

Modulation of β 1A Integrin Functions by Tyrosine Residues in the β 1 Cytoplasmic Domain

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Abstract. β 1A integrin subunits with point mutations of the cytoplasmic domain were expressed in fibroblasts derived from β 1-null stem cells. β 1A in which one or both of the tyrosines of the two NPXY motifs (Y783, Y795) were changed to phenylalanines formed active α 5 β 1 and α 6 β 1 integrins that mediated cell adhesion and supported assembly of fibronectin. Mutation of the proline in either motif (P781, P793) to an alanine or of a threonine in the inter-motif sequence (T788) to a proline resulted in poorly expressed, inactive β 1A. Y783,795F cells developed numerous fine focal contacts and exhibited motility on a surface. When compared with cells expressing wild-type β 1A or β 1A with

the D759A activating mutation of a conserved membrane-proximal aspartate, Y783,795F cells had impaired ability to transverse filters in chemotaxis assays. Analysis of cells expressing β 1A with single Tyr to Phe substitutions indicated that both Y783 and Y795 are important for directed migration. Actin-containing microfilaments of Y783,795F cells were shorter and more peripheral than microfilaments of cells expressing wild-type β 1A. These results indicate that change of the phenol side chains in the NPXY motifs to phenyl groups (which cannot be phosphorylated) has major effects on the organization of focal contacts and cytoskeleton and on directed cell motility.

INTEGRINS are transmembrane $\alpha\beta$ heterodimers that mediate organization of focal contacts, actin-containing cytoskeleton, and extracellular matrix (1, 11, 24, 35, 56, 70). Intracellular signals cause changes in integrin extracellular domains and thereby regulate affinity for extracellular ligands (22, 30, 33, 35, 38, 59). Regulation of the interaction of integrins with extracellular ligands allows control of cell adhesion and migration. Adhesion and migration, in turn, are important determinants of cell proliferation and differentiation (21, 24, 27, 34, 35, 39). Integrins are involved in a number of pathological processes (1, 30, 35, 56, 64). Therefore, a detailed understanding is needed of the complex “inside-out” and “outside-in” signal transduction pathways underlying and provoked by the association of integrins with extracellular ligands and intracellular effector molecules and how these pathways control cellular behavior.

Three regions of the A splice form of β 1 subunit (β 1A) cytoplasmic domains have been implicated in the function of β 1A integrins (see Fig. 1). The Asp of the membrane-proximal sequence KLLXXXXD likely forms a salt bridge

with a conserved Arg of the α subunit, thus stabilizing a default inactive conformation (32). The other regions are comprised of two NPXY motifs (54). These motifs are found in the cytoplasmic domains of β 1A and β 1D, β 2, β 3, β 5, β 6, and β 7 (see Fig. 1) (25, 49). In all except β 5, the motifs are separated by an intervening sequence of eight residues. Substitution of a Pro for a Ser in the intervening sequence of β 3 is associated with Glanzmann thrombasthenia, a bleeding disorder caused by abnormal function of platelet α IIb β 3 (16, 17). Mutagenesis of the Asn or Pro residues of the NPXY motifs results in β subunits that do not localize to focal contacts (19, 54). Such changes likely alter the tight β -turn predicted for such sequences (3, 28). When the cDNA for β 1A was first sequenced, the tyrosine residues of the NPXY motifs were identified as potential sites of phosphorylation (62). Subsequent studies demonstrated tyrosine phosphorylation of β 1 in transformed cells and of β 3 in activated and aggregated platelets and in α v β 3-expressing K562 cells (8, 9, 31, 40, 63). Tyrosine phosphorylated β 1 of *src*-transformed cells, detected by anti-phosphopeptide antibodies, localizes to podosomes rather than focal contacts (37). Transfected β 1As with one or both Tyr mutated to Glu, Ala, or Ser localize less well than wild-type β 1A to focal contacts (19, 54). Transfected β 1A with conservative mutations of Tyr to Phe, which cannot be phosphorylated, localizes normally to focal contacts (19, 29, 48, 54, 65).

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We have studied the effects of the Tyr to Phe substitutions in the NPXY motifs on cell behavior. Mutated β 1A was expressed in mouse GD25 cells, which lack β 1 integrin heterodimers because of targeted knockout of the β 1 gene (23, 67). The Tyr to Phe mutations resulted in β 1As that were active in GD25 cells as assessed by antibody binding and fibronectin matrix assembly assays. However, the Tyr to Phe mutations were associated with loss of ability of cells to undergo directed migration.

Materials and Methods

Mutation of β 1A

Mutant β 1A constructs were generated from pBS β 1A encoding full-length mouse β 1A integrin subunit (67). The β 1A was cloned into pGEM7Zf by the unique XbaI and Acc65I restriction sites. Mutants were generated by oligonucleotide-primed DNA synthesis using the U.S.E. mutagenesis kit (Pharmacia Biotech Sevrage, Uppsala, Sweden). Regions spanning the PmlI and Acc65I sites were analyzed by DNA sequence analysis. Mutagenized PmlI–Acc65I fragments were isolated and ligated into PmlI–Acc65I-digested pBS β 1A to generate cDNAs encoding full-length β 1A polypeptides containing the individual point mutations in the cytoplasmic tail. The plasmid was linearized with XbaI and transfected into β 1-deficient GD25 cells by electroporation. After 72 h, selection with 5 μ g/ml puromycin was started. Surviving clones were isolated and expanded. Clones stably expressing β 1A were identified for each mutation. Clones were monitored for expression of β 1A by flow cytometry. If necessary, the population of higher expressing cells was selected by fluorescence detected cell sorting.

Flow Cytometry

Cells were harvested and suspended in PBS containing 3% (wt/vol) BSA. Approximately 1.0×10^6 cells were incubated with primary antibody, and then treated with FITC-conjugated secondary antibody at 4°C. We confirmed by titration that the dilutions of antibodies used were saturating and gave maximal specific signals. Cells (8,000 per sample) were analyzed in a FACScan® flow cytometer (Becton and Dickinson Co., Mountain View, CA). Monoclonal antibodies 9EG7 to mouse β 1 (4, 42), MFR5 to mouse α 5, H9.2B8 to mouse α v, and 2C9.G2 to mouse β 3 were all from PharMingen (San Diego, CA). Antibody MB1.2 to mouse β 1 and GoH3 to α 6 were provided by Dr. B. Chan (University of Western Ontario, Ontario, Canada) and Dr. A. Sonnenberg (The Netherlands Cancer Institute, Amsterdam, Netherlands), respectively. Possible upregulation of the 9EG7 epitope by Mn²⁺ or recombinant adhesive modules III7-10 of fibronectin (41) was assessed by flow cytometry (4).

Metabolic Labeling and Immunoprecipitation

The cells were given methionine-free DMEM, then L-[³⁵S]methionine (100 μ Ci/ml medium) (Dupont NEN, Boston, MA) and incubated for various time periods. After incubation, conditioned media were collected, and cells were solubilized with nonionic detergent buffer (1% [vol/vol] Triton X-100, 0.05% [vol/vol] Tween 20, 150 mM NaCl, 2 mM PMSF, 5 μ g/ml leupeptin, 0.1 μ g/ml pepstatin A, 0.4 mM pefabloc SC, and 20 mM Tris-HCl, pH 7.4). Extracellular matrix proteins were immunoprecipitated with rabbit antibodies from metabolically labeled, conditioned media as previously described (58). β 1A integrins were immunoprecipitated from cellular lysates with MB1.2 or 9EG7 by an established protocol (60). The amounts of antibodies used were sufficient to precipitate all of the target antigen.

Cell Adhesion Assay

Cell adhesion assays were performed as previously described (15). Vitronectin (7), fibronectin (43), or laminin-1 (Sigma Chemical Co., St. Louis, MO) were coated at 2–10 μ g/ml onto wells of a 96-well plate, and the wells were blocked with 2% BSA in PBS containing Ca²⁺ and Mg²⁺. The cells were then allowed to attach to wells for 60 min. After adhesion, nonadhered cells were removed by washing, and adhered cells were quantified by colorimetric detection at 595 nm after staining with 1% bromophenol blue.

Binding Assays

Binding of iodinated or FITC-labeled human plasma fibronectin or the 70-kD NH₂-terminal fragment were assayed as described (43, 72).

