

# Requirement of the NPXY Motif in the Integrin $\beta 3$ Subunit Cytoplasmic Tail for Melanoma Cell Migration In Vitro and In Vivo

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**Abstract.** The NPXY sequence is highly conserved among integrin  $\beta$  subunit cytoplasmic tails, suggesting that it plays a fundamental role in regulating integrin-mediated function. Evidence is provided that the NPXY structural motif within the  $\beta 3$  subunit, comprising residues 744–747, is essential for cell morphological and migratory responses mediated by integrin  $\alpha v \beta 3$  in vitro and in vivo. Transfection of CS-1 melanoma cells with a cDNA encoding the wild-type integrin  $\beta 3$  subunit, results in de novo  $\alpha v \beta 3$  expression and cell attachment, spreading, and migration on vitronectin. CS-1 cells expressing  $\alpha v \beta 3$  with mutations that disrupt the NPXY sequence interact with soluble vitronectin or an

RGD peptide, yet fail to attach, spread, or migrate on immobilized ligand. The biological consequences of these observations are underscored by the finding that CS-1 cells expressing wild-type  $\alpha v \beta 3$  acquire the capacity to form spontaneous pulmonary metastases in the chick embryo when grown on the chorioallantoic membrane. However, migration-deficient CS-1 cells expressing  $\alpha v \beta 3$  with mutations in the NPXY sequence lose this ability to metastasize. These findings demonstrate that the NPXY motif within the integrin  $\beta 3$  cytoplasmic tail is essential for  $\alpha v \beta 3$ -dependent post-ligand binding events involved in cell migration and the metastatic phenotype of melanoma cells.

CELL adhesion and migration play a central role in diverse biological and pathological processes including embryogenesis, angiogenesis, wound healing, and metastasis. Although the mechanisms that regulate cell adhesion and motility are poorly understood, it is clear that in many instances, these events are initiated by the specific recognition of extracellular matrix proteins by a family of cell surface adhesion receptors known as integrins (Ruoslahti, 1991; Hynes, 1992). Upon ligand recognition, integrins typically cluster at the site of cell–substrate interface; this leads to polymerization of the actin cytoskeleton (Burrige et al., 1988). In this context, integrins form a physical linkage between the extracellular matrix and the actin cytoskeleton. Ultimately, these integrin-initiated adhesive interactions can lead to gross changes in cellular morphology orchestrated by the actin cytoskeleton that facilitate cell migration.

Structurally, integrins are heterodimers composed of noncovalently associated  $\alpha$  and  $\beta$  subunits (Hynes, 1992). Each subunit contains a single transmembrane domain that separates a large ectodomain from its cytoplasmic tail,

which in most cases is shorter than 50 amino acids. Although several studies have identified functionally important regions within the integrin ectodomain (Smith and Cheresh, 1988, 1990; D'Souza et al., 1988, 1990, 1994; Loftus et al., 1990; Takada et al., 1992; Lee et al., 1995) and cytoplasmic domain (Hibbs et al., 1991; O'Toole et al., 1991, 1995; Bauer et al., 1993; Filardo and Cheresh, 1994) that can regulate ligand-binding function, relatively little is known regarding structural motifs within the integrin that regulate morphological changes in cell shape which accompany cellular adhesion and migration.

Several observations indicate that, upon ligand binding, the integrin cytoplasmic tail mediates a physical interaction with cytoskeletal proteins (Otey et al., 1990, 1992; Miyamoto et al., 1995). These interactions are presumed to promote the organization of the actin cytoskeleton leading to changes in cellular structure and morphology associated with integrin receptor clustering at focal contacts. Moreover, several studies have reported that sequences within the  $\beta$  subunit cytoplasmic tails may play a role in the recruitment of integrins to cell–substrate attachment sites (Hayashi et al., 1990; La Flamme et al., 1992; Reszka et al., 1992; Ylänné et al., 1993; Cone et al., 1994). These sequences represent regions of structural homology between the individual integrin  $\beta$  subunit cytoplasmic tails including a membrane-proximal sequence

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that forms an amphipathic helix, and two distal well-conserved sequences, NPXY and NXXY, that comprise tight  $\beta$  turns. While these regions have been linked to the ability of integrins to localize to focal contacts, little is known about these domains in terms of postligand binding events.

Integrin  $\alpha\beta 3$  is highly expressed on various motile cells *in vivo*, including neural crest cells (Delannet et al., 1994), vascular endothelial cells (Brooks et al., 1994a,b), and malignant melanoma cells (Albelda et al., 1991). Therefore, to define structural regions within the  $\beta 3$  cytoplasmic tail that are required for  $\alpha\beta 3$  post-ligand binding function, we utilized a hamster cell line, CS-1, that fails to express endogenous  $\beta 3$  or  $\beta 5$  subunit proteins, and as a consequence does not display any vitronectin receptors on its surface (Thomas et al., 1993). Upon transfection of a cDNA-encoding wild-type  $\beta 3$  subunit protein, these cells express  $\alpha\beta 3$  enabling them to attach, spread, and migrate in response to vitronectin. Moreover, these cells gain the ability to form pulmonary metastases in the chick embryo after being grown on the chorioallantoic membrane (CAM)<sup>1</sup>. In contrast, mutations that disrupt the NPXY sequence (amino acids 744–747) present in the  $\beta 3$  cytoplasmic tail maintain their capacity to bind soluble ligand yet are unable to promote  $\alpha\beta 3$ -dependent cell morphological changes that are prerequisite for cell motility both *in vitro* and during tumor cell metastasis *in vivo*.

## Materials and Methods

### Cell Culture

The CS-1 hamster melanoma cell line was originally described by Knudsen et al., 1982. These cells do not express  $\alpha\beta 3$  or  $\alpha\beta 5$  vitronectin receptors, since they fail to express  $\beta 3$  or  $\beta 5$ , and as a consequence are nonadherent when cultured in the presence of serum (Thomas et al., 1993). CS-1 cells were propagated in RPMI media supplemented with 5% FBS, 2 mM L-glutamine, and 50  $\mu\text{g}/\text{ml}$  gentamicin.

### cDNA Constructs

Mutant and wild-type integrin  $\beta 3$  subunit proteins were generated from a molecular clone, p $\beta 3$ GEM-1, containing a 3.8-kb EcoRI cDNA fragment encoding a full-length human  $\beta 3$  polypeptide (Fitzgerald et al., 1987). To express wild-type  $\beta 3$  polypeptide, the EcoRI ends of the 3.8-kb p $\beta 3$ GEM-1 cDNA fragment were filled in with Klenow enzyme, and blunt-end ligated into the EcoRV site within the polylinker of the pcDNA-1neo vector (Invitrogen, La Jolla, CA). To facilitate the construction of cDNAs encoding mutant  $\beta 3$  cytoplasmic tails, an expression cassette encoding the amino-terminal two thirds of the  $\beta 3$  subunit polypeptide was created within the pcDNA-1neo expression vector. An oligonucleotide containing a HindIII restriction site, d(CCAAGCTTGG), was inserted into the 5' EcoRI site of the p $\beta 3$ GEM-1 cDNA and the 1,500-bp HindIII-BamHI  $\beta 3$  fragment was directionally cloned into pcDNA-1neo to generate pH3Bm1.5 $\beta 3$ neo.

Truncated  $\beta 3$  subunit proteins are named based upon the position of their COOH-terminal amino acid residue with respect to the mature  $\beta 3$  polypeptide and are designated by the symbol  $\Delta$ . To construct cDNAs encoding  $\beta 3.751 \Delta$ - and  $\beta 3.741 \Delta$ -truncated polypeptides, cDNA fragments were amplified from linearized p $\beta 3$ GEM-1 plasmid DNA by PCR using an upstream sense primer corresponding to nucleotides (nts) 2139–2165 and downstream anti-sense primers 5' CTAGTCTAGACTAGGGTGGCCTCTTTATACAGTGGGTTG 3',  $\beta 3.751(\Delta)$  and 5' CTAGTCTAGACTAGGGTGTCCATTTTGGCTCTGGCGCGTTC 3',  $\beta 3.741(\Delta)$  containing nts 2295–2320 and 2327–2351, respectively. In addition to the  $\beta 3$ -derived nucleotides, the downstream primers were designed to introduce

an amber stop codon (underlined) and an XbaI restriction endonuclease recognition site (bold) in order to facilitate the exchange of these PCR fragments into the pcDNA-1neo vector. EcoRI-XbaI-restricted PCR fragments encoding the truncated cytoplasmic tails were then purified by electrophoresis through 8% nondenaturing acrylamide gels, and ligated in a ternary reaction with a 774-bp BamHI-EcoRI fragment encoding the remainder of the ectodomain and the transmembrane anchor nt 1500–2274 and BamHI-XbaI-digested pH3Bm1.5 $\beta 3$ neo.

