Rolling Adhesion through an Extended Conformation of Integrin $\alpha_L\beta_2$ and Relation to $\alpha I$ and $\beta$ I-like Domain Interaction

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Summary

In vivo, $\beta_2$ integrins and particularly $\alpha_\beta L$ (LFA-1) robustly support firm adhesion of leukocytes, but can also cooperate with other molecules in supporting rolling adhesion. Strikingly, a small molecule $\alpha/\beta$ I-like allosteric antagonist, XVA143, inhibits LFA-1-dependent firm adhesion, while at the same time it enhances adhesion in shear flow and rolling both in vitro and in vivo. XVA143 appears to induce the extended conformation of integrins as shown by increased activation epitope exposure. Fab to the $\beta_2$ I-like domain converts firm adhesion to rolling adhesion, but does not enhance adhesion. Residue $\alpha_\gamma$-Glu-310 in the linker following the $I$ domain is critical for communication to the $\beta_2$ I-like domain, rolling, integrin extension, and activation by Mn$^{2+}$ of firm adhesion. The results demonstrate the importance of integrin extension in rolling, and suggest that rolling and firm adhesion are mediated by extended conformations of $\alpha_\beta L$ that differ in the affinity of the $\alpha I$ domain for ICAM-1.

Introduction

Integrins are a family of heterodimeric receptors that use bidirectional (inside-out and outside-in) signaling to integrate the intracellular and extracellular environments. Some integrins contain an inserted (I) domain in their $\alpha$ subunits, including LFA-1 (integrin $\alpha_\beta L$) (Humphries, 2000; Shimaoka et al., 2002). LFA-1 binds IgSF cell surface molecules known as intercellular adhesion molecules (ICAMs) and functions in lymphocyte recognition of other cells and in leukocyte trafficking between blood, tissue, and lymph (Springer, 1994). The ligand binding site of LFA-1 is wholly contained within the $\alpha I$ domain (Lu et al., 2001b; Shimaoka et al., 2001, 2003b). One- and two-turn axial displacements in the C-terminal direction of the $\alpha I$ domain $\alpha 7$-helix are linked to rearrangements around the ligand binding site at the metal ion dependent adhesion site (MIDAS) and to increases in affinity for ICAM-1 of up to 10,000-fold (Huth et al., 2000; Shimaoka et al., 2003b). The I-like domain in the integrin $\beta_2$ subunit undergoes conformational changes during activation that allosterically regulate binding through the $\alpha I$ domain (Lu et al., 2001c; Yang et al., 2004a, 2004b). Like the $\alpha I$ domain, the $\beta_2$ I-like domain contains a MIDAS that plays a key role in its function.

Although there are no overall structures for integrins that contain I domains, rapid progress has recently been made in understanding the structural basis for regulation of ligand binding in integrins that lack I domains. In these integrins, the $\beta$ subunit I-like domain and the $\alpha$-subunit $\beta$-propeller domain constitute the ligand binding site (Takagi et al., 2003; Xiong et al., 2002; Yahalom et al., 2002). A crystal structure for most of the extracellular domains of integrin $\alpha_\beta L$ revealed an unexpected overall V-shaped organization, in which the ligand binding domains in the headpiece are bent toward the cell membrane by extensive interactions with tailpiece domains (Takagi et al., 2002; Xiong et al., 2001). The extreme bend in the $\alpha$ and $\beta$ subunits occurs at a location termed the “genu.” Based on evidence from the NMR structure of $\beta_2$ subunit I-EGF domains (Beglova et al., 2002), together with mapping of activation-dependent or activation-inducing mAbs and sites of interaction with $\alpha$ that constrain $\beta_2$ integrins in the resting conformation (Lu et al., 2001a; Zang and Springer, 2001), the bent conformation was proposed to represent a low-affinity state of the integrin, and a switchblade-like opening was proposed to occur upon activation. Indeed, EM and mutational studies of integrins $\alpha_\beta L$ and $\alpha_\beta 3$ have directly demonstrated at least three distinct conformational states (Takagi et al., 2002, 2003). These correspond to the bent conformation seen in the crystal structure that has low affinity for ligand (Figure 1F), and two extended conformations that differ in the angle between the $\beta$ I-like and hybrid domains (Figures 1G and 1H). The extended conformations with the open (Figure 1H) and closed (Figure 1G) headpieces appear to have high and intermediate affinity for ligand, respectively. Recent mutagenesis, epitope mapping, and solution X-ray scattering studies have supported integrin extension and hybrid domain swing-out in the open headpiece conformation (Luo et al., 2003b; Mould et al., 2003a, 2003b) while ruling out an alternative model of headpiece separation (Luo et al., 2003a; Mould et al., 2003b).

The integrin $\alpha_\beta L$ appears to undergo similar conformational transitions, as shown with epitopes that become exposed upon LFA-1 activation and map to $\beta_2$ I-like EGF domains 2 and 3 (Beglova et al., 2002) (Figures 1A–1E). Work with a class of small molecules termed $\alpha/\beta L$-like allosteric antagonists also suggests analogous conformational changes in $\alpha_\beta L$. These compounds bind to the MIDAS of the $\beta_2$ I-like domain, and also to the $\alpha$-subunit (Shimaoka et al., 2003a). They structurally resemble small molecule RGD-based antagonists in containing an absolutely required carboxyl group that is thought to bind to the $\beta_2$ I-like domain MIDAS. The $\alpha/\beta$ I-like allosteric antagonists, like RGD-based $\alpha/\beta$ I-like competitive antagonists, also appear to bind across the interface between the $\alpha$-subunit $\beta$-propeller and $\beta$-subunit I-like domains (Shimaoka et al., 2003a). Furthermore, both classes of compounds induce the extended integrin conformation, as shown by exposure of activation epitopes (Kouns et al., 1992; Shimaoka et al., 2003a;
Figure 1. Conformational States for Integrins and Structure of $\alpha_3\beta_2$ Antagonists

(A–E) Model for $\alpha_3\beta_2$ integrin activation. The models for all of the extracellular domains except for the I domain (Springer, 2002) are based on conformational states of $\alpha_3\beta_1$ defined by negative stain electron microscopy (Takagi et al., 2002), crystallography (Xiong et al., 2002), NMR (Beglova et al., 2002), and mapping of activation epitopes (Lu et al., 2001a, 2001c). The $\alpha_3$ I domain is a cartoon based on crystal structures (Shimaoka et al., 2003b). The I domain is joined at the point of its insertion in the $\beta$-propeller domain but its orientation is arbitrary; the I domain is shown at slightly larger scale for emphasis. The C-terminal I domain $\alpha$-helix is represented by a red cylinder, and $\alpha_3$ Glu-310 in the linker as a blue sphere. The positions of the epitope recognized by the TS1/18 Fab used here, and activation epitopes, are circled. Epitopes are circled only in the conformations in which they are thought to be exposed. (A) Bent conformation with low affinity. (B and C) LFA-1 with a closed headpiece and closed I domain in partially (B) or fully (C) extended states. (D) Extended conformation with open headpiece, and closed I domain, in the presence of XVA143, represented by three red spheres. (E) Extended conformation with open headpiece and open I domain.

(F–H) Model for $\alpha_3\beta_2$ integrin activation, with at least three conformations of the extracellular domain (Takagi et al., 2002). (F) Bent, low-affinity conformation. (G) Extended conformation with closed headpiece. (H) Extended conformation with open headpiece. (I) Chemical structures of small molecule $\alpha_3\beta_2$ antagonists used in this study.

Takagi et al., 2002, and for $\alpha_3\beta_2$ by direct electron microscopic observation (Takagi et al., 2002). By contrast, $\alpha_3$ I allosteric antagonists of $\alpha_3\beta_2$, which bind under the $\alpha_3$-helix of the $\alpha_3$ I domain and stabilize it in the closed conformation (Kallen et al., 1999; Lu et al., 2001b; Weitz-Schmidt et al., 2001), stabilize the bent conformation,
as shown by suppression of epitope induction by Mn$^{2+}$
(Shimaoka et al., 2003a; Woska et al., 2001).

