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Effects of the Association between the α-Subunit Thigh and the β-Subunit EGF2 Domains on Integrin Activation and Signaling

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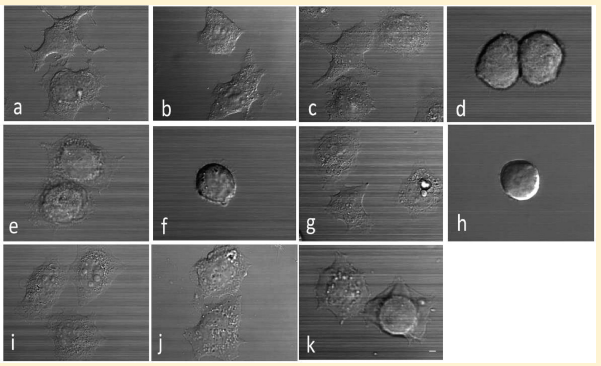
Supporting Information

ABSTRACT: Integrin bidirectional signaling is mediated by conformational change. It has been shown that the separation of the α- and β-subunit transmembrane/cytoplasmic tails and the lower legs is required for transmitting integrin bidirectional signals across the plasma membrane. In this study, we address whether the separation of the αβ knee is critical for integrin activation and outside-in signaling. By introducing three disulfide bonds to restrict dissociation of the α-subunit thigh domain and β-subunit I-EGF2 domain, we found that two of them could completely abolish integrin inside-out activation, whereas the other could not. This disulfide-bonded mutant, in the context of the activation mutation of the cytoplasmic domain, had intermediate affinity for ligands and was able to mediate cell adhesion. Our data suggest that there exists rearrangement at the interface between the thigh domain and the I-EGF2 domain during integrin inside-out activation. None of the disulfide-bonded mutants could mediate cell spreading upon adhering to immobilized ligands, suggesting that dissociation of the integrin two knees is required for integrin outside-in signaling. Disrupting the interface by introducing a glycan chain into either subunit is sufficient for high affinity ligand binding and cell spreading.



Integrins are heterodimeric type I transmembrane proteins consisting of two noncovalently associated α- and β-subunits, each with a large extracellular domain, a single transmembrane (TM) domain, and generally a short cytoplasmic domain.[1] They play essential roles in cell adhesion, spreading, migration, and other biological functions. Integrin signaling is bidirectional in which the specific intracellular molecules can interact with integrin cytoplasmic domain to increase ligand-binding affinity (known as inside-out activation), while extracellular ligand binding can transmit signals into the cell, leading to intracellular signaling events (known as outside-in signaling). It has been shown that conformational rearrangements are involved and critical in integrin signal transduction bidirectionally across the plasma membrane.[2,3]

Crystal structures of extracellular domains of αVβ3, αIIbβ3, and αXβ2 have revealed a V-shaped bent conformation.[4−7] The β-propeller noncovalently associates with the β I domains to form the globular head. The two legs are folded backward between the α-subunit thigh and calf-1 domain and at the β-subunit I-EGF2 domain. This bent conformation, stabilized by specific α/β interfaces existing in the extracellular, TM, and cytoplasmic domains, is believed to represent the physiologically low affinity state.[3,8−14] Negative-staining EM studies of integrins αVβ3,[3] αIIbβ3,[6] and αXβ2[7,15,16] showed that a substantial amount of integrin molecules were in the extended conformation in the presence of Mn^{2+} and ligand-mimetic peptide or compounds. Therefore, it is likely that large conformational changes occur during integrin activation and signaling.[8,11,15,17,18] At least two distinct extended conformational states have been visualized by EM: one with closed headpiece and the other with open headpiece,[3,7,16,19] which were proposed as the intermediate and active states, respectively.[2] During transition from the closed to the open headpiece, the hybrid domain swing-out is coupled with a crankshaft-like displacement of the βI domain α7 helix, converting the ligand-binding βI domain from the low affinity to the high affinity state.[3,20−23]

It has been shown that the association of the α- and β-subunit TM/cytoplasmic tails is critical for maintaining integrins in the low-affinity state, whereas intracellular signals that destabilize αβ TM/cytoplasmic association result in integrin activation.[24−28] However, the mechanism of how activation signals are transmitted from the TM domain through two long extracellular legs to the ligand-binding headpiece remains unclear. Mutagenesis studies showed that separation of two integrin lower legs is required for integrin activation and signaling.[10,29] This separation was proposed to be important for integrin activation because it leads to integrin extension.[6] However, the role of the integrin knee, i.e., the α-subunit thigh/calf-1 domains and the β-subunit I-EGF2 domain, remains elusive. In this study, we tested the role of association and

