β1 Integrin Cytoplasmic Domain Residues Selectively Modulate Fibronectin Matrix Assembly and Cell Spreading through Talin and Akt-1*

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The integrin β1 cytoplasmic domain (tail) serves as a scaffold for numerous intracellular proteins. The mechanisms by which the tail coordinates these proteins to facilitate extracellular matrix assembly and cell spreading are not clear. This study demonstrates that the β1 cytoplasmic domain can regulate cell spreading on fibronectin and fibronectin matrix assembly through Akt- and talin-dependent mechanisms, respectively. To identify these mechanisms, we characterized GD25 cells expressing the β1 integrin cytoplasmic domain mutants W775A and R760A. Although cell spreading appears normal in R760A mutant-integrin cells compared with wild type, it is inhibited in W775A mutant cells. In contrast, both mutant cell lines show defective fibronectin matrix assembly. Inhibition of cell spreading, but not matrix assembly, in the W775A mutant cells is due to a specific defect in Akt-1 activation. In addition, we find that both W775A and R760A mutant integrins have reduced surface expression of the 9EG7 epitope that correlates with reduced recruitment of talin to β1 integrin cytoplasmic complexes. Down-regulation of talin with small interfering RNA or expression of green fluorescent protein-talin head domain inhibits matrix assembly in β1 wild-type cells, mimicking the defect seen with the W775A and R760A mutant cells. These results demonstrate distinct mechanisms by which integrins regulate cell spreading and matrix assembly through the β1 integrin cytoplasmic tail.

Integrins are heterodimeric transmembrane receptors that are formed by the selective pairing of 8 β and 18 α subunits (1). Integrin receptors can rapidly and reversibly modulate the level of cell adhesiveness (2). The adhesive function of integrins can be regulated by extracellular factors, such as extracellular ligands, divalent cations, or monoclonal antibodies. Alternatively, integrin activation associated with high affinity ligand binding can be initiated from within the cell via modulation of the cytoplasmic tail and transmitted by conformational changes to the extracellular domain (inside-out signaling) (3). Specific amino acid sequences within the short integrin cytoplasmic tail are known to be important for integrin function and cell adhesion (4–12). However, the mechanisms by which these sequences selectively potentiate the many signals that affect adhesion-related functions are not clear.

The extracellular matrix (ECM) is a dynamic protein scaffold that provides structural support to cells and also induces cell signaling, in part through formation of cell-matrix adhesions. Fibronectin is an important and ubiquitous component of the ECM. Adhesion of cells to fibronectin can be regulated by changes in the repertoire of integrin expression or by modulation of integrin binding properties. Both α5β1 and αvβ3 integrin receptors can mediate cell adhesion to fibronectin and remodel it; however, the αvβ3 integrin is the primary receptor involved in fibronectin fibrillogenesis (13, 14). Previous studies have shown the importance of the β1 integrin in fibronectin matrix formation (15–19). However, how sites within the cytoplasmic tail of the β1 integrin can selectively control fibronectin matrix formation as opposed to other integrin-dependent processes such as cell spreading remains to be elucidated. GD25, a mouse cell line derived from the embryonic stem cell line G201 (20), is useful for studying β1 integrin function, because it is β1-deficient. Reconstitution of GD25 cells with wild-type or mutant β1 integrins provides an approach to study the functional contribution of particular integrin β1A cytoplasmic domain amino acid residues to different integrin-mediated functions.

We previously demonstrated that a point mutation of the β1A integrin cytoplasmic tail, W775A, influences cell survival and Akt signaling (21). In our current studies, we test the hypothesis that specific sites in the β1A tail can regulate cell spreading and matrix assembly. Reconstitution of β1A−/− GD25 cells with wild-type or mutant β1A integrins permitted us to identify β1A integrin cytoplasmic domain mutations (W775A and R760A) that disrupt regulation of Akt-1 and/or talin, leading to defects in cell spreading and fibronectin matrix assembly. The involve-

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3 The abbreviations used are: ECM, extracellular matrix; siRNA, small interfering RNA; GFP, green fluorescent protein; FN, fibronectin; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DOC, deoxycholate; bFN, biotinylated FN; talinH, talin-head domain; wt, wild type.
ment of Akt-1 in cell spreading and talin in fibronectin matrix assembly was further confirmed using an inhibitor of Akt activity and by disrupting interactions with intracellular talin using siRNA or the GFP-talin head domain. Taken together, our data suggest that specific $\beta_1$ integrin cytoplasmic domain residues can have both distinct and overlapping roles when regulating adhesion-dependent processes.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Antibodies against $\beta_1$ integrin were rat 9E7G (BD Pharmingen), mouse K20 (Immunotech), mouse 12G10 (22), mouse 4B4 (Beckman Coulter), and rabbit antibody Rab 4080 (23). Rat anti-$\alpha_5$ integrin (clone 5H10-27), rat anti-$\alpha_6$ (clone GoH3), hamster anti-$\beta_1$ (clone 2C9,G2), and the IgG controls were from BD Pharmingen. Rat anti-$\alpha_6$, integrin function-blocking antibody (clone C8F12) was a generous gift from Charles Streuli (24). Mouse anti-vinculin antibody (clone vin-11-5), mouse anti-talin antibody (clone 8d4), mouse anti-actin (clone AC-40), and mouse anti-vimentin (clone Vim-13.2) were from Sigma. Rabbit anti-phospho-Akt (Ser-473) was from Cell Signaling Technology. Rabbit polyclonal anti-fibronectin antibody was produced in our laboratory. Secondary antibodies were from Jackson ImmunoResearch Laboratories. Human plasma fibronectin (FN) was purified as previously described (25). Mouse laminin-1 was from Trevigen. EZ-Link Sulfo-NHS-Biotin was from Pierce. The Alexa dyes were from Molecular Probes. Linear GRGDS and linear GRGES peptides were from Bachem. The isozyme selective (Akti-1/2) Akt inhibitor VIII was from Calbiochem.