$\alpha_4\beta_2$, and the other $\beta_2$ integrins are far more facile in
supporting firm adhesion than rolling adhesion. In many
in vitro and in vivo systems in which $\beta_2$ integrins mediate
firm adhesion and selectins mediate tethering and rolling
adhesion, $\beta_2$ integrins are not seen to mediate tethering
in shear flow or rolling (Lawrence and Springer, 1991;
von Andrian et al., 1991). The $\alpha_i$ integrins have been
known for some time to mediate rolling as well as firm
adhesion (Alon et al., 1995; Berlin et al., 1995), although
they do not support rolling as efficiently as selectins (de
Chateau et al., 2001). Nonetheless, in vivo studies have
suggested for some time that while $\beta_2$ integrins cannot
on their own mediate rolling, they can contribute to roll-
ing together with selectins or $\alpha_i$ integrins. Thus, when
$\beta_2$ integrin function is blocked in vivo, and particularly
when $\beta_2$ integrins or $\alpha_i\beta_2$ are blocked or eliminated to-
gether with one or more selectins or $\alpha_i$ integrins, they
can be seen to contribute to the accumulation of rolling
cells, the stability of rolling, and the velocity of rolling
cells in inflamed tissues (Forlow and Ley, 2001; Forlow
et al., 2000; Henderson et al., 2001; Jung et al., 1998;
Perry and Granger, 1991). By contrast, LFA-1 has been
shown not to contribute to lymphocyte rolling in the high
endothelial venules (HEV) of noninflamed lymph nodes
(Warnock et al., 1998). In vitro, LFA-1 can mediate teth-
ering in flow of leukocytes to ICAM-2 on platelets in the
absence of selectin-mediated interactions (Weber and
Springer, 1997). Moreover, special attention to the way
in which ICAM-1 is coupled to the substrate, and to the
type of LFA-1-bearing cell that is used, enables rolling
solely through $\alpha_i\beta_2$ heterodimers to be detected in vitro
(Salas et al., 2002; Sigal et al., 2000). Surprisingly, the
wild-type $\alpha_i$ I domain expressed in isolation from other
integrin domains by fusing its C terminus to an artificial
membrane anchor supports rolling on ICAM-1 much
more efficiently than $\alpha_i\beta_2$ heterodimers (Knorr and Dus-
tin, 1997; Salas et al., 2002). Is an isolated, $\alpha_i$ I domain
expressed on the cell surface in a better orientation to
mediate tethering and rolling on ICAM-1 than a bent
$\alpha_i\beta_2$ heterodimer? Isolated I domains locked in the open
conformation with a disulfide-bond mediate firm adhe-
sion (Salas et al., 2002), as do $\alpha_i\beta_2$ integrins activated
by Mn$^{2+}$, activating antibodies, or physiologic activating
stimuli (Campbell et al., 1998; Lawrence et al., 1995;
Lawrence and Springer, 1991; Salas et al., 2002; Sigal
et al., 2000; von Andrian et al., 1991). However, the ability
of $\alpha_i\beta_2$ and other $\beta_2$ integrins to support rolling in vivo
raises the intriguing possibility that rolling might be me-
diated by an intermediate conformation of $\alpha_i\beta_2$ with an
extended conformation but with the I domain in a low or
intermediate affinity state, corresponding approxi-
mately to the conformation depicted in Figures 1B
and 1C.

How the overall conformation of $\alpha_i\beta_2$ relates to its
ability to support rolling adhesion remains to be eluci-
dated. The greater height above the cell surface of the
headpiece in the extended conformation, and its altered
orientation, appear more favorable for ligand encounter.
Although both $\alpha_i\beta_2$ and $\alpha_i$ integrins can mediate rolling,
nothing is currently known about the global conforma-
tion of these integrins during rolling, or how overall con-
formation influences rolling. Here, we demonstrate the
importance of integrin extension or conformational
breathing in regulating rolling, and the interrelationship
in $\alpha_i\beta_2$ between global conformation and the confor-
mation of the $\alpha_i$ I domain, the $\beta_2$ I-like domain, and the
coupling between these domains. We not only demon-
strate the importance of integrin extension in rolling,
but also make the surprising observation that $\alpha/\beta$ I-like
allosteric antagonists markedly enhance or induce roll-
ing adhesion while inhibiting firm adhesion.

Results

An $\alpha/\beta$ I-like Allosteric Antagonist Blocks Activation-
Dependent Adhesion and in Contrast Enhances Rolling Adhesion on ICAM-1

In conditions in which $\alpha_i\beta_2$ is in a low-affinity confor-
mation, such as in Ca$^{2+}$/Mg$^{2+}$, binding to soluble ICAM-1,
even as a multimeric complex of ICAM-1-Fc with FITC-
anti-IgA, is weak (Figure 2A). Activation, for example by
Mg$^{2+}$/EGTA or Mn$^{2+}$, is required to convert $\alpha_i\beta_2$ to a
form that can bind soluble, multimeric ICAM-1 with high
affinity (Figure 2A). However, appropriate conditions can
be found where $\alpha_i\beta_2$ transfectants in Ca$^{2+}$/Mg$^{2+}$ bind
to ICAM-1 substrates in shear flow at 0.3–0.4 dyn/cm$^2$
(Figure 2B) as previously described (Salas et al., 2002;
Sigal et al., 2000). Once cells are bound, their interactions
with ICAM-1 substrates in Ca$^{2+}$/Mg$^{2+}$ are relatively
shear resistant, with cells continuing to roll or adhere
at shear stresses ranging from 1 to 8 dyn/cm$^2$ (Figure 2B;
Salas et al., 2002). The fraction of cells that roll increases
markedly with increasing shear (Figure 2B). In contrast, in
the presence of Mg$^{2+}$/EGTA (Figure 2E) or Mn$^{2+}$ (data not shown), most cells adhere firmly to
the ICAM-1 substrate and remain attached even after
shear is increased to 16 dyn/cm$^2$.

Two classes of small molecule antagonists were used
to probe the effect of the overall conformation of $\alpha_i\beta_2$ on
its ability to mediate rolling. $\alpha_i$ allosteric antagonists,
represented here by BIRT377 (Figure 1I), bind in a hydro-
phobic pocket under the C-terminal $\alpha_i$-helix of the I
domain, stabilize the I domain in the closed, low-affinity
conformation (Kallen et al., 1999; Last-Barney et al.,
2001; Liu et al., 2001; Weitz-Schmidt et al., 2001), and
also inhibit activation epitope exposure in $\alpha_i\beta_2$ (Shi-
maoka et al., 2003b; Woska et al., 2001), i.e., stabilize
the bent conformation (Figure 1A). $\alpha/\beta$ I-like allosteric
antagonists, represented here by XVA143 (Figure 1I),
bind to the MIDAS of the $\beta_2$ I-like domain, as well as to
a region of the $\alpha_i$ subunit that does not include the I
domain (Shimaoka et al., 2003a; Welzenbach et al.,
2002). $\alpha/\beta$ I-like allosteric antagonists were identified
by their ability to inhibit high-affinity binding of $\alpha_i\beta_2$ to
ICAM-1 (Fotouhi et al., 1999; Gadek et al., 2002), as
subsequently confirmed (Shimaoka et al., 2003a; Wel-
zenbach et al., 2002). $\alpha/\beta$ I-like allosteric antagonists
bind to a critical interface for relaying conformational
signals within $\alpha_i\beta_2$ (Shimaoka et al., 2003a). By binding
to the $\beta_2$ I-like domain MIDAS, they stabilize the I-like
domain in the active conformation and induce the ex-
tended conformation of $\alpha_i\beta_2$, as shown by exposure of
activation epitopes, while blocking activation of the $\alpha_i$
I domain by the $\beta_2$ I-like domain (Figure 1D) (Shimaoka
et al., 2003a).
Figure 2. Effect of XVA143 on Binding to ICAM-1

(A) K562 cells expressing α5β2 were incubated with XVA143 in the presence of the indicated divalent cations. Binding of multimeric, soluble ICAM-1 was determined using flow cytometry and expressed as specific mean fluorescence intensity (MFI).