Immunofluorescence

Immunofluorescence studies were performed as described previously (57). Cells were seeded in serum-containing medium and grown on glass coverslips for 3 d. Alternatively, cells were seeded onto glass coverslips coated with vitronectin, fibronectin, or laminin-1 and incubated in DME containing 0.2% BSA for 4 h at 37°C. For double staining of fibronectin and β 1 integrin, cells were incubated with FITC-labeled fibronectin in the presence of 1-oleoyl-lysophosphatidic acid (LPA)¹ (Avanti Polar Lipids, Alabaster, AL) for an additional 1 h at 37°C. Cells were then fixed with 3.5% (wt/vol) paraformaldehyde in phosphate buffer, pH 7.4. Before staining of intracellular antigens, the cells were permeabilized with 0.2% Triton X-100 in TBS. After incubation with 3% BSA in TBS to block non-specific protein binding, the fixed cells were incubated with rabbit antisera to FITC or fibronectin, and/or monoclonal antibody to paxillin, vinculin, or β 1, and then treated with FITC anti-mouse IgG, FITC anti-rabbit IgG, lissamine rhodamine B sulfonyl chloride (LRSC)-labeled anti-rat IgG, or rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR). Monoclonal antibodies against human vinculin (clone hVIN-1) and chick paxillin were from Sigma Chemical Co. and Transduction Laboratories (Lexington, KY), respectively. β 1 integrin was identified with antibody MB1.2. FITC- or LRSC-conjugated, affinity-purified secondary antibodies were from Jackson ImmunoResearch Labs. Inc. (West Grove, PA). Controls were done to insure the species specificity of the secondary antibodies. Photographs were taken with an Olympus BX-60 epifluorescence microscope using additional emission filters that allowed the FITC and LRSC or rhodamine fluorochromes to be visualized individually or simultaneously (Chroma Technology, Brattleboro, VT).

Cell Migration

Cell migration assays were performed in modified Boyden chambers containing Nucleopore polycarbonate membranes (5- μ m pore size; Costar Corp., Cambridge, MA). The filters were soaked overnight in a 10 μ g/ml solution of vitronectin, fibronectin, or laminin-1 or 100 μ g/ml gelatin, briefly rinsed with PBS containing 0.2% BSA, air dried, and then placed in the chamber. EGF or PDGF at concentrations of 3–100 ng/ml in DME containing 0.2% BSA was added to the lower compartment of the chambers. Cells suspended in DME containing 0.2% BSA were introduced into the upper compartment of the chamber. The chambers were then incubated for 6 h at 37°C. The filters were fixed and stained, and the cells that had migrated to the lower surface were counted at 400 \times magnification. Two areas from each of two cells were counted. Values are the mean \pm SD of cells per 0.16-mm² field. EGF and PDGF were from Upstate Biotechnology (Lake Placid, NY) and R&D Systems, Inc. (Minneapolis, MN), respectively.

Cell Motility

The cell motility assay was adapted from an established method (50). Vitronectin, fibronectin or laminin-1, 10 μ g/ml, was coated onto wells of a 96-well plate, and the wells were blocked with 2% BSA in PBS containing Ca²⁺ and Mg²⁺. Yellow polystyrene monodispersed particles (1.0- μ m-diam polybeads; Polysciences, Inc., Warrington, PA) were suspended with DME, sonicated three times, and then added to wells. The plates were centrifuged at 1,400 rpm for 25 min and incubated overnight at 37°C. Cells (500 cells/well) suspended in DME containing 0.2% BSA in the absence or presence of motility factors, PDGF or EGF, 3–100 ng/ml, were introduced into wells and incubated for 16 h at 37°C. After incubation, the contents of the wells were fixed with 10% (vol/vol) glutaraldehyde, wells were washed, and clearing of beads by motile cells was assessed by phase microscopy.

Presentation of Results

Experiments were done on at least three separate occasions over an 8-month period on clones of cells selected to give maximal expression in the case of the P781A, T788P, P793A, or P781,793A mutants or expression similar to

1. *Abbreviations used in this paper:* FAK, focal adhesion kinase; LPA, lysophosphatidic acid; LRSC, lissamine rhodamine B sulfonyl chloride.

	7		7	7	7	7
	5		8	8	8	9
	9		1	3	8	3
	A		A	F	P	A
β1A	KLLMLIIHDRREFAKFEKEKMNKAWDTGENPIYKSAVTTVVNPKYEGK					
β1D	KLLMIIDRRREFAKFEKEKMNKAWDTQENPIYKSPINNFKNPNYGRKAGL					
β2	KALIHLSDLREYRRFEKEKLSQWNNND.NPLPKSATTTVMNPKFAES					
β3	KLLITIDHRKEFAKFEERARAKWDTANNPLYKEATSTFTNITYRGT					
β5	KLLVTIHDRREFAKFQSESRARAYEMASNPLYRKPISHTVDFTFNFKFNKSYNGITVD					
β6	KLLVSPHDKREKVAKFEAERSKAKWQTGTNPLYRGSTSTFKNVTYKHREKQKVDLSTDC					
β7	RLSVEIYDRREYSRFEKEQQQLNWKQDSNPLYKSAITTTINPRFQEADSPTE					

Figure 1. Deduced amino acid sequence (in single letter code) of the intracellular domain of murine $\beta 1A$ and comparable domains of $\beta 1D$, $\beta 2$, $\beta 3$, $\beta 5$, $\beta 6$, and $\beta 7$. The sequences are aligned with lysine (K) at position 752 of mature $\beta 1A$. The residues mutated in $\beta 1A$ and homologous residues in other β subunits are shown in bold. The mutations are depicted above the $\beta 1A$ sequence.

GD25- $\beta 1A$ cells in the case of the D759A, Y783F, Y795F, or Y783,795F mutants. The cell migration assays and microscopic analysis of microfilament bundles and focal contacts were repeated on independently derived clones of D759A and Y783,795F cells. The experiments and photomicrographs shown were chosen as representative of differences constantly observed among cells expressing the different $\beta 1A$ s.

Results

We characterized the effects of Tyr to Phe mutations in the NPXY motifs of $\beta 1A$ in mouse GD25 cells, which lack

expression of $\beta 1$ integrin heterodimers because of targeted knockout of the $\beta 1$ gene (23, 67). We also created point mutations that would be expected, on the basis of previous studies described in the Introduction, to enhance or interfere with integrin activity (Fig. 1).

Analysis of Integrins and Cell Attachment

Expression of cell surface $\beta 1A$ and associated $\alpha 5$ and $\alpha 6$ subunits was analyzed by flow cytometry (Fig. 2). Transfection of $\beta 1A$ mutants resulted in appearance of cell surface $\alpha 5$ and upregulation of cell surface $\alpha 6$, as previously reported for GD25- $\beta 1A$ cells expressing wild-type $\beta 1A$ (23, 67). Binding of monoclonal antibody 9EG7, which recognizes an extracellular epitope of $\beta 1$ that can be upregulated by Mn^{2+} or ligand, was compared with the binding of a second anti- $\beta 1$ monoclonal antibody, MB1.2, as an index for conformationally active integrins. For GD25- $\beta 1A$ cells and cells expressing D759A, Y783F, Y795F, or double Y783,795F mutant $\beta 1A$, fluorescence resulting from binding of 9EG7 was 80–100% of that due to MB1.2 binding. Attempts to upregulate the 9EG7 epitope further with Mn^{2+} or recombinant adhesive modules III-7-10 of fibronectin failed (not shown). For each of the four mutants, cell surface $\beta 1A$ was expressed at a level comparable to that of GD25- $\beta 1A$ cells (67) in at least 6 of the ~ 50 clones tested. Metabolic labeling and immunoprecipitation analysis confirmed that the production level of the D759A or Y783,795F

