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Transition From Rolling to Firm Adhesion Is Regulated by the Conformation of the I Domain of the Integrin Lymphocyte Function-associated Antigen-1*

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The integrin lymphocyte function-associated antigen-1 ($\alpha_L\beta_2$), which is known for its ability to mediate firm adhesion and migration, can also contribute to tethering and rolling in shear flow. The α_L I domain can be mutationally locked with disulfide bonds into two distinct conformations, open and closed, which have high and low affinity for the ligand intercellular adhesion molecule 1 (ICAM-1), respectively. The wild type I domain exists primarily in the lower energy closed conformation. We have measured for the first time the effect of conformational change on adhesive behavior in shear flow. We show that wild type and locked open I domains, expressed in $\alpha_L\beta_2$ heterodimers or as isolated domains on the cell surface, mediate rolling adhesion and firm adhesion, respectively. $\alpha_L\beta_2$ is thus poised for the conversion of rolling to firm adhesion upon integrin activation *in vivo*. Isolated I domains are surprisingly more effective than $\alpha_L\beta_2$ in interactions in shear flow, which may in part be a consequence of the presence of $\alpha_L\beta_2$ in a bent conformation. Furthermore, the force exerted on the C-terminal α-helix appears to stabilize the open conformation of the wild type isolated I domain and contribute to its robustness in supporting rolling. An allosteric small molecule antagonist of $\alpha_L\beta_2$ inhibits both rolling adhesion and firm adhesion, which has important implications for its mode of action *in vivo*.

Two distinct adhesive modalities are required for leukocyte accumulation at inflammatory sites and lymphocyte homing. Rolling adhesion greatly increases the time a cell spends in a post-capillary venule and enables surveillance of endothelium for activating signals such as chemoattractants. Firm adhesion results in the arrest of the leukocyte in the postcapillary venule, and sets the stage for diapedesis.

The integrin $\alpha_L\beta_2$ (lymphocyte function-associated antigen-1 (LFA-1)[1]) mediates adhesion and migration of leukocytes in immune and inflammatory processes by binding to intercellular adhesion molecules (ICAMs), which are members of the Ig superfamily (1). Dynamic regulation of ligand-binding activity by $\alpha_L\beta_2$ and other integrins in response to signals transmitted from inside the cell (inside-out signaling) activates β_2 integrin adhesiveness in response to engagement of the antigen receptor on T lymphocytes in immune responses, and in response to chemoattractant binding to G-protein-coupled receptors in leukocyte adhesion to endothelium (2–4). β_2 integrin-mediated arrest of rolling leukocytes within the vasculature occurs on a second time scale, enabling arrest to occur in the same postcapillary bed where chemoattractant is encountered.

The β_2 integrins are far more facile in mediating firm adhesion than rolling adhesion. In many *in vitro* and *in vivo* systems in which β_2 integrins mediate firm adhesion and selectins mediate rolling adhesion, β_2 integrins are not seen to mediate tethering in shear flow or rolling (2, 3). The α_4 integrins have been known for some time to mediate rolling as well as firm adhesion (5, 6), although they do not support rolling as efficiently as selectins (7).

Recently, β_2 integrins have also been found to be capable of contributing to rolling *in vivo* and in special cases to support on their own rolling *in vitro*. When multiple adhesion pathways are blocked *in vivo*, *i.e.* selectins together with β_2 integrins or ICAM-1, or LFA-1 together with α_4 integrins, β_2 integrins and in particular $\alpha_L\beta_2$ can be seen to contribute to accumulation of rolling cells, the stability of rolling, and the velocity of rolling cells (8–10). $\alpha_L\beta_2$ can mediate tethering in flow of leukocytes to ICAM-2 on platelets in the absence of selectin-mediated interactions (11). An important ligand-binding domain of $\alpha_L\beta_2$, the inserted (I) domain of the α_L subunit, has been expressed on the cell surface in isolation from other integrin domains and found to support rolling on immobilized ICAM-1 under shear flow (12). It was suggested that the I domain represents a transient ligand-binding domain and that cooperation with other ligand-binding domains was required for firm adhesion. However, recent studies have shown that the isolated I domain, when stabilized in a conformation that has high affinity for ligand, is sufficient for the same amount of adhesiveness in static binding assays as maximally activated $\alpha_L\beta_2$ (13), and binds with the same kinetics and affinity as activated $\alpha_L\beta_2$ in real-time soluble ligand-binding assays (14). $\alpha_L\beta_2$ expressed in K562 cells, which shows little basal activity in static adhesion assays (13, 15), has recently been shown to support rolling on ICAM-1 in shear flow, whereas $\alpha_L\beta_2$ expressed in Jurkat cells, which shows basal adhesion to ICAM-1 in static adhesion assays that is further inducible with activation, supported weak adhesion without rolling (16).