Received: May 13, 2011
Revised: September 20, 2011
Published: October 3, 2011

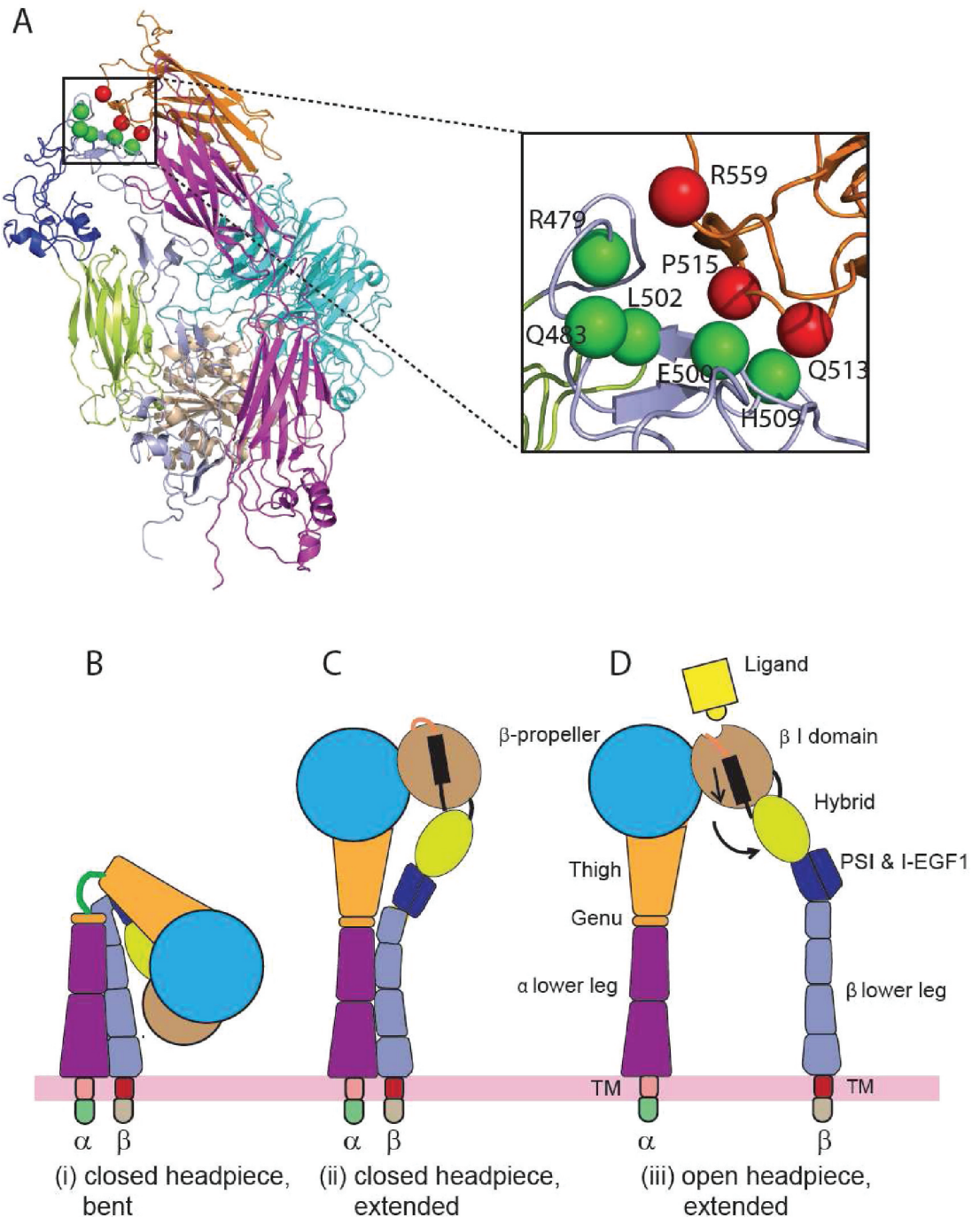


Figure 1. Conformational states of αIIbβ3 structure with designed mutations. (A) The bent conformation of αIIbβ3. The mutations are located at the interface between the thigh domain and the I-EGF2 domain, which are located near the integrin genu. The residues involved in the mutations are represented by spheres. Red balls represent residues from the αIIb-subunit, and green balls represent residues from β-subunit. (B) Cartoon representation of integrin bent conformational state. (C, D) Cartoon representation of integrin extended conformational states. (C) The extended conformation with closed headpiece showed that the integrin knee and legs are associated. (D) The extended conformation with open headpiece showed that integrin headpiece is open by hybrid domain swing-out with two separated legs. Figures were modified from the literature.[32,33]

dissociation of integrin knee in integrin activation and signaling by introducing mutations that either strengthen or disrupt the interface between the α-subunit thigh domain and β-subunit I-EGF2 domain (Figure 1). Our results showed that locking integrin knee by different disulfide bonds differentially regulates ligand binding affinity and cell adhesion, implying that the extended conformation with closed headpiece exists with intermediate affinity for ligand binding. However, separation of two subunit knees is necessary for cell spreading.

MATERIALS AND METHODS

Plasmid Construction, Expression, and Immunoprecipitation. Plasmids with sequences for full-length human αIIb and β3 were subcloned into pEF/V5-HisA and pcDNA3.1/Myc-His (+), respectively.[3] The αIIb mutants F992A/F993A (activating GAAKR mutant, denoted as α*), α*R513C, Q513N/P515T, and α*R559C and the β3 mutants R479C, Q483C, E500N/L502T, and H509C were made using site-

dx.doi.org/10.1021/bi200744g | Biochemistry 2011, 50, 9264–9272

directed mutagenesis with the QuikChange kit (Stratagene, La Jolla, CA). Constructs were transfected into HEK293T cells (American Type Culture Collection, Manassas, VA) using a FuGENE transfection kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. The expression levels of αIIb and β3 were detected by flow cytometry staining with the following monoclonal antibodies: AP3 (nonfunctional anti-β3 mAb, American Type Culture Collection), 7E3 (anti-β3 mAb), and 10E5 (anti- αIIb mAb, kindly provided by B. S. Coller, Rockefeller University, New York). To characterize disulfide-bond formation and glycosylation, transiently transfected cells were metabolically labeled with [^{35}S] cysteine/methionine as described.[26] Lysates in 20 mM Tris-buffered saline, pH 7.4 (TBS), supplemented with 1 mM Ca^{2+}, 1% Triton X-100, and 0.1% Nonidet P-40 were immunoprecipitated with 1 μg of anti-β3 mAb AP3 and protein G-sepharose at 4 °C for 1 h and eluted with 0.5% SDS. After the addition of 1% Nonidet P-40, the protein was treated with or without 500 units of PNGase F (New England BioLabs) at 37 °C for 1 h. Material was subjected to 7.5% nonreducing SDS-PAGE and fluorography.[26]

