

# Human Fibronectin Contains Distinct Adhesion- and Motility-promoting Domains for Metastatic Melanoma Cells

James B. McCarthy, Scott T. Hagen, and Leo T. Furcht

Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455. Address reprint requests to J. B. McCarthy.

**Abstract.** The active migration of tumor cells through extracellular matrices has been proposed to play a role in certain aspects of metastasis. Metastatic tumor cells migrate in vitro in response to substratum-bound adhesive glycoproteins such as fibronectin. The present studies use affinity-purified proteolytic fragments of fibronectin to determine the nature of adhesion- and/or motility-promoting domains within the protein. Two distinct fragments were identified with cell adhesion-promoting activities. By a number of criteria, the adhesive activity promoted by these two fragments was distinct. One fragment, a 75-kD tryptic fragment purified by monoclonal antibody chromatography, promoted the adhesion, spreading, and haptotactic motility of melanoma cells. Experiments using a synthetic cell attachment peptide in solution indicated that at least part of the attachment activity exhibited by the 75-kD fragment is mediated by the sequence arg-gly-asp-ser. It was not possible to demonstrate migration-stimulating activity using a

small (11.5 kD) peptic fragment containing this sequence (Pierschbacher, M.D., E. G. Hayman, and E. Ruoslahti, 1981, *Cell*, 26:259-267) suggesting that another cell-binding activity within the 75 kD fragment distinct from arg-gly-asp-ser might be required for motility. The second fragment that stimulated melanoma adhesion was a 33-kD tryptic/catheptic carboxyl-terminal heparin-binding fragment, which is localized to the A chain of fibronectin. This fragment promotes adhesion and spreading but not the motility of these cells. Melanoma adhesion to this heparin-binding fragment was sensitive to the effects of cycloheximide, which contrasted adhesion to the haptotaxis-promoting fragment. Importantly, these studies illustrate that haptotaxis in response to fibronectin is not due to simple adhesion gradients of this protein. The results are discussed in light of a model for multiple distinct cell surface constituents mediating cell adhesion and motility on fibronectin.

THE metastatic spread of certain malignant neoplasms has been associated with active cell motility. This association has been made both at the primary site of growth (43) as well as with model systems designed to study the entry and exit of tumor cells into and out of the vasculature (42, 46, 48 and references therein). Cells encounter a wide variety of extracellular matrix and basement membrane molecules as they penetrate and migrate through tissues. The vast amount of work on fibronectin has been important for developing an understanding of the molecular basis by which noncollagenous matrix glycoproteins promote the adhesion of many normal and transformed cell types in vitro (10, 12, 16, 50 and references therein). The interaction of cells with fibronectin results in cell adhesion and in the reorganization of the cytoskeleton leading to spreading. This spreading can then become polarized and lead to cell motility over this protein. A variety of cell types have been shown to manifest this response to fibronectin (for review see references 10, 12, 16, 50).

Work from our laboratory (23) as well as others (1, 20, 25) demonstrates that fibronectin can promote the migration of a variety of tumor cell types in vitro. Laminin (2, 23, 24, 39) and serum-spreading factor (2), two additional cell attachment/spreading factors, have also been shown to promote the directed motility of a variety of tumor cell types in vitro. At least for certain of these cell/protein combinations, the directed motility has been shown to be haptotactic in nature, meaning that the directional migration observed is in response to substratum-bound density gradients of these attachment proteins (2, 20, 23, 24).

Studies have shown that the diverse binding or functional activities of fibronectin exist in specific domains or regions of the molecule that can be isolated by proteolytic digestion and purification with various antibodies or other ligands. The work of Pierschbacher and co-workers was the first to elucidate the molecular nature of a cell attachment/spreading activity present within the fibronectin molecule (31-33). These studies initially used a monoclonal antibody, termed

3E3, to isolate an 11.5-kD pepsin-derived fragment of fibronectin shown to directly promote the attachment and spreading of fibroblastic cells in vitro (33). This fragment represents domain IV of fibronectin according to the nomenclature described in Furcht (10). Further studies using direct amino acid sequencing identified the active region of the cell attachment-promoting domain as a continuous determinant of the molecule containing the amino acids arginyl-glycyl-aspartyl-serine (RGDS) (31). This sequence has been shown to occur in a disulfide-independent type III homology (30) of the intact protein located to the amino-terminal side of the carboxyl-terminal heparin-binding domain (13, 31, 38, 47).

Yamada and Kennedy have examined the effect of soluble fibronectin or RGDS-containing synthetic peptides on the attachment and spreading of continuous cell lines (51). This study reported that high levels of soluble fibronectin (5–10 mg/ml) could inhibit the spreading of fibroblastic cells on substrates coated with 3  $\mu$ g/ml of plasma fibronectin. Increasing the concentration of substrate-bound fibronectin could overcome the inhibitory effect of soluble fibronectin, so inhibition was concluded to be competitive in nature. Similar inhibitory effects were also reported using a soluble 75-kD tryptic cell-binding peptide or synthetic RGDS-containing peptides (32, 51).

The present studies were performed in order to establish the nature of the domains in the fibronectin molecule that promote the attachment and motility of metastatic melanoma cells. These studies use a combination of limited proteolysis, affinity chromatography, and monoclonal antibody techniques to isolate biologically active fragments of fibronectin. The results show that although melanoma cells do in fact contain "receptors" for the RGDS peptide, the RGDS-containing domain alone does not appear to be sufficient to stimulate melanoma haptotaxis. Furthermore, while certain fragments with adhesion-promoting activities of fibronectin result in tumor cell motility, other fragments with adhesion-promoting activities do not stimulate movement. We conclude that melanoma cells interact with at least two and possibly three distinct domains on the intact protein, and that these interactions are mediated by distinct cell surface components each of which potentially has different consequences for tumor cell behavior.

## Materials and Methods

### Cell cultures

Murine melanoma cells, B16 F10, were cultured in vitro in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum (Dutchland Labs, Denver, PA) as previously described (23). Cells used for either attachment or migration assays were always 70–90% confluent. The lung-colonizing potential of these cells was verified using the tail vein experimental metastatic assay in C57B16 mice as previously described (34).