**Cell Culture and Generation of $\beta_1$ Integrin Mutants**—The $\beta_1^{-/-}$ GD25 cell line was generously provided by Reinhard Fassler (Max Planck Institute of Biochemistry). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone (complete medium). Generation of alanine point mutations in the cDNA of the $\beta_{1A}$ integrin cytoplasmic domain and subsequent stable expression in the $\beta_1^{-/-}$ GD25 cell line have been previously described (21). Briefly, amino acid residues that are conserved between six $\beta$ integrin cytoplasmic tails were identified and mutated to alanine. Plasmids containing cDNA encoding either the wild-type or mutated $\beta_{1A}$ integrins were cotransfected with the puromycin selection vector pHA262pur into $\beta_1^{-/-}$ GD25 cells. Stable cell lines containing similar levels of $\beta_{1A}$ integrin expression were obtained by culturing cells in medium containing 10 µg/ml puromycin and by fluorescence-activated cell sorting using K20, an anti-total $\beta_1$ integrin antibody. Generation of W775A $\beta_1$ integrin mutant cells expressing constitutively active Akt has also been previously described (26).

**Fibronectin Matrix Assays**—Stably transfected GD25 $\beta_{1A}$ integrin cell lines were plated in complete medium at 3 x 10^6 cells/well in 6-well plates. After culturing overnight, the medium was changed to serum-free DMEM containing 20 µg/ml biontinylated FN (bFN), 1% bovine serum albumin (BSA), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml fungizone. FN was biontinylated with EZ-Link Sulfo-NHS-Biotin according to the manufacturer’s protocol for biontinylating IgG. Following a 5-h incubation, cells were washed twice with Dulbecco’s phosphate-buffered saline plus calcium and magnesium (PBS-+), scapped into deoxycholate (DOC) buffer (1% sodium deoxycholate, 2 mM N-ethylmaleimide, 2 mM iodoacetatic acid (sodium salt), 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 20 µM Tris-HCl, pH 8.5) on ice, and passed five times through a 23-gauge needle. Insoluble material was collected by centrifugation at 20,000 x g for 20 min at 4°C. The DOC-insoluble material was washed once in DOC buffer, resuspended in Novex 2 x Tris-glycine SDS sample buffer (Invitrogen), heated to 95 °C for 5 min, and resolved on 4–12% Tris-glycine gels (Invitrogen). After electrophoresis to nitrocellulose membranes (Invitrogen), the filters were blocked (5% nonfat dry milk and 0.1% Tween 20 in 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) and probed with streptavidin-horseradish peroxidase to quantify bFN incorporation into DOC-insoluble FN matrix. Binding was visualized using the ECL system and Hyperfilm x-ray film (Amersham Biosciences). The same membranes were stripped and reprobed with anti-vimentin antibody (Sigma clone Vim 13.2) to provide an internal protein loading control.

**Cell Spreading Assays**—Cells were detached with 0.05% trypsin-EDTA, washed with Hanks’ balanced salt solution, and resuspended in serum-free DMEM containing 1% BSA. Cell density was adjusted to 2 x 10^5 cells/ml, and the cells were incubated for 10–15 min with or without inhibitors/antibodies at room temperature. The cells were replated on 35-mm diameter tissue-culture polystyrene plates coated with the indicated ECM protein. Cells were assayed for spreading at the times indicated in the figure legends. Microscope fields for each time point or treatment were chosen at random, and images were acquired using a Zeiss Axiovert 25 light microscope equipped with a Nikon Coolpix 4500 digital camera. An individual cell was counted as “spread” when there were cell protrusions or lamellae with loss of refractility over greater than 70% of the cell area. The percentages of spread cells were evaluated as (number of spread cells / total number of cells in field) x 100%.

**Flow Cytometry and Immunofluorescence**—Integrin expression profiles were determined for the parental $\beta_1^{-/-}$ GD25 and early passage (<5) GD25 integrin $\beta_{1A}$-sorted cell populations. The cells were cultured overnight in serum-free medium on a fibronectin-coated (10 µg/ml) substratum. The cells were detached by initially washing twice in PBS without calcium and magnesium (PBS-) followed by incubation with PBS- containing 1 mM EDTA, pH 8.0, in a 37°C incubator. The cells were washed by centrifugation and resuspended in cold PBS- buffer containing 1% BSA and 0.02% sodium azide (BSA buffer). 5 x 10^6 cells were incubated with an anti-integrin antibody or control IgG at 10 µg/ml final concentration for 40 min on ice, washed three times with BSA buffer, and stained with fluorescein isothiocyanate-conjugated secondary antibodies. The cells were fixed in PBS- containing 1% formaldehyde and stored at 4°C.

