The Relative Influence of Metal Ion Binding Sites in the I-like Domain and the Interface with the Hybrid Domain on Rolling and Firm Adhesion by Integrin α₄β₇

Received for publication, July 9, 2004, and in revised form, September 9, 2004

Published, JBC Papers in Press, September 24, 2004, DOI 10.1074/jbc.M407773200

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We examined the effect of conformational change at the β₇ I-like/hybrid domain interface on regulating the transition between rolling and firm adhesion by integrin α₄β₇. An N-glycosylation site was introduced into the I-like/hybrid domain interface to act as a wedge and to stabilize the open conformation of this interface and hence the open conformation of the α₄β₇ headpiece. Wild-type α₄β₇ mediates rolling adhesion in Ca²⁺ and Ca²⁺/Mg²⁺ but firm adhesion in Mg²⁺ and Mn²⁺. Stabilizing the open headpiece resulted in firm adhesion in all divalent cations. The interaction between metal binding sites in the I-like domain and the interface with the hybrid domain was examined in double mutants. Changes at these two sites can either counterbalance one another or be additive, emphasizing mutuality and the importance of multiple interfaces in integrin regulation. A double mutant with countervailing mutations could still be activated by Mn²⁺, confirming the importance of the adjacent to metal ion-dependent adhesion site (LIMBS) and activating wedge mutations could not do so. This conversion from the closed to the open conformation of the ligand-binding domains in the integrin headpiece also destabilizes the bent conformation and induces integrin extension in which the headpiece extends and breaks free from an interface with the leg domains that connect it to the plasma membrane. To stabilize the outward swing of the hybrid domain and the high affinity open headpiece conformation, glycans have been introduced into the interface between the hybrid and I-like domains of β₇ and β₇ integrins (9). The relation between hybrid domain swing-out and high integrin affinity has also been strongly supported by the study of an allosteric inhibitory β₇ integrin antibody SG19, which binds to the outer side of the I-like hybrid domain interface and prevents hybrid domain swing-out as shown by electron micrographic image averages (10). Allosteric inhibition by this mAb was confirmed because it did not inhibit ligand binding to the low affinity state but rather inhibited conversion to the high affinity state. Binding of SG19 mAb to the β₇ wedge mutant was dramatically decreased compared with wild-type, further supporting induction of hybrid domain swing-out by the wedge mutant. Conversely, allosteric activating mAbs have been shown to map to the face of the β hybrid domain that is closely opposed to the α subunit in the closed conformation and therefore appear to induce the high affinity state by favoring hybrid domain swing-out (11). Disulfide cross-links in the β₇-α₇ loop (12) and shortening of the α₇-helix in the I-like domain (13) also support the conclusion that downward displacement of the α₇-helix induces high affinity for ligand. A homologous α₇-helix displacement in integrin α subunit I domains similarly induces high affinity for ligand (14).

Integrins are a family of heterodimeric adhesion molecules with noncovalently associated α and β subunits that mediate cell-cell, cell-matrix, and cell-pathogen interactions and that signal bidirectionally across the plasma membrane (1, 2). The affinity of integrin extracellular domains is dynamically regulated by "inside-out" signals from the cytoplasm. Furthermore, ligand binding can induce "outside-in" signaling and activate many intracellular signaling pathways (3–6). Integrin extracellular domains exist in at least three distinct global conformational states that differ in affinity for ligand (5, 7); the cellular domains exist in at least three distinct global conformational states that differ in affinity for ligand (5, 7); the equilibrium among these different states is regulated by the binding of integrin cytoplasmic domains to cytoskeletal components and signaling molecules (4, 6).

