Localization and Chemical Synthesis of Fibronectin Peptides with Melanoma Adhesion and Heparin Binding Activities†

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ABSTRACT: Tumor cell adhesion to the extracellular matrix is an important consideration in tumor metastasis. Recent results show that multiple adhesion-promoting domains for melanoma cells can be purified from proteolytic digests of fibronectin [McCarthy, J. B., Hagen, S. T., & Furcht, L. T. (1986) *J. Cell Biol. 102*, 179–188]. Monoclonal antibodies were generated against a tryptic/catheptic 33K heparin binding fragment of fibronectin derived from the carboxyl terminal of the A chain. This region contains a tumor cell adhesion-promoting domain(s). The amino-terminal sequence was determined for this fragment, as well as a tryptic 31K fragment which is located to the carboxyl-terminal side of the 33K heparin binding fragment in A chains of fibronectin. The partial sequence data demonstrate that arginyl-glycyl-aspartyl-serine (RGDS) or the related arginyl-glutamyl-aspartyl-valine (REDV) is not present in the 33K heparin binding fragment, confirming earlier results which demonstrated that cells adhere to this fragment by an RGDS-independent mechanism. Two monoclonal antibodies, termed AHB-1 and AHB-2, recognized epitopes common to heparin binding fragments derived from the carboxyl terminus of both the A and B chains of fibronectin. Monoclonal antibody AHB-2 inhibited melanoma adhesion to the 33K heparin binding fragment of fibronectin in a concentration-dependent manner, whereas monoclonal antibody AHB-1 had no effect on adhesion to this fragment. Neither monoclonal antibody inhibited adhesion to intact fibronectin. However, monoclonal AHB-2 potentiated the inhibitory effect of suboptimal levels of exogenous RGDS on cell adhesion to intact fibronectin. AHB-2 recognized an epitope common to both the A- and B-chain carboxyl-terminal heparin binding region of fibronectin. Thus, synthetic peptides of this region were prepared in order to further localize the cell adhesion-promoting activity of this portion of the molecule. Two peptides were identified which promoted melanoma cell adhesion in a concentration-dependent manner. These peptides also bound [^{3}H]heparin in a solid phase binding assay. The studies support the concept that melanoma adhesion to intact fibronectin occurs as a result of multiple distinct adhesion-promoting domains, which interact with multiple, functionally discrete receptors on the surface of melanoma cells.

The invasion and metastasis of tumor cells is a complex process which involves numerous tumor- and host-related factors. One important approach for understanding the biology of metastasizing tumor cells is to develop tools which can be used to study the molecular basis of cell adhesion to components of the extracellular matrix. Work from several laboratories has shown that adhesion to the extracellular matrix is an integral part of the metastatic process. For example, coinjection of metastatic tumor cells with antibodies against laminin has been shown to inhibit the formation of pulmonary metastatic nodules (Terranova et al., 1982). More recently, isolated tumor cell adhesion-promoting fragments of laminin (Barsky et al., 1984) or synthetic adhesion-disrupting peptides from fibronectin (Furcht et al., 1985; Humphries et al., 1986) have been shown to inhibit the metastatic behavior of tumor cells when these cells were incubated ex vivo in the presence of these reagents prior to injection into mice. These and many other results suggest a role for tumor cell adhesion to molecules in plasma, basement membranes, or extracellular matrices in the arrest and extravasation of metastasizing tumor cells.

It has become apparent over the last several years that the molecular basis for the adhesion of both normal and transformed cells to extracellular matrix components is complex and probably involves several distinct cell-surface molecules. The extracellular matrix component which has been most intensively studied in this regard is fibronectin. Fibronectin contains several discrete domains which are involved in the binding of the molecule to other matrix constituents such as collagen or glycosaminoglycans, as well as to cell surfaces [reviewed in Furcht (1983), Yamada (1983), Hynes (1985), and McCarthy (1985)]. The primary structure of one cell adhesion domain within fibronectin was originally determined to be a tetrapeptide sequence consisting of arginyl-glycyl-aspartyl-serine (RGDS)[1] (Pierschbacher & Ruoslahtti, 1984). The RGDS peptide can directly promote the adhesion of certain cell types, and high levels of soluble RGDS will partially disrupt cell adhesion to intact fibronectin (Pierschbacher & Ruoslahti, 1984; Yamada & Kennedy, 1984; McCarthy et al., 1986). Cell adhesion to the RGDS sequence in fibronectin is thought to occur by the interaction of this sequence with a cell-surface glycoprotein complex termed integrin (Horwitz et al., 1985; Tamkun et al., 1986).

Despite the importance of the RGDS/integrin complex in fibronectin-mediated cell adhesion, several lines of evidence

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[1] Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; PBS, Dulbecco's phosphate-buffered saline, pH 7.2; RGDS, arginyl-glycyl-aspartyl-serine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; FCS, fetal calf serum; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kDa, kilodalton(s).

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point to the involvement of additional cellular receptors and different determinants within fibronectin in this process. Multiple adhesion- and/or neurite-promoting determinants within fibronectin have been identified for human neuroblastoma cells (Waite et al., 1987). Many cell types form focal adhesions on intact fibronectin which represent regions of close apposition between the plasma membrane and the substratum [reviewed in Lark et al. (1985) and Izzard et al. (1985)]. These sites also represent insertion points for actin-rich stress fibers and have been shown to contain several actin-associated cytoskeletal proteins. Focal adhesion sites also contain several classes of molecules implicated in cell adhesion, including integrin (Damsky et al., 1985), heparan sulfate and chondroitin sulfate proteoglycans (Lark et al., 1985), and gangliosides (Yamada, 1983). The action of multiple receptors has been implicated in adhesion plaque formation. Cells adherent on either RGDS-containing fragments or heparin binding, adhesion-promoting ligands (e.g., platelet factor 4 or heparin binding fragments of fibronectin) form only close contacts (Laterra et al., 1983; Woods et al., 1986). In contrast, cells adherent on both RGDS-containing fragments and heparin binding ligands display fully developed focal adhesions (Beyth & Culp, 1984; Woods et al., 1986). Additionally, antibodies against heparin binding fragments of fibronectin inhibit focal adhesion formation, although the level of cell adhesion on intact fibronectin is not reduced (Woods et al., 1986). Collectively, these results suggest a role for heparin binding domains of fibronectin in promoting normal and malignant cell adhesion, and in regulating phenotypic expression of cells.

