Integrin Cytoplasmic Domains Mediate Inside-Out Signal Transduction

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Abstract. We analyzed the binding of fibronectin to integrin $\alpha_5\beta_1$ 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 $\alpha_{IIb}\beta_3$ joined to the cytoplasmic domains of $\alpha_5\beta_1$. The affinity state of these chimeras was assessed by binding of fibrinogen or the monoclonal antibody, PAC1. The cytoplasmic domains of $\alpha_5\beta_1$ conferred an energy-dependent high affinity state on $\alpha_{IIb}\beta_3$ in CHO but not K562 cells. Three additional α cytoplasmic domains (α_2, α_6A, α_6B) conferred PAC1 binding in CHO cells, while three others (α_M, α_L, α_v) did not. In the high affinity α chimeras, cotransfection with a truncated ($\beta_3\Delta724$) or mutated ($\beta_3(S^{752}\rightarrow P)$) β_3 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 $\alpha_{IIb}\beta_3$. 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 β_3 (7), β_2 (4), and β_1 (26) integrins. Changes in adhesive function may also occur without changes in ligand-binding affinity. For example, phorbol esters stimulate the $\alpha_5\beta_1$-dependent adhesion of CHO cells (20) to fibronectin (Fn)[1] with no change in Fn-binding affinity. Similarly, certain β_3 mutations reduce $\alpha_{IIb}\beta_3$-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 $\alpha_2\beta_1$ 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.

$\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa) is a prototype integrin for analysis of changes in integrin affinity. As with all integrins, $\alpha_{IIb}\beta_3$ is a heterodimer of two Type I transmembrane protein subunits (43). $\alpha_{IIb}\beta_3$ is platelet-specific (88), and affinity state-specific antibodies, e.g., PAC1 (81), simplify analysis of recombinant $\alpha_{IIb}\beta_3$ in heterologous cells (67). Conformational changes in the extracellular domain of $\alpha_{IIb}\beta_3$ regulate its affinity (67, 82). Platelet agonists increase the affinity of $\alpha_{IIb}\beta_3$ ("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,

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

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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 $\alpha_{IIb}\beta_3$ is pretreated with RGD peptides, it subsequently binds Fg and PAC1 (23, 50, 85). Certain anti-β_3 antibodies directly increase the Fg-binding affinity of $\alpha_{IIb}\beta_3$ (29) and certain anti-β_1 antibodies activate $\alpha_5\beta_1$ 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 $\alpha_M\beta_2$ (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 α_{IIb} cytoplasmic domain after residue 990 ($\alpha_{IIb}\Delta 991$) results in $\alpha_{IIb}\beta_3$ that constitutively binds Fg and PAC1 (69). Conversely, truncations of α_4 (45), α_2 (46), and β_2 (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 Ser^{752} to Pro mutation in the β_3 cytoplasmic domain was associated with an apparent $\alpha_{IIb}\beta_3$ 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 β_1 and reduced binding of $\alpha_5\beta_1$ 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 $\alpha_{IIb}\beta_3$, 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 NH_2 terminus of the α subunit cytoplasmic domain, also resulted in high affinity binding of ligands to $\alpha_{IIb}\beta_3$. 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-$\alpha_{IIb}\beta_3$ antibody D57 was produced by Dr. Xiaoping Du (Scripps Research Institute) using previously described methods (28). It binds to CHO cells transfected with $\alpha_{IIb}\beta_3$ but not $\alpha_v\beta_3$, and does not block Fg binding to $\alpha_{IIb}\beta_3$. This antibody was biotinylated with biotin-N-hydroxysuccinimide (Sigma Chem. Co., St. Louis, MO) according to manufacturers directions. The $\alpha_{IIb}\beta_3$ 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 α_5 (PB1) and anti-β_1 (7E2) antibodies were obtained from Dr. Rudolph Juliano (10) (University of North Carolina, Chapel Hill), and the β_1 activating antibody, 8A2, was supplied by Drs. Nick Kovach and John Harlan (51) (University of Washington, Seattle). A human anti-α_5 antibody, BIIG2, was supplied by Dr. Caroline Damsky (95) (University of California, San Francisco) while a polyclonal anti-peptide antibody against the cytoplasmic domain of human α_5 (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-α_{IIb} 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 GRGDSP 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 (Sigma). 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), panning 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 with PBS, and combined with appropriate cDNAs (10 μg each subunit). 3×10^7 cells in 0.5 ml in growth media were electroporated at 350 V, 960 μF in a BTX (BTX, San Diego, CA) electroporator. Media were changed after 24 h, and cells analyzed for surface expression or PAC1 binding after 48 h. Stable CHO transfectants were established as above with cotransfection of 0.6 μg of CDNeo. After 48 h, these cells were selected for 2 wk in 700 μg/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 10^7 cells in 0.8 ml of PBS at 300 V and 500 μF. After 48 h, the cells were maintained in media 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, 5×10^5 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 (Tago, Burlingame, CA) 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 $MgCl_2$ and $CaCl_2$ 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, 5×10^5 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 D57. 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., Junction City, 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 $\alpha_{IIb}\beta_3$ 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 $\alpha_{IIb}\beta_3$ (8), a rightward shift in the histogram in the absence of peptide is indicative of the presence of high affinity $\alpha_{IIb}\beta_3$. 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:

$100*(F_0-F_R)/F_R$ where:
F_0 = Mean Fluorescence Intensity in the absence of inhibitor
F_R = Mean Fluorescence Intensity in the presence of GRGDSP

Expression of $\alpha_{IIb}\beta_3$ 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, $\alpha_{IIb}\Delta 991$, like a similar α_L 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 $\alpha_L\Delta$ 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 $\alpha_{IIb}\beta_3$, $\alpha_5\beta_3$, $\alpha_5\beta_3\Delta 724$, $\alpha_{Ra}\beta_3$, and $\alpha_{Ra}\beta_3\Delta 728$. The data with the stable cell lines confirmed the findings in transient analyses. All other combinations were tested in transient transfections only.

