Transition from Rolling to Firm Adhesion Can Be Mimicked by Extension of Integrin $\alpha_L\beta_2$ in an Intermediate Affinity State

Azucena Salas$^1$, Motomu Shimaoka, Uyen Phan, Minsoo Kim$^2$, and Timothy A. Springer$^3$

From the CBR Institute for Biomedical Research, Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

$\alpha_L\beta_2$ affinity for intercellular adhesion molecule-1 (ICAM-1) is regulated by the conformation of the $\alpha_L$ I domain, which is in turn controlled by the conformation and orientation of other adjacent domains. Additionally, overall integrin conformation (bent versus straightened) influences the orientation of the I domain and access to its ligands, influencing adhesive efficiency. The open or high affinity I domain conformation supports strong adhesion, whereas the closed, low affinity conformation mediates weak interactions or rolling. We have previously suggested that $\alpha_L\beta_2$ can also exist on the cell surface in an intermediate affinity state. Here we have studied the adhesive properties of integrin $\alpha_L\beta_2$ containing mutant I domains with intermediate affinities for ICAM-1. In an overall bent conformation, the intermediate affinity state of $\alpha_L\beta_2$ is hardly detected by conventional adhesion assays, but robust adhesion is seen when an extended conformation is induced by a small molecule $\alpha/\beta$ I allosteric antagonist. Intermediate affinity $\alpha_L\beta_2$ supports more stable rolling than wild-type $\alpha_L\beta_2$ under shear conditions. Moreover, antagonist-induced extension transforms rolling adhesion into firm adhesion in a manner reminiscent of chemokine activation of integrin $\alpha_L\beta_2$. These findings suggest the relevance of intermediate affinity states of $\alpha_L\beta_2$ to the transition between inactive and active states and demonstrate the importance of both I domain affinity and overall integrin conformation for cell adhesion.

Integrins are cell membrane proteins that integrate the extracellular and intracellular compartments by binding to ligands on other cells or on the extracellular matrix. Integrins contain two noncovalently associated, transmembrane glycoprotein $\alpha$ and $\beta$ subunits. A globular headpiece binds ligand, and two long leg regions connect the ligand binding headpiece to the transmembrane and C-terminal cytoplasmic domains. Half of integrin $\alpha$ subunits, including $\alpha_L$, contain a domain of ~200 amino acids, known as an inserted (I) or von Willebrand factor A domain that contains the major ligand binding site. Integrin $\alpha_L\beta_2$ also known as leukocyte function-associated antigen-1 (LFA-1), is expressed on leukocytes and participates in leukocyte trafficking in inflammation, lymphocyte homing, and T lymphocyte interactions with antigen-presenting cells in immune reactions.

Received for publication, November 21, 2005, and in revised form, February 22, 2006. Published, JBC Papers in Press, February 27, 2006, DOI 10.1074/jbc.M512472200

© 2006 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
the intermediate affinity state, integrin extension induces transition from rolling to firm adhesion in a way that is reminiscent of in situ activation by chemokines (10). Our results demonstrate that an intermediate affinity I domain, when in the context of extended α and β subunits, is sufficient to support cell arrest and firm adhesion on ICAM-1 surfaces.