We have recently published results identifying a 33K heparin binding fragment of fibronectin which promotes the adhesion and spreading of metastatic melanoma cells by an RGDS-independent mechanism (McCarthy et al., 1986). This fragment, which originates from the carboxyl terminal of the A chains of fibronectin (Hayashi & Yamada, 1983; Hynes, 1985; McCarthy et al., 1986), also promotes the adhesion of neurons and the extension of neurites by these cells (Rogers et al., 1985). The current studies were designed to further localize and characterize this adhesion-promoting activity within intact fibronectin. The 33K fragment was shown to lack the RGDS sequence by direct sequencing and was verified to contain part of the type IIIcs insert (Hynes, 1985; Kornblihtt et al., 1985). Monoclonal antibodies were generated against this fragment and characterized for the ability to inhibit cell adhesion to this fragment and to intact fibronectin. An adhesion-inhibiting antibody was identified which recognized an epitope common to both the A and B chain derived carboxyl-terminal heparin binding fragments of fibronectin. Synthetic peptides which represented structures common to the carboxyl-terminal heparin binding region of both A and B chains of plasma fibronectin were constructed and tested for the ability to promote tumor cell adhesion. Two nonoverlapping peptides were identified which promoted tumor cell adhesion. These peptides also bound heparin in a solid phase binding assay. The peptides were distinct from the type IIIcs cell adhesion sequences recently described by Humphries et al. (1986,1987), suggesting that melanoma adhesion to the carboxyl-terminal heparin binding region of fibronectin has a complex molecular basis.

Materials and Methods

Cells and Culture Conditions. The cells used for the current studies were the metastatic melanoma cell line K1735 M4, originally supplied as a generous gift from Dr. I. J. Fidler of Houston, TX. Cells were passaged in vitro in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) by methods which we have described previously (McCarthy et al., 1986). The high metastatic potential of this clonal line of cells was verified by lateral tail vein injection of C57B16♀ × C3HHeN♂ F1 mice. The injection of 3×10^5 tumor cells resulted in the production of greater than 250 pulmonary tumor nodules per mouse in 21 days. Care was taken to limit the number of in vitro passages to 12 in order to minimize drift of the metastatic phenotype.

Monoclonal Antibody 2-8. For certain studies, proteolytic fragments of fibronectin were affinity purified on columns to which monoclonal antibody 2-8 was affixed (McCarthy et al., 1986). This monoclonal antibody, which was originally used in our laboratory to localize free sulfhydryls within plasma fibronectin (Smith et al., 1982), recognizes an epitope on fibronectin which is near the carboxyl-terminal end of the molecule (see Figure 1, domain VI).

Protein and Fragment Preparation. Human plasma fibronectin was purified as a byproduct of factor VIII production by sequential ion-exchange and gelatin affinity chromatography as described (McCarthy et al., 1986). The protein migrates as a closely spaced doublet of 230K and 240K on reducing sodium dodecyl sulfate–polyacrylamide electrophoresis gels (SDS–PAGE).

Several proteolytic fragments of fibronectin were used for the current studies. A 2-min tryptic digest of intact fibronectin (McCarthy et al., 1986) served as a source of a 31K free sulfhydryl containing fragment which originates from the carboxyl–terminal end of one chain of fibronectin (Smith et al., 1982). Additionally, two large fragments which contain both the gelatin binding domain and the carboxyl-terminal heparin binding domain of fibronectin were purified from this digest. These two fragments, which have apparent molecular weights of 190K (t190) and 200K (t200), were purified from tryptic digests of the molecule by sequential gelatin and heparin affinity chromatography (McCarthy et al., 1986). This 190K/200K doublet was then cleaved with cathepsin D (Smith & Furcht, 1982). Cathepsin D digests of the t190/t200 fragments contain two major heparin binding fragments having apparent molecular weights of 33K (t/c 33) and 66K (t/c 66; Smith & Furcht, 1982; McCarthy et al., 1986). The digests also contain a minor 44K heparin binding fragment which represents a degradation product of the t/c 66 fragment, since it binds monoclonal antibody 2-8 along with the t/c 66 fragments (Smith & Furcht, 1982). The 33K fragment promotes the adhesion and spreading of cells by an arginyl-glycyl-aspartyl-serine (RGDS)-independent mechanism (McCarthy et al., 1986). These fragments were purified from the cathepsin D digests and from each other by affinity chromatography on monoclonal antibody 2-8 as described (McCarthy et al., 1986).

Amino Acid Sequencing. The amino-terminal ends of the t/c 33, t/c 66, and t31 purified proteolytic fragments of fibronectin were sequenced, as in Hewick et al. (1981), by Dr. James L'Italien at Molecular Genetics, Inc. (Edina, MN), and by Dr. Robert Wohlheuter at the Microchemical Facility of The University of Minnesota, using an Applied Biosystems gas phase sequenator (Model 470 A).

Peptide Synthesis. Peptides were synthesized at the Microchemical Facility of The University of Minnesota by Dr. Robert Wohlheuter using a Beckman System 990 peptide synthesizer, or by Dr. Bianca Conti-Tronconi of the Department of Biochemistry, University of Minnesota. The procedures used are based on the Merrifield solid phase system as described previously (Stewart & Young, 1984). Lyophilized crude peptides were purified by preparative reverse-phase high-performance liquid chromatography (HPLC) on a C-18

column, using an elution gradient of 0–60% acetonitrile with 0.1% TFA in water.

Monoclonal Antibody Production. Mouse monoclonal antibodies were generated against the t/c 33 heparin binding fragment of fibronectin by using a protocol similar to that published previously (Smith et al., 1982). Briefly, Balb/C immune splenocytes were fused with murine P3-NS-1-AGY-1 myelomas using poly(ethylene glycol). The fused cells were then dispensed into 24-well tissue culture plates (Costar, Cambridge, MA) in DMEM with 20% horse serum, supplemented with 22 mM hypoxanthine, 9 μM aminopterin, and 8 mM thymidine (HAT). Wells which contained actively secreting hybridomas were detected by direct ELISA assay on plates coated with the t/c 33 heparin binding fragment of fibronectin (Smith et al., 1982). Wells containing antibody-producing hybridomas were cloned in 96-well tissue culture plates by limiting dilution (one cell per two wells). Antibody-producing clones were injected into the peritoneal cavities of Balb/C mice which had been injected 1 month prior with 0.5 mL of pristane. The ascites which formed 2–4 weeks later were harvested and rescreened for the presence of immunoreactive antibody. This ascites then served as a source of immunoglobulin for the present studies.

Purification and Characterization of Monoclonal Antibodies. Monoclonal antibodies were purified by using a commercial kit available from Bio-Rad (Richmond, CA). This kit uses a protein A affinity column in order to purify the immunoglobulins from ascites. The protocol used for purification was that supplied by the manufacturer. Purified antibodies were then concentrated by dialysis and lyophilization, and the immunological reactivity of these antibodies was verified by ELISA. The antibodies were all characterized as IgG_1 using Ouchterlony plates and commercially available typing antisera (Cooper Biomedical/Cappel Laboratories, Malvern, PA).

