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**Recommended Citation**

Wang, W., Zhu, J., Springer, T., & Luo, B. (2011). Tests of integrin transmembrane domain homo-oligomerization during integrin ligand binding and signaling. *Journal of Biological Chemistry, 286* (3), 1860-1867. [https://doi.org/10.1074/jbc.M110.193797](https://doi.org/10.1074/jbc.M110.193797)
Tests of Integrin Transmembrane Domain Homo-oligomerization during Integrin Ligand Binding and Signaling*S

JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 3, pp. 1860 –1867, January 21, 2011

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Integrin transmembrane (TM) and/or cytoplasmic domains play a critical role in integrin bidirectional signaling. Although it has been shown that TM and/or cytoplasmic α and β domains associate in the resting state and separation of these domains is required for both inside-out and outside-in signaling, the role of TM homomeric association remains elusive. Formation of TM homo-oligomers was observed in micelles and bacterial membranes previously, and it has been proposed that homomeric association is important for integrin activation and clustering. This study addresses whether integrin TM domains form homo-oligomers in mammalian cell membranes using cysteine scanning mutagenesis. Our results show that TM homomeric interaction does not occur before or after soluble ligand binding or during inside-out activation. In addition, even though the cysteine mutants and the heterodimeric disulfide-bounded mutant could form clusters after adhering to immobilized ligand, the integrin TM domains do not form homo-oligomers, suggesting that integrin TM homomeric association is not critical for integrin clustering or outside-in signaling. Therefore, integrin TM homo-oligomerization is not required for integrin activation, ligand binding, or signaling.

Integrins are cell adhesion molecules that are essential for many biological functions such as cell migration, survival, and differentiation. These functions are accomplished by integrin bidirectional signaling across the cell membrane. Inside-out activation occurs when specific intracellular molecules such as talin and kindlin bind to the integrin cytoplasmic domain, leading to integrin conformational change and therefore high affinity for extracellular ligands. Then, binding of multimeric extracellular ligands results in outside-in signaling that is critical for many cellular processes. It has been shown that integrin bidirectional signal transduction requires integrin structural change and lateral distribution (clustering) (1, 2).

Integrins are type I transmembrane (TM)² proteins consisting of two noncovalently associated α and β subunits, each with a large extracellular domain, a single-span TM domain, and a short cytoplasmic domain. Recent structural studies have greatly advanced our understanding of integrin conformational change during inside-out activation (3–9). Even though relatively short, the integrin TM/cytoplasmic domains play a crucial role in this process. It has been shown that the association of α and β subunit TM/cytoplasmic domains is critical for stabilizing integrins in the resting state (10–16). When induced by binding of the β subunit cytoplasmic domain through talin or other intracellular molecules, the TM/cytoplasmic domains separate (17, 18), driving integrin extension and shifting the ligand binding α/β headpiece to an open, high affinity conformation (7, 19). Recently, the structure of the TM/cytoplasmic domains in the resting state was proposed based on Rosetta computational modeling and experimental data using intact integrins on mammalian cell surface (16). In this structure, the αIIb GXXG motif and its counterparts in the β3 TM domains associate with ridge-in-groove packing, and the αIIb GFFKR motif and the β3 Lys716 in the cytoplasmic segments play a critical role in α/β association. The structures of complex and monomeric α and β subunit TM/cytoplasmic domains have also been solved by NMR (8, 9, 20, 21). These studies have shed light on the structural basis of integrin TM/cytoplasmic domain signaling across the plasma membrane (22).

