Integrin $\alpha_v\beta_3$ Mediates Chemotactic and Haptotactic Motility in Human Melanoma Cells through Different Signaling Pathways*

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Active locomotion of tumor cells stimulated by cytokines and ECM* proteins contributes to invasion and metastasis of malignant neoplasms (1–3). Migration induced by ECM proteins is further defined as chemotaxis (CTX) when the ligand is soluble and haptotaxis (HTX) when substratum-bound. Tumor cells traversing blood vessels and tissue stroma interact with intact, substratum-bound as well as soluble, partially degraded ECM resulting in directional cell spreading and requires $\alpha_v\beta_3$-mediated tyrosine phosphorylation of paxillin.

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1 The abbreviations used are: ECM, extracellular matrix; VN, vitronectin; CTX, chemotaxis; HTX, haptotaxis; PT, pertussis toxin; FBS, fetal bovine serum; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; Ab, antibody; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride.

Distinctions between chemotaxis and haptotaxis of cells to extracellular matrix proteins have not been defined in terms of mechanisms or signaling pathways. Migration of A2058 human melanoma cells to soluble (chemotaxis) and substratum-bound (haptotaxis) vitronectin, mediated by $\alpha_v\beta_3$, was inhibited by a blocking antibody to $\alpha_v\beta_3$ (LM609), whereas haptotaxis was inhibited only by approximately 50% suggesting involvement of multiple receptors and/or signaling pathways. However, blocking antibodies to $\alpha_v\beta_3$ also present on A2058 cells, did not inhibit. Pertussis toxin treatment of cells inhibited chemotaxis by $>80\%$, but did not inhibit haptotaxis. Adhesion and spreading over vitronectin induced the phosphorylation of paxillin on tyrosine. In cells migrating over substratum-bound vitronectin, tyrosine phosphorylation of paxillin increased 5-fold between 45 min and 5 h. Dilutions of anti-$\alpha_v\beta_3$ that inhibited haptotaxis also inhibited phosphorylation of paxillin (by $50\%$) and modestly reduced cell spreading. In contrast, soluble vitronectin ($50–100 \mu g$/ml) did not induce tyrosine phosphorylation of paxillin. The data suggest that soluble vitronectin stimulates chemotaxis predominantly through a G protein-mediated pathway that is functionally linked to $\alpha_v\beta_3$. Haptotaxis is analogous to directional cell spreading and requires $\alpha_v\beta_3$-mediated tyrosine phosphorylation of paxillin.

Reagents and Antibodies—Monoclonal antibodies to integrins $\alpha_v\beta_3$ (clone LM609, IgG1; Ref. 18), $\alpha_v\beta_5$ (clone P1F6), and the $\beta_3$ subunit (clone J B1a) were from Chemicon International Inc. (Temecula, CA). Vitronectin was from Collaborative Biomedical Products/Becton Dickinson (Bedford, MA). Pertussis toxin was from List Biological Laboratories (Campbell, CA). Peptides GRGDS and GRGES were synthesized using a peptide synthesizer (model 9600; Biosearch, San Rafael, CA). [35S]-Methionine (1175 Ci/mmol) was from DuPont NEN. Affinity-purified goat anti-mouse IgG chromatography gel was from Cappel (West Chester, PA). Triton X-100 was from Boehringer Mannheim. Aprotinin, leupeptin, and AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; phenylmethylsulfonfyl fluoride substitute) were from ICN Biomedicals (Aurora, OH). Sodium orthovanadate was from Sigma. Precast gels for SDS-PAGE were from Novex (San Diego, CA). Monoclonal antibodies to vitronectin and phosphotyrosine (PY20) were from Transduction Laboratories (Lexington, KY). Horseradish peroxidase-conjugated goat anti-mouse IgG was from Pierce.

Cell Culture—The human melanoma cell line A2058 was previously established from a brain metastasis in a 43-year-old patient (24), and maintained in culture in DMEM supplemented with 10% FBS. Cells were used for the following studies at passages 13–21. Medium, serum, and trypsin/EDTA were from Biofluids, Inc. (Gaithersburg, MD). Cell Matity Assays—Cells in logarithmic phase of growth were detached by brief exposure to 0.05% trypsin, 0.02% EDTA and allowed to recover 1 h in DMEM, 10% FBS. Cells were then centrifuged at 800 $\times$ g for 5 min and resuspended at $2 \times 10^6$/ml in serum-free DMEM containing 0.1% BSA (DMEM/BSA). Where indicated, cells were pretreated at room temperature with PT, antibodies, or peptides after resuspension in DMEM/BSA. Pretreatment was 2 h for PT, 45 min for
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antibodies, and 30 min for peptides.

