

Characterization of a Synthetic Peptide from Type IV Collagen That Promotes Melanoma Cell Adhesion, Spreading, and Motility

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Abstract. The adhesion and motility of tumor cells on basement membranes is a central consideration in tumor cell invasion and metastasis. Basement membrane type IV collagen directly promotes the adhesion and migration of various tumor cell types in vitro. Our previous studies demonstrated that tumor cells adhered and spread on surfaces coated with intact type IV collagen or either of the two major enzymatically purified domains of this protein. Only one of these major domains, the pepsin-generated major triple helical fragment, also supported tumor cell motility in vitro, implicating the involvement of the major triple helical region in type IV collagen-mediated tumor cell invasion in vivo. The present studies extend our previous observations using a synthetic peptide approach. A peptide, designated IV-H1, was derived from a continuous collagenous region of the major triple helical domain of the human $\alpha 1(\text{IV})$ chain. This peptide,

which has the sequence GVKGDKGNPGWPGAP, directly supported the adhesion, spreading, and motility of the highly metastatic K1735 M4 murine melanoma cell line, as well as the adhesion and spreading of other cell types, in a concentration-dependent manner in vitro. Furthermore, excess soluble peptide IV-H1, or polyclonal antibodies directed against peptide IV-H1, inhibited type IV collagen-mediated melanoma cell adhesion, spreading, and motility, but had no effect on these cellular responses to type I collagen. The full complement of cell adhesion, spreading, and motility promoting activities was dependent upon the preservation of the three prolyl residues in the peptide IV-H1 sequence. These studies indicate that peptide IV-H1 represents a cell-specific adhesion, spreading, and motility promoting domain that is active within the type IV collagen molecule.

IN light of the involvement of tumor cell adhesion and motility in the process of basement membrane (BM)¹ invasion (Liotta, 1977), it appears that type IV collagen may play a role in the invasion of BM by various tumor types. In vitro, BM type IV collagen promotes the adhesion and motility of various normal and transformed cell types (Kurkinen et al., 1984; Aumailley and Timpl, 1986; Tomaselli et al., 1988; Herbst et al., 1988; Chelberg et al., 1989). In our previous studies using the highly metastatic murine melanoma cell line K1735 M4 (Fidler et al., 1981), the cells adhered and spread on surfaces coated with intact type IV collagen, as well as to surfaces coated with the isolated globular collagenase-generated major noncollagenous domain of type IV collagen (NC1) or the purified pepsin-generated major triple helical fragment (p-IV), which lacks the NC1 domain. However, motile behavior was observed only in response to the helical p-IV fragment (Chelberg et al., 1989). These findings are consistent with the involvement of multiple distinct regions on the type IV collagen molecule

in cell adhesion, spreading, and motility (Chelberg et al., 1989), and suggest that the p-IV fragment may be important for tumor cell invasion of BM.

To characterize further the molecular basis of the cellular responses promoted by the p-IV fragment, a series of synthetic peptides derived from the triple helical region of human type IV collagen was screened for the ability to promote the adhesion and spreading of the M4 murine melanoma cell line in vitro. The present studies provide evidence that the peptide designated IV-H1 (having the amino acid sequence GVKGDKGNPGWPGAP²; residues 1263–1277) represents a specific cell adhesion, spreading, and motility promoting domain within the major triple helical region of type IV collagen. Peptide IV-H1 promoted the adhesion and spreading of various cell types, and was a potent attractant for M4 melanoma cell motility. Preservation of the prolyl residues appears to be necessary for the full complement of adhesion, spreading, and motility promoting activities of the IV-H1 sequence. The data indicate that the activity associated with the IV-H1 sequence is active within, and specific to, type IV

1. *Abbreviations used in this paper:* BM, basement membrane; FN, fibronectin; KLH, keyhole limpet hemocyanin; NC1, collagenase-generated major noncollagenous domain of type IV collagen; p-IV, pepsin-generated major triple helical fragment of type IV collagen.

2. A, alanine; D, aspartate; E, glutamate; G, glycine; K, lysine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; V, valine; W, tryptophan; Y, tyrosine.

collagen, since the presence of excess soluble peptide IV-HI (or polyclonal antibodies generated against peptide IV-HI), inhibited type IV collagen-mediated (but not type I collagen-mediated) melanoma cell adhesion, spreading, and motility. These studies indicate that peptide IV-HI represents a cell-specific adhesion, spreading, and motility promoting domain that is active within the type IV collagen molecule.

Materials and Methods

Cell Culture

The highly metastatic murine melanoma cell line, termed K1735 M4, was a generous gift from Dr. I. J. Fidler (M. D. Anderson, Houston, TX). This cell line was routinely maintained in DME media (Sigma Chemical Co., St. Louis, MO) supplemented with 10% calf serum (CS). Cells were passaged twice weekly with trypsin as previously described (Chelberg et al., 1989), and care was taken to limit the number of in vitro passages to 8 to minimize phenotypic drift. Dr. Fidler also provided the highly metastatic human melanoma cell line A375 SM, which was maintained in MEM with added vitamins and 10% FBS; and the fibrosarcoma cell line UV2237 MM, which was maintained in DME with 10% FBS. Other cell lines studied include: the C6 rat glioma cell line (No. CCL107; American Type Culture Collection [ATCC], Rockville, MD), which was maintained in DME plus 10% CS; the SCC9 human squamous carcinoma cell line (No. CRL1629; ATCC), which was maintained in a 1:1 solution of DME and HAM F12 containing 10% FBS; the B65 and B104 murine neuroblastoma cell lines that were a generous gift from Dr. David Schubert at the Salk Institute (Schubert et al., 1974) and maintained in F12H media with supplements; and bovine aortic endothelial cells, which were isolated and maintained as described previously (Herbst et al., 1988).

Protein Purification and Preparation of Proteolytic Fragments of Type IV Collagen

Type IV collagen was extracted from the Englebreth Holm Swarm sarcoma grown in lathyrus mice according to a modification of the method by Kleinman et al. (1982) as previously described (Herbst et al., 1988; Chelberg et al., 1989). Type IV collagen purified by DEAE anion exchange chromatography was subjected to ultracentrifugation at 110,000 *g* for 90 min to clear aggregates >50S. The supernatant was decanted and stored in 2 M guanidine containing 2 mM DTT at 4°C until needed. The concentration of type IV collagen was determined spectrophotometrically as previously described (Waddell, 1956; Chelberg et al., 1989). Type I collagen (Vitrogen) was obtained from the Collagen Corporation (Palo Alto, CA). Human plasma fibronectin (FN) was purified by sequential ion exchange and gelatin affinity chromatography as described previously (McCarthy et al., 1986).

Peptide Synthesis and Characterization

Peptides representing amino acid sequences from human type IV collagen or FN were synthesized using a peptide synthesizer (990; Beckman Instruments, Inc., Palo Alto, CA), either by Dr. Robert Wohlhueter at the Microchemical Facility of the University of Minnesota, or by Dr. Bianca Conti-Tronconi (University of Minnesota at St. Paul). The procedures used were based on the Merrifield solid phase system as described previously (Stewart and Young, 1984). Lyophilized crude peptides were purified by preparative reverse-phase HPLC on a C-18 column, using an elution gradient of 0–60% acetonitrile with 0.1% trifluoroacetic acid in water. The purity and composition of the peptides was verified by HPLC analysis of acid hydrolysates of the peptides. Peptides, including peptide IV-HI (GVKGDKGNPWGAP) and a variant of peptide IV-HI (GVKGDKGNAGWAGAA, designated peptide IV-HIA), were synthesized from the sequence of the major triple helical domain of human type IV collagen (Table I). As an additional control, an unrelated FN-derived peptide I (YEKPGSPREVVPR-PRPGV², McCarthy et al., 1990) was studied. Certain peptides were synthesized with a tyrosyl residue to the carboxy terminal end to allow radioactive iodination of the peptide.

