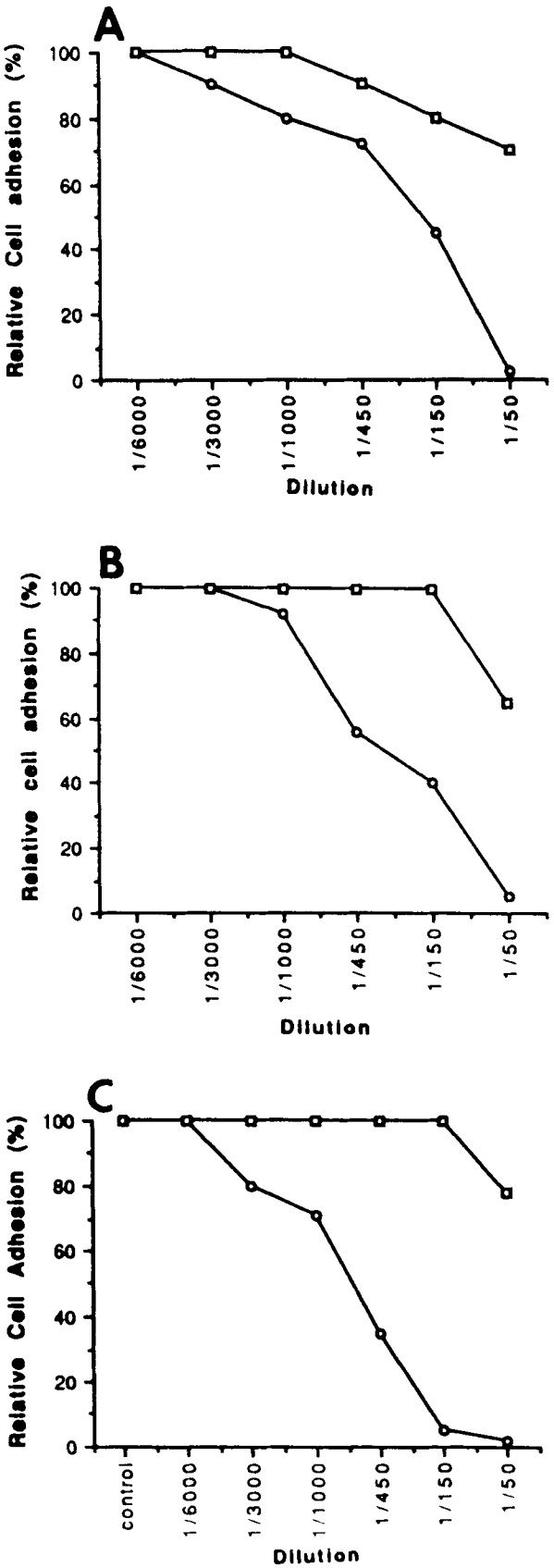


Figure 8. Melanoma cell adhesion to peptides FN-C/H-I, FN-C/H-II, and CS1 is sensitive to the inhibitory effects of anti-α4 integrin subunit mAb. Microtiter wells were coated with (*A*) CS1/OVA, (*B*) FN-C/H-I/OVA, or (*C*) FN-C/H-II/OVA at concentrations that promoted half-maximal cell adhesion, and then blocked with PBS/BSA. Cells were preincubated with anti-α4 integrin mAb (P4G9, ○), or anti-α5 integrin mAb (P1D6, □) for 30 min at 37°C, and then evaluated for their ability to adhere to the various peptide coated substrata in the continued presence of the mAbs. The assays were performed for 30 min, and each data point represents the mean of triplicate determinations. The SEM were <10% of mean values in each case.

