Specifications of the Direction of Adhesive Signaling by the Integrin β Cytoplasmic Domain*§

Received for publication, March 31, 2005, and in revised form, May 16, 2005
Published, JBC Papers in Press, June 3, 2005, DOI 10.1074/jbc.M503508200

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Integrin adhesion receptors can signal in two directions: first, they can regulate cellular behaviors by modulating cellular signaling enzymes (“outside-in signaling”); second, cells can regulate the affinity of integrins (“inside-out signaling”) by such pathways. Integrin β cytoplasmic domains (tails) mediate both types of signaling, and Src family kinases (SFKs) and talin, which bind to β tails, are important for integrin signaling. Here, we utilized “homology scanning” mutagenesis to identify β tail mutants selectively defective in c-Src binding and found that amino acid exchanges affecting a combination of an Arg and Thr residue in the integrin β3 tail control the binding specificity for SFKs but have no effect on talin binding. Using β tail mutants at these residues, we found that SFK binding to integrin β tails is dispensable for inside-out signaling but is obligatory for cell spreading, a marker of outside-in signaling. Conversely, we found that point mutations that disrupt talin binding abolish integrin activation, but they do not inhibit SFK binding to the β3 tail or the initiation of outside-in signaling once the integrins are in a high affinity form. Thus, we show that inside-out and outside-in integrin signaling are mediated by distinct and separable interactions of the integrin β tails. Furthermore, based on our results, it is possible to discern the relative contributions of the direction of integrin signaling on biological functions in cell culture and, ultimately, in vivo.

Cell-cell and cell-matrix adhesion, mediated by integrin adhesion receptors, is essential to the development and functioning of multicellular organisms (1–4). In addition to supporting adhesion, these receptors are bona fide signaling receptors that inform the cell about the physical and biochemical nature of its environment, leading to regulation of gene expression and cell proliferation, differentiation, and survival (1, 4–7). This process is frequently referred to as “outside-in signaling.” The ability of many integrins to bind ligands is regulated by cellular signaling mechanisms (8–11). This process, often called integrin “activation” or “inside-out signaling,” can arise through conformational changes in the integrin extracellular domain and/or changes in the physical distribution of these receptors on the cell surface (12). Thus, integrins can transmit information in both directions across the plasma membrane. A central question is to what extent each form of integrin signaling contributes to particular biological functions.

Integrins are heterodimers of non-covalently associated α and β subunits. The N terminus of each subunit is extracellular, and a single transmembrane domain separates the ~700–1200-residue extracellular domain from a usually short (5–65-residue) cytoplasmic domain, or tail (13). The cytoplasmic domains of integrins play an essential role in bi-directional signaling processes, and intensive work has sought to identify cellular proteins that interact directly with these domains (14). Recent studies have focused attention on two groups of proteins that bind to integrin β cytoplasmic domains and regulate integrin signaling: Src family kinases (SFKs)1 (15) and talin (16). SFKs are constitutively associated with integrins, and their activation is a proximal and early consequence of integrin clustering (17, 18). The presence of SFKs is obligatory for the majority of tyrosine phosphorylation events that follow integrin-mediated adhesion (19). Cell spreading, a measure of integrin signaling into cells, depends on the presence of SFKs. We recently found that SFKs can bind directly to integrin β cytoplasmic domains via their SH3 domains (15). This interaction requires the C terminus of the integrin β tail and promotes the activation of the SFKs (15) and downstream transcriptional factors such as nuclear factor-κB (20). Thus, the binding of SFKs to integrin β tails is important for outside-in signaling; however, its role in integrin activation remains to be clarified.

Talin 1 is required for many integrin functions (21–23), in part because it is a pivotal connection between integrins and the actin cytoskeleton. However, recent studies have also implicated talin as a key regulator of inside-out integrin signaling. Overexpression of the N terminus of talin leads to integrin activation (24, 25), and short hairpin RNA knockdown of talin expression in cultured cells (16) inhibits the activation of β1 and β3 integrins. Furthermore, in platelets and their megakaryocyte precursors, intracellular signals initiated by agonist binding to G-protein-coupled receptors result in integrin αIIbβ3 activation. Expression of talin short hairpin RNA, but not mismatched short hairpin RNA, blocked agonist-stimulated fibrinogen binding to megakaryocytes. Thus, talin is a downstream target of cellular signaling pathways that activate integrins. In addition, x-ray crystallography and NMR spectroscopy have indicated that a 96-residue F3 domain of talin interacts with multiple sites on the integrin β3 tail (26–28). Thus, binding of the talin F3 domain to the β3 cytoplasmic tail is a final common step in integrin activation; however, the role

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§ The on-line version of this article (available at http://www.jbc.org) contains Supplementary Fig. 1.

