The ligand-binding βI and αI domains of integrin are the best-studied von Willebrand factor A domains undergoing significant conformational changes for affinity regulation. In both βI and αI domains, the αI- and α7-helices work in concert to shift the metal-ion-dependent adhesion site between the resting and active states. An absolutely conserved Gly in the middle of the αI-helix of βI helps maintain the resting βI conformation, whereas the homologous position in the αI-helix contains a conserved Phe. A functional role of this Phe is structurally significant for affinity regulation. In both the αI domains, the metal-ion-dependent adhesion site (MIDAS) defined by a signature Asp-Xaa-Ser-D-Ser (DXSS) motif is connected to the N terminus of an αI-helix and also is seen in many other VWA domains. The different combinations of 18 αI and 8 βI subunits lead to 24 integrin αI/βI heterodimers that play important roles in diverse physiological and pathological conditions such as hemostasis, development, immune responses, thrombosis, inflammation, and cancer (1). All the βI integrin subunits contain a conserved Phe. A functional role of this Phe is structurally significant for affinity regulation. In both the αI domains, the metal-ion-dependent adhesion site (MIDAS) defined by a signature Asp-Xaa-Ser-D-Ser (DXSS) motif is connected to the N terminus of an αI-helix and also is seen in many other VWA domains. The different combinations of 18 αI and 8 βI subunits lead to 24 integrin αI/βI heterodimers that play important roles in diverse physiological and pathological conditions such as hemostasis, development, immune responses, thrombosis, inflammation, and cancer (1). All the βI integrin subunits contain a conserved Phe. A functional role of this Phe is structurally significant for affinity regulation.

Integrins are cell adhesion molecules that transmit both the mechanical and chemical signals into and out of the cells and thus allow the cells to communicate with their surroundings (1–4). The different combinations of 18 αI and 8 βI subunits lead to 24 integrin αI/βI heterodimers that play important roles in diverse physiological and pathological conditions such as hemostasis, development, immune responses, thrombosis, inflammation, and cancer (1). All the βI integrin subunits contain a conserved Phe. A functional role of this Phe is structurally significant for affinity regulation.

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2 The abbreviations used are: I-EGF, integrin epidermal growth factor; VWA, von Willebrand factor A; MIDAS, metal ion-dependent adhesion site; ICAM-1, intercellular adhesion molecular 1; ADMIDAS, adjacent to MIDAS; APC, allophycocyanin; MD, molecular dynamics; RMSD, root mean square deviation; RMSF, root mean square fluctuation; TRAP, thrombospondin repeat anonymous protein; PDB, Protein Data Bank.
domains (Fig. 1). In addition, the βl domains have two additional metal ion-binding sites flanking the MIDAS, namely adjacent to MIDAS (ADMIDAS) and synergetic metal ion-binding sites (Fig. 1H). Both αl and βl domains can shift between two conformations defined as the closed inactive and the open active states. In the active state, the β1-αl loop moves toward the MIDAS, increasing its potential for binding ligands (Fig. 1, H and I). For βl domain, this is accompanied by a bent-to-straight structural transition, resulting in an inward movement of the α1/α1'-helix (Fig. 1H), whereas for αl domain, it is associated with only a swing-in motion of the N terminus of α1-helix (Fig. 1I). For both βl and αl domains, such conformational changes of α1/(α1')-helix are linked with a downward displacement of β6-α7 loop and α7-helix, leading to hybrid domain swinging out (Fig. 1, C, F, and H) or the engagement of αl and βl domain via an internal ligand at the C terminus of α7-helix (Fig. 1, F and I). In our recent studies, we defined the role of an absolutely conserved Gly residue at the α1/α1'-helix (Fig. 1H), which is critical for ligand binding, is critical for ligand binding. 

Results

A bulky residue in the mid position of α1-helix of αl domain is critical for ligand binding

