The CC’ and DE Loops in Ig Domains 1 and 2 of MAdCAM-1 Play Different Roles in MAdCAM-1 Binding to Low- and High-affinity Integrin $\alpha_4\beta_7$ *\(^{\text{S}}\)

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Lymphocyte homing is regulated by the dynamic interaction between integrins and their ligands. Integrin $\alpha_4\beta_7$ mediates both rolling and firm adhesion of lymphocytes by modulating its affinity to the ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1). Although previous studies have revealed some mechanisms of $\alpha_4\beta_7$-MAdCAM-1 binding, little is known about the different molecular bases of the low- and high-affinity $\alpha_4\beta_7$-MAdCAM-1 interactions, which mediate rolling and firm adhesion of lymphocytes, respectively. Here, we found that two loops in immunoglobulin domains 1 and 2 (D1 and D2) of MAdCAM-1 played different roles in MAdCAM-1 binding to low-affinity (inactive) and high-affinity (activated) $\alpha_4\beta_7$. The Asp-42 in the CC’ loop of D1 was indispensable for MAdCAM-1 binding to both low-affinity and high-affinity $\alpha_4\beta_7$. The other CC’ loop residues except for Arg-39 and Ser-44 were essential for MAdCAM-1 binding to both inactive $\alpha_4\beta_7$ and activated $\alpha_4\beta_7$. Single amino acid substitution of the DE loop residues mildly decreased MAdCAM-1 binding to both inactive and activated $\alpha_4\beta_7$. Notably, removal of the DE loop greatly impaired MAdCAM-1 binding to inactive and SDF-1α- or talin-activated $\alpha_4\beta_7$, but only decreased 60% of MAdCAM-1 binding to Mn\(^{2+}\)-activated $\alpha_4\beta_7$. The crystal structure of MAdCAM-1 highlights two protruding loops from the two Ig domains, the CC’ loop in D1 and DE loop in D2, which are important for the interaction between MAdCAM-1 and $\alpha_4\beta_7$ (10, 11). The essential integrin-binding motif (LDTS) resides in the CC’ loop of MAdCAM-1 D1, and the Asp-42 in the LDTS motif serves as the primary $\alpha_4\beta_7$-binding site by directly interacting with the metal ion at the metal ion-dependent adhesion site (MIDAS) in the integrin $\beta_7$ I domain (8, 12–14). Mutational studies suggest that some other residues in the CC’ loop of MAdCAM-1 are also involved in MAdCAM-1-$\alpha_4\beta_7$ binding (9, 12, 13). The DE loop in D2 is predominated by negatively charged residues, which have been reported to play an important role in determining integrin binding specificity (15). Mutagenesis study has shown that some residues in the DE loop are important for MAdCAM-1 binding to integrin $\alpha_4\beta_7$ (9).

Integrins are $\alpha/\beta$ heterodimeric cell adhesion molecules that mediate cell-cell, cell-extracellular matrix, and cell-pathogen interactions and transmit signals bidirectionally across the plasma membrane (16–18). Cell adhesion through integrin is dependent on the dynamic regulation of integrin affinity. The low-affinity integrin $\alpha_4\beta_7$ mediates rolling adhesion of lymphocytes. Upon activation, $\alpha_4\beta_7$ converts to a high-affinity state, which mediates firm cell adhesion. Early studies on integrin structure have revealed that integrin extracellular domains exist in at least three distinct global conformational states: bent

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1 The abbreviations used are: MAdCAM-1, mucosal cell adhesion molecule-1; MIDAS, metal ion-dependent adhesion site; ADMIDAS, adjacent to MIDAS; PBL, peripheral blood lymphocyte; hu, human.

