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Integrin α4β1 Promotes Focal Adhesion Kinase-Independent Cell Motility via α4 Cytoplasmic Domain-Specific Activation of c-Src‡

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The fibronectin binding integrins α5β1 and α4β1 generate signals pivotal for cell migration through distinct yet undefined mechanisms. For α5β1, β1-mediated activation of focal adhesion kinase (FAK) promotes c-Src recruitment to FAK and the formation of a FAK-Src signaling complex. Herein, we show that FAK expression is essential for α5β1-stimulated cell motility and that exogenous expression of human α4 in FAK-null fibroblasts forms a functional α4β1 receptor that promotes robust cell motility equal to the α5β1 stimulation of wild-type and FAK-reconstituted fibroblasts. α4β1-stimulated FAK-null cell spreading and motility were dependent on the integrity of the α4 cytoplasmic domain, independent of direct paxillin binding to α4, and were not affected by PRNK expression, a dominant-negative inhibitor of Pyk2. α4 cytoplasmic domain-initiated signaling led to a ~4-fold activation of c-Src which did not require paxillin binding to α4. Notably, α4-stimulated cell motility was inhibited by catalytically inactive receptor protein-tyrosine phosphatase α overexpression and blocked by the p50Csk phosphorylation of c-Src at Tyr-529. α4β1-stimulated cell motility of triple-null $Src^{-/-}$, c-$Yes^{-/-}$, and $Fyn^{-/-}$ fibroblasts was dependent on c-Src reexpression that resulted in p130Cas tyrosine phosphorylation and Rac GTPase loading. As p130Cas phosphorylation and Rac activation are common downstream targets for α5β1-stimulated FAK activation, our results support the existence of a novel α4 cytoplasmic domain connection leading to c-Src activation which functions as a FAK-independent linkage to a common motility-promoting signaling pathway.

Integrins are a family of heterodimeric α/β transmembrane cell adhesion receptors that play important roles in the regulation of cell migration during development, wound healing, inflammation, and the spread of tumor cells. Integrins do not possess intrinsic catalytic activity, and thus, signaling events are mediated by either lateral association with other receptors (8, 52) or the clustering of signaling proteins with integrin cytoplasmic domains (44). As the composition of integrin signaling complexes is diverse and remains poorly defined (16), it is important to identify the molecular signaling signature of integrins that share a common β subunit, bind to a common substrate such as fibronectin (FN), and function to promote cell motility. The FN binding integrins α5β1 and α4β1 share these properties.

α5β1 is considered to be the classical FN receptor, with binding occurring within FN repeats III-9 and III-10 (33, 37). Rapid activation of protein-tyrosine kinases (PTKs) is one of the first signaling events associated with α5β1 binding to FN, and signals generated by the β1 cytoplasmic domain are important in promoting cell motility (12, 39). Focal adhesion kinase (FAK) is recruited to sites of α5β1 clustering through FAK C-terminal domain interactions with β1-integrin binding proteins such as talin and adaptor proteins such as paxillin (34). FN-stimulated FAK activation results in increased FAK tyrosine phosphorylation and the binding of Src-family PTKs to FAK, thus creating a dual FAK-Src signaling complex (28). Overexpression of the FAK C-terminal domain termed FRNK acts as a competitive inhibitor of FAK activation at sites of α5β1 integrin clustering (34).

Cellular FN contains an alternately spliced region called the type III connecting segment (IIICS) or connecting segment 1 (CS-1) region that contains binding sites for α4β1 and α4β7 (33, 37). α4β1 integrins also bind to vascular cell adhesion molecule 1 (VCAM-1), the expression of which is upregulated on activated endothelium during inflammation (40). α4β1 signaling promoting cell motility plays an important role in heart (46) and neural crest cell (21) development, as well as in hematopoietic cell homing to the bone marrow (45). Studies with chimeric α4 integrin subunits have shown that the α4 cytoplasmic domain can confer enhanced migratory properties to cells (3) and that α4β1 and α5β1 promote cell migration through distinct mechanisms in melanoma cells (30).

Null mutation of *fibronectin* or *fak* genes results in similar lethal developmental phenotypes (17). FAK-null ($FAK^{-/-}$) fibroblasts exhibit a small rounded morphology and refractory

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motility responses to both growth factor and integrin stimuli (47). The expression of the FAK-related Pyk2 PTK is elevated in FAK$^{-/-}$ cells (49). However, Pyk2 is not efficiently recruited to sites of β1 integrin clustering and does not substitute for the loss of FAK in promoting FN-stimulated cell motility (49). In other cell types, Pyk2 promotes cell migration (6, 32) and can be inhibited by the exogenous expression of the Pyk2 C-terminal domain termed PRNK (41, 51). FAK reexpression in FAK$^{-/-}$ cells fully rescues both the morphology and motility defects, and this is associated with enhanced α-actinin and p130Cas tyrosine phosphorylation, activation of mitogen-activated protein kinases, increased p21 Rac GTPase activity, and the modulation of RhoA GTPase activity (28). Whereas Src-mediated transformation also acts to promote FAK$^{-/-}$ cell motility (13, 29), the key molecular connections of Src or FAK in promoting these events remain poorly understood.

One unique feature of the α4 cytoplasmic domain is that it directly binds to paxillin (26), and this interaction is negatively regulated by α4 serine-988 phosphorylation (10, 11). It was originally proposed that paxillin binding to α4 facilitated outside-in signal transduction through the activation of paxillin-binding PTKs such as FAK, thereby leading to enhanced cell motility (26). However, a point mutation of α4 (Y991 to alanine, Y991A) that disrupts paxillin binding can still function to promote cell migration (36) and enforced paxillin association with α4 inhibits cell motility (7). Recent studies have linked paxillin binding to α4 to the negative regulation of Rac activity at the posterior end of migrating cells via the formation of an α4, paxillin, and Arf-GTPase-activating protein complex (31). To this end, it remains unclear how α4-associated signaling promotes Rac activation at the leading edge of migrating cells (15). Although α4β1 activation can promote the tyrosine phosphorylation of a broad range of proteins (14) including Pyk2 and FAK (6, 19, 41), it is unclear whether differences in α4β1- and α5β1-stimulated cell motility reflect a novel α4 cytoplasmic domain linkage to PTK activation as opposed to enhanced β1-mediated signaling.

To address this issue and to determine the role of FAK, Pyk2, or Src activation in α4β1- and α5β1-stimulated signaling responses, we evaluated FAK$^{-/-}$, FAK$^{+/+}$, FAK-reconstituted (DA2), and triple-null c-Src$^{-/-}$, c-Yes$^{-/-}$, and Fyn$^{-/-}$ (SYF) fibroblasts using recombinant FN ligands that specifically bind α4β1 or α5β1 integrins. As these murine fibroblasts express α5 and β1 but not α4 integrins, we used both the transient and stable expression of human α4 and α4 cytoplasmic domain mutants to create α4β1 heterodimeric receptors. Through a series of biochemical analyses and gain-of-function cell motility assays, herein, we show that α4β1 and α5β1 activate a common downstream motility-promoting pathway through distinct receptor-proximal connections to Src or FAK-Src signaling complexes, respectively.

MATERIALS AND METHODS

Antibodies and reagents. Antiphosphotyrosine (anti-pTyr) (4G10) monoclonal antibody (MAb) and Rac MAb (clone 23A8) were from UBI. Hemagglutinin (HA) epitope tag MAb (clone 16B12) and Myc epitope tag MAb (clone 9E10) were from Covance Research. p130Cas (clone 21), Pyk2 (clone 11), and FAK (clone 77) MAbs were from BD Biosciences. β-Actin MAb (clone AC-74) was from Sigma. Polyclonal antibodies to p130Cas (C20) and to c-Src (Src-2) were from Santa Cruz Biotechnology. Phosphospecific antibodies to the FAK pTyr-397 motif, the Pyk2 pTyr-402 motif, the c-Src kinase pTyr-418 region, and the c-Src C-terminal pTyr-529 motif were from BioSource International. Polyclonal antibodies to FAK (5904 and 5592), Pyk2 (5906), and p50Csk (5363) were generated as described previously (49). A MAb specific to c-Src (clone 2-17) was provided by W. Eckhart (The Salk Institute, La Jolla, CA). Mouse MAbs to human integrin α4 (clones HP2/1 and P1H4), integrin α5 (clones P1D6 and NKI-SAM-1), and integrin α6 (clone 4F10) and rat anti-human MAb to integrin α6 (clone NKI-GoH3) were from Chemicon. Rat anti-mouse MAbs to integrin α5 (clone 5H10-27), integrin αv (clone RMV-7), integrin α4 (clone 9C10), and integrin β1 (clone KMI6) were from BD Biosciences. Purified bovine plasma FN (F-1141) was from Sigma. The PP2 c-Src inhibitor and the PP3 Src-inactive control compounds were from Calbiochem.

Cells, retrovirus, and plasmids. FAK$^{-/-}$, wild-type FAK$^{+/+}$, and FAK-reconstituted (DA2) fibroblasts are p53-null and were maintained as described previously (48). SYF and SYF+c-Src fibroblasts immortalized by simian virus 40 large T antigen (American Type Culture Collection) were maintained in Dulbecco's modified Eagle medium (DMEM). Cell medium was supplemented with 10% fetal bovine serum (FBS), 1 mM nonessential amino acids, 1 mM sodium pyruvate, 1,000 U/ml penicillin, and 1,000 μg/ml streptomycin sulfate. Human integrin α4 wild type (WT) and α4 Y991A in pcDNA3.1 (41) were cloned into pBabe-puro, and retrovirus was produced via transfection of 293-Phoenix-Eco packaging cells as described previously (49). Target cells were infected for 24 h in retrovirus-containing medium with 5 μg/ml Polybrene and selected for growth in 2 μg/ml puromycin for 72 h, and pooled populations of human α4 integrin-expressing cells were sorted by flow cytometry using fluorescence-activated cell sorter (FACS) Vantage DiVa I (BD Biosciences). For FAK$^{-/-}$ cells, stable human α4 expression was established in clonal cell populations and maintained with repeated FACS using anti-integrin α4 (HP2/1).

