The membrane-distal regions of integrin α cytoplasmic domains contribute differently to integrin inside-out activation

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Functioning as signal receivers and transmitters, the integrin α/β cytoplasmic tails (CT) are pivotal in integrin activation and signaling. 18 α integrin subunits share a conserved membrane-proximal region but have a highly diverse membrane-distal (MD) region at their CTs. Recent studies demonstrated that the presence of α CTMD region is essential for talin-induced integrin inside-out activation. However, it remains unknown whether the non-conserved α CTMD regions differently regulate the inside-out activation of integrin. Using αIIbβ3, αLβ2, and α5β1 as model integrins and by replacing their α CTMD regions with those of α subunits that pair with β3, β2, and β1 subunits, we analyzed the function of CTMD regions of 17 α subunits in talin-mediated integrin activation. We found that the α CTMD regions play two roles on integrin, which are activation-supportive and activation-regulatory. The regulatory but not the supportive function depends on the sequence identity of α CTMD region. A membrane-proximal tyrosine residue present in the CTMD regions of a subset of α integrins was identified to negatively regulate integrin inside-out activation. Our study provides a useful resource for investigating the function of α integrin CTMD regions.

Integrins are cell adhesion receptors composed of α and β subunits, each containing a large extracellular domain, a single transmembrane (TM) domain and usually a short cytoplasmic tail (CT). In human, the combinations of 18 α and 8 β subunits form 24 integrin heterodimers that play essential roles in numerous biological activities such as hemostasis, immune responses, and development1. Aberrant activation of integrin is associated with many pathological conditions including thrombosis, inflammatory diseases, and tumor-driven cell growth, metastasis, and angiogenesis2-4. Therefore, tight regulation of integrin activation is important for normal integrin function. A unique feature of integrins is that they can transmit signals bidirectionally across the cell membrane, so called inside-out and outside-in signaling5,6. In the inside-out direction, the activating signals impinge on the integrin CT to transform integrin from a resting to an active state by inducing large-scale conformational changes of the extracellular domain7. In the outside-in direction, ligand binding to the extracellular domain of active integrin also induces long-range conformational changes that are transmitted to the CT to provoke the association and activation of the kinases and adaptor molecules in the cytosol8,9. As such, acting as both the receiver and the transmitter of signals, integrin CT is pivotal in integrin activation and signaling.

Largely based on the studies of β3, β2, and β1 integrins, great progress has been made in understanding how the β integrin CT contributes to integrin activation6,10. Most of the β integrin CTS contain the conserved binding motifs for the common integrin activators, talin and kindlin (Fig. 1A). Structural and functional studies suggested that binding of talin and kindlin to β integrin CT induces integrin activation by disrupting the α-β interactions at the TM and the CT domains10, which in turn leads to conformational changes of the extracellular domain7. A functional role of the α integrin CT in integrin activation had been focused on the highly conserved Gly-Phe-Phe-Lys-Arg (GFFKR) motif at the membrane proximal (MP) region (Fig. 1A), which helps maintain the α-β CT associations11,12. Notably, the membrane-distal (MD) regions of the α integrin CT differ significantly in both amino acid sequence and length (Fig. 1A) and their roles in integrin activation and signaling remain ill defined. Accordingly, the current available structures of α integrin CTS also show conformational diversity at the
MD regions (Fig. 1B). Moreover, even the same αintegrin CTMD region shows different conformations among the reported structures (Fig. 1B). Recent studies from our and other groups demonstrated that the presence of αintegrin CTMD region is essential in talin/kindlin induced integrin inside-out activation 13,14. In addition, our study also showed that the length and amino acids of the αintegrin CTMD region might be important in regulating integrin inside-out activation 13,14. Given that one β integrin subunit such as β3, β2, or β1 usually heterodimerizes with more than one α subunits (Fig. 1A), an intriguing question is whether the diverse αCTMD regions contribute differently to integrin activation and signaling, which may determine the specific and diverse integrin functions.

In this study, using the platelet-specific αIIbβ3, leukocyte-specific αLβ2, and the ubiquitously expressed α5β1 as model integrins, we attempted to compare the effect of 17 out of 18 total αintegrin CTMD regions on integrin inside-out activation. Our study revealed that the αCTMD regions contribute differently to talin head (TH)-induced integrin activation, evidenced by different levels of ligand binding and conformational changes. This was at least in part determined by the presence of specific residues in the αCTMD regions. Potential mechanisms by which the αCTMD regions participate in integrin activation were discussed.
Results

Design and generation of chimeric \( \alpha \) integrins to examine the contributions of the diverse \( \alpha \) CTMD regions in integrin inside-out activation. Studies from our group and others have demonstrated the requirement of the presence of \( \alpha \) CTMD region in integrin inside-out activation\(^{13}\). However, it remains elusive whether and/or how the diverse CTMD regions regulate integrin activation. It has been shown that the \( \alpha \) CTMD regions also contribute to maintaining integrin in the resting state possibly by interacting with the \( \beta \) CT\(^{13-15}\). Therefore, a simple mutagenesis or replacement of the \( \alpha \) CTMD region with an irrelevant sequence may result in complicated and uninterpretable results. To address this question, we took the advantage that one \( \beta \) subunit usually pairs with more than one \( \alpha \) subunits. For example, \( \beta_3, \beta_2, \) and \( \beta_1 \) subunit can heterodimerize with 2, 4, and 12 different \( \alpha \) subunits, respectively (Fig. 1A). Furthermore, in vitro activation assays of \( \alpha_{IIb} \beta_3, \alpha_L \beta_2, \) and \( \alpha_5 \beta_1 \) have been very well established\(^{13,16,17}\). Therefore, we used \( \alpha_{IIb}, \alpha_L, \) and \( \alpha_5 \) as model \( \alpha \) integrins, in which their CTMD regions were replaced by those of \( \alpha \) subunits that can pair with \( \beta_3, \beta_2, \) and \( \beta_1 \) subunits, respectively (Fig. 2A). In such way, we generated the \( \alpha \) chimeras that are denoted as \( \alpha_{IIb}-\alpha_V, \alpha_L-\alpha_X, \alpha_L-\alpha_D, \alpha_L-\alpha_M, \alpha_5-\alpha_V, \alpha_5-\alpha_1, \alpha_5-\alpha_2, \alpha_5-\alpha_3, \alpha_5-\alpha_4, \alpha_5-\alpha_6, \alpha_5-\alpha_7, \alpha_5-\alpha_8, \alpha_5-\alpha_9, \) and \( \alpha_5-\alpha_{10} \). When these \( \alpha \) chimeras are co-expressed with \( \beta_3, \beta_2, \) and \( \beta_1 \) subunits, respectively, the native associations of \( \alpha \) and \( \beta \) CT domains are maintained. In addition, comparisons can be made among the \( \alpha \) chimeras that share the same \( \beta \) subunit. Any differences seen in the integrin inside-out activation assay would attribute to the diverse CTMD regions. These 15 chimeras together with the wild type (WT) \( \alpha_{IIb}, \alpha_L, \) and \( \alpha_5 \) subunits allow our study to cover the CTMD regions of 17 out of 18 human \( \alpha \) integrins.