DNA Constructs

The generation of CDM8 constructs encoding α_{IIb}, $\alpha_{IIb}\Delta 991$, $\alpha_{IIb}\Delta 996$, β_3, and $\beta_3\Delta 728$ have been previously described (68, 69, 97). The β_3 truncation, $\Delta 724$, and amino acid substitution, $S^{752}\rightarrow P$, were first generated in BS3a (68) by oligonucleotide-directed mutagenesis (52), digested with HincII to isolate coding sequences, ligated to BstXI linkers, (Invitrogen, San Diego, CA) and subcloned into the BstXI sites of CDM8. The β_3 chimera, containing the β_1 cytoplasmic domain, was constructed by first generating an EcoRI site at bases 2387-2392 of β_1 cDNA sequence. After HindIII digestion, a 400-bp fragment containing the complete β_1 cytoplasmic domain and partial 3' non-coding sequences was isolated and subcloned into the HindIII site of CDM8. 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. β_2 cytoplasmic sequences were first isolated by the PCR from a β_2 cDNA, and then subcloned into the MluI and XhoI sites of CDM8. The β_2 cytoplasmic domain chimera was then generated by digestion with MluI and HindIII and ligation with a corresponding MluI–HindIII fragment from CD3a containing its extracellular and transmembrane sequences. Chimeric α subunits were generated using a previously described strategy (69). Cytoplasmic sequences from α_v, α_M, α_L, α_2, α_6A, and α_6B were isolated from the appropriate cDNA clones by PCR (57). Amplified products were digested with HindIII and XbaI and subcloned into HindIII and XbaI cut CDM8. After digesting with HindIII, these constructs were ligated with a HindIII fragment from CD2b (68) containing its extracellular and transmembrane domains. PCR oligonucleotides for $\alpha_L\Delta$ were designed to omit the VGFFK sequence. Its construction followed the procedure for other α chimeras. The α_{Ra} 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 XbaI and ligated to a SalI–XbaI 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 (Appl. Biosystems Inc., Foster City, CA).

Ligand Binding

The binding of ^{125}I-Fg or ^{125}I-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 $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mg/ml BSA, and 1 mg/ml dextrose). A typical assay included 120 μl of cells (2×10^6 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. ^{125}I-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 $CaCl_2$, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, and 10 mM N-ethylmaleimide). Cell extracts were immunoprecipitated with polyclonal antiserum directed against the α_{IIb} or α_5 cytoplasmic domains and a monoclonal antibody against the $\alpha_{IIb}\beta_3$ 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

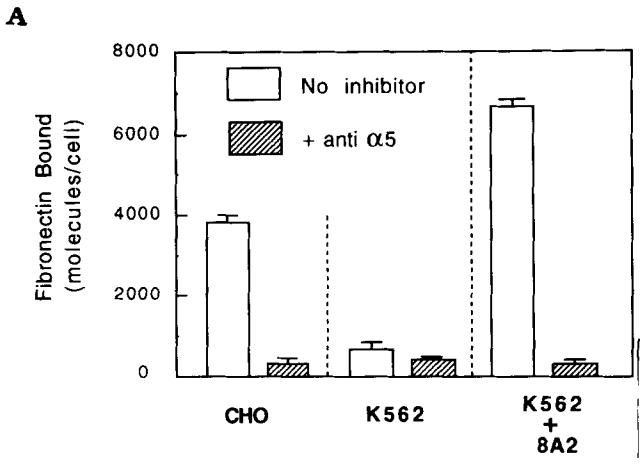
Total RNA was isolated from 10^6 transfected cells using the RNAzol reagent (Cinna Biotecx). First strand cDNA synthesis from 5 μg of RNA was performed with the cDNA cycle kit (Invitrogen) using oligo dT as a primer. Coding sequences downstream of the α_{IIb} transmembrane region were specifically amplified with a 5' primer specific for transmembrane α_{IIb} (2bsf: CGGGCCTTGGAGGAGAGGGCCATTC) and 3' primers specific for the cytoplasmic sequences of α_{IIb} (α_{IIb}cyt: CTCTGTTGGGAGGGAAACGA) and α_5 $\alpha_5$cyt: TGTAAACAAGGGTCCTTCAC). Amplified products were analyzed by agarose gel electrophoresis.

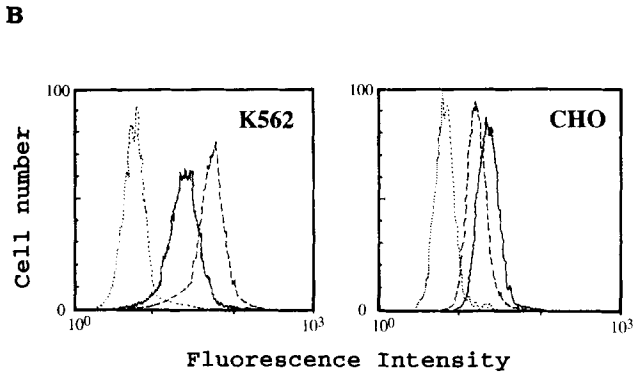
Results

Cell Type-Specific and Energy-dependent Affinity Modulation of Integrin $\alpha_5\beta_1$

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 $\alpha_5\beta_1$. The cells analyzed fell into two groups: those that bound Fn with only low affinity (Kd >1 μM), e.g., K562, THP1, U937, and peripheral blood T cells, and those that bound with moderate affinity (Kd ~100 nM), e.g., CHO, WI-38, and MG63 cells (Fig. 1 *A*). The low affinity $\alpha_5\beta_1$ 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 $\alpha_5\beta_1$ (Fig. 1 *B*). Specificity of Fn binding to high affinity $\alpha_5\beta_1$ was verified by inhibition with an anti α_5 antibody (Fig. 1 *A*).

To find out whether spontaneous high affinity Fn binding





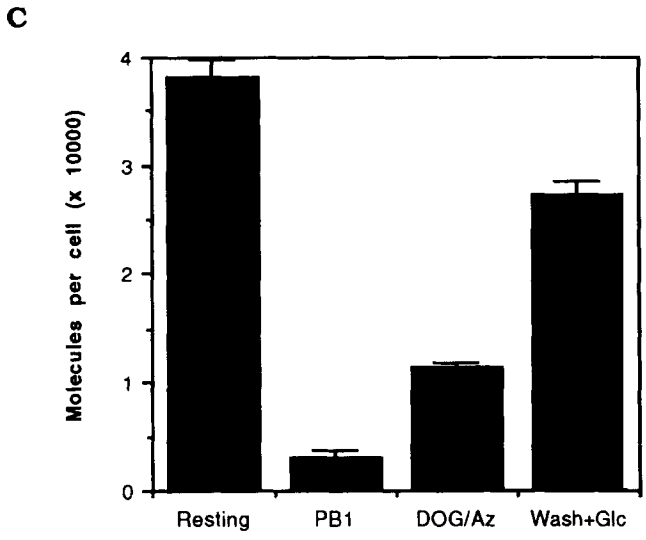


Figure 1. The high affinity state of $\alpha_5\beta_1$ is cell type-specific and energy-dependent. (*A*) ^{125}I-Fn (50 nM) was incubated at 22°C with CHO or K562 cells. After 30 min, bound Fn was assessed by centrifugation through a sucrose cushion as described in Materials and Methods. $\alpha_5\beta_1$-specific binding was established by blocking binding to the CHO cells with PB1, an anti-hamster α_5. Binding to K562 cells was induced by addition of 20 nM activating antibody (*8A2*) and was inhibited by the anti-α_5, BIIG2. (*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-β_1 antibody (*K562:8A2, CHO:7E2*) (*solid line*), or an anti-α_5 antibody (K562:BIIG2, CHO:PB1) (*dashed line*), and then analyzed by flow cytometry as described in Materials and Methods. (*C*) The binding of ^{125}I-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 PB1 antibody.

