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# Effect of Ligand Conformation on Melanoma Cell $\alpha_3\beta_1$ Integrin-Mediated Signal Transduction Events: Implications for a Collagen Structural Modulation Mechanism of Tumor Cell Invasion<sup>†</sup>

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**ABSTRACT:** The importance of three-dimensional interactions between receptors with their respective ligands has been extensively explored during the binding process, but considerably less so for postbinding events such as induction of signaling pathways. Tumor cell receptor association with basement membrane proteins is believed to facilitate the metastatic process. Melanoma and ovarian carcinoma cells have been shown to utilize the  $\alpha_3\beta_1$  integrin to bind to models of the  $\alpha 1(IV)531-543$  sequence from basement membrane (type IV) collagen [Miles, A. J., et al. (1994) *J. Biol. Chem.* 269, 30939–30945; Miles, A. J., et al. (1995) *J. Biol. Chem.* 270, 29047–29050]. In the present study, the effects of ligand three-dimensional structure on possible signal transduction pathways induced by  $\alpha_3\beta_1$  integrin binding have been evaluated. Human melanoma cell binding to type IV collagen resulted in Tyr phosphorylation of p125<sup>FAK</sup>, consistent with prior studies correlating  $\beta_1$  integrin subunit binding to collagen and p125<sup>FAK</sup> Tyr phosphorylation. Cross-linking of an anti- $\alpha_3$  integrin subunit monoclonal antibody also induced p125<sup>FAK</sup> Tyr phosphorylation. Incubation of melanoma cells with single-stranded or triple-helical peptide models of  $\alpha 1(IV)531-543$  induced Tyr phosphorylation of intracellular proteins. Immunoprecipitation analysis identified one of these proteins as pp125<sup>FAK</sup>. Induction of p125<sup>FAK</sup> Tyr phosphorylation was enhanced and the time of induction was shortened when the ligand was used in triple-helical conformation. Subsequent clustering of either the single-stranded or the triple-helical ligand also increased the level of p125<sup>FAK</sup> phosphorylation compared to unclustered ligand. The clustered triple-helical peptide ligand induced more rapid paxillin Tyr phosphorylation than the single-stranded ligand. In addition, the induction of activated proteases was found to be more rapid due to ligand triple helicity. Overall, these studies have shown that (i) a model of an isolated sequence from type IV collagen,  $\alpha 1(IV)531-543$ , can induce  $\alpha_3\beta_1$  integrin-mediated signal transduction in melanoma cells and (ii) ligand conformation (secondary, tertiary, and/or quaternary structure) can directly influence several  $\alpha_3\beta_1$  integrin-mediated signal transduction events. The effects of ligand conformation suggest that a “collagen structural modulation” mechanism may exist for tumor cell invasion, whereby triple-helical collagen promotes cell binding and induction of signal transduction, subsequently leading to collagen dissolution by proteases, decreased signal transduction, and enhanced tumor cell motility.

The metastatic process involves a coordinated series of tumor cell behaviors, including adhesion to and migration on extracellular matrix (ECM)<sup>1</sup> components and invasion of the basement membrane. Such interactions may be mediated by a great variety of cell surface biomolecules, including integrins and proteoglycans (1). The best characterized cell surface adhesion molecules are integrins, which are heterodimeric proteins composed of one  $\alpha$  and one  $\beta$  subunit.

During the invasion process, tumor cell integrins interact with basement membrane (type IV) collagen. The collagen-binding integrins include  $\alpha_1\beta_1$ ,  $\alpha_2\beta_1$ , and  $\alpha_3\beta_1$  (2). In addition to promoting adhesion, integrins provide a linkage between the ligand and intracellular proteins. The role of adhesion in directing intracellular events (signal transduction) and subsequent cellular behaviors, such as regulation of gene transcription, is of great interest (3). Each of the collagen-binding integrins have been implicated in modulating signal transduction events (4–8). For example, when bound to

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<sup>1</sup> Abbreviations:  $\alpha 1(IV)531-543$ , Gly-Glu-Phe-Tyr-Phe-Asp-Leu-Arg-Leu-Lys-Gly-Asp-Lys; BSA, bovine serum albumin; ECM, extracellular matrix; EMEM, Eagle's Minimum Essential Medium; FAK, focal adhesion kinase; mAb, monoclonal antibody; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline solution; SSP, single-stranded peptide; TBST, Tris-buffered saline solution; THP, triple-helical peptide.

collagen, the  $\alpha_1\beta_1$  integrin can mediate signals inducing downregulation of collagen gene expression (9), whereas the  $\alpha_2\beta_1$  integrin can mediate induction of MMP-1 (9, 10).

While the complete cell signaling pathway following collagen binding has not been elucidated, several early events have been described. Integrin-mediated binding to collagen results in the Tyr phosphorylation of numerous intracellular proteins. Tyr phosphorylation of a  $\sim 125$  kDa protein that is found at focal adhesions (focal adhesion kinase, or p125<sup>FAK</sup>) is a common signaling event (3, 11). For example, p125<sup>FAK</sup> phosphorylation is seen following adhesion of fibroblasts, fibrosarcoma cells, and platelets to type I collagen (5, 12, 13) and endothelial cells to types I and IV collagen (6). Tyr phosphorylation of p125<sup>FAK</sup> has been linked to  $\beta_1$  integrin subunit binding of collagen (5, 6). Autophosphorylation occurs at Tyr<sub>397</sub> of p125<sup>FAK</sup> (14), resulting in the association of the SH2 domain of pp60<sup>src</sup> with the pp125<sup>FAK</sup> autophosphorylation site (14) and additional pp125<sup>FAK</sup> phosphorylation at residues Tyr<sub>407</sub>, Tyr<sub>576</sub>, Tyr<sub>577</sub>, Tyr<sub>681</sub>, and Tyr<sub>925</sub> due to pp60<sup>src</sup> kinase (11, 15). Phosphorylation of pp125<sup>FAK</sup> Tyr<sub>925</sub> leads to Grb2 binding via its SH2 domain (11, 16). Paxillin binds directly to the C-terminal region of pp125<sup>FAK</sup> (17) or to the SH3 domain of pp60<sup>src</sup> (16, 17), thus linking it to the signaling pathway.

Most prior studies on signal transduction events are based on using either proteins that bind several integrins (i.e., collagen and fibronectin) or ligands that bind specific integrins (i.e., mAbs and Arg-Gly-Asp based peptides) but are structurally dissimilar from native proteins.<sup>2</sup> Thus, it has traditionally been difficult to elucidate both the pathways mediated by individual integrins and the effects of ligand conformation on integrin-mediated signaling events. For example, cell binding to collagen utilizes several different integrins, possibly resulting in multiple signal transduction pathways. Cell binding to specific sites within collagen and subsequent individual signaling pathways have not been correlated. Some signaling pathways are dependent upon collagen triple-helical structure (secondary and tertiary structural effects) (12, 18) and early signaling events mediated by integrins are different for smooth muscle cell binding to monomeric versus fibrillar collagen (quaternary structural effects) (19). It is thus desirable to use isolated collagen sites in both triple-helical and nontriple-helical form for dissecting integrin-mediated activities.