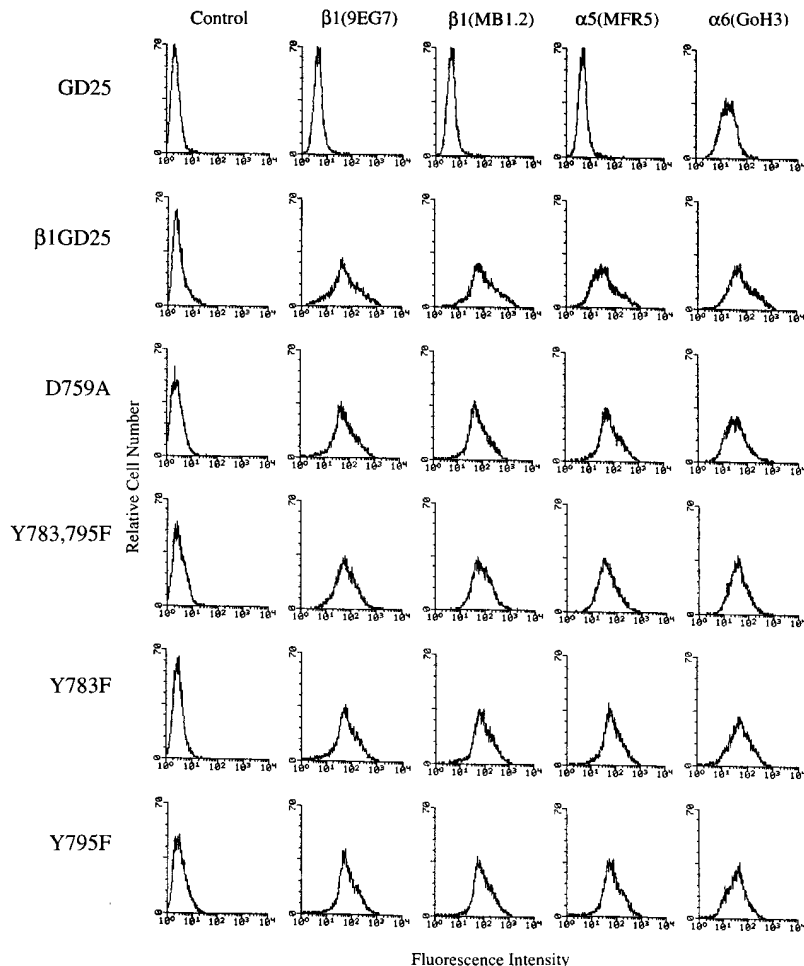


Figure 2. Expression of $\beta 1A$ and associated $\alpha 5$ and $\alpha 6$ subunits in $\beta 1$ -deficient GD25, wild-type, and mutant $\beta 1A$ cells by flow cytometry. *Control*, no primary antibody; *$\beta 1(9EG7)$* , and *$\beta 1(MB1.2)$* , antibodies to $\beta 1$; *$\alpha 5(MFR5)$* , antibody to $\alpha 5$; *$\alpha 6(GoH3)$* , antibody to $\alpha 6$; *GD25*, $\beta 1$ -deficient cells; *$\beta 1GD25$* , GD25 cells expressing wild-type $\beta 1A$; other cells are designated by mutation(s).

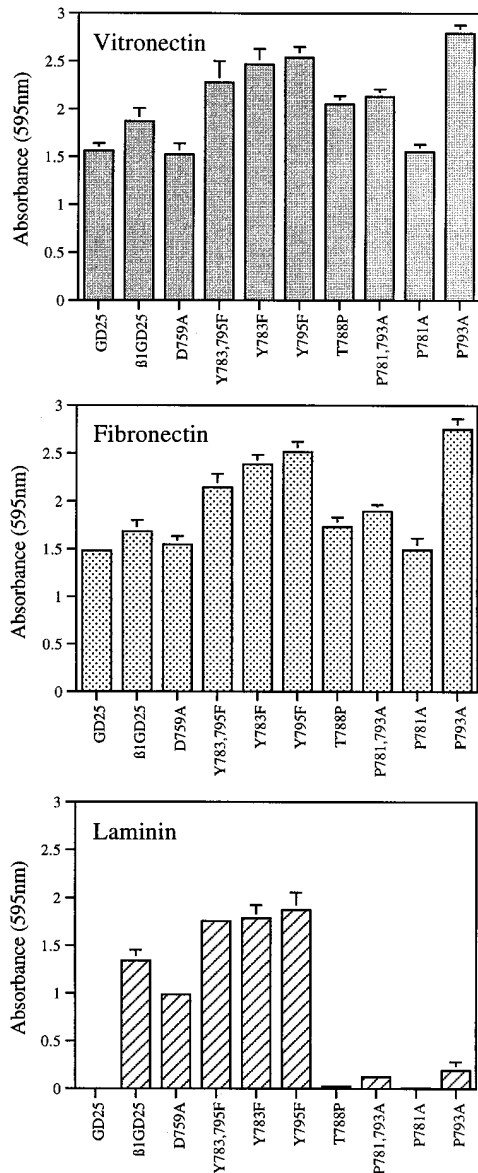


Figure 3. Attachment of wild-type and mutant β 1A cells on vitronectin, fibronectin, and laminin-1. Bars represent the mean of attachment activity quantified by spectrophotometric analysis at OD = 595 nm after staining of adherent cells with bromophenol blue. Error bars represent \pm SD of quadruplicate experiments. Absorbance resulting from nonspecific cell adhesion as measured on BSA-coated wells was \sim 0.05, and has been subtracted. *GD25*, β 1-deficient cells; *β 1GD25*, GD25 cells expressing wild-type β 1A; other cells are designated by mutation(s).

mutant β 1A was not different from production of wild-type β 1A in the GD25- β 1A cells (not shown).

Binding of 9EG7 to cells expressing P781A, T788P, P793A, or double P781,793A mutant β 1As, in contrast, was $<$ 50% of MB1.2 binding (not shown). In addition, we were unable to find cells expressing P781A, T788P, P793A or double P781,793A mutant β 1As that bound MB1.2 at $>$ 30% of the expression level on GD25- β 1A cells despite testing \sim 50 (P781A and P793A) or \sim 100 (T788P and P781,793A) clones. The flow cytometry results, therefore,

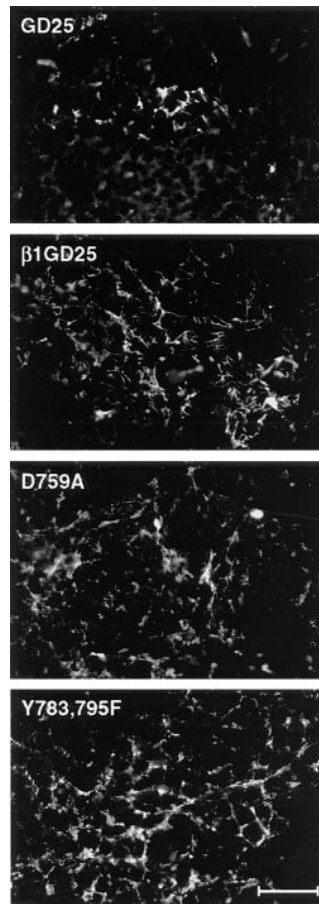


Figure 4. Immunofluorescence of fibronectin matrix after 3 d of culture in serum-containing medium. *GD25*, β 1-deficient cells; *β 1GD25*, GD25 cells expressing wild-type β 1A; other cells are designated by mutation(s). Bar, 60 μ m.

indicate that wild-type β 1A and the D759A, Y783F, Y795F, and double Y783,795F mutant β 1As are all expressed in an active state by GD25 cells. In contrast, the P781A, T788P, P793A, and double P781,793A mutant β 1As are expressed less well and in a less active state.

Transfection and selection of D759A or double Y783,795F mutant β 1A resulted in variable downregulation of cell surface α v and β 3 when compared with the parent GD25 cells (not shown). Similar results were obtained previously using surface iodination of GD25 and GD25 β 1A cells (67).

