

# Correction

## BIOPHYSICS AND COMPUTATIONAL BIOLOGY

Correction for “Unique transmembrane domain interactions differentially modulate integrin  $\alpha\beta3$  and  $\alpha11\beta3$  function,” by Rustem I. Litvinov, Marco Mravic, Hua Zhu, John W. Weisel, William F. DeGrado, and Joel S. Bennett, which was first published June 3, 2019; 10.1073/pnas.1904867116 (*Proc. Natl. Acad. Sci. U.S.A.* **116**, 12295–12300).

The authors note that, due to a printer’s error, the affiliation for Hua Zhu appeared incorrectly. It should instead appear as Division of Hematology/Oncology, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104. The corrected author and affiliation lines appear below. The online version has been corrected.

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# Unique transmembrane domain interactions differentially modulate integrin $\alpha\nu\beta3$ and $\alpha\text{IIb}\beta3$ function

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Contributed by William F. DeGrado, April 16, 2019 (sent for review March 21, 2019; reviewed by Renhao Li and Peter J. Newman)

**Lateral transmembrane (TM) helix–helix interactions between single-span membrane proteins play an important role in the assembly and signaling of many cell-surface receptors. Often, these helices contain two highly conserved yet distinct interaction motifs, arranged such that the motifs cannot be engaged simultaneously. However, there is sparse experimental evidence that dual-engagement mechanisms play a role in biological signaling. Here, we investigate the function of the two conserved interaction motifs in the TM domain of the integrin  $\beta3$ -subunit. The first motif uses reciprocating “large-large-small” amino acid packing to mediate the interaction of the  $\beta3$  and  $\alpha\text{IIb}$  TM domains and maintain the inactive resting conformation of the platelet integrin  $\alpha\text{IIb}\beta3$ . The second motif, S-x<sub>3</sub>-A-x<sub>3</sub>-I, is a variant of the classical “G-x<sub>3</sub>-G” motif. Using site-directed mutagenesis, optical trap-based force spectroscopy, and molecular modeling, we show that S-x<sub>3</sub>-A-x<sub>3</sub>-I does not engage  $\alpha\text{IIb}$  but rather mediates the interaction of the  $\beta3$  TM domain with the TM domain of the  $\alpha\nu$ -subunit of the integrin  $\alpha\nu\beta3$ . Like  $\alpha\text{IIb}\beta3$ ,  $\alpha\nu\beta3$  on circulating platelets is inactive, and in the absence of platelet stimulation is unable to interact with components of the subendothelial matrix. However, disrupting any residue in the  $\beta3$  S-x<sub>3</sub>-A-x<sub>3</sub>-I motif by site-directed mutations is sufficient to induce  $\alpha\nu\beta3$  binding to the  $\alpha\nu\beta3$  ligand osteopontin and to the monoclonal antibody WOW-1. Thus, the  $\beta3$ -integrin TM domain is able to engage in two mutually exclusive interactions that produce alternate  $\alpha$ -subunit pairing, creating two integrins with distinct biological functions.**

integrin activation | transmembrane domain | interaction motifs | force spectroscopy

**L**ateral transmembrane (TM) helix–helix interactions are central to the regulation of many proteins that transmit signals across membranes, including integrins (1). As a relevant example, a heterodimer composed by the TM helices of its  $\alpha\text{IIb}$ - and  $\beta3$ -subunits stabilizes the integrin  $\alpha\text{IIb}\beta3$  on circulating platelets in its resting inactive conformation, preventing the spontaneous formation of platelet aggregates in the circulation and ensuring that  $\alpha\text{IIb}\beta3$  is only activated at sites of vascular injury (2). Molecular modeling has provided insight into the structure of the  $\alpha\text{IIb}\beta3$  TM heterodimer (3). Initial models were built by combining rigid-body sampling and physics-based energy minimization subject to numerous restraints derived from biochemical experiments (4, 5). The resulting structural models were in remarkably good agreement with subsequent NMR structures of the TM domain heterodimer, highlighting the power of data-driven computer models of TM complexes in the absence of high-resolution structural information (6, 7).

The interface of the  $\alpha\text{IIb}\beta3$  TM domain heterodimer consists of a tightly packed structure in which small and large residues interdigitate by efficient van der Waals packing along the heterodimer interface. Thus, a sequence motif in the  $\alpha\text{IIb}$  TM domain, G-x<sub>3</sub>-G-x<sub>3</sub>-L, packs in a reciprocal manner with the  $\beta3$  TM

domain sequence V-x<sub>3</sub>-I-x<sub>3</sub>-G such that bulky residues from one TM helix contact a hole formed by a small Gly residue on the neighboring helix (8) (Fig. 1).