Bacterial expression vectors for glutathione *S*-transferase (GST) fusion proteins encompassing the human FN CS-1 region and FN repeats 9 through 11 (9-11) were provided by J. W. Smith (Burnham Institute, La Jolla, CA) and J. W. Ramos (Rutgers University, New Brunswick, NJ), respectively, and were purified as described previously (18, 38). VCAM-immunoglobulin (Ig), consisting of the seven extracellular Ig domains of VCAM-1 fused to the heavy chain of human IgG1, was expressed and purified as described previously (41). Expression, purification, and activated p21Rac binding assays using a GST fusion encompassing residues 67 through 150 from p65PAK kinase were performed as described previously (13). Protein purity was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (>90%) and protein concentrations determined by A_{280} or the D_C protein assay (Bio-Rad) using bovine serum albumin (BSA) as a standard. Murine paxillin was amplified by reverse transcriptase PCR and cloned into pEGFP-N3 (BD Biosciences), and β-galactosidase (LacZ) in pcDNA3 was used as described previously (48). HA-tagged receptor protein-tyrosine phosphatase α (RPTPα) and a catalytically inactive mutant (C433S and C723S) in the pSG expression vector were used as described previously (2). Cell transfection with expression plasmids (5 μg total DNA per 10-cm plate) was performed using Lipofectamine Plus (BD Biosciences) as described previously (48).

Ad production and infection. Recombinant adenovirus (Ad) expressing WT p50Csk and kinase-inactive (KD) p50Csk were created as described previously (50). The cDNA sequence for human α4 integrin (X4C4) and cytoplasmic domain-truncated (α4-ΔCyto) α4 integrin (X4C0) (20) were subcloned into pAd-Lox and recombinant viruses produced by cotransfection of 293-Cre cells with adenovirus DNA (Ad5-ψ5) that contains an E1A/E3-deleted adenovirus genome. Viruses were plaque purified, and PCR was used to screen for the incorporation of α4 cDNA. Immunodetection using human-specific α4 antibody (HP2/1) with Ad-infected rabbit synovial fibroblasts was used to verify Ad-mediated α4 integrin surface expression. Myc-tagged versions of human Pyk2, F402 Pyk2, kinase-inactive A457 Pyk2 (49), Myc-tagged PRNK (41), HA-tagged FRNK, and HA-tagged FRNK S-1034 in pcDNA3.1 were subcloned into pADtet7 containing Tet-responsive enhancer sequences within a minimal cytomegalovirus promoter. pADtet7 also contains the simian virus 40 late poly(A) cassette, adenovirus E1A, and a single *loxP* site. Recombinant viruses were produced by pADtet7-FAK cotransfection of 293-Cre cells as described above. Adenoviral Pyk2, F402 Pyk2, A457 Pyk2, FRNK, and PRNK protein expression were driven by coinfection with Ad-TA expressing the Tet transactivator as described previously (13). Cells were infected at a multiplicity of infection (MOI) of 5 PFU/cell for Ad-TA (mock) and with an MOI of 50 for Pyk2, FRNK, or PRNK. Ad-α4 WT, Ad-α4 ΔCyto, Ad-Csk WT, and Ad-Csk KD were used at an MOI of 100, and cells were analyzed 48 h postinfection.

Flow cytometric analysis. Cells were trypsinized, enumerated, and incubated with specific primary antibodies (10^6 cells/μg antibody) for 20 min on ice in 100 μl of phosphate-buffered saline (PBS) followed by pelleting and washing of cells. Alexa-488-conjugated goat anti-rat IgG (Molecular Probes) and Alexa-488-con-

jugated goat anti-mouse IgG (Molecular Probes) were used as labeled secondary antibodies for visualization. The conditions for secondary antibody incubation and washing were the same as described above. Analyses were performed using a FACScan or FACScalibur machine (BD Biosciences). Negative controls used mouse or rat IgG in the primary incubation.

Cell adhesion and migration. For cell adhesion, glass coverslips were precoated with GST-FN (9-11) or GST-FN (CS-1) at 5 μg/ml in PBS overnight at 4°C and then blocked with 1% heat-denatured BSA for 1 h at 37°C. Cells were used 48 h after Ad infection and were serum starved (0.5% FBS) between 36 and 48 h. Equal numbers of suspended cells in DMEM plus 0.5% BSA (migration medium) were plated on FN (9-11)- or FN (CS-1)-coated glass slides and incubated at 37°C. At 15-, 30-, and 60-min intervals, unattached cells were washed away with PBS and the attached cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature. Cell adhesion and spreading values were enumerated under a microscope at high magnification.

For FN-stimulated cell migration in the absence of serum (haptotaxis motility), Millicell chambers (12-mm diameter with 8-μm pores; Millipore) were precoated with the indicated concentration of FN ligand or VCAM-1 IgG in PBS for 2 h on the membrane underside (48). For random motility, the top and bottom membrane surfaces were precoated with 10 μg/ml FN ligand or VCAM-1. Cells were used 48 h after plasmid transfection or Ad infection and were serum starved (0.5% FBS) between 36 and 48 h. Adherent cells were collected by using 0.06% trypsin with 2 mM EDTA treatment, followed by the addition of soybean trypsin inhibitor (0.25 mg/ml with 0.25% BSA in DMEM), resuspended in migration medium, and held in suspension for 30 min at 37°C. Cells (1×10^5) in 300 μl were added to the upper Millicell chamber, placed into 400 μl migration medium in individual wells of a 24-well plate, and incubated at 37°C. Pharmacological inhibitors or antibodies were added to both chambers at the indicated concentration. After 3 h, cells were fixed in 3.7% paraformaldehyde for 5 min, cells on the upper membrane surface were removed using a cotton swab, and cells on the lower membrane surface were either stained with 0.1% crystal violet or analyzed for β-galactosidase activity using X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) as a substrate. Motile cells were viewed under a 40× lens objective, 10 nonoverlapping fields were counted per Millicell membrane, and all experiments were performed in triplicate. One-way analysis of variance was used to determine significance.

Cell lysis and blotting. Cells were solubilized in radioimmunoprecipitation assay lysis buffer containing 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS. Antibodies (2.5 μg) were incubated with lysates for 3 h at 4°C and collected by binding to Protein G Plus (Oncogene Research Products) or protein A (Repligen) agarose beads. SDS-PAGE, antibody blotting, and sequential membrane reprobing were performed as described previously (49).

In vitro kinase (IVK) assays. Cells were used 48 h after Ad infection and were serum starved (0.5% FBS) between 36 and 48 h. Lysates were prepared from equal numbers of serum-starved cells held in suspension for 45 min or replated on the indicated FN ligand for 45 min in migration medium. Anti-c-Src (MAb clone 2-17) immunoprecipitates (IPs) were washed in radioimmunoprecipitation assay lysis buffer, followed by lysis buffer without sodium deoxycholate and SDS, followed by HNTG buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 0.1% Triton, 10% glycerol), and washed twice in kinase buffer {20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 7.0], 10 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol}. Next, 20 μCi of [γ-^{32}P]ATP at 5 μCi/nmol ATP was added to the c-Src IPs along with 2 μg GST-FAK-CT as a substrate (containing FAK Y861 and Y925 phosphorylation sites) and the IPs were incubated at 32°C for 15 min. The reactions were stopped by the addition of 2× SDS sample buffer, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, visualized by autoradiography, and quantified using a phosphorimager (PI), and values are expressed as arbitrary PI units. The equal recovery of c-Src in the IPs was verified by blotting.

Immunofluorescence staining and scratch wound assays. For staining, cells were transiently transfected with pEGFP-paxillin using Lipofectamine Plus (Invitrogen) and, after 36 h, plated onto FN (CS-1) (5 μg/ml) glass coverslips for 2 h in migration medium, fixed with 4% paraformaldehyde in PBS for 15 min, and permeabilized for 10 min with 0.2% saponin in PBS containing normal goat serum (blocking solution). Primary MAb antibody incubation was performed for 2 h at room temperature, and slides were washed in PBS and then further incubated for 45 min in tetramethyl rhodamine isocyanate-conjugated goat anti-mouse antibody (Vector). For scratch wound assays, glass coverslips were coated with 2 μg/ml FN (CS-1) and cells were plated at a density of 10^6 cells/35-mm dish and serum starved overnight. The cell monolayer was scratched with a single pass of a pipette tip, washed twice with PBS, and then incubated in 5% FBS-containing medium. After 2 h, the medium was replaced with medium 199 (Invitrogen) and the glass-coverslip-associated cells were placed in an open chamber maintained at 37°C (20/20 Technology Inc.) and visualized in phase contrast using a 20× lens objective (Olympus IX51; numerical aperture, 0.5). Images were collected every 2 min for 14 h with a charge-coupled-device camera (Hamamatsu Orca-ER) and processed using OpenLab software (Improvision).

RESULTS

Human α4 integrin expression in FAK$^{-/-}$ cells creates a functional α4β1 receptor. Signals generated by α4β1 binding to VCAM or to the CS-1 region of FN promote the motility of various cell types. FAK$^{-/-}$ fibroblasts express α5β1 (Fig. 1A), and yet FAK$^{-/-}$ cells exhibit enhanced focal contact formation and refractory cell motility responses to cellular FN compared to FAK$^{+/+}$ cells (17). The FAK-related PTK Pyk2 is expressed in FAK$^{-/-}$ cells, but Pyk2 is not efficiently recruited to β1 integrin signaling complexes (49). It is FAK C-terminal domain binding to both paxillin and talin that is important for FAK activation, association of FAK with a β1 integrin complex, and the rescue of FAK$^{-/-}$ cell motility by either FAK or chimeric Pyk2-FAK constructs (22, 48). As α4 possesses the unique property of binding paxillin directly, we wished to test whether the unique signaling properties of α4 may be sufficient to overcome FAK$^{-/-}$ cell motility defects. Flow cytometry analyses of FAK$^{-/-}$ and FAK$^{+/+}$ fibroblasts revealed high surface expression levels of αv, α5, β1, and β5 integrins but no significant surface expression of the α4 subunit (Fig. 1A). As FAK$^{-/-}$ and FAK$^{+/+}$ fibroblasts are amenable to recombinant Ad infection (13), WT human α4 and cytoplasmic domain-truncated α4 (α4-ΔCyto) integrins were made as recombinant Ad constructs (Fig. 1B). Flow cytometry analyses of Ad-infected FAK$^{-/-}$ and FAK$^{+/+}$ cells revealed strong α4-WT expression with slightly less α4-ΔCyto surface expression as detected with a human-specific α4 antibody (Fig. 1C).