Iodination of Peptides and Determination of Binding Efficiency on Immulon 1 Plates

Labeling of peptides with Na¹²⁵I was performed as described by McCon-

Table I. Type IV Collagen-derived Synthetic Peptides

Peptide name	Peptide sequence*	Residue numbers‡
Peptide 15	GPKGEPGKIVPLPG(Y)	634–647
Peptide 16	GLPGKPGSNDKVDMSGMKG(Y)	930–948
Peptide 17	GVPKGDGQAGQPGQP(Y)	975–989
Peptide 18	GEKGDKGLPLGD(Y)	1115–1126
IV-HI	GVKGDKGNPWGAP(Y)	1263–1277
IV-HIA	GVKGDKGNAGWAGAA(Y)	—

* Using the single-letter amino acid code: A, alanine; D, aspartate; E, glutamate; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; S, serine; V, valine; W, tryptophan. Each peptide contained a tyrosine residue (Y) at the carboxyl terminal end so that the peptides could be iodinated.

‡ Residue numbers assigned beginning with amino terminal end, based upon the sequence presented in Muthukumaran et al. (1989).

hey and Dixon (1980). Briefly, 0.1 mg of each peptide in NaHPO₄ buffer (pH 7.2) was incubated for 2 min with 0.05 mg chloramine T and 0.5 mCi Na¹²⁵I (New England Nuclear, Boston, MA). The reaction was terminated by the addition of 0.2 mg of Na₂S₂O₅. Free iodine was removed by reverse-phase chromatography using Sep-pak C-18 columns (Waters Division of Millipore, Bedford, MA), from which the radiolabeled peptides were eluted with acetonitrile (50%) containing 0.1% trifluoroacetic acid. The labeled peptides were lyophilized and stored at –80°C until needed. The efficiency of peptide binding to the wells of 96-well polystyrene Immulon 1 plates (Dynatech Laboratories Inc., Chantilly, VA) was determined by drying down 100 μl aliquots of radioiodinated peptides, which had been diluted in Voller's carbonate buffer to concentrations ranging from 1–10 μg/ml. To simulate binding conditions of the peptides in adhesion and spreading assays, the wells were then washed and incubated for 2 h with 150 μl/well of a 10 mg/ml solution of BSA in Voller's carbonate buffer. The wells were then washed and the amount of peptides bound to the surfaces was quantitated by solubilizing the peptides with 150 μl/well of 0.5 N NaOH containing 1% SDS. Bound radioactivity was quantitated in a gamma counter (1193; Tm Analytic Inc., Elk Grove Village, IL).

Generation and Purification of Polyclonal Antibodies

Polyclonal antibodies were generated against peptide IV-HI coupled to keyhole limpet hemocyanin (KLH; Sigma Chemical Co., St. Louis, MO) using carbodiimide as a coupling agent, based on a procedure described previously (Bauminger and Wilchek, 1980). Briefly, equal amounts (by weight) of peptide and KLH were solubilized in water and mixed with a 10-fold excess (by weight) of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Sigma Chemical Co.) dissolved in water. New Zealand white rabbits were immunized on the back by multiple subcutaneous injections of ~2 mg/rabbit of peptide/KLH conjugate in complete Freund's adjuvant. Subsequent biweekly boosts were given intramuscularly in incomplete Freund's adjuvant. Sera were collected 14 d after the fourth immunization, and tested by RIA for reactivity against uncoupled peptide, the protein of origin, and various other ligands. Immune sera were also prepared from rabbits immunized with FN peptide I coupled to KLH as a control to confirm the specificity of the antibody response for peptide IV-HI, rather than for the KLH.

IgG was purified from normal rabbit sera and pooled immune sera by precipitation with a final concentration of 50% ammonium sulfate overnight at 4°C. The resolubilized precipitate was dialyzed against 0.035 M NaCl in 0.025 M Tris, pH 8.8, and the IgG was purified by DEAE column chromatography as described previously (Skubitz et al., 1987). Purity of the IgG was determined by SDS-PAGE. Retained immunoreactivity of the purified IgG was verified by RIA (Skubitz et al., 1987).

Assays for Antibody Specificity

Immune sera and purified anti-peptide IV-HI IgG were screened for specificity by an indirect solid-phase RIA in 96-well polystyrene Immulon 1 plates as described previously, with minor modifications (Skubitz et al., 1987). Briefly, 50 μl of proteins or peptides at various concentrations in Voller's carbonate buffer was added to each well and dried overnight at 29°C. The next day, 200 μl of PBS containing 5% BSA (fraction V, fatty acid free, Sigma Chemical Co.), 0.1% Triton X-100, and 0.02% Na₂S₂O₅ was

added to each well followed by a 60-min incubation at 37°C. After removal of this buffer, 100 μ l of purified IgG at various dilutions in PBS containing 5% BSA and 0.02% NaN₃, was added in triplicate and the wells were incubated 1 h at 37°C. After three washings with the above buffer, bound IgG was detected by the addition of 100 μ l of 5% BSA in PBS/NaN₃ containing \sim 100,000 cpm of ¹²⁵I-labeled donkey IgG directed against rabbit Ig (sp act, 5 μ Ci/ μ g; Amersham Corp., Arlington Heights, IL). After a 1-h incubation at 37°C, unbound antibody was removed by washing. After an incubation with 100 μ l of 2 M NaOH for 15 min at 60°C, the solubilized proteins were transferred to glass tubes and the radioactivity was measured in a gamma counter (1193; Tm Analytic Inc.).

Cell Adhesion and Spreading Assays

Assays for cell adhesion and spreading were performed as previously described (Chelberg et al., 1989). Briefly, subconfluent cultures of cells were radiolabeled overnight with 2 μ Ci/ml [³H]thymidine. Before the adhesion assay, cells were released from the culture flasks with 10 mM EDTA in Hank's media or, alternatively, in a trypsin solution (0.05% trypsin with 0.02% EDTA) to confirm that cell adhesion did not require endogenous cell surface proteins that would be retained after EDTA treatment. Cells were then washed and resuspended to a final concentration of 4–5 \times 10⁴ cells/ml in DME supplemented with 20 mM Hepes and 10 mg/ml BSA. The cells remained viable (>95%) after this procedure, based upon exclusion of trypan blue dye.

