The Protein Journal (2018) 37:280–289
https://doi.org/10.1007/s10930-018-9776-8

Differential Binding of Active and Inactive Integrin to Talin

Dongchuan Wang[1] · Qiang Guo[2] · Ailin Wei[3] · Ang Li[3]

Published online: 21 May 2018

Abstract
Bi-directional signaling of integrins plays an important role in platelet and leukocyte function. Talin plays a key role in integrin bi-directional signaling and its binding to integrin is highly regulated. The precise regulation of the recruitment and binding of talin to integrin is still being elucidated. In particular, the recruitment of talin to integrin is controlled by the RAP-1 and RIAM/lamellipodin signaling axis and the affinity between talin and integrin is regulated by the conformation or protease cleavage of talin. However, whether the binding between integrin and talin is also regulated by integrin conformation has not been thoroughly explored before. In this work, we used biochemical binding assays to study the potential role of integrin conformational changes in integrin–talin interactions. Constitutively active integrin αIIbb3 binds markedly stronger to talin than inactive αIIbb3. Inactive αIIbb3 markedly increases its binding to talin once activated, regardless of how αIIbb3 is activated. Further, the increased binding to talin is b3 tail dependent. Our results suggest that integrin conformation is another regulatory mechanism for integrin–talin interaction.

Keywords Integrin · Talin · Active integrin · Integrin signaling · Outside-in signaling · Integrin binding

1 Introduction

Bi-directional signaling of integrin to cytoskeleton plays vital roles in immune response, hemostasis, development, and cancer metastasis [1–3]. Integrins are type I transmembrane (TM) domain proteins consisting of α and b subunits, each having a large extracellular domain, a single pass transmembrane domain, and a short cytoplasmic tail [4]. Integrins are normally inactive and have low affinity for its ligand, e.g. extracellular matrix proteins, but undergo rapid activation upon various stimuli in a process termed "inside-out" signaling or "activation" [1–3]. Upon integrin engagement with extracellular matrix (ECM), integrins undergo further conformational changes, leading to the recruitment and the binding of cytoplasmic adaptor proteins, signaling molecules, and/or other transmembrane receptors to the integrin TM domains or cytoplasmic tails in a process termed "outside-in" signaling [1, 2, 5, 6]. Such integrin bi-directional signaling is often coupled: integrins are first activated through inside-out signaling, resulting in integrin-ECM binding; the integrin-ECM binding will then trigger outside-in signaling, leading to assembly of integrin adhesome, linkage of integrin cytoplasmic tail to cytoskeleton, formation of stress fiber, and formation of other adhesive signaling structures [1–3, 5–8]. The bi-directional inside-out and outside-in signaling together controls many cellular phenomenon such as cell adhesion, cell migration, cell survival, and immune synapse formation [1–3, 5–8].

Cytoplasmic protein talin participates in both the inside-out and the outside-in signaling [9–12]. Talin binds to integrin cytoplasmic tail and induces a tilting angle change of integrin b TM domain, which destabilizes the α and b TM domain interaction and activates integrin [13, 14]. Talin binding also sterically clashes with the membrane proximal region of the α cytoplasmic tail and further interferes with the α and b TM packing [15]. Talin will also binds to activated and ligand-engaged integrin in outside-in signaling

Dongchuan Wang and Qiang Guo contributed equally to this study.

✉ Ang Li
AngLi@scu.edu.cn

[1] Institute of Hospital Management, West China Hospital, Sichuan University, 37 Guoxue Rd., Chengdu, Sichuan Province, China

[2] Department of Vascular Surgery, West China Hospital, Sichuan University, 37 Guoxue Rd., Chengdu, Sichuan Province, China

[3] Department of Pancreatic Surgery, West China Hospital, Sichuan University, 37 Guoxue Rd., Chengdu 610041, Sichuan Province, China

and along with other integrin-binding adaptor proteins to induce clustering of integrins, mediate integrin linkage to cytoskeleton, and mediate the formation of focal adhesions or other strong adhesion structures [16–19]. Therefore, the binding between talin and integrin is a critical event in integrin bi-directional signaling.

As a critical event in integrin signaling, the binding of talin to integrins is regulated by at least two mechanisms: through the recruitment of talin to integrin tails and through the modulation of the affinity between talin and integrins. Talin recruitment to integrin is regulated by the RAP-1 and RIAM complexes and by vinculin [18, 20–22]. RIAM binds both to talin and RAP-1 and mediates the formation of the RAP-1–RIAM–Talin complex [18, 22]. This RAP-1–RIAM–Talin complex is targeted to nascent cell adhesion structure at the cell membrane via the RAP-1 membrane targeting sequences and localizes at the tips of growing actin filaments in lamellipodial and filopodial protrusions, thus corresponding to the tips of the "sticky fingers" [9, 18, 20–22]. Vinculin displaces RIAM by competing with RIAM for talin binding and release the auto-inhibitory interactions within talin, thereby inducing the integrin-activating capacity of talin [18, 20–22]. Tuned by RIAM and vinculin, talin acts as a molecular switch that mediates the transition of integrin-based adhesions from drivers of nascent lamellipodial protrusion to stable, force-bearing adhesions [18, 20–22]. Thus, talin binding to integrin is regulated temporally and spatially by other proteins through recruitment, targeting, and the conformational changes in talin.

The Affinity between talin and integrin is also regulated by the conformation of talin and by protease cleavage of talin. Calpain cleaves talin into a talin head domain and a talin rod domain [23]. The talin head domain from the calpain cleavage encompasses the functional fragments for integrin binding and activation and has a sixfold higher affinity to integrin b tail peptides [23]. Talin is also reported to be in an auto-inhibited state, where its membrane targeting and integrin binding is blocked by an interaction between talin head and talin rod [20, 24–27]. The removel of the head-rod interaction enables targeting of talin to plasma membrane and the activation of integrin by talin [20, 25–27].

The transition from nascent adhesion to stable focal adhesion is a complex process. Consequently, it is expected that multiple levels of regulation on talin-integrin interaction may work concertedly to switch the adhesions on and off while the cells migrate. Despite of this interest, whether other regulatory mechanisms on talin-integrin interaction may be at play is still unclear. Here we revealed a novel regulatory mechanism of regulation of the interaction between talin and integrin. We report that the affinity of talin to constitutively active integrin is markedly higher than that of talin to inactive integrin. The affinity of inactive integrin to talin can be "turned up" to a level comparable to that between talin and constitutively active integrin by a variety of integrin activating reagents, including Mn^{2+}, RGD-like ligand, activating antibodies, and DTT. Our results indicate that integrin activation can induce stronger binding with talin.