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with a closed headpiece, extended with a closed headpiece, and extended with an open headpiece, which correspond to the low-affinity, intermediate-affinity, and high-affinity states, respectively (19–21). The equilibrium among these different affinity states is regulated by integrin inside-out signaling and certain extracellular stimuli, such as divalent cations (22, 23). When compared with the low-affinity state in Ca$^{2+}$ + Mg$^{2+}$, removal of Ca$^{2+}$ or the addition of Mn$^{2+}$ strikingly increases ligand binding affinity and adhesiveness of almost all integrins (14, 24–26). Crystal structures of $\alpha_4\beta_2$ and $\alpha_{IIb}\beta_3$ integrins revealed three interlinked metal ion-binding sites in integrin $\beta_1$ domain (27, 28). The central MIDAS is flanked by two metal ion-binding sites, the adjacent to MIDAS (ADMIDAS) site and the synergistic metal ion-binding site. The divergent cation at MIDAS directly coordinates the acidic side chain shared by all integrin ligands and is essential for integrin-ligand binding (14, 29). The synergistic metal ion-binding site and ADMIDAS function as positive and negative regulatory sites, respectively (14, 25, 30–32). Upon activation, integrin with the bent conformation converts to the extended conformation coupled with a series of global and local conformational changes, including separation of cytoplasmic tails, extension of integrin ectodomains, swing-out of the hybrid domain, $\beta_1$ domain $\alpha_7$ helix downward movement, and conformational rearrangement at the integrin-ligand-binding site around MIDAS and ADMIDAS.

Despite the above advances in understanding of $\alpha_4\beta_7$-binding hotspots on MadCAM-1 and integrin conformational rearrangement during activation, the molecular basis for the recognition of MadCAM-1 by low- and high-affinity $\alpha_4\beta_2$ remains elusive. The mechanism of rolling and firm adhesion of lymphocyte mediated by $\alpha_4\beta_2$ on MadCAM-1 is not well understood.

In this study, we found that the CC’ loop in D1 and DE loop in D2 of MadCAM-1 exerted different functions in MadCAM-1 binding to low- and high-affinity $\alpha_4\beta_2$. In addition, we demonstrated that the inactive $\alpha_4\beta_2$, and $\alpha_4\beta_7$, activated by different stimuli might have distinct conformations with different structural requirements for MadCAM-1 binding.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—cDNAs of human $\alpha_4$ and $\beta_2$ were constructed in vector pcDNA3.1/Hygro(−) (Invitrogen). cDNA of mouse talin head domain (talin 1–435) with N-terminal-fused GFP (GFP-talin-head) in vector pcDNA3.1 was kindly provided by Dr. Minsoo Kim. All constructs were confirmed by DNA sequencing. Transient transfection of 293T cells was performed by Dr. Minsoo Kim. Site-directed mutations were introduced into MadCAM-1 cDNA using QuikChange (Stratagene). 293T cells were transiently transfected with huMadCAM-1/Fc construct and cultured for 3–4 days in a humidified atmosphere of 5% (v/v) CO$_2$ at 37°C. The huMadCAM-1/Fc was isolated from conditioned medium by protein A-Sepharose (Pierce) and further purified by gel filtration in 20 mM HEPES, 300 mM NaCl, pH 7.4 (HEPES-buffered saline).

**Flow Chamber Assay**—The flow chamber assay was performed as described (25). A polystyrene Petri dish was coated with a 5-mm diameter, 20-μl spot of 10 μg/ml purified huMadCAM-1/Fc in coating buffer (PBS, 10 mM NaHCO$_3$, 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 Ca$^{2+}$ + Mg$^{2+}$-free HEPES-buffered saline (20 mM HEPES, pH 7.4, 5 mM EDTA, 0.5% BSA), resuspended at 1 × 10$^7$/ml in buffer A (Ca$^{2+}$ + Mg$^{2+}$-free HEPES-buffered saline, 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 10 s at 0.4 dyne/cm$^2$. Then, shear stress was increased every 10 s from 1 dyne/cm$^2$ up to 16 dynes/cm$^2$ in 2-fold increments. The number of cells remaining bound at the end of each 10-s interval was determined. Rolling velocity at each shear stress was calculated from the average distance traveled by rolling cells in 3 s. To avoid confusing rolling with small amounts of movement due to tether stretching or measurement error, a velocity of 2 μm/s, which corresponds to a movement of one-half cell diameter during the 3-s measurement interval, was the minimum velocity required to define a cell as rolling instead of firmly adherent.

