Phosphorylation of β₃ Integrin Controls Ligand Binding Strength*

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The cytoplasmic domain of β₃ integrin contains tyrosines at positions 747 and 759 in domains that have been implicated in regulation of α₃β₃ function and that serve as potential substrates for Src family kinases. The phosphorylation level of β₃ integrin was modulated using a temperature-sensitive v-Src kinase. Increased β₃ phosphorylation abolished α₃β₃-, but not αventing β₃-mediated adhesion to fibronectin. α₃β₃-Mediated cell adhesion was restored by the expression of β₃ containing Y747F or Y759F mutations but not by wild type β₃ integrin. Thus, phosphorylation of the cytoplasmic domain of β₃ is a negative regulator of α₃β₃-fibronectin binding strength.

Integrins are heterodimeric cell surface receptors that are present in multicellular animals and serve as a major mechanical link to hold cells and tissues together (1). In most biological contexts, integrin receptors exist in an environment of ligand excess, and hence, binding between receptor and ligand is usually controlled by intracellular signals rather than by receptor or ligand availability. During normal development, α₃β₁ integrin becomes dispensable for basal epithelial cells undergoing differentiation to keratinocytes and for myoblasts differentiating into myotubes. In both cases there is a down-modulation of integrin function that precedes the down modulation of its synthesis (2, 3). Modulation of integrin function is important for cell migration to produce differences in functional integrin states at the leading and trailing edges (4). A highly specialized control of integrin function has evolved in platelets and lymphocytes to mediate the rapid response to injury or parasitic invasion (1, 5). The ability of integrin-mediated adhesion to be regulated by intracellular cues is critical to many facets of biology.

The activation of integrin-ligand binding requires metabolic energy and the actin cytoskeleton, but the exact mechanisms are poorly understood. In platelets and lymphocytes, G-protein-coupled receptors can serve as co-stimulatory receptors to activate integrin binding through intracellular signaling pathways (5). Outside the hemopoietic system, Ha-Ras and R-Raf have been identified in transfection assays as suppressors of integrin activation as measured by the binding of the PAC-1 activator of integrin function (7). The tetraspan protein CD98 has been implicated in regulation of integrin function that precedes the down modulation of integrin function has been more problematic. Measurement of integrin phosphorylation has been technically more difficult than for other focal adhesion-associated proteins. Hence, most reports have relied on tyrosine to phenylalanine point mutations to probe this issue. In most integrin function assays, these mutants were indistinguishable from wild type (12, 13, 15), although they could be distinguished in clot retraction and cell motility assays that involve complex cytoskeletal functions in addition to integrin function (15, 16). To more directly address the role of phosphorylation of the cytoplasmic domain of β integrins in the regulation of ligand binding function, we developed a system for conditional modulation of integrin phosphorylation and applied a newly developed method to measure directly the effect of this phosphorylation in the strength of the α₃β₃-fibronectin bond (17, 18).

MATERIALS AND METHODS

Cell Lines and Reagents—Human osteosarcoma cells (HOS cells)³; ATCC, Manassas, VA) were cultured in Dublecco's modified Eagle's medium with 10% fetal calf serum (Mediatech, Herndon, Virginia) and penicillin-streptomycin. Human plasma fibronectin and cell culture media were purchased from Invitrogen. Monoclonal antibody, LIBS-1, was a gift from M. Ginsberg. AIB2 and BII2 hybridomas were gifts from C. Damsky. Monoclonal antibodies LM609, P1D6, P1B5 and PM6/13 were purchased from Chemicon (Temecula, California). Fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody was purchased from Jackson ImmunoResearch (West Grove, Pennsylvania), and phycoerythrin-conjugated goat anti-rat antibody was purchased from Sigma. To generate HOSnsrc cells, HOS cells were transfected with the temperature-sensitive UP1-v-Src mutant using the HIT retroviral vector system (19, 20). Stable transfecants were selected with Geneticin. WT β₃, β₃(Y747F), β₃(Y759F), and β₃(Y747F,Y759F) DNAs were cloned into pREP9 (Invitrogen), and α₃ DNA was cloned into pCDM8 (Invitrogen) (from S. Blystone). The ptreLuc vector expressing hygromycin resistance was a gift from P. Bates. HOSnsrc cells were transfected with a mixture of a β₃ vector, an α₃ vector, and ptreLuc using LipofectAMINE Plus (Invitrogen). Stable and transient transfecants were selected by hygromycin resistance.

