**Breaking the Integrin Hinge**

A DEFINED STRUCTURAL CONSTRAINT REGULATES INTEGRIN SIGNALING*

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Integrins are heterodimeric (α, β) cell adhesion receptors. We demonstrate that point mutations in the cytoplasmic domains of both the α and β subunits promote constitutive signaling by the integrin α1β2. By generating charge reversal mutations, we show these "activating" mutations may act by disrupting a potential salt bridge between the membrane-proximal portions of the α and β subunit cytoplasmic domains. Thus, the modulation of specific interactions between the α and β subunit cytoplasmic domains may regulate transmembrane signaling through integrins. In addition, these activating mutations induce dominant alterations in cellular behavior, such as the assembly of the extracellular matrix. Consequently, somatic mutations in integrin cytoplasmic domains could have profound effects in vivo on integrin-dependent functions such as matrix assembly, cell migration, and anchorage-dependent cell growth and survival.

The integrin family of cell adhesion receptors are heterodimers of α and β transmembrane subunits that play key roles in important biological processes such as inflammation, wound healing, and cell growth and survival. Integrins modulate their affinity for ligands via a process termed "activation" or "inside-out" signaling (1, 2). Furthermore, ligand binding to integrins changes the activities of cytoplasmic kinases, GTPases, and phospholipases ("outside-in" signaling) (2-5). Thus, integrins are bidirectional signaling receptors conducting information both into and out of the cell.

Inside-out signaling may involve the propagation of a conformational change from the integrin cytoplasmic domains to the extracellular domains resulting in high affinity ligand binding (6, 7). Integrin α and β subunit cytoplasmic domains share a similar membrane-proximal organization with apolar and polar sequences following sequentially after the membrane-cytosol interface (Fig. 1A). The conserved sequences for the α and β subunits are -GFFKR and LLv-iHDR (highly conserved residues are uppercase, less conserved residues are lowercase, and dashes represent nonconserved residues). Deletion of these sequences in either α or β subunit cytoplasmic domain "activates" integrins, locking them in the high affinity state (8-11). Consequently, we termed this region the integrin "hinge." Hence, the capacity of the conserved membrane-proximal motifs to regulate the affinity state of integrins may depend on an interaction between them that constrains integrins to a low affinity state. Furthermore, studies on integrin assembly suggested that there may be an interaction between the membrane-proximal portions of the α and β subunit cytoplasmic domains (12).

In this paper, we describe point mutations in this hinge region that activate α1β2. Moreover, by generating complementary charge reversal mutations, we show these activating mutations may act by disrupting a potential salt bridge between the membrane-proximal portions of the α and β subunit cytoplasmic domains. In addition, we demonstrate that these active mutations can induce constitutive outside-in signaling as assayed by the phosphorylation of pp125FAK and the ligand independent recruitment of α1β2 to focal adhesions.

**MATERIALS AND METHODS**

Antibodies—The anti-α1β2 antibody D57 (9) was biotinylated with biotin-N-hydroxysuccinimide (Sigma) according to the manufacturer's directions. The isolation and characterization of anti-LIBS6 (13) and PAC1 (14) have been described previously.

cDNA Constructs—pCDM8 expression constructs encoding wild-type α1 and β3 were constructed as described (9). The generation of expression constructs in pCDM8 encoding point mutation α1 and β3 DNAs were undertaken using PCR mutagenesis (15). CDM8 expression vectors encoding β3 mutants were constructed by cloning a 0.9-kb MluI and PstI cut PCR fragment encompassing the mutations into MluI and PstI cut pCDM8. This construct was then cut with AflI and DraI, and the 3-kb fragment ligated with the 3.5-kb AflI-DraIII fragment of pCD8β3. pCD8β3 expression vectors encoding α1 mutants were constructed by tripartite ligation of a 0.6-kb XbaI-BamHI cut PCR fragment encompassing the mutations, the 4.1-kb BamHI-DraIII fragment of pCD8α and the 2.6-kb DraIII-XbaI fragment of pCDM8. All constructs were verified by DNA sequencing and purified by CsCl centrifugation before transfection. Mutagenic oligonucleotides were synthesized on a model 391 DNA synthesizer.

