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 α₅β₃ and α₅β₅ integrin receptors can mediate cell adhesion to fibronectin and remodel it; however, the α₅β₃ 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 β₅-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 β₁−/− 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-
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 β1 integrin cytoplasmic domain residues can have both distinct and overlapping roles when regulating adhesion-dependent processes.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Antibodies against β1 integrin were rat 9EG7 (BD Pharmingen), mouse K20 (Immunotech), mouse 12G10 (22), mouse 4B4 (Beckman Coulter), and rabbit antibody Rab 4080 (23). Rat anti-α5 integrin (clone 5H10-27), rat anti-α6 (clone GoH3), hamster anti-β1 (clone 2C9,G2), and the IgG controls were from BD Pharmingen. Rat anti-α1, integrin function-blocking antibody (clone C8F12) was a generous gift from Charles Streuli (24). Mouse anti-vein-culin 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 (Akt-1/2) Akt inhibitor VIII was from Calbiochem.

**Cell Culture and Generation of β1 Integrin Mutants**—The β1−/− GD25 cell line was generously provided by Reinhard Fässler (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 β1α integrin cytoplasmic domain and subsequent stable expression in the β1−/− GD25 cell line have been previously described (21). Briefly, amino acid residues that are conserved between six β integrin cytoplasmic tails were identified and mutated to alanine. Plasmids containing cDNA encoding either the wild-type or mutated β1α integrin were cotransfected with the puromycin selection vector pHA262pur into β1−/− GD25 cells. Stable cell lines containing similar levels of the wild-type or mutated β1α 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 β1 integrin antibody. Generation of W775A β1 integrin mutant cells expressing constitutively active Akt has also been previously described (26).

**Fibronectin Matrix Assays**—Stably transfected GD25 β1α integrin cell lines were plated in complete medium at 3 × 10⁶ cells/well in 6-well plates. After culturing overnight, the medium was changed to serum-free DMEM containing 20 μg/ml biotinylated FN (bFN), 1% bovine serum albumin (BSA), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone. FN was biotinylated with EZ-Link Sulfo-NHS-Biotin according to the manufacturer’s protocol for biotinylating IgG. Following a 5-h incubation, cells were washed twice with Dulbecco’s phosphate-buffered saline plus calcium and magnesium (PBS−), scraped 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 mM 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 × g for 20 min at 4 °C. The DOC-insoluble material was washed once in DOC buffer, resuspended in Novex 2 × Tris-glycine SDS sample buffer (Invitrogen), heated to 95 °C for 5 min, and resolved on 4 – 12% Tris-glycine gels (Invitrogen). After electrotransfer 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 × 10⁵ 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 Axiosvert 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 refractivity 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) × 100.

**Flow Cytometry and Immunofluorescence**—Integrin expression profiles were determined for the parental β1−/− GD25 and early passage (<5) GD25 integrin β1α-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 × 10⁵ 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 β1−/− GD25, integrin β1-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. β1−/− GD25 cells and integrin β1 sorted pop-
Beta 1 Integrin and Fibronectin Matrix Assembly

ulations were extensively washed with PBS$. Then 5 × 10^5 cells were resuspended in HEPES buffer (150 mM NaCl, 1% BSA, 20 mM HEPES, pH 7.4) supplemented with 1 mM calcium chloride and 1 mM magnesium chloride (integrin activation buffer) without or with 0.2 mM GRGDS or 0.2 mM GRGES peptides for 30 min on ice. Then 5 × 10^6 cells were then incubated with fluorescein isothiocyanate-conjugated K20 were then added to the cells and incubated for 30 min on ice. Following incubation, excess antibody was removed by washing three times with the integrin activation buffer containing 0.02% azide and the corresponding peptides. The cells were then fixed with PBS$^+$ 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$^−$/1% BSA and 50% complete medium. Then 5 × 10^6 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$^−$/1% BSA and resuspended in complete DMEM. The GD25-β1A cell lines were reconstituted 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 × 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 β2 antibody K20 at a ratio of 100 μg to 2 × 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 disrupter 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-

![Figure 1](https://example.com/f1.png)