Integrin affinity regulation is accompanied by a series of conformational rearrangements. Electron micrographic studies of integrins α₅β₃ and α₆β₄ demonstrate that ligand binding, in the absence of restraining crystal lattice contacts, induces a switchblade-like extension of the extracellular domain and a change in angle between the I-like and hybrid domains (5, 7). Recent crystal structures of integrin α₁β₃ in the open, high affinity conformation demonstrate that the C-terminal a₇-helix of the β I-like domain moves axially toward the hybrid domain, causing the β hybrid domain to swing outward by 60° (away from the α subunit) (8). This conversion from the closed to the open conformation of the ligand-binding domains in the integrin headpiece also destabilizes the bent conformation and induces integrin extension in which the headpiece extends and breaks free from an interface with the leg domains that connect it to the plasma membrane. To stabilize the outward swing of the hybrid domain and the high affinity open headpiece conformation, glycans have been introduced into the interface between the hybrid and I-like domains of β₇ and β₇ integrins (9). The relation between hybrid domain swing-out and high integrin affinity has also been strongly supported by the study of an allosteric inhibitory β₇ integrin antibody SG19, which binds to the outer side of the I-like hybrid domain interface and prevents hybrid domain swing-out as shown by electron micrographic image averages (10). Allosteric inhibition by this mAb was confirmed because it did not inhibit ligand binding to the low affinity state but rather inhibited conversion to the high affinity state. Binding of SG19 mAb to the β₇ wedge mutant was dramatically decreased compared with wild-type, further supporting induction of hybrid domain swing-out by the wedge mutant. Conversely, allosteric activating mAbs have been shown to map to the face of the β hybrid domain that is closely opposed to the α subunit in the closed conformation and therefore appear to induce the high affinity state by favoring hybrid domain swing-out (11). Disulfide cross-links in the β₇-α₇ loop (12) and shortening of the α₇-helix in the I-like domain (13) also support the conclusion that downward displacement of the α₇-helix induces high affinity for ligand. A homologous α₇-helix displacement in integrin α subunit I domains similarly induces high affinity for ligand (14).

It has long been known that integrin affinity for ligand is strongly influenced by metal ions, and recently the basis for this regulation has been deduced for the integrin α₄β₇ (15). The integrin α₄β₇ binds the cell surface ligand mucosal cell adhesion molecule-1 (MAdCAM-1) and mediates rolling adhesion by...
lymphocytes in postcapillary venules in mucosal tissues and the subsequent firm adhesion in endothelium and trans-endothelial migration. These key steps in lymphocyte trafficking involve the subsequent firm adhesion in endothelium and trans-endothelial migration. These key steps in lymphocyte trafficking involve the subsequent firm adhesion in endothelium and trans-endothelial migration.

Despite these advances in understanding the mechanism by which metal ions stabilize alternative conformations of integrin β1-like domains, several issues remain unresolved. How do the closed and open conformations of the αβ2 headpiece affect rolling and firm adhesion? Does metal ion occupancy at the MIDAMIDS and ADMIDAS or outward swing of the hybrid domain have the strongest effect on I-like domain conformation? If changes occur at both metal binding sites and the I-like/hybrid domain interface, does one dominate the other, or can they be counterbalancing or additive? Here we address these questions and the importance of allostery at the I-like/hybrid domain interface by introducing a glycan wedge mutation into the β2 subunit to stabilize the open conformation of this interface.

**MATERIALS AND METHODS**

**Monoclonal Antibodies**—The human integrin αβ2-specific monoclonal antibody Act-1 was described previously (20, 21). cDNA Construction, Transient Transfection, and Immunoprecipitation—The β2 site-directed mutations were generated by using QuikChange (Stratagene). Wild-type human β2 cDNA (22) in vector pcDNA3.1/Hygro+ (Invitrogen) was used as the template. All mutations were confirmed by DNA sequencing. Transient transfection of 293T cells using calcium phosphate precipitation was as described (23). Transfected 293T cells were metabolically labeled with [35S]cysteine and -methionine, and labeled cell lysates were immunoprecipitated with 1 μl of Act-1 mAb ascites and 20 μl of protein G agarose, eluted with 0.5% SDS, and subjected to non-reducing 7% SDS-PAGE and fluorography (24). The selected protein bands were quantified using a Storm PhosphorImager after 3 h of exposure to storage phosphor screens (Amersham Biosciences).

**Immunofluorescence Flow Cytometry**—Immunofluorescence flow cytometry was as described (23) using 10 μg/ml purified antibody.