By plating cells on recombinant fragments of FN spanning type III repeats 9 through 11 or the IIICS variable region of FN (CS-1) as GST fusion proteins (Fig. 1D), we could evaluate the selective activation of α5β1 after cell binding to GST-FN (9-11) or the functional expression and activation of α4β1 upon cell binding to GST-FN (CS-1), respectively. Without Ad-mediated α4 expression, the FAK$^{-/-}$ and FAK$^{+/+}$ cells did not bind to GST-FN (CS-1), whereas 75% of the cells bound to GST-FN (9-11) within 15 min (Fig. 1E). Ad-mediated α4-WT and α4-ΔCyto expression promoted the rapid adhesion of FAK$^{-/-}$ and FAK$^{+/+}$ to GST-FN (CS-1) equal to cell binding to GST-FN (9-11) (Fig. 1E). This binding was mediated by α4β1, as cell adhesion was blocked in the presence of either α4 or β1, but not α5, antibodies (data not shown). Interestingly, cells expressing α4-ΔCyto readily bound to FN (CS-1) but failed to spread and remained rounded and retractile after 1 h (Fig. 1F). FAK$^{-/-}$ and FAK$^{+/+}$ cells expressing α4-WT readily spread upon adhesion to FN (CS-1) and began to show signs of cell polarization within 1 h (Fig. 1F). These results show that Ad-mediated human α4 expression in murine fibroblasts creates a functional α4β1 receptor capable of promoting cell adhesion and spreading on FN (CS-1).

To determine whether the recombinant FN (9-11) and FN (CS-1) fragments would function to promote cell motility, FAK$^{-/-}$ and FAK$^{+/+}$ cells were evaluated in integrin-mediated haptotaxis assays. FN (9-11)-stimulated cell motility of FAK$^{+/+}$ cells was fourfold greater than that of FAK$^{-/-}$ cells

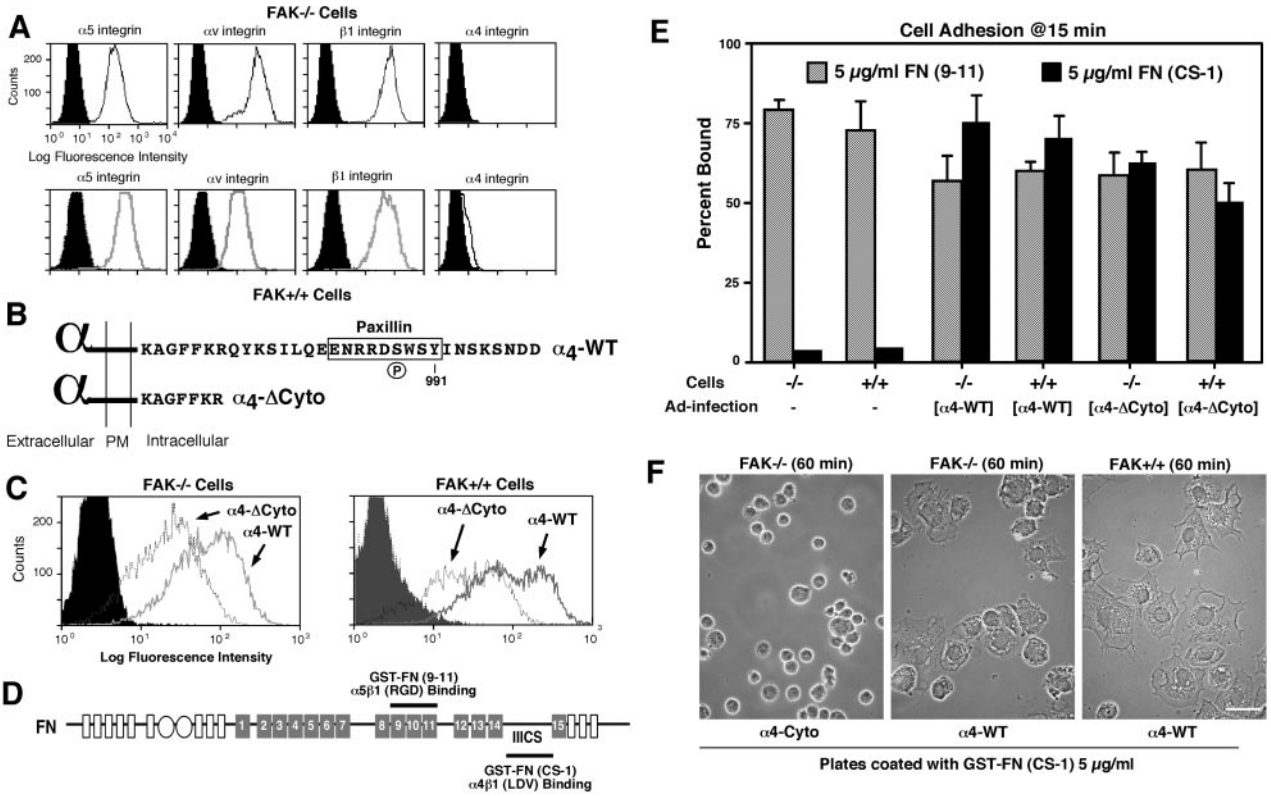


FIG. 1. Transient human α4 integrin expression creates a functional α4β1 receptor for FN (CS-1) in $FAK^{-/-}$ and $FAK^{+/+}$ fibroblasts. (A) Flow cytometry analyses of endogenous murine α5, αv, β1, and α4 integrin expression in $FAK^{-/-}$ and $FAK^{+/+}$ cells (open peaks). Staining with control MAb (shaded peaks). (B) Schematic representation and single-letter amino acid code for cytoplasmic domain residues of human α4 integrin (α4-WT) and a truncation mutant (α4-ΔCyto). Region of paxillin binding, site of protein kinase A phosphorylation, and the location of a point mutant (Tyr-991 to alanine, Y991A) disrupting paxillin binding are indicated. PM, plasma membrane. (C) Flow cytometry analyses of human integrin α4-WT (solid line) or α4-ΔCyto (dotted line) Ad-mediated expression in $FAK^{-/-}$ and $FAK^{+/+}$ cells as detected by anti-α4 MAb HP2/1 staining. Staining with control MAb (shaded peaks). (D) Schematic illustration of FN and recombinant GST fusion proteins. The type III FN repeats have been numbered 1 through 15. GST-FN (9-11) encompasses repeats 9 through 11 and contains the α5β1 binding motif (Arg-Gly-Asp, RGD). IIICS is an alternatively spliced region of FN that is also termed CS-1. GST-FN (CS-1) encompasses the IIICS region and contains the α4β1 binding motif (Leu-Asp-Val, LDV). (E) Percent $FAK^{-/-}$ and $FAK^{+/+}$ cell adhesion after 15 min to GST-FN (9-11) (gray bars) or to GST-FN (CS-1) (black bars) in noninfected or Ad-infected cells expressing human α4-WT or α4-ΔCyto. Mean values ± standard deviations from two experiments are given. (F) Phase contrast images of $FAK^{-/-}$ and $FAK^{+/+}$ cell adhesion and spreading on GST-FN (CS-1) after 60 min. Ad-mediated α4-ΔCyto expression promotes cell adhesion but not spreading. Bar, 65 μm.

(Fig. 2A), and $FAK^{+/+}$ cell motility was blocked in the presence of either α5 or β1 integrin antibodies (data not shown). This fourfold difference in $FAK^{-/-}$ and $FAK^{+/+}$ cell motility on FN (9-11) is similar to that measured for intact FN (17). Transient reexpression of FAK in $FAK^{-/-}$ cells or analyses of FAK-reconstituted (DA2) (48) fibroblasts resulted in equivalent FN (9-11)-stimulated cell motility compared to that for $FAK^{+/+}$ cells (data not shown), supporting the conclusion that α5β1-stimulated cell migration is FAK dependent.

When cells were evaluated using FN (CS-1) as a ligand, transient expression of α4-WT in both $FAK^{-/-}$ and $FAK^{+/+}$ cells promoted enhanced haptotaxis cell motility equal to α5β1-stimulated $FAK^{+/+}$ cell migration (Fig. 2A). α4-stimulated $FAK^{-/-}$ cell motility was specific for α4β1, as no increase in α5β1-stimulated $FAK^{-/-}$ cell migration was observed in α4-expressing cells, and antibodies to human α4 or murine β1 blocked α4β1 motility (data not shown). Even though α4-ΔCyto expression promoted cell adhesion to FN (CS-1) (Fig. 1E), truncation of the α4 cytoplasmic domain abrogated the ability of α4β1 to stimulate $FAK^{-/-}$ and $FAK^{+/+}$ cell motility (Fig. 2A). Expression of α4-WT expression led to the adhesion-dependent phosphorylation of Pyk2 at Tyr-402 and FAK at Tyr-397 in $FAK^{-/-}$ and $FAK^{+/+}$ cells, respectively (Fig. 2B and C). However, in cells expressing the α4-ΔCyto truncation, FN (CS-1)-stimulated Pyk2 and FAK tyrosine phosphorylation was significantly inhibited (Fig. 2C). Taken together, these results show that α4β1 but not α5β1 possesses a unique ability to promote $FAK^{-/-}$ cell motility and that the integrity of the α4 cytoplasmic domain is required for full tyrosine kinase activation and cell motility signaling events.

Stable α4 expression rescues the morphological and motility defects of $FAK^{-/-}$ cells. To determine whether paxillin binding to α4 was required for α4-stimulated $FAK^{-/-}$ cell motility, recombinant retroviruses producing human α4-WT or a α4 Y991A paxillin binding mutant (24) were used to infect $FAK^{-/-}$, $FAK^{+/+}$, and DA2 ($FAK^{-/-}$ reconstituted) fibroblasts. After growth selection in puromycin, cells were enriched for human α4 expression by FACS (Fig. 3A). For

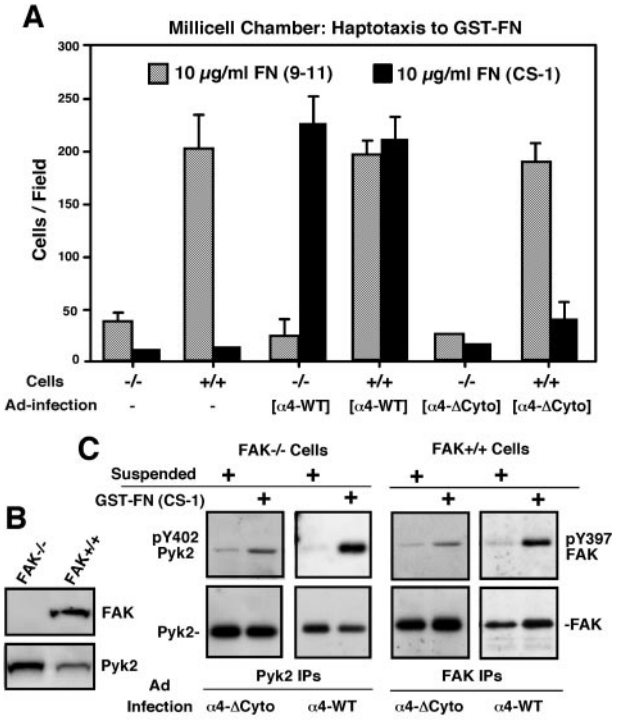


FIG. 2. α4 integrin cytoplasmic domain functions to promote $FAK^{-/-}$ cell motility at equivalent levels to α4β1- and α5β1-stimulated $FAK^{+/+}$ cells. (A) Haptotaxis assays were performed using $FAK^{-/-}$ and $FAK^{+/+}$ cells with or without human α4-WT or α4-ΔCyto expression. GST-FN (9-11) (gray bars) or GST-FN (CS-1) (black bars) at 10 μg/ml was used to stimulate cell migration (4 h) in the absence of serum. Values are means ± standard deviations of triplicate samples from three independent experiments. (B) Blotting of whole-cell lysates shows expression levels of Pyk2 and FAK in $FAK^{-/-}$ and $FAK^{+/+}$ cells. (C) The α4 cytoplasmic domain facilitates adhesion-dependent Pyk2 and FAK tyrosine phosphorylation at pY402 and pY397, respectively, in $FAK^{-/-}$ and $FAK^{+/+}$ cells. Cells were infected with the indicated α4 integrin, held in suspension for 45 min, or plated onto GST-FN (CS-1) for 45 min, and lysates were prepared and analyzed by Pyk2 or FAK immunoprecipitation. The IPs were blotted with phosphospecific antibodies to Pyk2 pY402 or FAK pY397, followed by reprobing with anti-Pyk2 or anti-FAK antibodies, respectively.

