The Unique Disulfide Bond-stabilized W1 β4-β1 Loop in the α4 β-Propeller Domain Regulates Integrin α4β7 Affinity and Signaling

Background: Integrin αβ7 is unique for mediating rolling and firm adhesion of lymphocytes pre- and post-activation. The two-phase cell adhesion suggests a unique molecular basis for the dynamic interaction of αβ7, with its ligand, mucosal addressin cell adhesion molecule 1 (MAdCAM-1). Here we report that a disulfide bond-stabilized W1 β4-β1 loop in the α4 β-propeller domain plays critical roles in regulating integrin αβ7 affinity and signaling. Either breaking the disulfide bond or deleting the disulfide bond-occluded segment in the W1 β4-β1 loop inhibited rolling cell adhesion supported by the low-affinity interaction between MAdCAM-1 and inactive αβ7, but negligibly affected firm cell adhesion supported by the high-affinity interaction between MAdCAM-1 and Mn2+-activated αβ7. Additionally, disrupting the disulfide bond or deleting the disulfide bond-occluded segment not only blocked the conformational change and activation of αβ7 triggered by talin or phorbol-12-myristate-13-acetate via inside-out signaling but also disrupted integrin-mediated outside-in signaling and impaired phosphorylation of focal adhesion kinase and paxillin. Thus, these findings reveal a particular molecular basis for αβ7-mediated rolling cell adhesion and a novel regulatory element of integrin affinity and signaling.

Integrins are a family of α/β heterodimeric cell adhesion molecules that mediate cell-cell, cell-matrix, and cell-pathogen interactions and signal bidirectionally across the plasma membrane (1). Different from most integrins that mediate only firm cell adhesion upon activation, a small subset of integrins, including α3β1, α4β1, α6β4, and αβγ, can mediate rolling and firm cell adhesion pre- and post-activation (2–5). Integrin α4β7 is expressed exclusively on lymphocytes, and its major ligand, mucosal addressin cell adhesion molecule 1 (MAdCAM-1)3, is specifically expressed on high endothelial venules of Peyers patches and postcapillary venules in intestinal laminae propriae (6). The unique two-phase cell adhesion mediated by integrin αβ7 makes it indispensable in the homing of lymphocytes to the intestine and the associated lymphoid tissues and plays critical roles in gut immune homeostasis and the pathogenesis of intestinal inflammatory disorders (7–9).

The rolling and firm cell adhesion mediated by αβ7 are dependent on the dynamic regulation of integrin affinity (2, 3). The inactive and activated αβ7 support the rolling and firm adhesion of lymphocytes via the low-affinity and high-affinity interaction with its ligand, MAdCAM-1, respectively (6, 10, 11). Integrin affinity transition is associated with the conformational rearrangement of the integrin molecule. In the resting state, integrin has a low-affinity bent conformation, with the headpiece facing down toward the cell membrane. Upon activation, integrin undergoes a series of conformational rearrangements and extends upward in a switchblade-like opening motion, which leads to the increased integrin affinity (12–14). Integrin affinity is dynamically regulated by inside-out signals from the cytoplasm. Several intracellular effector molecules, such as talin and kindlins, have been shown to activate integrin through the interaction with integrin cytoplasmic domains (15–18). In addition to inside-out signaling, extracellular metal ions can also regulate adhesion by integrins (19). Compared with the low-affinity state in Ca2+ + Mg2+, addition of Mn2+ or removal of Ca2+ strikingly increases the affinity and adhesiveness of almost all integrins (20–22). Studies have shown

3 The abbreviations used are: MAdCAM-1, mucosal addressin cell adhesion molecule 1; MIDAS, metal ion-dependent adhesion site; PMA, phorbol-12-myristate-13-acetate; FAK, focal adhesion kinase; ANOVA, analysis of variance.
that integrin affinity is regulated by divalent cations via a cluster of three divalent cation-binding sites in the integrin β7 I domain, with the metal ion-dependent adhesion site (MIDAS) at the center and flanked by the synergistic metal ion-binding site and the adjacent to MIDAS (10, 11, 19, 23–25). The divalent cation at MIDAS directly coordinates the acidic side chain of Asp-42 in MAdCAM-1 and is essential for both rolling and firm cell adhesion (26). Binding of Ca^{2+} at the adjacent to MIDAS stabilizes the closed β1 domain conformation to support rolling adhesion (10). On the contrary, the occupancy of the synergistic metal ion-binding site by divalent cation is required for integrin activation to support firm adhesion (10). In addition, the synergistic metal ion-binding site cation links the specificity-determining loop through a cation-π interaction with Phe-185 in the β7 I domain, which has been reported to be critical for αβ7-mediated firm cell adhesion and signaling (27).