Immunoblots were prepared according to a previously published protocol (Towbin et al., 1979). Briefly, SDS–PAGE gels were sandwiched against nitrocellulose (Millipore, type HAHY), and the transfer was performed by using a Transphor apparatus (Hoeffer, San Francisco, CA) at a potential of 6 V/cm. The nitrocellulose sheets were incubated overnight in phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA, Sigma) and 5% carrier (goat) serum. Individual sheets were removed from this buffer and incubated for 45 min to 1 h in the presence of various monoclonal antibodies which were diluted in the PBS/BSA carrier serum mixture. The sheets were washed in three 10-min changes of PBS/BSA with carrier serum and then incubated for an additional 45 min in the presence of commercially prepared ^{125}I-labeled goat anti-mouse antibody (Amersham, Arlington Heights, IL) diluted in PBS/BSA carier serum. The sheets were then washed extensively in PBS, air-dried, and sandwiched against Kodak X-Omat X-ray film with an intensifying screen. The film was exposed for 4 h to overnight at −70 °C.

Cell Adhesion Assay. The methodology for cell adhesion has been previously published (McCarthy et al., 1986). Briefly, cultures of cells which were 50–70% confluent were pulsed overnight in the presence of 2 μCi/mL [^3H]thymidine. The cells were harvested by trypsinization, washed in DMEM, and adjusted to a final concentration of (3–4) × 10^4 cells/mL in DMEM buffered with 15 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) containing 5 mg/mL fatty acid free BSA (Miles, Elkhart, IN). In certain situations, the cells were preincubated for 30 min in the presence of various concentrations of the synthetic tetrapeptide RGDS prior to the assay. The cells were then dispensed into wells (100 μL/well) which had previously been coated with intact fibronectin or the 33K fragment of fibronectin (McCarthy et al., 1986). The actual amount of 33K fragment or intact fibronectin bound to the wells was determined by using radioactively labeled proteins as previously described (McCarthy et al., 1986). Nonspecific adhesion to sites on the plastic wells was blocked by preincubating plates with 5 mg/mL BSA for 2 h prior to adding the cells. Some wells were also preincubated for 1 h in the presence of various concentrations of monoclonal antibodies which had been diluted in PBS/BSA. The assay was incubated for 30 min at 37 °C, at which time the wells were washed with warm (37 °C) PBS containing 10 mM $CaCl_2$ to remove nonadherent cells. The adherent cells were then solubilized with 0.5 N NaOH containing 1% SDS, and bound radioactivity was quantitated in a Beckman Model 3801 liquid scintillation counter. Determinations represent the mean of triplicates.

Cell adhesion to synthetic peptides was performed essentially as above except that Immulon C (Dynatech, Alexandria, VA) plates were used instead of 96-well tissue culture plates. These plates are designed with an activated surface which will permit the covalent attachment of ligands to the surface of the wells. Peptides were diluted to appropriate concentration in PBS, and 100 μL of each peptide was applied to wells in triplicate. The plates were then incubated overnight at 37 °C to allow the peptides to dry on the plates, and the following morning, the wells were incubated with PBS containing 1% BSA for 1 h to block nonspecific sites. The wells were then washed 4 times with deionized water prior to the addition of cells for the adhesion assay. Quantitation of cell adhesion to synthetic peptides was performed as described above.

Heparin Binding Assay. The ability of synthetic peptides to bind [^3H]heparin was also tested by using the Immulon C plates prepared as for the cell adhesion assay above. To quantitate heparin binding, 2 μg (10000 dpm/well) of [^3H]heparin (0.03 mCi/mg; ICN Immunobiologicals, Lisle, IL) in PBS with 1% BSA was added to each well. The plates were incubated at 37 °C for 2 h, at which time the plates were washed 5 times with PBS, and the contents of each well were solubilized with 0.5 NaOH containing 1% SDS. Bound radioactivity was quantitated in a liquid scintillation counter.

RESULTS

Origin of the 33-kDa Heparin Binding Fragment from the A Chain of Fibronectin. Limited tryptic digests of plasma fibronectin generated two high molecular weight proteolytic fragments of 190K and 200K which have both heparin and collagen binding activities (Smith & Furcht, 1982; Smith et al., 1982; Click & Balian, 1985; McCarthy et al., 1986). Purification by sequential gelatin and heparin affinity chromatography and further cleavage with cathepsin D produce (a) a 46K gelatin binding fragment, (b) a series of high molecular weight fragments which do not bind gelatin or heparin but do contain the RGDS cell adhesion sequence, and (c) 33K and 66K strong heparin binding fragments derived from the carboxyl-terminal third of the molecule [see Figure 1 and Smith and Furcht (1982), Smith et al. (1982), Click and Balian (1985), and McCarthy et al. (1986)]. In order to determine if the t190 or t200 fragments gave rise preferentially to either heparin binding fragment, the two fragments were chromatographed over affinity columns to which monoclonal antibody 2-8 (Smith & Furcht, 1982) had been coupled (Figure 2). Lane A of the gel shows the unbound material from the monoclonal antibody column, consisting of the t190 fragment. In contrast, the t200 fragment binds to this mo-

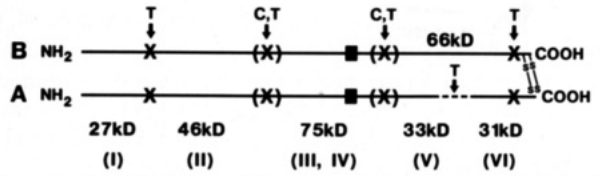

FIGURE 1: Model of plasma fibronectin. The two types of chains (A and B) of plasma fibronectin are shown as a disulfide-bonded heterodimer. The six domains of fibronectin (I–VI) are named according to Furcht (1983). Biological activities within each domain include (I) weak heparin binding, (II) collagen binding (noncovalent), (III) DNA binding, (IV) RGDS-mediated cell adhesion, indicated by the solid box, and (V) heparin binding, RGDS-independent cell adhesion. (VI) contains the epitope for monoclonal antibody 2-8 and a free sulfhydryl (Smith et al., 1982). The molecular weight estimates of proteolytic fragments containing each domain are based on a previously described digestion and purification scheme (McCarthy et al., 1986). Tryptic cleavage sites are designated T and cathepsin D cleavage sites C. By this scheme, domains V and VI isolated from digests of the B chain are located in a 66K fragment. In contrast, the A-chain digests contain a 33K fragment containing domain V and a 31K fragment containing domain VI. The difference between the A- and B-chain cleavage products is a result of a trypsin-sensitive site present in the A-chain-specific type IIIcs insert shown as a dashed line (Yamada, 1983; Hynes, 1985).

