Molecular Biology of the Cell
Vol. 7, 1939–1951, December 1996

Trans-dominant Inhibition of Integrin Function

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Submitted July 30, 1996; Accepted September 14, 1996
Monitoring Editor: Masatoshi Takeichi

Occupancy of integrin adhesion receptors can alter the functions of other integrins and cause partition of the ligand-occupied integrin into focal adhesions. Ligand binding also changes the conformation of integrin extracellular domains. To explore the relationship between ligand-induced conformational change and integrin signaling, we examined the effect of ligands specific for integrin $\alpha_{IIb}\beta_3$ on the functions of target integrins $\alpha_5\beta_1$ and $\alpha_2\beta_1$. We report that binding of integrin-specific ligands to a suppressive integrin can inhibit the function of other target integrins (trans-dominant inhibition). Trans-dominant inhibition is due to a blockade of integrin signaling. Furthermore, this inhibition involves both a conformational change in the extracellular domain and the presence of the β cytoplasmic tail in the suppressive integrin. Similarly, ligand-induced recruitment of $\alpha_{IIb}\beta_3$ to focal adhesions also involves a conformational rearrangement of its extracellular domain. These findings imply that the ligand-induced conformational changes can propagate from an integrin's extracellular to its intracellular face. Trans-dominant inhibition by integrin ligands may coordinate integrin signaling and can lead to unexpected biological effects of integrin-specific inhibitors.

INTRODUCTION

Integrins, adhesion receptors composed of two type 1 transmembrane subunits, α and β, transfer information in both directions across cell membranes (Hynes, 1992). Intracellular signals change the ligand-binding affinity of the integrin extracellular domain in a process termed inside-out signaling (Schwartz *et al.*, 1995). Conversely, integrins regulate intracellular biochemical events such as tyrosine phosphorylation of cytoplasmic proteins, activation of this sodium-hydrogen antiporter, and local accumulation of certain cytoskeletal proteins (Hemler *et al.*, 1994; Juliano, 1994; Ruoslahti and Reed, 1994; Schwartz *et al.*, 1995). Integrins often recognize small discrete peptide segments in their natural protein ligands (Loftus *et al.*, 1994). These recognition sites can be simulated by small peptide (Pierschbacher and Ruoslahti, 1984) or nonpeptide compounds (Alig *et al.*, 1992) or by mAbs (Tomiyama *et al.*, 1992a,b). Binding of natural protein ligands (Frelinger, *et al.*, 1990) or of ligand-mimetic peptides (Parise *et al.*, 1987), small molecules (Kouns *et al.*, 1992), or antibodies to integrins (Tomiyama *et al.*, 1992a) induces conformational changes in their extracellular domains. These changes can be detected by conformation-dependent mAbs, termed antiligand-induced-binding sites (LIBS; Frelinger *et al.*, 1988). Mapping of the epitopes of these antibodies indicates that conformational information is propagated from the ligand-binding site to the membrane proximal region of the integrin extracellular domain (Du *et al.*, 1993). It is uncertain whether the ligand-induced alteration can then be transmitted across the cell membrane.

Previous work suggests that occupancy of one integrin can suppress the function of other integrins (trans-dominant inhibition). For example, anti-$\alpha_v\beta_3$ antibodies suppress $\alpha_5\beta_1$-dependent phagocytosis (Blystone *et al.*, 1994) and ligation of $\alpha_4\beta_1$ inhibits $\alpha_5\beta_1$-dependent expression of metalloproteinases (Huhtala *et al.*, 1995). Similarly, isolated integrin β cytoplasmic domains inhibit the activation (Chen *et al.*, 1994), matrix assembly, and migratory functions (LaFlamme *et al.*, 1994) of intact integrins. Because

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unoccupied intact integrins lack this inhibitory activity, these results suggest that the α cytoplasmic domain masks an inhibitory function of the β cytoplasmic domain. We reasoned that trans-dominant inhibition could be caused by propagation of an occupancy-induced conformational change to the integrin cytoplasmic face, resulting in unmasking of the β cytoplasmic domain. In this work, we tested this idea by use of integrin $\alpha_{IIb}\beta_3$ as the initiator of the inhibitory signals. We found that ligation of $\alpha_{IIb}\beta_3$ induces trans-dominant suppression of the function of target integrins $\alpha_5\beta_1$ and $\alpha_2\beta_1$. The trans-dominant effect is manifested by suppression of signaling to the target integrins, is correlated with the capacity of ligands to induce conformational change in $\alpha_{IIb}\beta_3$, and requires an intact β_3 cytoplasmic domain. These data provide evidence for propagation of a ligand-induced conformational change to the integrin cytoplasmic face with consequent inhibitory effects. Moreover, these studies suggest that certain integrin-specific ligands provoke trans-dominant inhibition that could lead to unexpected effects on cell migration, gene expression, and cell viability.

MATERIALS AND METHODS

Antibodies and Reagents

The inhibitory anti-hamster α_5 and β_1 antibodies, PB1 and 7E2 respectively (Brown and Juliano, 1985; Brown and Juliano, 1988), were obtained from Dr. R.L. Juliano (University of North Carolina, Chapel Hill, NC). The inhibitory anti-human $\alpha_{IIb}\beta_3$ complex specific antibody, 2G12 (Woods *et al.*, 1984), was supplied by V.L. Woods, Jr. (University of California, San Diego, San Diego, CA). OPG2, a gift from Dr. Tom Kunicki (The Scripps Research Institute) is a function-blocking anti-$\alpha_{IIb}\beta_3$ that has ligand-mimetic properties (Tomiyama *et al.*, 1992a). The antiligand-induced binding site antibody, anti-LIBS1 (Frelinger *et al.*, 1990), and the noninhibitory anti-$\alpha_{IIb}\beta_3$ antibody D57 (O'Toole *et al.*, 1994) have been described previously. Monoclonal antibody (mAb) 15 is a noninhibitory monoclonal antibody against the human β_3 subunit (Frelinger *et al.*, 1990). R2-8C8 is a function-blocking mAb against the human α_2 integrin subunit (Faull and Ginsberg, unpublished results). Ab 2308 is a rabbit polyclonal antibody against the human α_{IIb} subunit (O'Toole *et al.*, 1994). Rat collagen type I was obtained from Sigma Chemical (St. Louis, MO). Human plasma fibronectin (Fn) and fibrinogen were prepared as described previously (Plow *et al.*, 1985). ^{125}I-labeled Fn or antibody was prepared by a chloramine-T procedure (Sigma Chemical; Plow *et al.*, 1985). The protein kinase inhibitors, staurosporin and calphostin C, were obtained from Calbiochem (San Diego, CA) and H7 from LC Laboratories (Woburn, MA). Ro43-5054 and Ro44-9883 (lamifiban) are two $\alpha_{IIb}\beta_3$-specific peptido-mimetic antagonists and have been described previously (Alig *et al.*, 1992).

cDNA Vectors, Cells, and Cell Transfection

Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (Rockville, MD). All cell lines were maintained in DMEM (BioWhittaker, Walkersville, MD) with 4.5 g/100 ml of glucose (Life Technologies, Grand Island, NY) containing 5% fetal calf serum, 2 mM glutamine, 1% nonessential amino acids (Sigma Chemical), and 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical). The construction of pCDM8 expression plasmids encoding the integrin subunit, α_{IIb}, β_3, and $\beta_3\Delta728$ (O'Toole *et al.*, 1989, 1991) have been described previously. CHO cells expressing human $\alpha_2\beta_1$ were a generous gift from Dr. Y. Takada (Scripps Research Institute). All cDNA constructs were verified by DNA sequencing and purified on CsCl gradients before transfection into CHO cells using the Lipofectamine reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's recommendations. Stable CHO cell lines expressing the human integrins $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\beta_3\Delta728$ were established as described above with cotransfection of a pCDM8 plasmid (0.6 μg) encoding the *Escherichia coli* neomycin resistance gene. After 48 h, these cells were selected for 2 wk in 700 μg/ml G418 (Life Technologies) and clonal lines were established by single-cell sorting in a FACStar (Becton Dickinson Immunocytometry Systems, Mountain View, CA). A CHO cell line expressing the human $\alpha_2\beta_1$ integrin was cotransfected with a mixture of α_{IIb}, β_3 cDNAs, and the Na^+-H^+ antiporter variant, NHE3 (Counillon *et al.*, 1993), as a selectable marker. Cells expressing both $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ were selected by acute acidification (Counillon *et al.*, 1993). Clonal cell lines were established by single-cell sorting using flow cytometry.