interfere with cell surface CSPG production) also caused significant (50%) inhibition of melanoma cell adhesion to the 33-kD fragment. The inhibition of cell adhesion observed in the presence of these antagonists of CSPG function was not due to a loss of cell surface α4 or β1 integrin subunits from the cell surface, as shown by flow cytometry; suggesting that CSPG might bind to and play a direct role in melanoma cell adhesion to the 33-kD fragment. Importantly, pretreatment of melanoma cells with either βDX or chondroitinase ABC could potentiate the inhibitory effect of suboptimal levels of anti-α4 integrin mAb, which is consistent with an involvement of both cell surface CSPG and α4 integrin subunits in mediating the initial recognition and adhesion of melanoma cells to the 33-kD fragment. Since certain anti-α4 and anti-β1 integrin mAbs can completely inhibit melanoma cell adhesion to the 33-kD fragment and antagonists of CSPG are only partially effective, it would appear that the α4β1 integrin complex is fundamentally important for supporting adhesion on the 33-kD fragment. In contrast, cell surface CSPGs, in the absence of functional α4β1 integrin, may not be as efficient in promoting adhesion to the fragment. Such differences may relate to the relative affinities of each receptor for recognition sites within the fragment, or may be a reflection of specific differences in intracellular signals transmitted by each type of receptor.

Evidence for the role of cell surface CSPG in promoting melanoma cell adhesion to the 33-kD fragment comes from the identification of FN-C/H-III, a novel cell adhesion promoting synthetic peptide contained within type III repeat #12 of FN, located near the amino terminus of the 33-kD fragment (McCarthy et al., 1988). As with FN-C/H-I and FN-C/H-II (two other previously identified cell adhesion promoting synthetic peptides from within this fragment), peptide FN-C/H-III is cationic, binds [^{3}H]heparin, and promotes melanoma cell adhesion in a concentration-dependent fashion. Both soluble FN-C/H-III/OVA and specific anti-FN-C/H-III IgG partially inhibited melanoma cell adhesion to the 33-kD fragment. These results are similar to those obtained using the same strategies to evaluate FN-C/H-I and FN-C/H-II (McCarthy et al., 1990; Haugen et al., 1990), and are consistent with the notion that the 33-kD fragment contains multiple distinct cell adhesion promoting sites.

FN-C/H-III is also a major CSPG binding site within the 33-kD fragment, as evidenced from the binding of CSPG (or alkaline reduced CS) to FN-C/H-III affinity columns, as well as by the ability of soluble FN-C/H-III to completely inhibit CSPG binding to affinity columns containing the 33-kD fragment. Furthermore, based on specific inhibition studies, the cell adhesion promoting activity of FN-C/H-III is dependent on cell surface CS, and independent of cell surface α4β1 integrin. While soluble FN-C/H-III completely inhibits the binding of CSPG to the 33-kD fragment, it only partially inhibits cell adhesion to the fragment, consistent with the notion that PG-independent, α4β1 integrin-dependent adhe-

sion mechanisms are also important for melanoma cell recognition of the fragment (Wayner et al., 1989; McCarthy et al., 1990; Mould et al., 1990; Haugen et al., 1990). Importantly, the primary sequence of FN-C/H-III is important for its biological activities, as demonstrated by the failure of scrambled FN-C/H-III, with the same amino acid composition, net hydropathy, and net charge as the parent peptide FN-C/H-III, to inhibit CSPG binding or melanoma cell adhesion to FN-C/H-III or to the 33-kD fragment.

Melanoma cell adhesion to the other three cell adhesion promoting synthetic peptides, FN-C/H-I, FN-C/H-II, and CS1, was distinct from, and molecularly more complex than FN-C/H-III–mediated cell adhesion, in that cell adhesion to these three peptides was sensitive to anti-α4 integrin mAbs. Furthermore, inhibition of CSPG production or expression had differential effects on melanoma cell adhesion to these three synthetic peptides. Cell adhesion to FN-C/H-I was only slightly (15%) sensitive, whereas peptide FN-C/H-II–mediated adhesion was inhibited by ~50%. Importantly, CS1-mediated melanoma cell adhesion was also inhibited 50% by chondroitinase ABC or βDX pretreatments, despite the fact that the level of cell surface $\alpha 4\beta 1$ integrin is not altered by these treatments and CS1 does not directly bind purified CSPG. This inhibition was not due to nonspecific toxic side effects of antagonists of CSPG function, as supported by trypan blue exclusion data, protein synthesis and ^{3}H-TdR incorporation analysis, as well as by the relative inability of either chondroitinase ABC or βDX to inhibit melanoma cell adhesion on FN-C/H-I. While the exact mechanism by which melanoma cell surface CSPG can modulate cell adhesion to CS1 remains to be determined, one possibility is that the CSPG may be important for modulating the affinity of $\alpha 4\beta 1$ integrin for specific ligands. Alternatively, it is possible that the CSPG may influence $\alpha 4\beta 1$ integrin in some other way, perhaps by altering intracellular signals that could change the functional capacity of $\alpha 4\beta 1$ integrin to mediate cell adhesion.