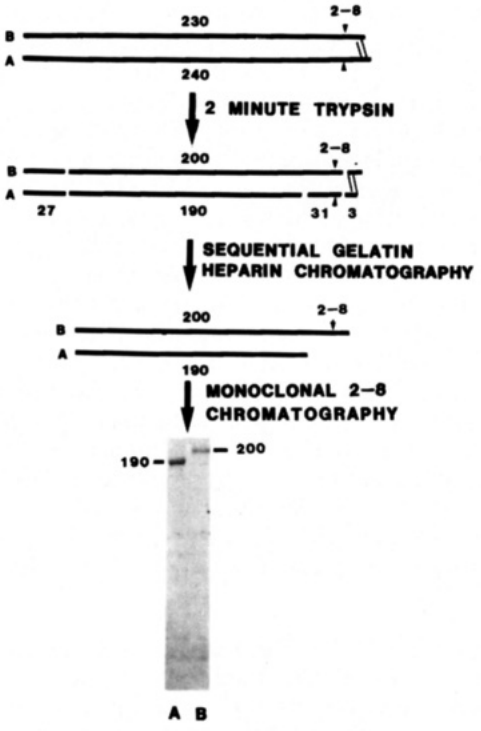

FIGURE 2: Purification of t190 and t200 fragments of fibronectin. The t190/t200 fragments of fibronectin were isolated from tryptic digests of fibronectin by sequential gelatin and heparin affinity chromatography (McCarthy et al., 1986). Monoclonal antibody 2-8, which recognizes the t200 chain, was used to affinity purify the t190 fragment (lane A) from the t200 fragment (lane B). Each lane contains 10 μg of protein.

noclonal antibody column (Figure 2, lane B). These two purified proteolytic fragments of fibronectin were then digested with cathepsin D, and the digests were subjected to heparin affinity chromatography and SDS–PAGE (Figure 3). The cathepsin D digest of the t190 fragment is shown in lane A. This digestion produces several high molecular weight fragments which are 100K or greater and two prominent lower molecular weight bands with apparent molecular weights of 46K and 33K. The cathepsin D digest of the t200 fragment (lane B) is quite similar in appearance to that of t190, with the exception that the 33K fragment is lacking and is replaced by a major fragment at 66K. The cathepsin D digests of the

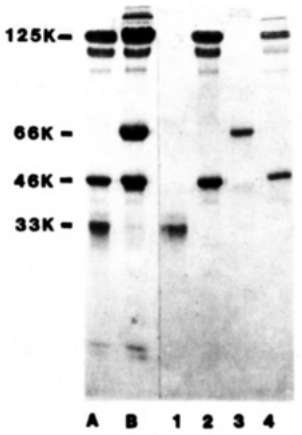

FIGURE 3: SDS–PAGE analysis of heparin binding fragments in catheptic digests of t190 and t200 fragments. The t190 and t200 fragments were digested with cathepsin D as described under Materials and Methods, and each digest was applied to heparin affinity columns. Lane A, 15-min digest of t190; lane B, 15-min digest of t200 fragments. Lanes 1 and 3 contain heparin-bound fragments, and lanes 2 and 4 contain heparin-unbound fragments of t190 and t200 digests, respectively. Each lane contains 20–30 μg of protein.

t190 or t200 fragments were subjected to heparin affinity chromatography (Figure 3, lanes 1–4). The heparin-bound fraction from the t190 and t200 digests is shown in lanes 1 and 3, respectively. The 33K fragment in the t190 digest binds to heparin, as does the 66K fragment from the t200 digest. The 46K fragment in each digest binds gelatin noncovalently (Smith & Furcht, 1982). These results demonstrate that the 33K heparin binding fragment originates from the t190 fragment and the 66K fragment originates from the t200 fragment in agreement with previously published results (Click & Balian, 1985). Thus, the size heterogeneity of these heparin binding fragments from the carboxyl-terminal third of the molecule does not result from cleavage by cathepsin D but rather is related to the initial trypsin treatment of intact fibronectin.

Amino-Terminal Sequence of the Tryptic/Catheptic 66K and 33K Heparin Binding Fragments and the Carboxyl-Terminal Tryptic 31K Free-Sulfhydryl-Containing Fragment. The entire primary structure of fibronectin either has been determined directly (Peterson et al., 1983) or has been predicted from recombinant DNA technology (Schwarzbauer et al., 1983; Kornblihtt et al., 1985; Bernard et al., 1985). The amino-terminal sequences of the t/c 33, t/c 66, and t 31 fragments were established to determine the exact location of these fragments relative to the known human sequence. The first 21 amino acids were determined for the t/c 66 heparin binding fragment (Figure 4, underlined residues which begin in line 1 and continue to line 2). This fragment starts with the amino acid alanine which corresponds to residue 1583 on intact plasma fibronectin (Kornblihtt et al., 1985). The presence of tyrosine to the amino-terminal side of this alanine in intact fibronectin is consistent with a preference of cathepsin D for peptide bonds involving aromatic residues. The sequence of the t/c 66 fragment does not contain the ED III insert, since the sequence proceeds from a threonine at residue 1599 (indicated by a slash at the end of line 1) to an alanine at residue 1689 (first residue, line 2) (Kornblihtt et al., 1985). This lack of the ED III region has been reported previously to be a characteristic feature which distinguishes plasma- or liver-derived fibronectin from cellular- or fibroblast-derived fibronectin (Schwarzbauer et al., 1983; Kornblihtt et al., 1985; Umezawa et al., 1985). The t/c 33 fragment also shares a common amino-terminal sequence with the t/c 66 fragment

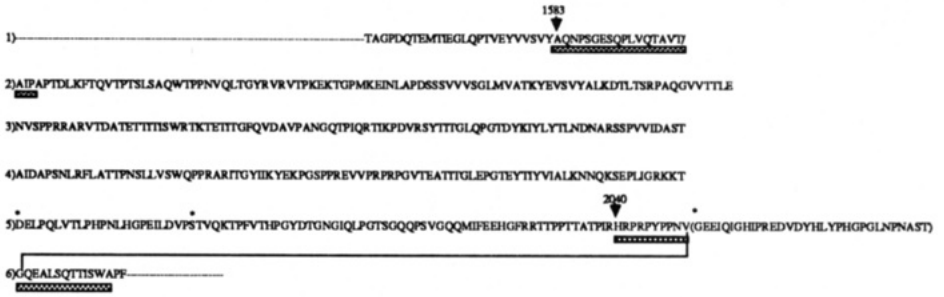

FIGURE 4: Partial amino acid sequence of t/c 33, t/c 66, and t31 fragments of fibronectin. The entire amino acid sequence of the carboxyl-terminal heparin binding region of human fibronectin is shown [based on Kornblihtt et al. (1985) and Bernard et al. (1985)]. The underlined residues in lines 1 and 2 represent sequence information obtained from the amino terminus of the t/c 33 and t/c 66 fragments. Lines 1–4 represent repeating type III homologies which are common to A- and B-chain isoforms of plasma fibronectin. The slash at the end of line 1 indicates a splice point for an extra type III homology (ED III; Kornblihtt et al., 1985) which is missing (denoted by the slash at the end of line 1) in plasma fibronectin but present in cellular fibronectin. The underlined residues in lines 5 and 6 represent the sequence information obtained from the t31 fragment of fibronectin. Line 5 represents the full 120 amino acid sequence which characterizes the type IIIcs region of the A chains of human plasma fibronectin and cellular fibronectin (Kornblihtt et al., 1985; Garcia-Pardo et al., 1985; Bernard et al., 1985). Line 6 depicts the continuation of the last type III homology of fibronectin located to the carboxyl-terminal side of the type IIIcs insert. The single asterisks in line 5 denote splice points for the differential insertion of segments of the type IIIcs insert (Schwarzbauer et al., 1983), which characterize subpopulations of the A chain of the molecule. On the basis of the sequence of the t31 fragment, the last 31 residues (shown in parentheses, line 5) of the potential 120 amino acid type IIIcs insert were absent from the t31 fragment. The residue numbers in lines 1 and 5 are based on Kornblihtt et al. (1985).

