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Regulation of Integrin αIIbβ3 Ligand Binding and Signaling by the Metal Ion Binding Sites in the β I Domain

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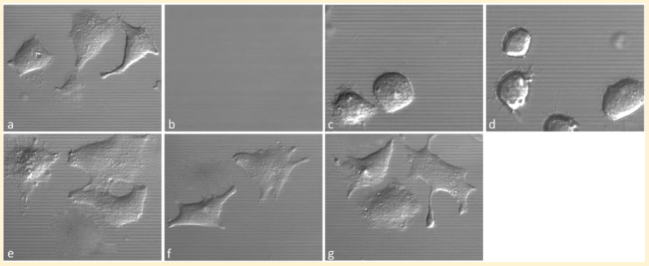
ABSTRACT: The ability of αIIbβ3 to bind ligands and undergo outside-in signaling is regulated by three divalent cation binding sites in the β I domain. Specifically, the metal ion-dependent adhesion site (MIDAS) and the synergistic metal binding site (SyMBS) are thought to be required for ligand binding due to their synergy between Ca^{2+} and Mg^{2+}. The adjacent to MIDAS (ADMIDAS) is an important ligand binding regulatory site that also acts as a critical link between the β I and hybrid domains for signaling. Mutations in this site have provided conflicting results for ligand binding and adhesion in different integrins. We have mutated the β3 SyMBS and ADMIDAS. The SyMBS mutant abolished ligand binding and outside-in signaling, but when an activating glycosylation mutation in the αIIb Calf 2 domain was introduced, the ligand binding affinity and signaling were restored. Thus, the SyMBS is important but not absolutely required for integrin bidirectional signaling. The ADMIDAS mutants showed reduced ligand binding affinity and abolished outside-in signaling, and the activating glycosylation mutation could fully restore integrin signaling of the ADMIDAS mutant. We propose that the ADMIDAS ion stabilizes the low-affinity state when the integrin headpiece is in the closed conformation, whereas it stabilizes the high-affinity state when the headpiece is in the open conformation with the swung-out hybrid domain.



Integrins are cell adhesion receptors involved in multiple biological processes including development, cancer, hemostasis, and immune response through cell−cell, cell−extracellular matrix (ECM), and cell−pathogen interactions.[1,2] They are composed of two noncovalently associated subunits α and β. Each subunit contains a large extracellular domain, a short transmembrane domain, and a short cytoplasmic domain. Integrins are unique because of their ability to transmit bidirectional signals across the membrane via inside-out and outside-in signaling. These receptors are usually in the low-affinity state with the extracellular domain in the bent conformation in normal physiological conditions. During inside-out signaling or integrin activation, the cell is stimulated by external agents that activate specific intracellular molecules to interact with the integrin cytoplasmic domain, resulting in conformational change leading to integrin extension into the higher affinity state.[3−7] Furthermore, ligand binding to the extracellular domain induces outside-in signaling and activates many intracellular signaling pathways.[8−11]

It has been known that integrin affinity for ligands is regulated by metal ions.[12−16] Some divalent cations such as Mn^{2+} can activate integrins for ligands, whereas Ca^{2+} typically favors the inactive state. Crystallographic studies of the αVβ3, αIIbβ3, and αXβ2 in the unliganded closed conformations[17−19] and of the αIIbβ3 in the liganded open conformation[3] have provided the structural basis for the cation dependence for ligand binding. Within the extracellular domain, the α I domain, which was found in half of the integrins, is responsible for ligand binding. In integrins that lack the α I domain such as the β3 integrins, the β I domain is the main binding site for ligands.[3−5,7,18,20,21] The β I domain contains three metal ion-coordinating sites that are important for ligand binding and signaling. The metal ion-dependent adhesion site (MIDAS) is at the center of these coordinating sites, while the other two sites on the peripheral are the synergistic metal binding site (SyMBS), previously known as ligand-associated metal binding site or LIMBS, and adjacent to MIDAS (ADMIDAS).[3,4,14,15,19−25] First, the MIDAS coordinates Mg^{2+} under normal physiological conditions and binds to a specific anionic sequence on ligands.[14,15,20−22] The MIDAS contains a conservative motif DXSXS (Figure 1). This DXSXS motif is essential for coordinating the metal ion, which is critical for ligand binding.[24,25] Structural studies have revealed that the carboxyl group of Asp in the RGD sequence of the ligand coordinates with the MIDAS Mg^{2+} of the β3 subunit during ligand binding, indicating that the MIDAS is essential for ligand binding.[3,4] This was supported by mutagenesis studies in which mutations in the MIDAS site completely abolished ligand binding.[3,4] Next, the SyMBS coordinates Ca^{2+} and allosterically activates integrins for ligand binding by stabilizing the MIDAS site.[19,23] The SyMBS in low Ca^{2+} concentration will synergize with low concentration of MIDAS Mg^{2+} to facilitate the ligand binding.[19] Mutations in the SyMBS in various integrin families have reduced or abolished ligand binding, while this abnormal ligand binding can be restored by the activating mutations of the α I domain or a glycosylation mutation that stabilized the