Flow Cytometry

Flow cytometry analysis was performed in a FACScan cytofluorometer (Becton Dickinson). Briefly, 5×10^5 cells were incubated on ice for 30 min with a primary antibody, washed, and then incubated on ice for 30 min with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse F(ab')$_2$ (Tago, Burlingame, CA) secondary antibody. Cells were then pelleted, resuspended, and analyzed on the FACScan. A total of 1×10^4 cells was analyzed for each sample. The fluorescence produced by D57 mAb on untransfected CHO cells was considered as background. Anti-LIBS1 binding was also assayed using flow cytometry but with some modifications. Anti-LIBS1 mAb was conjugated with fluorescein isothiocyanate on cellite (Calbiochem) using the manufacturer's instructions. Cells were incubated with FITC-conjugated anti-LIBS1 mAb (10 μg/ml) in a final volume of 50 μl. After 30 min at room temperature, cells were resuspended in 0.5 ml of phosphate-buffered saline (PBS) and analyzed by FACScan. To obtain a numerical estimate of the conformational change, we calculated the percentage of expression of the anti-LIBS1 epitope. This was defined as $100 \times [(Fr - Fo)/(Ao)]$, where Fr is the mean fluorescence intensity of anti-LIBS1 staining in the presence of inhibitor (either Ro43-5054 or Ro44-9883), Fo is the mean fluorescence intensity of anti-LIBS1 staining in the absence of inhibitor, and Ao is the mean fluorescence intensity of Ab15 staining (maximal fluorescence intensity).

Adhesion Assays

The adhesion of cells to immobilized Fn and collagen was assayed as described previously (Faull *et al.*, 1993). Briefly, tissue culture plates (96 wells; Costar Corp., Cambridge, MA) were coated with 150 μl of soluble collagen (0.3 μg/ml) or Fn (0.4 μg/ml or at the concentration indicated in each experiment) in PBS for 1 h at 37°C. After washing with PBS, the wells were blocked with 1% bovine serum albumin (BSA) (RIA grade, Sigma) in PBS for 1 h at 37°C. Wells coated with 1% BSA were used to determine background adhesion. Cells were detached with 0.5 g/l trypsin and 0.5 mM EDTA (Irvine Scientific, Santa Ana, CA), rinsed in PBS, resuspended in DMEM (BioWhittaker) at a concentration of 1×10^6 cells/ml, and incubated with mAbs, inhibitors, or media alone for 15 min at 4°C. Then, 100 μl of cell suspension (1×10^5 cells) were added to the wells in triplicate. The plates were incubated at 37°C for 30 min. After washing with PBS, the residual adherent cells were checked by visual inspection and quantified with a colorimetric reaction using endogenous cellular acid phosphatase activity. The numerical value of the color density was obtained in an enzyme-linked immunosorbent assay plate reader (Molecular Devices, Menlo Park, CA) with a 405-nm filter. Background values, determined in wells coated with 1% BSA alone, were subtracted from each point. Ad-

herence was expressed as percentage of adhesion or inhibition of the number of cells originally added to each well.

Radioligand-binding Assay

The binding of ^{125}I-labeled ligands (Fn, antibodies) to platelets and CHO cells was assayed as described previously (Ginsberg *et al.*, 1983). The standard buffer used in the assay was modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 1 mg/ml BSA, 1 mg/ml glucose, 2 mM MgCl2, pH 7.4). Briefly, binding assays were performed in a 1.5-ml microcentrifuge tube (Sarstedt, Germany) in a final volume of 200 μl composed of 120 μl of cells (2×10^6/tube), 40 μl of radiolabeled protein, and 40 μl of inhibitors (2 μM Ro43-5054 or Ro44-9883), antibody (PB1(100 nM in Fn-binding assays), or D57 or PB1 (1 μM in antibody-binding assays). After a 30-min incubation at room temperature, 50-μl aliquots were layered in triplicate onto 300 μl of 20% sucrose in microfuge tubes (West Coast Scientific, Hayward, CA) and centrifuged for 3 min at 12,000 rpm. The tips were cut off and counted in a gamma scintillation spectrometer (Searle, Chicago, IL). In some experiments, cells were preincubated for 30 min at room temperature with different protein kinase inhibitors. The specific binding of Fn was obtained by subtracting at each point the Fn-binding value in the presence of 100 nM anti-$\alpha_5\beta_1$ antibody, PB1, which was taken as nonspecific binding. Nonspecific binding of ^{125}I-labeled antibody was estimated as the fraction bound in the presence of 100-fold molar excess of unlabeled antibody.

Immunofluorescence Staining

Immunofluorescence staining was done on 18-mm circular glass coverslips (Fisher Scientific, Pittsburgh, PA). The coverslips were coated overnight at 4°C with 20 μg/ml Fn in PBS, followed by blocking with 1% BSA for 1 h at 37°C. Cells were detached with trypsin-EDTA solution (Irvine Scientific, Irvine, CA), washed twice with the same medium without serum, and seeded on the coverslips in serum-free medium. After 90 min at 37°C, 2 μM Ro43-5054 or Ro44-9883 were added for 30 min. Cells attached to the coverslips were fixed with 3.7% formaldehyde (methanol free; Polysciences, Warrington, PA) for 10 min at room temperature and permeabilized with 0.2% Triton X-100 for 5 min at room temperature. The coverslips were incubated with 10% normal goat serum for 1 h at room temperature to block nonspecific binding.

For immunofluorescence double staining, coverslips were incubated for 30 min at 37°C with the primary antibodies in 10% normal goat serum, washed five times with PBS, and incubated for another 30 min at 37°C with a mix of FITC-conjugated goat anti-mouse $F(ab')_2$ (Tago) and RITC-conjugated goat anti-rabbit $F(ab')_2$ (Tago) in 10% normal goat serum. The coverslips were then washed and mounted in FITC-Guard mounting media (Testog, Chicago, IL). The slides were examined with a Leitz Orthoplan microscope with a 100× oil immersion objective, and photographs were taken on Kodax Tmax 400 film (Eastman Kodak, Rochester, NY).

DNA Fragmentation Assay

Cells (1×10^6 cells) were plated on tissue culture plastic dishes (Costar, Cambridge, MA) in DMEM in the presence or absence of 2 μM ligand (e.g., Ro43-5054). Additional ligand was added at 3-h intervals. After 24 h of incubation at 37°C, adherent cells were detached with a solution of trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml; Sigma Chemical) and were combined with nonadherent cells. Cells were rinsed once in PBS and pelleted for 5 min at 13,200 rpm and resuspended in 300 μl of buffer containing 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, and 0.5 mg/ml proteinase K. After overnight incubation at 37°C, DNA was ethanol precipitated, resuspended in 200 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) containing 50 μg/ml RNase A, and incubated at 37°C for 2 h. DNA was extracted with phenol-chloroform followed by a chloroform extraction and precipitated with ethanol. The DNA was resuspended in TE and quantified by absorbance at 260 nm. Equal amounts of DNA were end labeled with [α-^{32}P]ddATP (Amersham, Arlington Heights, IL) using terminal transferase (Boehringer Mannheim, Indianapolis, IN) as described (Tilly and Hsueh, 1993). Equal aliquots of DNA (50 ng) were electrophoresed on a 2% agarose gel, which was dried under vacuum and autoradiographed to visualize the DNA laddering. Autoradiographs were digitized using a Scanjet II scanner (Hewlett Packard, San Diego, CA) and quantified utilizing the National Institutes of Health Image program running on a Macintosh Power PC 6100.

RESULTS

Trans-dominant Suppression of Integrin Function by an Integrin-specific Ligand

To determine whether ligands specific for one (suppressive) integrin might block the functions of another (target) integrin, we utilized recombinant unactivated (O'Toole *et al.*, 1990) human $\alpha_{IIb}\beta_3$ as the suppressive integrin in CHO cells and used endogenous hamster $\alpha_5\beta_1$ as the target integrin. Because the recombinant $\alpha_{IIb}\beta_3$ in these cells is not activated (O'Toole *et al.*, 1990), cell adhesion to Fn is mediated solely by endogenous $\alpha_5\beta_1$ (Kieffer *et al.*, 1991). Thus, anti-$\alpha_5\beta_1$(PB1) but not anti-$\alpha_{IIb}\beta_3$ (2G12) antibodies blocked adhesion of these cells to Fn (Figure 1A). To test for a potential trans-dominant effect, we added an $\alpha_{IIb}\beta_3$-specific ligand, Ro43-5054. This compound suppressed the adhesion of the $\alpha_{IIb}\beta_3$-transfected CHO cells to Fn (Figure 1A). Inhibition was not due to direct blockade of the ligand-binding site of $\alpha_5\beta_1$, since the inhibitor had no effect on the adhesion of untransfected CHO cells (Figure 1A). The inhibitory effect was proportional to the quantity of transfected $\alpha_{IIb}\beta_3$ (Figure 1B). Thus, in cells expressing $\alpha_{IIb}\beta_3$, an $\alpha_{IIb}\beta_3$-specific inhibitor blocked $\alpha_5\beta_1$-mediated cell adhesion.

