Structural determinants of the integrin transmembrane domain required for bidirectional signal transmission across the cell membrane

Received for publication, June 14, 2021, and in revised form, October 12, 2021 Published, Papers in Press, October 20, 2021, https://doi.org/10.1016/j.jbc.2021.101318
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Edited by Henrik Dohlman

Studying the tight activity regulation of platelet-specific integrin \( \alpha_{IIb}\beta_3 \) is foundational and paramount to our understanding of integrin structure and activation. \( \alpha_{IIb}\beta_3 \) is essential for the aggregation and adhesion function of platelets in hemostasis and thrombosis. Structural and mutagenesis studies have previously revealed the critical role of \( \alpha_{IIb}\beta_3 \) transmembrane (TM) association in maintaining the inactive state. Gain-of-function TM mutations were identified and shown to destabilize the TM association leading to integrin activation. Studies using isolated TM peptides have suggested an altered membrane embedding of the \( \beta_3 \) TM \( \alpha \)-helix coupled with \( \alpha_{IIb}\beta_3 \) activation. However, controversies remain as to whether and how the TM \( \alpha \)-helices change their topologies in the context of full-length integrin in native cell membrane. In this study, we utilized proline scanning mutagenesis and cysteine scanning accessibility assays to analyze the structure and function correlation of the \( \alpha_{IIb}\beta_3 \) TM domain. Our identification of loss-of-function proline mutations in the TM domain suggests the requirement of a continuous TM \( \alpha \)-helical structure in transmitting activation signals bidirectionally across the cell membrane, characterized by the inside-out activation for ligand binding and the outside-in signaling for cell spreading. Similar results were found for \( \alpha_1\beta_2 \) and \( \alpha_5\beta_1 \) TM domains, suggesting a generalizable mechanism. We also detected a topology change of \( \beta_3 \) TM \( \alpha \)-helix within the cell membrane, but only under conditions of cell adhesion and the absence of \( \alpha_{IIb} \) association. Our data demonstrate the importance of studying the structure and function of the integrin TM domain in the native cell membrane.

Integrins are a large family of cell surface receptors composed of \( \alpha \) and \( \beta \) subunits. The combination of 18 \( \alpha \) and eight \( \beta \) subunits in human forms 24 integrin heterodimers that interact with multiple extracellular or cell surface ligands and play diverse functions in different cell types (1). Both \( \alpha \) and \( \beta \) subunits contain a large extracellular domain, a single-pass transmembrane (TM) domain, and usually a short cytoplasmic tail (CT). As a cell adhesion molecule, integrin can sense and transmit both chemical and mechanical signals, which are associated with local small-scale as well as long-range large-scale conformational changes (2, 3). A unique feature of integrin signaling is the bidirectional signal transmission across cell membrane, namely outside-in and inside-out signaling (1). The integrin TM domain not only acts as a membrane anchor but also plays a pivotal role in regulating the bidirectional signal transduction (4).

Current model of integrin activation regulation is largely based on the structural and functional studies of platelet-specific integrin \( \alpha_{IIb}\beta_3 \) (5). The tight control of the on-off state of \( \alpha_{IIb}\beta_3 \) is critical for the normal function of platelets in hemostasis. The resting \( \alpha_{IIb}\beta_3 \) is maintained by a bent ectodomain structure involving extensive close contacts, which switches into an extended conformation upon activation (2, 5). Remarkably, the relatively simple and weak interactions at the TM and CT domains also contribute critically to the inactive state (4). The inside-out activation of \( \alpha_{IIb}\beta_3 \) is triggered by the binding of intracellular proteins talin and kindlin to the \( \beta_3 \) CT, leading to the disruption of \( \alpha_{IIb}\beta_3 \) heterodimerization at the TM and CT domains (6). The destabilized \( \alpha_{IIb}\beta_3 \) TM interaction then induces ectodomain extension on the cell surface, increasing affinity for binding with ligands such as fibrinogen in blood (5). Ligand binding to the ectodomain further induces conformational changes that are relayed to the CT through the TM domain, which induces the outside-in signaling for platelets adhesion, spreading, aggregation, and clot retraction (7). During this process, mechanical forces are generated by the cytoskeleton proteins binding to integrin CT and the extracellular ligands binding to integrin ectodomain. The integrin TM domain needs to resist the forces applied bidirectionally to the single-pass TM \( \alpha \)-helical structure. How the conformational rearrangements of ectodomain are coupled with the structural changes of TM domain has been an active area of research.

Great efforts have been made in understanding the structural basis of the TM-CT domain in regulating \( \alpha_{IIb}\beta_3 \) activation (8–13). Although the heterodimeric structures of \( \alpha_{IIb}\beta_3 \) TM-CT determined by different approaches share common structure features regarding the TM helix–helix packing (14), discrepancies remain about how the TM-CT
association is maintained in an inactive state and whether and how the TM α-helix change its conformation upon activation \((10, 11, 13, 15–18)\). The \(\beta_3\) TM domain was often studied as a free isolated fragment in model membrane or detergent micelles, but inconsistent results were obtained regarding the oligomerization state and membrane topology \((15, 18–20)\). In this study, we used proline scanning mutagenesis and cysteine accessibility by \textit{in situ} biotin-maleimide labeling to analyze the structure of \(\alpha_{IIb}\beta_3\) TM domain in native cell membrane. Our data reveal the structural requirement of TM α-helix in regulating integrin bidirectional signal transduction, which also provides an example of how a rigid α-helical conformation participates in the signal transduction of single-pass cell membrane receptors.