$FAK^{+/+}$ and DA2 fibroblasts, α4 expression remained constant for up to five cell passages. For $FAK^{-/-}$ cells, α4 expression in pooled populations was lost as the cells were passaged (data not shown). To circumvent this problem, α4 WT- and α4 Y991A-expressing $FAK^{-/-}$ cells were clonally expanded and two clones each with similar α4 expression levels to $FAK^{+/+}$ and DA2 α4 pools were further characterized (Fig. 3A). Analyses of cells growing in culture revealed that α4 WT expression was associated with the morphological conversion of small rounded $FAK^{-/-}$ cells to a polarized fibroblast phenotype and were comparable in shape to normal DA2 fibroblasts (Fig. 3B).

$FAK^{-/-}$ α4 Y991A cells exhibited a larger circumference than parental $FAK^{-/-}$ cells but only a weakly polarized phenotype compared to $FAK^{-/-}$ α4-WT and DA2 fibroblasts (Fig. 3B). No morphology differences were associated with stable α4 WT or α4 Y991A expression in either $FAK^{+/+}$ or DA2 cells (data not shown). To determine whether the α4 Y991A mutation disrupts the binding of paxillin to α4 in $FAK^{-/-}$ cells, coimmunoprecipitation analyses were performed in lysates from suspended and FN (CS-1)-replated $FAK^{-/-}$ cells (Fig. 3C). A strong α4-paxillin association was detected only from FN (CS-1)-plated cells, and this was disrupted by the α4 Y991A mutation. This result suggests that both loss of cell adhesion and site-specific mutagenesis can disrupt the α4-paxillin binding interaction.

To determine whether stable α4 expression in $FAK^{-/-}$ cells functioned to promote cell motility, haptotaxis assays were performed using FN (9-11) and FN (CS-1) (Fig. 4). α4 expression did not facilitate $FAK^{-/-}$ cell motility to the α5β1 FN (9-11) ligand (Fig. 4A). However, both α4 WT and α4 Y991A promoted $FAK^{-/-}$ cell motility to FN (CS-1) at equivalent levels to α4 WT-expressing $FAK^{+/+}$ and DA2 cells (Fig. 4B). α4 Y991A expression in $FAK^{+/+}$ and DA2 cells also functioned to promote FN (CS-1)-stimulated cell motility equal to α4 WT (data not shown). FN (CS-1) cell migration for both α4 WT- and α4 Y991A-expressing $FAK^{-/-}$ cells was blocked in the presence of an antibody to human α4 (Fig. 4C and D). α4 WT- and α4 Y991A-expressing cells also exhibited equivalent levels of random (data not shown) and haptotaxis cell motility on FN (CS-1)- or VCAM-1-coated membranes (Fig. 4C and D). To verify that α4 Y991A was functionally equivalent to α4 WT in promoting $FAK^{-/-}$ cell motility, scratch wound healing assays were performed as described previously for α4 motility studies in CHO cells (7). Time-lapse video microscopy revealed that both α4 WT- and α4 Y991A-expressing $FAK^{-/-}$ cells exhibited extensive lamellipodium formation along the wound edge and closed the ~350-μm wound region within 12 h (Fig. 4E; see also Videos S1 and S2 in the supplemental material). This rate of cell migration (~30 μm/h) is similar to that measured for α4-expressing CHO cells (7), whereas parental $FAK^{-/-}$ cells exhibit only partial wound closure activity when plated onto FN (48). Taken together, these results show that α4β1 expression is sufficient to rescue the morphological and motility defects of $FAK^{-/-}$ fibroblasts and that paxillin binding to α4 is not essential to promote leading-edge membrane ruffling or enhanced $FAK^{-/-}$ cell migration.

α4β1 cell motility is not affected by inhibitors of FAK and Pyk2. One of our original hypotheses was that paxillin binding to α4 may promote the recruitment of Pyk2 in $FAK^{-/-}$ cells to an α4β1 signaling complex. However, endogenous Pyk2 staining remains perinuclear localized and not coassociated with the peripheral distribution of green fluorescent protein (GFP)-paxillin in $FAK^{-/-}$ α4-WT cells plated on FN (CS-1) (Fig. 5A). In $FAK^{+/+}$ α4-WT cells, there is a strong colocalization of FAK with GFP-paxillin at cell adhesion contact sites (Fig. 5A). As both Pyk2 and FAK bind paxillin via conserved C-terminal domain interactions and the exogenous expression of the Pyk2 or FAK C-terminal domains (termed PRNK and FRNK, respectively) can inhibit cell motility, Ad-mediated expression of PRNK or FRNK was evaluated for effects on α4β1- and α5β1-stimulated phosphorylation and cell migration (Fig. 5B). Interestingly, high levels of PRNK or FRNK overexpression had little to no effect on FN (CS-1)-stimulated Pyk2 or FAK tyrosine phosphorylation in α4-WT $FAK^{-/-}$ and $FAK^{+/+}$ cells, respectively (Fig. 5B).

Additionally, PRNK or FRNK expression did not inhibit α4β1-stimulated $FAK^{-/-}$, $FAK^{+/+}$, or DA2 cell motility (Fig. 5C). In contrast, equivalent levels of FRNK expression potently blocked α5β1-stimulated FAK tyrosine phosphorylation

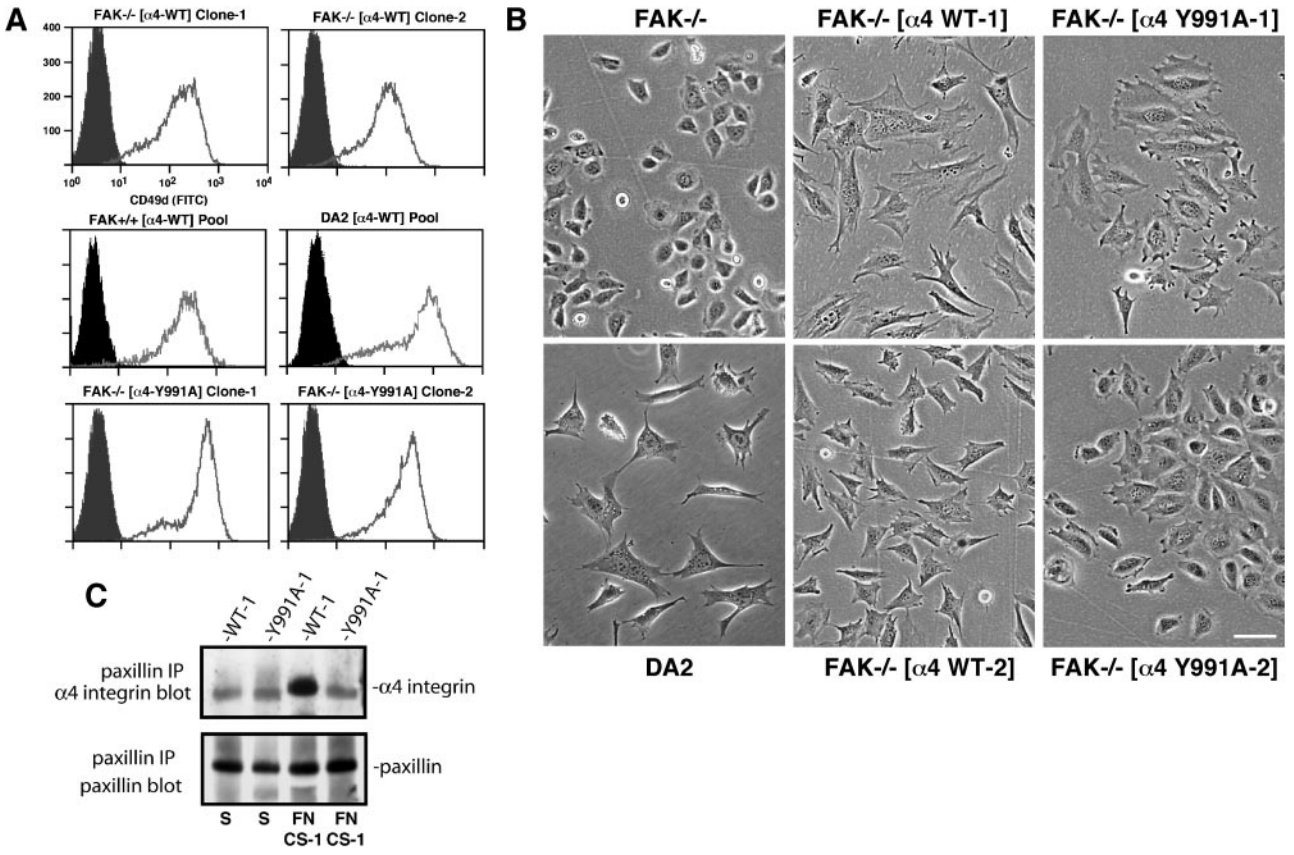


FIG. 3. Stable expression of α4 Y991A in $FAK^{-/-}$ cells disrupts paxillin binding to α4 and affects cell morphology compared to WT α4 expression. (A) Flow cytometry analyses of human α4 integrin surface expression (open peaks) in two $FAK^{-/-}$ cell clones (α4 WT-1 and α4 WT-2), in α4-expressing pooled populations of $FAK^{+/+}$ cells, and in $FAK^{-/-}$ cells reconstituted with FAK (DA2). Human α4 Y991A expression in two $FAK^{-/-}$ cell clones (α4 Y991A-1 and α4 Y991A-2). Staining with control MAb (shaded peaks). (B) Phase contrast image of parental $FAK^{-/-}$, $FAK^{-/-}$ reconstituted (DA2), $FAK^{-/-}$ α4 integrin-expressing, and α4 Y991A-expressing $FAK^{-/-}$ fibroblasts growing in culture in 10% serum. Scale bar, 65 μm. (C) Paxillin IPs were made either from $FAK^{-/-}$ α4 WT-1 or α4 Y991A-1 cells held in suspension or replated onto FN (CS-1) for 45 min and were blotted for α4 integrin coassociation (top) with paxillin (bottom).

