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# Differences in α–β transmembrane domain interactions among integrins enable diverging integrin signaling

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ABSTRACT

Integrins are transmembrane adhesion molecules composed of α and β subunits. In humans, 24 integrins are expressed in a tissue-specific manner. Each integrin plays a specific role within a tissue type to control cell adhesion. We previously found that the degree of transmembrane domain (TMD) interaction between the integrin αIIb and β3 subunits is reversely correlated with the affinity of integrin αIIbβ3 to its ligand. Here, we examined the TMD interactions of various integrins, including α4β1, αLβ2, α5β1, αVβ1, αIIbβ3, and αVβ3. Our findings revealed that the degree of the TMD interactions in integrins α4β1 and αLβ2 expressed in immune cells was low and in integrins αIIbβ3 and αVβ3 expressed in platelets was high, while integrins α5β1 and αVβ1 that are expressed in most adherent cells displayed intermediate TMD interactions. We identified sequence variation within the N-terminal TMD region as a factor responsible for the observed differential degree of TMD interaction among integrins. When the N-terminal interaction that was missing in integrin α5β1 was restored with mutagenesis, the increase in TMD interaction inhibited the outside-in but not inside-out signaling of integrin α5β1 and also accelerated the speed of cell migration. We suggest, therefore, that the degree of TMD interaction is designed to accommodate the specific, desired function of each integrin.



## 1. Introduction

Integrins are major cell adhesion molecules composed of α and β subunits, which are transmembrane proteins containing a large extracellular domain, the transmembrane domain (TMD), and relatively short tails. There are 18 α and 8 β subunits in human integrins, which in different combinations result in a total of 24 unique integrins that are expressed in a tissue-specific manner and bind to certain ligands [1].

Integrins are involved in a variety of cell adhesion processes that occur during biological and pathological situations [2]. Integrin-mediated cell adhesion is particularly important for cell migration, where it needs to be dynamically controlled to enable process such as migrating cell generation of new cell-substrate adhesions in the front and simultaneous disruption of mature adhesions in the rear [3]. Integrins control cell adhesion with conformational changes of their extracellular domains. Specifically, when these domains are folded, integrins are in a low-affinity state towards their ligands, extracellular matrices (ECMs). Conversely, when these domains are extended, integrin ligand binding sites are exposed and consequently can bind to ECMs, thereby mediating cell adhesion [4,5]. The intracellular signaling pathway that controls integrin affinity is called "inside-out" signaling. Inside-out signaling that induces integrin to be in a high-affinity state (integrin activation) is thought to rely on talin binding to the integrin β cytoplasmic tail [6].

The structural change of extracellular domain controlling cell adhesion has been shown to be regulated by the TMD interaction between α and β subunits [4]. An intact TMD interaction keeps integrins in a low-affinity state (inactive), whereas the disruption of this interaction results in integrins in a high-affinity state (active). Accordingly, many activating mutations identified in TMDs and the membrane-proximal regions of integrins can disrupt the TMD interaction [7–12]. Moreover, talin binding to the integrin β tail can also disrupt the TMD interaction [7]. The regulation of the TMD interaction is, therefore, key for controlling integrin affinity.

Upon binding to ECMs, integrins can also initiate the intracellular signaling pathway, called "outside-in" signaling, which leads to the activation of many signaling molecules, cell spreading, cell proliferation, and so forth [13,14]. Outside-in signaling also appears to be dependent on the separation of the α–β TMD interaction. Fluorescent resonance energy transfer (FRET) experiments revealed that the FRET efficiency between fluorophores attached to α and β cytoplasmic tails decreases when an integrin binds to

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its ligand, suggesting that tails and the TMD get separated by outside-in signaling [15]. Furthermore, clamping of the integrin α–β TMD interaction though the introduction of a disulfide bond between TMDs blocks outside-in signaling events such as cell spreading and focal adhesion kinase activation [16]. The regulation of the TMD interaction is, therefore, crucial not only in the inside-out but also in outside-in signaling of integrins.

We previously developed an affinity capture assay to measure the degree of TMD interaction and determined the extent of the α–β TMD interaction in the platelet integrin αIIbβ3 [7]. Since the TMD interaction has a pivotal role in the establishment of integrin affinity states, it would be of great interest to investigate TMD interactions in additional integrins as well to predict the basal affinity states of others. In the present study, therefore, we evaluated differences in the degree of TMD interaction between five different integrin α subunits and three different β subunits as representatives for integrins. By comparing the degree of TMD interaction of each integrin subunit with its amino acid sequence within the TMD region, we identified a novel binding interface in the N-terminal TMD region that varied in amino acid sequence among integrins. Moreover, we investigated the importance of this binding interface in integrin signaling. Finally, through analysis of these findings together, we propose a model of how the TMD interaction regulates integrin signaling and cell migration.