Alanine or phenylalanine substitution mutants are designated by the position of the amino acid residue at which the substitution has been introduced into the wild-type  $\beta 3$  polypeptide. The first letter following the number indicates the original residue and the second letter following the / denotes the substituted one. To create a mutagenesis cassette, the 1,136-kb BamHI-SacI fragment encoding the carboxyl-terminal one third of the  $\beta 3$  subunit protein, nt 1500–2636, was subcloned into pTZ18U (U.S. Biochem. Corp., Cleveland, OH). The 3' SacI site at nt 2636 in the untranslated region was converted to an XbaI site by the insertion of a synthetic oligonucleotide d(CTAGTCTAGACTAG) (New England Biolabs, Beverly, MA) to create 18U $\beta 31.1X$ . Amino acid substitutions were produced by oligonucleotide mutagenesis of uracylated phagemid DNA prepared from 18U $\beta 31.1X$  as described by Filardo and Cheresch (1994). Mutant cDNA clones were identified by DNA sequence analysis using the dideoxynucleotide chain termination method (Sanger et al., 1977). Mutagenized 1136-bp BamHI-XbaI fragments were isolated and ligated to BamHI-XbaI-digested pH3Bm1.5 $\beta 3$ neo to generate cDNAs encoding full-length  $\beta 3$  polypeptides containing individual point mutations within the cytoplasmic tail.

### Monoclonal Antibodies

mAb LM609 ( $\alpha\beta 3$ ) has been previously described (Cheresch and Spiro, 1987). mAb AP3, specific for the human  $\beta 3$  subunit, was a generous gift of Dr. Peter Newman (Blood Center of Southwestern Wisconsin, Milwaukee, WI). Fluoresceinated ligand induced binding site (LIBS)-1 detects a conformer-specific epitope on  $\beta 3$  integrins (Frelinger et al., 1990) and was kindly provided by Dr. Mark Ginsberg (Scripps Research Institute, La Jolla, CA). mAb 7E2 specific for hamster  $\beta 1$  integrins was graciously provided by Dr. Rudolph Juliano (University of North Carolina, Chapel Hill, NC).

### Transfection and Selection of Stable $\beta 3$ -expressing Cell Lines

$\beta 3$ -deficient CS-1 cells were transfected with pcDNA-1neo constructs encoding cDNAs encoding full-length or mutated versions of the  $\beta 3$  subunit protein using lipofectin (GIBCO BRL, Gaithersburg, MD). 3 d after transfection, 500  $\mu\text{g}/\text{ml}$  of geneticin (Sigma Chemical Co., St. Louis, MO) was added to the growth medium. Transfected cells expressing mutant or wild-type  $\beta 3$  integrins were enriched from the geneticin resistant population by fluorescence activated cell sorting using  $\beta 3$ -specific mAb, AP3. Transfectants were sorted based on their mean intensity fluorescence, where the highest staining (0.5%) cells were gated under sterile conditions, expanded in culture, and then resorted. This procedure was repeated until stable cell lines were obtained that displayed equivalent amounts of wild-type or mutated  $\beta 3$  integrins on their surface. The integrity of each mutation within the  $\beta 3$  cytoplasmic tail was verified by nucleotide sequence determination of PCR products generated from polyadenylated RNA synthesized in each transfected cell line. Throughout the course of these studies, all cell lines were maintained free of mycoplasma.

### Ligands and Cellular Adhesion Assays

Vitronectin was purified from human serum as previously described (Yatohgo et al., 1988). Mouse laminin was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Cellular adhesion assays were performed as previously described (Klemke et al., 1994).

### Spreading on Integrin Specific Antibodies

Glass coverslips (12 mm, No.1 thickness) were coated with 20  $\mu\text{g}/\text{ml}$  goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Birmingham, AL) for 1 h at room temperature. Coverslips were then washed twice in PBS, and blocked with 1% heat denatured BSA for 1 h at 37°C. The wells were then washed with PBS and incubated in 10  $\mu\text{g}/\text{ml}$  of AP3 mAb for 1 h at room temperature, and washed twice in adhesion buffer.  $\beta 3$  transfected cells were seeded at a density of 100,000 cells/ml and incu-

1. Abbreviations used in this paper: CAM, chorioallantoic membrane; LIBS, ligand induced binding site; nt, nucleotide; WT, wild-type.

bated at 37°C in a humidified atmosphere for 2 h. Samples were viewed using a microscope equipped with phase contrast (Nikon Inc., Instr. Group, Melville, NY).

### RGD-induced LIBS-1 Expression

CS-1 transfectants expressing wild-type or mutant  $\beta 3$  integrins were analyzed for RGD-induced LIBS-1 expression as previously reported (Filardo and Cheresch, 1994). GRGDSPK and SPGDRGK peptides were provided by E. Merck (Darmstadt, Germany).

### Cellular Migration Assays

Cell migration assays were performed using modified Boyden chambers containing polycarbonate membranes (tissue culture treated, 6.5- $\mu\text{m}$  diameter, 10- $\mu\text{m}$  thickness, 8- $\mu\text{m}$  pore, Transwell; Costar Corp., Cambridge, MA). Migration toward immobilized adhesive ligand (haptotaxis) was measured as described by Klemke et al. (1994). Migration toward soluble ligand (chemotaxis) was assessed in a similar manner. Except in this case, the undersurface of the membrane and all surfaces of the lower reservoir were preblocked with BSA. Excess BSA was removed and the lower reservoir was filled with 0.5 ml of FBM containing vitronectin or laminin (10  $\mu\text{g}/\text{ml}$ ). Cells that migrated into the lower chamber were enumerated by counting the number of cells on the floor of the lower reservoir per 200 $\times$  field.

### Chick Embryo Metastasis Assay

The chick embryo metastasis assay was performed as previously described with some minor modifications (Brooks et al., 1993). Five million CS-1 melanoma cells expressing wild-type or mutant  $\beta 3$  integrins were deposited on the CAM of 10-d-old chick embryos and incubated at 39°C in a humidified atmosphere. After 1 wk, tumors formed at the primary site were excised, trimmed free of surrounding CAM tissue, and wet weights were determined. Tumor invasion was assessed by determining the percentage of melanoma cells present in a single cell suspension of whole lung tissue by flow cytometry using mAb 7E2, specific for hamster  $\beta 1$  integrins.

## Results

### Residues 742–751 within the $\beta 3$ Cytoplasmic Tail Are Required for $\alpha v\beta 3$ -dependent Cellular Adhesion and Spreading

Comparison of the cytoplasmic tail sequences of the individual integrin  $\beta$  subunits reveals a striking degree of structural homology. A structural motif common to six of the eight  $\beta$  subunit cytoplasmic tails is the presence of two well-conserved predicted tight  $\beta$  turns (see Fig. 1). One of these  $\beta$  turns, defined by the NPXY sequence, has been shown to have an influence on the recruitment of transfected integrins into preformed focal contacts (Reszka et al., 1992; Cone et al., 1994). Therefore, to address the influence of the NPXY sequence with respect to its function in regulating integrin-mediated adhesion, we examined the effect of mutations that eliminate the NPXY structural motif from the cytoplasmic domain of integrin  $\alpha v\beta 3$ .

The adhesive properties of cells expressing  $\alpha v\beta 3$  containing full-length or truncated  $\beta 3$  subunit proteins were characterized by assessing the relative capacities of the  $\beta 3.741$  ( $\Delta$ ),  $\beta 3.751$  ( $\Delta$ ), and  $\beta 3.762$  (WT) cell lines to adhere to polystyrene wells coated with varying concentrations of vitronectin or the control protein, laminin. As shown in Fig. 2 A, nontransfected CS-1 cells, lacking  $\alpha v\beta 3$ , were incapable of attaching to vitronectin, yet attached well to the laminin-coated surface, consistent with their expression of  $\beta 1$  integrins (Thomas et al., 1993). CS-1 cells expressing  $\alpha v\beta 3$  composed of  $\beta 3.762$  (wild-type) or  $\beta 3.751$

$\beta 1$	I I H D R R E F A K F E K E K M N A K W D T G E	<b><i>NPXY</i></b>	K S A V T T V V	<b><i>NPKY</i></b>	E G K
$\beta 2$	H L S D L R E Y R R F E K E K L K S Q W N N D	<b><i>NPLF</i></b>	K S A T T T V M	<b><i>NPKF</i></b>	A E S
$\beta 3$	T I H D R K E F A K F E E E R A R A K W D T A N	<b><i>NPLY</i></b>	K E A T S T F T	<b><i>NITY</i></b>	R G T
$\beta 5$	T I H D R R E F A K F Q S E R S R A R Y E M A S	<b><i>NPLY</i></b>	R K P I S T H T V D F T F N K F	<b><i>NKSY</i></b>	N G T V D
$\beta 6$	S F H D R K E V A K F E A E R S K A K W Q T G T	<b><i>NPLY</i></b>	R G S T S T F K	<b><i>NVTY</i></b>	K H R E K Q K V D L S T D C
$\beta 7$	E I Y D R R E Y S R F E K E Q Q L N W K Q D S	<b><i>NPLY</i></b>	K S A I T T T I	<b><i>NPRF</i></b>	Q E A D S P T L

