Published in final edited form as:

J Thromb Haemost. 2014 July ; 12(7): 1145–1155. doi:10.1111/jth.12610.

Integrin α IIB Tail Distal of GFFKR Participates in Inside-out α IIB β 3 Activation

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Abstract

Background—Increases in ligand binding to integrins (“activation”) play critical roles in platelet and leukocyte function. Integrin activation requires talin and kindlin binding to integrin β cytoplasmic tails. Much research has focused on the conserved GFFKR motif in integrin α IIB tails for its importance in keeping integrins inactive, integrin β cytoplasmic tails and the binding partners of β tails. However, the roles of α IIB tail distal of GFFKR motif are unexplored.

Objective—To investigate the role of α IIB tail distal of GFFKR in talin-mediated inside-out integrin signaling.

Methods—We used model cell systems to examine the role of α IIB tail distal of GFFKR in bi-directional α IIB β 3 signaling and α IIB β 3-talin interactions.

Results—Deletion of amino acid residues after the GFFKR motif in α IIB tail moderately decreased β 3(D723R)-induced activation, abolished talin-induced α IIB β 3 activation in model cells and inhibited agonist-induced α IIB β 3 activation in megakaryocytic cells. Furthermore, residues in α IIB tail distal of GFFKR did not affect outside-in α IIB β 3 signaling or α IIB β 3-talin interaction. Addition of non-homologous or non-specific amino acids to the GFFKR motif restored α IIB β 3 activation in model cells and in megakaryocytic cells. Molecular modeling indicates that β 3-bound talin sterically clashes with the α IIB tail in the α IIB β 3 complexes, potentially disfavoring the α - β interactions that keep α IIB β 3 inactive.

Conclusion—The α IIB tail sequences distal of GFFKR participate in talin-mediated inside-out α IIB β 3 activation through its steric clashes with β 3-bound talin.

Keywords

Integrin α IIB β 3; talin; kindlin-2 protein; human; signal transduction; cell adhesion

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Authorship

F. Ye conceived the projects and designed the research. F. Ye and W. Hu directed the research. A. Li, Q. Guo, C. Kim and F. Ye performed experiments. F. Ye, C. Kim and A. Li analyzed data. F. Ye, C. Kim, A. Li and W. Hu wrote the manuscript.

The authors declare that they have no conflict of interest.

Introduction

Agonist-induced increases in soluble ligand binding (often referred to as integrin activation or inside-out integrin signaling) are important in development and play major roles in both platelet and leukocyte function [1–3]. Integrins are α and β heterodimers and each integrin subunit contains a large extracellular domain, a single transmembrane domain (TMD) and a short cytoplasmic domain (tail). The interactions between α and β TMD and cytoplasmic tails (TMD-tail) regulate the equilibrium between inactive and active integrins: optimal α - β TMD-tail interactions keep integrins inactive; whereas the separation of α and β TMD-tails results in large conformational changes in the extracellular domains, leading to integrin activation [2–4]. Agonist-stimulated integrin activation requires talin and kindlin binding to integrin β cytoplasmic tails [4–5]. Talin, through its interactions with phospholipids and two binding sites on the integrin β cytoplasmic tail [6], induces the transmembrane allostery that leads to integrin activation [7–8]. Kindlins cluster talin-activated integrins to increase the activation capacity of talin [9–11]. Thus far, research on α IIB β 3 activation has mostly focused on the α IIB TMD including the GFFKR motif, the β 3 TMD-tail and β 3 binding partners. Any requirement for the α IIB tail distal of GFFKR motif in α IIB β 3 inside-out activation is still unclear.

α IIB interacts with β 3 at the transmembrane GXXXG motif and cytoplasmic GFFKR motif, which are critical to keep α IIB β 3 in the inactive state [12–13]. An α IIB tail binding protein, calcium- and integrin-binding protein1 (CIB1), can contribute to the maintenance of resting α IIB β 3 by preventing talin from binding [14]. Another α tail binding protein, sharpin, was recently shown to play a similar inhibitory role as CIB1, by keeping talin and kindlin from binding to integrins [15]. It is unclear whether the α IIB tail is only required to keep α IIB β 3 inactive or it also plays a role in inside-out integrin activation, and if so, by what mechanism. The roles of α IIB tail distal of GFFKR in integrin activation have been speculated in a couple of recent reviews [16–17]. Here, we provided the first experimental evidence that α IIB tail distal of GFFKR participates in inside-out α IIB β 3 activation. We proposed that β 3-bound talin activates α IIB β 3 by weakening the α IIB(R995)- β 3(D723) electrostatic interaction in the cytoplasmic tails [18], altering β 3 TMD topology [7] and sterically clashing with α IIB tail.