Spinning Disc Assay—The spinning disc assay was performed essentially as described (17, 21, 22). Briefly, the cells were allowed to adhere for 7 min to fibronectin on glass coverslips in the chamber of the spinning disc device, which was partially filled with spinning buffer pre-warmed to 37 °C to keep the cells near this temperature during the spinning.

* This research was supported National Institutes of Health Grants CA16502 and GM57388. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: HOS cells, human osteosarcoma cells; WT, wild type; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.
adhesion period. Cells were spun for 5 min, fixed with 3.7% paraformaldehyde, and stained with ethidium homodimer. Cell density at different radial positions was determined by using a motorized stage and Phase 3 image analysis software Version 3.0, and the shear stress corresponding to 50% cell detachment \( (r_{50}) \) was calculated using SigmaPlot software version 5.0 (17).

Wash Adhesion Assay—Corning tissue culture microtiter plates were coated with different concentrations of fibronectin type III repeats 7–10 (23) and blocked with 1% bovine serum albumin. Cells were labeled with calcein AM, trypsinized, and plated in triplicate at 10^5 cells/well. After 1 h, the cells were washed 3 times using a microplate washer and shaken on a Vortex Genie (Fisher) at a setting of 3–5 min of shaking between washes. The shaking is the most stringent step in this procedure and, hence, regulates the strength of the washing. This produced plates with a uniform distribution of cells in the wells rather than a donut-shaped clearing due to differential shear at different points in the well. Plates were read using a modified Dynatech MicroFluor plate reader.

Flow Cytometry—Cells were trypsinized, resuspended in fluorescence-activated cell sorter buffer (0.1% bovine serum albumin and 0.01% sodium azide in phosphate-buffered saline), and incubated on ice for 15 min. Anti- \( \alpha_v \) and \( \beta_3 \) hybridoma supernatants BIIIG2 or AIIIB2, respectively, were added at 1:5 dilutions or P1B5 or LM609 purified monoclonal antibodies were added at 10 \( \mu \)g/ml. For LIBS-1 binding studies, cells were treated with 2 \( \mu \)g GRGDSP and stained with the monoclonal anti-LIBS-1 at a dilution in fluorescence-activated cell sorter buffer. Incubation of the primary antibody was carried out at 4°C for 30 min with shaking. Fluorescein isothiocyanate-conjugated anti-mouse or phycoerythrin-conjugated anti-rat antibodies (for AIIIB2 and BIIIG2) were added at a dilution of 1:100 and incubated for 30 min at 4°C. Cells were analyzed by flow cytometry.

Cross-linking of Bound Integrins—Cells were plated on 2 \( \mu \)g/ml fibronectin-coated dishes for 1 h, cross-linked with 1 \( \mu \)g [%sulfosuccinimidyl] (sulfo) 1, 4-benzenedicarboxylate (Pierce) in phosphate-buffered saline for 30 min. The cells were extracted with 0.1% SDS in phosphate-buffered saline containing protease inhibitors. The extracted protein concentration was determined by a protein assay reagent (Pierce) to ensure that same total numbers of cells were attached to the substrate.

The cross-linkers were cleaved in carbonate buffer (50 mM Na2CO3, 0.1% SDS, pH 11.6) for 2 h at 37°C, and the cross-linked pool of integrins was analyzed by Western blotting using polyclonal antibodies to the cytoplasmic domains of integrins \( \alpha_5, \alpha_v, \beta_1, \beta_3 \) (Chemicon). Blots were developed with ECL (Amersham Biosciences, Inc.) and analyzed using a Fuji LAS-1000 system and ScienceLab 2.5 software.