**SDF-1α Stimulation Assay under Flow**—A polystyrene Petri dish was coated with a 5-mm diameter, 20-μl spot of 10 μg/ml purified huMadCAM-1/Fc with SDF-1α (2 μg/ml) or huMadCAM-1/Fc alone in coating buffer for 1 h at 37°C followed by 2% BSA in coating buffer for 1 h at 37°C to block nonspecific binding sites. The PBLs (1 × 10$^6$/ml) were infused into the chamber, and then cells were allowed to settle for 2 min and to accumulate for 30 s at 0.3 dyne/cm$^2$ and 10 s at 0.4 dyne/cm$^2$. Then, shear stress was increased every 10 s from 1 dyne/cm$^2$ up to 16 dynes/cm$^2$ in 2-fold increments. Cells remaining bound under the wall shear stress of 1 dyne/cm$^2$ were counted. The PBLs (1 × 10$^6$/ml) preincubated with $\alpha_4\beta_2$-blocking mAb Act-1 (2 μg/ml) or treated with 5 mM EDTA were used as control.
Distinct Functions of Two Loops in MAdCAM-1 D1 and D2

RESULTS

To study the molecular basis for the recognition of MAdCAM-1 by low- and high-affinity integrin \( \alpha_4 \beta_7 \), we generated soluble human MAdCAM-1 protein (from Val-1 to Pro-315), which contains domain 1, domain 2, and mucin-like domain with C-terminal fused Fc1 and two regions of human IgG1. Because the CC’ loop in D1 of MAdCAM-1 possesses the LDTS motif, which has been implicated as the primary integrin-binding site, we first investigated the function of the CC’ motif, which has been implicated as the primary integrin-binding site, we first investigated the function of the CC’ loop in the binding of low- and high-affinity integrin \( \alpha_4 \beta_7 \) to MAdCAM-1 by introducing a series of single point mutations in the CC’ loop based on the MAdCAM-1 crystal structure (Fig. 1) (10, 11).

Asp-42 in CC’ Loop of MAdCAM-1 D1 Is Essential for MAdCAM-1 Binding to Both Low-affinity and High-affinity \( \alpha_4 \beta_7 \)—Adhesive behavior of 293T cells stably expressing human integrin \( \alpha_4 \beta_7 \) in shear flow was characterized in a parallel wall flow chamber by allowing them to adhere to MAdCAM-1 adsorbed to the lower wall. The shear stress was incrementally increased, and the velocity of the cells remaining bound at each increment was determined. Human \( \alpha_4 \beta_7 \) 293T transfectants behaved as described previously for lymphoid cells expressing \( \alpha_4 \beta_7 \) (26). In 1 mM \( \text{Ca}^{2+} + 1 \text{ mM Mg}^{2+} \), about 90% of the bound \( \alpha_4 \beta_7 \) transfectants rolled at the shear stress of 1 dyne/cm² (Fig. 2A). By contrast, cells were firmly adherent in 0.5 mM \( \text{Mn}^{2+} \) (Fig. 2B). Rolling and firm adhesion represent the low- and high-affinity interactions of \( \alpha_4 \beta_7 \) with MAdCAM-1, respectively. As control, \( \alpha_4 \beta_7 \)-transfectants treated with \( \alpha_4 \beta_7 \) blocking antibody Act-1 or with 5 mM EDTA did not accumulate on MAdCAM-1 substrates (Fig. 2, A and B). In contrast to the robust cell adhesion on WT MAdCAM-1, substitution of Asp-42 with Ala abolished both rolling and firm adhesion on MAdCAM-1, suggesting its essential role in integrin-ligand binding (Fig. 2, A and B).

In Addition to Asp-42, other CC’ Loop Residues Except for Arg-39 and Ser-44 Are Essential for MAdCAM-1 Binding to Low-affinity \( \alpha_4 \beta_7 \)—When compared with the efficient rolling cell adhesion on WT MAdCAM-1, single amino acid substitution of most residues in the MAdCAM-1 CC’ loop with Ala abolished the rolling cell adhesion on MAdCAM-1 mediated by low-affinity \( \alpha_4 \beta_7 \) in 1 mM \( \text{Ca}^{2+} + 1 \text{ mM Mg}^{2+} \) (Fig. 2A). Arg-39
and Ser-44 to Ala mutations showed less effect, which led to 70 and 80% decrease of cell adhesion to MAdCAM-1, respectively.

**CC’ Loop Residues Other than Asp-42 Are Not Important for MAdCAM-1 Binding to High-affinity αβ7, Activated by Mn^{2+}**

In contrast to rolling cell adhesion mediated by low-affinity αβ7, the firm cell adhesion mediated by Mn^{2+}-activated αβ7 was only slightly affected by the same mutations, except for Asp-42 (Fig. 2B). More than 70% of cell adhesion was retained when Leu-41, Thr-43, Leu-45, and Gly-46 were mutated to Ala, respectively. The rest of the mutations only caused less than 10% loss of cell adhesion in Mn^{2+}. Thus, except for the primary MAdCAM-1-αβ7-binding site Asp-42, the rest of the CC’ residues other than Arg-39 and Ser-44 in the CC’ loop are essential for the interaction between low-affinity αβ7 and MAdCAM-1, but not required for the binding of Mn^{2+}-activated αβ7 to MAdCAM-1.