The foregoing studies established that an $\alpha_{IIb}\beta_3$-specific ligand could suppress the adhesive function of $\alpha_5\beta_1$. To determine whether the function of other target integrins could also be suppressed, we cotransfected CHO cells with human α_{IIb}, β_3, β_1, and α_2. Transfection with $\alpha_2\beta_1$ rendered the cells competent to adhere to type I collagen; adhesion was inhibited by an α_2-specific (R2-8C8) but not $\alpha_{IIb}\beta_3$-specific (2G12) antibody. The $\alpha_{IIb}\beta_3$-specific inhibitor Ro43-5054 suppressed adhesion of the $\alpha_{IIb}\beta_3$-transfected cells to collagen (Figure 1C). In contrast, the inhibitor had no effect on cells transfected only with $\alpha_2\beta_1$. Thus, the binding of ligand to a suppressive integrin can inhibit the function of multiple target integrins.

Trans-dominant Inhibition Is Due to Disruption of Integrin Signaling

Integrin-dependent cell adhesion can be reduced by blocking ligand binding or by disrupting signaling processes that control integrin affinity (inside-out sig-

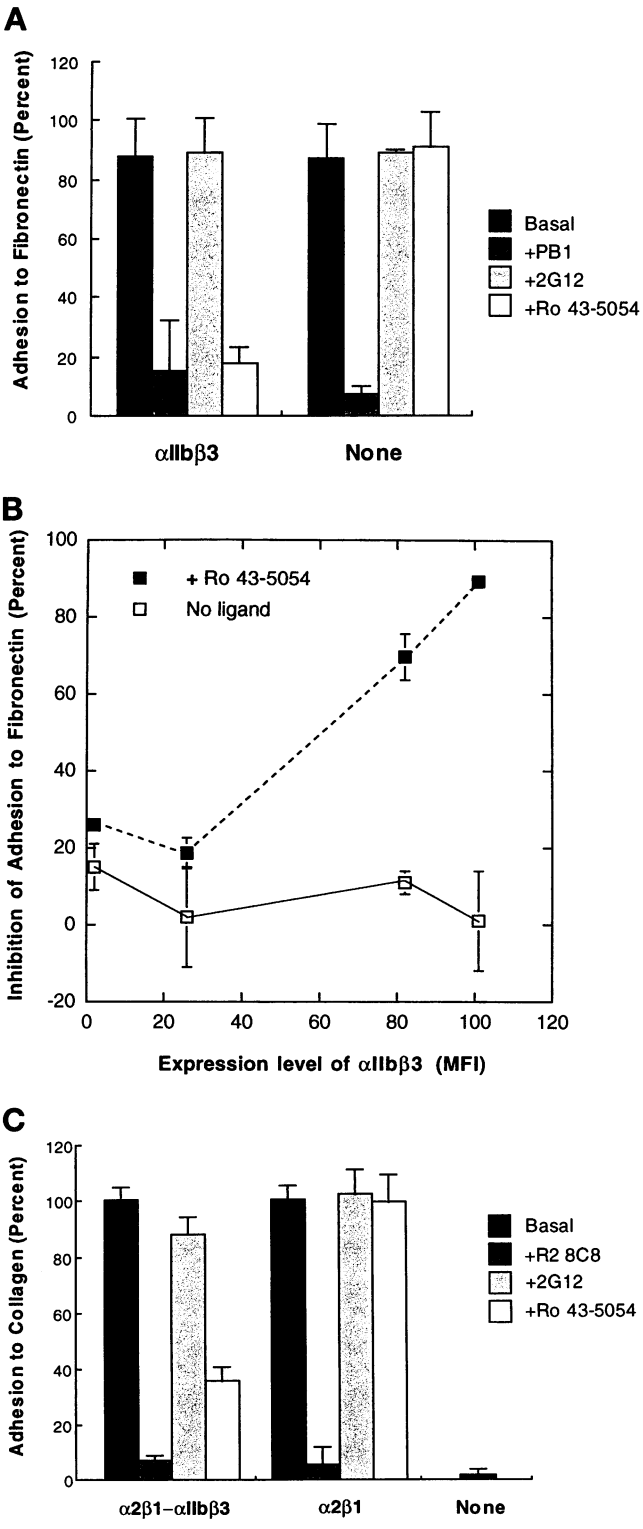


Figure 1. An $\alpha_{IIb}\beta_3$-specific ligand inhibits β_1 integrin-mediated cell adhesion. (A) $\alpha_{IIb}\beta_3$-bearing ($\alpha_{IIb}\beta_3$) or untransfected (None) CHO cells were permitted to attach to immobilized Fn in the absence (Basal) or presence of an inhibitory anti-hamster α_5(PB1) or $\alpha_{IIb}\beta_3$ (2G12) antibody (10 μg/ml) or 2 μM $\alpha_{IIb}\beta_3$-specific ligand (Ro43-5054). Cell adhesion was measured by endogenous acid phosphatase activity as described in MATERIALS AND METHODS and is expressed as the mean ± SE of three independent experiments in this and succeeding panels. (B) CHO cell lines expressing $\alpha_{IIb}\beta_3$ at different levels were plated on Fn in the presence or absence of saturating concentrations (2 μM) of Ro43-5054. $\alpha_{IIb}\beta_3$ expression was assessed by flow cytometry as described in MATERIALS AND METHODS, and cell adhesion was assessed as described in A. (C) Stable CHO cell lines expressing recombinant $\alpha_2\beta_1$ integrin and $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$, and no $\alpha_{IIb}\beta_3$, or no recombinant integrin (None) were plated on collagen (Basal). Adhesion was also assayed in the presence of 10 μg/ml inhibitory anti-α_2 antibody (R2-8C8), anti-$\alpha_{IIb}\beta_3$ antibody (2G12), or 2 μM $\alpha_{IIb}\beta_3$-specific ligand (Ro43-5054).

naling) or that mediate events that follow ligand binding (outside-in signaling). Ro43-5054 does not directly block $\alpha_5\beta_1$-dependent cell adhesion. Consequently, we tested the effect of binding of Ro43-5054 on signaling to integrin $\alpha_5\beta_1$.

In CHO cells, $\alpha_5\beta_1$ spontaneously binds Fn with high affinity through an energy-dependent process (O'Toole *et al.*, 1994). Ro43-5054 blocked the binding of soluble Fn to $\alpha_{IIb}\beta_3$-transfected CHO cells, whereas the compound had no effect in cells lacking $\alpha_{IIb}\beta_3$ (Figure 2A). The binding of Fn to the $\alpha_{IIb}\beta_3$-transfected CHO cells was inhibited by anti-$\alpha_5\beta_1$ antibody, but not by an $\alpha_{IIb}\beta_3$-specific (2G12) antibody (our unpublished results), confirming that in this cell type, the $\alpha_{IIb}\beta_3$ was not contributing to Fn binding. These cell expressed 1.57 (±0.03) × 10^6 molecules/cell of $\alpha_{IIb}\beta_3$ and 2.01 (±0.09) × 10^5 molecules/cell of $\alpha_5\beta_1$ as determined by direct binding of ^{125}I-labeled mAbs. Thus, the signaling required for maintenance of high-affinity binding of Fn to $\alpha_5\beta_1$ was disrupted by Ro43-5054.

$\alpha_5\beta_1$ in the low-affinity state can still mediate the adhesion of cells to Fn; conversion to high affinity reduces the surface density of Fn required for cell adhesion (Faull *et al.*, 1993). We examined the effect of the Fn-coating concentration on the trans-dominant inhibition of $\alpha_5\beta_1$-dependent adhesion. Increasing the Fn-coating concentration could overcome the inhibitory effect (Figure 2B) of the Ro43-5054. Because these cells adhere to a high-coating concentration of Fn in the presence of Ro43-5054, we were able to examine potential effects on cell spreading. Ro43-5054 blocked $\alpha_5\beta_1$-dependent spreading of $\alpha_{IIb}\beta_3$-transfected cells on Fn (Figure 3). In contrast, untransfected cells were unaffected by the presence of the ligand. Thus, in the presence of 2 μM Ro43-5054, 19 ± 4% of $\alpha_{IIb}\beta_3$-transfected cells were spread on Fn. In the absence of compound, 67 ± 13% of the cells were spread. Furthermore, in untransfected cells, 61 ± 7% and 57 ± 1% of the cells were spread in the presence and absence of compound, respectively. Consequently, the bind-

ing of Ro43-5054 to $\alpha_{IIb}\beta_3$ caused trans-dominant suppression of signaling to integrin $\alpha_5\beta_1$.

