Integrin Cytoplasmic Domains Mediate Inside-Out Signal Transduction

Timothy E. O'Toole, Yasuhiro Katagiri, Randall J. Faull, Karlheinz Peter, Richard Tamura,† Vito Quaranta,† Joseph C. Loftus, Sanford J. Shattil,§ and Mark H. Ginsberg

* Departments of Vascular Biology and † Cell Biology, Scripps Research Institute, La Jolla, California 92037; and § University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract. We analyzed the binding of fibronectin to integrin α5β1 in various cells; in some cells fibronectin bound with low affinity (e.g., K562 cells) whereas in others (e.g., CHO), it bound with high affinity (Kd ~ 100 nM) in an energy-dependent manner. We constructed chimeras of the extracellular and transmembrane domains of α5β1 joined to the cytoplasmic domains of αβ3. The affinity state of these chimeras was assessed by binding of fibrinogen or the monoclonal antibody, PAC1. The cytoplasmic domains of αβ3 conferred an energy-dependent high affinity state on αβ3 in CHO but not K562 cells. Three additional α cytoplasmic domains (α2, αA, αB) conferred PAC1 binding in CHO cells, while three others (αM, αL, αI) did not. In the high affinity α chimeras, cotransfection with a truncated (β3Δ724) or mutated (β3(S552P)) β3 subunit abolished high affinity binding. Thus, both cytoplasmic domains are required for energy-dependent, cell type-specific affinity modulation. In addition, mutations that disrupted a highly conserved α subunit GFFKR motif, resulted in high affinity binding of ligands to αβ3. In contrast to the chimeras, the high affinity state of these mutants was independent of cellular metabolism, cell type, and the bulk of the β subunit cytoplasmic domain. Thus, integrin cytoplasmic domains mediate inside-out signaling. Furthermore, the highly conserved GFFKR motif of the α subunit cytoplasmic domain maintains the default low affinity state.

Cells alter their adhesiveness in response to developmental events or environmental cues. These adaptations are often mediated through integrins, adhesion receptors composed of two transmembrane subunits, α and β (43). Rapid changes in integrin function are critical in cell migration, cellular aggregation, and leukocyte transmigration during inflammation (2, 24, 31, 36, 43, 75, 86). A given integrin may also manifest varying adhesive competence depending on its cellular environment (15, 25, 49, 62, 94), or the state of differentiation of the cell (1, 15, 35, 66). Such variations in function may be due to changes in ligand-binding affinity as occurs with certain β3 (7), β2 (4), and β1 (26) integrins. Changes in adhesive function may also occur without changes in ligand-binding affinity. For example, phorbol esters stimulate the αβ3-dependent adhesion of CHO cells (20) to fibronectin (Fn) with no change in Fn-binding affinity. Similarly, certain β3 mutations reduce αβ3-dependent stabilization of cell adhesion to fibrinogen (Fg) without changing intrinsic Fg-binding affinity (97). Such affinity-independent changes in integrin function are ascribed to "post receptor events" (20). Nevertheless, the host cell governs the capacity of solubilized αβ3 to bind to immobilized ligands (49). This last result suggests that some cell type-specific differences in integrin function may be due to differences in ligand-binding affinity.

αβ3 (platelet GPIb-IIIa) is a prototype integrin for analysis of changes in integrin affinity. As with all integrins, αβ3β3 is a heterodimer of two Type I transmembrane protein subunits (43). αβ3β3 is platelet-specific (88), and affinity state-specific antibodies, e.g., PAC1 (81), simplify analysis of recombinant αβ3β3 in heterologous cells (67). Conformational changes in the extracellular domain of αβ3β3 regulate its affinity (67, 82). Platelet agonists increase the affinity of αβ3β3 ("activation") via cytoplasmic signaling pathways. These pathways include heterotrimeric GTP-binding proteins, phospholipid metabolism, and serine-threonine kinases and may also involve calcium fluxes, tyrosine kinases.

1. Abbreviations used in this paper: Fg, fibrinogen; Fn, fibronectin.

© The Rockefeller University Press, 0021-9525/94/03/1047/13 $2.00
The Journal of Cell Biology, Volume 124, Number 6, March 1994 1047-1059 1047
and low molecular weight GTP-binding proteins (31, 32, 65, 79, 80, 82). How cytoplasmic signals result in changes in the conformation and ligand-binding affinity of the extracellular domain ("inside-out signal transduction") of the integrin remains obscure.

A variety of in vitro treatments may alter integrin affinity. When purified αmβ2 is pretreated with RGD peptides, it subsequently binds Fg and PAC1 (23, 50, 85). Certain anti-β2 antibodies directly increase the Fg-binding affinity of αmβ2 (29) and certain anti-β2 antibodies activate αmβ2 to bind Fn with high affinity (26). Changes in the divalent cation composition of the extracellular medium, proteolytic digestion, and treatment with reducing agents may also "activate" integrins (3, 30, 34, 48, 62, 94, 98). Thus, moieties that interact with the extracellular domain can modulate integrin affinity. Furthermore, lipid environment can alter an integrin's ligand-binding capacity (17, 85) and an apparently novel lipid, IMF-1, may regulate αmβ2 (37). Although many treatments may change integrin affinity in vitro, the mechanism(s) of physiological modulation has not been defined.