**CC’ Loop Residues Other than Arg-39 and Ser-44 Are Crucial for MAdCAM-1 Interaction with High-affinity Integrin αβ7, Activated by Talin or SDF-1α**—Besides the unphysiological strong activation by Mn^{2+}, integrin can be activated by more physiological pathways such as overexpression of intracellular talin or SDF-1α stimulation (34). Talin is a cytoskeletal protein that can interact with the cytoplasmic tail of the integrin β subunit and activate integrin. To investigate the influence of the CC’ loop mutations on the interaction between MAdCAM-1 and talin-activated αβ7, we overexpressed the GFP-talin-head in αβ7 293T transfectants (supplemental Fig. S1) and examined the cell adhesion behavior to WT and mutant MAdCAM-1 under flow (Fig. 2C). The firmly adherent αβ7 transfectants on WT MAdCAM-1 increased from 11 to 37% of total bound cells under the shear stress of 1 dyne/cm^2 after co-transfection with GFP-talin-head, suggesting the activation of integrin αβ7 by talin. Surprisingly, unlike the mild effects of the CC’ mutations on the cell adhesion mediated by Mn^{2+}-activated αβ7, the cell adhesion mediated by talin-activated αβ7 was greatly disrupted by most CC’ loop mutations. GFP-talin-head overexpression only slightly increased cell adhesion on R39A, G40A, S44A, L45A, G46A, and V48A mutants. R39A and S44A showed less effect, which led to 76 and 54% decrease of cell adhesion mediated by talin-activated αβ7 to MAdCAM-1. Thus, the above results suggest that the CC’ loop residues other than Arg-39 and Ser-44 are crucial for the recognition of MAdCAM-1 by talin-activated αβ7 and that integrin αβ7 activated by Mn^{2+} and talin could have different conformations with different structural requirements for MAdCAM-1 binding.

Because the CC’ loop might play different roles in MAdCAM-1 interaction with Mn^{2+} and talin-activated αβ7, we next tested its function in the interaction between MAdCAM-1 and αβ7 activated by SDF-1α. SDF-1α can induce integrin activation through the PI3 kinase pathway by binding to CXCR4, the G protein-coupled receptor of SDF-1α (35, 36). Human PBLs were used that express high levels of integrin αβ7 (37 and CXCR4 (38). In contrast to the robust cell adhesion to WT MAdCAM-1 in 1 mM Ca^{2+} + 1 mM Mg^{2+}, PBL adhered weakly to most CC’ loop mutants and did not adhere to D42A and T43A mutants at all (Fig. 2D). R39A and S44A mutations showed less effect, which led to 86 and 68% decrease of cell adhesion to MAdCAM-1, respectively. Activation of αβ7 by SDF1-α stimulation notably increased the number of PBLs bound to WT MAdCAM-1, but not to the MAdCAM-1 CC’ loop mutants, suggesting the importance of the CC’ loop residues in the interaction between MAdCAM-1 and αβ7 activated by SDF1-α (Fig. 2D). Interestingly, cell adhesion to MAdCAM-1 CC’ loop mutants could be increased by talin but not SDF1-α, indicating the subtle difference between integrins activated by talin and SDF-1α (Fig. 2, C and D).

Taken together, the above data demonstrate that Asp-42 in the CC’ loop is essential for MAdCAM-1 binding to both low-affinity and high-affinity αβ7, and the rest of the CC’ loop residues other than Arg-39 and Ser-44 are crucial for MAdCAM-1 binding to αβ7 activated by SDF1-α, suggesting the importance of the CC’ loop residues in the interaction between MAdCAM-1 and integrin activated by physiological stimuli as SDF-1α or talin, but not required for MAdCAM-1 binding to αβ7 activated by Mn^{2+}. Thus, integrins activated by distinct stimuli might have different high-affinity conformations with diverse structural requirements for MAdCAM-1 binding.

