Integrin Cytoplasmic Domains Mediate Inside-Out Signal Transduction

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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 αmβ3 joined to the cytoplasmic domains of αtβ1. The affinity state of these chimeras was assessed by binding of fibrinogen or the monoclonal antibody, PAC1. The cytoplasmic domains of α5β1 conferred an energy-dependent high affinity state on αmβ3 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, αv) did not. In the high affinity α chimeras, cotransfection with a truncated (β3Δ724) or mutated (β3(S552→P)) 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 αmβ3. 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 α5β1 (7), 55β1 (4), and 5β1 (26) integrins. Changes in adhesive function may also occur without changes in ligand-binding affinity. For example, phorbol esters stimulate the α5β1-dependent adhesion of CHO cells (20) to fibronectin (Fn) with no change in Fn-binding affinity. Similarly, certain β1 mutations reduce αmβ1-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 α5β1 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.

αmβ3 (platelet GPIIb-IIIa) is a prototype integrin for analysis of changes in integrin affinity. As with all integrins, αmβ3 is a heterodimer of two Type I transmembrane protein subunits (43). αmβ3 is platelet-specific (88), and affinity state-specific antibodies, e.g., PACI (81), simplify analysis of recombinant αmβ3 in heterologous cells (67). Conformational changes in the extracellular domain of αmβ3 regulate its affinity (67, 82). Platelet agonists increase the affinity of αmβ3 ("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.
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 α5β1 is pretreated with RGD peptides, it subsequently binds Fg and PAC1 (23, 50, 85). Certain anti-β3 antibodies directly increase the Fg-binding affinity of α5β1 (29) and certain anti-β3 antibodies activate α5β1 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 α1β3 (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 α5β3 cytoplasmic domain after residue 990 (α5β3991) results in α5β3, that constitutively binds Fg and PAC1 (69). Conversely, truncations of α5 (45), α2 (46), and β3 (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 Ser252 to Pro mutation in the β3 cytoplasmic domain was associated with an apparent α5β3 activation defect (16) in a single individual. Cytoplasmic domains of several integrins are phosphorylated coincidently 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 β3, and reduced binding of α5β3 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 α5β3, 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 α5β3. 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-α5β3 antibody D57 was produced by Dr. Xiaoping Du (Scripps Research Institute) using previously described methods (28). It binds to CHO cells transfected with α5β3 but not α5β3, and does not block Fg binding to α5β3. This antibody was biotinylated with biotin-N-hydroxysuccinimide (Sigma Chem. Co., St. Louis, MO) according to manufacturers' directions. The α5β3 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 α5 (FBI) and anti-β3 (1E10) antibodies were obtained from Dr. Rudolph Juliano (10) (University of North Carolina, Chapel Hill), and the β3 activating antibody, 8A2, was supplied by Drs. Nick Kovach and John Harlan (51) (University of Washington, Seattle). A human anti-α5 antibody, BIIG2, was supplied by Dr. Caroline Dansky (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-LIB6, anti-LIB82, anti-α5β3 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 spectrometry.

Cell Culture and Transfection

The human cell lines K562, U937, W138, 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), pan- 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 (Irvin Scientific), washed with PBS, and combined with appropriate cDNAs (10 μg each subunit). 3 x 10^6 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 about 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 10^6 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 x 10^6 cells were incubated on ice for 30 min with primary antibody, washed, and then in-}

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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 αmβ3 expression (phycoerythrin staining). To define affinity state, histograms depicting PAC1 staining in the absence or presence of 1 mM GRGDSP were superimposed. Since GRG peptides are inhibitors of PAC1 binding to αmβ3 (8), a rightward shift in the histogram in the absence of peptide is indicative of the presence of high affinity αmβ3. 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:

$$100\times(F_0-F_R)/F_R$$

where:

- $F_0$ = Mean Fluorescence Intensity in the absence of inhibitor
- $F_R$ = Mean Fluorescence Intensity in the presence of GRGDSP