Integrin cytoplasmic domains may be targets of cytoplasmic signals that alter integrin affinity. The cytoplasmic tails are ~180 Å from the ligand-binding site (93), but integrins can undergo propagated long-range conformational changes in situ (22). Truncation of the αm cytoplasmic domain after residue 990 (αm991) results in αmβ2 that constitutively binds Fg and PAC1 (69). Conversely, truncations of α5 (45), α2 (46), and β1 (39) profoundly reduce the capacity of these integrins to mediate cell adhesion, possibly due to effects on ligand-binding affinity. Such truncations may result in misfolded receptors that lack function or that bind ligands with an inappropriately high affinity. Furthermore, a Ser752 to Asp mutation in the β2 cytoplasmic domain was associated with an apparent αmβ2 activation defect (16) in a single individual. Cytoplasmic domains of several integrins are phosphorylated coincidentally with increases in adhesive function. Nevertheless, detailed studies (38, 40) have so far failed to establish a role for these phosphorylations in increased affinity. Conversely, the rounding of cells during mitosis is associated with phosphorylation of β1, and reduced binding of αmβ2 to Fn (43). Thus, physiological activation signals may be transmitted through integrin cytoplasmic domains, but definitive proof is lacking.

In the present work, we tested the hypothesis that integrin cytoplasmic domains are directly involved in physiological affinity modulation. Using chimeras containing the cytoplasmic domains of various α and β subunits joined to the transmembrane and extracellular domain of αmβ2, we found that integrin cytoplasmic domains transduce cell type-specific signals that modulate ligand-binding affinity. These signals require active cellular processes and both α and β cytoplasmic tails of the integrin, suggesting that they represent physiologically relevant signals. In addition, deletion of a highly conserved GFFKR motif, at the NH2 terminus of the α subunit cytoplasmic domain, also resulted in high affinity binding of ligands to αmβ2. In contrast to the chimeras, high affinity ligand binding to GFFKR deletion mutants was independent of cellular metabolism, cell type, and the bulk of the β subunit cytoplasmic domain. Thus integrin cytoplasmic tails are targets for the modulation of integrin affinity.

Materials and Methods

Antibodies and Reagents

The anti-αmβ2 antibody D57 was produced by Dr. Xiaoping Du (Scripps Research Institute) using previously described methods (28). It binds to CHO cells transfected with αmβ2 but not αmβ3, and does not block Fg binding to αmβ3. This antibody was biotinylated with biotin-N-hydroxysuccinimide (Sigma Chem. Co., St. Louis, MO) according to manufacturers' directions. The αmβ3 complex specific antibody, 2G12 (71), was supplied by Dr. Virgil Woods (University of California, San Diego) and used as dilutions of ascites fluid. The anti-hamster α1 (PBI) and anti-β1 (TEL) antibodies were obtained from Dr. Rudolph Juliano (10) (University of North Carolina, Chapel Hill), and the β1 activating antibody, B2A2, was supplied by Drs. Nick Kovach and John Harlan (51) (University of Washington, Seattle). A human anti-α5 antibody, BI1-G2, was supplied by Dr. Caroline Dansmyk (95) (University of California, San Francisco) while a polyclonal anti-peptide antibody against the cytoplasmic domain of human α5 (44) was obtained from Drs. Gene Marcantonio and Richard Hynes (Massachusetts Institute of Technology, Boston). The isolation and characterization of other antibodies (anti-LIBS6, anti-LIBS2, anti-αmβ2 cytoplasmic domain [28, 69]) and PAC1 (81) have been described. Glucose and 2-deoxyglucose were purchased from Sigma and sodium azide was from Fisher Scientific Co. (Pittsburgh, PA). The peptide GRGDS was obtained from Peninsula Laboratories (Belmont, CA). Its purity and composition were verified by high performance liquid chromatography and fast atom bombardment mass spectroscopy.

Cell Culture and Transfection

The human cell lines K562, U937, WI38, and MG63 were obtained from the Amer. Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 media (Biowhittaker, Walkersville, MD) containing 10% FBS (Biowhittaker), 1% glutamine (Sigma), and 1% penicillin and streptomycin. THP-1 cells (Amer. Type Culture Collection) were maintained in the same medium with the addition of 10 mM Hepes and 20 mM 2-mercaptoethanol. CHO cells (Amer. Type Culture Collection) were maintained in DMEM media (Biowhittaker) with 10% FCS, the above noted antibiotics, and 1% non-essential amino acids (Sigma). Human T lymphocytes were purified from peripheral blood of normal donors by centrifugation on a Ficoll-Paque gradient (Pharmacia Fine Chemicals, Piscataway, NJ, passing for monocytes on serum-coated dishes, and passage over a nylon wool column.

CHO cells were transiently transfected by electroporation. Cells in log phase growth were harvested with trypsin (Irvine Scientific), washed by harvesting with 3.5 mM EDTA, incubating for 5 rain in 1 mg/ml TPCK treated trypsin (Worthington) and diluting with an equal volume of Tyrode's containing 5 × 105 cells were incubated in a final volume of 50 μl containing 10% FCS and 0.1% soybean trypsin inhibitor (Sigma). After washing, 5 × 105 cells were incubated in a final volume of 50 μl containing Fab fragments of specific antibodies as described (57, 68). Briefly, 5 × 105 cells were incubated on ice for 30 min with an FITC-conjugated goat anti-mouse (IgG, Biotrend, Cologne, Germany) second antibody. Cells were pelleted, resuspended, and analyzed on a FACScan (Becton Dickinson Immunocytometry Sys., Mountain View, CA). Stable K562 transfectants were established by electroporation of 105 cells in 0.8 ml of PBS at 300 V and 500 μF after 48 h, the cells were maintained in medium containing 1 mg/ml G418 (GIBCO BRL, Gaithersburg, MD) and clonal lines were established by single cell sorting in a FACStar (Becton Dickinson Immunocytometry Sys., Mountain View, CA). Stable K562 transfectants were established by electroporation of 105 cells in 0.8 ml of PBS at 300 V and 500 μF. After 48 h, the cells were maintained in medium containing 1 mg/ml G418, and clonal lines established by limiting dilution cloning.