## Results

### Effect of \(\alpha_{IIb}\beta_3\) transmembrane proline mutations on the ligand binding at resting condition

Sequence alignment of the TM domains of 18 α and eight β human integrins shows typical TM features with hydrophobic residues that are rich in Leu, Ile, and Val (Fig. 1A). A conserved Gly residue (G708 in \(\beta_3\)) in β integrins and two conserved small amino acids (Ala, Ser, or Gly) that form the

![Figure 1. Proline scanning of \(\alpha_{IIb}\beta_3\) TM domains. A, sequence alignment of human integrin transmembrane domains. The predicted TM C-terminal boundary is marked with a red dashed line. The conserved small amino acids are highlighted in yellow. Shown in red are the residues that inhibit integrin inside-out activation when mutated to proline. B, backbone structure of \(\alpha_{IIb}\beta_3\) TM domain. Backbone hydrogen bonds are shown as dashed lines in yellow. Selected residues are marked as spheres of Ca atoms. C and D, ligand mimetic PAC-1 binding to \(\alpha_{IIb}\beta_3\) integrin with indicated proline substitutions. HEK293FT cells were transfected with indicated \(\alpha_{IIb}\beta_3\) constructs. The ligand-mimetic mAb PAC-1 binding was performed in the buffer containing 1 mM Ca\(^{2+}/\)Mg\(^{2+}\) and detected by flow cytometry. The level of PAC-1 binding was normalized to \(\alpha_{IIb}\beta_3\) surface expression detected by mAb AP3 and shown as mean ± SD \((n = 3)\). Unpaired two-tailed \(t\) test was performed between the control group without proline mutation and the group with proline mutation. Only \(p\) values less than 0.05 are shown. E, structural illustration of \(\alpha_{IIb}\beta_3\) TM domain within the cell membrane in the absence and presence of selected proline mutations. The proline mutations were introduced \textit{in silico} to the TM structure using PyMOL. The proline-induced broken of a rigid α-helical structure was indicated. The interfacial residues are shown as sticks or Ca spheres. MP, membrane proximal.

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\(J.\) Biol. Chem. (2021) 297(5) 101318
GXXXG motif in α integrins (G972XXXG975 in αIIb) were identified to be critical in maintaining the resting state of αIIbβ3 integrin (Fig. 1A) (21). Other conserved features that have been demonstrated to be important for the resting state include a conserved Lys at the TM inner boundary in both α and β integrins (11, 15), a conserved Asp in β membrane-proximal (MP) region (22), and a conserved GFFKR motif in the α MP region (23, 24) (Fig. 1A). Structure studies of αIIbβ3 TM-CT heterodimer show a cross-angled straight α-helical structure for both αIIb and β3 (10, 11, 13). The α-helical structure extends to the MP region in β3 subunit (Fig. 1B). A continued backbone hydrogen bonding network maintains the integrity of α-helical structure (Fig. 1B), which may be critical for the transmission of conformational signals across the cell membrane. To test this hypothesis, we performed proline scanning mutagenesis for αIIbβ3 TM domain (Fig. 1, C and D), given that when present in an α-helical structure, proline tends to disturb the α-helical conformation by introducing a break or kink due to the lack of backbone hydrogen bonding (25) (Fig. 1E). Among the 14 proline mutations tested for β3 TM domain, most of them showed similar level of ligand-binding activity when measured with ligand-mimetic mAb PAC-1 under the resting condition (Fig. 1C). Several proline mutations, including β3-V696P and β3-L713P, and the previously identified β3-G708P and β3-K716P (11, 21), significantly rendered αIIbβ3 more active than wild type (Fig. 1C). Among the ten proline mutations tested for αIIb TM domain, seven of them showed a similar level of ligand binding as wild type under the resting condition (Fig. 1D) and three of them significantly rendered αIIbβ3 constitutively active (Fig. 1D). All the active proline mutations, such as the β3-L706P and -G708P and the αIIb-G972P and -G976P, are located at or close to the αIIbβ3 TM heterodimer interface, which may disturb the αIIbβ3 TM interface leading to integrin activation.

Identification of TM proline mutations that attenuate αIIbβ3 inside-out activation

Having identified some TM proline mutations that reduced the basal level ligand binding of αIIbβ3 under resting condition, we next tested the effect of these proline mutations on the inside-out activation of αIIbβ3. Several activating mutations such as αIIb-R995D, αIIb-F993A, αIIb-F992A-F993A, and β3-K716A (or K716C), which are known to disturb the cytoplasmic domain association, have been widely used to mimic the inside-out activation of αIIbβ3. When coexpressed with αIIb-R995D, four of the β3 TM proline mutations, including L698P, L705P, L707P, and A711P, significantly reversed the activating effect of αIIb-R995D (Fig. 2A), while the rest of proline mutations either had no effect or further enhanced the activation by αIIb-R995D (Fig. 2A). Similarly, when coexpressed with αIIb-F993A that is more activating than αIIb-R995D, the β3 L698P, L705P, L707P, and A711P significantly reversed the activating effect of αIIb-F993A (Fig. 2B). We next focused on β3 L705P and A711P for further analysis since their deactivating effect is more dramatic than others (Fig. 2, A and B). To test if the proline mutation also reverses the activating effect of β3 mutation, we generated L705P and A711P mutations in the context of β3-K716C. Both proline mutations significantly reduced the activation of αIIbβ3 induced by β3-K716C (Fig. 3C). To test if the β3 L705P and A711P mutations have a synergistic effect, we coexpressed the single or double proline mutations with the highly active αIIb-F992A-F993A mutant. Both β3-L705P and β3-A711P reduced the activation by αIIb-F992A-F993A, while the β3-L705P-A711P double mutation further decreased the activation (Fig. 2C), demonstrating a synergistic effect. All the above activating mutations are expected to destabilize the αIIbβ3 cytoplasmic interaction, which also disturbs TM association. We then tested a TM interface disturbing mutation αIIb-G976L. The β3-L705P-A711P also significantly reduced αIIb-G976L-mediated αIIbβ3 activation (Fig. 2C). In contrast to β3 proline mutations, when coexpressed with the activating β3-K716A mutant, almost all the all β3 proline mutations further enhanced αIIbβ3 activation, except αIIb-1982P that reduced the activation (Fig. 2D), indicating that the αIIb TM domain tolerates proline mutation less than β3 TM domain.