A hallmark structural change of αl domain in the transition from the low to the high-affinity state is the inward movement of the N terminus of α1-helix and the piston-like movement of the entire α7-helix (Fig. 1, E, F, and I). Structural superimposition of the active conformation onto the resting conformation of the αl domain shows steric clashes between the N-terminal interfacial residues of α1-helix and α7-helix (Fig. 2A), indicating that these helices should mechanically work in concert to avoid the collisions. Interestingly, the invariant phenylalanine residue α1-Phe-153 at the middle of α1-helix in the active position has no van der Waals overlaps with the α7-helix in the resting position (Fig. 2A). To test whether the conserved α1-Phe-153 is important for the activation of αl integrin, we mutated it to Gly, Ala, Val, Leu, Met, Tyr, and Trp, respectively, ranging from the smallest Gly to the bulkiest Trp (Fig. 2B). The activation of α1β2 integrin was assessed by the ICAM-1-binding assay in the presence of the universal integrin activator Mn2+. Remarkably, the α1-F153G and α1-F153A mutations almost completely abrogated Mn2+-induced ICAM-1 binding to HEK293FT cells expressing α1β2 integrin (Fig. 2C). Remarkably, the level of ICAM-1 binding correlated well with the side chain volumes of the substituted amino acids for α1-Phe-153, i.e. Gly = Ala < Val < Met < Leu = Phe = Tyr < Trp (Fig. 2C). As a comparison, the α1-Glu-146 and α1-Lys-149 were mutated to Ala. These residues show steric clashes with the α7-helix Phe-292 when overlapping their high-affinity conformation with the low-affinity conformation of α7-helix (Fig. 2A). As expected, both α1-E146A and α1-K149A mutations greatly dampened Mn2+-induced ICAM-1 binding, but to a lesser extent compared with the α1-F153A mutation (Fig. 2D). Interestingly, the α1-F147A and α1-L151A mutations, located at the opposite site of the α1- and α7-helix interface (Fig. 2A), also decreased ICAM-1 binding (Fig. 2D). By contrast, the α1-K160A mutation at the bottom of α1-helix had no effect on Mn2+-induced ICAM-1 binding of α1β2 integrin (Fig. 2, A and D). Most of the mutations had little effect on the α1β2 cell surface expression except the Leu substitution decreased the expression by ~50% (Fig. 2, C and D). In addition, the α1-F153I mutation completely abolished α1β2 cell surface expression probably because the position of α1-Phe-153 cannot accommodate an Ile residue because all the Ile rotamers show severe steric clashes with the surrounding residues when mutated in silico (data not shown). These data demonstrated that a bulky amino acid in the mid α1-helix is critical for the high-affinity ligand binding of αl domain. In addition, the N-terminal residues of α1-helix also contribute to the high-affinity conformation of α1-helix.

Mutations that lock the high-affinity conformation of α7-helix of αl domain counteract the inactivating effect of the α1-helix Phe-153 mutation

It has been shown that the downward movement of the α7-helix is important for the activation of αl domain (15, 22, 23). We speculated that the inactivating effect of the α1-F153G or F153A mutation could be due to the destabilization of the downward movement of α7-helix. In line with this possibility, enforcing the downward movement of α7-helix, for example by mutations, may rescue the inactivating effect of the α1-F153 mutations. This hypothesis was tested using four activating mutations of α1β2 integrin. The β2-G128A/G129T mutation (Fig. 3A) renders α1β2 activation by facilitating the active conformation of βl domain α1-helix (19). The α1-F265S mutation induces the downward movement of α7-helix by facilitating the movement of the β5-a6 loop (Fig. 3, A and B) (24), whereas the α1-K287C/K294C mutation directly stabilizes the downward movement of the β6-a7 loop and α7-helix of αl domain by forming a disulfide bond (Fig. 3, A and C) (15). The α1-GFFKR/GAAKR (FFAA) mutation renders α1β2 constitutively active by disrupting the αβ cytoplasmic association, which mimics the inside-out activation of integrin (Fig. 3A) (25). As shown in Fig. 3D, the presence of the β2-G128A/G129T...
Function of the α1-helix bulky residue of αI domain

mutation did not rescue the inactivating effect of αI-L153A and αI-L153A. However, compared with the β2 wild type (Fig. 2C), the requirement of residue size at the position of αI-L153 for Mn2+-induced ICAM-1 binding was weakened by the β2-G128A/G129T mutation. With the β2-G128A/G129T mutation, even the αI-L153V rendered the same level of ICAM-1 binding as the αI wild type in the presence of Mn2+ but not Ca2+/Mg2+ (Fig. 3D). Consistently, the bulkiest amino acid Trp further enhanced the ICAM-1 binding at both metal ion conditions (Fig. 3D). There was ~20% decrease of the αI/β2 cell surface expression because of these mutations (Fig. 3D). In sharp contrast, both the αI-L153S and αI-L153S mutations completely rescued the inactivating effect of αI-L153A (Fig. 3E). Interestingly, the αI-L153A mutation even enhanced the activating effect of αI-L153C to K287C/K294C mutations completely rescued the inactivating effect of αI-L153A (Fig. 3E). Although the αI-L153A mutation induced the similar level of αI activation as the αI-L153C and αI-L153C mutations, it failed to rescue the inactivating effect of αI-L153A (Fig. 3E). All of these mutations clearly decreased the cell surface expression of αI/β integrin (Fig. 3E). These data suggest that the bulky αI-Phe-153 of α1-helix is critical for stabilizing the downward displacement of α7-helix, required for the high affinity ligand binding of αI/β integrin.

