The α-Subunit Regulates Stability of the Metal Ion at the Ligand-associated Metal Ion-binding Site in β3 Integrins*

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Background: Metal ions at LIMBS and MIDAS are essential for integrin-ligand interactions.

Results: MD simulations showed strikingly different and functionally relevant conformations of LIMBS in αVβ3 and αIIbβ3.

Conclusion: The α-subunit regulates metal ion coordination at LIMBS and hence function of β3 integrins.

Significance: The results reveal a new mechanism of integrin regulation by the α-subunit.

Integrins are αβ heterodimeric cell adhesion receptors that mediate divalent cation-dependent cell-matrix and cell-cell adhesion during morphogenesis, as well as the maintenance of tissues and organs in adult life. 18 α- and 8 β-subunits assemble into 24 integrin receptors in mammals. Integrins regulate fundamental aspects of cell behavior, including migration, adhesion, differentiation, growth, and survival, by communicating bidirectional signals between the extracellular environment and the intracellular cytoskeleton (1).

Integrins are unusual receptors as they do not engage physiologic ligand unless activated. This property allows patrolling blood leukocytes and platelets, for example, to circulate without aggregating or interacting with the vessel walls. Inappropriate activation of integrins contributes to the pathogenesis of common diseases including heart attacks, stroke, and cancer growth and metastasis. Thus understanding how these receptors are regulated is important in promoting health and treating disease (2).

The integrin heterodimer comprises a head segment that sits on top of two leg segments each spanning the plasma membrane once and ending with a short cytoplasmic tail. The ligand-binding head consists of a seven-bladed β-propeller domain from the α-subunit that associates noncovalently with a GTPase-like domain, βA, from the β-subunit (3). Contacts between the cytoplasmic tails and transmembrane segments hold the integrin in an inactive state (unable to bind physiologic ligand). Binding of talin to the β-propeller tail breaks these contacts, switching the ectodomain into the active (ligand-competent) conformation, a process called “inside-out” signaling (4). Ligand binding then triggers global conformational changes that propagate through the plasma membrane to the cytoplasmic tails, leading to “outside-in” signaling.

Integrin-ligand interactions are regulated in a complex manner by divalent cations (5–8). Although Mn2+ and, to a lesser extent, Mg2+ stimulate ligand binding, Ca2+ is typically inhibitory. The ligand-binding face of the βA domain is decorated by three metal ion-binding sites: a metal ion-dependent adhesion site (MIDAS),3 flanked on one side (facing the propeller domain of the α-subunit) by a ligand-associated metal ion-binding site (LIMBS), and on the opposite side by an adjacent to MIDAS (ADMISS) (9). A ligand aspartate completes the octahedral metal coordination of an Mg2+ or Mn2+ at MIDAS in ligand-
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FIGURE 1. α-subunit residues facing LIMBS in β₃ integrins. A ribbon diagram showing the residues from α₃β₃ and α₅ facing LIMBS loop residues Arg¹²⁶–Ala¹³⁸ is presented. The β₃ subunits of unliganded α₃β₃ (green, 3fcs.pdb) and α₅β₃ (yellow, 4g1e.pdb) ectodomains were superposed with Chimera. The metal ion (M⁺) at LIMBS (sphere) has the color of the respective integrin. α₃ and α₅ residues are labeled in green and black, respectively, and the LIMBS residues are labeled in red.

bound integrins. Ca²⁺ but not Mg²⁺ binds preferentially at LIMBS and ADMIDAS in physiologic buffer conditions, and both sites can coordinate Mn²⁺. The metal ion at LIMBS stabilizes the one at MIDAS (9–11), thus acting as a positive regulator of ligand binding to integrins, whereas the ADMIDAS metal ion can stabilize alternate inactive and active conformations of the integrin (2).