Chemotaxis—CTX was assayed in triplicate as described previously using 48-well microchemotaxis chambers with 8-μm pore polycarbonate Nucleopore filters (3, 25). VN was diluted to the indicated concentrations into DMEM/BSA and added to the lower wells. Chambers were incubated at 37 °C for 4 h, after which filters were removed, fixed and stained with Diff-Quick (Baxter Scientific, McGaw Park, IL), and then mounted on glass slides. After removal of non-migrated cells, cells that had migrated were quantitated with a 2202 Ultratraso laser densitometer (LKB Instruments, Gaithersburg, MD), as described previously (2, 25). Alternatively, where indicated, migrated cells were quantitated by light microscopy under high power field (magnification, ×500) (3). Untreated cells, as well as cells from every treatment group, were tested in each experiment for their unstimulated random motility to DMEM/BSA.

Results are expressed as stimulated motility, which represents the total motility response minus unstimulated random motility.

Haptotaxis—HTX was assayed in triplicate using individual modified Boyden chambers, with 8-μm pore polycarbonate Nucleopore filters (13-mm diameter) as described previously (3). Filters were precoted on one side with VN at 10 μg/ml, diluted in DPBS. Filters were then washed and placed in the chamber with the protein-coated side facing the lower compartment, which contained DMEM/BSA. After addition of cells, chambers were incubated at 37 °C for 5 h. Filters were processed and quantitated as for CTX. For HTX, random motility (uncoated filters) did not exceed 2–4 cells/high power field.

In one set of experiments (Fig. 7), cells were set up in four large single-well chambers (volume of lower wells is 12 ml, volume of upper wells is 9 ml, and they accommodate 25 × 80-mm filters). Triton X-100 lysates of migrating cells were collected at various time points for SDS-PAGE and immunoblotting. At 45 min, when cells had just attached to the upper filter surface, one chamber was disassembled, and all cells on the filter were collected in lysis buffer (see below). At all subsequent time points (90 min, 3 h, and 5 h), cells on the upper filter surface were first removed, allowing the separation of only the migrating cells for lysis.

Metabolic Labeling and Immunoprecipitation of Integrin αvβ3—A2058 cells were cultured in 10-cm dishes until subconfluent, then labeled for 1 h with 0.2 μCi/ml [35S]methionine. After labeling, cells were incubated overnight in DMEM, 10% FBS. Cells were then lysed at 4 °C in 25 mM Tris-HCl, pH 7.4, containing 0.5% Nonidet P-40, 4 mM AEBSF, 150 units/ml aprotinin, 1 mM EDTA, and 10 μg/ml leupeptin. Integrin αvβ3 was immunoprecipitated from the 15,000 × g supernatant as follows: lysate was precleared with mouse IgG1 (isotype control Ab, Chemicon International Inc.), followed by anti-mouse IgG agarose beads, then incubated with anti-integrin αvβ3 (or anti-αvβ3) at 1:10 dilution overnight, with end-over-end rocking. Anti-mouse IgG agarose beads were added for an additional 1 h, after which precipitate was washed extensively and eluted by boiling in SDS-containing sample buffer. Antibody incubations and washes were done at 4 °C. Samples were resolved by SDS-PAGE on a 7% polyacrylamide gel, which was processed for autoradiography. Approximately 12,000 cpm/ lane were loaded.

Preparation of Cell Lysates after Adhesion to ECM Proteins—35-mm tissue culture dishes (Costar, Cambridge, MA) were coated overnight at 4 °C with VN (10 μg/ml) or gelatin (100 μg/ml), diluted in DPBS. Before addition of cells, dishes were rinsed three times with DPBS and preincubated for 15 min with DMEM/BSA at 37 °C. Subconfluent cells were detached with trypsin-EDTA as for motility assays and resuspended in DMEM/BSA before plating. Approximately 1.5 × 105 cells/dish were plated. In some experiments, an equal number of cells was left in culture to determine CTX and HTX to VN (data not shown).