While CS1 has clearly been shown by several investigators to bind $\alpha 4\beta 1$ integrin directly (Mould, 1990; Guan, 1990), additional studies are required before definitive ligand-receptor relationships can be established for FN-C/H-I and FN-C/H-II. Although the current studies demonstrate that FN-C/H-I and FN-C/H-II affinity columns can directly bind purified CSPG from melanoma cells, this binding is of somewhat lower affinity than that observed for FN-C/H-III. Furthermore, inhibition of CSPG expression only partially inhibits melanoma cell adhesion on these two synthetic peptides, suggesting that FN-C/H-I or FN-C/H-II might interact with α4 (or β1) integrin subunits, or possibly that these two synthetic peptides may be capable of binding both CSPG and $\alpha 4\beta 1$ integrin.

Regardless of the exact mechanism(s) involved, the results suggest a working model for A375 SM human melanoma cell recognition of the 33-kD fragment from FN A-chains (Fig. 9). This model depicts two overlapping elements in the context of a larger cellular recognition domain within the 33-kD fragment, one of which is CSPG dependent, and a second which is α4 integrin subunit dependent. According to this model, the CSPG-dependent element contains at least four sites, characterized by FN-C/H-III, FN-C/H-I, and FN-C/H-II, CS-1, and possibly others (Bober-Barkalow and Schwarzbauer, 1991), all of which could directly bind CSPG

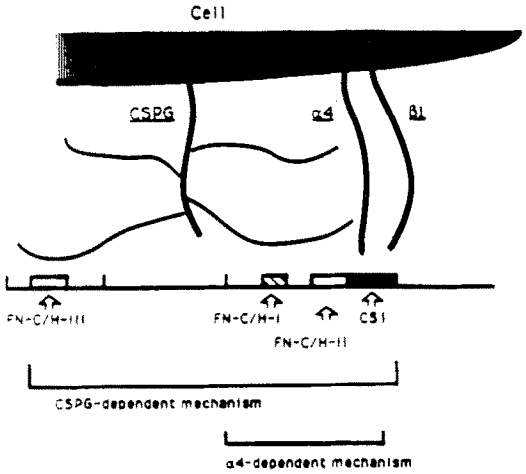


Figure 9. Schematic diagram illustrating a coordinated role for CSPG-dependent and $\alpha 4\beta 1$ integrin-dependent cellular recognition sites in mediating melanoma cell adhesion to the 33-kD fragment. Schematically depicted is a human melanoma cell initially recognizing substrata coated with the 33-kD fragment of human plasma FN-A-chains. The three type III repeating homologies (12, 13, and 14) and a portion of the type IIICS insert are depicted, as are the relative locations of the four synthetic peptides used in this study. According to this model, the proximity of the CSPG-dependent and $\alpha 4\beta 1$-integrin–dependent elements, within the context of a larger cellular recognition domain within this fragment, would act to focus cell surface CSPG and $\alpha 4\beta 1$ integrin on the ventral surface of the plasma membrane. This clustering of cell surface receptors would act to modulate early cellular events that contribute to melanoma cell adhesion on this fragment. Evidence for this model is summarized in the Discussion.

and/or be influenced by cell surface CSPG. Additionally, there is a multisite α4 integrin-dependent element within this larger domain, consisting of at least FN-C/H-I, FN-C/H-II, and CS1, and possibly other sites within the fragment (Mould and Humphries, 1991). The potential significance of the overlapping nature of these two elements is that each could act in concert to efficiently bring cell surface CSPG and $\alpha 4\beta 1$ integrin into close proximity during initial melanoma cell recognition of the 33-kD fragment. Ligand-induced clustering of cell surface ECM receptors on the plasma membrane could have a profound effect on altering the conformation and binding specificity of receptors within such clusters or it could influence the generation of intracellular signals that follow initial cellular recognition of the 33-kD fragment, thereby affecting cell adhesion, cytoskeletal reorganization, cell motility, and other diverse aspects of cell phenotype.