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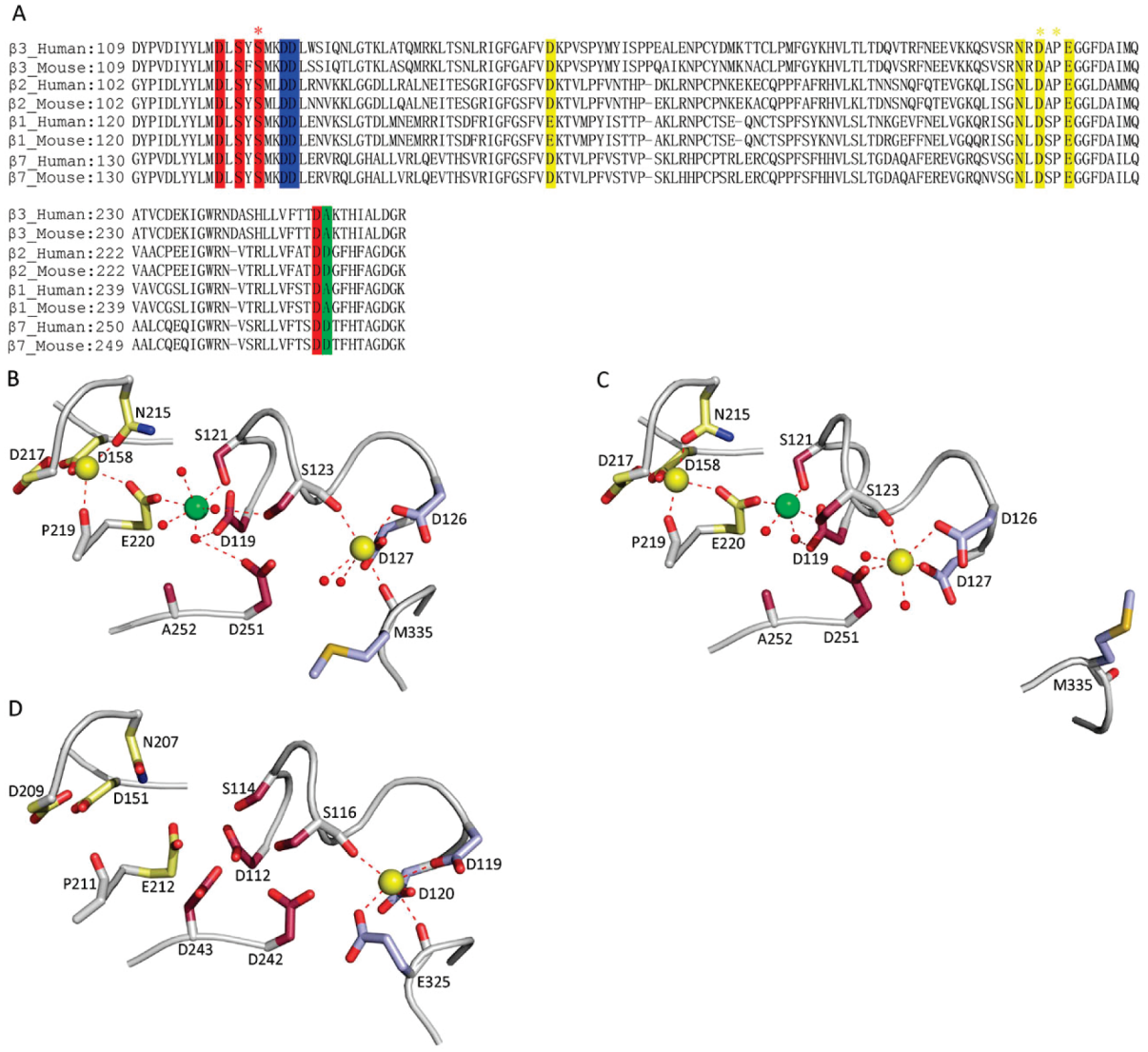

Figure 1. β subunit sequence alignment and structure of metal-binding sites in αIIbβ3 and αXβ2. (A) Sequence alignment of human β1, β2, β3, and β7 I domain metal ion binding sites. Residues with metal-coordinating side-chain oxygen atoms are highlighted, and residues with metal-coordinating backbone carbonyl oxygen atoms are asterisked. Highlighted or asterisked residues are colored as follows: MIDAS, red; SyMBS, yellow; ADMIDAS, light blue. (B) Structure of unliganded αIIbβ3 metal binding sites. (C) Structure of liganded αIIbβ3 metal binding sites. (D) Structure of unliganded αXβ2 metal binding sites. The metal binding sites are colored as follows: raspberry, MIDAS; pale yellow, SyMBS; light blue, ADMIDAS. N and O atoms involved in metal-coordinating side chains or carbonyl backbones are colored in blue and red, respectively. Ca^{2+} and Mg^{2+} ions are colored in yellow and green, respectively. Polar coordination between O atoms and metal ions is shown by dashed red lines.

integrin headpiece in the open conformation.[14,15,26−30] A recent study showed that the SyMBS coordinates with the specificity-determining loop and disruption of this interaction resulted in impaired bidirectional signaling.[31] Therefore, the exact biological roles of the SyMBS remain elusive. Finally, the ADMIDAS was proposed to allosterically inhibit ligand binding when coordinated with Ca^{2+}.[14,22,26,27] The linkage between the β I domain ADMIDAS and the hybrid domain essential for integrin signaling creates a unique biological role for the ADMIDAS.[27,32] This site was thought to orchestrate between ligand binding inhibition and propagation of the signal to the inside of the cell.[3,4,17,26,27] Mutations in the ADMIDAS have abolished integrin outside-in signaling, but either increased or decreased ligand binding affinity was observed in different integrins.[14,15,22,26,27,33] In studies with αLβ2 and α4β7, mutations in the ADMIDAS increase ligand binding and firm adhesion compared to the wild type (WT).[14,15,27] By contrast, the ADMIDAS mutations in α5β1 and α2β1 decreased ligand binding.[22,26] It remains elusive how to reconcile these conflicting observations.