Detection of \( \beta_3 \) Phosphorylation—Normal and transformed HOSnsrc cells were grown at 35°C or 39.5°C for 72 h before treatment. The protocols were washed, fresh Dulbecco’s modified Eagle’s medium containing 0.2% fetal calf serum and 75 mM sodium orthovanadate was added, and incubation was continued at 39.5°C or 35°C for 2 h. Cells were then lysed in CHAPS buffer (1% CHAPS, 10 mM Tris-HCl, pH 7.6, 2 mM sodium orthovanadate, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.01% NaN3, 10 \( \mu \)g/ml aprotinin, 350 \( \mu \)g/ml phenylmethylsulfonyl fluoride, 10 \( \mu \)g/ml leupeptin, 0.5 mg/ml DNase 1) at 20°C for 10 min. Lysates were spun at 15,000 \( \times \) g for 4°C for 10 min and precleared overnight with 50 \( \mu \)l of goat anti-mouse IgG beads (ICN Pharmaceuticals, Costa Mesa, CA). Lysates were then immunoprecipitated with a mixture of anti- \( \beta_3 \) antibody P1D6 (Chemicon), anti- \( \beta_1 \) antibody TS 2/16, and goat anti-mouse beads. The supernatant fraction was immunoprecipitated with anti- \( \beta_3 \) antibody P605/2 (Chemicon) and goat anti-mouse beads. The beads were washed 3 times with radioimmune precipitation buffer containing 2 mM sodium orthovanadate, separated on reducing 8% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and blotted with either 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY) for phosphorytosine or rabbit polyclonal antibody to either \( \beta_3 \) cytoplasmic domain (24) or \( \beta_1 \) cytoplasmic domain (AB 1932, Chemicon). Blots were developed with ECL (Amersham Biosciences, Inc.) and analyzed using a Fuji LAS-1000 system and ScienceLab 2.5 software.

RESULTS

v-Src Induces Phosphorylation of \( \beta_3 \) Integrin—HOS cells were transfected with a vector containing the temperature-sensitive mutant of v-Src (UP1 (19)) and neoR. This mutant is temperature-sensitive for kinase activity, and the protein remains stable at the non-permissive temperature (Ref. 19 and data not shown). Geneticin-resistant colonies were selected and screened for temperature-sensitive expression of v-Src kinase activity. Two clones that exhibited minimal phosphorylation of pp60v-Src at the non-permissive temperature of 39.5°C and high levels of pp60v-Src phosphorylation at the permissive temperature of 35°C were chosen for further analysis (Fig. 1A). HOS cells growing at 35°C and expressing active v-Src kinase will hereafter be referred to as HOSnsrc35 and HOS cells grown at 39.5°C expressing an inactive v-Src kinase will be referred to as HOSnsrc39.5.

Src-dependent Phosphorylation of \( \beta_3 \) and \( \beta_1 \) integrin subunits was analyzed by comparing the levels of phosphorytosine in \( \beta_1 \) and \( \beta_3 \) immunoprecipitates of HOSnsrc35 and HOSnsrc39.5 cells. Fig. 1B shows that activation of v-Src resulted in a >5-fold increase in the level of \( \beta_3 \) phosphorylation. Tyr-747 in the cytoplasmic domain of \( \beta_3 \) integrin is a likely site for this phosphorylation since it has been shown to be phosphorylated in vivo and is in a site homologous to Tyr-788 in chicken \( \beta_1 \), which can be phosphorylated by v-Src in vitro (25) (26). In contrast, \( \beta_1 \) integrin showed a moderate level of phosphorylation in HOSnsrc39.5 cells, and this level did not appear to increase substantially after the activation of v-Src kinase in HOSnsrc35 cells. The increased background seen in the HOSnsrc35 \( \beta_1 \) integrin blot is likely due to the increase in phosphorylation of many proteins after v-Src expression. This differs from previous reports using 32P-labeling that report increased phosphorylation of \( \beta_3 \) in v-Src-transformed chicken cells (27–29). The HOSnsrc cells express lower levels of v-Src compared with v-Src-transformed chicken embryo fibroblasts. Because Src co-localizes selectively with \( \beta_3 \) integrin as opposed to \( \beta_1 \) integrin (30), the reduced expression of v-Src would produce a selective phosphorylation of \( \beta_3 \) over \( \beta_1 \).