Although there is evidence suggesting that CSPG (and specifically CS GAG) is associated with cellular de-adhesion, the current studies argue that this may not be a universal property of all CSPG. Much of the data describing the ability of CSPG to weaken cell adhesion rely on cell adhesion assays in which CSPG from a variety of sources is used to compete for cell adhesion to substrata coated with various ligands (e.g., FN). These approaches have led to the hypothesis that exogenous CSPG may weaken cell adhesion by binding to ECM components and masking other cell adhesion promoting sites (e.g., RGD) within these ECM components (reviewed in Ruoslahti, 1988; Gallagher, 1989). How-

ever, other studies using melanoma cells have demonstrated that cell surface CSPG is enriched on cell surface microspikes (Garrigues et al., 1986), suggesting that cell surface CSPG may also play a role in mediating early recognition events in cell adhesion. The current studies are consistent with the latter hypothesis. While we cannot currently explain the discrepancies between these two apparently disparate notions, one possibility is that the biological activity of cell surface CSPG may depend on the nature, function, and intracellular signals transmitted by cell surface CSPG core proteins. More definitive identification of the CSPG core protein(s) produced by these human melanoma cells will be required to test this hypothesis directly.

Implicit in this more generalized "receptor cluster" model of ECM recognition is the prediction that information transmitted into the cell is closely related to the specific combinations of cell surface receptors that may come into proximity on the cell surface as a result of the influence of closely spaced cellular recognition domains within the ECM. Cell type-specific differences in ECM receptor expression could therefore alter the molecular composition of such complexes, resulting in profound differences in cell type-specific signals transmitted by the ECM. Changes that occur in the composition of these receptor complexes (e.g., presence or absence of specific integrins, type of PG core protein, etc.) as a function of malignant progression could be fundamentally important in generating aberrant signals that may modulate tumor cell invasion and metastasis. Future work on this model in the area of tumor cell biology should include a comparison of tumor cells at various stages of progression. Such studies may help in the understanding of the biology of ECM mediated-tumor cell adhesion, invasion, and metastasis.

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References

Albelda, S. M., and C. A. Buck. 1990. Integrins and other cell adhesion molecules. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2868–2880.

Bober Barkalow, F. J., and J. E. Schwarzbauer. 1991. Localization of the major heparin-binding site in fibronectin. *J. Biol. Chem.* 266:7812–7818.

Brennan, M. J., A. Oldberg, E. G. Hayman, and E. Ruoslahti. 1983. Effect of a proteoglycan produced by rat tumor cells on their adhesion to fibronectin-collagen substrata. *Cancer Res.* 43:4302–4307.

Buck, C. A., and A. F. Horwitz. 1987. Cell surface receptors for extracellular matrix molecules. *Annu. Rev. Cell Biol.* 3:179–205.

Bumol, T. F., and R. A. Reisfeld. 1982. Unique glycoprotein-proteoglycan complex defined by monoclonal antibody on human melanoma cells. *Proc. Natl. Acad. Sci. USA.* 79:1245–1249.

Chelberg, M. K., J. B. McCarthy, A. P. N. Skubitz, L. T. Furcht, and E. C. Tsilibary. 1990. Characterization of a synthetic peptide from type IV collagen that promotes melanoma cell adhesion, spreading and motility. *J. Cell Biol.* 111:261–270.

Couchman, J. R., and M. Höök. 1988. Proteoglycans and wound repair. *In* The Molecular and Cellular Biology of Wound Repair. R. A. F. Clark and P. Henson, editors. Plenum Press, New York. 437–470.

Fidler, I. J. 1986. Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. *Cancer Metastasis Rev.* 5:29–49.

Gallagher, J. T. 1989. The extended family of proteoglycans: social residents of the pericellular zone. *Curr. Op. Cell Biol.* 1:1201–1218.