In this paper, we introduced specific mutations into the SyMBS and ADMIDAS to study the role of these metal ion complexes in αIIbβ3 ligand binding and outside-in signaling. Our results showed that the SyMBS mutant abolished ligand binding and outside-in signaling, but when an activating glycosylation mutation was introduced into the lower legs to disrupt the $\alpha\beta$ association, the ligand binding and signaling were restored. The ADMIDAS mutants showed reduced ligand binding affinity and abolished outside-in signaling. The activating glycosylation mutation could fully restore the functions of one of the ADMIDAS mutants. The study provides insight into the role of these two metal ion binding sites in integrin ligand binding and signaling.

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EXPERIMENTAL PROCEDURES

Plasmid Construction and Expression. Plasmids with sequences for full-length human αIIb and β3 were subcloned into pEF/V5-HisA and pcDNA3.1/Myc-His (+), respectively.[34] The αIIb mutant F755T and the β3 mutants D126A, D127A, and D217A were made using site-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), 10E5 (anti- αIIb mAb, kindly provided by B. S. Coller, Rockefeller University, New York, NY), and LM609 (anti-αV mAb).

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.[35] 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 (BSA) were incubated on ice for 30 min with 10 μg/mL PAC-1 or 60 μg/mL Alexa Fluor 488-conjugated human fibrinogen in the presence of either 5 mM EDTA, 5 mM Ca^{2+}, or 1 mM Mn^{2+}. For PAC-1 binding, cells were washed and stained with FITC-conjugated anti-mouse 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.[35] Briefly, transfected cells suspended in HBS supplemented with 5.5 mM glucose and 1% BSA were incubated for 15 min at room temperature with or without 50 μM GRGDSP peptide in the presence of 5 mM Ca^{2+} or 5 mM Mn^{2+}. Then, cells were stained with 10 μg/mL anti-LIBS mAbs LIBS1 or LIBS6 and incubated on ice for 30 min. After incubation, 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 the expression levels of LIBS1 and LIBS6 epitope were represented 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.[11] Briefly, cells suspended in HBS supplemented with 5.5 mM glucose, 1% BSA, and 1 mM Ca^{2+} were added into flat-bottom 12-well plates (1×10^5 cells/well) precoated with 20 μg/mL fibrinogen and blocked with 1% BSA. After incubation at 37 °C for 30 min, 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 six-well plates (MatTek Corp., 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 for 1 h. The transiently transfected HEK293T cells were detached with trypsin/EDTA, washed three times with DMEM, and seeded on fibrinogen-coated plates. After incubation at 37 °C for 1 h, cells were washed three times with PBS and fixed with 3.7% formaldehyde in PBS at room temperature 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 and Expression of Mutant αIIbβ3 Integrins. Based on the crystal structure of the αIIbβ3 integrin, several point mutations in the SyMBS and ADMIDAS sites in the β3 β I domain were created to change Asp to Ala. By replacing the Asp with the nonpolar amino acid, the SyMBS and ADMIDAS were expected to be disrupted; accordingly, their functions could be eliminated. Also, a glycosylation point mutation was created in the αIIb Calf-2 domain, αIIb_F755T (denoted F755T), which results in N-glycosylation of αIIb N753 in the interface of Calf-2 domain and the β3 EGF-4 domains and thus disrupts the two leg association. The mutant has been shown to cause full activation of the αIIbβ3 integrin.[36] To determine the expression of integrins on the cell surface, the WT and mutant αIIbβ3 were cotransfected into HEK293T cells and subjected to immunostaining flow cytometry. To exclude the possible contribution of endogenous αV or β3 in HEK293T cell lines, anti-αV antibody LM609 was used, and we did not detect any endogenous αV or β3 expression. When αV and β3 subunits were cotransfected into this cell line, the αV expression can be detected using LM609, suggesting that the antibody is functional (data not shown). Furthermore, when the WT and mutant αIIbβ3 integrins were cotransfected, LM609 did not bind to the cells (Figure 2), confirming that no endogenous αV was expressed that could potentially complicate our experiments. Two anti-β3 antibodies AP3 and 7E3, which recognize the hybrid and the β3 I domains, respectively, and one anti-αIIb antibody 10E5, which recognizes the β-propeller domain, were used to monitor cell surface expression of the transfected αIIbβ3 integrins. Wild-type and mutant integrins bound to the three antibodies with similar level (Figure 2), suggesting that all mutations have little effect on protein expression, and they adopted a native structure on the cell surface.