## 2. Materials and methods

### 2.1. Plasmids and antibodies

Plasmids encoding αIIb™-TAP and Tac-β3™ were described previously [7]. To generate α4™-TAP, α5™-TAP, αL™-TAP, and αV™-TAP, nucleotide sequences encoding TMD and cytoplasmic tail regions of α4, α5, αL, or αV, as shown in Fig. 1A, were amplified by polymerase chain reaction (PCR), fused to a tandem affinity purification (TAP) tag, and cloned into pcDNA3.1 (Invitrogen) containing a part of the preprotrypsin leader sequence followed by 3 repeats of the FLAG sequence (Sigma–Aldrich) as previously described [7]. For the construction of Tac-β1 and Tac-β2, TMD and cytoplasmic regions of β1 or β2, as shown in Fig. 1A, were fused to a Tac extracellular domain by overlapping PCR and subsequently cloned into pcDNA3.1. Lentivirus constructs encoding integrin α5, α5 (I960W), β1, and β1(V733L) cloned in pRRL-SIN.cPPT.PGK–IRES–GFP WPRE (Addgene) were prepared as previously described [17].

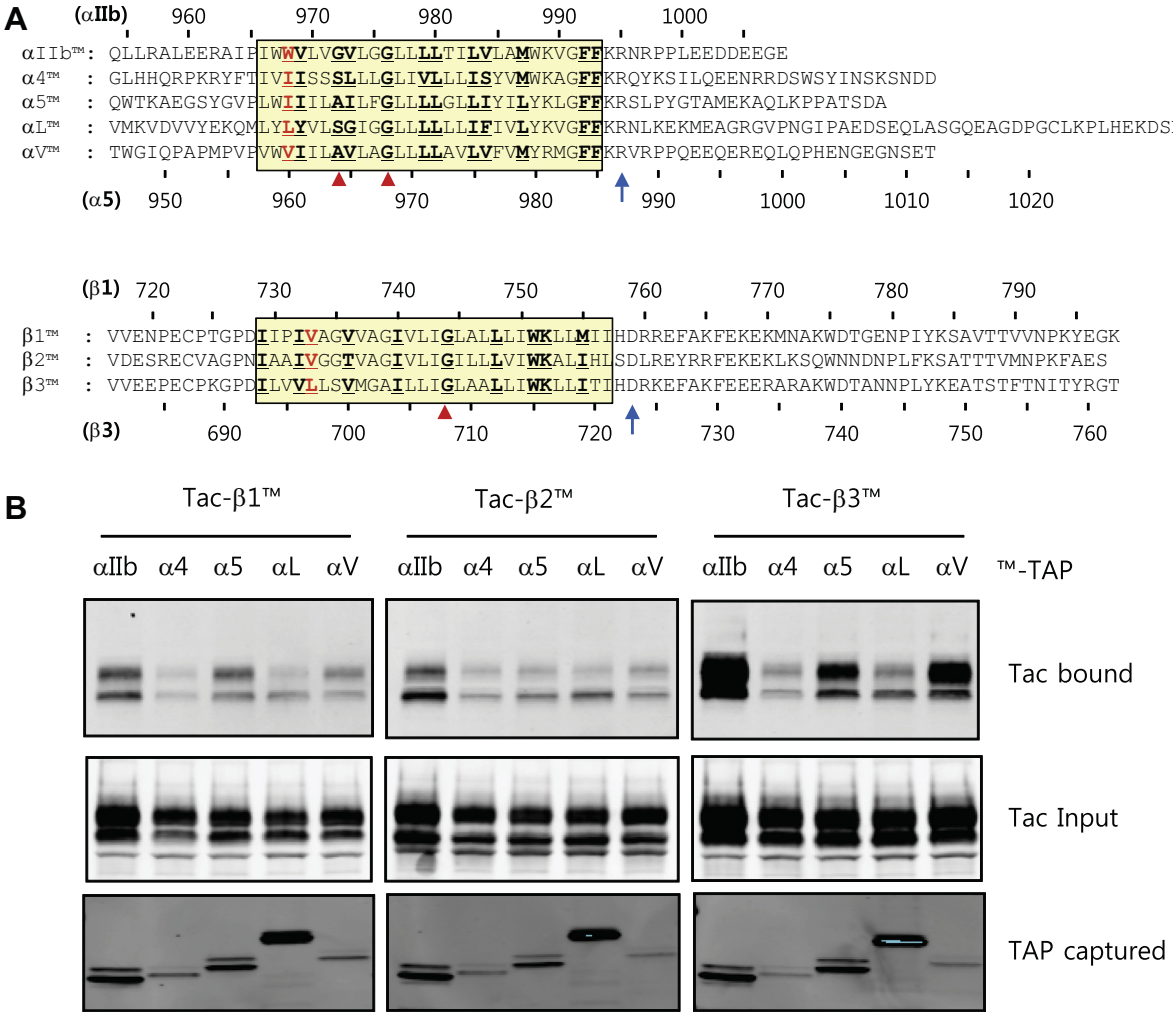


**Fig. 1.** (A) Sequence alignments of amino acids near TMDs of integrin α and β subunits. TMD regions are boxed. Amino acid residues in the α–β TMD interface, as based on αIIbβ3 TMD structure [21], are underlined. Amino acids residues essential for the outer membrane and inner membrane clasps are indicated with arrowheads and arrows, respectively. Amino acid numbering for αIIb, α5, β1, and β3 are shown. (B) Integrin α TMDs fused with a TAP tag (α™-TAP) and β TMDs fused with Tac (Tac-β™) were transfected into CHO cells as indicated. TAP-tagged α TMDs were pulled down and associated β TMDs were detected by Western blot using an anti-Tac antibody (upper panel). The total input of β TMDs (middle panel) and pulled-down α TMDs (lower panel) were analyzed by Western blot using an anti-Tac and anti-FLAG antibody, respectively.

### 2.2. Affinity capture

Affinity capture assay for measuring the α and β TMD interaction was performed as previously described [7]. Briefly, Chinese hamster ovary (CHO) cells transfected with the generated α and β TMD constructs were first lysed with lysis buffer (1% CHAPS, 150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM $CaCl_2$, protease inhibitor mixture). The resultant cell lysates were then clarified by centrifugation, incubated with calmodulin Sepharose (GE healthcare), and bound proteins were analyzed by Western blot.