(Fig. 5B) and $FAK^{+/+}$ α4-WT cell motility on FN (9-11) (Fig. 5D). As the FRNK-inhibitory effects on α5β1-stimulated FAK tyrosine phosphorylation and cell migration were inactivated by a point mutation that disrupts paxillin binding (FRNK S-1034) (Fig. 5B and D), our results support the conclusion that paxillin plays an important role in α5β1-stimulated FAK activation and cell migration. However, as α4 Y991A functions to promote $FAK^{-/-}$ and $FAK^{+/+}$ cell motility (Fig. 4) and Pyk2 and FAK tyrosine phosphorylation (data not shown) and as neither PRNK nor FRNK overexpression inhibits α4β1-stimulated cell motility (Fig. 5), our results support the conclusion that paxillin binding to α4 and signaling through FAK are not essential for the initiation events promoting α4β1 cell motility. Taken together, our combined results suggest that α5β1 and α4β1 use distinct receptor-proximal linkages in promoting tyrosine kinase activation. Moreover, results using the α4-ΔCyto truncation (Fig. 2) support a model whereby a novel route to tyrosine kinase activation is initiated by the α4 cytoplasmic domain and may act in a dominant manner over β1-mediated signals within an α4β1 complex.

The α4 cytoplasmic domain promotes α4β1-stimulated c-Src activation. In a variety of cells, stimuli that result in increased Pyk2 or FAK tyrosine phosphorylation are not always associated with the catalytic activation of these PTKs. It is known that Src-family PTK activation can lead to Pyk2 or FAK transphosphorylation (43), and accordingly, pharmacological inhibition of Src-family PTKs by PP2 blocked α4β1-stimulated $FAK^{-/-}$ cell motility in a dose-dependent manner (Fig. 6A). To evaluate whether α4-initiated signals can promote c-Src activation, Ad-mediated expression of α4-WT or α4-ΔCyto in $FAK^{-/-}$ cells was tested for effects on c-Src IVK activity (Fig. 6B). Only basal c-Src IVK activity was detected in suspended cells, whereas upon adhesion to FN (CS-1), c-Src showed a fourfold increase in activity in Ad-infected α4-WT $FAK^{-/-}$ cells (Fig. 6B). In contrast, α4-ΔCyto cells adhered to FN (CS-1), but there was no stimulation of c-Src activity compared to suspended cells. This result is consistent with only weak levels of Pyk2 and FAK tyrosine phosphorylation after plating cells expressing α4-ΔCyto on FN (CS-1) (Fig. 2C). Interestingly, the level of α4β1-stimulated c-Src activity in α4-WT-expressing $FAK^{-/-}$ cells was ~2-fold greater than the level of α5β1-stimulated c-Src activity in $FAK^{-/-}$ cells plated onto FN (9-11) (Fig. 6B). This result is consistent with the importance

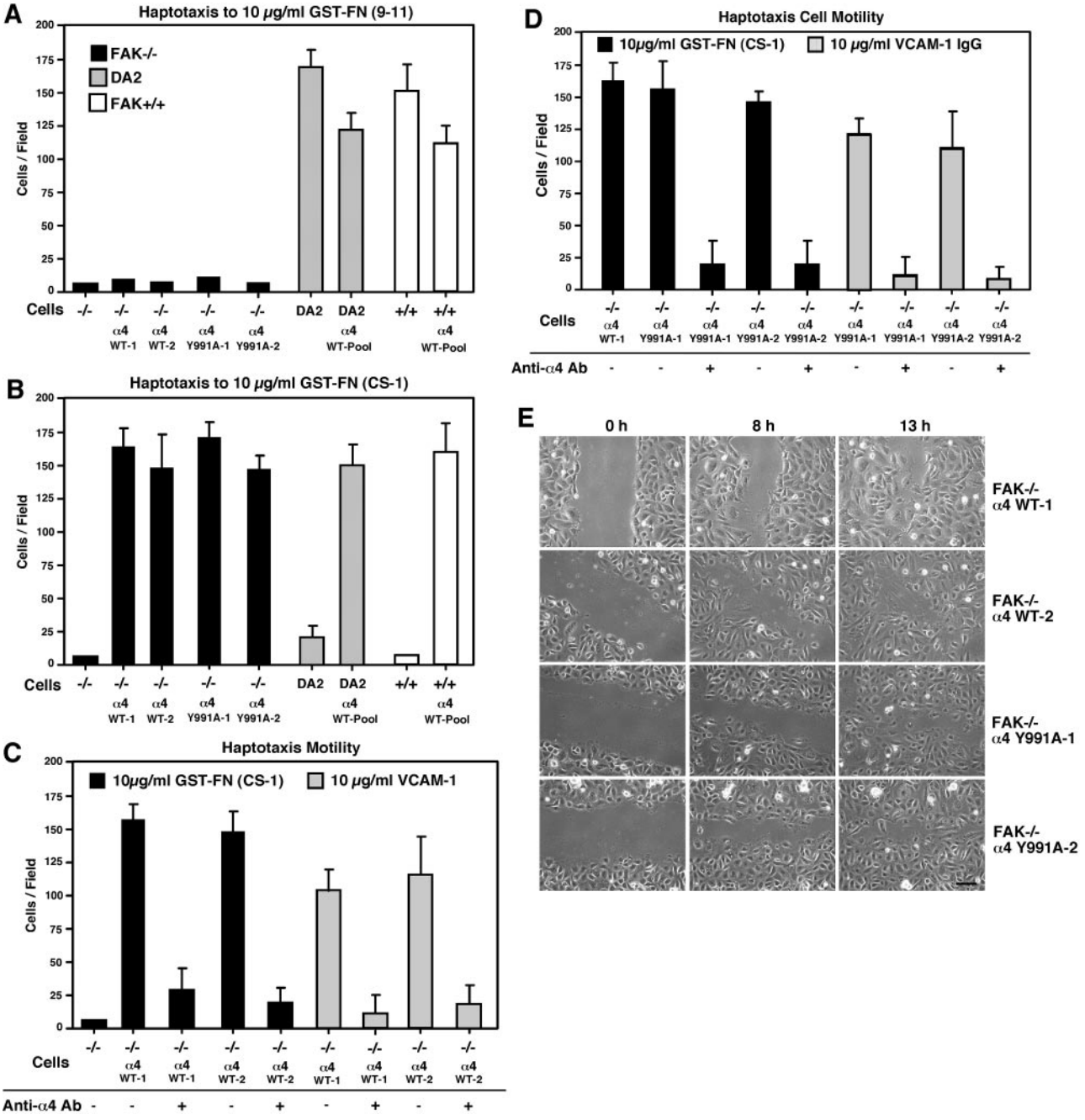


FIG. 4. α4 WT and α4 Y991A selectively and equally promote FAK$^{-/-}$ cell motility to FN (CS-1) and VCAM-1 but not to FN (9-11). (A) Haptotaxis motility assays using FN (9-11) as an α5β1 ligand. Stable α4 WT or α4 Y991A expression does not reverse α5β1-stimulated motility defects of FAK$^{-/-}$ cells. Stable α4 WT expression in DA2 and FAK$^{+/+}$ cells resulted in slightly reduced levels of α5β1-stimulated motility. (B) Haptotaxis motility assays using FN (CS-1) as an α4β1 ligand. Both α4 WT and α4 Y991A act to promote equivalent levels of FN (CS-1)-stimulated FAK$^{-/-}$, DA2, and FAK$^{+/+}$ cell motility. (A and B) Values are means ± standard deviations of triplicate samples from three independent experiments. (C and D) Haptotaxis motility assays using FN (CS-1) and VCAM-1 Ig fusion proteins as selective α4β1 ligands. Equivalent levels of α4 WT- and α4 Y991A-stimulated FAK$^{-/-}$ cell motility to FN (CS-1) (black bars) or to VCAM-1 (gray bars) are blocked by the addition of an inhibitory MAb to α4 (10 μg/ml). Values are means ± standard deviations of triplicate samples from two independent experiments. (E) Equal wound closure motility of α4 WT- and α4 Y991A-expressing FAK$^{-/-}$ cells. The indicated cells were plated onto FN (CS-1) at a confluent density and were wounded with a pipette tip. Time-lapse video microscopy images obtained at 0 h, 8 h, and 13 h after cell wounding showed extensive membrane ruffling and cell movement to fill in the open space. Scale bar, 100 μm. See Videos S1 (FAK$^{-/-}$ α4-WT) and S2 (FAK$^{-/-}$ α4-Y991A) in the supplemental material.