Soluble VN Stimulates Chemotaxis and Haptotaxis of A2058 Cells—Soluble VN stimulated a concentration-dependent motility response of A2058 human melanoma cells, which peaked at 50–100 μg/ml VN, reaching a value ~3.5–4 fold above unstimulated motility (Fig. 1). Typically the response remained near peak values at higher concentrations of VN. All subsequent CTX assays were done at 50 μg/ml to conserve VN, unless otherwise indicated.

We also observed a strong haptotactic response of A2058 cells to substratum-bound VN, maximal at a coating concentration of ~10 μg/ml (data not shown).

The adhesion of αv-containing integrins to VN is mediated by the RGD sequence at the amino terminus of VN (8, 27). To assess the importance of receptor binding to this sequence in VN-mediated motility, cells were pretreated with a pentapeptide containing the RGD sequence (GRGDSS) at 500 μM prior to CTX and HTX assays. The RGD-containing peptide abolished both CTX and HTX to VN (data not shown), indicating that integrin binding to the RGD site is an absolute requirement for both types of motility. Pretreatment with the control peptide GRGES at the same concentration resulted in 20–40% inhibition.

Pertussis toxin ADP-ribosylates the α subunit of certain classes of heterotrimeric GTP-binding proteins. This results in the functional uncoupling of G proteins from their receptors, blocking signal transduction (28). Since CTX of A2058 cells to laminin and type IV collagen was inhibited by treatment of cells with PT (3), we tested the effects of PT on CTX and HTX to VN (Fig. 2). Cells were treated with PT at the indicated concentrations, then tested for motility to soluble VN (50 μg/ml) (A) and substratum-bound VN (B). As illustrated, PT treatment results in a concentration-dependent inhibition of CTX, with maximal inhibition (~100%) at 0.5 μg/ml (Fig. 2A). This concentration of PT also completely inhibited CTX to higher

![Picture](https://example.com/f1.png)

**FIG. 1.** Soluble VN stimulated CTX of A2058 human melanoma cells, which peaked at ~100 μg/ml VN to a value ~4 fold above background migration (subtracted out in all figures). Graph illustrates one experiment, representative of four.

**RESULTS**

- VN Stimulates Chemotaxis and Haptotaxis of A2058 Cells—Soluble VN stimulates a concentration-dependent motility response of A2058 human melanoma cells, which peaked at 50–100 μg/ml VN, reaching a value ~3.5–4 fold above unstimulated motility (Fig. 1). Typically the response remained near peak values at higher concentrations of VN. All subsequent CTX assays were done at 50 μg/ml to conserve VN, unless otherwise indicated.

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Concentrations of VN (100–400 \( \mu \text{g/ml} \); data not shown). However, the same batch of cells migrated at or near control levels over substrate-bound VN at all PT concentrations (Fig. 2B). These results implicate a PT-sensitive G protein in transduction of the chemotactic signal through \( \alpha_3 \beta_3 \), whereas this signal transduction pathway does not regulate VN-mediated HTX. PT at 0.5 \( \mu \text{g/ml} \) did not inhibit adhesion or spreading of melanoma cells on VN- or gelatin-coated dishes (data not shown). Treatment with this concentration of PT is sufficient to completely ribosylate G proteins in these cells (data not shown).

Identification of VN Receptors on A2058 Cells—We next sought to identify and quantitate VN receptors on the surface of A2058 cells which may mediate the motility responses. Flow cytometry was performed on intact cells immunostained with anti-\( \alpha_3 \beta_3 \) and anti-\( \alpha_3 \beta_5 \), using a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) (data not shown). The VN receptor \( \alpha_3 \beta_3 \) stained with approximately 4-fold the staining intensity of \( \alpha_3 \beta_5 \), which indicates \(~4\) times the level of surface expression. Flow cytometry on A2058 cells has also revealed the presence of the \( \beta_5 \) subunit (data not shown), which can combine with the \( \alpha_3 \) chain to form \( \alpha_3 \beta_5 \), another VN receptor (6). However, it is not known if \( \alpha_3 \beta_1 \) exists as a heterodimer on A2058 cells.

The VN receptor \( \alpha_3 \beta_3 \) was also identified by immunoprecipitation from \(^{35}\text{S}\text{methionine-labeled lysates with monoclonal Ab LM609 (Fig. 3). SDS-PAGE under nonreducing conditions followed by autoradiography revealed bands of approximately 160 kDa (\( \alpha_3 \) chain) and 95 kDa (\( \beta_3 \) chain) in the immunoprecipitate (lane 4). Under reducing conditions (lane 2), the \( \alpha_3 \) chain migrates at \~140 kDa and the \( \beta_3 \) chain at \~110 kDa. Integrin \( \alpha_3 \beta_3 \) was not detectable by immunoprecipitation under the same conditions using anti-\( \alpha_3 \beta_3 \) (lanes 1 and 3).