(Figure 4, line 1), beginning with alanine at position 1583, and it also lacks the ED III domain. These results illustrate that the amino-terminal sequences of these fragments are identical and support the contention that the size heterogeneity of the t/c 33 and t/c 66 heparin binding fragments results from the action of trypsin within the type IIIcs insert of the A chains of plasma fibronectin.

The amino-terminal end of the t31 fragment, which arises from A chains of fibronectin, was determined to establish the limit of the sequence present in the carboxyl terminus of the t/c 33 heparin binding fragment (Figure 4, line 5). The amino-terminal end of the t31 fragment begins at histidine residue 2040, underlined, line 5 (Kornblihtt, 1985; Garcia-Pardo et al., 1985). This is consistent with the known specificities of trypsin, since the residue to the amino-terminal side of this histidine is an arginine. This sequence is present in the type IIIcs insert which occurs in a subset of fibronectin molecules (Schwarzbauer et al., 1983; Kornblihtt et al., 1985). This fragment contains 9 additional amino acids from the type IIIcs insert, skips the last 31 amino acids of this insert (Figure 4, line 5, parentheses), and then continues as a type III homology (Figure 4, line 6, underlined) up to the tryptophan at residue 2061 where the current sequence information ends. These results demonstrate that the t31 fragment contains a portion (the first 89 amino acids) of the maximum possible 120-residue inserted sequence (Bernard et al., 1985), in agreement with previously established sequence data for this region of plasma fibronectin (Kornblihtt et al., 1985; Garcia-Pardo et al., 1985). The sequence information indicates the maximum possible carboxyl-terminal limit of the t/c 33 heparin binding fragment at arginine residue 2039, within the type IIIcs insert (Figure 4, line 5).

Production of Monoclonal Antibodies against the 33K Heparin Binding Fragment of Fibronectin. A panel of monoclonal antibodies was prepared against the 33K heparin binding fragment of fibronectin. These monoclonal antibodies were purified from ascites and screened by immunoblots against the t190 and t200 fragments as well as the t/c 33 and t/c 66 heparin binding fragments of fibronectin. The results of immunoblots using two of these monoclonal antibodies are shown in Figure 5. Monoclonal antibody AHB-1 reacts with both the t190 and t200 fragments and also recognizes the t/c 33, t/c 44, and t/c 66 heparin binding fragments of fibronectin (Figure 5, lanes a and c). The t/c 44 fragment is a cleavage

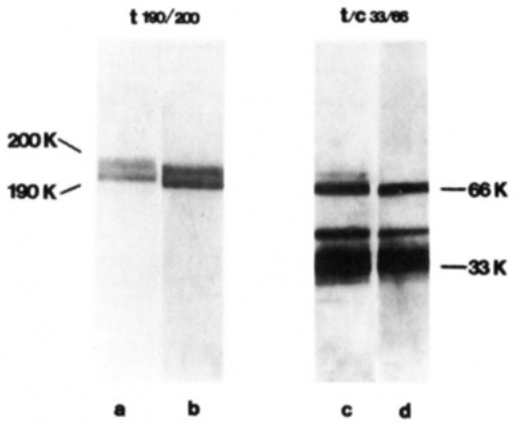

FIGURE 5: Immunoblot analysis of AHB-1 and AHB-2. The t190/t200 doublet or the t/c 33 and t/c 66 heparin binding fragments were electrophoresed on 5% or 12.5% SDS–polyacrylamide gels, respectively. Following transfer onto nitrocellulose, blots were reacted with 2 μg/mL AHB-1 (lanes a and c) or AHB-2 (lanes b and d) as described. Bound antibodies were detected by using ^{125}I-labeled secondary antibody. Each lane in the polyacrylamide gels contained 10 μg of protein.

product of the t/c 66 fragment, since both fragments recognize monoclonal antibody 2-8. This reactivity contrasts that of the t/c 33 heparin binding fragment, which fails to bind antibody 2-8 (Smith & Furcht, 1982; McCarthy et al., 1986). Thus, the epitope for monoclonal antibody AHB-1 is within a type III homology which is common to both the A-chain (t/c 33) and B-chain (t/c 66) fragments. The immunoblot using monoclonal antibody AHB-2 is also shown in Figure 5, lanes b and d. As with monoclonal antibody AHB-1, this antibody recognizes both the major t/c 33 and t/c 66 fragments of fibronectin, as well as the minor t/c 44 fragment present in these digests.

These two antibodies were adjusted to equivalent concentrations and assayed by ELISA to determine relative titers of each antibody (Table I). Both monoclonal antibodies AHB-1 and AHB-2 were reactive with intact fibronectin and the t/c 33 heparin binding fragment of fibronectin at antibody concentrations ranging from 1 to 100 μg/mL (Table I). It was necessary to determine this prior to performing any functional assays using these antibodies.

Inhibition of Cell Adhesion by Monoclonal Antibodies. The monoclonal antibodies shown above were used in cell adhesion

Table I

monoclonal antibody[a]	absorbance (490 nm)[b] FN	33K
AHB-1		
1 μg/mL	0.29	0.26
10 μg/mL	0.54	0.68
100 μg/mL	0.61	0.72
AHB-2		
1 μg/mL	0.14	0.09
10 μg/mL	0.30	0.23
100 μg/mL	0.37	0.81

[a] The indicated concentrations of monoclonal antibodies were added to the wells which had been coated with 100 ng of fibronectin or 200 ng of the t/c 33 fragment of fibronectin. The plates were incubated with these antibodies for 45 min at room temperature, washed, and incubated for an additional 45 min in the presence of a 1:1000 dilution of commercial peroxidase-conjugated secondary antibody. The wells were then washed prior to the addition of substrate. [b] Fifty microliters *o*-phenylenediamine was added to wells coated with 100 μL of solutions containing 1 μg/mL fibronectin or 2 μg/mL t/c 33 fragment. The reaction was terminated 15 min later with 50 μL of 2.5 M H_2SO_4, and the intensity of the reaction was determined by measuring the absorbance at 490 nm. The values shown are corrected for the background absorbance, which was 0.03.

assays to determine the ability to disrupt cell adhesion on intact fibronectin and the t/c 33 heparin binding fragment of fibronectin (Figure 6). The coating concentrations of fibronectin (10 μg/mL) and the t/c 33 fragment of fibronectin (3 μg/mL) were chosen on the basis that these coating concentrations yielded half-maximal adhesion of melanoma cells in a 30-min adhesion assay (not shown). Monoclonal antibody AHB-2 caused a concentration-dependent inhibition of cell adhesion to the t/c 33 heparin binding fragment of fibronectin (Figure 6A). This inhibition was virtually complete (approximately 80%) at the highest concentration of monoclonal antibody tested (1 mg/mL). Even at the lowest concentration of antibody tested (10 μg/mL), there was still a 50% inhibition of cell adhesion to the t/c 33 heparin binding fragment of fibronectin. In contrast, this antibody was virtually ineffective at inhibiting cell adhesion to wells coated with intact fibronectin (Figure 6A).