Platelets contain a second  $\beta3$ -integrin,  $\alpha\nu\beta3$ . Like  $\alpha\text{IIb}\beta3$ ,  $\alpha\nu\beta3$  on resting platelets is inactive until platelets are stimulated, after which it is able to bind to immobilized ligands such as the extravascular matrix proteins osteopontin (OPN) and vitronectin (9). Although  $\alpha\nu$  and  $\alpha\text{IIb}$  are homologous proteins, they share only 36.1% sequence identity (10). Nonetheless, compared with the  $\alpha\text{IIb}$  TM domain motif interfacing with  $\beta3$ , the homologous motif on  $\alpha\nu$  contains only one conservative Gly-to-Ala substitution (A-x<sub>3</sub>-G-x<sub>3</sub>-L) (8). On the other hand, most integrin  $\beta$ -subunits, including  $\beta3$ , also contain a highly conserved small residue-x<sub>3</sub>-small residue motif (8) (Fig. 1 and *SI Appendix, Table S1*)—a motif in which the close interaction of small residues (Gly, Ala, and Ser) between two TM domains stabilizes dimeric TM complexes (11). However, the small residue-x<sub>3</sub>-small residue motif S-x<sub>3</sub>-A on the  $\beta3$  TM helix is located on the face of the helix opposite the interface of the  $\alpha\text{IIb}\beta3$  TM heterodimer (12) (Fig. 1).

Platelets circulate in a milieu containing a high concentration of the principal  $\alpha\text{IIb}\beta3$  ligand fibrinogen (13). Thus,  $\alpha\text{IIb}\beta3$  function is tightly regulated (14). Given the specialized nature of

## Significance

Besides anchoring proteins in membranes, transmembrane (TM) helices facilitate the assembly of multisubunit proteins. Often, TM helices contain several TM–helix interaction sequences, arranged such that they cannot be simultaneously engaged. The TM helix of the  $\beta3$ -integrin subunit contains two different sequences that it uses to interact with two different  $\alpha$ -subunits, one with  $\alpha\text{IIb}$  and the other with  $\alpha\nu$ . Because integrins have distinct biological functions, the ability of a single TM helix to use different sequences to interact with different partners likely contributes to this functional specificity. Thus, the ability to achieve binding on two faces of a single TM helix provides a mechanism to evolve new binding partners for integrins and likely other membrane proteins as well.

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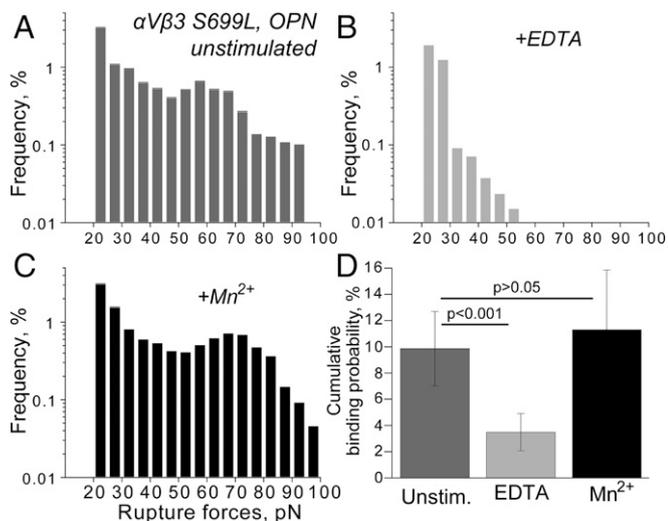
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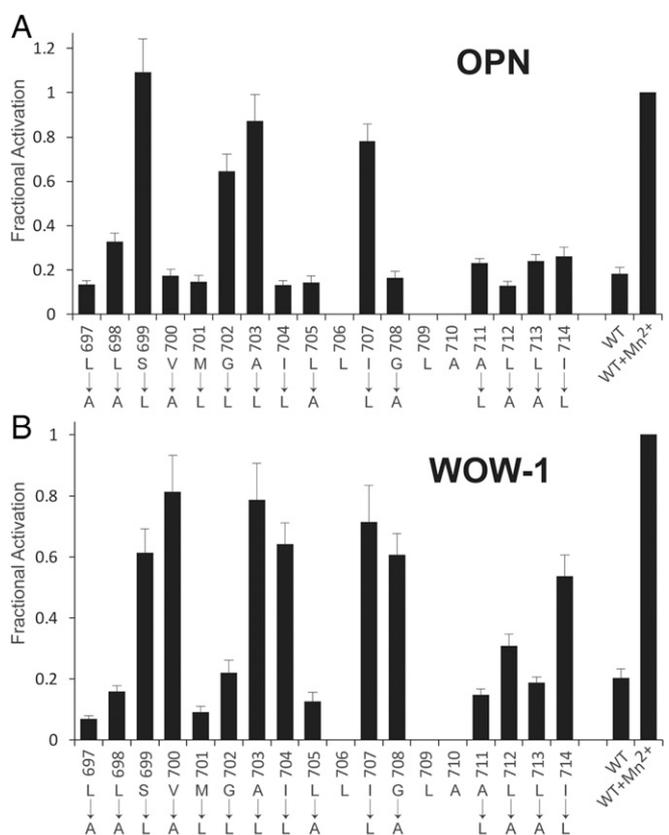
identical to that of WT  $\alpha\text{v}\beta 3$  fully activated by 1 mM  $\text{Mn}^{2+}$  (Fig. 2C). The range of rupture forces extended to 95 pN with a peak at 60–75 pN and a cumulative probability of rupture forces >20 pN of  $9.9 \pm 4.2\%$  (Fig. 3A and D). Addition of 1 mM  $\text{Mn}^{2+}$  to S699L induced only a modest increase in the cumulative binding probability for forces >20 pN,  $11.3 \pm 3.8\%$ , while the addition of 5 mM EDTA caused substantial inhibition of rupture forces in the range of 30–55 pN and complete abrogation of those >55 pN (Fig. 3B–D), consistent with the divalent cation dependence and specific binding between S699L  $\alpha\text{v}\beta 3$  and OPN.