(B) K562 cells were prepared in Buffer A/1 mM Ca2+/1 mM Mg2+ containing DMSO, 1 μM XVA143, or 10 μM LFA703. Cells were immediately infused into a flow chamber with ICAM-1-Fc immobilized on the lower wall. The number of firmly adherent or rolling cells was determined for each shear stress interval. Bars represent the average ± SEM of three to five independent experiments.
If integrin extension is important in supporting rolling, whereas the high-affinity conformation of the αι/β2 domain is not, then αι/β2 I-like allosteric antagonists should have the paradoxical effect of enhancing rolling adhesion through αι/β2. Indeed, in Ca\(^{2+}\)/Mg\(^{2+}\), XVA143 markedly increased the total number of αι/β2 transfectants that accumulated at 0.3 dyn/cm\(^2\), and remained adherent at 0.4 to 16 dyn/cm\(^2\) (Figure 2B). Furthermore, XVA143 increased the total number of cells that rolled, and decreased the number of cells that were firmly adherent, at all wall shear stresses in Ca\(^{2+}\)/Mg\(^{2+}\) (Figure 2B). The increase in rollingly adherent cells was particularly marked at 2 to 16 dyn/cm\(^2\) (Figure 2B). The agonistic effect in shear flow of XVA143 was further verified in accumulation assays (Figure 2C). Accumulation at all wall shear stresses tested, 0.4, 0.6, and 0.8 dyn/cm\(^2\), was enhanced by XVA143. In contrast, the αι allosteric antagonists LFA703 and BIRT377, which stabilize the closed conformation of the I domain, and the overall bent conformation of αι/β2 (Shimaoka et al., 2003a) greatly decreased the number of interacting cells (Figure 2D; data not shown) (Salas et al., 2002).

Although β2 integrins clearly contribute to leukocyte rolling in inflamed vessels in vivo (Forlow and Ley, 2001; Forlow et al., 2000; Henderson et al., 2001; Jung et al., 1998; Perry and Granger, 1991), rolling by peripheral blood cells on ICAM-1 substrates has not previously been demonstrated in vitro. Indeed, under control conditions we failed to detect rolling by peripheral blood lymphocytes (PBL) on ICAM-1 substrates (Figure 2D), consistent with the markedly lower expression of LFA-1 on PBL than K562 transfectants (not shown). However, XVA143 treatment induced readily detectable tethering in shear flow and rolling adhesion of PBL on ICAM-1 substrates (Figure 2D). By contrast, the αι I allosteric antagonist BIRT377 did not induce rolling adhesion (Figure 2D). In Mg\(^{2+}\)/EGTA (Figure 2E) and Mn\(^{2+}\) (not shown), LFA-1-bearing cells develop firm adhesion to ICAM-1 substrates, with hardly any cells rolling. Addition of increasing concentrations of XVA143 resulted in a decreasing number of firmly adherent cells and an increasing number of rolling cells (Figure 2E). At 1 μM XVA143, almost all of the adherent cells exhibited the rolling modality. At maximally effective XVA143 concentrations of 1 μM, more cells accumulated and rolled in Ca\(^{2+}\)/Mg\(^{2+}\) than in Mg\(^{2+}\)/EGTA (Figure 2B compared to 2E), suggesting that Ca\(^{2+}\) makes a contribution to rolling interactions in the XVA143-bound conformation of αι/β2. No interactions occurred in Ca\(^{2+}\) alone or Ca\(^{2+}\) + XVA143 (data not shown).

Contrasting results were obtained in assays of binding of multimeric soluble ICAM-1, which requires high-affinity αι/β2. XVA143 completely abolished binding of ICAM-1 to LFA-1 in Mn\(^{2+}\) and Mg\(^{2+}\)/EGTA, with no hint of agonism (Figure 2A).

**Dose Dependence of the Effect of XVA143 on Rolling Stability and Exposure of Activation Epitopes**

The IC\(_{50}\) values for inhibition by XVA143 of binding of soluble, multimeric ICAM-1 varied depending on the activating divalent cation conditions. XVA143 was most potent against the weakest activator (2 mM Mg\(^{2+}\)/1 mM EGTA, IC\(_{50}\) ~10^(-3) nM) and least potent against the strongest activator (1 mM Mn\(^{2+}\), IC\(_{50}\) ~0.3 nM) (Figure 2A). The IC\(_{50}\) value for 10 mM Mg\(^{2+}\)/1 mM EGTA was intermediate (~10^(-2) nM). All of these IC\(_{50}\) values for inhibition of soluble multimeric ICAM-1 binding are in the subnanomolar range. By contrast, in shear flow adhesion assays in Mg\(^{2+}\)/EGTA, 500 nM to 1 μM concentrations were required for inhibition of firm adhesion, and for stimulation of rolling adhesion (Figure 2E). This difference may reflect a higher affinity of XVA143 for activated receptors than for resting receptors, as demonstrated for αι/β2 I-like competitive antagonists of integrin αιβ3 (Bednar et al., 1998).

We hypothesized that exposure of activation epitopes and stimulation of rolling adhesion in Ca\(^{2+}\)/Mg\(^{2+}\) would each involve binding of XVA143 to the low-affinity resting receptor, and therefore should occur in similar concentration ranges. Increasing concentrations of XVA143 markedly stabilized rolling interactions, as shown by a higher number of rolling cells (Figure 3A) and slower rolling velocities (Figure 3B). The effect of XVA143 began at a concentration of about 10 nM and was maximal at about 1 μM. The dose responses were examined for exposure of activation epitopes recognized by mAbs to different αι/β2 extracellular domains. mAb K1M127 maps to residues in I-EGF domain 2 of the β2 subunit (Beglova et al., 2002; Lu et al., 2001a; Robinson et al., 1992). mAb m24 maps to residues that locate near the top of the β2 subunit I-like domain, not far from its MIDAS (Lu et al., 2001c). The exposure of the K1M127 and m24 epitopes by XVA143 followed a similar dose-response as for stabilization of rolling, with the largest increase in the range of 10 nM to 1 μM (Figure 3C). The EC\(_{50}\) values for stabilization of rolling adhesion and induction of activation epitopes each occurred within the range of 10 to 100 nM XVA143 (Figures 3A–3C).

**Comparison between mAbs to the αι I Domain and β2 I-like Domain for Effect on Adhesive Modality and Global Conformation**

Could mAbs also differentially affect firm adhesion as compared to rolling adhesion? TS1/22 Fab to the αι domain completely inhibited rolling and firm adhesion in both Ca\(^{2+}\)/Mg\(^{2+}\) and Mg\(^{2+}\)/EGTA (Figure 4). Similarly, the αι allosteric antagonist BIRT377 completely inhibited rolling and firm adhesion in both Ca\(^{2+}\)/Mg\(^{2+}\) and Mg\(^{2+}\)/EGTA (Figure 4), as previously shown for the αι allosteric antagonist LFA703 (Salas et al., 2002). TS1/18

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(C) αιβ3 K562 cells were resuspended in Buffer A/1 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\) in the presence of 0 or 1 μM XVA143. Cells were infused at 0.4, 0.6, or 0.8 dyn/cm\(^2\) into the flow chamber and the number of bound cells (rolling or firmly adherent) determined with time. Filled and open symbols are with and without 1 μM XVA143, respectively; squares, 0.4 dyn/cm\(^2\); circles, 0.6 dyn/cm\(^2\); triangles, 0.8 dyn/cm\(^2\). Average ± s.e.m. of three experiments.

(D) Freshly isolated human PBMCs resuspended in Buffer A/1 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\) in the presence of 1 μM XVA143, 1 μM BIRT377 or the equivalent amount of DMSO were infused at 0.3 dyn/cm\(^2\) and the number of rolling cells was determined at 1 and 2 dyn/cm\(^2\). Bars represent the average ± SEM of three independent experiments.