Cell adhesion studies were carried out to characterize further the activity of the mutant integrins. Cells transfected with any of the set of β 1As that resulted in higher ratios of 9EG7/MB1.2 binding adhered vigorously to laminin-1, whereas untransfected GD25 cells or GD25 cells transfected with the P781A, T788P, P793A, or P781,793A mutant attached and spread poorly on cell culture plastic coated with laminin-1 (Fig. 3). Adherence to laminin-1 was blocked by antibody GoH3 to α 6 β 1 (not shown). GD25 cells or each of the transfectants attached and spread on vitronectin or fibronectin (Fig. 3), consistent with previous studies showing that adhesion of GD25 cells to fibronectin can be mediated by either the α v β 3 vitronectin receptor or the α 5 β 1 fibronectin receptor (23, 67). These results indicate that substitution of Ala for Pro in the NPXY motifs or the thrombasthenia-like Thr-to-Pro substitution in the sequence between the two motifs results in poorly expressed integrins with little or no α 6 β 1-mediated cell adhesive activity for laminin-1. Integrins

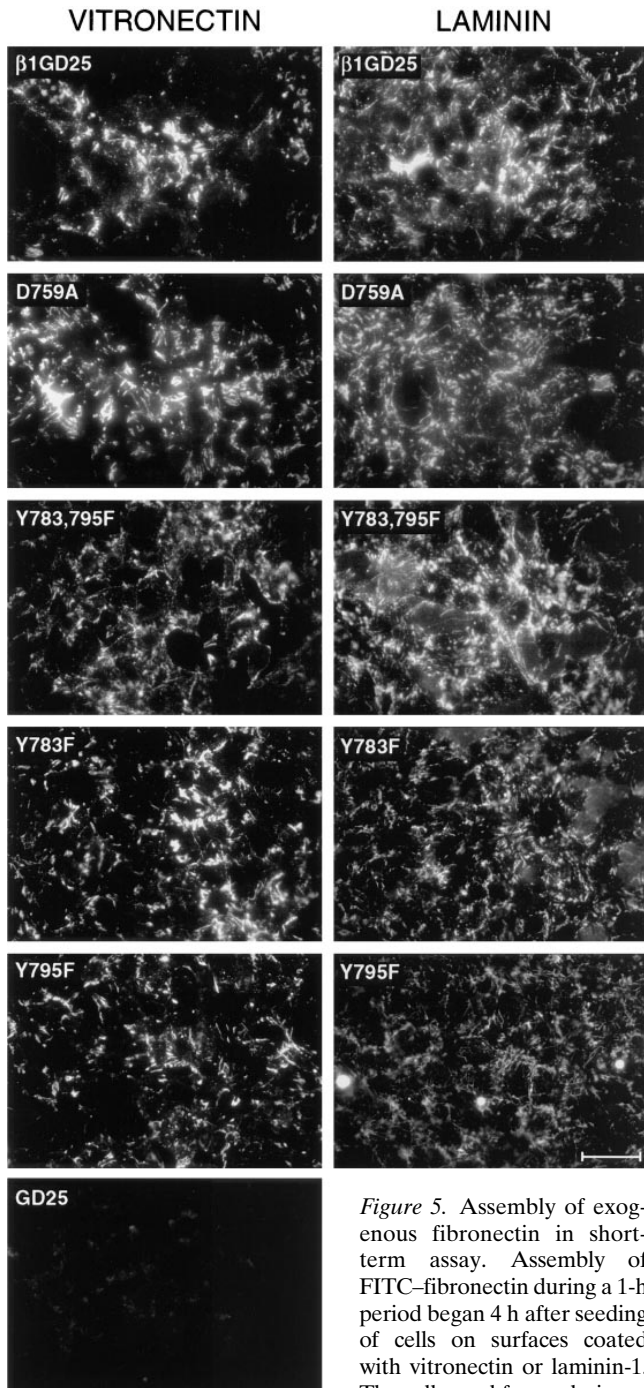


Figure 5. Assembly of exogenous fibronectin in short-term assay. Assembly of FITC-fibronectin during a 1-h period began 4 h after seeding of cells on surfaces coated with vitronectin or laminin-1. The cells used for analysis are indicated in each figure. Bar, 60 μm .

formed by $\beta 1A$ carrying the D759A mutations or substitution of Phe for Tyr in the NPXY motifs, in contrast, were at least as active as those formed by wild-type $\beta 1A$.

Fibronectin Matrix Assembly by Cells Expressing Mutant $\beta 1A$

Fibronectin assembly is enhanced by experimental manipulations that activate $\beta 1$ or $\beta 3$ integrins, including activating antibodies, truncation of the α subunit cytoplasmic do-

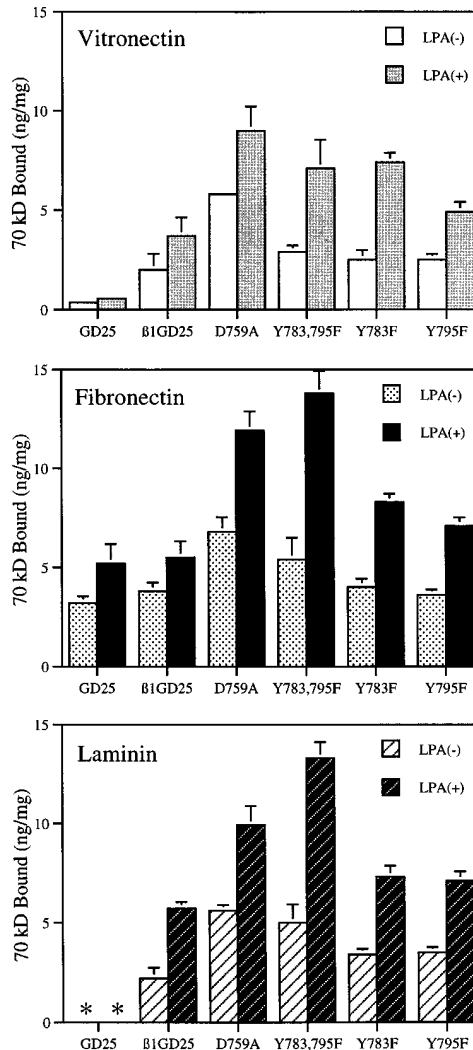


Figure 6. Binding of the 70-kD NH_2 -terminal fragment of fibronectin to cells seeded on surfaces coated with vitronectin, fibronectin, or laminin-1. Symbols represent the mean of specific binding. Bars represent mean \pm SD of triplicate experiments with duplicate determinations in each experiment ($n = 6$). GD25, $\beta 1$ -deficient cells; $\beta 1GD25$, GD25 cells expressing wild-type $\beta 1A$; other cells are designated by mutation(s). *, $\beta 1$ -deficient GD25 cells could not be studied on laminin-1 coating because too few cells adhered (see Fig. 3).

main, and mutation of the membrane-proximal Asp to disrupt the interaction between α and β cytoplasmic domains (32, 69). To learn whether expression of $\beta 1A$ s with Tyr to Phe mutations in the NPXY motifs also results in hyperactive integrins, we studied assembly of fibronectin by monolayers of transfected cells. After 3 d in 10% FBS, fibronectin was deposited in a fibrillar pattern around most cells expressing wild-type $\beta 1A$ or D759A or double Y783,795F mutant $\beta 1A$ (Fig. 4). Fibronectin deposition was more patchy in cultures of GD25 cells in that foci of cells had copious matrix whereas nearby cells had none (Fig. 4). No differences were found between GD25 cells and GD25 cells expressing wild-type $\beta 1A$ or among cells expressing wild-type or mutant $\beta 1A$ s in the secretion of fibronectin, or of laminin-1 or tenascin-C, when media of