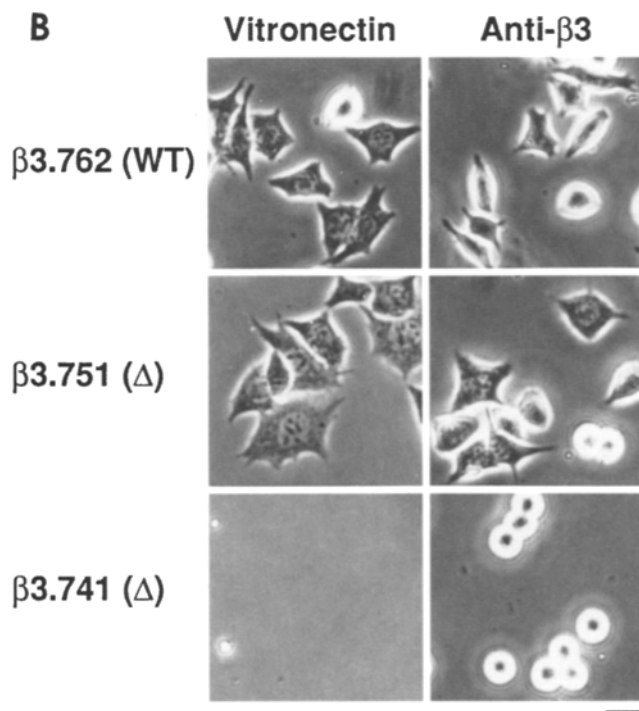
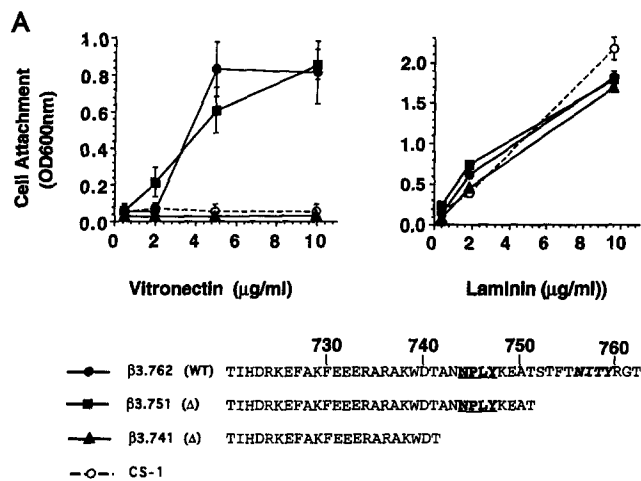
*Figure 1.* Alignment of the deduced amino acid sequences of several integrin  $\beta$  subunit cytoplasmic tails. The deduced amino acid sequences of the  $\beta$  subunit cytoplasmic tails listed above are obtained from the following published data:  $\beta 1$  (Argraves et al., 1987),  $\beta 2$  (Kishimoto et al., 1987),  $\beta 3$  (Fitzgerald et al., 1987),  $\beta 5$  (McLean et al., 1990),  $\beta 6$  (Sheppard et al., 1990), and  $\beta 7$  (Erle et al., 1991). For each  $\beta$  subunit, the first amino acid listed is the residue that follows the transmembrane anchor. Residues comprising the tight  $\beta$  turns encoded by the ***NPXY*** (*underscored*) and ***NXXY*** (*italicized*) sequences are denoted.

( $\Delta$ ) polypeptides adhered and spread well on vitronectin, indicating that elimination of 11 COOH-terminal amino acids, including the NXXY predicted  $\beta$  turn (residues 756–759) had little, if any, effect on  $\alpha v\beta 3$ -mediated cell attachment. Adhesion of either of these  $\beta 3$ -transfected cells to vitronectin was completely inhibited by mAb LM609 demonstrating that  $\alpha v\beta 3$  is responsible for this event (Filardo, E. J., and D. A. Cheresch, unpublished data). In contrast, CS-1 cells displaying  $\alpha v\beta 3$  containing the  $\beta 3.741$  ( $\Delta$ )-truncated subunit protein failed to attach to the vitronectin substrate, while they readily attached to laminin. These results suggest that the sequence between Thr<sup>741</sup> and Ala<sup>750</sup> is required for  $\alpha v\beta 3$ -mediated cell attachment to immobilized vitronectin.

Cellular adhesion to planar surfaces is greatly strengthened by cell spreading (Lotz et al., 1989). Therefore, to establish whether the failure of CS-1 cells expressing  $\beta 3.741$  ( $\Delta$ ) to attach to a vitronectin substrate may be due in part to an inability of these cells to organize their actin cytoskeleton and promote changes in cell shape, cells expressing wild-type or mutant  $\alpha v\beta 3$  were allowed to attach to coverslips coated with mAb AP3, specific for the  $\beta 3$  subunit protein. As shown in Fig. 2 B, all cell lines expressing  $\alpha v\beta 3$  attached to the anti- $\beta 3$  antibody, but only  $\alpha v\beta 3.762$  (WT) and  $\alpha v\beta 3.751$  ( $\Delta$ ) cells were able to spread on this ligand. In contrast,  $\alpha v\beta 3.741$  ( $\Delta$ ) cells attached but remained rounded, indicating their inability to engage the cytoskeleton and promote a shape change. These data suggest that amino acids 742–751 of the  $\beta 3$  subunit play a critical role in regulating  $\alpha v\beta 3$ -dependent cell spreading, and may explain why cells expressing this form of  $\alpha v\beta 3$  fail to maintain an adherent phenotype on vitronectin.

### Residues Asn<sup>744</sup> or Tyr<sup>747</sup> within the NPXY Sequence in the $\beta 3$ Cytoplasmic Tail Are Essential for $\alpha v\beta 3$ -dependent Cellular Shape Changes

Theoretical calculations that predict protein secondary structure (Chou and Fasman, 1978; Rose, 1978) indicate that the NPXY sequence within the integrin  $\beta$  subunit cytoplasmic tails forms a tight turn. To determine whether this structural motif, defined by residues Asn<sup>744</sup>-Tyr<sup>747</sup> of the  $\beta 3$  cytoplasmic tail, is necessary for  $\alpha v\beta 3$ -mediated



**Figure 2.** The effect of  $\beta 3$  truncation on CS-1 cell attachment to vitronectin. (A) CS-1 cells expressing  $\alpha\beta 3$  consisting of full-length ( $\beta 3.762$ ) or truncated ( $\beta 3.751\Delta$  or  $\beta 3.741\Delta$ )  $\beta 3$  subunit proteins were allowed to attach for 2 h to wells coated with varying concentrations of vitronectin or laminin. After adhesion, unattached cells were removed by washing, and the attached cells were stained with crystal violet. Cell attachment was quantified by spectrophotometric analysis at OD 600 nm of the crystal violet dye eluted from the adherent cells. Each data point represents the mean  $\pm$  the standard deviation of quadruplicate samples. Nonspecific cell adhesion as measured on BSA-coated wells has been subtracted. (B) Phase-contrast micrographs of CS-1 cells expressing wild-type,  $\beta 3.762$  (WT) or truncated,  $\beta 3.751$  ( $\Delta$ ) and  $\beta 3.741$  ( $\Delta$ ) polypeptides plated on 10  $\mu\text{g/ml}$  of either vitronectin or anti- $\beta 3$  mAb, AP3. 300 $\times$ . Bar, 10  $\mu\text{m}$ .

cellular adhesion to vitronectin, individual amino acid residues within this region were substituted by alanine-scanning mutagenesis. The adhesive properties of cells expressing  $\alpha\beta 3$  containing single alanine substitution between amino acid residues 741–750 in the  $\beta 3$  subunit cyto-