In contrast to monoclonal antibody AHB-2, the addition of monoclonal antibody AHB-1 was ineffective at inhibiting cell adhesion to either the 33K fragment of fibronectin or the intact molecule (Figure 6B). Even at the highest concentration of antibody, less than a 20% inhibition of cell adhesion was observed on either intact fibronectin or the t/c 33 fragment. These data demonstrate that the inhibition of cell adhesion observed on the 33K fragment in the presence of monoclonal antibody AHB-2 was not related to potential nonspecific effects of immunoglobulin.

Potentiation of RGDS-Mediated Inhibition of Cell Adhesion by Monoclonal Antibody AHB-2. Previous work from several laboratories (Beyth & Culp, 1984; Izzard et al., 1985; McCarthy et al., 1986; Woods et al., 1986) has suggested that cell adhesion to fibronectin is mediated by multiple interactions of the cell surface with distinct cell adhesion domains of fibronectin. It was therefore of interest to determine if the inability of monoclonal antibody AHB-2 to inhibit cell adhesion to intact fibronectin was due to adhesion to alternative domains on fibronectin, e.g., RGDS, in the presence of this antibody. To test this hypothesis, the adhesion of melanoma cells to intact fibronectin and the t/c 33 heparin binding fragment of the molecule in the presence of both RGDS and monoclonal antibody AHB-2 was examined. Previous studies (Pierschbacher & Ruoslahtti, 1984) have shown that high

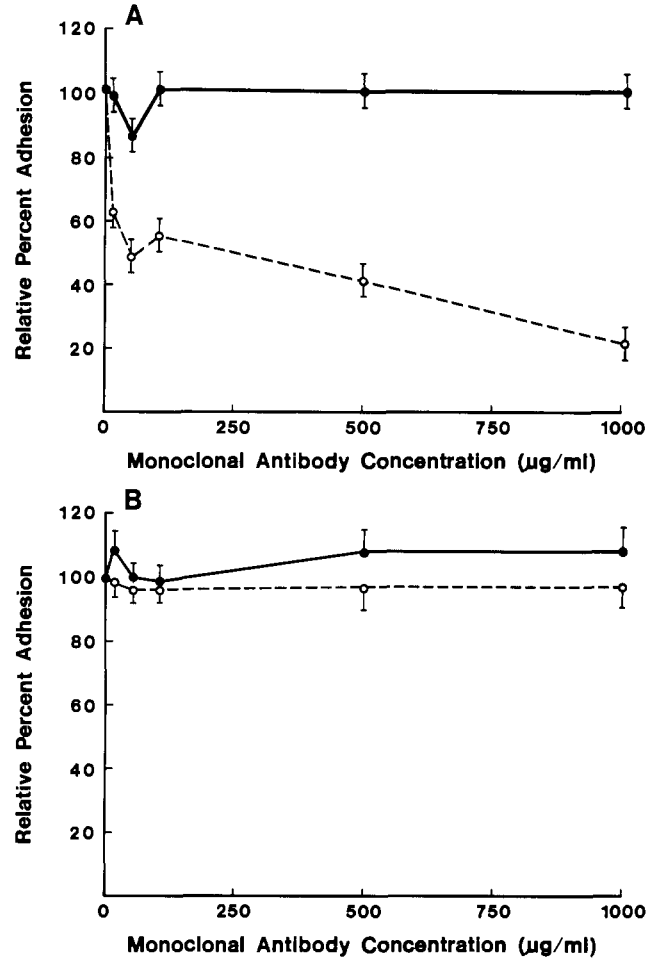

FIGURE 6: Effect of monoclonal antibodies on cell adhesion. Effect of AHB-1 and AHB-2 on cell adhesion to the t/c 33 heparin binding fragment (dashed lines) and intact fibronectin (solid lines). Substrata were coated with 3 μg of t/c 33 or 10 μg of fibronectin as described. The actual amount of 33K fragment or intact fibronectin bound to the plates was 1.3 and 1.25 μg, respectively. Coated wells were then incubated with the indicated concentrations of AHB-2 (A) or AHB-1 (B), and 3×10^3 radiolabeled cells were added to the wells for the adhesion assay. Nonadherent cells were aspirated after 30 min and bound cells quantitated as described. Data points represent the means of triplicate determinations, plus or minus the standard errors of the means.

levels (10^{-3}–10^{-2} M) of exogenous RGDS can virtually eliminate fibroblast adhesion to intact fibronectin. For the present studies, a suboptimal concentration of RGDS (5×10^{-4} M) was chosen, which was far less effective than higher concentrations at inhibiting cell adhesion to intact fibronectin (Pierschbacher & Ruoslahtti, 1984; McCarthy et al., 1986).

The adhesion of cells to the t/c 33 fragment was sensitive to the addition of 500 μg/mL monoclonal antibody AHB-2 to the adhesion assay (Figure 7). In contrast, cell adhesion to the t/c 33 fragment was completely resistant to the effects of 5×10^{-4} M RGDS, consistent with results published previously (McCarthy et al., 1986). In some experiments, the adhesion of cells to the 33K fragment was slightly potentiated by the addition of this tetrapeptide sequence (data not shown). Furthermore, the addition of both the monoclonal antibody and the RGDS synthetic peptide had no additive effect on inhibiting cell adhesion to the t/c 33 fragment.

Melanoma adhesion to intact fibronectin was little affected in the presence of 500 μg/mL AHB-2 (less than 10%, Figure 7). The addition of a suboptimal dose (5×10^{-4} M) of RGDS to the adhesion assay was somewhat more effective at inhibiting adhesion to the intact protein (greater than 20%).