Materials and Methods

Materials

Lentiviral expression constructs for α IIB and β 3, and transient expression constructs for THD (talin residue 1–433), kindlin-1 and kindlin-2 have been previously described [8, 19]. Anti- α IIB-tail (Rb8276), anti- β 3-tail (Rb8275), anti- α IIB β 3 antibodies (D57 and Rb8053) have been described [20–21]. PAC-1 was from BD Biosciences (San Jose, CA, USA). Latrunculin A and blebbistatin were from Millipore (Billerica, MA, USA). NHS-activated agarose beads were from Pierce (Rockford, IL USA). Calmodulin-conjugated sepharose beads were from GE Life Sciences (Pittsburgh, PA, USA). Anti-GAPDH and anti-Tac were from Santa Cruz Biotechnology (Dallas, Texas, USA). Mutations were generated using a Quikchange mutagenesis kit (Agilent, Santa Clara, CA, USA). Anti-Akt pS473 and anti-Akt

were from Cell Signaling (Danvers, MA, USA). Anti-FAK and anti-FAK pY576 were from Life Technologies (Carlsbad, CA, USA). Complete protease and phosphatase inhibitor tablets were from Roche (Basel, Switzerland).

Cell line construction and cell culture

CHO cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Fetal Bovine Serum (FBS), L-glutamate, non-essential amino acid, and antibiotics. CHO Cells stably expressing α Iib β 3, α Iib 1001 β 3, α Iib 996 β 3, α Iib_5A β 3 were established by co-infecting CHO cells with lentiviruses encoding respective α Iib and β 3 construct, each with GFP marker controlled by an internal ribosome entry site (IRES) [8]. CHO cells expressing wild type (wt) α Iib β 3 (A5 cells) and CHO cells expressing α Iiba5 β 3 or α IibaL β 3 have been described [21–22]. Human Acute Megakaryocytic Leukemia Cells (CMK) cells were generous gifts from Dr. Tomiyama [23–24] and were cultured in RPMI 1640 supplemented with FBS, Sodium Pyruvate, L-glutamate, non-essential amino acid, and antibiotics. CMK Cells stably expressing α Iib β 3, α Iib 1001 β 3, α Iib 996 β 3, α Iib_5A β 3 were established as described for the integrin-expressing CHO cells.

Integrin Activation assays

To measure β 3(D723R)-induced activation, α Iib or α Iib mutants as indicated were co-transfected to CHO cells with either wt β 3 or β 3(D723R). 24 hours after transfection, cells were harvested and co-stained with an activation-sensitive, ligand-mimetic antibody, PAC1 [25], and an anti- α Iib β 3 antibody, D57. PAC-1 and D57 were then detected by alexa647-conjugated anti-IgM and FITC-conjugated anti-IgG respectively. The amount of PAC-1 binding to cells with similar α Iib β 3 expression (high D57 staining) was analyzed by FACS and compared (supplemental figure 1). Normalized PAC-1 binding was calculated as $(\text{MFI-MFI}_0)/\text{MFI}_{\text{D57}}$, where MFI was the mean fluorescence intensity of bound PAC-1 and MFI_0 was that in the presence of 20 μ M α Iib β 3-specific antagonist, eptifibatide, and MFI_{D57} was the mean fluorescence intensity of D57. All staining steps were carried out in Tyrode's buffer (137mM NaCl, 12mM NaHCO₃, 2.6mM KCl, 1mM MgCl₂, 1mM CaCl₂, 5.6mM Glucose, 5mM HEPES, pH7.4).

To measure talin/kindlin-induced activation, CHO cells stably expressing α Iib β 3, α Iib 996 β 3, α Iib 1001 β 3, or α Iib_5A β 3 were bulk sorted using their IRES GFP marker, transfected with Td-tomato (1/60 of the total DNA) as a transfection marker, and pcDNA vector, THD (talin residue 1–433), THD+ kindlin-1 or THD+kindlin-2 as indicated. 24 hours later, cells were harvested and stained separately with either ligand-mimetic PAC-1 or anti- α Iib β 3, D57, followed by alexa647-conjugated anti-IgM or anti-IgG. PAC-1 and D57 binding to transfected cells were analyzed by FACS. Integrin activation was calculated as $(\text{MFI-MFI}_0)/\text{MFI}_{\text{D57}}$, where MFI was the mean fluorescence intensity of PAC-1, MFI_0 was that in the presence of 20 μ M α Iib β 3-specific antagonist, eptifibatide, and MFI_{D57} was the mean fluorescence intensity of D57. To measure THD-induced α Iiba5 β 3, and α IibaL β 3 activation, CHO cells stably expressing α Iib β 3, α Iiba5 β 3 or α IibaL β 3 were transfected with EGFP (1/60 of the total DNA) as transfection marker and either pcDNA or THD. The cells were stained and analyzed as described above. To assess the effects of latrunculin A and blebbistatin on α Iib β 3 activation, the α Iib β 3-expressing CHO cells were transfected

with DNA constructs as indicated, treated with vehicle (DMSO), 2 μ M latrunculin A, or 50 μ M blebbistatin for 30~60 minutes prior to harvesting. The inhibitors were then maintained at these concentrations throughout the FACS staining.