**FIGURE 1.** β1A integrin tail mutations affect cell spreading on fibronectin. A, conserved amino acid residues (boxed) in integrin β subunits that are present at comparable sites in human β1, β2, β3, β4, and β5 cytoplasmic sequences were mutated to alanine and transfected into the β1A,GD25 cell line. B, the β1A,GD25 cell line, with or without reconstituted β1A wild-type integrin, was plated on tissue-culture dishes coated with 10 μg/ml FN. The spreading kinetics of the cells in the presence or absence of a β1A blocking antibody (40 μg/ml 4B4) was assessed. The β1A blocking antibody significantly inhibited GD25-β1A wt spreading at 10 and 20 min (p < 0.05 and p < 0.01, respectively). C, the β1A GD25 cell line reconstituted with β1A wild-type or mutant integrins was allowed to spread for 20 min on 10 μg/ml FN to assess β1A integrin function. For β1A wt versus T789A, p = 0.1; for β1A wt versus L754A, p = 0.001; for β1A wt versus E769A, p = 0.7; for β1A wt versus E767A, p = 0.5; for β1A wt versus F766A, p = 0.05; for β1A wt versus L754A, p = 0.3; for β1A wt versus D759A, p = 0.8; for β1A wt versus R760A, p = 1.0; for β1A wt versus T789A, p = 0.05; for β1A wt versus L745A, p = 0.2; and for β1A wt versus T789A, p < 0.05. Graphs represent pooled data from three independent experiments. Error bars represent ± S.E.*; statistical significance (p < 0.05).
The ability of GD25 cells expressing mutant integrin tail mutations to assemble fibronectin matrix was quantified as fold change compared with GD25 cells expressing wild-type integrin. The graphs show 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 β1A wt versus T789A, p = 0.1; for β1A wt versus W775A, p < 0.001; for β1A wt versus E769A, p < 0.001; for β1A wt versus E767A, p < 0.01; for β1A wt versus T789A, p < 0.001; for β1A wt versus E762A, p < 0.001; for β1A wt versus R760A, p < 0.001; for β1A wt versus D759A, p = 0.06; and for β1A wt versus L754A, p < 0.001. Error bars represent ± 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 transfected using a 6:1 ratio of Fugene6 (Roche Applied Science) reagent to DNA according to the manufacturer’s protocol. The targeting sequences for the talin-1 SMARTpool siRNA were CGAAGACCGCUACAUCAAC, GCACAUACGUGAGAAGC, and GAGGGAGAGCGUGAGAGA. The targeting sequences for the talin-2 SMARTpool siRNA were GAGGGAGAGCGUGAGAGA, GAACGUUUUUGUUGCUACA, UGGCAGGGAUUUCAGAA, and CGAAGGCGGCUACAGCAA. After 48 h, the cells were harvested, and protein levels were quantified using Western blots developed as described previously.

**FIGURE 2.** Multiple β1A integrin tail mutations affect fibronectin matrix assembly. The ability of GD25 cells expressing wild-type β1A integrin to assemble fibronectin matrix was quantified as fold change compared with GD25 cells expressing mutant β1A integrins. 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 β1A wt versus T789A, p = 0.1; for β1A wt versus W775A, p < 0.001; for β1A wt versus E769A, p < 0.001; for β1A wt versus E767A, p < 0.01; for β1A wt versus T789A, p < 0.001; for β1A wt versus E762A, p < 0.001; for β1A wt versus R760A, p < 0.001; for β1A wt versus D759A, p = 0.06; and for β1A wt versus L754A, p < 0.001. Error bars represent ± S.E., statistical significance (p < 0.05).