Immulon 1 microtiter plates were prepared by drying down 100 μ l/well of protein or peptide that had been diluted to various concentrations (1–500 μ g/ml) in Voller's carbonate buffer. The relative abilities of the peptides to bind the plates was determined using radiolabeled peptides to be certain that any differences in cell adhesion or spreading were not due to differential binding to surfaces. Nonspecific cell adhesion to sites on the plastic was blocked by the use of a 10 mg/ml solution of BSA in Voller's carbonate buffer at 37°C for 2 h. The Voller's/BSA solution was then aspirated and 5–6 \times 10³ cells were added to each well in 150 μ l of adhesion media (DME containing 20 mM Hepes and 10 mg/ml BSA). The cells were incubated for 30–40 min at 37°C (or up to 90 min for certain assays), at which time cells were either visualized for spreading or harvested to determine adhesion. Adhesion was quantitated, after washing to remove nonadherent and weakly adherent cells, by solubilizing the cells with 150 μ l/well of 0.5 N NaOH containing 1% SDS. Bound radioactivity was quantitated in a liquid scintillation counter (LS 3801; Beckman Instruments). Spreading determinations were made by two different individuals, in double blind studies by visualizing at least 100 cells/well and calculating the percentage of spread cells. Each experiment was repeated a minimum of three times, and within a given experiment, each experimental point was determined in triplicate.

Inhibition of M4 melanoma cell adhesion and spreading on surfaces coated with type IV collagen was monitored in the presence of excess soluble peptides or polyclonal antibodies generated against synthetic peptides. In these assays, Immulon 1 plates were coated as described above for cell adhesion assays using concentrations of peptide IV-H1, type IV collagen, type I collagen, or FN (50 μ g/ml of the peptide and 5 μ g/ml of the intact proteins) which yielded half-maximal M4 melanoma cell adhesion in previous dose-response experiments. In the peptide inhibition studies, cells were preincubated for 20 min at 37°C in the presence (or absence) of various concentrations of peptide IV-H1 to occupy the cell surface binding "receptors" recognizing these peptides. As controls, two other peptides were studied for the ability to inhibit collagen-mediated cell adhesion. The first, peptide 17 (Table I), was derived from type IV collagen and has a length and amino acid content similar to peptide IV-H1, but does not promote melanoma cell adhesion or spreading. The second, the FN-derived synthetic peptide I, promotes adhesion, spreading, and motility of the M4 melanoma cells (McCarthy et al., 1990). In the antibody inhibition studies, the protein-coated surfaces were incubated with various concentrations of purified normal rabbit IgG or purified IgG against peptide IV-H1 or peptide I to block the corresponding sequence within the surface-bound protein. Cells were then dispensed into the wells, in the continued presence of peptide or IgG, and incubated at 37°C for 30 min. Spreading and adhesion determinations were quantitated as described above.

Assay for Cell Migration

Peptide-mediated M4 melanoma cell motility was examined in 48-well microchemotaxis chambers (Neuro Probe, Inc., Cabin John, MD) equipped with 8- μ m pore size polyvinylpyrrolidone-free polycarbonate filters by a modification of the procedure described previously (Herbst et al., 1988;

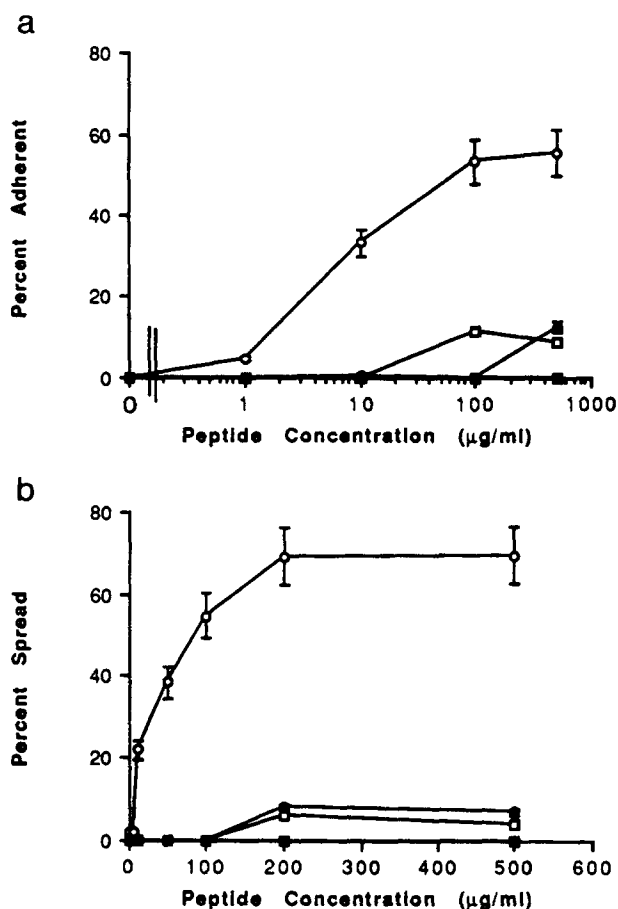


Figure 1. Melanoma cell adhesion and spreading in response to synthetic peptides. Surfaces were coated with 100 μ l/well of various synthetic peptides ranging in concentration from 1–500 μ g/ml as described in Materials and Methods. ³H-Thymidine-labeled M4 melanoma cells were added to wells coated with peptide IV-H1 (○), peptide 15 (■), peptide 17 (●), or peptide 18 (□). (a) Cell adhesion was quantitated and data are presented as the percent of added cells that were adherent. (b) Data are presented as the percent of added cells that were spread on surfaces coated with the peptides. All data represent the means of at least three experiments in which triplicate determinations were made \pm SEM.

Chelberg et al., 1989). Briefly, peptides were diluted in Voller's carbonate buffer and added in triplicate 50 μ l aliquots to the lower wells of the chambers. The chambers were assembled with the filters in place and incubated overnight at 37°C to allow the peptides to adsorb to the lower surfaces of the filters. The filters were then washed and transferred to fresh chambers that contained only media (DME containing 20 mM Hepes, pH 7.4). Cells were then added to the upper wells at 20,000/well in DME/Hepes media and incubated for 6 h at 37°C in a humidified CO₂ chamber. Filters were then washed, fixed, and stained, and the number of cells that had migrated through the filters was quantitated using an Optomax Image Analysis System, as previously described (Herbst et al., 1988; Chelberg et al., 1989). Each experiment was repeated a minimum of three times.

The ability of peptide IV-H1 or anti-peptide IV-H1 polyclonal IgG to inhibit type IV collagen-mediated haptotactic motility was determined by pre-coating filters on the underside with either type IV collagen or type I collagen in Voller's carbonate buffer as previously described (McCarthy et al., 1989; Chelberg et al., 1989). The collagen coating concentration (5 μ g/ml) was chosen based on the concentration which yielded half-maximal levels of cellular motility (Chelberg et al., 1989). As a control for collagen specificity, certain studies included filters coated with a 5 μ g/ml solution of FN, a potent inducer of M4 melanoma cell migration chemotactic activity. The inhibitory effect of peptides on cell motility was determined by

Table II. Cell Adhesion and Spreading to Substratum-Bound Peptide IV-H1 or Type IV Collagen

Cell types	Species of origin	Peptide IV-H1*		Type IV collagen adhesion (%)
		Adhesion (%) [‡]	Spreading (%)	
Group 1				
K1735 M4 melanoma	Mouse	22.4	70	61.9
A375 M melanoma	Human	16.3	20	50.3
C6 glioma	Rat	35.9	82	100
B104 neuroblastoma	Rat	18.5	20	18.6
Group 2				
Bovine aortic endothelial cells	Bovine	0.8	0	52.7
Squamous cell carcinoma	Human	1.9	0	39.6
Group 3				
UV 2237 fibrosarcoma	Murine	1.1	0	7.9
B65 neuroblastoma	Rat	4.6	0	7.7

* Surfaces were coated with 100 μ l/well of peptide IV-H1 or intact type IV collagen at 100 μ g/ml in Voller's carbonate buffer.