The αI-L153A mutation has little effect on the overall conformational change of β2 integrin

Integrin activation is associated with the long-range conformational rearrangements that are relayed among the connecting domains (5). Such conformational changes on the cell surface can be measured by the exposure of epitopes that are masked in the bent inactive conformation (26). Next, we asked whether the inactivating αI-L153A mutation could affect the global conformational change of αI/β integrin. Two conformation-specific mAbs were used to report the conformational states of αI/β integrin. The mAb m24 binds to the β2/β domain and is specific to the open headpiece conformation (27) (Fig. 3A). The mAb KIM127 binds to the I-EGF2 domain of β2 leg and is specific to the extended conformation of β2 (28, 29) (Fig. 3A). Mn2+ induced the binding of both m24 and KIM127 to the αI/β integrin expressed in HEK293FT cells (Fig. 4, A and B), indicating the headpiece opening and leg extension. The inactivating mutations αI-K149A and αI-L153A decreased ICAM-1 binding by more than 90% (Fig. 2B). By contrast, these mutations had no effect on Mn2+-induced binding of m24 (Fig. 4A) or KIM127 (Fig. 4B) binding to the β2 wild type. The αI-L153A mutation had no obvious effect on the binding of
In line with these findings, neither the almost 100% decrease of ICAM-1 binding (Fig. 3) with the activating FFAA mutation, which mimics the integrin inside-out activation, induced spontaneous binding of m24 and KIM127, which was further enhanced by Mn$^{2+}$ (Fig. 4, A and B). Although the $\alpha_{1}$-F153A mutation significantly reduced the binding of both mAbs when combined with the $\alpha_{1}$-FFAA mutation, it only decreased the mAb binding by 14 or 18% (Fig. 4, A and B), which is in sharp contrast with the almost 100% decrease of ICAM-1 binding (Fig. 3E). In line with these findings, neither $\alpha_{1}$-K149A nor $\alpha_{1}$-F153A affected the binding of m24 and KIM127 in the presence of the activating mutant $\beta_{2}$-G128A/G129T that stabilizes the active conformation of $\beta_{2}$ $\alpha_{1}$-helix (Fig. 4, A and B). As seen in our previous study on $\beta_{1}$ and $\beta_{2}$ integrins (19), the $\beta_{2}$-G128A/G129T mutation increased the binding of both m24 and KIM127 compared with the wild type (Fig. 4). These data demonstrated that the inactivating mutations at the $\alpha_{1}$ $\alpha_{2}$-helix had little effect on the headpiece opening and extension induced by the signals of outside-in or inside-out activation. This is consistent with the previous study showing that locking the $\alpha_{2}$ domain in a high-affinity conformation by the $\alpha_{2}$-K287C/K294C mutation did not induce the binding of m24 or KIM127 (22). Thus, the conformational changes between the $\alpha_{2}$ and the $\beta_{2}$ subunit can be uncoupled during the transmission of conformational changes.

To further define the structure requirement for $\beta_{2}$ headpiece opening and extension, we introduced a $\beta_{2}$-KKGG mutation at the $\alpha_{1}$-helix of $\beta_{2}$ domain (Fig. 3A). This mutation has been shown in our previous study to dramatically reduce ICAM-1 binding to $\alpha_{1}\beta_{2}$ integrin by stabilizing the inactive conformation of $\alpha_{1}$-helix (19). In opposition to the $\beta_{2}$-G128A/G129T mutation of $\alpha_{1}$-helix, the $\beta_{2}$-KKGG mutation greatly impaired the binding of m24 and KIM127 when co-expressed with the $\alpha_{1}$ wild type (Fig. 4). Moreover, it reduced the m24 binding by more than 90% (Fig. 4A), and the KIM127 binding by more than 50% (Fig. 4B), even when combined with the activating $\alpha_{1}$-FFAA mutation. These data clearly demonstrated that the active conformation of $\beta_{1}$ $\alpha_{1}$-helix; i.e., the inward movement toward the MIDAS (Fig. 1, F and H), is essential for $\beta_{2}$ headpiece opening and extension.