Trans-dominant Inhibition Correlates with a Conformational Change in the Extracellular Domain of the Suppressive Integrin

The foregoing experiment established that occupancy of $\alpha_{IIb}\beta_3$ can block signaling to other target integrins such as $\alpha_5\beta_1$. To determine whether conformational changes in the suppressive integrin were involved in trans-dominant inhibition, we assessed the effect of varying doses of Ro43-5054 on Fn binding to $\alpha_5\beta_1$. At the same time, ligand-induced changes in the conformation of $\alpha_{IIb}\beta_3$ were assayed by the binding of the occupancy-dependent antibody anti-LIBS1. Suppression of Fn binding to $\alpha_5\beta_1$ and the conformational change in $\alpha_{IIb}\beta_3$ were precisely correlated (Figure 4A). Another $\alpha_{IIb}\beta_3$-specific inhibitor, Ro44-9883 (lamifiban), is reported not to change the conformation of $\alpha_{IIb}\beta_3$ on the surface of platelets (Steiner *et al.*, 1993). This compound was completely without effect on Fn binding to $\alpha_5\beta_1$ and failed to elicit anti-LIBS1 binding (Figure 4A). Nevertheless, Ro44-9883 was a potent inhibitor of ligand binding to $\alpha_{IIb}\beta_3$ as manifest by its capacity to inhibit $\alpha_{IIb}\beta_3$-dependent adhesion to fibrinogen (Figure 4B) or Fn binding to platelet $\alpha_{IIb}\beta_3$ (our unpublished results). The binding of the two compounds was mutually exclusive, since Ro44-9883 could reverse both anti-LIBS-1 binding and inhibition of Fn binding induced by Ro43-5054 (Figure 4C).

The foregoing experiment suggested the general principle that trans-dominant blockade of integrin function involves a conformational change in a suppressive integrin. To test further the relationship between conformational change and trans-dominant suppression, we compared the effects of different $\alpha_{IIb}\beta_3$-specific antibodies on this process. OPG2 is a ligand-mimetic antibody reported to induce a conformational change in $\alpha_{IIb}\beta_3$ (Tomiyama *et al.*, 1992a; Kunicki *et al.*, 1995). In contrast, 2G12, although it blocks ligand binding, is not ligand mimetic (Loftus *et al.*, 1990) and D57 does not influence binding function. OPG2 but not the other two antibodies block the adhesion of $\alpha_{IIb}\beta_3$-transfected CHO cells to Fn (Figure 4D). Thus, of the chemically distinct $\alpha_{IIb}\beta_3$ ligands, only ligands with the capacity to induce a conformational change in the suppressive integrin provoked trans-dominant suppression of integrin signaling.

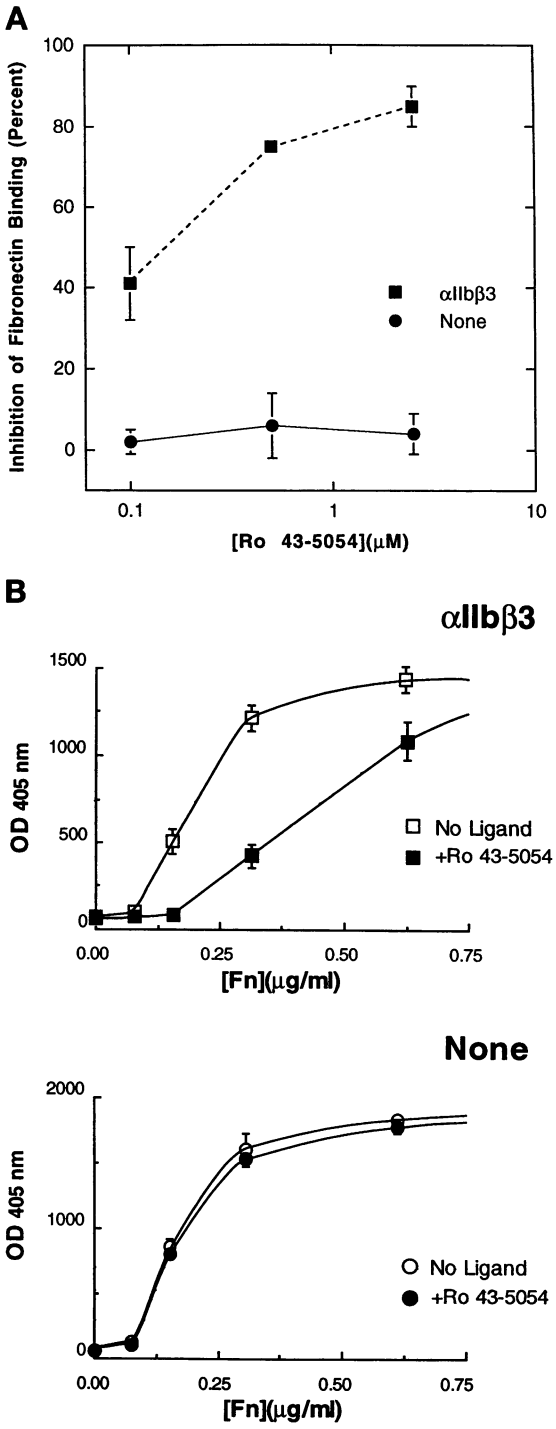


Figure 2. (A) An $\alpha_{IIb}\beta_3$-specific ligand inhibits the binding of soluble Fn to $\alpha_5\beta_1$. CHO cells (●, None) or $\alpha_{IIb}\beta_3$-transfected CHO cells (■) were incubated in the presence of increasing concentrations of Ro43-5054 and 100 nM ^{125}I-labeled Fn. Specific Fn binding was

(Figure 2 cont.) a 30-min incubation as described in MATERIALS AND METHODS. Data are presented as percentages of inhibition of the basal Fn binding and represent the means ± SE of three independent experiments. (B) CHO cells (None) or $\alpha_{IIb}\beta_3$-transfected CHO cells ($\alpha_{IIb}\beta_3$) were plated on wells coated with the indicated concentration of Fn in the presence (+Ro43-5054) or absence (No Ligand) of 2 μM Ro43-5054. Adhesion was measured as described in MATERIALS AND METHODS. Data are expressed as means ± SE of triplicate measures of optical density at 405 nm.

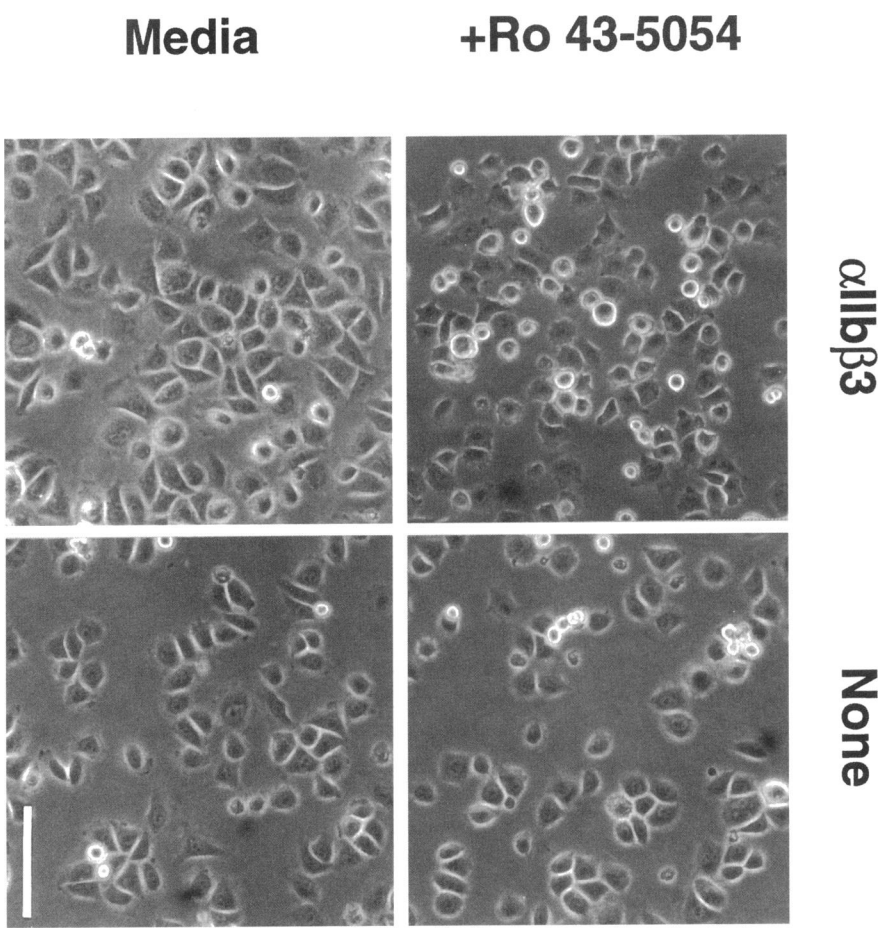


Figure 3. An $\alpha_{IIb}\beta_3$-specific ligand blocks $\alpha_5\beta_1$-dependent cell spreading. CHO cells (None) or $\alpha_{IIb}\beta_3$-transfected CHO cells ($\alpha_{IIb}\beta_3$) were plated on wells coated with 0.4 μg/ml Fn in the presence (+Ro43-5054) or absence (Media) of 2 μM Ro43-5054. After incubation for 30 min at 37°C, cells were fixed and visualized using phase-contrast microscopy. Spread cells, defined as those that were flattened with regular margins (Ylanne *et al.*, 1993), were enumerated. Bar, 100 μm.