[‡] Percentage of input cells that adhered or spread as described in Materials and Methods section. Data is expressed as the average of three experiments; each experiment was performed in triplicate.

preincubating the cells with increasing concentrations of the peptides for 20 min before the assay to allow for binding to cell surface receptors. Cells in the continued presence of peptides were then added to the upper wells of chambers containing the protein-coated filters. The inhibitory effect of anti-peptide IV-H1 IgG on haptotaxis was determined by incubation of the protein-coated filters for 20–30 min with antibody in the lower wells to bind (and thus block) the IV-H1 sequence within the type IV collagen molecule. Cells were then added to the upper wells and the assay proceeded as described above. In addition to IgG purified from normal rabbit sera, IgG generated against peptide I coupled to KLH was also used as a negative control to confirm that antibodies generated against KLH did not contribute to the inhibitory effects of the anti-peptide IV-H1 IgG.

Results

Melanoma Cell Adhesion and Spreading on Peptide IV-H1

A number of type IV collagen-derived synthetic peptides were screened for the ability to promote melanoma cell adhesion (Table I). As described previously (Koliakos et al., 1989), 17 peptides were initially selected for synthesis from the published sequence of the major triple helical region of the α 1(IV) chain based upon their potential to bind heparin, as a model for glycosaminoglycan binding. The criteria used to select the sequences to be synthesized included the presence of the positively charged amino acid residues arginine and lysine, as well as the presence or absence of discontinuities of the triple helical motif. Although the charges of these peptides were potentially predictive of heparin binding abilities, only a few of the peptides actually did bind heparin (these peptides have been extensively characterized; Koliakos et al., 1989). In the present study, several of those peptides that did not bind heparin were screened for the ability to promote the adhesion of melanoma cells, assuming that the pro-

motion of cell adhesion by these peptides would likely involve a mechanism(s) distinct from binding a cell surface glycosaminoglycan. Only one of the peptides studied (designated IV-H1) promoted significant levels of both adhesion and spreading. This peptide promoted melanoma cell adhesion in a concentration-dependent manner within a coating range of 1–500 μ g/ml (Fig. 1 a). Significant cell adhesion (\sim 35% of input cells) occurred at a coating concentration of 10 μ g/ml of peptide IV-H1 and maximal cell adhesion ($>$ 50% of input cells) was observed in wells coated with a 100 μ g/ml solution of peptide IV-H1. The other peptides studied did not promote significant levels of cell adhesion ($<$ 10% over BSA controls), even at the highest concentration of peptide studied (500 μ g/ml).

Melanoma cell spreading also occurred on surfaces coated with peptide IV-H1 in a concentration-dependent manner. Maximal spreading (\sim 70% of input cells) was observed within 40 min at the 200 μ g/ml coating concentration of peptide IV-H1 (Fig. 1 b). The other type IV collagen-derived peptides promoted insignificant melanoma cell spreading within the time frame studied.

To ensure that peptide IV-H1-mediated cell adhesion and spreading was not unique to these melanoma cells, various other cell types were screened for adhesion and spreading on surfaces coated with the peptide. Cells of different embryonic origin and from various species were studied for the ability to adhere and spread on surfaces coated with peptide IV-H1. The cell types comprise three major groups (Table II): (Group 1) cells that adhered and spread on both peptide IV-H1 and type IV collagen which, in addition to including the high metastatic murine K1735 M4 melanoma, included a highly metastatic human melanoma (A375 M), a rat glioma (C6), and a rat neuroblastoma (B104); (Group 2) cells that

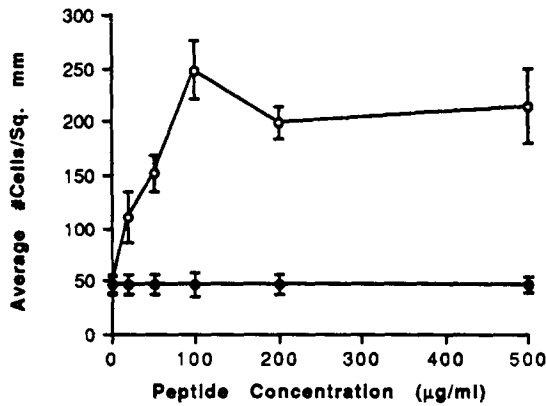


Figure 2. Melanoma cell motility in response to peptide IV-HI. Increasing concentrations of peptide IV-HI were used to coat polycarbonate filters as described in Materials and Methods. Cells were added to the upper wells of a chamber containing the precoated filter. The number of cells that migrated was quantitated using an image analysis system. Data are presented as the average number of cells per square millimeter of filters coated with peptide IV-HI (○) or BSA (●). All data represent the means of at least three experiments in which triplicate determinations were made \pm SEM.

adhered and spread on type IV collagen but not on peptide IV-HI, including bovine aortic endothelial cells and the human squamous carcinoma cell line (SCC9); and (Group 3) cells that did not adhere to either type IV collagen or peptide IV-HI, including the murine UV2237 MM fibrosarcoma and the rat B65 neuroblastoma. In all cases, cell adhesion and spreading on surfaces coated with BSA or control peptides was <5%.

Melanoma Cell Motility on Peptide IV-HI

In light of the involvement of cell adhesion and spreading in motility (see Chelberg et al., 1989), peptide IV-HI was studied for its ability to directly promote melanoma cell motility. Peptide IV-HI promoted a concentration-dependent increase in melanoma cell motility in microchemotaxis chambers (Fig. 2). Whereas a significant level of motility (twice BSA control levels) was observed at a coating level as low as 20 μ g/ml, maximal cell motility (5 \times BSA control levels) was observed when the filter was coated with a 100–200 μ g/ml solution of peptide IV-HI. This compares with melanoma cell motility of 10-fold background levels in response to a 10 μ g/ml solution of intact type IV collagen as previously reported (Chelberg et al., 1989).

Inhibition of Cellular Adhesion, Spreading, and Motility by Exogenous Peptide IV-HI

Since peptide IV-HI was observed to promote cell adhesion, spreading, and motility, it was necessary to demonstrate the activity of the IV-HI sequence within the intact type IV collagen molecule. Thus, studies were performed to determine the ability of the peptide to inhibit type IV collagen-mediated M4 melanoma cell adhesion, spreading, and migration. The specificity of the peptide IV-HI sequence within type IV collagen was determined by including type I collagen and FN in the inhibition studies. As additional controls, two other peptides, type IV collagen peptide 17 and FN peptide I, were

studied for the ability to inhibit collagen-mediated cell activity. Cells remained viable in the presence of all concentrations of peptides studied, as demonstrated by exclusion of trypan blue dye (data not shown).