Regulation of PAC-1 and Fibrinogen Binding by the SyMBS and ADMIDAS Sites. Two-color flow cytometry was used to determine the fibrinogen and ligand-mimetic PAC-1 binding of the WT and mutants. In the presence of Ca^{2+}, WT αIIbβ3 bound very little ligand-mimetic PAC-1 antibody or fibrinogen. This is consistent with a low-affinity state under the physiological condition. In the presence of Mn^{2+}, WT αIIbβ3 bound PAC-1 and fibrinogen with higher affinity, suggesting that Mn^{2+} could activate integrin αIIbβ3 for ligand binding, even though the activation effect is lower than the glycosylated mutant F755T. This F755T mutant caused a 3−4-fold increase in ligand binding compared to the WT αIIbβ3. Consistent with our

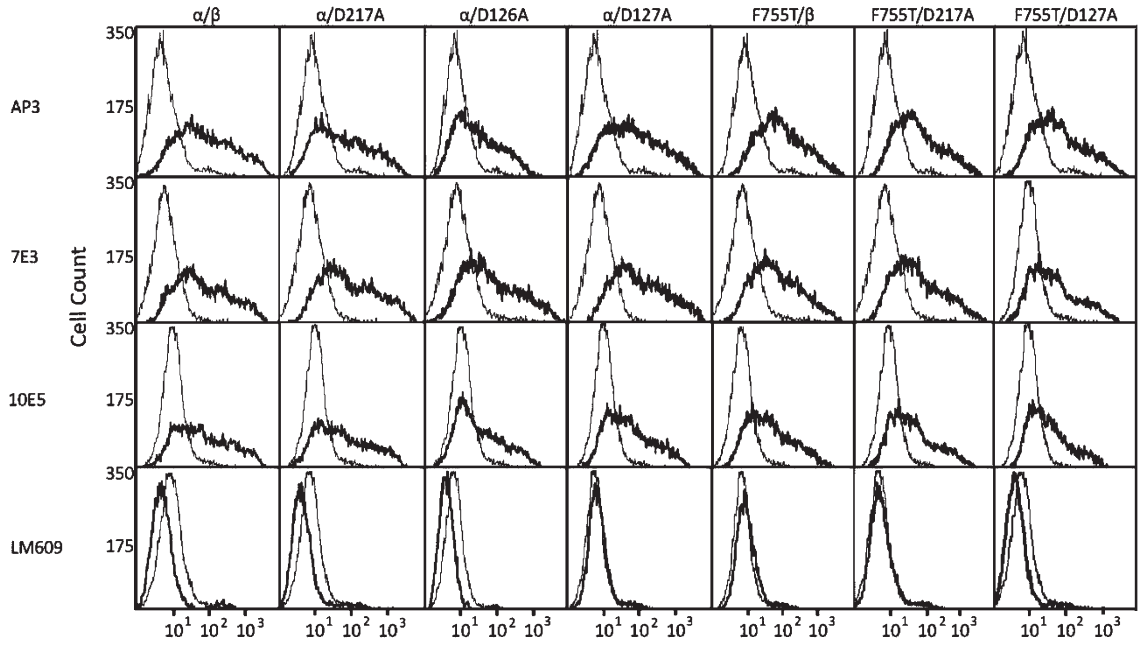

Figure 2. Expression of WT and mutant αIIbβ3 integrins. Immunofluorescent flow cytometry study of the HEK293T transfectants stained with AP3 (anti-β3), 7E3 (anti-β3), 10E5 (anti-αIIb), and LM609 (anti-αV). Thick and thin lines show labeling of the αIIbβ3 transfectant and the mock transfectant, respectively.

previous observation, the F755T mutant appeared to have maximum ligand binding in Ca^{2+}, and Mn^{2+} could not further enhance its binding (Figure 3).[36]

By contrast, the D217A SyMBS mutant severely inhibited ligand binding, and even Mn^{2+} could not activate the mutant for PAC-1 or fibrinogen binding (Figure 3). The data suggest that the SyMBS site is important for ligand binding, and integrin is inactivated when the SyMBS site is abolished. However, this mutation, when combined with the F755T glycosylation mutation, appears to recover the ability of ligand binding. The F755T/D217A double mutant showed higher binding to PAC-1 in Ca^{2+} compared to the WT, and the presence of Mn^{2+} could not further increase its binding. In soluble fibrinogen binding, the F755T/D217A double mutant exhibited much higher binding compared to the WT both in Ca^{2+} and in Mn^{2+}. Therefore, a mutation that disrupts the association of the two integrin legs could restore the SyMBS mutant, suggesting that SyMBS is important but not absolutely required for integrin ligand binding.

Two ADMIDAS mutations D126A and D127A showed almost no binding to soluble PAC-1 or fibrinogen in Ca^{2+}, similar to the WT, suggesting that the mutants do not enhance binding affinity. In the presence of Mn^{2+}, these mutants showed slightly higher affinity for ligand binding, indicating that integrins with ADMIDAS abolishment are still sensitive to Mn^{2+} activation. However, both mutants exhibited reduced ligand binding affinity compared to the WT in Mn^{2+}. These mutants bound soluble PAC-1 with significantly lower binding affinity compared to the WT, whereas their binding to fibrinogen was only slightly reduced. Interestingly, the glycosylated double mutant F755T/D127A showed almost maximal affinity to both PAC-1 and fibrinogen, in either Ca^{2+} or Mn^{2+} conditions (Figure 3). Our results indicate that the ADMIDAS is important for regulating integrin ligand binding affinity, but its role is more complicated.