2 Materials and Methods

2.1 Preparation of Integrin αIIbb3

Integrin αIIbb3 was purified from outdated human platelets based on a modified protocol [28, 29]. Briefly, outdated platelets were centrifuged first at 300×*g* to remove the red and white blood cells. The supernatant was subsequently centrifuged at 1800×*g* to pellet the platelets. The platelets were washed three times with Tris-buffered saline and then extracted overnight with lysis buffer: 20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM PMSF, 0.5 mM $CaCl_2$, 10 μM Leupeptin, 10 μM protease inhibitor E64 (Sigma), 2.76 μM Calpeptin. The extracted integrin αIIbb3 was purified with a Con A column. The fibrinogen and thrombospondin-1 in the extracted αIIbb3 were then removed by absorbing against a Heparin column. αIIbb3 was further purified by gel filtration chromatography using a Superdex-200 column. The purified integrins were kept in a buffer of 20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM MgCl2 and 1 mM $CaCl_2$ and stored in − 80 °C until use.

The outdated platelets used for biochemical integrin purification were from commercial source (Chengdu Blood Center, Chengdu, China) and have been de-linked from identities of the donors. The use of outdated platelets for integrin purification has been approved by West China Hospital review board. Two independent preparations of αIIbb3 integrin were performed and tested in this study.

2.2 Separation of Inactive and Active αIIbb3

The active integrin was separated from inactive integrin according to a modified protocol [28–32]. Briefly, purified total αIIbb3 was passed through an immobilized KYGRGDS affinity matrix and the bound active αIIbb3 was eluted by competitive binding with 20 μM of RGD peptide mimetic ligand, epitifibatide (also named integrilin) [33, 34]. The KYGRGDS peptide column was regenerated by alternating three washes of low (pH = 3.5) and high pH (pH = 8.5) buffers with 0.5M NaCl. The KYGRGDS peptide column was re-equilibrated and flowthrough of the 1st KYGRGDS-column affinity purification was passed for the 2nd time through the KYGRGDS-column. The bound active αIIbb3 was again eluted by competitive binding with 20 μM of RGD peptide mimetic ligand, epitifibatide. The area under elution peak

in the two affinity purification was measured using the Unicorn 4.1 program. The purified active αIIbb3 was dialyzed extensively to remove the epitifibatide used for elution. Both inactive and active αIIbb3 was analyzed by SDS–PAGE and coomassie staining to assess the purity of the αIIbb3.

2.3 Purification of Recombinant Talin Head

The recombinant human talin head expression plasmid construct was a generous gift from Dr. Mark Ginsberg [28]. R358A and W359A double mutations were introduced to the talin head domain by performing sited directed mutagenesis using commercial kit (Agilent, Santa Clara, CA, USA). The R358A and W359A mutations and their effects were previously described [35]. The recombinant talin head domain (wild type and mutant) was expressed in E. coli BL21-DE and purified with His-binding beads according to manufacture's instruction (Novagen). The purified proteins were dialyzed thoroughly in two buffers: first against 20 mM Tris, 150 mM NaCl, pH 7.4 (TBS buffer) with 2 mM EDTA to completely remove the residual Ni^{2+} on the his6 tag; and then against EDTA-free TBS buffer.

2.4 Assay for Integrin Binding to Talin

ELISA assay for measuring binding between αIIbb3 and talin head domain was performed using a protocol modified from the previous report [28, 36, 37]. High capacity ELISA plates (Thermo Fisher, USA) were coated with 5 µg/ml AP3 antibody overnight at 4 °C and blocked with BSA for 1 h at 37 °C. The plates were thoroughly washed, 6 µg/ml integrin was added and incubated for 2 h at room temperature. The wells were again thoroughly washed and purified V5-tagged talin head domain was added to the wells and incubated with captured αIIbb3 for 2 h at 37 °C. Unbound talin head domain was washed off and bound talin head domain was detected by a mouse anti-V5 antibody, further detected by an HRP-conjugated anti-mouse-IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA), and quantitated by chemi-luminescence HRP substrate, Enhanced Chemi-Luminescence (ECL) reagent (BD Bioscences). The luminescence was measured on a plate reader (PerkinElmer) and analyzed.

To test binding of αIIbb3 to talin head domain when integrins were activated, various integrin activators were added along with talin head domain in the above described binding assay. The activators used include 1 mM $MnCl_2$, 20 µM of anti-LIBS6 (generous gift from Dr. Mark Ginsberg), 1 mM of DTT, and 1 mM KYGRGDS peptide. For competitive inhibition of talin binding to αIIbb3, 10 µM of recombinant b1 cytoplasmic tail peptide (generous gift from Dr. Mark Ginsberg) was used because b1 tail peptide has much higher solubility than b3 cytoplasmic tail peptide.

2.5 Assay for Calpain Cleavage

b3 tail can be cleaved off from the purified αIIbb3 by calpain [38]. To assay cleavage of b3 tail, protocols from published studies were adopted and modified [28]. Briefly, 25 µg of recombinant calpain-II (CalBiochem) were incubated with 1 mg purified αIIbb3 in a buffer of TBS plus 0.1% Triton, 1 mM $CaCl_2$ and 1 mM MgCl2 at room temperature overnight. The calpain was neutralized by E-64 (Sigma) at a final concentration of 10 µM and specific calpain inhibitor calpeptin at a final concentration of 5 µM. An ELISA assay was adopted to ascertain that the tail has been effectively cleaved. Briefly, high capacity ELISA plates were coated with 100 µl of 5 µg/ml AP3 antibody overnight at 4 °C, blocked with BSA for 1 h at 37 °C, and incubated with 6 µg/ml of cleaved or uncleaved αIIbb3 for 1 h at room temperature. The wells were thoroughly washed and either Ab8053 against the whole αIIbb3 protein or Ab8275 against the b3 tail was added. The mixture was incubated with the captured αIIbb3 for 1 h at room temperature and thoroughly washed. The washed wells were then incubated with HRP-conjugated goat anti-rabbit Ig's antibody for one hour at room temperature. The amount of antibody binding was quantitated with peroxidase substrate Enhanced ChemiLuminescence (ECL) reagent (BD Biosciences). The luminescence of the test wells was read on a plate reader (PerkinElmer).