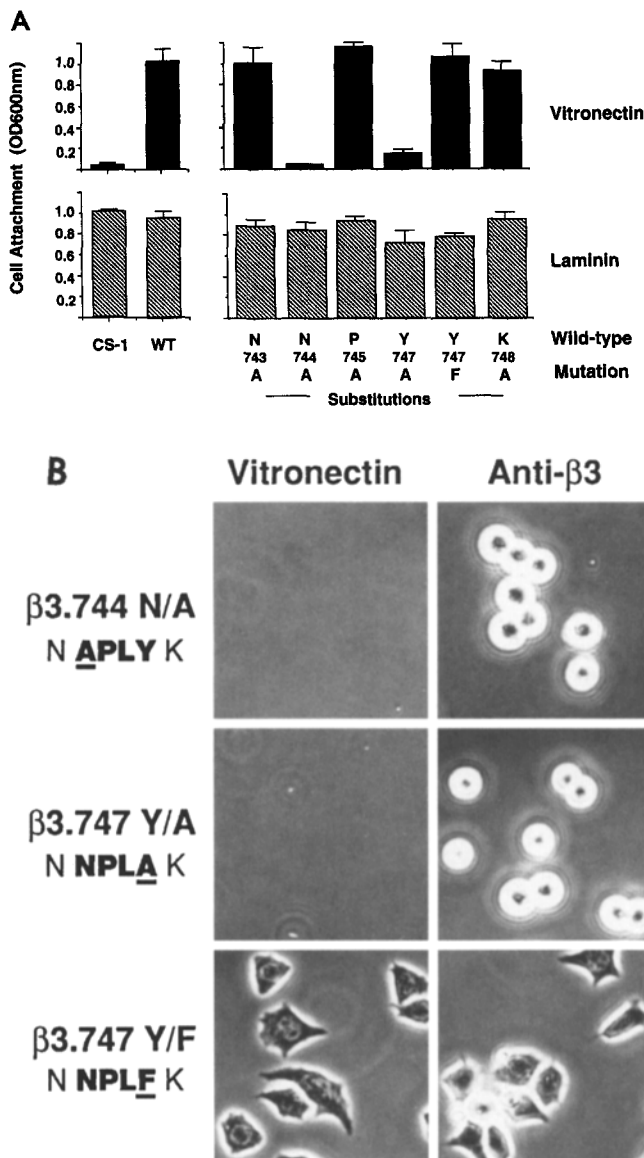
plasmic tail were examined on varying concentrations of immobilized vitronectin. As shown in Fig. 3 A, CS-1 cells expressing  $\alpha\beta 3$  with alanine substitutions in residues immediately upstream, Asn<sup>743</sup> or downstream, Lys<sup>748</sup> of the NPXY<sup>744–747</sup> sequence, exhibited vitronectin adhesion activity comparable to cells expressing wild-type  $\alpha\beta 3$ . Similarly, conversion of Pro<sup>745</sup> to an alanine had no effect on vitronectin binding. In contrast, replacement of Asn<sup>744</sup> with an alanine resulted in a total loss of vitronectin adhesion behaving identically to truncation mutant  $\beta 3.741$  ( $\Delta$ ) (Fig. 2). This mutant cell line did not adhere to surfaces coated with as much as 20  $\mu\text{g/ml}$  of vitronectin (data not shown). In addition, an alanine substitution in Tyr<sup>747</sup> exhibited a substantial but not complete loss in vitronectin adhesive activity (Fig. 3 A). The finding that two independent point-mutations in noncontiguous residues within the NPXY sequence impacted vitronectin adhesive function suggests that the structural integrity of this region is required for  $\beta 3$  integrin function.

Reduced vitronectin adhesivity displayed by  $\alpha\beta 3.747$  Y/A cells may be due either to alterations in the structure of this region or a loss of phosphorylation at this site. To address this issue, the tyrosine at position 747 was substituted with phenylalanine, an amino acid which is structurally homologous to tyrosine, but lacks the hydroxyl group which is the acceptor site for phosphorylation. As shown in Fig. 3 A, cells expressing  $\alpha\beta 3.747$  Y/F containing a phenylalanine in place of Tyr<sup>747</sup> exhibited wild-type vitronectin adhesion, supporting the idea that it is the structure of this region rather than its phosphorylation status that is essential for adhesion to this substrate.

These same point mutants were examined for their ability to spread on immobilized anti- $\beta 3$  antibody. Cells expressing  $\alpha\beta 3$  containing individual amino acid substitutions that were capable of adhering to vitronectin were able to spread on this substrate or anti- $\beta 3$  antibody (Fig. 3 B). Conversely, point mutants defective in their capacity to adhere to vitronectin (Fig. 3 A) were unable to spread on immobilized anti- $\beta 3$  antibody (Fig. 3 B), consistent with their inability to engage the actin cytoskeleton and mediate alterations in cell shape. Collectively, these findings demonstrate that the structure defined by the NPXY sequence within the  $\beta 3$  cytoplasmic tail is required to induce  $\alpha\beta 3$ -dependent cellular shape change.

#### **Effect of Mutations within the $\beta 3$ Cytoplasmic Tail on Cell Interaction with Soluble Vitronectin or an RGD Peptide**

To determine whether mutations within the  $\beta 3$  NPXY sequence may alter the ligand binding properties of  $\alpha\beta 3$ , cells expressing these mutant receptors were examined for their ability to bind soluble ligand. Ligand occupancy of  $\beta 3$  integrins can be measured by a conformational change in the  $\beta 3$  subunit as detected by the LIBS-1 monoclonal antibody (Frelinger et al., 1990). Therefore, expression of the LIBS-1 epitope on  $\alpha\beta 3.741$  ( $\Delta$ ),  $\alpha\beta 3.744$  N/A, or  $\alpha\beta 3.762$  (WT) cells was measured in the presence or absence of soluble GRGDSPK heptapeptide, a known ligand for  $\alpha\beta 3$  (Spiro and Chersesh, 1987). As shown in Fig. 4 A, all three cell lines expressed equivalent LIBS-1 reactivity in the presence of 500  $\mu\text{M}$  GRGDSPK peptide, yet failed



**Figure 3.** Adhesive properties of CS-1 cells expressing  $\alpha\beta 3$  with alanine or phenylalanine substitutions within the  $\beta 3$  subunit cytoplasmic tail. (A) CS-1 cells expressing  $\alpha\beta 3$ -containing amino acid substitutions in the residues encompassing the  $\beta$  turn encoded by the NPXY sequence (744–747) in the  $\beta 3$  subunit cytoplasmic tail were allowed to attach for 2 h to wells coated with 10  $\mu\text{g}/\text{ml}$  of vitronectin (black) or laminin (gray). After adhesion, the attached cells were quantified as described in Fig. 2. (B) Phase-contrast micrographs of CS-1 cells expressing  $\beta 3$  polypeptides containing individual amino acid substitutions,  $\beta 3.744$  N/A,  $\beta 3.747$  Y/A, or  $\beta 3.747$  Y/F plated on coverslips coated with either vitronectin or anti- $\beta 3$  mAb, AP3. 300 $\times$ . Bar, 10  $\mu\text{m}$ .

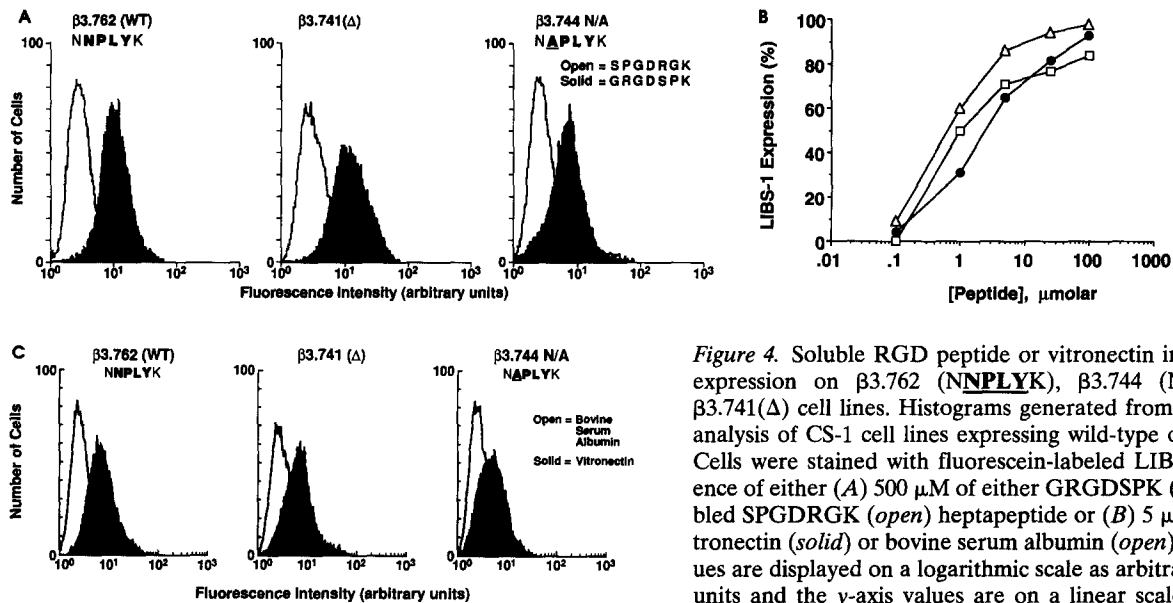
to show LIBS-1 binding in the presence of the same concentration of the scrambled control peptide SPGDRGK. More importantly, each of these  $\beta 3$ -transfected cell lines showed an equivalent RGD dose-dependent LIBS-1 activation with half-maximal concentrations between 1 and 2  $\mu\text{M}$ , suggesting that RGD binds to each of these receptors with comparable affinity (Fig. 4 B). We next examined whether intact soluble vitronectin binding to these cells would induce expression of the LIBS-1 epitope. As dem-

onstrated in Fig. 4 C,  $\alpha\beta 3.741\Delta$ ,  $\alpha\beta 3.744$  N/A, or  $\alpha\beta 3.762$  (WT) cells bound soluble vitronectin as measured by LIBS-1 expression. These data demonstrate that mutations in the  $\beta 3$  cytoplasmic tail that eliminate or disrupt the NPXY sequence abolish  $\alpha\beta 3$  cell attachment to immobilized vitronectin, yet do not perturb the ability of  $\alpha\beta 3$  to interact with soluble ligands.