Flow Cytometry

Surface expression of integrins was analyzed by flow cytometry with specific antibodies as described (57, 68). Briefly, 5 × 105 cells were incubated on ice for 30 min with primary antibody, washed, and then incubated on ice for 30 min with an FITC-conjugated goat anti-mouse (IgG, Biotrend, Cologne, Germany) second antibody. Cells were pelleted, resuspended, and analyzed on a FACScan (Becton Dickinson Immunocytometry Sys.). PAC1 binding was analyzed by two color flow cytometry. Cell staining was carried out in Tyrode's (33) buffer containing 2 mM MgCl2 and CaCl2 and 1 mg/ml BSA (Sigma) and dextrose. Single cell suspensions were obtained by harvesting with 3.5 mM EDTA, incubating for 5 min in 1 mg/ml TPCK trypsin (Worthington) and diluting with an equal volume of Tyrode's containing 10% FCS and 0.1% soybean trypsin inhibitor (Sigma). After washing, 5 × 105 cells were incubated in a final volume of 50 μl containing...
0.1% PAC1 ascites in the presence or absence of 1 mM GRGDSP peptide. After a 30-min incubation at room temperature, cells were washed with cold Tyrode’s solution and then incubated on ice with biotinylated antibody DS7. After 30 min, cells were washed and then incubated on ice with Tyrode’s containing 10% FITC-conjugated goat anti-mouse IgM (Tago) and 4% phycoerythrin-streptavidin (Molecular Probes Inc., Eugene, OR). Thirty minutes later cells were diluted to 0.5 ml with Tyrode’s solution and analyzed on a FACScan (Becton Dickinson) flow cytometer as described (67). PAC1 binding (FITC staining) was analyzed only on a gated subset of cells positive for αmβ2 expression (phycoerythrin staining). To define affinity state, histograms depicting PAC1 staining in the absence or presence of 1 mM GRGDSP were superimposed. Since RGD peptides are inhibitors of PAC1 binding to αmβ2 (8), a rightward shift in the histogram in the absence of peptide is indicative of the presence of high affinity αmβ2. To compare the effects of multiple α subunits, pooling of data involving experiments from different days, was required. To do this, a numerical activation index was defined as:

\[
100(F_o-F_a)/F_o \times \%
\]

where:

- \(F_o\) = Mean Fluorescence Intensity in the absence of inhibitor
- \(F_a\) = Mean Fluorescence Intensity in the presence of GRGDSP

Expression of αmβ2 in transiently transfected K562 cells was too low to permit affinity state analysis. Thus, all data reported with these cells is from stable lines. The truncation mutant, αmΔ991, like a similar αl truncation (39), was expressed at low levels in transient transfections, so that all data obtained with this mutant refer to stable cell lines. In contrast the αA728 variant was well expressed, so that data from both transient and stable experiments are reported. Stable cell lines were also prepared with the following cytoplasmic domain combinations αmβ3, αα3, αs/β2, αα3, and αs/β2A99 (TGTAAACAAGCTCTTCAC). Amplified products were digested with HindIII and XbaI and ligated into the HindIII site of CDMS. This construct was then digested with EcoRI and ligated with a 2.2-kb EcoRI fragment from CD3a (68) containing its transmembrane and extracellular domains. β2 cytoplasmic domains were isolated by the PCR from a β2 cDNA, and then subcloned into the Mful and Xhol sites of CDM8. The β2 cytoplasmic domain chimera was then generated by digestion with Mful and HindIII and ligation with a corresponding Mful and HindIII fragment from CD3a (68) containing its extracellular and transmembrane sequences. Chimeric α subunits were generated using a previously described strategy (69). Cytoplasmic sequences from αs, αl, αα3, αs/αA, and αs/β were isolated from the appropriate cDNA clones by PCR (57). Amplified products were digested with HindIII and Xhol and subcloned into HindIII and Xbal cut CDM8. After digestion with HindIII, these constructs were ligated with a HindIII fragment from CD2b (68) containing its extracellular and transmembrane domains. PCR oligonucleotides for αΔ were designed to omit the VGFK sequence. Its construction followed the procedure for other α chimeras. The αs variant was made by first generating a SalI site in CD2b coding sequences corresponding to bases 3061-3066. This vector was then digested with SalI and Xhol and ligated to a SalI-Xhol Bluescript vector sequence (bases 674-731). All constructs were verified by DNA sequencing and purified by CsCl centrifugation before transfection. Oligonucleotides were synthesized on a model 391 DNA Synthesizer (Applied Biosystems Inc., Foster City, CA).

### Ligand Binding

The binding of 125I-Fg or 125I-Fn to cultured cells was accomplished as described (26, 67). Cells were harvested with EDTA and trypsin as described above for flow cytometry and resuspended in a modified Tyrode’s buffer (150 mM NaCl, 2.5 mM KCl, 2 mM NaHCO3, 2 mM MgCl2, 2 mM CaCl2, 1 mg/ml BSA, and 1 mg/ml dextrose). A typical assay included 120 μl of cells (2 X 106 cells per tube), 40 μl of radiolabeled protein, and 40 μl of inhibitor (GRGDSP peptide, blocking antibodies) or agonist (activating antibody). After 30 min at room temperature, 50-μl aliquots were layered in triplicate on 0.3 ml of 20% sucrose and centrifuged for 3 min at 12,000 rpm. 125I-labeled protein associated with the cell pellet was determined by scintillation spectrometry. Nonsaturable binding was determined in the presence of 2 mM GRGDSP peptide. Data were fit to equilibrium binding models by the nonlinear least squares curve-fitting LIGAND program (64). In binding experiments using metabolic inhibitors, the cells were first incubated with 2 mg/ml 2-deoxyglucose and 0.1% sodium azide for 30 min at room temperature before addition of radiolabeled ligand. In washout experiments, cells treated in this way were washed, incubated with Tyrode’s containing 1 mg/ml dextrose for 30 min at room temperature, and then analyzed for ligand binding.