v-Src Modulates \( \alpha_\beta_3 \) Fibronectin Bond Strength—A modified wash-type adhesion assay was used to determine which integrins were responsible for adhesion of HOSnsrc35 and HOSnsrc39.5 cells to fibronectin. In the modified assay, the stringency is controlled by the mechanical shaking device rather than by the force of the buffer during fluid changes. This provided a more reproducible assay and reduced the effects of the well geometry on the assay. The remaining cells following the washing procedure were uniformly distributed over the well rather than the donut-shaped detachment pattern common to wash assays. Adhesion was determined for a range of fibronectin densities, and the proportion of cells remaining was
The strength of the individual integrin ligand bonds depends on activation processes within the cell (18, 32). Thus, this approach provides a quantitative measure of the relative strength of the integrin-ligand bonds. Fig. 3A shows a combined cell detachment profile for HOSnsrc35 and HOSnsrc39.5 cells from fibronectin as a function of applied shear stress at 7 min after plating. The data show about a 40% reduction (leftward shift, cells detach at lower shear stress) in adhesion strength as a result of temperature-dependent activation of v-Src. Analysis of the levels of cell surface expression of integrin by flow cytometry showed no difference in the levels of α5, α5, α5, β1, or β3 between HOSnsrc35 and HOSnsrc35 cells (data not shown). Thus the reduction of adhesion after the activation of v-Src cannot be explained by altered integrin expression levels. To determine whether the difference was due to differences in incubation temperature, HOSnsrc and parental HOS cells incubated at 35 and 39.5 °C were assayed using the spinning disc. Fig. 3B shows a summary of the τ50 values (shear stress for 50% adhesion) for several experiments similar to that shown in Fig. 3A. Incubation temperature had no effect on the adhesion of parental HOS cells but showed a 40% reduction for HOSnsrc cells at 35 °C.

For cells that express the same number of integrin receptors, differences in the slope of the mean cell detachment force (as determined form analyses shown in Fig. 3A) as a function of fibronectin density reflect differences in the strength of the integrin-ligand interaction. Fig. 3C shows that the HOSnsrc35 and HOSnsrc39.5 cells had different slopes. For the HOSnsrc39.5, there is a suggestion of a two-component curve as it deviates from linearity at higher fibronectin densities, again raising the possibility that the two receptors with different binding strengths were involved. To determine which integrin receptors were responsible for these differences, spinning disc experiments were performed on HOSnsrc cells treated with antibodies to α5 (BIIG2), β1 (AIIB2), or β3 (LM609) integrin. Fig. 3D shows that the adhesion of HOSnsrc35 cells was reduced to background levels by either anti-α5 or anti-β1 but was unaffected by anti-β3. In contrast, HOSnsrc39.5 cells showed a significant but partial reduction in the presence of either anti-α5 or anti-β3 and required the mixture of anti-β1 and anti-β3 to reduce adhesion to background levels. Thus, both α5β1 and α5β3 mediated the adhesion of HOSnsrc35 cells. Specific adhesion strengths do not appear to be directly additive; i.e., the sum of the adhesion strength in the presence of anti-α5 representing α5β3-mediated adhesion plus the adhesion strength in the presence of anti-β3 representing α5β3-mediated adhesion, was more than the adhesion strength in the absence of antibody. This result is not unexpected since cross-talk between these receptors had been described (33).

Unlike the HOSnsrc39.5 cells, the HOSnsrc35 cells showed no α5β3-mediated adhesion to fibronectin. Attempts to use vitronectin to provide an alternative means of distinguishing α5β3 from α5β1-mediated adhesion were limited by the expression of vitronectin receptors by the HOSnsrc35 cells in addition to α5β3. Thus, activation of v-Src in the HOSnsrc cells results in the inactivation of α5β3 integrin function as determined by its ability to support a mechanical connection to fibronectin. This α5β3 function could be switched on and off by switching the incubation temperature of the cells, thereby activating or inactivating the v-Src kinase enzymatic function.