Despite the advances in understanding the mechanism by which αβ7 regulates its affinity to support rolling and firm adhesion of lymphocytes, the precise molecular basis for the regulation of low- and high-affinity αβ7–MAdCAM-1 interactions remains elusive because of lack of the αβ7-MAdCAM-1 complex structure. As shown from the crystal structure of the αβ7 headpiece, the β-propeller domain of α7 differs from those of the previously characterized αIIb, αv, and α7 integrins, especially in the loops on the face of β-propeller domain that bind the β1 domain and contribute to the formation of the αβ7 ligand-binding pocket (28) (Fig. 1A). A particular one is the W1 β4-β1 loop, which is stabilized by a disulfide bond that exists exclusively in α7/α7 subfamily (28) (Fig. 1). Considering the structure specificity of this loop, we hypothesize that this disulfide bond-stabilized W1 β4-β1 loop might contribute to unique two-phase cell adhesion mediated by αβ7. Here we demonstrated that the disulfide bond-stabilized W1 β4-β1 loop is essential for rolling cell adhesion mediated by the low-affinity interaction between inactive αβ7 and MAdCAM-1 but not for firm cell adhesion supported by the high-affinity interaction between Mn^{2+}-activated αβ7 and MAdCAM-1. Either breaking the disulfide bond or deleting the disulfide bond-occluded segment in the W1 β4-β1 loop not only blocked the global conformational rearrangement and activation of αβ7, triggered by talin or phorbol-12-myristate-13-acetate (PMA) via inside-out signaling but also disrupted integrin outside-in signaling. Thus, the disulfide bond-stabilized W1 β4-β1 loop is a novel regulatory element of integrin affinity and bidirectional signaling and plays an essential role in supporting the αβ7-mediated rolling adhesion.

**EXPERIMENTAL PROCEDURES**

**cDNA Construction and Expression**—The α7 site-directed mutations were generated using QuikChange (Stratagene). WT human α7 cDNA in vector pcDNA3.1/Hygro(-) (Invitrogen) was used as the template. cDNA of the human talin head domain (talin 1–435) was cloned into vector pMcCherry-C1 (modified from vector pEGFP-C1) to generate a construct of the talin head domain with N-terminal fused mCherry. All constructs were confirmed by DNA sequencing.

Transient Transfection of 293T cells was performed as described (10). CHO-K1 cells stably expressing WT and mutant human αβ7 were established by cotransfection of human αβ7 cDNAs and selection by 0.2 mg/ml hygromycin (Amresco).

**Antibodies and Reagents**—The human integrin αβ7-specific blocking mAb Act-1 was as described previously (29, 30). The rat mAb FIB504 against human β7, was prepared using a hybridoma (Developmental Studies Hybridoma Bank, University of Iowa). Phycocerythrin (PE)–conjugated FIB504 and mAb to paxillin were from BD Biosciences. mAb to pY118-paxillin was from Cell Signaling Technology, Inc., and mAbs to FAK and pY397-FAK were from Upstate Biotechnology, Inc. Alexa Fluor 488–conjugated goat anti-rat IgG mAb was from Invitrogen. Human MAdCAM-1/Fc fusion protein (h-MAdCAM-1/Fc) was prepared as described previously (10).

**Flow Cytometry**—Immunofluorescence flow cytometry was done as described (31). The expression level of integrin αβ7 on transient 293T transfectants was determined by staining with mAb FIB504 and, subsequently, staining with Alexa Fluor 488–conjugated mAb goat anti-rat IgG. The expression level of integrin αβ7 on stable CHO-K1 transfectants was determined by staining with PE–conjugated FIB504. Stained cells were then measured using FACSCalibur (BD Biosciences) and analyzed using WinMDI software.

**Flow Chamber Assay**—The flow chamber assay was performed as described (10, 11). A polystyrene Petri dish was coated with a 5-mm-diameter, 20-μl spot of 10 μg/ml purified h-MAdCAM-1/Fc in coating buffer (PBS, 10 mM NaHCO3 (pH 9.0)) for 1 h at 37 °C, followed by 2% BSA in coating buffer for 1 h at 37 °C to block nonspecific binding sites. Cells were washed twice with HBS (20 mM Hepes (pH 7.4)) containing 5 mM EDTA and 0.5% BSA and resuspended at 1 × 10^6/ml in buffer A (HBS, 0.5% BSA) and kept at room temperature. Cells were diluted to 1 × 10^6/ml in buffer A containing different divalent cations immediately before infusion in the flow chamber using a Harvard apparatus programmable syringe pump. Cells were allowed to accumulate for 30 s at 0.3 dyne/cm^2 and for 10 s at 0.4 dyne/cm^2. Then, shear stress was increased every 10 s from 1 dyne/cm^2 up to 32 dyne/cm^2 in 2-fold increments. The number of cells remaining bound at the end of each 10-s interval was determined. The rolling velocity at each shear stress was calculated from the average distance traveled by rolling cells in 3 s. A velocity of 1 μm/s, which corresponds to a movement of 1/2 cell diameter during the 3 s measurement interval, was the minimum velocity required to define a cell as rolling instead of firmly adherent. For the experiment of stimulation, 0.1 μM PMA (final concentration) was added and incubated for 10 min at 37 °C before cells were infused into the flow chamber.