EXPERIMENTAL PROCEDURES

Cell Lines, Antibodies, and Small Molecule Inhibitors—Overlap extension PCR was used to generate cysteine substitution mutations in intact αL, as described previously (3). K562 cells were stably transfected with K160C/F299C-αL, K160C/T300C-αL, L161C/F299C-αL, or L161C/T300C-αL in pcDNA3.1(hygro) together with wild-type β2 and then selected in 200 μg/ml hygromycin. The 1% highest expressing cells were sorted by immunofluorescence flow cytometry into 96-well plates at −1 cell/well. Wells with an outgrowth of a single colony were restreaked by immunofluorescence, and clones with similar expression to wild-type K562 cells were maintained in medium containing 200 μg/ml hygromycin. For each mutant, all results shown are for the same clone, and only one clone was tested. Similar αL β2 surface expression as wild-type was selected and routinely verified by immunofluorescence flow cytometry. K562 cells stably transfected with wild-type αL β2 or K287C/K294C-αL/β2 (high affinity) in pEF(puro) were as described previously (3). K562 transfecteds were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin/streptomycin, and 3 μg/ml puromycin (wild-type αL β2 and high affinity αL β2) or 200 μg/ml hygromycin for the rest of the mutants. A non-binding mouse IgG1 (X63) as control, an anti-human αL domain mAb (TS1/22), an αL β2-propeller domain mAb (TS2/4), and an anti-β2 I-like domain mAb (TS1/18) were used to determine surface expression of the transfecteds by immunofluorescence flow cytometry or to specifically block αL β2 surface expression as wild-type was selected and routinely verified by immunofluorescence flow cytometry. K562 cells stably transfected with wild-type αL β2 or K287C/K294C-αL/β2 (high affinity) in pEF(puro) were as described previously (3). K562 transfecteds were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin/streptomycin, and 3 μg/ml puromycin (wild-type αL β2 and high affinity αL β2) or 200 μg/ml hygromycin for the rest of the mutants. A non-binding mouse IgG1 (X63) as control, an anti-human αL domain mAb (TS1/22), an αL β2-propeller domain mAb (TS2/4), and an anti-β2 I-like domain mAb (TS1/18) were used to determine surface expression of the transfecteds by immunofluorescence flow cytometry or to specifically block αL β2-ICAM-1 interactions (11, 12). CBR LFA-1/2 was employed to induce activation of αL β2 (11). Activation-dependent mAbs KIM127 (13) and m24 (4, 14) were kindly provided by Dr. M. Robinson (Celltech, Slough, UK) and Dr. N. Hogg (Imperial Cancer Research Fund, London, UK), respectively. XVA143 (15) was obtained from Dr. Paul Gillespie (Hoffman-La Roche, Nutley, NJ). BIRT377 (16) was from Dr. Terence Kelly (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgway, CT).

Soluble ICAM-1 Binding Assay—K562 transfecteds were washed with HBS (20 mM HEPES, 150 mM NaCl, pH 7.3) containing 5 mM EDTA and resuspended in HBS buffer containing the indicated concentration. To induce activation, wild-type αL β2 cells were preincubated with 10 μg/ml CBR LFA-1/2 for 15 min. In some experiments, cells were preincubated with 10 μg/ml of blocking mAb TS1/22 or isotype mouse IgG control X63 before the addition of ICAM-1. Binding of dimeric soluble ICAM-1 was assayed as follows. A chimera containing the five Ig domains of human ICAM-1 fused to the Fc portion of IgA (ICAM-1-Fca) (17) was added to the cells at 50 μg/ml and incubated at 37 °C for 30 min. To test the effect of XVA143 and BIRT377 on soluble ICAM-1-Fca binding to the K562 transfecteds, cells were preincubated with 0.001% Me2SO, 1 μM XVA143, or 10 μM BIRT377 for 10 min at 22 °C. The cells were washed and incubated with a 1:100 dilution of goat anti-human IgA-FITC (Zymed Laboratories Inc.) for 30 min at room temperature, washed, and analyzed by flow cytometry.

In another series of experiments, multimeric ICAM-1 complexes were prepared by mixing ICAM-1-Fca with affinity-purified goat anti-human IgA antibody labeled with FITC (1:10 w/w) and incubated at 22 °C for 30 min. The mixture (4 μl) was then added to 50 μl of transfected cells to yield final concentrations of 50 μg/ml anti-human IgA-FITC and 5 μg/ml ICAM-1-Fcα and incubated for 30 min at 22 °C. The cells were washed and subjected to immunofluorescence flow cytometry.

Cell Adhesion to Immobilized ICAM-1 under Shear Flow—Cell adhesion in a parallel wall flow chamber was quantified as described previously (9). A chimera containing the five immunoglobulin domains of human ICAM-1 fused to the Fc portion of IgG (ICAM-1-Fcy) was a kind gift from Dr. Lloyd Klickstein (Brigham and Women’s Hospital, Boston, MA). When indicated, Me2SO (0.001%) as control or XVA143 (1 μM) was added to the cells immediately before infusion into the flow chamber.