The integrin activation-state experiments used $\beta_1^{-/-}$ GD25, integrin $\beta_{1A}$-sorted wild-type, W775A, and R760A cells. Cells cultured in complete medium were washed once with Hanks’ balanced salt solution and cultured for 5 h in serum-free DMEM at 37°C. Cells were harvested with PBS- /EDTA as described above. $\beta_1^{-/-}$ GD25 cells and integrin $\beta_1$ sorted pop-
ulations were extensively washed with PBS. Then $5 \times 10^5$ cells were resuspended in HEPES buffer (150 mM NaCl, 1% BSA, 20 mM HEPES, pH 7.4) supplemented with 1 mM calcium chloride and 1 mM magnesium chloride (integrin activation buffer) without or with 0.2 mM GRGDS or 0.2 mM GRGES peptides for 30 min on ice. Alexa 488-conjugated 9E10 or fluorescein isothiocyanate-conjugated K20 were then added to the cells and incubated for 30 min on ice. Following incubation, excess antibody was removed by washing three times with the integrin activation buffer containing 0.02% azide and the corresponding peptides. The cells were then fixed with PBS and 1% formaldehyde and stored at 4 °C. Fluorescence intensity was determined by flow cytometry on a FACSCalibur interfaced with CellQuest Pro software (BD Biosciences).

Cell sorting was performed as previously described (21). Briefly, the GD25-β1A cell lines were detached with trypsin and washed in complete medium. The cells were resuspended in a mixture of 50% PBS/BSA and 50% complete medium. Then $5 \times 10^5$ cells were incubated with fluorescein isothiocyanate-conjugated K20 (anti-total β1 integrin antibody) for 30 min on ice. After the antibody incubation, the cells were washed with PBS/BSA and resuspended in complete DMEM. The GD25-β1A integrin wild-type, W775A, and R760A cell populations with similar integrin β1 surface expression were sorted and collected using a Mo-Flo cell sorter (Dako) and Summit (Dako) software and were subsequently resuspended and cultured in complete medium.

For immunofluorescence, samples were fixed/permeabilized in PBS containing 4% paraformaldehyde, 5% sucrose, and 0.1% Triton X-100 for 1 min. The samples were then washed and fixed for an additional 15 min in PBS containing 4% paraformaldehyde and 5% sucrose. For staining only the fibronectin matrix and cell nuclei with SYBR green, cells were fixed for 15 min with 4% paraformaldehyde and 5% sucrose in PBS without permeabilization. For staining cells transfected with the GFP-talin head domain, cells were fixed for 10 min with 2% paraformaldehyde and 5% sucrose in PBS and permeabilized for 20 min with 0.5% Triton-X-100 in PBS. Non-specific binding sites were blocked with 20% donkey serum and 2% BSA in PBS before staining with primary and secondary antibodies. Slides were mounted with Gel/Mount (Biomedra Corp.) supplemented with 20% glycerol. Images were acquired using a Zeiss LSM 510 confocal microscope.

Isolation of Integrin-associated Protein Complexes—To isolate integrin-associated complexes, GD25 cell lines were harvested with trypsin, washed, and resuspended in DMEM with 1% calf serum. To recover from the trypsinization, the cells were maintained in suspension for 3 h with constant rocking on a platform shaker. The suspended cells at a density of $2 \times 10^6$ cells/ml were then incubated for 1 h at 37 °C with magnetic beads (Dynabeads, 4.5-µm diameter from Dynal/Invitrogen) at a bead-to-cell ratio of 5:1. The magnetic beads were pre-coated with mouse anti-integrin β1 antibody K20 at a ratio of 100 µg to $2 \times 10^6$ goat anti-mouse IgG conjugated beads. Cells with bound magnetic beads were positively sorted using a magnetic separator and washed with cold CSK buffer 50 (50 mM sodium chloride, 300 mM sucrose, 3 mM magnesium chloride, 1 mM sodium vanadate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, Complete protease inhibitor mixture (Roche Applied Science), and 10 mM PIPES buffer, pH 6.8). Integrin-associated protein complexes bound to the anti-integrin antibody beads were extracted with CSK buffer containing 0.5% Triton X-100 and 300 mM NaCl at 4 °C. To isolate the complexes, lysate and beads were bath-sonicated in a Branson 1510 ultrasonic cleaner twice for 10 s at room temperature. The bead complexes were subsequently washed five times with cold CSK buffer containing 0.5% Triton X-100 and 300 mM NaCl. To elute the proteins from the beads, 2× SDS sample buffer (Invitrogen) was added, and the samples were probe-sonicated with a Microson XL ultrasonic cell disruptor at setting 2–3 for three 5-s periods to reduce the viscosity of the samples. The levels of talin were determined by Western blot using sample volumes containing equal levels of β1 integrin. The Western blots were developed using the ECL system (Amersham Biosciences). The chemiluminescence was detected with a Fuji-
\( \beta_1 \) Integrin and Fibronectin Matrix Assembly