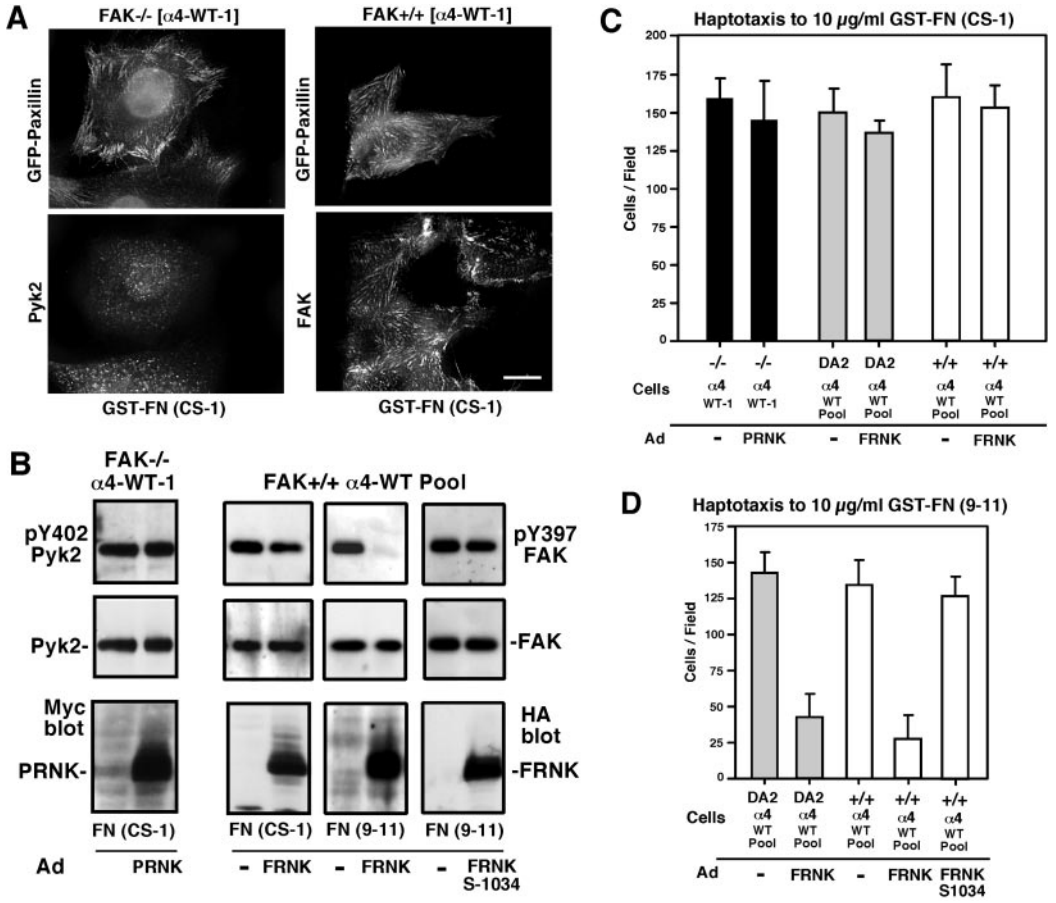
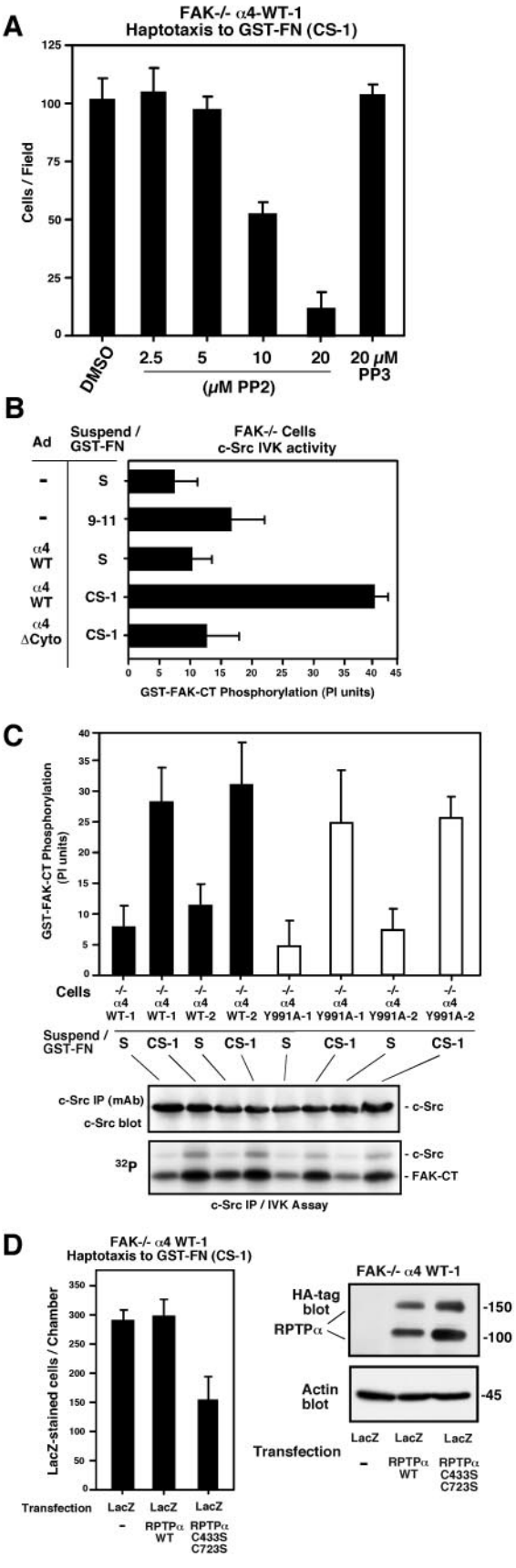


FIG. 5. Adenovirus-mediated expression of FRNK blocks α5β1 signaling in $FAK^{+/+}$ cells, but neither PRNK nor FRNK inhibit α4β1-stimulated tyrosine phosphorylation or cell motility in $FAK^{-/-}$ or $FAK^{+/+}$ cells, respectively. (A) Cells were plated onto FN (CS-1) in the absence of serum for 2 h, fixed, stained for Pyk2 or FAK, and covisualized for GFP-paxillin expression or indirect antibody immunofluorescence (IF). In $FAK^{-/-}$ α4 cells, Pyk2 IF was primarily perinuclear distributed and not associated with GFP-paxillin at cell adhesion sites. In $FAK^{+/+}$ α4 cells, FAK IF was strongly coassociated with GFP-paxillin. Scale bar, 20 μM. (B) The indicated cells were preinfected with either Ad-Myc-PRNK, Ad-HA-FRNK, or Ad-HA-FRNK S-1034 and plated onto FN (CS-1)- or FN (9-11)-coated plates for 45 min. Pyk2 or FAK IPs were analyzed by phosphospecific blotting, followed by anti-Pyk2 or FAK blotting. PRNK and FRNK expression was verified by epitope tag blotting of whole-cell lysates. (C) Haptotaxis assays using FN (CS-1) revealed no significant differences in α4-expressing $FAK^{-/-}$, DA2, and $FAK^{+/+}$ cell motility with or without PRNK or FRNK overexpression. (D) α5β1 haptotaxis assays using FN (9-11) showed that FRNK, but not FRNK S-1034, expression potently inhibited α4-expressing DA2 and $FAK^{+/+}$ cell motility. (C and D) Values are means ± standard deviations of triplicate samples from two separate experiments.

of α5β1-stimulated FAK activity in promoting the formation of an activated FAK-Src complex.

In stable α4-expressing $FAK^{-/-}$ cells, α4β1 adhesion led to a ~3-fold activation of c-Src compared to suspended cells and this was independent of paxillin binding to α4, as c-Src was equally activated in $FAK^{-/-}$ α4 Y991A cells in an adhesion-dependent manner (Fig. 6C). The mechanisms of integrin-mediated c-Src activation are complex and may involve either direct binding to β3 integrin subunits (1) or be mediated through the activation of protein-tyrosine phosphatases that act to dephosphorylate the regulatory Tyr-529 (murine numbering) site in c-Src (42). As c-Src does not bind to β1 integrin (1) but can be activated via RPTPα-mediated dephosphorylation of Tyr-529 after the FN stimulation of cells (53), WT or the catalytically inactive RPTPα mutant (C433S and C723S) was overexpressed in $FAK^{-/-}$ α4-WT cells and evaluated for the effects on α4β1-stimulated cell motility (Fig. 6D). Whereas WT RPTPα did not enhance α4-stimulated cell motility, catalytically inactive RPTPα inhibited FN (CS-1)-asssociated cell migration by 50%. Although cotransfection studies with RPTPα C433S/C723S and c-Src did not reveal an inhibitory mechanism due to complications of elevated c-Src IVK activity upon overexpression (data not shown), the RPTPα results point to the importance of Src-family PTK activity in promoting α4-initiated cell motility.

To further support the role of c-Src activation after α4β1 stimulation, an adenovirus-mediated strategy of overexpressing various inhibitors was employed. First, Ad-FRNK and Ad-PRNK were tested for effects on FN (9-11)- or FN (CS-1)-stimulated c-Src activity in $FAK^{+/+}$ and α4 WT-1 $FAK^{-/-}$ cells, respectively (Fig. 7A). As expected, FRNK blocked FN (9-11)-stimulated c-Src activity, thus confirming the importance of FAK in α5β1-mediated c-Src activation. In contrast, PRNK (or FRNK) (data not shown) did not significantly affect



adhesion-dependent c-Src activation upon cell binding to FN (CS-1) (Fig. 7A). In an opposite manner to RPTPα, p50Csk phosphorylates c-Src at Tyr-529, thus promoting intramolecular binding of the c-Src SH2 domain with Tyr-529 and facilitating an inactive c-Src conformation (42). Ad-mediated p50Csk overexpression potently blocked α4β1-increased c-Src activity (Fig. 7A), whereas similar levels of Ad-mediated KD p50Csk expression had no effect on FN (CS-1)-stimulated c-Src activity (Fig. 7A). These biochemical changes in c-Src IVK activity were confirmed by blotting with phosphospecific antibodies to c-Src Tyr-418 (pY418) within the catalytic domain and to the p50Csk phosphorylation site at c-Src Tyr-529 (pY529). Notably, WT Csk overexpression inhibited c-Src Tyr-418 phosphorylation and promoted Tyr-529 phosphorylation, thus preventing α4β1-stimulated c-Src activation (Fig. 7B). This inactivation of c-Src by WT but not KD Csk was correlated with the inhibition of α4-mediated $FAK^{-/-}$ cell motility on FN (CS-1) (Fig. 7C). Together, these results support the conclusion that the α4 cytoplasmic domain can facilitate c-Src activation independent of paxillin binding to α4 and in a manner that does not require FAK.

Src expression is required for α4β1-stimulated SYF cell motility. To verify the importance of c-Src in α4β1 cell migration, comparisons were performed with SYF fibroblasts and SYF cells stably reconstituted with c-Src (Fig. 8). Flow cytometry revealed that these cells expressed α5, αv, and β1 integrins but very little α4 (Fig. 8A). Haptotaxis assays using FN (9-11) and bovine plasma FN showed that FN promotes greater levels of cell motility than FN (9-11) and that SYF+c-Src cells possess a twofold-elevated level of α5β1-stimulated cell migration compared to SYF cells (Fig. 8B). These results are consistent with the required role of Src in FN-initiated cell motility (23). When FN (CS-1) was used as a ligand, no significant haptotaxis motility was detected in SYF or SYF+c-Src cells (Fig. 8B) consistent with the low level of endogenous α4 expression in these cells. Stable human α4 expression was achieved in pooled

FIG. 6. The α4 cytoplasmic domain facilitates adhesion-dependent, α4β1-mediated c-Src catalytic activation in a manner that does not require paxillin binding to α4. (A) Haptotaxis assays using FN (CS-1) revealed a dose-dependent inhibition of cell motility with the PP2 pharmacological inhibitor of Src activity but not with dimethyl sulfoxide (DMSO) or the PP3 Src-inactive compound. (B) $FAK^{-/-}$ cells were mock treated (−) or preinfected with Ad-mediated α4-WT or Ad-mediated α4-ΔCyto, serum starved, and then held in suspension (S) or plated onto FN (9-11) or FN (CS-1) for 45 min prior to cell lysis. c-Src-associated IVK activity was determined using GST-FAK-CT as a substrate. (C) c-Src IVK activity was evaluated from $FAK^{-/-}$ cells expressing α4 WT (black bars) or α4 Y991A (open bars) as indicated above. (Bottom) Isolation of c-Src by IP and the associated autoradiograph showing c-Src autophosphorylation and GST-FAK C-terminal domain (CT) substrate phosphorylation. (B and C) Values are means ± standard deviations of duplicate samples from two separate experiments. (D) Inhibition of $FAK^{-/-}$ α4β1-stimulated haptotaxis cell motility by overexpression of a catalytically inactive mutant of RPTPα (C433S and C723S) compared to WT RPTPα and LacZ controls. On the right, expression of transfected HA-tagged RPTPα (the 150-kDa protein is the mature and glycosylated form) and β-actin in whole-cell lysates of cells not used in the motility assay is shown. (A and D) Values are means ± standard deviations of triplicate samples from two separate experiments.