### 2.3. Lentivirus preparation

Lentivirus was prepared according to an established protocol [18]. Briefly, HEK293T cells were first transfected with a lentivirus construct encoding each integrin, as well as pCMVΔR8.2 (Addgene) and pMD2.G (Addgene). After a 3 h incubation, the transfection medium was replaced with UltraCULTURE medium (BioWhittaker) and then the cells were incubated for another 48 h. Media were subsequently harvested, and clarified by centrifugation at 1000 rpm for 10 min. Media were then concentrated using Centricon (Millipore) and finally used to infect CHO-B2 cells to generate CHO/α5β1 and CHO/α5(I960W)β1(V733L).

### 2.4. Soluble fibronectin binding assay

CHO/α5β1 and CHO/α5(I960W)β1(V733L) were transfected with cDNA encoding the talin head domain or an empty vector as well as tdTomato as an expression marker, using Lipofectamine (Invitrogen) and Plus reagent (Invitrogen). After a 24 h incubation, cells were harvested by trypsinization and incubated with a biotinylated fibronectin FN9–11 fragment for 30 min as described previously [19]. Cells were subsequently fixed with 3.7% formaldehyde for 10 min and then washed with Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) three times. Fixed cells were further incubated with streptavidin conjugated with allophycocyanin and washed with DMEM twice, before being finally analyzed by flow cytometry.

### 2.5. Spreading assay

Plates containing 12 wells were coated with fibronectin overnight at 4 °C at the indicated concentrations. After being washed twice with phosphate buffered saline (PBS), each well was blocked with 1% bovine serum albumin in PBS for 1 h at 37 °C. CHO/α5β1 and CHO/α5(I960W)β1(V733L) detached by trypsinzation, were washed twice with DMEM and then added into the fibronectin coated wells. Cells were first incubated for 1 h at 37 °C in a $CO_2$ incubator, then fixed with 3.7% formaldehyde for 10 min, and finally stained with crystal violet for observation with a microscope.