V-bottom Cell Adhesion Assay—Cell adhesion to a V-bottom-well plate was assayed as described previously (18). Briefly, V-bottom 96-well plates (Corning) were coated with affinity-purified human tonsil ICAM-1 or with bovine serum albumin at 4 °C overnight and then blocked with 2% bovine serum albumin for 1 h at 37 °C. Cells were labeled for 15 min at 37 °C with 2’7’-bis-(carboxyethyl)-5-(and -6)-carboxyfluorescein acetoxymethyl ester (Molecular Probes), washed, resuspended in L-15 medium/2.5% fetal calf serum (3 × 104 cells in 50 μl), and when indicated, incubated for 15 min with 1 mM MnCl2. The cells were added to the plates and immediately centrifuged at 200 × g for 15 min at room temperature.

Flat Bottom Cell Adhesion Assay—Binding of the transfecteds to immobilized ICAM-1 was as described previously (12). Briefly, cells and plates (96-well flat bottom plates; Corning) were prepared as described above. In some experiments, wild-type αL β2 transfecteds were preincubated with 10 μg/ml CBR LFA-1/2, Me2SO (0.001%), BIRT377 (10 μM), or XVA143 (1 μM) for 15 min. Cells were added to the plates in 100 μl of L15/2.5% fetal calf serum with the indicated agents. After incubation at 37 °C for 30 min, unbound cells were washed off using a plate washer and fluorescence read.

Statistical Analysis—Data were analyzed when indicated using Student’s unpaired t test. Values are reported as mean ± S.E. Statistical significance was defined as p < 0.05.

RESULTS

Characterization of Intermediate Affinity αL β2 Mutants and Adhesion to ICAM-1—Measurements of the solution affinity for ICAM-1 of different double cysteine mutants of the αL I domain (1, 2) show that they can be classified as low affinity (wild-type and K160C/T300C), intermediate affinity (L161C/T300C, K160C/F299, L161C/F299C), and high affinity (K287C/K294C) (Table 1). To correlate the solution affinity with adhesive behavior, the mutations were introduced into the full-length αL subunit. K562 cells were stably transfected with the double cysteine αL I domain substitution mutants together with wild-type β2. In preliminary experiments, two to three clonal cell lines expressing each mutant were tested in shear flow assays. No significant differences in rolling versus firm adhesion behavior were seen among clones of the same mutant. In all subsequent experiments, one clone for each mutant with the most similar expression to the wild-type αL β2 as revealed by staining with an anti-β2 β-propeller mAb TS2/4 (Fig. 1) and an anti-β2 I-like domain mAb TS1/18 (data not shown), was selected. The previously described high affinity (K287C/K294C) αL/β2 mutant (3) was expressed on transfecteds 50–70% as well as the others. Transfectants were tested for their ability to bind to immobilized human ICAM-1 in a flat bottom or a V-well assay. As previously described, adhesion of K562 transfecteds expressing wild-type αL β2 is hardly detectable in flat bottom adhesion assays when cells are incubated at room temperature (22 °C) (data not shown) (19), and <10% of
Integrin $\alpha_L\beta_2$ in an Intermediate Affinity State