(E) As in (B) except in 2 mM Mg\(^{2+}\)/1 mM EGTA and the indicated XVA143 concentrations.
Figure 4. Effect of Different LFA-1 Inhibitors on Rolling on ICAM-1 Substrates

Wild-type \( \alpha_4 \beta_2 \) K562 cells were treated with 10 \( \mu \)g/ml TS1/22 Fab against the \( \alpha_4 \) I domain, 10 \( \mu \)g/ml TS1/18 Fab to the \( \beta_2 \) I-like domain, 1 \( M \) BIRT377, or 1 \( M \) XVA143 in 1 mM Ca\(^{2+}\)/Mg\(^{2+}\) or 2 mM Mg\(^{2+}\)/1 mM EGTA for 30 min at room temperature, and then accumulated on an ICAM-1-Fc-coated substrate at 0.3 dyn/cm\(^2\). Wall shear stress was incremented every 10 s and the number of rolling and firmly adherent cells measured at 1 dyn/cm\(^2\). Mean and s.e.m. for three to four experiments.

2001c). Interestingly, in Ca\(^{2+}/\)Mg\(^{2+}\) TS1/18 Fab increased the number of rolling cells, while it decreased by a similar amount the number of firmly adherent cells (Figure 4). Furthermore, whereas in Mg\(^{2+}/\)EGTA almost all cells were firmly adherent, TS1/18 Fab resulted in an almost complete adhesive phase transition, converting \(~90\%\) of the cells to rolling adhesion.

Although TS1/18 Fab and XVA143 each inhibit firm adhesion, they differ in other respects. In Ca\(^{2+}/\)Mg\(^{2+}\), TS1/18 Fab did not increase the total number of adherent cells, whereas XVA143 consistently increased the total number of adherent cells (Figure 4). Furthermore, in contrast to XVA143, TS1/18 Fab did not decrease rolling velocity (data not shown). TS1/18 Fab and XVA143 were further compared for their effect on activation epitope exposure (Table 1). Although there was little KIM127 or m24 epitope exposure in Ca\(^{2+}/\)Mg\(^{2+}\), pretreatment with TS1/18 Fab further reduced epitope exposure. (A and B) \( \alpha_4 \beta_2 \) K562 cells were resuspended in Buffer A/1 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\) in the presence of 0–1000 nM XVA143. Cells were introduced into a flow chamber and allowed to accumulate for 30 s at 0.3 dyn/cm\(^2\) onto an ICAM-1-Fc substrate (see Experimental Procedures). Shear was then increased and the number of rolling cells (A) as well as their rolling velocity (B) was measured at 1, 2, and 4 dyn/cm\(^2\). Average \pm s.e.m. of three experiments.

Figure 3. XVA143 Dose-Dependent Stabilization of Rolling Adhesion Mirrors \( \alpha_4 \beta_2 \) Straightening

(A and B) \( \alpha_4 \beta_2 \) K562 cells were resuspended in Buffer A/1 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\) in the presence of 0–1000 nM XVA143. Cells were introduced into a flow chamber and allowed to accumulate for 30 s at 0.3 dyn/cm\(^2\) onto an ICAM-1-Fc substrate (see Experimental Procedures). Shear was then increased and the number of rolling cells (A) as well as their rolling velocity (B) was measured at 1, 2, and 4 dyn/cm\(^2\). Average \pm s.e.m. of three experiments.

(C) \( \alpha_4 \beta_2 \) K562 cells in HBS/1 mM Ca\(^{2+}\)/1 mM Mg\(^{2+}\) were incubated with different concentrations of XVA143. Binding to activation-dependent mAbs KIM127 and m24 was detected by flow cytometry. Exposure of the epitopes at 1,000 nM XVA143 appeared maximal, because it was equivalent to that in 1 mM Mn\(^{2+}\). Average \pm s.e.m. of two experiments.

and other mAbs that bind to the \( \beta_2 \) I-like domain are allosteric inhibitors, since they inhibit ligand binding by wild-type \( \alpha_4 \beta_2 \) but not \( \alpha_4 \beta_2 \) containing an I domain locked in the high-affinity state with a disulfide bond (Lu et al., 2001c). Interestingly, in Ca\(^{2+}/\)Mg\(^{2+}\) TS1/18 Fab increased the number of rolling cells, while it decreased by a similar amount the number of firmly adherent cells (Figure 4). Furthermore, whereas in Mg\(^{2+}/\)EGTA almost all cells were firmly adherent, TS1/18 Fab resulted in an almost complete adhesive phase transition, converting \(~90\%\) of the cells to rolling adhesion.

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A Single Mutation in the I Domain Linker, E310A, Abrogates Both Integrin Activation and \( \alpha_5\beta_2 \) Straightening, Resulting in Complete Inhibition of Rolling and Firm Adhesion

Mutation of Glu-310 of \( \alpha_5 \) to Ala has been shown to abrogate binding of \( \alpha_5\beta_2 \) to ICAM-1 in a static adhesion assay (Huth et al., 2000). Moreover, this mutation does not respond to activation by an activating mAb (Huth et al., 2000). Residue \( \alpha_5 \)-Glu-310 locates to the linker that follows the C-terminal \( \alpha_7 \)-helix of the I domain, connects to the \( \beta \)-propeller domain, and must be nearby in the 3-dimensional structure to the MIDAS of the \( \beta_2 \) I-like domain. As proposed (Alonso et al., 2002; Shimao et al., 2002), Glu-310 has been demonstrated (Yang et al., 2004b) to function as an intrinsic ligand that binds to the \( \beta_2 \) I-like MIDAS when it is activated, thereby exerting a downward pull on the C-terminal \( \alpha_5 \)-helix of the \( \alpha \) I domain, and activating its MIDAS. Remarkably, experiments with K562 transfectants, which expressed wild-type and mutant \( \alpha_5\beta_2 \) at comparable densities as shown with TS2/4 mAb to a constitutively exposed epitope (Figure 5A), showed that the \( \alpha_5 \)-E310A mutation completely abolished rolling adhesion, firm adhesion, and transient tethering, whether measured in Ca\(^{2+}\)/Mg\(^{2+}\) (Figure 5B), Mn\(^{2+}\) (Figure 5C), Mg\(^{2+}\)/EGTA, or low wall shear stresses of 0.3 dyn/cm\(^2\) (data not shown).

**Table 1. Effect of TS1/18 Fab and XVA143 on Exposure of Activation-Dependent Epitopes**

<table>
<thead>
<tr>
<th></th>
<th>Mouse Fab</th>
<th>TS1/18 Fab</th>
<th>DMSO</th>
<th>XVA143</th>
<th>TS1/18 + XVA143</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM127</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca(^{2+}) + Mg(^{2+})</td>
<td>7.3 ± 0.45</td>
<td>5.2 ± 0.5</td>
<td>9.3 ± 0.7</td>
<td>85.8 ± 1.4</td>
<td>33.2 ± 1.8</td>
</tr>
<tr>
<td>Mg(^{2+}) + EGTA</td>
<td>27.8 ± 3.7</td>
<td>17.6 ± 0.3</td>
<td>21.6 ± 5.0</td>
<td>87.3 ± 2.8</td>
<td>74.7 ± 3.8</td>
</tr>
<tr>
<td>Mn(^{2+}) m24</td>
<td>48.9 ± 3.3</td>
<td>35.8 ± 2.05</td>
<td>54.2 ± 6.1</td>
<td>75.5 ± 3.6</td>
<td>75.0 ± 3.5</td>
</tr>
</tbody>
</table>

Binding of KIM127, m24, and control mouse IgG was determined after incubation with 10 \( \mu \)g/ml control mouse Fab or TS1/18 Fab, 1:10 dilution of DMSO or 1 \( \mu \)M XVA143 in the presence of the indicated divalent cations. KIM127 and m24 binding was measured with FITC anti-mouse IgG, Fc specific, and background with control mouse IgG was subtracted. Values are specific mean fluorescence intensity of three to four independent experiments ± S.D.