Garrigues, H. J., M. W. Lark, S. Lara, I. Hellström, K. E. Hellström, and T. N. Wight. 1986. The melanoma proteoglycan: restricted expression on microspikes, a specific microdomain of the cell surface. *J. Cell Biol.* 103: 1699–1710.

Guan, J.-L., and R. O. Hynes. 1990. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha4\beta1$. *Cell.* 60: 53–61.

Guan, J.-L., J. E. Trevithick, and R. O. Hynes. 1991. Fibronectin/integrin interaction induces tyrosine phosphorylation of a 120-kDa protein. *Cell Reg.* 2:951–964.

Haugen, P. K., J. B. McCarthy, A. P. N. Skubitz, L. T. Furcht, and P. C. Letourneau. 1990. Recognition of the A chain carboxy-terminal heparin binding region of fibronectin involves multiple sites: two contiguous sequences act independently to promote neural cell adhesion. *J. Cell Biol.* 111:2733–2745.

Höök, M., L. Kjellen, S. Johansson, and J. Robinson. 1984. Cell surface proteoglycans. *Annu. Rev. Biochem.* 53:847–869.

Humphries, M. J. 1990. The molecular basis and specificity of integrin-ligand interactions. *J. Cell Sci.* 97:585–592.

Humphries, M. J., A. Komoriya, S. K. Akiyama, K. Olden, and K. M. Yamada. 1987. Identification of two distinct regions of the type IIICS connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J. Biol. Chem.* 262:6886–6892.

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

Kato, Y., K. Kimata, K. Ito, K. Karasawa, and S. Suzuki. 1978. Effect of β-D-xyloside and cycloheximide on the synthesis of two types of proteochondroitin sulfate in chick embryo cartilage. *J. Biol. Chem.* 253:2784–2789.

Klein, D. M., D. M. Brown, and T. R. Oegema. 1986. Partial characterization of heparan and dermatan sulfate proteoglycans synthesized by normal rat glomeruli. *J. Biol. Chem.* 261:16636–16652.

Kornberg, L. T., H. S. Earp, C. E. Turner, C. Prockop, and R. L. Juliano. 1991. Signal transduction by integrins: increased protein tyrosine phosphorylation caused by clustering of $\beta1$ integrins. *Proc. Natl. Acad. Sci. USA.* 88:8392–8396.

Kornblihtt, A. R., K. Umezawa, K. Vibe-Pedersen, and F. E. Baralle. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755–1759.

Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.

Lark, M. W., J. Laterra, and L. A. Culp. 1985. Close and focal contact adhesions of fibroblasts to a fibronectin-containing matrix. *Fed. Proc.* 44: 394–403.

Liao, N. S., J. St. John, J. B. McCarthy, L. T. Furcht, and H. T. Cheung. 1989. Adhesion of lymphoid cells to the carboxyl-terminal heparin-binding domains of fibronectin. *Exp. Cell Res.* 181:348–361.

Lohmander, S., K. Madsen, and A. Hinek. 1979. Secretion of proteoglycans by chondrocytes: influence of colchicin, cytochalasin B and β-D-xyloside. *Arch. Biochem. Biophys.* 192:148–157.

McCarthy, J. B., S. T. Hagen, and L. T. Furcht. 1986. Human fibronectin contains distinct adhesion and motility promoting domains for metastatic melanoma cells. *J. Cell Biol.* 102:179–188.

McCarthy, J. B., M. K. Chelberg, D. J. Mickelson, and L. T. Furcht. 1988*a*. Localization and chemical synthesis of fibronectin peptides with melanoma adhesion and heparin binding activities. *Biochemistry.* 27:1380–1388.

McCarthy, J. B., A. P. N. Skubitz, S. L. Palm, and L. T. Furcht. 1988*b*. Metastasis inhibition of different tumor types by purified laminin fragments and a heparin binding fragment of fibronectin. *J. Natl. Cancer Inst.* 80: 108–116.

McCarthy, J. B., A. P. N. Skubitz, Q. Zhao, X.-Y. Yi, D. J. Mickelson, and L. T. Furcht. 1990. RGD-independent cell adhesion to the carboxy-terminal heparin binding fragment of fibronectin involves heparin-dependent and -independent activities. *J. Cell Biol.* 110:777–787.