**TABLE 1**

<table>
<thead>
<tr>
<th>Domain</th>
<th>$k_{\text{on}}^*$</th>
<th>$k_{\text{off}}^*$</th>
<th>$K_D^*$</th>
<th>Class</th>
<th>Phcotype under shear stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>$3.1 \pm 0.1$ s$^{-1} \times 10^{10}$</td>
<td>$4.6 \pm 0.36$ s$^{-1}$</td>
<td>$1500 \pm 200$ $\mu$M</td>
<td>Low</td>
<td>Rolling/Firm adhesion</td>
</tr>
<tr>
<td>K160C/T300C</td>
<td>$3.4 \pm 0.9$</td>
<td>$1.2 \pm 0.08$</td>
<td>$450 \pm 210$</td>
<td>Low</td>
<td>Rolling</td>
</tr>
<tr>
<td>L161C/T300C</td>
<td>$89 \pm 12$</td>
<td>$0.76 \pm 0.07$</td>
<td>$9.4 \pm 2.4$</td>
<td>Intermediate</td>
<td>Rolling/Firm adhesion</td>
</tr>
<tr>
<td>K160C/F299C</td>
<td>$103 \pm 15$</td>
<td>$0.77 \pm 0.07$</td>
<td>$8.4 \pm 2.4$</td>
<td>Intermediate</td>
<td>Rolling/Firm adhesion</td>
</tr>
<tr>
<td>L161C/F299C</td>
<td>$133 \pm 10$</td>
<td>$0.43 \pm 0.07$</td>
<td>$3.0 \pm 0.44$</td>
<td>Intermediate</td>
<td>Rolling/Firm adhesion</td>
</tr>
<tr>
<td>K287C/K294C</td>
<td>$115 \pm 7$</td>
<td>$0.014 \pm 0.001$</td>
<td>$0.15 \pm 0.016$</td>
<td>High</td>
<td>Firm adhesion</td>
</tr>
</tbody>
</table>

* Determined using $\alpha_L$ domains with immobilized ICAM-1 in the presence of Mg$^{2+}$ with surface plasmon resonance (1).

**FIGURE 1.** Exposure of epitopes in the presence or absence of an allosteric antagonist. K562 cells transfected with the indicated $\alpha_L\beta_2$ mutants were incubated with 0.001% Me2SO as control (white histogram) or 1 $\mu$M of the allosteric antagonist XVA143 (gray histogram). Mouse IgG control, TS2/4 mAb to a constitutive $\alpha_L$-propeller domain epitope, and KIM127 mAb to an activation-dependent $\beta_2$ I domain epitope, and KIM127 mAb to an activation-dependent epitope in the $\beta_2$ integrin epidermal growth factor-like 2 domain were detected by flow cytometry using anti-mouse IgG-FITC antibody. The affinity class of the $\alpha_L$ domains is shown in parentheses. DMSO, Mn2SO (dimethylsulfoxide).

the cells adhere at 37 °C (Fig. 2A) (4). Activation of wild-type $\alpha_L\beta_2$ with Mn$^{2+}$ (4, 19) or an activating antibody, CBR LFA-1/2, greatly increases adhesion to levels seen with high affinity (K287C/K294C)-$\alpha_L/\beta_2$ (Fig. 2A) (4, 19).

As expected, adhesion to immobilized ICAM-1 of K562 cells expressing the low affinity K160C/T300C-$\alpha_L/\beta_2$ mutant was as low as that of wild-type $\alpha_L\beta_2$ (Fig. 2A). Moreover, none of the cells transfected with intermediate affinity $\alpha_L\beta_2$ mutants showed significantly higher binding to immobilized ICAM-1 in this assay (Fig. 2A). This contrasted with the higher affinity for ICAM-1 of the same mutations in isolated $\alpha_L$ domains as measured with surface plasmon resonance (Table 1).

We next explored the ability of the K562 transfectants expressing $\alpha_L\beta_2$ mutants to bind to immobilized ICAM-1 under less stringent conditions than those of the flat bottom assay. In the V-well assay, cells are spun down at 200 × g and are never subjected to washing. Non-adherent cells roll or slide to the bottom of the V under centrifugal force, whereas adherent cells remain evenly distributed on the V-well bottom. The V-well assay detects lower affinity or avidity states that cannot be detected by conventional flat bottom assay or soluble ligand binding (6).

18). In the V-well assay, ~40–50% of the high affinity $\alpha_L\beta_2$ transfectants or Mn$^{2+}$-treated wild-type $\alpha_L\beta_2$ transfectants bound to the ICAM-1-coated wells, whereas the wild-type and low affinity K160C/T300C-$\alpha_L/\beta_2$ transfectants were significantly less adherent (Fig. 2B). Despite the less stringent conditions of this assay, among the intermediate affinity mutants, significant adhesion ($p < 0.05$) was seen only with L161C/F299C (Fig. 2B), which has the highest affinity for ICAM-1 of the three intermediate affinity mutants (Table 1).