Conformational Changes Induced by SyMBS and ADMIDAS Mutants. 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.[34,37] To investigate the conformational state of the αIIbβ3 mutants, binding of anti-β3 LIBS mAb LIBS1 and LIBS6[38] was measured in various conditions of Ca^{2+}, Ca^{2+}/RGD, Mn^{2+}, and Mn^{2+}/RGD. The LIBS1 and LIBS6 bound poorly to wild-type αIIbβ3 in the presence of Ca^{2+} alone. The presence of Mn^{2+} or the ligand-mimetic peptide GRGDSP could increase the antibody binding, whereas the binding significantly increased when both Mn^{2+} and RGD were added (Figure 4), suggesting that the RGD peptide with Mn^{2+} stabilizes integrins in the more open conformation. The glycosylated mutant F755T bound LIBS1 and LIBS6 antibodies even in Ca^{2+}, comparable to the WT in the presence of Mn^{2+}/RGD (Figure 4). The data confirm that the separation of the two legs stabilizes integrins in a more open, high-affinity conformation, consistent with our previous observation.[36]

The SyMBS mutant D217A exhibited lower binding to LIBS1 and LIBS6 under Ca^{2+} than the WT, suggesting that this mutant is likely in the more bent conformation. The presence of Ca^{2+}/RGD, Mn^{2+}, or Mn^{2+}/RGD had almost no effect on LIBS1 and LIBS6 binding. Therefore, the SyMBS mutation not only inhibited ligand binding but also caused the integrin to remain in the bent conformation, regardless of whether Mn^{2+} or RGD was added. The glycosylated double mutant F755T/D217A showed significantly higher binding to LIBS1 and LIBS6 compared to the WT and the D217A mutant (Figure 4), suggesting that separation of the two legs shifts the equilibrium of integrin conformations toward a more open conformation even though the SyMBS was abolished. This global conformational change induced by the glycosylation may explain the higher binding affinity of this double mutant to soluble PAC-1 and fibrinogen as shown above.

The ADMIDAS mutants D126A and D127A showed similar LIBS1 and LIBS6 binding as the WT in the presence of Ca^{2+},

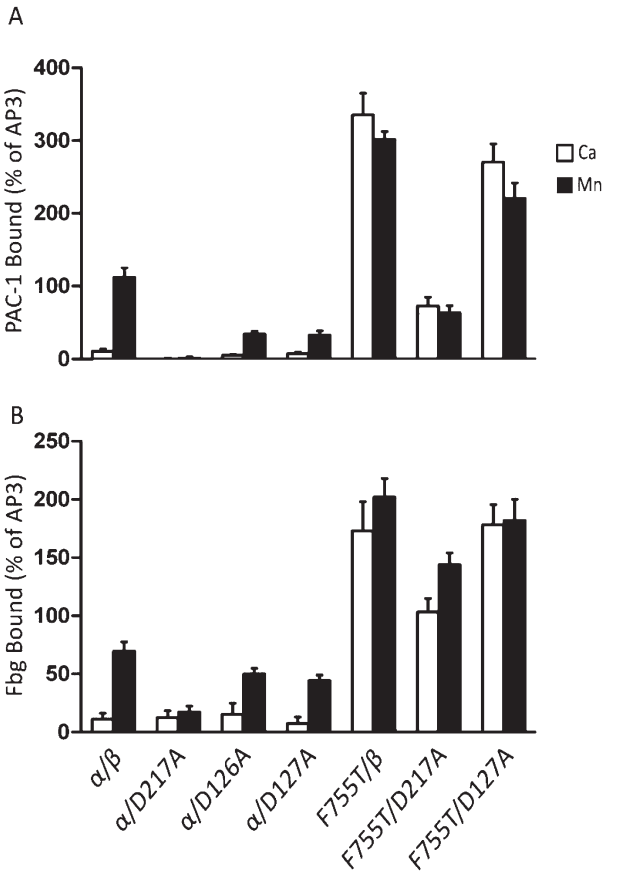

Figure 3. Soluble ligand binding with PAC-1 and fibrinogen. Cells were incubated with (A) PAC-1 in the presence of 5 mM Ca^{2+} or 1 mM Mn^{2+} or (B) FITC-fibrinogen in the presence of 5 mM Ca^{2+} or 1 mM Mn^{2+} as indicated. Binding activities were determined by flow cytometry and expressed as described in Experimental Procedures. Error bars are standard deviation (SD).