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2 The abbreviations used are: SFK, Src family kinase; SH, Src homology; GST, glutathione S-transferase; GFP, green fluorescent protein.
of talin binding to integrin β cytoplasmic tails in outside-in signaling is less clear.

Relationships between inside-out and outside-in integrin signaling remain uncertain. Structural and immunochemochemical studies have suggested that the similar conformational rearrangements in the extracellular domain might lead to both forms of signaling (29, 30). In contrast, truncation of the last 3 residues of the β3 cytoplasmic domain can inhibit cell spreading, with less of an effect on integrin activation (31), suggesting that the two processes might be separable, at least at the cytoplasmic face of the integrin. Furthermore, talin deficiency impacts the force-dependent reorganization of initial integrin-cytoskeleton bonds with little effect on cell spreading or SFK activation (32). These results suggested that the interaction of SFKs and talin with integrin β tails might have separable roles in integrin signaling in the two directions.

Here we report that amino acid exchanges affecting a combination of an Arg and Thr residue in the integrin β3 tail control the binding specificity for SFKs but have no effect on talin binding. Using mutants at these residues and cells engineered to express only c-Src among SFKs, we find that SFK binding to integrin β tails is not required for inside-out integrin signaling but is obligatory for cell spreading. Conversely, we show that point mutations that disrupt talin binding abolish integrin activation, but they do not inhibit SFK binding to the β3 tail or the initiation of outside-in signaling by SFKs. Thus, we provide decisive evidence that inside-out and outside-in integrin signaling are biochemically distinct.

MATERIALS AND METHODS

Antibodies and Reagents—Antibodies against the integrin β3 subunit (S9A6 and 9053) were described previously (15). Monoclonal antibody 227 is specific for the c-Src SH3 domain. Monoclonal antibody 7E2 to the β1 integrin subunit was from Rudolph Juliano (33). Polyclonal antibody 828 is specific for the β2 integrin subunit (34). Human αIIbβ3-specific monoclonal antibody, D57, and activating anti-β3 antibody, anti-LIBS6, have been described previously (35–37). Monoclonal antibody 8d4 to talin was from Sigma. Antibodies to His6, GST (B14), Fyn, and Lyn were from Santa Cruz Biotechnology. Antibody to c-Yes was from Upstate Biotechnology. Horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. Monoclonal antibody, PY20, to phosphotyrosine was from BD Biosciences. Purified human fibrinogen was from Enzyme Research Laboratories, Inc. Rhodamine-phalloidin was from Molecular Probes. Protein A-Sepharose was from Amersham Biosciences. All other reagents were obtained from Sigma.

Cell Lines, Plasmids, and Transfections—Cell lines stably expressing human wild-type αIIbβ3 have been described (38). SYF cells (mouse embryonic fibroblasts deficient in c-Src, Fyn, and c-Yes) have been described previously (35–37). Monoclonal antibody, PY20, to phosphotyrosine was from BD Biosciences. Purified active human c-Src (5 μg/ml) was from Escherichia coli. CHO cells expressing recombinant SFKs and bacterial expression constructs for recombinant SFKs and talin with integrin αIIbβ3 tails might have separable roles in integrin signaling in the two directions. Reactions were normalized to the percentage of maximal binding to account for fluctuations in maximal absorbance signal at saturation between experiments.

Enzyme Activity of Purified c-Src—Purified active human c-Src (5 units/reaction; Upstate Biotechnology) was incubated with β cytoplasmic domain (20 μg) bound to neutravidin beads (Pierce) in the presence of 150 μM Src-specific peptide substrate. Reactions were carried out using a Src kinase assay kit (Upstate Biotechnology). Briefly, tyrosine phosphorylation of substrate peptide KVKIEGTVYVVK by recombinant c-Src was measured by incorporation of [γ-32P]ATP in the presence or absence of beads. In the cases where the enzyme was added and transfected, the hem of the cells was transferred in without cell lysis. For the spreading experiments, transfected cells were transiently cotransfected with plasmids encoding αIIbβ3, native or mutant β3, and enhanced GFP (as a marker of transfection). After 72 h binding, the activation-specific monoclonal IgM antibody PAC1 was assayed by flow cytometry as previously described (41). PAC1 binding was only analyzed on a gated subset of live (propidium iodide-negative) and transfected (GFP-positive) cells. Integrin surface expression levels of each transfection were analyzed with αIIbβ3-specific monoclonal antibody D57.