Immunofluorescent staining revealed the differential distribution of \( \alpha_3 \beta_3 \) and \( \alpha_3 \beta_5 \) on attached and spread A2058 cells (data not shown). \( \alpha_3 \beta_3 \) is localized to focal adhesions, which were predominant along the cell margins, whereas \( \alpha_3 \beta_5 \) had a more diffuse, nonfocal contact distribution throughout the cell, similar to previous observations in other human tumor cell lines (19).

CTX and HTX to VN Are Mediated by \( \alpha_3 \beta_3 \)—Results after treatment with a variety of blocking antibodies indicate that \( \alpha_3 \beta_3 \) is the primary receptor mediating CTX of A2058 cells to VN. The effects of anti-\( \alpha_3 \beta_3 \) (LM609) on CTX and HTX to VN is illustrated in Fig. 4. Anti-\( \alpha_3 \beta_3 \) inhibited CTX to VN in a concentration-dependent manner (Fig. 4A), with nearly 100% inhibition of stimulated motility at 1:10,000 dilution of Ab. In the experiment illustrated, a gelatin-coated filter was used in the CTX assay; gelatin is also an adhesive ligand for \( \alpha_3 \beta_3 \). However, stimulated CTX to VN was also inhibited by this Ab using filters coated with type IV collagen, which does not bind \( \alpha_3 \beta_3 \) (data not shown). Isotype control Ab at a wide range of dilutions had no inhibitory effect on CTX (data not shown).

Whereas anti-\( \alpha_3 \beta_3 \) treatment of A2058 cells near abolished CTX to VN, the same dilutions of Ab did not inhibit CTX to type IV collagen or laminin (data not shown), confirming the involvement of distinct receptors in CTX to these matrix components.

A2058 cells also express \( \alpha_3 \beta_5 \) on their surfaces, although at lower levels. This integrin mediates migration of keratinocytes (29) and pancreatic carcinoma cells (30) over VN-coated substrata. As expected, however, treatment of A2058 cells with blocking Abs to \( \alpha_3 \beta_3 \) (P1F6) or to the \( \beta_3 \) subunit (B 1a) did not inhibit stimulated CTX to VN (at 1:200–1:10,000) (data not shown). As with CTX, HTX to VN was inhibited by treatment of cells with anti-\( \alpha_3 \beta_3 \) (Fig. 4B), and this inhibition was maximal at an antibody dilution of 1:10,000 (data not shown). Unlike CTX, inhibition of HTX by anti-\( \alpha_3 \beta_3 \) ranged from only 40% to 60% in several experiments. To test the possibility that \( \alpha_3 \beta_3 \) and/or a \( \beta_3 \) integrin contributed to HTX on VN, cells were treated (at

**Fig. 2.** Pertussis toxin treatment results in concentration-dependent inhibition of CTX, but not HTX, to VN. Cells were pre-treated with PT at the indicated concentrations, then assayed for motility to soluble (50 \( \mu \text{g/ml} \)) (A) or substratum-bound (B) VN. Graph illustrates one experiment, representative of three.

**Fig. 3.** Immunoprecipitation of \( \alpha_3 \beta_3 \) from A2058 cells. Cells were pulse-labeled with \(^{35}\text{S}\text{methionine, after which Nonidet P-40-extracted lysates were immunoprecipitated with anti-\( \alpha_3 \beta_3 \) (lanes 2 and 4). Under nonreducing conditions (lane 4), a heterodimer of \~160 kDa (\( \alpha_3 \) chain) and \~95 kDa (\( \beta_3 \) chain) was detected. Under reducing conditions (lane 2), the \( \alpha_3 \) chain migrated at \~140 kDa and the \( \beta_3 \) chain at \~110 kDa. Integrin \( \alpha_3 \beta_3 \) was not detectable by immunoprecipitation under the same conditions using anti-\( \alpha_3 \beta_3 \) (lanes 1 and 3).
$\alpha_3\beta_3$ is the primary CTX receptor and is a major receptor for HTX to VN. A, anti-$\alpha_3\beta_3$ inhibits CTX to VN in a concentration-dependent manner. Cells were treated with anti-$\alpha_3\beta_3$ (LM 609) at the indicated dilutions, then assayed for CTX to 50 $\mu$g/ml VN in the continued presence of antibody. Maximal inhibition of stimulated motility (~90%) was observed at 1:10,000. Graph illustrates one experiment, representative of three. B, anti-$\alpha_3\beta_3$ inhibits HTX. Cells were treated with antibodies to $\alpha_3\beta_3$, $\alpha_3\beta_1$, or the $\beta_1$ subunit at 1:1000. One aliquot of cells was given a combination of anti-$\alpha_3\beta_3$, plus anti-$\beta_1$, each at 1:1000. Cells were then assayed for motility to substrate-bound VN in the continued presence of antibody. Anti-$\alpha_3\beta_3$ inhibited HTX by ~49%, relative to untreated cells. Graph illustrates one experiment, representative of four.