Trans-dominant Inhibition Is Associated with a Change in the Cytoplasmic Face of the Suppressive Integrin

The foregoing studies established that a conformational change of the extracellular domain of the suppressive integrin is involved in inhibition. To determine whether this change was also associated with an intracellular change in the suppressive integrin, we examined the effect of $\alpha_{IIb}\beta_3$ ligands on the recruitment of $\alpha_{IIb}\beta_3$ to focal adhesions. This process is known to require intracellular interactions of the β_3 cytoplasmic domain (Ylanne *et al.*, 1993). CHO cells transfected with $\alpha_{IIb}\beta_3$ adhere to Fn and form $\alpha_5\beta_1$-containing focal adhesions; however, the $\alpha_{IIb}\beta_3$ remains diffusely distributed (Ylanne *et al.*, 1993). Ro43-5054 caused $\alpha_{IIb}\beta_3$ to be localized to these focal adhesions. In sharp contrast, Ro44-9883 lacked this effect (Figure 5). Thus, this transmembrane effect of integrin occupancy was correlated with the capacity of a ligand to induce a conformational change in the integrin.

The β cytoplasmic domain is necessary and sufficient for integrin localization to focal adhesions (LaFlamme *et al.*, 1992; Salomon *et al.*, 1992; Ylanne *et al.*, 1993). To test the role of the β cytoplasmic domain in trans-dominant suppression, we examined the capacity of $\alpha_{IIb}\beta_3$ bearing a truncated β_3 cytoplasmic domain to act as a suppressive integrin. In cells expressing comparable quantities of either $\alpha_{IIb}\beta_3$ or the truncated $\alpha_{IIb}\beta_3\Delta728$ variant (Figure 6C), addition of Ro43-5054 resulted in trans-dominant suppression of Fn binding (Figure 6A) and adhesion to Fn (our unpublished results) only in cells transfected with intact wild-type $\alpha_{IIb}\beta_3$. The ligand produced a comparable conformational change in the extracellular domain of both integrins (Figure 6B). Thus, the trans-dominant effect depends on the cytoplasmic domain of the β subunit of the suppressive integrin.

Other workers report that certain protein kinase inhibitors reverse suppressive effects of anti-$\alpha_v\beta_3$ antibodies on phagocytosis (Blystone *et al.*, 1994, 1995). Consequently, we considered the possibility

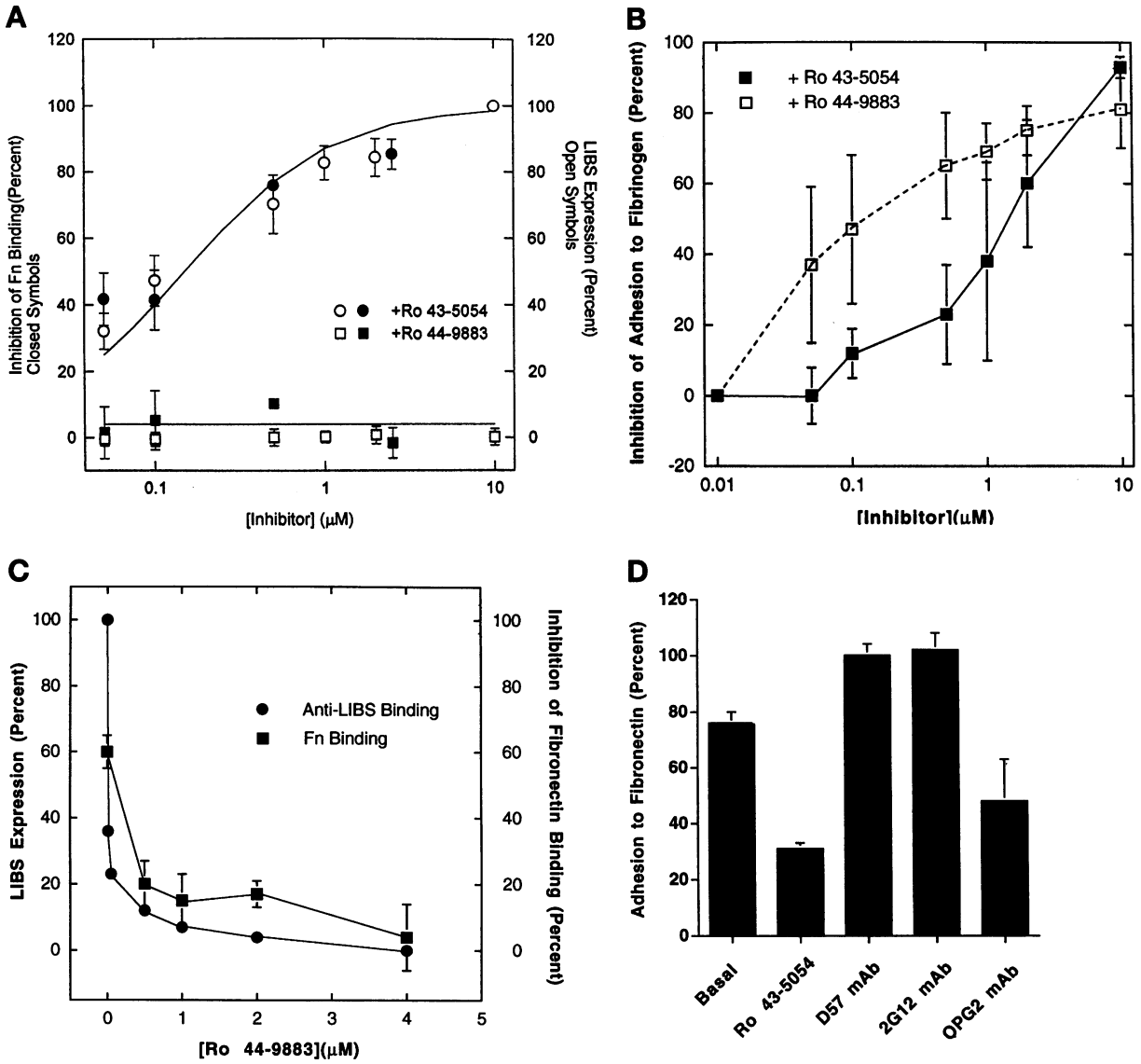


Figure 4. Trans-dominant inhibition correlates with a conformational change in the extracellular domain of the suppressive integrin. (A) Correlation between conformational change in $\alpha_{IIb}\beta_3$ and suppression of Fn binding to $\alpha_5\beta_1$. Stable $\alpha_{IIb}\beta_3$-bearing CHO cells were incubated at room temperature for 30 min in the presence of the indicated concentrations of Ro43-5054 (Ro43, ○, ●) or Ro44-9883 (Ro44, □, ■). The change in the conformation of $\alpha_{IIb}\beta_3$ was assessed by anti-LIBS1 binding (○, □) as described in MATERIALS AND METHODS. In a separate incubation, inhibition of the binding of 100 nM soluble ^{125}I-labeled Fn (●, ■) was measured in parallel. Depicted here and in B and C are the means ± SE of triplicate determinations. (B) Effect of Ro43-5054 (■) and Ro44-9883 (□) on cell adhesion to immobilized fibrinogen. $\alpha_{IIb}\beta_3$-transfected CHO cells were plated on fibrinogen-coated (10 μg/ml) wells in the presence of the indicated ligand concentration, and adhesion was measured as described in MATERIALS AND METHODS. Adhesion was $\alpha_{IIb}\beta_3$ dependent since it was completely inhibited by mAb 2G12 (10 μg/ml). (C) Mutually exclusive binding of Ro43-5054 and Ro44-9883. $\alpha_{IIb}\beta_3$-transfected CHO cells were incubated in the presence of 100 nM Ro43-5054 and the indicated concentration of Ro44-9883 for 30 min. Anti-LIBS1 binding (●) and inhibition of soluble ^{125}I-labeled Fn binding (■) were assessed as described in A. (D) Trans-dominant suppression of $\alpha_5\beta_1$-mediated adhesion by certain anti-$\alpha_{IIb}\beta_3$ antibodies. $\alpha_{IIb}\beta_3$-transfected CHO cells were plated on Fn-coated wells in the presence or absence (Basal) of the indicated $\alpha_{IIb}\beta_3$ ligands. The mAbs were at saturating concentrations as assessed by flow cytometry, the Ro43-5054 was at 2 μM. Adhesion was assessed as described in MATERIALS AND METHODS and are expressed as the mean ± SD of three independent experiments. Inhibition by OPG2 was statistically significant as judged by a two-tailed t test ($p < 0.02$).