**FRET Assay**—FRET was measured as described (27). For detecting the orientation of the integrin ectodomain relative to the cell membrane, 293T transient transfected cells were seeded on a poly-L-lysine-coated (100 μg/ml) surface in serum-free DMEM and incubated for 30 min at 37 °C. 0.5 mM Mn^{2+} or 0.1 μM PMA was added to activate integrin. Adherent cells were fixed with 3.7% paraformaldehyde for 15 min at room temperature, and nonspecific sites were blocked by incubation with 10% serum-rich medium for 10 min at room temperature. Then, cells were stained with 20 μg/ml Alexa Fluor 488–conju-
gated Act-1 Fab for 40 min at 37 °C. After two washes, cells were labeled with 10 μM FM4–64 FX (Invitrogen) for 4 min on ice, washed once, and mounted immediately with Mowiol® 4–88 (Polysciences, Inc.) mounting solution under a coverslip. The mounted slides were kept in the dark and subjected to photobleach FRET acquisition by a confocal microscope (TCS SP5, Leica). FRET efficiency (E) was calculated as E = 1-(Fdonor(d)_post/Fdonor(d)_pre), where Fdonor(d)_pre and Fdonor(d)_post are the mean donor emission intensities of pre- and post-photobleaching.

Cell Spreading and Microscopy—Cell spreading was described as described (32). Glass coverslips were coated with 100 μg/ml poly-L-lysine or 10 μg/ml h-MAdCAM-1/Fc overnight at 4 °C and blocked by 2% BSA for 1 h at 37 °C. CHO-K1 stable cells were plated on coated coverslips for 2 h at 37 °C and then fixed by 3.7% paraformaldehyde. 0.5 mM Mn²⁺ was added to the Ham’s F12 medium during the spreading if needed. Differential interference contrast and interference reflection microscopy were conducted on an Olympus IX71 microscope with a ×63 oil objective coupled to the Retiga Exi Fast 1394 camera (Q-imaging). For the quantification of cell spreading, outlines of 50 randomly selected adherent cells from each of three separate experiments were generated, and the number of pixels contained within each of these regions was measured by using Image-Pro® plus v. 6.0.

Western Blotting—CHO-K1 stable cells were plated on 100 μg/ml poly-L-lysine-coated or 10 μg/ml h-MAdCAM-1/Fc-coated (with 1 mM Ca²⁺/Mg²⁺ or 0.5 mM Mn²⁺) 6-well plates for 2 h at 37 °C. After washing with ice-cold TBS (20 mM Tris-HCl, 150 mM NaCl (pH 7.4)), cells were lysed with 100 μl of lysis buffer (TBS containing 1% Triton X-100, 0.05% Tween 20, complete protease inhibitor mixture tablets, and PhosSTOP phosphatase inhibitor mixture tablets) for 30 min on ice. Cell lysates were prepared by centrifuge for 15 min at 12,000 rpm. Supernatants were fractionated by reducing SDS-PAGE. pY397-FAK, FAK, pY118-paxillin, paxillin, and β-actin were detected by immunoblotting with the corresponding mAbs. Immunoreactive bands were visualized by SuperSignal West Pico chemiluminescent substrate (Thermo Scientific) and exposed to x-ray films (Kodak).

RESULTS

The Disulfide Bond in the W1 β4-β1 Loop Is Required for Rolling Cell Adhesion Mediated by Low-affinity α₄β₇-MAdCAM-1 Interaction—The crystal structure of the α₇β₇ headpiece has shown that the W1 β4-β1 loop in the β-propeller domain of the α7 subunit contains a unique disulfide bond between Cys-81 and Cys-85 (Fig. 1, A and B). To investigate the role of this disulfide bond-stabilized loop in rolling and firm cell adhesion mediated by human α₄β₇, we first substituted Cys-81 and Cys-85 with Ser individually (C81S and C85S) or together (C2S) to break the disulfide bond. The WT and mutant α₄β₇ were transiently expressed in 293T cells at comparable levels (data not shown), and the adhesive behaviors of these transfectants in shear flow were characterized in a parallel wall flow chamber with human MAdCAM-1 (h-MAdCAM-1/Fc) absorbed to its lower wall. The shear stress was increased incrementally, and the velocity of the cells remaining bound at each increment was determined. The WT α₄β₇ transfectants behaved as described previously for lymphoid cells expressing α₄β₇ (22). In 1 mM Ca²⁺/Mg²⁺, about 88% of the bound cells rolled at a wall shear stress of 2 dynes/cm² (Fig. 2A). In contrast, cells were firmly adherent in 0.5 mM Mn²⁺ (Fig. 2B). As controls, α₄β₇ transfectants treated with α₄β₇ blocking antibody Act-1 or with EDTA did not accumulate on MAdCAM-1 substrates (Fig. 2, A and B). All three disulfide bond mutations (C81S, C85S, and C2S) significantly impaired rolling adhesion in 1 mM Ca²⁺/Mg²⁺. Compared with WT α₄β₇ transfectants, the number of bound cells decreased by 84% for C81S, 90% for C85S, and 90% for C2S, respectively (Fig. 2A). In contrast, these mutations showed much less effect on firm cell adhesion in 0.5 mM Mn²⁺ (Fig. 2B). Thus, the disulfide bond in the W1 β4-β1 loop of the α7 subunit is essential for the inactive α₄β₇ to support rolling cell adhesion but not indispensable for the activated α₄β₇ to mediate firm cell adhesion, suggesting a critical role of the W1 β4-β1 loop in the low-affinity α₄β₇-MAdCAM-1 interaction.

