Interactions of platelet integrin α_{IIb} and β_3 transmembrane domains in mammalian cell membranes and their role in integrin activation

Chungho Kim,[1] Tong-Lay Lau,[2] Tobias S. Ulmer,[2] and Mark H. Ginsberg[1]

[1]Department of Medicine, University of California San Diego, La Jolla; and [2]Department of Biochemistry and Molecular Biology and Zilkha Neurogenetic Institute, Keck School of Medicine, University of Southern California, Los Angeles

Clustering and occupancy of platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) generate biologically important signals: conversely, intracellular signals increase the integrins' affinity, leading to integrin activation; both forms of integrin signaling play important roles in hemostasis and thrombosis. Indirect evidence implicates interactions between integrin α and β transmembrane domains (TMDs) and cytoplasmic domains in integrin signaling; however, efforts to directly identify these associations have met with varying and controversial results. In this study, we develop mini-integrin affinity capture and use it in combination with nuclear magnetic resonance spectroscopy to show preferential heterodimeric association of integrin $\alpha_{IIb}\beta_3$ TMD-tails via specific TMD interactions in mammalian cell membranes and in lipid bicelles. Furthermore, charge reversal mutations at α_{IIb}(R995)β_3(D723) confirm a proposed salt bridge and show that it stabilizes the TMD-tail association; talin binding to the β_3 tail, which activates the integrin, disrupts this association. These studies establish the preferential heterodimeric interactions of integrin $\alpha_{IIb}\beta_3$ TMD-tails in mammalian cell membranes and document their role in integrin signaling. (Blood. 2009;113: 4747-4753)

Introduction

Integrin-mediated cell adhesion modulates signaling pathways that control many biologic functions,[1] and these signals involve both integrin clustering and integrin occupancy[2]; the latter changes integrin conformation, leading to allosteric rearrangements that propagate across the membrane and modify intracellular interactions.[3] A second form of integrin signaling, initiated intracellularly, leads to increased affinity for ligands, a process termed integrin activation.[1] Both forms of integrin signaling are central to the functions of platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) in hemostasis and thrombosis.[4] Integrin activation is also essential for functions such as inflammation and assembly of the extracellular matrix. Integrins are noncovalent α-β heterodimeric, type-1 transmembrane (TM) receptors formed from combinations of 18 α and 8 β subunits. Many studies indirectly implicate interactions between integrin α and β TM domains (TMDs) and cytoplasmic domains (tails) in both forms of integrin signaling[5-11]; however, studies to directly identify such interactions have met with contradictory results.

Mutational studies of platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), based on sequence alignments, suggested an interaction between β_3 Asp^{723} and α_{IIb} Arg^{995}.[5] Charge reversal mutation of either residue resulted in constitutive bidirectional integrin signaling (ie, integrin activation and constitutive phosphorylation of $pp125^{FAK}$); however, a double-charge reversal in α_{IIb}(R995D)β_3(D723R) did not exhibit constitutive signaling, suggesting that a salt bridge between these residues was a defined structural constraint that limited the integrin signaling.[5] A spontaneous activating mutation (β_3(D723H)) found in patients also suggests the importance of the β_3 Asp^{723} residue for stabilization of the inactive state of the integrin.[12] This constraint gained support from elegant protein engineering studies in which clasping the cytoplasmic domains or TMDs together inhibited integrin activation,[9] and joining of the α and β TMDs with disulfide bonds limited bidirectional integrin signaling.[7] Despite this mutational data, our initial efforts to identify interactions of isolated α and β integrin tails by nuclear magnetic resonance (NMR) spectroscopy were unsuccessful in aqueous solution.[13] Two laboratories reported NMR studies of these tails that suggested the existence of such interactions, albeit different structures for the αβ dimer were reported.[14,15] Structures of the individual α_{IIb} and β_3 TMDs in phospholipid bicelles[16,17] show that the β_3 TMD adopts an elongated, tilted membrane helix[17] and the α_{IIb} TMD folds into a short, straight helix, followed by a surprising backbone reversal that packs Phe^{992} and Phe^{993} against the TM helix.[16] This structure demonstrated an unexpected complexity in the α_{IIb} TMD and membrane-proximal cytoplasmic domain that was not observed in the studies of cytoplasmic domains in aqueous solution,[14,15] thus calling NMR studies that suggested the interactions into question.

Whereas mutational studies and molecular modeling have strongly suggested that integrin αβ TMD interactions are important in regulating integrin signaling,[8,10,11,18,19] efforts to identify direct interactions between α and β TMDs (either biochemically or genetically) have had contradictory results.[20-23] Indeed, αα and ββ interactions were proposed to make a major contribution to the clustering that occurs in integrin signaling[22]; however, later studies have strongly challenged this idea.[8,11] Furthermore, a 20 Å reconstruction of detergent-solubilized integrin $\alpha_{IIb}\beta_3$ revealed a cylindrical density interpreted as a TM segment that suggests that the TMDs are associated in a parallel, α-helical coiled-coil[24]; however, as noted above, higher resolution structures of the individual $\alpha_{IIb}\beta_3$ TMDs in a lipid environment are not consistent with a purely coiled-coil architecture. In this study, we use a novel mini-integrin affinity capture approach to establish the preferential heteromeric interaction of integrin α_{IIb} and β_3 TMDs and cytoplasmic domains

Submitted October 28, 2008; accepted January 27, 2009. Prepublished online as *Blood* First Edition paper, February 13, 2009; DOI 10.1182/blood-2008-10-186551.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