that the trans-dominant inhibitory effect observed here was kinase dependent. The Ro43-5054 inhibition of Fn binding to $\alpha_{IIb}\beta_3$-transfected CHO cells was not interrupted by protein kinase inhibitors H7 (20 μM), staurosporin (100 nM), and calphostin C (2 μM), even though each inhibitor was active as

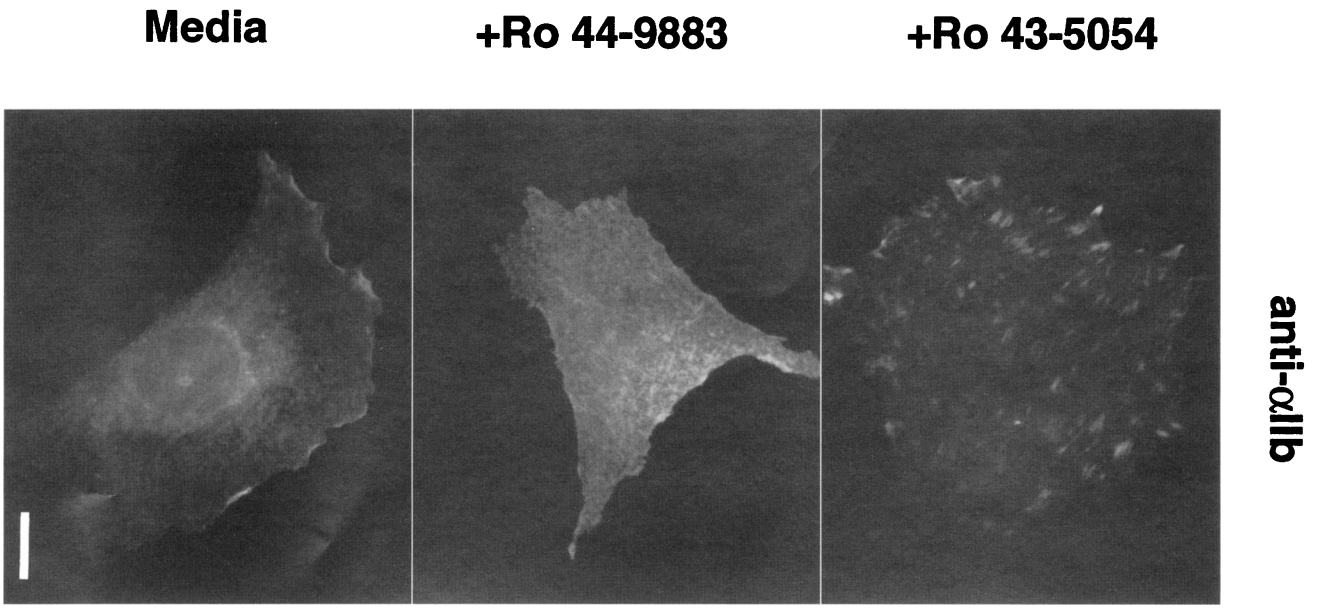


Figure 5. Trans-dominant inhibition is associated with a change in the cytoplasmic face of the suppressive integrin. $\alpha_{IIb}\beta_3$-transfected CHO cells were permitted to adhere to Fn substratum at 37°C for 90 min. Subsequently, 2 μM Ro43-5054, Ro44-9883, or control media were added and the incubation was continued for an additional 30 min. Cells were then fixed and stained with an anti-α_{IIb} polyclonal (Ab 2308) antibody as described in MATERIALS AND METHODS. Addition of Ro43-5054 but not Ro44-9883 provoked redistribution of $\alpha_{IIb}\beta_3$ to focal adhesions. In double staining experiments, the same focal adhesions also contained endogenous hamster $\alpha_5\beta_1$ (Ylanne *et al.*, 1993 and our unpublished results). Bar, 10 μm.

judged by reduction of cell spreading on Fn (our unpublished results).

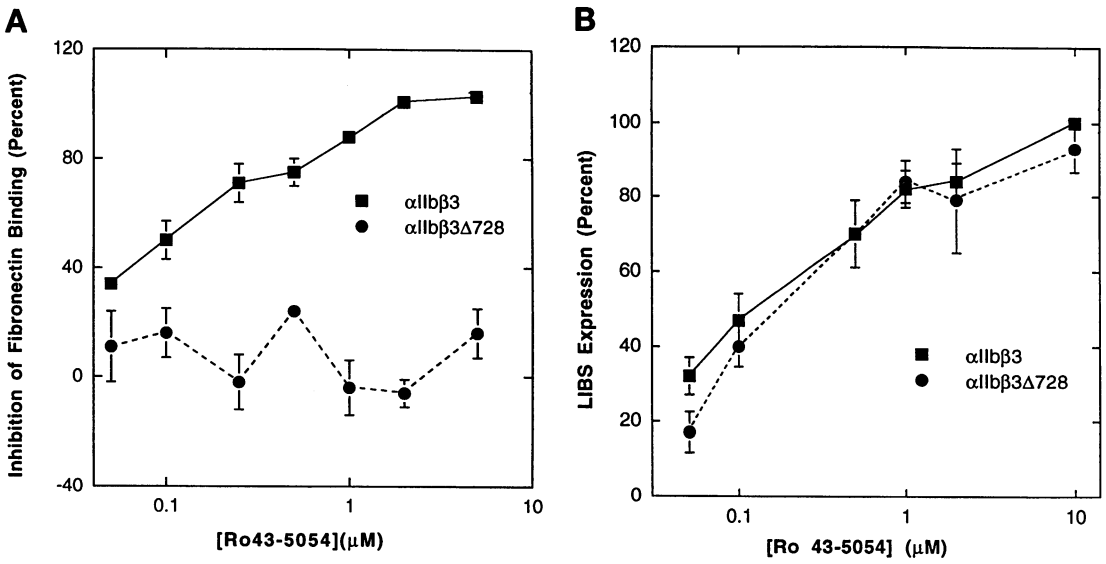
Induction of Apoptosis by Trans-dominant Suppression of Integrin Signaling

Signals to and from integrins not only control integrin function, but also regulate a variety of biological processes including programmed cell death as manifested by apoptosis (Meredith *et al.*, 1993; Frisch and Francis, 1994). To test the effect of trans-dominant integrin suppression on apoptosis, CHO cells were grown in low serum, conditions under which their survival becomes adhesion dependent (Zhang *et al.*, 1995). Under these conditions, Ro43-5054 caused $\alpha_{IIb}\beta_3$-bearing cells to develop irregular shrunken profiles (Figure 7A). Moreover, those cells exhibited DNA laddering diagnostic of internucleosomal cleavage of DNA and apoptosis (Figure 7B). In sharp contrast, untransfected CHO cells treated with Ro43-5054 did not manifest these changes (Figure 7). Thus, trans-dominant suppression of integrin signaling can result in induction of programmed cell death as assessed by these two criteria.

DISCUSSION

Our major findings are as follows: 1) binding of integrin-specific ligands to a suppressive integrin can inhibit the function of other target integrins in the same cell (trans-dominant inhibition); 2) trans-dominant inhibition is due to a blockade of integrin signaling; and 3) this process involves both a conformational change in the extracellular domain and the presence of the β cytoplasmic tail of the suppressive integrin. These findings imply that the ligand-induced conformational changes in an integrin can propagate from the extracellular to the intracellular face of the receptor. Trans-dominant inhibition by integrin ligands could spatially and temporally coordinate integrin signaling and can lead to unexpected biological effects of integrin-specific inhibitors.