Several cell-binding collagen-derived sequences have been identified previously (20) and can potentially be used for identifying specific pathways and correlating ligand conformation with signaling events. A peptide incorporating the  $\alpha 1(IV)531-543$  sequence promotes keratinocyte, corneal epithelial, melanoma, ovarian carcinoma, and Jurkat cell adhesion (21-24) as well as the migration of corneal epithelial cells and keratinocytes (22, 25). Recently, we described the tumor cell adhesion promoting activity of the  $\alpha 1(IV)531-543$  sequence from type IV collagen (24) and demonstrated that this activity was due to cell binding via the  $\alpha_3\beta_1$  integrin of human melanoma and ovarian carcinoma cells (26). In the present study, we have examined the

possible induction of melanoma cell and melanocyte signal transduction by the  $\alpha 1(IV)531-543$  sequence to determine if this sequence has a function beyond promotion of cell adhesion. The effect of triple-helical conformation on signaling has been studied using a triple-helical peptide model of  $\alpha 1(IV)531-543$ . We have also studied the effects of substrate "clustering" on signaling activities. Substrate clustering and subsequent receptor clustering promotes cytoskeletal reorganization which can impact signaling pathways (27, 28). As a measure of signal transduction, Tyr phosphorylation of p125<sup>FAK</sup> and paxillin have been examined. p125<sup>FAK</sup> Tyr phosphorylation is a common early signaling event, and overexpression of p125<sup>FAK</sup> appears to correlate with the invasive potential of tumor cell lines (29). Paxillin phosphorylation has been proposed to lead to divergent signaling activities (16, 17). Finally, we have used fluorogenic substrate assays (30) to quantitate the production of activated proteases in response to  $\alpha_3\beta_1$  integrin binding to single-stranded and triple-helical peptide models of  $\alpha 1(IV)531-543$ .

## EXPERIMENTAL PROCEDURES

**Materials.** All standard peptide synthesis chemicals were of analytical reagent grade or better and purchased from Applied Biosystems, Inc. (Foster City, CA) or Fisher (Pittsburgh, PA). The synthesis, purification, and characterization of the single-stranded peptide (SSP) and triple-helical peptide (THP) incorporating the  $\alpha 1(IV)531-543$  sequence, as well as the Asp<sup>541</sup>→Leu-substituted SSP, have been described (26, 31). Peptide concentrations were determined spectrophotometrically. Intact type IV collagen (molecular mass  $\approx 500$  kDa) was isolated from mouse Engelbreth-Holm-Swarm tumor as described (32, 33). MAb P5D2 was prepared against the  $\beta_1$  integrin subunit using methods described previously (34). MAb P1B5, prepared against the  $\alpha_3$  integrin subunit, was purchased from Chemicon International (Temecula, CA). The anti- $\alpha_3$  mAb was obtained as ascites fluid ( $\sim 2.5$  mg of IgG/mL of ascites), while the anti- $\beta_1$  mAb was a purified IgG. The anti-p125<sup>FAK</sup>/pp125<sup>FAK</sup> and anti-phosphoTyr mAbs were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-paxillin mAb was purchased from Transduction Laboratories (Lexington, KY). Goat anti-mouse and sheep anti-rabbit antibodies conjugated to horseradish peroxidase were purchased from Amersham (Arlington Heights, IL).

**Cells.** M14 clone 5 human melanoma cells were propagated as described previously (24, 26). Briefly, melanoma cells were cultured in EMEM supplemented with 10% fetal bovine sera, 1 mM sodium pyruvate, 0.1 mg/mL gentamicin (Boehringer Mannheim, Indianapolis, IN), 50 units/mL penicillin, and 0.05 mg/mL streptomycin. Cells were passaged 8 times and then replaced from frozen stocks of early passage cells to minimize phenotypic drift. All cells were maintained at 37 °C in a humidified incubator containing 5% CO<sub>2</sub>. All media reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

NHEM685 normal human melanocytes were obtained from Clonetics Normal Human Cell Systems (San Diego, CA). Cells are cultured as described by Clonetics using the MelanoPack, which contains HEPES-buffered saline solution, 0.025% trypsin/0.01% EDTA in HBSS, trypsin-neutralizing

<sup>2</sup> Although mAbs against integrin subunits have been used to dissect signaling pathways, they are not true substrates. Subtle differences have been found between pathways induced by anti-integrin mAbs and native substrates (76).

solution, and confidential melanocyte-proliferating and melanocyte growth medium products.

**Cell Assay.** Melanoma cell and melanocyte adhesion to substrate-coated Falcon Petri dishes (Becton Dickinson & Co., Lincoln Park, NJ) was performed as described previously for substrate-coated Immulon plates (24). Briefly, type IV collagen (10  $\mu\text{g/mL}$ ; 0.20 nM) or  $\alpha 1(\text{IV})531-543$  SSP (10  $\mu\text{g/mL}$ ; 5.7  $\mu\text{M}$ ) dissolved in PBS was added to Petri dishes and allowed to adsorb overnight at 4 °C for type IV collagen or 37 °C for the SSP. Nonspecific-binding sites were blocked with 2 mg/mL ovalbumin in PBS for 2 h at 37 °C. Cells were added to the Petri dishes and allowed to adhere for 30–60 min at 37 °C. Nonadherent cells were removed by washing the plate with PBS three times. Adherent cells were lysed with lysis buffer (see below). Alternatively, cell binding to peptides was performed in solution by a novel binding assay. Cells were harvested at 80–90% confluence with 5 mM EDTA, and  $\text{Mg}^{2+}$  was added after cells were released. Cells were washed, suspended in PBS, added to the peptide [either  $\alpha 1(\text{IV})531-543$  SSP or  $\alpha 1(\text{IV})531-543$  THP], and incubated at 37 °C with mixing. The cells were pelleted at 10000g for 5 s and lysed with lysis buffer (0.25% Triton X-100, 75 mM NaCl, 25 mM Tris-HCl, 0.5 mM sodium vanadate, 2.5 mM EDTA, 5  $\mu\text{g/mL}$  aprotinin, 5  $\mu\text{g/mL}$  leupeptin, 5  $\mu\text{g/mL}$  pepstatin, and 5  $\mu\text{g/mL}$  PMSF). This lysis buffer has been shown previously to solubilize intracellular proteins including signaling molecules (13, 35, 36). Lysates were microfuged (13500g) at 4 °C for 30 min to remove particulates.