## 3. Results and discussion

### 3.1. Degree of transmembrane domain interaction varies among different integrins

We previously developed an efficient method to detect the TMD interaction between integrin αIIb and β3 subunits [7]. Using this method, in the present study we investigated the TMD interaction in other integrins. The TMD-tail regions of subunits αIIb, α4, α5, αL, and αV, which were chosen as representative α subunits of integin, were PCR-amplified (Fig. 1A) and then fused to the N-terminal signal sequence, a FLAG tag, and a C-terminal tandem-affinity purification (TAP) tag containing the calmodulin binding region and an imunoglobulin G binding region. Subunits β1, β2, and β3 were chosen as representative β subunits of integrin, and fused the TMD-tail regions of those integrins (Fig. 1A) to the extracellular domain of Tac (interleukin-2 receptor). We then transfected each α and β TMD-tail construct into CHO cells in all the possible combinations. Among the 15 resultant combinations that were tested, 6 can make functional integrins (α4β1, α5β1, αVβ1, αLβ2, αIIbβ3, and αVβ3). The findings of this experiment revealed that TMD interactions of the platelet integrins, αIIbβ3 and αVβ3, are stronger than those of other integrins, consistent with the notion that integrins expressed in platelets should be kept in a low affinity state to ensure that platelet adhesion in normal conditions is not induced, as it would lead to fatal diseases such as thrombosis or stroke [20]. Integrin α5β1 and αVβ1, which are expressed in a number of adherent cells such as epithelial and endothelial cells, displayed a intermediate TMD interaction, suggesting that the basal affinity of these cells to ECMs is not as inhibited as it is in platelets. On the other hand, the TMD interactions of integrins α4β1 and αLβ2, which are known to be expressed in immune cells, were weaker than those observed for any other integrins in this study (Fig. 1B). Just like platelets, since immune cells circulate in the blood stream, their adhesion to a ligand expressed on endothelial cell surfaces should be tightly regulated so not to induce any unwanted immune responses. The TMD interactions of integrins α4β1 and αLβ2, however, were as low as that of integrin αIIbβ3 containing a ΔGFFKR mutation (data not shown) that abolishes the TMD interaction of this integrin altogether and also activates the integrin better than any other activating mutations (data not shown). Our data, therefore, suggests that the affinity of those particular integrins that are expressed in immune cells is not regulated only by a separation of the TMD interaction but also by another mechanism, such as the adjustment of surface level expression of those integrins or their ligands.

The TMD interaction between αIIb and β3 subunits is known to be mediated by two different clasps. One is outer membrane clasp mediated by αIIb(Gly972, Gly976) and β3(Gly708) (Fig. 1A, arrowheads), and the other is inner membrane clasp mainly mediated by electrostatic interaction between αIIb(Arg995) and β3(Asp723) (Fig. 1A, arrow) [21]. When the degree of each α TMD interaction to the β3 TMD (Fig. 1B, right panels) was compared with the associated sequence of the α TMD for each integrin (Fig. 1A), the alanine residue in α5 and αV, located at the region equivalent to Gly(972) in αIIb, seemed to be involved in generating the relatively stable TMD interaction, as suggested before [21], while the corresponding serine residue in α4 and αL did not permit this interaction (Fig. 1B, right panels). However, β1 TMD binding to all of the α TMDs were significantly reduced when compared to β3 TMD binding to those α TMDs (Fig. 1B, left panels), although the β1 TMD contained all the amino acid residues responsible for the outer membrane clasp, β1(Gly744), and the inner membrane clasp, β1(Asp759). Thus, another region involved in maintaining the stable TMD interaction may exist.

### 3.2. α5(I960W) and β1(V733L) enhance the α5-β1 TMD interaction

To determine the additional region responsible for the TMD interaction, we focused on integrin α5β1. The major sequence differences between the α5 and αIIb TMD at the αβ binding interface were identified to be α5(Ile960), corresponding to αIIb(Trp968), and α5(leu979), corresponding to αIIb(Met987). In terms of the β subunit, sequence differences were observed at β1(Ile732, Val733), corresponding to β3(Val696, Leu697), and β1(Met755), corresponding to β3(Ile719) (Fig. 1A). To determine if those resultant residue alterations could affect the structural integrity of the TMD interaction, we examined the αIIbβ3 TMD structure in detail [21]. Structural analysis revealed that three residues in αIIb, Trp968, Val971, and Gly972, make up a hydrophobic pocket, which