**FIGURE 2. Multiple \( \beta_1A \) integrin tail mutations affect fibronectin matrix assembly.** The ability of GD25 cells expressing wild-type \( \beta_1A \) integrin to assemble fibronectin matrix was quantified as fold change compared with GD25 cells expressing wild-type \( \beta_1A \) integrin. The graphs shown represent pooled data from three independent experiments. The blot shown is from one experiment where the lanes have been joined so that the sequence of mutations is consistent with that of Fig. 1. For \( \beta_1A \) wt versus T78A, \( p = 1.0 \); for \( \beta_1A \) wt versus W775A, \( p < 0.001 \); for \( \beta_1A \) wt versus E769A, \( p < 0.001 \); for \( \beta_1A \) wt versus E767A, \( p < 0.001 \); for \( \beta_1A \) wt versus F766A, \( p < 0.001 \); for \( \beta_1A \) wt versus D759A, \( p < 0.001 \); for \( \beta_1A \) wt versus L754A, \( p < 0.001 \); for \( \beta_1A \) wt versus R760A, \( p < 0.001 \); for \( \beta_1A \) wt versus D759A, \( p = 0.06 \); and for \( \beta_1A \) wt versus L754A, \( p < 0.001 \). Error bars represent \( \pm \) S.E., statistical significance \( (p < 0.05) \).

**FIGURE 3. W775A \( \beta_1A \) integrin mutant cells have a selective defect in spreading on fibronectin compared with R760A, but they share a defect in spreading on laminin-1.** \( A, \beta_1A^+/+ \) GD25 cells and cells reconstituted with wild-type, R760A, or W775A mutant \( \beta_1A \) integrins were allowed to spread on culture dishes coated with 10 \( \mu \)g/ml FN for 60 min in the presence of 10 \( \mu \)g/ml C8F12, an \( \alpha_5 \) integrin blocking antibody. C8F12 significantly inhibited cell spreading of \( \beta_1A^+/+ \), GD25 and W775A cells \( (p = 0.002 \) and \( p = 0.001 \), respectively) but not of GD25- \( \beta_1A \) wt or R760A cells \( (p > 0.05) \). B, in contrast, the ability of \( \beta_1A^-/+ \) GD25, W775A, and R760A cells to spread on 10 \( \mu \)g/ml laminin-1 was substantially decreased compared with GD25- \( \beta_1A \) wt cells \( (p < 0.001) \). Graphs shown represent pooled data from three independent experiments. Error bars represent \( \pm \) S.E., statistical significance \( (p < 0.05) \).

The plasmid encoding the GFP-mouse talin head domain was a generous gift from Michael Sheetz. For cDNA transfection, cells were plated for 2 h on tissue-culture dishes in complete medium. Immediately before transfection, the medium was changed to DMEM containing 10% fetal bovine serum. Cells were transected using a 6:1 ratio of Fugene6 (Roche Applied Science) reagent to DNA according to the manufacturer’s instructions. The cells were incubated overnight in a 37 °C, 10% CO\(_2\) incubator. The next morning the cells were passaged and re-plated on glass coverslips. The cells were fixed and stained 48 h post-transfection.

**Image Processing and Statistics**—All confocal images were prepared and processed using LSM Image Browser and NIH Image J software by adjusting brightness/contrast and applying a median filter. Graphs and statistics were prepared using SigmaPlot and SigmaStat software. \( p \) values were derived using...
one-way analysis of variance with pairwise multiple comparisons made using the Tukey-Kramer test.

RESULTS

Specific Sites in the $\beta_1$ Integrin Cytoplasmic Domain Are Required for Cell Spreading—DNA plasmids encoding wild-type $\beta_1$ integrin or cytoplasmic mutant integrins were expressed in $\beta_1^{-/-}$ GD25 cells, and stable cell lines were generated as previously described (21). Specific amino acid residues in the cytoplasmic domain of the $\beta_1$ integrin were selected for mutation (Fig. 1A), because they were conserved between comparable sites of six different $\beta_1$ integrin subunits. To compare the role of these specific amino acid residues in a key function of cells, cell spreading was examined on a fibronectin-coated substrate. The $\beta_1$ and $\beta_3$ integrins are major mediators of cell spreading on fibronectin. Because $\beta_1^{-/-}$ GD25 cells, which primarily use the $\beta_1$ integrin to spread on fibronectin, displayed slower spreading kinetics than the GD25-$\beta_1$wt cells ($p < 0.05$ and $p < 0.01$ at 10 and 20 min, respectively). Treatment of the GD25-$\beta_1$wt cells with the $\beta_1$ blocking antibody 4B4 inhibited spreading rates to the levels observed with the $\beta_1^{-/-}$ GD25 cells, indicating that the difference in spreading kinetics is due to $\beta_1$ integrin function. As expected, 4B4 had no effect on the spreading rate of the $\beta_1^{-/-}$ GD25 cells.