In the adhesion assays, exogenous soluble peptide IV-HI strongly inhibited IV-HI and type IV collagen-mediated melanoma cell adhesion (Fig. 3 a). Melanoma cell adhesion on surfaces coated with peptide IV-HI was inhibited by nearly 40% in the presence of soluble peptide IV-HI at 20 μ g/ml, and by \sim 80% at the highest concentration of peptide IV-HI tested (500 μ g/ml). Similarly, melanoma cell adhesion to surfaces coated with type IV collagen was inhibited by \sim 20% in the presence of only 20 μ g/ml soluble peptide IV-HI, and by as much as 70% at the highest concentration of peptide studied (500 μ g/ml). In contrast, melanoma cell adhesion on surfaces coated with type I collagen was not affected by the presence of soluble peptide IV-HI. In addition, no effect of the control peptide 17 (Fig. 3 a) or the FN peptide I (data not shown) on type IV collagen-mediated melanoma cell adhesion was observed, even at the highest concentration tested (500 μ g/ml). In all cases using type IV collagen-derived peptides, no inhibition of adhesion was observed on surfaces coated with FN, whereas peptide I specifically inhibited FN-mediated adhesion (data not shown).

Since soluble peptide IV-HI inhibited type IV collagen-mediated melanoma cell adhesion, its effect on cell spreading was next studied. Cell spreading on surfaces coated with peptide IV-HI was inhibited by up to 70% in the presence of soluble peptide IV-HI at 200 μ g/ml (Fig. 3 b). Similarly, cell spreading on type IV collagen-coated surfaces was inhibited by 20% at the 100 μ g/ml concentration of peptide IV-HI and the level of cell spreading decreased by \sim 50% at the highest concentration studied (200 μ g/ml). In contrast, pretreatment of cells with peptide IV-HI had no effect on the percent of cells spreading on surfaces coated with type I collagen. In control studies, excess peptide 17 (Fig. 3 b) or the FN peptide I (data not shown) had no effect on cell spreading on surfaces coated with type IV collagen or peptide IV-HI. In all cases using type IV collagen-derived peptides, no inhibition of FN-mediated spreading was observed, whereas the FN peptide I specifically inhibited FN-mediated spreading (data not shown).

Finally, type IV collagen-mediated haptotactic melanoma cell motility was studied in the presence of the synthetic peptides. Type IV collagen-mediated cell motility was inhibited by peptide IV-HI in a dose-dependent manner with maximal inhibition of \sim 80% at the 50 μ g/ml level of peptide IV-HI (Fig. 3 c). Melanoma cell motility through filters precoated with type I collagen, however, was not inhibited by the presence of peptide IV-HI. Peptide 17 had no effect on type IV collagen-mediated cell motility. In additional control experiments, the FN peptide I specifically inhibited FN-mediated motility, whereas no inhibition was observed using type IV collagen-derived peptides (data not shown).

Polyclonal IgG Inhibition of Type IV Collagen-mediated Cell Adhesion, Spreading, and Motility

Additional studies were designed to confirm the activity of the IV-HI sequence within intact type IV collagen. In these studies, polyclonal antibodies were generated against pep-

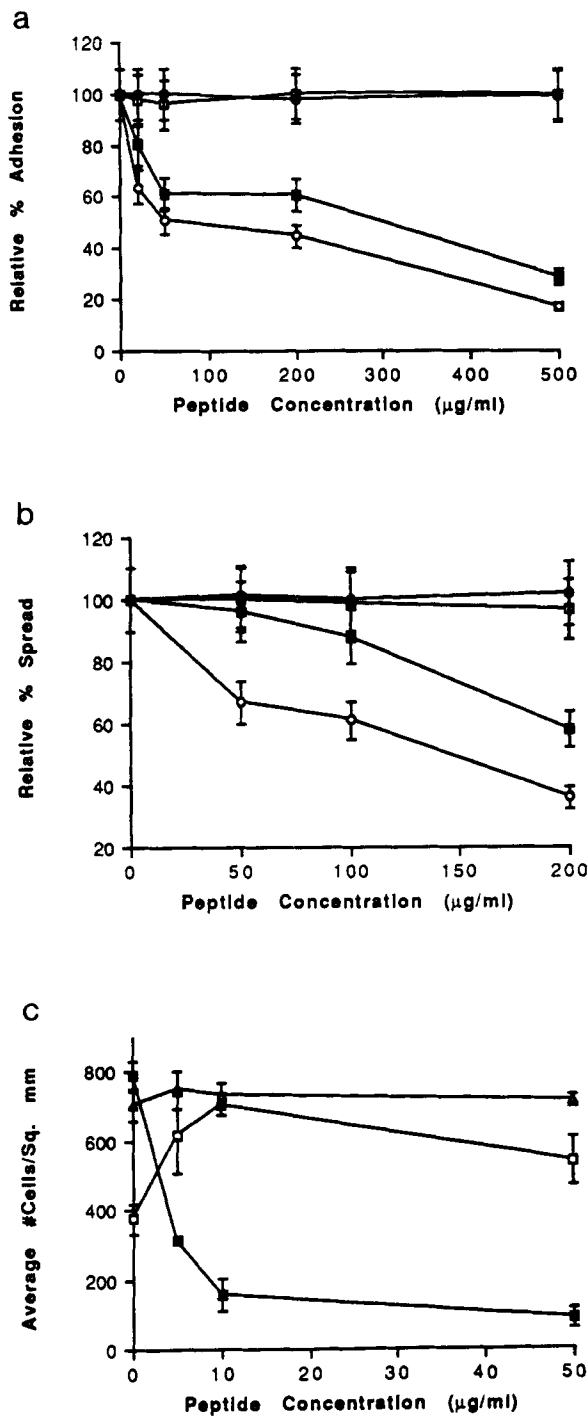


Figure 3. Inhibition of adhesion, spreading, and motility on type IV collagen by peptide IV-H1. ^3H -Thymidine-labeled M4 melanoma cells were preincubated with increasing concentrations of soluble peptides and cell adhesion or spreading on peptide- or protein-coated surfaces was determined in the continued presence of the peptides. Cell adhesion (a) or spreading (b) in the presence of soluble peptide IV-H1 was determined on surfaces coated with 5 $\mu\text{g}/\text{ml}$ type IV collagen (■) or type I collagen (□), or 50 $\mu\text{g}/\text{ml}$ peptide IV-H1 (○); or in the presence of peptide 17 on surfaces coated with 5 $\mu\text{g}/\text{ml}$ type IV collagen (●). Data are presented as the percent of cells adherent or spread in the presence of peptide relative to adhesion/spreading in the absence of soluble peptide. (c) Filters were coated on the underside with either type IV collagen or type I collagen as described in Materials and Methods. Cell migration was quantitated in the presence of soluble peptide IV-H1

Table III. Reactivity of Antibody Raised against Peptide IV-H1

Protein or peptide (ligand)	IgG Concentration ($\mu\text{g}/\text{ml}$)			
	0.001*	0.004	0.02	1.0
Peptide IV-H1	2,996‡	3,179	3,705	3,927
Type IV collagen	127	290	779	1,747
Type I collagen	69	71	110	198
Fibronectin	88	73	88	257

* Purified anti-peptide IV-H1 IgG was screened for specificity by an indirect solid-phase RIA. Briefly, surfaces were coated with proteins or peptides as described in Materials and Methods. Purified IgG at various dilutions were added. Bound IgG was detected by the addition of ^{125}I -labeled secondary IgG (sp act, 5 $\mu\text{Ci}/\mu\text{g}$).