Effect of disturbing the rigidity of α-helical structures of TM and CT domains on talin1-head-induced αIIbβ3 activation

Overexpression of the talin1 head domain is a well-established method to induce integrin inside-out activation (26). We used EGFP-tagged talin1 head (EGFP-TH) to induce αIIbβ3 activation in HEK293FT cells (14). Compared with the EGFP control, EGFP-TH induced substantial αIIbβ3 activation reported by PAC-1 binding (Fig. 3A). In contrast, the β3-L705P, β3-A711P, and β3-L705P-A711P mutations all completely abolished EGFP-TH-induced αIIbβ3 activation (Fig. 3A). The αIIb-1982P mutation also significantly reduced EGFP-TH-induced αIIbβ3 activation, while αIIb-L974P by contrast further enhanced αIIbβ3 inside-out activation (Fig. 3A). The expression of EGFP-TH was comparable among all the transfections as determined by flow cytometry. These data are consistent with the above results obtained using the activating mutations that mimic αIIbβ3 inside-out activation. Talin1 head induces αIIbβ3 activation through binding to β3 cytoplasmic tail (CT) and disturbing α-β CT as well as TM associations (4). Our proline mutagenesis data suggest that the integration of TM α-helical structure is critical for talin1-head-induced conformational change of TM-CT domain. Since the α-helical structure of β3 TM extends to the cytoplasmic region (Fig. 1B), we asked if disturbing the integration of cytoplasmic α-helix also affects αIIbβ3 activation. Instead of using proline mutagenesis, we inserted a flexible loop composed of GS or GGGS into the MP region of β3 CT (Fig. 3B). The insertion site is right before the conserved D723, given that the K716-H722 sequence is the minimal requirement for maintaining the resting state (14), and the sequence after H722 contains the talin1-binding sites (27). Compared with wild-type β3, both β3-GS and β3-GGGS mutants significantly reduced EGFP-TH-induced αIIbβ3 activation (Fig. 3C). The longer GGGS insertion
had more attenuated effect than the shorter GS insertion on αIIbβ3 activation (Fig. 3C). The αIIb-R995A mutation was used to synergistically enhance the activation by EGFP-TH (14). The GS and GGGS mutations did not affect the binding of EGFP-TH to β3 CT as shown by the EGFP-TH pull-down assay (Fig. 3D). As a control, truncation of β3 CT after H722 (β3-H722Tr) completely abolished EGFP-TH binding (Fig. 3D). Altogether, these data suggest that the integrity of the α-helical structure at both the TM and CT domains of β3 is required for the inside-out activation of αIIbβ3.

### TM proline mutations that attenuate the inside-out activation of β2 and β1 integrins

We expanded our studies to β2 integrin to ask if certain TM proline mutations also affect the inside-out activation of αIβ2 integrin. We screened 13 proline mutations for the C-terminal half of the β2 TM domain, which includes the residues β2-V691 and -L697 that are equivalent to β3-L705 and -A711, respectively (Figs. 1A and 4A). Using the αI-GFFKR to GAAKR mutation to induce inside-out αIβ2 activation reported by soluble ICAM-1 binding, we found that six β2 TM proline mutations reduced αIβ2 activation (Fig. 4A). Most of the inhibitory proline mutations of β2 are equivalent to those found in β3 integrin, such as β2-I690P, -V691P, and -I693P that are equivalent to β3-I704P, -L705P, and -I707P, respectively (Fig. 1A). Similar to β3-L705P, the equivalent β2-V691P has much profound negative effect. However, the β3-A711P equivalent mutation, β2-L697P, only slightly reduced αIβ2 activation (Fig. 4A). To correlate the effect of proline mutations on ligand binding with large-scale conformational changes of ectodomain, we used two mAbs KIM127 and m24 to report β2 extension and headpiece open, respectively (28, 29). Consistent with the ICAM-1 binding results, β2-V691P and -I693P mutations significantly reduced the αI-GAAKR-induced binding of KIM127 and m24 (Fig. 4, B and C), suggesting that the TM proline mutations restrained the conformational activation signal transmitted across the cell membrane. In contrast, Mn2+, an integrin activator for the extracellular domain, still stimulates KIM127 and m24 binding in the presence of the inhibitory proline mutations (Fig. 4, B and C). We also tested the β2-L705P and -A711P equivalent mutations on β1 integrin, namely β1-V721P and -L727P. The fibronectin-binding assay showed that the β1-V721P mutation significantly reduced while the β1-L727P mutation significantly enhanced EGFP-TH-induced αIβ1 activation (Fig. 4D). These
data suggest that the integrity of TM α-helical structure is also important for the inside-out activation of β1 and β2 integrins.

**The β3-L705P-A711P mutation dampens αιββ3-mediated outside-in signaling**

Integrin conformational signal is transmitted bidirectionally across the TM domain. Having found that the β3-L705P-A711P mutation greatly reduced the inside-out activation of αιββ3 integrin, we further asked whether such mutation also affects αιββ3 outside-in signaling, in which extracellular ligand binding induces large-scale conformational changes that are transmitted to the CT through the TM α-helix (2). We generated HEK293 cell lines stably expressing comparable levels of αιββ3 wild type, αιββ3-L705P-A711P, or αιββ3-1982P/β3. The αιββ3-mediated cell spreading on the immobilized ligand, a hallmark of integrin outside-in signaling, was comparably measured among the stable cell lines. When seeded on the plate coated with ligand-mimetic mAb PAC-1, the HEK293-αιββ3-WT cells showed substantial cell spreading (Fig. 5A). By contrast, the HEK293-αιββ3/β3-L705P-A711P cells had dramatic defects of cell spreading, showing much smaller spreading area than the WT cells (Fig. 5, B and F), although there were no obvious differences in the number of attached cells. Interestingly, the HEK293-αιββ3-1982P/β3 cells spread as well as the WT (Fig. 5, C and F). Consistently, the HEK293-αιββ3/β3-L705P-A711P cells also showed defective cell spreading on the physiological ligand fibrinogen (Fig. 5, D–F). These data suggest that the integrity of the α-helical structure of β3 TM domain is required for the outside-in signaling.