Thrombin-induced α IIB β 3 activation in CMK cells has been previously described [24]. To measure thrombin-induced α IIB β 3 activation in CMK cells, CMK cells stably expressing α IIB β 3, α IIB 996 β 3, α IIB 1001 β 3, or α IIB_5A β 3 were stimulated with thrombin (1Unit/ml) for 10 minutes in the presence of anti-GPIIb and PAC1, fixed with 3.7% formaldehyde, and detected with PE conjugated anti-IgG (1:100 final dilution) and alexa647 conjugated anti-IgM (u chain specific). For integrin expression, the same procedure was carried out except that PAC1 was replaced by rabbit polyclonal antibody Rb8053 and alexa647 conjugated anti-rabbit IgG. α IIB β 3-transduced (GFP positive) and GPIIb-high CMK cells were analyzed for PAC1 and Rb8053 binding in FACS.

α IIB and β 3 TMD-tail interaction assay

Integrin TMD-tail binding assays have been previously described [26]. Briefly, CHO cells were co-transfected with the indicated α IIB and β 3 TMD-tail constructs. 24 hours after transfection, the cells were lysed in a lysis buffer (20 mM HEPES pH 7.4, 1% CHAPS, 150 mM NaCl, 2 mM CaCl₂, and EDTA) and clarified by centrifugation at 14 000 rpm for 15 minutes. The clarified lysates were incubated with calmodulin Sepharose for 2 hours at 4°C. Bound proteins were eluted with sodium dodecyl sulfate (SDS) reducing sample buffer, subjected to SDS–polyacrylamide gel electrophoresis (PAGE), and analyzed by Western blotting.

Pulldown assay for α IIB β 3-THD affinities

Anti- α IIB β 3 antibody D57 was conjugated to NHS-activated agarose beads according to manufacturer's instructions and final conjugated D57 is about 10mg/ml. 700 μ l of D57-agarose (50% slurry) was incubated with lysates from CHO cells stably expressing α IIB β 3 or α IIB 996 β 3 (twenty 150cm²-plates of confluent cells for each integrin) for 2 hours at 4°C and washed thoroughly to produce integrin-coated beads. 30 μ l of the immobilized integrin agarose (50% slurry) was then incubated overnight at 4°C with various concentrations of purified THD [8]. The bound THD was then washed 3 times with a buffer (20mM Tris, 150mM NaCl, 0.1% triton, 1mM MgCl₂, 1mM CaCl₂ pH7.4) and eluted with non-reducing SDS-PAGE loading buffer for 5 minutes without boil (to prevent the elution of antibody heavy chain that blocks THD (~50kDa) in western blots). The eluates were transferred to fresh eppendorf tubes, reduced with 2-mercaptoethanol, boiled for 5 minutes and analyzed by western blotting. Bound THD was detected by anti-His6 antibody and analyzed with the one site binding model to determine the K_d value in Prism 3.0 (GraphPad Software).

Microscopy—For fluorescent microscopy, cells were fixed with 3.7% formaldehyde, permeabilized with 0.2% triton-100, stained with alexa488-phalloidin for actin, and imaged at 60 $\times$ with a Nikon Diaphot inverted fluorescence microscope equipped with a 100W mercury light source and a Sony XCD-X900 IEEE 1394 camera. Phase contrast images were taken on the same microscope using a 10 $\times$ objective lens.

Outside-in Signaling Assays

Outside-in signaling assays were performed as described [27]. Briefly, cells were serum-starved overnight, harvested, and freshly plated on plates that have been coated with 10 µg/ml fibrinogen and blocked with 2% BSA. After 0, 15, 30, 45 or 60 minutes of adhesion, cells were washed and lysed with lysis buffer (RIPA+1%CHAPS+protease inhibitor +phosphatase inhibitor). 8 µg of total proteins was loaded onto each lane of PAGE gel and analyzed by western blotting with antibodies as indicated.

Statistical Analysis

All statistical analyses were performed in Prism 3.0 (GraphPad Software) with two-tail, unpaired t-tests.

Results

The presence, not the sequence, of residues distal of GFFKR in the αIIb tail is necessary for integrin activation

To determine whether the αIIb tail distal of GFFKR is required for inside-out integrin signaling, we tested the effects of αIIb truncation on integrin activation induced by β3(D723R), a widely used activating mutation that destabilizes αIIb and β3 TMD-tail interactions and shifts the equilibrium towards activated integrins in a talin-dependent manner [10, 28]. Deletion of the αIIb tail c-terminus at residue 996 (αIIb₉₉₆) decreased β3(D723R)-induced αIIbβ3 activation by 54% as measured by eptifibatide-inhibitable binding of an activation-specific antibody PAC1 [25] or the physiological αIIbβ3 ligand fibrinogen, whereas truncation of αIIb at residue 1005 (αIIb₁₀₀₅) or 1001 (αIIb₁₀₀₁) had little effect (Fig 1A, supplemental figure 1, 2, 3 and 4). Furthermore, PAC1 binding to αIIb₉₉₆β3(D723R) was not maximal because addition of Mn²⁺ markedly increased binding (supplemental Figure 5), indicating that αIIb₉₉₆ inhibited the responsiveness to β3(D723R)-induced activation. Addition of 5 alanines to the c-terminus of αIIb₉₉₆ (αIIb₉₉₆_5A) completely restored PAC1 binding (Fig 1A), suggesting that the additional residues from 996–1001 contribute to αIIbβ3 activation by a sequence-independent mechanism.