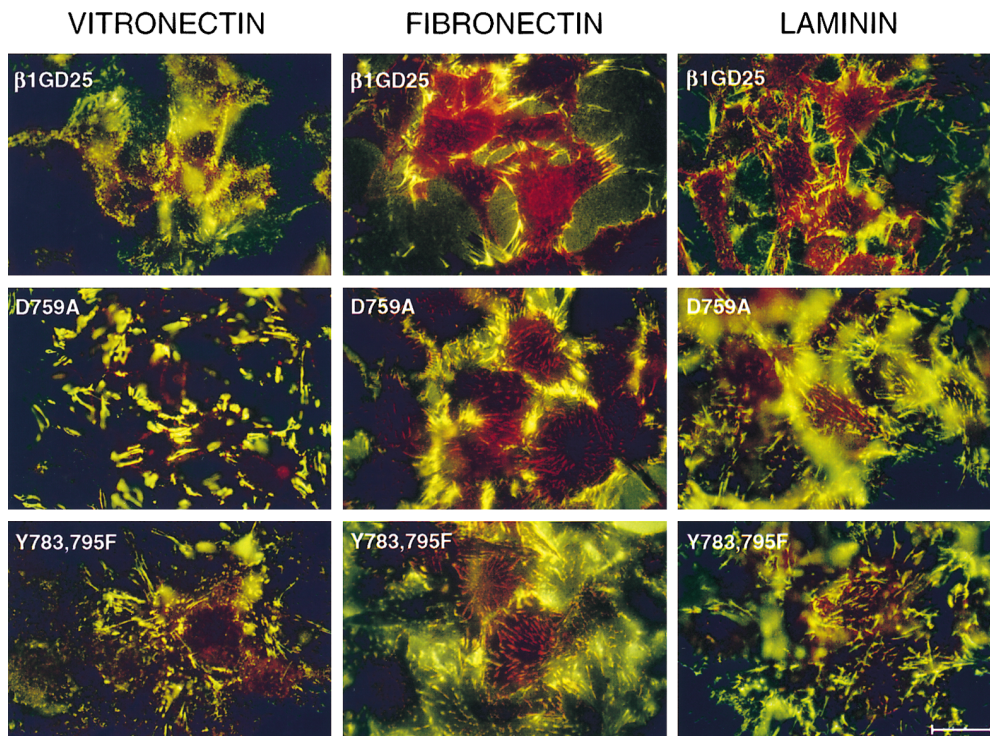


Figure 7. Double fluorescence of $\beta 1$ integrin (red) and FITC-labeled fibronectin (green). Cells were incubated for 4 h on a fibronectin-, laminin-, or vitronectin-coated substratum and additional 1 h with FITC-fibronectin. After fixation with paraformaldehyde, staining for $\beta 1$ integrin was performed using $\beta 1$ antibody MB1.2 and LRSC-labeled anti-rat IgG. Samples were viewed with an emission filter that allowed visualization of both fluorochromes and photographed focusing on the interface of cells and substrate. *GD25*, $\beta 1$ -deficient cells; *$\beta 1GD25$* , *GD25* cells expressing wild-type $\beta 1A$; other cells are designated by mutation(s). Bar, 25 μm .

cells incubated with [^{35}S]methionine were analyzed by immunoprecipitation (not shown).

We also analyzed fibronectin matrix deposition in short-term experiments. Fig. 5 shows fluorescence due to deposition over a 1-h period of FITC-labeled plasma fibronectin added to cells in plates coated with vitronectin or laminin-1. The vitronectin coating supported extensive network of assembled fibronectin formed around cells expressing wild-type $\beta 1A$ or *D759A*, *Y783F*, *Y795F*, or *Y783,795F* mutant $\beta 1A$ (Fig. 5). In contrast, untransfected *GD25* (Fig. 5) cells or cells expressing inactive *T788P* or *P781,793A* mutant $\beta 1A$ (not shown) did not assemble fibronectin over this time period when cultured on vitronectin. Thus, assembly of a matrix by *GD25* cells cultured short-term on vitronectin requires active $\beta 1$ integrins. Fibronectin deposition around cells expressing $\beta 1A$ or the *D759A* mutation or the Phe for Tyr substitutions, whether cultured on vitronectin or laminin-1, consistently stained at least as intensely as cells expressing wild-type $\beta 1A$ (Fig. 5).

The qualitative fluorescence microscopic observations in Fig. 5 were buttressed by assays of binding of [^{125}I]-labeled, 70-kD NH_2 -terminal fragment of fibronectin (Fig. 6). This fragment is a suitable probe to quantify the ability of cells to assemble fibronectin, inasmuch as the fragment and intact fibronectin bind to cell layers with the same affinities, cross-compete for binding, and can be cross-linked to the same large apparent molecular mass target in cell layers (46, 71). Cells expressing *D759A* or *Y783,795F* mutant $\beta 1A$ bound 1.5- to 2.5-fold more 70-kD fragment than cells expressing wild-type $\beta 1A$. Binding to cells expressing $\beta 1A$ with the single Tyr mutation was intermediate between binding to cells expressing $\beta 1A$ with the double Tyr mutations and cells expressing wild-type $\beta 1A$. Increased binding was found regardless of whether cells were cul-

tured on vitronectin, fibronectin, or laminin-1 and was enhanced by LPA, which causes a coordinated change in cell shape, and upregulation of binding of fibronectin or the 70-kD fragment (45, 72).

Cellular Localization of $\beta 1A$ in Relationship to Assembled Fibronectin

Activated integrins are required in the process of fibronectin matrix assembly (69). We therefore analyzed cellular localization of wild-type $\beta 1A$ or *D759A* or *Y783,795F* mutant $\beta 1A$ in relationship to assembled fibronectin by fluorescence microscopy of cells cultured on vitronectin, fibronectin, or laminin-1 and incubated with FITC-fibronectin before fixation and staining for $\beta 1$ (Fig. 7). These three cell types in general behaved in the same manner on a given substratum. However, they showed different subcellular localization of $\beta 1A$ that depended upon the different substratum. In cells adherent to vitronectin, $\beta 1A$ did not localize to focal contacts but rather to assembled fibronectin. In cells adherent to fibronectin, some $\beta 1A$ localized to focal contacts of cells, and some was associated with assembled fibronectin, especially around cells expressing wild-type $\beta 1A$ and, to a lesser extent, around cells expressing *D759A* or *Y783,795F* mutant $\beta 1A$. $\beta 1A$ localized to focal contacts of all three cell types when cultured on laminin-1. These focal contacts were linear and often associated with FITC-fibronectin.

Cell Motility and Migration of Cells Expressing Wild-Type $\beta 1A$ or Mutant $\beta 1A$ s

Motility of *GD25* cells and the various transfectants was assessed by an assay in which moving cells displace polystyrene beads layered onto surfaces coated with vitrone-

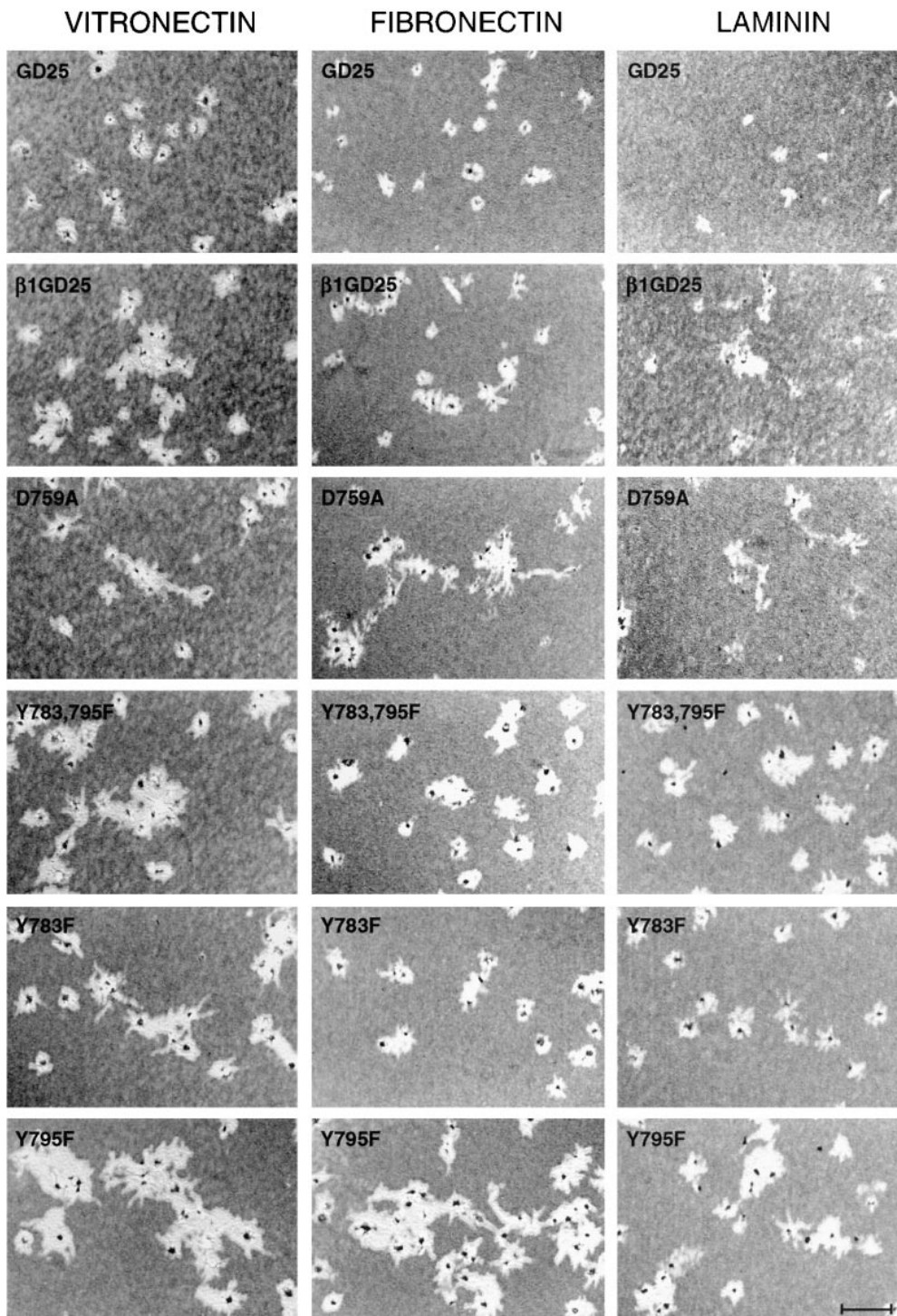


Figure 8. Cell motility as assessed by clearing of beads layered on vitronectin-, fibronectin-, or laminin-1-coated surfaces. Cells were added in the presence of PDGF, 10 ng/ml. Wells were fixed 16 hours after seeding and photographed by phase microscopy. Cells, which were covered with beads, appear as dark specks in cleared-out areas. *GD25*, $\beta 1$ -deficient cells; *$\beta 1GD25$* , *GD25* cells expressing wild-type $\beta 1A$; other cells are designated by mutation(s). Bar, 240 μm .