Fig. 4. $\alpha_3\beta_3$ is the primary CTX receptor and is a major receptor for HTX to VN. A, anti-$\alpha_3\beta_3$ inhibits CTX to VN in a concentration-dependent manner. Cells were treated with anti-$\alpha_3\beta_3$ (LM 609) at the indicated dilutions, then assayed for CTX to 50 $\mu$g/ml VN in the continued presence of antibody. Maximal inhibition of stimulated motility (~90%) was observed at 1:10,000. Graph illustrates one experiment, representative of three. B, anti-$\alpha_3\beta_3$ inhibits HTX. Cells were treated with antibodies to $\alpha_3\beta_3$, $\alpha_3\beta_1$, or the $\beta_1$ subunit at 1:1000. One aliquot of cells was given a combination of anti-$\alpha_3\beta_3$, plus anti-$\beta_1$, each at 1:1000. Cells were then assayed for motility to substrate-bound VN in the continued presence of antibody. Anti-$\alpha_3\beta_3$ inhibited HTX by ~49%, relative to untreated cells. Graph illustrates one experiment, representative of four.

1:1000 with the corresponding blocking Abs and assayed for migration (Fig. 4B); one aliquot of cells was treated with a combination of Abs to $\alpha_3\beta_3$ and $\beta_1$, each at 1:1000, to determine if additive inhibition would be observed. However, only anti-$\alpha_3\beta_3$ significantly inhibited HTX to VN (49%). Cells treated with anti-$\alpha_3\beta_3$, plus anti-$\beta_1$, were not inhibited to any additional degree relative to those treated with anti-$\alpha_3\beta_3$ alone. A range of dilutions of anti-$\alpha_3\beta_3$ and isotypic control antibody had no inhibitory effect on HTX (data not shown). Therefore, although anti-$\alpha_3\beta_3$ did not inhibit HTX as completely as it inhibited CTX, $\alpha_3\beta_3$ still appears to be the major motility-promoting receptor in HTX; the other VN receptors $\alpha_3\beta_3$ and $\alpha_3\beta_1$ do not play a direct role.

Adhesion and Spreading over VN Induce Tyrosine Phosphorylation of Paxillin—We then wanted to determine if soluble and/or substrate-bound VN induced tyrosine phosphorylation of proteins in A2058 cells, at the concentrations that induced motility. In Fig. 5A, cells were plated onto dishes coated with VN (10 $\mu$g/ml) (lane 1) or gelatin (100 $\mu$g/ml) (lane 2) and allowed to adhere for 90 min before lysates were prepared for electrophoresis and immunoblotting. Another aliquot of cells was plated on tissue culture plastic for 4 h before lysis (lane 3). The anti-phosphotyrosine immunoblot (10 $\mu$g/lane) shows that relatively few proteins were phosphorylated on tyrosine under these conditions, including a cluster of ~110–130 kDa in each lane, and a broad band of ~68 kDa in lysate of VN-adherent cells (Fig. 5A, lane 1). One of the bands in the 110–130-kDa cluster was subsequently identified as p125FAK by immunoprecipitation with anti-FAK antibody followed by immunoblotting with anti-phosphotyrosine or anti-FAK (data not shown).