Next we sought to determine whether the αIIb tail distal of GFFKR has similar functions when integrins are directly activated by talin and kindlin. The fragment containing talin residues 1–433, known as the talin head domain (THD) [6], activated and synergized with kindlin-1 or kindlin-2 to activate αIIbβ3 or αIIb₁₀₀₁β3, but not αIIb₉₉₆β3 (Fig 1B,C). Moreover, αIIb₉₉₆β3 activation was not increased at the level of THD overexpression that induced maximal activation in αIIbβ3 (Fig 1D). The capacity of THD and kindlin to activate αIIbβ3 was restored in αIIb₉₉₆_5Aβ3 (Fig 1B,C). Thus, the presence, not the sequence of residues after GFFKR in the αIIb tail is necessary for talin-mediated integrin activation.

The presence, not the sequence, of residues distal of GFFKR in the αIIb tail is necessary for agonist-induced αIIbβ3 activation in megakaryocytic cells

Next, we sought to determine whether residues distal of GFFKR in the αIIb tail are similarly required for agonist-induced αIIbβ3 activation in human acute megakaryocytic leukemia

cells (CMK cells). CMK cells expressing high level of GPIb, a marker for megakaryocytes and platelets, activate α IIB β 3 upon thrombin stimulation and this thrombin-induced α IIB β 3 activation is talin-dependent [23–24]. As reported, thrombin stimulated α IIB β 3 activation in GPIb-high CMK cells (Fig 2A). CMK cells express endogenous α IIB β 3 (Fig 2C, uninfected) and thus can respond to thrombin (Fig 2B, uninfected). However, thrombin-induced α IIB β 3 activation was increased in CMK cells transduced with wt α IIB β 3, α IIB 1001 β 3 or α IIB_5A β 3 but not with α IIB 996 β 3 when compared to that of the uninfected (Fig 2B). Furthermore, Lentiviral transduction of wt α IIB β 3, α IIB 1001 β 3, α IIB 996 β 3 or α IIB_5A β 3 into CMK cells increased α IIB β 3 expression to similar high levels above that of the uninfected CMK cells (Fig 2C), indicating that the defects of α IIB 996 β 3 activation is a direct result of lost responsiveness of α IIB 996 β 3 to thrombin stimulation. Thus residues distal of GFFKR in the α IIB tail are required for thrombin-induced α IIB β 3 activation in megakaryocytic cells.

The α IIB tail distal of GFFKR contributes to keep α IIB β 3 in resting state

α IIB 996 β 3 loses responsiveness to THD but the basal activation of α IIB 996 β 3 is elevated comparing to that of wt α IIB β 3 or α IIB 1001 β 3 (Fig 1A,B), suggesting that α IIB residues from 996 to 1000 help maintain α IIB and β 3 interaction and keep α IIB β 3 inactive. Indeed, β 3 TMD-tail bound less to α IIB 996 or α IIB_5A than to α IIB or α IIB 1001 in our validated integrin TMD-tail interaction assay [26, 29], indicating that first few residues in α IIB tail distal of GFFKR contribute to optimal α IIB and β 3 TMD-tail interactions (Fig 3A,B). Thus, α IIB residues from 996 to 1000 help maintain α IIB β 3 in resting state by contributing to the optimal TMD-tail interactions between α IIB and β 3. As expected from this regulatory function of α IIB residues distal of GFFKR, the sequences of α IIB tails after GFFKR are conserved across different species (supplemental figure 6).

The α IIB tail distal of GFFKR is not required for α IIB β 3-talin interaction or α IIB β 3 outside-in signaling

To investigate the mechanism by which α IIB 996 causes loss of inside-out integrin activation, we sought to determine whether α IIB tail distal of GFFKR is required for talin binding. α IIB 996 β 3 bound similar amount of talin as wt α IIB β 3 did in a co-immunoprecipitation assay (Fig 4A). To better quantify this result, we captured α IIB β 3 or α IIB 996 β 3 from cell lysate using antibody-conjugated agarose beads. We then used these immobilized recombinant integrins to quantitatively pull down purified THD. THD bound to α IIB 996 β 3 ($K_d=205.7\pm35.5$ nM) as well as to wt α IIB β 3 ($K_d=213.5\pm38.7$ nM) (Fig 4B). Thus α IIB tail after GFFKR is not required for α IIB β 3-talin the interaction w is controlled by the membrane distal region of β 3 tail [30–31].