The $\alpha_{1}$-F153A mutation abolishes $\alpha_{1}\beta_{2}$-mediated cell adhesion on immobilized ICAM-1

Having established the critical role of $\alpha_{1}$-F153 in the binding of $\alpha_{1}\beta_{2}$ integrin to soluble ICAM-1, we asked whether it is also
The locations of the indicated mutations and the epitopes of mAbs m24 and KIM127 are shown.

important in $\alpha_4\beta_2$-mediated cell adhesion and spreading on immobilized ICAM-1, given that the receptor-ligand binding kinetics could be different in solution and in the solid phase. When seeded onto the ICAM-1-coated dish, the HEK293FT cells transfected with wild-type $\alpha_4\beta_2$ or $\alpha_4\beta_2$-F153Y/$\beta_2$ integrin spontaneously adhered and spread on the surface (Fig. 5A). However, the $\alpha_4$-F153A mutation completely abolished cell adhesion under the tested coating concentration of ICAM-1 (Fig. 5A), despite its comparable cell surface expression with the wild type (Fig. 5B). This is in agreement with the pivotal role of the bulky residue of $\alpha_4$-F153 in the high-affinity ligand binding of $\alpha_4\beta_2$.

The $\alpha_{M}$-F156A mutation reduces $\alpha_{M}\beta_2$-mediated cell adhesion on immobilized fibrinogen

We next tested whether the conserved Phe residue plays the same role in $\alpha_{M}$ (Mac-1) integrin that binds multiple ligands including fibrinogen. The $\alpha_{M}$-Phe-156 was mutated to the small amino acid Ala and the bulky amino acid Trp. The $\alpha_{M}\beta_2$-mediated cell adhesion on immobilized human fibrinogen was induced by Mn$^{2+}$ ions. The $\alpha_{M}$-F156A mutation significantly reduced the $\alpha_{M}\beta_2$-mediated cell adhesion (Fig. 6A). By contrast, the bulky $\alpha_{M}$-F156W mutation significantly enhanced the cell adhesion (Fig. 6A). This is not due to the differences in the cell surface expression of $\alpha_{M}\beta_2$ because both the $\alpha_{M}$-F156A and $\alpha_{M}$-F156W mutations decreased the $\alpha_{M}\beta_2$ expression to a similar extent compared with the wild type (Fig. 6B). In addition, the cell adhesion could be blocked by the anti-$\alpha_{M}$ inhibitory mAb ICRF44 (data not shown). These data demonstrated that a bulky residue is also required for the $\alpha_1$-helix of the $\alpha_{M} \alpha_1$ domain to support ligand binding.

Molecular dynamics simulations suggest a structural role of the bulky Phe residue of the $\alpha_1 \alpha_1$-helix in maintaining the high affinity conformation

Our experimental data demonstrated an important role of the conserved Phe in the $\alpha_1$-helix of $\alpha_1 \alpha_1$ domain in high-
affinity ligand binding. To gain a structural insight into how the Phe affects the conformation of αl domain, we performed the all-atom molecular dynamics (MD) simulation for wild-type and mutant αl domains in water solvent. We used the crystal structure of αl domain in the open conformation for the MD simulation in line with our hypothesis that the bulky Phe may stabilize the active conformation, whereas a small amino acid substitution like Ala would have the opposite effect. A 60-ns MD simulation was performed for both αl wild type and αl-F153A mutant using exactly the same parameters. To analyze the overall structural similarity of the recorded snapshots to the starting structure, the root mean square deviations (RMSDs) of backbone atoms were calculated. The αl wild type showed stable RMSD values over the 60-ns simulation. However, the RMSD values of the αl-F153A increased substantially after the first 30-ns simulation, indicating the structure instability (data not shown). To evaluate the conformational dynamics for each residue, we calculated the root mean square fluctuation (RMSF, i.e. standard deviation) of Ca positions in the snapshots relative to the starting structure. As shown in Fig. 7A, most of the residues of the wild-type αl domain are relatively stable over the 60-ns simulation. Remarkably, in the presence of F153A mutation, the regions of β5-α6 and β6-α7 loops showed significant fluctuations (Fig. 7A). These differences became more obvious when the RMSF values were converted to B-factors and represented as cartoon models (Fig. 7B). We next performed the same MD simulations for the open-conformation crystal structure of αl domain. The simulation time was extended to 100 ns. Similar to αl-F153A, the αlM-F156A mutation rendered the β5-α6 and β6-α7 loops more flexible than the wild type (Fig. 7, C and D), although to a lesser extent compared with the αl domain (Fig. 7, A–D). These data clearly demonstrated that the bulky Phe residue of αl-helix contributes to the high affinity state of αl domain by stabilizing the active conformation of β5-α6 and β6-α7 loops.