**High-affinity soluble ligand binding does not induce conformational changes of αιββ3 transmembrane domain detected by biotin-maleimide (BM) labeling**

Having found that the rigid α-helical structure of TM domain is critical for the bidirectional integrin activation, we next asked if the α-helix performs conformational change within the cell membrane. Such conformational changes may be disrupted due to the helix-breaking effect of a proline mutation (Fig. 1E). To measure the potential conformational change of TM α-helix in the cell membrane, we performed the cysteine scanning accessibility assay (30). The membrane-permeable sulfhydryl-specific reagent, biotin-labeled maleimide (BM), was used to label the substituted cysteine residues. Since the reaction only occurs in an aqueous environment, the cysteines residing in the lipid bilayer cannot be labeled (Fig. 6A), which will show the burial/exposure status of TM residues. By comparing the resting and active states, the changes of labeling accessibility of the substituted cysteines indicate the changes of membrane embedding of TM α-helix. We first tested whether ligand-induced large-scale conformational change at the ECD induces structural rearrangement of TM domains. The RGD-mimetic high-affinity drug epifibatide was used to induce the headpiece opening and extension of αιββ3 (31, 32). Both the N-terminal and C-terminal membrane-proximal cysteine mutations of αιββ3 TM domains were analyzed by BM labeling in the absence or presence of epifibatide. 2-Bromopalmitate (2-BP) was used to inhibit the potential palmitoylation of C-terminal cysteines (11). The signals of BM labeling and total protein were simultaneously detected by western blot after immunoprecipitation (Fig. 6, B–E). BM
labeling of αIIbβ3 TM N-terminal cysteine mutations showed that the β3-I693C and αIIb-W697C are the last residues giving detectable BM signal (Fig. 6, B and C), suggesting they are the N-terminal boundary residues of αIIbβ3 TM domains. BM labeling of β3 TM C-terminal cysteine mutations suggested that the C-terminal TM boundary of β3 is at the residues I721H722 (Fig. 6D). The BM labeling of αIIb TM C-terminus showed an interesting pattern, suggesting that the residues G991C, F992C, F993C, and R995C are all embedded in the cell membrane, while K994C and the residues after R995 are exposed (Fig. 6E).

This data is consistent with the loop conformation of αIIb GFFKR motif as determined by structural studies (11, 13) (Fig. 1, B and E). Compared with the results without epitifibatide, epitifibatide did not induce obvious changes in the BM labeling patterns for both αIIb and β3 subunits, suggesting no changes in membrane embedding (Fig. 6, B–D).

**BM labeling of αIIb cytoplasmic membrane-proximal region reveals no obvious conformational changes related to activation**

Previous studies showed that the cysteine mutations of αIIb cytoplasmic GFFKR motif rendered αIIbβ3 constitutively active (11), suggesting that the BM labeling pattern of GFFKR cysteine mutations might be related to the active conformation (17). To reverse the activating status of GFFKR cysteine mutations, we introduced additional αIIb-L959C and β3-P688C mutations at the N-terminal MP region of TM domain, which was known to form an interchain disulfide bridge that restrains the α/β TM separation (33). Western blot under nonreducing conditions demonstrated 100% disulfide bond formation for all the mutants tested (Fig. 7A). Ligand-mimetic PAC-1 binding showed that αIIb-L959C/β3-P688C mutations reversed the activating effect of the GFFKR cysteine mutations (Fig. 7B). However, there are no obvious changes in the BM labeling for the GFFKR motif in the presence of αIIb-L959C/β3-P688C disulfide bond (Fig. 7, C and D), suggesting that the activating cysteine mutations do not change the membrane embedding of the αIIb GFFKR motif. Thus, the membrane embedding of GFFPR revealed by BM labeling should represent the resting state.

**BM labeling of β3 TM N-terminal membrane-proximal region under cell adhesion or overexpression of EGFP-TH**

During integrin-mediated cell adhesion and spreading, in addition to the ligand-induced long-range conformational changes that are transmitted to the CT through TM domain,
the tensile force generated by ectodomain interacting with immobilized extracellular ligand and CT interacting with intracellular cytoskeleton is also applied to TM domain (34), which may affect the membrane embedding of TM α-helix. We performed the BM labeling of the N-terminal MP region of β3 TM domain for the cells spreading on immobilized fibrinogen. Compared with the BM labeling of suspension cells, we consistently observed a decrease of BM labeling for the residues of β3-K689C, β3-P691C, β3-D692C, and β3-I693C that are at the β3 TM outer boundary (Fig. 8, A and B), suggesting a change of membrane embedding of β3 TM α-helix in the spreading cells. Talin1 head has been reported to induce conformational change of β3 TM domain (35). We also did BM labeling of β3 TM N-terminal cysteine mutants in the presence of overexpression of EGFP-TH. αIIb-R995A mutant was used to synergize the activating effect of EGFP-TH (14). EGFP-TH induced comparable activation of all the tested β3 cysteine mutants (data not shown). However, compared with the cells with EGFP expression, no obvious changes of BM labeling were detected for the N-terminal MP residues of β3 TM

Figure 5. Effect of TM proline mutations on αIIbβ3-mediated cell spreading. A–C, HEK293 cells stably expressing αIIb/β3 WT, αIIb/β3-L705P-A711P, or αIIb-I982P/β3 spread on immobilized PAC-1. D and E, HEK293 cells stably expressing αIIb/β3 WT or αIIb/β3-L705P-A711P spread on immobilized fibrinogen. Cells were allowed to adhere on platelets coated with 5 μg/ml PAC-1 or 25 μg/ml fibrinogen at 37 °C for 1 h and then fixed and stained with Alexa-Fluor-488-labeled AP3 for β3 detection and Alexa-Fluor-564-labeled phalloidin for F-actin detection. Nuclei were stained with DAPI in panels A–C. F, quantification of cell spreading areas in panels A–E. The averaged cell areas were calculated based on 30 to 50 cells for each of three independent repeats. Data are mean ± SD. Unpaired two-tailed t test was performed between the WT and the mutant cells.
α-helix (Fig. 8, C and D). These data suggest that mechanical force but not talin1 head may induce a change of β3 membrane embedding.