### Reagents

Pepsin-generated 11.5-kD cell-binding fragment (31) and monoclonal antibody 3E3 (31) were generously supplied to us by Dr. Michael Pierschbacher of the La Jolla Cancer Research Foundation, La Jolla, CA. *N*-Tosyl-L-phenylalanine chloromethyl ketone-trypsin was obtained from CooperBiomedical, Inc. (Malvern, PA). Cathepsin D, pepsin, cycloheximide, heparin-Sepharose, and 3,3'-diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO). Pentex bovine serum albumin (BSA) was from Miles Laboratories Inc. (Elkhart, IN). Insolubilized protease inhibitors, soybean trypsin inhibitor, and pepstatin A were from Pierce Chemical Co. (Rockford, IL), and Affi-Gel 10 was from Bio-Rad Laboratories (Richmond, CA). Nitrocellulose blotting paper,

type HAHY, was purchased from Millipore Corporation (Bedford, MA). Peroxidase-coupled goat anti-mouse IgG heavy and light chains was purchased from CooperBiomedical/Cappel (Malvern, PA). The synthetic fibronectin cell attachment peptide, RGDS, (32) was purchased from Peninsula Laboratories, Inc., Belmont, CA and was also kindly supplied by Dr. Alan Day, Genesis Labs (Minneapolis, MN).

### Purification of Fibronectin and Proteolytic Fragments

Human plasma fibronectin was purified from plasma by gelatin and ion exchange chromatography as previously described (40). Tryptic/catheptic fragments of fibronectin were prepared as previously described (40, 41) by sequential affinity chromatography over gelatin, heparin, and monoclonal antibody 180-8 affinity columns.

Additional proteolytic fragments were also prepared from fibronectin. Either the tryptic/catheptic 80–125-kD fragments or intact fibronectin were trypsinized extensively (1% enzyme to substrate for 2 h at 37°C) and fractionated over a monoclonal antibody 180-8 affinity column. The 180-8 bound fragments were then chromatographed over a Spherogel TSK 3000 column (Altex 7.5 mm  $\times$  30 cm) at a flow rate of 0.75 ml/min in 0.01 M phosphate, pH 6.5, containing 0.25 M NaCl. Fractions were collected every minute, and fractions containing molecular mass peptides ranging from 60 to 100 kD were pooled, concentrated, and used for further analysis in biological assays. SDS PAGE analysis of this pool demonstrated the presence of one major peptide at 75 kD and occasionally lesser amounts of a fragment at 70 kD which was closely related to the 75-kD fragment, since both bound monoclonal antibodies 180-8 and 3E3 and could not be separated by a variety of chromatographic techniques. The 180-8 unbound fragments consisted of a series of poorly defined low molecular mass proteolytic fragments. These peptides were used in the cell attachment and motility assays as described below.

Further proteolysis of the 180-8-bound 75-kD fragment was also performed with pepsin. Solutions of purified 75-kD fragment were dialyzed against 2% acetic acid and digested at 37°C with pepsin at an enzyme to substrate ratio of 1% for various times. The reaction was terminated by the addition of insolubilized pepstatin A, and the digests were characterized for cell attachment and motility activities. The concentration of intact fibronectin was estimated using the extinction coefficient previously reported for the intact protein (26). The concentration of fragments was determined assuming an extinction coefficient of 10.0 for a 1% solution and a 1-cm path length at 280 nm.

Purified fragments of fibronectin or the intact protein were radioactively labeled using the reductive methylation technique as previously described (23). Tritiated fragments or intact fibronectin were qualitatively assessed by the use of autoradiography of SDS polyacrylamide gels. The quantitative binding of these peptides to either tissue culture plastic or to polycarbonate filters used in the attachment and migration assays was analyzed as previously described (24, 36).

### Immunolocalization of Electrophoretic Transfers

Purified fibronectin fragment preparations were electrophoresed in SDS polyacrylamide gels (36) and electrophoretically transferred to nitrocellulose as described (44). After overnight incubation in PBS with 3% bovine serum albumin (PBS/BSA), the filters were incubated for 1 h in the presence of monoclonal antibodies 180-8 (40), 2-8 (41), or 3E3 (31) that were diluted from ascites at a 1:500 ratio in PBS/BSA containing 10% normal goat serum. After 30-min rinse in PBS, peroxidase-coupled goat anti-mouse antibodies, diluted 1:500 in PBS/BSA, were incubated with the sheets for an additional hour. Finally, blots were rinsed and color was developed using diaminobenzidine dissolved in PBS at a concentration of 120  $\mu$ g/ml with 0.001% H<sub>2</sub>O<sub>2</sub> for 5 min. The reaction was terminated by flushing with water. Parallel blots were also stained immediately after transfer with Amido black to visualize all transferred proteins or proteolytic fragments.

### Cell Migration Assays and Attachment

The migration of cells in response to fibronectin or proteolytic fragments of the molecule was measured in blind well microchambers using 8.0 pore size polycarbonate filters as previously described (23, 24). The chambers were incubated for 4 h at 37°C, at which time the filters were removed, fixed, stained, and the cells were visualized on a Zeiss Universal Microscope at 400 $\times$  magnification. Migrated cells were quantitated with the aid of an Optomax Image System IV automatic image analysis system (Optomax, Inc., Hollis, NH).

The basic cell attachment methodologies have also been described previously (9, 27). Late log phase cultures of melanoma cells were incubated overnight in the presence of 2  $\mu$ Ci/ml of tritiated thymidine (specific activity, 6 mCi/ml; Amersham Corp., Arlington Heights, IL). In some cases the cells were then incubated with 10  $\mu$ g/ml cycloheximide for 2 h prior to harvest. Cells were

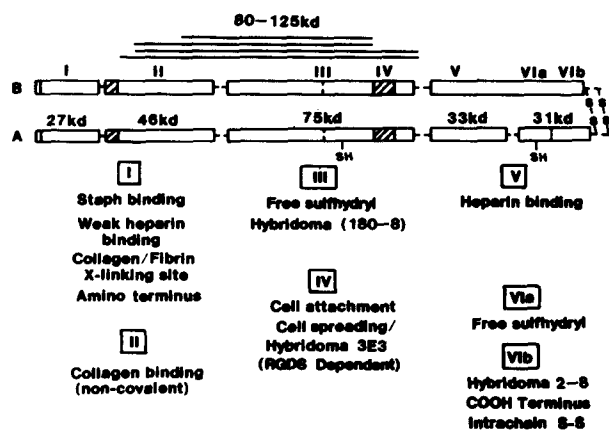
trypsinized, washed in serum-free medium, and adjusted to a final concentration of  $5 \times 10^4$ /ml in Dulbecco's modified Eagle's medium buffered with 0.015 M HEPES, pH 7.2. The cell attachment buffer contained 2 mg/ml BSA. The cells were added in a volume of 0.5 ml ( $2.5 \times 10^4$  cells) to 24-well tissue culture plates (Costar, Cambridge, MA) coated with the appropriate attachment factor, and the plates were incubated for 90 min to 2 h at 37°C. Non-attached cells were washed away and attached cells were solubilized with 0.5 N NaOH and 1% SDS. Solubilized radioactivity was determined by liquid scintillation counting. In experiments using cycloheximide, it was necessary to add the compound back to the cell attachment assay at a concentration of 10  $\mu$ g/ml in order to prevent the resumption of protein synthesis during the assay.