At the ligand-binding face of β₃α domain, the LIMBS loop residues Arg¹²⁶–Asp¹³⁷, and Ala¹³⁸ contact residues in the α-subunit propeller domain (Fig. 1), but a potential role of the α-subunit in regulating metal ion occupancy in the β₃ domain has not been explored. In this study, we carried out computational and functional studies on the two β₃ integrins α₃β₃ and α₅β₃. Our studies reveal an important role of the α-subunit in regulating metal ion stability at LIMBS in β₃ integrins. The significance of this finding is discussed.

EXPERIMENTAL PROCEDURES

Molecular Dynamics Simulations Design—The crystal structures of the ectodomains of α₃β₃ (Protein Data Bank (PDB) 3fcs) (12) and α₅β₃ (PDB 4g1e) (13) were downloaded from the Protein Data Bank. In α₅β₃, LIMBS (also known as SymBS, synergistic metal-binding site), MIDAS, and ADMIDAS were occupied by Ca²⁺, Mg²⁺, and Ca²⁺ respectively. Because only LIMBS was metal-occupied (by Ca²⁺) in unliganded α₅β₃ structure, Mg²⁺ and Ca²⁺ were respectively placed at MIDAS and ADMIDAS such that their initial distances (i.e. before minimization) from all the proximal oxygen atoms were between 2.4 and 4 Å. Mutations were made to the native structures using the software Swiss-Pdb Viewer 4.1.0 (14). All non-protein atoms were removed from α₅β₃ and α₅β₃ structures, leaving α- and β-subunits with nearly 24,000 atoms for each, including hydrogens. Proteins were solvated in water boxes of sizes 168 × 169 × 207 Å (for α₅β₃) and 180 × 144 × 226 Å (for α₃β₃), adding ~183,000, and 185,000 water molecules to α₅β₃ and α₃β₃ respectively, and ionized with 150 mM KCl. To investigate the effects of Phe¹¹⁹ in α₃ and Trp¹⁷⁹ in α₅ on coordinating divalent cations at LIMBS, six distinct combinations of cation type and mutation for α₃β₃ and four for α₅β₃ were tested. To investigate the effects of each cation arrangement, the α₃β₃ and α₅β₃ structures were equilibrated with LIMBS-MIDAS-ADMIDAS occupancies of Ca²⁺·Mg²⁺·Ca²⁺ and Mn²⁺·Mn²⁺·Mn²⁺.

Molecular Dynamics Simulations—MD simulations of the integrin ectodomain were performed with NAMD cvs_20130828 software package (15). The CHARMM27 force field parameter (16) was used to model the protein. The TIP3P model (17) was used for water molecules. Structures were visualized with Visual Molecular Dynamics (VMD) (18) or Chimera (19). The crystal structures of the ligand-free forms of α₃β₃ and of α₅β₃ ectodomains were used without modification, except for the removal of the sugar and water molecules before solvation. All simulations were carried out at the computing facilities of the National Energy Research Scientific Computing Center (NERSC). Periodic boundary conditions were applied in all three directions. Afterward, the entire system was minimized for 20,000 steps followed by 20 or 40 ns of equilibration. A time step of 2 fs was used in all simulations. The temperature and pressure of the systems were held constant at 1 atmosphere and 310 K respectively, using the isothermal-isobaric ensemble with the Langevin piston and Hoover method, as successfully used for modeling integrins (20–22). We performed 20 or 40 ns of MD simulation for each run, and the trajectories were used for all analyses. The cutoff distance for non-bonded interactions was 1.2 nm, and the particle mesh Ewald method was used for electrostatic force calculations (15). All B-factors were set at zero. The hydrogen atom bond length was constrained using the SHAKE algorithm (23).