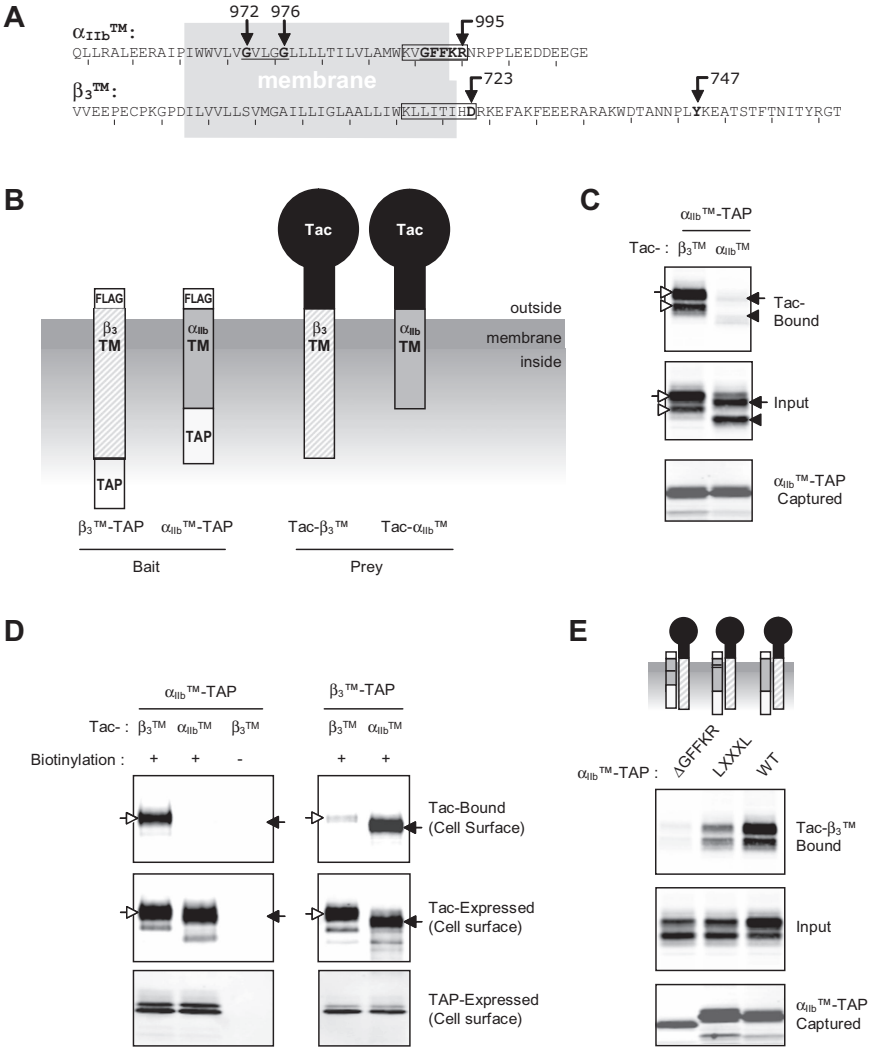

Figure 1. Integrin α_{IIb} and β_3 TMD-tail mini-integrins interact with each other via the TMDs. (A) Amino acid sequences of α_{IIb}™ and β_3™ used in this study. Sequences in the gray box represent amino acid residues in membrane region. GXXXG motif and GFFKR motif in α_{IIb} are underlined. Point mutations used in this study are indicated with arrows. The previously designated membrane-proximal regions containing hydrophobic stretches in both α and β integrin are boxed.[25] (B) Schematic diagram of TMD-tail constructs. For β_3™-TAP and α_{IIb}™-TAP baits, β_3™ and α_{IIb}™ (A) were fused to TAP tag for purification and an N-terminal FLAG tag for detection. Tac-α_{IIb}™ and Tac-β_3™ were made by fusion of α_{IIb}™ and β_3™, respectively, with Tac extracellular domain. (C) CHO cells were transiently transfected with α_{IIb}™-TAP (bait) and Tac-α_{IIb}™ or Tac-β_3™ (preys), and cells were lysed and incubated with calmodulin beads to capture the baits. Bound Tac constructs were analyzed by Western blot using anti-Tac antibody (top panels). Expression of Tac preys (middle panel) and captured α_{IIb}™-TAP (bottom panel) were verified by Western blot using anti-Tac antibody and anti-FLAG antibody, respectively. The arrows indicate mature cell-surface proteins, and the arrowheads incompletely glycosylated intracellular proteins. Open symbols represent Tac-β_3™, and closed symbols represent Tac-α_{IIb}™. (D) CHO cells were transiently transfected with baits and preys, as indicated, and cell-surface proteins were biotinylated before cell lysis. Ten percent of the lysates were incubated with neutravidin beads to determine the input of biotinylated proteins in the lysates (middle and bottom panels). The remaining lysates were first incubated with calmodulin beads to capture the baits, and the bound proteins were eluted with 10 mM EDTA. The eluates were then incubated with NeutrAvidin beads to capture the biotinylated surface proteins and the presence of Tac preys was analyzed with Western blot using anti-Tac antibody (top panel). (E) α_{IIb}™-TAP constructs containing deletion of GFFKR motif or mutations of 2 Gly in GXXXG motif to Leu were tested for their binding to Tac-β_3 as in (C).

in mammalian cells, and show by NMR spectroscopy that heterodimerization takes place analogously in small bicelle model membranes. Thus, we show that integrin TMDs and cytoplasmic domains preferentially interact in a heterodimeric rather than homodimeric fashion in mammalian cell membranes, interaction between the highly conserved β_3 Asp^{723} and α_{IIb} Arg^{995} stabilizes this interaction, and talin binding to the β_3 cytoplasmic domain disrupts it.

Methods

Plasmids, antibody, and cell lines

Tac-α_{IIb}™, Tac-β_3™ were generated by ligation of polymerase chain reaction (PCR)–generated fusion sequences consisting of extracellular domain of Tac and TM tail regions of each integrin subunit (Figure 1A) into pcDNA3.1 (Invitrogen, Carlsbad, CA). For the construction of α_{IIb}™- TAP (tandem affinity purification), the sequences of the preprotrypsin leader sequence followed by 3 repeats of FLAG sequence (Sigma-Aldrich, St Louis, MO) were first cloned into pcDNA3.1, and then the PCR-generated fusion sequences containing α_{IIb}™ and TAP were ligated. Point mutations were performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Anti–Tac 7G7B6 (ATCC, Manassas, VA), anti–Tac N19 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti–FLAG M2 (Sigma-Aldrich) were obtained commercially. Rabbit polyclonal anti-β_3 antibody (Rb8053), mouse monoclonal antibody specific for the human integrin α_{IIb} subunit (PMI-1), activation-specific anti-$\alpha_{IIb}\beta_3$ antibody (PAC1), and $\alpha_{IIb}\beta_3$ activating antibody (anti-LIBS6) have been described previously.[26] Chinese hamster ovary (CHO) cells and CHO cells expressing recombinant $\alpha_{IIb}\beta_3$ (A5) were maintained, as described previously.[26] Lipofectamine and Lipofectamine Plus reagents (Invitrogen) were used according to the manufacturer's recommendation for transient transfections.

Affinity capture

Twenty-four hours after transfection, cells were lysed with CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) lysis buffer (20 mM HEPES [N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid] pH 7.4, 1% CHAPS, 150 mM NaCl, 2 mM $CaCl_2$, and EDTA [ethylenediaminetetraacetic acid]–free protease inhibitor mixture [Roche, Basel, Switzerland]) and clarified by centrifugation at 14 000 rpm for 15 minutes, and then the clarified lysates were incubated with calmodulin Sepharose (GE Healthcare, Piscataway, NJ) 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 blot. For capturing biotinylated intact integrin subunits, A5 cells expressing TAP constructs were detached and surface proteins were biotinylated using EZ-Link Sulfo-NHS-Biotin (Thermo Scientific, Rockford, IL) according to the manufacturer's recommendation. The cells were washed twice with Tris-buffered saline (TBS; pH 8.4), and incubated for 30 minutes at 37°C with TBS containing 5 mM EDTA. Resulting cells were washed with HEPES-buffered saline (20 mM HEPES, 150 mM NaCl, pH 7.4) before

being lysed with CHAPS lysis buffer. Lysates were incubated with calmodulin-beads to capture TAP constructs for 2 hours, bound proteins were eluted with 10 mM EDTA, and then the eluates were further incubated with NeutrAvidin agarose resin (Thermo Scientific) overnight to capture biotinylated proteins. Bound proteins were eluted with SDS reducing sample buffer, subjected to SDS-PAGE, and analyzed by Western blot.