## Immunoprecipitation

Transfectants were surface labeled by the iodogen method according to the manufacturer’s instructions (Pierce Chem. Co., Rockford, IL) and solubilized in lysis buffer (10 mM Hepes (pH 7.5), 0.15 M NaCl, 50 mM octylglucoside, 1 mM CaCl2, 1 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 10 mM N-ethylmaleimide). Cell extracts were immunoprecipitated with polyclonal antisera directed against the αm or αs cytoplasmic domains and a monoclonal antibody against the αmβ2 complex (2G12). The antibodies were attached onto preswollen protein A-Sepharose beads (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) by incubation at 4°C overnight. The antibody-conjugated Sepharose beads were washed, pelleted by centrifugation, and then incubated with the detergent lysates from the surface labeled cells overnight with shaking. The Sepharose beads were washed extensively in lysis buffer, resuspended in sample buffer (53), and boiled for 5 min. After centrifugation, the precipitated protein was resolved by SDS-PAGE (non-reducing, 7.5% acrylamide gels). Gels were dried, and radiolabeled polypeptides were visualized by autoradiography.

## Polymerase Chain Reaction

Total RNA was isolated from 106 transfected cells using the RNAzol reagent (Cinna Biotech). First strand cDNA synthesis from 5 μg of RNA was performed with the CDNA cycle kit (Invitrogen) using oligo dT as a primer. Coding sequences downstream of the αm transmembrane region were specifically amplified with a 5′ primer specific for transmembrane αmΔ (2basf: CGGGCCGGAGAATTC) and 3′ primers specific for the cytoplasmic sequences of αm (αmβ2: CTCCTGTGGAGGGAAAA-CGA) and αs/αα3: TGGAAAAGGCTTCTCAC). Amplified products were analyzed by agarose gel electrophoresis.

## Results

### Cell Type-Specific and Energy-dependent Affinity Modulation of Integrin αmβ2

As noted above, there is evidence for cell type-specific control of the adhesive function of integrins. To begin to investigate the cell type-specific control of ligand-binding affinity, we first analyzed the binding of soluble Fn to cells expressing integrin αmβ2. The cells analyzed fell into two groups: those that bound Fn with only low affinity (Kd >1 mM), e.g., K562, THP1, U937, and peripheral blood T cells, and those that bound Fn with only low affinity (Kd >1 mM), e.g., K562, WI-38, and MG63 cells (Fig. 1 A). The low affinity αm/β2 was intrinsically functional since it bound Fn after activation with the 8A2 monoclonal antibody (26) (Fig. 1 A) and was expressed at comparable levels to high affinity αmβ2 (Fig. 1 B). Specificity of Fn binding to high affinity αmβ2 was verified by inhibition with an anti αm antibody (Fig. 1 A).

To find out whether spontaneous high affinity Fn binding
Figure 1. The high affinity state of \( \alpha_\beta_1 \) is cell type-specific and energy-dependent. (A) \( ^{125}\text{I}-\text{Fn} \) (50 nM) was incubated at 22°C with CHO or K562 cells. After 30 min, bound Fn was assessed by centrifugation through a sucrose cushion as described in Materials and Methods. \( \alpha_\beta_1 \)-specific binding was established by blocking binding to the CHO cells with PB1, an anti-hamster \( \alpha_\beta_1 \). Binding to K562 cells was induced by addition of 20 nM activating antibody (8A2) and was inhibited by the anti-\( \alpha_5 \) antibody (K562:BIIG2, CHO:PBI) (dashed line), and then analyzed by flow cytometry as described in Materials and Methods. (B) The level of surface expression of \( \alpha_5 \beta_1 \) in the two cell types. CHO and K562 cells were stained with irrelevant mouse IgG (dotted line), an anti-\( \beta_1 \) antibody (K562:8A2, CHO:7E2) (solid line), or an anti-\( \alpha_5 \) antibody (K562:BIIG2, CHO:PBI) (dashed line), and then analyzed by flow cytometry as described in Materials and Methods. (C) The binding of \( ^{125}\text{I}-\text{Fn} \) to CHO cells (Resting), to cells incubated in medium containing 2 mM deoxyglucose and 0.1% sodium azide (DOG/Az), or to cells washed free of these inhibitors and returned to glucose-containing medium (Wash + Glc) was determined. Specificity of binding to \( \alpha_5 \beta_1 \) was verified by inhibition with the PBl antibody.
Chimeric integrin constructs manifest cell type-specific affinity states. (A) FACS analysis. CHO or K562 cells were stably transfected with the chimeras containing the cytoplasmic domains of α5 and β1 and the affinity state of the α5β1 extracellular domain was assayed by its ability to bind PAC1 in the absence (solid line) or presence (dotted line) of 1 mM GRGDSP. Depicted are flow cytometry histograms. The K562 transfectants specifically bound PAC1 only after incubation with 6 μM activating antibody, anti-LIBS6. (B) Immunoprecipitation analysis of K562 transfectants. Wild-type K562 cells (None) or stable transfectants expressing the α5 subunit noted (α5ib, α5ibβ1) were surface iodinated, lysed, and immunoprecipitated with polyclonal antibodies specific for the α5 and α5ib cytoplasmic domains or with a monoclonal antibody reactive with the extracellular domain of α5β1 (2G12). Immunoprecipitates were resolved by SDS-PAGE and constituent polypeptides were visualized by autoradiography. (C) Reverse transcriptase-polymerase chain reaction (RT-PCR). The location of the 2bsf, 2bcyt and α5cyt primers used for PCR bound after addition of an activating antibody, anti-LIBS6, confirming that the ligand-binding site was intact (Fig. 3 A). Thus, the capacity of cell type-specific elements to modulate affinity depends on the integrin cytoplasmic domains.