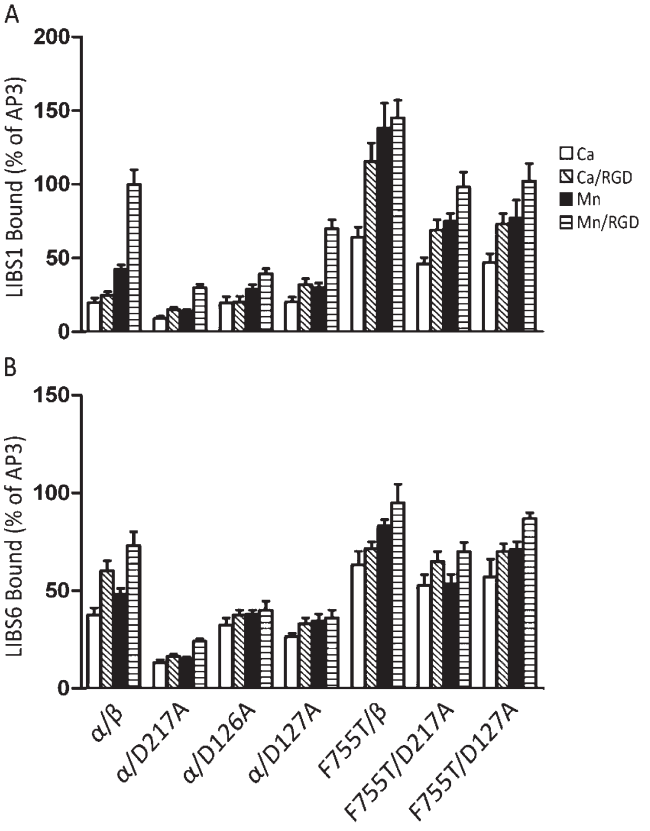

Figure 4. Exposure of the LIBS1 and LIBS6 epitope. Cells were stained with (A) anti-LIBS antibody LIBS1 and (B) anti-LIBS antibody LIBS6 in the presence of 5 mM Ca^{2+}, 5 mM Ca^{2+} plus 50 μM RGD peptides (GRGDSP), 5 mM Mn^{2+}, or 5 mM Mn^{2+} plus 50 μM RGD peptides. LIBS epitope exposure was determined as the percentage of MFI of anti-LIBS1 antibodies relative to nonfunctional anti-β3 mAb AP3. Error bars are SD.

whereas these two mutants bound to LIBS1 and LIBS6 with significantly lower levels compared to the WT in the presence of Mn^{2+} and Mn^{2+}/RGD. These data suggest that the ADMIDAS mutants remain in the bent conformation even in the Mn^{2+} and/or RGD conditions. The glycosylated double mutant F755T/D127A showed higher binding to LIBS1 and LIBS6 than the WT in Ca^{2+}, confirming that the F755T/D127A mutant is in the more open conformation (Figure 4).

Effects of SyMBS and ADMIDAS Mutants on Adhesion and Spreading. Cell adhesion on the immobilized fibrinogen was assessed using the cytotoxicity detection kit of LDH. The adhesion percentage was calculated by the ratio of adherent cells to the total input cells. About 50% of the HEK293T cells transiently transfected with the WT adhered to the immobilized fibrinogen, while the glycosylated mutant F755T showed slightly increased adhesion (Figure 5A). In contrast, the SyMBS mutant D217A almost abolished the adhesion. The double mutant F755T/D217A could adhere to the immobilized fibrinogen even though with lower ratio compared to the WT (Figure 5A), suggesting that the separation of the two legs could partially restore the cell adhesion ability of the SyMBS mutant. The adhesion of ADMIDAS mutants D126A and D127A was about 60% of the WT, which correlated to their decreased ligand binding ability and increased tendency toward the bent conformation compared to the WT. The double mutant F755T/D127A increased the adhesion to a similar level to the WT (Figure 5A). Compared to the WT αIIbβ3, the glycosylation mutation showed different effects on soluble ligand binding and cell adhesion. This is not surprising since the adhesion strength is dependent not only on the affinity of the receptors but also on the spreading of the cells on the immobilized ligands.[36]

To test if mutations of metal ion sites could affect integrin outside-in signaling, HEK293T transient transfectants were coated on immobilized fibrinogen at 37 °C for 1 h, followed by fixation and microscopic analysis to determine cell spreading of the mutants. Cells transfected with the WT showed normal cell adhesion and spreading (Figure 5B). No spreading data were obtained for the SyMBS mutation, since there was no adhesion. The glycosylated double mutant F755T/D217A partially restored the cell adhesion, and those cells that adhered to immobilized fibrinogen showed significant spreading (Figure 5C). The cell area was quantified, and the data showed that this double mutant had larger cell size compared to the two ADMIDAS mutants but was slightly smaller than the WT (Figure 5B).

Two ADMIDAS mutants D126A and D127A exhibited defective spreading on fibrinogen (Figure 5C), and even though some cells could adhere to the immobilized fibrinogen, they remained round and did not change size. Quantification of the cell area showed that these two mutants had a significant decrease in adherent cell size comparing to that of the WT (Figure 5B). The glycosylated double mutant F755T/D127A could adhere