We directly compared cell spreading by the $\beta_1^{-/-}$ GD25, GD25-$\beta_1$wt, and nine $\beta_1$ integrin cytoplasmic mutant cell lines on 10 $\mu$g/ml fibronectin for 20 min, the time at which wild-type $\beta_1$ integrin function was most apparent. Cell spreading varied widely between the mutants, with seven point mutations, including R760A and T789A ($p < 0.05$ for both mutants), having little to no effect on spreading compared with that of the GD25-$\beta_1$wt cells, and the W775A ($p < 0.001$) and F766A ($p < 0.05$) mutants having the most significant defect in cell spreading (Fig. 1C).

Comparison of Specific Sites in the $\beta_1$ Integrin Cytoplasmic Domain Required for Fibronectin Matrix Assembly and Cell Spreading

FIGURE 4. W775A and R760A $\beta_1$ integrin mutant cells express similar levels of $\alpha_\nu$, $\alpha_6$, and $\beta_3$ integrin subunits, but increased levels of $\alpha_5$ subunits compared with $\beta_1$wt cells. GD25 cell lines sorted by flow cytometry for similar expression levels of wild-type and mutant $\beta_1$ integrins were examined for surface expression of integrin $\alpha_\nu$, $\alpha_6$, $\alpha_5$, and $\beta_3$ subunits. Shown are the fluorescence-activated cell sorting histograms in which the x axis is fluorescence intensity and the y axis indicates cell count. The experiment was performed three times with different sorted cell populations, and similar results were observed. The results from a representative experiment are shown.
Another key function of β1 integrins is to mediate fibronectin fibrillogenesis or matrix assembly. To compare the effect of each cytoplasmic domain integrin mutant on fibronectin fibrillogenesis, 20 μg/ml bFN was added to cells adherent to glass coverslips. The ability of the cells to assemble fibronectin fibrils was assessed by measuring incorporation of the added soluble bFN into a DOC-insoluble matrix as described under “Experimental Procedures.” Greater than 95% of the production of FN matrix could be attributed to the β1 integrin, because very little bFN was incorporated into the DOC-insoluble fraction by the β1Δ integrin mutant cells (data not shown). As observed for cell spreading on fibronectin, the effects of the β1Δ integrin cytoplasmic domain integrin mutant on fibronectin fibrillogenesis were selective (Fig. 2). For example, cells expressing the T789A mutation compared with the W775A mutation showed no effect (Fig. 2A) versus a dramatic decrease (Fig. 2B) in fibronectin matrix formation, respectively. Interestingly, the R760A mutation also showed a striking decrease in fibronectin matrix formation, even though it showed little or no inhibition of spreading as seen in Fig. 2C (p < 0.001 for fibronectin matrix formation versus p = 1.0 for cell spreading on fibronectin). The magnitudes of the effects of each point mutation on matrix assembly compared with cell spreading revealed no direct correlation, indicating that mechanisms regulating fibronectin matrix assembly and cell spreading may not be coupled. To elucidate mechanisms that selectively regulate fibronectin matrix formation and cell spreading through the β1 integrin cytoplasmic domain, we further characterized the molecular and cellular phenotypic differences between the W775A and R760A β1Δ integrin mutations.

W775A and R760A β1Δ Integrin Mutants Support Different Cell Spreading Phenotypes on Fibronectin versus Laminin-1—We first compared cell spreading of the two mutant cell lines on fibronectin and laminin-1. Both cell lines expressing wild-type β1 integrin were incubated for 15 min at room temperature with the indicated concentrations of Akt inhibitor VIII. Cell spreading was quantified at two different time points for spreading were used, 20 min after plating on tissue-culture dishes coated with 10 μg/ml FN to assess cell spreading activity. Error bars represent ±S.E. *, statistical significance (p < 0.05). B, to confirm the importance of Akt in β1 integrin-mediated cell spreading, GD25 cells expressing wild-type β1 integrin were incubated for 15 min at room temperature with the indicated concentrations of Akt inhibitor. Cell spreading was quantified 20 min after plating on tissue-culture dishes coated with 10 μg/ml FN. Graphs shown represent pooled data from three independent experiments. C, GD25 cells reconstituted with β1Δ wild-type or W775A mutant integrins, with or without stable transfection of constitutively active Akt-1, were assayed for their ability to assemble a DOC-insoluble fibronectin matrix as described under “Experimental Procedures.” The blot shown is from one experiment where the bands have been joined so that the sequence is consistent with that of panel A. Blots are representative of three independent experiments.

Surface Expression of αv, αv, α5, and β3 Integrins in W775A and R760A β1Δ Integrin Mutant Cells—Ectopic expression of the β1Δ integrin in β1ΔΔ cells induces expression of the integrin αv subunit (18), which permits spreading of the GD25-β1Δwt cells on laminin-1. Both β1ΔΔ GD25 cells and GD25-β1Δwt cells treated with the αv integrin function-blocking antibody GoH3 could not spread on laminin-1 (data not shown), indicating that the αvβ3 integrin receptor is the primary mediator of cell spreading on laminin-1. Because the cells expressing the W775A and R760A β1Δ integrin mutations failed to spread on laminin-1 yet displayed a selective cell spreading defect on
fibronectin, we quantified the surface expression of integrin subunits that bind to fibronectin and laminin-1 to rule out defects due to decreased surface levels. As shown in Fig. 4, expression of these β1A mutations did not decrease, and in fact increased, the surface expression of the integrin αι subunit. Interestingly, expression of the β1A integrin subunit also induces expression of the β4 integrin subunit in this cell type (18); therefore, the increased expression of the αι subunit may represent an increase in the expression of the αιβ4 integrin. It is possible that increased expression of the αι subunit could also induce expression of the αιβ4 integrin at the cell surface. However, this appears unlikely because expression of the αι integrin subunit, which only partners with the β1 integrin subunit, does not decrease on the surface of the W775A and R760A integrin mutant cells. In fact, αι integrin subunit levels correlate closely with β1 integrin subunit levels for each mutant cell type.