2.6 Assay for the Activation State of Purified Integrin αIIbb3

Integrin activation assay was adopted from published a protocol [28]. Briefly, high capacity ELISA plates were coated with 5 µg/ml AP3 antibody overnight at 4 °C, blocked with BSA for 1 h at 37 °C. The plates were thoroughly washed, 6 µg/ml integrin was added and incubated for 2 h at room temperature. The wells were again thoroughly washed and incubated with 5 µg/ml PAC-1 antibody [39] to measure the activity of αIIbb3. Either anti-LIBS6 or anti-LIBS6 plus 20 µM eptifibatide were added with PAC-1 as positive activation controls and negative controls. After 2 h of PAC-1 binding, the wells were again thoroughly washed and horse-radish-peroxidase (HRP) conjugated µ-chain-specific anti-mouse IgM were added for 1 more hour of incubation. Following the final wash, HRP substrate, ECL reagent, was added to the wells and the plate was read on a plate reader.

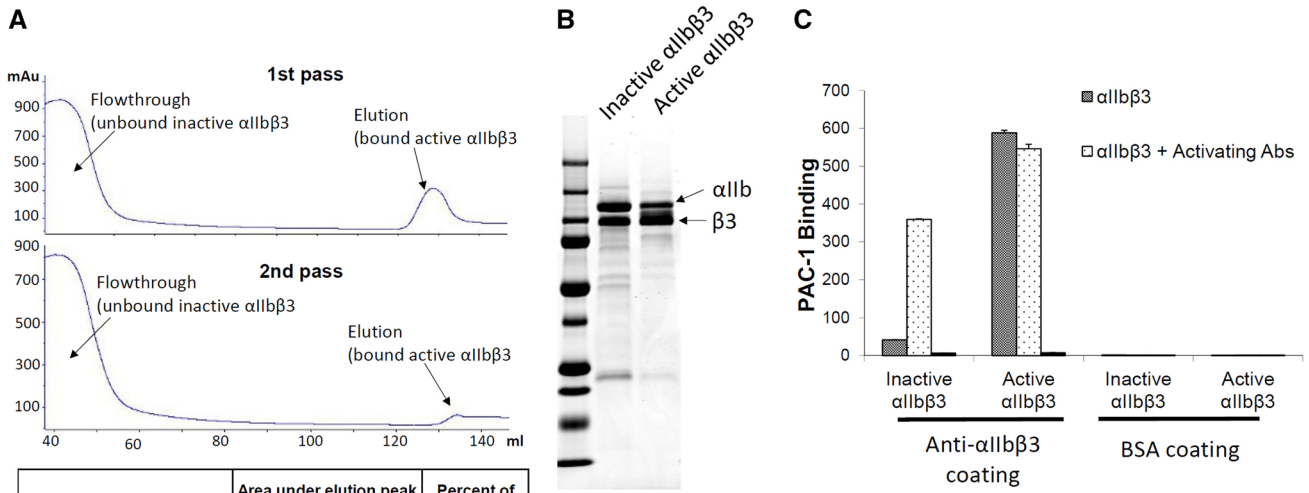
3 Results

3.1 Preparation of Inactive and Constitutively Active Integrins

We purified integrins from outdated human platelets. The vast majority of integrin αIIbb3 from these outdated resting platelets is inactive but a small fraction is in a constitutively active state [28, 31]. We therefore separated the active integrins from inactive integrins using a RGD-peptide conjugated column [32]. The total purified αIIbb3 integrin was passed through the RGD-peptide affinity column twice to completely deplete the active αIIbb3 from inactive integrin αIIbb3. The amount of active αIIbb3 bound to the RGD-column in the second passage as shown by the area under elution peak was only 6% of that bound in the first passage, indicating that the vast majority of the active αIIbb3 had been depleted by the RGD-column affinity purification (Fig. 1a). Both active and inactive integrin αIIbb3 were purified to high purity as shown by the SDS–PAGE analysis (Fig. 1b). Consistent with previously reports by others [30, 40], the active integrin and inactive integrins have slightly different appearance in SDS–PAGE gel, likely due to difference in structures and post-translational modification.

The activation states of the purified integrins were validated in an ELISA format integrin activation assay using an activation-specific ligand-mimetic antibody, PAC-1 [28, 39]. The inactive αIIbb3 bound PAC-1 minimally (Fig. 1c). Upon activation by an activating antibody, anti-LIBS6 [41], binding of the αIIbb3 to PAC-1 increased dramatically (Fig. 1c). The increase was specific as such increase was inhibited by a mimetic αIIbb3 ligand, eptifibatide (Fig. 1c). Consistent with previous reports that activation of integrin is not binary but rather a shift in the equilibrium between inactive and active integrins [1, 2, 41–43], activating antibody appears to further shifting the equilibrium by locking more active integrins in the active conformation. Thus, the purified inactive αIIbb3 is inactive and can be specifically activated.

The purified constitutively active αIIbb3 bound PAC-1 at a high level, and the high PAC-1 binding was not further increased upon stimulation of an activating antibody, indicating that these constitutively active αIIbb3 was maximally active (Fig. 1c). The binding of active αIIbb3 to PAC-1 was specific as it was completely inhibited by a mimetic αIIbb3 ligand, eptifibatide (Fig. 1c). Thus the inactive and active integrin αIIbb3 has been separated.



	Area under elution peak (mAu*ml)	Percent of total
1st Run with RGD Column	1925	94%
2nd Run with RGD Column	124	6%

Fig. 1 Preparation of integrin αIIbb3 and separation of active from inactive integrin αIIbb3. **a** Purified total integrin αIIbb3 was passed through the RGD-peptide column twice. The upper panel shows the chromatogram of the 1st pass and the lower panel shows the second pass. The table at the bottom shows the area under the elution peak of the 1st and the 2nd pass. The amount of active integrins bound to the RGD column was only a 6% of the amount bound in the first pass, indicating that the active integrin αIIbb3 has been depleted. **b** SDS–PAGE analyses followed by Coomassie staining showing that both inactive and active integrin αIIbb3 has been purified to high purity. **c** Integrins were captured on an high capacity ELISA plate by an immobilized anti-b3 antibody, AP3. Ligand mimetic antibody PAC-1 was added to the integrins alone, with an activating antibody anti-LIBS6, or with anti-LIBS6 plus epitifibatide. The bound PAC-1 was then quantified by an HRP-conjugated μ-chain specific anti-IgM antibody and the HRP luminescence substrate ECL and shown in the Y-axis in arbitrary luminescence units. Results show that inactive integrins are inactive and activatable; and active integrins are maximally active. In **c**, error bar represents SEM from 3 experiments