PAC1 Binding—The cDNAs expressing α1β2 variants were transiently transfected into Chinese hamster ovary (CHO-K1) as described (16). PAC1 binding was then analyzed by two-color flow cytometry as described (9, 10). Briefly, FITC-PAC1 binding was analyzed on a gated subset of cells positive for α1β2 expression detected with a biotinylated non-function blocking antibody to α1 and β3 (D57) and phycoerythrin/streapavidin. To define affinity state, histograms depicting PAC1 staining in the absence or presence of the competitive inhibitor Ro 43-5054 (32) were compared. The activation index (AI) was defined as 100 × (Fo - Fc)/(Fc - LIBS6 - Fc - LIBS6), where Fc is the median fluorescence intensity of PAC1 binding and Fo is the median fluorescence intensity of PAC1 binding in the presence of competitive inhibitor Ro 43-5054 (1 μM). Fc-LIBS6 is the median fluorescence intensity of PAC1 binding in the presence of competitive inhibitor Ro 43-5054 (1 μM).

Analysis of pp125FAK Phosphorylation—100-mm tissue culture plates were coated with 5 mg/ml BSA or 100 μg/ml fibronectin and blocked with 5 mg/ml BSA. The cells were harvested and resuspended...
in incubation buffer (137 mM NaCl, 2.7 mM MgCl₂, 5.6 mM glucose, 3.3 mM NaH₂PO₄, and 20 mM HEPES, pH 7.4). 1 × 10⁵ cells were added to each coated dish and incubated at 37 °C for 90 min. Cells were then washed in PBS and lysed with RIPA buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, and 100 kallikrein inactivating units/ml aprotinin. Lysates were clarified by centrifugation, and the protein content was determined with the BCA reagent. 300 μg of protein from each lysate were incubated overnight at 4 °C with the anti-pp125Ak rabbit polyclonal antibody B3C (a generous gift from J. Thomas Parsons, Charlottesville, VA). Immune complexes were precipitated at 4 °C with protein A-Sepharose and washed extensively in ice-cold RIPA buffer containing 1 mM Na₃VO₄. The immune complexes were extracted into Laemmli sample buffer containing 10% β-mercaptoethanol, subjected to SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels, and electrotransferred. Western blots were prepared and analyzed for phosphotyrosine-containing proteins as described (17) using a mixture of the anti-phosphotyrosine antibodies PY20 and PY72 (a generous gift from Bart Sefton, La Jolla, CA).

**RESULTS AND DISCUSSION**

To define those membrane-proximal residues of the α subunit important for affinity modulation, we substituted specific residues in α₁β₃ with Ala. These variants were then co-expressed in Chinese hamster ovary (CHO) cells with a wild-type β₃ and the binding of PAC1, an antibody specific for the active conformation of α₁β₃ was used to define affinity states (14) (Fig. 1B). The Ala substitution of α₁(F992), α₁β₃(F993), and α₁β₃(R995) activated α₁β₃ as determined by high affinity PAC1 binding (Fig. 1, B and C). In contrast, the Ala substitution of α₁(G991) and α₁β₃(K994) had minimal effect (Fig. 1B). Therefore, Ala substitution of specific residues in the conserved membrane-proximal GFFKR motif of the α subunit can activate an integrin.

The Ala substitution of α₁(F992A), α₁β₃(F993A), and α₁β₃(R995A) activated PAC1 binding in the presence of the competitive inhibitor (anti-LIBS6) that binds to the extracellular domain of β₃ (13). C, activation indices of the α₁β₃ mutants. To obtain numerical estimates of integrin activation, an activation index (AI) was calculated for each of the α₁β₃ mutants. PAC1 binding was measured in CHO cells expressing wild-type α₁β₃, α₁β₃(R995A), α₁β₃(F993A), α₁β₃(K994A), α₁β₃(F992A), and α₁β₃(D723A). Depicted are the mean activation indices ± S.D. of three independent experiments for each mutation. For each experiment, wild-type α₁β₃ was included as a control. Transient transfection of CHO cells with activated forms of α₁β₃ had surface expression levels similar to wild-type α₁β₃. In 3 determinations, 5 different activated mutants were expressed at 75 ± 16% of wild-type α₁β₃ (not shown).