**Peptide Coupling to Magnetic Beads and Cell Assay.** Tosyl-activated magnetic beads (M-450, substitution level = 25  $\mu\text{mol/g}$ , Dynal, Oslo, Norway) were coated with peptide as described previously (36). Briefly, the beads were washed with PBS and incubated for 24 h with the desired peptide dissolved in PBS using bidirectional mixing. Fresh peptide solution was added and the incubation step was repeated. Unoccupied binding sites were blocked with Tris by incubating the beads in 1 M Tris-HCl for 4 h. Peptide beads were washed and stored in PBS at 4 °C until use. Binding efficiency was measured by performing amino acid analysis on a specific volume of PBS containing the peptide-coated beads and quantitating the amount of loaded peptide and the total amount of peptide used to coat the beads. Amino acid analyses were performed on a Beckman 6300 Analyzer with a sulfated polystyrene cation-exchange column (0.4 cm  $\times$  25 cm). Peptides and peptide beads were hydrolyzed with 6 N aqueous HCl at 110 °C for 18–24 h.

Cells were harvested at 80–90% confluence, washed, and suspended in PBS as described in the *Cell Assay* section. They were added to the peptide-coated beads and incubated at 37 °C with intermittent mixing. The magnetic beads and cells were pelleted and the cells lysed with lysis buffer (see above). Lysates were microfuged at 4 °C for 30 min to remove particulates.

**Anti-Integrin Subunit MAb Assay.** Cells were harvested at 80–90% confluence, washed, and suspended in PBS as described in the *Cell Assay* section. The anti- $\alpha_3$  integrin subunit mAb was added and allowed to incubate for 30 min at 4 °C. The cells were washed and incubated with goat anti-mouse IgG (Cappel, West Chester, PA) for 15 min at 4 °C. Cross-linking of the anti- $\alpha_3$  integrin subunit mAb with the IgG was initiated by placing the cells at 37 °C and was

stopped by placing the cells on ice and immediately adding lysis buffer. Lysates were microfuged at 4 °C for 30 min to remove particulates.

**Immunoprecipitation and Blotting Analysis.** Goat anti-mouse sepharose beads (Zymed, San Francisco, CA) were incubated with the appropriate mAb for at least 4 h at 4 °C with mixing. The beads were washed three times with lysis buffer. Cell lysate was added to the beads and incubated overnight (18 h) at 4 °C with mixing. The lysate was removed, and the beads were washed 3 times with lysis buffer. Reducing buffer (37) was added, and the beads were heated at 100 °C for 5 min. The samples were electrophoresed on a 4 to 20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) and transferred to nitrocellulose (Micron Separations, Inc, Westboro, MA). The nitrocellulose was incubated in TBST (10 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 0.5% tween-20) with 2% BSA for at least 4 h and incubated in anti-phosphoTyr mAb diluted in TBST with 2% BSA overnight at 4 °C. The membrane was washed with TBST and incubated with horseradish peroxidase conjugated secondary antibody diluted in TBST with 2% BSA for 1 h and washed with TBST. Enhanced chemiluminescence reagents (Amersham) were used to develop the membrane. Initially, control experiments were run to check the reproducibility of immunoprecipitations. In these assays, the amount of immunoprecipitated protein was split in half and run on separate gels. One gel was blotted with the appropriate anti-protein antibody and the other was blotted with the anti-phosphoTyr mAb. These assays were discontinued when reproducibility between samples was established.

**Fluorogenic Substrate Assay.** A cell-binding assay was performed in solution as indicated in the *Cell Assay* section, with the following exceptions. Cells were treated with the SSP or THP for 24 h, then pelleted. The supernatant was removed and checked for enzymatic activity as follows. Control or treated samples were added to enzyme buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM  $\text{CaCl}_2$ , 0.02%  $\text{NaN}_3$ , and 0.05% Brij, pH 7.5) containing the fluorogenic substrate NFF-1 [Mca-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Lys-(Dnp)-Gly, where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is 2,4-dinitrophenyl] (30) and incubated at 37 °C. NFF-1 has been shown previously to be hydrolyzed by MMP family members (30). Fluorescence was monitored after 20 h at  $\lambda_{\text{excitation}} = 325$  nm,  $\lambda_{\text{emission}} = 393$  nm using a Perkin-Elmer luminescence spectrophotometer LS50B.

## RESULTS

Several laboratories have demonstrated that cell binding to triple-helical collagen induces p125<sup>FAK</sup> Tyr phosphorylation (5, 6, 12, 13). Signal transduction events, specifically p125<sup>FAK</sup> Tyr phosphorylation, were thus examined for human melanoma cell binding to type IV collagen. Melanoma cells were incubated on Petri dishes either coated with 10  $\mu\text{g/mL}$  (0.20 nM) type IV collagen or ovalbumin blocked (control). Following 30 min of melanoma cell adhesion to type IV collagen, immunoprecipitation of the whole melanoma cell lysate with a p125<sup>FAK</sup>/pp125<sup>FAK</sup> mAb and immunoblotting with an anti-phosphoTyr mAb indicated that one of the proteins phosphorylated was p125<sup>FAK</sup> (Figure 1). Melanoma cell adhesion to uncoated (ovalbumin blocked) dishes (0 min)

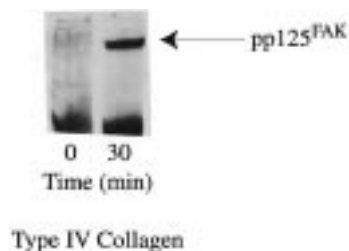


FIGURE 1: p125<sup>FAK</sup>/pp125<sup>FAK</sup> immunoprecipitation analysis of melanoma whole cell lysates following binding to Petri dish surfaces adsorbed with 10 µg/mL of type IV collagen. Cells were incubated with type IV collagen coated dishes for 0 or 30 min, then adherent cells were lysed, immunoprecipitated with an anti-p125<sup>FAK</sup>/pp125<sup>FAK</sup> mAb, and immunoblotted with an anti-phosphoTyr mAb. Phosphorylation of a ~125 kDa protein was seen. Cells incubated for 0 min on type IV collagen coated/ovalbumin-blocked Petri dishes showed low levels of p125<sup>FAK</sup> phosphorylation. Conditions are given in the Experimental Procedures.