Expression of αmβ3 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 α 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 αLΔ 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 αmβ8β2, αmβ8β5, αmβLΔ724, αmβ8β3, and αmβ8β2Δ78. 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 CD83 constructs encoding αm, αmΔ991, αmΔ996, β3, and β3Δ728 have been previously described (68, 69, 97). The β3 truncation, Δ724, and amino acid substitution, S1024F, were first generated in B53a (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 CD83. The β3 chimera, containing the β1 cytoplasmic domain, was constructed by first generating an EcoRI site at bases 2387-2392 of β3 cDNA sequence. After HindIII digestion, a 400-bp fragment containing the complete β1 cytoplasmic domain and partial 3’ non-coding sequences was isolated and subcloned into the HindIII site of CD83. The β3 cytoplasmic domain was then ligated with EcoRI and with a 2.2-kb EcoRI fragment from CD3α (68) containing its transmembrane and extracellular domains. β3 cytoplasmic sequences were first isolated by the PCR from a β3 cDNA, and then subcloned into the MufI and Xhol sites of CD83. The β2β3 cytoplasmic domain chimera was then generated by digestion with MufI and HindIII and ligation with a corresponding MufI HindIII fragment from CD83 containing its extracellular and transmembrane sequences. Chimeric α subunits were generated using a previously described strategy (69). Cytoplasmic sequences from αm, αmβ3, αL, αLβ2, αA, and αB were isolated from the appropriate cDNA clones by PCR (57). Amplified products were digested with HindIII and Xbal and subcloned into HindIII and Xbal cut CD83. After digestion with HindIII, these constructs were ligated with a HindIII fragment from CD2β (68) containing its extracellular and transmembrane domains. PCR oligonucleotides for αLΔ were designed to omit the VGGFK sequence. Its construction followed the procedure for other α chimeras. The αLβ2 variant was made by first generating a SalI site in CD2β coding sequences corresponding to bases 3061-3066. This vector was then digested with SalI and Xbal and ligated to a SalI-Xbal 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 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 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. 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 mM NaCl, 50 mM acetylglucoside, 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 antiserum directed against the αm or αL cytoplasmic domains and a monoclonal antibody against the αmβ3 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 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 αm (2bsf: CGGCCCTTGAGGAGGGCCTTACCC) and 3’ primers specific for the cytoplasmic sequences of αm (αm5ct: CTCTGGCTGGAGGGAAAGCGA) and αL (αLct: TGAAAACAGGGCTCC1C). Amplified products were analyzed by agarose gel electrophoresis.

Results

Cell Type-Specific and Density-dependent Affinity Modulation of Integrin αmβ3

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 αmβ3. 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 Fn with only low affinity (Kd >1 μM), e.g., CHO, WI-38, and MG63 cells (Fig. 1 A). The low affinity αmβ3 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 αmβ3 (Fig. 1 B). Specificity of Fn binding to high affinity αmβ3 was verified by inhibition with an anti αL antibody (Fig. 1 A).

To find out whether spontaneous high affinity Fn binding
Figure 1. The high affinity state of α5β1 is cell type-specific and energy-dependent. (A) 125I-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. α5β1-specific binding was established by blocking binding to the CHO cells with PB1, an anti-hamster α5. Binding to K562 cells was induced by addition of 20 nM activating antibody (8A2) and was inhibited by the anti-α5, BIIG2. (B) The level of surface expression of α5β1, in the two cell types. CHO and K562 cells were stained with irrelevant mouse IgG (dotted line), an anti-β1 antibody (K562:8A2, CHO:7E2) (solid line), or an anti-α5 antibody (K562:BIIG2, CHO:PB1) (dashed line), and then analyzed by flow cytometry as described in Materials and Methods. (C) The binding of 125I-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 α5β1 was verified by inhibition with the PBl 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 αβ (residue 990) and β (residue 727) sequences denote the position at which chimeric cytoplasmic domains were joined to the extracellular and transmembrane domains of α5 and β. The position of stop codons producing cytoplasmic truncations are noted by triangles, while the S752→P point mutation in β is indicated. The residues deleted in the α5Δ cytoplasmic domain are overlain by the heavy line.