Figure 2. Integrin \( \alpha_{IIb} \)–chimeras bearing various \( \alpha \) CTMD regions responded differently to talin head (TH)-induced integrin activation. (A) Design of \( \alpha \) integrin chimera. Integrin \( \alpha \) chimera is constructed by replacing the MD region of a model \( \alpha \) integrin with those of indicated \( \alpha \) integrins. (B) TH-induced binding of the ligand–mimetic mAb PAC-1 to the \( \alpha_{IIb} \) chimeras. (C) PAC-1 binding to \( \alpha_{IIb} \)-WT and \( \alpha_{IIb}-\alpha_V \) in response to the different expression levels of GFP-TH. PAC-1 binding was measured with the HEK293FT cells transfected with the \( \alpha_{IIb} \) integrins plus the \( \beta_3-D723A \) mutant and the indicated amounts of GFP-TH plasmids. (D) TH-induced binding of the active conformation-specific mAb 370.3 to the \( \alpha_{IIb} \) chimeras. Binding of the mAbs was measured by flow cytometry with HEK293FT cells co-transfected with the indicated integrin constructs and GFP or GFP-TH. The GFP and integrin double-positive cells were analyzed. \( \beta_3-D723A \) was used to increase the sensitivity of the assay. Data are presented as the MFI of the GFP-binding normalized to the MFI of integrin expression. Data are presented as mean ± s.e.m. (n ≥ 3) and two-tailed Student’s t-test was performed to compare the \( \alpha_{IIb} \)-chimeras to \( \alpha_{IIb} \)-WT under the GFP-TH condition in B and D or under same GFP-TH concentration in C. * \( P < 0.05 \), ** \( P < 0.01 \). Integrin and GFP-TH expression levels were presented in MFI in the lower panel in B. n = 2 for \( \alpha_{IIb}-\alpha_1 \) in D.
Integrin $\alpha_{\text{M}}$–chimeras bearing various $\alpha$ CTMD regions respond differently to talin head (TH) stimulation. The $\beta_3$ integrins partners $\alpha_{\text{M}}$ and $\alpha_{\text{V}}$ subunits share 6 consensus residues at their CTMD regions, but the $\alpha_\text{M}$ CTMD region is about two times longer than that of the $\alpha_{\text{V}}$ (Fig. 1A). We have shown that complete deletion of the CTMD regions of $\alpha_{\text{V}}$ and $\alpha_{\text{M}}$ subunits abolished TH-induced $\alpha_{\text{M}}$–$\beta_3$ and $\alpha_{\text{V}}$–$\beta_3$ activation. Here, we asked whether the CTMD regions of $\alpha_{\text{M}}$ and $\alpha_{\text{V}}$ subunits could be exchangeable and whether they could exert different effect on $\beta_3$ integrin inside-out activation. The ligand-mimetic mAb PAC-1 was used to access the $\alpha_{\text{M}}$–$\beta_3$ activation induced by the overexpression of GFP-TH in the presence of the $\beta_3$ cytoplasmic mutation $\beta_3$–D723A, which has been shown to greatly enhance the responsiveness of TH-induced $\alpha_{\text{M}}$–$\beta_3$ activation.

When the CTMD region of $\alpha_{\text{M}}$ was replaced by the $\alpha_3$ CTMD region, the $\alpha_{\text{M}}$–$\alpha_3$–$\beta_3$–D723A chimeric integrin still remained responsive to GFP-TH-induced activation (Fig. 2B). However, the activation of $\alpha_{\text{M}}$–$\alpha_3$ was significantly decreased compared with the $\alpha_{\text{M}}$–WT (Fig. 2B). The reduced activation of $\alpha_{\text{M}}$–$\alpha_3$-integrin was not due to the differences in GFP-TH expression since the lower activity of $\alpha_{\text{M}}$–$\alpha_3$ compared with $\alpha_{\text{M}}$–WT was consistently seen at various levels of GFP-TH expression (Fig. 2C). As a comparison, we replaced the $\alpha_{\text{M}}$ CTMD region with those of $\alpha_3$ and $\alpha_5$ integrins that do not heterodimerize with $\beta_3$ integrin. Remarkably, the presence of both the $\alpha_3$ and $\alpha_5$ CTMD regions significantly enhanced the GFP-TH-induced activation of $\alpha_{\text{M}}$–$\beta_3$ integrin (Fig. 2B). The increased activation of $\alpha_{\text{M}}$–$\alpha_3$ and $\alpha_{\text{M}}$–$\alpha_5$ was also obvious in the absence of TH expression (Fig. 2B), indicating that the mismatch of the $\alpha_{\text{M}}$ CTMD mutant with the $\beta_3$ CT renders $\alpha_{\text{M}}$–$\beta_3$ more active than the wild type. This may be due to the destabilization of $\alpha_{\text{M}}$–$\beta_3$ CT interaction, being consistent with the previous observations that the $\alpha$ CTMD regions contribute to maintaining integrin in the resting state.

Replacing the $\alpha_\text{M}$ CTMD region with that of $\alpha_X$, $\alpha_D$, or $\alpha_M$ subunit reduced TH-mediated $\alpha_\beta_3$ integrin activation. Integrin $\beta_3$ subunit forms heterodimers with $\alpha_2$, $\alpha_5$, $\alpha_\text{M}$, and $\alpha_\text{V}$ subunits. $\alpha_\text{L}$ has the longest while $\alpha_{\text{M}}$ has the shortest CTMD sequence among the four subunits (Fig. 1A). The NMR structures of $\alpha_2$, $\alpha_5$, and $\alpha_{\text{M}}$ CTs show great structural heterogeneities at their MD regions (Fig. 1B). In addition, we found that deletion of the $\alpha_\text{M}$ CTMD region abolished, while truncation of the $\alpha_2$ CTMD region dampened TH-induced $\alpha_\beta_3$ activation, arguing a potential regulatory role of the CTMD region. Similar to the $\alpha_{\text{M}}$ chimeras, we made the $\alpha_2$ chimeras by replacing the $\alpha_\text{M}$ CTMD region with that of $\alpha_2$ in the $\alpha_\text{M}$–$\beta_3$ integrin. The active conformation-specific mAb 370.3 was used to report the extension of $\alpha_\text{M}$-integrin. Consistent with the PAC-1 binding assay, the $\alpha_{\text{M}}$–$\alpha_2$ chimeras showed increased while the $\alpha_{\text{M}}$–$\alpha_2$ and $\alpha_{\text{M}}$–$\alpha_2$ chimeras showed increased binding of mAb 370.3 either in the presence or absence of TH expression (Fig. 2D). This data demonstrates that the CTMD regions of $\alpha_{\text{M}}$ and $\alpha_2$ are not completely interchangeable. They can exert different effect on $\beta_3$ integrin activation at least in part through regulating the conformational change of integrin.