Peptide inhibition studies of attachment were performed as follows. Cell suspensions were adjusted to a concentration of  $3 \times 10^4$ /ml in Dulbecco's modified Eagle's medium with 2 mg/ml BSA and 0.015 M HEPES, pH 7.2. Synthetic peptide was added at the indicated concentrations to radioactive cell suspensions and the samples were incubated at a 37°C water bath for 10–15 min. 100- $\mu$ l aliquots of these cell suspensions ( $3 \times 10^3$  total cells) were added to pre-coated microtiter wells and cells were incubated for 90 min in the cell attachment assays. The attached cells were examined visually before harvesting the assay.

## Results

### Immunochemical Analysis of Tryptic Catheptic Fragments of Fibronectin

Tryptic/catheptic fragments of plasma fibronectin were prepared as described in Materials and Methods and in previous reports (37, 40, 41). This particular fragment preparation was chosen to start these studies since previous work in our laboratory indicated these fragments collectively could account for essentially the entire molecular mass as well as many of the biological activities of the intact protein (40, 41). These fragments were further characterized by immunoblotting SDS PAGE gels of the fragment preparations (not shown), the results of which are summarized in Fig. 1. The fragments will be summarized here for the sake of clarity. The initial tryptic/catheptic digestion scheme generates the following proteolytic fragments (37, 40, 41): (a) a 27-kD tryptic fragment, designated t27, that binds heparin weakly;



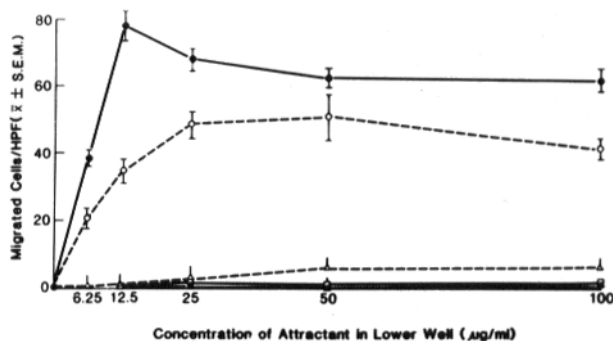
**Figure 1.** The schematic shown above depicts the arrangement of biologically active domains within fibronectin as well as the alignment and apparent molecular masses of proteolytic fragments used in this study. Details are summarized in Results. Hatched boxes indicate the actual location of the domain delineated by the roman numerals. The 75-kD fragment represents a cell-binding fragment previously described by Hayashi and Yamada (13). Domain IV represents the pepsin-generated 11.5-kD fragment that binds monoclonal antibody 3E3 and contains the RGDS sequence described by Pierschbacher and co-workers (31, 33). Domain nomenclature is based on the review of Furcht (10).

(b) a 46-kD tryptic/catheptic fragment (tc 46) that binds gelatin noncovalently; (c) a series of four proteolytic fragments, ranging in molecular mass from 80 to 125 kD (tc 80–125), that do not bind gelatin or heparin; (d) two tryptic/catheptic fragments that bind heparin strongly (tc 33/66); and (e) a tryptic 31-kD fragment (t31) that contains a free sulfhydryl. Fig. 1 also shows the reactivity of these fragments with three monoclonal antibodies. The 180-8 and 2-8 monoclonal antibodies have previously been used to immunolocalize two free sulfhydryl-containing sites along the chain of the fibronectin molecule (40, 41). Monoclonal antibody 3E3 has previously been used to locate a pepsin fragment within fibronectin that contains a cell attachment/spreading promoting sequence, RGDS (31). The four main peptides inserted above the main drawing of the molecule with apparent molecular masses of 80, 105, 120, and 125 kD all contain the 180-8 determinant. In contrast, the 2-8 determinant is localized within the 66-kD fragment, which binds heparin strongly, and the 31-kD free sulfhydryl-containing fragment. The nomenclature of the domains as shown in this figure was previously described in Furcht (10).

### Migration Response of Tumor Cells to Purified Proteolytic Fragments of Fibronectin

The fragment preparations shown in Fig. 1 were added to the lower well of a modified Boyden chamber, and cells were added to the upper wells. The migration of cells in response to these fragments is shown in Fig. 2. Melanoma cells migrate in a concentration-dependent manner to increasing levels of intact fibronectin, with a maximum response of 80-fold over control level migration observed at 12.5  $\mu$ g/ml in the lower well. The only fragments observed to promote haptotactic motility in these chambers were the tryptic/catheptic 80–125-kD fragments that contained the 180-8 and 3E3 determinants. All other fragments were observed to be ineffective at promoting melanoma cell migration in this assay.

Fragments were isotopically labeled with tritium using reductive methylation and added to the lower wells of migration



**Figure 2.** Migration of tumor cells in response to affinity-purified tryptic/catheptic fragments of fibronectin. Purified tryptic/catheptic fragments of fibronectin, shown in Fig. 1, were adjusted to a concentration equivalent on a molar basis to the indicated concentration of intact fibronectin and added to the lower well of a Boyden chamber assay. B16F<sub>10</sub> murine melanoma cells were added to the upper well and the chambers were incubated for 4 h. The assay was then terminated and quantitated as described in Materials and Methods. The results are presented as the mean number of migrated cells per high power field (HPF)  $\pm$  SEM. (●) Intact fibronectin; (○) tc80–125 fragments; (△) tc46 fragment; (□) tc33/66 heparin-binding fragments, t27 or t31 fragments of fibronectin.

chambers to determine that all fragments bound to the filter surface. This was done to establish that failure to stimulate migration in this system by any given preparation of fragments was not due to a failure of the fragments to bind to the filter under the assay conditions used, since earlier studies showed that cells migrated primarily in response to substratum-bound fibronectin (23, 24). The results of this experiment are shown in Table I. These data show that all of the fragments used in this assay bound to the filter surface in amounts that were within or exceeded the range of the binding of intact fibronectin to the filter surface, demonstrating that failure of cells to migrate to a given fragment was not due to a failure of the fragment to bind to the filter surface.