The Disulfide Bond-occluded Segment in the W1 β4-β1 Loop Is Essential for Low-affinity α₄β₇-MAdCAM-1 Binding—The disulfide bond in the W1 β4-β1 loop occludes a short segment consisting of three amino acid residues (Gly-82, Lys-83, and Thr-84), which is unique for the α₄/α7 subfamily (Fig. 1, B and C). To investigate the roles of these residues in α₄β₇-MAdCAM-1 interaction, we mutated them to Ala individually and examined the adhesive behaviors of the mutant α₄β₇ 293T transfectants. In 1 mM Ca²⁺/Mg²⁺, G82A and T84A led to about 72 and 20% decreases of cell adhesion on MAdCAM-1 at 2 dynes/cm², respectively, whereas the K83A mutation hardly affected α₄β₇-mediated rolling cell adhesion (Fig. 2C). In contrast, to their effects on rolling adhesion, all the three mutations barely affected the firm cell adhesion on MAdCAM-1 in 0.5 mM Mn²⁺ (Fig. 2D). Thus, among the three amino acid residues, Gly-82 in particular and Thr-84 are required for the low-affinity α₄β₇-MAdCAM-1 interaction, whereas all three residues are dispensable for Mn²⁺-activated, high-affinity α₄β₇-MAdCAM-1 binding.

Next we investigated the role of the disulfide bond-occluded segment by deleting the three residues simultaneously (Del). As shown in Fig. 2, C and D, the Del mutation abolished the rolling cell adhesion on MAdCAM-1 at 2 dynes/cm² in 1 mM Ca²⁺/Mg²⁺ (Fig. 2C). In contrast, it could still mediate decent firm cell adhesion in 0.5 mM Mn²⁺ (Fig. 2D). Thus, the disulfide bond-occluded segment in the W1 β4-β1 loop is critical for the low-affinity α₄β₇-MAdCAM-1 interaction but not for the high-affinity α₄β₇-MAdCAM-1 interaction.

The Disulfide Bond-stabilized W1 β4-β1 Loop Is Required for Stable Interaction between Low-affinity α₄β₇ and MAdCAM-1—To study the influence of the W1 β4-β1 loop on the strength of α₄β₇-mediated cell adhesion to MAdCAM-1, we examined the resistance to detachment by increasing wall shear stress (Fig. 3, A–D). In 1 mM Ca²⁺/Mg²⁺, C81S, C85S, and C2S mutant transfectants detached much more rapidly from MAdCAM-1 than WT α₄β₇ transfectants (Fig. 3A), suggesting a less stable interaction between low-affinity α₄β₇ and MAdCAM-1 because of the loss of the disulfide bond in the W1 β4-β1 loop. Consistent with the effects of the W1 β4-β1 loop single-residue mutations
on cell adhesion (Fig. 2C), cell resistance to detachment in 1 mM Ca\(^{2+}\)/Mg\(^{2+}\) was mostly decreased by G82A and least affected by K83A, with T84A in between (Fig. 3C). In contrast to the results in 1 mM Ca\(^{2+}\)/Mg\(^{2+}\), all of the above mutant-expressing cells showed a similar shear resistance as WT \(\alpha_4\beta_7\) transfectants in 0.5 mM Mn\(^{2+}\) (Fig. 3, B and D). Thus, these data indicate that the disulfide bond-stabilized W1\(4\)/W9252-4-propeller W1\(4\)/W9252-1 loop is required for stable interaction between low-affinity \(\alpha_4\beta_7\)-transfectants and MAdCAM-1 to support efficient rolling adhesion but not indispensable to maintain the stable high-affinity \(\alpha_4\beta_7\)-MAdCAM-1 interaction.