3.2 Active Integrin Binds Stronger to Talin than Inactive Integrin

To test whether the binding between integrin and talin is regulated by integrin activation, we compared the binding between active and inactive αIIbb3 to talin head domain, the functional fragment in talin that binds and activates integrins [28, 44]. The inactive αIIbb3 bound weakly to talin (Fig. 2a); in sharp contrast, active αIIbb3 bound markedly stronger to talin (Fig. 2a). To ascertain that the markedly higher binding between active αIIbb3 and talin was specifically attributable to the b3 tail that contains the known talin binding sites but not due to other conformational changes in active αIIbb3, we prepared tailless active and inactive αIIbb3 by subjecting αIIbb3 integrins to calpain digestion, which specifically cleaves off b3 tails. The b3 tails in both inactive and active integrins were efficiently removed, as the about 80% of the binding of an anti-b3-tail antibody Ab8275 was lost; whereas an anti-whole-αIIbb3 antibody was unaffected (Fig. 2b, c). Part of the membrane proximal region may be inaccessible to calpain cleavage due to the detergent micelle formed around the transmembrane domain. The increase in binding between active αIIbb3 and talin over that of talin and inactive integrin was lost in a tailless active αIIbb3, indicating that the binding between active αIIbb3 and talin was specifically b3 tail dependent (Fig. 2a). As previously reported, there are two talin binding sites in integrin b tails: a strong membrane distal site that contributes to the majority of the free energy gain from the binding and a weak membrane proximal binding site [45]. The weak membrane proximal site and/or the incomplete cleavage may contribute to some of the residual talin binding in the tailless integrin.

To confirm that the binding between active αIIbb3 and talin was specifically attributable to b3 tail, we tested whether an integrin b tail peptide could competitively block the binding between active αIIbb3 and talin. The binding between active αIIbb3 and talin was almost completely abolished in the presence of the b tail peptide, indicating that the binding between active αIIbb3 and talin was specifically attributable to b3 tail (Fig. 2d). To further ascertain that the increased binding between active integrin and talin over that between inactive integrin and talin is specific, we tested the

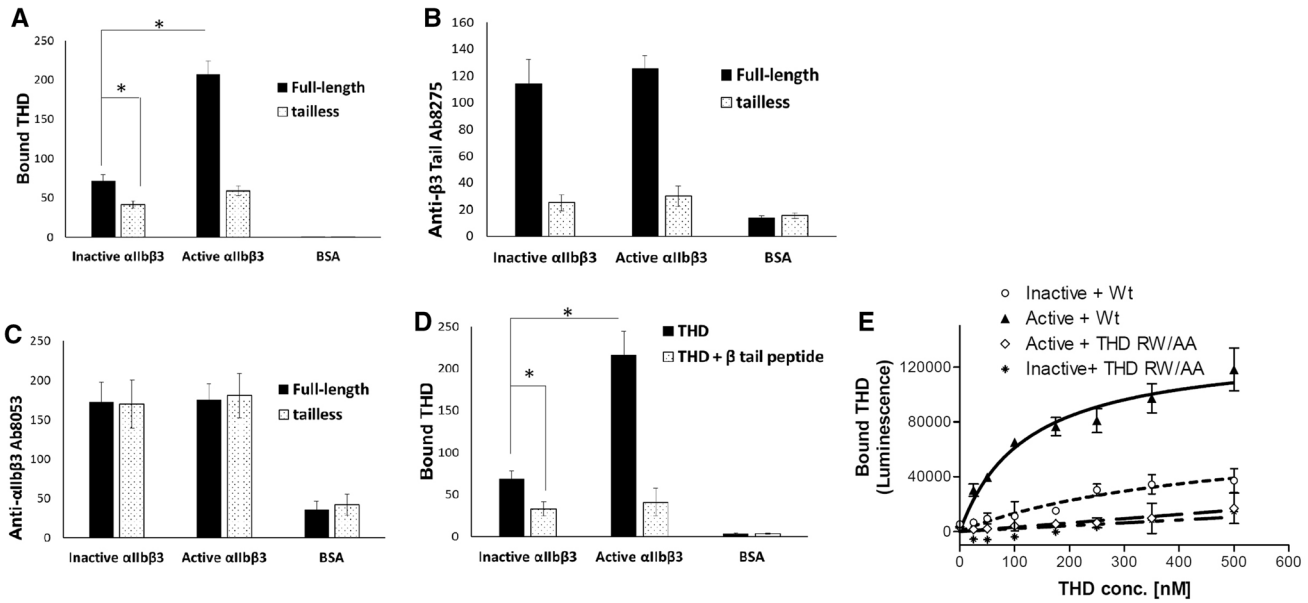


Fig. 2 Differential binding of inactive and active αIIbb3 to talin head domain. **a** Either intact or tailless Integrin αIIbb3 was captured on an high capacity ELISA plate by an immobilized anti-b3 antibody, AP3. V-5 tagged talin head domain was added and the amount of bound talin head domain was assessed by an HRP conjugated anti-V5 antibody and the luminescent HRP substrate ECL. Results show that inactive integrin binds weakly to talin head domain; binding between talin and active integrin is markedly stronger; and the markedly higher talin binding with active αIIbb3 was lost in tailless αIIbb3. **b** Tailless αIIbb3 has no b3 cytoplasmic tails. Integrin αIIbb3 was subject to calpain digestion and neutralized with calpeptin. Intact or tailless αIIbb3 was captured on a high capacity ELISA plate by an immobilized anti-b3 antibody, AP3. The presence or absence of b3 tail was then assessed by a b3-tail specific polyclonal antibody Ab8275. Results indicate that calpain completely cleaved off the b3 tail from the integrin αIIbb3. **c** Similar to B, except that an anti-whole-αIIbb3 antibody, AB8053, was used as a control. **d** Similar to (**a**), but showing the binding of inactive and active αIIbb3 to talin head domain in the presence of a b1 tail peptide as competitive inhibitor. The binding between active integrin and talin is inhibitable by the integrin b cytoplasmic domain peptide. **e** Similar to (**a**) except that an increasing concentration of wild type (wt) talin head domain or talin head domain with R358A and W359A double mutations (RW/AA) were tested. Results in (**e**) were plotted and fitted with one site binding curve in GraphPad Prism. All error bars represents SEM from 3 experiments. In **a**–**e**, arbitrary luminescence units were shown in Y-axis and asterisks indicate statistical significant difference at $P < 0.05$ in a two tailed t-test

binding between active integrin and talin at various concentration of talin. At every concentration tested, higher binding between active integrin and talin was observed (Fig. 2e); whereas no significant difference in talin binding was seen with a talin double mutant (R358A and W359A, referred to as RW/AA) that is defective in integrin binding (Fig. 2e) [35]. Active integrin binds to talin with a K_D value of 121 ± 30 nM. Such an affinity is consistent with the K_D value observed between talin and synthetic b tail peptide, which is often considered to mimic the active integrin [23, 45]. Inactive integrin binds to talin with a much lower K_D value of 472 ± 288 nM. Thus, active integrin binds stronger to talin than inactive integrin and the increased binding is specifically attributable to the b3 tail of active αIIbb3.