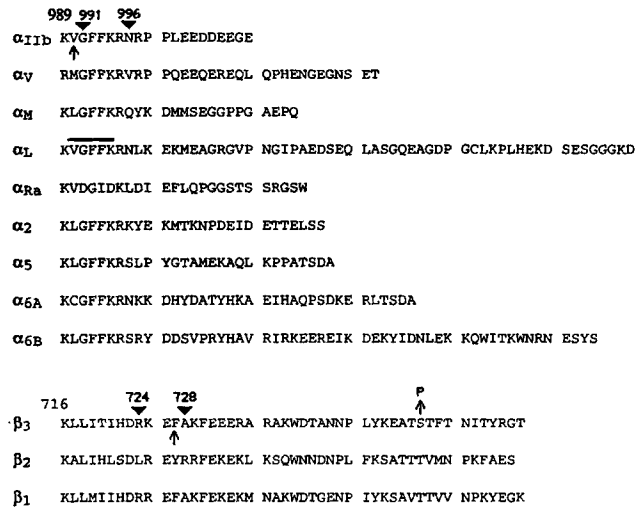


Figure 2. Amino acid sequences of wild-type and variant integrin cytoplasmic domains. Single letter amino acid code is used. The arrows underneath the α_{IIb} (residue 990) and β_3 (residue 727) sequences denote the position at which chimeric cytoplasmic domains were joined to the extracellular and transmembrane domains of α_{IIb} and β_3. The position of stop codons producing cytoplasmic truncations are noted by triangles, while the $S^{752}\rightarrow P$ point mutation in β_3 is indicated. The residues deleted in the $\alpha_L\Delta$ cytoplasmic domain are overlain by the heavy line.

to $\alpha_5\beta_1$ is an active process, we treated CHO cells with a combination of inhibitors of oxidative phosphorylation (NaN_3) and anaerobic glycolysis (2-deoxyglucose). This resulted in loss of specific high affinity Fn binding. This effect was partially reversible since washout of the metabolic inhibitors resulted in restoration of 75% of the high-affinity binding (Fig. 1 *C*). Thus, high affinity Fn binding to integrin $\alpha_5\beta_1$ is cell type-specific and an active cellular process.

The Cytoplasmic Domains of $\alpha_5\beta_1$ Confer an Energy-dependent High Affinity State on $\alpha_{IIb}\beta_3$ in Some Cells but Not Others

To decide whether the cytoplasmic domains of $\alpha_5\beta_1$ were involved in cell type-specific affinity modulation, we generated chimeras in which the cytoplasmic domains of α_{IIb} and β_3 were replaced with the corresponding sequences from α_5 and β_1 (Fig. 2). The α and β chimeras were then cotransfected into CHO or K562 cells, and the affinity state of the extracellular $\alpha_{IIb}\beta_3$ reporter group was assayed by binding of PAC1, an antibody specific for the high affinity state of $\alpha_{IIb}\beta_3$ (81). The double chimera bound PAC1 when it was expressed in CHO cells (Fig. 3). Since wild-type $\alpha_{IIb}\beta_3$ does not bind PAC1 when expressed in CHO cells (67), it is concluded that the $\alpha_5\beta_1$ cytoplasmic domains conferred the high affinity state on $\alpha_{IIb}\beta_3$. In sharp contrast, PAC1 did not bind to the double chimera in K562 cells. However, PAC1