A preserved tyrosine residue in the CTMD regions of $\alpha_M$, $\alpha_X$, and $\alpha_D$ subunits negatively regulates the inside-out activation of $\beta_3$ integrin. Sequence alignment revealed a conserved tyrosine residue at the second position of all the CTMD regions of $\alpha_5$, $\alpha_X$, and $\alpha_D$ subunits (Fig. 1A). We asked whether this tyrosine contributes to the reduced activation of $\alpha_{\text{M}}$ chimeras. We first tested the tyrosine mutation on $\alpha_2$–$\alpha_{\text{X}}$ chimeras with the co-expression of $\beta_3$–D709A mutant. Compared with the $\alpha_2$–$\alpha_{\text{X}}$, the tyrosine to phenylalanine mutation at the $\alpha_\text{X}$ CTMD region, $\alpha_2$–$\alpha_{\text{X}}$–Y1117F, significantly increased the TH-induced ICAM-1 binding and restored it to the WT $\alpha_2$ level (Fig. 4A). Similarly, the $\alpha_2$–$\alpha_{\text{X}}$–Y1115F mutation also increased the TH-induced ICAM-1 binding although it was not to the WT level and not statistically significant (Fig. 4B). We further tested the tyrosine mutation on the $\alpha_3$–$\alpha_M$ chimeras. Mutating the tyrosine in the $\alpha_3$ CTMD sequence to phenylalanine (Y1121F), glutamic acid (Y1121E), and alanine (Y1121A) all significantly increased the TH-induced ICAM-1 binding of $\alpha_3$–$\alpha_M$ chimeras (Fig. 4C). Interestingly, the Y1121A mutation exerted a higher level of activation than the Y1121E and Y1121E mutations (Fig. 4C), indicating that both the bulky side chain and the hydroxyl group of tyrosine are important for its negative effect on integrin activation. Sequence alignment also shows a conserved methionine at the fifth position of all the $\alpha_5$, $\alpha_X$, and $\alpha_D$ regions (Fig. 1A). We tested whether this methionine residue contributes to the negative effect of CTMD region using the $\alpha_3$–$\alpha_M$ chimeras. We found that the $\alpha_3$–$\alpha_M$–M1124A mutation did not significantly increase TH-induced ICAM-1 binding (Fig. 4C). The expression levels of integrin and GFP-TH were comparable among the transfections within the same experimental group (Fig. 4A–C). These data clearly demonstrate that the negative effects of the CTMD regions of $\alpha_5$, $\alpha_X$, and $\alpha_D$ subunits on TH-induced $\beta_3$ integrin activation at least in part attribute to the presence of a conserved tyrosine.

The position of the tyrosine mutation at the CTMD region is critical for its negative regulation of the $\alpha_3$–$\beta_3$ integrin inside-out activation. Interestingly, there is only one tyrosine in the CTMD region...
of αM, αX, or αD subunit and no tyrosine in the αL CTMD region (Fig. 1A). To further demonstrate the important regulatory role of a tyrosine residue in the CTMD region, we performed a tyrosine scanning mutagenesis for the αL CTMD region. A tyrosine mutation was placed at the 1st, 2nd, 3rd, 4th, or 6th position of the CTMD region after the conserved GFFKR motif (Fig. 5A). As shown in the TH-induced ICAM-1 binding assay, the tyrosine mutation at the 1st position, αL-N1095Y, significantly enhanced ICAM-1 binding (Fig. 5B). This is probably due to the disturbance of αβ association at the GFFKR motif, which was known to be important in maintaining integrin in the resting state 11. In contrast, the tyrosine mutation at the 2nd position, αL-L1096Y, which is equivalent to the native tyrosine of the αX, αD and αM CTMD regions, significantly reduced TH-induced ICAM-1 binding.
(Fig. 5B). This is consistent with the above data. Remarkably, the tyrosine mutations at the 3rd and 4th positions, αL-K1097Y and αL-E1098Y, also significantly reduced ICAM-1 binding (Fig. 5B). However, the tyrosine mutation at the 6th position, αL-M1100Y, had no significant effect on ICAM-1 binding (Fig. 5B). Among the single tyrosine mutations, the tyrosine mutation at the 3rd position, αL-K1097Y, had the most negative effect (Fig. 5B). We next asked whether the presence of multiple tyrosine mutations at the CTMD region has a synergistic effect on its negative regulation of integrin activation. A triple tyrosine repeat was introduced into the 2nd to 4th positions of αL CTMD region (Fig. 5A). The αL-YYY mutation significantly reduced ICAM-1 binding compared with the wild type, but had no significant difference with the single αL-K1097Y mutation (Fig. 5B). Furthermore, the negative
Figure 5. The position of a tyrosine mutation at the \( \alpha_L \) CTMD region determines its negative effect on \( \alpha_L \) integrin inside-out activation. (A) Tyrosine mutations introduced into the \( \alpha_L \) CTMD region. (B-D-E) TH-induced ICAM-1 binding of the \( \alpha_L \) tyrosine mutations co-expressed with the \( \beta_2 \)-D709A mutant (B,D) or \( \beta_2 \)-WT (E). Binding of ICAM-1 was measured by flow cytometry with HEK293FT cells co-transfected with the indicated integrin constructs and GFP or GFP-TH. The GFP and integrin double-positive cells were analyzed. ICAM-1 binding is presented as the MFI of ICAM-1 normalized to integrin expression, and shown as mean ± s.e.m. (n ≥ 3). Two-tailed Student’s t-test was performed to compare the \( \alpha_L \) mutants to \( \alpha_L \)-WT under GFP-TH condition, or as indicated. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. Integrin and GFP-TH expression levels were presented in the lower panel. (C) Dose response curves of ICAM-1 binding to selected tyrosine mutant of \( \alpha_L \) integrin. The \( \alpha_L \) constructs were co-expressed with \( \beta_2 \)-D709A and GFP-TH in HEK293FT cells. ICAM-1 binding is measured by flow cytometry and analyzed for the GFP and integrin double-positive cells. Data are presented as the percentage of maximum ICAM-1 binding of each experimental repeat and shown as mean ± s.e.m. (n = 2).

effect of the tyrosine mutation did not depend on the ICAM-1 concentration (Fig. 5C). These data demonstrate that the presence and the position but not the number of tyrosine mutations at the CTMD region are important in the negative regulation of \( \alpha_L/\beta_2 \) activation.
The α1-K1097Y mutation coincidently formed an Yxxϕ motif (x is any amino acid, ϕ is hydrophobic residue), which has been found recently in a subset of α integrins to play a role in the regulation of integrin endocytosis. It has been shown that the presence but not the position of the Yxxϕ motif is important for its function in integrin endocytosis. To test whether the negative effect of α1-K1097Y mutation was due to the formation of Yxxϕ motif, we generated another tyrosine mutation, α1-G1103Y, which formed an Yxxϕ motif (Fig. 5A). In contrast to the α1-K1097Y mutation, the α1-G1103Y mutation showed no difference with the α3-WT in ICAM-1 binding (Fig. 5D), suggesting that the negative effect of α1-K1097Y mutation is not due to the presence of an Yxxϕ motif.

In our TH-induced ICAM-1 binding assay, all the α3 constructs were co-expressed with the β3-D709A mutation. To demonstrate that the differences we observed among the α3 mutations in the integrin activation assay are not due to the presence of the β3-D709A mutation, we did the same assay in the presence of β3-WT for several representative tyrosine mutations. The results show that all the selected α3 tyrosine mutations reduced TH-induced ICAM-1 binding although not as significant as in the presence of the β3-D709A mutation (Fig. 5E).

The α CTMD regions contribute to integrin activation by regulating the conformational change of integrin. TH-induced integrin activation is coupled with the large-scale conformational changes of integrin extracellular domain. We used two mAbs, KIM127 and m24, which report α5 to the decrease in TH-induced α5 extracellular domain 5,10. We used two mAbs, KIM127 and m24, which report α5 suggesting that swapping the β3 on the TH and α5 data indicate that the regulatory function of TH-induced ICAM-1 binding although not as significant as the presence of the 2-D709A mutation (Fig. 5E).

Having found that introducing a tyrosine residue into the specific position of α3 CTMD region negatively regulates α3β3 ligand binding and conformational change, the next question is whether the native tyrosine residue present in the α3α5 or α3β3 CTMD region plays a role in regulating the activation of these integrins. To answer this question, we performed the TH-induced activation assay for α3β3 integrin by detecting the β3 integrin headpiece opening using m24. Consistent with the α3β3, the presence of β3-D709A mutation greatly enhanced TH-induced binding of m24 to α3β3 (Fig. 6C). When replacing the conserved tyrosine at the 2nd position of α3 CTMD region (Y1121) with either leucine or alanine, the TH-induced binding of m24 was further increased significantly in the presence of β3-D709A (Fig. 6C). The same effect was found when the α3-Y1121L or α3-Y1121A was co-expressed with the β3-WT (Fig. 6D). Interestingly, the enhanced activation by α3-Y1121A is more obvious than α3-Y1121L when they were co-expressed with β3-WT (Fig. 6D), again suggesting that the bulky side chain of tyrosine is important for the negative effect on integrin activation. The expression level of integrin or GFP-TH is comparable among the transfections (Fig. 6C,D). These data, in addition to the α3β3 tyrosine mutations that exerted the opposite effect on α3β3 integrin activation, clearly demonstrate that a specific tyrosine residue present in a subset of α integrin CTMD regions negatively regulates β3 integrin inside-out activation.