**FIGURE 3.** W775A β1A 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, β1A wt GD25 cells and cells reconstituted with wild-type, R760A, or W775A mutant β1A integrins were allowed to spread on culture dishes coated with 10 μg/ml FN for 60 min in the presence of 10 μg/ml C8F12, an αv integrin blocking antibody. C8F12 significantly inhibited cell spreading of β1A wt GD25 and W775A cells (p = 0.002 and p = 0.001, respectively) but not of GD25-β1A wt or R760A cells (p > 0.05). In contrast, the ability of β1A wt GD25, W775A, and R760A cells to spread on 10 μg/ml laminin-1 was substantially decreased compared with GD25-β1A wt cells (p < 0.001). Graphs shown represent pooled data from three independent experiments. Error bars represent ± 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 transfected using a 6:1 ratio of Fugene6 (Roche Applied Science) reagent to DNA according to the manufacturer’s protocol. The targeting sequences for the talin-1 SMARTpool siRNA were CGAAGACCGCUACAUCAAC, GCACAUACGUGAGAAGC, and GAGGGAGAGCGUGAGAGA. The targeting sequences for the talin-2 SMARTpool siRNA were GAGGGAGAGCGUGAGAGA, GAACGUUUUUGUUGCUACA, UGGCAGGGAUUUCAGAA, and CGAAGGCGGCUACAGCAA. After 48 h, the cells were harvested, and protein levels were quantified using Western blots developed as described previously.

**FIGURE 3.** W775A β1A 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, β1A wt GD25 cells and cells reconstituted with wild-type, R760A, or W775A mutant β1A integrins were allowed to spread on culture dishes coated with 10 μg/ml FN for 60 min in the presence of 10 μg/ml C8F12, an αv integrin blocking antibody. C8F12 significantly inhibited cell spreading of β1A wt GD25 and W775A cells (p = 0.002 and p = 0.001, respectively) but not of GD25-β1A wt or R760A cells (p > 0.05). In contrast, the ability of β1A wt GD25, W775A, and R760A cells to spread on 10 μg/ml laminin-1 was substantially decreased compared with GD25-β1A wt cells (p < 0.001). Graphs shown represent pooled data from three independent experiments. Error bars represent ± S.E., statistical significance (p < 0.05).
one-way analysis of variance with pairwise multiple comparisons made using the Tukey-Kramer test.

RESULTS

Specific Sites in the β1A Integrin Cytoplasmic Domain Are Required for Cell Spreading—DNA plasmids encoding wild-type β1A integrin or cytoplasmic mutant integrins were expressed in β1A/GD25 cells, and stable cell lines were generated as previously described (21). Specific amino acid residues in the cytoplasmic domain of the β1A integrin were selected for mutation (Fig. 1A), because they were conserved between comparable sites of six different β1 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 β1A/GD25 and GD25 cells are major mediators of cell spreading on fibronectin.

Because β1A/GD25 cells reconstituted with the β1A integrin (GD25-β1A/wt) still express the α5β1 integrin function, the kinetics of spreading on fibronectin were first compared to identify differences in rates of β1 versus β3 integrin-mediated spreading. The β1-/- GD25 and GD25-β1A/wt cell lines were plated on substrates coated with 10 μg/ml fibronectin and allowed to spread. The number of cells spread at the indicated time points was counted in the presence and absence of 40 μg/ml β1 integrin-blocking antibody 4B4 (Fig. 1B). The β1-/- GD25 cells, which primarily use the α5β1 integrin to spread on fibronectin, displayed slower spreading kinetics than the GD25-β1A/wt cells (p < 0.05 and p < 0.01 at 10 and 20 min, respectively). Treatment of the GD25-β1A/wt cells with the β1 blocking antibody 4B4 inhibited spreading rates to the levels observed with the β1A/GD25 cells, indicating that the difference in spreading kinetics is due to β1 integrin function. As expected, 4B4 had no effect on the spreading rate of the β1A/GD25 cells.

We directly compared cell spreading by the β1-/- GD25, GD25-β1A/wt, and nine β1A integrin cytoplasmic mutant cell lines on 10 μg/ml fibronectin for 20 min, the time at which wild-type β1A integrin function was most apparent. Cell spreading varied widely between the mutants, with seven point mutations, including R760A and T789A (p < 1.0 for both mutants), having little to no effect on spreading compared with that of the GD25-β1A/wt cells, and the W775A (p = 0.001) and F766A (p < 0.05) mutants having the most significant effect in cell spreading (Fig. 1C).