The results of scanning the entire  $\beta 3$  TM domain with alanine or leucine replacements on  $\alpha\text{v}\beta 3$  binding to OPN are shown in Fig. 4A and SI Appendix, Table S2. They reveal that mutating either S699 or A703 that comprises the small residue- $x_3$ -small residue motif in the  $\beta 3$  TM domain ( $^{699}\text{S-x}_3\text{-A}^{703}$ ) causes constitutive OPN binding to  $\alpha\text{v}\beta 3$ . By contrast, mutating the same residues did not cause constitutive fibrinogen binding to  $\alpha\text{IIb}\beta 3$  (19). To directly compare the TM domain interfaces of  $\alpha\text{v}\beta 3$  and  $\alpha\text{IIb}\beta 3$ , we plotted the fractional activation of  $\alpha\text{v}\beta 3$  (OPN binding) versus the fractional activation of  $\alpha\text{IIb}\beta 3$  (fibrinogen binding) caused by mutating  $\beta 3$  TM domain residues 697–705 in Fig. 5A. The curves for constitutive  $\alpha\text{v}\beta 3$  and  $\alpha\text{IIb}\beta 3$  activation are completely out of phase, indicating that the residues critical for the interaction of  $\alpha\text{IIb}$  versus  $\alpha\text{v}$  with  $\beta 3$  reside on opposite sides of the  $\beta 3$ -helix (Fig. 5C). Besides the high degree of  $\alpha\text{v}\beta 3$  activation caused by S699L and A703L, G702L and I707L are also highly activating. G702 is adjacent to A703, and I707 is one full  $\alpha$ -helical turn down. Both of these residues lie on the same face of the  $\alpha$ -helix as the  $^{699}\text{S-x}_3\text{-A}^{703}$  motif (Fig. 4A). Thus, the  $\beta 3$  residues whose mutations cause constitutive  $\alpha\text{v}\beta 3$  activation (S699, G702, A703, I707) define a contiguous interaction interface on  $\beta 3$  with  $\alpha\text{v}$ , with a different helical register and clearly distinct from the  $\alpha\text{IIb}\beta 3$  interface.

To confirm these results, we repeated the optical trap-based force spectroscopy measurements using the activation-dependent monovalent monoclonal antibody WOW-1 as a second  $\alpha\text{v}\beta 3$  ligand (16). As shown in Fig. 4B and SI Appendix, Table S2, force spectroscopy measurements using WOW-1 as the  $\alpha\text{v}\beta 3$  ligand were generally in agreement with those using OPN. As with OPN, large effects were seen for the S699L, A703L, and I707L mutants.