Treatment with XVA143 Restores Rolling of the \( \alpha_5\)-E310A \( \beta_2 \) Mutant by Inducing Extension of \( \alpha_5\beta_2 \)

Despite the complete absence of adhesive interactions by the \( \alpha_5\)-E310A mutant, treatment of this mutant with XVA143 restored its ability to support rolling at all wall shear stresses tested (Figure 5B). The complete lack of adhesion of the \( \alpha_5\)-E310A mutant in Mn\(^{2+}\) (Figure 5C) was in excellent agreement with the observation that Mn\(^{2+}\) induced KIM127 epitope exposure in wild-type but not \( \alpha_5\)-E310A mutant (Figure 5A). Similarly, induction of rolling by XVA143 of the \( \alpha_5\)-E310A mutant was in agreement with induction by XVA143 of KIM127 exposure in the \( \alpha_5\)-E310A mutant as efficiently as with wild-type \( \alpha_5\beta_2 \) (Figure 5A). The S114A mutation in the \( \beta_2 \) I-like domain has previously been shown to abolish all tested effects of \( \alpha_5\beta_2 \) I-like allosteric antagonists, suggesting that the \( \beta_2 \) I-like domain MIDAS is part of the binding site for these compounds (Shimaoka et al., 2003a). Additionally, the \( \beta_2\)-S114A mutation completely abolished XVA143-induced rolling (Figure 5B) and KIM127 epitope exposure, i.e., extension (Figure 5A).

**Regulation of Integrin Extension by \( \alpha_5 \) I and \( \beta_2 \) I-like Interdomain Communication**

Overall, the data suggest that communication between the I domain linker and the I-like domain MIDAS is not only crucial for stabilizing the high-affinity conformation of the I domain, but also regulates the equilibrium between the bent and extended \( \alpha_5\beta_2 \) conformers. These interactions are important in Ca\(^{2+}\)/Mg\(^{2+}\) as well as Mn\(^{2+}\), and appear to explain the complete absence in Ca\(^{2+}\)/Mg\(^{2+}\) of rolling interactions in the \( \alpha_5\)-E310A and \( \beta_2\)-S114A mutants (Figure 5B). In support of the suggestion that in the absence of interactions between the \( \alpha_5 \) linker and \( \beta_2 \) I-like domains, \( \alpha_5\beta_2 \) is locked in a bent conformation with virtually no equilibration to the extended conformation, the \( \alpha_5\)-E310A and \( \beta_2\)-S114A mutants reproducibly showed in three of three experiments lower basal binding in Ca\(^{2+}\)/Mg\(^{2+}\) of KIM127 mAb than wild-type transfectants (Figure 5A).

To further test the role of \( \alpha_5\)-Glu-310 in interdomain communication and ligand binding in \( \alpha_5\beta_2 \), its influence on ligand-induced exposure of the KIM127 and m24 epitopes was examined. In the presence of control human IgA complexes with anti-IgA, wild-type \( \alpha_5\beta_2 \) transfectants showed significantly more basal binding of KIM127 mAb than \( \alpha_5\)-E310A \( \beta_2 \) transfectants (Figure 6A), confirming that the E310A mutation pushed the equilibrium between the bent and extended conformations toward the bent conformation with no KIM127 epitope exposure. As shown above (Figure 2A), in Ca\(^{2+}\)/Mg\(^{2+}\), binding of 20 \( \mu \)g/ml sICAM-1-Fc\(\alpha\) complexed with FITC anti-IgA to wild-type \( \alpha_5\beta_2 \) was only moderately above background. Coupling of ligand binding to the KIM127 and m24 epitopes was examined using a high concentration of 100 \( \mu \)g/ml sICAM-1-Fc\(\alpha\) complexed with anti-IgA and coincubation with KIM127 or m24 mAb followed by FITC anti-mouse IgG (Figure 6). For wild-type \( \alpha_5\beta_2 \), multimeric ICAM-1 increased both KIM127 epitope exposure (p < 0.05) and m24 epitope exposure (p < 0.0005), demonstrating ligand binding and its coupling to conformational change. By contrast, multimeric ICAM-1 had no effect on epitope exposure in mutant \( \alpha_5\)-E310A \( \beta_2 \) integrins. This experiment confirms that the equilibria relating ligand binding and exposure of
Figure 5. Straightening of αL-E310Aβ2 by XVA143 Restores Rolling Interactions under Flow

(A) K562 cells expressing wt αL-β2, αL-E310A-β2, or αL-S114A-β2 were incubated with 1 μM XVA143 or an equivalent concentration of DMSO in HBS/Ca2+ + Mg2+, or HBS/Mn2+. Binding of a control mouse IgG (open curves), the αL activation-independent mAb TS2/4, or the αL activation-dependent mAbs KIM127 or m24 (closed curves) was detected using flow cytometry. Numbers in the panels are the mean specific fluorescence intensities (averages of three experiments).

(B) K562 cells expressing wt αL-β2, αL-E310A-β2, or αL-S114A-β2 were incubated in HBS/Ca2+ + Mg2+ with 1 μM XVA143 or the equivalent concentration of DMSO. Cells were allowed to accumulate on an ICAM-1-Fc-coated substrate at 0.3 dyn/cm², and the flow rate was increased every 10 s. The number of cells bound, either firmly adherent or rolling, was analyzed at different wall shear stresses.

(C) Same as (B), except in 1 mM Mn2+. Data are mean ± s.e.m. of three experiments.

KIM127 and m24 epitopes are coupled through αL residue Glu-310, and does not define whether affinity for ICAM-1, extension, or both are affected. Binding of control IgG or TS2/4 (an activation-independent β-propeller mAb) was unchanged by incubation of multimeric ICAM-1 with wild-type αL-β2 or mutant αL-E310A-β2 (data not shown).

XVA143 Treatment Increases the Fraction of Murine Lymphocytes that Roll in Lymph Node Microvessels, and Decreases the Speed at which They Roll

Physiological lymphocyte rolling in peripheral lymph node high endothelial venules (HEV) is mediated primarily by L-selectin binding to its endothelial ligand, peripheral node addressin (PNAd), while the chemokine-triggered binding of activated αLβ2 to endothelial ICAM-1 leads to subsequent firm adhesion (von Andrian and Mackay, 2000). Intravital microscopy (IVM) in mouse intestinal lymph nodes was used to test the effects of XVA143 on lymphocyte rolling and firm adhesion in vivo. Treatment of wild-type murine lymphocytes with 100 μM XVA143 induced a 50% increase in rolling fraction compared to vehicle-treated control cells (from 27 ± 5% to 41 ± 7%) (Figure 7A). This effect of XVA143 was largely eliminated by antibody blockade of αLβ2 function (Figure 7A). Cooperation with L-selectin was required for detection of LFA-1-dependent, XVA143-enhanced lymphocyte rolling, because XVA143-treated lymphocytes from L-selectin−/− mice did not roll in HEV (data not shown). Lymphocytes treated with XVA143 rolled at a markedly slower velocity than cells treated with DMSO vehicle (Figure 7B). Again, this effect of XVA143 was completely reversed by LFA-1 mAb (Figure 7B). The effects on lymphocyte rolling velocity were highly significant (Figure 7 legend). These effects were not due to changes in microvascular hemodynamics between treatment groups (data not shown). Despite the substan-
K562 cells expressing wt or αLβ2, αL-E310A β2 in HBS/Ca\(^{2+}\)/Mg\(^{2+}\) were incubated with ICAM-1-Fc/goat anti-human IgA or human IgA/goat anti-human IgA in the presence of control IgG, TS2/4, m24, or KIM127 mAbs. Binding of the mAbs was detected by FITC-anti-mouse IgG antibody. Binding of control mouse IgG or mAb TS2/4 was unchanged by the presence of multimeric ICAM-1 (not shown). Data are mean ± s.e.m. of three to five experiments. *, p value of IgA versus ICAM-1-Fcα. **, p value of wild-type αLβ2 versus αL-E310A β2.