Comparison of Specific Sites in the β1A Integrin Cytoplasmic Domain Required for Fibronectin Matrix Assembly and Cell Spreading

![Figure 4](image-url)
Spreading—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 β1Awt GD25 cells (data not shown). As observed for cell spreading on fibronectin, the effects of the β1A integrin cytoplasmic mutants on fibronectin fibrillogenesis were selective (Fig. 2). For example, cells expressing the T789A mutation compared with the W775A mutation showed no effect (p = 1.0) versus a dramatic decrease (p < 0.001) 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. 1C (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 β1A integrin mutations.

W775A and R760A β1A 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 to test whether the differential spreading phenotype could be reproduced on another extracellular matrix component. Later time points for spreading were used, because the GD25-β1Awt cells spread more slowly on laminin-1. We also used a specific αv-blocking antibody, C8F12 (24), to inhibit the compensating effect of the αvβ3 integrin on cell spreading on fibronectin. In the presence of the αv-blocking antibody, spreading was significantly reduced in the W775A β1A integrin mutant cells when compared with the R760A β1A integrin mutant cells (Fig. 3A). These results reproduced the selective spreading defect observed previously (Fig. 1C). In striking contrast, when the cells were plated on 10 μg/ml laminin-1, neither the W775A nor the R760A β1A integrin mutant cells were able to spread (Fig. 3B). This finding indicates that different mutations in the cytoplasmic domain of a single integrin subunit can lead to markedly different ligand adhesive specificities at the extracellular face and that the selective defect in cell spreading between the W775A and R760A integrin was characteristic of interactions with fibronectin but not laminin-1.

Surface Expression of αv, αv, and β3 Integrins in W775A and R760A β1A Integrin Mutant Cells—Ectopic expression of the β1A integrin in β1Awt GD25 cells induces expression of the integrin αv subunit (18), which permits spreading of the GD25-β1Awt cells on laminin-1. Both β1Awt GD25 cells and GD25-β1Awt 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 β1A 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 effects 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 α5 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 α5β4 subunit may represent an increase in the expression of the α5β4 integrin. It is possible that increased expression of the α5 subunit could also induce expression of the α5β1 integrin at the cell surface. However, this appears unlikely because expression of the α5 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, α5 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 α5, αv, 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 constitutively 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-β1Awt 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 Cytoplasmic 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 cytoplasmic 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 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 \( \beta_1 \) 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 \( \beta_1 \) 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 \( \beta_1 \) integrin cytoplasmic domain. 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 \( \beta_1A \) 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 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 \( \beta_1A \) 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 consequences of talin perturbation on fibronectin matrix assembly. This is the first study to show an effect of \( \beta_1A \) integrin cytoplasmic mutations on the formation of fibronectin matrix-associated fibrillar adhesions and consequences of talin perturbation on fibronectin matrix assembly. The conclusions of this study are illustrated in Fig. 10.
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 α5β1 integrin-mediated fibronectin matrix assembly (40). In support of this finding, the α5 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 β3 wt background. These cells showed reduced adhesion and integrin acti-

![Fibronectin, GFP-talinH, Vinculin, Merged](image)

**FIGURE 9. Expression of the talin head domain in β1 wild-type cells inhibits fibronectin matrix assembly.** β1−/− GD25 cells reconstituted with β1A wild-type integrin were transiently transfected with a plasmid encoding the GFP-talin head domain (GFP-talinH) as described under “Experimental Procedures.” At 48 h post-transfection, 20 μg/ml bFN was added to the cells. At 48 h post-transfection the cells were fixed and stained with mouse anti-vinculin/Cy3 anti-mouse and Cy5-conjugated anti-streptavidin antibodies. Cells in A show cells that are not expressing GFP-talinH. Cells in B and C show cells that are either expressing or not expressing GFP talin-H. White arrowheads indicate residual fibronectin fibrils formed at the cell periphery (B) or between cells (C). Cells in D show cells that are expressing different levels of GFP-talinH (white asterisks, high expression; orange asterisk, low expression). Scale bar indicates 10 μm. Images represent one slice of a confocal z-series. The images shown are representative of three independent experiments.