‡ Quantitation of ^{125}I -labeled secondary IgG bound, expressed as counts per minute.

peptide IV-H1 and tested for the ability to inhibit type IV collagen-mediated M4 melanoma cell adhesion, spreading, and motility. Purified IgG generated against KLH-coupled peptide IV-H1 was screened for immunoreactivity by RIA (Table III). Reactivity of anti-peptide IV-H1 IgG was type IV collagen-specific, since the IgG recognized type IV collagen and peptide IV-H1 but not type I collagen or FN. The controls, purified normal rabbit IgG or purified IgG generated against the FN peptide I coupled to KLH did not react with type IV collagen or any of the synthetic type IV collagen-derived peptides, although anti-peptide I IgG specifically reacted with FN (data not shown).

Melanoma cell adhesion on surfaces coated with peptide IV-H1, intact type IV collagen, or type I collagen was monitored in the presence of increasing concentrations of anti-peptide IV-H1 IgG. Melanoma cell adhesion on surfaces coated with 50 $\mu\text{g}/\text{ml}$ of peptide IV-H1 was inhibited ~40% by anti-IV-H1 IgG at 20 $\mu\text{g}/\text{ml}$ (Fig. 4 a). Maximal inhibition (70%) was observed at the highest concentration of antibody tested (500 $\mu\text{g}/\text{ml}$). Cell adhesion to type IV collagen was also reduced in a concentration-dependent manner by preincubation with anti-IV-H1 IgG. Significant inhibition (20%) was observed in the presence of only 20 $\mu\text{g}/\text{ml}$ of anti-peptide IV-H1 IgG, and a 40% inhibition of adhesion was observed at 500 $\mu\text{g}/\text{ml}$ of anti-peptide IV-H1 IgG. In contrast, preincubation with the anti-peptide IV-H1 IgG did not reduce the levels of melanoma cell adhesion to surfaces coated with type I collagen. No inhibition of cell adhesion to surfaces coated with any of the collagenous proteins was observed in the presence of normal rabbit IgG or anti-FN peptide I IgG, even at the highest concentration used (500 $\mu\text{g}/\text{ml}$); demonstrated by the lack of inhibition of cell adhesion to peptide IV-H1 with normal rabbit IgG (Fig. 4 a). As expected, anti-peptide I IgG reduced cell adhesion to FN by up to 60% (data not shown).

Melanoma cell spreading on surfaces coated with peptide IV-H1, intact type IV collagen, or type I collagen was moni-

using filters coated with type IV collagen (■) or type I collagen (□), or in the presence of soluble peptide 17 using filters coated with type IV collagen (Δ). All data represent the means of at least three experiments in which triplicate determinations were made \pm SEM.

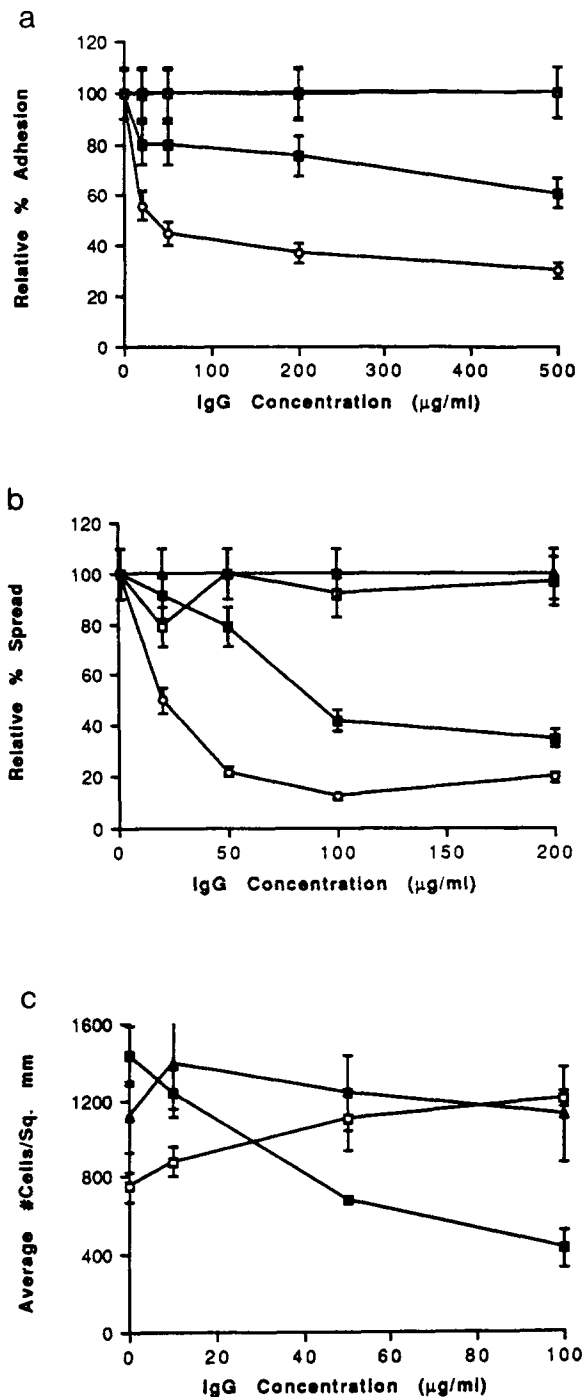


Figure 4. Anti-IV-H1 IgG inhibition of melanoma cell adhesion, spreading, and motility on type IV collagen. (a) Immulon 1 plates were coated, as described in Materials and Methods, with peptide IV-H1, type IV collagen, or type I collagen (50 µg/ml, 5 µg/ml, and 5 µg/ml, respectively). Coated surfaces were preincubated with increasing concentrations of anti-IV-H1 IgG or normal rabbit IgG, then cells were added in the continued presence of IgG. Data are presented as the percent of cells adherent in the presence of anti-peptide IV-H1 IgG on surfaces coated with type IV collagen (■), type I collagen (□), or peptide IV-H1 (○), or in the presence of normal rabbit IgG on type IV collagen (Δ). (b) Assay proceeded as in a. Data are presented as the percent of cells spreading in the presence of anti-peptide IV-H1 IgG on surfaces coated with type IV collagen (■), type I collagen (□), or peptide IV-H1 (○), or in the presence of normal rabbit IgG on type IV collagen (Δ). Data are expressed

tored in the presence of increasing concentrations of anti-peptide IV-H1 IgG. Cell spreading on surfaces coated with peptide IV-H1 was significantly decreased (50%) at levels of anti-peptide IV-H1 IgG as low as 20 µg/ml, and maximal inhibition (80%) was observed at 50 µg/ml (Fig. 4 b). Similarly, cell spreading on surfaces coated with type IV collagen was decreased by 20% in the presence of 50 µg/ml of anti-peptide IV-H1 IgG and by as much as 60% when 100 µg/ml of this IgG was present. In contrast, cell spreading on surfaces coated with type I collagen was not affected by this IgG (Fig. 4 b). The controls, normal rabbit IgG and anti-peptide I IgG, did not affect cell spreading on surfaces coated with peptide IV-H1, type IV collagen, or type I collagen.