Ligation of one integrin can modify the function of other integrins in the same cell. Ro43-5054 (Alig *et al.*, 1992) is $\alpha_{IIb}\beta_3$ specific, yet it suppressed the function of both $\alpha_2\beta_1$ and $\alpha_5\beta_1$. The suppressive effect was not observed in the absence of $\alpha_{IIb}\beta_3$ expression in the same cell. Consequently, inhibition was not due to direct inhibition of ligand binding to $\alpha_5\beta_1$ or $\alpha_2\beta_1$. Furthermore, the surface expression of $\alpha_5\beta_1$ and $\alpha_2\beta_1$ did not change upon Ro43-5054 treatment. The possibility that $\alpha_{IIb}\beta_3$ was responsible for some of the adhesion to collagen or Fn and that Ro43-5054 directly blocked this function can be excluded because of the following: 1) Adhesion could be completely blocked by anti-$\alpha_5\beta_1$ or anti-$\alpha_2\beta_1$ antibodies. 2) An $\alpha_{IIb}\beta_3$-specific inhibitor (Ro44-9883) was without effect on



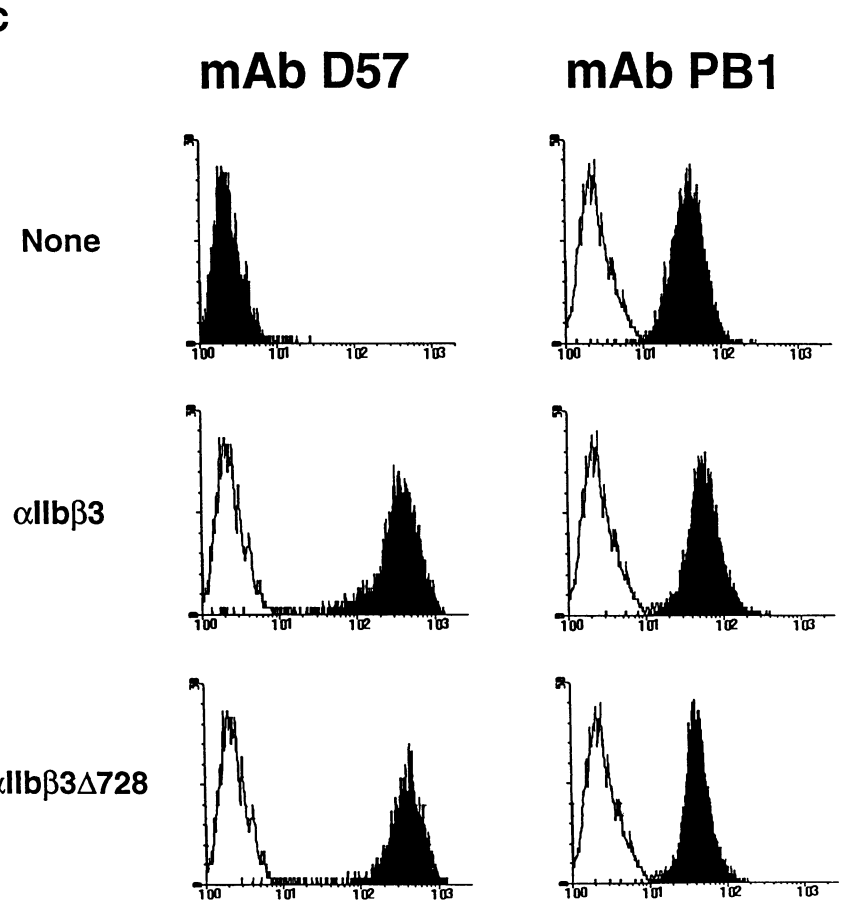


Figure 6. The β cytoplasmic domain of the suppressive integrin is required for trans-dominant inhibition. (A) Inhibition of soluble Fn binding to $\alpha_5\beta_1$. CHO cell lines expressing a β subunit truncation mutant (●, $\alpha_{IIb}\beta_3\Delta728$) or wild-type (■) $\alpha_{IIb}\beta_3$ were incubated for 20 min in the presence of 100 nM ^{125}I-labeled Fn and the indicated concentration of Ro43-5054. Inhibition of Fn binding was calculated as described in MATERIALS AND METHODS and expressed as the mean ± SE of three independent determinations. (B) $\alpha_{IIb}\beta_3$ ligands alter the conformation of a β cytoplasmic domain truncation mutant. CHO cells expressing the truncation mutant $\alpha_{IIb}\beta_3\Delta728$ (●) or wild-type $\alpha_{IIb}\beta_3$ (▲) were incubated for 30 min in the presence of the indicated concentration of Ro43-5054 and 100 nM ^{125}I-labeled anti-LIBS1. Anti-LIBS1 binding was assessed as described in MATERIALS AND METHODS and is expressed as the mean ± SE of triplicated determinations. (C) Integrin expression in cell lines. The expression of hamster $\alpha_5\beta_1$ (PB1) and human $\alpha_{IIb}\beta_3$ (D57) were estimated by flow cytometry as described in MATERIALS AND METHODS. Note the similar expression levels of endogenous $\alpha_5\beta_1$ in untransfected (None) as well as wild-type ($\alpha_{IIb}\beta_3$) and truncation mutant ($\alpha_{IIb}\beta_3\Delta728$) transfected CHO cells. Ro43-5054 (2 μM) had no effect on expression levels of either $\alpha_5\beta_1$ or $\alpha_{IIb}\beta_3$ (our unpublished results).

adhesion. 3) Ro43-5054 also blocked soluble Fn binding to $\alpha_{IIb}\beta_3$-transfected CHO cells. Soluble Fn binding was not inhibited by Ro44-9883 nor 2G12, reagents that block Fn binding to $\alpha_{IIb}\beta_3$. Consequently, the occupancy of one integrin can suppress the functions of other integrins in the same cell.

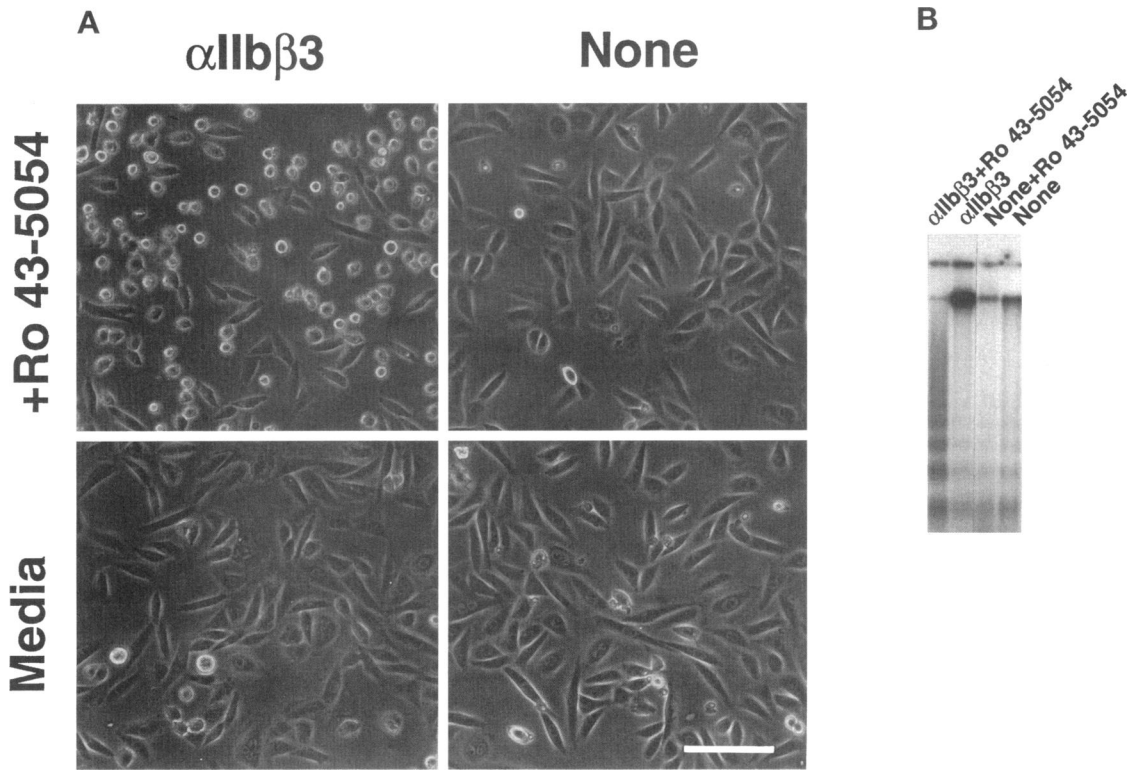


Figure 7. Induction of apoptosis by trans-dominant suppression of integrin signaling. $\alpha_{IIb}\beta_3$-transfected CHO cells or untransfected (None) cells were grown in medium containing 0.25% fetal calf serum in the presence or absence of 2 μM Ro43-5054 for 24 h. (A) Cell morphology was assessed using phase-contrast microscopy. (B) DNA fragmentation. The total DNA was isolated, ^{32}P end labeled, and analyzed by agarose gel electrophoresis as described in MATERIALS AND METHODS. Addition of Ro43-5054 to the transfected cells resulted in a reduction in radioactivity in high-molecular-weight DNA from 73% to 7% of the total present as assessed by scanning densitometry.