**BM labeling of free β3 TM domain in the absence of αIib subunit**

The current model suggests a separation of α and β TM domains upon integrin activation (2). The β3 α-helix has been studied as an isolated single-chain peptide (15, 20), which may represent an active form separated from αIib TM association. To investigate the membrane embedding of β3 TM α-helix by BM labeling in the absence of αIib subunit, we generated a β3 construct containing the β3-tail, TM, and CT domains with an N-terminal protein C (PC) tag, namely β3-tail-TMCT (Fig. 9 A). The β3-tail-TMCT construct could be expressed on the cell surface without αIib subunit as determined by flow cytometry (data not shown), suggesting that it may mimic a free state of β3 TM domain that is fully separated from αIib subunit. Both the N- and C-terminal MP regions of β3 TM domain were comparably studied by BM labeling for β3-tail-TMCT and β3 full-length constructs (Fig. 9, B–E). BM labeling of β3 full-length cysteine mutants coexpressed with αIib subunit shows that the N-terminal boundary of β3 TM domain is at residue β3-I693 (Fig. 9B). In contrast, the elevated BM labeling of β3-tail-TMCT suggested the changes of TM boundary and membrane embedding (Fig. 9, C and F). Consistently, compared with the β3 full-length, there was an obvious increase of BM labeling for the C-terminal residues, such as β3-R724C and β3-K725C in β3-tail-TMCT (Fig. 9, D, E, and G), suggesting a change of β3 membrane embedding. Our BM labeling data for the full-length β3 complexed with αIib indicate that the β3 TM α-helix needs to be tilted in the lipid bilayer to accommodate membrane embedding (Fig. 1E). Upon disassociation from the αIib TM domain, the β3 TM α-helix may adopt a different tilt within the cell membrane as shown by our BM labeling analysis.

**Discussion**

Mutagenesis studies have identified numerous activating mutations at the αIibβ3 TM domains (8, 11, 21), which were known to destabilize the αIibβ3 TM association. In this study, we used proline scanning mutagenesis to identify TM proline mutations that either reduce or enhance αIibβ3 inside-out activation. Proline residues present in an α-helix tend to disrupt the integrity of the rigid α-helical structure by
introducing a kink due to the interruption of backbone hydrogen bonds (25). As a result, it is expected that some proline mutations may disturb the TM association and render αIIbβ3 more active than wild type. However, it is remarkable that proline mutations at certain positions also greatly reduced αIIbβ3 inside-out activation triggered by either activating mutations or EGFP-TH. Such proline mutations are distributed at the N-terminus (β3-L698P), middle (β3-L705P and -I707P), and C-terminus (β3-A711P) of β3 TM domain (Fig. 1A), which displayed different levels of inhibitory effect on αIIbβ3 inside-out activation (Fig. 2B). Similarly, the β3 equivalent TM proline mutations also inhibited the inside-out activation of α2β1 and α5β1 integrins (Figs. 1A and 4), suggesting a generalizable mechanism. A remarkable difference between β3 and αIIb TM domains is that most of the tested αIIb TM proline mutations increased αIIbβ3 inside-out activation except one inhibitory proline mutation, αIIb-I982P present in the C-terminal portion of αIIb TM α-helix, suggesting that the αIIb TM domain is less tolerant to the α-helix disrupting proline mutations than β3 TM domain. These data demonstrate that a continuous α-helical structure is required for both α and β integrin TM domains to maintain a normal function of integrin inside-out activation.

The inhibitory β3-A711P mutation was previously identified by random mutagenesis (15). Structural studies by NMR demonstrated a kink conformation induced by β3-A711P in the isolated β3 TM fragment and in complex with αIIb TM domain (15, 36). The structural and thermodynamic analysis suggests that the β3-A711P mutation stabilizes the αIIbβ3 association probably due to the kink-mediated repacking of αIIbβ3 TM heterodimer (36). Besides β3-A711P, we identified three more β3 TM proline mutations, β3-L698P, β3-L705P, and β3-L707P, all of which reduce the inside-out activation of αIIbβ3. We also found that these proline mutations have different levels of inhibitory effect, which is similar to the effect of activating mutations. Similar to β3-A711P, these inhibitory proline mutations are likely to stabilize the αIIbβ3 TM association by altering the packing of β3 TM α-helix on αIIb. The stabilized αIIbβ3 TM interaction then increases the energy barrier for activation. This is consistent with our data showing that the αIIbβ3 activation in the presence of β3-L705P-A711P mutant requires the highly activating mutation αIIb-F992A-F993A (Fig. 2C).

The physiological inside-out activation of integrin is initiated by the binding of talin head domain to the β CT, which disrupts the α-β TM association leading to conformational activation of ectodomain (37). It was suggested that the binding of talin head may change the topology of β3 TM domain, as determined using the isolated β3 TM-CT fragment in model membranes (35). We found that the β3-L705P and -A711P mutations
completely abolished talin1-head-induced αIIbβ3 activation. Given that the proline-mediated kink formation disrupts the integrity and rigidity of β3 TM α-helix, it may interrupt the talin1-head-induced topology change of β3 TM domain. We tested this possibility by introducing a flexible loop insert at the intracellular boundary of β3 TM domain, where it does not affect talin1 head binding but interrupts the rigid α-helical structure. These α-helix disruption mutations also dramatically reduced talin1-head-induced αIIbβ3 activation, suggesting that a rigid α-helical structure is required for talin head-induced conformational change of β3 TM domain.