### Attachment of Metastatic Melanoma Cells to Surfaces Coated with Purified Proteolytic Fragments of Fibronectin

Migration of tumor cells in this assay system is via substratum-bound attractant (20, 23), so it was of interest to determine the attachment-promoting activities of the fragment populations used in the migration assay. To do this, attachment of tumor cells to tissue culture surfaces coated with either intact fibronectin or the various fragment preparations was measured as described. The results of this experiment are shown in Fig. 3. As has been shown repeatedly for this protein (for review see references 10, 16, 50), many cell types attach and spread very well to surfaces coated with the intact fibronectin molecule. Results in general indicate that within 90 min, 85% of the cells attach to tissue culture surfaces coated with 25  $\mu\text{g}/\text{ml}$  of intact fibronectin (Fig. 3). High levels (>70%) of cell attachment were also observed to surfaces adsorbed with the molar equivalent of 25  $\mu\text{g}/\text{ml}$  of the 80–125-kD and 33/66-kD heparin-binding fragments (designated tc 80–125 and tc

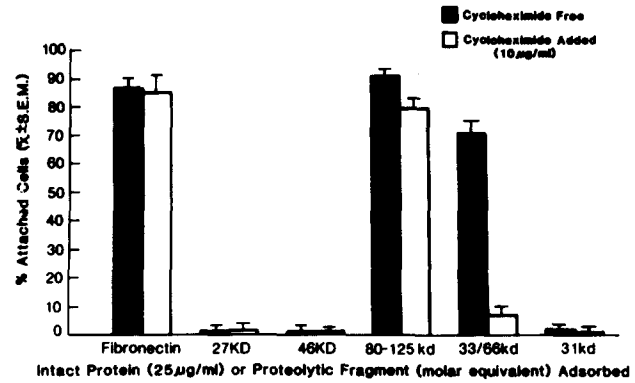


Figure 3. Cycloheximide inhibition of cell attachment to the carboxyl-terminal heparin-binding domains of fibronectin. Wells were coated with 25  $\mu\text{g}/\text{ml}$  intact fibronectin or the molar equivalent of 25  $\mu\text{g}/\text{ml}$  of the particular proteolytic fragments. Radioactive fragments were bound to plastic in parallel to determine that all fragments bound sufficiently (not shown). Radioactively labeled cells were added to wells as described in the presence or absence of 10  $\mu\text{g}/\text{ml}$  cycloheximide. The assay was terminated and adhesion values were determined after a 90-min incubation period. Background adhesion to BSA-coated substrata was 2.5% of the applied counts. Values shown are the triplicate determination of the mean percentage of attached cells plus or minus the SEM.

33/66, respectively). Attachment of these cells to intact fibronectin or the centrally located haptotaxis-promoting region(s) was resistant to the effects of cycloheximide. In contrast, cell adhesion to the 33/66-kD heparin-binding fragments was sensitive to cycloheximide, with a ~90% decrease in levels of adhesion compared to that observed in the cycloheximide-free conditions.

### Identification of Proteolytic Heparin-Binding Fragment That Promotes Melanoma Attachment

The 33/66-kD heparin-binding fragments (tc 33/66) could be further resolved by affinity chromatography on monoclonal antibody 2-8. Previous reports using this monoclonal antibody (40, 41) demonstrated that the 66-kD heparin-binding fragment contains a determinant for this antibody while the 33-kD fragment does not (see Fig. 1). This difference was used to separate these two tryptic/catheptic heparin-binding fragments from each other. A gel of the isolated peptides is shown in Fig. 4. Cell attachment activity was then measured for the purified 33-kD and 66-kD fragments (Table II). The majority of the cell attachment activity present in the 33/66-kD carboxyl-terminal heparin-binding fragments of fibronectin is associated with the 33-kD heparin-binding fragment (containing domain V) and not the 66-kD fragment (which contains both domains V and VI). Curiously, the combination of fragments plated at the lowest concentration tested (25  $\mu\text{g}/\text{ml}$ ) exhibited much greater adhesion activity (about fivefold) than either the 33-kD or 66-kD fragment alone at this concentration.

### Further Definition of the Haptotaxis-promoting Region of the Molecule

The initial studies indicated that the haptotaxis-promoting domain(s) of the molecule is located within the region of the protein which has been shown to contain the determinant for the 3E3 monoclonal antibody and the RGDS sequence. This

Table I. Binding of Purified Fibronectin Fragments to Polycarbonate Filter Surfaces

Sample	Protein in lower well*	Amount bound to filter surface <sup>‡</sup>	Molar equivalent to intact fibronectin bound <sup>§</sup>
	$\mu\text{g}/\text{ml}$	ng	ng
Fibronectin	100	120	120
Fibronectin	1.0	2.3	2.3
27 kD (t27)	100	123	1025
27 kD	10	14.4	120
46 kD (tc46)	100	70	350
46 kD	10	20.5	100
80–125 kD (tc80–125)	100	115	230
80–125 kD	10	38.7	78
33/66 kD (tc33/66)	100	97.5	325
33/66 kD	10	24.7	82
31 kD (t31)	100	59.5	425
31 kD	10	15	107

\* Intact radioactively labeled fibronectin, at the indicated concentrations, or purified proteolytic fragments, at the molar equivalent, were diluted in the medium used for the migration assay and incubated in the chambers as described in Materials and Methods. The filters were removed and processed, and bound radioactivity was determined.

<sup>‡</sup> The actual amount of bound protein to the filter surface is the mean of triplicate wells in the migration chamber. The surface area of one side of the filter in a well is 7.9 mm<sup>2</sup>.

<sup>§</sup> The molar equivalent bound relative to intact fibronectin was calculated as follows. The ratio of the molecular mass between each fragment and intact fibronectin monomer (assumed molecular mass of 230 kD) was calculated. The actual amount bound was divided by this ratio to represent the molar equivalent of this peptide relative to intact fibronectin monomer.

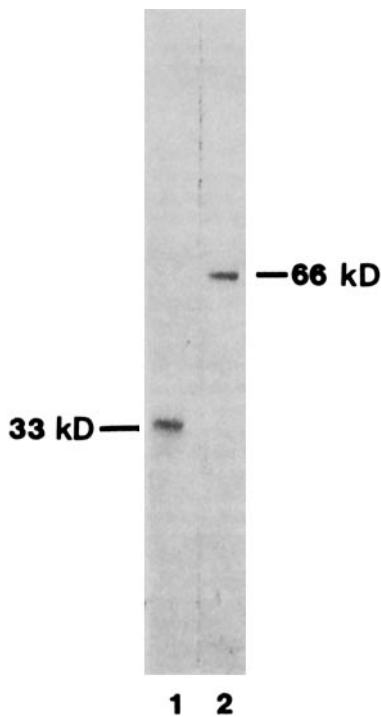


Figure 4. SDS PAGE gel of purified carboxyl-terminal heparin-binding fragments of fibronectin. The 33/66-kD carboxyl-terminal heparin-binding fragments were chromatographed over a monoclonal antibody 2-8 column. The 2-8 unbound (lane 1) and bound (lane 2) depict the 33-kD and 66-kD fragments, respectively.