As shown in Fig. 4, surface expression of the fibronectin-binding integrin subunits αι, αι, and β3 subunits were similar in the wild-type and mutant β1A integrin-expressing cells. Therefore, the spreading defects exhibited by the W775A β1A integrin mutation on fibronectin and both mutants on laminin-1 cannot be attributed to a reduction in cell surface levels of these integrin subunits. To specifically investigate the cause of the differential spreading phenotype, we focused on studying mechanisms involved in β1-integrin mediated cell spreading on fibronectin.

Constitutively Active Akt-1 Rescues Defective Spreading on Fibronectin but Not Defective Fibronectin Matrix Assembly in W775A Mutant Cells—We had previously reported that, out of the nine mutants studied here, only the W775A β1 integrin mutant has a significant Akt signaling defect, which leads to an increase in apoptosis induced by serum deprivation (21). We therefore hypothesized that a defect in Akt-1 activation could be the cause of the selective defects in cell spreading and/or fibronectin matrix assembly. We therefore tested whether expression of constitutive active Akt-1 in the W775A β1 integrin mutant cell line could rescue the cell spreading defect. We used two stable cell lines, W775A Akt-1 clone 1 and W775A Akt-1 clone 3, that expressed 1.6 times and 3.4 times the level of activated Akt than the parental W775A mutant cell line, respectively (26). We demonstrate that constitutively active Akt-1 rescues the cell spreading defect of the W775A β1 integrin mutant (Fig. 5A). Furthermore, we confirmed the role of Akt in mediating cell spreading in the β1 wild-type cells by using a specific inhibitor of Akt activity, which inhibited cell spreading in a dose-dependent manner (Fig. 5B). Interestingly, expression of constitutively active Akt-1 did not rescue the defect in fibronectin fibrillogenesis (Fig. 5C). This finding suggests that a different mechanism may be involved in regulating fibronectin matrix assembly that is independent of Akt signaling and cell spreading.

W775A and R760A β1A Mutant Integrin Cells Form Fewer Fibrillar Adhesions and Show Decreased Fibronectin Matrix Assembly—Because fibronectin matrix assembly was decreased in both W775A and R760A β1 integrin mutant cells (Fig. 6A), we tested the ability of each cell type to form fibrillar adhesions. Fibrillar adhesions are elongated or dot-like structures that initiate from focal adhesions and are closely associated with ECM fibrils (27–29). Like focal adhesions, they are mediated by integrins and interact with the actin cytoskeleton at the cell interior (27). They differ from focal adhesions in that they are normally located in the central region of the cell and contain fewer proteins than reside in focal adhesions, such as decreased levels of vinculin. Fibrillar adhesions are thought to be sites of fibronectin matrix formation (30). We stained for the presence of fibrillar adhesions using 9EG7, an anti-CLIBS (cation-and-ligand-influenced binding site) antibody (31) against the β1 integrin and vin-11-5, an antibody against vinculin, a focal adhesion marker. The white arrowheads in Fig. 6B show the presence of fibrillar adhesions in the GD25-β1A wt cell line. Both the R760A and the W775A β1A integrin mutant cells, however, showed substantially reduced fibrillar adhesion formation. This finding...
indicates that the cytoplasmic tail of the $\beta_{1A}$ integrin can control the ability of cells to form fibrillar adhesions.

W775A and R760A $\beta_{1A}$ Integrin Mutants Both Show Decreased Exposure of the 9EG7 Epitope and Reduced Recruitment of Talin to $\beta_1$, Integrin Cytosolic Complexes—Because we could not detect fibrillar adhesions using the 9EG7 antibody in the mutant cells, we hypothesized that the ability of the mutant integrins to undergo inside-out signaling and to express the 9EG7 epitope was reduced. We therefore quantitatively assessed the ability of each mutant integrin to bind the 9EG7 antibody in the absence and presence of a soluble ligand. Binding of the 9EG7 antibody in the absence of ligand was reduced by $\sim$40% for both W775A and R760A $\beta_{1A}$ integrins compared with the wild-type $\beta_{1A}$ integrin (Fig. 7A). This result suggests that the two mutant integrins exhibit different conformational states compared with the wild-type integrin as defined by exposure of the 9EG7 epitope on the extracellular domain. Interestingly, binding of 12G10, another anti-CLIBS antibody (22) was reduced by $\sim$80% for the W775A mutant $\beta_{1A}$ integrin, but no effect on 12G10 binding was observed for the R760A mutant integrin (data not shown). Thus, the 9EG7 epitope may be more sensitive to defects in integrin inside-out signaling that affect matrix assembly in both mutants. Furthermore, an RGD peptide (GRGDS), but not an RGE peptide control (GRGES), enhanced 9EG7 binding to the wild-type $\beta_{1A}$ integrin but not to the two mutant integrins. Thus, in contrast to the differences in capacity to mediate adhesion/spreading, the defects in RGD peptide-induced 9EG7 epitope expression correlated with defective fibronectin fibrillogenesis for both of these mutants. These results therefore suggest that $\beta_{1A}$ integrin inside-out signals controlling affinity for ligand may be disrupted by both W775A and R760A $\beta_{1A}$ mutations.