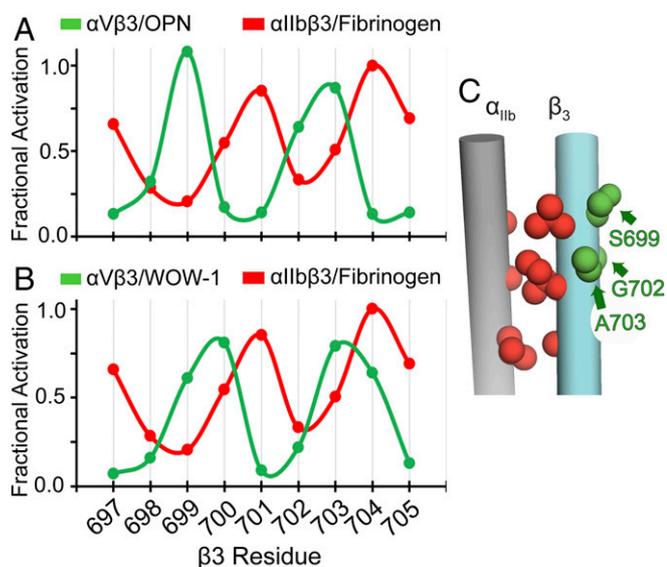
**Fig. 3.** Rupture force histograms of the interaction of OPN-coated latex beads with CHO cells expressing human  $\alpha\text{v}\beta 3$  containing the  $\beta 3$  TM helix mutation S699L. (A) Rupture force histograms resulting from OPN binding to unstimulated CHO cells expressing the  $\beta 3$  S699L  $\alpha\text{v}\beta 3$  mutant. (B) Histograms in the presence of 5 mM EDTA. (C) Histograms in the presence of 1 mM  $\text{Mn}^{2+}$ . (D) Bar graph derived from the histograms shown in A–C indicating the cumulative probability  $\pm$  SEM of detecting rupture forces >20 pN. Unstim., unstimulated  $\alpha\text{v}\beta 3$  S699L.



**Fig. 4.** Effects of leucine or alanine replacements in the  $\beta 3$  TM helix on the cumulative probability of constitutive OPN (A) and WOW-1 (B) binding to  $\alpha\text{v}\beta 3$  expressed by CHO cells. The  $\beta 3$  mutants were coexpressed with WT human  $\alpha\text{v}$  in CHO cells, and the cumulative probabilities of constitutive OPN and WOW-1 binding to  $\alpha\text{v}\beta 3$  was measured by optical trap-based force spectroscopy. The cumulative probabilities of OPN and WOW-1 binding to WT  $\alpha\text{v}\beta 3$  in the absence and presence of 1 mM  $\text{Mn}^{2+}$  were used as negative and positive controls, respectively. The bars and error bars correspond to the mean and SE generated from rupture force histograms representing  $10^4$ – $10^5$  bead–cell contacts in at least 10 independent experiments.

Moreover, the subtle isomeric replacement of Ile707 with Leu resulted in  $\alpha\text{v}\beta 3$  activation, demonstrating that the native van der Waals packing interactions are highly stereochemically specific. It is noteworthy that mutating residues V700, I704, and G708 that neighbor S699, A703, and I707 was more activating when WOW-1 binding was measured compared with OPN binding. This might reflect a higher affinity of WOW-1 for  $\alpha\text{v}\beta 3$  such that marginally activated mutants were detected more easily. By contrast, replacing G702 with leucine was less activating when WOW-1 was the  $\alpha\text{v}\beta 3$  ligand. Likewise, it is also possible that WOW-1 binds to a different range of activated  $\alpha\text{v}\beta 3$  conformations from the physiological  $\alpha\text{v}\beta 3$  ligand OPN. Despite these differences, the overall trend is similar between mutants binding to OPN and WOW-1. These results confirm that the conserved  $\beta 3$  S- $x_3$ -A- $x_3$ -I motif is critical for gating  $\alpha\text{v}\beta 3$  activation, and that the TM domain surface on  $\beta 3$  used to bind  $\alpha\text{v}$  is quite distinct from that used in  $\alpha\text{IIb}\beta 3$ .

**Modeling and Molecular Dynamics Simulation of  $\alpha\text{v}\beta 3$ .** To gain a better structural understanding of the  $\alpha\text{v}\beta 3$  TM domain interface, we compared the helical register implied from our scanning mutagenesis of  $\alpha\text{v}\beta 3$  to the interfaces calculated for two independently published solution NMR structures of  $\alpha\text{IIb}\beta 3$  (6, 7). First, we analyzed the per-residue interhelical distances between each residue in  $\beta 3$  and the closest residues in  $\alpha\text{IIb}$  by C $\alpha$ -atom for the lowest-energy model reported in each NMR study.