**FIG. 1.** The Ala substitution of specific residues in the membrane-proximal regions of the α₃ and β₃ cytoplasmic domains activates α₃β₃. A, schematic representation of the topology of the transmembrane and cytoplasmic domains of α₃ and β₃. The conserved membrane-proximal sequences of the α₃ and β₃ are illustrated (highly conserved residues are uppercase, less conserved residues are lowercase). Integrin α and β cytoplasmic domains share a similar membrane-proximal organization with apolar and polar sequences following on sequentially after the W-K membrane-cytosolm interface. The conserved sequences for the α and β subunits are GFFKR and LL-HDR (dashes represent unconserved residues), respectively. Deletion of these sequences lock integrins in the high affinity state. The β₃ and β₃ subunits lack these conserved membrane-proximal sequences which suggests that they may signal via a different mechanism to other integrins. B, flow cytometry histograms illustrating PAC1 binding to α₁β₃ and α₁β₃(F992A). Depicted are flow cytometry histograms illustrating PAC1 binding in the presence (open histogram) or absence (filled histogram) of competitive inhibitor, Ro 43-5054 to CHO cells expressing wild-type α₁β₃ and α₁β₃(F992A). The peptidomimetic Ro 43-5054, is a selective inhibitor of ligand binding to α₁β₃ (32). The α₁β₃(F992A) transfectants specifically bind PAC1, illustrating that this mutation activates α₁β₃(F992A). In contrast, cells expressing wild-type α₁β₃ which is in the low affinity state, can only bind PAC1 in the presence of an activating antibody (anti-LIBS6) that binds to the extracellular domain of β₃ (13). C, activation indices of the α₁β₃ mutants. 2 M. H. Ginsberg, unpublished data.
a_{11b}(R995A) may activate a_{11b}β3 by the disruption of an interaction between the a_{11b} and β3 cytoplasmic domains. The β3 cytoplasmic domain contains a highly conserved Asp residue that is at a similar displacement from the proposed cytoplasm-membrane interface as the highly conserved Arg-995 of a_{11b}. This raises the possibility that the membrane-proximal regions may interact via a salt bridge formed between a_{11b}(R995) and β3(D723). To test this idea, we expressed a_{11b}β3(D723A); this integrin bound PAC1 with high affinity (Fig. 1C). To further test the proposed salt bridge we constructed the “charge-reversal” mutants, a_{11b}(R995D) and β3(D723R). Both the single mutations, a_{11b}(R995D)β3 and a_{11b}β3(D723R), were in the high affinity state and exhibited spontaneous PAC1 binding (Fig. 2). However, the double charge reversal mutant a_{11b}(R995D)β3(D723R) complemented the activating effect of the individual mutations. We suggest that this double mutation may restore the potential salt bridge between the a and β subunits, reforming the structural constraint which prevents the activation of the integrin. As a control, we examined the affinity state of double mutants a_{11b}(R995A)β3(D723A), a_{11b}(R995A)β3(D723R), and a_{11b}(R995D)β3(D723A). All of these variants bound PAC1 spontaneously (data not shown).

Ligand binding to integrins induces changes in cytoskeletal organization, intracellular pH, and protein tyrosine phosphorylation (outside-in signaling) (2–5). Above we described cytoplasmic domain mutations that result in constitutive inside-out signaling. To determine if these mutations caused constitutive intracellular signaling, we examined the tyrosine phosphorylation of focal adhesion kinase (pp125FAK) (19, 20). Stable CHO cell lines expressing the mutants a_{11b}(F992A)β3 and a_{11b}β3(D723A) exhibited the constitutive phosphorylation of pp125FAK when in suspension (Fig. 3A). In contrast, cells expressing wild-type a_{11b}β3 only phosphorylated pp125FAK when adherent to a fibrinogen matrix (Fig. 3A).