Since K562 cells express endogenous α5b1 under certain conditions (12), it was necessary to verify that all of the α5b1 expressed in the α chimera transfectants contained the α5 cytoplasmic domain. Immunoprecipitation of α chimera transfectants with an anti-α5 cytoplasmic domain antibody isolated polypeptides corresponding to transfected α5b1 and β3 chimeras and endogenous α5b1 (Fig. 3 B). In contrast, an anti-α5 cytoplasmic domain antibody immunoprecipitated no labeled polypeptides. An anti-α5 cytoplasmic domain antibody precipitated only endogenous α5b1 from wild-type α5b1 transfectants. In addition, we confirmed fidelity of expression at the mRNA level. Reverse transcriptase PCR was performed using a 5' primer specific for the extracellular domain of α5b1 and 3' primers specific for cytoplasmic domains of α5b1 or α5 (Fig. 3 C). A specific 393-bp band was observed from α5 chimera transfectants when primed with the 3'α5 oligonucleotide. A specific 294-bp band was observed with wild-type α5b1 transfectants when primed with the 3'α5 oligonucleotide. No bands were observed when inappropriate 3' primers were used.

As was shown in Fig. 1, high affinity Fn binding to α5b1 depends on active cellular metabolism. We therefore analyzed the effects of NaN3 and 2-deoxyglucose on the affinity state of the double chimera in CHO cells. These inhibitors blocked both PAC1 (Fig. 4 A) and Fg (Fig. 4 B) binding. Anti-LIBS2, an activating antibody (29), restored high affinity binding. Furthermore, the metabolic blockade was reversible since high affinity ligand binding reappeared after the inhibitors were washed out (Fig. 4 A). These results show that α5b1 cytoplasmic sequences confer a cell type-specific, energy-dependent, high affinity state on the extracellular domain of α5b1.

Both α and β Cytoplasmic Domains Are Involved in Affinity Modulation

To learn which cytoplasmic domain specified the high affinity state in CHO cells, we transfected each subunit chimera with a complementary wild-type subunit. Transfectants expressing both α and β chimeras or expressing the cemic α but wild-type β subunits bound PAC1 (Fig. 5 A). In contrast, cells expressing the β chimera with wild-type α5b1 were in a low affinity state and bound PAC1 only after addition of anti-LIBS2 (Fig. 5 A). These results show that α5b1 cytoplasmic sequences are involved in specifying affinity state.

To find out if the β subunit was also involved in specifying the high affinity state in CHO cells, we constructed two β,
cytoplasmic variants, \( \beta_\Delta \Delta 724 \) and \( \beta_\Delta (S^{751}\rightarrow P) \). The former is a truncation mutant that ends at D^{721} while the latter contains a single nucleotide alteration resulting in a Ser^{751}\rightarrow Pro substitution (Fig. 2). These \( \beta_\Delta \) cytoplasmic domain mutants were then cotransfected with the \( \alpha \) chimera. In contrast to wild-type \( \beta_\Delta \), coexpression of either \( \beta_\Delta \) variant with chimeric \( \alpha \) resulted in a receptor that failed to bind PAC1 constitutively (Fig. 5 B). Thus, the cytoplasmic domain of the \( \beta \) subunit as well as the \( \alpha \) subunit is involved in affinity modulation.

Regulation of Integrin Affinity by the \( \alpha \) Subunit Cytoplasmic Domain Is \( \alpha \) Subunit-Specific

These data established that the cytoplasmic domains of \( \alpha_{6\beta} \) and \( \alpha_\Delta \) specify different affinity states in CHO cells; \( \alpha_m \) the low and \( \alpha_5 \) the high affinity state. To learn whether there are consensus activation sequences, we constructed chimeras with the cytoplasmic domains of six additional \( \alpha \) subunits and analyzed their affinity state after cotransfection with \( \beta_\Delta \) into CHO cells. The \( \alpha \) cytoplasmic domains of three other \( \beta \) family members (\( \alpha_2, \alpha_5 \), \( \alpha_6 \)) conferred PAC1 binding (Fig. 6 a), while those chimeras containing \( \alpha_5 \) cytoplasmic domains from \( \beta_\Delta \) (\( \alpha_5\), \( \alpha_6 \)) (data not shown) or \( \beta_\Delta (\alpha_2) \) (Fig. 6 A) families did not. The same result was obtained with the \( \beta_\Delta \) chimeras containing cytoplasmic domains of the relevant \( \beta \) subunit partner (\( \beta_\Delta \), for \( \alpha_2, \alpha_5, \alpha_6 \), and \( \alpha_6 \) or \( \beta_\Delta \) for \( \alpha_5 \) and \( \alpha_{6M} \)). Similar to the \( \alpha_5 \) chimera, constitutive PAC1 binding was also dependent upon the \( \beta \) cytoplasmic domain. It was lost when the \( \alpha_2, \alpha_5 \), or \( \alpha_6 \) chimeras were cotransfected with \( \beta_\Delta \Delta 724 \) or \( \beta_\Delta S752P \) (Fig. 6 B). Thus, the \( \beta \) subunit cytoplasmic domain designates integrin-specific affinity differences. The \( \beta \) subunit cytoplasmic domain may be permissive for the high affinity state.