Structure Analysis—Two parameters, root mean square deviation (r.m.s.d.) values of the LIMBS metal ion and the energy of interaction of the metal ion with the LIMBS pocket, were used to evaluate stability of the metal ion at LIMBS. The r.m.s.d. of a single atom is a measure of its distance at each time step from the initial position of the ion. Hence, higher r.m.s.d. values represent fluctuations with larger amplitudes and less stable ion pocket bonds. Higher interaction energies reflect higher bond stability between the ion and LIMBS or between the α-subunit and the LIMBS loop comprising residues Arg¹²⁶, Asp¹³⁷, and Ala¹³⁸. To let systems equilibrate for a considerable time span before starting to take samples for r.m.s.d. measurements, r.m.s.d. values for the ion were averaged over time steps between t = 16–20 ns for all simulations (n = 40). Energies of interaction between the divalent ion and the LIMBS pocket were estimated using Langevin dynamics. As this energy of interaction did not show significant fluctuations throughout the simulations, values for energy were averaged over t = 0–20 ns (n = 200).

Reagents and Site-directed Mutagenesis—Restriction and modification enzymes were obtained from New England Bio-labs Inc. (Beverly, MA), Invitrogen Life Technologies, or Fisher Scientific. All cell culture reagents were obtained from Invitrogen Life Technologies. The non-inhibitory monoclonal antibody (mAb) AP3 (American Type Culture Collection, ATCC) detects the β₃ subunit in all conformations. The heterodimer-
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specific mouse mAbs CD41P2 to αιββ3 and LM609 to αιββ3 were from Millipore (Danvers, MA). The function-blocking anti-β3 mAb P5D2 was from R&D Systems, Inc. (Minneapolis, MN). Mouse mAb AP5 detects the N-terminal sequence in the PSI domain only in high affinity/ligand-bound states. The Fab fragment of AP5 was prepared by papain digestion followed by anion exchange and size-exclusion chromatography. The allophycocyanin-labeled goat anti-mouse Fc-specific IgG antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA). Recombinant αιββ3-specific high affinity fibronectin 10 domain (hFN10) (24) was expressed and purified from Escherichia coli as described (25), and fibronectin-depleted human fibrinogen (FB) was obtained from Enzyme Research Laboratories (South Bend, IN). Wild-type ligands FN10 and FB and mAbs AP5 (Fab) and AP3 (IgG) were labeled respectively with N-hydroxysuccinimidy esters of Fluor 488 (Alexa Fluor 488) or Alexa Fluor 647 (Invitrogen) according to the manufacturer’s instructions. Excess dye was removed using Centri-Spin size-exclusion microcentrifuge columns (Princeton Separations, Adelphia, NJ). The final hFN10, FB, AP5, and AP3 concentrations and dye-to-protein molar ratios (F/P) were determined spectrophotometrically, giving dye:protein molar ratios of 1–5.

Ligands FN10 and FB and mAbs AP5, AP3, and AP3 (Fab) were transiently co-transfected with pcDNA3 plasmids encoding constitutively active forms of these integrins by subcloning different combinations of wild type and mutant integrins in HEK293T, αιββ3-expressing HEK293T cells in 100 μl of HBSS buffer containing 0.5% BSA and incubated in the presence of a saturating amount of Alexa Fluor 488-labeled FB (160 μg/ml) or Alexa Fluor 488-labeled wild-type FN10 (12.6 μg/ml) for 30 min at 25 °C. To block any potential interaction of FN10 with endogenous β3 integrins in HEK293T, αιββ3-expressing HEK293T cells were preincubated with the function-blocking anti-β3 mAb P5D2 before adding Alexa Fluor 488-labeled FN10. Saturating amounts of each ligand were derived from dose-response curves, where labeled ligand was added in increasing concentrations to HEK293T cells expressing constitutively active β3 integrins in the presence of 1 mM MnCl2. Integrin-ligand interactions in the presence of varying concentrations of Mn2+ were measured by adding increasing amounts of MnCl2 to a mixture of β3 integrin-expressing cells and saturating amounts of Alexa Fluor 488-labeled ligand. Treated cells were then incubated with Alexa Fluor 467-labeled AP3 (10 μg/ml; 30 min; 4 °C) followed by washing once in HBSS containing the corresponding concentration of Mn2+. Cells were then fixed with 1% paraformaldehyde and analyzed by flow cytometry. Binding of soluble ligand to β3 integrin-expressing cells was normalized by dividing mean fluorescence intensity by that for Alexa Fluor 467-labeled AP3 and multiplying by 100. Mean and S.D. values from three independent experiments were calculated and compared using Student’s t test. Non-linear curve fittings of the dose-response curves were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA).