3.3 Inactive Integrin Binds Stronger to Talin Upon Activation

Next we investigated the whether the binding of integrins to talin is increased when integrins are activated. Work from the past three decades have shown that integrins can be activated by a variety of well-established, routinely-used means, including Mn^{2+} [42, 46, 47], small molecule ligand [40, 42], reducing reagent [30, 31, 48], or activating antibodies [28, 41, 49], all of which are widely and commonly used in the literature. The binding of αIIbb3 to talin increased markedly upon αIIbb3 activation, regardless the activation was induced by Mn^{2+}, eptifibatide (integrillin), DTT, or an activating antibody (Fig. 3). Moreover, such increased talin binding upon activation was specifically attributable to b3 tail, as the increased binding between activated αIIbb3 and talin was lost when a tailless inactive αIIbb3 was used (Fig. 2a). Furthermore, the increased binding between activated αIIbb3 and talin was specifically inhibited by a b1 tail peptide. Therefore, inactive integrin binds weakly to talin and the binding is markedly increased upon integrin activation in a b3-tail dependent manner (Fig. 4).

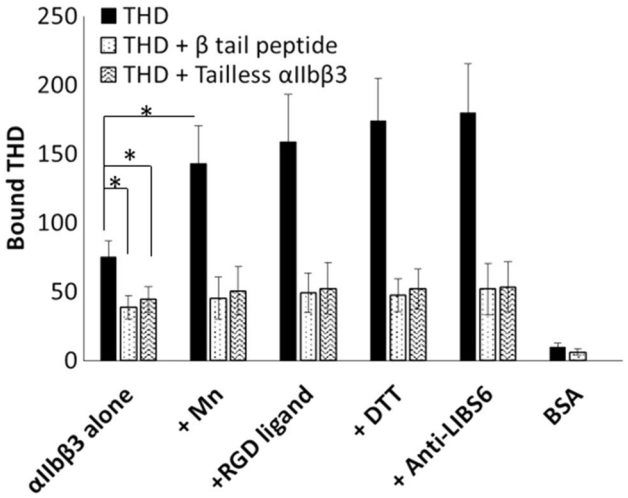


Fig. 3 Inactive integrin binds markedly stronger to talin upon activation. Similar to Fig. 2a, except that inactive αIIbb3 or inactive tailless αIIbb3 was used in the presence of various activators as denoted in the x-axis. The binding between intact αIIbb3 and talin head domain was also assessed in the presence of a b1 tail peptide as competitive inhibitor. The inactive αIIbb3 binds markedly stronger to talin upon activation regardless of the activator used; and the increased talin binding is b3-tail dependent and inhibitable by a integrin b cytoplasmic domain peptide. Error bars represents SEM from three experiments. Arbitrary luminescence units were shown in Y-axis and asterisks indicate statistical significant difference at $P < 0.05$ in a two tailed *t* test

4 Discussions

Talin is a pivotal regulator in the bi-directional integrin signaling. In the inside-out signaling, talin binds to integrin b tail and activate integrins together with co-activators such as kindlins [10, 28, 47, 50–53]. In the outside-in signaling, talin scaffolds between integrins and cytoskeleton, forming the probing "sticky fingers" in the lamellipodium, switching between transient and more stable integrin adhesion structure, and sensing and signaling the adhesive tension sustained by the integrin adhesion structure [16–21, 26]. Therefore, the regulatory mechanisms for the binding between talin and integrin has been a focal point in the research of integrin signaling. At least two mechanisms have been proposed: (1) through the targeting of talin via the RAP-1–RIAM–Talin complex [18, 20–22, 26]; and (2) through the regulation of talin-integrin binding affinity via talin conformational changes or protease cleavage [20, 23, 26, 27]. Here we report a novel mechanism of regulating talin-integrin interactions: regulating talin-integrin binding affinities via integrin conformational changes. We show that integrin binds weakly to talin when inactive but markedly increases its binding to talin upon activation.

Our finding is consistent with previous reports on integrin–talin binding affinity. Yan et al. previously reported that the b3 cytoplasmic tail peptide binds to talin head domain with a K_D value of ~ 100 nM [23]. Wegener et al. reported a similar K_D value of 140 nM between a mimetic b3 cytoplasmic tail peptide and talin F3 subdomain, a PTB-like domain within talin head [45]. These reported affinities of talin and b3 cytoplasmic tail peptides are consistent with the K_D value of 121 ± 30 nM between active integrin and talin determined here. Thus the studies in Yan et al. and Wegner et al. most likely reflect the interaction between active integrin and talin, as b3 cytoplasmic tail peptides mimic the freely available b3 cytoplasmic tail in the separated integrin α and b transmembrane and cytoplasmic subunits in the activated integrins [42]. We speculate that such separation of α and b cytoplasmic domains removes the inhibition of α subunits on the interaction between b cytoplasmic tail and talin.

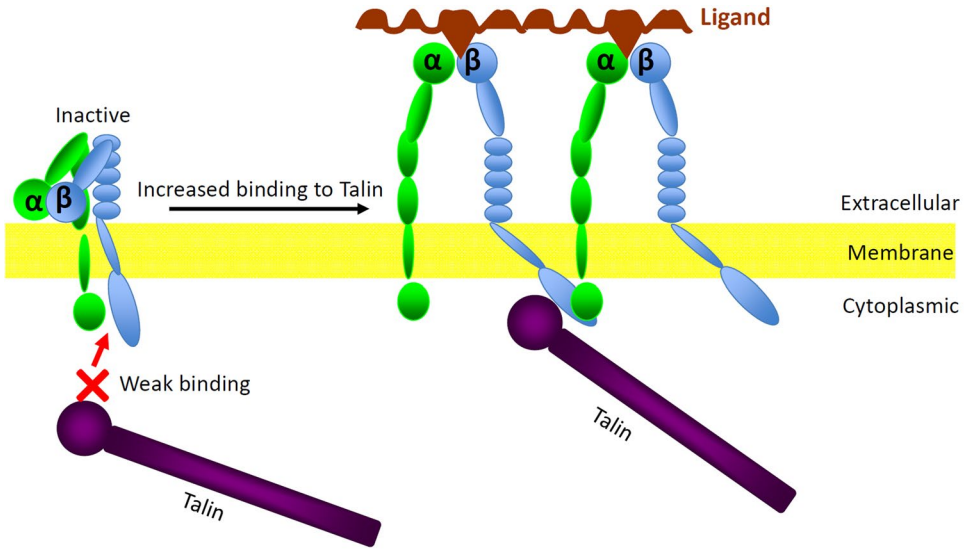


Fig. 4 Schematic representation of the hypothesis. In inactive integrin, αIIb and b3 cytoplasmic tails interact with each other thus reducing the affinity between b3 tail and talin. In active integrins, αIIb and b3 tails are separated, making b3 tail freely accessible to talin, and providing stronger interaction between αIIbb3 and talin