**Binding of Soluble Dimeric and Multimeric ICAM-1-Fcα to Intermediate Affinity $\alpha_L\beta_2$ Mutants**—For comparison to the cell adhesion assay results, we examined binding of soluble ligands using dimeric or multimeric soluble ICAM-1-Fcα and fluorescent flow cytometry. In con-
shown (22) that wild-type standard adhesion assays (Figs. 2 and 3) (2, 3). Previously, we have shown that low shear forces are applied (20–22), despite the lack of binding in activated/L/H9251 mutants, which exhibit a 3-fold affinity increase over wild-type, exhibited more rolling cells than wild-type (Fig. 4). Furthermore, the three intermediate affinity mutants all showed a mixture of rolling and firmly adherent cells under basal conditions, in contrast to rolling by the low affinity mutant and firm adhesion by the high affinity mutant (Fig. 4).

Differential Effect of the αβ1 Allosteric Antagonist XVA143 on Low, Intermediate, and High Affinity Mutants—The small molecule αβ2 antagonist XVA143 binds in a metal-dependent fashion to the β2 I-like domain and induces extension of the integrin legs, as shown by activation epitope exposure. XVA143 blocks activation of the αL domain by the β2 I domain and therefore inhibits ligand binding by wild-type αβ2 but not high affinity mutant αβ2 (8), or as shown below, intermediate affinity I domain mutants. Binding of XVA143 and extension of αβ2 is reflected by the exposure of activation-dependent epitopes in the β2 I-like and integrin epidermal growth factor-like 2 domains detected by the antibodies m24 and KIM127, respectively (8, 9). The mutations used here to introduce disulfide bonds into the αL I domain did not interfere with exposure by XVA143 of the m24 activation epitope in the β2 I domain or the KIM127 activation epitope in the integrin epidermal growth factor-like 2 domain in the β2 leg (Fig. 1). Thus, binding of this small molecule and extension of αβ2 are not impaired by the αL I domain cysteine substitutions.

<table>
<thead>
<tr>
<th>Binding of dimeric ICAM-1-Fcα</th>
<th>High affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low affinity</td>
<td>Intermediate affinity</td>
</tr>
<tr>
<td>αLβ2</td>
<td>K160C/T300C-αLβ2</td>
</tr>
<tr>
<td>X63</td>
<td>3.8%</td>
</tr>
<tr>
<td>TS1/22</td>
<td>0.7%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Binding of multimeric ICAM-1-Fcα</th>
<th>High affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low affinity</td>
<td>Intermediate affinity</td>
</tr>
<tr>
<td>αLβ2</td>
<td>K160C/T300C-αLβ2</td>
</tr>
<tr>
<td>X63</td>
<td>1.4%</td>
</tr>
<tr>
<td>TS1/22</td>
<td>0.8%</td>
</tr>
</tbody>
</table>

FIGURE 3. Dimeric and multimeric ICAM-1 binds to intermediate affinity αβ2. Transfectants were treated with TS1/22 mAb to the αI domain or an isotype control (X63) and then incubated with ICAM-1-Fcα, washed and stained with FITC-anti-human IgA (A), or stained with immune complexes formed by pre-incubating ICAM-1-Fcα-FITC and anti-IgA followed by immunofluorescence flow cytometry (B). Histograms with the number of cells on the y-axis and fluorescence intensity on the x-axis are shown for one representative experiment of two or three. The percentage of positive cells is shown for each histogram.
XVA143 had an effect on the intermediate affinity mutants, unlike that seen on other classes of mutants (Fig. 5). For all three intermediate affinity mutants, XVA143 promoted a marked increase in the total number of firmly adherent cells that were bound and remained attached under shear forces (Fig. 5). As previously reported (9), XVA143 enhanced rolling by wild-type L/H9252 and had no effect on the high affinity mutant. These results suggest that, when L/H9252 has intermediate affinity for ligand, extension is a major factor influencing the strength of the adhesion. This may be similar to the case with wild-type L/H9252, where extension greatly promotes rolling (Fig. 5) (9). However, in contrast to intermediate affinity mutants, extension of the wild-type or low affinity mutant was not sufficient to promote firm adhesion (Fig. 5).