### The Role of the $\beta 3$ NPXY Sequence in Melanoma Cell Motility In Vitro and Metastasis In Vivo

Integrin  $\alpha\beta 3$  has been shown to promote tumor cell migration (Leavesley et al., 1992). Therefore, to investigate the role of the  $\beta 3$  NPXY sequence in cell motility, cells expressing wild-type or mutant  $\alpha\beta 3$  were tested for their chemotactic or haptotactic response to soluble or immobilized vitronectin, respectively. As shown in Fig. 5, A and B, CS-1 cells lacking  $\alpha\beta 3$  fail to migrate toward soluble or immobilized vitronectin while they readily migrate in response to the control protein laminin. Cells expressing either  $\alpha\beta 3.762$  (WT) or  $\alpha\beta 3.751$  ( $\Delta$ ) acquire the ability to migrate toward lower chambers coated with vitronectin (Fig. 5 A) or containing soluble vitronectin (Fig. 5 B). In contrast, CS-1 cells expressing  $\alpha\beta 3.741$  ( $\Delta$ ) fail to migrate in response to either immobilized or soluble vitronectin. These findings demonstrate that although the  $\alpha\beta 3.741$  ( $\Delta$ ) cells bind soluble vitronectin, they are unable to chemotax toward this protein.

Integrin-mediated cell motility events have been linked to cell invasion in vivo (Juliano, 1987; Cheresch, 1991). Furthermore, expression of  $\alpha\beta 3$  on the surface of human melanoma cells has been associated with tumorigenicity and progression toward malignancy (Albelda et al., 1991; Felding-Habermann et al., 1992; Nip et al., 1993). To examine whether the NPXY sequence within the  $\beta 3$  cytoplasmic tail can regulate cellular migration in vivo, CS-1 cells expressing wild-type or mutant  $\alpha\beta 3$  were examined for their growth and metastatic properties in the chick embryo model (Brooks et al., 1993). CS-1 melanoma cells were deposited onto the CAM of 10-d-old chick embryos and allowed to grow for 1 wk, after which the primary tumor nodule was surgically excised and weighed. In addition, the lungs were harvested, and a single cell suspension was prepared by collagenase treatment. Pulmonary metastasis was quantified by determining the percentage of tumor cells present in this cell suspension by staining with a monoclonal antibody specific for hamster cells. As shown in Fig. 6, CS-1 cells lacking  $\alpha\beta 3$ , formed large tumors at the primary site, yet these animals contained few, if any, detectable tumor cells in the lungs. In contrast,  $\alpha\beta 3.762$  (WT) cells produced small primary tumors which, on average, were fivefold smaller than CS-1 tumors (Fig. 6 A). However, as shown in Fig. 6 B, this lack of primary tumor size was associated with an approximate fivefold increase in pulmonary metastasis ( $P \leq 0.001$ ), suggesting that expression of  $\alpha\beta 3$  on CS-1 cells promoted migration from the primary tumor site. In contrast, cells expressing  $\alpha\beta 3.744$  N/A (Fig. 6) or  $\beta 3.747$  Y/A (data not shown), which exhibited deficient migration in vitro, produced tumors whose growth properties and metastatic behavior were identical to the parental,  $\alpha\beta 3$ -negative CS-1 cell line. CS-1 cells expressing  $\alpha\beta 3.747$  Y/F possessing



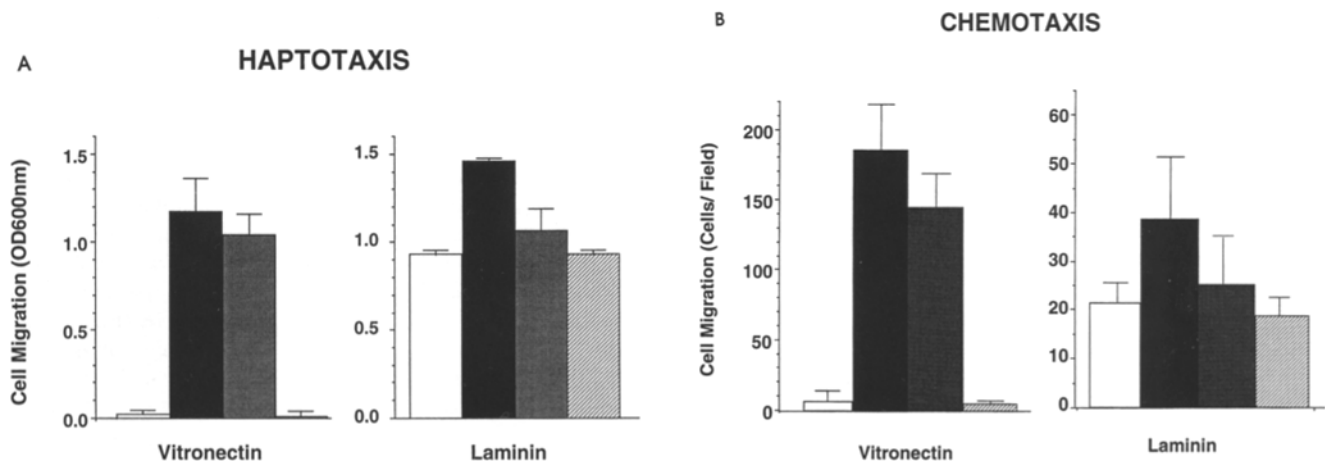
**Figure 4.** Soluble RGD peptide or vitronectin increases LIBS-1 expression on  $\beta 3.762$  (**NNPLYK**),  $\beta 3.744$  (**NAPLYK**) and  $\beta 3.741(\Delta)$  cell lines. Histograms generated from flow cytometry analysis of CS-1 cell lines expressing wild-type or mutant  $\alpha v\beta 3$ . Cells were stained with fluorescein-labeled LIBS-1 in the presence of either (A) 500  $\mu\text{M}$  of either GRGDSPK (solid) or scrambled SPGDRGK (open) heptapeptide or (B) 5  $\mu\text{M}$  of soluble vitronectin (solid) or bovine serum albumin (open). The x-axis values are displayed on a logarithmic scale as arbitrary fluorescence units and the y-axis values are on a linear scale and represent number of cells. WT, wild-type. Bold-face indicates the NPXY  $\beta$  turn motif. The alanine substitution at position 744 is underscored. ●-,  $\beta 3.762$  (WT); - $\Delta$ -,  $\beta 3.741$  ( $\Delta$ ); -□-,  $\beta 3.744$  N/A. (C) Line graph derived from flow cytometry analysis of cells stained with fluoresceinated LIBS-1 antibody in the presence of varying concentrations of GRGDSPK peptide. The x-axis values are displayed on a logarithmic scale as arbitrary fluorescence units and the y-axis values are on a linear scale and represent the percentage of the increase in LIBS-1 expression between no peptide and 500  $\mu\text{M}$  of GRGDSPK.

wild-type migration properties in vitro, completely restored the metastatic phenotype of these cells. The observed inverse correlation between the size of the primary tumor and its ability to metastasize suggests that CS-1 cells lacking the metastatic phenotype are unable to migrate from the primary lesion. In support of this contention,  $\alpha v\beta 3$  with mutations that disrupt CS-1 melanoma cell motility in vitro also prevent pulmonary metastasis in vivo. Collectively, these data provide evidence that the NPXY

sequence within the  $\beta 3$  cytoplasmic tail is a critical structure in regulating cellular migration in vitro and in vivo.

### Discussion

Integrin-mediated adhesion events are associated with a wide range of biological activity both in vitro and in vivo. In fact, integrin gene “knock-outs” lead to embryonic lethal mutations (Yang et al., 1995) and altered cell biologi-



**Figure 5.** Cell migration of CS-1 cells expressing  $\alpha v\beta 3$  consisting of full-length or truncated  $\beta 3$  polypeptides. Cellular motility toward vitronectin or laminin was determined using a modified Boyden chamber separated by a 6.5  $\mu\text{m}$  porous membrane. CS-1 (white),  $\beta 3.762$  (WT) (black),  $\beta 3.751$  ( $\Delta$ ) (gray), or  $\beta 3.741$  ( $\Delta$ ) (hatched) cells were seeded into the upper chambers and allowed to migrate for 24 h toward lower chambers containing 10  $\mu\text{g}/\text{ml}$  of immobilized adhesive ligand (haptotaxis) (A) or soluble ligand (chemotaxis) (B). Cells which had migrated to the lower reservoir were enumerated by dye uptake (A) or by microscopic evaluation (B) as described in the experimental procedures. Each bar represents the mean  $\pm$  the standard deviation of quadruplicate samples.

cal responses (Stephens et al., 1993) while integrin antagonists block platelet aggregation (Savage et al., 1990), tumor cell migration (Humphries et al., 1986), angiogenesis (Brooks et al., 1994), and viral infection (Wickham et al., 1993). The biological role of integrins depends on their ability to bind extracellular matrix proteins resulting in the generation of intracellular signals. This leads to cell spreading and migration, events which have been linked to the organization of the actin cytoskeleton (Burrige et al., 1988). Therefore, it has been proposed that ligand recognition by integrins leads to structural alterations in the integrin cytoplasmic domain facilitating postligand binding events important for cell spreading and motility. While extensive studies have identified sequences within the extracellular domain of integrins involved in ligand binding (Smith and Cheresch, 1988, 1990; D'Souza et al., 1988, 1990; 1994; Loftus et al., 1990; Takada et al., 1992; Lee et al., 1995), little is known about the structural regions within the integrin cytoplasmic domain that mediate postligand binding events.