Many reports support that there is an integrin α and b cytoplasmic domain interaction that is altered upon integrin signaling. This interaction between αIIb and b3 cytoplasmic tail is indispensable in maintaining the inactive state of αIIbb3 integrin and deletion or mutation of either the αIIb or b3 cytoplasmic domain residues results in constitutive integrin activation [43]. Biochemical studies further support an interaction between the α and b tails [54–56]. Tethering of the α and b cytosolic tail with coiled-coil forming peptides or covalent cross-linking results in inactive integrin that cannot be physiologically activated but becomes active upon release of the tethering [57, 58]. Also, peptides corresponding to the αIIb and b3 cytosolic tails are dynamically unstructured on their own but show conformational propensities for membrane-proximal helical structure [59, 60]. Additionally, heterodimeric αIIb-b3 cytosolic tails NMR structures show membrane-proximal αIIb and b3 interactions [61, 62].

Besides studies with modeled α and b tail peptides, the α and b cytoplasmic domain interactions have also been observed full-length integrins in cells. The disulfide bond cross-linking experiments demonstrate that αIIb and b3 cytoplasmic tail come into interaction at many residues throughout the cytoplasmic tail with multiple residues on either αIIb and b3 cytoplasmic tail producing 80–100% cross-linking efficiency [63]. Moreover, outside-in integrin signaling stimuli lead to a decrease in fluorescence resonance energy transfer between fluorophore-linked α and b cytosolic domains indicative of their dissociation [64]. Furthermore, α and b subunit transmembrane and cytoplasmic domain separation is indispensable for integrin outside-in signaling as tethering of the transmembrane and cytoplasmic domain with a disulfide bonds abolished outside-in integrin signaling [65]. Therefore, our results, taking together with these previous reports, suggest that that α and b cytoplasmic domain is in close interaction in the inactive state that kept the binding to talin low and b cytoplasmic tail is freed from α cytoplasmic tail upon integrin activation, resulting in stronger binding for cytoplasmic talin.

The weak interaction between inactive integrin and talin may be a mechanism of the cells to control unwanted or "false" inside-out and outside-in integrin signaling. Multiple levels of regulation then come into play to coordinate integrin signaling. First, talin is recruited to the membrane and the site of integrin, thus increasing the local concentration of talin to overcome the initial weak binding between talin and integrin [18, 20, 22, 26]. The integrins become activated by talin as a result of the high local concentration of talin. Activated integrins then binds stronger to talin, forming a stable integrin–talin complex that serves the nucleation site for the assembly of integrin adhesome. Multiple reports further comport with our hypothesis. First, active integrins are concentrated in the focal adhesions, sites where integrins are connected to talin [66]. Second, active integrins are at the leading edge where nascent integrin–talin complex is [18, 20]. Third, conformational changes, i.e. the separation between integrin α and b subunits, by themselves, are sufficient to induce outside-in integrin signaling [67]. Although it is conceivable that the binding between other intracellular integrin binding proteins and integrin is similarly regulated by the activation state of integrin because the binding sites on integrin tails are shared among various integrin binding proteins [68, 69], our studies are limited to talin and further studies are need to extend the present work to other intracellular integrin binding proteins.

We used ELISA assays to measure the binding between active integrin and talin as well as that between inactive integrin and talin. Major limitations of an ELISA assay include the non-specific adsorption of antibodies or other protein reagents to the solid phase contributing to false positivity

and the non-preferential measurement of protein interactions in the solid phase as opposed to in a solution mimicking the cytoplasm. We minimized the risk of non-specific binding by using at least three controls: the tailless active integrin, inhibition of the binding by a b tail peptide, and a talin mutant that does not bind to integrin. Future work is needed to develop model peptides that can mimic both inactive and active α-b integrin cytoplasmic domain dimers in order to perform the binding studies in solution, such as by isothermal titration calorimetry and by NMR.

In conclusion, we demonstrate that the integrin αIIbb3 binds weakly to talin when inactive but markedly increases its binding to talin upon activation. This novel mechanism of regulating integrin–talin interaction may be another level of regulation in interin signaling that contributes to the dynamic nature of integrin adhesome formation and integrin adhesive signaling.

Acknowledgements The authors greatly indebted to Dr. Mark Ginsberg for his generous gifts of many research reagents. All of the authors contributed to the collection and analysis of the data and to the preparation of the report. The corresponding author had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Funding This work is supported by the Sichuan Provincial Department of Science and Technology Supporting Project (No. 2015SZ0074).