**Discussion**

Integrin is an excellent example of protein machinery in which local conformational changes in one site are propagated to a distal site, altering the ligand-binding affinity and function. Structure-based mutagenesis studies have determined certain residues that are critical for the ligand-binding activity of β and α domains (19, 22, 24, 30, 31). Most of the studies focused on the residues that either directly participate in the coordination with the metal ion and/or ligand or that are involved in the movements of the loops or α-helices surrounding the active site. The importance of these residues is obvious according to the structural changes because the gain-of-function mutations are predicted to shift the conformation to the open state, whereas the loss-of-function mutations are predicted to shift it to the closed state (15, 19, 24). However, a functional role of the conserved Phe of αl α-helix is unpredictable because this residue seems not directly participate in the movement of either αl-helix or α7-helix based on the structural comparison between the active and inactive conformations. Thus, our findings of the loss-of-function mutation of αl-Phe-153 are unexpected. Our current study further advanced our understanding of the conformational requirement for integrin ligand binding.

How does the αl-Phe-153 contribute to the ligand binding of αl domain? It has been indicated that the downward movement of α7-helix is required for the high affinity ligand binding of αl domain because mutations constraining it at the upward position blocked, whereas mutations facilitating the downward movement enhanced ligand binding (22, 23). In addition, small molecule inhibitors bound to the cavity under the α7-helix allosterically block ligand binding of αl β₂ by restraining the downward movement of α7-helix (32–37). Our mutagenesis study showed that the function of a residue at the position of αl-Phe-153 is determined by its size (or volume). A bulky residue is specifically required to maintain the ligand-binding capability of αl domain. At the downward position, the β6-α7 loop moves close to the middle of αl-helix (Figs. 11 and 3, B and C), where a hydrophobic and bulky side chain would act as a pawl to anchor the downward position of β6-α7 loop and α7-helix during their ratchet-like movement (15). This was indicated by the MD simulation data showing that the αl-F153A or αM-F156A mutation rendered the β5-α6 and
Function of the α1-helix bulky residue of α1 domain

β6-α7 loops less stable in the active conformation (Fig. 7). The bulky α1-Phe-153 is solely required for the downward movement of β6-α7 loop and α7-helix but dispensable for the inward movement of α1-helix because once the β6-α7 loop is enforced to the downward position, the trimmed α1-helix by the α1-F153A mutation still supports ligand binding. Crystal structures revealed the flexibility of α7-helix C terminus both in the isolated form of α1 domain and in the context of integrin ecto-domain (38–41), indicating that the α1-helix and the connecting β6-α7 loop can sample rapidly between the closed and the open conformational states. We did not observe the β6-α7 loop and α7-helix moving upward to the closed conformation in our MD simulation even in the presence of α1-F153A mutation. This is very likely due to the short time scale used for the simulation. Such a large conformational change may require much longer time of simulation to be observed. Indeed, the hydrogen-deuterium exchange kinetics measured via NMR revealed a large conformational fluctuation of α7- but not α1-helix in the resting state of the α1 integrin α1 domain (42). The conserved bulky residue in the middle of α1-helix is critical to regulate the shifting between the two states of α7-helix by facilitating the transition of the α1 domain to the high affinity conformation.

It should be noted that although Leu and Met have very close side chain volumes, the α1-F153L mutation exerted a similar level of ICAM-1 binding as the α1 WT, whereas the α1-F153M mutation greatly reduced ICAM-1 binding (Fig. 2C). This is consistent with the numbers of rotamers that Leu and Met have. Leu can only sample four rotamer conformations, which is likely to stabilize the downward position of β6-α7 loop and α7-helix. In contrast, Met is more flexible by having more than

Figure 5. Effect of α1-Phe-153 mutations on α1β2-mediated cell adhesion and spreading. A, HEK293FT cells transfected with the indicated integrin α1β2 constructs were seeded onto the plates coated with human ICAM-1-Fc (5 μg/ml coating concentration) at 37 °C for 1 h. The cells were fixed and immunostained with anti-α1 mAb TS2/4 shown in green. The nuclei were stained with DAPI. Scale bar, 200 μm. B, flow cytometry plots showing integrin α1β2 expression reported by mAb TS2/4 in the corresponding HEK293FT transfectants used in A. One representative experiment of more than three repeats is shown.
ten rotamer conformations, one of which may adapt to the free movements of $\beta$-$\alpha$-$\gamma$ loop and $\alpha$-$\gamma$-helix and thus lack the activation supporting function.