In contrast to the role of heterodimeric TM/cytoplasmic domain association and dissociation, that of homo-oligomerization of integrin TM domains in integrin signaling remains elusive. In 2001, an NMR study of this region by Li et al. failed to detect heterodimeric association between the α and β subunit TM/cytoplasmic domains using TM/cytoplasmic fragment peptides in micelles. Instead, they observed that the α and β fragments tend to form homodimers and homotrimers, respectively (23). Later, αIIb and β3 TM helices were confirmed to form homo-oligomers in bacterial membranes using the TOXCAT assay (24, 25). The α and β homomeric interactions have been widely studied by computational modeling (26–28). These studies showed that the homo-oligomerization interface and the heterodimerization motifs largely overlap, but it seems that homomeric interaction is less specific than the heterodimeric interaction (25). In 2003, asparagine mutagenesis in the TM region of β3 subunit (with most experiments on the mutation β3G708N) suggested that TM homo-oligomerization contributes to integrin activation and clustering (29). However, the mutation β3G708N, which was reported to enhance trimerization in detergent and increase ligand binding avidity in the transfected CHO cells (29), was later found to activate the integrin by changing ligand binding
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affinity rather than valence (13). Furthermore, mutations that disrupted homodimerization of integrin TM domains, which also disrupted heterodimerization because the two interfaces overlap, were shown to activate integrins for ligand binding, suggesting that TM domain separation is sufficient to activate integrins (14). Therefore, it was proposed that integrin TM homo-oligomerization is not a critical step for inside-out activation, but instead, it may help to stabilize the integrin in the high affinity state and be important for outside-in signaling (24).

It has been shown that integrin outside-in signaling requires both conformational change and clustering of integrins. However, the mechanism of how integrins transmit these signals across the plasma membrane through the TM/cyttoplasmic domains remains unknown. More specifically, it is unclear whether integrin TM homo-oligomerization plays any role in integrin clustering and signaling, even though it has been proposed that it provides structural basis for this process (29, 30). Importantly, although α and β TM homo-oligomerization was found in micelles and bacterial cell membranes (14,23,25,29,31), it has never been observed in mammalian cell membrane using full-length integrins. On the contrary, the TM heterodimeric but not homomeric association of integrin fragments was detected using mini-integrin affinity capture method in mammalian cell membranes (32). In this paper, we tested whether integrin TM domains form homo-oligomers in mammalian cell membranes using disulfide scanning of intact integrin αIIbβ3. Our results show that integrin TM domains do not form homomeric integrins before or after soluble ligand binding or during integrin inside-out and outside-in signaling.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Transient Transfection—Plasmids coding for full-length human αIIb and β3 were sub-cloned into pEF/V5-HisA and pcDNA3.1/Myc-His(+), respectively (33). Amino acid substitution in TM and TM proximal regions to cysteine was made using site-directed mutagenesis with the QuikChange kit (Stratagene). Constructions were transfected into 293T cells (American Type Culture Collection) using a FuGENE transfection kit (Roche Applied Science) according to the manufacturer’s instructions.

Immunoprecipitation—Twenty-four hours after transfection, 293T cells were treated with 20 μg/ml 2-bromopalmitate, metabolically labeled with [35S]cysteine/methionine for 1.5 h before adding chase medium containing 500 μg/ml cysteine and 100 μg/ml methionine, and cells were cultured for 17 h overnight (12). Then, cells were detached and suspended in Tris-buffered saline (TBS) (106 cells in 100 μl) supplemented with 5 mM Ca2+, 1 mM Mn2+ with 3 mg/ml fibrinogen or 1 mM Mn2+ with 50 μM RGD peptide and incubated at room temperature for 30 min. Then, saponin was added as control before seeding. 400 nM cytochalasin D was added 3.7% formaldehyde in PBS at room temperature for 10 min or 3.7% formaldehyde in PBS at pH 7.4 followed by blocking with 1% BSA at room temperature for 1 h. The transiently transfected HEK293T cells were detached by trypsin/EDTA and washed three times with DMEM. Cells were seeded on fibrinogen-coated dishes. After incubation at 37 °C for 1 h, cells were washed 3 times with PBS and fixed with 3.7% formaldehyde in PBS at room temperature for 10 min or for integrin clustering, 10 μg/ml anti-β3 mAb AP3 was added at room temperature for 30 min followed by staining with 10 μg/ml FITC-conjugated goat anti-mouse IgG for 30 min at room temperature before fixation. 400 nM cytochalasin D was added as control before seeding.