Compliance with Ethical Standards

Conflict of interest We reports grant from Sichuan Provincial Department of Science and Technology, Grant No. 2015SZ0074.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Kim C, Ye F, Ginsberg MH (2011) Regulation of integrin activation. Annu Rev Cell Dev Biol 27:321–345
2. Shattil SJ, Kim C, Ginsberg MH (2010) The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol 11:288–300
3. Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110:673–687
4. Shimaoka M, Takagi J, Springer TA (2002) Conformational regulation of integrin structure and function. Annu Rev Biophys Biomol Struct 31:485–516
5. Hu P, Luo BH (2013) Integrin bi-directional signaling across the plasma membrane. J Cell Physiol 228:306–312
6. Abram CL, Lowell CA (2009) The ins and outs of leukocyte integrin signaling. Annu Rev Immunol 27:339–362
7. Legate KR, Wickstrom SA, Fassler R (2009) Genetic and cell biological analysis of integrin outside-in signaling. Genes Dev 23:397–418
8. Luo BH, Springer TA (2006) Integrin structures and conformational signaling. Curr Opin Cell Biol 18:579–586
9. Lagarrigue F, Kim C, Ginsberg MH (2016) The Rap1-RIAM-talin axis of integrin activation and blood cell function. Blood 128:479–487
10. Ye F, Snider AK, Ginsberg MH (2014) Talin and kindlin: the one-two punch in integrin activation. Front Med,
11. Calderwood DA, Campbell ID, Critchley DR (2013) Talins and kindlins: partners in integrin-mediated adhesion. Nat Rev Mol Cell Biol 14:503–517
12. Critchley DR (2009) Biochemical and structural properties of the integrin-associated cytoskeletal protein talin. Annu Rev Biophys 38:235–254
13. Kim C, Ye F, Hu X, Ginsberg MH (2012) Talin activates integrins by altering the topology of the beta transmembrane domain. J Cell Biol 197:605–611
14. Kim C, Schmidt T, Cho EG, Ye F, Ulmer TS, Ginsberg MH (2012) Basic amino-acid side chains regulate transmembrane integrin signalling. Nature 481:209–213
15. Li A, Guo Q, Kim C, Hu W, Ye F (2014) Integrin alphaII b tail distal of GFFKR participates in inside-out alphaII b beta3 activation. J Thromb Haemost 12:1145–1155
16. Kumar A, Ouyang M, Van den Dries K, McGhee EJ, Tanaka K, Anderson MD, Groisman A, Goult BT, Anderson KI, Schwartz MA (2016) Talin tension sensor reveals novel features of focal adhesion force transmission and mechanosensitivity. J Cell Biol 213:371–383
17. Liu J, Wang Y, Goh WI, Goh H, Baird MA, Ruehland S, Teo S, Bate N, Critchley DR, Davidson MW, Kanchanawong P (2015) Talin determines the nanoscale architecture of focal adhesions. Proc Natl Acad Sci USA 112:E4864–E4873
18. Lagarrigue F, Anekal V, Lee P, Bachir HS, Ablack AI, Horwitz JN, A.F., and Ginsberg MH (2015) A RIAM/lamellipodin–talin–integrin complex forms the tip of sticky fingers that guide cell migration. Nat Commun 6:8492
19. del Rio A, Perez-Jimenez R, Liu R, Roca-Cusachs P, Fernandez JM, Sheetz MP (2009) Stretching single talin rod molecules activates vinculin binding. Science 323:638–641
20. Lee HS, Anekal P, Lim CJ, Liu CC, Ginsberg MH (2013) Two modes of integrin activation form a binary molecular switch in adhesion maturation. Mol Biol Cell 24:1354–1362
21. Goult BT, Zacharchenko T, Bate N, Tsang R, Hey F, Gingras AR, Elliott PR, Roberts GC, Ballestrem C, Critchley DR, Barsukov IL (2013) RIAM and vinculin binding to talin are mutually exclusive and regulate adhesion assembly and turnover. J Biol Chem 288:8238–8249
22. Lee HS, Lim CJ, Puzon-McLaughlin W, Shattil SJ, Ginsberg MH (2009) RIAM activates integrins by linking talin to ras GTPase membrane-targeting sequences. J Biol Chem 284:5119–5127
23. Yan B, Calderwood DA, Yaspan B, Ginsberg MH (2001) Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. J Biol Chem 276:28164–28170
24. Goult BT, Xu XP, Gingras AR, Swift M, Patel B, Bate N, Kopp PM, Barsukov IL, Critchley DR, Volkmann N, Hanein D (2013) Structural studies on full-length talin1 reveal a compact auto-inhibited dimer: implications for talin activation. J Struct Biol 184:21–32
25. Wang JH (2012) Pull and push: talin activation for integrin signaling. Cell Res 22:1512–1514
26. Banno A, Goult BT, Lee H, Bate N, Critchley DR, Ginsberg MH (2012) Subcellular localization of talin is regulated by inter-domain interactions. J Biol Chem 287:13799–13812
27. Goksoy E, Ma YQ, Wang X, Kong X, Perera D, Plow EF, Qin J (2008) Structural basis for the autoinhibition of talin in regulating integrin activation. Mol Cell 31:124–133
28. Ye F, Hu G, Taylor D, Ratnikov B, Bobkov AA, McLean MA, Sligar SG, Taylor KA, Ginsberg MH (2010) Recreation of the