NMR spectroscopy

Peptides encompassing human integrin α_{IIb}(Ala^{958}-Pro^{998}) and β_3(Pro^{685}-Phe^{727}) residues, respectively, including β_3(Cys^{687}Ser), were prepared as described previously.[16,17] In addition, an α_{IIb} peptide incorporating Arg^{995}Ala and a β_3 peptide with an Asp^{723}Ala substitution were prepared analogously. The peptides were reconstituted in 385 mM 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine, 83 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 41 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine], 25 mM HEPES·NaOH, pH 7.4, 6% D_2O, and 0.02% wt/vol NaN_3. Transverse relaxation optimized–heteronuclear single quantum coherence (TROSY-HSQC) NMR experiments were conducted on a cryoprobe-equipped Bruker Avance 700 spectrometer at 23°C.

Flow cytometry

PAC1-binding assay and fibronectin-binding assay were performed essentially as described.[26,27] In brief, 1 day after transfection, suspended cells were incubated with 7G7B6 (anti-Tac) in combination with either PAC1 or biotinylated glutathione *S*-transferase-FN9-11, followed by staining with fluorescein isothiocyanate (FITC)–conjugated anti–mouse immunoglobulin (Ig) G and with R-phycoerythrin (PE)–conjugated anti–mouse IgM or PE-conjugated streptavidin. Five minutes before analysis, propidium iodide (PI) was added, and PI-negative live cells were analyzed on FACSCalibur (BD Biosciences, San Jose, CA). The data were analyzed using MATLAB R2007a software to calculate the geometric means and generate dot plots.

Cell imaging

A5 cells expressing Tac-α_{IIb}^{TM} or Tac-α_{IIb} were detached and stained with anti–Tac N19 antibody and PAC1 in the presence or absence of anti-LIBS6, as indicated, followed by staining with FITC-conjugated anti–rabbit IgG and rhodamine-conjugated anti–mouse IgM. The stained cells were fixed, mounted in Prolong Gold (Invitrogen), and observed under a Nikon Eclipse TE2000-U inverted microscope. Images were acquired using 60×/1.4 NA oil-objective lens with 1.5× intermediate magnification, a Coolsnap HQ camera (Photometrics, Tucson, AZ), and QED InVivo imaging software (Media Cybernetics, Silver Spring, MD) at room temperature. Postacquisition image analysis was performed using ImageJ (National Institutes of Health, Bethesda, MD).

Results

Integrin α_{IIb} and β_3 TMDs mediate heterodimeric interactions in mammalian cell membranes

To test whether the TMDs of α_{IIb} and β_3 interact with each other, we constructed an α_{IIb} mini-integrin containing the TMD and cytoplasmic tail of α_{IIb} (Figure 1A) joined to an N-terminal flag tag for detection and a C-terminal TAP tag[28] for rapid and efficient purification (Figure 1B). We expressed this α_{IIb}^{TM}-TAP bait in combination with preys comprising the extracellular domain of the Tac (interleukin-2 receptor α) joined to the TMD and tail of α_{IIb} (Tac-α_{IIb}^{TM}) or β_3 (Tac-β_3^{TM}; Figure 1B). The cells were lysed, and baits were captured using calmodulin beads. In the reaction, approximately 20% of expressed baits were usually captured. We detected bound prey by Western blotting with anti-Tac antibody and found that α_{IIb}^{TM} bait bound preferentially to Tac-β_3^{TM} rather than Tac-α_{IIb}^{TM} (17-fold more in quantification; Figure 1C). This interaction required the β_3 TMD, because a prey containing the Tac extracellular and TMD joined to the β_3 tail failed to bind to the α_{IIb}^{TM} bait (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). We noted multiple bands for each Tac construct and enzymatic deglycosylation and cell-surface biotinylation experiments showed that the top band observed in Figure 1C is a glycosylated form found predominantly on the cell surface, whereas the bottom band is a nonglycosylated intracellular form (Figure S1B,C). When we surface labeled the cells, we again found that α_{IIb}^{TM}-TAP bait interacts with Tac-β_3^{TM} (Figure 1D left panels) and only the top band was observed. Conversely, bait containing the TMD and tail of β_3 preferentially bound the Tac-α_{IIb}^{TM} rather than Tac-β_3^{TM} on the cell surface, although subtle ββ homomeric interactions were also observed (Figure 1D right panels). Thus, we observed preferential heteromeric interactions between α_{IIb} and β_3 TMD-tail in both cell surface and intracellular membranes.

The foregoing results showed preferential heteromeric interactions between baits and preys containing the TMDs and cytoplasmic domains of integrin $\alpha_{IIb}\beta_3$, and that the interaction required the β_3 TMD. To test the role of the α_{IIb} TMD, we changed 2 Gly in the α_{IIb} TMD GXXXG motif to Leu (Figure 1A). This motif is buried in the hydrophobic core of the lipid bilayer and is predicted to be important in αβ TMD packing.[8,19] As a second test of specificity, we deleted the membrane-proximal GFFKR motif; both this deletion and the Leu substitutions activate integrin $\alpha_{IIb}\beta_3$.[8] Both of these mutations markedly reduced interaction of α_{IIb} bait with β_3 prey (Figure 1E). Thus, the bait-prey interactions detected in this study are mediated by specific packing interactions of the TMDs. The strong preference for heteromeric αβ interactions in mammalian cell membranes is in sharp contrast to the homodimeric and trimeric interactions previously observed in detergent micelles,[21] in electrophoresis,[21,22] and in the *Escherichia coli* inner membrane.[23] Schneider and Engelman[20] did observe promiscuous weak heterodimeric interactions of central hydrophobic 17-residue fragments of the integrin TMDs using transcriptional repression of LacZ by dimer formation in *E coli* membranes; those authors observed extensive homo-oligomerization as well. Thus, the present data provide the first direct evidence for the heteromeric association of $\alpha_{IIb}\beta_3$ TMD-tails in mammalian cell membranes.