tin, fibronectin, or laminin-1 (Fig. 8). *GD25* cells cultured in the presence of PDGF (Fig. 8) or EGF (not shown) demonstrated little motility. *GD25* cells expressing wild-type $\beta 1A$ or various mutants of $\beta 1A$ were not motile in the absence of PDGF or EGF (not shown). Addition of PDGF (Fig. 8) or EGF (not shown) caused greater motility of $\beta 1A$ -expressing cells, that is, a greater area around cells was cleared of beads. The areas cleared by cells expressing wild-type $\beta 1A$ or the *D759A* mutant tended to be asymmetrical and irregular whereas areas cleared by cells

expressing the Phe for Tyr substitutions, especially *Y795F*, were rounder. These patterns were found regardless of which adhesive protein (vitronectin, fibronectin, or laminin-1) was present on the surface and which motility agent (EGF or PDGF) was present in the medium.

Transwell migration assays were performed in blind-well chambers fitted with filters coated with vitronectin, fibronectin, or laminin-1. In the absence of EGF or PDGF, 4- to 10-fold more cells moved across filters coated with fibronectin (Fig. 9) than filters coated with gelatin (<10

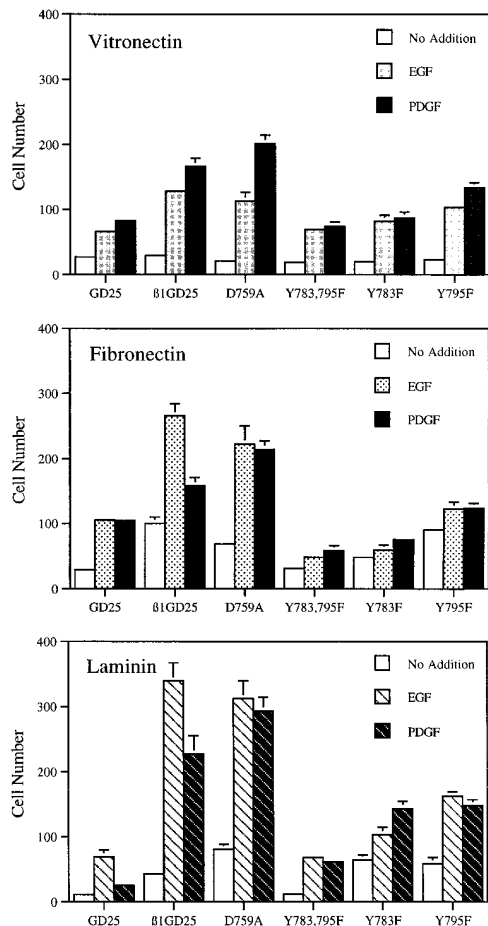


Figure 9. Cell migration through vitronectin-, fibronectin-, or laminin-1-coated filters in response to EGF or PDGF. EGF (100 ng/ml) or PDGF (10 ng/ml), was in the lower chamber. Each bar represents the mean of cell number per 0.16-mm² field. Error bars indicate \pm SD of quadruplicate determinations. GD25, β 1-deficient cells; β 1GD25, GD25 cells expressing wild-type β 1A; other cells are designated by mutation(s).

cells/0.16 mm²; not shown). This increase is presumed to represent a haptotactic response. The haptotactic responses to vitronectin were both less in magnitude and less variable than the haptotactic response to fibronectin, whereas the haptotactic responses to laminin-1 were equally variable (Fig. 9). GD25 and Y783,795F cells did not demonstrate haptotaxis through laminin-1-coated filters.

In response to EGF or PDGF, GD25 cells expressing wild-type β 1A or the D759A mutant migrated two- to fourfold more than GD25 cells lacking β 1A (Fig. 9). These results indicate that migration is enhanced by β 1A integrins interacting with the adhesive substrates coating the filter, probably α v β 1A with vitronectin, α 6 β 1A with laminin-1, and α 5 β 1A with fibronectin. In response to EGF, D759A cells and cells expressing wild-type β 1A migrated equivalently. In response to PDGF, D759A cells migrated better than cells expressing wild-type β 1A. Y783,785F cells, in contrast, migrated much less than cells expressing wild-type β 1A and no more than cells lacking β 1A. Y783F and Y795F cells had migratory behavior that was intermediate between Y783,795F cells and cells expressing wild-type β 1A. In all experiments, however, migration of cells

expressing the Y783F mutation was less than migration of cells expressing the Y795F mutation. Differences in migration ability among cells expressing various mutant β 1As were found over a 30-fold range of concentrations (3–100 ng/ml) of both of the chemotactic agents (not shown).

In the bead clearing assay, Y795F cells were most active, and the Y783,795F cells were as active as cells expressing wild-type β 1A (Fig. 8). The discrepancy in behavior between the bead clearing assay and the chemotaxis assay shown in Fig. 9 was investigated by checkerboard analysis in which different concentrations of chemotactic agent are added to the upper and lower chamber of the apparatus (Table I). Such an analysis differentiates directed migration across the filter in response to a gradient of chemotactic agent (chemotaxis) from increased random motility because of the presence of the chemotactic agent per se (chemokinesis). Cells expressing wild-type β 1A and the D759A mutant responded to a gradient of PDGF by chemotaxis but demonstrated only a low and variable chemokinetic response to increasing concentrations of PDGF in both chambers. The chemokinetic responses of wild-type and D759A cells were more than twofold less than the chemotactic responses. Migration of Y795F cells, in contrast, was greater when PDGF was present in both chambers than when there was a gradient of PDGF, indicating that the chemokinetic response was the main effect. Migration of Y783,795F and Y783F cells was low in magnitude and variable between fibronectin- and vitronectin-coated filters, making it difficult to classify the migration as chemotaxis or chemokinesis.

Focal Contact Formation and Actin Cytoskeleton Organization in Cells Expressing Wild-Type β 1A or Mutant β 1As

Cells expressing wild-type β 1A and the D759A and Y783,795F mutants were analyzed for focal contacts with antipaxillin (Fig. 10 A) and antivinculin (Fig. 10 B) and for F-actin-containing cytoskeleton with rhodamine phalloidin (Fig. 10 B). As assessed by paxillin or vinculin staining, focal contacts formed by D759A cells on fibronectin were larger, coarser, and more numerous than focal contacts formed by cells expressing wild-type β 1A. More numerous focal contacts were also formed by Y783,795F cells on fibronectin. Compared with the focal contacts formed by D759A cells, focal contacts of Y783,795F cells were finer and more uniform in size. The differences in the patterns of paxillin and vinculin staining among the three cell types on fibronectin were similar to the difference in staining patterns of β 1A (Fig. 7; more obvious in studies not shown in which only single fluorescence for β 1 was done). The difference between vinculin-containing focal contacts of D759A and Y783,795F cells was also found when the cells were cultured on vitronectin-coated surfaces (Fig. 10 B) even though β 1A could not be demonstrated in focal contacts (see Fig. 7). On the vitronectin-coated substratum, vinculin-staining focal contacts of cells expressing wild-type β 1A were coarser and more heterogeneous than wild-type cells on the other substrates. On a laminin-1-coated substratum, more vinculin-staining focal contacts were present in cells expressing wild-type β 1A or the D759A mutant than in Y783,795F cells.