In integrins that contain I domains, conformational changes within the I domain regulate ligand binding (17). A downward movement of the C-terminal α-helix of the I domain is linked to structural rearrangements in the metal ion adhesion site and surrounding loops that constitute the ligand-binding site of the I domain, as shown by crystallographic studies of α_M and α_2

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[1] The abbreviations used are: LFA, lymphocyte function-associated antigen-1; ICAM, intercellular adhesion molecule; PDGFR, platelet-derived growth factor receptor; TNF-α, tumor necrosis factor α; DTT, dithiothreitol; dyn, dynes; HUVEC, human umbilical vein endothelial cells; NMR, nuclear magnetic resonance.

(18, 19). Movements in the same regions of the α_L I domain occur in the presence of an ICAM-1 fragment as shown by NMR chemical shift experiments (20). Two conformations of the I domain termed open and closed have been shown to have high and low affinity for ligand, respectively. An engineered disulfide bond in the α_L I domain that locks the loop between the C-terminal β-strand and α-helix into the open conformation has been shown to activate cell adhesiveness in static binding assays and to increase the affinity of the locked open I domain in soluble ligand-binding assays 9,000-fold relative to the wild type I domain (13–15). The increase in affinity was the result of a 50-fold increase in on-rate to 140,000 $M^{-1}s^{-1}$ and a 200-fold decrease in off-rate from 5 s^{-1} to 0.025 s^{-1}. A mutant I domain that was analogously locked in the closed conformation was similar in affinity to the wild type I domain, and all evidence suggests that the closed conformation is lower energy than the open conformation and is the predominant conformation assumed by isolated I domains.

Small molecule antagonists directed to the I domain of $\alpha_L\beta_2$ have been developed. Crystal and NMR structures show that they bind to the closed conformation of the I domain, between the C-terminal α-helix and the body of the domain, distal from the ligand-binding site on the "top" of the I domain (21, 22). These antagonists are allosteric modulators that stabilize the closed conformation of the I domain, as confirmed by failure to antagonize I domains locked in the open conformation with disulfide bonds (15).

The effect of conformational alterations in integrins on rolling interactions has not been studied, although it is known in general that activation can convert rolling to firm adhesion for both α_4 and β_2 integrins. It has long been hypothesized that rapid kinetics for bond association and dissociation are important in rolling (23), and that slower bond dissociation would favor firm adhesion. Although there exists now an extensive body of literature on the kinetics of bond dissociation for receptors that mediate rolling (24), there is no data on how conformational change, with accompanying alterations in bond association and dissociation kinetics, would affect adhesive behavior in shear flow. Recent crystal structure, NMR, and electron microscopic studies have revealed that integrins, including $\alpha_L\beta_2$, assume a highly bent conformation in the resting state, and that activation results in a dramatic switchblade-like opening (25–27). In the bent conformation the headpiece is close to the membrane, whereas in the active, extended conformation it moves ~15 nm upwards and into an orientation much more favorable for ligand binding. Furthermore, these conformations are in rapid equilibrium, and activation should be viewed as a shift in the equilibrium rather than fixing a particular conformation. The I domain, although not directly visualized in these studies, is connected to the headpiece and would become dramatically more accessible to ligand in the extended conformation. To examine the effect of only conformational change within the I domain, which is likely to be linked to global conformational change in native integrin heterodimers, we study I domains expressed in isolation from other integrin domains on the cell surface. We demonstrate that α_L integrin I domain conformation, as influenced by disulfide bonds that lock in specific conformations, or binding of a small molecule antagonist that stabilizes the closed conformation, regulates rolling interactions in shear flow. Furthermore, we compare for the first time the efficacy of cell surface $\alpha_L\beta_2$ heterodimers and isolated α_L I domains in rolling assays, and describe some surprising differences.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—K562 cells stably transfected with $\alpha_L\beta_2$ containing wild type, locked open (K287C/K294C), or locked closed (L289C/K294C) I domains, or these I domains expressed in the absence of other integrin domains using a platelet-derived growth factor receptor (PDGFR) transmembrane domain and the first five amino acid residues of the PDGFR cytoplasmic domain were described previously (13). All mutant constructs were verified here to be expressed at similar levels on the cell surface as previously shown (13, 15). $\alpha_L\beta_2$ and I domain transfected cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin/streptomycin, and 3 μg/ml puromycin or 100 μg/ml hygromycin, respectively. The mouse anti-human α_L monoclonal antibodies TS2/6 (28) and MHM24 (DAKO, Carpinteria, CA) were used to block LFA-1-mediated interactions. A non-binding mouse IgG1 (X63) as control and two different anti-human α_L I domain monoclonal antibodies, TS1/11 and TS1/12, were used to determine surface expression of the transfectants by immunofluorescence flow cytometry (29).