Integrin α_{IIb} and β_3 TMD interaction is stabilized by interaction of α_{IIb} Arg^{995} and β_3 Asp^{723}

The plasma membrane is a complex environment; that is, it contains many proteins and lipids. Whereas this complexity is representative of the environment of integrin $\alpha_{IIb}\beta_3$ in blood platelets,[29] it raises the possibility of indirect α_{IIb}-β_3 interactions. Using NMR spectroscopy, we therefore assessed the association of peptides corresponding to α_{IIb}(Ala^{958}-Pro^{998}) and β_3(Pro^{685}-Phe^{727}), which contain the TMDs[16,17] and possible cytosolic heterodimerization sites in the controlled lipid environment of small bicelle model membranes. Within this environment, the presence of the α_{IIb} peptide led to the appearance of a second set of NMRs for the β_3 TMD residues in addition to the previously observed monomeric signals,[16,17] as exemplified for β_3(G702) and β_3(G708) (Figure 2A-C). This second set of resonances arises from α_{IIb}-β_3 association (heterodimerization), which creates new chemical environments for all $\alpha_{IIb}\beta_3$ TMD resonances (Figure S2A) as a consequence of direct α_{IIb}-β_3 contacts and indirect, propagated effects.

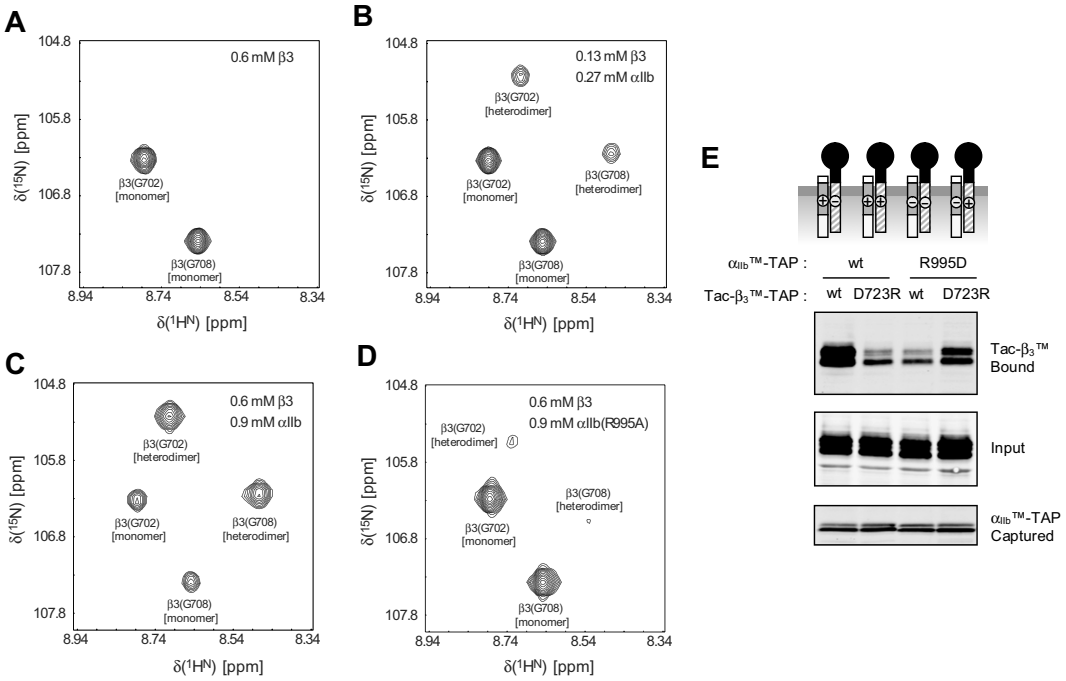

Figure 2. In vitro α_{IIb}-β_3 TM heterodimerization. (A-C) Spectral region of TROSY-HSQC spectra showing Gly^{702} and Gly^{708} of the $^2H/^{13}C/^{15}N$-labeled β_3 TM segment in the absence and presence of unlabeled α_{IIb} TM peptide. One set of signals corresponds to the monomeric signals (A). The signal intensity ratios of monomeric-to-heterodimeric signals, but not their positions, depend on the α_{IIb} and β_3 peptide concentrations, demonstrating slow exchange kinetics on the NMR timescale between the 2 species. As evidenced by the disappearance of the monomer signals at higher peptide-to-bicelle ratios (C), heterodimerization is predominant. (D) In the presence of mutant α_{IIb}(R995A) peptide, heterodimerization is weakened. The signal intensity ratio of heterodimer-to-monomer signals drops from 1.56 to 0.14 (C,D), and the heterodimeric signals are shifted compared with the interaction with wild-type α_{IIb} peptide. (E) The effects of charge reversal mutations in the membrane-proximal regions of α_{IIb} or β_3 on the TMD interaction were analyzed, as in Figure 1.

For example, β_3(G708) is expected to form the dimerization interface, whereas β_3(G702) is peripheral and most likely experiences predominantly indirect, next-neighbors changes. To test the existence of the proposed α_{II}b(R995)-β_3(D723) salt bridge and verify the specificity of the α_{IIb}-β_3 interaction, wild-type α_{IIb} TMD peptide was substituted by α_{IIb}(R995A).[5] This mutant α_{IIb} peptide, whose backbone fold is indistinguishable from wild-type α_{IIb} (Figure S2B), failed to induce a significant second set of β_3 signals (Figure 2D), demonstrating that heterodimerization weakened considerably. Analogously, mutant β_3(D723A) peptide did not induce a second set of α_{IIb} resonances in contrast to wild-type peptide (data not shown). Thus, in the defined model membrane environment of phospholipid bicelles, specific TM α_{IIb}-β_3 interactions, leading to heterodimerization, were observed and were dependent on α_{IIb}(R995) and β_3(D723) electrostatic interactions.

To assess whether α_{IIb}(R995)-β_3(D723) electrostatic interaction also takes place in cell membranes, we introduced charge reversal mutations in these residues creating α_{IIb}(R995D) bait and β_3(D723R) prey. The combination of α_{IIb}(R995D) bait with β_3 prey, or of β_3(D723R) prey with α_{IIb} bait, resulted in marked reduction in the interaction (Figure 2E). The αβ interaction was largely rescued when a combination of α_{IIb}(R995D) bait and β_3(D723R) prey was used (Figure 2E). Thus, both NMR in synthetic membranes and affinity capture in mammalian cell membranes confirm the importance of the α_{IIb}(R995)-β_3(D723) interaction in stabilizing the association of the $\alpha_{IIb}\beta_3$ TMD-tails. Our previous inability to detect specific $\alpha_{IIb}(Arg^{995})$-$\beta_3(Asp^{723})$ contacts in aqueous solution[13] indicates their weakness in the absence of a lipid milieu that provides a lowered dielectric constant and reduced solvent water concentration.