Discussion

β2 integrins and αLβ2 especially are best known for supporting firm leukocyte adhesion to endothelium and transendothelial migration. Nonetheless, as reviewed in the Introduction, there is extensive in vivo evidence that β2 integrins including αLβ2 also contribute to rolling adhesion by neutrophils, by affecting the efficiency of accumulation of rolling cells or the velocity of rolling cells. How can β2 integrins mediate both rolling and firm adhesion, which can be viewed as phases, analogously to the liquid and crystalline states, with markedly different requirements for receptor-ligand bond formation and dissociation kinetics? Here, we demonstrate that distinct conformational states of αLβ2 regulate rolling and firm adhesion, and that an extended conformation of αLβ2 favors rolling. Furthermore, we demonstrate that the overall conformation of αLβ2 and its ability to support rolling are regulated by interactions between the αL I
and $\beta_2$ I-like domains, and make the surprising observation that an $\alpha/\beta$ I-like allosteric integrin antagonist can inhibit firm adhesion and enhance or induce rolling adhesion.

The findings with XVA143 demonstrate that rolling is induced by $\alpha_1\beta_2$ expansion in the absence of activation of high-affinity ligand binding by the I domain. In Ca$^{2+}$/Mg$^{2+}$, XVA143 enhanced the number of cells rolling through LFA-1 both by increasing the rate of accumulation of cells in shear flow and hence the total number of adherent cells, and also by inhibiting firm adhesion through LFA-1. Mg$^{2+}$/EGTA and Mn$^{2+}$ activate firm adhesion through $\alpha_1\beta_2$. In Mg$^{2+}$/EGTA, XVA143 altered the phenotype from firm adhesion to rolling adhesion. Comparison between XVA143-treated cells in Mg$^{2+}$/Ca$^{2+}$ and Mg$^{2+}$/EGTA shows that Ca$^{2+}$ enhances the stability of rolling interactions. Ca$^{2+}$ is also important for rolling interactions through $\alpha_1$ integrins (de Chateau et al., 2001) and stabilizes the ADMIDAS of integr $\alpha_2\beta_1$, in a conformation that supports rolling (Chen et al., 2003). Peripheral blood lymphocytes failed to mediate rolling on ICAM-1 substrates, consistent with the absence to date of any publications demonstrating rolling solely through $\beta_2$ integrin-ICAM-1 interactions by peripheral blood leukocytes. However, XVA143 treatment enabled LFA-1 on PBL to mediate tethering and rolling in shear flow on ICAM-1 substrates. Together with our in vivo studies, this demonstrates that the physiological density of $\alpha_1\beta_2$ on PBL is sufficient to contribute to rolling, when the conformation of LFA-1 is appropriate.

Direct observations on integrin conformation have thus far come from crystal, EM, and X-ray scattering studies on integrins that lack I domains (Adair and Yeager, 2002; Mudie et al., 2003; Takagi et al., 2003; Xiong et al., 2001, 2002). Three conformations have been revealed for $\alpha_1\beta_2$ (Figures 1F–1H), and similar conformations have been suggested for the I domain-containing integr $\alpha_1\beta_2$ (Beglova et al., 2002; Takagi et al., 2002) (Figures 1A–1E). XVA143 induces exposure of the KIM127 epitope in 1-EGF module 2; strongly suggesting a conversion of $\alpha_1\beta_2$ from a bent to an extended conformation. In Mn$^{2+}$ or Mg$^{2+}$/EGTA, XVA143 blocked soluble, multimeric ICAM-1 binding at subnanomolar concentrations. However, in Ca$^{2+}$/Mg$^{2+}$, XVA143 induced accumulation of rolling cells, a decrease in rolling velocity, and an increase in KIM127 and m24 epitopes, with half-maximal effects between 10 and 100 nM. The finding that XVA143 induces both rolling and integrin expression as measured by KIM127 epitope exposure, and that the dose-response is similar, strongly supports a direct relationship between the extension of LFA-1 and its enhanced ability to support adhesion in shear flow and rolling.

In vivo data suggest that $\beta_2$ integrins and LFA-1 on neutrophils contribute to rolling interactions in inflamed venules (Forlow and Ley, 2001; Forlow et al., 2000; Henderson et al., 2001; Jung et al., 1998; Perry and Granger, 1991), whereas LFA-1 on lymphocytes does not contribute to lymphocyte rolling interactions in uninfamed HEV (Warnock et al., 1998). The latter result is confirmed by our findings, but we also demonstrate that when the extended conformation of LFA-1 with a low-affinity I domain is induced by XVA143, it enhances rolling interactions in HEV. Differences in the contribution of LFA-1 to rolling in vivo may reflect differences in regulation of LFA-1 between lymphocytes or neutrophils, or differences in the concentration and duration of exposure to activating stimuli such as chemokines in inflamed tissues and HEV. Although lower rolling velocity is usually associated with a higher fraction of cells that become firmly adherent, XVA143 markedly decreased the fraction that became firmly adherent in HEV. This finding is in agreement with in vitro observations that XVA143 enhances rolling adhesion while blocking firm adhesion. The effect on firm adhesion in vivo fell short of statistical significance; however, we caution that the pharmacokinetics of XVA143 or any other $\alpha/\beta$ I-like allosteric antagonist are not yet published, and we do not know if XVA143 remained saturating in vivo.

Recently, a “humanized” mAb to the LFA-1 I domain has been approved by the FDA for treatment of moderate to severe psoriasis (Gordon et al., 2003), and $\alpha/\beta$ I-like allosteric antagonists have been proposed as small molecule follow-ons to this antibody (Gadek et al., 2002). We have shown that a mAb to the $\alpha_1$ I domain blocks both firm adhesion and rolling adhesion, whereas $\alpha/\beta$ I-like allosteric antagonists of LFA-1 block firm adhesion while inducing or enhancing rolling adhesion. Because of the importance of firm adhesion by $\beta_2$ integrins, this difference in mechanism of action certainly does not preclude the therapeutic utility of $\alpha/\beta$ I-like allosteric antagonists; however, it does need to be taken into account during preclinical and clinical development. Furthermore, attention should be paid in medicinal chemistry to the difference in potency between blocking high-affinity ligand binding and inducing rolling adhesion, which likely reflects a higher affinity of these antagonists for the active conformation of the $\beta_1$-like domain. It might be possible to find compounds that differ in the ratios of the IC50 values for different conformational states of $\beta_2$ integrins, as has been demonstrated for $\alpha/\beta$ I-like competitive antagonists of $\alpha_{\alpha_1\beta_3}$ (Bednar et al., 1998).

The TS1/18 Fab has a different mechanism of action from XVA143. TS1/18 did not induce rolling by PBL or enhance adhesion in shear flow to ICAM-1 substrates. However, TS1/18 almost completely switched the adhesive phenotype from firm or mixed adhesion to rolling adhesion. Consistent with lack of enhancement of adhesiveness by TS1/18 Fab, it did not increase KIM127 expression. Rather, TS1/18 appears to stabilize the inactive conformation of the $\beta_1$-like domain, inhibiting firm adhesion and enabling rolling adhesion to occur instead.

Overall, the results with XVA143 and TS1/18 Fab suggest that extension of $\alpha_1\beta_2$ favors adhesion, and that the conformation of the $\beta_2$-like domain regulates the affinity of the $\alpha_1$ I domain and whether it mediates rolling adhesion or firm adhesion. Previous studies on mutant I domains expressed as isolated $\alpha_1$ I domains with an artificial transmembrane domain or within intact $\alpha_1\beta_2$ heterodimers have shown that the wild-type, unconstrained $\alpha_1$ I domain mediates rolling, whereas the $\alpha_1$ I domain locked in the high-affinity conformation mediates only firm adhesion (Salas et al., 2002). This finding suggests that XVA143 and TS1/18 each block firm adhesion by blocking the conversion of the $\alpha_1$ I domain to the high-affinity conformation, although they do so by
different mechanisms because XVA143 enhances while T51/18 Fab inhibits induction of the m24 epitope in the $\beta_2$ I-like domain. In terms of currently understood integrin conformations, the conformation mediating rolling appears to most closely correspond to $\alpha_{\text{L}}\beta_2$ in an extended conformation with a low-affinity I domain (Figures 1B and 1C), whereas the conformation that mediates firm adhesion appears to most closely correspond to an extended conformation with a high-affinity $\alpha_{\text{L}}$ I domain (Figure 1E).

