Breaking the Integrin Hinge

A DEFINED STRUCTURAL CONSTRAINT REGULATES INTEGRIN SIGNALING*

(Received for publication, January 22, 1996)

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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 αIIbβ3. 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-cytoplasm interface (Fig. 1A). The conserved sequences for the α and β subunits are -GFFKR and LL-v-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 suggest that there may 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 αIIbβ3. 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 the constitutive outside-in signaling as assayed by the phosphorylation of pp125FAK and the ligand independent recruitment of αIIbβ3 to focal adhesions.

MATERIALS AND METHODS

Antibodies—The anti-αIIbβ3 antibody D57 (9) was biotinylated with biotin-N-hydroxysuccinimide (Sigma) according to the manufacturers' directions. The isolation and characterization of anti-LIBS6 (13) and PAC1 (14) have been described previously.

cDNA Constructs—pCM8 expression constructs encoding wild-type αIIb and β3 were constructed as described (9). The generation of expression constructs in pCM8 encoding point mutation αIIb and β3 CDNAs 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 pCM8. This construct was then cut with AflIII and DraIII, and the 3-kb fragment ligated with the 3.5-kb AflIII-DraIII fragment of pCDβ3. pCM8 expression vectors encoding αIIb mutants were constructed by tripartite ligation of a 0.6-kb Xba-BamHI cut PCR fragment encompassing the mutations, the 4.1-kb BamHI-DraIII fragment of pCDβ3, and the 2.6-kb DraIII-XbaI fragment of pCD8B. 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 αIIbβ3 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 only on a gated subset of cells positive for αIIbβ3 expression detected with a biotinylated non-function blocking antibody to αIIbβ3 (D57) and phycoerythrin/strepavidin. 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 × (Fm - Fc)/(Fm LIBS6 - Fc LIBS6), where Fm is the median fluorescence intensity of PAC1 binding and Fc is the median fluorescence intensity of PAC1 binding in the presence of the competitive inhibitor Ro 43-5054 (1 μM). Fm LIBS6 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 2 μM anti-LIBS6 and competitive inhibitor.

Analysis of pp125FAK Phosphorylation—100-mm tissue culture plates were coated with 5 mg/ml BSA or 100 μg/ml fibrinogen 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 × 10⁶ 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 at 4°C with protein A-Sepharose and washed extensively in ice-cold RIPA buffer containing 1 mM Na₃VO₄. The 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).

Immunofluorescence—CHO cells were transiently transfected as described (16) and, after 48 h, were cultured on fibronectin-coated coverslips for 2 h at 37°C. The cells were fixed with 3.7% paraformaldehyde (methanol-free) and permeabilized with 0.2% Triton X-100. The coverslips were blocked in 10% normal goat serum, incubated with the rabbit polyclonal antibody 2308 (anti-human αIIb) and the anti-hamster β₃ mAb 7E2 (18) in 10% normal goat serum for 60 min, washed with PBS, and then incubated with the secondary antibodies, FITC-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit in 10% normal goat serum for an additional 30 min. The coverslips were washed in PBS and mounted using FITC-guard mounting medium. The slides were examined with a Leitz Orthoplan microscope with a 100x oil immersion objective. Photographs were taken on Kodak T-Max 400 film.

Fibronectin Matrix Assembly—Cells were suspended in α-minimal essential medium containing 10% fetal calf serum depleted of fibronectin by gelatin affinity adsorption and supplemented with purified human plasma fibronectin. All reagents were dialyzed against three changes of 100 volumes of α-minimal essential medium and passed through a 0.2-μm filter prior to addition to the culture medium. Cells were plated in 12-well HTS slides at a final density of 2.3 × 10⁵ cells/ml and cultured for 41 h. The cells were then fixed with 3.7% paraformaldehyde and stained with polyclonal rabbit anti-human plasma Fn antibody MC54 (32 μg/ml) and Cy3-conjugated goat anti-rabbit IgG antibodies (7.5 μg/ml). Stained cell monolayers were observed using a Nikon FXA epifluorescence microscope, and representative fields were photographed using Kodak T-Max 400 or Ektachrome 1600 direct positive slide film.

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 α₁β₃(F992) and 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 α₁β₃(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 α₁β₃(F992A), α₁β₃(F993A), and α₁β₃(R995A) results in the decrease of binding to PAC1, as determined by high affinity PAC1 binding (Fig. 1, B and C). In contrast, the Ala substitution of α₁β₃(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.

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 co-expressing wild-type α₁β₃ and the activating mutants. The AI was calculated for each mutant in the presence of an activating antibody (anti-LIBS6) that binds to the extracellular domain of β₃(13). Cells expressing wild-type 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).

2 M. H. Ginsberg, unpublished data.
Fig. 2. The membrane-proximal regions of the αιβ and β3 cytoplasmic domains may interact via a salt bridge between αιβ(R995) and β3(D723). The activation indices of αιβ(R995D)β3, αιββ3(D723R), and αιβ(R995D)β3(D723R) are illustrated. The activation index of αιβ(R995D)β3(D723R) is significantly less (p ≤ 0.001) than αιβ(R995)β3 and αιββ3(D723R). Depicted are the mean activation indices ± S.D. of three independent experiments for each mutation.