Talin binding to the β_3 tail disrupts the interaction of the α_{IIb} and β_3 TMD-tails

Talin binding to integrin β cytoplasmic domains is a final step in integrin activation.[30,31] Transfection of cells with the talin head domain (THD) inhibits Förster resonance energy transfer (FRET) between donors and acceptors fused to the cytoplasmic tails of α_L and β_2 integrins,[6] indicating a change in the orientation and/or distance between the tails. Having developed a direct measure of the association of the integrin α and β TMD-tails, we asked whether talin binding can inhibit the interaction. THD reduced the α_{IIb}™-TAP-Tac-β_3™ interaction by 40% relative to a β_3 binding-defective mutant THD, THD(W395A)[32] (Figure 3A). Conversely, THD did not block binding of Tac-β_3™(Y747A), a mutation that inhibits talin binding,[32] to α_{IIb}™-TAP (Figure 3B). In the absence of THD, Tac-β_3™(Y747A) also showed increased association with α_{IIb}™-TAP, probably due to the presence of endogenous talin in the CHO cells (data not shown). Thus, talin binding to the β_3 tail inhibits the interaction of the α_{IIb} and β_3 TMD-tails. The partial dissociation of the αβ complex by THD may be due to variation in the ratio of THD to Tac-β_3™ and α_{IIb}™-TAP on a per cell basis in these triple transfection experiments.

It is notable that mutations in the α_{IIb} GXXXG motif, located near the outer leaflet of the membrane, and mutations that disrupt the salt bridge or talin binding, which would act near the inner leaflet of the membrane, both decrease the TMD-tail association. These results raise the intriguing possibility that both outer membrane interaction (including the α_{IIb} GXXXG motif) and inner membrane interaction (involving α_{IIb}(F992,933 and R995) and β_3(D723) are required for stabilization of the TMD interface and the inactive state of the integrin.

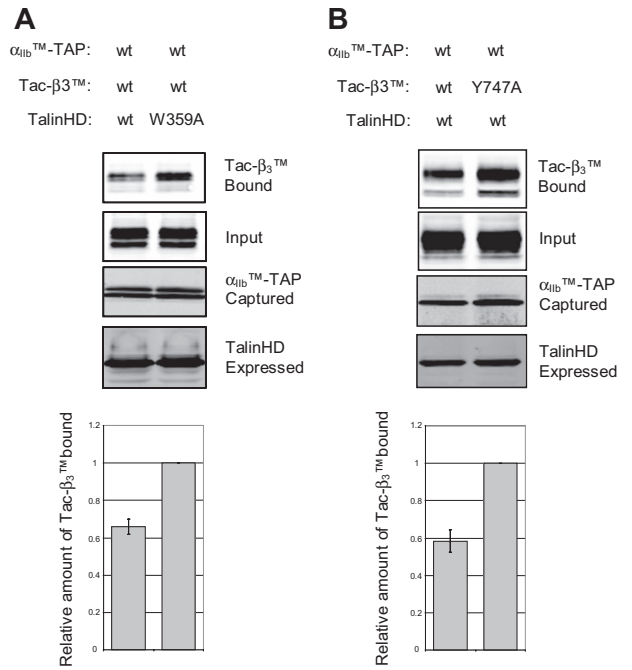

Figure 3. Talin binding to the β_3 tail inhibits the $\alpha\beta$ TMD-tail interaction. (A) α_{IIb}™-TAP and Tac-β_3™ were cotransfected with THD or THD(W359A) mutant into CHO cells, and their interaction was analyzed, as described in Figure 1C. Ratio of the amount of Tac-β_3™ bound to α_{IIb}™-TAP in presence of wild-type THD to that in the presence of THD W359A is shown as a bar graph. The data are the mean ± SE of 4 experiments. (B) α_{IIb}™-TAP and THD were cotransfected with Tac-β_3™ or Tac-β_3™(Y747A) mutant into CHO cells and their interaction was analyzed, as described in panel A. The data are mean ± SE of 3 experiments.

The TMD-tail of α_{IIb} or β_3 activates integrin $\alpha_{IIb}\beta_3$

We reasoned that should these TMD-tails interact with native $\alpha_{IIb}\beta_3$, then they might disrupt the interaction of the endogenous α_{IIb} and β_3, leading to integrin activation. Indeed, overexpression of Tac-α_{IIb}™ induced a dose-dependent increase in the binding of an activation-specific anti-$\alpha_{IIb}\beta_3$, PAC1,[33] whereas a construct lacking the α_{IIb} TMD (Tac-α_{IIb}) exhibited no such effect (Figure 4A). The Tac-α_{IIb}™ failed to activate $\alpha_5\beta_1$ (Figure S3), supporting the integrin class specificity of interactions of integrin TMDs.[34] Additional evidence for specificity of this effect was provided by mutants that disrupt the TMD-tail interaction of integrin α_{IIb} and β_3; Tac-α_{IIb}™ constructs in which we deleted the GFFKR motif or mutated the 2 Gly in the GXXXG motif failed to activate $\alpha_{IIb}\beta_3$ (Figure 4B).

Expression of the free β_3 cytoplasmic domain inhibited integrin activation (Figure 4C) probably due to sequestration of endogenously expressed talin.[33] Addition of the β_3 TMD to the cytoplasmic domain led to reversal of the inhibitory effect and integrin activation at higher levels of expression (Figure 4C), possibly resulted from combination of inhibitory effect by sequestering talin and activating effect by binding to the TMDs of the intact integrin. Furthermore, the β_3(Y747A) mutation, which blocks talin binding,[32] increased the ability of the β_3 TMD-tail to activate integrin $\alpha_{IIb}\beta_3$ and reduced the inhibitory effect (Figure 4C). Thus, overexpression of the TMD-tail of either α_{IIb} or β_3 activated the native integrin, and activation depended on the presence of the TMDs.

TMD-tail of α_{IIb} interacts with native $\alpha_{IIb}\beta_3$ integrin by binding the β_3 subunit

To examine the interaction of α_{IIb} TMD-tail with native $\alpha_{IIb}\beta_3$, we captured the α_{IIb}™-TAP bait, resulting in isolation of native $\alpha_{IIb}\beta_3$ (Figure 5A), whereas deletion of the GFFKR motif inhibited the association (Figure 5A). We also examined the association of Tac-α_{IIb}™ with activated $\alpha_{IIb}\beta_3$ in cells. Most PAC1-positive clusters of activated $\alpha_{IIb}\beta_3$ also contained the Tac-α_{IIb}™ (Figure 5B). Cells transfected with Tac-α_{IIb}, which lacks the TMDs, failed