Table I. Checkerboard Analysis of PDGF-induced Cell Migration of $\beta 1$ -deficient GD25, and GD25 Wild-Type or Mutant $\beta 1A$

	Vitronectin			Fibronectin			
	Upper compartment			Upper compartment			
Concentration of PDGF	0	10	30	Concentration of PDGF	0	10	30
GD25				GD25			
Lower compartment	<i>ng/ml</i>			Lower compartment	<i>ng/ml</i>		
0	34 ± 4	37 ± 4	35 ± 2	0	27 ± 2	31 ± 2	34 ± 4
10	87 ± 10	88 ± 8	90 ± 5	10	90 ± 2	60 ± 3	56 ± 3
30	105 ± 6	104 ± 5	114 ± 3	30	72 ± 6	67 ± 4	75 ± 5
$\beta 1$GD25				$\beta 1$GD25			
Lower compartment				Lower compartment			
0	37 ± 6	48 ± 3	55 ± 7	0	84 ± 4	50 ± 6	32 ± 2
10	155 ± 12	76 ± 12	56 ± 3	10	129 ± 7	57 ± 3	36 ± 6
30	198 ± 10	91 ± 9	63 ± 6	30	105 ± 3	78 ± 4	58 ± 5
D759A				D759A			
Lower compartment				Lower compartment			
0	36 ± 2	43 ± 3	52 ± 3	0	73 ± 4	53 ± 3	37 ± 5
10	206 ± 4	127 ± 13	96 ± 5	10	172 ± 17	109 ± 12	83 ± 8
30	221 ± 13	144 ± 7	104 ± 4	30	242 ± 14	165 ± 8	103 ± 6
Y783, 795F				Y783, 795F			
Lower compartment				Lower compartment			
0	25 ± 2	28 ± 4	18 ± 2	0	31 ± 2	26 ± 2	31 ± 2
10	51 ± 2	32 ± 2	25 ± 2	10	59 ± 3	48 ± 2	46 ± 5
30	44 ± 3	34 ± 4	37 ± 3	30	56 ± 3	50 ± 3	57 ± 3
Y783F				Y783F			
Lower compartment				Lower compartment			
0	25 ± 2	29 ± 3	31 ± 5	0	44 ± 3	41 ± 2	38 ± 4
10	87 ± 6	43 ± 4	35 ± 3	10	70 ± 2	60 ± 3	52 ± 5
30	61 ± 4	42 ± 5	32 ± 3	30	62 ± 6	61 ± 3	69 ± 3
Y795F				Y795F			
Lower compartment				Lower compartment			
0	30 ± 3	54 ± 4	62 ± 4	0	58 ± 8	84 ± 8	92 ± 4
10	117 ± 8	123 ± 5	114 ± 4	10	102 ± 3	122 ± 7	130 ± 9
30	120 ± 3	125 ± 8	129 ± 16	30	112 ± 7	125 ± 5	144 ± 12

Cell migration assays through fibronectin- or vitronectin-coated filters were performed as described in Materials and Methods except that variable concentrations of PDGF were added to the upper as well as the lower chamber. Results are expressed as the mean ± SD ($n = 4$) of cells that had migrated to the lower surface of the filter. GD25, $\beta 1$ -deficient cells; $\beta 1$ GD25, GD25 cells expressing wild-type $\beta 1A$; other cells are designated by mutation(s).

Cells expressing wild-type $\beta 1A$ or the D759A mutant, when stained with rhodamine phalloidin, contained thick cables that ran through most of the length of the cells (Fig. 10 B). These cables terminated in the coarse vinculin-staining focal contacts. The F-actin network of Y783,795F cells was more peripheral than in cells expressing wild-type $\beta 1A$ or the D759A mutant and terminated in the fine vinculin-staining focal contacts. The differences in F-actin distribution were noted on all three substrata. For each of the transfected cell types, however, the F-actin network was less when cells were on vitronectin. Each of the transfected cell types had a better developed F-actin network than nontransfected GD25 cells on vitronectin or fibronectin (Fig. 10 B).

Discussion

We studied eight cytoplasmic mutant of $\beta 1A$ —four active (D759A, Y783F, Y795F, and Y783,795F) and four inactive (P781A, T788P, P793A, and P781,793A) as judged by reactivity with the 9EG7 anti- $\beta 1$ antibody, adhesion to laminin-1, and ability to support fibronectin assembly. (Recently, it was reported that human foreskin fibroblasts do not assemble fibronectin when cultured on vitronectin

[18]. In our hands, human foreskin fibroblasts do assemble fibronectin when cultured on vitronectin, and the inability to assemble fibronectin when cultured on vitronectin is a specific property of cells deficient in active $\beta 1$ integrin [Zhang, Q., T. Sakai, R. Fässler, J. Nowlen, and D.F. Mosher, manuscript in preparation]). The active $\beta 1A$ s were associated with distinctive cellular phenotypes. D759A cells had thick F-actin-containing microfilaments that terminated in coarse vinculin-containing focal contacts. Y783,795F cells had thinner and more peripheral F-actin-containing microfilaments that terminated in fine focal contacts. The cytoskeletal phenotypes were found when cells were on vitronectin-coated substrates, in which $\beta 1A$ was associated with assembling fibronectin, as well as on fibronectin- or laminin-1-coated substrates, in which $\beta 1A$ was associated both with focal contacts and assembling fibronectin. Motility phenotypes were associated with the cytoskeletal phenotypes. D759A cells were as active as cells expressing wild-type $\beta 1A$ in chemotaxis assays whereas Y783,795F cells were no more active than cells lacking $\beta 1A$ and Y795F demonstrated a strong chemokinetic response.

Cell migration is a complex process that involves lamellipodial extension, generation of intracellular force, inte-

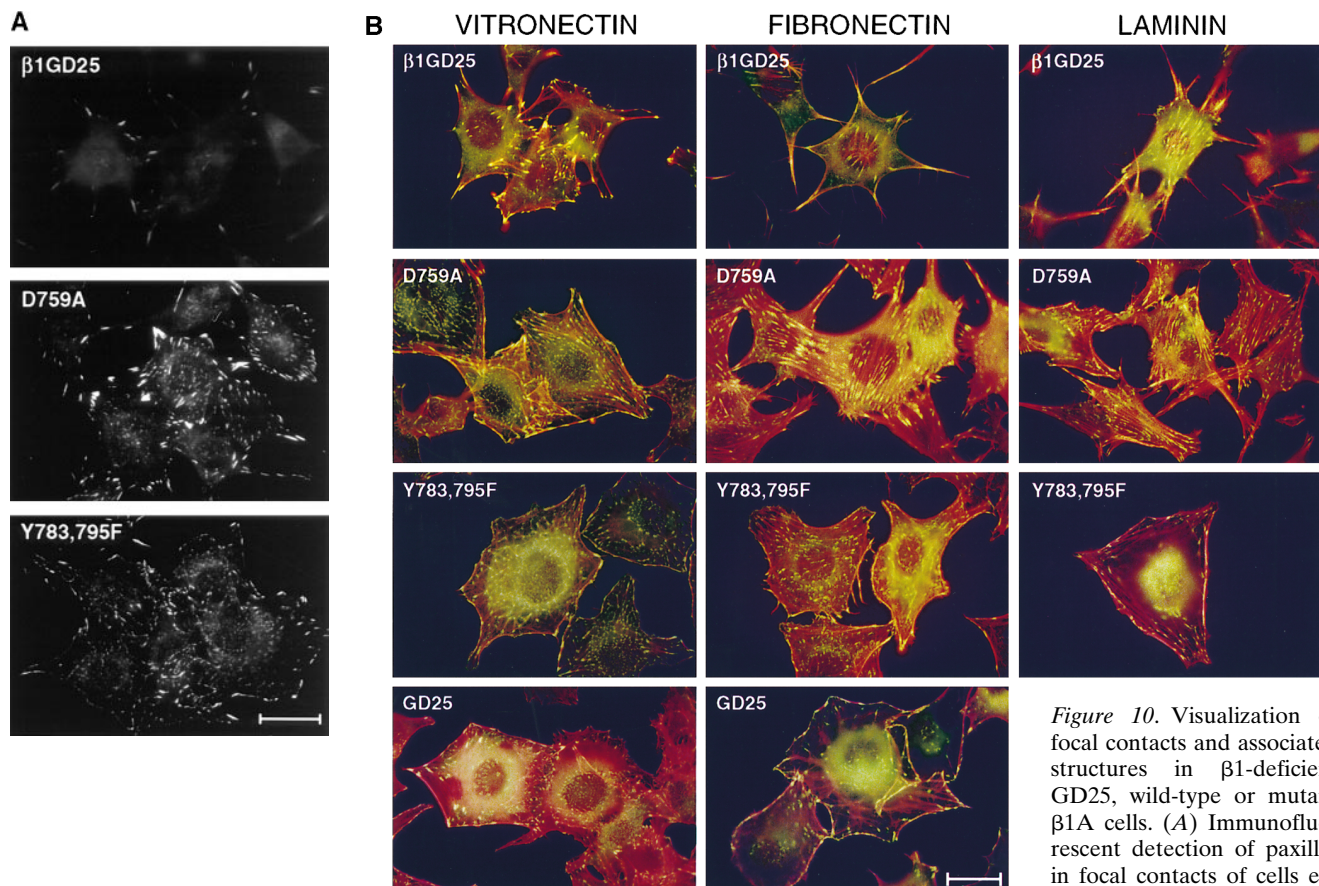


Figure 10. Visualization of focal contacts and associated structures in $\beta 1$ -deficient GD25, wild-type or mutant $\beta 1A$ cells. (A) Immunofluorescent detection of paxillin in focal contacts of cells expressing wild-type $\beta 1A$, or