Mutagenesis of the αCTMD and αCTMD regions does not affect the TH binding to the integrin β3 and β3 CTs. Structural and functional studies have demonstrated that TH binds to the integrin β CT to induce integrin activation 18. To test whether the α CTMD region contributes to integrin activation by affecting the TH association with the β CT, we did co-immunoprecipitation assay for the GFP-TH and integrin β subunit co-expressed with the α CTMD mutants. For the αCTMD transfectants, the cell lysates were precipitated with anti-GFP antibody and the associated β integrins were detected by the anti-β antibody. The β3 WT or β3-D723A was robustly detected in the anti-GFP pull-down only when the GFP-TH but not GFP was present, demonstrating the specific interaction between TH and β3 subunit (Figs 7A and S1). The expression levels of GFP-TH and β3 integrin were comparable among the transfectants according to the Western blot of whole cell lysate (Figs 7A and S1). No obvious differences for the TH-bound β3-D723A were observed among the α3WT and α3 chimera, suggesting that swapping the αCTMD region with that of α3α5, α3, or α3 does not affect the association of TH with β3 subunit.

We next performed the same assay for the α3 CTMD mutations. To simultaneously detect both the α3 and β3 subunits in the anti-GFP pull-down assay, we did the cell surface biotinylation before lysing the cells for co-immunoprecipitation. The presence of α3 and β3 bands was confirmed by anti-α3 pull-down using the anti-α3 specific mAb TS2/4. As shown in Fig. 7B, two bands that correspond to the α3 and β3 subunits were readily detected in the anti-GFP pull-down only when both the α3β3 and GFP-TH were co-expressed. No α3 and β3 bands were detected in the anti-GFP pull-down in the transfections of GFP-TH alone or α3β3 plus GFP (Figs 7B and S1). The expression levels of integrin and GFP-TH were comparable among the transfections detected by flow cytometry (data not shown). To compare the association of GFP-TH and the α3β3 constructs, we quantified the Western blots by normalizing the α3 and β3 signals to the GFP-TH signals (Fig. 7C). Compared with the α3 WT, only the α3α5, α3α5 chimera shows an obvious increase in the association of integrin and GFP-TH (Fig. 7C). This is in contrast to the decrease in TH-induced α3β3 activation as shown above. No significant differences were found among the α3 WT, α3α5, α3α5, α3α3, and the β3 tyrosine mutations, α3-Y1106Y, α3-K1097Y, and α3-M1100Y (Fig. 7C). These data indicate that the regulatory function of α CTMD region on integrin activation should not be affected by the effect on the TH and β CT association.

Integrin αCTMD-chimeras bearing various α CTMD regions respond differently to TH stimulation when paired with the same integrin β3 subunit. Twelve integrin α subunits share the integrin β3 subunit, making the largest β3 integrin subfamily (Fig. 1A). The major fibronectin receptor, integrin α5β3, has been relatively well studied structurally and functionally. Like α3β3 and α3β3, the α3β3 integrin can be activated by the overexpression of TH 18. We have demonstrated the important role of the CTMD regions of α5α5, α5α3 and α5α3.

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subunits in TH-induced integrin activation\(^\text{13}\). Here, we extended our study to \(\alpha_5\beta_1\) integrin and asked whether the \(\alpha_5\) CTMD region follows the same rule. Using the \(\alpha_5\beta_1\)-deficient CHO-B2 cell line\(^\text{21}\), we found that complete deletion of the \(\alpha_5\) CTMD region abolished GFP-TH-induced binding of fibronectin fragment Fn9–10 to \(\alpha_5\beta_1\) integrin.

Figure 6. The \(\alpha\) CTMD regions contribute to integrin activation by regulating the conformational change of integrin. (A, B) TH-induced integrin conformational change. Binding of mAb KIM127 (reports integrin extension) or m24 (reports integrin headpiece opening) was assessed with (A) \(\alpha\)L-chimeras, or with (B) selected \(\alpha\)L tyrosine mutants co-expressed with \(\beta_2\)-D709A in HEK293FT cells. (C, D) TH-induced integrin conformational change of \(\alpha_M\) integrin constructs co-expressed with \(\beta_2\)-D709A or \(\beta_2\)-WT. Binding of m24 or KIM127 mAb was measured by flow cytometry with HEK293FT cells co-transfected with the indicated integrin constructs and GFP or GFP-TH. Data are presented as the MFI of bound m24 or KIM127 normalized to the MFI of integrin expression, and shown as mean \(\pm\) s.e.m. (\(n \geq 3\)). Two-tailed Student’s t-test was performed to compare the \(\alpha\)L mutants to \(\alpha\)L-WT in A and B, or to compare the \(\alpha_M\) mutants to \(\alpha_M\)-WT, or as indicated under the GFP-TH condition in C and D (*\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\)). Integrin and GFP-TH expression levels were presented in MFI in lower panel of C and D. One representative experiment was shown for the KIM127 binding in B.
(Fig. 8A), demonstrating the requirement of \( \alpha_5 \) CTMD region in \( \alpha_5 \beta_1 \) inside-out activation. The next question is whether the diverse \( \alpha \) CTMD regions of the \( \beta_1 \) integrin family also differently regulate \( \beta_1 \) integrin activation. To answer this question, we compared the function of all the \( \alpha \) CTMD regions of the \( \beta_1 \) integrin family in the context of \( \alpha_5 \) subunit. Eleven \( \alpha_5 \) chimeras were generated by replacing the \( \alpha_5 \) CTMD region with that of \( \alpha \) subunits as indicated in Figs 2A and 8B. The \( \beta_1 \)-induced fibronectin-binding assay for \( \alpha_5 \beta_1 \) was performed using the \( \alpha_5 \beta_1 \)-knockout HEK293FT cells. The activating \( \beta_1 \)K732E mutation, located at the transmembrane domain22, was used to enhance the sensitivity of the assay. As shown in Fig. 8B, the \( \beta_1 \)K732E significantly increased TH-induced fibronectin binding compared with the \( \beta_1 \) WT. Among the \( \alpha_5 \) chimeras, two groups were identified: one group has no but the other has significant effect on TH-induced fibronectin binding compared with the \( \alpha_5 \) WT (Fig. 8B). Remarkably, all the \( \alpha_5 \) chimeras, including \( \alpha_5 \alpha_4, \alpha_5 \alpha_6, \alpha_5 \alpha_7, \alpha_5 \alpha_8, \) and \( \alpha_5 \alpha_9 \), which have a tyrosine residue adjacent to the GFFKR motif (Fig. 1A), showed comparable fibronectin binding with the \( \alpha_5 \) WT that also contains a tyrosine at the CTMD region (Figs 1A and 8B). In contrast, all the \( \alpha_5 \) chimeras, including \( \alpha_5 \alpha_1, \alpha_5 \alpha_2, \alpha_5 \alpha_3, \) and \( \alpha_5 \alpha_10 \), which lack the tyrosine residue adjacent to the GFFKR motif (Fig. 1A), showed no such effect (Fig. 8B). However, an exception is the \( \alpha_5 \)\( \alpha_5 \) chimera, which has an equivalent tyrosine at the \( \alpha_5 \) CTMD region but significantly increased \( \alpha_5 \beta_1 \) activation (Figs 1A and 8B). The similar results were obtained when the selected \( \alpha_5 \) chimeras were co-expressed with the \( \beta_1 \) WT in the TH-induced fibronectin binding assay (Fig. 8C). The \( \alpha_5 \alpha_3 \) and \( \alpha_5 \alpha_10 \) significantly increased \( \alpha_5 \beta_1 \) activation while the \( \alpha_5 \alpha_2 \) had no obvious effect (Fig. 8C). By contrast, the enhanced effect of \( \alpha_5 \alpha_3 \) was not detectable when the \( \beta_1 \) WT was used (Fig. 8C), indicating the low sensitivity of the assay. Finally, we correlated the TH-induced \( \alpha_5 \beta_1 \) activation with the large-scale conformational change of \( \beta_1 \) ectodomain using the active conformation dependent mAb 9EG7. The presence of \( \beta_1 \)K732E significantly enhanced the TH-induced 9EG7 binding. Consistent with the fibronectin binding assay, the \( \alpha_5 \alpha_2, \alpha_5 \alpha_7, \) and \( \alpha_5 \alpha_10 \) significantly increased 9EG7 binding, but the \( \alpha_5 \alpha_8 \) had no such
effect compared with the $\alpha_5$ WT (Fig. 8D). These data suggest that the $\alpha$ CTMD regions of the $\beta_1$ family could contribute differently to $\beta_1$ integrin inside-out activation. They may also follow the rule of the negative regulation by a tyrosine residue.