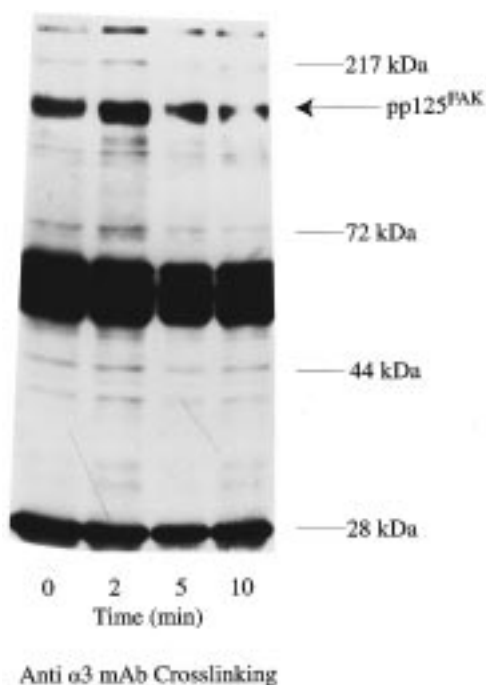


FIGURE 2: Immunoprecipitation and immunoblotting with anti-phosphoTyr mAbs was performed on melanoma whole cell lysates following binding to 5 µg/mL of a mAb against the  $\alpha_3$  integrin subunit. Cells were incubated with the  $\alpha_3$  mAb for 30 min, washed, and incubated with a goat anti-mouse IgG antibody for 15 min. Cross-linking was initiated by placing the cells at 37 °C for 0, 2, 5, or 10 min, and stopped by lysing the cells. Conditions are given in the Experimental Procedures.

resulted in low levels of p125<sup>FAK</sup> phosphorylation (Figure 1). Although three different integrins ( $\alpha_1\beta_1$ ,  $\alpha_2\beta_1$ , and  $\alpha_3\beta_1$ ) can bind to type IV collagen, our interest was whether the  $\alpha_3\beta_1$  integrin induces p125<sup>FAK</sup> Tyr phosphorylation. This is based on our prior studies that demonstrate that  $\alpha 1(IV)$ -531–543 is a specific  $\alpha_3\beta_1$  integrin binding sequence (24, 26). Treatment of melanoma cells with a mAb against the  $\alpha_3$  integrin subunit, followed by immunoprecipitation and immunoblotting with the anti-phosphoTyr mAb, indicated Tyr phosphorylation of a protein of ~125 kDa (Figure 2). Levels of Tyr phosphorylation of the substrate peaked at 2 min (190% of control). Immunoprecipitation analysis using the p125<sup>FAK</sup>/pp125<sup>FAK</sup> mAb confirmed the ~125 kDa protein as pp125<sup>FAK</sup> (data not shown).

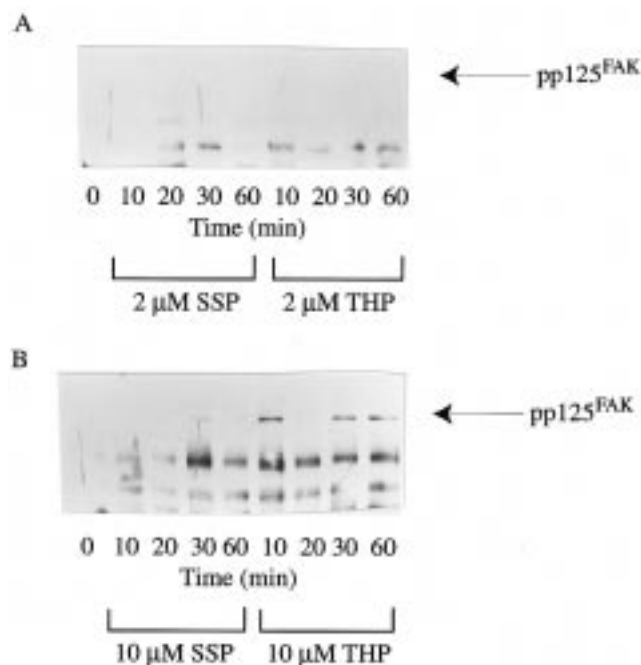


FIGURE 3: Immunoprecipitation and immunoblotting analysis of melanoma whole cell lysates following adhesion to (A) 2 µM  $\alpha 1(IV)$ 531–543 SSP or THP or (B) 10 µM of the  $\alpha 1(IV)$ 531–543 SSP or THP. Cells were incubated with peptide for 0, 10, 20, 30, or 60 min, then lysed, immunoprecipitated with an anti-p125<sup>FAK</sup>/pp125<sup>FAK</sup> mAb, and immunoblotted with an anti-phosphoTyr mAb. Phosphorylation of a ~125 kDa protein was seen. Conditions are given in the Experimental Procedures.

Tyr phosphorylation of p125<sup>FAK</sup> was induced by melanoma cell binding to type IV collagen or via an anti- $\alpha_3$  integrin subunit mAb. The  $\alpha_3\beta_1$  integrin binding sequence  $\alpha 1(IV)$ -531–543 was thus examined for induction of p125<sup>FAK</sup> phosphorylation. Levels of phosphorylation were examined initially for both solid-phase and solution cell binding to peptide models. However, we found that 30 min of melanoma cell attachment to ovalbumin-blocked dishes results in some upregulation of intracellular protein Tyr phosphorylation (data not shown). Thus, for peptide assays, we utilized only a solution-phase binding protocol which minimized nonspecific induction of signaling pathways.

We had shown previously that 40–60% maximum cell adhesion is achieved with 5–10 µM of either triple-helical or single-stranded peptide analogues of the  $\alpha 1(IV)$ 531–543 sequence (24). Cell-binding studies were thus performed at two peptide concentrations: 2 µM, which is well below the level where maximum adhesion occurs but comparable to the bead-coupled peptide concentration (see below), and 10 µM, which should promote a high level of cell binding.

Melanoma cells were incubated with 2 µM  $\alpha 1(IV)$ 531–543 SSP for 10, 20, 30, or 60 min. Immunoprecipitation using an anti-pp125<sup>FAK</sup> mAb was performed on whole cell lysates. Tyr phosphorylation of p125<sup>FAK</sup> was not detected (Figure 3A). Increasing the  $\alpha 1(IV)$ 531–543 SSP concentration to 10 µM resulted in low levels of p125<sup>FAK</sup> phosphorylation (140% of control) following cell binding for 30 min (Figure 3B).

Triple-helical structure has been shown to influence cellular recognition of native collagens (20). Signal transduction experiments were thus repeated using a triple-helical peptide model of  $\alpha 1(IV)$ 531–543. Melanoma cells were

incubated with 2  $\mu$ M  $\alpha$ 1(IV)531–543 THP for 10, 20, 30, or 60 min. Immunoprecipitation using an anti-pp125<sup>FAK</sup> mAb was performed on whole cell lysates. Tyr phosphorylation of p125<sup>FAK</sup> was not detected (Figure 3A). When the  $\alpha$ 1(IV)531–543 THP concentration was increased from 2 to 10  $\mu$ M, Tyr phosphorylation of p125<sup>FAK</sup> was detected at 10 min (320% of control) (Figure 3B). Phosphorylation levels decreased at 20 min, then increased at 30 min and remained elevated at 60 min (280% of control). The  $\alpha$ 1(IV)531–543 THP produced higher levels of p125<sup>FAK</sup> phosphorylation as well as a decreased time of induction (10 versus 30 min) compared with the SSP (see Figure 3B). Overall trends shown for Tyr phosphorylation of p125<sup>FAK</sup>, such as times of peak phosphorylation induced by both the SSP and THP ligands and apparent cyclic activity, were readily reproduced between experiments.

