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

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**Abstract**

$\alpha_L\beta_2$ 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 $\sim$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),$^4$ is expressed on leukocytes and participates in leukocyte trafficking in inflammation, lymphocyte homing, and T lymphocyte interactions with antigen-presenting cells in immune reactions.

The conformational state of the I domain in the $\alpha_L$ subunit strongly influences its affinity for ligand. A downward movement of the C-terminal $\alpha$-helix of the I domain, linked to

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$^4$The abbreviations used are: LFA-1, leukocyte function-associated antigen-1; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule-1.
conformational rearrangements at the metal ion-dependent adhesion site that constitutes the binding site, is seen in the ICAM-1-bound structure of the αI domain (1). This conformation is termed open and has been engineered by locking the loop between the C-terminal β-strand and α-helix into the open conformation using a disulfide bond. The open I domain, both in isolation or in the context of intact αLβ2, can support firm adhesion to ICAM-1 that is comparable with activated, wild-type αLβ2 (2–4). Intermediate affinity I domain mutants were also engineered by mutationally introducing disulfide bonds between the C-terminal α-helix and other portions of the I domain, resulting in affinities for ICAM-1 between 3 and 9.4 μM (Table 1) (1). Crystalization of one of the intermediate affinity mutants showed that the C-terminal α7-helix, which regulates ligand binding affinity, was intermediate in position between the low affinity closed and high affinity open conformations (1).

Besides the α I domain, integrin ectodomains contain four other domains in the α subunit and eight domains in the β subunit. In addition to conformational change in the I domain, integrins undergo large changes in the overall shape of the ectodomain. On physiological cell surfaces, integrins that lack I domains have been shown to assume multiple conformations with distinct affinity states, with the bent conformation in which the head folds over the legs because of a bend at the knees, being the predominant conformation for integrins in the resting, low affinity state (5). Physiological signals that impinge on integrin cytoplasmic domains, as well as certain activating mAbs, alter the equilibrium between different conformational states and trigger extension. For integrins that lack I domains, two extended conformations that differ in the conformation of their heads and in affinity for ligand have been visualized by electron microscopy and in crystal structures (5). On the cell surface, I domains may exist in the three conformations termed closed, intermediate, and open, as seen in crystal structures. Indeed, we have recently demonstrated that αLβ2 can mediate adhesive interactions even when high affinity ligand binding cannot be detected (6). This cannot be explained by avidity regulation, implying the physiologic importance of intermediate affinity states. However, intermediate affinity states (with μM K_D), which were first suggested in phorbol myristyl acetate-stimulated T cells, are difficult to detect using conventional ligand binding assays (7).

Here we introduce three different intermediate affinity mutations into αLβ2 expressed in K562 cells and assess their impact on αLβ2 adhesion to ICAM-1. The affinity and kinetics for the binding to ICAM-1 of isolated I domains with the same mutations have previously been measured (1). We have demonstrated that intermediate affinity, in contrast to high affinity, is on its own insufficient to result in strong adhesion. Nevertheless, intermediate affinity increases the frequency of tethering and supports more stable rolling interactions. By using an αLβ2 α/β I allosteric inhibitor that induces αLβ2 extension (8) and has been shown to facilitate rolling by wild-type αLβ2 (9), we have demonstrated that, for 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 immunofluorescent flow cytometry into 96-well plates at ~1 cell/well. Wells with an outgrowth of a single colony were retested by immunofluorescence, and clones with similar expression to wild-type and high affinity transfectants were expanded and frozen in liquid.
Clones 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 αLβ2 surface expression as wild-type was selected and routinely verified by immunofluorescence flow cytometry. K562 cells stably transfected with wild-type αLβ2 or K287C/K294C-αL/β2 (high affinity) in pEF(puro) were as described previously (3). K562 transfectants were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin/streptomycin, and 3 μg/ml puromycin (wild-type αLβ2 and high affinity αLβ2) 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 αLβ-propeller domain mAb (TS2/4), and an anti-β2 I-like domain mAb (TS1/18) were used to determine surface expression of the transfectants by immunofluorescent flow cytometry or to specifically block αLβ2-ICAM-1 interactions (11,12). CBR LFA-1/2 was employed to induce activation of αLβ2 (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., Ridgeway, CT).