Talin is an integrin cytosolic protein that is known to be important for integrin activation and function (10). Recently, 9EG7 antibody binding has been shown to be particularly sensitive to intracellular depletion of talin protein, because talin-deficient cells showed very little staining with 9EG7 compared with cells reconstituted with wild-type talin (32). We therefore hypothesized that a defect in talin binding to the $\beta_1$ integrin cytoplasmic domain might be responsible for the decrease in fibronectin matrix assembly. To test this hypothesis, we biochemically isolated protein complexes associated with the $\beta_1$ integrin cytoplasmic domain after clustering of the extracellular domain with the anti-$\beta_1$ integrin antibody K20. We found that both the W775A and the R760A $\beta_1$ integrin mutations inhibited recruitment of talin to $\beta_1$ integrin cytoplasmic complexes (Fig. 7B). This result supports the observation that expression of the 9EG7 epitope is reduced in these mutants and provides an explanation for the inability of these mutant cells to form fibrillar adhesions involving decreased connection to the actin cytoskeleton through talin.

Protein Depletion of Talin Inhibits Fibronectin Matrix Assembly—To confirm the role of talin in fibronectin matrix assembly, we used talin siRNA to knock down talin in the $\beta_1$ wild-type cells. With this cell line, we found it necessary to use a combination of talin-1 and talin-2 siRNA to obtain consistent talin knockdown; using only talin-1 siRNA produced an anomalous up-regulation of total talin levels most likely due to talin-2 up-regulation (data not shown). As shown in Fig. 8, a reduction of total talin protein levels by $\sim$80% using dual talin-1/talin-2 knockdown in $\beta_1$ wild-type cells decreases the ability of these cells to assemble a fibronectin matrix. In contrast, talin knockdown had no apparent effect on cell morphology or early fibronectin-induced cell spreading (data not shown). These results demonstrate a role for talin in mediating fibronectin matrix assembly through the $\beta_1$ integrin.
Expression of the Talin Head Domain in β1 Wild-type Cells Inhibits Fibronectin Matrix Assembly—As an independent, complementary approach to elucidate the mechanism(s) by which talin functions in fibronectin matrix assembly, we transiently transfected into β1 wild-type cells a plasmid that encodes the head domain of talin fused to GFP. Because there are actin-binding sites within the C-terminal rod domain of talin (33, 34), we hypothesized that even though expression of the talin head domain may activate integrins (35), the talin-head domain could also act as a dominant negative inhibitor by competing for interactions of full-length talin with the β1 integrin cytoplasmic domain, thereby weakening the connection of the cytoplasmic domain to the cytoskeleton. In fact, it has recently been found that re-expression of the talin head domain alone in talin-deficient cells is not sufficient to restore defective ECM-cytoskeleton linkage resulting from talin depletion (32). As shown in Fig. 9, expression of the GFP talin-head domain (GFP-talinH) in β1 wild-type cells (Fig. 9, B–D) results in reduced matrix assembly compared with cells not expressing GFP-talinH (Fig. 9, A–C). Interestingly, even in cells expressing GFP-talinH, residual fibronectin fibrils can still be observed between cells and at the cell periphery (Fig. 9, B and C, white arrowheads) with the reductions occurring at the main cell body. These peripheral fibrils may still form as cells migrate away from each other.

In addition, the cells that express the highest levels of GFP-talinH (Fig. 9D, white asterisks) show smaller vinculin-containing adhesions compared with adjacent cells expressing less GFP-talinH (Fig. 9D, orange asterisk). This result demonstrates a dose-dependent effect of the talin-head domain and agrees with recent findings that talin is also needed for formation of vinculin-containing adhesions (32). Nonetheless, reduced fibronectin matrix assembly was observed even in the cells expressing lower levels of the GFP talin-head domain (Fig. 9D, orange asterisk), indicating that fibronectin matrix assembly is more sensitive to expression of the talin head domain. Taken together, these results support the conclusion that talin associated with the β1 integrin cytoplasmic domain is important for fibronectin fibrillogenesis and suggest that the talin rod domain is important for mediating the cytoskeletal linkage that facilitates the assembly of vinculin-containing adhesions and the formation of fibronectin matrix.