The effect of anti-peptide IV-H1 IgG on melanoma cell haptotaxis mediated by type IV collagen was monitored by incubating the collagen-coated filter with anti-peptide IV-H1 IgG before the addition of cells to the motility assay (Fig. 4 c). Motility was decreased in a concentration-dependent manner with maximal inhibition of 70% at 100 µg/ml of IgG. In contrast, cell motility through filters precoated with type I collagen (Fig. 4 c) or FN (data not shown) was not inhibited by preincubation of the filters with anti-peptide IV-H1 IgG. Normal rabbit IgG had no effect on type IV collagen-mediated cell motility.

Melanoma Cell Adhesion, Spreading, and Motility on Alanyl-substituted Peptide IV-H1

In light of the reported importance of prolyl residues in mediating cell adhesion on collagen-coated surfaces (Rubin et al., 1981), a substituted version of peptide IV-H1 was synthesized to determine the relative importance of these residues in the ability of peptide IV-H1 to promote M4 melanoma cell adhesion, spreading, and motility. This peptide, designated IV-H1A, contained alanyl residues (A) in place of the three prolyl residues of the IV-H1 sequence (GVKGDKNAGWAGAA). Melanoma cell adhesion was essentially unaffected by the alanyl-substitution of peptide IV-H1, since cells adhered to nearly the same extent on surfaces coated with either peptide IV-H1 or IV-H1A (Fig. 5). However, cells exhibited a substantially decreased ability to spread on surfaces coated with peptide IV-H1A compared to spreading on peptide IV-H1 (Fig. 6). This occurred at all concentrations of the peptides (5–500 µg/ml) and at all time points monitored (20–90 min). Studies in which these two peptides were radiolabeled and bound to the plates indicated that pep-

as the percent of cell adhesion/spreading in the presence of IgG, relative to adhesion/spreading in the absence of IgG (represented as the 0 concentration). (c) Polycarbonate filters were coated on the underside with either type IV collagen or type I collagen, as described in Materials and Methods. Anti-peptide IV-H1 or normal rabbit IgG was added to the lower wells of the motility chambers in increasing concentrations and allowed to bind to the coated filters for 30 min. Cell motility was quantitated after a 4-h incubation. Data are presented as the average number of cells per square millimeter of filters coated with type IV collagen (■) or type I collagen (□) in the presence of anti-peptide IV-H1 IgG; or on type IV collagen in the presence of normal rabbit IgG (Δ). All data represent the means of at least three experiments in which triplicate determinations were made ± SEM.

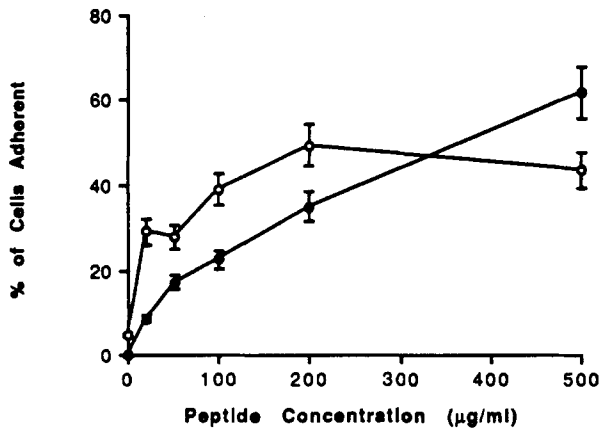


Figure 5. Adhesion of melanoma cells on alanyl-substituted peptide IV-H1. Peptide IV-H1 (○) or peptide IV-H1A (●) were coated to wells as described in Materials and Methods. Cell adhesion data are presented as the percent of cells added that were adherent. All data represent the means of at least three experiments in which triplicate determinations were made \pm SEM.

peptide IV-H1A binds to the plates as well as or better than native peptide IV-H1 at all concentrations tested (Table IV). These findings indicate that the reduced spreading of cells that was observed on surfaces coated with peptide IV-H1A was not due simply to failure of this peptide to bind to the plates.

In addition to decreasing the ability of peptide IV-H1 to promote melanoma cell spreading, the alanyl substitutions also decreased the ability of peptide IV-H1 to promote melanoma cell motility. Cell motility was not observed through filters coated with peptide IV-H1A, even at the highest concentrations tested (Fig. 7), whereas peptide IV-H1-coated filters promoted significant cell motility at levels as low as 10 μ g/ml. The relative abilities of the peptides to bind to the filters was determined using radioiodinated peptides to be certain that the differences in motility were not due to differential binding of the peptides to the filters. The studies demonstrated that peptide IV-H1A and peptide IV-H1 bound to the filters to similar extents (Table IV).

Table IV. Relative Binding of Peptides to Surfaces

Peptide*	Polystyrene plates [‡]		Polycarbonate filters [§]	
	Input (μ g/well)	Bound	Input (μ g/well)	Bound
IV-H1	0.1	0.094	1.75	0.020
	0.5	0.130	3.5	0.052
	1.0	0.364	7.0	0.221
IV-H1A	0.1	0.120	1.75	0.005
	0.5	0.420	3.5	0.071
	1.0	0.479	7.0	0.196

* The relative abilities of the peptides to bind to the plates or filters were determined using radioiodinated peptides. Radioiodinated peptides were dried to the plastic plates (100 μ l/well at each concentration) or polycarbonate filters (35 μ l/well at each concentration) as described in Materials and Methods.

[‡] Using a specific activity of 4.4×10^5 or 1.1×10^5 cpm/ μ g for peptide IV-H1 or IV-H1A, respectively, the amount of label bound per well was converted to μ g/well bound.

[§] Using a specific activity of 1.3×10^6 or 1.1×10^6 cpm/ μ g for peptide IV-H1 or IV-H1A, respectively, the amount of label bound to the filter per well was converted to μ g/well bound.

Discussion

A growing body of evidence, including the identification of type IV collagen-specific integrin-like receptors on the surface of tumor cells (Tomaselli et al., 1988; Kramer and Marks, 1989) indicates that BM type IV collagen may play a major role in tumor cell invasion of BM. Cell adhesion and motility on BM components have been described as necessary steps in BM invasion (Liotta, 1977). Our earlier studies (Chelberg et al., 1989) presented data that indicated that cell adhesion and spreading on surfaces coated with type IV collagen involve multiple, distinct domains of the molecule. Whereas both the purified helical p-IV fragment and the NC1 domain of type IV collagen supported in vitro M4 melanoma cell adhesion and spreading, only the p-IV fragment promoted cell motility. In addition, our findings indicated that these cell activities (adhesion and spreading) are necessary, albeit insufficient, for successful cell motility. Since cell motility is instrumental in tumor cell invasion of BM, the triple helical portion of type IV collagen has become a major focus of our further investigation. The current studies