Another key observation in our study is that the inhibitory β3 TM proline mutations block αIIbβ3-mediated cell spreading, a hallmark of integrin outside-in signaling. Our previous studies using disulfide cross-linking demonstrated that the separation or conformational change of αIIbβ3 TM domain is required for integrin outside-in signaling (38). Given that the proline mutations stabilize the TM association as discussed above, the extracellular ligand-induced separation or conformational change of the TM domain may be inhibited by the stabilizing TM proline mutations. However, the stabilizing αIIb-I982P mutation exerted no effect on cell spreading. These data suggest that the integrity of β3 TM α-helical structure is critical for the outside-in signal transmission. During outside-in signaling, integrin transmits both chemical and mechanical signals across the cell membrane (34). The mechanical stress generated by the binding of extracellular ligands to the ectodomain and cytoskeleton molecules to the β CT exerts tensile forces.
force to the TM domain. Our data suggest that a continuous and rigid α-helical structure of β3 TM domain is critical for the sensing and regulation of molecular tension.

The structures of αIIbβ3 TM-CT domains have been studied as isolated fragments in the model membrane or detergent micelles (10, 12, 13, 19, 20). However, complexed results were obtained about the TM topology within the membrane and the potential structural changes upon integrin activation. This is possibly due to the absence of integrin extracellular domains and the non-native membrane environment in those studies. An elegant study using a cysteine scanning accessibility method investigated the topology of intracellular borders of both αIIb and β3 TM domains in the context of full-length integrin on the cell membrane (17). Using the same approach, we analyzed both the extracellular and intracellular membrane interfaces of αIIbβ3 TM domains in the resting and active conditions. In our study, we used both membrane-permeable and -impermeable biotin-maleimide and performed the labeling on ice or at 4 °C to minimize the effect of membrane thermodynamics. Also, we used 2-BP to inhibit the potential intracellular cysteine palmitoylation that is known to block the -SH group for maleimide labeling. This enables us to detect the BM labeling of the intracellular cysteine mutations such as β3 D723C, R724C, K725C, and E726C, which were not detected in the previous study when 2-BP was not used (17). Our study allows us to define both the outer and inner membrane boundaries of αIIbβ3. For β3 integrin, the membrane-embedded portion is I693-H722, which is seven residues longer than the predicted 23-residue TM domain. Given the continuous α-helical structure of this 30-residue fragment, it requires a tilt of β3 TM domain to match the thickness of lipid bilayer (Fig. 1E). This is in general consistent with the model suggested based on structural studies of αIIbβ3 TM heterodimer by NMR in lipid bicelles (13).

Our BM labeling results suggest the membrane-embedded portion for αIIb is W968-R995, showing that the conserved

Figure 9. BM labeling of the cysteine substitutions in the β3 TM domain that was fully separated from the αIIb subunit. A, design of β3-tail-TMCT construct. A protein C tag was added to the N-terminus of β3-tail-TMCT construct. B and D, BM labeling of the cysteine substitutions in the TM domain of full-length β3 coexpressed with αIIb in HEK293FT cells. C and E, BM labeling of the cysteine substitutions in the TM domain of β3-tail-TMCT expressed in HEK293FT cells. F, quantification of western blot data of panels B and C. G, quantification of western blot data of panels D and E. Data are presented as BM signal as a percentage of total β3 signal.
Topology of integrin transmembrane α-helix

GFFKR motif is masked by a membrane except for K994. This is consistent with a reverse turn conformation of GFFKR as determined by structural studies (11, 13), which allows the GFF and R to be buried while exposing K994 (Fig. 1E). However, due to the activating effect of GFFKR cysteine substitutions, it was suggested by the previous study that the BM labeling pattern of the GFFKR motif reflects an active conformation (17). To compensate for the activating effect of GFFKR cysteine mutations, we introduced an interchain disulfide bridge at the extracellular membrane-proximal portion to restore αιββ3 to the resting state by stabilizing TM association. This activation-reversing mutation did not change the pattern of BM labeling of the GFFKR motif, suggesting that the GFFKR motif is membrane embedded in the resting state. In contrast to the β3 TM domain, the reverse turn conformation of the GFFKR motif allows the αιβ TM α-helix to adopt a perpendicular orientation in the lipid bilayer (Fig. 1E).

The membrane tilt of β3 TM domain has been suggested to play a role in αιββ3 inside-out activation (15, 35). How the TM tilt is determined is still a debate (18). NMR studies of the monomeric β3 TM-CT fragment with and without β3-K716E mutation revealed a change of membrane embedding, leading to the conclusion that the β3 TM tilt is determined by the snorkeling of β3-K716 side chain to form a lysine/phospholipid ion pair (15). However, the observed change of membrane embedding in the presence of β3-K716E was demonstrated to be an experimental artifact by another NMR study (18), questioning the β3-K716 snorkeling model in defining β3 TM tilt. Using a monomeric β3-tail-TMCT construct composed of β3 β-tail and TM-CT domains expressed on the cell surface without αιβ, our BM labeling experiments revealed a different membrane embedding compared with αιββ3 heterodimer, which shows an increased exposure of both extracellular and intracellular borders, indicating the change of TM tilt. This data suggests that the β3 TM tilt is very likely to be determined by the association with αιβ domain. Structural analysis of αιββ3 TM heterodimer reveals two close contacts within cell membrane, one formed by the αιβ GXXG motif, the other formed by the αιβ GFKKR (Fig. 1E). The β3-K716 side chain hydrogen bonding with the backbone oxygens of αιβ GFKKR motif (11), along with the close packing at the αιβ GXXG motif favors the tilt topology of β3 TM domain (Fig. 1E).

The activation inhibitory proline mutations may further stabilize the β3 TM tilt and αιββ3 TM association. A perpendicular and rigid α-helical structure of αιβ TM domain is critical in maintaining the resting state of αιββ3 since our mutagenesis study shows that αιβ is much less tolerant to TM proline mutations than β3.