D759A, or Y783,795F mutations. Cells were cultured for 4 h on a fibronectin-coated substratum. (B) Double fluorescence of vinculin (green) and rhodamine-phalloidin (red). Cells were incubated for 4 h on a fibronectin-, laminin-1-, or vitronectin-coated substratum. GD25, $\beta 1$ -deficient cells; $\beta 1GD25$, GD25 cells expressing wild-type $\beta 1A$; other cells are designated by mutation(s). Bar, 25 μm .

grin clustering in focal contacts, integrin avidity, and integrin signaling (34, 39, 51, 61). Focal contacts are enriched in a large number of kinases, at least one phosphatase, and various adaptor and connector proteins (11, 22, 27, 38, 44, 70). Phe lacks the hydroxyl group that is the acceptor site for tyrosine phosphorylation. The results suggest, therefore, that conversion of tyrosines between nonphosphorylated and phosphorylated states is critical for movement on adhesive substrates (Fig. 11). Phosphorylated integrins are hypothesized to initiate a pathway leading to changes in F-actin-containing cytoskeleton and generation of the polarity required for directional movement. NPXY motifs are also hypothesized to regulate cellular localization of $\beta 1A$ integrins. Cells lacking focal adhesion tyrosine kinase (FAK) exhibit reduced motility and increased numbers of focal contacts whereas overexpression of FAK causes increased motility (14, 36). Enhancement of tyrosine phosphorylation causes loss of focal contacts (20, 47). Phosphorylated $\beta 1$ integrins of *src*-transformed fibroblasts do not localize to focal contacts (37). Solubilized phosphorylated integrins from *src*-transformed fibroblasts, in contrast to integrins from normal fibroblasts, fail to bind to an extracellular ligand, fibronectin, or cytoplasmic protein, talin (63). These observations all suggest that upon phosphorylation of the NPXY motifs by tyrosine kinase(s), the integrin loses its affinity for both extracellular ligand and cytoplasmic components of the focal contacts

and exits the focal contact. Dephosphorylation of the motifs by unknown tyrosine phosphatase(s) would allow the integrin to participate in a new round of ligation and focal contact formation. The two hypotheses shown in Fig. 11 could be linked in the sense that the cytoskeleton may polarize the cycling of integrins.

For phosphorylated integrins to initiate a pathway leading to changes in F-actin-containing cytoskeleton, one or more adaptor molecules specific for the phosphorylated NPXY motifs would be required. Tyrosine-phosphorylated intracellular domains interact with Grb2 and Shc in the case of $\beta 3$ (40) and with the P85 of phosphoinositide-3 kinase (PI-3) kinase in the case of $\beta 1A$ (37). However, the double Phe for Tyr substitution did not impair the ability of human $\beta 1A$ transfected into mouse 3T3 cells to signal via Shc when cross-linked by mouse anti-human $\beta 1$ antibody (66). D759A and Y783,795F cells each had increased numbers of focal contacts and upregulation of fibronectin assembly. Increased focal contacts also correlate with increased assembly when cells are treated with LPA (2, 45, 55, 72, 73). The patterns of focal contacts in D759A and Y783,795F cells, however, were strikingly different. The different patterns and the divergent behavior of the two cells in the migration assay suggest that the mutations influence focal contacts by impinging at different points in the pathway shown in Fig. 11. The D759A mutation likely increases the avidity of $\beta 1A$ integrins for extracellular

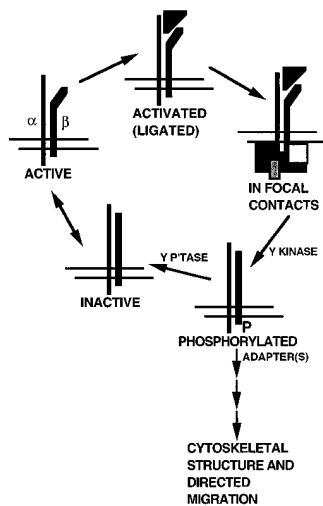


Figure 11. Models whereby directed cell migration may be accomplished by phosphorylation/dephosphorylation of tyrosines in the cytoplasmic domain of $\beta 1A$. The integrin heterodimer is depicted as being in an equilibrium between inactive and active forms. Upon binding of extracellular ligand (triangle), the integrin complexes with cytoplasmic components of focal contacts (various boxes). Phosphorylation (P) of $\beta 1A$ by a tyrosine kinase (Y Kinase) causes the integrin to dissociate from both extracellular ligand and focal contact components. Dephos-

phorylation by a tyrosine phosphatase (Y P'tase) regenerates the integrin in an inactive form. Alternatively or additionally, binding of adapter molecule(s) to phosphorylated NPXY motifs may initiate a pathway leading to cytoskeletal rearrangement, polarization, and directional integrin cycling.

ligands and the probability that integrins will bind ligand and enter focal contacts (32). Increased avidity of integrins for ligands does not slow migration but rather lowers the density of ligands required for maximum migration (12, 34, 39, 51). The Y783,795F mutations, as above, are hypothesized to cause persistence of ligated $\beta 1A$ integrins in focal contacts.

$\beta 3$ in which the tyrosine homologous to Y783 in $\beta 1A$ was mutated to phenylalanine failed to induce adhesion and clot retraction by cells in which $\beta 3$ integrins are constitutively inactive (9). Our tyrosine mutants of $\beta 1A$ were constitutively active in GD25 cells. We do not know whether expression of the mutant $\beta 1A$ s in cells in which the integrins are constitutively inactive would reveal additional functions for the phosphorylation. Indeed, the argument that phosphorylation rather than the simple absence of the hydroxyl group explains the observed phenotype of transfected GD25 cells is circumstantial. The argument is based on the fact that $\beta 1A$ can, in principle, be phosphorylated as evidenced by studies of transformed cells and presumes that only a small, undetectable fraction of $\beta 1A$ is phosphorylated at any one time in untransformed cells.

Several of the substitutions generated in the present investigation are found naturally in other β subunits (Fig. 1). In $\beta 1D$, the conserved Thr in the sequence between the NPXY motifs is an Asn, and a Pro is present in the sequence. $\beta 1D$ is a muscle-specific integrin found in junctions where there is high tensile strength (5). $\beta 1D$ -transfected GD25 display retarded spreading, reduced cell migration, enhanced contractility, and robust ability to assemble fibronectin matrix when compared with $\beta 1A$ -expressing counterparts (6). Transfected $\beta 1D$ is targeted to focal contacts and is also more strongly associated with detergent-insoluble cytoskeleton than $\beta 1A$. These results suggest that alternative splicing of $\beta 1$ is a means to strengthen the cytoskeleton-matrix link where an extremely stable association is required for contraction (6). In $\beta 2$, Tyr of both

NPXY motifs are replaced by Phe. $\beta 2$ integrins interact with counter receptors on other cells, thus localizing lymphocytes and leukocytes in inflammatory and immunological reactions and facilitating interactions among these cells (13, 26, 30, 35, 56). These and other (10, 24, 30, 35, 52, 53, 64, 68) natural variations in NPXY sequences in β subunits may determine the kinetics with which integrins enter and exit focal contacts and the relative abilities of integrins to mediate cell migration, stable adhesions, endocytosis, and assembly of extracellular matrix.

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