Table II. Adhesion of Metastatic Melanoma Cells to Purified Fibronectin Fragments

Protein or fragment tested*	Percent total cells bound to increasing concentrations of protein <sup>†</sup>		
	25 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$	100 $\mu\text{g/ml}$
Fibronectin	54.2	69.7	62.4
tc 33/66	50.0	58.6	53.1
tc 33 (2-8 unbound)	8.6	25.5	50.4
tc 66 (2-8 bound)	1.9	2.7	7.0

\* The 33/66-kD heparin-binding fragments were obtained from a tryptic/catheptic limited digest of fibronectin as described in Materials and Methods. These fragments, which both bind heparin strongly, could be segregated on the basis of monoclonal antibody 2-8 chromatography.

<sup>†</sup> Cell adhesion was performed as described in Materials and Methods in the absence of cycloheximide. Intact fibronectin or purified proteolytic fragments were added to wells for coating at the indicated concentration ( $\mu\text{g/ml}$  or molar equivalent). The data are presented as mean percent adhesion, and represent the mean of triplicate determinations. SEMs were <10% of the reported value. (Background adhesion to BSA-coated plastic was 1% of the total counts applied.)

sequence has been reported to promote the attachment and spreading of fibroblasts in vitro (31, 33). Furthermore, these fragments also contained the determinant for monoclonal antibody 180-8, a monoclonal antibody that has been previously used to localize a free sulfhydryl within these fragments (41). Different proteases were used with monoclonal antibody chromatography to further define the region of the molecule that was responsible for promoting the haptotaxis of melanoma cells. The first approach was to perform long-term (2-h) trypsinization of either the 80-125-kD fragments or of intact fibronectin (Fig. 5). In either case a predominant end

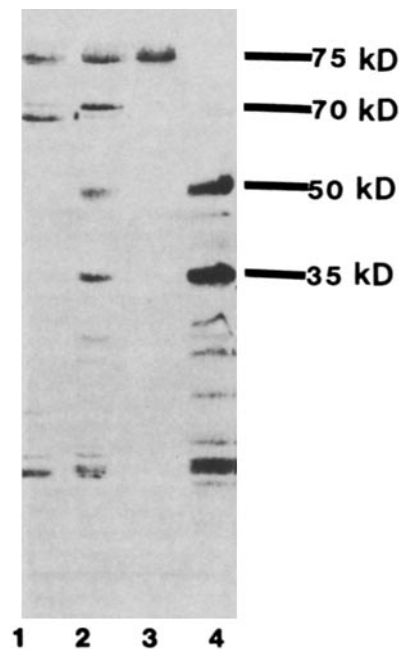
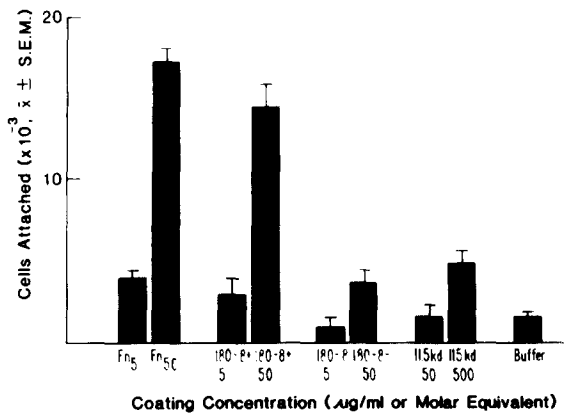


Figure 5. Long-term trypsinization of the 80-125 kD haptotaxis-promoting fragments of fibronectin. The haptotaxis-promoting fragments (tc 80-125) or intact fibronectin were trypsinized for 2 h at 37°C and the digests were examined by gradient (6-20%) SDS PAGE in the presence of 2-mercaptoethanol. Lane 1 contains a 2-h digest of the 80-125 kD fragments and lane 2 shows a 2-h digest of intact fibronectin. Lane 3 contains the 180-8 bound/high performance liquid chromatography-purified 75-kD fragment from the fibronectin digest. The fragments in lane 4 represent the 180-8 nonbound fragments from a 2-h tryptic digest of fibronectin. Approximately 20  $\mu\text{g}$  of protein was applied to each lane.

product was a 75-kD fragment. Both monoclonal antibody and ligand-binding activities were used to place the 75-kD tryptic fragment within fibronectin as shown in Fig. 1. This fragment consists of domains III and IV of fibronectin (10). The 180-8 affinity column was used to bind the 75-kD fragment from the tryptic digest of fibronectin (see Fig. 5, lane 3). The unbound fragments from the 180-8 affinity column included a series of smaller fragments (Fig. 5, lane 4). The 180-8 bound and unbound fragments were tested for biological activity in the cell attachment and haptotaxis assays.

The results of the attachment assay on the 180-8 bound and unbound fractions are shown in Fig. 6. Fibronectin-coated surfaces are active at promoting attachment of melanoma cells in a concentration-dependent manner. At the higher coating concentration (50  $\mu\text{g/ml}$ ), almost 75% of the cells were attached to the substratum. Similar values of cell attachment were observed on surfaces coated with the molar equivalent of the 180-8-bound fragments of the tryptic digest. In contrast, minimal attachment of the melanoma cells was observed on surfaces coated with the fractions of the tryptic digest that did not bind the 180-8 affinity column. The localization of the 3E3 monoclonal antibody determinant to the 180-8 bound tryptic fragments prompted us to examine the pepsin-derived 11.5-kD fragment (domain IV) for melanoma cell attachment activity. This purified peptide, kindly supplied to us by Dr. Michael Pierschbacher, has been previously demonstrated to promote the attachment of fibroblasts to surfaces having this peptide adsorbed to it (31, 33). In