Immunochemistry—Immunoprecipitations were performed with plasma membranes and protein A-Sepharose beads. Immunoprecipitates were subjected to SDS-PAGE and Western blotting with the indicated antibodies. Immunoreactive bands were detected by chemiluminescence (Pierce).

Affinity Chromatography—Recombinant integrin cytoplasmic tail model proteins containing both a His6 and an in vivo biotinylation peptide at the N terminus were expressed and purified from Escherichia coli extracts as previously described (15). A scrambled sequence preserving the same amino acid composition of the integrin β3 tail sequence (KLATNEPTTAFIELGHKHTRREKTNSYRWFTAEERFKILDATNKYA) was produced in the same manner and used as control for nonspecific binding. Affinity chromatography of cell lysates was performed using integrin tail model proteins immobilized on neutravidin-agarose (Pierce) as previously described (15).

Enzyme-linked Immunosorbent Assay—Biotinylated recombinant integrin tails (200 ng) were bound at saturating concentrations (20 μg/ml) to various neutrinodulin-coated microsubstrates (H9251:GST-SH3 domain of c-Src, Lyn, Fyn, and c-Yes were expressed in E. coli strain BL21(DE3)pLys (Novagen, Inc.). Increasing concentrations of GST-c-Src SH3 (in 100 μl) were added to wells in triplicate and incubated for 1 h at room temperature. After three washes with phosphate-buffered saline-0.05% Tween 20 (PBS-T), mouse anti-GST antibody (0.4 μg/ml) was added and incubated for an additional hour, followed by another wash and incubation with PBS-T and 1 μg/ml peroxidase-conjugated mouse monoclonal antibody 327 (anti-c-Src-SH3). Loading density of integrin cytoplasmic tail constructs onto neutravidin-coated plates was determined using mouse monoclonal anti-His6. Binding of GST-c-Src-SH3 to the β3 tail was determined using GraphPad Prism Software. Non-saturable binding was estimated by performing parallel binding curves with randomized β3 or by competition with an excess (150 μM) of peptide KGGRSLRLPLLPPPG previously found to abrogate the binding of c-Src-SH3 to β3 (15). After subtraction of the nonsaturable binding component, binding data were fit to a single site-binding model in which Y = Bsat⋅(X/Kd + X) by non-linear regression. Results were normalized to the percentage of maximal binding to account for fluctuations in maximal absorbance signal at saturation between experiments.

Cell Spreading—Covergels were coated with 5 μg/ml fibrinogen overnight at 4°C, washed twice with phosphate-buffered saline, and blocked with heat-denatured bovine serum albumin (5 mg/ml) for 1 h at room temperature. For the spreading experiments, transfected cells...
FIG. 1. Mapping of the Src-binding sequences in the β3 cytoplasmic tail. A, amino acid sequence of the β3 and β1A cytoplasmic tails, including the scrambled β3 sequence used as control for nonspecific binding (random β3), and representation of the recombinant exchanges made between β3 and β1A cytoplasmic tails. Residues from β3 are indicated by gray boxes, and residues from β1 are indicated by white boxes. B, pull-down analysis from platelet lysates using native and chimeric β3 cytoplasmic tails immobilized on neutravidin-agarose. Binding of c-Src was assayed by Western blot. Monoclonal anti-His6 was used to check for tail loading. C, binding curves of GST-c-Src-SH3 to neutravidin-bound biotinylated β3 and β1A cytoplasmic tail and their respective C-terminal exchange chimeras (20 μg/ml) on microtiter plates, developed with anti-GST antibodies. Results were normalized to the percentage of maximal binding after subtraction of nonspecific binding to randomized β3 cytoplasmic tail. D, effect of the β cytoplasmic tail type on relative Src activity. Results were expressed as the percentage increase in activity relative to basal Src activity in the absence of β3 to permit pooling of data from three independent experiments.
were resuspended at 10^6 cells/ml in Tyrode’s buffer and incubated on coated coverslips at 37 °C for 15–60 min. After removal of unbound cells by washing with phosphate-buffered saline, adherent cells were fixed with 3.7% formaldehyde and permeabilized with 0.2% Triton X-100. Platelets were stained with rhodamine-phalloidin and antibody PY20 followed by a fluorescein isothiocyanate-conjugated anti-mouse IgG secondary antibody. Fluorescence images were acquired with a laser scanning confocal microscope (MRC 1024; Bio-Rad). The surface areas of transfected cells (GFP-positive) were measured using Image Pro Plus software (Media Cybernetics, Inc.).