Following up on these findings, we tested the hypothesis that XVA143 could induce adhesion by the intermediate affinity mutants in the flat bottom assay described above. Indeed, incubation with XVA143 induced a significant increase in adhesion mediated by the intermediate affinity α1β2 mutants but had no effect on the low affinity mutant K160C/T300C-α1/β2 (Fig. 6). In keeping with our previous findings (9), XVA143 abrogated binding mediated by resting and activated wild-type α1β2, but as expected for an allosteric inhibitor, had no effect on adhesion mediated by the high affinity K287C/K294C-α1/β2-transfected cells (Fig. 6).

As a contrast, we tested the effect of the small molecule BIRT377, which belongs to the α1 allosteric class of inhibitors that stabilize the closed conformation of the α1 I domain and do not induce extension of α1β2 (8, 16). BIRT377 did not increase binding by the intermediate affinity α1β2 transfectants and, if anything, slightly decreased their binding (Fig. 6). As expected, BIRT377 completely blocks adhesion mediated by the resting or activated wild-type α1β2 and had no effect on the high affinity K287C/K294C-α1/β2 mutant (Fig. 6).

**DISCUSSION**

A structural picture of integrin activation is emerging from crystal, NMR, and electron microscopy studies (5). Studies on integrins that lack I domains demonstrate at least three distinct conformational states: a bent conformation with the I domain in the closed conformation, an extended conformation with the I domain in the closed conformation, and an extended conformation with the I domain in the open conformation. Integrins that contain I domains appear to undergo analogous extension and opening of the I domain, with additional complexities because of the presence of the α I domain and evidence that it can exist in three conformational states (1, 23). In wild-type α1β2, it is likely that extension and β I domain opening would be a requirement for or coupled in a yet-undefined manner to α I domain conformational transitions.

Currently, there is highly suggestive, but indirect, evidence in favor of an intermediate affinity state during physiologic activation of wild-type α1β2. That is, adhesion and binding to multivalent soluble ligands through α1β2 is stimulated in the absence of any change in clustering and in the absence of high affinity monomeric ligand binding (6). Highly sensitive ligand binding...
assays do detect a stimulated increase in monovalent affinity by cell surface
$\alpha_\beta_2$; however, they do not discriminate between a small subpopulation of
high affinity $\alpha_\beta_2$ or a larger population of intermediate affinity $\alpha_\beta_2$ mol-
ecules (7). Here, modeling the intermediate affinity state with mutants has
allowed us to demonstrate that an intermediate affinity state is capable of
supporting cell adhesion, and use of an antagonist that induces extension
has also enabled us to show that I domain affinity and integrin extension
each contribute to integrin adhesiveness.

Remarkably, the presence within an $\alpha_\beta_2$ heterodimer of an interme-
diate affinity ($K_D \sim 3–10$ nM) I domain per se does not result in high
adhesion to ICAM-1 as measured here by flat bottom and V-well bot-
tom adhesion assays. By contrast, soluble dimeric and multimeric ligand
binding was readily detected. These differences are attributable to the
greater importance of integrin orientation for adhesion than soluble
ligand binding. The KIM127 mAb recognizes an epitope in the $\beta_2$ leg in
integrin epidermal growth factor-like 2 domain that is buried in the bent
conformation and exposed in the extended conformation (11, 13, 24); m24 recognizes an activation epitope in the $\beta_2$ I domain (4, 14, 25).
Staining with these mAbs suggested that the low affinity and interme-
diate affinity mutants studied here, similar to the previously character-
ized high affinity mutant, basally assume predominantly the bent con-
formation with a closed $\beta_2$ I domain. An important contrast was noted
with $\alpha_\beta_2$ containing a high affinity mutant I domain; even though it
adopted predominantly a bent conformation, it supported adhesion in
the flat well and V-well assays that was strong and indistinguishable
from activated wild-type $\alpha_\beta_2$.