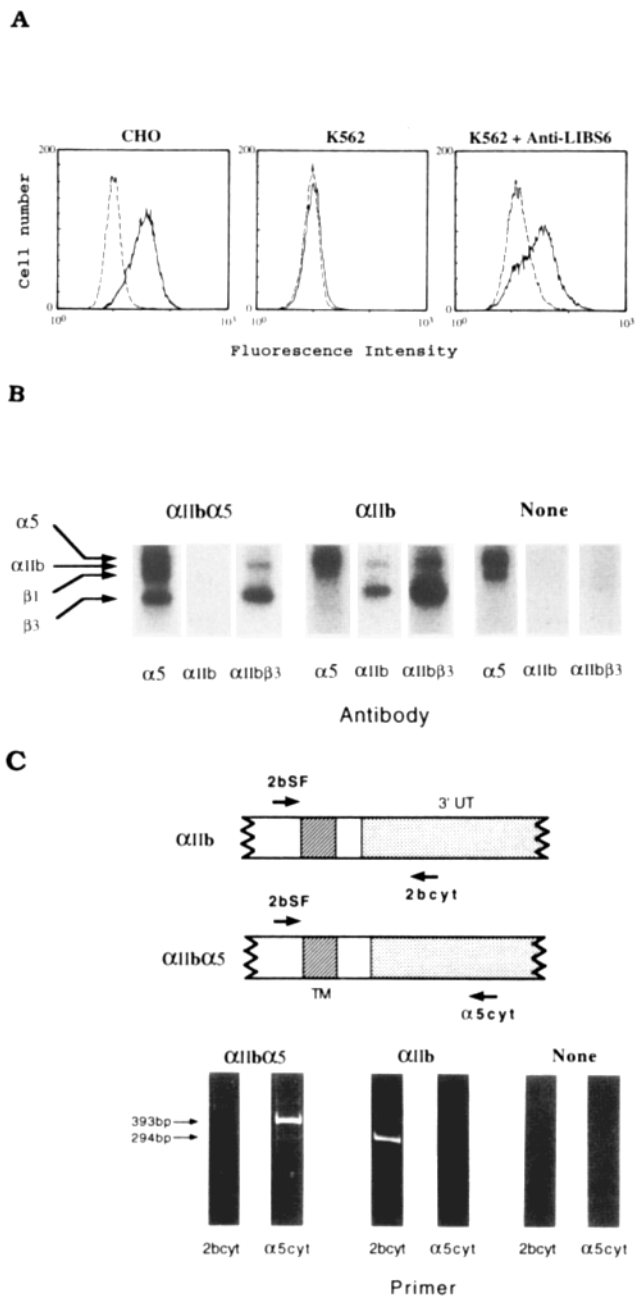


Figure 3. Chimeric integrin constructs manifest cell type-specific affinity states. (*A*) FACS analysis. CHO or K562 cells were stably transfected with the chimeras containing the cytoplasmic domains of α_5 and β_1 and the affinity state of the $\alpha_{IIb}\beta_3$ extracellular domain was assayed by 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-LIBS 6. (*B*) Immunoprecipitation analysis of K562 transfectants. Wild-type K562 cells (*None*) or stable transfectants expressing the α subunit noted ($\alpha_{IIb}\alpha_5$ = α_5 cytoplasmic domain chimera) were surface iodinated, lysed, and immunoprecipitated with polyclonal antibodies specific for the α_5 and α_{IIb} cytoplasmic domains or with a monoclonal antibody reactive with the extracellular domain of $\alpha_{IIb}\beta_3$ (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 $\alpha_5$cyt primers used for PCR analysis are illustrated. The transmembrane (*TM: crosshatched*), 3′ untranslated (*3′UT: stippled*), and cytoplasmic and extracellular domain (*clear*) sequences are indicated. RT-PCR was performed as described in Materials and Methods with the 5′ 2bsf primer and 3′ primers specific for α_{IIb} or α_5 3′ untranslated sequences. Amplified products were analyzed by agarose gel electrophoresis (*below*) with arrows denoting the position of the 393 and 294 bp bands. Transfectant type is listed above while the 3′ primer used is noted below.

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 α_{IIb} under certain conditions (12), it was necessary to verify that all of the α_{IIb} expressed in the α chimera transfectants contained the α_5 cytoplasmic domain. Immunoprecipitation of α chimera transfectants with an anti-α_5 cytoplasmic domain antibody isolated polypeptides corresponding to transfected α_{IIb} and β_3 chimeras and endogenous $\alpha_5\beta_1$ (Fig. 3 *B*). In contrast, an anti-α_{IIb} cytoplasmic domain antibody immunoprecipitated no labeled polypeptides. An anti-α_5 cytoplasmic antibody precipitated only endogenous $\alpha_5\beta_1$ from wild-type $\alpha_{IIb}\beta_3$ 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 α_{IIb} and 3′ primers specific for cytoplasmic domains of α_{IIb} or α_5 (Fig. 3 *C*). A specific 393-bp band was observed from α chimera transfectants when primed with the 3′ α_5 oligonucleotide. A specific 294-bp band was observed with wild-type α_{IIb} transfectants when primed with the 3′ α_{IIb} oligonucleotide. No bands were observed when inappropriate 3′ primers were used.

As was shown in Fig. 1, high affinity Fn binding to $\alpha_5\beta_1$ depends on active cellular metabolism. We therefore analyzed the effects of NaN_3 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 $\alpha_5\beta_1$ cytoplasmic sequences confer a cell type-specific, energy-dependent, high affinity state on the extracellular domain of $\alpha_{IIb}\beta_3$.

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 chimeric α but wild-type β_3 subunits bound PAC1 (Fig. 5 *A*). In contrast, cells expressing the β chimera with wild-type α_{IIb} were in a low affinity state and bound PAC1 only after addition of anti-LIBS2 (Fig. 5 *A*). These results show that α 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_3

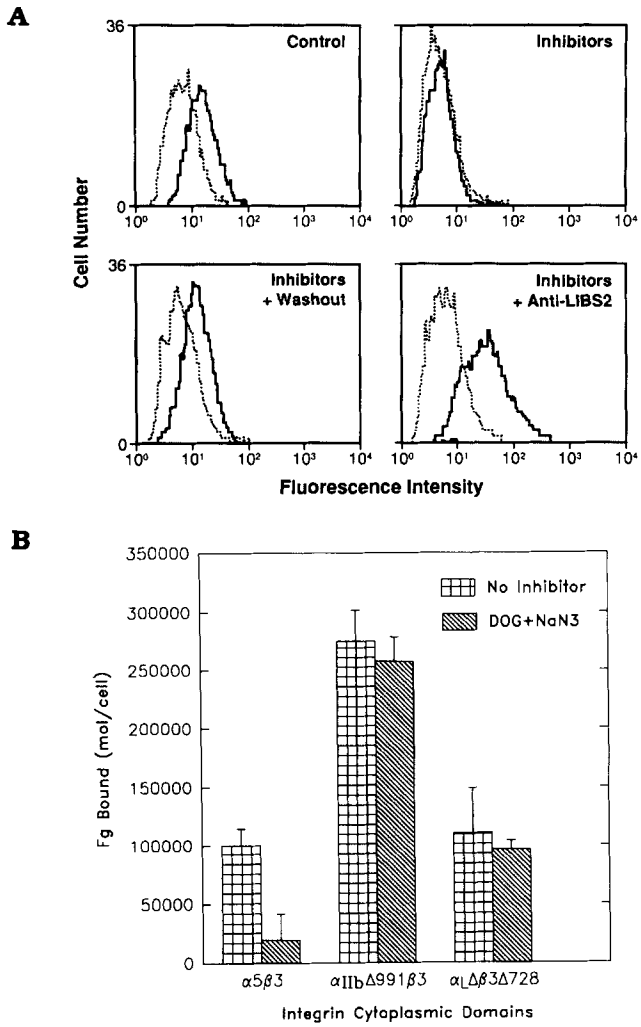


Figure 4. The high affinity state of the α_5 cytoplasmic domain chimera is energy-dependent. (*A*) PAC1 binding. Stable CHO transfectants expressing the α_5 and β_1 cytoplasmic domain chimeras were assayed for PAC1 binding in the absence (*solid line*) and presence (*dotted line*) of 2 mM GRGDSP by flow cytometry. Transfectants incubated with 2 mg/ml deoxyglucose and 0.1% NaN_3 (Inhibitors) as described in Materials and Methods manifested loss of specific binding. Addition of 6 μM anti-LIBS2 (*Inhibitors + Anti-LIBS2*) or washout of these inhibitors (*Inhibitors + Washout*) and return to glucose-containing medium reconstituted specific PAC1 binding. (*B*) Fibrinogen binding. Stable CHO transfectants expressing the cytoplasmic domains noted below were analyzed for fg binding as described in Materials and Methods. Constitutive binding to transfectants expressing the α_5 chimera with wild-type β_3 was inhibited by 2 mg/ml deoxyglucose plus 0.1% NaN_3; binding to transfectants expressing the $\alpha_{IIb}\Delta991$ or $a_L\Delta$ variant was not (see below).

cytoplasmic variants, $\beta_3\Delta724$ and $\beta_3(S^{752}\rightarrow P)$. The former is a truncation mutant that ends at D^{723}, while the latter contains a single nucleotide alteration resulting in a $Ser^{752}\rightarrow$ Pro substitution (Fig. 2). These β_3 cytoplasmic domain mutants were then cotransfected with the α chimera. In contrast to wild-type β_3, coexpression of either β_3 variant with chimeric α resulted in a receptor that failed to bind PAC1 constitutively (Fig. 5 *B*). Thus, the cytoplasmic domain of the β subunit as well as the α subunit is involved in affinity modulation.