RESULTS AND DISCUSSION

c-Src and Talin Bind to Distinct Sites in the β3 Cytoplasmic Domain—We sought to discern the relative biological roles of c-Src and talin binding to integrin β cytoplasmic domains. c-Src binding to integrin β involves the last 14 residues of β3 (15), a region partially overlapping with the talin binding site (26, 27). To identify β tail mutants selectively defective in c-Src binding, we utilized a previously described (43) homology scanning approach by creating chimeras between the β3 and β1A cytoplasmic tails (Fig. 1A). We had previously found that c-Src within platelet lysates binds to the β3 cytoplasmic tail, but not to the β1A or β2 tails, and that binding to the β3 tail is abrogated upon elimination of the 4 C-terminal amino acid residues (15). Upon exchanging the C-terminal 3 residues between β1A and β3 (Fig. 1B), we observed that whereas the β3 tail with the β1A substitution at positions 760–762 (β3β1(C3R)) lost its ability to bind c-Src, β1A containing the C-terminal 3 residues of β3 (RGRT) (β1β3(C3R)) exhibited a gain of binding toward c-Src (Fig. 1B). We previously found that the related SFKs Yes, Hck, and Lyn bound to β1 and β3 (15); each of these kinases bound to all of the chimeras shown in Fig. 1B. In contrast, Fyn, which is β3-specific (15), exhibited an identical binding pattern to c-Src (data not shown). Individual β3 mutations, R760E or T762K, were incapable of suppressing c-Src binding to the β3 tail, and individual β1A mutations (E760R or K762T) were unable to promote c-Src binding to the β1A tail (data not shown). To routinely control for nonspecific binding, a randomized sequence version of the β3 cytoplasmic tail (rβ3) failed to interact with c-Src (Fig. 2B) or any of the other SFKs (data not shown). Taken together, these results indicate that the binding of c-Src is dependent on the combined presence of the Arg and Thr residues of the C-terminal β3 RGT motif.

Because c-Src interacts with β3 via its SH3 domain (15), we examined the effect of integrin β tail exchange on the binding of recombinant c-Src-SH3 to immobilized native or chimeric β1A and β3 tails (Fig. 1C). The β1β3(C3R) chimera bound c-Src SH3 with an EC50 similar to the native β3 tail (~5–10 μM). Conversely, the β3β1(C3R) chimera showed complete loss of saturable binding to c-Src-SH3. Interaction of full-length c-Src with β3 activates c-Src (15). Similarly, the β1β3(C3R) chimera gained the capacity to activate c-Src in

![Diagram](image-url)
vitro, whereas the β3β1(C3R) chimera lost this capacity (Fig. 1D). Thus, the last 3 residues of β3 and β1 control binding to c-Src and the capacity of the integrin tail to regulate c-Src activity.

Having established that an exchange of 2 amino acid residues could regulate c-Src binding to and activation by integrins, we assessed the effects of this exchange on the binding of talin, which interacts with integrin β3 tails principally through its N-terminal F23 subdomain (44). Whereas exchange of the last 3 residues of β1A with those of β3 (β3β1(C3R)) markedly reduced c-Src binding, it had no effect on the binding of the F23 fragment of talin (Fig. 2A) or full-length talin (Fig. 2B) to the β3 tail. Conversely, β3 mutations known to abolish talin binding (β3(W739A), β3(L746A/K748A)) (16, 26) had no effect on the binding of c-Src SH3 (Fig. 2A) or full-length c-Src (Fig. 2B) to the integrin β3 tail. Moreover, saturating concentrations of the talin F23 fragment (26, 44) failed to block the binding of the c-Src SH3 domain (Fig. 3A) to β3 or the capacity of β3 to activate c-Src (Fig. 3B). Thus, the interaction sites in β3 integrins for talin and c-Src are distinct, and talin does not compete with c-Src for binding to the integrin.