**CC’ Loop Is Required for the Stable Interaction between MAdCAM-1 and Low-affinity Integrin αβ7**—To test the effect of the CC’ loop on the strength of αβ7-mediated cell adhesion to MAdCAM-1, we examined the resistance of adherent cells to detachment by increasing shear stress (Fig. 3). MAdCAM-1 R39A and S44A mutants were chosen for studies on low-affinity αβ7 in Ca^{2+} + Mg^{2+} and high-affinity αβ7, activated by talin or SDF-1α because they were the only CC’ loop mutants

![Figure 3](image-url)

**Distinct Functions of Two Loops in MAdCAM-1 D1 and D2**

- **A**: 1 mM Ca^{2+} + 1 mM Mg^{2+}
- **B**: 0.5 mM Mn^{2+}
- **C**: Talin
- **D**: SDF-1α

The figure shows the effects of shear stress on cell adhesion to WT MAdCAM-1 and mutant MAdCAM-1, with the number of cells remaining bound at each shear stress as a percentage of total bound cells determined before transfection with GFP-talin-head (C) or SDF-1α (D). Error bars are ± S.D. (n = 3).
Distinct Functions of Two Loops in MAdCAM-1 D1 and D2

The Intact DE Loop Is Essential for MAdCAM-1 Binding to Low-affinity α4β7, but Not for Its Binding to High-affinity α4β7—Activated by Mn2+. To further define the different structural requirements for MAdCAM-1 binding to inactive and activated integrin α4β7, we generated a series of single amino acid substitutions and a partial deletion (ΔDE, from Glu-149 to Asp-158) of the DE loop in MAdCAM-1 D2. MAdCAM-1 with deletion of the whole DE loop (from Glu-149 to Asp-158) could not be expressed. All of the single amino acid substitutions by Ala in the DE loop (residue 149–158) decreased the cell adhesion mediated by both low-affinity and high-affinity α4β7 on MAdCAM-1, but to a different extent (Fig. 4A). For the rolling cell adhesion mediated by low-affinity α4β7 on MAdCAM-1 in 1 mM Ca2+ + 1 mM Mg2+, substitution of Glu-152 and Glu-153 by Ala led to an ∼70 and 60% decrease of adherent cells, respectively. E150A, E154A, and D158A showed less effect, which resulted in an ∼50% decrease (Fig. 4A). Other point mutations had even milder impact, retaining from 60 to 80% of adherent cells. For the firm cell adhesion mediated by high-affinity α4β7 in Mn2+, most DE loop point mutations showed much milder effects, except for Glu-150 and Glu-154, which led to 50 and 40% decrease of bound cells, respectively (Fig. 4B). Notably, the DE loop residues that mostly affected MAdCAM-1 interaction with low-affinity and high-affinity α4β7, are different, suggesting the different roles of the DE loop residues in the recognition of integrin α4β7 before and after activation. Different from single amino acid substitutions, the partial deletion of the DE loop abolished the interaction between low-affinity α4β7 and MAdCAM-1, but only caused about 60% loss of cell adhesion to MAdCAM-1 mediated by the Mn2+-activated α4β7. The data demonstrate that the intact DE loop is essential to MAdCAM-1 binding to low-affinity α4β7, but not to high-affinity α4β7 activated by Mn2+.

The Intact DE Loop Is Required for MAdCAM-1 Binding to Integrin α4β7 Activated by Talin and SDF-1α—To further study the function of the DE loop in MACAM-1 binding to activated α4β7, we examined the impact of DE loop deletion on the interaction between MAdCAM-1 and α4β7, activated by talin and SDF-1α. Opposite to the partial rescued cell adhesion to ΔDE MAdCAM-1 after α4β7 was activated by Mn2+, the activation of α4β7 by either talin or SDF-1α did not rescue the abolishment of cell adhesion by DE loop deletion in 1 mM Ca2+ + 1 mM Mg2+ (Fig. 5). Thus, the intact DE loop is important for MAdCAM-1 interaction with both low-affinity and high-affinity integrin α4β7 activated by talin or SDF-1α. On the other hand, α4β7 activated by Mn2+ could support decent cell adhesion to MAdCAM-1 in the absence of the intact DE loop, suggesting that the conformation of Mn2+-activated α4β7 may be different from those of the low-affinity and high-affinity α4β7 activated by more physiological stimuli. The overexpression of GFP-talin-head augmented the firm adhesion of α4β7, 293T transfectants on both WT and DE loop single residue mutant MAdCAM-1 (Fig. 5A). In contrast, SDF-1α stimulation increased the PBL adhesion only to the WT MAdCAM-1, but not to the DE loop mutants (Fig. 5B). These data suggest that the residues in the DE loop might be involved in distinguishing...
the subtle difference between α₄β₇ activated by talin and α₄β₇ activated by SDF-1α.