The association of integrins with specialized cytoskeletal structures termed focal adhesions, is regulated by ligand binding to their extracellular domains (21). As another assay for outside-in signaling, we analyzed the effect of the activating mutations on integrin targeting to focal adhesions. To ensure that the targeting of the a_{11b}β3 variants to focal adhesions was independent of ligand binding, each was expressed with a ligand binding-deficient β3 mutant, β3(D119Y) (22). When expressed in CHO cells plated on fibronectin, both a_{11b}(R995D)β3(D119Y) and a_{11b}β3(D723R)(D119Y) were spontaneously recruited to focal adhesions formed by endogenous hamster integrins (Fig. 3B). In contrast, the distribution of a_{11b}(R995D)β3(D723R)(D119Y) was diffuse, similar to that of a_{11b}β3(D119Y), and it was not recruited to focal adhesions formed by the endogenous integrins (Fig. 3B). Therefore, we conclude that these activating point mutations allow a spontaneous association of the integrin with the cytoskeleton in the absence of ligand binding. Thus, activating membrane-proximal point mutations in both integrin a and β subunits can induce constitutive bidirectional transmembrane signaling.
integrated transmembrane signaling. Signals from integrins can
to the release of a “constraint” that maintains the receptor in
For this reason, fibronectin matrix assembly could be perturbed by
activating integrin mutations. To test this idea, we used CHO
B2 cells that are unable to assemble a fibronectin matrix due to
To be interesting to determine if such mutations could account for some of the increased depo-
sion of extracellular matrix that characterizes certain patho-
logical states.

There presently exists no three-dimensional structure of the
native cytoplasmic domains of $\alpha_{11} \beta_3$. Furthermore, a high res-
olution structure of this transmembrane protein may be diffi-
cult to acquire. Consequently, a mutational analysis, similar to
those conducted in bacterial chemoattractant receptors (24)
and G protein-coupled receptors (23, 26), can provide a viable
alternative to develop a structural hypothesis of transmem-
brane signaling. The approach described here has led us to
propose a plausible and testable mechanism for integrin sig-
naling. Indeed, the present studies may provide insight into a
general mechanism of signaling mediated by a variety of trans-
membrane receptors. Point mutations can constitutively acti-
vate such structurally diverse receptors such as G protein-
coupled receptors, growth factor receptors, and bacterial
chemoattractant receptors (23–26). However, only in the $\beta_3$
adrenergic receptor has constitutive bidirectional signaling
been reported (26). In common with integrins, the ability of
specific mutations to activate these receptors has been ascribed
to the release of a “constraint” that maintains the receptor in
an off state. As the topography of integrins is comparatively
simple, having two parallel membrane-spanning subunits, we
have been able to identify such a constraint. Utilizing charge
reversal mutations, we provide direct mutational evidence for a
salt bridge constraining integrins into a nonsignaling state.

As reported here, membrane-proximal point mutations in
both integrin $\alpha$ and $\beta$ subunits can cause constitutive bidir-
ectonal transmembrane signaling. Signals from integrins can
influence cell growth and death, and the assembly of the extrac-
cellular matrix (27–29). This raises the intriguing possibil-
ity that activating integrin mutations may produce dominant
phenotypes in vivo. The assembly of a fibronectin matrix, a
process important in wound healing and cell migration during
development, is regulated by integrin affinity state (29, 30).
Therefore, fibronectin matrix assembly could be perturbed by
activating integrin mutations. To test this idea, we used CHO
B2 cells that are unable to assemble a fibronectin matrix due to
a lack of the appropriate integrins (31). Transfection of these
cells with the constitutively active mutant $\alpha_{11} \beta_3 (D723R)$ en-
abled them to assemble a fibronectin matrix (Fig. 4). In con-
trast, CHO B2 cells expressing wild-type $\alpha_{11} \beta_3$ failed to make
a fibronectin matrix (Fig. 4). Thus, activating point mutations
in the integrin cytoplasmic domains can influence the assembly
of the extracellular matrix. It will be interesting to determine if
such mutations could account for some of the increased depo-
sion of extracellular matrix that characterizes certain patho-
logical states.

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