The studies with XVA143 and the $\alpha_{\text{L}}$-E310A mutation yield insights into communication between the $\alpha_{\text{L}}$ I and $\beta_2$ I-like domains and how it regulates global conformation and rolling. The $\alpha_{\text{L}}$-Glu-310 residue in the linker between the $\alpha_{\text{L}}$ subunit I and $\beta$-propeller domains appears to bind to the $\beta_2$ I-like MIDAS when it is activated, and through displacement of the C-terminal $\alpha$-helix of the I domain, to transduce activation to the I domain (Figures 1B and 1C). The $\alpha_{\text{L}}$-E310A mutation not only blocks firm adhesion, but also completely blocks rolling adhesion through $\alpha_{\text{L}}\beta_2$.Expression under basal conditions of the KIM127 epitope was significantly decreased, demonstrating coupling of residue $\alpha_{\text{L}}$-E310 in the $\alpha_{\text{L}}$ I domain-$\beta$-propeller linker to a distal site in the 3-dimensional structure in I-EGF2 in the $\beta_2$ genu. These results suggest that under basal conditions, some equilibration between the bent and extended structures occurs, i.e., breathing at the $\beta_2$ genu, and that this breathing is linked to $\alpha_{\text{L}}$ residue Glu-310, possibly by an interaction of this residue with the $\beta_2$ I-like domain MIDAS. Furthermore, the mutation $\alpha_{\text{L}}$-E310A completely abolished increase by soluble, multimeric ICAM-1 of the m24 and KIM127 epitopes, demonstrating either that linkage through $\alpha_{\text{L}}$-Glu-310 is required for the I domain to have sufficient affinity to bind ligand, or that this linkage is required for ligand binding by the I domain to be communicated to the $\beta_2$ I-like domain and the genu. Remarkably, XVA143 rescued the negative effect of the $\alpha_{\text{L}}$-E310A mutation on rolling adhesion. Whereas the $\alpha_{\text{L}}$-E310A mutation ablated rolling adhesion, addition of XVA143 restored rolling almost to wild-type levels, and induction of the m24 and KIM127 epitopes was similar to wild-type. These results further emphasize the importance of the extended integrin conformation for rolling adhesion. They suggest that the $\alpha_{\text{L}}$-E310A $\beta_2$ heterodimer is in a conformation incapable of supporting rolling, likely because it is in the bent conformation and does not appreciably equilibrate with the extended conformation, as suggested by the lower expression of the KIM127 epitope on $\alpha_{\text{L}}$-E310A $\beta_2$ than wild-type $\alpha_{\text{L}}$-$\beta_2$ transfectants.

XVA143 was completely unable to rescue rolling by the $\beta_2$ I-like MIDAS mutant S114A, or as previously described for this mutant, m24 and KIM127 epitope expression (Shimaoka et al., 2003a). This supports the previous conclusion that XVA143 and related $\alpha/\beta$ I-like antagonists utilize as part of their binding site the $\beta_2$ I-like domain MIDAS. The efficacy of XVA143 with the $\alpha_{\text{L}}$-E310A mutant demonstrates that $\alpha_{\text{L}}$-Glu-310 is not an important part of the XVA143 binding site, and is consistent with the model that XVA143 is a mimic of $\alpha_{\text{L}}$-Glu-310 and inhibits its binding to the $\beta_2$ I-like MIDAS.

In contrast to XVA143, Mn$^{2+}$ did not induce straightening of the $\alpha_{\text{L}}$-E310A mutant as shown by lack of induction of the KIM127 epitope, and the mutant was completely incapable of interactions in shear flow in Mn$^{2+}$. Mn$^{2+}$ is a strong activator of wild-type $\alpha_{\text{L}}$-$\beta_2$, inducing opening of the I-like domain as measured with m24 mAb, extension of the integrin as measured with KIM127 mAb, and firm adhesion. Mn$^{2+}$ activates by binding to the I-like domain ADMIDAS, as recently demonstrated with the $\alpha_{\text{L}}$-$\beta_2$ integrin (Chen et al., 2003). Together with the positive control of the effect of XVA143 on the $\alpha_{\text{L}}$-E310A mutant, these results suggest that Mn$^{2+}$ activates adhesiveness of the $\alpha_{\text{L}}$ I domain through interaction of $\alpha_{\text{L}}$-Glu-310 with the I-like domain. The results further demonstrate that the interaction through $\alpha_{\text{L}}$-Glu-310 activated by Mn$^{2+}$ is required for $\alpha_{\text{L}}$-$\beta_2$ extension as measured by KIM127 exposure. Some induction of m24 expression in the I-like domain by Mn$^{2+}$ is noted in the $\alpha_{\text{L}}$-E310A mutant, although it is markedly attenuated compared to wild-type. This is consistent with the ability of Mn$^{2+}$ to bind to the $\beta_2$ I-like domain and partially stabilize localized conformational change, with a partial requirement for linkage through $\alpha_{\text{L}}$-Glu-310 to stabilize this altered local conformation, and a complete requirement for $\alpha_{\text{L}}$-Glu-310 for linkage of local, weak effects of Mn$^{2+}$ to the $\alpha_{\text{L}}$ I domain and exposure of the KIM127 epitope in $\beta_2$ I-EGF module 2.

In summary, our results show that $\alpha_{\text{L}}$-$\beta_2$ mediates rolling when it is in an extended conformation and the $\alpha_{\text{L}}$ I domain is not in a high-affinity state. We demonstrate that equilibration between a bent conformation in which the KIM127 epitope is buried and an extended conformation in which the KIM127 epitope is exposed, i.e., breathing at the headpiece-tailpiece interface, is important for rolling interactions mediated by $\alpha_{\text{L}}$-$\beta_2$. The interface between the I domain linker region and the I-like domain functions as a switch that can regulate the affinity of the $\alpha_{\text{L}}$ I domain as well as the switchblade-like extension of $\alpha_{\text{L}}$-$\beta_2$. In vivo, $\alpha_{\text{L}}$-$\beta_2$ is sufficient by itself to support firm adhesion, and can contribute in combination with other adhesion receptors to rolling adhesion. We demonstrate that a small molecule antagonist can induce rolling adhesion through $\alpha_{\text{L}}$-$\beta_2$, and that distinct conformational states of $\alpha_{\text{L}}$-$\beta_2$ mediate rolling adhesion and firm adhesion. This raises the intriguing possibility that in vivo, distinct conformational states of $\alpha_{\text{L}}$-$\beta_2$ may also be responsible for supporting rolling and firm adhesion. This could occur if intermediate levels of activating stimuli induced an intermediate conformation of LFA-1 that was extended but did not have a fully activated I domain, as depicted in Figures 1B and 1C. Analogous intermediate states of $\alpha_{\text{L}}$-$\beta_2$ have been directly observed (Figure 1G). A novel intermediate conformational state of the $\alpha_{\text{L}}$ I domain has recently been demonstrated structurally, further emphasizing the complexity of the regulation of $\alpha_{\text{L}}$-$\beta_2$ (Shimaoka et al., 2003b).

Experimental Procedures

Cell Lines, Antibodies, and Small Molecules

K562 cells stably transfected with wild-type $\alpha_{\text{L}}$-$\beta_2$ were described previously (Lu et al., 2001b). $\alpha_{\text{L}}$-310A and $\beta_2$-S114A mutants and $\alpha_{\text{L}}$-$\beta_2$, K562 transfectants were as described (Shimaoka et al., 2003a). Similar $\alpha_{\text{L}}$-$\beta_2$ surface expression as wild-type was routinely verified by immunofluorescence flow cytometry. The mouse anti-human $\alpha_{\text{L}}$...
mAbs TS2/6 (Huang and Springer, 1997) and MmH24 (DAKO; Carpinteri, CA), Fab TS2/8, and anti-α5 Fab TS1/22 (Sanchez- Madrid et al., 1982), and matching isotype IgG or Fab controls were used to block LFA-1-mediated interactions. A nonbinding mouse IgG1 (X63 control, anti-human α5 mAbs, TS1/11, TS1/12, TS2/4, and anti-β1 mAb TS1/18 (all IgG1) were used in immunofluorescent flow cytometry (Lu et al., 2001a; Lu and Springer, 1997). Activation-dependent mAbs KIM127 (Robinson et al., 1992) and m24 (Dransfield and Hogg, 1988; Lu et al., 2000c) were kindly provided by M. Robinson (Celltech, Slough, U.K.) and N. Hogg (Imperial Cancer Research Fund, London), respectively. Purified azide-free, low endotoxin anti-mouse CD11a and anti-mouse CD18 (anti-α1, M17/4; anti-β2, GAME46; BD Pharmingen, San Diego, CA) were used in intravitral microscopy experiments. XVA143 (Welzenbach et al., 2002) was from Dr. Paul Gillespie, Roche, Nutley, N.J. BIRT377 (Last-Barney et al., 2001) was from Dr. Terence Kelly, Boehringer Ingelheim Pharmaceuticals Inc., Ridgeway, CT. LFA703 (Weitz-Schmidt et al., 2001) was from Dr. G. Weitz-Schmidt, Novartis Pharma AG (Basel, Switzerland).