Mould, A. P., and M. J. Humphries. 1991. Identification of a novel recognition sequence for the integrin $\alpha4\beta1$ in the COOH-terminal heparin-binding domain of fibronectin. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:4089–4095.

Mould, A. P., L. A. Wheldon, A. Komoriyama, E. A. Wayner, K. M. Yamada, and M. J. Humphries. 1990. Affinity chromatographic isolation of the melanoma cell adhesion receptor for the IIICS region of fibronectin and its identification as the integrin $\alpha4\beta1$. *J. Biol. Chem.* 265:4020–4024.

Obara, M., M. S. Kang, and K. M. Yamada. 1988. Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. *Cell.* 53:649–657.

Robinson, J., and D. Gospodarowicz. 1984. Effect of p-nitrophenyl-β-D-xyloside on proteoglycan synthesis and extracellular matrix formation by bovine corneal endothelial cell cultures. *J. Biol. Chem.* 259:3818–3824.

Rogers, S. L., P. C. Letourneau, B. A. Peterson, L. T. Furcht, and J. B. McCarthy. 1987. Selective interaction of peripheral and central nervous system cells with two distinct cell-binding domains of fibronectin. *J. Cell Biol.* 105:1435–1442.

Ross, A. H., G. Cossu, M. Herlyn, J. R. Bell, Z. Steplewski, and H. Koprowski. 1983. Isolation and chemical characterization of a melanoma-associated proteoglycan antigen. *Arch. Biochem. Biophys.* 225:370–383.

Ruoslahti. E. 1988. Structure and biology of proteoglycans. *Ann. Rev. Cell Biol.* 4:229–257.

Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87:1–5.

Saunders, S., and M. Bernfield. 1988. Cell surface proteoglycan binds mouse mammary epithelial cells to fibronectin and behaves as a receptor for interstitial matrix. *J. Cell Biol.* 106:423–430.

Schwartz, N. B. 1977. Regulation of chondroitin sulfate synthesis. *J. Biol. Chem.* 252:6316–6321.

Skubitz, A. P. N., J. B. McCarthy, A. S. Charonis, and L. T. Furcht. 1988. Localization of three distinct heparin-binding domains of laminin by monoclonal antibodies. *J. Biol. Chem.* 263:4861–4868.

Skubitz, A. P. N., J. B. McCarthy, Q. Zhao, X.-Y. Yi, and L. T. Furcht. 1990. Definition of a sequence, RYVVLPR, within laminin peptide F-9 that mediates metastatic fibrosarcoma cell adhesion and spreading. *Cancer Res.* 5:7612–7622.

Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (Lond.)* 346:425–434.

Stewart, J. M., and J. D. Young. 1984. Solid Phase Peptide Synthesis. 2nd ed. Pierce Chemical Co. Rockford, IL.

Visser, M. R., G. M. Vercellotti, J. B. McCarthy, J. L. Goodman, T. J. Herbst, L. T. Furcht, and H. S. Jacob. 1989. Herpes simplex virus inhibits endothelial cell attachment and migration to extracellular matrix proteins. *Am. J. Pathol.* 134:223–230.

Wait, K. A., G. Mugnai, and L. A. Culp. 1987. A second cell binding domain on fibronectin (RGDS-independent) for neurite extension of neuroblastoma cells. *Exp. Cell Res.* 169:311–327.

Wayner, E. A., and N. L. Kovach. 1992. Activation-dependent recognition by hematopoietic cells for the LDV sequence in the V region of fibronectin. *J. Cell Biol.* 116:489–497.

Wayner, E. A., A. Garcia-Pardo, M. J. Humphries, J. A. McDonald, and W. G. Carter. 1989. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. *J. Cell Biol.* 109:1321–1330.

Woods, A., J. R. Couchman, S. Johanssen, and M. Höök. 1986. Adhesion and cytoskeletal organization of fibroblasts in response to fibronectin fragments. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:665–670.

Yamagata, M., S. Suzuki, S. K. Akiyama, K. M. Yamada, and K. Kimata. 1989. Regulation of cell-substrate adhesion by proteoglycans immobilized on extracellular substrate. *J. Biol. Chem.* 264:8012–8018.