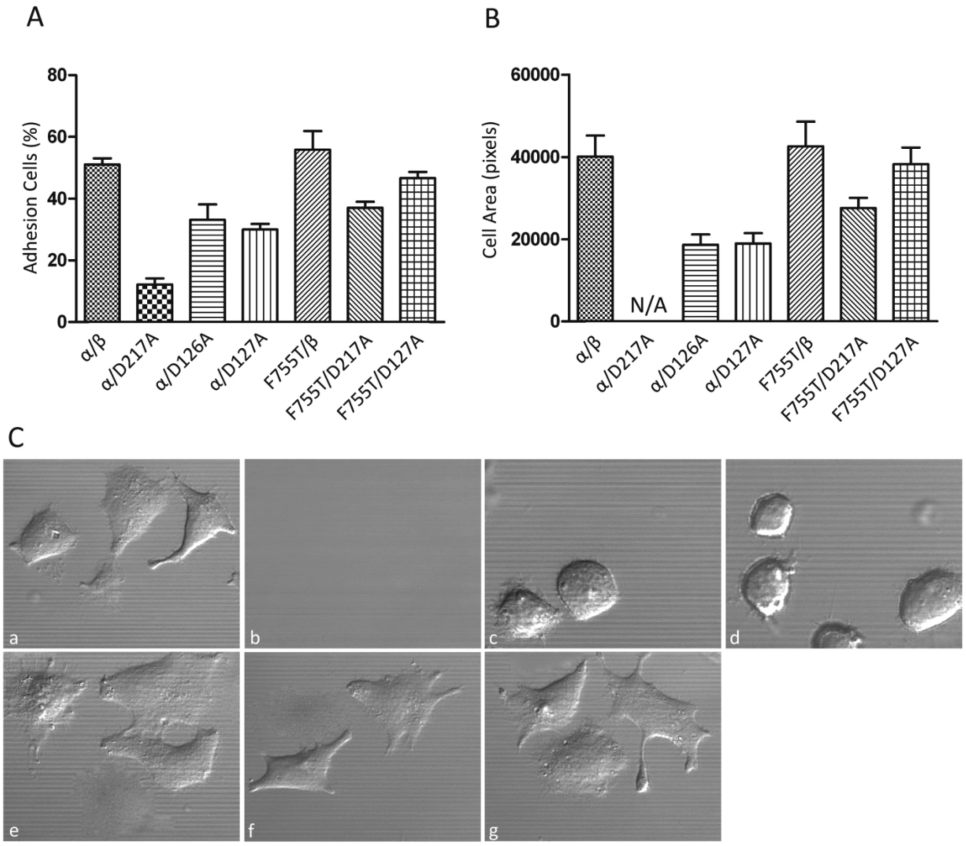

Figure 5. Cell adhesion and spreading. (A) Adhesion of HEK293T transfectants to surfaces coated with 20 μg/mL fibrinogen. The amount of bound cells was determined by measuring LDH activity as described in Experimental Procedures. Data are representative of three independent experiments, each in triplicate. (B) Quantification of the areas of adhering/spreading cells as described in Experimental Procedures. Error bars are SD. (C) DIC images of HEK293T transfectants after adhering to immobilized fibrinogen at 37 °C: a, WT; b, D217A; c, D126A; d, D127A; e, F755T/WT; f, F755T/D217A; g, F755T/D127A. The images are representatives of three independent experiments.

and spread on immobilized fibrinogen almost as well as the WT. The data suggest that separation of two legs could restore cell spreading capability of the ADMIDAS mutant. Thus, the glycosylated mutation that disrupts the two leg association was able to reestablish the cell adhesion and spreading in the SyMBS and ADMIDAS mutations.

DISCUSSION

We made several β3 integrin SyMBS and ADMIDAS mutants and studied their effect on αIIbβ3 ligand binding and signaling. The SyMBS mutation almost completely abolished ligand binding, conformational change into the active state, and integrin outside-in signaling, whereas the glycosylated mutant that disrupts integrin lower leg association could restore its function. The ADMIDAS mutant decreased integrin ligand binding and abolished outside-in signaling, but this mutant was sensitive to Mn^{2+} activation. Therefore, the role of the ADMIDAS is more complicated, and the glycosylated mutant could fully restore the function of the ADMIDAS mutant.

It has been shown that the side-chain carboxyl of β3 subunit Glu^{220} coordinates the SyMBS Ca^{2+} and MIDAS Mg^{2+} at the same time in both the unliganded low-affinity state and the liganded high-affinity state (Figure 1B,C).[3,19] Therefore, the presence of the SyMBS Ca^{2+} may favor the specific orientation of this side-chain carboxyl to stabilize the MIDAS ion occupancy. Consequently, SyMBS acts as a stimulatory site of Ca^{2+} as shown in α2β1.[26] Our study confirmed that the metal ion in the SyMBS is critical for integrin αIIbβ3 bidirectional signaling. Similar results have been reported that the SyMBS mutants abolish ligand binding and conformational change into the extended state in the α2β1,[26] α5β1,[22] α4β7,[14,15] and αLβ2[27] integrins, and the effects of SyMBS mutations in shear flow have resulted in rolling rather than firm adhesion.[14,15,27] In αIIbβ3, the β3 SyMBS mutations were also reported to abolish ligand binding.[28,39−41] Together, these studies demonstrate the similar function of the SyMBS among different β subunit I domains and between integrins with and without the α I domain. Our study showed that the SyMBS mutant, when paired with a glycosylated mutant that disrupts integrin lower leg association, was able to restore the ability for ligand binding and signaling. We have shown that this glycosylation caused global conformational change resulting in increased ligand binding affinity.[36] It is possible that separation of the two legs leads to integrin extension with an open headpiece conformation, causing metal ion occupied in the MIDAS to facilitate the ligand binding and signaling. Similar results were obtained in α4β7 integrin where glycan wedge was introduced at the interface of the β I and hybrid domains to stabilize the integrin in the open headpiece conformation.[15] This glycan wedge mutant could restore the SyMBS mutants for ligand binding, conformational change, and cell adhesion. Therefore, it seems that the SyMBS is important,

but not absolutely required, for integrin signaling, and integrins in the extended conformation with open headpiece could counteract the SyMBS mutant.

It was proposed that the ADMIDAS is an allosteric inhibitor of ligand binding when bound to Ca^{2+} and functions as a critical connection between the β I domain with hybrid domain in outside-in signaling.[14,15,22,26] However, ADMIDAS mutants produced conflicting results by either increasing or decreasing ligand binding in different integrins.[14,15,22,26,33] Our study showed that the β3 ADMIDAS mutants were sensitive to Mn^{2+} activation for both fibrinogen and PAC-1 binding, consistent with the $\alpha2\beta1$ and $\alpha5\beta1$ ADMIDAS mutants.[22,26] In addition, similar to $\alpha2\beta1$ and $\alpha5\beta1$ integrins, the β3 ADMIDAS mutants bound PAC-1 and fibrinogen with lower affinity than the WT and remained in the bent conformation. Conversely, while remaining in the bent conformation, the ADMIDAS mutants of $\alpha4\beta7$ and $\alpha L\beta2$ showed ligand binding at higher levels than the WT.[14,15,27] These studies altogether suggest a significant difference in the allosteric properties of the ADMIDAS, and the degree of regulation by the ADMIDAS seems to vary among integrin families.