Differential interference contrast imaging was conducted on a Leica TCS SP2 spectral confocal system, coupled to a DM IRE2 inverted microscope with 63× oil objective. For the quantification of cell spreading area, outlines of 100 randomly selected adherent cells were generated, and the number of pixels contained within each of these regions was measured using ImageJ software (National Institutes of Health, Bethesda, MD).
Cell Adhesion—Cell adhesion on immobilized human fibrinogen was assessed by the measurement of cellular lactate dehydrogenase activity as described (1). Briefly, cells suspended in HEPES-buffered saline supplemented with 5.5 mM glucose, 1% bovine serum albumin, and 1 mm Ca$^{2+}$ were added into flat bottom 12-well plates (1 $\times$ 10$^5$ cells/well) that had been precoated with 20 $\mu$g/ml fibrinogen and blocked with 1% BSA. After incubation at 37 °C for 1 h, wells were washed three times with HEPES-buffered saline supplemented as indicated above. Remaining adherent cells were lysed with 1% Triton X-100, and lactate dehydrogenase activity was assayed using the Cytotoxicity Detection Kit (lactate dehydrogenase) (Roche Applied Science) according to the manufacturer’s instructions. Cell adhesion was expressed as a percentage of bound cells relative to total input cells.

RESULTS

Integrin TM Domains Do Not Form Homo-oligomers before or after Soluble Ligand Binding—Previously, cysteine mutagenesis and heterodimeric disulfide scanning were used to identify the integrin $\alpha$IIb and $\beta$3 TM regions formed homomeric disulfide bonds before or after soluble ligand binding. Here, we used single-cysteine mutations of the $\alpha$IIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\alpha$IIbW967C, none of the cysteine mutants of the $\alpha$IIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$3 TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$891C $\beta$892C $\beta$693C $\beta$894C lysates from $^{35}$S-labeled HEK293T cell transfectants were immunoprecipitated with mAb AP3. Precipitates were subjected to nonreducing 7.5% SDS-PAGE and fluorography. Except for $\alpha$IIbW967C, none of the cysteine mutants of the $\alpha$IIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$3 TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$891C $\beta$892C $\beta$693C $\beta$894C lysates from $^{35}$S-labeled HEK293T cell transfectants were immunoprecipitated with mAb AP3. Precipitates were subjected to nonreducing 7.5% SDS-PAGE and fluorography. Except for $\alpha$IIbW967C, none of the cysteine mutants of the $\alpha$IIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$3 TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$891C $\beta$892C $\beta$693C $\beta$894C lysates from $^{35}$S-labeled HEK293T cell transfectants were immunoprecipitated with mAb AP3. Precipitates were subjected to nonreducing 7.5% SDS-PAGE and fluorography. Except for $\alpha$IIbW967C, none of the cysteine mutants of the $\alpha$IIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$3 TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$891C $\beta$892C $\beta$693C $\beta$894C lysates from $^{35}$S-labeled HEK293T cell transfectants were immunoprecipitated with mAb AP3. Precipitates were subjected to nonreducing 7.5% SDS-PAGE and fluorography. Except for $\alpha$IIbW967C, none of the cysteine mutants of the $\alpha$IIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$3 TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$891C $\beta$892C $\beta$693C $\beta$894C lysates from $^{35}$S-labeled HEK293T cell transfectants were immunoprecipitated with mAb AP3. Precipitates were subjected to nonreducing 7.5% SDS-PAGE and fluorography. Except for $\alpha$IIbW967C, none of the cysteine mutants of the $\alpha$IIb TM regions formed homomeric disulfide bonds before or after soluble ligand binding. $\beta$3 TM regions formed homomeric disulfide bonds before or after soluble ligand binding.
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suggested by the TOXCAT assay (24, 25) and predicted by computer modeling (26–28), the \( \alpha \)IIb TM helix forms homodimers, and the \( \beta \)3 TM helix forms heterotrimers, using surfaces that are similar to those in the heterodimeric interface. If this homomeric interface is actually formed in the mammalian cell membrane, we expected that some single-cysteine mutations of these residues would form homodimeric disulfide bonds in the presence of an oxidation catalyst such as Cu-phenanthroline. In addition, 2-bromopalmitate was used to block cysteine palmitoylation, which can inhibit disulfide formation; and saponin was used to increase the permeability of Cu-phenanthroline and the efficiency of disulfide bond formation as described previously (16). To confirm the efficiency of disulfide bond formation in the presence of Cu-phenanthroline, several cysteine pairs (one from \( \alpha \) and the other from \( \beta \)) at the TM or TM proximal region, which have been shown to form heterodimeric disulfide bonds (12, 16), were used as controls.