The trans-dominant inhibition reported here was due to disruption of integrin signaling. After attachment to Fn, CHO cells undergo active, $\alpha_5\beta_1$-dependent cell spreading; binding of Ro43-5054 to $\alpha_{IIb}\beta_3$ disrupted this spreading reaction. Furthermore, high-affinity binding of soluble Fn to CHO cells requires energy-dependent activation of $\alpha_5\beta_1$, i.e., inside-out signaling (O'Toole *et al.*, 1994). Binding of Ro43-5054 to $\alpha_{IIb}\beta_3$ blocked soluble Fn binding to $\alpha_5\beta_1$ also. In both cases, inhibition required the presence of the cytoplasmic domain of the β_3 subunit of the suppressive integrin. Suppression of integrin signaling could account for the inhibitory effect of $\alpha_4\beta_1$ ligation on $\alpha_5\beta_1$-dependent metalloproteinase gene expression (Huhtala *et al.*, 1995) or for the capacity of LFA-1 to dominate VLA-4 in T cell adhesion to endothelium (van Kooyk *et al.*, 1993). In addition, ligation of β_3 integrins can enhance or suppress phagocytosis. In K562 cells, ligation of $\alpha_v\beta_3$ blocks $\alpha_5\beta_1$-mediated phagocytosis, a process that depends on the affinity state of $\alpha_5\beta_1$ (Blystone *et al.*, 1994). In contrast to the present work, there were no reported effects of $\alpha_v\beta_3$ ligation on soluble Fn binding to K562 cells (Blystone *et al.*, 1994). Since phagocytosis is a complex cellular response requiring coordinated adhesion, deadhesion, and assembly of cytoskeletal proteins, it is possible that the inhibitory action of anti-$\alpha_v\beta_3$ on K562 cell phagocytosis may have been due to downstream effects. Indeed, the complexity of phagocytosis is underscored by the promotion rather than inhibition of β_2-dependent phagocytosis by ligation of a β_3-related integrin in neutrophils (Gresham *et al.*, 1989). Because direct measures of integrin function were inhibited here, we conclude that occupancy of the suppressive integrin ($\alpha_{IIb}\beta_3$) induces inhibition of signaling to a target integrin ($\alpha_5\beta_1$).

Occupancy initiates transmembrane signals by changing the conformation of the integrin extracellular domain. The trans-dominant inhibitory effects of Ro43-5054 directly correlated with its capacity to alter the conformation of $\alpha_{IIb}\beta_3$. Furthermore, another $\alpha_{IIb}\beta_3$-specific inhibitor, Ro44-9883, did not induce a detectable conformational change and did not cause trans-dominant inhibition. In addition, a ligand-mimetic anti-$\alpha_{IIb}\beta_3$ antibody but not another anti-$\alpha_{IIb}\beta_3$ antibody also provoked trans-dominant inhibition. It is possible that the capacity of function-blocking anti-$\alpha_v\beta_3$ antibodies to block $\alpha_5\beta_1$-dependent phagocytosis

may also require a conformational change in $\alpha_v\beta_3$. Furthermore, integrin occupancy can promote recruitment of integrins to focal adhesions (LaFlamme *et al.*, 1992) and local accumulation of cytoskeletal proteins such as α actinin and talin at sites of integrin clustering (Miyamoto *et al.*, 1995). The present work suggests that altered conformation of the extracellular domain may be involved in these effects of integrin occupancy.

Conformational changes in the integrin extracellular domain are transmitted to the cytoplasmic domain. In the present work, β cytoplasmic domain-dependent activities, such as trans-dominant inhibition and focal adhesion recruitment, were provoked by binding of ligands that alter the conformation of the extracellular domain. Both of these activities are spontaneously exhibited by isolated integrin β cytoplasmic domains (LaFlamme *et al.*, 1992; Chen *et al.*, 1994; LaFlamme *et al.*, 1994; Lukashev *et al.*, 1994; Blystone *et al.*, 1995), suggesting that the α cytoplasmic domain might mask these activities. In fact, deletion of the α cytoplasmic domain causes spontaneous integrin recruitment to focal adhesions (Briesewitz *et al.*, 1993; Ylanne *et al.*, 1993). A change in conformation of the extracellular domain must traverse the conserved membrane proximal cytoplasmic domain to propagate to the cell interior (Williams *et al.*, 1994). Alterations in the conserved sequences in this region, which may alter interactions between the α and β subunits (Briesewitz *et al.*, 1995; Hughes *et al.*, 1996), can either block (Briesewitz *et al.*, 1995) or promote (Hughes *et al.*, 1996) intracellular integrin signals or increase the affinity of the extracellular domain (Hughes *et al.*, 1995). Thus, a quaternary structural change could propagate through the integrin hinge to uncover the β cytoplasmic domain. The unmasked β cytoplasmic domain could then compete with endogenous integrins for limiting cytoplasmic factors involved in integrin signaling. In addition, the conformational change could promote integrin clustering; such clustering could also be involved in trans-dominant inhibition. Furthermore, Blystone *et al.* (1995) reported that isolated β_3 cytoplasmic domains may generate protein kinase-dependent signals that suppress phagocytosis. The failure of the same inhibitors used by Blystone *et al.* (1995) to reverse trans-dominant inhibition in our system neither supports nor precludes this mechanism. In either case, the present results implicate transmembrane allosteric rearrangements as a component of outside-in integrin signaling.

Trans-dominant inhibition of integrin functions may serve to regulate integrin-dependent cellular activities. The present experiments employed a suppressive integrin ($\alpha_{IIb}\beta_3$) present at a 7.9-fold excess relative to a target integrin ($\alpha_5\beta_1$). This excess is less than the >10:1 natural ratio of $\alpha_{IIb}\beta_3$:β_1 integrins in platelets (Kunicki *et al.*, 1993). Furthermore, endothelial cells assemble a Fn matrix only on their abluminal surfaces, even though they express potential matrix assembly competent integrins on both surfaces (Conforti *et al.*, 1992). Fn matrix assembly requires integrin activation via inside-out signaling in addition to cytoskeletal interactions (Wu *et al.*, 1995). Thus, the polarization of the Fn matrix in this cell may be due to trans-dominant inhibition of the activation of luminal integrins by occupied abluminal integrins. Similarly, cell migration involves both adhesion and deadhesion mediated by integrins (Lauffenburger, 1991). Inhibitory signals emanating from occupied integrins may be involved in coordinating these events.

Soluble integrin-specific ligands can block the function of a specific integrin; the present work suggests that such ligands may also cause trans-dominant suppression of the function of other integrins when three circumstances apply: 1) The ligand induces a conformational change in the suppressive integrin. 2) The suppressive integrin possesses an appropriate β cytoplasmic domain. 3) The suppressive integrin is abundant. When these conditions are met, trans-dominant inhibition could have unexpected consequences. For example, signals from integrins serve as a survival factor to prevent programmed cell death (Meredith *et al.*, 1993; Frisch and Francis, 1994). In particular, $\alpha_5\beta_1$ may provide survival signals in CHO cells (Zhang *et al.*, 1995). As illustrated here, trans-dominant inhibition of $\alpha_5\beta_1$ function with an $\alpha_{IIb}\beta_3$-specific inhibitor caused loss of $\alpha_5\beta_1$-dependent attachment. At the same time, the occupied $\alpha_{IIb}\beta_3$ failed to prevent apoptosis. The same phenomenon could explain the capacity of anti-$\alpha_v\beta_3$ antibodies to promote apoptosis of angiogenic endothelial cells (Brooks *et al.*, 1995), cells that bear multiple other integrins. Furthermore, angiogenic endothelial cells are enriched in $\alpha_v\beta_3$ (Brooks *et al.*, 1994), which may account for their peculiar susceptibility to this apoptotic signal. In general, the principles enumerated above should permit prediction of specific situations in which trans-dominant inhibition might occur with resultant nonspecific blockade of integrin functions. These principles may have a bearing on the design of therapeutic integrin inhibitors.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Drs. Eric Brown, Joe Ramos, Martin Schwartz, and Sanford Shattil for critical review of the manuscript. We also thank our colleagues for the generous gifts of reagents described in MATERIALS AND METHODS. Virginia M. Keivens provided excellent technical support and Ruby Larson and Mary Lawhead provided helpful secretarial assistance. This work was supported in part by grants HL-48728 and AR-27214 from the National Institutes of Health and from Thrombosys. Dr. Díaz-González was supported by a fellowship from Ministerio de Educación y Ciencia of SPAIN 95/42063593. This is publication number 9831-VB from The Scripps Research Institute.

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