**Fig. 5.** Motifs in the  $\beta_3$  TM helix that mediate its association with  $\alpha v$  and  $\alpha IIb$  are completely out of phase and are located on opposite sides of the  $\beta_3$ -helix. (A) The curves compare the effect of replacing  $\beta_3$  residues 697–705 with leucine or alanine on constitutive  $\alpha v\beta_3$  binding to OPN using data from Fig. 4A and the effect of the same replacements on constitutive  $\alpha IIb\beta_3$  to fibrinogen using data from ref. 4. (B) The curves compare the effect of replacing  $\beta_3$  residues 697–705 on constitutive  $\alpha v\beta_3$  binding to WOW-1 using data from Fig. 4B and the effect of these replacements on constitutive  $\alpha IIb\beta_3$  to fibrinogen using data from ref. 4. (C) The  $\beta_3$  residues whose mutation causes constitutive binding of  $\alpha v\beta_3$  to OPN (S699, G702, and A703) are shown as green spheres on the  $\alpha IIb\beta_3$  TM structure cylinders. This demonstrates that the residues responsible for the interaction of  $\beta_3$  with  $\alpha IIb$  versus  $\alpha v$  reside on opposite sides of the  $\beta_3$ -helix.

To make comparisons to the per-residue change associated with  $\alpha v\beta_3$  mutant binding to OPN, the interhelical distances were converted to a normalized interhelical closeness (NIC) value, as described in *SI Appendix, Materials and Methods*, where  $NIC = 1$  for the closest  $\beta_3$  residue to the  $\alpha IIb$  backbone and  $NIC = 0$  for the farthest  $\beta_3$  residue (*SI Appendix, Fig. S1A*). When the NIC values for the two  $\alpha IIb\beta_3$  NMR structures are plotted alongside the fractional activation values for  $\alpha v\beta_3$  binding to OPN shown in Fig. 4, it is clear that mutating residues in the  $\beta_3$  TM helix that are closest to the  $\alpha IIb$ -helix would have no effect on the activation state of  $\alpha v\beta_3$  (*SI Appendix, Fig. S1B*). Further, this analysis indicates that any attempt to model the  $\alpha v\beta_3$  TM dimer from an  $\alpha IIb\beta_3$  NMR structure would require large-scale conformational rearrangements.

Next, we sought to build a model for all-atom molecular dynamics simulations to sample the energetic landscape of the  $\alpha v\beta_3$  TM and cytoplasmic domains. An initial model was built by threading the sequences of  $\alpha v$  and  $\beta_3$  onto the coordinates of the first conformation of the reported  $\alpha IIb\beta_3$  NMR model (PDB ID code 2knc). This model was first structurally relaxed in an implicit lipid bilayer using the Rosetta modeling suite, applying rounds of side-chain rotamer sampling, cartesian minimization, and rigid-body reorientation. The resultant lowest-energy model was inserted into a POPC lipid bilayer, solvated, and equilibrated with constraints on the initial  $\alpha$ -atomic positions. Constraints were then released for 400 ns of unbiased simulation time. Thereafter, each frame was structurally clustered using a 2.6-Å cutoff to identify unique conformations. Additionally, for each simulation frame, a Pearson correlation was calculated between the per-residue interhelical distances across the  $\alpha v\beta_3$  TM domain (i.e., NIC of  $\beta_3$  residues) and the per-residue fractional activation datasets for both OPN and WOW-1 binding (Fig. 6A).

Within the first 20 ns of unrestrained simulation, the  $\alpha v$  and  $\beta_3$  TM helices rapidly reoriented to a new geometry (*Movies S1 and S2*) with S699, A703, and I707 at the interface, resulting in a marked increase in the correlation between the model and the scanning mutagenesis results (Fig. 6A and B). After remaining in this associated state for 75 ns, the TM helices dissociated into a conformation that persisted for an additional 70 ns. Meanwhile, the cytoplasmic domains remained in contact, including the  $\alpha v$  R995- $\beta_3$  E723 salt bridge (*SI Appendix, Fig. S2*). The  $\alpha v\beta_3$  TM heterodimer was reconstituted with an interhelical geometry similar to that before dissociation, persisting in this conformation for the remaining 245 ns.

The major TM domain conformation (cluster medoid, 80.4% of 400 ns) had a consistently low structural deviation within the cluster (mean  $C\alpha$  rmsd =  $1.20 \pm 0.01$ , SEM) suggesting that it is energetically stable. Further, there was a statistically significant correlation between frames in this cluster and the rupture force spectroscopy results ( $P < 0.05$ ). An overlay of the rupture force spectroscopy results for each  $\beta_3$  mutation and the interhelical distance for each  $\beta_3$  residue in the  $\alpha v\beta_3$  TM domain, as measured from a representative model of the major conformation, is shown in Fig. 6B. It is noteworthy that the modeled interface agrees well with both datasets. Further, while the average correlation with simulation frames is greater for the WOW-1 data than for the OPN data, the change in correlation coefficient between the initial and the medoid frames is greater for the OPN data than for the WOW-1 data.