Certain Src Family Kinases Bind to Integrin β1 Cytoplasmic Tails—The foregoing results establish that exchange of the last

**Fig. 3.** Talin does not inhibit c-Src binding to or increased Src activity upon binding to integrin β3 cytoplasmic tail. A, increasing concentrations of c-Src-SH3 were added to microtiter wells coated with immobilized β3 tail model protein in the presence or absence of 1 μM talin-F23 protein. Bound c-Src-SH3 was detected with monoclonal antibody 327, and binding was expressed as the percentage of maximal binding. **Inset,** binding of talin-F23 fusion with GST to β3 cytoplasmic tail detected with anti-GST antibodies. B, talin-F23 at 1 μM does not inhibit the increase in Src activity upon incubation with β3 cytoplasmic tail bound to neutravidin-agarose. c-Src-SH3 (20 μM) was used as inhibition control. Activity is expressed relative to basal Src activity in the absence of β3. All experiments were performed in triplicate.
FIG. 4. Binding specificity of different SFKs to various β cytoplasmic tails. A, enzyme-linked immunosorbent assay binding curves for GST-SH3 domains of the indicated SFKs using neutravidin-bound biotinylated β1, β2, and β3 cytoplasmic tails (20 μg/ml) and developed with anti-GST. Results were normalized to the percentage of maximal binding after subtraction of nonspecific binding to randomized β3 cytoplasmic tail.
3 residues of β3 and β1 can regulate the binding and activation of c-Src. Most cells express multiple Src family kinases, and we previously found in pull-down studies with platelet lysates (15) that Lyn and c-Yes kinases are capable of binding to integrin β1A, β2, and β3 tails. We now find that the purified SH3 domains of c-Yes and Lyn can bind to β1A, β2, and β3 tails (Fig. 4A), whereas Fyn binds only to β3. Furthermore, native Lyn and c-Yes associated with heterodimeric integrins bearing the β1 and β2 tails in cells (Fig. 4B). These results suggest that although exchange of the last 3 C-terminal residues between β1A and β3 can regulate the binding of c-Src, it would not change the binding of c-Yes or Lyn. Indeed, this proved to be the case (Supplementary Fig. 1, A and B).

Biological Role of the c-Src-Integrin Interaction—To analyze the functional importance of the c-Src-integrin interaction, we sought a cellular system in which c-Src was the only SFK expressed. Klinghoffer et al. (19) have derived fibroblasts from c-Src, Fyn, and c-Yes triple-knockout embryos and showed that integrin signaling and cell spreading are dramatically depressed in these cells (SFK cells). They have also reconstituted these cells with c-Src (SYF + c-Src cells). Immunoblotting confirmed that all three kinases were absent from SYF cells and that c-Src was the only one present in the SYF + c-Src cells (data not shown). The SYF + c-Src cells were then transiently transfected with the αIIb subunit in combination with integrin β3 or with the talin binding-defective β3(L746A/K748A) mutant. Alternatively, the cells were transfected with αIIb and chimeras of β3 containing the β3 extracellular and transmembrane domains joined to either the β1 cytoplasmic domain (34, 45) or one of the chimeric cytoplasmic domains described above, β3β1(C3R) or β1β3(C3R). c-Src was present in β3 immunoprecipitates when either the wild-type β3, β3(L746A/K748A), or β1β3(C3R) chimeric tail was present (Fig. 4C). In contrast, no c-Src was associated with integrins bearing either the β1A tail or the β3β1(C3R) tail. Thus, these various SYF + c-Src cells provide us with a tool to examine the biological role of the c-Src-integrin interaction.