The $\beta$ and $\alpha$ domains are the best-studied VWA domains that undergo conformational changes for affinity regulation. Although their $\beta$-$\alpha$-$\gamma$ loops and $\alpha$-$\gamma$-helices move in a similar fashion to accommodate for the active position of MIDAS loop and to communicate with the neighboring domains, their $\alpha$-$\gamma$-helices change in a different fashion (Fig. 1, H and I). This is in accordance with a conserved Gly and a conserved Phe at the homologous position of $\alpha$-$\gamma$-helix, which play opposite roles in the $\beta$ and $\alpha$ domains. As we found for a conserved negative role of the Gly in the $\beta$-$\alpha$-$\gamma$-helix (19), the positive role of the Phe in the $\alpha$-$\gamma$-helix could also be generalized to all the $\alpha$-containing integrins, given that the same conformational changes of $\alpha$-$\gamma$-helix have been observed in the crystal structures of the $\alpha$ domains of $\alpha_{L}$, $\alpha_{M}$, $\alpha_{X}$, $\alpha_{I}$, and $\alpha_{E}$ integrins (12–15, 40, 43, 44). This was demonstrated in the current study on $\alpha_{L}$ and $\alpha_{M}$ integrins. Interestingly, an equivalent Phe but not Gly is also present in the $\alpha$-$\gamma$-helix of many VWA domains other than integrins (Fig. 1J). Moreover, most of the VWA domains have only one or lack an intact MIDAS, whereas the integrin $\beta$ domains have three metal ion-binding sites that regulate ligand binding. Given the different structural features and conformational regulations of $\beta$ domains, it is tempting to speculate that the $\beta$ domains might be evolved differently from a typical VWA domain and could be classified as VWA variants.

Although a conserved Phe residue at the equivalent position of $\alpha$-$\gamma$-helix is found in many non-integrin VWA domains, generalizations for the similar function of this residue as seen in the $\alpha$ domains can only be made with caution. For example, the VWF A1, A2, and A3 domains and the collagen VI $\alpha$3N5 domain all have an equivalent Phe at the $\alpha$-$\gamma$-helix, but they lack an intact MIDAS (Fig. 1J), and no conformational changes were observed in their $\alpha$-$\gamma$-helixes (45–49). Similarly, the presence of an intact MIDAS does not seem to correlate with the presence of the equivalent Phe in the $\alpha$-$\gamma$-helix. Several examples are found in the complement components C2a and factor B, in the thrombospordin repeat anonymous proteins (TRAPs) of the Plasmodium vivax and Plasmodium falciparum, and in the pilus-related adhesion RrgA of Streptococcus pneumoniae, in which a non-Phe residue is present at the equivalent position of $\alpha$-$\gamma$-helix (Fig. 1J). We compared the crystal structures of several representative VWA domains that have an intact MIDAS, which has been suggested or confirmed to participate in ligand binding (Fig. 8). Crystal structures of the VWA domain of human anthrax toxin receptor 2 (ANTR2) in complex with anthrax toxin or pseudo-ligands highly resemble the active open conformation of integrin $\alpha$ domain (Fig. 8A) (50). Although a closed conformation has yet to be seen for the ANTR2 VWA domain, mutagenesis studies suggested that it might undergo conformational changes for affinity regulation and signaling (51). Other VWA domains that have similar conformational regulations as integrin $\alpha$ domains are from the adhesins of the parasites Toxoplasma gondii micronemal protein 2 (TgMIC2) and the P. vivax TRAP or P. falciparum TRAP as mentioned above. The TRAP VWA domain has been seen in both the closed and open conformations (Fig. 8B) (52). The MIC2 VWA domain has only been crystalized in the closed conformation so far (Fig. 8C), but the transition between the closed and the open states is highly predictable (53). A recent crystal structure of the Blue Mussels adhesion protein, the PTMP1 (proximal thread matrix protein 1) revealed two tandem VWA domains both in the closed conformation with the A1 domain MIDAS occupied by Zn$^{2+}$ (Fig. 8D) (54). It was suggested that the MIDAS of PTMP1 might participate in collagen binding (54), which resembles the interaction of the $\alpha_{L}$ integrin $\alpha$ domain and collagen. All the above-mentioned VWA domains contain the equivalent Phe at the $\alpha$-$\gamma$-helix except that some TRAP proteins have a Leu or Met (Figs. 1J and 8, A–D). However, our data showed that a Met or Leu residue at the equivalent position of $\alpha$-$\gamma$-helix could still support the ligand binding of $\alpha$ domain (Figs. 2C and 3D). Therefore, it is tempting to speculate that these VWA domains may follow the activating mechanism of integrin $\alpha$ domains.