Two-Color Ligand Binding Assay on HEK293T Transfectants. Soluble binding of ligand mimetic IgM PAC-1 (BD Biosciences, San Jose, CA) and Alexa Fluor 488-labeled human fibrinogen (Enzyme Research Laboratories, South Bend, IN) was determined as previously described.[30] Briefly, transfected cells suspended in 20 mM HEPES-buffered saline, pH 7.4 (HBS), supplemented with 5.5 mM glucose and 1% bovine serum albumin were incubated on ice for 30 min with PAC-1 or Alexa Fluor 488-conjugated human fibrinogen in the presence of either 5 mM EDTA, 5 mM Ca^{2+}, 100 μM Ca^{2+}/1 mM Mn^{2+}, or 5 mM Ca^{2+} with 2 mM DTT (Preincubated for 15 min at RT). For PAC-1 binding, cells were washed and stained with FITC-conjugated antimouse IgM on ice for another 30 min before being subjected to flow cytometry. Cells were also stained in parallel with Cy3-conjugated anti-β3 mAb AP3. Binding activity is presented as the percentage of the mean fluorescence intensity (MFI) of PAC-1 or fibrinogen staining after background subtraction of the staining in the presence of EDTA, relative to the MFI of the AP3 staining.

Ligand-Induced Binding Site (LIBS) Epitope Expression. LIBS epitope expression was measured as previously described.[30] Briefly, transfected cells suspended in HBS supplemented with 5.5 mM glucose and 1% bovine serum albumin were incubated with or without 2 mM DTT at room temperature for 15 min, followed by incubation of 50 μM GRGDSP (RGD) peptide in the presence of 1 mM Mn^{2+} for 15 min. After incubation with AP3 on ice for 30 min, cells were washed and stained with FITC-labeled anti-mouse IgG on ice for 30 min. The stained cells were subjected to flow cytometry, and LIBS epitope expression was expressed as the percentage of MFI of anti-LIBS antibody relative to MFI of the conformation-independent anti-β3 mAb AP3.

Cell Adhesion Assays. Cell adhesion on immobilized human fibrinogen was assessed by the measurement of cellular lactate dehydrogenase (LDH) activity as previously described.[31] Briefly, cells suspended in HBS supplemented with 5.5 mM glucose and 1% bovine serum albumin and 1 mM Ca^{2+} with or without 2 mM DTT were added into flat bottom 12-well plates (1×10^5 cells/well) precoated with 20 μg/mL fibrinogen and blocked with 1% bovine serum albumin. After incubation at 37 °C for 1 h, wells were washed three times with HBS supplemented as indicated above. Remaining adherent cells were lysed with 1% Triton X-100, and LDH activity was assayed using the Cytotoxicity Detection Kit (Roche Applied Science) according to the manufacturer's instructions. Cell adhesion was expressed as a percentage of bound cells relative to total input cells.

Cell Spreading and Microscopy. Glass bottom 6-well plates (MatTek Corporation, Ashland, MA) were coated with 20 μg/mL human fibrinogen in phosphate-buffered saline at pH 7.4 (PBS) overnight at 4 °C and then blocked with 1% BSA at room temperature (RT) for 1 h. The transiently transfected HEK293T cells were detached with trypsin/EDTA, washed three times with DMEM, and seeded on fibrinogen-coated plates with or without 1 mM DTT. After incubation at 37 °C for 1 h, cells were washed three times with PBS and fixed with 3.7% formaldehyde in PBS at RT for 10 min for microscopy.

Differential interference contrast (DIC) imaging was conducted on a Leica TCS SP2 spectral confocal system coupled to a DM IRE2 inverted microscope with a 63× oil objective. For the quantification of cell spreading area, outlines of 100 randomly selected adherent cells were generated, and the number of pixels contained within each of these regions was measured using ImageJ software (Bethesda, MD).

RESULTS

Design of αIIbβ3 Mutants Stabilize or Disrupt αβ Knee Association. In the crystal structures of the αVβ3[4] and αIIbβ3,[6] there exists the interface between the α-subunit thigh domain and the β-subunit I-EGF2 domain which are located near the integrin genu in the bent conformation (Figure 1A,B). According to the EM images of the αVβ3[3] and αXβ2,[7] the thigh domain also interacts with the I-EGF2 domain in the extended conformation with closed headpiece, even though the atomic interaction may differ (Figure 1C). But in the extended conformation with the open headpiece, this interface is completely lost since the swing-out of the hybrid domain pulls the I-EGF2 domain away from the α-subunit (Figure 1D). It has been proposed that these three conformers (Figure 1B–D) correspond to low-affinity, intermediate-affinity, and high-affinity states, respectively, based on the ligand-binding ability.[32] By studying this interface in detail, we would provide structural information on integrin regulation and signaling. On the basis of the crystal structure and computer modeling of αIIbβ3,[6,33] we designed mutations at this interface in the bent conformation (Figure 1A). To mimic integrin inside-out activation, site-directed mutagenesis was used to mutate two phenylalanines in the GFFKR motif of the αIIb cytoplasmic domain to alanines (αIIb_F992A/F993A/β3, denoted α*/β).[24,29,34] Previously, cysteine residues were introduced into the α*/β construct to test the effects of disulfide-bridged mutants on integrin signaling.[26,29] Here we introduced three pairs of cysteine residues into this construct (α*513C/β509C, α*559C/β479C, α*559C/β483C). Since the cysteine residues were introduced into the interface of αβ Knee (Figure 1A), they were expected to form disulfide bonds and therefore preventing the dissociation of the thigh and the I-EGF2 domains. We expected that these disulfide bonds would "lock" the integrin in the closed headpiece conformation, since with the disulfide bridge the hybrid domain would not be able to swing out from the βI domain.