The DE Loop Is Required for the Stable Interaction between MAdCAM-1 and Low-affinity Integrin α₄β₇—Next, we investigated the function of the DE loop in the strength of α₄β₇-mediated cell adhesion to MAdCAM-1 (Fig. 6). In 1 mM Ca²⁺ + 1 mM Mg²⁺, the DE loop mutations significantly decreased the shear resistance of adherent cells bearing low-affinity α₄β₇ (Fig. 6A). In contrast, the same mutations showed little effect on the stability of adhesion mediated by high-affinity α₄β₇ activated by Mn²⁺, talin, or SDF-1α (Fig. 6, B–D). Thus, the residues in the DE loop of MAdCAM-1 are important for stabilizing the interaction between low-affinity α₄β₇ and MAdCAM-1.

**FIGURE 6.** Effect of the DE loop on the strength of α₄β₇-mediated adhesion to MAdCAM-1. A–C, resistance to detachment of α₄β₇ 293T transfectants at increasing wall shear stresses in 1 mM Ca²⁺ + 1 mM Mg²⁺ (A), in 0.5 mM Mn²⁺ (B), or in 1 mM Ca²⁺ + 1 mM Mg²⁺ after transfection with GFP-talin-head (C). D, resistance to detachment of human PBL stimulated with SDF-1α. The total number of cells remaining rolling and firmly adherent at increasing wall shear stress was determined as a percentage of adherent cells at 1 dyne/cm². The experiment was performed on the surface coated with WT or mutant MAdCAM-1 (10 μg/ml). Error bars are ± S.D. (n = 3).

**FIGURE 7.** Schematic illustration of distinct interactions between MAdCAM-1 and integrin α₄β₇ at different activation states. Integrin α₄β₇ at different activation states has distinct binding interfaces for the MAdCAM-1 CC’ and DE loops. A, binding of MAdCAM-1 to low-affinity (inactive) integrin α₄β₇. B, binding of MAdCAM-1 to high-affinity integrin α₄β₇ activated by physiological stimuli (talin or SDF-1α). C, binding of MAdCAM-1 to high-affinity integrin α₄β₇ activated by Mn²⁺.

**DISCUSSION**

Lymphocyte homing to gut is dependent on the interaction between integrin α₄β₇ and MAdCAM-1. The resting (low-affinity) and activated (high-affinity) integrin α₄β₇ can mediate rolling and firm adhesion of lymphocytes, respectively, which are two of the critical steps in lymphocyte homing. Previous studies have shown that integrin undergoes global and local conformational changes upon activation, resulting in the distinct conformations of low-affinity and high-affinity integrins. Thus, it is tempting to speculate that the low-affinity and high-affinity α₄β₇ binds MAdCAM-1 differently, which might play a fundamental role in supporting the rolling and firm cell adhesion. The integrin α₄β₇-MAdCAM-1 interaction is dependent on a conserved acidic peptide motif in the first Ig-like domain of MAdCAM-1, which is present as a surface-exposed structure. The Asp-42 in this motif forms the primary interaction with the divalent cation at β₇ MIDAS. Because the primary interaction between Asp-42 and the MIDAS metal ion is shared by both low-affinity and high-affinity α₄β₇-MAdCAM-1 binding, there should be other interactions between MAdCAM-1 and α₄β₇ that determine rolling or firm adhesion.

Although previous studies have revealed that some residues in MAdCAM-1 are important for MAdCAM-1-α₄β₇ binding (9, 12, 13, 15), the structural basis for supporting MAdCAM-1-α₄β₇-mediated rolling and firm cell adhesion remains elusive because the static cell adhesion assay used in those studies is unable to distinguish rolling and firm cell adhesion. In this study, we used a flow chamber assay to screen the critical residues in MAdCAM-1, which are important for supporting rolling and firm cell adhesions, respectively. Our results demonstrate that the CC’ and DE loops play distinct roles in the recognition of MAdCAM-1 by low- and high-affinity α₄β₇, and suggest that the inactive α₄β₇ and α₄β₇ activated by different stimuli have distinct conformations with different structural requirements for MAdCAM-1 binding (Fig. 7).