As shown in Fig. 1, the cysteine pair \( \alpha 971C/\beta 967C \) formed a heterodimeric disulfide bond in the presence of Cu-phenanthroline (Fig. 1B), as described previously (12). By contrast, none of the single-cysteine mutants of \( \alpha \)IIb TM regions formed homomeric disulfide bonds under these conditions except \( \alpha \)IIbW967C (Figs. 1B and 2). As reported previously (12, 13), \( \alpha \)IIbW967C, when co-transfected with wild-type \( \beta \)3, formed a homomeric disulfide bond in the resting state, and therefore, the \( \alpha \)IIb\( \beta \)3 integrin associated to form a disulfide-bridged tetramer (\( \alpha \)IIbW967C/\( \beta \)3). This residue is located outward, away from the heterodimeric interface or predicted homodimeric interface (12, 13). When DTT was added, the disulfide bond was reduced. After DTT was removed and Cu-phenanthroline was added to catalyze disulfide formation, only a trace amount of homomeric disulfide was formed on the cell surface (Fig. 2A). We further traced the disulfide bond formation of \( \alpha \)IIbW967C mutant after \( \beta \)3S labeling by lysing cells and immunoprecipitating the protein at different time points (Fig. 2B). After 30 min of labeling, \( \alpha \)IIb precursor was formed. Then after 1 h, mature \( \alpha \)IIb subunit was formed, and a trace amount of disulfide-bonded \( \alpha \)IIb was observed. In 1.5 h, a significant amount of disulfide-bonded dimeric \( \alpha \)IIbW967C was formed. This suggests that the homomeric disulfide bond is formed during biosynthesis and post-transla-

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**FIGURE 2.** The homomeric disulfide bond of the \( \alpha \)IIbW967C was formed during biosynthesis. A, formation of the homomeric disulfide bond after DTT treatment. The \( \alpha \)35S-labeled cells were treated with or without 2 mM DTT at 37 °C for 10 min. After washing with TBS, DTT-treated cells were incubated with or without Cu-Phenanthroline. B, tracing the formation of disulfide bond of \( \alpha \)IIbW967C mutant after labeling. The cells were lysed at different periods of time after \( \beta \)3S labeling, and integrin \( \alpha \)IIb\( \beta \)3 was immunoprecipitated and subjected to 7.5% nonreducing SDS-PAGE. The additional bands of the \( \alpha \) and \( \alpha / \alpha \) are probably due to the precursors of the \( \alpha \)IIb subunit.
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FIGURE 3. Integrin TM domains do not form homo-oligomers during inside-out activation. Samples were processed as in Fig. 1 and subjected to nonreducing 7.5% SDS-PAGE and fluorography. A, cysteine mutations of the αIIb GAAKR mutant were co-transfected with wild-type β3 integrin. No homomeric disulfide bond was formed for any of the αIIb cysteine mutants. B, cysteine mutations of the β3 TM region were co-transfected with the αIIb GAAKR mutant. No homomeric disulfide bond was formed for any of the β3 cysteine mutants.

gest that during integrin inside-out activation across the mammalian cell membrane, TM homomeric association does not occur. Therefore, the TM homo-oligomerization does not play any role in this process.