**Discussion**

Compared with the extensive structural and functional studies of the relatively conserved $\beta$ integrin CT that serve as docking sites for many signaling molecules, little is known about the role of $\alpha$ integrin CT especially the non-conserved MD regions. Since many $\alpha$ integrin subunits share the same $\beta$ subunit, it is tempting to speculate that on one side the $\alpha$ CTMD regions may be interchangeable; on the other side, they may provide the specificity for integrin function. One of the difficulties in studying the $\alpha$ CTMD regions is the sequence diversity among 18 $\alpha$ subunits. Another challenge is the lack of well-established activation assays for many integrin members, which limits the functional studies of the CTMD regions for many $\alpha$ integrins. Our approach in the current study provides a useful tool to thoroughly examine the potential functions of the CTMD regions of almost all integrin $\alpha$ subunits. By putting the different $\alpha$ CTMD regions in the context of the $\alpha_{IIb}$, $\alpha_L$, or $\alpha_5$ subunit, this approach made it possible to compare the function of different $\alpha$ CTMD regions.

The potential function of several individual $\alpha$ integrin CTMD regions in integrin activation had been indicated in previous studies more than 20 years ago. They showed that deletion of the $\alpha$ CTMD region diminished cell adhesion or migration mediated by $\alpha_1$, $\alpha_2$, $\alpha_4$, $\alpha_6$, and $\alpha_5$ integrins and dampened PMA-induced activation of $\alpha_{IIb}\beta_3$ integrin. Direct evidence for a role of $\alpha$ CTMD region in integrin inside-out activation was provided by the observation that complete deletion of the CTMD region of $\alpha_{IIb}$, $\alpha_5$, or $\alpha_6$ integrin abolished...
talin and kindlin-mediated integrin ligand binding and conformational changes\textsuperscript{13,14}. It was suggested that the presence but not the sequence of specific residues was required for the \(\alpha\) CTMD region to support talin-induced integrin activation\textsuperscript{34}. In the current study, we found that replacing the \(\alpha\) CTMD region of \(\alpha_{\text{M}}\), \(\alpha_{\text{L}}\), or \(\alpha_{\text{M}}\) integrin with those of other \(\alpha\) integrins still maintained the capability of integrin inside-out activation mediated by the overexpression of TH, suggesting that the \(\alpha\) CTMD region can be interchangeable for this common activation supportive function. However, we observed significant variations of the activation levels among the same \(\alpha\) integrins carrying different CTMD regions, indicating that the \(\alpha\) CTMD region also plays a regulatory role in integrin inside-out activation.

We found that replacing the \(\alpha_{\text{M}}\) CTMD region with that of \(\alpha_{\text{L}}\) integrin markedly reduced TH-induced activation of \(\alpha_{\text{M}}\). By contrast, the \(\alpha_{\text{L}}\) and \(\alpha_{\text{M}}\) CTMD regions rendered \(\alpha_{\text{M}}\) more active than WT, consistent with the previous observation that replacing the \(\alpha_{\text{L}}\) CT with those of \(\alpha_{\text{M}}, \alpha_{\text{V}}, \alpha_{\text{L}},\) or \(\alpha_{\text{M}}\) that do not natively pair with \(\beta_{3}\) subunit enhanced \(\alpha_{\text{M}}\) activation despite they share the same GFFKR motif\textsuperscript{32}. Thus, the native pair between the \(\alpha\) CTMD region and \(\beta\) CT is important to maintain the resting state of integrin. Although the \(\alpha_{\text{M}}\) CTMD region shares six consensus residues with \(\alpha_{\text{L}}\) CTMD region, which is half of the length of \(\alpha_{\text{M}}\) CTMD region (Fig. 1A), it exerts different effect on \(\alpha_{\text{M}}\) activation. A predicted \(\beta\) turn structure formed by the PPQEE motif of \(\alpha_{\text{V}}\) CTMD region was suggested to regulate the conformation and ligand binding of \(\alpha_{\text{V}}\)\textsuperscript{35,36}. A similar motif PPLEE was also found in the \(\alpha_{\text{M}}\) CTMD region (Fig. 1A). Peptides containing this motif of \(\alpha_{\text{V}}\) or \(\alpha_{\text{M}}\) CTMD region could inhibit the activation of \(\alpha_{\text{M}}\) or \(\alpha_{\text{V}}\) CTMD region, indicating that it could not be the reason for the different regulation by the \(\alpha_{\text{M}}\) and \(\alpha_{\text{V}}\) CTMD regions. Although the structure of the \(\alpha_{\text{M}}\) CT has been determined, it shows large conformational variations (Fig. 1B). It is not known if the structural flexibility of \(\alpha_{\text{M}}\) CT is functionally relevant. The \(\alpha_{\text{M}}\) CTMD region has a unique tandem acidic residue motif, EEDDEEGE, which is conserved among the \(\alpha_{\text{M}}\) from different species and not seen in the \(\alpha_{\text{V}}\) or \(\alpha_{\text{L}}\) CTMD region (Fig. 1A).Negatively charged residues might regulate the conformation of \(\alpha_{\text{M}}\) CT through repulsive interactions with the acidic phospholipid head groups at the cytosolic face of cell membrane, or through ionic interactions with the positively charged residues at the membrane-proximal region of \(\alpha_{\text{M}}\) CT as suggested by a NMR study\textsuperscript{34}. The membrane-permeable peptides containing the \(\alpha_{\text{M}}\) CTMD region were shown to block \(\alpha_{\text{M}}\) activation in platelets\textsuperscript{15,35}, and to inhibit the association of talin with \(\alpha_{\text{M}}\) in thrombin-activated platelets\textsuperscript{36}. In addition, the TH domain contains several positively charged surface residues that have been shown to be important for its integrin activating function through interacting with the cell membrane\textsuperscript{37–39}. It is possible that the negatively charged \(\alpha_{\text{M}}\) CTMD region may also affect the orientation of TH domain when TH encounters with \(\beta_{3}\) CT, but a direct interaction between TH and \(\alpha_{\text{M}}\) remains to be confirmed. Being critical in hemostasis and thrombosis, the activation of \(\alpha_{\text{M}}\) is strictly regulated in platelets\textsuperscript{39}. Our data demonstrated that the unique feature of \(\alpha_{\text{M}}\) CTMD region renders \(\alpha_{\text{M}}\) more susceptible to the signals of inside-out activation, which is in line with the high extent of activation required for \(\alpha_{\text{M}}\) function.