We also designed mutations to disrupt this interface to determine whether disrupting the αβ knee association affected integrin activation and signaling. N-Glycosylation sites were introduced on the αIIb-subunit thigh domain and on the β3-

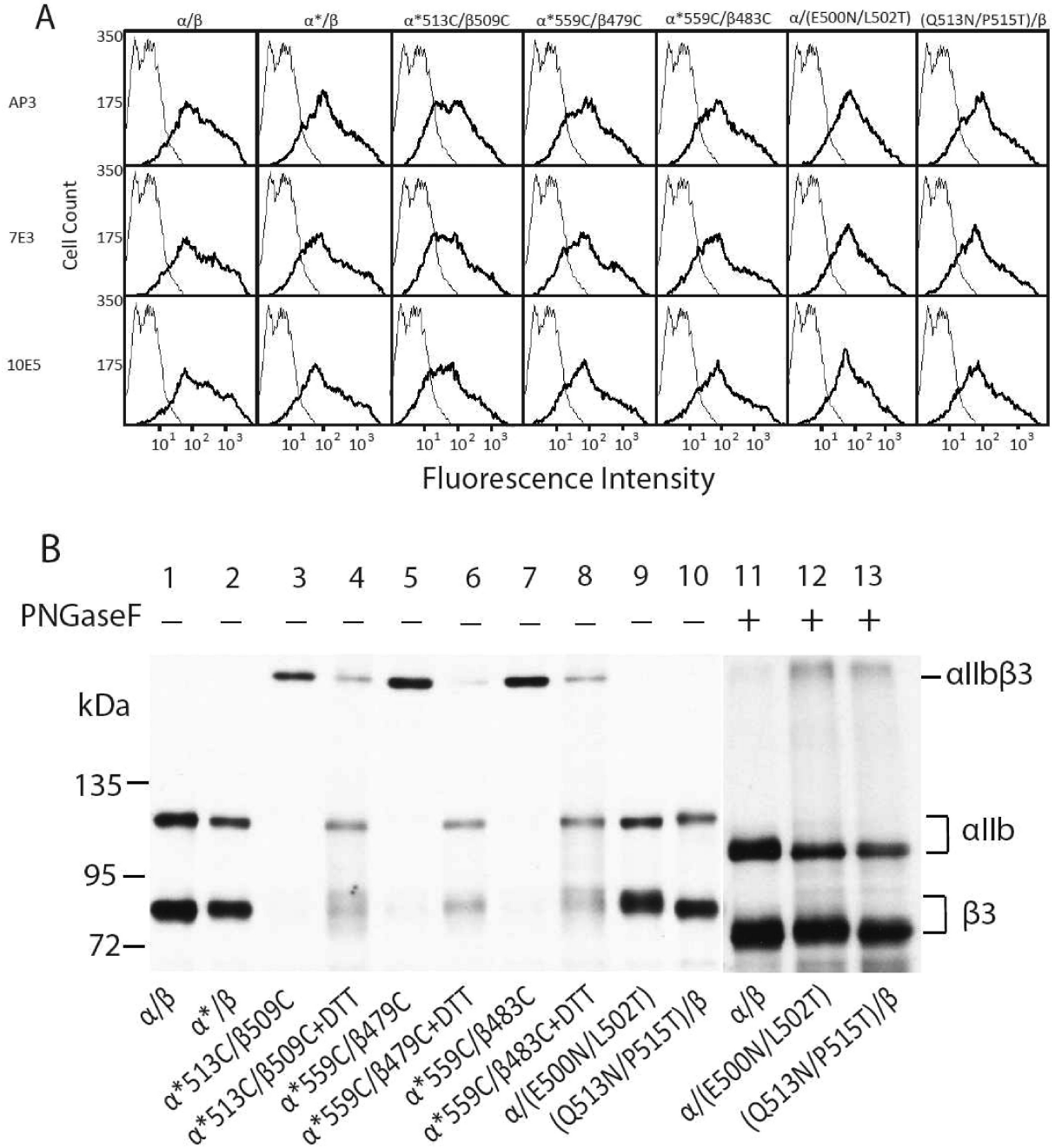


Figure 2. Expression and immunoprecipitation of wild-type and mutant αIIbβ3 integrins. (A) Immunofluorescent flow cytometry. HEK293T transfectants were labeled with AP3 (anti-β3), 7E3 (anti-β3), and 10E5 (anti-αIIb). Thick and thin lines show labeling of the αIIbβ3 transfectant and the mock transfectant, respectively. (B) Immunoprecipitation. Lysates from ^{35}S-labeled HEK293T cell transfectants were immunoprecipitated with mAb AP3. Precipitates were subjected to nonreducing 7.5% SDS-PAGE and fluorography.

subunit I-EGF2 domain. In the crystal structure, the residues αIIb_Q513 and β3_E500 are at the interface between the thigh and I-EGF2 domains (Figure 1A) and were expected to be important for the $\alpha\beta$ association. Therefore, introducing an N-glycan chain to either residue was expected to disrupt the $\alpha\beta$ association. The following mutants were constructed to test this hypothesis: αIIb_Q513N/P515T/β3 (denoted (Q513N/P515T)/β, resulting in N-glycosylation of Q513N in αIIb), and αIIb/β3_E500N/L502T (denoted α/(E500N/L502T), resulting in N-glycosylation of E500N in β3).