The binding of integrin ligands on magnetic beads has been used previously to cluster integrins and form focal adhesion complexes (38). The use of tosyl-activated magnetic beads allows for specific attachment of peptides and proteins via free primary amino groups. Prior studies have shown that the primary amino groups from  $\alpha$ 1(IV)531–543 SSP are not essential for cellular recognition of this sequence (24). Following attachment to magnetic beads, amino acid analysis per milliliter of suspended beads indicated that the effective concentrations of the bound SSP [designated  $\alpha$ 1(IV)531–543 SSP<sub>b</sub>] and THP [designated  $\alpha$ 1(IV)531–543 THP<sub>b</sub>] were 1.88 and 1.63  $\mu$ M, respectively. Immunoprecipitation analysis following cell incubation with  $\alpha$ 1(IV)531–543 SSP<sub>b</sub> and  $\alpha$ 1(IV)531–543 THP<sub>b</sub> was performed.  $\alpha$ 1(IV)531–543 SSP<sub>b</sub> induced maximum p125<sup>FAK</sup> phosphorylation at 30 min (180% of control) (Figure 4A). At a similar concentration (2  $\mu$ M),  $\alpha$ 1(IV)531–543 SSP in solution did not induce detectable levels of phosphorylation (see Figure 3A). Even at a peptide concentration of 10  $\mu$ M, the level of phosphorylation induced by  $\alpha$ 1(IV)531–543 SSP in solution was slightly lower than that induced by solid-phase  $\alpha$ 1(IV)531–543 SSP<sub>b</sub> at 1.88  $\mu$ M (compare Figures 3B and 4A). An additional protein of  $\sim$ 130 kDa was coimmunoprecipitated following  $\alpha$ 1(IV)531–543 SSP<sub>b</sub> treatment (Figure 4A). This could be p130<sup>Cas</sup>, which is known to associate with pp125<sup>FAK</sup> (39, 40). The clustered THP,  $\alpha$ 1(IV)531–543 THP<sub>b</sub>, induced maximum p125<sup>FAK</sup> phosphorylation at 20 min (160% of control) (Figure 4B). As was the case for  $\alpha$ 1(IV)531–543 SSP<sub>b</sub>, 1.63  $\mu$ M  $\alpha$ 1(IV)531–543 THP<sub>b</sub> produced higher levels of phosphorylation compared with a 2  $\mu$ M concentration of the unbound THP (compare Figure 4B to Figure 3A). For melanocytes,  $\alpha$ 1(IV)531–543 SSP<sub>b</sub> did not induce p125<sup>FAK</sup> phosphorylation, but  $\alpha$ 1(IV)531–543 THP<sub>b</sub> did (Figure 5). The time course of induction showed an increase at 30 min (135% of control), which stayed at the same level at 60 min (140% of control). Melanocyte response to  $\alpha$ 1(IV)531–543 was clearly different than for melanoma cells, as (i) no enhancement of p125<sup>FAK</sup> phosphorylation was seen upon binding to the single-stranded ligand, (ii) the time of maximum induction in response to the ligand was shifted to 30 min, and (iii) the relative level of Tyr phosphorylation was slightly lower. Again, the overall trends in p125<sup>FAK</sup> Tyr phosphorylation were consistent between experiments.

An SSP analogue of the  $\alpha$ 1(IV)531–543 sequence was next studied. The analogue contains a Leu in position 541

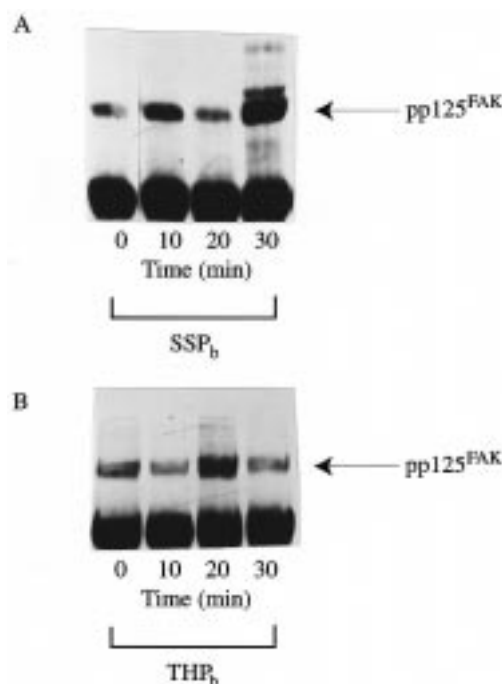


FIGURE 4: Immunoprecipitation and immunoblotting analysis of melanoma whole cell lysates following adhesion to (A)  $\alpha$ 1(IV)531–543 SSP or (B)  $\alpha$ 1(IV)531–543 THP covalently bound to magnetic beads (SSP<sub>b</sub> or THP<sub>b</sub>, respectively). Cells were incubated with peptide beads for 0, 10, 20, or 30 min, then lysed, immunoprecipitated with an anti-p125<sup>FAK</sup>/pp125<sup>FAK</sup> mAb, and immunoblotted with an anti-phosphoTyr mAb. Phosphorylation of a  $\sim$ 125 kDa protein was seen. Conditions are given in the Experimental Procedures.

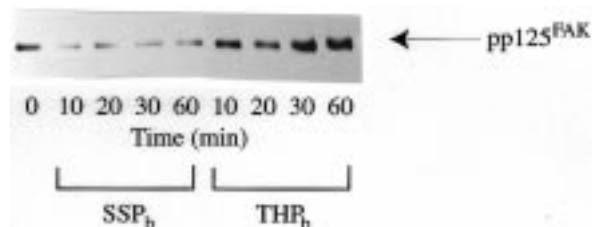


FIGURE 5: Immunoprecipitation and immunoblotting analysis of melanocyte whole cell lysates following adhesion to  $\alpha$ 1(IV)531–543 SSP or  $\alpha$ 1(IV)531–543 THP covalently bound to magnetic beads (SSP<sub>b</sub> or THP<sub>b</sub>, respectively). Cells were incubated with peptide beads for 0, 10, 20, 30, or 60 min, then lysed, immunoprecipitated with an anti-p125<sup>FAK</sup>/pp125<sup>FAK</sup> mAb, and immunoblotted with an anti-phosphoTyr mAb. Phosphorylation of a  $\sim$ 125 kDa protein was seen. Conditions are given in the Experimental Procedures.

rather than Asp from the native  $\alpha$ 1(IV)531–543 sequence. Melanoma cell adhesion to the Asp<sup>541</sup>→Leu SSP is  $28 \pm 2\%$  lower than the parent molecule (data not shown). Inhibition studies using anti-integrin mAbs indicate that melanoma cells utilize the same receptor, the  $\alpha_3\beta_1$  integrin, for binding to both peptides (data not shown). Since the only difference between the cellular recognition of each peptide appears to be in relative binding affinity, we decided that this analogue could serve as an appropriate negative control for some of the signaling studies. When the Asp<sup>541</sup>→Leu SSP was used as a clustered ligand for binding assays, no appreciable increase in the Tyr phosphorylation of p125<sup>FAK</sup> was seen up to 60 min. Induction of cellular-signaling processes was thus specific for the  $\alpha$ 1(IV)531–543 sequence.