The Asp-42 in the CC’ loop is required for both low-affinity α₄β₇-mediated rolling adhesion and high-affinity α₄β₇-mediated firm adhesion. In addition to Asp-42, most other CC’ loop residues other than Arg-39 and Ser-44 are essential for the low-affinity α₄β₇-MAdCAM-1 interaction, suggesting the potential binding sites at the CC’ loop for low-affinity α₄β₇. In contrast, the same CC’ loop mutations only slightly decreased cell adhesion mediated by Mn²⁺-activated α₄β₇, suggesting the different inter-
actions between MAdCAM-1 and Mn2+-activated integrin other than with low-affinity integrin. The reasons that make the CC’ loop subsidiary in supporting αβ2-MAdCAM-1 binding could be due to the stronger interaction between Asp-42 and Mn2+ at MIDAS of β2, and/or there could be additional interactions formed between Mn2+-activated αβ2 and MAdCAM-1.

The DE loop in D2 of MAdCAM-1 is another αβ2 binding interface. Our results showed that the DE loop was less important than the CC’ loop in supporting MAdCAM-1 binding to low-affinity αβ2. Only the removal of the DE loop abolished the interaction between MAdCAM-1 and low-affinity αβ2. Thus, the major function of the DE loop could be to stabilize the interaction between MAdCAM-1 and αβ2, especially the low-affinity integrin. The DE loop is a long and flexible loop exposed on the MAdCAM-1 surface, and its orientation and conformation should be crucial to the MAdCAM-1-αβ2 interaction. Among all of the DE loop single amino acid substitution mutations tested, E152A resulted in the maximal decrease of MAdCAM-1 binding to the low-affinity or talin or SDF-1α-activated αβ2. The MAdCAM-1 crystal structure revealed that the side chain of Glu-152 faces inside the DE loop and protrudes to Glu-158 (9, 10). The Glu-152 side-chain oxygen could form a hydrogen bond with the Asp-158 main-chain oxygen, which might be important to maintain the proper conformation of the DE loop. E152A mutation could lead to loss of this hydrogen bond, which disrupts the optimal conformation of the DE loop for low-affinity αβ2 binding.

Asp-150 is another important residue in the DE loop. E150A mutation decreased the firm cell adhesion mediated by Mn2+-activated CC’ binding, at least at the ligand-binding interface, than CC’ binding. Another notable finding of our study is the distinct structural requirements for MAdCAM-1 binding to αβ2, activated by Mn2+ and more physiological stimuli, such as talin and SDF-1α. In our study, we found that the CC’ loop and the intact DE loop were crucial for MAdCAM-1 binding to integrin αβ2, activated by talin and SDF-1α; however, the MAdCAM-1 binding to αβ2, activated by Mn2+ was mostly dependent on Asp-42 and only partially affected by some CC’ and DE loop mutations. Thus, the high-affinity αβ2, activated by Mn2+ should have distinct conformation, at least at the ligand-binding interface, than αβ2, activated by talin or SDF-1α through the inside-out signaling.