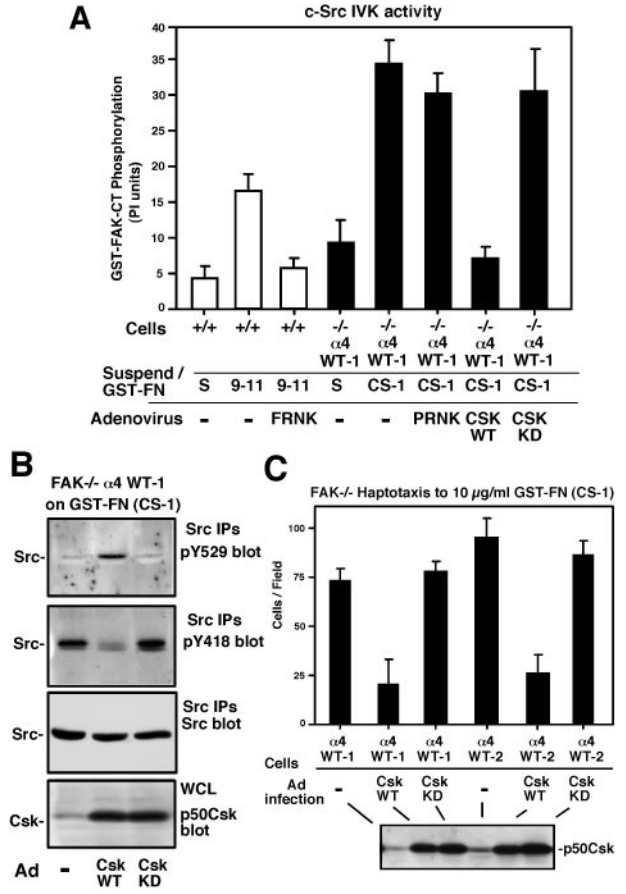


FIG. 7. α4β1 stimulation of c-Src activity and cell motility is inhibited by p50Csk phosphorylation of c-Src at Tyr-529. (A) c-Src IVK activity was evaluated for normal $FAK^{+/+}$ cells (open bars) held in suspension (S) or plated on FN (9-11) and for α4-expressing $FAK^{-/-}$ cells (black bars) held in suspension or plated on FN (CS-1) for 45 min. As indicated, cells were preinfected with Ad constructs for FRNK, PRNK, Csk WT, or Csk KD. Values are means ± standard deviations of duplicate samples from two separate experiments. (B) α4-expressing $FAK^{-/-}$ cells were preinfected with Ad-Csk WT or Ad-Csk KD, serum starved, and plated onto FN (CS-1) for 45 min prior to cell lysis. c-Src IPs were sequentially analyzed by phosphospecific blotting to c-Src pY529 and pY418 within the c-Src catalytic region. Equal c-Src recovery was verified by anti-Src blotting of c-Src IPs, and Csk overexpression was verified by anti-Csk blotting of whole-cell lysates (WCL). (C) Haptotaxis motility assays revealed that overexpression of Csk WT but not Csk KD inhibited α4-expressing $FAK^{-/-}$ cell motility to FN (CS-1). Values are means ± standard deviations of triplicate samples from two separate experiments. Csk overexpression was verified by blotting lysates from equal numbers of cells not used in the motility assay.

populations of SYF and SYF+c-Src cells by retrovirus-mediated infection, and flow cytometry analyses verified equivalent levels of surface α4 expression in SYF and SYF+c-Src (Fig. 8C). Human α4 expression promoted SYF and SYF+c-Src adhesion to FN (CS-1) (data not shown). However, α4-SYF fibroblasts exhibited only low levels of haptotaxis motility to FN (CS-1), whereas α4-SYF+c-Src cell motility was ~5-fold greater than that for α4-SYF cells (Fig. 8D). As α4-SYF+c-Src and $FAK^{-/-}$ α4 WT-1 cells showed equivalent levels of FN (CS-1) cell migration, these results confirm the importance of c-Src expression and activity in promoting α4β1-stimulated cell motility.

To further examine the intracellular signaling role of c-Src in α4-mediated motility, α4-SYF and α4-SYF+c-Src cells were plated onto FN (CS-1). Anti-pTyr blotting of cell lysates revealed increased protein tyrosine phosphorylation in α4-SYF+c-Src compared to α4-SYF cells (Fig. 8E). Moreover, high levels of α4β1-stimulated FAK Y397, Src Y418, and p130Cas tyrosine phosphorylation were observed only in α4-SYF+c-Src cells (Fig. 7E). As p130Cas phosphorylation is associated with Crk adaptor protein binding, p21 Rac GTPase activation, and enhanced cell motility in a variety of cells (4), the amount of GTP-bound Rac was visualized by an affinity binding assay to the N-terminal region of p65PAK. Rac-GTP loading was observed in α4-SYF+c-Src cells but not in α4-SYF cells (Fig. 8E). These data support a model whereby Src activation is a common and important upstream component of both α4β1 and α5β1 signaling, leading to enhanced motility.

DISCUSSION

The FN binding integrins α5β1 and α4β1 generate signals promoting cell migration through distinct mechanisms. Here, using $FAK^{-/-}$ and $FAK^{+/+}$ fibroblasts stimulated with a recombinant fragment of FN (9-11) that binds to α5β1, we show that FAK is essential for α5β1-stimulated cell motility. By using the Ad-mediated expression of FRNK as a dominant-negative inhibitor of FAK activity, we found that FRNK but not FRNK S-1034 (a mutation that disrupts paxillin binding) expression blocks FN (9-11)-stimulated FAK phosphorylation at Y397 and adhesion-dependent c-Src activity upon binding FN (9-11) and that FRNK potently inhibits α5β1-stimulated cell motility. These results support a model (Fig. 9) whereby a paxillin-mediated linkage to α5β1 facilitates FAK activation, the formation of an activated FAK-Src complex, and increased cell motility, in part due to the tyrosine phosphorylation of p130Cas, generating signals leading to Rac activation and lamellipodium formation at the leading edge of migrating cells (28, 43).

To address molecular signaling differences between α5β1 and α4β1, we compared cell motility responses after cell binding to FN (9-11) or to FN (CS-1), a recombinant FN fragment that binds α4β1. We found that human α4 expression in murine $FAK^{-/-}$ fibroblasts formed a functional α4β1 receptor and promoted cell spreading and robust cell motility equivalent to α5β1-stimulated $FAK^{+/+}$ cell migration. Although the expression of the FAK-related Pyk2 kinase is elevated in $FAK^{-/-}$ cells, α4-mediated $FAK^{-/-}$ binding to FN CS-1 did not promote the redistribution of Pyk2 to cell adhesion sites of paxillin clustering. Neither α4-stimulated cell motility nor Pyk2 tyrosine phosphorylation was affected by overexpression of the Pyk2 C-terminal domain (PRNK) that binds paxillin. Notably, expression of an α4 mutant (Y991A) that does not bind paxillin still functioned to promote both $FAK^{-/-}$ and $FAK^{+/+}$ cell motility to FN (CS-1) and VCAM-1 ligands. Additionally, FRNK expression selectively blocked α5β1- but not α4β1-stimulated FAK tyrosine phosphorylation in $FAK^{+/+}$ cells. These results, along with the fact that α4β1 expression effectively rescued the $FAK^{-/-}$ cell motility defects, support the