Regulation of Integrin Affinity by the α Subunit Cytoplasmic Domain Is α Subunit-Specific

These data established that the cytoplasmic domains of α_{IIb} and α_5 specify different affinity states in CHO cells; α_{IIb} the low and α_5 the high affinity state. To learn whether there are consensus activation sequences, we constructed chimeras with the cytoplasmic domains of six additional α subunits and analyzed their affinity state after cotransfection with β_3 into CHO cells. The α cytoplasmic domains of three other β_1 family members (α_2, α_6A, α_6B) conferred PAC1 binding (Fig. 6 *a*), while those chimeras containing α subunit cytoplasmic domains from β_2 (α_M, α_L) (data not shown) or $\beta_3(\alpha_v)$ (Fig. 6 *A*) families did not. The same result was obtained with the β chimeras containing cytoplasmic domains of the relevant β subunit partner (β_1 for α_2, α_v, α_6A, and α_6B or β_2 for α_L and α_M). Similar to the α_5 chimera, constitutive PAC1 binding was also dependent upon the β cytoplasmic domain. It was lost when the α_2, α_6A, or α_6B chimeras were cotransfected with $\beta_3\Delta724$ or β_3S752P (Fig. 6 *B*). Thus, the α subunit cytoplasmic domain designates integrin-specific affinity differences. The β subunit cytoplasmic domain may be permissive for the high affinity state.

Deletion of Conserved α Cytoplasmic Sequences Results in High Affinity Ligand Binding That Is Independent of Metabolic Energy and the β Subunit Cytoplasmic Domain

We previously reported that constitutive ligand binding to $\alpha_{IIb}\beta_3$ results from a truncation of the cytoplasmic domain of α_{IIb} (69). To identify the important deleted α_{IIb} cytoplasmic residues, we generated additional variants. Integrin α subunit cytoplasmic domains contain a highly conserved GFFKR sequence at their NH_2 termini (Fig. 2). As previously reported (69, 97), the $\alpha_{IIb}\Delta991$ truncation eliminates this motif and results in constitutive PAC1 binding (Fig 7 *A*; panel *A*) whereas a truncation after the GFFKR ($\alpha_{IIb}\Delta996$) 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 α_L cytoplasmic domain chimera (Fig. 2). This chimera was selected because it possesses the longest α cytoplasmic domain. Coexpression of this chimeric internal deletion mutant ($\alpha_L\Delta$) in CHO cells with β_3 resulted in high affinity PAC1 binding (Fig. 7 *B*, panel *A*). Finally, to further exclude contributions from downstream α sequences, we generated a variant that contains a 24-residue random cytoplasmic sequence (Fig 2). This construct (α_{Ra}) also conferred high affinity binding when expressed in CHO cells with wild-type β_3 (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 β 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 β_3 subunit (Fig. 7 *B*; panel *B*). In addition, in contrast to transfectants expressing constitutively active α