Expression of Wild-Type and Mutant αIIbβ3 on HEK293T Cells. To determine the expression of integrin receptors on the mammalian cell surface, wild-type and mutant αIIb and β3 subunits were cotransfected into HEK293T cells. Previously, we showed that in this HEK293T cell line neither αV nor β3 integrin is expressed endogenously,[29] excluding any possible contribution of endogenous αV to the expression and functional analysis in our study. Therefore, this cell line is ideal for studying functions of the transfected β3 integrin family. After 24−48 h of transfection, cells were subjected to immunostaining flow cytometry. Two anti-β3 antibodies 7E3 and AP3, which recognize the β3 I and hybrid domains, respectively, and one anti-αIIb antibody 10E5, which recognizes the β-propeller domain, were used to monitor cell surface

dx.doi.org/10.1021/bi200744g | Biochemistry 2011, 50, 9264−9272

expression. All wild-type and mutant integrins bound to the three antibodies similarly (Figure 2A), suggesting that they adopted a native conformation on the cell surface, and mutations have no effect on integrin expression or domain folding on the mammalian cell surface.

Nonreducing SDS-PAGE of ^{35}S-labeled, immunoprecipitated receptors showed that in the activating mutant (Figure 2B, lane 2), the α*- and the β3-subunits migrated in a similar pattern to the wild-type receptor (denoted α/β, Figure 2B, lane 1). In comparison, the receptors with the pairs of cysteine mutants α*_Q513C/β3_H509C, α*_R559C/β3_R479C, and α*_R559C/β3_Q483C formed disulfide bonds (Figure 2B, lanes 3, 5, and 7), and the efficiency of the disulfide-bond formation was close to 100%. Most of the disulfide bonds were reduced by DTT treatment (Figure 2B, lanes 4, 6, and 8). The β3-subunit of β3-glycosylated mutant α/(E500N/L502T) (Figure 2B, lane 9) migrated slightly slower than that of the wild-type (Figure 2B, lane 1), suggesting that there was an additional glycan chain added to the β3-subunit. For the αIIb-glycosylation mutant (Q513N/P515T)/β (Figure 2B, lane 10), the β3-subunit migrated in a similar pattern to the wild-type β3 (Figure 2B, lane 1), whereas the mutated αIIb-subunit (Figure 2B, lane 10) migrated slightly slower than its wild-type counterpart (Figure 2B, lane 1), consistent with the presence of an additional glycan chain. Furthermore, these differences between the wild-type and glycosylation mutants disappeared after deglycosylation by PNGase F (Figure 2B, lanes 11−13), confirming the attachment of extra glycan chains.

Mutations Introduced into the Integrin Knee Differentially Affect Ligand Binding. To study the effect of the association or separation of the integrin knee located between the thigh domain and the I-EGF2 domain on integrin activation, two-color flow cytometry was used to determine the binding of the soluble ligand-mimetic antibody PAC-1 and fibrinogen to the wild-type and mutant receptors on the HEK293T cell surface. In the presence of Ca^{2+}, the wild-type αIIbβ3 bound very little ligand-mimetic antibody PAC-1 or fibrinogen. Addition of Mn^{2+} or DTT slightly increased the ligand binding affinity of wild-type αIIbβ3 (Figure 3). The GAAKR mutant receptor (α*/β) bound PAC-1 or fibrinogen with high affinity even in the presence of Ca^{2+}, confirming the previous study that this mutation activates integrin for ligands, mimicking inside-out activation.[29] The addition of the Mn^{2+} did not increase the ligand binding further (Figure 3). Interestingly, three disulfide bonds introduced into this activating mutant showed different ligand binding affinity in the presence of Ca^{2+}. The mutant α*513C/β 509C had much higher PAC-1 or fibrinogen binding in Ca^{2+} comparing to that of the wild-type, but slightly lower than the GAAKR mutant. Furthermore, addition of DTT or Mn^{2+} could further increase PAC-1 and fibrinogen binding, suggesting that this disulfide-bonded mutant has intermediate affinity for ligands. By contrast, the other two disulfide-bonded mutants (α*559C/β479C and α*559C/β483C) almost did not bind PAC-1 or fibrinogen in Ca^{2+}. The addition of Mn^{2+} could only slightly increased ligand binding for the mutant α*559C/β483C, but not the mutant α*559C/β479C. But the DTT treatment significantly increased the ligand binding for both mutants, suggesting that the lack of ligand binding of the disulfide-bonded mutants was not due to the cysteine mutation itself, but to the formation of a disulfide bridge (Figure 3). These results indicate that preventing the separation of the $\alpha\beta$ knee by disulfide bonds differentially regulate the integrin inside-out activation.