Having established that the α IIB tail participates in α IIB β 3 activation by a sequence-independent mechanism, we sought to determine whether the α IIB tail is required for integrin outside-in signaling. Initial cell adhesion to immobilized fibrinogen is α IIB β 3 activation-independent [32–33], possibly due to increased ligand density and altered fibrinogen conformation [34], and matrix-induced outside-in signaling can occur in the absence of inside-out integrin activation [35], enabling us to examine effects of α IIB truncation independent of its effects on integrin activation, per se. Integrin-mediated

adhesion activates signaling molecules such as focal adhesion kinase (FAK), phosphatidylinositol 3-kinase (PI3-kinase) and its downstream target, Akt [27]. Neither adhesion-dependent phosphorylation of FAK or Akt was affected by α IIB 996 truncation (Fig 4B). Furthermore, cell spreading on immobilized fibrinogen was not affected by α IIB 996 truncation (Fig 4C). Therefore, the α IIB tail distal of GFFKR is selectively required for inside-out signaling but dispensable for outside-in signaling.

The β 3 tail-bound talin sterically clashes with the α IIB tail in the inactive α IIB β 3 TMD complex structures

α IIB β 3 is kept in the inactive form by the interactions between the α IIB and β 3 TMD-tails as shown by recent structures [12–13]. Talin binding to the β 3 tail is both necessary and sufficient for α IIB β 3 activation [8, 36–37]. We modeled a structure of the talin- α IIB β 3 complex by aligning the β 3 tail from a talin- β 3 tail structure [18] with the β 3 tail in a α IIB β 3 TMD complex structure determined in cellular membrane [13]. The resulting talin- α IIB β 3 complex structure showed substantial steric clash between the F3 subdomain in the THD and residues after the GFFKR motif in the α IIB cytoplasmic tail (Fig 5A). Since α IIB tail has some structural flexibility, we examined the individual structures in the ensemble of both the NMR [12] and the Rosetta [13] α IIB β 3 TMD structures. α IIB clashes with β 3-bound talin in 60 of the 73 lowest energy structures. Therefore, by clashing with α IIB tail and disfavoring a large proportion of low energy structures in which α IIB can optimally dimerize with β 3, talin shift the equilibrium toward an active integrin conformation where α IIB and β 3 TMDs are separated.

Swapping α IIB tail with non-homologous α tail preserves inside-out α IIB β 3 activation

The model proposed above does not require sequence specificity in the α IIB tail (Fig 3A, 4C). Indeed, unlike the sequence-conserved integrin β tails, integrin α tails display no significant similarities in sequence after the GFFKR motif (Fig 5B), yet a number of them are regulated by talin and kindlins [4–5]. Furthermore, swapping α IIB tail with non-homologous sequences of α 5 or α L tails did not affect the ability of THD to activate these chimeric integrins (Fig 5C,D). Thus, as predicted by our model, the presence, not the sequence of amino acids after GFFKR in α IIB tail is required for talin to activate integrin α IIB β 3.

Neither an intact cytoskeleton nor myosin II-mediated contractility is required for THD-mediated α IIB β 3 activation

Integrin-cytoskeleton connections and forces exerted on integrin tails contribute to the activation of some integrins, such as α 4 β 1 [38] and β 2 integrins [39]. To assess this alternative interpretation, we examined the possibility of α IIB tail contributing to α IIB β 3 activation via potential unknown connections with cytoskeletons and force generating machinery. The THD-induced α IIB β 3 activation was independent of an intact actin cytoskeleton, as latrunculin A did not inhibit it in spite of inhibiting actin polymerization (Fig. 6A,B). Similarly, myosin II-mediated contractility was not required as a myosin II ATPase inhibitor, blebbistatin, did not affect α IIB β 3 activation (Fig 6A,B). Thus, the ability of THD to activate α IIB β 3 requires neither an intact actin cytoskeleton nor myosin II-driven contractility.

Both THD-induced $\beta 3$ TMD topology change and the steric clash between THD and α IIB tail are required for effective integrin activation

We have previously reported that $\beta 3$ topology change is required for THD-induced α IIB $\beta 3$ activation [7]. $\beta 3$ (A711P) mutation, which blocks the transmission of $\beta 3$ TMD topology change, blocks α IIB $\beta 3$ activation [7, 29]. Therefore, to investigate whether steric clash between THD and α IIB tail alone is sufficient to activate α IIB $\beta 3$, we examined the effects of $\beta 3$ (A711P) on α IIB $\beta 3$ activation in the presence of expected THD- α IIB-tail clash. $\beta 3$ (A711P) completely abolished $\beta 3$ (D723R)-induced activation of α IIB $\beta 3$, α IIB 1001 $\beta 3$ and α IIB_5A $\beta 3$ (supplemental figure 7A), where steric clash between α IIB and $\beta 3$ -bound talin is expected. $\beta 3$ (A711P) also blocked THD-induced wt α IIB $\beta 3$ activation, where the THD- α IIB-tail clash is expected, at the level of THD overexpression that induce maximal activation in α IIB $\beta 3$ (supplemental figure 7B). Furthermore, we showed that change of $\beta 3$ TMD topology alone is not sufficient to effectively activate α IIB $\beta 3$, as α IIB 996 $\beta 3$ completely lost its responsiveness to talin without affecting talin- α IIB $\beta 3$ interactions (Fig 1A,B,D). Therefore, both THD-induced $\beta 3$ TMD topology change and the steric clash between THD and α IIB tail are required for effective integrin activation (Fig 6C).