Fig. 5A illustrates that adhesion is sufficient to induce tyrosine phosphorylation of the 110–130-kDa cluster, but the 68-kDa band is detectably phosphorylated on tyrosine only in VN-adherent cells (lane 1). We suspected the 68-kDa band to be paxillin, based on its molecular mass and phosphorylation on tyrosine upon adhesion (31). Paxillin is phosphorylated on tyrosine in response to a variety of signals, all involving cytoskeletal remodeling (32). From the lysate of VN-adherent cells (Fig. 5A, lane 1), we immunoprecipitated paxillin with monoclonal Ab to paxillin. In Fig. 5B, the immunoprecipitate was divided into two portions for Western immunoblotting with anti-phosphotyrosine (lanes 1–3) and anti-paxillin (lanes 4–6). In lane 1, the anti-paxillin immunoprecipitate stains prominently at 68 kDa (arrow) with anti-phosphotyrosine. In lane 2, non-immunoprecipitated lysate was run alongside for comparison. Lane 4 is immunoprecipitated paxillin, which stains with anti-paxillin; this band co-migrates with a band from the nonfractionated lysate (lane 5). (Lanes 3 and 6 are immunoprecipitates of this lysate using isotype control mAb.) These results show that...
A2058 cells do contain paxillin, and that paxillin is a protein phosphorylated as cells migrate over VN. We then wanted to determine if tyrosine phosphorylation of paxillin increased over time in VN-adherent cells, coincident with cell spreading (Fig. 6). To determine the time course of phosphorylation, cells were plated on VN-coated dishes; at the indicated times after plating, lysates were collected as described under "Materials and Methods." A, 10 μg/lane of each lysate was immunoblotted with PY20 (68-kDa region illustrated). B, duplicate immunoblot was stained with anti-paxillin. Lanes 1 and 2, cells in suspension for 90 min at room temperature (lane 1) and 37 °C (lane 2); lanes 3–7, cells adherent to VN for 10 min (lane 3), 20 min (lane 4), 45 min (lane 5), 90 min (lane 6), and 4 h (lane 7). C, table showing the integrated densities of phosphotyrosine bands (panel A), normalized as described under "Results." Lanes are numbered as in panels A and B. This experiment was repeated three times, with similar results. Lower panels, cells were fully spread on VN-coated dishes within 90 min. At 10 min (A), 20 min (B), 45 min (C), and 90 min (D) after plating, adherent cells were processed and photographed with a Zeiss Axiohot microscope under phase contrast. Magnification, ×100.
lin would continuously increase in cells migrating over substratum-bound VN during the course of a 5-h assay, since cells would be in a constant dynamic state. Lysates of migrating cells were collected at various time points, as described under "Materials and Methods." 11 µg/lane of lysate was immunoblotted with anti-phosphotyrosine. Integrated densities of phosphotyrosine bands, normalized as described under "Results," are as follows: lane 1, 0.35; lane 2, 0.79; lane 3, 1.22; lane 4, 1.43. This experiment was repeated twice, with similar results.

In contrast, soluble VN at concentrations that stimulate CTX (50–100 µg/ml) did not induce detectable tyrosine phosphorylation of paxillin in gelatin-adherent cells (data not shown), when examined over a time course of 5 min to 5 h. We also did not detect tyrosine phosphorylation of paxillin in cells migrating to gradients of soluble VN, in experiments using single-well chambers as in Fig. 7 (data not shown).

Cell Spreading and Tyrosine Phosphorylation of Paxillin Are Partly Mediated by αβ3—Since HTX to VN was reduced ~50% by treatment of cells with anti-αβ3, we wanted to determine whether this Ab would have corresponding effects on cell spreading and tyrosine phosphorylation (Fig. 8). Untreated cells (lane 1) and cells treated with (1:1000) anti-αβ3 (lane 2) or anti-αβ2 (lane 3) were plated on VN-coated dishes, and lysates were collected after 90 min for immunoblotting with anti-phosphotyrosine (A) and anti-paxillin (B). In C, bands were quantitated by densitometry and normalized as for Fig. 6. In Fig. 8 (A and C), comparison of lane 2 with lane 1 shows that anti-αβ3 reduced tyrosine phosphorylation of paxillin (arrow) by ~2-fold, relative to untreated cells. The 68-kDa band in lane 3 of panel A (anti-αβ3) also appears to be reduced relative to control cells; however, this could be due to underloading, since normalization of the phosphotyrosine band as described above gives a reading of 0.64, which is close to control levels. Photicoptographs A–D on right show morphology of cells that were pretreated with the indicated Abs (at 1:1000) and fixed after 90 min of adhesion to VN: A, untreated cells; B, mouse IgG2; C, anti-αβ3; D, anti-αβ2. Note the modest reduction in degree of cell spreading in anti-αβ3 treated cells (C) relative to cells in panels A, B, and D. Collectively, the data suggest that tyrosine phosphorylation of paxillin is required for cell spreading and haptotactic migration on VN, and that these events are at least partly mediated by αβ3.