Current model suggests that αιββ3 activation involves a disruption of α-β TM association, which may alter the TM topology of either αιβ or β3, or both (4, 17). The TM association can be disturbed by many modifications including disruptive mutations, talin and kindlin binding, and even membrane tension (39), which all lead to integrin activation. Unexpectedly, our BM labeling experiments failed to detect any significant topology changes upon soluble ligand binding or overexpression of talin1 head domain. A previous study using the same approach also did not show the change of BM labeling for the intracellular portion of β3 TM domain in the presence of the activating αιβ-F992A-F993A mutation (17). A possible reason is that the BM labeling approach is not sensitive enough to detect the subtle and/or transient topology changes of the TM domain. The change of membrane embedding depends on the type of movement of TM α-helix relative to the lipid bilayer. A tilt or piston-like movement can lead to the change of membrane embedding, while a small rotation may not affect the embedding. All of these structural changes are expected to disrupt α-β TM interaction, leading to integrin activation. A study using membrane-embedded monomeric β3 TM-CT fragment bearing environment-sensitive fluorophores showed an increase of membrane embedding upon talin1 head binding (35). However, our BM labeling of monomeric β3-tail-TMCT shows a change of membrane embedding even without talin head, suggesting that the β3 TM domain adopts a different topology in the absence of αιβ TM domain. The observation of talin-induced topology change of monomeric β3 TMCT peptide in the absence of αιβ may not represent the physiological condition. Nonetheless, the topology changes may happen when tension applied to the β3 TMCT during cell adhesion and spreading as shown by our current study. A topology change of αιβ TMCT may also contribute to αιββ3 activation as suggested by the previous study using BM labeling (17). The membrane distal region of αιβ CT may be required for the topology change (14). A topology change of αιβ TM α-helical structure is more prone to disturb the α-β TM interaction according to our proline mutagenesis study. Alerting the αιβ TM structure may require a lower energy cost than changing the β3 TM tilt. A subtle rotation of TM α-helix will be more efficient to disturb TM association than changing the membrane embedding. Unfortunately, this type of TM structural change cannot be detected by BM labeling. Despite the substantial efforts on αιββ3 TM studies, more questions remain to be answered using innovative approaches.

Experimental procedures
DNA constructs and mutagenesis

The plasmids for αιββ3, β3, αι, α3, and β3 integrins were as described before (14, 40–42). The DNA construct of EGFP-tagged mouse talin1-head domain was as described (43). The β3-tail-TMCT construct composed of β3 β-tail, TM, and CT domains (residues P605-T762) was cloned into a modified pIREs2-EGFP vector with an N-terminal signal peptide derived from murine IgG kappa V followed by a protein C epitope tag. Mutations were made using site-directed mutagenesis with the QuikChange kit (Agilent Technologies). All the introduced mutations were confirmed by DNA sequencing.

Antibodies and ligands

PAC-1 (BD Bioscience) is a ligand-mimetic mAb (IgM) for the activated αιββ3 integrin (44). AP3 is a conformation-independent anti-β3 mAb (45) and was conjugated with
Alexa Fluor 488 (Thermo Fisher Scientific). 10E5 is an anti-αILβ3 complex specific mAb (31, 46). 314.5 is an anti-αIL mouse mAb that binds to calf-2 domain. H-96 is a rabbit anti-β3 polyclonal IgG (Santa Cruz Biotechnology). H-160 is a rabbit anti-αIL polyclonal IgG (Santa Cruz Biotechnology). PE-labeled MAR4 (BD Bioscience) is a nonfunctional anti-β1 mAb. PE-labeled TS2/4 (BioLegend) is a nonfunctional anti-αL mAb (47). KIM127 that binds to I-EGF-2 domain and mAb 24 (m24) that binds to β1 domain are anti-β2 conformation-dependent mAbs (28, 29, 48, 49). Rabbit anti-protein C matrix beads were from Enzyme Research Laboratories. Human fibrinogen (Plasminogen, von Willebrand Factor, and Fibronectin Depleted) was from Enzyme Research Laboratories. Human fibrinogen (Plasminogen, von Willebrand Factor, and Fibronectin Depleted) was from Enzyme Research Laboratories. Human fibrinectin and human ICAM-1 with a C-terminal Fc tag of human IgG1 (ICAM-1-Fc) were from Sigma-Aldrich and Sino-Biological, respectively.

**Ligand and antibody-binding assay**

The detailed protocol of ligand and antibody-binding assay for integrin was as we published before (50). HEK293FT cells were used for the transient transfection of αILβ3 and αIL β2 integrins. HEK293FT- αILβ1-KO cells were used for αILβ1 integrin (51). The ligand binding to integrin-transfected cells was performed in HBSSG buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 5.5 mM glucose, and 1% BSA) plus 1 mM CaCl2 and 1 mM MgCl2 (Ca/Mg). The KIM127 and m24 binding were from Sigma-Aldrich. IRDye 800-labeled streptavidin and IRDye 680-labeled goat anti-rabbit (or mouse) IgG were from LI-COR Biosciences. Human fibrinogen (Plasminogen, von Willebrand Factor, and Fibronectin Depleted) was from Enzyme Research Laboratories. Human fibrinectin and human ICAM-1 with a C-terminal Fc tag of human IgG1 (ICAM-1-Fc) were from Sigma-Aldrich and Sino-Biological, respectively.