Discussion

The work reported here provides the first experimental proof that α IIB tail distal of GFFKR participates in inside-out α IIB $\beta 3$ activation. Integrins exist in an equilibrium between inactive and active state, and thus integrin activation can be viewed as the increased percentage of time (or the increased probability) of the integrins being in active than in the inactive state. The measured integrin affinity reflects the net effects of all the relevant factors on the activation equilibrium [40]. $\beta 3$ -bound talin clashes with the residues distal of GFFKR in the α IIB tail and disfavors the α IIB $\beta 3$ TMD interactions, thereby shifting the equilibrium towards activated α IIB $\beta 3$ (Fig 6C). α IIB tail residues distal of GFFKR have dual effects: (1) they contribute to α IIB and $\beta 3$ TMD-tail interactions and thus keep basal integrin activation low; (2) they enable talin- α IIB-tail steric clash that contributes to integrin activation. The combined effects of (1) and (2) is that the residues in α IIB tail distal of GFFKR maximize the responsiveness to talin-induced integrin activation. However, the α IIB-talin steric clash alone is not sufficient to activate α IIB $\beta 3$, as talin F3 subdomain by itself, which can clash with α IIB upon binding to $\beta 3$ tail, is a weak α IIB $\beta 3$ activator [18]. Furthermore, blocking talin-induced $\beta 3$ topology change abolished α IIB $\beta 3$ activation in the presence of expected α IIB-talin steric clash (supplemental figure 7). Therefore, the combined effects of talin- α IIB steric clash, talin-induced $\beta 3$ topology change [7], and talin-lipids and talin- $\beta 3$ interactions [17–18] are required for efficient α IIB $\beta 3$ activation.

We demonstrated that truncation of α IIB tail after the GFFKR motif (α IIB 996) does not affect talin- $\beta 3$ binding or outside-in signaling but completely abolished agonist-induced α IIB $\beta 3$ activation or the capacity of talin to activate α IIB $\beta 3$, both of which can be restored by attaching a non-specific or non-homologous sequence after the GFFKR motif. There are two talin binding sites in $\beta 3$ cytoplasmic tail: a strong binding site in the first NPXY⁷⁴⁷ motif that contributes all the binding free energy and determines the binding affinity [30–31]; and a membrane proximal, weak binding site that does not contribute to the binding free energy

[30–31]. Because no known α IIB and β 3 cytoplasmic tail interaction is expected to affect the NPxY⁷⁴⁷ motif, the presence or absence of α IIB tail is not expected to affect the affinity between talin and the strong β 3 binding site, consistent with our results. Structural modeling indicates that talin, when recruited to β 3 tail, sterically clashes with the residues after the GFFKR motif of the α IIB tail, potentially disfavoring the α IIB- β 3 TMD-tail interactions that keep α IIB β 3 inactive. Conversely, loss of α IIB sequences after the GFFKR motif may enable simultaneous α IIB- β 3 TMD packing and talin- β 3 binding, two otherwise incompatible events, thus abolishing talin's capacity to activate α IIB β 3. Furthermore, the α IIB 996 β 3 truncation mutant might increase the accessibility of the β 3 tail to other cytoplasmic factors, increasing its likelihood of being recruited to focal adhesions. Indeed, the α IIB 996 β 3 integrin mutant is indiscriminately recruited to focal adhesions formed by other integrins, whereas wild type α IIB β 3 is not [41]. Our results may also help explain the interesting observation that truncation of the α 4 tail after GFFKR caused complete loss of α 4 β 1 adhesive activity, while attaching non-conserved α 2 or α 5 cytoplasmic tail to the truncated α 4 completely restores α 4 β 1 adhesive function [42–43]. However, truncating the α 4 tail also reduced diffusion and clustering of α 4 β 1 [42], indicating possible integrin-specific mechanisms of α tail function. For example, the α 4 tail can bind to paxillin [44–45] while the α IIB tail cannot.

The alternative interpretation that α IIB tail may contribute to α IIB β 3 activation through potential unknown connections to cytoskeletons and contractile machineries, as with α 4 β 1 [38] and β 2 integrins [39], is not likely to account for our results. Inside-out activation of α IIB β 3 is force and cytoskeleton independent because neither disruption of actin-cytoskeleton organization nor blocking myosin II ATPase activity affected THD-induced α IIB β 3 activation. Thus, our results showed that, besides changing the β 3 TMD topology [7] and weakening the α IIB(R995)- β 3(D723) electrostatic interaction in the cytoplasmic tails [18], β 3-bound talin sterically clashes with α IIB tails and shift the equilibrium toward activated integrins (Fig 6C). Our results establish a role for the α IIB tail distal of GFFKR in inside-out α IIB β 3 activation and open the door for future mechanistic studies on the relationship between β -tail-bound factors (such as talin and kindlin) and α subunit, and vice versa for the α -tail-bound factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We express our deep appreciation to Dr. Mark H. Ginsberg for his gifts of many reagents and his advice on the research. We thank Drs Yoshiaki Tomiyama and Hisashi Kato for sharing CMK cells. Supported by grants from National Institute of Health to Mark H. Ginsberg.