β3(Leu697) inserts into to generate the hydrophobic interaction between the N-terminal regions of TMDs (Fig. 2A). α5, however, contains isoleucine (α5(Ile960)) at the region corresponding to αIIb(Trp968). In addition, β1 contains valine (β1(Val733)) at the position corresponding to β3(Leu697) (Fig. 1A). We therefore hypothesized that the amino acid residue variations observed in α5β1 may not be able to provide the proposed N-terminal hydrophobic interaction efficiently. To examine the involvement of those residues in the TMD interaction, we mutated them in α5 and β1 to create the residues found in αIIb and β3, and then determined the ability of the changes to enhance the α5β1 TMD interaction. Mutation of α5(Ile960) to generate Trp induced a small but consistent increase in the TMD interaction, suggesting that the hydrophobic pocket in the N-terminal α TMD is indeed involved in the TMD interaction (Fig. 2B). Moreover, mutation of β1(Val733) to create Leu significantly enhanced the TMD interaction, compared to the effects of wild type α5β1 on the TMD interaction (Fig. 2B). Furthermore, when the residues in both the α5 and β1 subunits were mutated, the TMD interaction was comparable to or higher than that observed for αIIbβ3 (Fig. 2B). We concluded, therefore, that the N-terminal hydrophobic interaction in TMDs is involved in the αβ TMD interaction and determines the degree of the TMD interaction within each integrin.

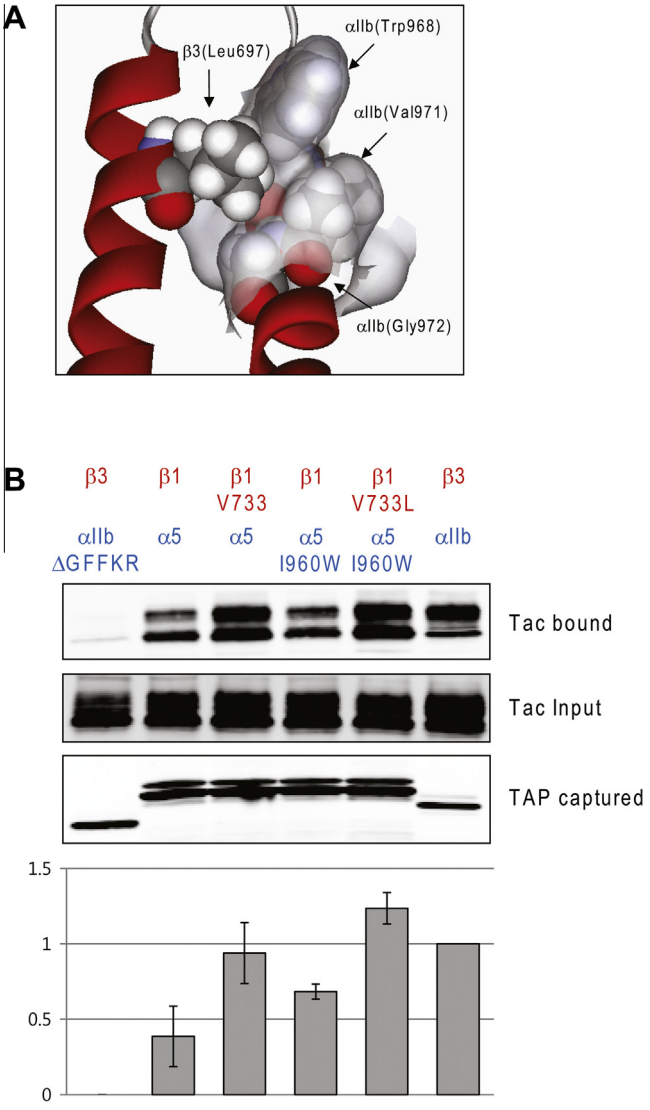


**Fig. 2.** (A) The N-terminal region of the αIIbβ3 TMD structure (PDB ID: 2K9J) is shown. Space filling models of β3(Leu697) and αIIb(Trp968, Val971, Gly972) are shown. (B) The TMD interaction between various α TMDs and β TMDs, as indicated, were measured with pull down experiments as in Fig. 1B. Quantification of TMD interaction results from three independent experiments is shown as a bar graph. In the graph, the TMD interactions of αIIbΔGFFKR-β3 and αIIb-β3 are set to 0 and 1, respectively. Error bars represent standard errors.