To further address the role of the W1\(4\)/W9252-1 loop in \(\alpha_4\beta_7\)-mediated rolling adhesion, we examined the rolling velocity of \(\alpha_4\beta_7\)-293T transfectants on MAdCAM-1 at different wall shear stresses (Fig. 3, E and F). In 1 mM Ca\(^{2+}\)/Mg\(^{2+}\), WT \(\alpha_4\beta_7\) transfectants rolled with increasing velocity from 4 to 8 \(\mu\)m/s as wall shear stress was increased from 1 to 4 dynes/cm\(^2\) (Fig. 3, E and F). Compared with WT \(\alpha_4\beta_7\) transfectants, the C81S, C85S, C2S, and G82A mutant transfectants showed an obviously increased rolling velocity at each wall shear stress (Fig. 3, E and F). In addition, the T84A transfectants exhibited a milder increase in rolling velocity, whereas the K83A transfectants showed a slightly increased rolling velocity (Fig. 3F). Taken together, the above data demonstrate that the disulfide bond-stabilized W1\(4\)/W9252-1 loop is required to stabilize the low-affinity \(\alpha_4\beta_7\)-MAdCAM-1 interaction for efficient rolling cell adhesion.

The Disulfide Bond-stabilized W1\(4\)/W9252-1 Loop Is Required for the Activation of \(\alpha_4\beta_7\) by Inside-Out Signaling—In addition to the activation by extracellular Mn\(^{2+}\), integrin can also be activated by intracellular effector proteins, such as talin, via inside-out signaling (17, 18, 33). To investigate the role of the W1\(4\)/W9252-1 loop in the activation of \(\alpha_4\beta_7\) by inside-out signaling, the talin head domain with N-terminal fused mCherry (mCherry-talin) was overexpressed at a comparable level in WT and mutant \(\alpha_4\beta_7\)-transfectants (Fig. 4A), and the cell adhesion at 2 dynes/cm\(^2\) was examined (Fig. 4B). In 1 mM Ca\(^{2+}\)/Mg\(^{2+}\), the total and firmly adherent cell numbers of WT \(\alpha_4\beta_7\) transfectants were both increased significantly after overexpression
of mCherry-talin, indicating the activation of αβ7 by talin (Fig. 4B). In contrast, the number of bound cells expressing either the C2S or Del mutant was not increased by mCherry-talin overexpression (Fig. 4B), suggesting impaired integrin activation via inside-out signaling.

To further confirm the impaired activation of the C2S and Del mutants by inside-out signaling, we tested αβ7 activation by PMA in WT, C2S, or Del mutant αβ7 293T transfectants. PMA is reported to activate integrins through inside-out signaling by activating the protein kinase C kinase pathway (34). Consistently, WT αβ7, but not the C2S and Del mutants, could be activated by PMA (Fig. 4C). Thus, these results suggest that the disulfide bond-stabilized W1 β4-β1 loop is crucial for the activation of integrin αβ7 by inside-out signaling.

The Disulfide Bond-stabilized W1 β4-β1 Loop Is Required for the Conformational Rearrangement in αβ7 during Integrin Activation—Integrin activation is accomplished by global conformational rearrangements, including the switchblade-like extension of the integrin ectodomain and headpiece opening (12, 35). We next used FRET to study the contribution of the W1 β4-β1 loop to integrin conformation. To assess the orientation of the αβ7 ectodomain relative to the plasma membrane, αβ7 was labeled with Alexa Fluor 488-conjugated Act-1 Fab fragment as a donor, which binds to the top of the β7 I domain (36). The plasma membrane was labeled with FM4–64 FX as an acceptor (24). In 1 mM Ca2+/Mg2+, all WT and mutant (C2S and Del) αβ7 transfectants showed a similar and relatively high FRET efficiency, suggesting the bent conformation of inactive integrins (Fig. 5). Activation of WT αβ7 with 0.5 mM Mn2+ significantly decreased the FRET efficiency, suggesting the extension of the αβ7 ectodomain (Fig. 5A). Interestingly, the FRET efficiency of the C2S and Del mutant αβ7 transfectants was much higher than that of the WT αβ7 transfectants in Mn2+, suggesting that Mn2+ induces less of an extension of the C2S and Del mutants than WT αβ7 (Fig. 5A). Thus, removal of either the disulfide bond or the disulfide bond-occluded segment impairs the global conformational changes of integrin αβ7 induced by Mn2+.

Additionally, we examined the conformational change of αβ7 activated by talin or PMA via inside-out signaling (Fig. 5, B and C). Both overexpression of mCherry-talin and stimulation with PMA significantly decreased the FRET efficiency of WT αβ7 transfectants in 1 mM Ca2+/Mg2+ (Fig. 5, B and C). In contrast, the FRET efficiency of the C2S and Del mutant αβ7 transfectants did not show a significant decrease after mCherry-talin overexpression or PMA treatment (Fig. 5, B and C). These results clearly suggest that the disulfide bond-stabilized W1 β4-β1 loop is essential for the global conformational change during integrin activation via inside-out signaling. Collectively, these data demonstrate that the disulfide bond-stabilized W1 β4-β1 loop in the αβ7-propeller domain is crucial for the global conformational rearrangement coupled with αβ7 activation.