RESULTS

LIMBS Structure and Stability of the Metal Ion in β3 Integrins—Measurements of the energy of interaction of αι and αιιιι subunits with the α-subunit-facing LIMBS loop residues Arg216-Asp217-Ala218 revealed a stronger (2–3-fold) interaction of αι with the LIMBS loop when compared with αιιιι. This difference was first detected at 8 ns of simulations and maintained through 40 ns (Fig. 2A).

MD simulations after a few nanoseconds of equilibration showed that Ca2+ coordination at LIMBS in αιββ3 comprised six “primary” oxygens (i.e. the two carboxyl oxygens of Asp158 and Asp217, one carboxyl oxygen of Glu220, and the carboxyl oxygen of Pro219) (Fig. 2B), which hold a mean distance of 2.2–2.3 Å from the encapsulated cation for the entire simulation, along with two “secondary” oxygens (i.e. the carboxyl oxygens of Asp217 and Ala218), whose mean distance from the cation was no more than 4.5 Å over the whole trajectory. The primary oxygens remained in close contact with the Ca2+ at LIMBS,
forming highly stable bonds with a mean length of 2.2–2.3 Å and fluctuation amplitude below 0.5 Å. The primary coordinating oxygens formed an octahedral arrangement, with a planar surface formed by OD2 of Asp158, OE1 of Glu220, OD1 of Asp217, and the carbonyl oxygen of Pro219, with OD1 of Asp158 and OD2 of Asp217 at the top and bottom of the plane, respectively (Fig. 2B), restricting cation fluctuations. Replacing Ca2+ with Mn2+ in LIMBS, MIDAS, and ADMIDAS of αivβ3 increased the energy of interaction of Mn2+ with LIMBS by 5–8% and had a small but significant effect on r.m.s.d. (Table 1).

In contrast, the LIMBS pocket is distorted to a planar shape in αivβ5 (Fig. 2C), the result of the stronger interaction that pulled the LIMBS loop toward αiv, changing metal ion coordination at this site. MD simulations showed that Ca2+ at LIMBS is coordinated by six primary oxygens (the two carboxyl oxygens of Asp158 and Asp217, the carbonyl oxygen of Pro219, and the carbonyl oxygen of Asn215), but only one secondary coordinating oxygen, the carbonyl oxygen from Asp217 (Fig. 2C). The carboxyl oxygens of Asp158 and Asp217 as well as the carbonyl oxygen of Pro219 all form one planar surface that surrounds the cation, with only the side chain oxygen of Asn215 interacting with the cation at the bottom of the plane (Fig. 2C). These changes made Ca2+ at LIMBS significantly less stable in αivβ3 when compared with αivβ5, as reflected by the significantly higher r.m.s.d. and lower energy of interaction of Mn2+ in LIMBS, MIDAS, and ADMIDAS of αivβ3 that had minimal effects on r.m.s.d. or on the energy of interaction of Mn2+ with LIMBS (Table 1).

**Structural Basis for the Stronger Interaction of αiv with the LIMBS Loop**—In αivβ3, all three LIMBS loop residues were involved in more extensive interactions with αiv: Arg216 side chain was engaged in strong ionic bonds with Glu123 and Glu123, and its carbonyl oxygen occasionally H-bonded the side chain of Tyr178 (H-bond probability 0.5%). Arg216 also formed van der Waals contacts with Phe154 and with the indole group of Trp179 (Fig. 3A and B). In addition, the main and side chains of Arg216 formed van der Waals contacts with the indole group of Trp179, and the side chain of Ala218 contacted the carbonyl oxygen of Asp219 in αiv. These interactions stretched the LIMBS loop toward αiv, distorting LIMBS.