Deletion of Conserved \( \alpha \) Cytoplasmic Sequences Results in High Affinity Ligand Binding That Is Independent of Metabolic Energy and the \( \beta \) Subunit Cytoplasmic Domain

We previously reported that constitutive ligand binding to \( \alpha_m\beta_\Delta \) results from a truncation of the cytoplasmic domain of \( \alpha_m \) (69). To identify the important deleted \( \alpha_m \) cytoplasmic residues, we generated additional variants. Integrin \( \alpha \) subunit cytoplasmic domains contain a highly conserved GFFKR sequence at their NH\(_2\) termini (Fig. 2). As previously reported (69, 97), the \( \alpha_m\Delta 991 \) truncation eliminates this motif and results in constitutive PAC1 binding (Fig. 7 A; panel A) whereas a truncation after the GFFKR (\( \alpha_m\Delta 996 \)) does not (Fig. 7 A; panel C). This pinpoints the conserved motif as a regulator of integrin affinity. To test this idea, we removed the LGFFK residues from the cytoplasmic domain of an \( \alpha_5 \) cytoplasmic domain chimera (Fig. 2). This chimera was selected because it possesses the longest \( \alpha \) cytoplasmic domain. Coexpression of this chimeric internal deletion mutant (\( \alpha_m\Delta \)) in CHO cells with \( \beta_\Delta \) resulted in high affinity PAC1 binding (Fig. 7 B, panel A). Finally, to further exclude contributions from downstream \( \alpha \) sequences, we generated a variant that contains a 24-residue random cytoplasmic sequence (Fig. 2). This construct \( (\alpha_m\Delta) \) also conferred high affinity binding when expressed in CHO cells with wild-type \( \beta_\Delta \) (Fig. 7 A; panel B).

To gain insight into the mechanisms of high affinity binding conferred by the GFFKR deletion mutants, we examined the requirements for cellular metabolism and \( \beta \) cytoplasmic sequences. In contrast to the constitutively active chimeras, high affinity PAC1 binding in the GFFKR deletion variants was maintained when they were coexpressed with the truncated \( \beta_\Delta \) subunit (Fig. 7 B; panel B). In addition, in contrast to transfecants expressing constitutively active \( \alpha \)
chimeras, transfectants expressing the GFFKR deletions retained high affinity for Fg (Fig. 4 B) and PAC1 (Fig. 7 B; panel C') when treated with the metabolic inhibitors NaN₃ and 2-deoxyglucose. Finally, the α₅Δ mutant conferred cell-type independent activation, since it was active in K562 (Fig. 7 B; panel D) and COS (not shown) as well as CHO cells. Thus, deletions in the highly conserved GFFKR motif resulted in a cell type-independent high affinity state that was resistant to metabolic inhibitors and truncation of the β subunit.

Discussion
The major findings of this work are (a) the affinity state of integrin αβ₁ is regulated by cell type-specific factors. The high affinity state requires active cellular processes suggest-
Figure 6. Affinity state is α subunit cytoplasmic domain specific. (A) Effect of different α cytoplasmic domains. Chimeric α subunits consisting of extracellular and transmembrane αm with the indicated cytoplasmic domain were transiently cotransfected with β3 into CHO cells. PAC1 binding was quantified by flow cytometry and the activation index was calculated as: 100*(F₀-Fᵣ)/Fᵣ where: 
F₀ = Mean Fluorescence Intensity in the absence of inhibitor; Fᵣ = Mean Fluorescence Intensity in the presence of 2 mM GRGDS. Depicted are the mean ± SD of at least three independent experiments for each α chimera. (B) β Subunit cytoplasmic domain dependence of the high affinity state. β Subunit chimeras containing the indicated cytoplasmic sequences were cotransfected with a α5 subunit whose cytoplasmic domain was truncated (β₃Δ724), contained the S752→P mutation (S752P), or had been exchanged for the homologous region of β₁. PAC1 binding was analyzed as described in panel A. Mean ± SD of at least three independent experiments for each α β pair.

Figure 7. GFFKR deletion variants confer an energy and β subunit cytoplasmic domain-independent high affinity state. (A) GFFKR sequence deletion activates αmβ₁. Stable CHO cell lines were established by cotransfection of αm containing the noted α cytoplasmic domain with wild-type β₁. PAC1 binding in the absence (solid line) and presence (dotted line) of GRGDS was assessed by flow cytometry. The αmΔ991 transfectant, which lacks GFFKR, specifically binds PAC1 (panel A). In contrast, the αmΔ996 transfectant, which retains GFFKR, binds only after activation with anti-LIBS2 (panels C and D). Replacement of the αm cytoplasmic domain with random sequence also induces PAC1 binding (panel B, αm). (B) Energy, β subunit cytoplasmic domain, and cell type-independent high affinity state of GFFKR deletion variants. CHO cells were transiently transfected with chimeras of the extracellular and transmembrane domains of αm/β₃ joined to the indicated cytoplasmic domains. Specific PAC1 binding to the population of cells expressing αmβ₃ was detected as described in Fig. 7 A. A GFFKR "loop out" mutant manifested PAC1 binding (panel A) that was maintained in the presence of 0.1% NaN₃ and 2 mM 2-deoxyglucose (panel C). This treatment abolished ligand binding to an αmβ₁ chimera bearing the cytoplasmic domain of αmβ₁ (not shown, but cf. Fig. 4 A). High affinity state was also maintained despite an extensive deletion of the β₃ cytoplasmic domain (panel B) that disrupted PAC1 binding to the αmβ₁ chimera (not shown but cf. Fig. 5 B). Similar results were obtained with αmΔ991 and αm transfectants. A stable K562 cell line bearing the GFFKR deletion mutant specifically bound PAC1 (panel D), but the αmβ₁ chimera was not active in these cells (cf. Fig. 3 A).
Table I. Summary of Affinity States of the Extracellular Domain: Physiological Modulation