Our data demonstrate that A2058 cells have a high ratio of αβ3 to αβ2 integrins on their surfaces and migrate rapidly toward concentration gradients of soluble and substratum-bound VN. While both CTX and HTX are mediated primarily by interaction of αβ3 with the RGD sequence in VN, there are differences in signal transduction mechanisms and phosphorylation patterns elicited by soluble versus substratum-bound VN, which may differentially affect the motility machinery. Specifically, CTX to VN is inhibited 80–100% by pertussis toxin, implicating a heterotrimeric Gα-like protein in signal transduction. In contrast, transduction of signals for HTX is largely independent of such a G protein. Blocking antibodies to αβ3 completely inhibited VN-stimulated CTX, while HTX is inhibited by only ~50%. Finally, the focal adhesion protein paxillin becomes tyrosine phosphorylated upon melanoma cell spreading and migration over substratum-bound VN in an αβ3-dependent manner, whereas soluble VN (at concentrations stimulating CTX) does not induce detectable tyrosine phosphorylation of paxillin. These differences may reflect quantitative and qualitative differences in the signals that regulate the motility apparatus.

Studies of agonist-stimulated eukaryotic cell migration have not revealed a unifying intracellular signaling pathway, despite overall similarities in crawling mechanisms (33). Even within the same cell type, different attractants can induce motility through distinct signaling pathways (2, 17, 25, 34). Previous studies with A2058 cells distinguished CTX from HTX by signal transduction through a PT-sensitive G protein (3). The receptor(s) involved were not identified; however, these studies showed that the same ECM protein could stimulate motility through distinct pathways when soluble versus substratum-bound. There has remained uncertainty as to whether CTX and HTX are truly distinguishable; other studies have not defined differences between them (23). Study of VN-mediated migration of A2058 cells allowed further investigation of the following questions. What additional signals distinguish CTX from HTX? How could the soluble and substratum-bound forms of an ECM protein stimulate different signaling pathways in the same cell? Would this result in distinct mechanisms of migration?

ECM proteins induce intracellular signals in large part through integrin receptors (4, 7). In this report, identification of integrin αβ3 as the motility-promoting receptor (particularly for CTX) through the use of blocking antibodies allowed additional insights into these questions. Ligand binding to αβ3 in other systems induces elevation of intracellular Ca2+ (17), protein tyrosine phosphorylation (35–37), and collagenase secretion (15). Many integrin-generated signals require clustering of integrins by adhesion to ECM, or with anti-integrin antibodies (38–40), as opposed to simple occupancy by monovalent, soluble ligand. However, Miyamoto et al. (39) identified distinct and increasing effects of integrin occupancy, aggregation, and both combined. This illustrates the diverse and graded nature of integrin-generated signals, and the importance of the form in which ligand is presented. Extrapolating to our studies, soluble and substratum-bound VN may cluster and activate αβ3 to different degrees, generating distinct signals. Differential signaling could also be due to different fates of the ligand-receptor complexes. Integrins cross-linked by soluble antibodies are rapidly internalized, leading to termination of signals (38, 40–42). If soluble VN is internalized more rapidly than substratum-bound VN (43, 44), chemotactic signals may not be as strong or as sustained as those for HTX.

Although HTX to VN is mediated by αβ3, additional receptors of varying affinities and specificities may also contribute,
resulting in multiple motility signals that could act synergistically. CTX, in which the soluble attractant can diffuse in three dimensions, would favor only the highest affinity ligand-receptor interaction, and therefore one predominant signaling pathway would be expected. HTX, in which the attractant is immobilized in two dimensions, would be more permissive for multiple low affinity interactions of VN with the cell surface. This would explain the inability of one agent to completely inhibit HTX. HTX was not inhibited by blocking Abs to \( \alpha_\nu\beta_3 \) or to the \( \beta_3 \) subunit, or with exogenous heparin (data not shown). This indicates that binding of these integrins and cell surface proteoglycans to VN does not directly induce HTX; however, it does not rule out secondary, modulatory roles. An example of cooperativity between integrins was reported (20) in which the “collaborating” integrin did not bind to the substrate.