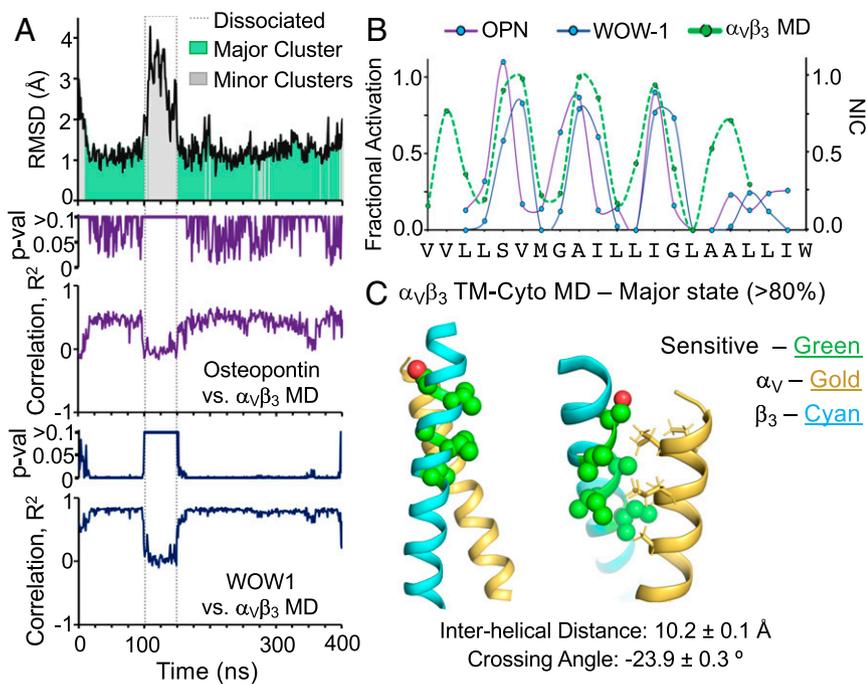
A representative simulation snapshot depicting the  $\alpha v\beta_3$  TM dimer interface is shown in Fig. 6C. The helices adopt a geometry that is similar to the canonical  $GAS_{Right}$  motif (1) in its parallel right-handed crossing ( $-23.9 \pm 0.3^\circ$ , SEM), but differs in its larger interhelical distance of  $10.2 \pm 0.1$  Å. We find  $\alpha v$  side chains packing against the  $\beta_3$  residues most sensitive to mutation. Overall, the agreement of the major conformation from the simulation with the rupture force spectroscopy results is remarkable, given that no additional forces were applied to the system to bias helix association or geometry.

## Discussion

TM domain interactions, mediated by specific sequence motifs, stabilize integrins in their inactive conformations (2, 22, 23). In the best characterized example, a canonical G-x<sub>3</sub>-G motif in the  $\alpha IIb$  TM helix packs against a reciprocating large residue-small residue motif in the  $\beta_3$  TM helix to stabilize the integrin  $\alpha IIb\beta_3$  in its inactive state (Fig. 1). Given the high degree of sequence conservation in both  $\alpha$ - and  $\beta$ -chains, it seemed likely that this TM domain complex might be common to all integrins. This notion was supported by our previous measurements of heterodimeric interaction strength between the TM domain segments of integrins in *Escherichia coli* using the dominant-negative TOXCAT assay (8). In striking contrast, the results presented here using the full-length protein in mammalian membranes indicate that the second platelet integrin  $\alpha v\beta_3$  is instead regulated by a distinct TM domain interface, which is also highly conserved (*SI Appendix, Table S1*).

When expressed in bacterial cell membranes, the  $\beta_3$  residues most sensitive to mutations were residues at the  $\alpha IIb\beta_3$  interface (8). Thus, the geometry of the isolated  $\alpha v\beta_3$  TM domain heterodimer assembled in bacterial membranes appears to differ from the geometry of the TM domain heterodimer when it is present in the context of the full-length receptor and expressed in mammalian membranes. This suggests that either the lipid environment or contiguous extracellular or intracellular sequences influence the conformation of the  $\alpha v$  TM domain (24, 25).