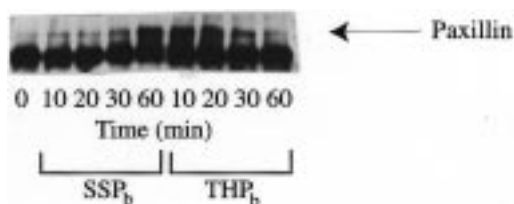


FIGURE 6: Immunoprecipitation and immunoblotting analysis of melanoma whole cell lysates following adhesion to  $\alpha 1(\text{IV})531-543$  SSP or  $\alpha 1(\text{IV})531-543$  THP covalently bound to magnetic beads ( $\text{SSP}_b$  or  $\text{THP}_b$ , respectively). Cells were incubated with peptide beads for 0, 10, 20, 30, or 60 min, then lysed, immunoprecipitated with an anti-paxillin mAb, and immunoblotted with an anti-phosphoTyr mAb. Phosphorylation of a  $\sim 68$  kDa protein was seen. Conditions are given in the Experimental Procedures.

The effects of triple-helical structure on melanoma cell signaling were next studied by monitoring paxillin Tyr phosphorylation. Substrate clustering and subsequent receptor clustering has been shown previously to enhance paxillin Tyr phosphorylation and accumulation (28). We have thus used  $\text{SSP}_b$  and  $\text{THP}_b$ , representing clustered ligands, for comparison of the time and level of paxillin Tyr phosphorylation induction. Melanoma cells were incubated with  $\alpha 1(\text{IV})531-543$   $\text{SSP}_b$  for 10, 20, 30, or 60 min. Immunoprecipitation using an anti-paxillin mAb was performed on whole cell lysates. An increase in Tyr phosphorylation of paxillin was detected after 60 min (175% of control) (Figure 6). Experiments were repeated using  $\alpha 1(\text{IV})531-543$   $\text{THP}_b$ . An increase in Tyr phosphorylation of paxillin was detected after 10 min (178% of control) (Figure 6). Thus, ligand triple helicity greatly shortened the time of paxillin Tyr phosphorylation induction. As paxillin has been proposed in some circumstances to bind directly to  $\text{pp125}^{\text{FAK}}$  (17), we examined the interaction of  $\text{pp125}^{\text{FAK}}$  and paxillin in response to  $\text{SSP}_b$  and  $\text{THP}_b$  by using immunoprecipitation of whole cell lysates with an anti- $\text{p125}^{\text{FAK}}/\text{pp125}^{\text{FAK}}$  mAb and analysis with an anti-paxillin mAb. In response to binding to  $\text{SSP}_b$ , increased levels of paxillin binding to  $\text{pp125}^{\text{FAK}}$  were found after 30 min (280% of control), which remained constant at 60 min (Figure 7). Cell binding to  $\text{THP}_b$  resulted in induction of a paxillin- $\text{pp125}^{\text{FAK}}$  complex after 10 min (166% of control), with levels increasing further at 20 min (300% of control) and remaining constant at 30 min (Figure 7). The time of induction of  $\text{pp125}^{\text{FAK}}$ -bound paxillin was shortened by cellular binding to the clustered triple-helical substrate.

Examination of  $\text{p125}^{\text{FAK}}$  and paxillin Tyr phosphorylation has clearly shown that ligand conformation can have a significant effect on  $\alpha_3\beta_1$  integrin-mediated cellular signaling. We next studied the effects of ligand conformation on one of the results of cellular-signaling pathways, protease production. Antibodies against the  $\alpha_3\beta_1$  integrin have been shown to stimulate the expression of MMP-2 (72 kDa type IV collagenase) (41, 42) and MMP-9 (92 kDa type IV collagenase) (43) after 24–48 h. We have previously described a fluorogenic substrate, NFF-1, that can be used to monitor MMP activity (30). Melanoma cells were incubated with either  $\alpha 1(\text{IV})531-543$  SSP or  $\alpha 1(\text{IV})531-543$  THP for 24 h, then the cell supernatant was assayed for 20 h using NFF-1. In response to  $\alpha 1(\text{IV})531-543$  SSP, melanoma cell production of activated protease was only slightly increased (120% of control). In contrast, melanoma cell production of activated protease was increased more

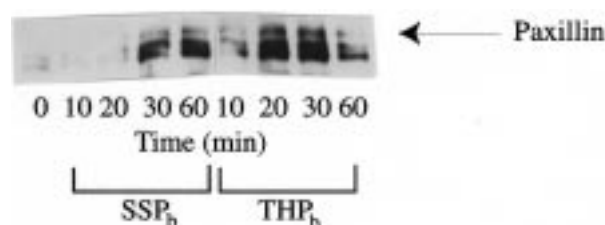


FIGURE 7: Immunoprecipitation and immunoblotting analysis of melanoma whole cell lysates following adhesion to  $\alpha 1(\text{IV})531-543$  SSP or  $\alpha 1(\text{IV})531-543$  THP covalently bound to magnetic beads ( $\text{SSP}_b$  or  $\text{THP}_b$ , respectively). Cells were incubated with peptide beads for 0, 10, 20, 30, or 60 min, then lysed, immunoprecipitated with an anti- $\text{p125}^{\text{FAK}}/\text{pp125}^{\text{FAK}}$  mAb, and immunoblotted with an anti-paxillin mAb. A  $\sim 68$  kDa protein coimmunoprecipitated with  $\text{pp125}^{\text{FAK}}$ . Conditions are given in the Experimental Procedures.

substantially (161% of control) in response to  $\alpha 1(\text{IV})531-543$  THP. The effects of ligand conformation on  $\alpha_3\beta_1$  integrin-mediated signaling are reflected in both early ( $\text{p125}^{\text{FAK}}$  and paxillin Tyr phosphorylation) and late (active protease production) events.

## DISCUSSION

Normal and metastatic cellular interactions with type IV collagen can be mediated by integrins and/or cell surface proteoglycans (1). We had demonstrated previously that the melanoma cell  $\alpha_3\beta_1$  integrin binds directly to peptide models of the  $\alpha 1(\text{IV})531-543$  sequence (26). In the present study, we have found that melanoma cell binding to type IV collagen induces  $\text{p125}^{\text{FAK}}$  phosphorylation, and that cross-linking of an anti- $\alpha_3$  integrin subunit mAb has a similar effect.