The β3 Integrin-Talin Interaction, But Not the β3-c-Src Interaction, Is Required for Integrin Activation—Integrin activation is regulated by the interaction of talin with the integrin β tail. To examine the relative roles of talin and c-Src binding to the integrin β tail on integrin activation, we transiently transfected SYF + c-Src cells with plasmids encoding the integrin β3 subunit, a talin binding-deficient mutant (β3(L746A/K748A)), or a c-Src binding-deficient mutant (β3β1(C3R)). To produce an activated integrin, we cotransfected a chimeric integrin α subunit in which the extracellular and transmembrane domains of αIIb were joined to the cytoplasmic domain of α5 (41). Integrin activation was assessed with the αIIb3 activation-specific monoclonal antibody, PAC1 (46). The c-Src binding-deficient mutant supported specific PAC1 binding to the same extent as wild-type αIIb3 (activation index, 12.2 and 12.3, respectively) (Fig. 5). In sharp contrast and as expected, the talin binding-deficient αIIb3(L746A/K748A) mutant failed to support specific PAC1 binding. Importantly, all three integrins were equally well expressed, as judged by the binding of an αIIb3-specific antibody, D57 (data not shown). Thus, whereas talin binding is required, c-Src binding is dispensable for integrin activation.

The β3 Integrin-c-Src Interaction, But Not the β3-Talin Interaction, Is Required for Integrin-Mediated Cell Spreading—Integrin-mediated adhesion initiates the SFK-dependent spreading of cells. We exploited the fact that β3 integrins, but not the widely expressed β1 integrins, mediate cell spreading on fibrinogen-coated surfaces (47). SYF + c-Src cells failed to spread on fibrinogen but did spread when transiently transfected with cDNAs encoding the β3 integrin subunit (Fig. 6). Importantly, β3 transfection failed to induce spreading of the SYF cells (data not shown), confirming that the spreading was both β3- and c-Src-dependent. Transfection of SYF + c-Src cells with the β3β1 chimera or the β3β1(C3R) chimera failed to induce cell spreading beyond that seen in untransfected cells, indicating that c-Src binding to the integrin tail was required for spreading. Conversely, the gain of c-Src binding function in the β1β3(C3R) chimera enabled that integrin subunit to mediate cell spreading (Fig. 6).

To quantify the results depicted in Fig. 6, the cell areas for 50–100 randomly sampled positively transfected cells were measured using Image Pro Plus software. The β3 integrins that promoted visual spreading (β3, β1β3(C3R)) on average resulted in a 2-fold increase in cell area relative to the chimeras that failed to promote visual spreading (β1, β3β1(C3R)) (Fig. 7). Thus, SFK binding to the integrin β tail is required for and mediates cell spreading.

To analyze the role of talin binding to the β3 tail in cell spreading, we examined the behavior of cells transfected with β3(L746A/K748A) upon attachment to fibrinogen. As expected from the fact that deletion of the β3 tail only modestly inhibits adhesion to fibrinogen (47), cells bearing this mutant adhered;
however, they failed to spread as judged visually and by quantitative image analysis (Fig. 7A). We hypothesized that the lack of activation of the β3 integrin could lead to reduced engagement of αIIbβ3, resulting in a secondary inhibition of cell spreading. Indeed, addition of the activating monoclonal antibody, anti-anti-LIBS6 (35, 37), restored cell spreading in αIIbβ3(L746A/K748A)-expressing cells (Fig. 7B). By contrast, even in the presence of anti-anti-LIBS6, αIIbβ3(C3R)-expressing cells spread poorly on fibronogen, indicating that the reduced spreading of these cells was not due to a defect in integrin activation. These results establish that talin contributes to cell spreading by activating the integrin, but once the integrin is activated, talin binding to the integrin β tail is no longer required.

**Perspective and Conclusions**—The studies described here provide unambiguous evidence that inside-out and outside-in integrin signaling are mediated by distinct and separable interactions of the integrin cytoplasmic domains. Talin binding is required for integrin activation, and the binding of c-Src kinases is required for the signaling events that lead to cell spreading. Thus, it is possible to exert precise mutational control over the vectorial direction of integrin signaling. Furthermore, previous studies have suggested that integrin activation could precede and regulate the ability of these receptors to generate intracellular signals (48, 49). The present studies directly establish this point by showing that the spreading defect caused by loss of talin binding (and hence, loss of activation) can be rescued by antibody-mediated increase in ligand binding affinity. This result also provides additional evidence that cells can undergo spreading even when talin binding to the integrin tail is blocked by mutation (present studies) or by lack of talin (32). Many studies have examined integrin signaling in development, the immune response, hemostasis, and a wide variety of pathological states. The present studies will provide a paradigm to dissect the relative contributions of the direction of integrin signaling on these processes in cell culture and, ultimately, in vivo.