Integrin  $\alpha v\beta 3$  is highly expressed on various motile cell types *in vivo*. For example, this integrin is preferentially expressed on invasive melanoma cells (Albelda et al., 1991; Nip et al., 1993), angiogenic vascular cells (Brooks et al., 1994*a,b*) and on migratory neural crest cells (Delannet et al., 1994). Also, integrin  $\alpha v\beta 3$  readily promotes migration of primary endothelial cells or various transformed cells *in vitro* (Leavesley et al., 1992, 1993). Therefore, we designed experiments to identify regions within the cytoplasmic domain of  $\alpha v\beta 3$  that were involved in the cell motility response. To accomplish this objective, we used CS-1 melanoma cells that fail to express any vitronectin binding integrin due to their lack of  $\beta 3$  or  $\beta 5$  protein. We show in this report that cells transfected with a cDNA encoding full-length  $\beta 3$  express  $\alpha v\beta 3$  and thereby gain the ability to attach, spread, and migrate on a vitronectin substrate. To identify functional domains within the  $\beta 3$  cytoplasmic tail critical for these vitronectin adhesion events, CS1 cells were transfected with truncated or mutant forms of  $\beta 3$ . In particular, we focused on two structural motifs, NPXY (744–747) and NXXY (756–759), which are predicted to form  $\beta$  turns in the cytoplasmic tails of, not only  $\beta 3$ , but most of the other integrin  $\beta$  subunits (Fig. 1).

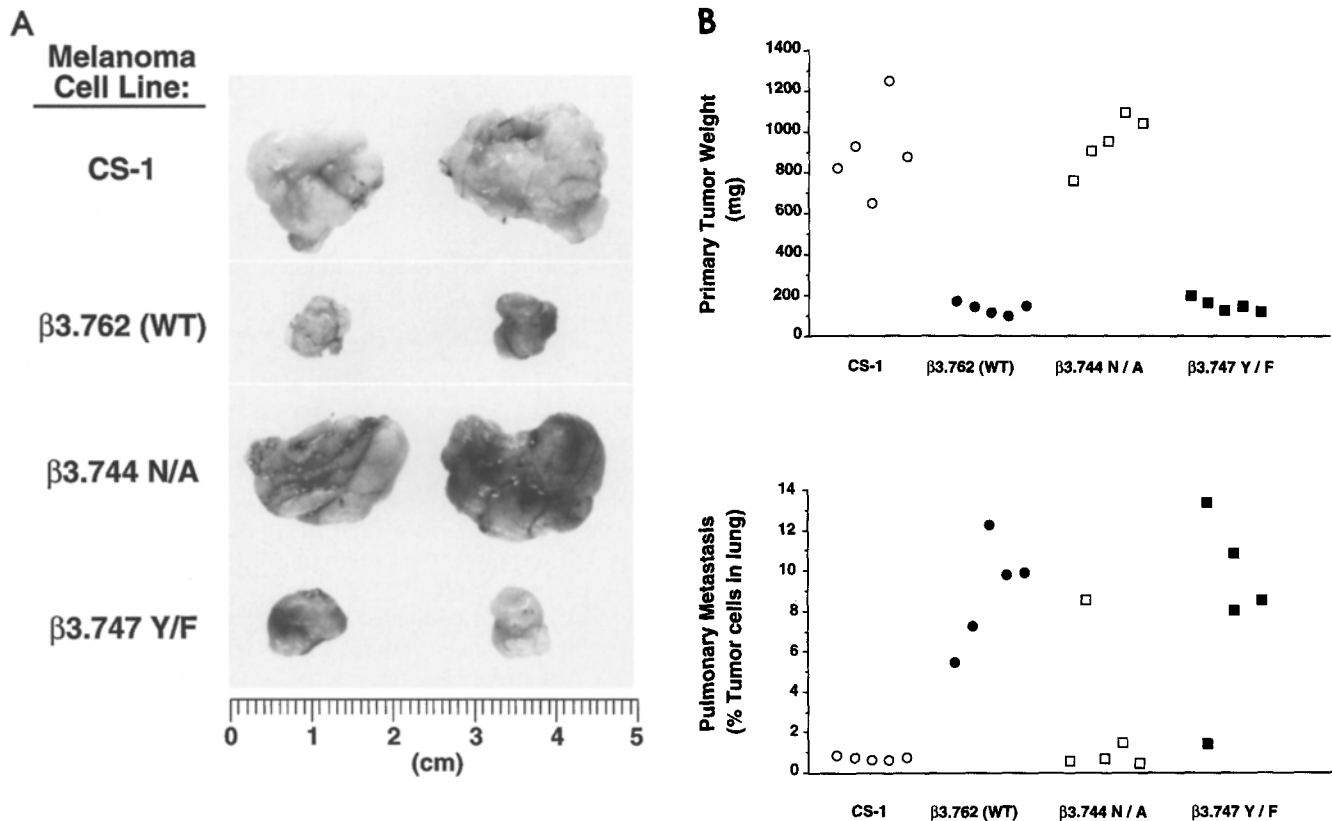
This mutational analysis of the  $\beta 3$  subunit demonstrates that disruption or elimination of the NPXY sequence prevents  $\alpha v\beta 3$ -dependent CS-1 cell attachment or migration on a vitronectin substrate. We provide evidence that these mutations do not influence the soluble ligand binding properties of this receptor since either vitronectin- or RGD-containing peptides were able to interact equally well with all mutant forms of  $\alpha v\beta 3$  tested. Therefore, we propose that mutations in the NPXY motif of  $\beta 3$  perturb postligand binding events involved in  $\alpha v\beta 3$ -dependent cytoskeletal assembly required for cell spreading and migration on a vitronectin substrate. This contention is supported by the finding that cells expressing a mutant or deleted NPXY sequence attached, but failed to spread or migrate on immobilized anti- $\beta 3$  antibody.

Prior reports have shown that mutations in the  $\beta$  subunit cytoplasmic tail impact cell adhesion to immobilized ligand. Mutations in the  $\beta 2$  subunit cytoplasmic tail abolish  $\alpha L\beta 2$ -mediated cell adhesion to purified ICAM (Hibbs

et al., 1991). More recently, it was shown that deletion of the NPXY sequence expressed in the context of the platelet receptor  $\alpha IIb\beta 3$  altered the affinity state of this receptor for its ligand (O'Toole et al., 1995). Our studies suggest that NPXY mutations in  $\alpha v\beta 3$  have little or no effect on the affinity of this receptor for soluble ligand as measured by LIBS epitope expression in the presence of varying concentration of soluble RGD. Rather, this mutation appears to abolish the interaction of cells with immobilized ligand. These results may be explained by the inability of these mutant receptors to organize the actin cytoskeleton and promote a shape change on immobilized vitronectin or anti- $\beta 3$  antibody.

The NPXY sequence is present in the cytoplasmic domain of a number of non-integrin receptors such as the low density lipoprotein receptor where it has been linked to receptor internalization since mutations in NPXY result in reduced receptor internalization (Chen et al., 1990). CS-1 cells expressing  $\alpha v\beta 3.741(\Delta)$  lacking the NPXY sequence were tested for their ability to internalize integrin  $\alpha v\beta 3$  in response to cross-linking with anti- $\beta 3$  antibodies. Interestingly, both the wild-type and NPXY-deleted receptors showed equivalent internalization (Filardo, E. J., unpublished data). These data suggest that while the NPXY is critical for the internalization of some cell surface receptors this motif does not appear to be required for  $\alpha v\beta 3$ -dependent internalization.

Integrin-mediated cell adhesion is characterized by integrin clustering at discrete membrane sites known as focal contacts, which serve to strengthen the interaction between cells and their substratum (Burrige et al., 1988). In fact, focal contacts serve to tether actin filaments and stress fibers facilitating cell spreading. Based on previous studies, it is apparent that specific regions of the integrin  $\beta$  subunit cytoplasmic domain are critical for focal contact assembly (Reszka et al., 1992; Cone et al., 1994). This process likely depends on the ability of the integrin cytoplasmic tail to associate with various cytoplasmic protein such as talin and  $\alpha$ -actinin (Tapley et al., 1989; Otey et al., 1992). In addition, there are several nonreceptor tyrosine kinases that are associated with focal contacts, including focal adhesion kinase, pp125FAK (Hanks et al., 1992; Kornberg et al., 1992; Schaller et al., 1992), src kinase, pp60v-src (Rohrschneider et al., 1990), type IV c-abl kinase (Van Etten et al., 1994), as well as a variety of SH2- and SH3-containing proteins (Miyamoto et al., 1995). These observations suggest that the integrin cytoplasmic domain is critical in recruiting signaling molecules that not only regulate cell shape, but also impact other events such as cell migration, invasion, and proliferation. To this end, we observed that the same mutations in  $\beta 3$  that prevent cell motility *in vitro* directly impact the metastatic properties of these cells *in vivo*. We showed that CS-1 melanoma cells lacking  $\alpha v\beta 3$  form large primary tumors when allowed to grow on the chick embryo CAM, yet these cells fail to establish lung metastases in these animals. However, transfection of CS-1 cells with wild-type  $\beta 3$  enables them to invade and colonize the lungs of these animals. Interestingly, primary tumors comprised of  $\alpha v\beta 3$  expressing CS-1 cells are fivefold smaller than their nontransfected counterparts, suggesting that the metastasis observed was due to the ability of CS-1 cells expressing  $\alpha v\beta 3$  to migrate from the