We presented two examples of VWA domains from the bacteria adhesin RrgA (Fig. 8E) (55) and the complement factor B (Fig. 8F) (56), which have a small amino acid at the equivalent position of $\alpha$-$\gamma$-helix. The crystal structure of RrgA VWA domain shows a closed conformation of MIDAS and $\alpha$-$\gamma$-helix and an Ala at the Phe-equivalent position of $\alpha$-$\gamma$-helix (Fig. 8E). Notably, the loop connecting the MIDAS motif and the $\alpha$-$\gamma$-helix in RrgA is much longer than that of $\alpha_{L}$ $\alpha$ domain (Figs. 1J and 8E). This longer MIDAS loop may move freely toward the metal ion to accommodate with the high affinity ligand binding even without the inward movement of the $\alpha$-$\gamma$-helix and the downward movement of the $\alpha$-$\gamma$-helix. As such, a bulky residue in the mid $\alpha$-$\gamma$-helix is not essential. The VWA domain of complement factor B was crystallized in both the closed and open
Function of the α1-helix bulky residue of α1 domain

conformations (56, 57). It has a Cys at the equivalent position of α1-helix (Figs. 1 and 8F). Although there is a swung-in motion at the N terminus of α1-helix, the scale of the downward movement of α7-helix is very small in the open conformation (Fig. 8F). The movement of α7-helix seems to be induced by the binding of factor D (56). In addition, the factor B VWA domain has an extra α1-helix, which interacts with the α7-helix and influences its conformation (Fig. 8F). All these differences may minimize the requirement of a bulky residue at the α1-helix. In summary, our study revealed a unique feature of integrin α1-α1-helix in regulating ligand binding, which may be applicable to certain VWA domains of other proteins that undergo large conformational changes for signal transduction.

Another important finding in the current study is that integrin conformational transmission can be uncoupled between the α1 and the β domains. We found that although the conserved Phe of α1-helix is critical for ICAM-1 binding to α1 domain, it is dispensable for β2 extension and headpiece opening. The downward displacement of α7-helix that requires the Phe of α1-helix is not essential for the conformational changes of β2 induced by Mn2+ or by inside-out activation. In addition, previous studies also showed that locking the downward conformation of α7-helix of α1 domain did not result in β2 extension or headpiece opening (22). Interestingly, the internal ligand α1-Glu-310 at the C terminus of α7-helix is required for the headpiece opening of β2 subunit (58). Recent crystal structures of α1β2 and α1β2 suggested the intrinsic flexibility of α1 domain on the platform formed by the β-propeller and β1 domains (39–41). This is consistent with the loose conformational linkage between the β1 and α1 domains. The α1β2 integrin mediates both the rolling adhesion and migration of leukocytes by binding the ICAM-1 expressed on the endothelial cells (59). Under the rolling condition, the loose linkage between the α1 and β1 enables the cells to quickly bind and release ICAM-1. When the cell signals for firm adhesion and migration are turned on, the tight engagement of the α1 and β1 domains could be enforced by the mechanical forces generated by the actin polymerization and ICAM-1 binding at each end of integrin (60).
Experimental procedures

DNA constructs

DNA constructs of human $\alpha_1$ and $\beta_2$ integrins were as described previously (19). The full-length cDNA of human $\alpha_M$ integrin was cloned into the pcDNA3.1 vector using the 5′ KpnI and 3′ XbaI sites. Mutations were introduced by site-directed mutagenesis with the QuikChange kit (Agilent Technologies).