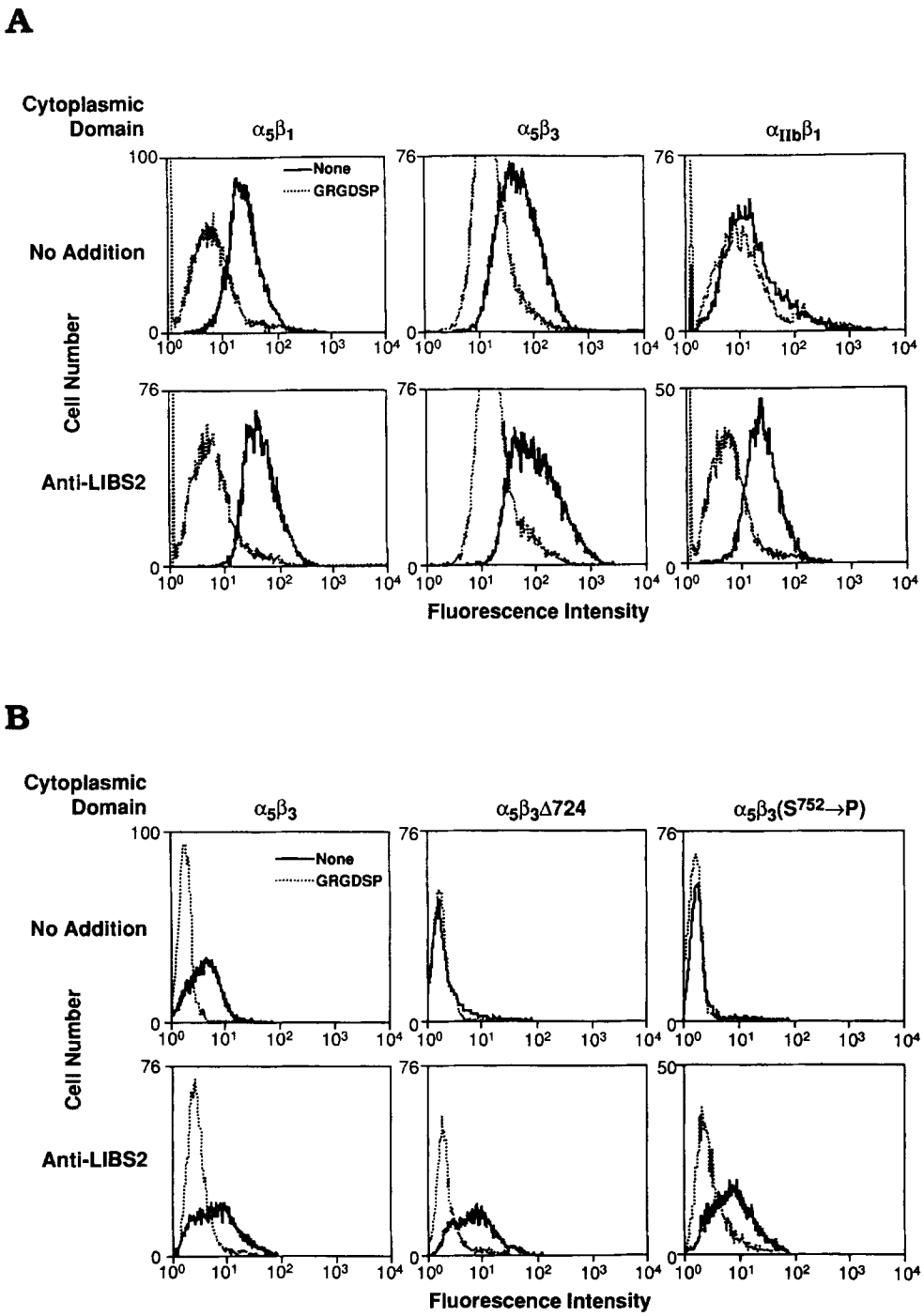


Figure 5. Role of both α and β cytoplasmic domains in affinity modulation. (*A*) The α subunit cytoplasmic domain specifies affinity state. CHO cells were transiently transfected with subunits comprised of the extracellular and transmembrane domains of α_{IIb} and β_3 joined to the indicated cytoplasmic domains. The affinity state of the extracellular portion of $\alpha_{IIb}\beta_3$ was assessed by PAC1 binding. Binding was analyzed in the absence (*solid line*) or presence (*dotted line*) of GRGDSP peptide. Histograms of cells incubated in the presence of 6 μM anti-LIBS2 are depicted in the lower panels. Specific PAC1 binding is present in both transfectants containing the α_5 cytoplasmic domain irrespective of the presence of either the β_3 or β_1 cytoplasmic domain on the β_3 subunit. In contrast, PAC1 specifically bound to those transfectants containing the α_{IIb} cytoplasmic domain only in the presence of the activating antibody, anti-LIBS2. (*B*) Affinity state is dependent upon the β_3 cytoplasmic domain. Stable CHO cell lines expressing recombinant $\alpha_{IIb}\beta_3$ chimeras containing the noted cytoplasmic domains were reacted with PAC1 and bound antibody was detected by flow cytometry as described in Materials and Methods. Binding was analyzed in the absence (*solid line*) or presence (*dotted line*) of GRGDSP peptide. The intrinsic functionality of each construct was assessed by PAC1 binding in the presence of 6 μM anti-LIBS2 (*lower panels*). A β_3 cytoplasmic truncation (Δ724) and single amino acid substitution($S^{752}\rightarrow P$) both abolished the constitutive high affinity state conferred by the cytoplasmic domain of α_5.

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 NaN_3 and 2-deoxyglucose. Finally, the $\alpha_L\Delta$ 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 $\alpha_5\beta_1$ is regulated by cell type-specific factors. The high affinity state requires active cellular processes suggest-

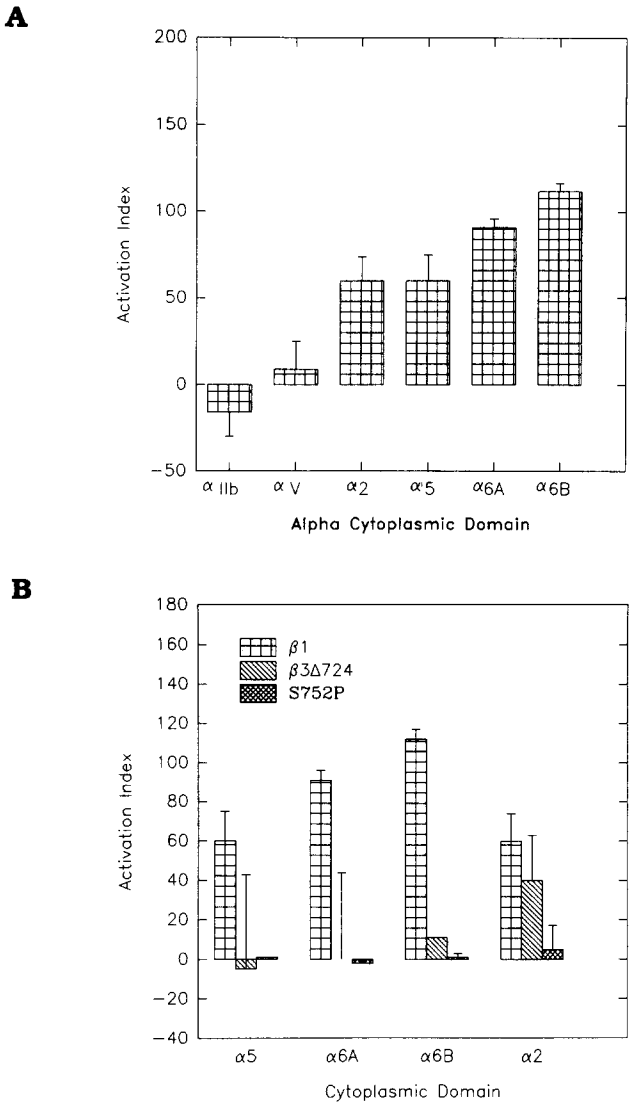


Figure 6. Affinity state is α subunit cytoplasmic domain specific. (*A*) Effect of different α cytoplasmic domains. Chimeric α subunits consisting of extracellular and transmembrane α_{IIb} with the indicated cytoplasmic domain were transiently cotransfected with β_3 into CHO cells. PAC1 binding was quantified by flow cytometry and the activation index was calculated as: $100*(F_O-F_R)/F_R$ where: F_O = Mean Fluorescence Intensity in the absence of inhibitor; F_R = Mean Fluorescence Intensity in the presence of 2 mM GRGDSP. 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. α Subunit chimeras containing the indicated cytoplasmic sequences were cotransfected with a β_3 subunit whose cytoplasmic domain was truncated ($\beta_3\Delta724$), contained the $S^{752}\rightarrow P$ mutation (*S752P*), or had been exchanged for the homologous region of β_1. PAC1 binding was analyzed as described in panel *A*. Mean ± SD of at least three independent experiments for each α β pair.