The  $\text{p125}^{\text{FAK}}$  Tyr phosphorylation results are consistent with other studies of  $\alpha_3\beta_1$  integrin binding. For example, cross-linking the  $\alpha_3\beta_1$  integrin from human epidermal carcinoma cells induces Tyr phosphorylation of  $\text{p125}^{\text{FAK}}$  (44), while human neuroblastoma cell adhesion to type IV collagen or mAbs against either the  $\alpha_3$  or  $\beta_1$  integrin subunit induces Tyr phosphorylation of a group of proteins that includes  $\text{p125}^{\text{FAK}}$  (8). Since melanoma cells bind to single-stranded and triple-helical peptide models of the  $\alpha 1(\text{IV})531-543$  sequence via the  $\alpha_3\beta_1$  integrin, postadhesion events mediated by integrin binding to the  $\alpha 1(\text{IV})531-543$  sequence were evaluated. Single-stranded and triple-helical peptide models of  $\alpha 1(\text{IV})531-543$  induced Tyr phosphorylation of intracellular proteins. Immunoprecipitation analysis identified one of these proteins as  $\text{p125}^{\text{FAK}}$ . The effects of triple helicity on shortening the time and enhancing the level of  $\text{p125}^{\text{FAK}}$  phosphorylation indicate that, although  $\alpha_3\beta_1$  integrin binding to  $\alpha 1(\text{IV})531-543$  appears to be independent of substrate conformation (24), signaling via this receptor does seem to be influenced by the three-dimensional interactions of receptor with ligand. In the case of the triple-helical peptide model,  $\text{p125}^{\text{FAK}}$  phosphorylation was induced at 10 min, decreased at 20 min, then increased at 30 min. It is possible that this sequence of events represents a regulatory mechanism, whereby autophosphorylation of Tyr<sub>397</sub> is followed by phosphatase activity (45) and subsequent  $\text{p125}^{\text{FAK}}$  reautophosphorylation.

Receptor occupancy without clustering can induce receptor redistribution, but only minimal Tyr phosphorylation of most

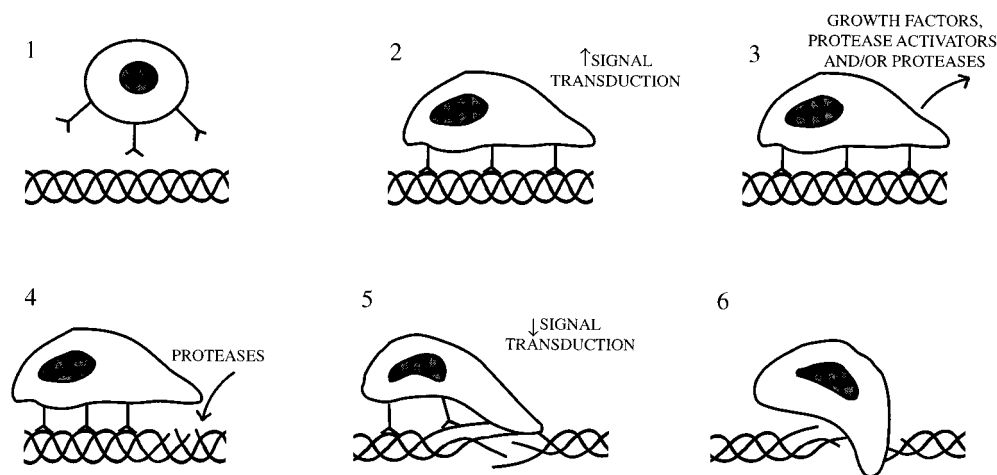


FIGURE 8: Proposed "collagen structural modulation" model based on melanoma cell interaction with  $\alpha 1(\text{IV})531-543$  via the  $\alpha_3\beta_1$  integrin. The complete sequence of events are described in the text.

intracellular proteins is seen (27, 28). One exception is  $p125^{\text{FAK}}$ , which is phosphorylated independent of receptor clustering (27, 28). However, optimal cytoskeletal protein redistribution is seen with a combination of receptor occupancy and clustering (28, 46). We thus studied the combined effects of receptor occupancy and ligand clustering on  $\alpha 1(\text{IV})531-543$  induction of  $p125^{\text{FAK}}$  phosphorylation. Clustering of either single-stranded or triple-helical ligand using peptide-coated magnetic beads, and hence clustering of the integrin, did increase the level of  $p125^{\text{FAK}}$  phosphorylation compared to ligand in solution at similar molar concentrations. Weaker binding by the  $\alpha_3\beta_1$  integrin to the clustered Asp<sup>541</sup>→Leu SSP was not sufficient to induce appreciable Tyr phosphorylation of  $p125^{\text{FAK}}$ . The difference in adhesion affinity between the SSP analogues is probably due to the removal of one negatively charged residue. This decreases the strength of the electrostatic interaction between the  $\alpha_3\beta_1$  integrin and the peptide ligand. Apparently, the removal of one of the negative charges within the  $\alpha 1(\text{IV})531-543$  sequence also affects cellular signaling. This result is not surprising since it has been shown that a common key feature of many integrin ligands is the presence of acidic residues, usually Asp, within their integrin-binding motifs (47).

In contrast to  $p125^{\text{FAK}}$ , paxillin Tyr phosphorylation is dependent upon both receptor occupancy and clustering (28). Consistent with this prior result, we found that paxillin Tyr phosphorylation were only slightly increased when  $\alpha 1(\text{IV})531-543$  was used in nonclustered form (data not shown). The combined effects of receptor occupancy and ligand clustering on  $\alpha 1(\text{IV})531-543$  induction of paxillin phosphorylation were much more significant. The time of induction of paxillin phosphorylation was shortened by the triple-helical ligand compared to the single-stranded one, and paxillin appeared to be bound directly to  $p125^{\text{FAK}}$ . It is not clear if the enhanced activities based upon multivalency are relevant to native collagen structures, as type IV collagen forms an ordered network *in vivo* which may present itself as a multivalent ligand to cell surface receptors.

Although  $\alpha_3\beta_1$  binding to a type IV collagen model sequence induces signal transduction events, the ultimate role of this integrin is not known. It has been shown previously that (i)  $\alpha_2\beta_1$  and  $\alpha_1\beta_1$  are high-affinity receptors for type IV

collagen, while  $\alpha_3\beta_1$  is a low-affinity receptor at best (2) and (ii) mAb P1B5 against the  $\alpha_3$  subunit cannot inhibit cell adhesion to type IV collagen but can inhibit adhesion to the  $\alpha 1(\text{IV})531-543$  site (26, 48). We believe that binding to type IV collagen is initiated via the  $\alpha_2\beta_1$  and  $\alpha_1\beta_1$  integrins, and then  $\alpha_3\beta_1$  integrin is recruited to sites on the substrate including  $\alpha 1(\text{IV})531-543$ . This model is consistent with that of DiPersio et al. (49), who demonstrated that the  $\alpha_3\beta_1$  integrin is localized to focal contacts following cell adhesion to type IV collagen, but does not initiate cell adhesion to this ligand. The primary role of  $\alpha_3\beta_1$  would then be in initiating postadhesion signal transduction events. One scenario that could be envisioned is that metastatic melanoma  $\alpha_3\beta_1$  integrin binding to the  $\alpha 1(\text{IV})531-543$  site in type IV collagen is part of a "collagen structural modulation" mechanism for tumor cell invasion involving the following steps (Figure 8):

(1) Melanoma cells adhere initially to triple-helical type IV collagen via the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrins. The  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrins bind to type IV collagen with reasonably high affinity (2, 50), and we and others have shown that highly metastatic melanoma cells use these integrins to bind type IV collagen (51–53). Interestingly, melanocytes and poorly metastatic melanoma cells have poor affinity for type IV collagen (54). We have found that melanocytes also differ from melanoma cells in their signaling responses to  $\alpha 1(\text{IV})531-543$ .