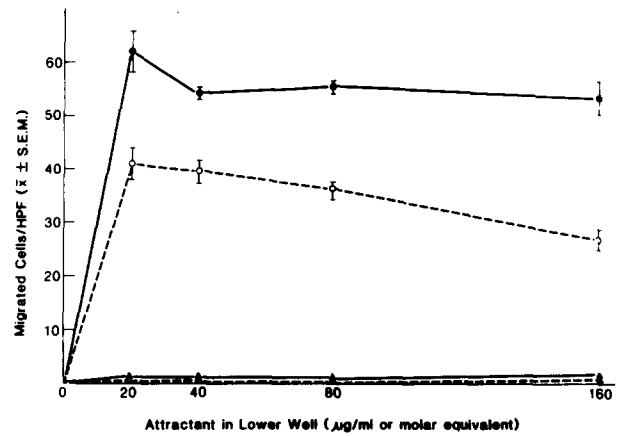


**Figure 6.** Purification of attachment activity from 2-h tryptic digests of fibronectin by monoclonal antibody 180-8. The 180-8-bound and -unbound fractions from a 2-h tryptic digest of fibronectin were used to coat tissue culture wells to monitor attachment of melanoma cells. Wells were coated at the indicated concentration of intact fibronectin or the molar equivalent of the 75-kD tryptic fragment or 11.5-kD RGDS-containing fragment. The molar equivalent of the series of low molecular mass 180-8 unbound fragments was estimated assuming an average molecular mass of 25 kD for this sample. The buffer control represents cells attached to tissue culture plastic coated with BSA alone. Data represent the mean number of cells attached  $\pm$  SEM. Determinations were in triplicate.

contrast to these reported results (31, 33), we had success in promoting only low levels of attachment of melanoma cells to surfaces coated with even very high concentrations of this peptide (see Fig. 6). Even at coating concentrations of a molar equivalent of 500  $\mu\text{g}/\text{ml}$  of intact fibronectin, only  $\sim 20\%$  of the cells applied were observed to attach to surfaces coated with the 11.5-kD pepsin-generated fragment. This was in contrast to the higher levels ( $>70\%$ ) of adhesion observed on either fibronectin or the 75-kD tryptic fragment coated at a 10-fold lower molar equivalent concentration. Data using tritium-labeled 11.5-kD fragment to monitor binding demonstrated that the molar equivalent amount bound was equal to or greater than the amount of intact fibronectin bound (not shown).

These fragment preparations were also tested for the stimulation of haptotaxis activity in the Boyden chamber assay. The results are shown in Fig. 7. As with the attachment assay, both intact fibronectin and the 180-8-bound tryptic fragments are effective at promoting the haptotactic migration of melanoma cells *in vitro*. The 180-8 unbound fragments are inactive at promoting melanoma migration. The purified 11.5-kD pepsin-derived fragment (domain IV) is also inactive at promoting the haptotactic migration of metastatic tumor cells. The failure of this peptide to stimulate migration is not due to the inability of the peptide to bind to the filter surface, since immunolocalization of the fragment on the filter surface could be established using monoclonal antibody 3E3 (not shown). Additionally, the radiolabeled 11.5-kD peptide verified binding to the filter surface (not shown). This 11.5-kD peptide added to the lower well at molar equivalent concentrations of up to 1 mg/ml fibronectin was observed to be ineffective at stimulating tumor cell migration in this system.

The 180-8 purified 75-kD cell-binding/haptotaxis-promoting fragment was subjected to further proteolytic digestion with pepsin for 10 or 30 min. The resulting digest is shown



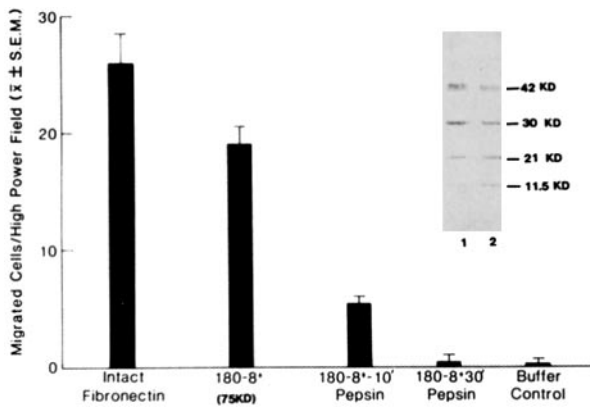
**Figure 7.** Purification of haptotaxis activity from long-term tryptic digests of fibronectin by monoclonal antibody 180-8. Fibronectin (●), the 11.5-kD RGDS-containing pepsin-derived cell attachment fragment (▲), or 180-8-bound (○) and unbound (△) fractions of a 2-h tryptic digest were adjusted to the indicated concentrations and dispensed into the lower well of a Boyden microchamber as described. Melanoma cells were added to the upper well and the chambers were incubated as described. Data represent the mean number of migrated cells per high power field (HPF)  $\pm$  SEM.

in the inset in Fig. 8 and this confirms our observation in Fig. 7. Within 10 min of treatment with pepsin, there is the appearance of four major peptides in the preparation that range in molecular mass from 11.5 to 42 kD. As indicated by immunoblots (not shown), the 180-8 and 3E3 determinants segregate to the 21- and 11.5-kD peptide fragments, respectively. By the end of 30 min of pepsinization, the four major peptides are all that remain of the 75-kD tryptic haptotaxis-promoting region. The data in Fig. 8 show that the migration-promoting activity of 75-kD fragment preparation is virtually destroyed by complete digestion with pepsin.

#### **Addition of Soluble Peptide to Cells Attaching to Substratum-bound Fibronectin or to Purified Cell Attachment Domains**

The next studies determined the role of the RGDS tetrapeptide in mediating the attachment and spreading of cells on fibronectin and the various purified fragments. In the first set of experiments, the effect of a high concentration of soluble RGDS was examined on cell attachment to substrates coated with various concentrations of fibronectin, the 80-125-kD fragments (tc 80-125), or the purified 75-kD haptotaxis-promoting fragment (t75) (Fig. 9, top). These results show that  $10^{-3}$  M soluble RGDS inhibits the attachment of melanoma cells by 80% to substrates coated with 1  $\mu\text{g}/\text{ml}$  of intact fibronectin compared to attachment in the absence of this synthetic peptide. Similarly, the attachment of cells to the haptotaxis-promoting fragments was inhibited dramatically (almost 90%) by the presence of this concentration of RGDS in wells coated with the molar equivalent of 5  $\mu\text{g}/\text{ml}$  of these fragments. The inhibitory effect of this peptide could be overcome by increasing the amount of fibronectin or haptotaxis-promoting fragment adsorbed to the surface. The use of 5  $\mu\text{g}/\text{ml}$  fibronectin or 25  $\mu\text{g}/\text{ml}$  of the haptotaxis-promoting fragments for coating the wells almost completely eliminated the inhibitory effects of  $10^{-3}$  M soluble RGDS.