Crystal structures of the  $\alpha v\beta_3$  and  $\alpha IIb\beta_3$  ectodomains and negatively stained electron microscopy images of the full-length proteins are similar (26–29). However, in the more membrane-distal regions of the  $\beta_3$  ectodomain, the interactions with either



**Fig. 6.** Molecular dynamics simulations of  $\alpha_v\beta_3$  TM and cytoplasmic domain interactions during  $\alpha_v\beta_3$  binding to OPN and WOW-1. (*A, Upper*) Rmsd of the  $\alpha_v\beta_3$  TM domain to the centroid of the major structural cluster. The color of each frame indicates whether it belongs to the major cluster (green) or a minor cluster (gray). (*A, Middle and Bottom*) Pearson correlation coefficients and *P* values of  $\alpha_v\beta_3$  TM domain interhelical distances for each  $\beta_3$  mutation and the cumulative probability of OPN (*Middle*) or WOW-1 (*Bottom*) binding. (*B*) Overlay of the normalized interhelical distance values (interhelical closeness) for the major  $\alpha_v\beta_3$  TM domain conformation observed in the MD simulations and the fractional activation of  $\alpha_v\beta_3$  caused by  $\beta_3$  TM helix mutations using the results shown in Fig. 4. (*C*) Representative model of the major conformation and the mean geometric parameters  $\pm$  SEM of the  $\alpha_v\beta_3$  TM domain heterodimer observed during the MD simulations.

$\alpha_v$  or  $\alpha_{IIb}$  that help maintain the inactive conformations of  $\alpha_v\beta_3$  and  $\alpha_{IIb}\beta_3$  differ (25). Consequently, the orientation of the distal  $\beta_3$ -stalk and the contiguous  $\beta_3$  TM helix might be expected to be shifted with respect to complementary portions of  $\alpha_v$  and  $\alpha_{IIb}$  as well. While the composition of the  $\alpha_{IIb}\beta_3$  TM domain interface heterodimer is well-established, there has been until now a paucity of information about the structure of the  $\alpha_v\beta_3$  TM domain. Although the  $\alpha_{IIb}$  and  $\alpha_v$  TM domains each contain a small residue- $x_3$ -small residue motif in approximately the same position, their van der Waal surfaces are certainly distinct and distinguishable, since computationally designed TM domain peptides were able to interact exclusively with either  $\alpha_v$  or  $\alpha_{IIb}$  (30). Then, it is not surprising that  $\beta_3$  can interact with  $\alpha_v$  and  $\alpha_{IIb}$  via distinct interfaces, varying in affinity (17, 31).

The distinct TM domain interface between  $\alpha_v\beta_3$  and  $\alpha_{IIb}\beta_3$  might be rationalized by their varying requirements for stringent regulation of the high-affinity ligand-binding conformation given their different physiological roles.  $\alpha_v\beta_3$  is widely expressed, mediating cell adhesion and migration on a variety of immobilized ligands containing an RGD motif (32), while  $\alpha_{IIb}\beta_3$  expression is limited to platelets. Moreover,  $\alpha_{IIb}\beta_3$  readily interacts with soluble ligands, whereas  $\alpha_v\beta_3$  does not. Previously, using optical trap-based force spectroscopy, we found that under essentially identical conditions, the average adhesion strength for OPN- $\alpha_v\beta_3$  interactions was  $47 \pm 7$  pN, whereas the average adhesion strength of fibrinogen binding to  $\alpha_{IIb}\beta_3$  was nearly twofold greater at 80–90 pN (20, 33). This may be a reflection of the physiological requirement that fibrinogen binding to  $\alpha_{IIb}\beta_3$  on aggregated platelets must be sufficiently strong to resist the shear forces present in arterial blood, which would not be the case for the adherence and spreading of single platelets mediated by ligand binding to  $\alpha_v\beta_3$ . Further, we found that a substantial fraction of unstimulated platelets exhibit a distribution of OPN-mediated rupture forces similar to ADP-stimulated platelets (20), whereas the fraction of unstimulated platelets that interact spontaneously with fibrinogen is negligible (33). This implies that  $\alpha_{IIb}\beta_3$  is less prone to activation than  $\alpha_v\beta_3$ , perhaps a consequence of the differences in the interfaces of their TM domains.