(2) (a) Following adhesion, the  $\alpha_3\beta_1$  integrin is recruited to the triple-helical  $\alpha 1(\text{IV})531-543$  site in type IV collagen. The  $\alpha_3\beta_1$  integrin is upregulated in certain tumor cell types, such as metastatic melanoma (55, 56). DiPersio et al. (49) and Menter et al. (57) have shown that the  $\alpha_3\beta_1$  integrin is recruited to the site of focal contacts, and we have demonstrated direct binding of melanoma and ovarian carcinoma cells to the  $\alpha 1(\text{IV})531-543$  site in type IV collagen (24, 26). (b) Once the  $\alpha_3\beta_1$  integrin binds  $\alpha 1(\text{IV})531-543$ , the cytoplasmic tail (C-terminus) of the  $\beta_1$  integrin subunit may interact directly with the N-terminal region of  $p125^{\text{FAK}}$  (58) or with talin, which binds to the C-terminus of  $p125^{\text{FAK}}$  (59).  $p125^{\text{FAK}}$  is autophosphorylated at Tyr<sup>397</sup>, and the SH2 domain of pp60<sup>src</sup> associates with the pp125<sup>FAK</sup> autophosphorylation site (14, 58). Phosphorylation of Tyr<sup>925</sup> within pp125<sup>FAK</sup> by pp60<sup>src</sup> (11, 17) leads to Grb2 binding via its SH2 domain



(11, 16). Paxillin may then bind directly to the C-terminal region of pp125<sup>FAK</sup> (17) or to the SH3 domain of pp60<sup>src</sup> (16, 58). We have found that, in response to  $\alpha_3\beta_1$  integrin recognition of  $\alpha 1(IV)531-543$ , p125<sup>FAK</sup> and paxillin are Tyr phosphorylated and paxillin binds directly to pp125<sup>FAK</sup>.

(3) Induction of signaling pathways leads to production of proteases, protease activators, or factors that induce protease production from neighboring cells, including induction of MMPs. Others have demonstrated that antibodies against the  $\alpha_3\beta_1$  integrin stimulate the expression of MMP-2 (72 kDa type IV collagenase) (41, 42) and MMP-9 (92 kDa type IV collagenase) (43) after 24–48 h. MMP-2 (60) and MMP-1 (61) activation, at the transcriptional level, has been shown to be downstream of src kinase activity. We have found that melanoma cell binding to the triple-helical  $\alpha 1(IV)531-543$  sequence results in the production of active proteases.

(4) Protease dissolution of the basement membrane results in denaturation of the type IV collagen triple helix. Several members of the MMP family, including MMP-2, MMP-3, and MMP-9, have been demonstrated to efficiently hydrolyze type IV collagen (62, 63). Proteases besides MMP family members can also use type IV collagen as a substrate (64, 65).

(5) The levels of p125<sup>FAK</sup> phosphorylation are reduced due to integrin binding to denatured collagen. We have demonstrated that the level of p125<sup>FAK</sup> phosphorylation decreases upon melanoma cell binding to the linear version of  $\alpha 1(IV)531-543$  compared with the triple-helical ligand.

(6) Integrin binding to denatured collagen results in increased tumor cell motility and invasion. Although p125<sup>FAK</sup> expression appears important for cell motility (66), decreased levels of p125<sup>FAK</sup> phosphorylation results in increased cell motility on ECM proteins (67, 68), and cells move readily on the denatured (linear) form of  $\alpha 1(IV)531-543$  (25). Denaturation reduces the affinity of the  $\alpha_1\beta_1$  and  $\alpha_2\beta_1$  integrins for type IV collagen (50), and the lower attachment strength of the cells to the collagen should enhance migration potential (69). MAbs to the  $\alpha_3$  subunit inhibit melanoma cell motility on type IV collagen (55, 70, 71), correlating a role for the  $\alpha_3\beta_1$  integrin to cell motility. The denaturation of the triple helix may also expose cryptic sites that promote cell motility. In a related study, the cleavage of a different extracellular matrix protein, laminin, by MMP-2 has been shown to induce breast epithelial cell migration (72).

The results reported here are the first example of a signaling pathway being correlated to integrin-mediated recognition of a specific collagen-like sequence,<sup>3</sup> as well as elucidation of the effects of ligand conformation on melanoma cell signaling. It has been noted previously that collagen structure can be an important factor in signal transduction. Differences in the upregulation of protein

synthesis have been shown based on fibroblast binding to a triple-helical substrate (collagen fibrils) versus denatured collagen (gelatin) (73). Smooth muscle cell spreading is delayed on fibrillar collagen compared with monomeric collagen (19). On the basis of our results, we propose a mechanism by which collagen structure and signal transduction are coordinated to facilitate the tumor cell invasion process.

As cellular-binding sites for the both the  $\alpha_3\beta_1$  (24, 26) and  $\alpha_2\beta_1$  (50, 53, 74) integrins exist within type IV collagen, these ligands can be used to determine if  $\alpha_2\beta_1$  and  $\alpha_3\beta_1$  integrin binding sites act in concert, or independently, with regard to signaling pathways. Complex regulation of signaling has been observed for  $\alpha_5\beta_1$  and  $\alpha_4\beta_1$  integrin binding to fibronectin and metalloproteinase gene expression (75); whether a similar circumstance exists for integrin binding to type IV collagen is important for fundamental understanding of the mechanisms of tumor cell invasion.

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<sup>3</sup> Prior researchers have shown that disulfide-cross-linked Gly-Cys-Hyp-(Gly-Pro-Hyp)<sub>10</sub>-Gly-Cys-Hyp-Gly can induce Tyr phosphorylation of several platelet intracellular proteins, one of which may be p125<sup>FAK</sup> (76, 77). No specific cell receptor was correlated to this activity, but it was independent of the  $\alpha_2\beta_1$  integrin (77). Aggregates of triple helices can cause platelet activation (78). Our circumstance is clearly different, however, as p125<sup>FAK</sup> phosphorylation was induced by a nontriple-helical sequence, with triple helicity enhancing the level and altering the time of induction.

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