**Flow Chamber Assay**—A polystyrene Petri dish was coated with a 5-mm diameter, 20-μl spot of 5 μg/ml purified h-MAdCAM-1/Fc in coating buffer (phosphate-buffered saline, 10 mM NaHCO3, pH 9.0) for 1 h at 37 °C followed by 2% human serum albumin in coating buffer for 1 h at 37 °C to block nonspecific binding sites (16). The dish was assembled as the lower wall of a parallel plate flow chamber and mounted on the stage of an inverted phase-contrast microscope (25).

293T cell transfectants were washed twice with Ca2+- and Mg2+-free Hanks’ balanced salt solution, 10 mM Hepes, pH 7.4, 5 mM EDTA, 0.5% bovine serum albumin and resuspended at 5 × 106/ml in buffer A (Ca2+-

**Table I**

<table>
<thead>
<tr>
<th>Name</th>
<th>Mutation</th>
<th>Expression</th>
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<tbody>
<tr>
<td>Wild type</td>
<td></td>
<td>% WT</td>
</tr>
<tr>
<td>Glycan wedge</td>
<td>Q324T</td>
<td>100 ± 12</td>
</tr>
<tr>
<td>LIMBS</td>
<td>D237A</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Wedge/LIMBS</td>
<td>Q324T/D237A</td>
<td>99 ± 20</td>
</tr>
<tr>
<td>ADMIDAS</td>
<td>D147A</td>
<td>38 ± 10</td>
</tr>
<tr>
<td>Wedge/ADMIDAS</td>
<td>Q324T/D147A</td>
<td>70 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35 ± 8</td>
</tr>
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Integrin αβ2 cell surface expression in 293T transient transfectants was determined with Act-1 mAb and immunofluorescence flow cytometry. The data are mean specific fluorescence intensity as percent of wild type (WT) ± difference from the mean for two independent experiments.
and Mg\(^{2+}\)-free Hanks’ balanced salt solution, 10 mM Hepes, and 0.5% bovine serum albumin) and kept at room temperature. Cells were diluted to 1 × 10⁶/ml in buffer A containing different divalent cations immediately before infusion in the flow chamber using a syringe pump.

Cells were allowed to accumulate for 30 s at 0.3 dyne cm⁻². Then, shear stress was increased every 10 s from 1 up to 32 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 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 (26). Microscopic images were recorded on Hi8 videotape for later analysis.

Surface Calculations—Accessible surfaces were calculated with probes of the indicated radii using the buried surface routine of Crys-7 with a Glycan Wedge Mutation—

TABLE II

<table>
<thead>
<tr>
<th>Radioactivity in each α₄ subunit band</th>
<th>Cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>α₄/180</td>
<td>α₄/150</td>
</tr>
<tr>
<td>WT</td>
<td>10</td>
</tr>
<tr>
<td>Q324T</td>
<td>2</td>
</tr>
</tbody>
</table>

Fig. 2. Adhesion in shear flow of wild-type and glycan wedge mutant α₄β₇ cell transfectants on MAdCAM-1 substrates. Cells were infused into the flow chamber in buffer containing 1 mM Ca\(^{2+}\), 1 mM Ca\(^{2+}\) + 1 mM Mg\(^{2+}\), 1 mM Mg\(^{2+}\), or 0.5 mM Mn\(^{2+}\). Cells transfected with α₄ cDNA alone (Mock) or α₄ transfectants treated with 5 mM EDTA did not accumulate on MAdCAM-1 substrates. Rolling velocities of individual cells were measured at a series of increasing wall shear stresses, and cells within a given velocity range were enumerated to give the population distribution. dyn, dynes.
ness was more activated than in Mg²⁺ because more cells accumulated and fewer cells detached at the highest wall shear stress of 32 dynes/cm². By contrast with wild type, the α₄β₇ Q324T glycan wedge mutant mediated firm adhesion regardless of the divalent cation present (Fig. 2). Furthermore, the accumulation efficiency and shear resistance of the wedge mutant was identical in Ca²⁺, Ca²⁺/Mg²⁺, Mg²⁺, and Mn²⁺ and similar to that of the wild-type α₄β₇ 293T transfectants in Mn²⁺. Thus, integrin α₄β₇ was constitutively activated by the glycan wedge introduced into the hybrid/I-like domain interface.