**Cell adhesion and spreading assay**

HEK293 cells were used to generate stable transfections of wild type and proline mutants of αILβ3. The single cell clones that have comparable surface expression of αILβ3 were selected for cell adhesion and spreading assay as described before (38, 40). In brief, the Delta T dish (Biotuchs) was coated with 5 μg/ml PAC-1 or 25 μg/ml fibronogen in PBS buffer at 4 °C overnight and then blocked with 1% BSA at 37 °C for 1 h. The cells in suspension were washed once with DMEM without serum and seeded onto the Delta T dish at 37 °C for 1 h. The attached cells were washed with DMEM and fixed with 3.7% formaldehyde in PBS at 25 °C for 5 min. The fixed cells were first immunostained with mAb AP3 and then permeabilized with 0.05% Triton X-100 in PBS, followed by staining with Alexa Fluor 546 labeled phalloidin (Thermo Fisher Scientific) and DAPI. Cells were imaged with EVOS digital inverted fluorescence microscope. The cell areas of 40 to 50 total cells for each independent experiment were measured using ImageJ and averaged.

**Biotin maleimide labeling and immunoprecipitation**

The cell-membrane-permeable biotin-maleimide was from Sigma-Aldrich. The cell-impermeant maleimide-PEG11-biotin was from Thermo Fisher Scientific. The concentrations of biotin-maleimide and maleimide-PEG11-biotin and the labeling time were optimized using the well-exposed and well-embedded cysteine mutants. The labeling temperature was also compared between 25 °C and 0 °C (or 4 °C). We also compared the maleimide-PEG11-biotin and biotin-maleimide for labeling the cysteine mutations at the extracellular portion of the TM domain in intact cells, or the cysteine mutations at the intracellular portion of the TM domain after physically breaking the cell membrane. No obvious differences were found among the different conditions. We used maleimide-PEG11-biotin for labeling the extracellular TM cysteine mutations and biotin-maleimide for the intracellular TM-CT cysteine mutations. The labeling was performed following the published protocol with some modifications (17). HEK293FT cells cultured in a 12-well plate were transfected with integrin constructs. For the transfection of intracellular cysteine mutants, the cells were pretreated with 15 μg/ml 2-bromopalmitate (Sigma-Aldrich) to inhibit the potential cysteine palmitoylation (11). The transfected cells were suspended and washed with 0.5 ml PBSCM (140 mM NaCl, 3 mM KCl, 6.5 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.0 plus 1 mM CaCl2, and 1 mM MgCl2). The cells suspended in 0.5 ml PBSCM were labeled with 0.2 mM biotin-maleimide or 0.1 mM maleimide-PEG11-biotin on ice for 30 min and then stopped by adding 1 mM glutathione and washed once with 0.5 ml PBSCM. The cells were lysed in 0.5 ml IPB buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100), 0.5% sodium deoxycholate, plus 1 mM CaCl2, and 1 mM MgCl2 containing 0.2% BSA and protease inhibitors cocktail (Roche Applied Science) on ice for 10 min. The cell lysates were cleared by centrifugation at 15,000 rpm for 15 min. The proteins were immunoprecipitated for 3 h at 4 °C by mAb 10E5 and protein A agarose beads for αILβ3 or anti-PC matrix beads for β3-tail-TMCT.

For the labeling in the presence of the RGD-mimetic ligand epitifibatide, the cells were incubated with 20 μM epitifibatide at 25 °C for 15 min and then labeled on ice for 30 min. For the labeling in the presence of EGFP-TH, the cells were cotransfected with αILβ3 plus EGFP-TH or EGFP. For the labeling of spreading cells, the cells suspended in DMEM without serum were allowed to spread on the plate coated with 50 μg/ml fibrinogen at 37 °C for 1 h and then washed with PBSCM and labeled on-site at 4 °C for 30 min.

**SDS-PAGE and immunoblotting**

The immunoprecipitated proteins were resolved on 7.5% or 12% SDS–polyacrylamide gels under reducing or nonreducing conditions and transferred to PVDF membranes. The membranes were blocked with Intercpt (PBS) blocking buffer.


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(LI-COR Biosciences) at 25 °C for 1 h and then incubated for 1 h at 25 °C with 1 μg/ml each of 314.5 (for αIIb), H-160 (for αIIa), H-96 (for β3), AP3 (for β3 under nonreducing condition), or anti-PC (for β3-tail-TMCT) diluted in Interceptor (PBS) blocking buffer plus 0.1% SDS and 0.1% Tween 20. The membranes were washed once with 10 ml TBS-T buffer for 15 min and then washed three times with TBS-T buffer for 5 min each and then incubated at 25 °C for 1 h with IRDye 800 streptavidin plus IRDye 680 goat anti-rabbit (or mouse) IgG diluted in Interceptor (PBS) blocking buffer plus 0.1% SDS and 0.1% Tween 20. The membranes were washed and scanned with the Odyssey Infrared Imaging System (LI-COR Biosciences).

**Statistical analysis**

Statistical analysis was carried out on at least three individual datasets and analyzed with GraphPad Prism software. Unpaired two-tailed t test was performed between control and treated experimental groups. p-values ≤0.05 were considered significant.

**Data and materials availability**

All data are available in the main text.

**Acknowledgments**—We thank Drs Chuanmei Zhang and Aye Myat Myat Thinn for preliminary assays at the early stage of this study. We thank Drs Barry Coller for providing 10E5 mAb, Peter Newman for AP3 mAb, Dan Bougie and Richard Aster for 314.5 mAb. We thank Dr David Calderwood for providing EGFP-TG construct.

**Author contributions**—J. Z. conceptualization; Z. W. and J. Z. data curation; Z. W. and J. Z. formal analysis; J. Z. funding acquisition; Z. W. and J. Z. investigation; Z. W. and J. Z. methodology; J. Z. project administration; J. Z. resources; J. Z. supervision; J. Z. validation; J. Z. writing—original draft; Z. W. and J. Z. writing—review and editing.

**Funding and additional information**—This research was supported by NIH R01 HL131836 (to J. Z.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Conflict of interest**—Authors declare that they have no competing interests.

**Abbreviations**—The abbreviations used are: 2-BP, 2-bromopalmitate; BM, biotin-labeled maleimide; CT, cytoplasmic tail; MP, membrane-proximal; TM, transmembrane.

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