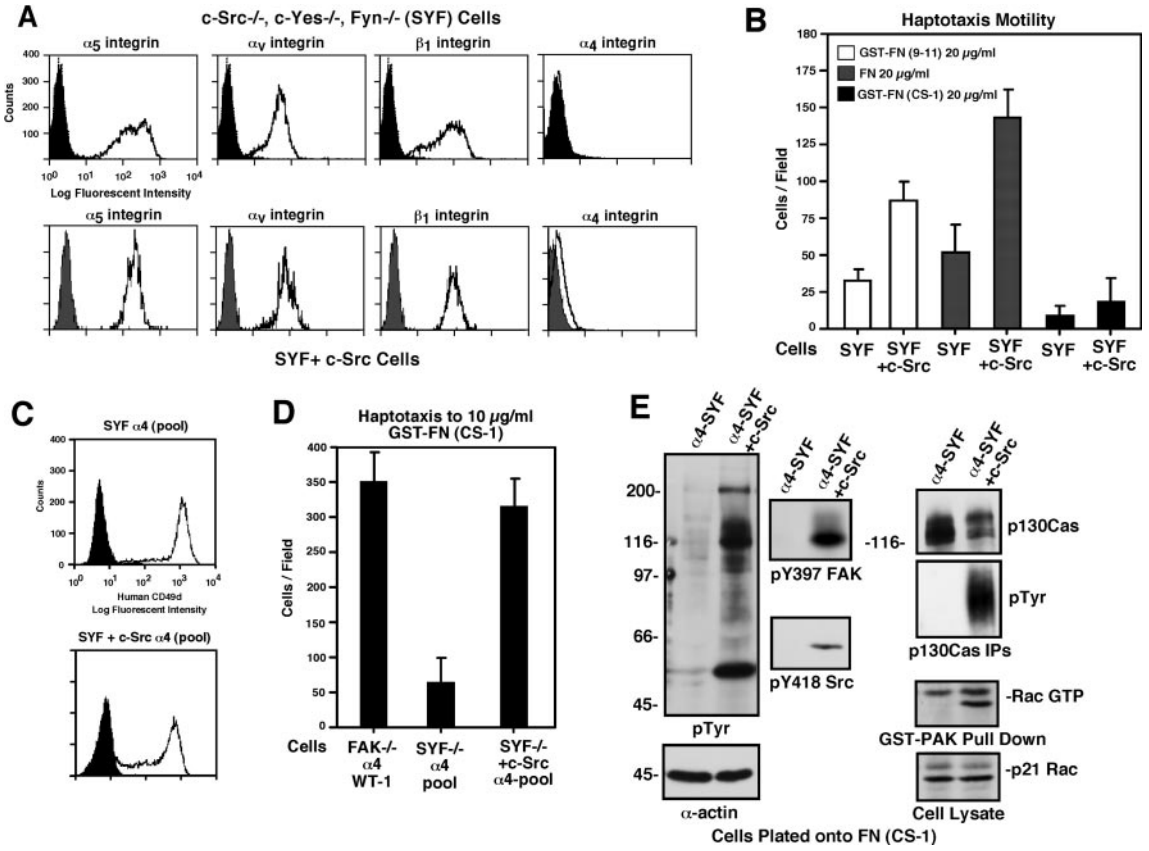


FIG. 8. Human α4 expression in SYF and SYF+c-Src fibroblasts reveals that c-Src expression is essential for α4β1-stimulated cell motility. (A) Flow cytometry analyses of endogenous murine α5, αv, β1, and α4 integrin expression in SYF and SYF+c-Src cells (open peaks) and staining with control MAb (shaded peaks). (B) Haptotaxis comparisons between SYF and SYF+c-Src cells confirm the importance of c-Src expression for FN (9-11) (open bars)- and plasma FN (black bars)-stimulated cell motility. Low values for FN (CS-1) cell motility (black bars) were obtained, as endogenous α4 is only weakly expressed. (C) Flow cytometry analyses of stable human α4 integrin expression in pooled populations of SYF and SYF+c-Src (open peaks) and staining with control MAb (shaded peaks). (D) c-Src stimulates equivalent levels of haptotaxis motility to FN (CS-1) in α4-expressing SYF+c-Src cells compared to FAK$^{-/-}$ α4-WT-1 cells. α4 expression in SYF cells does not function to promote cell motility. (B and D) Values are means ± standard deviations from triplicate samples of three separate experiments. (E) Anti-pTyr and phosphospecific FAK and Src blotting of whole-cell lysates from α4-expressing SYF or SYF+c-Src cells plated onto FN (CS-1) for 45 min reveals increased tyrosine phosphorylation in α4-SYF+c-Src but not α4-SYF cells. β-Actin blotting shows equal protein loading. Increased p130Cas tyrosine phosphorylation and Rac GTP loading in α4-SYF+c-Src but not α4-SYF cells as determined by p130Cas IP and GST-PAK affinity pull down, respectively.

conclusion that a paxillin-FAK/Pyk2 linkage is not essential for α4β1-stimulated cell motility.

This lack of a functional role for FAK is contrary to the original α4-stimulated signaling linkage model (25, 26). For paxillin, signaling linkage studies have been inconclusive, as cells expressing α4 Y991A retain enhanced membrane ruffling and wound closure activity (36). As serine phosphorylation of α4 disrupts paxillin binding (10, 11), as phosphorylated α4 is preferentially localized to leading lamellipodia (7), and as enforced paxillin association with α4 inhibits cell migration and lamellipodium formation (7, 11), paxillin binding to α4 is likely to play a regulatory role in the generation of motility-promoting intracellular signaling events. In particular, the α4-paxillin linkage has been connected to the negative regulation of Rac activity at the posterior end of migrating cells (31). Our studies with α4 WT and α4 Y991A expression in FAK$^{-/-}$ cells showed equal levels of membrane ruffling as analyzed in time-lapse wound healing assays as well as equal levels of motility in haptotaxis and random motility assays. However, α4 WT FAK$^{-/-}$ cells exhibited a polarized fibroblast phenotype, whereas α4 Y991A FAK$^{-/-}$ cells exhibited a more rounded phenotype and enhanced spreading compared to parental FAK$^{-/-}$ cells. Our findings that α4 Y991A-expressing FAK$^{-/-}$ cells exhibit a nonpolar morphology compared to α4-WT cells are consistent with a role for paxillin binding to α4 in the generation of cell polarity (15) (Fig. 9).

If a α4-paxillin linkage is not essential in promoting signaling events at the leading edge of cells, how does α4β1 function in a FAK-independent manner to promote tyrosine kinase activation? Integrins can generate intracellular signals through the lateral association with other receptors (8, 52) or the clustering of signaling proteins with integrin cytoplasmic domains (44). The α4 truncation mutant (α4-ΔCyto) promoted cell adhesion but not tyrosine kinase activation, cell spreading, or motility. Although many integrin signaling events have been attributed to β subunit-initiated events (1, 44), our results with α4-ΔCyto do not support a dominant role for the β1 subunit within an α4β1 complex. Moreover, overexpression of FRNK that

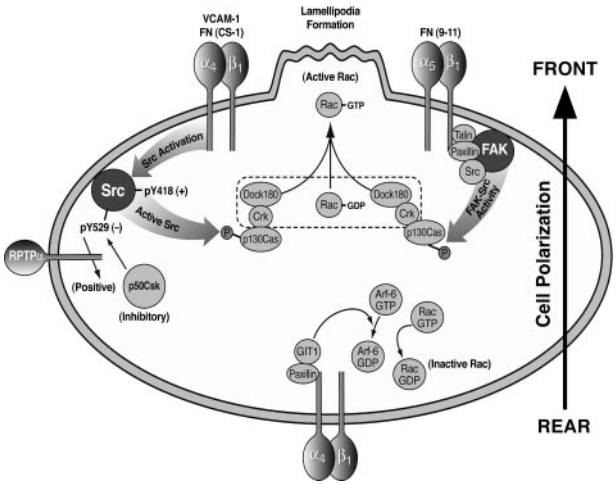


FIG. 9. Model of receptor-proximal differences in α4β1 and α5β1 signaling events promoting p130Cas phosphorylation associated with Rac activation in migrating cells. α4β1 binding to VCAM-1 or FN (CS-1) stimulates c-Src-family PTK phosphorylation at Y418 and promotes Src catalytic activation in a FAK-independent manner. This linkage between α4 and Src does not require paxillin binding to α4, which has been shown to down-regulate Rac activation at posterior regions of migrating cells through a signaling linkage involving paxillin binding to the ADP ribosylation factor GTPase-activating protein (Arf-6 GAP) GIT1. However, α4β1-mediated Src activation requires the integrity of the α4 cytoplasmic domain, implying that Src activation does not reflect enhanced β1-mediated signaling. α4β1-mediated c-Src activation is inhibited by p50Csk PTK phosphorylation of c-Src at Y529, and α4β1-stimulated cell motility is inhibited by a catalytically inactive mutant of RPTPα, a phosphatase that can dephosphorylate Src pY529. This α4β1-Src linkage promotes p130Cas tyrosine phosphorylation and Rac activation and is associated with enhanced lamellipodium formation as well as the reversal of $FAK^{-/-}$ cell motility defects. Crk adaptor protein binding to phosphorylated p130Cas coupling to the guanine nucleotide exchange factor Dock180 is one of several pathways that can promote Rac activation. It is speculated that the α4 linkage to Src and lamellipodium formation at the front of cells coupled with the α4-paxillin-GIT1 linkage at the rear of cells may act to promote the establishment of cell polarity. α5β1 binding to FN (9-11) also promotes p130Cas tyrosine phosphorylation, Rac activation, and cell motility. This linkage is dependent on FAK expression and activity and is inhibited by overexpression of the FAK C-terminal domain that binds to β1 integrin-associated proteins such as talin and paxillin. Thus, FN binding α4β1 and α5β1 integrins use distinct receptor-proximal mechanisms to activate c-Src or a FAK-Src complex, respectively. Phosphorylation of p130Cas by c-Src or a FAK-Src complex occurs via a shared pathway associated with enhanced Rac activation, lamellipodium formation, and cell motility.

blocked α5β1-stimulated FAK phosphorylation and c-Src activation had little effect on these α4β1-stimulated events. Whereas it remains possible that the α4-ΔCyto truncation disrupts a lateral association of α4 with another cell surface receptor, our results point to a unique role for the α4 cytoplasmic domain in generating a strong motility-promoting stimulus that is not shared by the α5 subunit. This conclusion was first proposed through the study of chimeric integrin subunits (3), and yet the molecular basis for this effect remains unknown. We found that signals initiated by α4 and α4 Y991A, but not α4-ΔCyto, promoted a ~3- to 4-fold increase in c-Src PTK activity that was unaffected by PRNK or FRNK overexpression.

Our results support a novel linkage between the α4 cytoplasmic domain leading to c-Src activation (Fig. 9). Although studies have postulated that an α-specific integrin connection to Fyn activation (8) and the Src-family PTKs (Hck, Fgr, and Lyn) are important for α4-stimulated signaling events in neutrophils (35), no studies to date have connected α4 to c-Src activation. The importance of this α4-to-Src linkage was supported by the fact that $SYF^{-/-}$ cells expressing α4 require c-Src reexpression for α4β1-stimulated migration. Whereas previous studies have shown that c-Src activity is important for α5β1-stimulated motility (23, 28), this α5β1 signaling connection involves FAK and paxillin (9). Our results support a model whereby Src activation is a common signaling component promoting α4β1- and α5β1-stimulated cell motility. However, unlike α5β1, α4β1-induced Src activity does not involve FAK. In SYF cells reexpressing c-Src, α4β1 stimulation promoted p130Cas tyrosine phosphorylation and Rac GTP activation. Notably, phosphorylation of p130Cas by c-Src or a FAK-Src complex occurs via a shared pathway associated with enhanced Rac activation, lamellipodium formation, and cell motility (Fig. 9). Thus, the α4 cytoplasmic domain connection to c-Src activation is a FAK-independent linkage to a common motility-promoting signaling pathway.

Although we did not elucidate the molecular connection between α4 and c-Src activation, we showed that p50Csk overexpression leads to elevated c-Src Y529 phosphorylation and that p50Csk inhibits α4-stimulated c-Src activation. This result points to the importance of protein-tyrosine phosphatase action in promoting c-Src Y529 dephosphorylation and conformational c-Src activation. To this end, we found that the overexpression of a catalytically inactive mutant of RPTPα (C433S and C723S) inhibits α4-stimulated cell motility compared to WT PTPα overexpression. As RPTPα functions to promote c-Src activation and FN-stimulated cell motility (53) and as other α integrin subunits bind protein-tyrosine phosphatases (5, 27), future studies will address the possibility of an α4 and PTPase association or the potential for direct c-Src binding to the α4 cytoplasmic domain as mechanisms connecting α4 to c-Src activation.

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