The Cytoplasmic Domains of αβ Confer an Energy-dependent High Affinity State on α5β1 in Some Cells but Not Others

To decide whether the cytoplasmic domains of αβ were involved in cell type-specific affinity modulation, we generated chimeras in which the cytoplasmic domains of α5 and β were replaced with the corresponding sequences from α5 and βA (Fig. 2). The α and β chimeras were then cotransfected into CHO or K562 cells, and the affinity state of the extracellular α5β reporter group was assayed by binding of PAC1, an antibody specific for the high affinity state of α5β (81). The double chimera bound PAC1 when it was expressed in CHO cells (Fig. 3). Since wild-type α5β does not bind PAC1 when expressed in CHO cells (67), it is concluded that the α5β cytoplasmic domains conferred the high affinity state on α5β. In sharp contrast, PAC1 did not bind to the double chimera in K562 cells. However, PAC1
Fig. 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 α5 and β1 and the affinity state of the αmβ3 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 chimera noted (as = α5 cytoplasmic domain chimera) were surface iodinated, lysed, and immunoprecipitated with polyclonal antibodies specific for the α5 and αm cytoplasmic domains or with a monoclonal antibody reactive with the extracellular domain of αmβ3 (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 αm under certain conditions (12), it was necessary to verify that all of the αm expressed in the α chimera transfectants contained the αm cytoplasmic domain. Immunoprecipitation of α chimera transfectants with an anti-α5 cytoplasmic domain antibody isolated polypeptides corresponding to transfected αm and β3 chimeras and endogenous αmβ3 (Fig. 3 B). In contrast, an anti-αm cytoplasmic domain antibody immunoprecipitated no labeled polypeptides. An anti-α5 cytoplasmic domain antibody precipitated only endogenous αmβ3 from wild-type αmβ3 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 αm and 3′ primers specific for cytoplasmic domains of αm or α (Fig. 3 C). A specific 393-bp band was observed from α chimera transfectants when primed with the 3′α3 oligonucleotide. A specific 294-bp band was observed with wild-type αm transfectants when primed with the 3′ αm oligonucleotide. No bands were observed when inappropriate 3′ primers were used.

As was shown in Fig. 1, high affinity Fn binding to αmβ3 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 αmβ3 cytoplasmic sequences confer a cell type-specific, energy-dependent, high affinity state on the extracellular domain of αmβ3.

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 subunits bound PAC1 (Fig. 5 A). In contrast, cells expressing the β chimera with wild-type αm 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,
cytoplasmic variants, $\beta_1\Delta 724$ and $\beta_4(S^{792}\rightarrow P)$. The former is a truncation mutant that ends at $D^{721}$ while the latter contains a single nucleotide alteration resulting in a Ser$^{792}$$\rightarrow$ Pro substitution (Fig. 2). These $\beta_1$ cytoplasmic domain mutants were then cotransfected with the $\alpha$ chimera. In contrast to wild-type $\beta_1$, coexpression of either $\beta_1$ 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 $\alpha_{1b}$ and $\alpha_5$ specify different affinity states in CHO cells; $\alpha_{1b}$ 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 $\beta_3$ into CHO cells. The $\alpha$ cytoplasmic domains of three other $\beta_1$ family members ($\alpha_2$, $\alpha_4$, $\alpha_6$) conferred PAC1 binding (Fig. 6 a), while those chimeras containing $\alpha_5$ cytoplasmic domains from $\beta_3$ ($\alpha_5\alpha$, $\alpha_6$) (data not shown) or $\beta_5$ ($\alpha_5$) (Fig. 6 A) families did not. The same result was obtained with the $\beta$ cytoplasmic domains of the relevant $\beta$ subunit partner ($\beta_3$ for $\alpha_2$, $\alpha_3$, $\alpha_4$, and $\alpha_5$ or $\beta_5$ for $\alpha_6$ and $\alpha_7$). Similar to the $\alpha_5$ chimera, constitutive PAC1 binding was also dependent upon the $\beta$ cytoplasmic domain. It was lost when the $\alpha_5\alpha$, $\alpha_6\alpha$, or $\alpha_7\beta_3$ chimeras were cotransfected with $\beta_3\Delta 724$ or $\beta_5S^{752}P$ (Fig. 6 B). Thus, the $\alpha_5$ 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 $\alpha_{1b}\beta_3$ results from a truncation of the cytoplasmic domain of $\alpha_{1b}$ (69). To identify the important deleted $\alpha_{1b}$ 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 $\alpha_5\Delta 991$ truncation eliminates this motif and results in constitutive PAC1 binding (Fig 7 A; panel A) whereas a truncation after the GFFKR ($\alpha_5\Delta 996$) 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 ($\alpha_5\Delta$) in CHO cells with $\beta_3$ 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 ($\alpha_5\Delta$) also conferred high affinity binding when expressed in CHO cells with wild-type $\beta_3$ (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 $\beta$ 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 $\beta_3$ subunit (Fig. 7 B; panel B). In addition, in contrast to transfectants expressing constitutively active $\alpha$