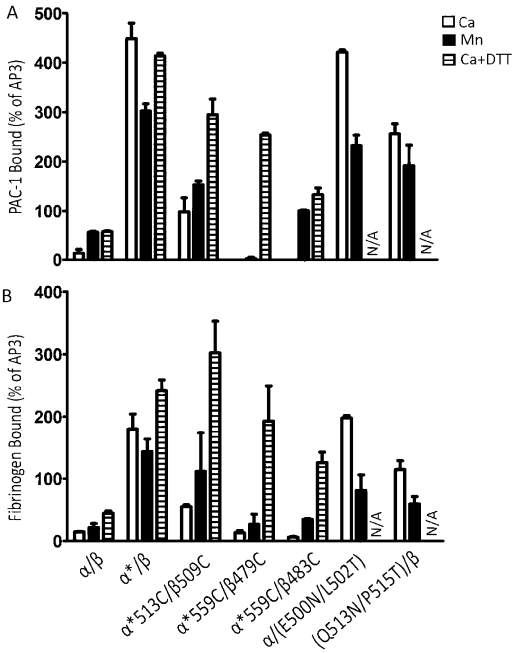


Figure 3. Ligand-binding activity of wild-type and mutant αIIbβ3 integrins. (A, B) Quantified soluble ligand-binding affinity. Cells were incubated with PAC-1 (A) or FITC-fibrinogen (B) in the presence of 5 mM Ca^{2+}, 1 mM Mn^{2+}, or 5 mM Ca^{2+} with 2 mM DTT. Binding activities were determined by flow cytometry and expressed as described in Materials and Methods. N/A stands for not available.

When an N-glycan chain was introduced into the $\alpha\beta$ interface of either subunit, receptors bound PAC-1 or soluble fibrinogen with high affinity in the presence of Ca^{2+} (Figure 3). The addition of Mn^{2+} did not increase their ligand binding, suggesting that these two mutants constitutively bound ligands with maximal affinity (Figure 3). Therefore, our experiments suggest that separation of the $\alpha\beta$ knee can fully activate integrin for ligand binding.

Integrin Conformational Change Caused by Mutations. Priming and ligand binding induce αIIbβ3 conformational changes that expose the LIBS epitopes. LIBS epitopes are at the interfaces between the headpiece and tailpiece and between the α- and β-legs so that they are buried in the bent conformation but exposed in the extended conformation.[3,35] To investigate the conformational states of the αIIbβ3 mutants, binding of anti-β3 LIBS mAb LIBS1[36] was analyzed. The LIBS1 bound poorly to wild-type αIIbβ3 in the presence of Ca^{2+}. The binding significantly increased when Mn^{2+} and the ligand mimetic peptide RGD (Figure 4) were added, consistent with the EM images which showed that the ligand mimetic peptide stabilizes integrins in the more extended conformation.[3] Treatment of DTT had little effect on global conformation of wild-type αIIbβ3. The GAAKR mutant (α*/β) bound LIBS1 better than the wild-type in the presence of Ca^{2+}, suggesting that the mutation mimicking inside-out signaling shifts the integrin toward a more extended conformation. Addition of Mn^{2+} and RGD peptide further increased binding of the GAAKR mutant to LIBS1. In comparison, introducing the disulfide bonds into this mutant decreased LIBS1 binding in varied extent in the presence of Ca^{2+} (Figure 4), suggesting that the disulfide bonds have different effects on the conformational change induced by inside-out activation. The mutant α*513C/β 509C bound LIBS1 much better than the wild-type in Ca^{2+},

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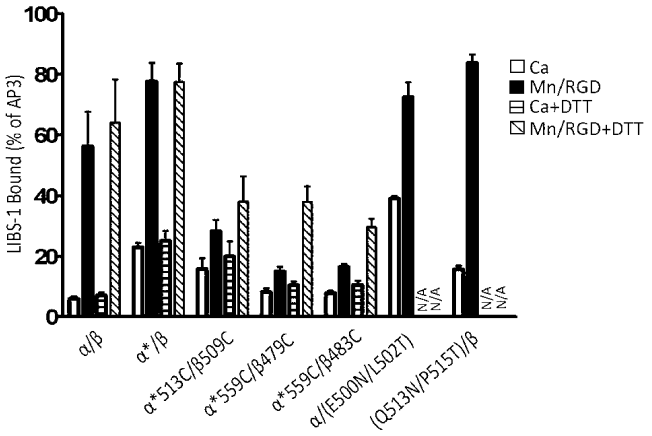

Figure 4. Exposure of the LIBS1 eptitope. Cells were stained with anti-LIBS antibody LIBS1 in the presence of 5 mM Ca^{2+} with or without 2 mM DTT or 1 mM Mn^{2+} plus 50 μM RGD peptides (RGD) with or without 2 mM DTT. LIBS epitope exposure was determined as the percentage of MFI of anti-LIBS1 antibody relative to nonfunctional anti-β3 mAb AP3. Error bars are standard deviation (SD). N/A stands for not available.

and addition of Mn^{2+} and RGD peptide only slightly increased the binding, but much lower than the wild-type in the same condition. It is interesting that this mutant bound soluble ligands better than the wild-type in Ca^{2+}. Therefore, it is likely that this mutant receptor, which has intermediate affinity for ligands, is in a different conformation comparing to the low affinity, bent conformation of the wild-type. Since the disulfide bridge prevents two legs separation, the addition of Mn^{2+} and RGD peptide could not increase the LIBS1 binding to a similar level as the wild-type. By contrast, the other two mutants, α*559C/β479C and α*559C/β483C, bound little to the LIBS1 in Ca^{2+}, similar to the wild-type. These data are consistent with their soluble ligand binding, suggesting that they may in the bent conformation with low affinity for ligands. After DTT treatment, addition of Mn^{2+} and RGD peptide to all three mutant receptors further increased binding to LIBS1.