terminal events in physiological integrin activation. J Cell Biol 188:157–173
29. Ye F, Liu J, Winkler H, Taylor KA (2008) Integrin alpha IIb beta 3 in a membrane environment remains the same height after Mn^{2+} activation when observed by cryoelectron tomography. J Mol Biol 378:976–986
30. Yan B, Smith JW (2000) A redox site involved in integrin activation. J Biol Chem 275:39964–39972
31. Yan B, Hu DD, Knowles SK, Smith JW (2000) Probing chemical and conformational differences in the resting and active conformers of platelet integrin alpha(IIb)beta(3). J Biol Chem 275:7249–7260
32. Beer JH, Springer KT, Coller BS (1992) Immobilized Arg-Gly-Asp (RGD) peptides of varying lengths as structural probes of the platelet glycoprotein IIb/IIIa receptor. Blood 79:117–128
33. Tcheng JE (1996) Glycoprotein IIb/IIIa receptor inhibitors: putting the EPIC, IMPACT II, RESTORE, and EPILOG trials into perspective. Am J Cardiol 78:35–40
34. Scarborough RM, Naughton MA, Teng W, Rose JW, Phillips DR, Nannizzi L, Arfsten A, Campbell AM, Charo IF (1993) Design of potent and specific integrin antagonists. Peptide antagonists with high specificity for glycoprotein IIb-IIIa. J Biol Chem 268:1066–1073
35. Garcia-Alvarez B, de Pereda JM, Calderwood DA, Ulmer TS, Critchley D, Campbell ID, Ginsberg MH, Liddington RC (2003) Structural determinants of integrin recognition by talin. Mol Cell 11:49–58
36. Li A, Guo Q, Wei A, Zhou Y, Hu W (2017) Role of the helix in Talin F3 domain (F3 helix) in talin-mediated integrin activation. Cell Biochem Biophys 75:79–86
37. Arias-Salgado EG, Lizano S, Shattil SJ, Ginsberg MH (2005) Specification of the direction of adhesive signaling by the integrin beta cytoplasmic domain. J Biol Chem 280:29699–29707
38. Du X, Saido TC, Tsubuki S, Indig FE, Williams MJ, Ginsberg MH (1995) Calpain cleavage of the cytoplasmic domain of the integrin beta 3 subunit. J Biol Chem 270:26146–26151
39. Shattil SJ, Hoxie JA, Cunningham M, Brass LF (1985) Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. J Biol Chem 260:11107–11114
40. Du XP, Plow EF, Frelinger 3rd AL, O'Toole TE, Loftus JC, Ginsberg MH (1991) Ligands "activate" integrin alpha IIb beta 3 (platelet GPIIb-IIIa). Cell 65:409–416
41. Frelinger 3rd AL, Du XP, Plow EF, Ginsberg MH (1991) Monoclonal antibodies to ligand-occupied conformers of integrin alpha IIb beta 3 (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. J Biol Chem 266:17106–17111
42. Takagi J, Petre BM, Walz T, Springer TA (2002) Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell 110:599–511
43. Hughes PE, Diaz-Gonzalez F, Leong L, Wu C, McDonald JA, Shattil SJ, Ginsberg MH (1996) Breaking the integrin hinge. A defined structural constraint regulates integrin signaling. J Biol Chem 271:6571–6574
44. Calderwood DA, Zent R, Grant R, Rees DJ, Hynes RO, Ginsberg MH (1999) The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. J Biol Chem 274:28071–28074
45. Wegener KL, Partridge AW, Han J, Pickford AR, Liddington RC, Ginsberg MH, Campbell ID (2007) Structural basis of integrin activation by talin. Cell 128:171–182
46. Gailit J, Ruoslahti E (1988) Regulation of the fibronectin receptor affinity by divalent cations. J Biol Chem 263:12927–12932
47. Ye F, Petrich BG, Anekal P, Lefort CT, Kasirer-Friede A, Shattil SJ, Ruppert R, Moser M, Fassler R, Ginsberg MH (2013) The mechanism of kindlin-mediated activation of integrin alphaIIb-beta3. Curr Biol 23:2288–2295
48. Yan B, Smith JW (2001) Mechanism of integrin activation by disulfide bond reduction. Biochemistry 40:8861–8867
49. Chen X, Xie C, Nishida N, Li Z, Walz T, Springer TA (2010) Requirement of open headpiece conformation for activation of leukocyte integrin alphaXbeta2. Proc Natl Acad Sci USA 107:14727–14732
50. Moser M, Legate KR, Zent R, Fassler R (2009) The tail of integrins, talin, and kindlins. Science 324:895–899
51. Moser M, Bauer M, Schmid S, Ruppert R, Schmidt S, Sixt M, Wang HV, Sperandio M, Fassler R (2009) Kindlin-3 is required for beta2 integrin-mediated leukocyte adhesion to endothelial cells. Nat Med 15:300–305
52. Malinin NL, Zhang L, Choi J, Ciocea A, Razorenova O, Ma YQ, Podrez EA, Tosi M, Lennon DP, Caplan AI, Shurin SB, Plow EF, Byzova TV (2009) A point mutation in KINDLIN3 ablates activation of three integrin subfamilies in humans. Nat Med 15:313–318
53. Moser M, Nieswandt B, Ussar S, Pozgajova M, Fassler R (2008) Kindlin-3 is essential for integrin activation and platelet aggregation. Nat Med 14:325–330
54. Laplantine E, Vallar L, Mann K, Kieffer N, Aumailley M (2000) Interaction between the cytodomains of the alpha 3 and beta 1 integrin subunits regulates remodelling of adhesion complexes on laminin. J Cell Sci 113(Pt 7):1167–1176
55. Haas TA, Plow EF (1996) The cytoplasmic domain of alphaIIb beta3. A ternary complex of the integrin alpha and beta subunits and a divalent cation. J Biol Chem 271:6017–6026
56. Muir TW, Williams MJ, Ginsberg MH, Kent SB (1994) Design and chemical synthesis of a neoprotein structural model for the cytoplasmic domain of a multisubunit cell-surface receptor: integrin alpha IIb beta 3 (platelet GPIIb-IIIa). Biochemistry 33:7701–7708
57. Takagi J, Erickson HP, Springer TA (2001) C-terminal opening mimics 'inside-out' activation of integrin alpha5beta1. Nat Struct Biol 8:412–416
58. Lu C, Takagi J, Springer TA (2001) Association of the membrane proximal regions of the alpha and beta subunit cytoplasmic domains constrains an integrin in the inactive state. J Biol Chem 276:14642–14648
59. Li R, Babu CR, Valentine K, Lear JD, Wand AJ, Bennett JS, DeGrado WF (2002) Characterization of the monomeric form of the transmembrane and cytoplasmic domains of the integrin beta 3 subunit by NMR spectroscopy. Biochemistry 41:15618–15624
60. Ulmer TS, Yaspan B, Ginsberg MH, Campbell ID (2001) NMR analysis of structure and dynamics of the cytosolic tails of integrin alpha IIb beta 3 in aqueous solution. Biochemistry 40:7498–7508
61. Weljie AM, Hwang PM, Vogel HJ (2002) Solution structures of the cytoplasmic tail complex from platelet integrin alpha IIb- and beta 3-subunits. Proc Natl Acad Sci USA 99:5878–5883
62. Vinogradova O, Velyvis A, Velyviene A, Hu B, Haas T, Plow E, Qin J (2002) A structural mechanism of integrin alpha(IIb) beta(3) "inside-out" activation as regulated by its cytoplasmic face. Cell 110:587–597
63. Zhu J, Luo BH, Barth P, Schonbrun J, Baker D, Springer TA (2009) The structure of a receptor with two associating transmembrane domains on the cell surface: integrin alphaIIbbeta3. Mol Cell 34:234–249
64. Kim M, Carman CV, Springer TA (2003) Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science 301:1720–1725
65. Zhu J, Carman CV, Kim M, Shimaoka M, Springer TA, Luo BH (2007) Requirement of alpha and beta subunit transmembrane helix separation for integrin outside-in signaling. Blood 110:2475–2483

66. Askari JA, Tynan CJ, Webb SE, Martin-Fernandez ML, Ballestrem C, Humphries MJ (2010) Focal adhesions are sites of integrin extension. J Cell Biol 188:891–903
67. Lefort CT, Hyun YM, Schultz JB, Law FY, Waugh RE, Knauf PA, Kim M (2009) Outside-in signal transmission by conformational changes in integrin Mac-1. J Immunol 183:6460–6468
68. Shattil SJ (2009) The beta3 integrin cytoplasmic tail: protein scaffold and control freak. J Thromb Haemost 7(Suppl 1):210–213
69. Shattil SJ, Newman PJ (2004) Integrins: dynamic scaffolds for adhesion and signaling in platelets. Blood 104:1606–1615