**Figure 6.** Effect of individual amino acid substitutions in the NPXY sequence within the  $\beta$ 3 cytoplasmic tail on tumor growth and metastasis. (A) Representative examples of tumors excised from 17-d-old chick CAM after seven d of growth. (B) Primary tumor weight and percentage of metastatic melanoma cells present in chick lung after 7 d of tumor growth. Tumor weight is expressed in mg. The percentage of metastatic melanoma cells was determined by flow cytometry analysis of a single cell suspension prepared from whole lung using mAb, 7E2 specific for the hamster integrin  $\beta$ 1 subunit. The difference in growth rate and metastasis are significant ( $P \leq 0.001$ ) as determined by the Student  $t$  test.

site of the primary tumor. This contention is supported by the fact that these cell lines show identical growth rates in culture (data not shown) suggesting that the observed difference in the size of these primary tumors is not due to an intrinsic difference in the growth rate of these cells. More importantly, mutations in the NPXY sequence that prevent cell motility in vitro, show minimal metastasis in vivo, while forming large primary tumors identical to those formed by  $\alpha$ v $\beta$ 3-negative CS-1 cells. These findings are consistent with the observation that vertically invasive primary as well as metastatic human melanoma tissue expresses  $\alpha$ v $\beta$ 3, while noninvasive primary lesions lack  $\alpha$ v $\beta$ 3 (Albelda et al., 1991). Moreover, Nip and co-workers (1993) showed that melanoma cells isolated from nude mouse metastases express elevated levels of  $\alpha$ v $\beta$ 3 compared with their primary tumor cell counterparts.

The structural motif defined by the NPXY sequence in  $\beta$ 3 is likely to play a major role in other integrin heterodimers since this motif is found in the same region of most other integrin  $\beta$  subunits. However, it is clear from our studies that the NPXY motif in the  $\beta$ 3 subunit regulates postligand binding events that are critical to cellular morphology and migration in vitro. More importantly, we provide the first evidence of an integrin structural motif that plays a critical role in the metastasis of tumor cells in vivo. This is underscored by the finding that a single point

mutation in the  $\beta$ 3 subunit prevents  $\alpha$ v $\beta$ 3-dependent cellular post-ligand binding events leading to the metastatic behavior of tumor cells in vivo.

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#### References

- Albelda, S. M., S. M. Mente, D. E. Elder, R. Stewart, L. Damjanovich, M. Herlyn, and C. A. Buck. 1991. Integrin distribution in malignant melanoma: association of the  $\beta$ 3 subunit with tumor progression. *Cancer Res.* 50:6757-6764.
- Argraves, W. S., S. Suzuki, H. Arai, K. Thompson, M. D. Pierschbacher, and E. Ruoslahti. 1987. Amino acid sequence of the human fibronectin receptor. *J. Cell Biol.* 105:1183-1190.
- Bauer, J. S., J. Varner, C. Schreiner, L. Kornberg, R. Nicholas, and R. L. Juliano. 1993. Functional role of the cytoplasmic domain of the integrin  $\alpha$ 5 subunit. *J. Cell Biol.* 122:209-221.
- Brooks, P. C., J.-M. Lin, D. L. French, and J. P. Quigley. 1993. Subtractive immunization yields monoclonal antibodies that specifically inhibit metastasis. *J. Cell Biol.* 122:1351-1359.
- Brooks, P. C., R. A. F. Clark, and D. A. Cheresh. 1994a. Requirement of vascu-