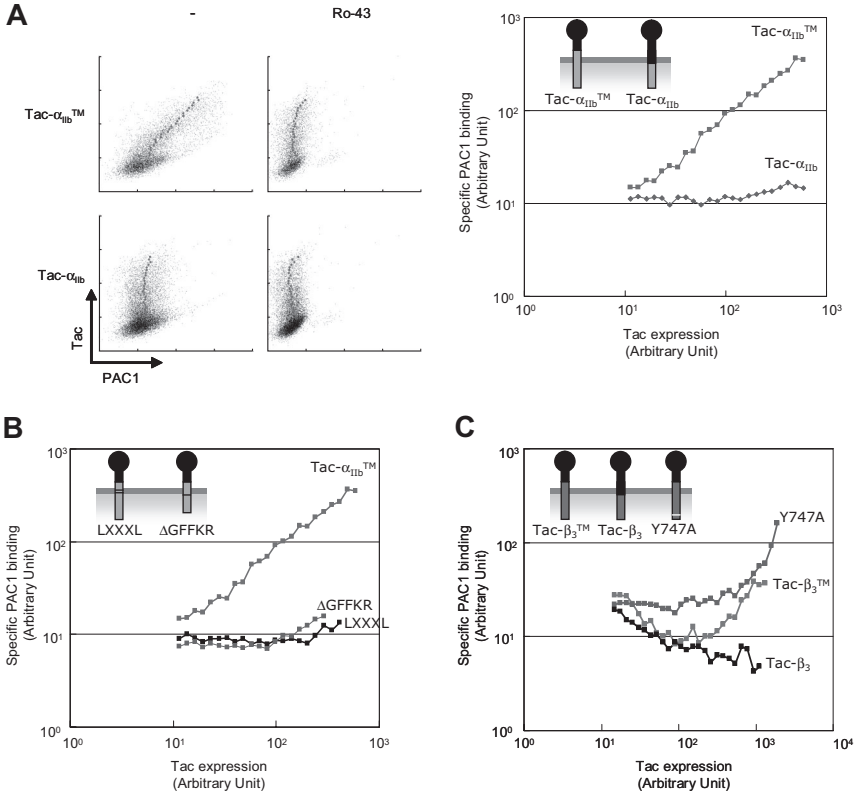

Figure 4. TMD-tail mini-integrins activate native integrin $\alpha_{IIb}\beta_3$. (A) Tac-α_{IIb}™ or Tac-α_{IIb} was transfected into CHO cells expressing $\alpha_{IIb}\beta_3$ (A5 cells). Twenty-four hours after transfection, cells were stained with PAC1 (activation-specific anti-$\alpha_{IIb}\beta_3$ antibody) and 7G7B6 (anti-Tac antibody) in the presence or absence of a selective inhibitor of ligand binding to $\alpha_{IIb}\beta_3$, Ro-43-5054, and then analyzed by flow cytometry to generate the dot plots shown (left). Geometric means of PAC1 binding in cells expressing different quantities of Tac constructs were plotted as larger red dots. Specific PAC1 binding was calculated by subtracting the geometric means of PAC1 binding in the presence of Ro-43-5054 from that in the absence of Ro-43-5054 in each range, and plotted as a line graph (right). (B) The effects of Tac-α_{IIb}™ΔGFFKR and Tac-α_{IIb}™LXXXL on $\alpha_{IIb}\beta_3$ activation were analyzed, as in panel A. (C) The effects of Tac-β_3™, Tac-β_3, and Tac-β_3™(Y747A) on $\alpha_{IIb}\beta_3$ activation were analyzed, as in panel A. The activating effects of each Tac constructs were tested at least 3 times, and representative data are shown.