Soluble ICAM-1 Binding Assay

K562 transfectants 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 cation concentrations. To induce activation, wild-type αLβ2 cells were pre-incubated with 10 μg/ml CBR LFA-1/2 for 15 min. In some experiments, cells were pre-incubated with 10 μg/ml αI 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-Fcα) (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-Fcα binding to the K562 transfectants, cells were pre-incubated 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-Fcα 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 IgA (ICAM-1-Fcγ) 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 × 10^4 cells in 50 μl), and when indicated, incubated for 15 min with 1 mM MnCl_2. 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 transfectants 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 αLβ2 transfectants were pre-incubated 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 αLβ2 Mutants and Adhesion to ICAM-1**

Measurements of the solution affinity for ICAM-1 of different double cysteine mutants of the αL-1 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-1 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 was 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 αLβ2, as revealed by staining with an anti-β2β-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 transfectants 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 transfectants expressing wild-type αLβ2 is hardly detectable in flat bottom adhesion assays when cells are incubated at room temperature (22 °C) (data not shown) (19), and <10% of the cells adhere at 37 °C (Fig. 2A) (4). Activation of wild-type αLβ2 with Mn^{2+} (4,19) or an activating antibody, CBR LFA-1/2, greatly increases adhesion to levels seen with high affinity (K287C/K294C)-αL/β2 (Fig. 2A) (4,19).

As expected, adhesion to immobilized ICAM-1 of K562 cells expressing the low affinity K160C/T300C-αL/β2 mutant was as low as that of wild-type αLβ2 (Fig. 2A). Moreover, none of the cells transfected with intermediate affinity αLβ2 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 I domains as measured with surface plasmon resonance (Table 1).

We next explored the ability of the K562 transfectants expressing αLβ2 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 ($p < .05$) 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 contrast to the cell adhesion assays, soluble ligand binding by $\alpha_L\beta_2$ transfectants (Fig. 3) correlated well with previous affinity measurements on purified I domains (Table 1). The panels in Fig. 3, A and B, are arranged in the same order as the affinities in Table 1. The rank order of these affinities in Table 1 is entirely concordant with the rank order of binding of dimeric and multimeric ICAM-1-Fcα in Fig. 3. Activation of wild-type $\alpha_L\beta_2$ with CBR LFA-1/2 mAb induced binding of ICAM-1-Fcα similar to the K287C/K294C mutant. Blocking by TS1/22 abrogated binding of dimeric and multimeric ICAM-1-Fcα to all of the transfectants studied, supporting the specificity of the binding.

### Binding of Intermediate Affinity Mutants to Immobilized ICAM-1 in the Presence of Shear Forces

Both the isolated wild-type $\alpha_I$ I domain and $\alpha_L\beta_2$ on the cell surface can mediate weak adhesion and rolling when low shear forces are applied (20–22), despite the lack of binding in standard adhesion assays (Figs. 2 and 3) (2,3). Previously, we have shown (22) that wild-type $\alpha_L\beta_2$ mediates mostly weak, rolling interactions under shear forces and that activated $\alpha_L\beta_2$ or the high affinity mutant supports primarily firm adhesion that is resistant to high shear forces, as confirmed here (Fig. 4).

Examination of the low and intermediate affinity mutants showed that adhesion in shear flow was correlated with affinity (Fig. 4). The low affinity mutant, which exhibits 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 $\alpha/\beta$ I Allosteric Antagonist XVA143 on Low, Intermediate, and High Affinity Mutants

The small molecule $\alpha_L\beta_2$ antagonist XVA143 binds in a metal-dependent fashion to the $\beta_2$ I-like domain and induces extension of the integrin legs, as shown by activation epitope exposure. XVA143 blocks activation of the $\alpha_I$ I domain by the $\beta_2$ I domain and therefore inhibits ligand binding by wild-type $\alpha_L\beta_2$ but not high affinity mutant $\alpha_L\beta_2$ (8), or as shown below, intermediate affinity I domain mutants. Binding of XVA143 and extension of $\alpha_L\beta_2$ is reflected by the exposure of activation-dependent epitopes in the $\beta_2$ 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 $\alpha_I$ I domain did not interfere with exposure by XVA143 of the m24 activation epitope in the $\beta_2$ I domain or the KIM127 activation epitope in the integrin epidermal growth factor-like 2 domain in the $\beta_2$ leg (Fig. 1). Thus, binding of this small molecule and extension of $\alpha_L\beta_2$ are not impaired by the $\alpha_I$ I domain cysteine substitutions.
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 αLβ2 and had no effect on the high affinity mutant. These results suggest that, when αLβ2 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 αLβ2, 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 αLβ2 mutants but had no effect on the low affinity mutant K160C/T300C-αL/β2 (Fig. 6). In keeping with our previous findings (9), XVA143 abrogated binding mediated by resting and activated wild-type αLβ2, but as expected for an allosteric inhibitor, had no effect on adhesion mediated by the high affinity K287C/K294C-αL/β2-transfected cells (Fig. 6).