The Disulfide Bond-stabilized W1 β4-β1 Loop Is Essential for αβ7-mediated Outside-In Signaling—Integrin ligand binding can trigger outside-in signaling to activate multiple intracellular signaling proteins, which leads to cytoskeleton rearrangements to support cell spreading (1, 33, 37–39). To assess the requirement of the W1 β4-β1 loop for integrin-mediated outside-in signaling, CHO-K1 cells stably expressing a similar level of WT or mutant (C2S and Del) αβ7 were established that exhibited similar adhesive behaviors on immobilized MadCAM-1 at a wall shear stress of 2 dynes/cm2 as 293T transient transfectants, suggesting that the effects of W1 β4-β1 loop mutations on αβ7-mediated cell adhesion were not cell type-specific (Fig. 6, A and B). Then, αβ7-mediated cell spreading was studied (Fig. 6, C–E). WT αβ7-expressing cells spread substantially on MadCAM-1 in 1 mM Ca2+/Mg2+, with an extensive area of close cell substrate contact (Fig. 6C). In contrast, both C2S and Del mutant αβ7-expressing cells did not spread on MadCAM-1 and showed the same area of projection as cells in suspension under this condition (Fig. 6, C and E). Addition of 0.5 mM Mn2+ further enhanced the spreading of WT αβ7-expressing cells and enabled the mutant αβ7 transfectants to spread on MadCAM-1 but with a much smaller cell contact area on the substrates (Fig. 6D). In addition, mutant αβ7-expressing cells exhibited a significantly decreased area of projection compared with that of the WT αβ7-expressing cells (Fig. 6E). Taken together, these results show that either breaking the disulfide bond or deleting the disulfide bond-occluded segment in the W1 β4-β1 loop leads to deficient cell spreading, suggesting disrupted αβ7 outside-in signaling.

FAK and paxillin are important integrin downstream proteins that are involved in the regulation of cell spreading (37, 38). The phosphorylation of Tyr-397-FAK and Tyr-118-paxillin was increased substantially in WT αβ7 CHO-K1 transfect-
tants after spreading on MadCAM-1 in 1 mM Ca$^{2+}$/Mg$^{2+}$ compared with the same cells on poly-L-lysine, indicating the activation of integrin downstream signaling upon ligand binding (Fig. 6F). In comparison, the phosphorylation of Tyr-397-FAK and Tyr-118-paxillin in C2S mutant $\alpha_\beta_7$ transfectants was much lower than that of WT $\alpha_\beta_7$ transfectants, suggesting impaired integrin downstream signaling (Fig. 6F). The data of Del mutant $\alpha_\beta_7$ transfectants on MadCAM-1 in 1 mM Ca$^{2+}$/Mg$^{2+}$ was not available because no cells adhered to MadCAM-1 under this condition. Compared with the phosphorylation of Tyr-397-FAK and Tyr-118-paxillin on MadCAM-1 in 1 mM Ca$^{2+}$/Mg$^{2+}$, addition of 0.5 mM Mn$^{2+}$ further enhanced the phosphorylation of FAK and paxillin in WT $\alpha_\beta_7$-expressing cells but not in C2S mutant $\alpha_\beta_7$ transfectants. Del mutant $\alpha_\beta_7$-expressing cells showed similar levels of phosphorylated Tyr-397-FAK and Tyr-118-paxillin, as seen in C2S mutant $\alpha_\beta_7$ transfectants (Fig. 6F). These results demonstrate that removal of either the disulfide bond or the disulfide bond-occluded short segment in the W1 $\beta_4$-$\beta_1$ loop impairs integrin outside-in signaling, which leads to decreased phosphorylation of FAK and paxillin. Thus, the disulfide bond-stabilized W1 $\beta_4$-$\beta_1$ loop is required for integrin $\alpha_\beta_7$-mediated outside-in signaling.

**DISCUSSION**

The ability of integrin $\alpha_\beta_\gamma$ to mediate rolling and firm cell adhesion after activation. In this study, we demonstrate that a unique disulfide bond-stabilized W1 $\beta_4$-$\beta_1$ loop in the $\beta$-propeller domain of the $\alpha_\gamma$ subunit plays an essential role in low-affinity $\alpha_\beta_\gamma$-MadCAM-1 interaction that supports rolling cell adhesion but is not dispensable for firm cell adhesion supported by high-affinity interaction between Mn$^{2+}$-activated $\alpha_\beta_7$ and MadCAM-1. Moreover, removal of either the disulfide bond or the disulfide bond-occluded short segment not only blocked the global conformational rearrangement and activation of $\alpha_\beta_7$ triggered by talin or PMA via inside-out signaling but also disrupted integrin outside-in signaling and led to deficient $\alpha_\beta_7$-mediated cell spreading. Thus, the disulfide bond-stabilized W1 $\beta_4$-$\beta_1$ loop in the $\alpha_\gamma$-propeller domain plays an essential role in supporting the rolling cell adhesion mediated by $\alpha_\gamma\beta_\gamma$ and functions as a novel regulatory element of integrin affinity and bidirectional signaling.