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**Figure 4.** The high affinity state of the $\alpha_5$ cytoplasmic domain chimera is energy-dependent. (A) PAC1 binding. Stable CHO transfectants expressing the $\alpha_5$ and $\beta_3$ 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) or washout of these inhibitors (Inhibitors + Washout) were assayed for binding to transfected PAC1. (Inhibitors + Anti-LIBS2) were incubated with 2 mg/ml deoxyglucose and 0.1% NaN$_3$ (Inhibitors) or washout of these inhibitors (Inhibitors + Washout) were assayed for binding to transfected PAC1.

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**Figure 5.** The $\alpha_5$ cytoplasmic domain chimera is a truncation mutant that ends at $D^{721}$ while the latter contains a single nucleotide alteration resulting in a Ser$^{792}$$\rightarrow$ Pro substitution (Fig. 2). These $\beta_1$ cytoplasmic domain mutants were then cotransfected with the $\alpha$ chimera. In contrast to wild-type $\beta_1$, coexpression of either $\beta_1$ 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.

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These data established that the cytoplasmic domains of $\alpha_{1b}$ and $\alpha_5$ specify different affinity states in CHO cells; $\alpha_{1b}$ 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 $\beta_3$ into CHO cells. The $\alpha$ cytoplasmic domains of three other $\beta_1$ family members ($\alpha_2$, $\alpha_4$, $\alpha_6$) conferred PAC1 binding (Fig. 6 a), while those chimeras containing $\alpha_5$ cytoplasmic domains from $\beta_3$ ($\alpha_5\alpha$, $\alpha_6$) (data not shown) or $\beta_5$ ($\alpha_5$) (Fig. 6 A) families did not. The same result was obtained with the $\beta$ cytoplasmic domains of the relevant $\beta$ subunit partner ($\beta_3$ for $\alpha_2$, $\alpha_3$, $\alpha_4$, and $\alpha_5$ or $\beta_5$ for $\alpha_6$ and $\alpha_7$). Similar to the $\alpha_5$ chimera, constitutive PAC1 binding was also dependent upon the $\beta$ cytoplasmic domain. It was lost when the $\alpha_5\alpha$, $\alpha_6\alpha$, or $\alpha_7\beta_3$ chimeras were cotransfected with $\beta_3\Delta 724$ or $\beta_5S^{752}P$ (Fig. 6 B). Thus, the $\alpha_5$ 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 $\alpha_{1b}\beta_3$ results from a truncation of the cytoplasmic domain of $\alpha_{1b}$ (69). To identify the important deleted $\alpha_{1b}$ 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 $\alpha_5\Delta 991$ truncation eliminates this motif and results in constitutive PAC1 binding (Fig 7 A; panel A) whereas a truncation after the GFFKR ($\alpha_5\Delta 996$) 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 ($\alpha_5\Delta$) in CHO cells with $\beta_3$ 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 ($\alpha_5\Delta$) also conferred high affinity binding when expressed in CHO cells with wild-type $\beta_3$ (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 $\beta$ 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 $\beta_3$ subunit (Fig. 7 B; panel B). In addition, in contrast to transfectants 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 NaN_3 and 2-deoxyglucose. Finally, the αΔ 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 αβ, 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 subunit cytoplasmic domains. Chimeric α subunits consisting of extracellular and transmembrane αm3 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 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 β3 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 αmβ3. Stable CHO cell lines were established by cotransfection of αmβ3 containing the noted α cytoplasmic domain with wild-type β3. PAC1 binding in the absence (solid line) and presence (dotted line) of GRGDSP 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, αm3). (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 αmβ3 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 αmβ3 chimera bearing the cytoplasmic domain of α5β3 (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 αmβ3 chimera (not shown but cf. Fig. 5 A). Similar results were obtained with αmΔ991 and αm6a transfectants. A stable K562 cell line bearing the GFFKR deletion mutant specifically bound PAC1 (panel D), but the αsβ3 chimera was not active in these cells (cf. Fig. 3 A).
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 main-
tains the default low affinity of the extracellular domain.