**Ligand-Bound α5β3 and α5β3—**Chemical cross-linking has been used for the analysis of many receptor-ligand interactions. We have taken the approach of using cell-impermeant cross-linkers to cross-link cell surface integrins to substrate-immobilized ligands. After cross-linking with Bis[2-(sulfosuccinimidyl)oxycarbonyloxy]ethyl]sulfone, the cells were extracted with a
strong detergent, leaving the cross-linked integrin behind. The cross-linker was cleaved at high pH, releasing the cross-linked integrin for analysis by Western blot. Control experiments have shown that the recovery of integrin from the cross-linked fraction requires the proper ligand and that the integrin be activated (17, 24). In addition, for $\alpha_5\beta_1$, the amount of cross-linked integrin recovered was directly proportional to the strength of the interaction measured by the spinning disc assay and the number of receptor-ligand bonds formed (31). This cross-linking assay provides an alternative assay for the presence of specific integrin-ligand bonds. The data in Fig. 4 show both the supernatant fraction (non-cross-linked) and the cross-linked fraction for each integrin subunit. Based on quantitation of three independent experiments, the higher v-Src kinase activity in the HOSnsrc35 cells had no significant effect on the level of cross-linked $\alpha_5$ or $\beta_1$. This is consistent with the adhesion data, which showed that v-Src activation had minimal effect on $\alpha_5\beta_1$-mediated adhesion. In contrast, the level of $\alpha_5$ was reduced about 2-fold, and the level of $\beta_1$ was reduced about 4-fold in the HOSnsrc35 cells compared with the HOSnsrc39.5 cells. The levels of $\alpha_5$ in the cross-linked fraction are low, and the antibody used does not give as clear a Western blot as the others. Nevertheless, these results support the conclusion that $\alpha_5\beta_1$ function is reduced in the HOSnsrc35 cells.

Restoration of $\alpha_5\beta_1$ Binding by Tyr $\rightarrow$ Phe Mutants of $\beta_3$—
Previous experiments show that activation of v-Src kinase in HOS cells increased the level of $\beta_3$ phosphorylation and blocked the ability of $\alpha_5\beta_3$ to mediate adhesion to fibronectin. Both the use of a temperature-sensitive v-Src kinase activity mutant and the relatively low level of v-Src expressed in these cells contribute to the argument that the effect on adhesion is specific to v-Src kinase function. Nevertheless, the correlation between the increase in $\beta_3$ phosphorylation and reduction of $\alpha_5\beta_3$ function could be due to the phosphorylation of $\beta_3$ integrin-associated proteins rather than $\beta_3$ integrin itself by v-Src kinase. To determine whether phosphorylation of $\beta_3$ itself was critical for the reduction of $\alpha_5\beta_3$-mediated adhesion, non-phosphorylatable mutants of $\beta_3$ integrin were transfected into HOSnsrc35 cells. Both transient transfections and selection of stable transformants were performed. There are two tyrosines in the cytoplasmic domain of $\beta_3$ at positions 747 and 759 (in the human sequence). Both of these tyrosines are embedded in sequences that are similar to the Tyr-788 region in the cytoplasmic domain of chicken $\beta_3$ (Tyr-783 human integrin), which can be phosphorylated by v-Src kinase in vitro (26). It is likely that both Tyr-747 and Tyr-759 in $\beta_3$ integrin can be phosphorylated in vivo, but definitive data exist only for Tyr-747 (25).

The transient transfection assays gave the best expression levels at 48 h after transfection, but we found that the cells recovering from various transfection protocols were too fragile.
Phosphorylation Controls \( \beta_3 \) Integrin