The two glycosylation mutants (α/E500N/L502T and Q515N/P515T/β) bound LIBS1 in the presence of Ca^{2+} much better than the wild-type (Figure 4), suggesting that separation of the integrin knee stabilizes integrins in a more extended conformation. The addition of Mn^{2+} and RGD peptide further increased the LIBS1 binding.

Separation of the $\alpha\beta$ Knee Is Required for Cell Spreading. We further determined the effect of the association or separation of the thigh and the I-EGF2 domains on outside-in signaling by assaying cell adhesion and spreading. HEK293T cells transiently transfected with wild-type and mutant αIIbβ3 were seeded on fibrinogen-precoated dish surfaces at 37 °C for 1 h. The amount of adherent cells was assessed by quantifying the cellular lactate dehydrogenase (LDH) activity. The results showed the disulfide-bonded mutant α*513C/β509C adhered to immobilized fibrinogen similarly to the wild-type cells. By contrast, HEK293T cells transfected with the other two disulfide-bonded αIIbβ3 (α*559C/β479C and α*559C/β483C) showed little adhesion (Figure 5A). When these two disulfide-bonded mutants were treated with DTT, the cell adhesion ability was recovered to the similar level of the wild-type with DTT.

It is interesting that all disulfide-bonded mutants in HEK293T cells exhibited defective spreading on fibrinogen (Figure 5B), regardless of their distinct cell adhesion ability. The cell area was quantified and showed that the disulfide-bonded mutants had a significant decrease in adherent cell size comparing to that of the wild-type (Figure 5C). Most adherent cells remained round with the same size (Figure 5B). To demonstrate that the defect in spreading was due to the disulfide linkage, we treated the cells with 1 mM DTT. The DTT treatment of the disulfide-bonded integrins led to a rescue of cell spreading (Figure 5B,C). HEK293T cells transfected with either glycosylation mutant could adhere to immobilized fibrinogen and demonstrated substantial spreading (Figure 5). Similar results were obtained when cell adhesion was carried out using transfectants of CHO cells (Supporting Information Figure 1), which have been widely used to study integrin αIIbβ3 outside-in signaling.[31,37−40] It suggests that the effect of disulfide-bonded mutants on integrin-mediated cell signaling is not cell-type specific. When disulfide bonds were introduced into wild-type integrins as controls, the disulfide-bonded mutants in HEK293T showed defective spreading as well (Supporting Information Figure 2). Our results indicate that separation of the α/β lower leg is crucial for cell spreading.

DISCUSSION

In the present study, we designed a series of mutations at the interface between the αIIb-subunit thigh domain and β3-subunit I-EGF2 domain to determine the effect of this interface during integrin inside-out activation and outside-in signaling. We demonstrate that locking the interface of integrin knee by three different disulfide bonds differentially regulates integrin inside-out activation reflected by ligand binding affinity. The disulfide-bonded mutant α*513C/β509C has intermediate affinity for ligand binding, since addition of Mn^{2+} or the treatment of DTT could further enhance ligand binding. By contrast, the other two disulfide-bonded mutants (α*559C/β479C and α*559C/β483C) have low ligand-binding affinity. Our study also shows that disrupting the association of this region by glycan chains results in integrin activation and signaling.

At least three distinct conformational states have been visualized by EM and crystallographic studies: the bent conformation with a closed headpiece, the extended conformation with a closed headpiece, and the extended conformation with an open headpiece.[3,7,16,19,41] Previously, locking integrins in the bent conformation by disulfide bonds abolished integrin ligand binding, indicating that the bent conformation is in the low affinity for ligands.[3,8,9,42] Glycan wedge mutations introduced into integrins to stabilize the open headpiece conformation resulted in maximal binding for ligands, suggesting that the extended state with an open headpiece has high affinity for ligands.[26,30,43] In this study, we introduced cysteines to three pairs of residues which are located at the interface between the thigh domain and the I-EGF2 domain based on the crystal structures of the bent conformation with a closed headpiece. By restricting the dissociation of this interface by these disulfide bonds, integrins showed different effects on transmitting inside-out signaling as mentioned above. Our data suggest that during inside-out activation conformational change does occur at the interface of the thigh domain and the I-EGF2 domain. It is likely that in the extended conformation with a closed headpiece the interface between the thigh domain and the I-EGF2 domain differs somewhat from that in the bent conformation. Unlike residue pairs α559/β479 and α559/β483, α513/β509 is located more