The ligand-binding affinity of integrin αβ1 depends on
the cell type in which it is found. Such differences in ligand-
binding affinity could well account for previous reports of
the cell type in which it is found. Such differences in ligand-

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

| α/β | β3 | β3Δ724 | β3S752P | β1 | β2 |
|-----|----|--------|---------|----|----|
| αLb | LO | LO     | LO      | LO | –  |
| a2  | HI | LO     | LO      | HI | –  |
| a5  | HI | LO     | LO      | HI | –  |
| a6A | HI | LO     | LO      | HI | –  |
| a6B | HI | LO     | LO      | HI | –  |
| aL  | LO | –      | –       | –  | LO |
| aM  | LO | –      | –       | –  | LO |
| αV  | LO | –      | –       | –  | –  |

Summary of the affinity states, as assayed by PAC1 binding to CHO cells transiently transfectd with chimeras of the extracellular and transmembrane domains of αmβ1 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.

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 ele-
ments might exist.

As summarized in Table I, the α subunit cytoplasmic do-
main designated integrin-specific differences in affinity state.
In CHO cells, α, αA and B, and α2 cytoplasmic tail chimeras specified the high affinity state. The αmΔ996
truncation mutant leaves the common (Fig. 2) KXGGFKR
motif and results in a low affinity state. This suggests that act-
ivation signaling sequences important for conversion of the
integrin to a high affinity state reside carboxyterminal of
GFFKR in α3, αA 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 α2 (46) cytoplasmic
tail. In addition, truncations of αm or α1 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 adhe-
sions. In CHO cells, the full-length αm cytoplasmic do-
main specifies a low affinity state but still constrains the lo-
galization of αmβ3 to focal adhesions. Thus, we suspect that the α cytoplasmic domain elements that control targeting to
d 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 β1 or β2 were required
for the activation of the α subunit chimeras. Moreover, the
capacity of β2(S752→P) to disrupt activation, underscores
the specificity of the β subunit requirement. Interestingly,
β1 Ser752 is homologous to a Thr (Fig. 2) involved in the
adhesive function of αmβ2 (38). S752 is not extensively phos-
phorylated in platelets (40). We have found no obvious
difference in the phosphorylation of αm cytoplasmic domain
chimeras in CHO cells and K562 cells (unpublished results).
Thus, the mechanism of the effect of the S752→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

|          | β3   | β3Δ724 | β3S752P |
|----------|------|--------|---------|
| αllb     | LO   | LO     | LO      |
| αllbΔ991 | HI*  | HI     | HI      |
| αllbΔ996 | LO*  | LO     | LO      |
| αLa      | HI*  | HI     | HI      |
| αL       | LO*  | -      | -       |
| αRa      | HI*  | HI     | HI      |

Summary of the affinity states of the extracellular domains of αllbβ3 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 (αllbΔ996) 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 αl, lacking GFFKR, is more efficient at assembling a Fn matrix than wild-type αl (96). This effect could be due to increased Fn binding, since matrix assembly may be regulated by the affinity state of αlβ3 (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 αllbβ3 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 subunits.
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 though 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. This shift may result in outside-in signaling. In integrins, the 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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