DISCUSSION

Integrins are crucial mediators of contact with the extracellular environment. In this study, we demonstrate that integrin function in fibronectin matrix assembly and cell spreading can be selectively modulated based on mutation of specific amino acid residues in the cytoplasmic tail of the β1A integrin (Table 1). In contrast to the selective modulation of Akt-1 signaling and cell spreading by the W775 residue, we show that both cytoplasmic amino acid residues Trp-775 and Arg-760 are required for recruitment of talin to integrin cytoplasmic complexes. Furthermore, we demonstrate that talin is important for fibronectin matrix assembly. Interestingly, regulation of cell spreading by Akt-1 seems to occur independently of talin and cell spreading by the W775 residue, we show that both cytoplasmic amino acid residues Trp-775 and Arg-760 are required for recruitment of talin to integrin cytoplasmic complexes. Furthermore, we demonstrate that talin is important for fibronectin matrix assembly. Interestingly, regulation of cell spreading by Akt-1 seems to occur independently of talin and cell spreading by the W775 residue, we show that both cytoplasmic amino acid residues Trp-775 and Arg-760 are required for recruitment of talin to integrin cytoplasmic complexes. Furthermore, we demonstrate that talin is important for fibronectin matrix assembly.
Despite having normal Akt activity (21), the R760A β1A integrin mutant cells fail to support cell spreading on laminin-1, indicating that spreading may be regulated differently on laminin-1 compared with fibronectin. In fact, differences in signaling have been previously reported for cells spreading on fibronectin compared with laminin-10/11 (36, 37). Although it is not clear in this study why this mutant cannot spread on laminin-1, the concentration of cytoskeletal linker proteins, including talin, are reduced in adhesions of fibroblasts spreading on laminin-1 compared with fibronectin (38). These earlier findings may help explain why in our study β1 integrin-mediated cell spreading on laminin-1 is more sensitive to β1 integrin cytoplasmic domain perturbations.

Focusing on the mechanisms of cell spreading on fibronectin, we demonstrate that Akt-1 activity is important for cell spreading downstream of β1 integrin signaling. We previously reported that constitutively active Akt-1 stimulates random migration by regulating Rac activity and numbers of peripheral lamellae (26). Because the extension of lamellae is critical for cell spreading, it seems likely that Akt-1 regulates β1 integrin-mediated cell spreading on fibronectin using this mechanism.

Akt-1 has also recently been shown to regulate fibronectin fibrillogenesis by modulating the activity of the αvβ3 integrin (39). In addition, Somanath et al. show that cell adhesion and migration are impaired if Akt-deficient fibroblasts are plated on fibronectin. We had previously demonstrated that, of all the mutants tested in this study, only the W775A β1A integrin mutant had a significant, selective defect in Akt-1 signaling (21). Although we show here that expression of constitutively active Akt-1 (myristoylated Akt) in W775A β1A integrin mutant cells can rescue cell spreading activity, it did not reverse the defective fibronectin fibrillogenesis in these cells. Consequently, talin recruitment to an intact β1 integrin cytoplasmic domain may be required for matrix assembly downstream of Akt-1 signaling.

The RGD cell-binding sequence of fibronectin is essential for initiation of αvβ3 integrin-mediated fibronectin matrix assembly (40). In support of this finding, the αv integrin does not localize to the fibronectin fibrils assembled by cells isolated from mice with a fibronectin RGE knock-in (41). In our study, binding of the wild-type β1 integrin to an RGD peptide increased exposure of the 9EG7 epitope, but the W775A and the R760A mutant β1A integrins were unresponsive to the RGD peptide. This defect is likely due, at least in part, to the decreased recruitment of talin to the β1 integrin cytoplasmic domain of both mutants.

Previously, the W775A mutant β1A integrin was studied in the context of a double mutation (W775A and D776A) within an interleukin-2 receptor β1 tail chimera in a β1wt background. These cells showed reduced adhesion and integrin act-

### Summary of Localization and Functional Perturbations of W775A and R760A β1A Integrin Mutants

<table>
<thead>
<tr>
<th>β1A cytoplasmic domain mutations</th>
<th>Cell spreading</th>
<th>FN matrix assembly, FN fibrils</th>
<th>Fibrous adhesion formation, β1</th>
<th>Integrin β1 9EG7 epitope expression, 9EG7</th>
<th>Recruitment to cytoplasmic domain, talin</th>
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</thead>
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<tr>
<td>β1A</td>
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<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β1A/−</td>
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<td>−</td>
</tr>
<tr>
<td>β1R760A/−</td>
<td>+/−</td>
<td>−</td>
<td>−</td>
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</tbody>
</table>

* αv-dependent.
* αv-independent.
We have demonstrated that specific \( \beta_1 \) integrin cytoplasmic mutations can selectively affect Akt-1 signaling and cell spreading on fibronectin, differentially affect spreading on fibronectin versus laminin-1, and disrupt fibrillar adhesions and fibronectin matrix assembly. The ability to control integrin function based on modulation of the cytoplasmic domain has been well studied in platelets and immune cells where rapid integrin activation is necessary to carry out its biological functions. However, in cells such as fibroblasts, in which integrins are often relatively constitutively activated, modulation of the integrin cytoplasmic domain could regulate signals that affect not only the ability of integrins to interact with the extracellular matrix but also the ability of integrins to apply force and remodel the matrix. Understanding these different aspects of integrin function may provide better insight into the pathology of diseases such as fibrosis and cancer that are commonly associated with aberrant integrin signaling and matrix formation.

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**REFERENCES**