PT sensitivity has been noted for a number of chemotactic signaling pathways, notably in neutrophils (45) and melanoma cells, mentioned above. Heterotrimeric G proteins transduce chemotactic signals in neutrophils in part by activation of phosphatidylinositol metabolism (9, 45–48). The products of these pathways are then thought to mediate actin polymerization, cross-linking, and pseudopod protrusion (33, 46, 49, 50). The mechanism by which integrin \( \alpha_\nu\beta_3 \) couples to a G protein in VN-mediated CTX is not known. However, several reports describe systems in which heterotrimeric G proteins are functionally linked to “non-classic” receptors including cell adhesion molecules (51, 52), epidermal growth factor receptor (53, 54), and others (55–58). Additional studies will address the identity of the \( \alpha_\nu\beta_3 \)-like protein mediating CTX to VN, the nature of its coupling to \( \alpha_\nu\beta_3 \), and downstream effectors.

Tyrosine phosphorylation of paxillin, a 68-kDa focal adhesion protein (32), accompanies cell spreading and HTX on VN. Upon \( \alpha_\nu\beta_3 \) treatment, tyrosine phosphorylation of paxillin and cell spreading are both reduced by \( \sim 50\% \) observed after 90 min on VN. The corresponding inhibition of HTX is observed at a later time (5 h), indicating that paxillin phosphorylation and

**Fig. 8.** Anti-\( \alpha_\nu\beta_3 \) treatment of A2058 cells results in reduced tyrosine phosphorylation of paxillin (arrow in panel A) and modestly reduces cell spreading. Cells were treated with anti-\( \alpha_\nu\beta_3 \) or anti-\( \alpha_\nu\beta_5 \) (1:1000), then plated onto VN-coated dishes, and allowed to adhere for 90 min. Lysates were prepared as described, and 10 \( \mu \)g/lane of lysate was immunoblotted with anti-phosphotyrosine (A) or duplicate blot probed with anti-paxillin (B). Lane 1, untreated cells; lane 2, anti-\( \alpha_\nu\beta_3 \); lane 3, anti-\( \alpha_\nu\beta_5 \). C, table showing integrated densities of the 68-kDa phosphotyrosine bands (panel A), normalized as described under “Results.” This experiment was repeated twice, with similar results. Photomicrographs on right: cells were left untreated (A) or were treated with (1:1000) mouse IgG1 (B), anti-\( \alpha_\nu\beta_3 \) (C), or anti-\( \alpha_\nu\beta_5 \) (D); after adhesion to VN for 90 min, adherent cells were processed and photographed as in Fig. 6. Magnification, \( \times \) 100.
migration predominates in CTX.

Focal adhesions (10, 32, 60) make paxillin a likely candidate for a key regulator of cell spreading and HTX.

In conclusion, the data suggest that different mechanisms of migration predominate in CTX versus HTX. HTX involves an initial interaction of $\alpha_\beta_3$ with the RGD sequence of substratum-bound VN, clustering of $\alpha_\beta_3$, and phosphorylation of paxillin. Additional signals generated by $\alpha_\beta_3$ ligation (and perhaps other receptors) promote actin polymerization and linkage of filament bundles with integrins (65). The gradient of VN favors formation of adhesive contacts and polymerized actin at the "leading edge" of cells. HTX could therefore be described as continuous, directional spreading, with the VN gradient maintaining directional cues. Detachment of the trailing edge to allow forward locomotion may require a Ca$^{2+}$-activated phosphatase (34). In CTX, $\alpha_\beta_3$ adheres to gelatin-coated filters for traction, but the gradient of soluble VN provides a directional motility cue. The predominant signaling pathway is G protein-mediated; this may involve localized phosphatidylinositol metabolism and cycles of pseudopod protrusion and cell translocation in a manner analogous to that described for neutrophils and Dictyostelium (9, 33). Apparently the tyrosine phosphorylation of paxillin is not involved in CTX. Our preparation of VN is purified using urea and, therefore, is multimeric (8, 66, 67). Soluble, multimeric VN at high concentrations (400–600 mg/ml) clusters integrin $\alpha_\beta_3$ and stimulates tyrosine phosphorylation in endothelial cells (36). In our system, however, soluble VN at optimal concentrations for CTX did not stimulate detectable tyrosine phosphorylation. The versatility of integrins as signaling receptors has implications for a wide range of biological processes, including invasive tumor cell migration.

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