Production of Fab

Fab fragments of mouse IgG (control) and mAbs TS1/22 and TS1/18 were prepared via papain cleavage using the ImmunoPure Fab Preparation Kit (Pierce; Rockford, IL) according to the manufacturer's instructions, and used to block adhesion at 10 or 20 μg/ml, which was saturating for the specific Fab.

Peripheral Blood Lymphocytes

Peripheral blood mononuclear cells were prepared by Ficoll-Hypaque centrifugation (Ulmer and Flad, 1979). Monocytes were depleted by diluting mononuclear cells from 43 ml blood in 60 ml of Hank's balanced salt solution supplemented with 1% human serum albumin and 20 mM Hepes (pH 7.2) (HBBS-HSA) supplemented with 1 mM Ca2+/Mg2+ and plating in three gelatin-coated 150 mm cell culture dishes at 37°C for 45 min. The nonadherent lymphocytes were then recovered, pelleted, and resuspended in HBSS-HSA and used in flow chamber experiments.

Soluble ICAM-1 Binding Assay

Cells were harvested, washed with HBSS (20 mM HEPES and 150 mM NaCl [pH 7.3]), and incubated with XVA143 (10 to 105 mM in DMSO) or DMSO alone (1:10 to 1:103 dilution) for 15 min at room temperature (22°C). A mixture of 20 μg/ml of IC1-5/IgA chimera containing the five Ig domains of human ICAM-1 fused to the Fc portion of IgA (ICAM-1-Fc) (Martin et al., 1993) and 25 μg/ml of affinity-purified goat anti-human IgA antibody labeled with FITC (Zymed) was then added to an equal volume (25 μl) of cells and incubated at room temperature. Cells were spun down and resuspended in 2% paraformaldehyde in PBS. Fluorescence was detected using flow cytometry.

Binding of Activation-Dependent mAbs in the Presence of sICAM-1

Wt or α5/E310A β3 transfected K562 cells were resuspended in HBSS/1 mM Ca2+ /1 mM Mg2+. The cell pellet was incubated with a mixture of 100 μg/ml of human IgA or ICAM-1-Fc and 333 μg/ml of affinity purified goat anti-human IgA antibody for 15 min at 37°C. Thereafter, mouse isotype control IgG, TS2/4, m24, or KIM127 in HBSS/Ca2+ /Mg2+ was added at 3 μg/ml for 30 min at 37°C. Cells were washed and stained with a FITC-goat anti-mouse IgG for 30 min at 4°C. After washing, cells were fixed with 2% paraformaldehyde and subjected to immunofluorescence flow cytometry.

Cell Adhesion to Immobilized ICAM-1 under Shear Flow

A chimera containing the five Ig domains of human ICAM-1 fused to the Fc portion of IgG (ICAM-1-Fc) at 10 or 60 μg/ml in PBS and 20 mM bicarbonate (pH 9.0) (Coating Buffer) for K562 transfectants and PBL, respectively, was spotted on a dish previously coated with 20 μl [20 μg/ml] protein A (Zymed; San Francisco, CA) in Coating Buffer and blocked with 2% human serum albumin (HSA). ICAM-1 substrates were assembled as the lower wall in a parallel-wall flow chamber and mounted on an inverted phase-contrast microscope (Lawrence and Springer, 1991). Cells were washed twice with Ca2+/Mg2+-free HBSS/10 mM Hepes (pH 7.4)/5 mM EDTA/0.5% bovine serum albumin (BSA), resuspended at 6 x 106/ml in Ca2+/Mg2+-free HBSS/10 mM Hepes (pH 7.4)/5 mM EDTA/0.5% BSA (buffer A), and kept at room temperature (22°C) throughout the experiment. Cells were diluted to 5 x 105/ml in buffer A containing 1 mM Ca2+/1 mM Mg2+ or 2 mM Ca2+/1 mM EGTA, immediately before infusion in the flow chamber using an automated syringe pump. Cells were incubated with XVA143 (1 μM in DMSO); DMSO alone, or mAbs (as IgG or Fab fragments) at saturating concentrations, for 15 min at room temperature. Images were captured using a CCD camera mounted on an inverted microscope with a 10 x objective and recorded on Hi-8 videotape.

Quantification of Interactions under Increasing Shear Forces

Cells were allowed to accumulate at 0.3 dyn/cm2 for 30 s. Shear stress was then increased to 0.4 dyn/cm2 and incremented every 10 s up to 36 dynes/cm2. Rolling velocity at each shear stress was calculated from the average distance traveled by rolling cells in 3 s. The number of cells interacting for more than 3 s with the coated surface was measured at each shear stress. To avoid confusing rolling with small amounts of movement due to tether stretching or measurement error, a velocity of 1.5 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.

Accumulation of Cells under a Fixed Shear Force

Cells were prepared as described above, resuspended in buffer A containing 1 mM Ca2+/1 mM Mg2+, and as indicated 1 μM XVA143. The cells were infused into the flow chamber, and immediately subjected to a fixed wall shear stress. The number of cells bound (rolling or firm adherent) was determined at different time points.

Statistical Analysis

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

Intravitral Microscopy

WT and L-selectin-deficient (L-selectin−/−) mice on a mixed C57/B6 × 129 background were used as lymphocyte donors, and P-selectin-deficient mice (P-selectin−/−; also on a C57/B6 × 129 background) were used as recipients in intravitral microscopy experiments (Weninger et al., 2000). Mice were given intra-arterial injections of XVA143 (10 μl of 10 μM) and 50 μg blocking anti-α5, or anti-β3 antibody at 37°C for at least 10 min. Small boluses of control lymphocytes were then injected intra-arterially and their adhesive behavior in LN HEV was recorded. Subsequently, recipient mice were given intra-arterial injections of XVA143 (10 μl of 100 μM) and XVA143-treated cells were injected and recorded passing through the same microvessels. In two experiments, recipient mice were then treated with either anti-α5 or anti-β3 antibody (50 μg) and XVA143 plus antibody-treated cells were injected. Labeled cells were visualized via epifluorescence (von Andrian and M’Rini, 1998).

Interactions between circulating cells and lymph node HEV were analyzed from recorded video images. The rolling fraction for each HEV was calculated as the percentage of labeled cells rolling on the endothelial surface among all labeled cells passing through the HEV. The sticking fraction was calculated as the percentage of cells arresting for >30 s among all rolling cells. Distances traversed by
rolling cells were measured using frame-by-frame video image analysis with specialized software (Velocity Imaging Software, Velocity-Meter, A.R. Preis, Berlin), and then divided by the elapsed time to obtain average rolling velocities ($v_{\text{roll}}$). Cumulative velocity curves were as described (Stein et al., 1999).

Means are given ± SEM. Statistical differences between two data sets were analyzed by paired or unpaired two-tailed Student's $t$ tests, as indicated. Differences between multiple data sets assuming Gaussian distributions (e.g., rolling fraction) were analyzed by repeated measurements ANOVA with Bonferroni correction. For multiple data sets with non-Gaussian distributions (e.g., rolling velocities), the nonparametric Kruskal-Wallis/Dunn’s Multiple Comparison test was used.

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