| β1A cytoplasmic domain mutations | Cell spreading | FN matrix assembly, FN fibrils | Fibrillar adhesion formation, β1 | Integrin β1 | Recruitment to cytoplasmic domain, talin |
|----------------------------------|---------------|-------------------------------|-------------------------------|------------|--------------------------------------|
| Fibronecin                       | Laminin-1     |                               |                               |            |                                      |
| +                                | +             | +                             | +                             | +          | +                                    |
| −                                | −             | −                             | −                             | −          | −                                    |
| +/−                              | +/−           | +/−                           | +/−                           | +/−        | +/−                                  |

Table 1

Summary of localization and functional perturbations of W775A and R760A β1A integrin mutants

*αv-dependent.
*αv-independent.
We have demonstrated that specific βι 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.

Acknowledgments—We are grateful to Kazue Matsumoto for generating the βιА integrin cytoplasmic mutant plasmids. We are also grateful to members of the Yamada laboratory for their valuable comments during preparation of this manuscript and to Harry Grant for his excellent proofreading. We appreciate the help of Derek Hewgill in providing assistance with cell sorting and flow cytometry. We would especially like to thank Charles Streuli for providing the C8F12 αι integrin-blocking antibody.

REFERENCES

1. Hynes, R. O. (2002) Cell 110, 673–687
2. Arnaout, M. A., Mahalingam, B., and Xiong, J. P. (2005) Annu. Rev. Cell Dev. Biol. 21, 381–410
3. Ginsberg, M. H., Partridge, A., and Shattil, S. J. (2005) Curr. Opin. Cell Biol. 17, 509–516
4. Bodeau, A. L., Berrier, A. L., Mastrangelo, A. M., Martinez, R., and Fassler, R. (1996) Exp. Cell Res. 235, 471–481
5. LaFlamme, S. E. (2001) J. Cell Sci. 114 (Pt 9), 1719–1729
6. Kaapa, A., Peter, K., and Ylanne, J. (1999) J. Biol. Chem. 274, 1321–1330
7. Kaapa, A., Peter, K., and Ylanne, J. (1999) J. Biol. Chem. 274, 1321–1330
8. Tadokoro, S., Shattil, S. J., Eto, K., Tai, V., Liddington, R. C., de Pereda, J. M., Ginsberg, M. H., and Calderwood, D. A. (2003) Science 302, 103–106
9. Vignoud, L., Albigez-Rizo, C., Frachet, P., and Block, M. R. (1997) J. Cell Sci. 110, 1421–1430
10. Ylanne, J., Chen, Y., O’Toole, T. E., Loftus, J. C., Takada, Y., and Ginsberg, M. H. (1993) J. Cell Biol. 122, 227–238
11. Larsen, M., Artyom, V. V., Green, J. A., and Yamada, K. M. (2006) Curr. Opin. Cell Biol. 18, 463–471
12. Mao, Y., and Schwarz, J. E. (2005) Matrix Biol. 24, 389–399
13. Danen, E. H., Sonneveld, P., Brakebusch, C., Fassler, R., and Sonnenberg, A. (2002) J. Cell Biol. 159, 1071–1086
14. Retta, S. F., Balzac, F., Ferraris, P., Belkin, A. M., Fassler, R., Humphries, M. J., De Leo, G., Silengo, L., and Tarone, G. (1998) Mol. Biol. Cell 9, 715–731
15. Wennerberg, K., Fassler, R., Warmegard, B., and Johansson, S. (1998) J. Cell Sci. 111, 1117–1126
16. Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S., and Fassler, R. (1996) J. Cell Biol. 132, 227–238
17. Zhang, Q., Sakai, T., Nowlen, J., Hayashi, I., Fassler, R., and Mosher, D. F. (1999) J. Biol. Chem. 274, 368–375
20. Fassler, R., Pfaff, M., Murphy, J., Noegel, A. A., Johansson, S., Timpl, R., and Albrecht, R. (1995) J. Cell Biol. 128, 979–988
21. Pankov, R., Cukierman, E., Clark, K., Matsumoto, K., Hahn, C., Poulin, B., and Yamada, K. M. (2003) J. Biol. Chem. 278, 18671–18681
22. Mould, A. P., Garratt, A. N., Askari, J. A., Akiyama, S. K., and Humphries, M. J. (1995) FEBS Lett. 363, 118–122
23. Tran, H., Pankov, R., Tran, S. D., Hampton, B., Burgess, W. H., and Yamada, K. M. (2002) J. Cell Sci. 115, 2031–2040
24. Delcommenne, M., and Streuli, C. H. (1999) Methods Mol. Biol. 129, 19–34
25. Miekka, S. I., Ingham, K. C., and Menache, D. (1982) Thromb. Res. 27, 1–14
26. Pankov, R., Endo, Y., Even-Ram, S., Araki, M., Clark, K., Cukierman, E., Matsumoto, K., and Yamada, K. M. (2005) J. Cell Biol. 170, 793–802
27. Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001) Nat. Rev. Mol. Cell. Biol. 2, 793–805
28. Zamir, E., Katz, B. Z., Aota, S., Yamada, K. M., Geiger, B., and Kam, Z. (1999) J. Cell Sci. 112, 1655–1669
29. Zamir, E., Katz, M., Posen, Y., Erez, N., Yamada, K. M., Katz, B. Z., Lin, S., Lin, D. C., Bershadsky, A., Kam, Z., and Geiger, B. (2000) Nat. Cell Biol. 2, 191–196
30. Humphries, M. J., Travis, M. A., Clark, K., and Mould, A. P. (2004) Biochem. Soc. Trans. 32, 822–825
31. Bazzoni, G., Shih, D. T., Buck, C. A., and Hemler, M. E. (1995) J. Biol. Chem. 270, 25570–25577
32. Zhang, X., Jiang, G., Cai, Y., Monkley, S. J., Critchley, D. R., and Sheetz, M. P. (2008) Nat. Cell Biol. 10, 1062–1068
33. Critchley, D. R., and Gingras, A. R. (2008) J. Cell Sci. 121, 1345–1347
34. Wegener, K. L., Partridge, A. W., Han, J., Pickford, A. R., Liddington, R. C., Ginsberg, M. H., and Campbell, I. D. (2007) Cell 128, 171–182
35. Bouaouina, M., Lad, Y., and Calderwood, D. A. (2008) J. Biol. Chem. 283, 6118–6125
36. Gu, J., Fujibayashi, A., Yamada, K. M., and Sekiguchi, K. (2002) J. Biol. Chem. 277, 19922–19928
37. Gu, J., Sumida, Y., Sanzen, N., and Sekiguchi, K. (2001) J. Biol. Chem. 276, 27090–27097
38. Sondermann, H., Dogic, D., Pesch, M., and Aumailley, M. (1999) Cell Adhes. Commun. 7, 43–56
39. Somanath, P. R., Kandel, E. S., Hay, N., and Byzova, T. V. (2007) J. Biol. Chem. 282, 22964–22976
40. Sechler, J. L., Takada, Y., and Schwarzauer, J. E. (1996) J. Cell Biol. 134, 573–583
41. Takahashi, S., Leiss, M., Moser, M., Ohashi, T., Kitao, T., Heckmann, D., Pfeifer, A., Kessler, H., Takagi, I., Erickson, H. P., and Fassler, R. (2007) J. Cell Biol. 178, 167–178
42. Lim, J., Wiedemann, A., Tzircotis, G., Monkley, S. J., Critchley, D. R., and Caron, E. (2007) Mol. Biol. Cell 18, 976–985
43. Garcia-Alvarez, B., de Pereda, J. M., Calderwood, D. A., Ulmer, T. S., Critchley, D., Campbell, I. D., Ginsberg, M. H., and Liddington, R. C. (2003) Mol. Cell 11, 49–58
44. Humphries, M. J. (2004) Biochem. Soc. Trans. 32, 407–411
45. Jiang, G., Giannone, G., Critchley, D. R., Fukumoto, E., and Sheetz, M. P. (2003) Nature 424, 334–337