In the structure of the unliganded low-affinity state of the integrin $\alpha IIb\beta3$ (Figure 1B),[19] the ADMIDAS metal ion coordinates with the main-chain carbonyl Met^{335} in the β3 I domain. This interaction helps to stabilize integrin headpiece in the closed conformation. During inside-out activation, the hybrid domain swings out, leading to the β I domain $\alpha7$ helix downward displacement, thus breaking the interaction of the Met^{335} and ADMIDAS ion. In this high-affinity state with the open headpiece,[3] the side-chain carboxyl of the Asp^{251} moves toward the ADMIDAS site, resulting in direct coordination with the ADMIDAS ion. This shift makes the MIDAS become more positive, resulting in higher affinity for ligands (Figure 1C).[19] Therefore, the role of ADMIDAS ion in regulating integrin ligand binding is complicated. We propose that the ADMIDAS ion stabilizes the low-affinity state when the integrin headpiece is in the closed conformation, whereas it stabilizes the high-affinity state when the headpiece is in the open conformation with the swung-out hybrid domain. In the cases of $\beta1$ and $\beta3$ integrins, mutating the ADMIDAS residues leads to destabilizing both the high- and low-affinity states. Therefore, the ADMIDAS mutants reduced but did not abolish ligand binding as shown here and previously.[22,26]

But when one ADMIDAS residue of the $\alpha4\beta7$ and $\alpha L\beta2$ integrins was mutated, integrins were activated, leading to firm adhesion. It is interesting that in physiological conditions both $\alpha4\beta7$ and $\alpha L\beta2$ integrins mediate cell rolling and firm adhesion, whereas $\alpha2\beta1$, $\alpha5\beta1$, and $\alpha IIb\beta3$ integrins only mediate firm adhesion.[42] When we studied the residues that are potentially important for regulatory effects on ligand binding, we found that one specific amino acid may contribute to these differences (Figure 1A). The residue after $\beta3$ Asp^{251} is Ala (and the same for $\beta1$ integrins), but the corresponding position in $\beta2$ and $\beta7$ integrins is Asp. In the open conformation of the $\alpha IIb\beta3$ crystal structures,[3,19] Ala^{252} moves approximately 0.7 Å toward the ADMIDAS site along with the Asp^{251}. This Ala residue in $\beta1$ and $\beta3$ may have no effect on ligand binding affinity, while the corresponding $\beta2$ residue Asp^{243} points toward the MIDAS ion and therefore may decrease ligand binding by increasing the negativity of the MIDAS site (Figure 1B−D).[17] It has been shown that integrins in the extended conformation with low to intermediate affinity mediate cell rolling, whereas those with high affinity mediate firm adhesion.[43] It is likely that the presence of this additional Asp residue in $\beta2$ and $\beta7$ integrins makes these two families lower affinity for ligands, capable of mediating cell rolling. It is possible that when one ADMIDAS residue Asp is mutated in the $\beta2$ and $\beta7$ integrins, the binding of ADMIDAS ion is not abolished, but instead, residues Asp^{242} and Asp^{243} shift toward the ADMIDAS and both coordinate with the metal ion, leading to a more positive MIDAS able to bind ligands with a higher affinity than the WT. This may explain that mutating ADMIDAS $\alpha L\beta2$ and $\alpha4\beta7$ leads to high affinity for ligands, resulting in firm adhesion,[14,15,27] contrary to the previous $\alpha5\beta1$ and $\alpha2\beta1$ studies[22,26] and the present $\alpha IIb\beta3$ work. Additional evidence is needed to confirm this hypothesis.

Our study also showed that, by eliminating the ADMIDAS function, integrin outside-in signaling from the ligand binding site to the lower leg in $\beta3$ integrins was abolished. Therefore, even though the ADMIDAS mutants were able to adhere to immobilized ligand, they did not exhibit cell spreading. It has been shown in $\alpha L\beta2$ that the ADMIDAS binds to ICAM-1 but does not trigger the cytoplasmic tail separation and outside-in signaling.[27] Therefore, it seems that the ADMIDAS in all integrin families provides a key structural link between the binding site in the β I domain and the hybrid domain for outside-in signaling. It is likely that, in order for integrins to transmit signals from the ligand binding site to the lower portion of the molecules, the β I domain must be stabilized in the open conformation in which the ADMIDAS plays a significant role.

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ABBREVIATIONS

ADMIDAS, adjacent to MIDAS; BSA, bovine serum albumin; DIC, differential interference contrast; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; HPS, HEPES-buffered saline; LDH, lactate dehydrogenase; LIBS, ligand-induced binding site; LIMBS, ligand-associated metal binding site; MFI, mean fluorescence intensity; MIDAS, metal ion-dependent adhesion site; PBS, phosphate-buffered saline; SyMBS, synergistic metal binding site; WT, wild type.

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