The parallel wall flow assay was more sensitive than the flat well or
V-well assays in detecting adhesiveness. The K160C/T300C mutant
with 3-fold higher affinity than wild-type, classed here as low affinity,
showed enhanced rolling compared with wild-type and few or no firmly
adherent cells. The three intermediate affinity mutants showed a
marked increase in accumulation on the substrate at 0.3 dyn/cm$^2$
compared with wild-type and the presence of both firmly adherent and
rolling cells over a wide range of shear stresses. The percentage of roll-
ingly adherent cells increased with shear. By contrast, essentially only
firm adhesion was seen with the high affinity mutant at all wall shear
stresses. These findings demonstrated a clear correlation between adhe-
Integrin $\alpha_L\beta_2$ in an Intermediate Affinity State

sive phenotype in shear flow and I domain affinity, predominant rolling with low affinity, mixed rolling and firm adhesion with intermediate affinity, and only firm adhesion with high affinity.

We found that integrin extension also makes an important contribution to adhesive phenotype. The $\alpha/\beta$ 1 allosteric antagonist XVA143 induced $\beta$ I domain opening and extension of all of the mutants studied here, as shown with the m24 and KIM127 epitopes. This antagonist binds to the $\beta$ I domain and blocks activation of the $\alpha$ I domain in wild-type $\alpha_L\beta_2$; it has no direct effect on I domains (8, 15). Modulation of I domain affinity, but not induction of extension, are abolished when the position of the $\alpha$ I domain C-terminal helix is stabilized with a mutagenically introduced disulfide bond (8). When predominantly in the bent conformation, $\alpha_L\beta_2$ heterodimers containing intermediate affinity I domains were inactive in conventional flat well adhesion assays. After small molecule-induced extension, all three intermediate affinity mutants mediated robust adhesion, whereas the low affinity mutant did not. Thus intermediate affinity and integrin extension alone are insufficient (but together are sufficient) to mediate adhesion.

The effect of extension on adhesiveness in shear flow of intermediate affinity $\alpha_L\beta_2$ mutants was unique. The low affinity mutant mediated rolling adhesion with or without small molecule-induced extension, and the high affinity mutant supported only firm adhesion with or without extension. By contrast, small molecule-induced extension triggered a substantial shift of all three intermediate affinity mutants from rolling adhesion to firm adhesion. Previously, we had shown that extension substantially increased rolling through wild-type $\alpha_L\beta_2$, which exists basally in a low affinity state. In contrast, the current results demonstrate that extension alters the adhesive phenotype for the intermediate affinity state.

Our findings have several important implications for integrin regulation in vivo. To date, it has generally been assumed that there should be a correlation between the activity of integrins in cell adhesion assays and integrin avidity as measured by binding of soluble, multivalent ligands. Our results show that this is not the case when overall integrin conformation changes; integrin extension greatly increases firm adhesion, particularly for integrins with intermediate affinity.

Integrin extension appears to be the first step in activation of the extracellular domain in inside-out signaling. Separation of the cytoplasmic and transmembrane domains causes separation of the lower $\alpha$ and $\beta$ subunit legs, which destabilizes their interface with the headpiece and converts the bent conformation to the extended conformation (5, 18). Opening of the $\beta$ I domain is not hindered in the extended conformation as it is in the bent conformation; however, neither is there any mechanism in the extended conformation to favor opening of the $\beta$ I domain. Other processes or interactions would be required to actively stabilize the open $\beta$ I domain, which in turn would stabilize the open, high affinity $\alpha$ I domain. In vivo and in vitro, chemokines activate the arrest of rolling cells in $<1$ s (10). The available structural data and the results described here suggest that extension of $\alpha_L\beta_2$ and an intermediate affinity, intermediate conformation of the $\alpha_L$ I domain are sufficient to mediate the arrest of rolling cells and that a high affinity, open conformation of the $\alpha_L$ I domain is not required.

Acknowledgment—We thank Charlotte Harwood for excellent technical assistance.

REFERENCES