<table>
<thead>
<tr>
<th></th>
<th>β3</th>
<th>β3Δ724</th>
<th>β3S752P</th>
<th>β1</th>
<th>β2</th>
</tr>
</thead>
<tbody>
<tr>
<td>αIIb</td>
<td>LO</td>
<td>LO</td>
<td>LO</td>
<td>LO</td>
<td>-</td>
</tr>
<tr>
<td>α2</td>
<td>HI</td>
<td>LO</td>
<td>LO</td>
<td>LO</td>
<td>HI</td>
</tr>
<tr>
<td>α5</td>
<td>HI</td>
<td>LO</td>
<td>LO</td>
<td>HI</td>
<td>-</td>
</tr>
<tr>
<td>α6A</td>
<td>HI</td>
<td>LO</td>
<td>LO</td>
<td>HI</td>
<td>-</td>
</tr>
<tr>
<td>α6B</td>
<td>HI</td>
<td>LO</td>
<td>LO</td>
<td>HI</td>
<td>-</td>
</tr>
<tr>
<td>αL</td>
<td>LO</td>
<td>-</td>
<td>-</td>
<td>LO</td>
<td>-</td>
</tr>
<tr>
<td>αM</td>
<td>LO</td>
<td>-</td>
<td>-</td>
<td>LO</td>
<td>-</td>
</tr>
<tr>
<td>αv</td>
<td>LO</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Summary of the affinity states, as assayed by PAC1 binding to CHO cells transiently transfected with chimeras of the extracellular and transmembrane domains of αIIbβ3 joined to the indicated cytoplasmic domains. Affinity states were defined as HI = Activation Index ≥60 and LO = Activation Index ≤45. Double underlining indicates the experiments that establish the importance of the β subunit cytoplasmic domain in maintenance of the high affinity state. In all instances of the high affinity state, treatment with NaN₃ and 2-deoxyglucose resulted in reversion to the low affinity state.

In CHO cells, α5, α6A and B, and α2 cytoplasmic tail chimeras specified the high affinity state. The αmΔ996 truncation mutant leaves the common (Fig. 2) KXGFFKR motif and results in a low affinity state. This suggests that activation signaling sequences important for conversion of the integrin to a high affinity state reside carboxyterminal of GFFKR in α5, α6A and B, and α2. Thus, deletion of related sequences could account for the reduction of cell adhesion by certain truncations of the α (45) or α (46) cytoplasmic tail. In addition, truncations of αm or αl that retain GFFKR lose the capacity to constrain integrin localization to focal adhesions (9, 97). This result suggests the existence of elements within the carboxyl terminal portion of the α subunit that inhibit targeting of the integrin to focal adhesions. In CHO cells, the full-length αm cytoplasmic domain specifies a low affinity state but still constrains the localization of αmβ3 to focal adhesions. Thus, we suspect that the α cytoplasmic domain elements that control targeting to focal adhesions and ligand-binding affinity are not identical. These elements may also be involved in the role of α subunit cytoplasmic domains in more complex cellular functions such as collagen gel contraction, cell migration, and cell adhesion (14, 45, 46).

The cytoplasmic domains of either β, or β2, were required for the activation of the α subunit chimeras. Moreover, the capacity of β(575→P) to disrupt activation, underscores the specificity of the β subunit requirement. Interestingly, β2 Ser575 is homologous to a Thr (Fig. 2) involved in the adhesive function of α2β1 (38). S575 is not extensively phosphorylated in platelets (40). We have found no obvious difference in the phosphorylation of αmβ3 cytoplasmic domain chimeras in CHO cells and K562 cells (unpublished results). Thus, the mechanism of the effect of the S575→P mutation on inside-out signaling remains to be resolved.

Mutations that delete portions of the conserved GFFKR sequence appear to reset αmβ3 to a default high affinity state. As summarized in Table II, a truncation mutant that removes this sequence (αmΔ991) is constitutively active,
Table II. Summary of Affinity States of the Extracellular Domain: Hinge Mutants

<table>
<thead>
<tr>
<th></th>
<th>B3</th>
<th>β3Δ724</th>
<th>β3S752P</th>
</tr>
</thead>
<tbody>
<tr>
<td>αIb</td>
<td>LO</td>
<td>LO</td>
<td>LO</td>
</tr>
<tr>
<td>αIbΔ991</td>
<td>HI*</td>
<td>HI</td>
<td>HI</td>
</tr>
<tr>
<td>αIbΔ996</td>
<td>LO*</td>
<td>LO</td>
<td>LO</td>
</tr>
<tr>
<td>αΔ</td>
<td>HI*</td>
<td>HI</td>
<td>HI</td>
</tr>
<tr>
<td>αL</td>
<td>LO*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>αRc</td>
<td>HI*</td>
<td>HI</td>
<td>HI</td>
</tr>
</tbody>
</table>