As a contrast, we tested the effect of the small molecule BIRT377, which belongs to the αI allosteric class of inhibitors that stabilize the closed conformation of the αI domain and do not induce extension of αLβ2 (8,16). BIRT377 did not increase binding by the intermediate affinity αLβ2 transfectants and, if anything, slightly decreased their binding (Fig. 6). As expected, BIRT377 completely blocks adhesion mediated by the resting or activated wild-type αLβ2 and had no effect on the high affinity K287C/K294C-αL/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 βI domain in the closed conformation, an extended conformation with the βI domain in the closed conformation, and an extended conformation with the βI domain in the open conformation. Integrins that contain I domains appear to undergo analogous extension and opening of the βI domain, with additional complexities because of the presence of the αI domain and evidence that it can exist in three conformational states (1,23). In wild-type αLβ2, it is likely that extension and βI domain opening would be a requirement for or coupled in a yet-undefined manner to αI domain conformational transitions.

Currently, there is highly suggestive, but indirect, evidence in favor of an intermediate affinity state during physiologic activation of wild-type αLβ2. That is, adhesion and binding to multivalent soluble ligands through αLβ2 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 αLβ2; however, they do not discriminate between a small subpopulation of high affinity αLβ2 or a larger population of intermediate affinity αLβ2 molecules (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 αLβ2 heterodimer of an intermediate affinity (KD ~3–10 μM) I domain per se does not result in high adhesion to ICAM-1 as measured here by flat bottom and V-well bottom 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 β2 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 β2 I domain (4,14,25). Staining with these mAbs suggested that the low affinity and intermediate affinity mutants studied here, similar to the previously characterized high affinity mutant, basally assume predominantly the bent conformation with a closed βI domain. An important contrast was noted with αLβ2 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 αLβ2.

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/cm² compared with wild-type and the presence of both firmly adherent and rolling cells over a wide range of shear stresses. The percentage of rollingly 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 adhesive 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 α/β I allosteric antagonist XVA143 induced β 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 β I domain and blocks activation of the α I domain in wild-type αLβ2; 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 α I domain C-terminal helix is stabilized with a mutationally introduced disulfide bond (8). When predominantly in the bent conformation, αLβ2 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 αLβ2 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 αLβ2, 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 α and β subunit legs, which destabilizes their interface with the headpiece and converts the bent conformation to the extended conformation (5,18). Opening of the β 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 β I domain. Other processes or interactions would be required to actively stabilize the open β I domain, which in turn would stabilize the open, high affinity α 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 αLβ2 and an intermediate affinity, intermediate conformation of the αL I domain are sufficient to mediate the arrest of rolling cells and that a high affinity, open conformation of the αL I domain is not required.

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FIGURE 1. Exposure of epitopes in the presence or absence of an allosteric antagonist
K562 cells transfected with the indicated αLβ2 mutants were incubated with 0.001% Me₂SO
as control (white histogram) or 1 μM of the allosteric antagonist XVA143 (gray histogram).
Mouse IgG control, TS2/4 mAb to a constitutive αL β-propeller domain epitope, m24 mAb to
an activation-dependent β2 I domain epitope, and KIM127 mAb to an activation-dependent
epitope in the β2 integrin epidermal growth factor-like 2 domain were detected by flow
cytometry using anti-mouse IgG-FITC antibody. The affinity class of the mutant I domains is
shown in parentheses. DMSO, Me₂SO (dimethylsulfoxide).
FIGURE 2. Binding of αLβ2 transfectants to ICAM-1 immobilized on a flat well bottom plate or V-well plate

A, K562 transfectants labeled with bis-carboxyethylfluorescein were allowed to bind to ICAM-1-coated flat bottom wells in L-15 medium/2.5% fetal calf serum for 30 min at 37 °C. The wells were washed, and adherent cells were detected. Each experiment was performed in triplicate.