The ability of integrin-mediated adhesion to be regulated by intracellular signals is critical to the function of integrins in processes that include cell migration, cell differentiation, cell survival, and cell proliferation (1). Direct analysis of the mechanisms by which the integrin function is controlled have been limited by both the biological systems that have been studied and by the assays used. We developed a quantitative assay to measure the relative strength of the integrin-ligand bonds in intact cells as a means of providing a more direct measurement of integrin activation than was available using “activation-specific” monoclonal antibodies (17, 34). Using that approach we have identified additional states of the integrin-ligand binding interaction that could not be identified using previous approaches (17, 18). In this study, we also developed a cell line that expresses a temperature-sensitive Src kinase and that displays a temperature-sensitive phosphorylation of \( \beta_3 \) integrin. This provides the first instance in which the phosphorylation of a specific integrin can be controlled without using specific phosphorylation-blocking integrin mutants. This is important because this phosphorylation event is likely to be transient, as suggested by the difficulty of observing phosphorylation of either \( \beta_1 \) or \( \beta_3 \) integrin in the absence of vanadate pretreatment. It is possible that the regulation is accomplished through a phosphorylation-dephosphorylation cycle. The second critical element of the model system is the focus on \( \beta_3 \) rather than \( \beta_1 \) integrin (discussed below). This is the first report of v-Src-induced phosphorylation of \( \beta_3 \) integrin. Remarkably, blocking \( \beta_3 \) phosphorylation of Tyr-747 by mutation complemented the adhesion defect showing that failure to phosphorylate \( \beta_3 \) resulted in failure to block \( \beta_3 \) function. This is also the first case of dominant suppression of a v-Src function by a single Tyr \( \rightarrow \) Phe mutation in a candidate v-Src target.

Sequence analysis of \( \beta_1 \), \( \beta_2 \), \( \beta_3 \) integrin revealed a high degree of homology in their cytoplasmic domains. The presence of tyrosines at 783 and 795 in human \( \beta_1 \) and 747 and 759 in human \( \beta_3 \) correspond to phenylalanines in \( \beta_2 \) integrin (35). Conformational disruption by the introduction of mutations into the domains containing these tyrosines in \( \beta_1 \) and \( \beta_3 \) integrin compromises its function in adhesion and/or spreading assays, suggesting that these sequences are critical for normal function (36, 37). In most analyses, the Tyr \( \rightarrow \) Phe mutations had a weaker phenotype than Tyr \( \rightarrow \) Ala mutants, and mutations near Tyr-747 had a stronger phenotype than mutations

**Table I**

| Cells           | Treatment | T50 ± S.D. |
|-----------------|-----------|------------|
| HOSnsrc35      | None      | 4.3 ± 1.3  |
| HOSnsrc35      | WT \( \beta_3 \) | 4.1 ± 0.6  |
| HOSnsrc35      | \( \beta_3(Y747F) \) | 22.1 ± 1.6 |
| HOSnsrc35      | \( \beta_3(Y759F) \) | 13.8 ± 7.3 |
| HOSnsrc35      | \( \beta_3(Y747,759F) \) | 22.9 ± 2.2 |

**DISCUSSION**

**FIG. 5. Rescue of \( \beta_3 \) binding by the expression of a \( \beta_3 \) phosphorylation mutant.** WT \( \beta_3 \) and \( \beta_3(Y747F) \) mutant expression plasmids were transiently transfected into HOSnsrc35 along with an expression plasmid for \( \alpha_3 \) integrin. Flow cytometry was used to analyze the level of \( \beta_3 \) expression on the surface of WT \( \beta_3 \) transfectants (A), \( \beta_3(Y747F) \) transfectants (B), and parental HOSnsrc35 cells (C). MFI is mean fluorescent index or geometric mean of \( \beta_3 \) fluorescence-negative control (secondary antibody alone)/negative control. D, Western blot showing the level of cross-linked \( \beta_3 \) for WT \( \beta_3 \) and \( \beta_3(Y747F) \) transient transfectants of HOSnsrc35 cells.

HOStsrc cells at 39.5°C increased the level of LIBS-1 binding about 2-fold, whereas this temperature-shift had no effect on the binding of LIBS-1 to parental HOS cells lacking the v-Src gene. In addition, the two v-Src kinase inhibitors herbimycin A and PP2 produced a significant increase in the level of LIBS-1 binding, whereas the inactive PP3 had only a modest effect. The 2-fold difference in binding is similar to that reported for the effect of Ras on the binding of the PAC-1 monoclonal antibody to \( \alpha_{1b},\beta_3 \) (6). Thus, the phosphorylation of \( \beta_3 \) cytoplasmic domain led to a detectable change in the conformation of the extracellular domain of \( \alpha_3,\beta_3 \).