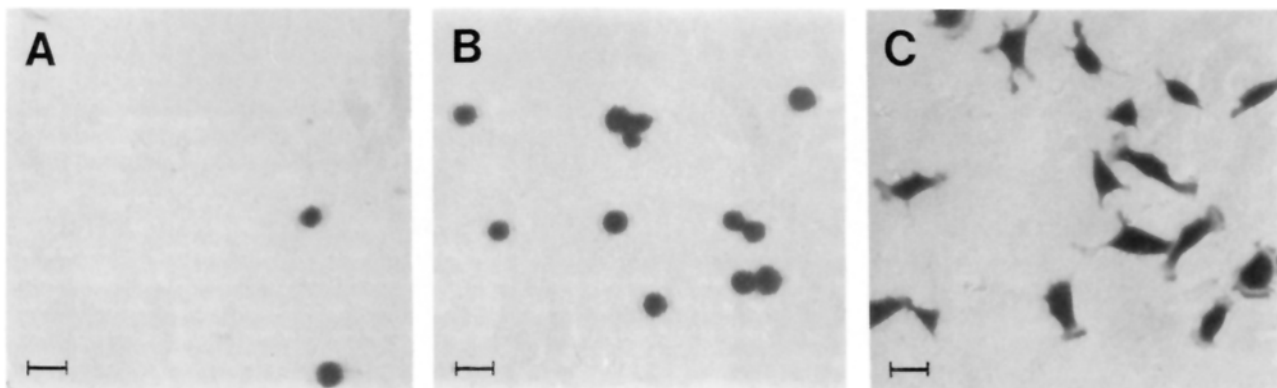


Figure 6. Spreading of melanoma cells on native peptide IV-H1 and alanyl-substituted peptide IV-H1A. BSA (A), peptide IV-H1A (B), or peptide IV-H1 (C) were coated to wells as described in Materials and Methods and M4 melanoma cell spreading was monitored for 80 min. The photomicrographs demonstrate the portion of adherent cells that were spread at 80 min. Bar, 50 μ m.

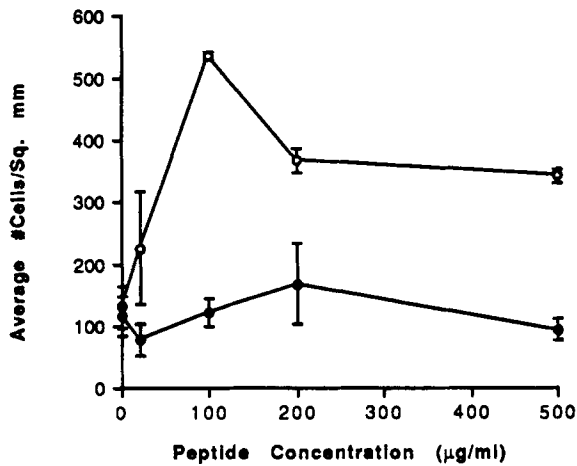


Figure 7. Directional motility of melanoma cells in response to the alanyl-substituted peptide IV-H1. Polycarbonate filters were coated as described in Materials and Methods with peptide IV-H1 (○) or peptide IV-H1A (●). Cell motility in response to these peptides was quantitated by image analysis microscopy. All data represent the means of at least three experiments in which triplicate determinations were made \pm SEM.

have been designed to further understand the molecular basis of the cellular responses promoted by the p-IV fragments. To this end, several synthetic peptides, derived from the α 1 chain of the major triple helical region of human type IV collagen, have been screened for the ability to promote cell adhesion and spreading.

The present studies indicate that a type IV collagen-derived sequence, designated peptide IV-H1, represents a cell adhesion, spreading, and motility promoting domain within the type IV collagen molecule. This is supported in part by the observations that peptide IV-H1 promoted these cellular activities in a concentration-dependent manner in studies using the highly metastatic M4 melanoma cell line. In addition, peptide IV-H1, or a polyclonal antibody directed against peptide IV-H1, inhibited type IV collagen-mediated melanoma cell adhesion, spreading, and motility. The latter results indicate that the sequence represented by peptide IV-H1 is active within the intact type IV collagen molecule.

Current studies have demonstrated receptors that recognize both type IV and either native type I collagen or gelatin (Kurkinen et al., 1984; Sugrue, 1987; Kramer and Marks, 1989). However, the findings presented here indicate that the effects of the peptide IV-H1 sequence are specific to type IV collagen. This is supported, in part, by our observations that type I collagen-mediated melanoma cell adhesion, spreading, and motility were not affected in competition studies by peptide IV-H1 or anti-peptide IV-H1 IgG, and is consistent with the observed absence of a IV-H1-like sequence within type I collagen in computer searches (using the ALIGN option of the PEP software or the QUEST program of the Intelligent Software package). It will be necessary, in future studies, to isolate a cell surface receptor that recognizes the sequence of peptide IV-H1 to test this hypothesis directly.

Studies of the adhesion and spreading of various normal and tumor cell types to surfaces coated with type IV collagen or peptide IV-H1 support a cell type-specific interaction with the IV-H1 sequence. In addition to the murine K1735 M4

melanoma cell line, cell lines derived from a human melanoma, a rat glioma, and a rat neuroblastoma were all observed to adhere and spread extremely well on surfaces coated with the human-derived peptide IV-H1. These findings support the possibility that the activity of the IV-H1 sequence in type IV collagen is conserved across species. This may be expected since the amino acid sequence of the entire type IV collagen molecule is highly conserved between the human and murine species (Muthukamaram et al., 1989). It will be of interest to further define the minimal sequence within peptide IV-H1 that supports cellular adhesion and motility, and to determine the sequence homology across species of this determinant.

Cell type specificity of peptide IV-H1-mediated cell adhesion and spreading was demonstrated by other cell types that strongly adhere to surfaces coated with type IV collagen, but which exhibited no adhesion to surfaces coated with peptide IV-H1. These cell types are represented in the current studies by the human squamous carcinoma cell line and bovine aortic endothelial cells. These observations suggest that different mechanisms for cell adhesion and spreading on type IV collagen may be used by different cell types. Additional cells, including a fibrosarcoma and neuroblastoma cell line, did not adhere to either intact type IV collagen or peptide IV-H1. It is worth noting that no cells were found to adhere to peptide IV-H1 which also did not adhere to intact type IV collagen. Taken with the results of the inhibition studies, this observation supports our assumption that the activity of peptide IV-H1 was not created upon removal of the sequence from the context of the intact type IV collagen molecule.

A peptide was synthesized, designated IV-H1A, in which alanyl residues were substituted for the three prolyl residues within the IV-H1 sequence, to confirm that the prolyl residues alone were not responsible for the activity of peptide IV-H1. Previous studies by Rubin et al. (1981) indicated that multiple prolyl residues presented in the collagenous helical motif promoted hepatocyte adhesion. In their studies, the tripeptide gly-X-pro repeat sequence promoted significant levels of adhesion, although reduced relative to intact collagens of various types or the cyanogen bromide-derived fragments of type I collagen. The conservative substitutions of alanyl residues for prolyl residues in the present studies drastically decreased the spreading and motility promoting activities of peptide IV-H1A relative to the parent IV-H1. These observations suggest that the prolyl residues are crucial for maintenance of the full repertoire of functions of the active sites within peptide IV-H1, in agreement with the findings of Rubin et al. (1981). Melanoma cell adhesion, however, was not affected by the amino acid substitutions. Taken together, these observations indicate that conservation of the prolyl residues is crucial for the full complement of IV-H1 sequence activities, but that other factors within peptide IV-H1 are also involved in promoting cell attachment. Although we do not presently have an explanation for these observations, the collagenous helix would be predicted to be more closed (<3.2 Å/turn) in the absence of the prolyl residues, which would alter the conformation of an active site. Additional studies are currently being performed to further establish the active site within peptide IV-H1. These studies, in combination with the isolation of a receptor(s) recognizing this sequence, will shed new light on the molecular basis of type IV collagen-mediated cell adhesion, spreading, and motility.

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