The effect of RGDS on attachment to the 33-kD heparin-

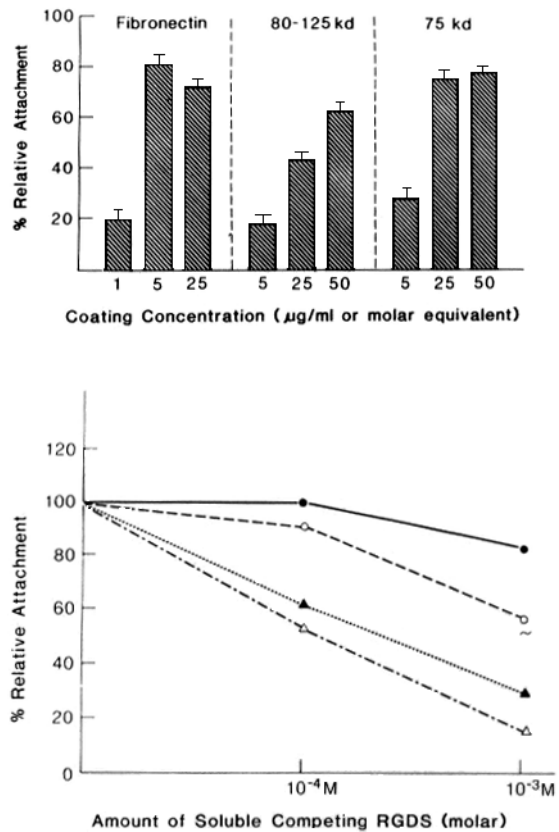


**Figure 8.** Pepsin sensitivity of haptotactic activity in the 75-kD fragment of fibronectin. The 180-8-purified 75-kD fragment (Fig. 4, lane 3) pepsinized for either 10 min (shown in gel insert in lane 1) or 30 min (lane 2). These samples, along with intact fibronectin, were added at a concentration (or molar equivalent) of 100  $\mu\text{g}/\text{ml}$  to the lower well of a Boyden chamber and tested as described for the ability to stimulate melanoma cell migration. Data represent the mean number of migrated cells per high power field  $\pm$  SEM. Determinations are in triplicate.

binding fragment was also studied (Fig. 9, *bottom*). In this experiment, substrates were coated with 5  $\mu\text{g}/\text{ml}$  of fibronectin or the molar equivalent (relative to intact fibronectin) of the 33-kD heparin-binding/cell attachment fragment. This coating level of 33-kD fragment was chosen since it was at the lower range of concentrations observed to promote melanoma attachment. The haptotaxis-promoting fragments of fibronectin were also included for comparison's sake in the assay and were coated at a molar equivalent concentration of 5  $\mu\text{g}/\text{ml}$  intact fibronectin. Concentrations of  $10^{-4}$  and  $10^{-3}$  M RGDS inhibited the attachment of melanoma cells to the 75-kD haptotaxis-promoting fragment by 50 and 85%, respectively, compared to attachment in the absence of any competing peptide. Similar levels of inhibition were also observed on substrates coated with the tryptic/catheptic 80-125-kD fragments. In contrast, the attachment of melanoma cells to substrates coated with 5  $\mu\text{g}/\text{ml}$  intact fibronectin was relatively resistant to the effects of RGDS. Similarly, the attachment of melanoma cells to the 33-kD heparin-binding/cell attachment domain was unaffected by  $10^{-4}$  M RGDS with <10% inhibition in relative attachment. Melanoma attachment to the 33-kD fragment was somewhat more sensitive to the presence of  $10^{-3}$  M RGDS (almost 40% relative inhibition); however, this level of RGDS also inhibited the relative nonspecific attachment to BSA-coated substrata by a similar amount (~45%). Therefore it is difficult to attribute this effect of  $10^{-3}$  M RGDS on the 33-kD fragment as a specific inhibitory effect of the peptide, since it also decreased nonspecific adhesion to BSA.

## Discussion

The interaction of the cell surface with extracellular matrices has wide-ranging consequences for numerous developmental and pathological processes. Constituents of the extracellular matrix participate in several aspects of cell behavior ranging from simple adhesion, to promoting cytoskeletal reorganization and active cell motility (10, 12, 16, 50). Furthermore, the phenotypic behavior of cells can be dramatically



**Figure 9.** Effect of soluble RGDS on fibronectin-mediated attachment of melanoma cells. (*Top*) Microtiter wells were coated with the indicated concentrations of fibronectin or the molar equivalent concentrations of the fragment preparations. Adherence of melanoma cells was then determined in the presence or absence of  $10^{-3}$  M soluble competing RGDS. Actual cell adhesion values for the various coating levels (1–50  $\mu\text{g}/\text{ml}$ ) in the absence of competing peptide ranged as follows. Fibronectin ranged from 22% (1  $\mu\text{g}/\text{ml}$  coating) to 60% (25  $\mu\text{g}/\text{ml}$ ), the 80–125 kD fragment ranged from 15% (molar equivalent of 5  $\mu\text{g}/\text{ml}$ ) to 50% (molar equivalent of 50  $\mu\text{g}/\text{ml}$ ), and adhesion to the 75-kD fragment ranged from 18–50%. Control (background) adherence to BSA was 3%. Data represent the mean percentage of cells ( $\pm$  SEM) adhering, relative to control adherence in the absence of competing peptide. Determinations were in triplicate. (*Bottom*) Microtiter wells were coated with 5  $\mu\text{g}/\text{ml}$  of intact fibronectin (●) or the molar equivalent of 5  $\mu\text{g}/\text{ml}$  of the 33-kD heparin-binding fragment (○), the 80–125 kD (▲) fragment, or the 75-kD (△) fragment. Adhesion was then measured in the presence or absence of the indicated concentrations of RGDS. Actual adherence in the absence of competing peptide was 35% for fibronectin, 18% for the 33-kD fragment, 15% for the 80–125-kD fragment, and 18% for the 75-kD fragment. The relative inhibition of nonspecific attachment to BSA (which was 3% of the applied counts) in the presence of competing peptide are shown (~). The data are expressed as the mean percent of cells attached to each fragment relative to control adherence in the absence of any competing soluble peptide. The SEMs were all <10% of the indicated values, and determinations were all in triplicate.

affected by the extracellular matrix, indicating an important role for the matrix in modulating cellular growth and differentiation (12). Although the importance of cell-matrix interactions has been appreciated for some time, the molecular basis for these interactions is complex and currently poorly understood.