### 3.3. Increased αβ interaction in N-terminal TMDs inhibits outside-in signaling but not inside-out signaling

To determine the effect of the observed increased interaction of integrin α5β1 N-terminal TMD regions in integrin-dependent cellular behaviors, we generated stable cell lines that express integrin α5β1 harboring the stabilizing mutations. cDNA from integrinα5 (I960W), β1(V733L), as well as their wild type versions were cloned into a lentivirus vector, and viruses generated from those constructs were infected into the CHO-B2 cell line which is known to have a defect in integrin expression [22]. In the end, two different cell lines were generated, CHO/α5β1 and CHO/α5(I960W)β1(V733L). Evaluation of integrin β1 expression by flow cytometry did not identify any differences in expression levels between those cell lines (Fig. 3A). As an initial test to further investigate the differential effects of the TMD stabilizing mutations, we compared the degree of soluble fibronectin binding between those cell lines as a measurement of integrin inside-out signaling [19]. The findings of this experiment revealed that both integrins have virtually the same basal affinity to fibronectin, irrespective of whether or not the TMD interaction is enhanced (Fig. 3B, upper panels). Even when cells were transfected with the talin head domain which is known to activate integrin [6], no significant differences between these cells were detected (Fig. 3B, lower panels), indicating that stabilizing the TMD interaction at the TMD N-terminal region does not affect integrin inside-out signaling. When we investigated the effects of TMD interaction stabilization on cell spreading as a measurement of integrin outside-in signaling [19], however, we found that cell spreading on a fibronectin-coated surface was significantly reduced in CHO/α5(I960W)β1(V733L) cells compared with CHO/α5β1 cells (Fig. 3C and D). We conclude, therefore, that stabilizing the N-terminal TMD interaction inhibits integrin outside-in signaling, but not inside-out signaling.

As mentioned above, the separation of the TMD interaction is involved in both integrin inside-out signaling [7] and outside-in signaling [16]. Inside-out signaling would disrupt the TMD interaction from the inside, as it is initiated by talin binding to the integrin β tail, which disrupts the electrostatic interaction between α(Arg) and β(Asp) in the inner membrane clasp [23] and induces the tilt of the β TMD [24]. With this type of signaling, therefore, we assume that talin-binding to the integrin would exert enough force to break the whole TMD, whether or not the N-terminal TMD interaction is stabilized in integrin α5β1 (Fig. 3E, upper panel). With outside-in signaling, however, ligand binding to the integrin extracellular domain would separate TMDs from outside. If the N-terminal TMDs bind more tightly to each other, then, separation of the N-terminal region should require more forces (Fig. 3E, lower panel), which would inhibit outside-in signaling. Furthermore, as the binding force is stronger, the elastic energy transmitted to the cell membrane would dissipate owing to the rupturing of bonds, to consequently inhibit cell spreading [25]. This interpretation of our findings explains the differential effects of N-terminal TMD interaction stabilization on inside-out and outside-in signaling.

### 3.4. Increased αβ interaction in N-terminal TMDs speeds up cell migration

Lastly, we investigated the effects of the observed stabilized TMD interaction on cell migration. Monolayers of CHO/α5β1 and