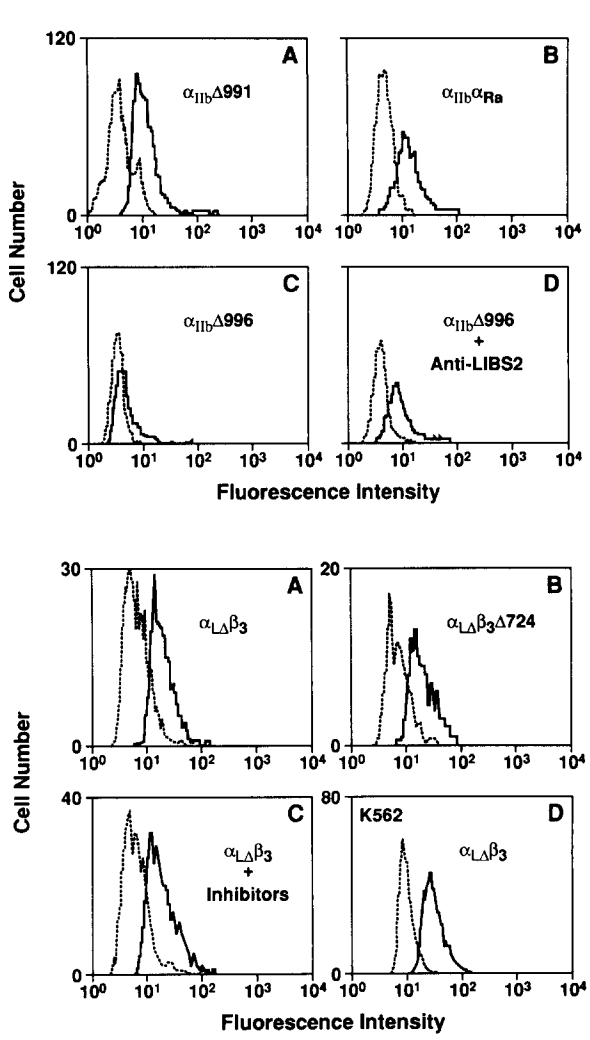


Figure 7. GFFKR deletion variants confer an energy and β subunit cytoplasmic domain-independent high affinity state. (*A*) GFFKR sequence deletion activates $\alpha_{IIb}\beta_3$. Stable CHO cell lines were established by cotransfection of α_{IIb} containing the noted α cytoplasmic domain with wild-type β_3. PAC1 binding in the absence (*solid line*) and presence (*dotted line*) of GRGDSP was assessed by flow cytometry. The $\alpha_{IIb}\Delta991$ transfectant, which lacks GFFKR, specifically binds PAC1 (panel *A*). In contrast, the $\alpha_{IIb}\Delta996$ transfectant, which retains GFFKR, binds only after activation with anti-LIBS2 (panels *C* and *D*). Replacement of the α_{IIb} cytoplasmic domain with random sequence also induces PAC1 binding (panel *B*, α_{Ra}). (*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 $\alpha_{IIb}\beta_3$ joined to the indicated cytoplasmic domains. Specific PAC1 binding to the population of cells expressing $\alpha_{IIb}\beta_3$ 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% NaN_3 and 2 mM 2-deoxyglucose (panel *C*). This treatment abolished ligand binding to an $\alpha_{IIb}\beta_3$ chimera bearing the cytoplasmic domain of $\alpha_5\beta_1$ (not shown, but cf. Fig. 4 *A*). High affinity state was also maintained despite an extensive deletion of the β_3 cytoplasmic domain (panel *B*) that disrupted PAC1 binding to the $\alpha_5\beta_1$ chimera (not shown but cf. Fig. 5 *B*). Similar results were obtained with $\alpha_{IIb}\Delta991$ and α_{Ra} transfectants. A stable K562 cell line bearing the GFFKR deletion mutant specifically bound PAC1 (panel *D*), but the $\alpha_5\beta_1$ chimera was not active in these cells (cf. Fig. 3 *A*).

ing that low affinity may be the default state. (*b*) Cell type-specific signals that modulate integrin affinity are transmitted through the cytoplasmic domains. Both cytoplasmic domains are involved in these signaling events. (*c*) Certain deletions in the conserved GFFKR sequence in the α subunit cytoplasmic domain reset the default state to high affinity. In

Table I. Summary of Affinity States of the Extracellular Domain: Physiological Modulation

	β3	β3Δ724	β3S752P	β1	β2
αIIb	LO	LO	LO	LO	–
α2	HI	LO	LO	HI	–
α5	HI	LO	LO	HI	–
α6A	HI	LO	LO	HI	–
α6B	HI	LO	LO	HI	–
αL	LO	–	–	–	LO
αM	LO	–	–	–	LO
αv	LO	–	–	–	–

Summary of the affinity states, as assayed by PAC1 binding to CHO cells transiently transfected with chimeras of the extracellular and transmembrane domains of $\alpha_{IIb}\beta_3$ 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 NaN_3 and 2-deoxyglucose resulted in reversion to the low affinity state.

these deletion mutants, the high affinity state is independent of cell type, and is not blocked by metabolic inhibitors or by near complete truncation of the β subunit tail. These studies show that the cytoplasmic domains of integrins control their affinity state. Thus, these domains become attractive targets for approaches to alter cell adhesion. Further, the conserved GFFKR motif of the α subunit cytoplasmic domain maintains the default low affinity of the extracellular domain.

The ligand-binding affinity of integrin $\alpha_5\beta_1$ depends on the cell type in which it is found. Such differences in ligand-binding affinity could well account for previous reports of cellular variation in the adhesive function of integrins (1, 15, 25, 35, 49, 55, 62, 66, 94). It is possible that cell type-dependent affinity differences are due to structural variations in $\alpha_5\beta_1$ synthesized in the different cell types. Permanent structural variants (e.g., alternative splicing [5, 11, 41, 42, 92], alternative proteolytic processing [58]) can be excluded as the sole cause of the affinity differences reported here because the affinity of $\alpha_5\beta_1$ was reversibly decreased by metabolic inhibitors. Consequently, the increased affinity of $\alpha_5\beta_1$ in CHO cells seems the result of active cellular signals. Since the metabolic inhibitors caused the reversion of $\alpha_5\beta_1$ to the low affinity state, it seems that low affinity is the default state of $\alpha_5\beta_1$. Similarly $\alpha_{IIb}\beta_3$ and $\alpha_M\beta_2$ usually require active cellular processes for high affinity ligand binding (4, 61). Furthermore, purified $\alpha_{IIb}\beta_3$ in liposomes spontaneously reverts to low affinity after removal of activating ligands (84). Thus, default low affinity state may be a general property of integrins.