Summary of the affinity states of the extracellular domains of αIbβ when joined to the indicated cytoplasmic domains and transiently expressed in CHO cells as assayed by PAC1 binding. Affinity states were defined as HI = Activation Index > 60 and LO = Activation Index < 45. Asterisks indicate the experiments establishing the role of the GFFKR motif in maintenance of the low affinity state. Double underlining indicates the experiments that establish the lack of importance of the β subunit cytoplasmic domain in supporting the high affinity state in these mutants. In all instances of the high affinity state, treatment with NaN₃ and 2-deoxyglucose did not result in reversion to the low affinity state.

whereas a truncation that retains this sequence (αIbΔ996) remains inactive. Moreover, a KLGFF loop out mutation in the α, cytoplasmic domain or replacement of the α cytoplasmic domain with a 24-residue random sequence also resulted in high affinity Fg and PAC1 binding. The nature of the high affinity state in these GFFKR mutants differed markedly from that in the cytoplasmic domain chimeras. Specifically, the deletions resulted in default high affinity in all cells tested. In addition, high affinity was maintained despite addition of metabolic inhibitors and truncation of the β cytoplasmic domain. Of note in this context, a truncated α, lacking GFFKR, is more efficient at assembling a Fn matrix than wild-type α (96). This effect could be due to increased Fn binding, since matrix assembly may be regulated by the affinity state of α, (26). The GFFKR sequence probably resides in the cell interior because there are generally about 20 hydrophobic residues preceding the charged Lys of the KXGFFKR motif (6, 18, 19, 54, 72, 83, 88-91). Mutations of intramembrane or cytoplasmic (13, 56, 73) residues can lead to constitutive transmembrane conformational changes in other receptors. In integrins, certain cytoplasmic domain mutations also initiate such a constitutive transmembrane alteration.

The high affinity state of αIbβ, provoked by deletion of GFFKR suggests possible mechanisms for transmembrane signaling through integrins. Transmembrane domains of type I membrane proteins such as integrin α and β subunits are presumably constrained into helices (83). Consequently, transmission of conformational information across the membrane probably involves changes in the spatial relationships of the α and β subunits. The bacterial aspartate receptor, like integrins (63), has ligand contact sites in each of its two

Figure 8. Working hypothetical model of affinity modulation of integrins. Depicted is a schematic that accommodates the results reported here. Cell-type specific energy-dependent cytoplasmic signals target the integrin cytoplasmic domains. The unidentified factor(s) responsible for these signals (represented as Integrin Activator Complex) interacts with the cytoplasmic domains to provoke changes in the spatial relationships or conformations of the α and β subunit cytoplasmic tails. Such changes then traverse the membrane-proximal GFFKR sequence to alter the relationship of α and β subunit transmembrane domains and ultimately the conformation of the extracellular domain. Although the action is depicted as a scissors, other motions such as pistons, seesaws, or rotation (47, 87) of the subunits are also possible. Since these motions must traverse GFFKR to reach the transmembrane domains, GFFKR could be viewed as a component of a hinge that connects the cytoplasmic and the transmembrane domains. Thus, deletions in GFFKR lock the integrin hinge in an irreversible high affinity state.
membrane-spanning subunits. Ligand binding results in a 1.4 Å shift of membrane-adjacent helices relative to each other. This shift may result in outside-in signaling. In integrins, the cytoplasmic GFFKR sequence could regulate the spatial relationships of the α and β subunits through interactions with the β subunit resulting in inside-out signaling. Alternatively, GFFKR could bind membrane lipids and perturbations of such interactions could explain lipid modulation of integrin function (17, 37, 84). Finally, cytoplasmic proteins could bind to this sequence. Calreticulin, a protein usually reported to bind to a short synthetic peptide containing GFFKR, could bind to this sequence. Calreticulin, a protein usually thought to reside in the endoplasmic reticulum, has been reported to bind to a short synthetic peptide containing GFFKR (74). If this association occurs in vivo, then the integrin's default affinity state. A search of the Swissprot Database, using Wordsearch (21), identified the GFFKR sequence in several C4 zinc finger ("steroid fingers") transcription factors (e.g., retinoic acid receptor [60], Drosophila tailless [70], Nur/77 [76]). The sequence is in the most part of an alpha helix that is initiated by two zinc-coordinating cysteines (59). This helix forms contacts with DNA that are critical for activity (27, 59, 77). In integrins, KKGFFKR presumably adjoins an ε helix. By analogy with the steroid fingers, GFFKR may therefore possess ε helical structure and participate in functionally important interactions.

The data presented here suggest a working hypothesis for affinity modulation of integrins (Fig. 8). Cell-type specific and energy-dependent cytoplasmic signals target the integrin cytoplasmic domains. The unidentified factor(s) responsible for these signals (represented as IAC in Fig. 8) require the presence of elements of both cytoplasmic domains. The IAC-cytoplasmic domain interaction could provide changes in the spatial relationships or conformations of the α and β subunit cytoplasmic tails. Such changes then probably traverse the membrane-proximal GFFKR sequence to influence the relationship of α and β subunit transmembrane domains and ultimately the formation of the extracellular domain. Although the action is depicted as a "scissors" in Fig. 8, other motions such as "pistons," "seesaws," or rotation (47, 87) of the subunits are also possible. Since these motions must traverse GFFKR to reach the transmembrane domains, GFFKR could be viewed as a component of a "hinge" that connects the cytoplasmic and the transmembrane domains. Thus, deletions in GFFKR might lock the integrin hinge in an irreversible high affinity state (Fig. 8). In theory, other mutations might lock the hinge in a low affinity state.

We thank Ms. Susan Wynant and Ruby Larson for able secretarial assistance and Jane Forsyth for excellent technical assistance. This is publication number 8232-CVB from The Scripps Research Institute.

Received for publication 18 November 1993 and in revised form 29 December 1993.

References

1057