According to the crystal structures of integrins, the ligand-binding site of $\alpha_\gamma$-prop is in an overall distinct shape from those of $\alpha_\beta_3$, $\alpha_\beta_\delta$, and $\alpha_\delta\beta_\gamma$. The crevice running along the $\alpha_\beta$ subunit in $\alpha_\beta_\gamma$ ligand-binding site is longer, wider, and deeper than those in $\alpha_\beta_3$, $\alpha_\beta_\delta$, and $\alpha_\delta\beta_\gamma$, which is contributed largely by the loops on the face of the $\beta$-propeller domain that bind the $\beta$ domain and form the ligand-binding site (28). Among the loops, the W1 $\beta_4$-$\beta_1$ loop is unique, as it contains a disulfide bond existing only in the integrin $\alpha_\gamma\beta_\gamma$ subfamily. Here we demonstrated that breaking the disulfide bond or deleting the disulfide bond-occluded segment of the W1 $\beta_4$-$\beta_1$ loop significantly impaired rolling adhesion mediated by low-affinity $\alpha_\beta_7$ on MadCAM-1 (Fig. 2). Considering the importance of disulfide bonds in stabilizing the three-dimensional...
In addition to MAdCAM-1, integrin α4β7 can also bind another ligand, vascular cell adhesion molecule 1 (VCAM-1), and mediate rolling adhesion on this substrate before activation (2, 41). To address whether the W1 β4-β1 loop is also involved in α4β7-VCAM-1 binding, we tested the influence of the W1 β4-β1 loop mutations on α4β7-mediated cell adhesion on immobilized VCAM-1 in shear flow. Consistent with the results on MAdCAM-1, either breaking the disulfide bond or deleting the disulfide bond-occluded segment inhibited the rolling cell adhesion on VCAM-1 in 1 mM Ca2+/Mg2+ but barely affected the firm cell adhesion in 0.5 mM Mn2+ (Fig. 7A). These results indicate that the disulfide bond-stabilized W1 β4-β1 loop is also essential for the binding of low-affinity α4β7 to VCAM-1.

It is noteworthy that the disulfide bond in the W1 β4-β1 loop is only present in the α4/α4 integrin subfamily, which consists of three integrins, α4β7, α4β1, and α4β1 (Fig. 1). To reveal whether the disulfide bond-stabilized W1 β4-β1 loop could also regulate α4β7 and α4β1 ligand binding, we disrupted the disulfide bond (C2S), deleted the disulfide bond-occluded segment (Del) in α4β1 and α4β1, respectively, and examined the influence of these mutations on α4β7- or α4β1-mediated cell adhesion to their ligand, VCAM-1 (Fig. 7, B and C). Consistent with the results of integrin α4β7, either breaking the disulfide bond or deleting the disulfide bond-occluded segment in the W1 β4-β1 loop in α4β1 abolished rolling cell adhesion mediated by the low-affinity α4β7-VCAM-1 interaction but hardly affected Mn2+-stimulated firm cell adhesion mediated by high-affinity integrin-ligand binding (Fig. 7B). Different from integrin α4β7 and α4β1, α4β1 could not support rolling adhesion before activation (in 1 mM Ca2+/Mg2+). The same mutations in the α4 W1 β4-β1 loop barely affected the adhesion of α4β1 to VCAM-1 in either 1 mM Ca2+/Mg2+ or 0.5 mM Mn2+ (Fig. 7C). Thus, the disulfide bond-stabilized W1 β4-β1 loop plays an essential role in supporting the rolling cell adhesion mediated by the low affinity α4 integrins but is not indispensable for the firm cell adhesion mediated by either low-affinity α4β7 or high-affinity α4β7, α4β1, and α4β1 integrins.