Integrin TM Domains Do Not Form Homo-oligomers after Adhering to Immobilized Fibrinogen—After binding to immobilized ligands, integrins on the mammalian cell surface will transmit outside-in signals. We carried out a series of experiments to address whether TM homomers form during this process. We randomly chose two αIIb and two β3 cysteine mutants and used them for cell adhesion and cell spreading assay. HEK293T cells transiently transfected with wild-type and mutant αIIbβ3 were seeded on fibrinogen precoated dish surfaces at 37 °C for 1 h. The amount of adherent cells was assessed by quantifying the cellular lactate dehydrogenase activity. All selected mutants could adhere to the immobilized fibrinogen similarly to the wild-type receptor, whereas the disulfide-bounded mutant (α968C/β693C) showed much less adhesion, and DTT treatment which was used to disrupt the disulfide bond was able to recover its adhesion to the similar level to the wild-type with DTT (Fig. 4A). To test whether the single-cysteine mutations could affect cell spreading, HEK293T transient transfectants were coated on the immobilized fibrinogen at 37 °C for 1 h, followed by fixation and microscopic analysis. Cells transfected with all single-cysteine mutants demonstrated cell spreading to a level similar to those transfected with the wild-type receptor. By contrast, the disulfide-bounded mutant (α968C/β693C) showed no spreading, and DTT treatment restored cell spreading (Fig. 4B and C). These results confirmed that dissociation of the TM heterodimers is required for integrin outside-in signaling as described previously using CHO cell transfectants (1). We also chose four cysteine mutants (αG972C, αG976C, βL705C, and βL712C), which were proposed to be critical for the homomeric interactions (25, 31), and used them for the cell spreading assay. Cells transfected with all mutants showed cell spreading similar to those transfected with the wild-type integrin (data not shown). Taken together, our results indicate that the single-cysteine mutations in this region do not affect the integrin adhesion to immobilized fibrinogen, nor do they affect outside-in signaling.

It is widely believed that lateral association (i.e. “clustering”) of integrin heterodimers, which occurs as a consequence of multivalent ligand binding (38, 39), plays a major role in outside-in signaling (40–42) (for review, see Ref. 43). It was also shown that ligand binding can directly lead to and stabilize separation of integrin cytoplasmic domains (44), and this integrin conformational change is critical for outside-in signaling as well (1). However, it remains unclear whether the integrin TM homomeric interaction after the TM helix separation is critical for integrin clustering. To assess formation of integrin clustering, HEK293T transfectants were allowed to adhere to fibrinogen-coated substrates followed by fixation and staining with fluorescent anti-αIIbβ3 antibodies. Under these conditions, wild-type integrins and all cysteine mutants could be readily detected in clustered patterns (Fig. 5). Interestingly, although the disulfide-bounded α968C/β693C mutant is defective for cell spreading, it formed similar cell clusters as others, in contrast to the negative control in which the actin filament disruptor cytochalasin D prevented integrin clustering (Figs. 4 and 5).

We then further determined, under the same conditions, whether integrin TM domains formed homo-oligomers in the plasma membrane. HEK293T cells transfected with a variety of αIIb and β3 cysteine mutants were seeded on a fibrinogen substrate at 37 °C for 1 h. After cells fully adhered and spread on immobilized fibrinogen, Cu-phenanthroline was added to catalyze disulfide bond formation. Four cysteine mutation pairs, α968C/β693C, α971C/β697C, α972C/β697C, and α955C/β723C, were used as controls to confirm the efficiency
of oxidation. Under this condition, four cysteine pairs formed disulfides efficiently. By contrast, none of the αIb or β3 TM cysteine mutations formed a homomeric disulfide bond (Figs. 6 and supplemental Fig. 3). Because these integrins adhered to the immobilized fibrinogen, clustered on the cell surface and transmitted outside-in signaling leading to cell spreading, the results suggest that homomeric association of the integrin TM domains is not important for integrin functions. Similar results were obtained when CHO cells were used (data not shown), suggesting that integrin TM/cytoplasmic domains do not form homomeric interaction during integrin outside-in signaling in mammalian cells.