The 2-fold difference in binding is similar to that reported for the effect of Ras on the binding of the PAC-1 monoclonal antibody to \( \alpha_{1b},\beta_3 \) (6). Thus, the phosphorylation of \( \beta_3 \) cytoplasmic domain led to a detectable change in the conformation of the extracellular domain of \( \alpha_3,\beta_3 \).
Phosphorylation Controls $\beta_3$ Integrin

**TABLE II**

Analysis of $\alpha_\beta_3$ conformation by LIBS-1 antibody binding

MFI is the geometric mean fluorescent intensity. Activation index is normalized MFI to HOSnsrc35 for four separate experiments done at different times.

| Cells      | Temperature | Treatment | MFI ± S.D. ($n = 4$) | Activation index ± S.D. |
|------------|-------------|-----------|----------------------|-------------------------|
| HOSnsrc    | 35          | None      | 98 ± 7               | 1.00 (base)             |
| HOSnsrc    | 39.5        | None      | 176 ± 10             | 2.2 ± 0.46              |
| HOSnsrc    | 35          | Herbimycin A | 426 ± 27          | 3.5 ± 1.0               |
| HOSnsrc    | 35          | PP2       | 289 ± 16             | 2.6 ± 0.7               |
| HOSnsrc    | 35          | Herbimycin A | 171 ± 8            |                        |
| HOS        | 35          | None      | 383 ± 21             |                        |
| HOS        | 39.5        | None      | 396 ± 19             |                        |

near Tyr-759. (13, 14). The complementation data presented here also show a stronger phenotype for the Y747F as compared with the Y759F mutant. The absence of tyrosines in $\beta_3$ integrin cytoplasmic domain suggests that the mechanisms of regulation of $\beta_3$ integrin-mediated adhesion will be different from $\beta_1$ and $\beta_3$. Despite the sequence homology between $\beta_3$ and $\beta_1$ integrin, several lines of evidence suggest that they are regulated differently.

First, analysis of the compartmentalization of phosphorylated and non-phosphorylated $\beta_3$ integrin in v-Src-transformed chicken embryo fibroblasts demonstrated that the increase in $\beta_1$ phosphorylation caused by v-Src expression was mostly in the soluble and not the adhesion-associated pool of $\beta_1$ (28). Second, mutants of $\beta_1$ in which the cytoplasmic tyrosine corresponding to Tyr-747 in $\beta_3$ was mutated to glutamate to simulate the phosphorylated form were distributed away from focal adhesions, whereas the phenylalanine substitutions tended to accumulate in focal adhesions (10). Third, analysis of the function of phenylalanine substitutions in $\beta_1$ using GD25 cells, which express no endogenous $\beta_3$ integrin, led to defects in the organization of focal adhesions, cytoskeleton, and cell motility (15). Each of these studies led to a model in which phosphorylation of $\beta_1$ resulted in the dissociation of connections between $\beta_1$ cytoplasmic domain and the cytoskeleton and dissociation between $\beta_1$ and its ligand. The results are also consistent with a model in which $\beta_1$ integrin is phosphorylated outside the focal adhesion, and the phosphorylated forms do not cycle into focal adhesions. Hence, phosphorylation would reduce the available $\beta_1$ integrin pool. This interpretation would be favored, at least for the case of Src kinase, by the demonstration that Src colocalizes with $\beta_1$ but not with $\beta_1$ in mouse fibroblasts (30). In the present study, varying the level of v-Src kinase activity had no substantial effect on the level of $\beta_3$ phosphorylation but had a large effect on $\beta_3$ phosphorylation. Does this difference between $\beta_1$ and $\beta_3$ extend to cells other than the HOSnsrc cells? GD25 cells lacking $\beta_1$ integrin adhere to fibronectin use $\alpha_\beta_3$ integrin (38). Transformation of the GD25 cells by v-Src resulted in the complete loss of adhesion to fibronectin (29). Also, chicken embryo fibroblasts plated on fibronectin cannot be detached by the addition of CSAT monoclonal antibody, which blocks $\beta_1$ integrin function; however, after transformation by v-Src, these cells can be detached by CSAT (39). Because the chicken embryo fibroblasts also express $\alpha_\beta_3$, inactivation of $\alpha_\beta_3$ by v-Src could lead to detachment by CSAT. Thus, $\beta_3$ integrin phosphorylation as a result of Src kinase appears to have a direct effect on its ability to mediate adhesion to fibronectin, whereas the effects of Src kinase on $\beta_1$ integrin appear to be less direct. In chicken embryo fibroblasts, expression of v-Src had no effect on the strength of the $\alpha_\beta_3$-fibronectin bond in the short term (15 min) assays. The longer term reductions in adhesion could be explained by increases in both protease secretion, resulting in ligand removal, and hyaluronic acid, which insulates $\alpha_\beta_3$ from the surface-bound fibronectin (39). There is no evidence that either of these effects can be mediated through effects of the activation levels of $\alpha_\beta_3$ by v-Src.