The lymphocyte-specific \(\alpha_{\text{L}}\) is another integrin whose activation is highly regulated by the inside-out signals\textsuperscript{40,41}. The \(\alpha_{\text{L}}\) CT has the second longest MD region that folds into \(\alpha\)-helical conformation as shown in an NMR structure (Fig. 1A,B). Our recent study showed that deletion of the \(\alpha_{\text{L}}\) CTMD region completely abolished TH-induced ICAM-1 binding to \(\alpha_{\text{L}}\) integrin\textsuperscript{15}. Here we found that replacing the \(\alpha_{\text{L}}\) CTMD region with that of \(\alpha, \alpha_{\text{M}}, \alpha_{\text{V}}, \alpha_{\text{L}},\) or \(\alpha_{\text{M}}\) all significantly reduced ICAM-1 binding mediated by TH. This was not determined by the differences in the length but by a common tyrosine residue seen in the \(\alpha_{\text{L}}\) CTMD regions. Remarkably, we found that the position of the tyrosine at the \(\alpha\) CTMD region is important to exert the negative effect on integrin inside-out activation. The bulky side chain and the hydroxyl group of tyrosine are critical for this regulatory function. Interestingly, the same negative effect was observed when introducing the tyrosine mutations into the equivalent position of the \(\alpha_{\text{M}}\) CTMD region\textsuperscript{15}. One caveat of our study is that the negative effect of the tyrosine was found in the context of \(\alpha_{\text{L}}\) integrin in which no native tyrosine is present in the CTMD region. However, the physiological relevance of our discovery was built on the same observations on the \(\alpha_{\text{M}}\) integrin, in which mutating the native tyrosine residue greatly enhanced the inside-out activation of \(\alpha_{\text{M}}\), suggesting that the tyrosine residue negatively regulates the activity of \(\alpha_{\text{M}}\). The tyrosine residue is also conserved among the \(\alpha_{\text{M}}, \alpha, \alpha_{\text{L}},\) or \(\alpha_{\text{V}}\) integrins from different species (data not shown). Indeed, in contrast to the \(\alpha_{\text{L}}\) integrin that only binds selectively to ICAMs, the \(\alpha_{\text{M}}\), \(\alpha_{\text{L}},\) and \(\alpha_{\text{V}}\) integrins all exhibit multiligand-binding properties\textsuperscript{42–44}. The negative regulation by a tyrosine residue in their \(\alpha\) CTMD regions may exert a restraint to avoid hyperactivity (or to balance the activation) of these \(\beta\) integrins, in accordance with their less-selective ligand binding functions.

Several studies have provided evidence demonstrating that the activation of \(\alpha_{\text{L}}\) and \(\alpha_{\text{M}}\) are differently regulated. Different chemokines and chemoattractants were shown to stimulate inside-out activation of \(\alpha_{\text{L}}\) and \(\alpha_{\text{M}}\), which was suggested to be mediated by distinct pathways via the \(\alpha\) CT.\textsuperscript{45,46} Given the \(\beta_{3}\) subunit shares the common integrin activation pathway mediated by talin and kindlin, other signaling molecules may be involved in the different regulation of \(\alpha_{\text{L}}\) and \(\alpha_{\text{M}}\) probably through direct interaction with the \(\alpha\) CT. An example is the Rap-1 interacting effector molecule RapL that specifically binds to the \(\alpha\) CTMD region to support Rap-1-mediated \(\alpha_{\text{L}}\) activation\textsuperscript{47}. It was known that the \(\alpha_{\text{L}}\)-K1097 is involved in RapL binding\textsuperscript{48}. Our results show that the \(\alpha_{\text{L}}\)-K1097Y mutation rendered \(\alpha_{\text{L}}\) the least active among the tyrosine mutations tested. However, RapL is predominantly expressed in immune cells\textsuperscript{49}. It should not account for the decreased activation by the \(\alpha_{\text{L}}\)-K1097Y mutation since integrin activation was measured in HEK293FT cells lacking RapL expression. A Ser phosphorylation was found in the CTMD regions of both \(\alpha_{\text{L}}\) and \(\alpha_{\text{M}}\) subunits\textsuperscript{50,51}. However, mutations of the Ser residue only blocked the conformational changes involved in \(\alpha_{\text{L}}\) activation but not \(\alpha_{\text{M}}\) activation, indicating different regulation by the \(\alpha_{\text{L}}\) CT. Here, we identified a tyrosine residue at the \(\alpha_{\text{L}}\) CTMD region that is also involved in the \(\alpha_{\text{M}}\) CTMD region may exert a restraint to avoid hyperactivity (or to balance the activation) of these \(\beta\) integrins, in accordance with their less-selective ligand binding functions.

It remains unknown whether the activation of \(\beta_{3}\) integrin family members are all subjected to inside-out regulation. Recent structural studies demonstrated that the conformational activation of \(\alpha_{\text{L}}\) integrin could be modulated by many components including the transmembrane and cytoplasmic domains\textsuperscript{45–53}. We found that similar to \(\alpha_{\text{L}}\), \(\alpha_{\text{V}}\), and \(\alpha_{\text{M}}\) integrins the \(\alpha_{\text{L}}\) CTMD region is also required for TH-induced inside-out activation,
rationalizing the use of $\alpha_\beta$ as a model integrin to study the function of $\alpha$ CTMD regions. Our studies on all the $\alpha$ CTMD regions of the $\beta_1$ subfamily suggest a potential regulatory function of these regions in $\beta_1$ integrin inside-out activation. Remarkably, a tyrosine residue at the $\alpha$ CTMD region seems to play a similar negative role as seen in $\alpha_\beta$ integrin in regulating $\alpha_\beta$ inside-out activation. All the $\alpha$ CTMD sequences lacking a tyrosine proximal to the GFFKR motif promoted $\alpha_\beta$ inside-out activation, while the $\alpha$ CTMD sequences having the tyrosine had no such effect, with the exception of $\alpha_5$ CTMD region. Notably, the $\alpha_5$ CTMD region contains a tyrosine at the 11th position distal from the GFFKR motif (Fig. 1A), but the $\alpha_5$-$\alpha_5$ exhibits a high level of integrin activation, consistent with the hypothesis that the membrane-proximal location of the tyrosine is important to exert an inhibitory effect. Interestingly, the $\alpha_{10}$ CTMD region that is abundant in negatively charged residues as seen in $\alpha_m$ CTMD region exerted the most dramatic effect on $\alpha_{10}$ activation. Consistently, the $\alpha_5$ CTMD region increased $\alpha_{10}$ activation due to the lack of the tyrosine residue when compared with $\alpha_5$ WT, but it decreased $\alpha_{10}$ activation due to the lack of the cluster of acidic residues when compared with $\alpha_{10}$ WT. We found that $\alpha_2$ CTMD region rendered $\alpha_{2}$ more active despite having the consensus tyrosine, suggesting that other residues of the $\alpha_2$ CTMD region may counteract with the negative effect of tyrosine in regulating integrin activation.