One interesting finding of our study is that the disulfide bond-stabilized W1 β4-β1 loop is required for talin- or PMA-mediated α4β7 activation but not indispensable for Mn2+-induced α4β7 activation and firm cell adhesion. This difference could be attributed to the fact that the mechanisms of integrin activation induced by Mn2+ and talin/PMA are different. Mn2+ activates integrins by direct binding to the metal ion binding sites in the β1 domain, which triggered integrin activation independently of cytoplasmic signaling (19, 42, 43), whereas talin or PMA activate integrin via inside-out signaling by regulating the binding of intracellular effector molecules to integrin cytoplasmic domains, which triggers the global conformational rearrangement and activation of integrin (15, 17, 18, 33, 34). In addition, FRET analysis of the distance between the integrin head domain and the cell membrane in this study also demonstrated that integrin α4β7, stimulated by PMA or overexpression of talin were less extended than Mn2+-activated α4β7 (Fig. 5), suggesting the different conformations of these integrins. Thus, it implies that integrin α4β7 activated by Mn2+ or talin/PMA might have distinct requirements for the W1 β4-β1 loop.
Another notable finding of our study is that the disulfide bond-stabilized W1 β4-β1 loop in the α4β7-propeller domain is crucial for the global conformational rearrangement of α4β7 triggered by inside-out signaling. Studies have shown that the local conformational changes in the integrin head domain are closely associated with its global conformational rearrangements (27, 31, 44–46). Mutations around the ligand binding site of integrin molecules (27, 31, 44–46). In this study, as the W1 β4-β1 loop is located on the face of the β-propeller domain, which binds the β1 domain and forms the ligand-binding site of integrin, it is tempting to speculate that deletion or mutations of this loop could exert some effects on the ligand-binding interface formed between the α subunit β-propeller domain and the β1 domain, which might affect the local conformational changes in the head domain of integrin that are required by the global conformational rearrangements triggered by inside-out signaling.

This study also finds that the disulfide bond-stabilized W1 β4-β1 loop is required for α4β7-mediated cell spreading and outside-in cell signaling (Fig. 6). Integrin-mediated cell spreading is a complex process that involves diverse signaling networks (47). One of the early steps in transducing extracellular cues through integrins to the cytoskeleton is the activation of FAK and paxillin (47). Our study showed that the W1 β4-β1 loop mutations decreased the activation of both FAK and pax-

FIGURE 5. Influence of the W1 β4-β1 loop mutations on integrin conformation. A–C, FRET between the β1 domain and the plasma membrane. The FRET efficiency of WT and mutant α4β7-293T transient transfectants under the indicated conditions: 1 mM Ca2+/Mg2+ and 0.5 mM Mn2+ (A), 1 mM Ca2+/Mg2+ and 1 mM Ca2+/Mg2+ with expression of mCherry-talin (B), and 1 mM Ca2+/Mg2+ and 1 mM Ca2+/Mg2+ with stimulation by 0.1 μM PMA (C). Data are mean ± S.E. (n = 20), p values were calculated by two-way ANOVA with Bonferroni post-tests. ***, p < 0.001.

FIGURE 6. Influence of the W1 β4-β1 loop mutations on α4β7-mediated outside-in signaling and cell spreading. A and B, rolling and firm adhesions of CHO-K1 stable transfectants on immobilized MadCAM-1 substrates (10 μg/ml) in 1 mM Ca2+/Mg2+ (A) or in 0.5 mM Mn2+ (B). The number of rolling and firmly adherent WT and mutant α4β7-transfectants was measured in the indicated divalent cations at a wall shear stress of 2 dynes/cm2. Cells treated with 5 mM EDTA were used as a control. Data are mean ± S.E. (n = 3), p values were calculated by one-way ANOVA with Dunnett post-tests. ***, p < 0.001. C and D, differential interference contrast and interference reflection microscopy images of CHO-K1 stable transfectants that adhered to immobilized MadCAM-1 in 1 mM Ca2+/Mg2+ (C) or 0.5 mM Mn2+ (D). The images are representatives from one of three independent experiments. Scale bars = 50 μm. E, quantification of cell spreading area (projection on substrates) of α4β7 CHO-K1 transfectants on the basis of differential interference contrast images. Data are mean ± S.E. (n = 50). p values were calculated by two-way ANOVA with Bonferroni post-tests. ***, p < 0.001. F, CHO-K1 cells stably expressing WT or mutant α4β7, were plated on poly-L-lysine in serum-free Ham’s F12 medium or on MadCAM-1 in Ham’s F12 medium containing 1 mM Ca2+/Mg2+ or 0.5 mM Mn2+ for 2 h, lysed, and blotted for indicated molecules. Phosphorylated Y397-FAK and Y118-paxillin were blotted as indications of FAK and paxillin activation, respectively. A representative result of three independent experiments is shown.
illin, indicating that α₆β₇-mediated outside-in signaling also requires the normal function of the W1 β4-β1 loop. Thus, the disulphide bond-stabilized W1 β4-β1 loop might contribute to a critical interaction between α₆β₇ and the ligands, which is important for integrin outside-in signaling. In conclusion, our findings reveal that the unique disulphide bond-stabilized W1 β4-β1 loop in α₆β₇-propeller domain functions as a novel regulatory element of integrin affinity and signaling and uncover a particular molecular basis for α₆β₇-mediated rolling cell adhesion.

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