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REFERENCES
1. Springer, T. A. (1994) Cell 76, 301–314
2. Butcher, E. C. (1991) Cell 67, 1033–1036
3. Butcher, E. C., and Picker, L. J. (1996) Science 272, 60–66
4. Gorfu, G., Rivera-Nieves, J., and Ley, K. (2009) Curr. Mol. Med. 9, 836–850
5. Briskin, M. J., McEvoy, L. M., and Butcher, E. C. (1993) Nature 363, 461–464
6. Adams, D. H., and Eksteen, B. (2006) Nat. Rev. Immunol. 6, 244–251
7. Eksteen, B., Liaskou, E., and Adams, D. H. (2008) Inflamm. Bowel Dis. 14, 1298–1312
8. Shyjan, A. M., Bertagnolli, M., Kenney, C. J., and Briskin, M. J. (1996) J. Immunol. 156, 2851–2857
9. Green, N., Rosebrook, J., Cochran, N., Tan, K., Wang, J. H., Springer, T. A., and Briskin, M. J. (1999) Cell Adhes. Commun. 7, 167–181
10. Tan, K., Casasnovas, J. M., Liu, J. H., Briskin, M. J., Springer, T. A., and Wang, J. H. (1998) Structure 6, 793–801
11. Dando, J., Wilkinson, K. W., Ortlepp, S., King, D. J., and Brady, R. L. (2002) Acta Crystallogr. D Biol. Crystallogr. 58, 233–241
12. Viney, J. L., Jones, S., Chiu, H. H., Lagrimas, B., Renz, M. E., Presta, L. G., Jackson, D., Hillan, K. J., Lew, S., and Fong, S. (1996) J. Immunol. 157, 2488–2497
13. Newman, P., Craig, S. E., Seddon, G. N., Schofield, N. R., Rees, A., Edwards, R. M., Jones, E. Y., and Humphries, M. J. (1997) J. Biol. Chem. 272, 19429–19440
14. Chen, J., Salas, A., and Springer, T. A. (2003) Nat. Struct. Biol. 10, 995–1001
15. Briskin, M. J., Rott, L., and Butcher, E. C. (1996) J. Immunol. 156, 719–726
16. Hynes, R. O. (2002) Cell 110, 673–687
17. Luo, B. H., Carman, C. V., and Springer, T. A. (2007) Annu. Rev. Immunol. 25, 619–647
18. Springer, T. A., Wang, J. H., and Tidswell, M. (2004) Adv. Protein Chem. 68, 29–63
19. Arnaout, M. A., Goodman, S. L., and Xiong, J. P. (2007) Curr. Opin. Cell Biol. 19, 495–507
20. Xiao, T., Takagi, J., Coller, B. S., Wang, J. H., and Springer, T. A. (2004) Nature 432, 59–67
21. Luo, B. H., and Springer, T. A. (2006) Curr. Opin. Cell Biol. 18, 579–586
22. Arnaout, M. A., Mahalingam, B., and Xiong, J. P. (2005) Annu. Rev. Cell Dev. Biol. 21, 381–410
23. Carman, C. V., and Springer, T. A. (2003) Curr. Opin. Cell Biol. 15, 547–556
24. Leitinger, B., McDowall, A., Stanley, P., and Hogg, N. (2000) Biochim. Biophys. Acta 1498, 91–98
25. Chen, J., Takagi, J., Xie, C., Xiao, T., Luo, B. H., and Springer, T. A. (2004) J. Biol. Chem. 279, 55556–55561
26. de Château, M., Chen, S., Salas, A., and Springer, T. A. (2001) Biochemistry 40, 13972–13979
27. Zhu, J., Luo, B. H., Xiao, T., Zhang, C., Nishida, N., and Springer, T. A. (2008) Mol Cell 32, 849–861
28. Xiong, J. P., Mahalingham, B., Alonso, J. L., Borrelli, L. A., Rui, X., Anand, S., Hyman, B. T., Rysiosk, T., Müller-Pomplaaa, D., Goodman, S. L., and Arnaout, M. A. (2009) J. Cell Biol. 186, 589–600
29. Lee, J. O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) Cell 80, 631–638
30. Chen, J., Yang, W., Kim, M., Carman, C. V., and Springer, T. A. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 13062–13067
31. Valdravidou, D., Humphries, M. J., and Mould, A. P. (2008) J. Biol. Chem. 283, 32704–32714
32. Pan, Y., Zhang, K., Qi, J., Yue, J., Springer, T. A., and Chen, J. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 21388–21393
33. Tidswell, M., Pachynski, R., Wu, S. W., Qiu, S. Q., Dunham, E., Cochran, N., Briskin, M. J., Kishaw, P. J., Lazarovits, A. L., Andrew, D. P., Butcher, E. C., Vednocks, T., and Erle, D. J. (1997) J. Immunol. 159, 1497–1505
34. Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O., and Ginsberg, M. H. (1999) J. Biol. Chem. 274, 28071–28074
35. Kunkel, E. J., and Butcher, E. C. (2002) Immunity 16, 1–4
36. Wright, N., Hidalgo, A., Rodríguez-Frade, J. M., Soriano, S. F., Mellado, M., Parro-Cañás, M., Briskin, M. J., and Teixidó, J. (2002) J. Immunol. 168, 5268–5277
37. Erle, D. J., Briskin, M. J., Butcher, E. C., García-Pardo, A., Lazarovits, A. L., and Tidswell, M. (1994) J. Immunol. 153, 517–528
38. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A., and Mackay, C. R. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 1925–1930