Fig. 3. Membrane-proximal mutations in both the αιβ and β3 cytoplasmic domains promote constitutive intracellular signaling. a, CHO cells expressing the activating mutations αιβ(F992A)β3 and αιββ3(D723A) constitutively phosphorylate pp125FAK. CHO cell lines expressing αιββ3, αιβ(F992A)β3, and αιββ3(D723A) were incubated for 90 min at 37°C on plates coated with either fibrinogen or BSA. The cells were in suspension on BSA-coated plates, but adhered to and spread on the fibrinogen-coated plates. The cells were then processed for analysis of pp125FAK tyrosine phosphorylation (17). In contrast to cells expressing wild-type αιββ3, those expressing αιβ(F992A)β3 and αιββ3(D723A) exhibited tyrosine phosphorylation of pp125FAK when incubated in suspension over BSA. As expected, cells expressing αιββ3 phosphorylated pp125FAK when plated on fibrinogen-coated plates, as did cells expressing αιβ(F992A)β3 or αιββ3(D723A) (data not shown). Untransfected CHO cells did not phosphorylate pp125FAK when plated on BSA or fibrinogen-coated plates. b, recruitment of αιβ(R995D)β3 and αιββ3(D723R) to focal adhesions in a ligand independent manner. CHO cells were transiently transfected and, after 48 h, were cultured on fibronectin for 2 h. The cells were then stained with a mixture of anti-human αιβ (panels A, B, C, and D) and anti-hamster β3 (mouse monoclonal 7E2). In all experiments, β3 was localized in punctate structures along the cell edge and along the ventral surface, characteristic of focal adhesions (data not shown). αιββ3(D119Y) had a uniform cell-surface distribution when plated on fibronectin (panel A) and was not localized to focal adhesions. In contrast, αιβ(R995D)β3(D119Y) (panel B) and αιββ3(D723R)(D119Y) (panel C) were recruited to the focal adhesions. αιβ(R995D)β3(D723R)(D119Y) (panel D) behaved similarly to αιββ3(D119Y) and was not present in focal adhesions.

Outside-in signaling, we analyzed the effect of the activating mutations on integrin targeting to focal adhesions. To ensure that the targeting of the αιββ3 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 αιβ(R995D)β3(D119Y) and αιββ3(D723R)(D119Y) were spontaneously recruited to focal adhesions formed by endogenous hamster integrins (Fig. 3). In contrast, the distribution of αιβ(R995D)β3(D723R)(D119Y) was diffuse, similar to that of αιββ3(D119Y), and it was not recruited to focal adhesions formed by the endogenous integrins (Fig. 3). 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 α and β subunits can induce constitutive bidirectional transmembrane signaling.

αιβ(R995A) may activate αιββ3 by the disruption of an interaction between the αιβ 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 αιβ. This raises the possibility that the membrane-proximal regions may interact via a salt bridge formed between αιβ(R995) and β3(D723R). To test this idea, we expressed αιββ3(D723A); this integrin bound PAC1 with high affinity (Fig. 1C). To further test the proposed salt bridge we constructed the “charge-reversal” mutants, αιββ3(R995D) and β3(D723R). Both the single mutations, αιββ3(R995D)β3 and αιββ3β3(D723R), were in the high affinity state and exhibited spontaneous PAC1 binding (Fig. 2). However, the double charge reversal mutant αιββ3(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 α 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 αιββ3(R995A)β3β3(D723A), αιββ3(R995A)β3β3(D723R), and αιββ3(R995D)β3β3(D723A). All of these variants bind 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 αιββ3(F992A)β3 and αιββ3β3(D723A) exhibited the constitutive phosphorylation of pp125FAK when in suspension (Fig. 3A). In contrast, cells expressing wild-type αιββ3 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
There presently exists no three-dimensional structure of the native cytoplasmic domains of $\alpha_{IIb}\beta_3$. Furthermore, a high resolution structure of this transmembrane protein may be difficult to acquire. Consequently, a mutational analysis, similar to those conducted in bacterial chemoreceptor receptors (24) and G protein-coupled receptors (23, 26), can provide a viable alternative to develop a structural hypothesis of transmembrane signaling. The approach described here has led us to propose a plausible and testable mechanism for integrin signaling. Indeed, the present studies may provide insight into a proposed mechanism for integrin signaling. The approach described here has led us to propose a plausible and testable mechanism for integrin signaling.

As reported here, membrane-proximal point mutations in both integrin $\alpha$ and $\beta$ subunits can cause constitutive bidirectional transmembrane signaling. Signals from integrins can influence cell growth and death, and the assembly of the extracellular matrix (27–29). This raises the intriguing possibility 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 $\alpha_{IIb}\beta_3$ (D723R) enabled them to assemble a fibronectin matrix (Fig. 4). In contrast, CHO B2 cells expressing wild-type $\alpha_{IIb}\beta_3$ 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 deposition of extracellular matrix that characterizes certain pathological states.
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doi: 10.1074/jbc.271.12.6571

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