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Figure 1.

The presence, not the sequence, of αIIb tail distal of GFFKR is required for inside-out αIIbβ3 activation. (A) Truncation of αIIb tail after GFFKR significantly inhibited β3(D723R)-induced αIIbβ3 activation, while presence of irrelevant residues after GFFKR restores such activation. CHO cells were transiently transfected with αIIb and β3 construct as indicated. Integrin activation was expressed as normalized PAC1 binding and calculated as $(\text{MFI} - \text{MFI}_0) / \text{MFI}_{D57}$ where MFI was the mean fluorescence intensity of bound activation-specific antibody PAC-1 and MFI_0 was that in the presence of an αIIbβ3-specific antagonist, eptifibatide, and MFI_{D57} was the mean fluorescence intensity of anti-αIIbβ3 antibody, D57. (B) Truncation of the αIIb tail after GFFKR completely abolished talin- and kindlin-induced αIIbβ3 activation, while presence of irrelevant residues after GFFKR restores such activation. CHO cells stably expressing αIIbβ3 or αIIbβ3 mutant were transfected with a transfection marker (tomato) and pcDNA, THD, THD+kindlin-1 or THD+kindlin-2. Integrin activation in transfected cells was expressed as normalized PAC1 binding as described in (A). In (A) and (B), asterisks indicate statistical significance at 95% confidence level with two-tail, unpaired t-test. (C) Protein expression of transfected cells from (B) were analyzed by western blotting with anti-Flag antibody for kindlins, anti-HA

for THD, anti- α IIb tail (Rb8276), anti- β 3 tail (Rb8275), and anti-GAPDH. **(D)** CHO cells stably expressing α IIb β 3 or α IIb 996 β 3 were transfected with increasing amount of cDNA encoding THD and a transfection marker (tomato). Integrin activation in transfected cells was expressed as normalized PAC1 binding as described in (A). Right panel of (D) shows the expected THD expression in transfected cells. Error bars in (A), (B) and (C) are standard errors of at least 3 independent experiments.

Figure 2.

The presence, not the sequence, of αIIb tail distal of GFFKR is required for thrombin-induced αIIbβ3 activation in human acute megakaryocytic leukemia cells (CMK). **(A)** CMK cells activate αIIbβ3 in response to thrombin stimulation. Unstimulated, EDTA-treated, and thrombin-stimulated CMK cells were double stained with GPIb, a marker for megakaryocytes and platelets, and activation specific antibody PAC1 as described in methods. As previously reported [23–24], PAC1 binding was increased in GPIb-high CMK cells upon thrombin stimulation. Histogram shows the overlay of the gated regions (box) from the FACS plots. **(B)** CMK cells were transduced with αIIbβ3 or mutant as indicated, double stained with GPIb and PAC1, and analyzed as in (A). The thrombin-induced PAC1 binding was used to measure the activation of each integrins upon thrombin stimulation and was calculated as $(MFI_{thrombin} - MFI_{unstimulated})$, where $MFI_{thrombin}$ was the mean fluorescence intensity of bound PAC-1 in the presence of 1U thrombin and $MFI_{unstimulated}$ was that before stimulation. CMK cells express endogenous αIIbβ3 and thus can respond to thrombin (bar of the uninfected). Thrombin-induced αIIbβ3 activation was increased in CMK cells transduced with wt αIIbβ3, αIIb 1001β3 or αIIb_5Aβ3 but not with αIIb 996β3. **(C)** CMK cells from (B) were double stained with GPIb and a rabbit polyclonal

anti- α IIb β 3 antibody to measure the expression level of α IIb β 3 in GPIb-high cells. CMK cells transduced with α IIb β 3, α IIb 996 β 3, α IIb 1001 β 3 or α IIb_5A β 3 had similar high level of α IIb β 3 expression over the uninfected, indicating that the defects of α IIb 996 β 3 seen in (B) is a direct result of lost responsiveness of α IIb 996 β 3 to thrombin stimulation. Error bars in B and C indicate standard errors of 3 experiments.

Figure 3.

The first few residues distal of GFFKR are required for optimal αIIb and β3 TMD-tail interactions. (A) Schematic representation of the αIIb and β3 TMD-tail interaction assays as previously described [26]. Flag tagged αIIb-TMD-tail-TAP and Tac-β3-TMD-tail fusion proteins were co-expressed in CHO cells. αIIb fusion protein was captured by calmodulin beads and amount of β3 TMD tail bound to the αIIb was analyzed by western blotting. (B) Western blots of the input and captured αIIb or β3 were shown. αIIb TMD-tail was blotted with anti-Flag and β3 TMD-tail was blotted with anti-Tac. αIIb 996 and αIIb_5A bind less to β3 than wt αIIb and αIIb 1001 do.

Figure 4.