In contrast, interaction of αiv with LIMBS loop residues Arg216–Asp217–Ala218 was primarily limited to Arg216. The side chain of Arg216 formed intermittent H-bonds with the hydroxyl group of Tyr190 (H-bond probability 5.0%), and its carbonyl oxygen contacted the side chain of Tyr190 (Fig. 3C). Arg216 side chain also formed occasional ionic interactions with Glu123 (Fig. 3D), but made no contacts with Phe191. Additionally, Asp217 made intermittent van der Waals contacts with the side chain of Ala218.

**TABLE 1**

Summary of mean r.m.s.d. and interaction energy values for tested β3 integrins

| Integrin and β3 metal ion occupancy state | αivβ3 (Ca2+-Mg2+-Ca2+) | αivβ3 (Mn2+-Mn2+-Mn2+) | αivβ5 (Ca2+-Mg2+-Ca2+) | αivβ5 (Mn2+-Mn2+-Mn2+)d |
|-----------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Wild type                               | 2.0                     | 3.2                     | 2.7                     | 12.3                    |
| F191/W                                  | 8.3                     | 14.5                    | 11.8                    | 6.1                     |
| D158/N                                  | 14.5                    | 14.5                    | 11.1                    | 0.8                     |
| p valuea                                | <0.001                  | <0.001                  | <0.001                  | <0.001                  |


Energy of interaction between LIMBS and metal ion (kcal/mol)

|                                      | αiv               | αiv               | αiv               | αiv               |
|-------------------------------------|-------------------|-------------------|-------------------|-------------------|
|                                      | 859               | 930               | 775               | 779               |
| Mean                                | 865               | 913               | 732               | 780               |
| S.D.                                | 620               | 752               | 15                | 15                |
| p valuea                            | <0.001            | <0.001            | <0.001            | <0.001            |


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a Integron mutation.

b All p values were compared to wild-type αivβ3 (Ca2+-Mg2+-Ca2+) structure.
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Effects of Modifying αIII and αV on Stability of the Metal Ion at LIMBS—Of the α-subunit residues contacting the LIMBS loop residues Arg216, Asp217, Ala218, the side chains of Phe191 in αIII and Trp197 in αV are superimposable (Fig. 1). We evaluated the impact of interchanging these two residues on stability of the metal ion at LIMBS in the two β3 integrins. As an internal control, we measured the effects of destabilizing the metal ion at LIMBS through the D158/N substitution (which removes one of the main coordinating oxygens from the metal ion). MD simulations showed that implementing the D158/N mutation in αIIIβ3 yielded a significant increase in r.m.s.d. (≈2-fold) and a reduction in the energy of interaction (Table 1), both reflecting destabilization of the metal ion at LIMBS.

Implementing the F191/W substitution in αIII significantly increased the energy of interaction of the LIMBS loop with the bulkier Trp191 in αIII F/W when compared with Phe191 in wild-type αIII (Fig. 4A). Snapshot of the structure at $t = 20$ ns showed that the side chain of Arg216 of β3 releases its interaction with Tyr219 and forms van der Waals contacts with the indole group of Trp197 in αIII F/W (compare Fig. 4B with Fig. 3C), pulling the LIMBS loop region toward the αIII F/W propeller domain. With Trp197 and Tyr219 pulling the LIMBS loop in the same direction, Ala218 is brought closer to Asp232 to form more contacts, increasing the energy of interaction of αIII F/W with the LIMBS loop and deforming the octahedral shape of the pocket. These movements displaced the oxygens forming the LIMBS pocket from their native pattern toward a more planar configuration. Although the energy of interaction of Ca$^{2+}$ or Mn$^{2+}$ with the LIMBS pocket in the αIII F/W β3 structure did not change, r.m.s.d. increased by ≈4-fold (Table 1). Hence, it appears that the energetic component of the free energy remains unchanged upon applying αIII β3.