The functional assays used in this report rely on the analysis of the strength and relative number of integrin-ligand bonds. For cultured cells, strong adhesion is maintained by a linkage from the inert plastic or glass substrate to the adsorbed extracellular matrix proteins, including predominantly fibronectin, to integrins, and to the actin cytoskeleton. The two regulated links in this chain are the integrin-ligand and the integrin-cytoskeleton connections. Interestingly, the regulation of these two linkages appears to be coupled. First, disruption of the actin cytoskeleton with agents such as cytochalasin D cause a reduction in adhesion strength (17, 40). Second, changes in the mechanical strength of the integrin-ligand bond correlate with changes in the mechanical strength of the integrin-cytoskeleton linkage (18, 41). Thus, changes in the mechanical strength of the interactions of the integrin cytoplasmic domain with cytoskeleton-associated proteins could affect the strength of the integrin-ligand bond. Indeed, such changes are thought to provide the basis for the regulation of integrin function by intracellular signals (9, 11).

The cytoskeletal proteins talin and filamin bind to the cytoplasmic domains of $\beta_1$, $\beta_2$, and $\beta_3$ integrins, and the binding to $\beta_1$ and $\beta_3$ integrins can be inhibited by a Tyr → Ala mutation in the membrane-proximal tyrosine (37, 42–44). This suggests that talin and filamin binding may be regulated by phosphorylation of Tyr-747. Phosphorylated $\beta_3$ integrin, produced by overexpression of v-Src kinase in vivo, showed a reduced binding affinity for talin in a gel filtration assay (26). Myosin was shown to bind to a phosphorylated $\beta_3$ peptide, suggesting that the binding of myosin to $\beta_3$ could also be affected by $\beta_3$ phosphorylation (45). This list is unlikely to be inclusive, and other cytoskeletal proteins have been found to bind $\beta_3$ cytoplasmic domain peptides, but the specific binding sites have not yet been mapped, e.g. skelemin (46). These proteins can also serve to link other cytoskeletal and cytoskeletal regulatory proteins including vinculin, zyxin, Mena/VASP, and FAK (11).

Although phosphorylated Tyr-747 and Tyr-759 in $\beta_3$ integrin could provide sites for the binding of specific cytoskeletal proteins, it must be noted that the phosphorylated forms are likely to be quite transient since they are only easily visualized after treatment of the cells with phosphatase inhibitors for several hours before lysis. In contrast, stable integrin-mediated adhesion requires that the cytoskeletal connections be maintained. We propose a model in which the role of $\beta_3$ phosphorylation is to alter the conformation of the cytoplasmic domain and provide for the dissociation of one protein complex and the formation of a new protein complex. $\beta_3$ integrin containing a phosphorylated Tyr-747 would represent a transition state, and hence, it would not be attached to the cytoskeleton. The absence of the cytoskeletal connection would limit the strength of the mechanical linkage required for cell adhesion and could retain $\alpha_\beta_3$ in an inactive conformation. A precedent for this...
can be found in analyses of $\beta_2$ integrin (42). In the absence of ligand, talin was bound to the cytoplasmic domain of $\beta_2$. After the binding of $\beta_2$ to ligand, talin was cleaved, released from $\beta_2$, and replaced by $\alpha$-actinin.

Acknowledgments—We thank Scott Blystone, Paul Bates, Mark Ginsberg, and Caroline Damsky for generous donations of reagents. We thank G. Steven Martin and Mark Ginsberg for critical comments on the manuscript.

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