B, binding of αLβ2 transfectants to ICAM-1 immobilized on a V-well bottom plate. Wild-type and mutant αLβ2 transfectants labeled as described above for A were added to ICAM-1-coated V-shaped wells, and the plates were immediately centrifuged. Adherence was measured as described under “Results.” Each experiment was performed in triplicate. Error bars represent the average of three independent experiments ± S.E.; *, p < 0.05; **, p < 0.005 versus wild-type αLβ2.
FIGURE 3. Dimeric and multimeric ICAM-1 binds to intermediate affinity αLβ2
Transfectants were treated with TS1/22 mAb to the αL 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.
FIGURE 4. Adhesive behavior of $\alpha_4\beta_2$ transfectants on immobilized ICAM-1 in shear flow is related to I domain affinity

Transfectants were allowed to accumulate on the ICAM-1-Fc/protein A surface in a parallel wall flow chamber at 0.3 dyn/cm$^2$ for 30 s. Shear was then increased every 10 s, and the number of rolling (white bar) and firmly adherent (gray bar) cells was quantified at each shear interval. Error bars represent the average of two to three independent experiments ± S.E.
FIGURE 5. Effect of extension of $\alpha_4\beta_2$ induced by the $\alpha/\beta$ I domain allosteric antagonist XVA143 on adhesive behavior in shear flow

Transfectants pretreated with 0.001% Me$_2$SO as control or 1 $\mu$M XVA143 were allowed to accumulate on an ICAM-1-Fcγ/protein A surface at 0.3 dyn/cm$^2$ for 30 s, and shear was increased every 10 s exactly as shown in Fig. 4. The number of rolling (white bar) and firmly adherent (gray bar) cells at 4 dyn/cm$^2$ was quantified. Error bars represent the average of two to three independent experiments ± S.E.
FIGURE 6. Effect of the $\alpha/\beta$ I allosteric antagonist XVA143 and the $\alpha$ I allosteric antagonist BIRT377 on $\alpha I$ $\beta_2$ transfectant binding to ICAM-1 immobilized on a flat well plate
Transfectants labeled with bis-carboxyethyl fluorescein were incubated with 0.001% Me$_2$SO (DMSO) as control, 10 $\mu$M BIRT377, or 1 $\mu$M XVA143 and allowed to bind to ICAM-1-coated wells in L-15 medium/2.5% fetal calf serum for 30 min at 37 °C. The wells were washed extensively, and adherent cells were detected. Each experiment was performed in triplicate. Error bars represent the average of two to three independent experiments ± S.E.
### TABLE 1

| I Domain            | $k_{on}$\(^{a}\) | $k_{off}$\(^{a}\) | $K_D$\(^{a}\) | Class          | Phenotype under shear stress         |
|---------------------|------------------|-------------------|--------------|----------------|-------------------------------------|
|                     | $M^{-1} s^{-1} \times 10^{-3}$ | $s^{-1}$ | $\mu M$   |                | Basal\(^{b}\) | XVA143\(^{b}\)               |
| Wild-type           | 3.1 ± 0.1        | 4.6 ± 0.36        | 1300 ± 200   | Low            | Rolling/Firm adhesion ↑ Rolling     |
| K160C/T300C         | 3.4 ± 0.9        | 1.2 ± 0.08        | 450 ± 210    | Low            | Rolling                            |
| L161C/T300C         | 89 ± 12          | 0.76 ± 0.07       | 9.4 ± 2.4    | Intermediate   | Rolling/Firm adhesion ↑ Firm adhesion |
| K160C/F299C         | 103 ± 15         | 0.77 ± 0.07       | 8.4 ± 2.4    | Intermediate   | Rolling/Firm adhesion Rolling/Firm adhesion |
| L161C/F299C         | 133 ± 10         | 0.43 ± 0.07       | 3.0 ± 0.44   | Intermediate   | Rolling/Firm adhesion ↑ Firm adhesion |
| K287C/K294C         | 115 ± 7          | 0.014 ± 0.001     | 0.15 ± 0.016 | High           | Firm adhesion                      |

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

\(^{b}\) Results of this study.