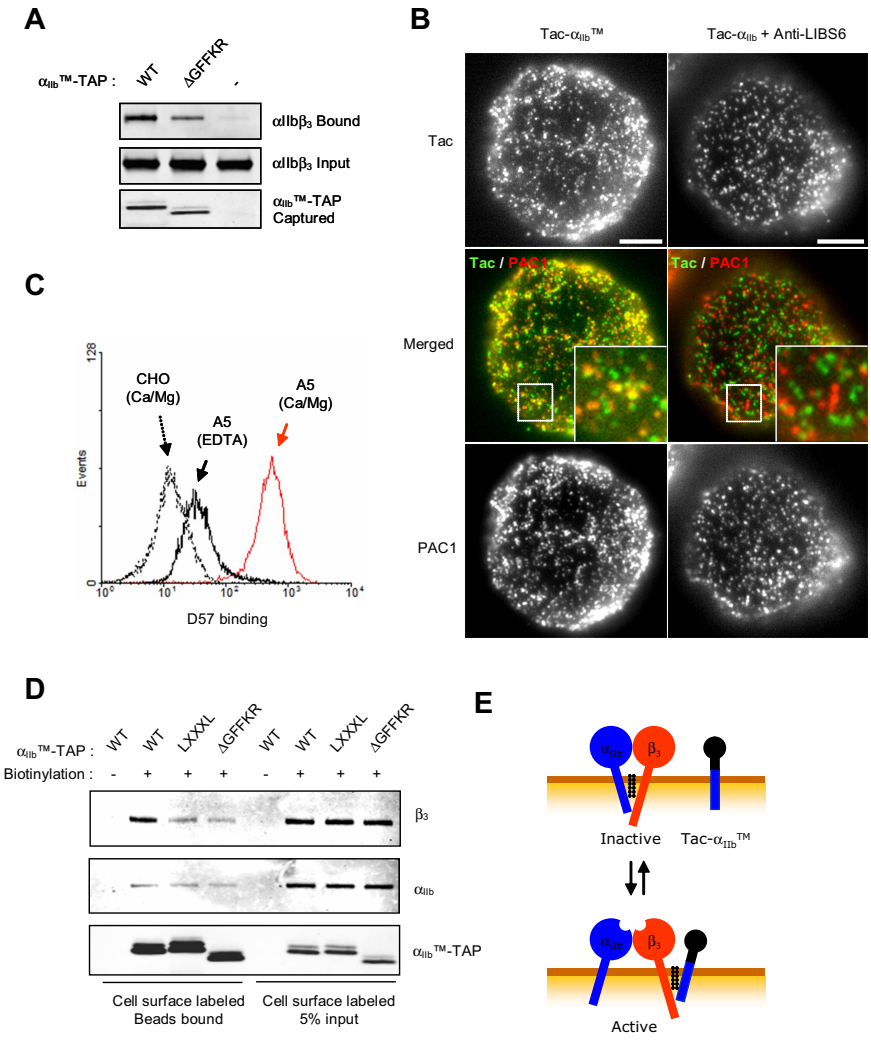

Figure 5. α_{IIb} TMD-tail binds to the β_3 subunit of native integrin $\alpha_{IIb}\beta_3$. (A) α_{IIb}™-TAP, α_{IIb}™ΔGFFKR-TAP, or empty vector was transfected into A5 cells, and after 24 hours, cells were lysed and incubated with calmodulin beads to capture TAP constructs. β_3 was detected by Western blot using anti-β_3 antibody as representative of the bound $\alpha_{IIb}\beta_3$ (top). Expression level of β_3 in lysates (middle) and captured TAP constructs (bottom) was assessed by Western blot. (B) A5 cells transfected with Tac-α_{IIb}™ or Tac-α_{IIb} were detached and stained with anti-Tac antibody and PAC1. In the merged image, green and red represent Tac and active integrin, respectively. The 2.7-fold digital enlargement of areas in small rectangles is shown in insets. Bar = 5 μm. (C) A5 cells were incubated for 30 minutes at 37°C with TBS (20 mM Tris, pH 8.4, 150 mM NaCl) containing either 1 mM Ca/Mg or 5 mM EDTA, and stained with $\alpha_{IIb}\beta_3$ complex-specific antibody (D57). D57 binding was measured by flow cytometry and plotted as histogram. (D) A5 cells transfected with TAP constructs were detached, and surface proteins were biotinylated and treated with EDTA to dissociate $\alpha_{IIb}\beta_3$ complex before the cells were lysed. Five percent of the lysates were incubated with neutravidin beads to determine the input of biotinylated proteins in the lysates (lanes 5-8). The remaining lysates were first incubated with calmodulin beads to capture TAP tag, and the bound proteins were eluted with 10 mM EDTA. The eluates were further incubated with NeutrAvidin beads to capture the biotinylated protein (lanes 1-4). Anti-α_{IIb} antibody (PMI-1), anti-β_3 antibody (Rb8053), and anti-FLAG antibody (M2) were used for the Western blot, as indicated. Schematic procedure for this experiment is shown in Figure S4. (E) Model of how the α_{IIb} TMD-tail induced $\alpha_{IIb}\beta_3$ activation. α_{IIb} TMD-tail interacts with TMD-tail region of β_3 in the native integrin and competes for the heterodimeric interaction between α_{IIb} and β_3, resulting in rearrangement of the TMD and activation.

to stain with PAC1; however, when the $\alpha_{IIb}\beta_3$ was activated with an activating antibody, anti-LIBS6,[35] Tac-α_{IIb} was not associated with the clusters of activated integrin (Figure 5B). Thus, the α_{IIb} TMD-tail interacts with native $\alpha_{IIb}\beta_3$, and this physical association activates the integrin.

Consistent with the findings described above, a peptide containing a partial sequence of α_{IIb} TMD (Trp^{968}-Lys^{989}) can bind and activate $\alpha_{IIb}\beta_3$[36]; however, this effect was ascribed to a homodimeric interaction of the α_{IIb} peptide with the full-length α_{IIb}. To assess which subunit of full-length $\alpha_{IIb}\beta_3$ binds to the α_{IIb} TMD-tail, we used EDTA to dissociate the subunits and verified the dissociation by the loss of binding of a complex-specific antibody (Figure 5C). Because the EDTA treatment can affect only cell surface–expressed integrin, we surface biotinylated cells expressing $\alpha_{IIb}\beta_3$ in combination with α_{IIb}™-TAP to exclude the intracellular integrin from this assay, treated the cells with EDTA to dissociate the $\alpha_{IIb}\beta_3$ on cell surface, and then captured α_{IIb}™-TAP with immobilized calmodulin (Figure S4). Biotinylated surface proteins were then affinity purified with NeutrAvidin beads and detected by antibody against α_{IIb} (PMI-1) and β_3 (Rb8053). We found that β_3 subunit was captured by α_{IIb}™-TAP (Figure 5D top, lane 2), and the LXXXL and ΔGFFKR mutations markedly reduced the association (Figure 5D top, lanes 3,4). In sharp contrast, only nonspecific (as judged by equal interactions of wt, LXXXL, or ΔGFFKR baits and by the ratio of captured [Figure 5D lanes 2-4] to input of the cell surface–expressed integrin subunits [Figure 5D lanes 6-8]) were observed with the α_{IIb} subunit (Figure 5D middle panel). This result shows that the α_{IIb}™-TAP preferentially captures full-length integrin β_3 in the presence of an equimolar amount of native full-length α_{IIb}. Thus, the unpaired α_{IIb} TMD-tail induces equilibrium shift in favor of activation of integrin $\alpha_{IIb}\beta_3$ by interacting with the β_3 subunit and separating the TMD-tail interaction of the intact integrin (Figure 5E).

Discussion

Bidirectional TM integrin signaling is central to integrin-mediated biological functions. Mutational studies suggested that this signaling involves heterodimeric interactions of the TMD-tails; frustratingly, efforts to directly demonstrate these interactions produced conflicting results. In this study, we took advantage of the high efficiency and rapidity of TAP tag purification to show preferential heterodimeric interactions of the α_{IIb} and β_3 TMD-tails in mammalian cell membranes. Previous studies in *E coli* inner cell membranes showed primarily homodimeric interactions of the α_{IIb} TMDs; the variance from our results is most likely ascribable to the

fact that we used a full-length cytoplasmic domain and included the GFFKR motif, shown in this study to be critical for stabilization of the association of the TMD-tail mini-integrins. Similarly, previous studies in detergent micelles suggested primarily homomeric α_{IIb} and β_3 TMD-tail interactions[21]; however, phospholipid bicelles are a more accurate representation of the bulk mammalian plasma membrane because they contain lipids in a bilayer arrangement. Indeed, embedding in dodecylphosphocholine micelles distorts the helical structure of the β_3 membrane-proximal domain,[17] a region important in regulating integrin signaling. The remarkable agreement of biochemical and biophysical methods in identifying heterodimeric associations, combined with previous mutational data, provides compelling evidence for preferential heterodimeric TMD-tail interactions of $\alpha_{IIb}\beta_3$ in the lipid environment and the mammalian cell membrane. Furthermore, we now provide direct proof that talin binding disrupts the TMD-tail association and that an $\alpha_{IIb}(R995)\beta_3(D723)$ interaction stabilizes it. Moreover, the new approaches described in this study now enable analysis of TMD-tail interactions among many type I membrane proteins, such as tyrosine kinase growth factor receptors, immunoreceptors, or cytokine receptors that transduce signals as homo- and heteromultimers.

Acknowledgments

This work was supported by grants from the National Institutes of Health (Bethesda, MD) to M.H.G. (HL70784 and AR27214) and T.S.U. (HL089726). C.K. and T.-L.L. are recipients of postdoctoral fellowships from the American Heart Association (Dallas, TX).

Authorship

Contribution: C.K. designed and performed the mammalian cell experiments and wrote the paper; T.-L.L. designed and performed the NMR experiments; T.S.U. designed the NMR experiments and edited the paper; and M.H.G. designed the mammalian cell experiments and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Dr Mark H. Ginsberg, Department of Medicine, University of California San Diego, 9500 Gilman Dr, La Jolla, CA 92093; e-mail: mhginsberg@ucsd.edu.