Mutation of the α₄ cleavage site residue Arg-558 abolishes α₄ subunit cleavage and has no effect on α₄β₇ adhesion on fibronectin or VCAM-1 (29, 35). We tested the effect of the same mutation in α₄β₇ transfectants, and we found it to have no effect on adhesion in shear flow to MadCAM-1 (data not shown).

As described previously (15), mutation of LIMBS residues stabilizes integrin α₄β₇ in the low affinity state. For example, the LIMBS mutant D237A mediates rolling adhesion regardless of the divalent cations that are present (Fig. 3A). The wedge/LIMBS double mutant (Q324T/D237A) was expressed as well as the wedge mutant in 293T transfectants (Table I). Compared with the LIMBS mutation, the wedge/LIMBS double mutation reproducibly increased the number of firmly adherent cells at low shear (1 and 2 dynes cm⁻²) in Ca²⁺, Ca²⁺/Mg²⁺, and Mg²⁺ (Fig. 3). In Mn²⁺, the wedge/LIMBS mutant mediated firm adhesion, whereas the LIMBS mutant mediated rolling adhesion (Fig. 3). These data show that the LIMBS is required for full activation by the wedge mutation in Ca²⁺, Ca²⁺/Mg²⁺,
Conversion of Rolling to Firm Adhesion

**Fig. 4. Interaction of glycan wedge and ADMIDAS mutations.** Adhesive modality and resistance to detachment in shear flow of ADMIDAS (D147A) and double wedge/ADMIDAS (Q324T/D147A) mutant α7β1 transfectants on the MAdCAM-1 substrates in the presence of the indicated divalent cations. The divalent cation concentrations are the same as in Fig. 2, dyn, dynes.

and Mg\(^{2+}\) (Q324T/D237A mutant in Fig. 3A compared with Q324T mutant in Fig. 2). Furthermore, activation by Mn\(^{2+}\) of the double wedge/LIMBS Q324T/D237A mutant definitively establishes that the LIMBS is not required for activation by Mn\(^{2+}\).

*Increased Firm Adhesion by Double ADMIDAS/Wedge Mutant*—Mutation of the negative regulatory ADMIDAS activates firm adhesion even in Ca\(^{2+}\) (15) (D147A mutant in Fig. 4 compared with wild type in Fig. 2). The double wedge/ADMIDAS Q324T/D147A mutant was somewhat less well expressed than the ADMIDAS D147A mutant (Table I). Nonetheless, the double Q324T/D147A mutant showed more firmly adherent cells in Ca\(^{2+}\) and Mn\(^{2+}\) than did the single D147A mutant (Fig. 4) or the single Q324T mutant (Fig. 2).

**DISCUSSION**

Allosteric transition to the high affinity integrin headpiece conformation is proposed to involve rearrangement of the β I-like LIMBS, MIDAS, and ADMIDAS (LMA) sites, downward displacement of the β I-like α7-helix that connects to the hybrid domain, and outward swing of the β hybrid domain (5, 7–9, 11, 12, 36). Outward swing of the hybrid domain has been demonstrated by electron microscopic studies of liganded αβ integrins and crystal studies of liganded αβ subunits, but not in an αβ crystal structure in which crystal lattice and head-piece-leg interactions presumably prevented swing-out when a ligand was soaked into crystals (8, 18). Conversely, introduction of a glycan wedge into the β1 and β3 subunits has been demonstrated to induce high affinity for ligand by αβ integrins and αβ integrins (9). Both of these integrins recognize ligands with RGD sequences. We have extended these results here to the αβ integrin, which does not recognize RGD in its ligands and which mediates both rolling and firm adhesion. In αβ, the glycan wedge converted rolling adhesion in Ca\(^{2+}\) and Ca\(^{2+}\)/Mg\(^{2+}\) to firm adhesion, demonstrating that stabilizing the open conformation at the I-like/hybrid domain interface is sufficient to stabilize high affinity firm adhesion.