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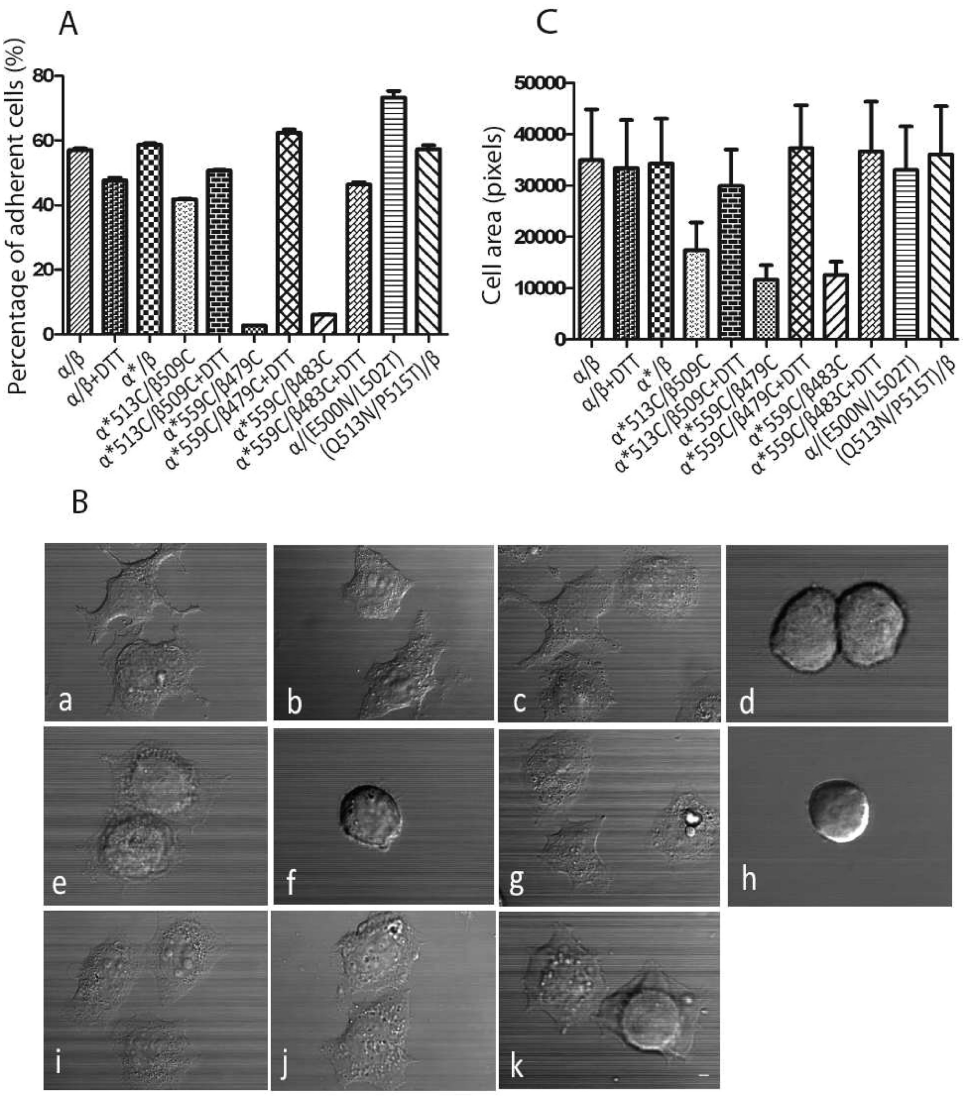

Figure 5. Cell adhesion and spreading. (A) Adhesion of HEK293T transfectants in the presence of 1 mM Ca^{2+} with or without DTT (2 mM) to surfaces coated with 20 μg/mL fibrinogen. The amount of bound cells was determined by measuring LDH activity as described in Materials and Methods. Data are representative of three independent experiments, each in triplicate. (B) DIC images of HEK293T transfectants after adhering to immobilized fibrinogen at 37 °C. a, α/β; b, α/β + DTT; c, α^*/β; d, α^*513C/β509C; e, α^*513C/β509C + DTT; f, α^*559C/β479C; g, α^*559C/β479C + DTT; h, α^*559C/β483C; i, α^*559C/β483C + DTT; j, α/(E500N/L502T); k, (Q513N/P515T)/β. The images are representatives of three independent experiments. Scale bar represents 10 μm. (C) Quantification of the areas of adhering/spreading cells as described in Materials and Methods. Error bars are SD.

deeply in the inner hinge of the "knee"; it may undergo relatively less local conformational change during integrin extension and thus is more likely associated in the extended conformation with a closed headpiece. Therefore, the mutant α^*513C/β509C could bind to ligands with intermediate affinity, but neither α^*559C/β479C nor α^*559C/β483C could. More experimental evidence is required to confirm this hypothesis. Recently, Ye et al. showed that integrins in nanodisks in the presence of the talin head domain were mainly in the extended conformation with closed headpiece.[19] Therefore, the intermediate state may be sufficient for αIIbβ3 to mediate ligand binding.

Outside-in signaling is induced by binding of integrins to multimeric ligands, which results in integrin conformational change and clustering, both of which are critical for signaling.[27,29,31,44,45] Our data indicate that the separation of integrin knee is required for outside-in signaling, since cell spreading was greatly reduced when disulfide bonds were introduced at the interface of this region (Figure 5B,C). Binding of ligands to integrin headpiece leads to conformational change of the β I domain, resulting in the swing-out of the hybrid domain, leading to separation of the interface between the thigh domain and the I-EGF2 domain. Therefore, the dissociation of this interface is critical for outside-in signaling. In our study, the mutant α^*513C/β509C could bind to ligands with higher affinity than the WT and could adhere to the immobilized ligands, but the cell spreading was defective. Together with the previous studies,[29,31] our studies suggest that dissociation of two heterodimeric knees, lower legs and TM/cytoplasmic domains is required for transmitting outside-in signals from the integrin extracellular headpiece to intracellular kinases.

dx.doi.org/10.1021/bi200744g | *Biochemistry* 2011, 50, 9264–9272

■ ASSOCIATED CONTENT

Supporting Information

CHO-K1 and HEK293T cell spreading of mutant αIIbβ3 integrin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

We thank the American Heart Association (#10GRNT3960011) and the Louisiana Board of Regents (LEQSF(2009-12)-RD-A07) for financial support.

■ ABBREVIATIONS

DIC, differential interference contrast; DTT, dithiothreitol; EM, electron microscopy; HPS, HEPES-buffered saline; LIBS, ligand-induced binding site; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; MFI, mean fluorescence intensity.

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