References

1. Hynes RO. Integrins: bidirectional, allosteric signaling machines. Cell. 2002;110:673-687.
2. Miyamoto S, Akiyama SK, Yamada KM. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science. 1995;267:883-885.
3. Diaz-Gonzalez F, Forsyth J, Steiner B, Ginsberg MH. Trans-dominant inhibition of integrin function. Mol Biol Cell. 1996;7:1939-1951.
4. Leisner TM, Yuan W, DeNofrio JC, Liu J, Parise LV. Tickling the tails: cytoplasmic domain proteins that regulate integrin $\alpha_{IIb}\beta_3$ activation. Curr Opin Hematol. 2007;14:255-261.
5. Hughes PE, Diaz-Gonzalez F, Leong L, et al. Breaking the integrin hinge: a defined structural constraint regulates integrin signaling. J Biol Chem. 1996;271:6571-6574.
6. Kim M, Carman CV, Springer TA. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. Science. 2003;301:1720-1725.
7. Zhu J, Carman CV, Kim M, Shimaoka M, Springer TA, Luo BH. Requirement of α and β subunit transmembrane helix separation for integrin outside-in signaling. Blood. 2007;110:2475-2483.
8. Luo BH, Carman CV, Takagi J, Springer TA. Disrupting integrin transmembrane domain heterodimerization increases ligand binding affinity, not valency or clustering. Proc Natl Acad Sci U S A. 2005;102:3679-3684.
9. Lu C, Takagi J, Springer TA. Association of the membrane proximal regions of the α and β subunit cytoplasmic domains constrains an integrin in the inactive state. J Biol Chem. 2001;276:14642-14648.
10. Li W, Metcalf DG, Gorelik R, et al. A push-pull mechanism for regulating integrin function. Proc Natl Acad Sci U S A. 2005;102:1424-1429.
11. Partridge AW, Liu S, Kim S, Bowie JU, Ginsberg MH. Transmembrane domain helix packing stabilizes integrin $\alpha_{IIb}\beta_3$ in the low affinity state. J Biol Chem. 2005;280:7294-7300.
12. Ghevaert C, Salsmann A, Watkins NA, et al. A nonsynonymous SNP in the ITGB3 gene disrupts the conserved membrane-proximal cytoplasmic salt bridge in the $\alpha_{IIb}\beta_3$ integrin and cosegregates dominantly with abnormal proplatelet formation and macrothrombocytopenia. Blood. 2008;111:3407-3414.
13. Ulmer TS, Yaspan B, Ginsberg MH, Campbell ID. NMR analysis of structure and dynamics of the cytosolic tails of integrin $\alpha_{IIb}\beta_3$ in aqueous solution. Biochemistry. 2001;40:7498-7508.
14. Vinogradova O, Velyvis A, Velyviene A, et al. A structural mechanism of integrin $\alpha_{IIb}\beta_3$ "inside-out" activation as regulated by its cytoplasmic face. Cell. 2002;110:587-597.
15. Weljie AM, Hwang PM, Vogel HJ. Solution structures of the cytoplasmic tail complex from platelet integrin α_{IIb}- and β_3-subunits. Proc Natl Acad Sci U S A. 2002;99:5878-5883.
16. Lau TL, Dua V, Ulmer TS. Structure of the integrin α_{IIb} transmembrane segment. J Biol Chem. 2008;283:16162-16168.
17. Lau TL, Partridge AW, Ginsberg MH, Ulmer TS. Structure of the integrin β_3 transmembrane segment in phospholipid bicelles and detergent micelles. Biochemistry. 2008;47:4008-4016.
18. Gottschalk KE. A coiled-coil structure of the $\alpha_{IIb}\beta_3$ integrin transmembrane and cytoplasmic domains in its resting state. Structure. 2005;13:703-712.
19. Luo BH, Springer TA, Takagi J. A specific interface between integrin transmembrane helices and affinity for ligand. PLoS Biol. 2004;2:e153.
20. Schneider D, Engelman DM. Involvement of transmembrane domain interactions in signal transduction by α/β integrins. J Biol Chem. 2004;279:9840.
21. Li R, Babu CR, Lear JD, Wand AJ, Bennett JS, DeGrado WF. Oligomerization of the integrin $\alpha_{IIb}\beta_3$: roles of the transmembrane and cytoplasmic domains. Proc Natl Acad Sci U S A. 2001;98:12462-12467.
22. Li R, Mitra N, Gratkowski H, et al. Activation of integrin $\alpha_{IIb}\beta_3$ by modulation of transmembrane helix associations. Science. 2003;300:795-798.
23. Li R, Gorelik R, Nanda V, et al. Dimerization of the transmembrane domain of integrin α_{IIb} subunit in cell membranes. J Biol Chem. 2004;279:26666-26673.
24. Adair BD, Yeager M. Three-dimensional model of the human platelet integrin $\alpha_{IIb}\beta_3$ based on electron cryomicroscopy and x-ray crystallography. Proc Natl Acad Sci U S A. 2002;99:14059.
25. Williams MJ, Hughes PE, O'Toole TE, Ginsberg MH. The inner world of cell adhesion: integrin cytoplasmic domains. Trends Cell Biol. 1994;4:109.
26. Han J, Lim CJ, Watanabe N, et al. Reconstructing and deconstructing agonist-induced activation of integrin $\alpha_{IIb}\beta_3$. Curr Biol. 2006;16:1796-1806.
27. Feral CC, Nishiya N, Fenczik CA, Stuhlmann H, Slepak M, Ginsberg MH. CD98hc (SLC3A2) mediates integrin signaling. Proc Natl Acad Sci U S A. 2005;102:355-360.
28. Puig O, Caspary F, Rigaut G, et al. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. Methods. 2001;24:218-229.
29. Garcia-Guerra R, Garcia-Dominguez JA, Gonzalez-Rodriguez J. A new look at the lipid composition of the plasma membrane of human blood platelets relative to the GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$) content. Platelets. 1996;7:195-205.
30. Tadokoro S, Shattil SJ, Eto K, et al. Talin binding to integrin β tails: a final common step in integrin activation. Science. 2003;302:103-106.
31. Petrich BG, Marchese P, Ruggeri ZM, et al. Talin is required for integrin-mediated platelet function in hemostasis and thrombosis. J Exp Med. 2007;204:3103-3111.
32. Garcia-Alvarez B, de Pereda JM, Calderwood DA, et al. Structural determinants of integrin recognition by talin. Mol Cell. 2003;11:49-58.
33. Calderwood DA, Tai V, Di Paolo G, De Camilli P, Ginsberg MH. Competition for talin results in trans-dominant inhibition of integrin activation. J Biol Chem. 2004;279:28889-28895.
34. Yin H, Slusky JS, Berger BW, et al. Computational design of peptides that target transmembrane helices. Science. 2007;315:1817-1822.
35. Frelinger AL III, Du XP, Plow EF, Ginsberg MH. Monoclonal antibodies to ligand-occupied conformers of integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) alter receptor affinity, specificity, and function. J Biol Chem. 1991;266:17106-17111.
36. Yin H, Litvinov RI, Vilaire G, et al. Activation of platelet $\alpha_{IIb}\beta_3$ by an exogenous peptide corresponding to the transmembrane domain of αIIb. J Biol Chem. 2006;281:36732-36741.