Signals that modulate the affinity of $\alpha_5\beta_1$ are targeted through the integrin's cytoplasmic domains. Joining the cytoplasmic domains of $\alpha_5\beta_1$ onto the extracellular and transmembrane domains of $\alpha_{IIb}\beta_3$, an exchange involving less than 5% of the total mass of the integrin, caused the $\alpha_{IIb}\beta_3$ to assume the high affinity state in CHO cells. Biologically relevant signals mediated the increased affinity of the chimera since: (*a*) it was cell-type specific, (*b*) it could be reset to the default low affinity by addition of inhibitors of oxidative phosphorylation and anaerobic glycolysis, and (*c*) a β_3 cytoplasmic domain mutation ($S^{752}\rightarrow P$) returned the chimeric integrin to the low affinity state. This naturally occurring mutation is associated with defective activation of $\alpha_{IIb}\beta_3$ in platelets (16). Cell-type specific signaling elements that interact with the integrin cytoplasmic domains could readily explain the distinctive cellular influences on integrin affinity. In view of the marked sequence divergence of the α subunit tails (Fig. 2), multiple such integrin-specific elements might exist.

As summarized in Table I, the α subunit cytoplasmic domain designated integrin-specific differences in affinity state. In CHO cells, α_5, α_6A and B, and α_2 cytoplasmic tail chimeras specified the high affinity state. The $\alpha_{IIb}\Delta996$ 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 α_5, α_6A and B, and α_2. Thus, deletion of related sequences could account for the reduction of cell adhesion by certain truncations of the α_4 (45) or α_2 (46) cytoplasmic tail. In addition, truncations of α_{IIb} or α_1 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 α_{IIb} cytoplasmic domain specifies a low affinity state but still constrains the localization of $\alpha_{IIb}\beta_3$ 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_1 or β_3 were required for the activation of the α subunit chimeras. Moreover, the capacity of $\beta_3(S^{752}\rightarrow P)$ to disrupt activation, underscores the specificity of the β subunit requirement. Interestingly, β_3 Ser^{752} is homologous to a Thr (Fig. 2) involved in the adhesive function of $\alpha_L\beta_2$ (38). S^{752} is not extensively phosphorylated in platelets (40). We have found no obvious difference in the phosphorylation of α_6 cytoplasmic domain chimeras in CHO cells and K562 cells (unpublished results). Thus, the mechanism of the effect of the $S^{752}\rightarrow P$ mutation on inside-out signaling remains to be resolved.

Mutations that delete portions of the conserved GFFKR sequence appear to reset $\alpha_{IIb}\beta_3$ to a default high affinity state. As summarized in Table II, a truncation mutant that removes this sequence ($\alpha_{IIb}\Delta991$) is constitutively active,

Table II. Summary of Affinity States of the Extracellular Domain: Hinge Mutants

	β3	β3Δ724	β3S752P
αIIb	LO	LO	LO
αIIbΔ991	HI*	HI	HI
αIIbΔ996	LO*	LO	LO
αLΔ	HI*	HI	HI
αL	LO*	–	–
αRα	HI*	HI	HI

Summary of the affinity states of the extracellular domains of $\alpha_{IIb}\beta_3$ 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 NaN_3 and 2-deoxyglucose did not result in reversion to the low affinity state.

whereas a truncation that retains this sequence (α_{IIb}Δ996) remains inactive. Moreover, a KLGFF loop out mutation in the α_L 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_5, lacking GFFKR, is more efficient at assembling a Fn matrix than wild-type α_5 (96). This effect could be due to increased Fn binding, since matrix assembly may be regulated by the affinity state of $\alpha_5\beta_1$ (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 $\alpha_{IIb}\beta_3$ 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

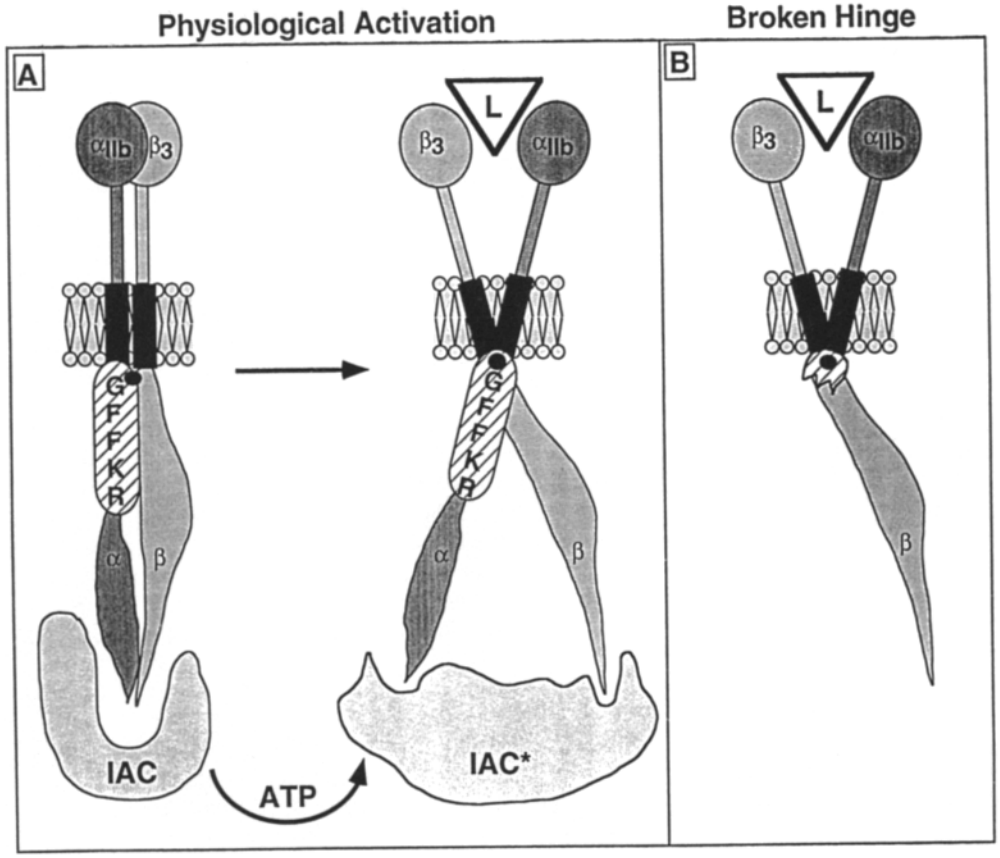


Figure 8. Working hypothetical model of affinity modulation of integrins. Depicted is a schematic that accommodates the results reported here. Cell-type specific energy-dependent cytoplasmic signals target the integrin cytoplasmic domains. The unidentified factor(s) responsible for these signals (represented 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 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 calreticulin-GFFKR interaction or one like it may set 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 highly conserved site in this receptor family (27, 77, 78). Moreover, in the glucocorticoid receptor, this region forms 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, KXGFFKR presumably adjoins an α helix. By analogy with the steroid fingers, GFFKR may therefore possess α 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 conformation 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.

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