FIGURE 3. α-subunit residues interacting with the LIMBS loop. A, snapshot of MD simulations at 20 ns showing interactions of the LIMBS loop Arg216-Ala218 residues (shown as ball and stick) with αIII subunit residues (shown as stick). The structures in A and C are shown in the same orientation after superposing LIMBS of each. LIMBS residues are labeled red in A and C (also in Figs. 4B and 5B). In αIII, the LIMBS loop interacts with the Phe191, Tyr190, Trp197, Asp232, Glu121, and Glu123 of αV. B, MD simulations showing van der Waals energy of interaction between Arg216 of the LIMBS loop with Trp197 and Phe191 of αV. C, snapshot of MD simulations at 20 ns showing interactions of the LIMBS loop Arg216-Ala218 residues with αIII subunit residues (shown as stick). In αIII, interactions are limited to the corresponding residues Tyr190, Tyr197, Phe191, and Asp232 and a transient interaction with Glu121. D, electrostatic energy of interaction between Arg216 of the LIMBS loop and Glu123 of αV. Occasional jumps to higher energy levels represent ionic bonds between Arg216 and Glu123. The energy peaks at $t = 7$ and 16 ns show large increases to the same value of about 100 kcal/mol, suggesting that the ionic bond occurs at a local energy minimum that the system continues to take, whereas the Arg216-Glu123 bond spends most of the simulation time in a lower electrostatic energy state (i.e., longer bond distance).

FIGURE 4. Effect of F191/W change in αIIIβ3 on interaction energies and shape of LIMBS. A, computed energy of interaction between LIMBS loop residues Arg216-Ala218 and Trp197 in αIII F/W. B, snapshot at $t = 20$ ns of the interactions of LIMBS loop with αIII F/W residues Tyr190, Tyr197, Trp197, Asp232, and Glu123. Mutating Phe191 to Trp in αIII F/W enhanced interactions of the larger Trp197 side chain with the LIMBS loop, especially with Arg216, modifying the conformation of the LIMBS pocket (see “Results”). F191/W, whereas the entropic component is highly changed upon deformation of the pocket as represented by a 4-fold increase in r.m.s.d.

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Implementing the W179/F substitution in αvWF did not change the energy of interaction of the LIMBS loop with αvWF (Fig. 5A). However, it reduced fluctuations of the Ca2+ at LIMBS (r.m.s.d. reduced by 2-fold, Table 1). The W179/F mutation weakened interaction of the LIMBS loop with αvWF, reshaping the LIMBS pocket. The structure is shown in the same orientation as in Fig. 4B.

**FIGURE 5. Effect of W179/F in αvβ3 on interaction energies and shape of LIMBS.** A, computed energy of interaction between the LIMBS loop residues Arg216, Ala218 and Phe179 in αvWF. Mutating Trp179 to Phe does not change the energy of interaction with the LIMBS loop significantly. B, snapshot at t = 20 ns of interactions of the LIMBS loop with αv subunit residues Glu121, Glu123, Phe154, Phe179, and Asp219. The W179/F mutation weakens interaction of LIMBS loop with αvWF, reshaping the LIMBS pocket. The structure is shown in the same orientation as in Fig. 4B.

Binding of Wild-type and F191/W Mutant αIbβ3 to Soluble Ligand—We next sought experimental validation of the computational studies. We expressed recombinant αIbWF/β3 in its resting and mutationally activated (αIbWF/FF/AAβ3, αIbWF/Δ-genu) states in HEK293T cells and compared its surface expression, structure, and ligand binding capacity with that of constitutively active wild-type αIbWFβ3. As shown in Fig. 6A, surface expression of the mutant resting or constitutively active heterodimeric receptor was comparable with that of resting or constitutively active wild-type αIbWFβ3 and the F191/W mutation did not change the recognition of the constitutively active integrin by the ligand-induced binding site (LIBS) mAb AP5 (Fig. 6B).