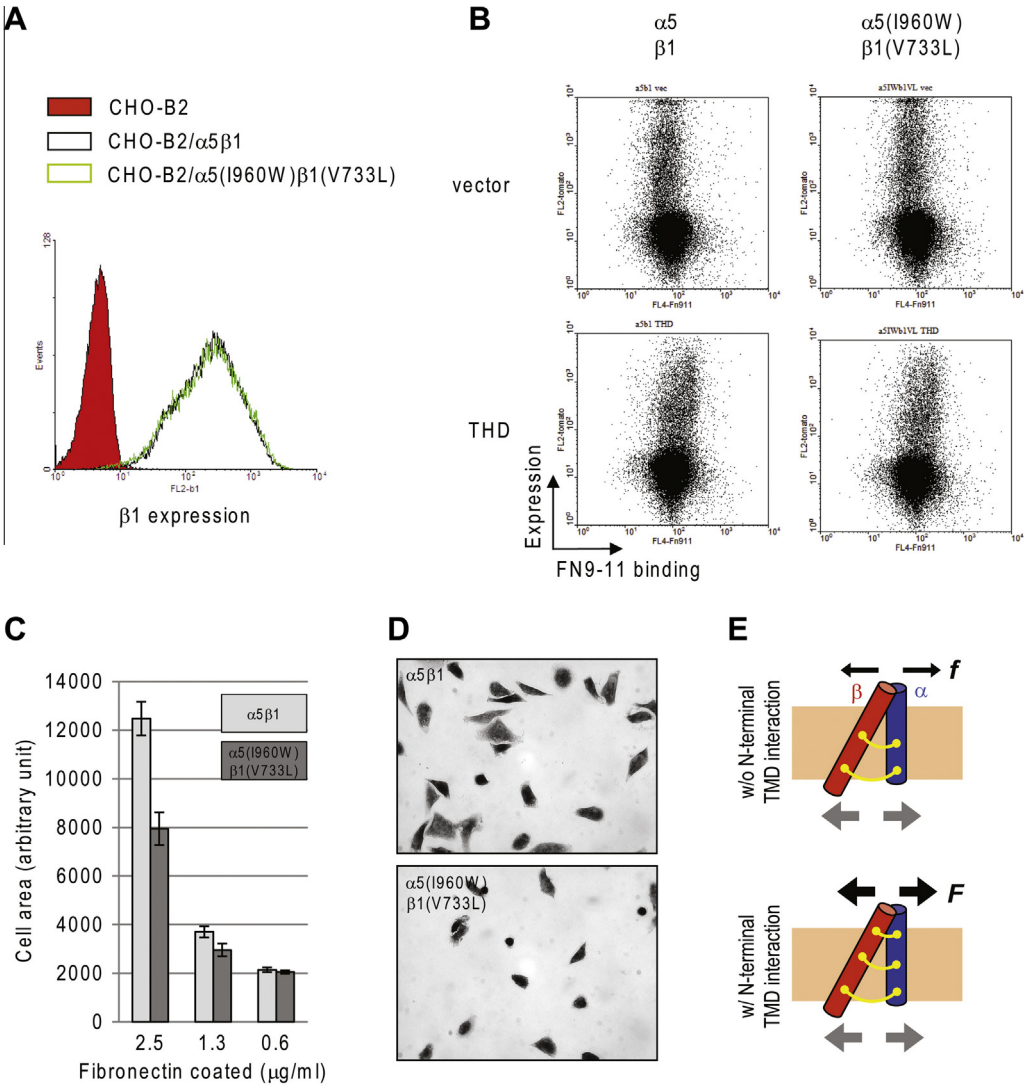


**Fig. 3.** (A) Expression levels of human integrin β1 in CHO-B2, CHO-B2/α5β1, and CHO-B2/α5(I960W)/β1(V733L) cells were determined by flow cytometry with an anti-human β1 antibody conjugated to phycoerythrin. (B) CHO-B2/α5β1 and CHO-B2/α5(I960W)/β1(V733L) cells were transfected with the talin head domain (THD) or empty vector, as well as the transfection marker, tdTomato. Their affinities toward the fibronectin fragment, FN9–11, were measured by flow cytometry. The *Y* axis represents the expression level of tdTomato, while the *X* axis represents FN9–11 binding. (C) CHO-B2/α5β1 and CHO-B2/α5(I960W)/β1(V733L) cells were allowed to adhere to a fibronectin-coated surface for an hour. Cell surface areas containing at least 43 cells within 4 different fields of each condition were measured and plotted on a bar graph. Error bars represent standard errors. (D) Representative images of the cell spreading on surfaces coated with 2.5 μg/ml fibronectin are shown. (E) Forces required to separate TMDs outside (black arrows) and inside (gray arrows) are illustrated. The N-terminal TMD interaction, outer membrane clasp, and inner membrane clasp are indicated with lines. Note that more force would be required to separate TMDs in the presence of a N-terminal TMD interaction as indicated by the black arrows.

CHO/α5(I960W)β1(V733L) cells spread to fibronectin were scratched to generate a wound, and their speed at which they covered the wounded surface was determined using a microscope equipped with a temperature controlling device that took cell images every hour (Fig. 4A). The region not covered with cells was measured (Fig. 4A, right panel), and the speed of cell migration was subsequently calculated by subtracting the areas of empty space at each time point from the initial area of empty space at time 0. The findings of this assay revealed that the rate of migration is increased approximately by 50% when the TMD interaction is stabilized (Fig. 4B). This result was very surprising, as we expected the reduced cell spreading observed in CHO/α5(I960W)β1(V733L) cells would inhibit cell migration. We, therefore, interpret this unexpected observation in terms of the turnover rate between active and inactive integrins. The activation of an integrin from inside-out signaling would separate the TMD interaction, making the integrin active irrespective of the presence or absence of a TMD interaction within the N-terminal region. Once an integrin is activated by the separation of TMDs, then, an increased TMD interaction generated from the presence of stabilizing N-terminal mutations would enhance the rate of the spontaneous inactivation integrin process because of the greater tendency to making the TMD interaction again, thereby increasing the turn-over rate (Fig. 4C). As a result, the rate of integrin activation at the leading edges of cells would be the same, whereas at the trailing edges of cells the rate of spontaneous deactivation of integrin or the disassembly of integrin-mediated mature adhesions would increase, making cells with stronger TMD interactions migrate faster (Fig. 4D).