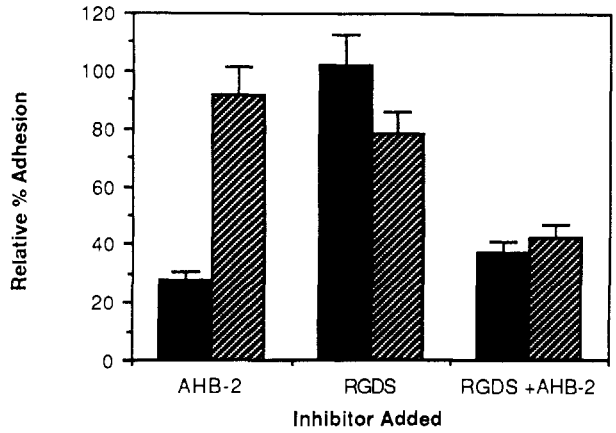

FIGURE 7: Effect of RGDS and AHB-2 on melanoma adhesion to intact fibronectin and t/c 33 heparin binding fragment. Substrata were coated with 10 μg/mL fibronectin (hatched bars) or 3 μg/mL t/c 33 (solid bars) as described. Coated substrata were then preincubated with AHB-2 prior to assaying cell adhesion; 3×10^3 radiolabeled cells which had been preincubated in the presence or absence of 5×10^4 M RGDS were then added to the assay. Nonadherent cells were aspirated 30 min later, and bound cells were solubilized and quantitated. Values are expressed as the percent cell adhesion relative to adhesion observed in the absence of any inhibitor. Actual values for total cell adhesion in the absence of inhibitors to intact fibronectin and the 33K fragment were 43% and 37%, respectively. Data represent the means of triplicates, plus or minus the standard errors of the means.

However, the addition of 500 μg/mL monoclonal antibody AHB-2 in the presence of this concentration of RGDS resulted in a potentiation of the inhibitory effects of RGDS. The combination of both of these reagents in the assay resulted in a 55% inhibition of adhesion to intact fibronectin, which is more than twice that observed with RGDS alone.

Construction of Synthetic Peptides with Cell Adhesion Activity. On the basis of immunoblot analysis, the epitope recognized by AHB-2 was common to the carboxyl-terminal heparin binding region of both A and B chains of plasma fibronectin, suggesting that it was distinct from the two type IIIcs specific cell adhesion promoting sequences recently described in fibronectin (Humphries et al., 1986, 1987). Therefore, it was of interest to determine if additional synthetic peptides from this region could be identified which promoted melanoma cell adhesion. To approach the problem, a hydropathy plot of this region was constructed according to the method of Kyte and Doolittle (1982). Several regions of the fragment which had a low (hydrophilic) hydropathy index were identified. As an additional criterion, hydrophilic stretches which were rich in cationic residues (e.g., lysine or arginine) were chosen for synthesis. This additional criterion was imposed on the basis of the potential association of heparin binding activity in this region of fibronectin with cell adhesion activity. According to this approach, two peptides were synthesized and characterized for the ability to promote melanoma cell adhesion. Peptide I, corresponding to fibronectin residues 1906–1924, consisted of the sequence Tyr-Glu-Lys-Pro-Gly-Ser-Pro-Pro-Arg-Glu-Val-Val-Pro-Arg-Pro-Arg-Pro-Gly-Val (YEKPGSPPREVVPRPRPGV, using the single-letter amino acid code). Peptide II represented residues 1946–1960 and consisted of the sequence Lys-Asn-Asn-Gln-Lys-Ser-Glu-Pro-Leu-Ile-Gly-Arg-Lys-Lys-Thr (KNNQKSEPLIGRKKT). Both peptides were hydrophilic, having an overall hydropathy index of −24.3 (peptide I) and −29.3 (peptide II).

Peptides I and II were then tested for the ability to promote the adhesion of melanoma cells (Figure 8A). Both peptides were observed to promote melanoma cell adhesion in a concentration-dependent manner. At the lowest coating level (1.56 μg/well), peptide I promoted cell adhesion at a level of 13-fold over control values. This value increased to a maximum of 31-fold over control values at the highest coating level tested (100 μg/well). Peptide II exhibited slightly greater adhesion activity at lower coating levels but promoted cell adhesion to approximately the same degree as peptide I when higher amounts of peptide were used. Both peptides also bound [^{3}H]heparin in solid phase binding assays (Figure 8B) in a concentration-dependent manner. As with cell adhesion, peptide II appeared slightly more effective at binding [^{3}H]-heparin than peptide I. At the highest level of peptides tested, peptide I bound approximately 14% of the [^{3}H]heparin added to the assay, while peptide II bound approximately 30% of the [^{3}H]heparin added.

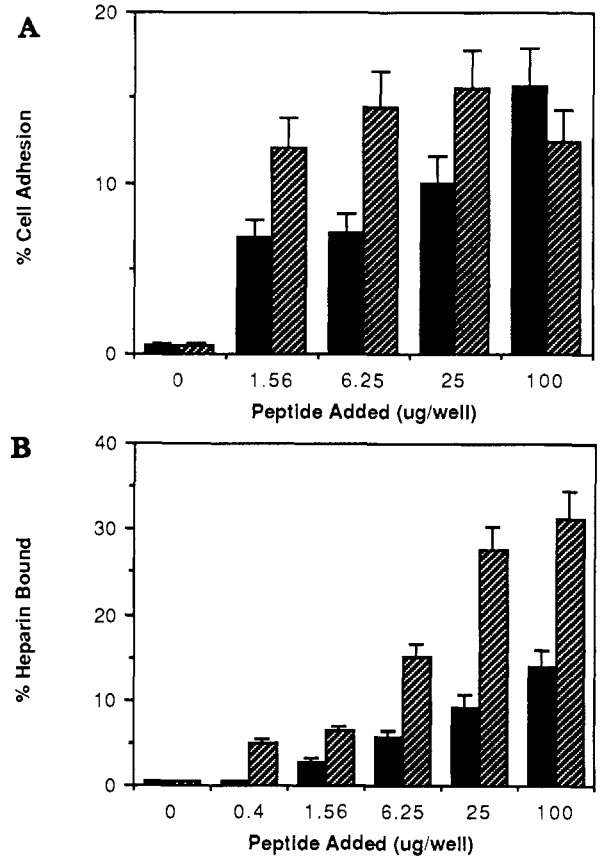

FIGURE 8: Melanoma cell adhesion and heparin binding properties of synthetic peptides from fibronectin. Immulon C wells were coated with the indicated concentrations of peptides I (solid bars) and II (hatched bars) as described under Materials and Methods and tested for the ability to promote tumor cell adhesion and for binding of [^{3}H]heparin. (A) For the cell adhesion assays, 5×10^3 radiolabeled tumor cells were added to the wells, and the plates were incubated for 90 min at 37 °C. At the conclusion of the assay, the wells were washed, and the adherent cells were solubilized and quantitated. Adhesion to wells coated with 50 μg/well of intact fibronectin in this assay (as a positive control) promoted the adhesion of 47.6% of the cells. Background adhesion represented 0.5% of the cells applied. Data represent the mean of triplicate determinations plus or minus the standard errors of the means. (B) Wells coated with the indicated amount of peptides I and II were incubated for 2 h at 37 °C in the presence of 0.8 μg/well of [^{3}H]heparin (4.4×10^3 dpm). The wells were then washed, and bound heparin was solubilized and quantitated as described. The background binding in this assay was 0.5% of the applied radioactivity. Data represent the mean of triplicate determinations.