**FIGURE 6. Effect of αIbWF F191/W mutation in αvβ3 on cell surface expression, activation, and binding to soluble ligand.** A, histograms (mean ± S.D.; n = 3) comparing cell surface expression and heterodimer formation of αIbWFβ3 and constitutively active αIbWF/FF/AAβ3, αIbWF/Δ-genu, αIbWF/FF/FAβ3, and αIbWF/F/AAβ3. Constitutive activation reduced expression of the wild type and F191/W integrin to equivalent degrees. B, histograms (mean ± S.D.; n = 3) showing binding of the LBS mAb AP5 to αIbWFβ3 and to constitutively active αIbWF/FF/AAβ3, αIbWF/Δ-genu, αIbWF/FF/FAβ3, and αIbWF/F/AAβ3. Binding was expressed as a percentage of binding of the heterodimer-specific mAb CD41-P2. C, histograms (mean ± S.D.; n = 3) showing binding of wild-type and constitutively active αIbWF/FF/AAβ3 to saturating amounts of soluble Alexa Fluor 488-FB (Alex488-FB) in 1 mM Ca2+ plus 1 mM Mg2+ (Ca2+/Mg2+) or 1 mM Mn2+. Binding is expressed as a percentage of Alexa Fluor 647-AP3 mAb staining. F191/W did not significantly impair ligand binding to constitutively active αIbWFβ3 in Mn2+. However, ligand binding to constitutively active αIbWFβ3 in Mn2+ was abolished when the ligand contact residue Tyr185 was simultaneously mutated to Ala.

αIbWF/Δ-genu bound constitutively to Alexa Fluor 488-FB in Ca2+/Mg2+–containing buffer, as expected, with 1 mM Mn2+ further increasing ligand binding by ~1.5-fold (Fig. 6C).

Cellular αIbWFβ3 showed minimal binding to soluble Alexa Fluor 488-FB in Ca2+/Mg2+ buffer, with 1 mM Mn2+ increasing binding by ~2-fold (Fig. 6C). Introduction of the F191/W muta-
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F191/W substitution in \( \alpha_{\text{IIb}}^{\text{FF/AA}} \beta_3 \) yielded an almost identical value (34.6 ± 6.2 \( \mu \text{M} \)) (Fig. 7B). In contrast, the W179/F mutation in \( \alpha_v \) significantly increased the apparent \( K_{d(app)} \) of Mn\(^{2+}\) to the \( \alpha_v^{\text{W/F}}/\text{FF/AA} \beta_3 \) heterodimer (as judged by mAb LM609 binding, not shown) by ~2-fold (mean ± S.D., 5.0 ± 1.9 \( \mu \text{M} \)) from 10.2 ± 3.5 \( \mu \text{M} \) to \( \alpha_v^{\text{FF/AA}} \beta_3, p = 0.018 \) (Fig. 7, C and D).