Furthermore, we examined here for the first time the interplay between the LMA metal binding sites at the ligand-binding interface on the “top” of the β I-like domain and the interface with the hybrid domain on the opposite, “bottom” face of the I-like domain. We asked whether one of these two interfaces would dominate regulation of rolling or firm adhesion, or whether there would be mutuality in which mutations in each of these interfaces influenced the equilibrium between rolling and firm adhesion. The results demonstrate the latter. That is, stabilization of rolling adhesion by LIMBS mutation was partially counteracted by the wedge mutation in Ca\(^{2+}\)/Mg\(^{2+}\) and Mg\(^{2+}\) and fully counteracted in Mn\(^{2+}\), where firm adhesion occurred. Conversely, stabilization of firm adhesion by the wedge mutation was fully counteracted by the LIMBS mutation in Ca\(^{2+}\), where rolling occurred, and largely counteracted in Ca\(^{2+}\)/Mg\(^{2+}\) and Mg\(^{2+}\). Therefore, the equilibrium at the LMA sites strongly influences that at the β I-like/hybrid domain interface and vice versa, and changes in equilibrium at one site can counterbalance those at the other. The combined effects of the ADMIDAS and wedge mutations also demonstrated additive effects at the LMA sites and I-like/hybrid interface because changes at both of these sites stabilized firm adhesion more strongly than changes at either alone.

Another notable finding of these studies is that Mn\(^{2+}\) can still activate firm adhesion when the LIMBS is mutated. Previously, the LIMBS and ADMIDAS were found to be positive and negative regulatory sites, respectively, and positive regulation by low Ca\(^{2+}\) concentrations was found to be intact when the ADMIDAS was mutated (15). This, together with structural considerations, suggested that negative regulation by high Ca\(^{2+}\) concentrations was effected at the ADMIDAS. Scatchard plots showed competitive rather than noncompetitive inhibition by Ca\(^{2+}\) of stimulation by Mn\(^{2+}\), suggesting that the ADMIDAS was also the stimulatory site for Mn\(^{2+}\). However, it was not possible to confirm the role of the ADMIDAS in stimulation of firm adhesion by Mn\(^{2+}\) because rolling adhesion occurred in LIMBS mutants even in Mn\(^{2+}\). By contrast, in the double LIMBS/wedge mutant, the equilibrium between rolling and firm adhesion is not far from that in wild type, and it is regulated by divalent cations. Mn\(^{2+}\) was found to fully activate...
firm adhesion by the LIMBS/wedge mutant, showing that the LIMBS is not required for regulation by Mn$^{2+}$ and providing strong support for the previous conclusion that the ADMIDAS is the site for activation by Mn$^{2+}$.

Although much progress has been made recently in defining different integrin conformational states, questions remain about how signals are transduced from the cytoplasm to the ligand binding site and whether intermediate conformational states have intermediate affinity for ligand. It appears that to mediate rolling adhesion, integrins must be in one of the extended conformations rather than in the bent conformation (15, 37). The extended conformation with the closed headpiece is an intermediate in the conformational pathway between the bent conformation, which contains a closed headpiece, and the extended conformation with the open headpiece (5). The current study demonstrates that stabilization of the open headpiece by a glycan wedge at the β1-like/hybrid interface is sufficient to convert low affinity rolling adhesion to high affinity firm adhesion. It appears that the glycan wedge converts the extended conformation with the closed headpiece to the extended conformation with the open headpiece. Therefore, this study strongly suggests that within the extended integrin conformation, conversion of the closed to the open headpiece is sufficient to convert rolling adhesion to firm adhesion. In an intact integrin, marked separation in the plane of the membrane of the transmembrane domains of the integrin α and β subunits would also stabilize the open headpiece and therefore may be the mechanism for converting rolling adhesion to firm adhesion.

Acknowledgment—We thank Dr. Michael J. Briskin for providing the human MadCAM-1/Fc.

REFERENCES