Antibodies and protein ligands

TS2/4 (BioLegend) is a non-functional anti-$\alpha_1$ mAb (61). KIM127 (binds to I-EGF2 domain) and mAb 24 (m24, binds to I domain) are anti-$\beta_2$ conformation-specific mAbs that report $\beta_2$ integrin extension and headpiece opening, respectively (27, 29, 62, 63). ICRF44 (BioLegend) is an inhibitory anti-$\alpha_M$ mAb. Human ICAM-1 (with a C-terminal tag of human IgG1Fc, ICAM-1-Fc) was purchased from Sino Biological. Biotin-labeled mouse anti-human IgG1 Fc in the presence of 5 mM EDTA or 1 mM Ca$^{2+}$/Mg$^{2+}$ or 0.2 mM Ca$^{2+}$ plus 2 mM Mn$^{2+}$ at 25 °C for 30 min. The cells were then washed and incubated on ice for 30 min with 10 $\mu$g/ml FITC-labeled TS2/4 and 10 $\mu$g/ml Alexa Fluor 647-labeled streptavidin. TS2/4-positive cells (expressing $\alpha_1$ integrin) were acquired for calculating the mean fluorescence intensity (MFI) by flow cytometry using BD Accuri™ C6 flow cytometer. ICAM-1 binding was presented as normalized MFI, i.e. ICAM-1 MFI (after subtracting the ICAM-1 MFI in the EDTA condition) as a percentage of TS2/4 MFI (integrin expression).

Conformation-specific antibody binding

Binding of the active conformation-specific anti-$\beta_2$ mAbs KIM127 and m24 to the HEK293FT transfectants was performed as described before (19, 21). In brief, the cells were first incubated with 10 $\mu$g/ml of biotin-labeled KIM127 or m24 in HBSGB buffer containing 5 mM Ca$^{2+}$/Mg$^{2+}$ or 0.2 mM Ca$^{2+}$ plus 2 mM Mn$^{2+}$ at 25 °C for 30 min and then washed and incubated with 10 $\mu$g/ml FITC-labeled TS2/4 and Alexa Fluor 647-labeled streptavidin on ice for 30 min. TS2/4-positive cells were acquired for calculating the MFI by flow cytometry. The KIM127 or m24 binding was presented as normalized MFI, i.e. streptavidin MFI as a percentage of TS2/4 MFI.

Cell adhesion assay and fluorescence microscopy

For $\alpha_1$-$\beta_2$-mediated cell adhesion assay, the Delta T dishes (Biorad) or 24-well cell culture plate (BD Falcon) were first
coated with 5 μg/ml mouse anti-human IgG in pH 7.4, PBS, at 4 °C for 12 h, and then blocked with 1% BSA at 37 °C for 1 h before finally coated with 5 μg/ml ICAM-1-Fc at 37 °C for another 1 h. Transfected HEK293FT cells suspended in DMEM without FBS were allowed to adhere on the ICAM-1-Fc-coated Delta T dishes at 37 °C for 1 h. The unattached cells were washed off by DMEM, and the attached cells were fixed with DMEM containing 3.7% formaldehyde, blocked with 5% nonfat dry milk in PBS, and stained with 10 μg/ml mAb TS2/4 and Alexa Fluor 488-labeled goat anti-mouse IgG. The nuclei were stained with 5 μg/ml DAPI. The stained cells were fixed with 3.7% formaldehyde in PBS and imaged with an EVOS digital inverted fluorescence microscope with a 20× objective. The level of integrin expression of the transfected cells was accessed by the mAb TS2/4 staining, followed by flow cytometry.

For αMβ2-mediated cell adhesion assay, the 12-well cell culture plate was coated with 3 mg/ml human fibrinogen in PBS at 37 °C for 1 h. HEK293FT cells transfected with αMβ2 plus EGFP were seeded onto the fibrinogen-coated plate in HEPES buffer for 10 min before adding 0.2 mM Ca2+ plus 2 mM Mn2+. After incubation at 37 °C for 1 h, the plate was washed with PBS buffer for three times and fixed with 3.7% formaldehyde in PBS. The adhered cells were imaged with an EVOS digital inverted fluorescence microscope with a 10× objective. At least 20 images were randomly taken for each sample, and the EGFP-positive cells were counted for each image. The data were presented as the averaged cell numbers that were normalized to the WT level. The cell surface expression of αMβ2 was measured by flow cytometry after staining with the allophycocyanin (APC)-labeled anti-αM mAb ICRF44 (BioLegend).

Molecular dynamics simulation

The crystal structures of the αL, αL (PDB code 1TOP) and the αM, αL domains (PDB code 1IDO) in the open conformation were used for MD simulation. αL-Phe-153 and αM-Phe-156 were mutated to Ala in silico using PyMOL. For the αL, αL structure (PDB code 1TOP), the two cysteine mutations, K287C and K294C, were mutated back to their native Lys residue by in silico

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