**DISCUSSION**

In this study, we provide computational and functional evidence that the \( \alpha \)-subunit plays an essential role in stability of the metal ion coordination at LIMBS in \( \beta_3 \) integrins. By combining these two approaches, we demonstrated the following. 1) interaction of the LIMBS loop with \( \alpha_v \) is more extensive than with \( \alpha_{\text{IIb}} \); 2) metal ion coordination at LIMBS after 20 ns of equilibration becomes planar in \( \alpha_v \beta_3 \); 3) changing the \( \alpha_{\text{IIb}} \)-LIMBS loop interface residue Phe\(^{191} \) to Trp destabilized the metal ion at LIMBS, whereas a Trp\(^{197} \) to Phe mutation in \( \alpha_v \) produced opposite but weaker effects; and 4) introducing F191/W in cellular \( \alpha_{\text{IIb}}^{\text{FF/AA}} \beta_3 \) reduced the apparent affinity of Mn\(^{2+} \) to this integrin; the reverse was observed upon introducing the W179/F mutation in cellular \( \alpha_{\text{IIb}} \). The higher energy of interaction of \( \alpha_v \) with the LIMBS loop residues Arg\(^{216} \), Ala\(^{218} \) was directly related to the more extensive contacts \( \alpha_v \) made with this loop when compared with \( \alpha_{\text{IIb}} \). The stronger contacts increased fluctuations of the metal ion at LIMBS in \( \alpha_v \beta_3 \) as reflected by the ~4-fold increase in r.m.s.d., and also increased the mean distances between the coordinating oxygens and the metal ion at LIMBS, as reflected by the reduction in total energy of interaction of the metal ion with the pocket. These observations may offer an explanation for the variable occupancy of LIMBS by metal ion in crystal structures of unliganded \( \alpha_v \beta_3 \) ectodomains, where LIMBS was metal-occupied in one (4Gl1e.pdb, used in this study) (13) but not in four other unliganded \( \alpha_v \beta_3 \) ectodomain structures (9, 26, 29, 30). The lack of metal occupancy at LIMBS was attributed to unfavorable crystallization conditions (13). However, LIMBS is metal ion occupied in \( \alpha_v \beta_3 \) under the same crystallization conditions when \( \alpha_v \beta_3 \) is ligand-bound (9, 25). The data produced in this study suggest that variability in LIMBS occupancy by metal is the result of the different contacts the LIMBS loop makes with \( \alpha_v \) in contrast to \( \alpha_{\text{IIb}} \). In the presence of ligand, Glu\(^{220} \) of the \( \beta \)A domain provides an extra primary oxygen, stabilizing the metal ion at LIMBS. In the absence of ligand, this stabilizing influence is lost, and the metal ion is freer to escape LIMBS. This scenario is reflected in the higher r.m.s.d. and lower energy of interaction of the metal ion with LIMBS in unliganded \( \alpha_v \beta_3 \) when compared with \( \alpha_{\text{IIb}} \beta_3 \) (Table 1).

\( \alpha_{\text{IIb}} \beta_3 \) is widely expressed in tissues including bone, where it mediates dynamic cell adhesion (31, 32). The majority of Mn\(^{2+} \) in the body is sequestered in bone (33) to levels that approach 10.2 ppm, and mM concentrations of the divalent cations Ca\(^{2+} \) and Mg\(^{2+} \) in blood containing high levels of its physiologic ligands, mainly fibrinogen, and Mn concentrations of the divalent cations Ca\(^{2+} \) and Mg\(^{2+} \) maintaining \( \alpha_{\text{IIb}} \beta_3 \) in a dormant inactive state in this environment is therefore essential to prevent pathologic thrombosis. The present data provide insights into how regu-
ulation can be tailored to the particular environment where an integrin is expressed. Occupancy of LIMBS, MIDAS, and ADMIDAS by metal ions in unliganded $\alpha_{IIb}\beta_3$ may explain the rapid ligand association rates to activated $\alpha_{IIb}\beta_3$ (7). This potential proactivation tendency at the ligand-binding site must be counteracted by energy barrier(s) to activation elsewhere to effectively keep $\alpha_{IIb}\beta_3$ in an inactive state on circulating resting platelets. One such barrier may exist in the integrin leg segments, between the $\alpha$-subunit Calf-2 domain and the $\beta_3$ subunit EGF-like 4 (IE4) and $\beta$TD domains (34). Disruption of this interface renders $\alpha_{IIb}\beta_3$ as susceptible to Mn$^{2+}$-induced ligand binding as $\alpha_{IIb}\beta_3$ (34). In $\alpha_\text{v}\beta_3$, where this Calf-2/IE4-$\beta$TD barrier is weak or absent (34), a relatively stronger $\alpha_\text{v}$-LIMBS interface may help favor the inactive $\alpha_\text{v}\beta_3$ conformation, thus limiting stable occupancy of the metal ion in this integrin to conditions when ligand is also accessible.

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