Deletion of αIIb tail after GFFKR does not affect $\alpha\text{IIb}\beta 3$ -talin interactions or outside-in integrin signaling. **(A)** CHO cells stably expressing $\alpha\text{IIb}\beta 3$ wt or $\alpha\text{IIb} \ 996\beta 3$ were transfected with HA-talin and lysed after culturing for 24 hours. $\alpha\text{IIb}\beta 3$ was immunoprecipitated with anti- $\alpha\text{IIb}\beta 3$ (Rb8053) and precipitants were eluted with SDS-PAGE loading buffer, and detected by western blotting with anti-HA for talin or anti- $\beta 3$. $\alpha\text{IIb} \ 996$ truncation did not affect talin binding. **(B)** Recombinant $\alpha\text{IIb}\beta 3$ or $\alpha\text{IIb} \ 996\beta 3$ was captured onto D57-conjugated agarose beads. The immobilized integrin beads were then incubated with various concentrations of purified THD, washed, eluted and analyzed by SDS-PAGE and western blotting. Bound THD was detected by blotting with anti-His6 tag and immobilized integrins were detected by coomassie staining (bottom two panels). There is no statistically significant difference between $\alpha\text{IIb}\beta 3$ and $\alpha\text{IIb} \ 996\beta 3$ in their affinity for THD. Error bars indicate standard deviation of 3 independent experiments. **(C)** CHO cells stably expressing $\alpha\text{IIb}\beta 3$ wt or $\alpha\text{IIb} \ 996\beta 3$ were serum starved overnight and plated on fibrinogen-coated plates for the specified periods of time, lysed and probed for signaling and control proteins as indicated in the western blots. $\alpha\text{IIb} \ 996$ truncation did not affect signaling as assessed by phosphorylation of Akt or FAK. **(D)** CHO cells stably expressing $\alpha\text{IIb}\beta 3$ or $\alpha\text{IIb} \ 996\beta 3$ were plated on fibrinogen-coated coverslips for 60 minutes, fixed with formaldehyde and imaged using phase-contrast microscope. There is no significant

difference of cell spreading between cells expressing $\alpha\text{IIb}\beta 3$ and $\alpha\text{IIb } 996\beta 3$. Scale bar is 50 μm .

Figure 5. Binding of talin to β3 tail results in steric clashes between talin and αIIb. **(A)** Shown in red and blue are the structure of integrin αIIb and β3 TMD complex in cellular membranes [13]. The F3 subdomain of THD is shown in green. The structure of the entire complex was assembled by building a homology model of β3 tail after the β1D in the β1D-Talin F2F3 complex structure [18] and aligning the resulting β3-Talin F2F3 model to the β3 of αIIbβ3 TMD complex structure determined in cellular membrane [13]. Talin head overlaps with αIIb tail in the model, indicating potential steric hindrance. **(B)** Sequence alignment of α tails. The α tails display no significant sequence similarity after GFFKR motif, suggesting no requirement for sequence specificity despite a number of them are regulated by talin and kindlins. **(C)** The capacity of THD to activate αIIbβ3 was not affected when αIIb tail was swapped with non-homologous sequences from α5 or αL. Integrin activation was expressed as normalized PAC1 binding and calculated as $(MFI - MFI_0) / MFI_{D57}$ where MFI was the mean fluorescence intensity of bound PAC-1 and MFI_0 was that in the presence of an αIIbβ3-specific antagonist, eptifibatide, and MFI_{D57} was the mean fluorescence intensity of D57. Asterisks indicate statistical significance at 95% confidence level with two-tail,

unpaired t-test. Error bars are standard errors of at least 3 experiments. **(D)** Protein expressions of the transfected cells from (C).

Figure 6.

Models of α IIb tail participating in talin-mediated α IIb β 3 activation. **(A)** CHO cells stably expressing α IIb β 3 (A5 cells) were transfected with a transfection marker (GFP), and pcDNA, or THD as indicated. Cells were treated with latrunculin A (2 μ M) to inhibit actin polymerization or blebbistatin (50 μ M) to inhibit myosin II ATPase activity. α IIb β 3 activation was expressed as normalized PAC1 binding and calculated as $(\text{MFI} - \text{MFI}_0) / \text{MFI}_{\text{D57}}$ where MFI was the mean fluorescence intensity of bound PAC-1 and MFI_0 was that in the presence of an α IIb β 3-specific antagonist, eptifibatide, and MFI_{D57} was the mean fluorescence intensity of D57. The lower panel showed the protein expressions of the transfected cells. Asterisks indicate statistical significance at 95% confidence level with two-tail, unpaired t-test. Error bars are standard errors of 4 experiments. **(B)** Cells from (A) were fixed with 3.7% formaldehyde, permeabilized with 0.2% triton-100, stained with alexa488-phalloidin for actin, and imaged at 60 $\times$ with a fluorescence microscope as described in the methods. At the concentration used in (A), latrunculin A demonstrably disrupted the cellular F-actin and blebbistatin markedly changed cell morphology, presumably as a result of blocked myosin II activity and loss of contractility. Scale Bar is 10 μ m. **(C)** Model of talin-induced integrin activation. Besides causing topological changes in the β 3 TMD, talin also clashes with the residues distal of GFFKR in α IIb tail, potentially

displacing the α II tail. Combined effects from both mechanisms result in the loss of α - β interactions and integrin activation.