DISCUSSION

In previous studies (Rogers et al., 1985; McCarthy et al., 1986), we have identified two proteolytic fragments of fibronectin which actively promote neuronal and melanoma cell

adhesion. These fragments include a 75K tryptic fragment of fibronectin, which contains the RGDS sequence, and a 33K tryptic/catheptic heparin binding fragment of fibronectin, which originates from a subset of fibronectin molecules which contain the type IIIcs insert (Click & Balian, 1985; McCarthy et al., 1986). Despite the fact that both the 75K and 33K fragments of fibronectin promote adhesion, several lines of evidence suggest that adhesion to each fragment occurs by a distinct mechanism(s) (McCarthy et al., 1986). First, adhesion of trypsin-released melanoma cells to the 33K heparin binding fragment is sensitive to the effects of protein synthesis inhibitors, whereas cell adhesion to the 75K fragment is resistant. Second, adhesion to the 75K RGDS-containing fragment results in the haptotactic motility of melanoma cells, whereas cells adherent to the heparin binding fragment of fibronectin do not exhibit this motile response (McCarthy et al., 1986). Finally, adhesion to the 75K fibronectin fragment is inhibited by soluble RGDS, whereas cell adhesion to the 33K fragment is resistant to inhibition by this peptide. The current study extends these earlier findings using direct amino acid sequencing to demonstrate that the RGDS sequence is not present within the 33K heparin binding fragment of fibronectin, consistent with the lack of effect of RGDS on cell adhesion to this fragment.

Two monoclonal antibodies generated against this 33K heparin binding fragment were characterized. Although both monoclonal antibodies recognized determinants on the A- and B-chain heparin binding fragments, only one of these antibodies inhibited melanoma cell adhesion to the 33K heparin binding fragment. This antibody, termed AHB-2, had no effect on the adhesion of melanoma cells to intact fibronectin but could potentiate the inhibitory effects of soluble RGDS on intact fibronectin. The failure of monoclonal antibody AHB-2 to inhibit adhesion to intact fibronectin was not due to a failure of the antibody to bind to the protein. Instead, we conclude that this result supports our previous study (McCarthy et al., 1986) demonstrating that multiple adhesion domains on intact fibronectin are utilized by the K1735 melanoma cell line.

A recent study (Humphries et al., 1986) has suggested that the effects of RGDS on B16 melanoma cell adhesion to fibronectin occur by inhibiting the interaction of the cell surface with the REDV sequence present within the type IIIcs insert of certain isoforms of human fibronectin (Bernard et al., 1985). Importantly, the REDV sequence is absent from the fibronectin fragments used in the current study, indicating that the inhibitory effects of RGDS in this system are due to a direct inhibition of this peptide on cell interaction with the RGDS sequence within fibronectin. Furthermore, the amino acid sequence data indicate that if the REDV sequence were present, it would not be in the 33K heparin binding fragment but rather it would be contained within the tryptic 31K fragment. The 31K fragment has previously been shown to be inactive at promoting melanoma cell adhesion (McCarthy et al., 1986). The lack of the REDV sequence in plasma fibronectin has been reported previously, based on nucleotide seqeunce data obtained from hepatocyte mRNA for fibronectin (Kornblihtt et al., 1985) as well as direct sequencing of this region (Garcia-Pardo et al., 1985). The presence of the epitope for monoclonal antibody AHB-2 on both the A- and B-chain-derived heparin binding fragments suggested that the adhesion domain recognized by this antibody is not related to the A-chain specific type IIIcs insert, which is present only in A chains, but rather is common to both chains. Thus, the epitope recognized by this antibody does not appear to be alternatively spliced and is therefore different from the sequences recently reported by Humphries et al. (1986, 1987). Further evidence for the presence of adhesion determinants which are distinct from the type IIIcs sites was obtained by identifying synthetic peptides, common to both A and B chains, with cell adhesion promoting activity. Although antibody AHB-2 inhibited cell adhesion on the 33K fragment, it did not bind to either synthetic adhesion-promoting peptide (data not shown), suggesting that the inhibitory effects of this antibody may be steric in nature.

On the basis of results using peptides I and II, as well as the recently published study of Humphries et al. (1987), we conclude that melanoma adhesion and spreading on the isolated 33K heparin binding fragment of fibronectin occur as a result of multiple determinants within this region of the molecule. Although the two heparin binding peptides identified in this study both promoted melanoma adhesion, they were not as active at promoting tumor cell spreading as the intact 33K fragment (our unpublished observation). This could be explained by the presence of the type IIIcs sequence (CS-I) in the 33K fragment which has recently been reported to promote melanoma cell spreading (Humphries et al., 1987). Indeed, preliminary results indicate that the CS-I synthetic peptide present in the type IIICs sequence is active at promoting melanoma cell adhesion and spreading. However, this CS-I synthetic peptide is hydrophobic and does not bind heparin in the solid phase binding assay (our unpublished result). Thus, we conclude that cell adhesion to the heparin binding region of fibronectin involves multiple determinants within this region of the molecule, some of which are A chain specific, and some of which involve determinants which are common to both chains.

These results demonstrate that adhesion of K1735 melanoma cells to intact fibronectin involves the RGDS sequence within fibronectin as well as additional domain(s) within the heparin binding fragment of fibronectin. This result is consistent with the work of several other investigators studying the effect of heparin binding and "cell binding" activities on the formation of focal contacts by cells. Previous studies have demonstrated that both the cell binding (i.e., RGDS-mediated) and heparin binding activities of fibronectin are required for focal adhesion formation (Beyth & Culp, 1984; Izzard et al., 1985; Woods et al., 1986). Furthermore, polyclonal antibodies generated against the heparin binding fragment of fibronectin will prevent focal adhesion formation by cells on intact fibronectin (Wood et al., 1986). The RGDS sequence within fibronectin has been shown to interact with a family of cell surface molecules which are termed integrins (Tamkun et al., 1986). Additional cell surface molecules which have been implicated in adhesion to fibronectin include other cell surface glycoproteins (Aplin et al., 1981; Urushihara & Yamada, 1986), cell surface proteoglycans (Lark et al., 1985; Woods et al., 1986), and gangliosides (Yamada et al., 1983). It will be important to utilize tools such as monoclonal antibodies or synthetic peptides with adhesion-promoting activity to identify receptors for these regions of fibronectin in order to establish the biological significance (i.e., growth, motility, expression of cell phenotype) of cell adhesion to these domains.

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Registry No. Peptide I, 111771-60-9; peptide II, 111793-75-0; heparin, 9005-49-6.

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