Our study raised the question of the mechanism by which the $\alpha$ CTMD regions contribute to integrin inside-out activation. Previous and our current data demonstrated that the integrin $\alpha$ CTMD region is involved in the associations at the $\alpha/\beta$ transmembrane and CT domains, required to maintain integrin in the resting state\(^\text{11}\). In line with this function, our current data suggest two functional aspects of $\alpha$ CTMD region in integrin inside-out activation, i.e. the activation-supportive function and the activation-regulatory function. Structure analysis suggested that when binding to the $\beta_1$ CT, the talin 1 head domain might encounter steric hindrance with the $\alpha_{10}$ CTMD residues immediately following the GFFKR motif\(^\text{31,46-48}\). Such interactions may disrupt the integrin $\alpha/\beta$ association at the cytoplasmic as well as transmembrane domains, leading to integrin conformation capable of high-affinity ligand binding. This non-specific interaction is required for the activation-supportive function of $\alpha$ CTMD region, which could be independent of the amino acid sequences. In addition, a minimal length of two amino acids of the $\alpha$ CTMD region could support the integrin inside-out activation although at a reduced level\(^\text{13}\). This model is consistent with our observation that the $\alpha$ CTMD regions are interchangeable for the activation-supportive function that is not sensitive to the diversities of sequence and length.

In contrast, the activation-regulatory function of $\alpha$ CTMD region is dependent on certain amino acids. Several potential mechanisms are involved in this regulation. We found that the differences of the $\alpha$ CTMD regions in regulating integrin activation were not due to the effect of talin binding to the $\beta_1$ CT since truncation or swapping the $\alpha$ CT since truncation $\beta_1$ regions in regulating integrin activation were not due to the effect of talin binding to the $\alpha_\beta$ CTMD regions induced by the binding of integrin activators such as talin and kindlin. Recent studies provided evidence suggesting the conformational change of $\alpha_{10}$ transmembrane and cytoplasmic domains in the context of full-length integrin on the cell surface\(^\text{49}\). In addition, different levels of integrin activation had been observed when introducing mutations into the transmembrane or cytoplasmic domains, which correlate with the different levels of ectodomain conformational changes (unpublished data). Certain amino acids such as the acidic residue clusters present in $\alpha_m$ and $\alpha_\beta$ CTMD regions and the tyrosine present in a subset of $\alpha$ CTMD regions may either facilitate or restrain the conformational change of $\alpha$ subunit as demonstrated by the active conformation-specific mAbs. The regulation of receptor activity by a tyrosine residue at the cytoplasmic domains has been seen in many cell surface receptors\(^\text{50}\). It was proposed that the tyrosine residue could be buried in the cell membrane to restrain the movement of the cytoplasmic domain in the resting state. Such restraints could be released upon the tyrosine phosphorylation. This mechanism may also be applied to the specific tyrosine of $\alpha$ integrin CTMD regions. Structures of $\alpha_5$, $\alpha_3$, and $\alpha_3$ CT indicate that the tyrosine could be buried in the cell membrane (Fig. 1B). The tyrosine may restrain the piston like movement of $\alpha$ CT or the conformational plasticity of the GFFKR region as suggested by structural studies\(^\text{59,61-63}\). This negative effect depends on the position of tyrosine as shown by our current data. Whether the tyrosine can be phosphorylated to release its negative effect on integrin activation clearly requires further investigation on individual integrins. Another potential mechanism by which the $\alpha$ integrin CTMD regions regulate the level of integrin activation is through their interacting proteins. Several $\alpha$ integrin CT binding proteins have been identified to function as either negative or positive regulators for integrin activation\(^\text{64}\). Some of the regulators such as SHARPIN, MDGI, and filamin interact with a subset of $\alpha$ integrins while the others bind to specific $\alpha$ integrins, such as Nischarin for $\alpha_5$ and CIB1 for $\alpha_{10}\text{54,62-65,67}$. Interestingly, most of the current $\alpha$ CT binding proteins interact with the membrane-proximal region containing the conserved GFFKR motif\(^\text{7}\). More novel integrin activation regulators interacting with the $\alpha$ CTMD region are yet to be identified.

There are accumulating data showing that the diverse $\alpha$ integrin CTMD regions can specify the cellular function of integrins. Novel functions of the $\alpha$ CTMD regions have been identified in recent years. For example, a subset of $\alpha$ integrin CTMD regions was found to regulate integrin internalization through interacting with the endocytic clathrin adaptor AP2\(^\text{22}\). Specific interaction between integrin $\alpha_\beta$ CTMD region with phosphodiesterase-4D5 (PDE4D5) was found to regulate endothelial inflammatory signaling\(^\text{48}\). It is tempting to speculate that more novel functions of $\alpha$ integrin CTMD regions are yet to be identified. Our large-scale analysis of the function of $\alpha$ integrin CTMD regions provokes new hypotheses that need to be tested on individual integrins. In addition, our approach provides a valuable tool and resource to study the integrin signaling events that are specified by the $\alpha$ CTMD regions.

Materials and Methods

DNA constructs. Plasmid DNA constructs for human $\alpha_{10}$, $\alpha_2$, $\alpha_5$, and EGFP-tagged mouse talin-1 head (GFP-TH) were as described\(^\text{30,62,79}\). Mutations were introduced by PCR following the protocol of the QuikChange XL site-directed mutagenesis kit (Agilent Technologies). $\alpha_5$-CRISPR/Cas9 and $\beta_1$-CRISPR/Cas9
plasmids were purchased from Santa Cruz Biotechnology. Human ICAM-1 cDNA was obtained from Addgene. The cDNA of ICAM-1 extracellular domain was amplified by PCR and subcloned into a modified pRES2-EGFP vector with a tag of human IgG1Fc region at the C-terminus (denoted as ICAM-1-Fc).

The integrin αmβ2-chimeras were generated by the overlap PCR to replace the cDNA of αmCTMD region with the cDNA of the CTMD region of α1, α3, or α5 integrin. The chimeric full-length αm cDNA was cloned into the pEF1/V5-HisA vector using the 5′ EcoRV and 3′ XbaI restriction sites. A stop codon was added right before the 3′ XbaI site. The integrin αmβ2-chimeras were constructed using the 5′ BspI407I site right before the cDNA of KVGGFR motif and the 3′ XbaI site preceded by a stop codon. The extra BspI407I and XbaI sites in the WT α1 vector were silenced by site-directed mutagenesis. A set of sense and antisense overlapping primers were designed to encode the sequence of KVGGFR followed by the CTMD region of α5, α6, or α1 integrin. The 5′ BspI407I and 3′ XbaI sites as well as a stop codon were included in the primers. The chimeric cDNA fragments were obtained by mixing the primers for PCR amplification and subcloned into the αm construct using the BspI407I and XbaI sites. The full-length cDNA of human αm integrin was cloned into the pcDNA3.1 vector using the 5′ KpnI and 3′ XbaI sites. Mutations at the αm CTMD region were introduced by PCR. The integrin αmβ2-chimeras was constructed using a 5′ HindIII site preceding the GFFKR-coding sequence and a 3′ MluI site preceded by a stop codon. A set of sense and antisense overlapping primers were designed to encode the sequence of GFFKR followed by the CTMD region of α5, α6, or α1 integrin. The WT α1 vector. All the DNA constructs were validated by DNA sequencing.