DISCUSSION

It has been shown that TM heterodimeric association stabilizes integrins in the resting state; when integrins are activated by intracellular signals, two TM/cytoplasmic tails separate, leading to conformational change of the ligand binding extracellular regions, resulting in high affinity for ligands (5, 8, 9, 12, 13, 16–18, 45). Therefore, equilibrium between the dissociated monomers and associated heterodimers of the TM domains is critical for integrin inside-out activation. Based on the observation that integrin TM helices formed homo-oligomers using recombinant peptides in detergent (23, 29, 31), the GALLEX assay in bacteria (45), the TOXCAT assay in bacteria (24, 25), and the computational modeling (26–28), TM homomeric association has been suggested to be important for integrin activation (14, 29, 31). Mutagenesis studies on the proposed TM homomeric interface residues showed that these mutations could lead to integrin activation, suggesting that heterodimer dissociation is sufficient to activate integrins (14). Therefore, a push-pull model was proposed in which after dissociation of the TM heterodimer, integrins are stabilized by TM homomeric association (14).

Here, our experiments on mammalian cell transfectants using full-length integrin αIbβ3 showed that the αIb and β3 TM domains do not form any homomeric association in the resting state, nor do they form after soluble ligand binding. The only mutant that formed a homomeric disulfide is αIbβW967C, as reported previously (12, 13). We found that this homomeric disulfide was formed during biosynthesis and in the integrin resting state, and dissociation of the TM heterodimer is not required for its formation. Therefore, we do not think that the same homomeric association occurs during integrin inside-out activation or outside-in signaling. We found that except for this mutant, none of the other 30 αIb and 34 β3 cysteine mutants formed a homomeric disulfide bond before or after binding to soluble ligands. In addition, during integrin inside-out activation, the integrin TM domains do not form any homomeric association that can be detected by disulfide scanning. Integrins must be regulated to be activated and deactivated quickly in mammalian cells, even within seconds. It has been shown that detachment of integrin from ligands in the trailing edge is critical for integrin functions (46), and mutations that constitutively active integrins result in malfunctions (47–50). Therefore, it is unlikely that integrin TM domains form more stable homomeric association during inside-out signaling.

We further showed that TM homomers do not form upon binding to ligands on a substrate and subsequent integrin clustering. Although integrin clustering is known to participate in integrin signaling pathways (40, 51–53), the exact mechanisms of integrin clustering remain elusive. One mechanism proposes that the integrin TM homo-oligomerization promotes integrin clustering (29). Studies have established that extracellular ligand binding triggers integrin conformational changes that promote oligomer assembly through TM fragments (54–56). In this study, we observed that after adhering to immobilized fibrinogen integrins clustered on the cell surface, but we did not detect any integrin TM homomeric interaction under this condition. Interestingly, the heterodimeric disulfide-bonded integrin formed clusters, suggesting that even the dissociation of integrin α/β TM domains is not required for integrin clustering. In addition, no evidence has been shown that the integrin extracellular domains form homomeric interaction, and therefore, it is unlikely that they induce integrin clustering during signaling.
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Taken together, our study suggests that integrin clustering is likely induced by bringing several integrins physically close together by multimeric ligands, and the TM/cytoplasmic domain homomeric association is not required for this process. Additional evidence is needed to define the mechanisms of integrin clustering further.

Even though the TM separation is not required for integrin clustering, it is critical for integrin outside-in signaling (1). We showed that all cysteine mutants could adhere and spread on the fibrinogen-coated surface, suggesting that they could transmit signals into the cells after binding to immobilized ligands. However, we did not observe any TM homomeric disulfide formation in the same condition, indicating that the TM homomeric association does not occur during integrin outside-in signaling. Based on the previous FRET studies (44), we assume that ligand-induced TM separation is likely coupled to cytoplasmic domain separation, implying that αβ cytoplasmic domain interactions somehow constrain or inhibit kinase activation (1, 13, 17, 44, 57). One possibility is that separation of TM and cytoplasmic domains induces dissociation of the TM and cytoplasmic domains, but not the homomeric association, is critical for integrin outside-in signaling. Therefore, clumping, it is critical for integrin outside-in signaling (1). Additional evidence is needed to define the mechanisms of integrin homo-oligomerization (2).

In conclusion, our study shows that the integrin TM homo-oligomerization does not occur in mammalian cell membranes before or after soluble ligand binding, during inside-out activation or outside-in signaling. Therefore, conformational change induced by intracellular signals or extracellular ligands, more specifically, separation of TM and cytoplasmic domains, but not the homomeric association, is critical for integrin bidirectional signaling.

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