From a biophysical perspective, the structural consequences of the mutations observed here are consistent with the literature

but also offer new insights. Employing small-to-large or large-to-small mutations successfully induced major disruptions in van der Waals packing at the TM heterodimer interface. However, deviation of this pattern to much more subtle mutants, namely Leu-Ile or Ile-Leu isomers, also had dramatic effects. For example, Ile707Leu had a marked effect on both OPN and WOW-1 binding, despite only one methyl group being stereochemically repositioned in the entire protein complex. This indicates a strict steric requirement for van der Waals packing interactions between TM domain side chains that dictates the stability and regulation of the  $\alpha_v\beta_3$  resting state. Similar Ile-Leu mutations have been observed to abrogate select TM domain interactions for shorter engineered TM peptides (34, 35), yet it is noteworthy to find a similar result within a large protein complex interacting simultaneously through several extramembrane domains. The mutational consequences might also be rationalized in the context of previous reports for small- $x_3$ -small motifs. Replacement of the neighboring  $\beta$ -branched residues V700 and I704 with alanine and leucine, respectively, caused constitutive WOW-1 binding to  $\alpha_v\beta_3$  as well. In a statistical analysis of amino acid patterns in TM helices, Senes et al. (11) found that not only were small- $x_3$ -small residue motifs frequent but these motifs were commonly associated with  $\beta$ -branched aliphatic residues in neighboring  $i \pm 1$  positions. Further, they posited that because the side chains of valine and isoleucine are constrained to only one rotamer when they are present in a helix, these residues contribute to the stability of transmembrane helix dimers by minimizing entropy loss upon helical packing. Thus, it is possible that by mutating either V700 to alanine or I704 to leucine, the  $\alpha_v\beta_3$  TM domain is destabilized sufficiently to permit WOW-1 binding to a different range of activated  $\alpha_v\beta_3$  conformations but not the more stringent binding of OPN.

Our MD simulations are also consistent with numerous experimentally reported features of the  $\alpha_v\beta_3$  cytosolic and juxtamembrane regions (24, 36). The homology model began with the entire cytoplasmic domains of both  $\alpha_v$  and  $\beta_3$  as  $\alpha$ -helical, yet we observed that only  $\alpha_v$  V993–V1024 and  $\beta_3$  D718–N769 had persistent  $\alpha$ -helical structure with the remaining regions in extended conformations. This is consistent with NMR measurements of a  $\beta_3$  TM-cytoplasmic construct in DHPC:DMPC bicelles where

$\beta 3$  was helical through residue A737, well beyond the TM domain and into the cytoplasm (24). Also consistent with this NMR study, K713 (numbered K716 in ref. 24) snorkeled its amine moiety to the cytoplasmic lipid head-group region in the majority of simulations. We also found that the R995-to-E723 salt bridge previously suggested to stabilize the low-affinity state of  $\alpha\beta 3$  (36) formed during the initial structural relaxation of the  $\alpha\beta 3$  homology model and was maintained through the first 260 ns of simulation, even during the TM helix dissociation event (*SI Appendix, Fig. S2*). Nonetheless, in the last 60 ns, we observed a switch to a K994–E723 salt bridge. Although the simulation does not completely sample the orientational and conformational energy landscapes available to  $\alpha\beta 3$ , in this short timescale, we observed behavior largely consistent with previous structural and functional experiments of  $\alpha\beta 3$ , implying that the simulated conformation may be physiologically relevant. Thus, the MD simulations are consistent with a unique interface for the  $\alpha\beta 3$  TM heterodimer. Our final model, derived entirely from unbiased simulations, shown in Fig. 6C, provides atomic-level understanding of the TM domain interactions that constrain  $\alpha\beta 3$  in its inactive state, as well as insight into how homologous integrins uniquely regulate cellular signal transduction.

## Conclusion

TM helix–helix interactions are known to play important roles in the function of many type 1 membrane proteins, including integrins and receptor-linked tyrosine kinases. Often the TM helices contain two TM–helix interaction motifs, arranged such that both motifs

cannot simultaneously engage a single target without large structural distortions. At the outset of our investigation, we expected that the different helix–helix interaction motifs might be alternatively engaged during the dynamics of signaling, as has been proposed for members of the EGF receptor family (37, 38). Therefore, we were surprised to find that the two motifs found in the  $\beta 3$  TM helix mediate interactions with two different  $\alpha$ -subunits. Given the combinatorial assembly of integrin subunits into heterodimers with distinct biological functions, the ability of a single TM domain to use two different faces of its helix for different interaction partners is noteworthy. This phenomenon might contribute to both the specificity of interaction and the different activation energies required for different receptors. Moreover, the ability to achieve binding on two faces of a single helix provides an attractive mechanism to evolve new partners in the integrin family as well as other membrane proteins.

## Materials and Methods

DNA cloning, cell culture, and force spectrometry materials were obtained from commercial sources and used as described in *SI Appendix*. Molecular modeling and dynamics simulations were conducted using publicly available software and analyzed as described in *SI Appendix*.

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