The work presented in this manuscript examined cell adhesion and haptotactic motility of malignant melanoma cells on fibronectin and purified proteolytic fragments of the molecule. Using the approaches outlined, we have identified two distinct proteolytic fragments of fibronectin with cell adhesion-promoting qualities. One of these adhesion-promoting fragments, a tryptic 75-kD fragment (t75), also promotes the haptotaxis of tumor cells *in vitro*. This fragment, which contains domains III and IV, includes the RGDS tetrapeptide sequence that promotes cell adhesion in a number of other systems (31, 33, 51). The second adhesion-promoting fragment, which is the 33-kD heparin-binding fragment, contains domain V. Adhesion of cells to domain V does result in cell spreading but not in tumor cell motility. This indicates that haptotactic migration in this system, which is due to substrate-bound fibronectin, is not due to simple adhesion gradients of this protein. High levels of RGDS in solution have little effect on cell adhesion to this heparin-binding fragment, which contrast the results seen on the 75-kD haptotaxis-promoting fragment. One further distinction between domain III/IV-mediated adhesion and domain V-mediated adhesion is that the former is cycloheximide resistant while the latter is sensitive to protein synthesis inhibition, suggesting that distinct receptors on the cell surface are involved in adhesion to each fragment.

The tryptic 75-kD cell-binding fragment, representing domains III and IV, is the smallest portion of the molecule that we could isolate with tumor cell haptotactic activity. It would appear from these data that although the RGDS sequence is involved in the adhesion and spreading of melanoma cells on the 75-kD fragment that it is insufficient to promote tumor cell haptotaxis. This assertion is supported by the following observations. First, the purified 11.5-kD pepsin fragment (domain IV), which contains RGDS, does not stimulate motility and only partially promotes adherence. These negative results were not due to a failure of the 11.5-kD fragment to bind to the surfaces used in the respective assays. Furthermore, it was difficult to totally inhibit adhesion of melanoma cells to the 75-kD fragment with high levels of soluble RGDS. Increasing the amount of bound 75-kD fragment virtually eliminates the inhibitory effect of soluble RGDS. Additionally, cells incubated for longer times in the presence of RGDS will also overcome RGDS inhibition of spreading (51). These results are consistent with the possibility that an adhesion site in addition to RGDS is present in domain III of the 75-kD fragment although definitive isolation of this putative site is necessary to prove this. Multiple spreading/motility activities in this region of the molecule have also been alluded to by Donaldson et al. (8), in studies designed to study active domains of fibronectin responsible for epithelial migration during wound healing.

There have been many attempts to define cell surface receptors for fibronectin (see reviews 10, 15, 16, 50). Gangliosides, which have been reported to inhibit cell attachment to fibronectin (17, 52) and to bind weakly to fibronectin (29), could interact with the type III homologies (30) present in the 75-kD fragment that contain clusters of positively charged residues (38). Similarly, a cell surface glycoprotein complex has been identified by several groups which seems to be involved in cell adhesion to fibronectin (5, 6, 49). This glycoprotein complex is apparently trypsin resistant (11), and recent work by Pytela et al. (35) suggests that this complex

may interact in part with the RGDS sequence on the fibronectin molecule. Studies using an adhesion-disrupting monoclonal antibody that recognizes this complex (7) demonstrates that not all cell types use this complex to the same degree in adhering to intact fibronectin. The current findings are also consistent with the possibility that binding of multiple constituents on the cell surface by the 75-kD fragment is a prerequisite for cell motility. Perhaps cross-linking of cell surface constituents by this fragment stimulates cell movement, a mechanism suggested by examining other model systems for motility (for review see reference 45).

These results also clearly demonstrate the attachment of melanoma cells to a carboxyl-terminal heparin-binding domain that is RGDS independent. The heparin-binding quality of the 33-kD fragment suggests that this adhesion activity might be mediated by cell-associated proteoglycan and preliminary data supports this (unpublished observations). The localization of the cell attachment activity to the 33-kD and not the 66-kD tryptic/catheptic heparin-binding fragment localizes this activity to the A chain of the molecule (5) since a tryptic site occurs within this region of the A chain that is lacking in the B chain (13). To our surprise, the combination of heparin-binding fragments exhibited much greater activity at lower coating concentrations (i.e., 25  $\mu\text{g}/\text{ml}$ ) than either the 33- or 66-kD fragments alone (approximately fivefold). We have no immediate explanation for this data and at this point feel that the reason might be trivial. As an example, the amount or conformation of the 33-kD fragment (the active fragment) might be altered when co-plated with the 66-kD fragment compared to adherence of this peptide directly onto plastic. Identification of the active adhesion structure in the 33-kD fragment will help to clarify this point.

The region of the molecule in the A chain that this 33-kD fragment arises from has been shown to represent a structurally dynamic portion of the protein (13, 19, 38). Schwarzbauer et al. (38) have shown that the A chain of rat hepatocyte fibronectin selectively contains one of two possible non-type I, II, or III homology inserts of 90 or 125 amino acids that, because of the extended conformation, could create new proteolytic sites on the molecule between domains V and VI. Based on the amino acid composition of these inserts, it has also been postulated that this insert may participate in modifying proteoglycan-binding activity by this region of the molecule (38). The results using human fibronectin cDNA also demonstrate heterogeneity in primary structure of this region of plasma fibronectin, with extra non-type III inserts found between domains V and VI (18). The results of the present study suggest a possible explanation for the biological significance at the cellular level for this structural heterogeneity. These variations in structure may have consequences regarding the interaction of fibronectins with specific cell surface constituents, and thus may be important in determining the phenotypic expression of cells attached to the various forms of this protein.

Several lines of evidence point to an important role for intercalated heparin sulfate proteoglycan (for review see references 3, 14) in cell surface interactions with fibronectin. Cell surface heparan sulfate can be cross-linked to fibronectin at the surface of cells attached to fibronectin (28). Proteoglycans, especially heparan sulfate, have also been identified in substrate-attached material (28), which is left behind following cell detachment from substrate by chelating agents (for review



see reference 21). Cell attachment via platelet factor 4 has previously been used by Laterra et al. (22) as a model to examine cell attachment via cell surface heparan sulfate. Using this model, cell attachment via cell surface heparan sulfate proteoglycan has been reported to have effects on cytoskeletal reorganization that are distinct from those observed on intact fibronectin (21).

Importantly, recent work from this group has indicated that cell attachment to the cell-binding (RGDS) region of fibronectin versus platelet factor 4 has different consequences for contact formation and cytoskeletal architecture (4). This study reported that attachment to either fragment alone results only in close contact formation, whereas cell adhesion to both fragments co-plated fully duplicates focal contact adhesion and stress fiber formation seen with cells adherent to intact fibronectin. These results suggest that multiple cell surface receptors interact with distinct domains and serve to regulate total phenotypic expression on intact fibronectin. The identification and further characterization of multiple attachment sites for cell surfaces should help to explain the biochemical basis for the myriad of effects that fibronectin has on behavior of both normal and malignant cells.

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