Cell Adhesion to Immobilized ICAM-1 under Static Conditions—Adhesion of K562 cell transfectants to ICAM-1 purified from human tonsil (30) and coated at 6 μg/ml on 96-well plates was assayed as described (29). Cells were labeled with 2′,7′-bis-(carboxyethyl)-5-(and -6)-carboxyfluorescein, acetoxymethyl ester, and resuspended in Hank's balanced salt solution, 10 mM HEPES, pH 7.4, 0.5% bovine serum albumin containing either 1 mM Ca^{2+} + 1 mM Mg^{2+}, or 2 mM Mg^{2+} + 1 mM EGTA. Cells were added to the ICAM-1-coated wells and incubated for 30 min at 37 °C. After incubation wells were washed and the fluorescence read.

Cell Adhesion to Immobilized ICAM-1 or HUVECs Under Shear Flow—Three different forms of ICAM-1 were immobilized on substrates. Human tonsil ICAM-1 was directly coated on polystyrene Petri dishes for 1 h at 37 °C in coating buffer (phosphate-buffered saline, 20 mM bicarbonate, pH 9.0). Substrates were washed and blocked with 2% human serum albumin in coating buffer for 1 h at 37 °C. Soluble IC1–5/IgA chimera containing the five Ig domains of human ICAM-1 fused to the Fc portion of IgA (ICAM-1-Fcα) was described previously (31). ICAM-1-Fcα (10 μg/ml in coating buffer) was spotted on a dish previously coated with 20 μg/ml goat anti-human IgA in coating buffer (Zymed Laboratories Inc., San Francisco, CA) and blocked with 2% human serum albumin in coating buffer. ICAM-1-Fcγ (10 μg/ml in coating buffer unless noted otherwise) was spotted on a dish previously coated with protein A (20 μg/ml) in coating buffer for 1 h at 37 °C and blocked with 2% human serum albumin in coating buffer (32). HUVECs (American Type Culture Collection) were maintained in medium 199 modified Earle's salt solution containing 20% fetal bovine serum, 100 mg/ml endothelial growth supplement (Sigma), 1% Nutridoma-NS (Roche), and 100 μg/ml heparin at 37 °C in humidified air containing 5% CO_2. Cells were grown on polystyrene cell culture dishes pre-coated with 10 μg/ml fibronectin and used for no more than five passages. For flow experiments, HUVECs were seeded onto fibronectin-coated wells of six-well cell culture dishes at 90% confluency and cultured for 3–4 days prior to use. Cells either received no treatment or were activated with TNF-α (100 ng/ml) for 5 or 24 h prior to each experiment. ICAM-1 surface expression was determined by flow cytometry using IC1/12, a mouse anti-human ICAM-1 monoclonal antibody (33) directly conjugated with Alexa488, and CBRM1/23-Alexa488, an anti-human α_M monoclonal antibody (34) as a negative control.

ICAM-1 substrates or HUVEC monolayers were assembled as the lower wall in a parallel wall flow chamber and mounted on an inverted phase-contrast microscope (2). Cells were washed twice with Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution, 10 mM Hepes, pH 7.4, 5 mM EDTA, 0.5% bovine serum albumin and resuspended at 5×10^6/ml in Ca^{2+} and Mg^{2+}-free Hank's balanced salt solution, 10 mM Hepes, 0.5% bovine serum albumin (buffer A) and kept at room temperature (22 °C) throughout the experiment. Cells were diluted to 5×10^5/ml in buffer A containing 1 mM Ca^{2+} + 1 mM Mg^{2+} or 2 mM Mg^{2+} + 1 mM EGTA immediately before infusion in the flow chamber using an automated syringe pump. Images were captured using a CCD camera mounted on an inverted microscope with a 10× objective and recorded on Hi-8 videotape.

Accumulation in Shear Flow and Rolling Velocity—Cells were allowed to accumulate at 0.3 dynes/cm^2 for 30 s. Shear stress was increased every 10 s up to 36 dynes/cm^2. 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.

Shear Detachment Assays—To evaluate the strength of the $\alpha_L\beta_2$ and I domain interactions with ICAM-1, cells were infused into the flow chamber and allowed to settle onto the substrate in stasis for 2 or 5 min as indicated. Flow was initiated at a shear stress of 0.3 dyn/cm^2 and increased every 10 s. The number of cells that remained attached at the end of each shear interval was counted.

The Effect of LFA703 on LFA-1-ICAM-1 Interactions—The statin-like LFA-1 antagonist LFA703 (35) was kindly provided by Novartis (Basel, Switzerland). LFA703 (100 mM in Me_2SO) was diluted in assay buffer (Hank's balanced salt solution/Hepes/bovine serum albumin). Cells were preincubated with LFA703 (0–100 μM) at room temperature for 15–30 min prior to infusion in the flow chamber. The 0 μM LFA703 or Me_2SO control used the same concentration of Me_2SO (0.1%) as in the highest LFA703 concentration.