- lar integrin  $\alpha v\beta 3$  for angiogenesis. *Science (Wash. DC)*. 264:569–571.
- Brooks, P. C., A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier, and D. A. Cheresh. 1994b. Integrin  $\alpha v\beta 3$  antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell*. 79:1157–1164.
- Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner. 1988. Focal adhesion: transmembrane junctions between the extracellular matrix and the cytoskeleton. *Annu. Rev. Cell Biol.* 4:487–525.
- Chen, W. J., J. L. Goldstein, and M. S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* 265:3116–3123.
- Cheresh, D. A. 1991. Structure, function, and biological properties of integrin  $\alpha v\beta 3$  on human melanoma cells. *Cancer Metastasis Rev.* 10:3–10.
- Cheresh, D. A., and R. C. Spiro. 1987. Biosynthetic and functional properties of an arg-gly-asp-directed receptor involved in human melanoma cell attachment to vitronectin, fibrinogen, and von Willebrand factor. *J. Biol. Chem.* 262:17703–17711.
- Chou, P. Y., and G. D. Fasman. 1978. Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol. Relat. Areas Mol. Biol.* 47:45–165.
- Cone, R. I., A. Weinacker, A. Chen, and D. Sheppard. 1994. Effects of  $\beta$  subunit cytoplasmic domain deletions on the recruitment of the integrin  $\alpha v\beta 6$  to focal contacts. *Cell Adhesion Comm.* 2:101–113.
- D'Souza, S. E., M. H. Ginsberg, T. A. Burke, S. C. T. Lam, and E. F. Plow. 1988. Localization of an arg-gly-asp recognition site within an integrin adhesion receptor. *Science (Wash. DC)*. 242:91–93.
- D'Souza, S. E., M. H. Ginsberg, T. A. Burke, and E. F. Plow. 1990. The ligand binding site of the platelet integrin receptor GPIIb-IIIa is proximal to the second calcium binding domain of its  $\alpha$  subunit. *J. Biol. Chem.* 265:3440–3446.
- D'Souza, S. E., T. A. Haas, R. S. Piotrowicz, V. Byers-Ward, D. E. McGrath, H. Soule, C. Cierniewski, E. F. Plow, and J. W. Smith. 1994. Ligand and cation binding are dual functions of a discrete segment of the integrin  $\beta 3$  subunit: cation displacement is involved in ligand binding. *Cell*. 79:659–667.
- Delannet, M., F. Martin, B. Bossy, D. A. Cheresh, L. F. Reichardt, and J.-L. Duband. 1994. Specific roles of the  $\alpha v\beta 1$ ,  $\alpha v\beta 3$ , and  $\alpha v\beta 5$  integrins in avian neural crest cell adhesion and migration on vitronectin. *Development (Camb.)* 120:2687–2702.
- Erle, D. J., C. Rugg, D. Sheppard, and R. Pytela. 1991. Complete amino acid sequence of an integrin  $\beta$  subunit (beta 7) identified in leukocytes. *J. Biol. Chem.* 266:11009–11016.
- Felding-Habermann, B., B. M. Mueller, C. A. Romerdahl, and D. A. Cheresh. 1992. Involvement of integrin  $\alpha v$  gene expression in human melanoma tumorigenicity. *J. Clin. Invest.* 89:2018–2022.
- Filardo, E. J., and D. A. Cheresh. 1994. A beta turn in the cytoplasmic tail of the integrin  $\alpha v$  subunit influences the conformation and ligand-binding of  $\alpha v\beta 3$ . *J. Biol. Chem.* 269:4641–4647.
- Fitzgerald, L. A., B. Steiner, S. C. Rall, Jr., S.-S. Lo, and D. R. Phillips. 1987. Protein sequence of endothelial glycoprotein IIIa derived from a cDNA clone. *J. Biol. Chem.* 262:3936–3939.
- Frelinger, A. L., I. Cohen, E. F. Plow, M. A. Smith, J. Roberts, S. C.-T. Lam, and M. H. Ginsberg. 1990. Selective inhibition of integrin function by antibodies specific for ligand-occupied receptor conformers. *J. Biol. Chem.* 265:6346–6352.
- Hanks, S. K., M. B. Calalb, M. C. Harper, and S. K. Patel. 1992. Focal adhesion protein tyrosine kinase phosphorylated in response to cell spreading on fibronectin. *Proc. Natl. Acad. Sci. USA*. 89:8487–8489.
- Hayashi, Y., B. Haimovich, A. Reszka, D. Boettiger, and A. Horowitz. 1990. Expression and function of chicken integrin  $\beta 1$  subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. *J. Cell Biol.* 110:175–184.
- Hibbs, M. L., S. Jakes, S. A. Stackler, R. W. Wallace, and T. A. Springer. 1991. The cytoplasmic domain of lymphocyte function-associated antigen I: sites required for binding to intercellular adhesion molecule 1 and the phorbol ester-stimulated phosphorylation site. *J. Exp. Med.* 174:1227–1238.
- Horowitz, A., K. Duggan, A. C. Buck, M. C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin—a transmembrane linkage. *Nature (Lond.)*. 320:531–533.
- Humphries, M. J., K. Olden, and K. Yamada. 1986. A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. *Science (Wash. DC)*. 233:467–470.
- Hynes, R. O. 1992. Integrins: versatility, modulation, and signalling in cell adhesion. *Cell*. 69:11–25.
- Juliano, R. 1987. Membrane receptors for extracellular matrix macromolecules: relationship to cell adhesion and tumor metastasis. *Biochim. Biophys. Acta*. 907:261–278.
- Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer. 1987. Cloning of the  $\beta$  subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. *Cell*. 48:681–690.
- Klemke, R. L., M. Yebra, E. Bayna, and D. A. Cheresh. 1994. Receptor tyrosine kinase signalling required for integrin  $\alpha v\beta 5$ -directed cell motility but not adhesion on vitronectin. *J. Cell Biol.* 127:859–866.
- Knudsen, K. A., C. H. Damsky, and C. A. Buck. 1982. Expression of adhesion-related membrane components in adherent vs. nonadherent melanoma cells. *J. Cell. Biochem.* 18:157–167.
- Kornberg, L. J., H. S. Earp, J. T. Parsons, M. D. Schaller, and R. L. Juliano. 1992. Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J. Biol. Chem.* 267:23439–23442.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367–382.
- LaFlamme, S. E., L. A. Thomas, S. S. Yamada, and K. M. Yamada. 1994. Single subunit chimeric integrins as mimics and inhibitors of endogenous integrin functions in receptor localization, cell spreading and migration, and matrix assembly. *J. Cell Biol.* 126:1287–1298.
- Leavesley, D. I., G. D. Ferguson, E. A. Wayner, and D. A. Cheresh. 1992. Requirement of the integrin  $\beta 3$  subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. *J. Cell Biol.* 117:1101–1109.
- Leavesley, D. I., M. A. Schwartz, M. Rosenfeld, and D. A. Cheresh. 1993. Integrin  $\beta 1$ - and  $\beta 3$ -mediated endothelial cell migration is triggered through distinct signalling mechanisms. *J. Cell Biol.* 121:163–170.
- Lee, J.-O., P. Rieu, M. A. Arnaout, and R. Liddington. 1995. Crystal structure of the A domain from the  $\alpha$  subunit of integrin CR3 (CD11b/CD18). *Cell*. 80:631–638.
- Loftus, J. C., T. E. O'Toole, E. F. Plow, A. Glass, A. L. Frelinger III, and M. H. Ginsberg. 1990. A  $\beta 3$  integrin mutation abolishes ligand binding and alters cation-dependent conformation. *Science (Wash. DC)*. 249:915–918.
- Lotz, M. M., C. A. Burdsal, H. P. Erickson, and D. R. McClay. 1989. Cell adhesion to fibronectin and tenascin: quantitative measurements of initial binding and subsequent strengthening response. *J. Cell Biol.* 109:1795–1805.
- McLean, J. W., D. J. Vestal, D. A. Cheresh, and S. C. Bodary. 1990. cDNA sequence of the human integrin  $\beta 5$  subunit. *J. Biol. Chem.* 265:17126–17131.
- Miyamoto, S., S. K. Akiyama, and K. M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science (Wash. DC)*. 267:883–885.
- Nip, J., H. Shibata, D. J. Loskutoff, D. A. Cheresh, and P. Brodt. 1992. Human melanoma cells derived from lymphatic metastases use integrin  $\alpha v\beta 3$  to adhere to lymph node vitronectin. *J. Clin. Invest.* 90:1406–1413.
- O'Toole, T. E., D. Mandelman, J. Forsyth, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1991. Modulation of the affinity of integrin  $\alpha IIb\beta 3$  (GPIIb-IIIa) by the cytoplasmic domain of  $\alpha IIb$ . *Science (Wash. DC)*. 254:845–847.
- O'Toole, T. E., J. Ylänne, and B. M. Culley. 1995. Regulation of integrin affinity states through an NPXY motif in the  $\beta$  subunit cytoplasmic domain. *J. Biol. Chem.* 270:8553–8558.
- Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between  $\alpha$ -actinin and the  $\beta 1$  integrin subunit in vitro. *J. Cell Biol.* 111:721–729.
- Otey, C. A., G. B. Vasquez, K. Burridge, and B. W. Erickson. 1992. Mapping of the  $\alpha$ -actinin binding site within the  $\beta 1$  integrin cytoplasmic domain. *J. Biol. Chem.* 268:21193–21197.
- Ramaswamy, H., and M. E. Hemler. 1990. Cloning, primary structure, and properties of a novel integrin  $\beta$  subunit. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1561–1568.
- Reszka, A. A., Y. Hayashi, and A. F. Horowitz. 1992. Identification of amino acid sequences in the integrin  $\beta 1$  cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol.* 117:1321–1329.
- Rose, G. D. 1978. Prediction of chain turns in globular proteins on a hydrophobic basis. *Nature (Lond.)*. 272:586–590.
- Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87:1–5.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*. 74:5463–5467.
- Savage, B., U. M. Marzec, B. H. Chao, L. A. Harker, J. M. Maraganore, and Z. M. Ruggeri. 1990. Binding of the snake venom-derived proteins applaggin and echistatin to the arginine-glycine-aspartic acid recognition site(s) on platelet glycoprotein IIb/IIIa complex inhibits receptor function. *J. Biol. Chem.* 265:1766–1774.
- Schaller, M. D., C. A. Borgman, B. C. Cobb, A. B. Reynolds, and J. T. Parsons. 1992. pp125<sup>FAK</sup>, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA*. 89:5192–5196.
- Sheppard, D. C., C. Rozzo, L. Starr, L. Quaranta, D. J. Elre, and R. Pytela. 1990. Complete amino acid sequence of a novel integrin  $\beta$  subunit ( $\beta 6$ ) identified in epithelial cells using the polymerase chain reaction. *J. Biol. Chem.* 265:11502–11507.
- Smith, J. W., and D. A. Cheresh. 1988. The arg-gly-asp binding domain of the vitronectin receptor: photoaffinity cross-linking implicates amino acid residues 61–203 of the  $\beta$  subunit. *J. Biol. Chem.* 263:18726–18731.
- Smith, J. W., and D. A. Cheresh. 1990. Integrin ( $\alpha v\beta 3$ )-ligand interaction: identification of a heterodimeric RGD binding site on the vitronectin receptor. *J. Biol. Chem.* 265:2168–2172.
- Stephens, L. E., J. E. Sonne, M. L. Fitzgerald, and C. H. Damsky. 1993. Targeted deletion of  $\beta 1$  integrins in F9 embryonal carcinoma cells affects morphological differentiation but not tissue-specific gene expression. *J. Cell Biol.* 123:1607–1620.
- Takada, Y., J. Ylänne, D. Mandelman, W. Puzon, and M. H. Ginsberg. 1992. A point mutation of integrin  $\beta 1$  subunit blocks binding of  $\alpha 5\beta 1$  to fibronectin and invasion but not recruitment to adhesion plaques. *J. Cell Biol.* 119:913–921.
- Tapley, P., A. Horowitz, C. Buck, K. Duggan, and L. Rohrschneider. 1989. Inte-

- grins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Oncogene*. 4:325-333.
- Thomas, L., P. W. Chan, S. Chang, and C. Damsky. 1993. 5-Bromo-2-deoxyuridine regulates invasiveness and expression of integrins and matrix-degrading proteinases in a differentiated hamster melanoma cell. *J. Cell Sci.* 105:191-201.
- Van Etten, R. A., P. K. Jackson, D. Baltimore, M. C. Sanders, P. T. Matsadaira, and P. A. Jamney. 1994. The COOH terminus of the c-Abl tyrosine kinase contains distinct F- and G-actin binding domains with bundling activity. *J. Cell Biol.* 124:325-340.
- Wickham, T. J., P. Mathias, D. A. Cheresh, and G. R. Nemerow. 1993. Integrins  $\alpha\beta 3$  and  $\alpha\beta 5$  promote adenovirus internalization but not virus attachment. *Cell*. 73:309-319.
- Yamada, K. M., D. W. Kennedy, S. S. Yamada, H. Grainick, W.-T. Chen, and S. K. Akiyama. 1990. Monoclonal antibody and synthetic peptide inhibitors of human tumor cell migration. *Cancer Res.* 50:4485-4486.
- Yang, J. T., H. Rayburn, and R. O. Hynes. 1995. Cell adhesion events mediated by  $\alpha 4$  integrins are essential in placental and cardiac development. *Development*. 121:549-560.
- Yang, L. J., S. G. Rhee, and J. R. Williamson. 1994. Epidermal growth factor-induced activation and translocation of phospholipase C- $\gamma 1$  to the cytoskeleton in rat hepatocytes. *J. Biol. Chem.* 269:7156-7162.
- Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparan affinity chromatography. *Cell. Struct. Funct.* 13:281-292.
- Ylänne, J., Y. L. Chen, T. E. O'Toole, J. C. Loftus, Y. Takada, and M. H. Ginsberg. 1993. Distinct functions of integrin  $\alpha$  and  $\beta$  subunit cytoplasmic domains in cell spreading and formation of focal adhesions. *J. Cell Biol.* 122: 223-234.