**Antibodies, inhibitors and ligands.** PAC-1 (BD Biosciences) is a ligand-mimetic IgM mAb that is specific to activated αmβ2. AP3 is non-blocking anti-β1 mAb. 9EG7 (BD Biosciences) is a rat anti-β1 conformation-specific mAb that reports β1 integrin headpiece opening and extension, respectively. 2LPm19c is an anti-α1 mAb (Santa Cruz Biotechnology). PE-labeled MAR-4 (BD Biosciences) is a non-blocking anti-β1, mAb. 9EG7 (BD Biosciences) is a rat anti-β1 conformation-specific mAb that reports β1 integrin extension. Rabbit anti-AFP antibody was from Immunology Consultants Laboratory. Rabbit anti-β1 antibody (H-96) was from Santa Cruz Biotechnology. Anti-β1-actin mAb was purchased from Sigma. 9EG7 were conjugated with Alexa Fluor-647 (Life Technologies). AP3 was conjugated with R-PE using the R-PE antibody conjugation kit (Solulink). 370.3, m24, and KIM127 were biotinylated using the EZ-Link Sulfo-NHS-Biotin (Thermo Scientific). A286982 (Santa Cruz Biotechnology) is a α5β1-specific inhibitor. Eptifibatide acetate (Santa Cruz Biotechnology) is a αmβ2-specific inhibitor.

Human ICAM-1-Fc was expressed as the secreted form in HEK293FT cells via transient transfection using polyethylenimine (PEI). The transfected cells were cultured for 10 days before the culture supernatant was collected. The concentration of ICAM-1-Fc in the supernatant was determined by ELISA using the anti-human ICAM-1 mAb (SinoBiological, Inc.) and the peroxidase-conjugated anti-human IgG1 (Fc specific) (Jackson ImmunoResearch Laboratories, Inc.). The culture supernatant was used for the ICAM-1 binding assay. Human fibronectin type III domains 9–10 fragment (Fn9–10) was expressed in E. coli and purified as described before. Human fibronectin (Fn) was purchased from Sigma. Fn9–10 and Fn were conjugated with Alexa Fluor-647.

**Cell lines.** HEK293FT cells (ThermoFisher Scientific) were cultured in DMEM plus 10% FBS at 37 °C supplied with 5% CO₂. The α1, and β1 integrin double-knockout HEK293FT (HEK293FT-α1β1KO) cells were generated by the CRISPR/Cas9 gene editing technology as described in our previous study. α3β1 integrin deficient CHO-B2 cells were as described before.

**Soluble ligand binding assay by flow cytometry.** GFP-TH induced ligand binding assay of HEK293FT cells transfected with αmβ2, α5β1, or α1 integrin was as described previously. In brief, HEK293FT cells were co-transfected with integrin constructs and GFP or GFP-TH for at least 24 hours. Ligand binding was performed in HBSGB buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 2.75 mM glucose, and 0.5% BSA) plus 10 μg/mL ephitibatide acetate (for αmβ2), 50 μg/mL A286982 (for α5β1), or 1 mM Ca²⁺ and 1 mM Mg²⁺ (Ca/Mg). The cells were first incubated at 25 °C for 30 min with 5 μg/mL PAC-1 and 10 μg/mL biotinylated AP3 for αmβ2, 42 μg/mL of ICAM-1-Fc and 45 μg/mL biotin-conjugated goat anti-human IgG Fc (Novex, Life Technologies) for α5β1, and then washed and incubated in Ca/Mg on ice with the detecting reagents: PE-labeled streptavidin and Alexa Fluor-647-labeled goat anti-mouse IgM for αmβ2, PE-labeled TS2/4 and Alexa Fluor 647-labeled streptavidin for α5β1. For α1 integrin, the CHO-B2 or HEK293FT-α5β1KO cells were transfected with α5β1, integrin plus GFP or GFP-TH. The cells were first incubated with 30 μg/mL Alexa Fluor-647-labeled Fn9–10 or 15 μg/mL Alexa Fluor-647-labeled Fn in HBSGB buffer plus 5 mM EDTA or 1 mM Ca/Mg, and then washed and incubated in Ca/Mg on ice with PE-labeled MAR-4. Integrin and GFP double-positive cells were acquired for calculating the mean fluorescence intensity (MFI) by flow cytometry. Ligand binding was presented as the normalized MFI, that is ligand MFI (after subtracting the ligand MFI in the inhibitor or EDTA condition) as a percentage of integrin MFI.

**Conformation-specific antibody binding.** Integrin conformational changes detected by the conformation-specific mAbs on the cell surface was as described previously. In brief, HEK293FT transfecants were first incubated with the biotinylated conformation-specific mAb in Ca/Mg at 25 °C for 30 mins, washed and then incubated with the detecting reagents: Alexa-Fluor-647-labeled streptavidin plus R-P-E labeled AP3 for αmβ2 or PE-labeled TS2/4 for α5β1. For α5β1 integrins, HEK293FT-α5β1KO transfecants were incubated with rat 9EG7 mAb in Ca/Mg at 25 °C for 30 mins, washed and then incubated with PE-labeled MAR-4 and Alexa-Fluor-647-labeled goat anti-rat IgG (cross-absorbed) (Abcam). Integrin and GFP double-positive cells were analyzed...
for calculating the MFI of mAb binding. The binding of conformation-specific mAb is presented as their MFI normalized to the MFI of integrin expression.

For m24 binding with α3β1 integrin, HEK293FT transfectants were incubated with m24 or mouse anti-α3 mAb (2LPM19c) separately, washed and incubated with Alexa-Fluor-647-labeled anti-mouse IgG. GFP-positive cells were analyzed for calculating the MFI of bound m24 or 2LPM19c. The m24 binding was presented as the m24 MFI normalized to the 2LPM19c MFI reporting α3β1 expression.

**Co-immunoprecipitation.** To detect the TH-binding to integrin β1 CT, the α1β1β3 constructs were co-transfected with GFP-TH or GFP into HEK293FT cells. The cells were lysed to perform co-immunoprecipitation with rabbit anti-β3 antibody. The GFP-TH-associated β3 subunit was detected by immunoblot using rabbit anti-β3 antibody H-96 and HRP-labeled anti-rabbit IgG antibody. The amount of GFP or TH in pulldown samples was detected by rabbit anti-GFP antibody and HRP-labeled anti-rabbit IgG antibody. Total expression levels of integrin β3 and GFP-TH in the whole cell lysates were detected by immunoblot. β3-actin was blotted with anti-β3-actin mAb as a loading control.

To detect the TH binding to integrin β3 CT, the α5 constructs were co-transfected with β3-D709A and GFP-TH into HEK293FT cells. GFP-TH only or α5β3, plus GFP-transfectants were used as negative controls. The cells were washed with PBS at pH 8.0 and the cell surface molecules were biotinylated using 2 μM biotin reagent (EZ-Link™ Sulfo-NHS-Biotin, Thermo Scientific) in PBS at 25°C for 30 min. The cells were washed and lysed for co- immunoprecipitation with anti-GFP antibody. As a positive control for integrin α5 and β3 bands, α5β3 was immunoprecipitated with anti-α5 mAb TS2/4. Integrin α5 and β3 subunits that associated with GFP-TH were quantitatively detected by blotting with IRDye® 800CW Streptavidin (LI-COR). The immunoprecipitated GFP or GFP-TH were detected by Western blot using anti-GFP antibody. Integrin α5 and β3 signals were normalized to the precipitated GFP or GFP-TH and shown as a percentage of the normalized α5-WT signal. The cell surface expression of α5β3 integrins and GFP-TH were detected by flow cytometry.

**Statistical Analysis.** Data are expressed as mean ± s.e.m from at least three independent experiments (n ≥ 3 in each group) unless specified. Statistical analyses were performed with GraphPad Prism using parametric Student’s t-test (two-tailed).

**Data Availability.** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**
A. Thinn performed the experiments, analyzed data, and wrote the manuscript. Z. Wang performed the experiments and analyzed data. J. Zhu designed the study, analyzed data and wrote the manuscript.

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