In the present study, we found that the degree of the TMD interaction varies among integrins, suggesting that each integrin has a different basal affinity state. More importantly, we determined that the N-terminal TMD interaction mediated by αIIb(Trp968) and β3(Leu697) in integrin αIIbβ3 is missing in integrin α5β1. The lack of the N-terminal TMD interaction enabled integrin α5β1 to respond more efficiently to a low concentration of

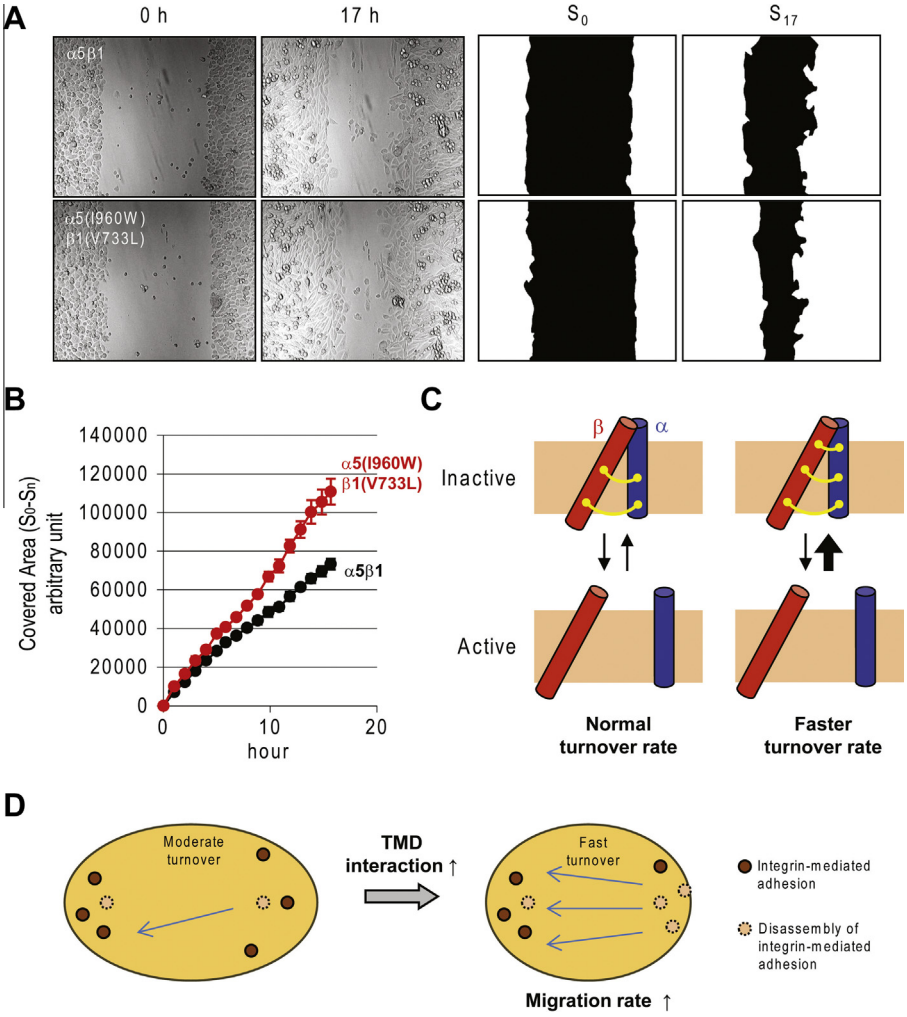


**Fig. 4.** (A) Representative images of the wounding migration of CHO-B2/α5β1 and CHO-B2/α5(I960W)/β1(V733L) cells at times 0 and 17 h (left). Empty spaces were identified and filled with black color, while regions covered with cells are shown with white (right). (B) At each time point, areas of black colored space in (A) were calculated and subtracted from the area present at the initial starting point of time 0 ($S_0$) to calculate the covered area. Error bars represents standard errors ($n = 4$). (C) Turn-over rates between the inactive and active conformation are illustrated as in Fig. 3E. The presence of the N-terminal TMD interaction would induce a faster association between TMDs as indicated by black arrows, thereby increasing the turnover rate. (D) Increased turnover rates of integrin-mediated adhesion may induce faster migration, by accelerating the disassembly of integrin-mediated adhesions and providing integrin pools to the front.

fibronectin, and thus facilitated outside-in signaling while maintaining the same degree of talin-mediated inside-out integrin activation. Integrin α5β1, therefore, seems to adopt a method that stabilizes the adhesion to existing ECMs and also compromises the regulation of activation as tightly as platelet integrin.

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