The Effect of Latrunculin A on LFA-1 and I Domain-ICAM-1 Interactions—Wild type and high affinity $\alpha_L\beta_2$ and I domain expressing K562 cells were incubated with 1 μM latrunculin A (Calbiochem) or Me_2SO for 20 min at RT. (22 °C).

Cells were resuspended in Buffer A containing 2 mM Mg^{2+}. Cells were infused at 10^7/ml and accumulated as described above. Shear stress was increased every 10 s, and the number of cells adherent at the end of each 10 s interval was counted.

RESULTS

Comparison of Static and Shear Flow Adhesion Assays—Binding of transfected cells expressing wild type $\alpha_L\beta_2$ heterodimer or the wild type or locked closed isolated α_L I domain to substrates coated with native ICAM-1 purified from tonsils is barely detectable if at all in conventional static adhesion assays in Ca^{2+} and Mg^{2+} (Fig. 1*A*) (13). However, locking the I domain in the open conformation by mutational introduction of a disulfide bond, or activation of wild type $\alpha_L\beta_2$ heterodimers with Mg^{2+}/EGTA results in marked binding to ICAM-1 (Fig. 1*A*) (13). Contrasting results in Ca^{2+} and Mg^{2+} were obtained when cells were allowed to settle in stasis for 5 min on native ICAM-1 substrates and then subjected to controlled shear flow forces. Laminar flow was initiated at a wall shear stress of 0.3 dyn/cm^2 and then incremented every 10 s. The number of cells that were initially in the field of view at stasis was determined, and then the percentage remaining at the end of each 10 s step was determined (Fig. 1*B*). In agreement with the data in the static adhesion assays in Ca^{2+} and Mg^{2+}, K562 transfectants expressing locked open $\alpha_L\beta_2$ heterodimers and the locked open α_L I domain were able to bind to ICAM-1. In both cases the cells were firmly adherent; the average velocity of all adherent cells was close to 0 μm/s (Fig. 1*C*), and detachment from the substrate was not preceded by rolling. In further agreement with the results of the static assays, transfectants with closed I domains or closed heterodimers were not adherent, and transfectants with wild type $\alpha_L\beta_2$ heterodimers were also not adherent. However, in marked contrast to the results of the static assays, transfectants expressing the wild type isolated I domain were adherent in the shear flow assay (Fig. 1*B*). Furthermore, this was accompanied by a contrasting adhesive behavior: the cells rolled (12) (Fig. 1*C*). Excluding the highest shear stress, at which few cells remained, the cells rolled at a velocity of 15 to 30 μm/s, corresponding to several cell diameters per second.

Effect of Presentation of ICAM-1 on the Substrate on Adhesion in Shear Flow—Although we found that native ICAM-1 adsorbed to a substrate did not support adhesion by wild type $\alpha_L\beta_2$ transfectants, these transfectants have been reported to support rolling adhesion on ICAM-1-Fcγ chimeras bound to protein A substrates. Therefore, we compared different types of ICAM-1 substrates for support of adhesion in shear flow in the presence of 1 mM Ca^{2+} + 1 mM Mg^{2+}. The initial cell binding to the substrate occurred in shear flow at 0.3 dyn/cm^2 rather than in stasis. ICAM-1 directly adsorbed to substrates or ICAM-1 fused to the Fc portion of IgA (ICAM-1-Fcα) and immobilized by binding to anti-IgA on a substrate supported adhesion in shear flow and rolling of cells expressing the α_L I domain, but not adhesion in shear flow and rolling of cells expressing $\alpha_L\beta_2$ (data not shown). However, ICAM-1 fused to the Fc portion of IgG (ICAM-1-Fcγ) and immobilized by binding to protein A on a substrate supported rolling of both I domain and $\alpha_L\beta_2$ transfectants (16) (Fig. 2). Rolling through $\alpha_L\beta_2$ was faster in velocity and less shear-resistant than rolling through the α_L I domain (Fig. 2). The ability of unactivated $\alpha_L\beta_2$ to support rolling was not nearly as robust as the ability of activated $\alpha_L\beta_2$ to support firm adhesion but was consistent with its ability to contribute, in combination with other adhesion pathways, to tethering and rolling *in vivo* and on cellular substrates *in vitro* (see Introduction). The great sensitivity of native wild type $\alpha_L\beta_2$ but not the isolated α_L I domain to the mode of ICAM-1 presentation on the substrate is consistent with adoption of the bent conformation by resting $\alpha_L\beta_2$, in which the I domain would have limited accessibility to ligand (26).

To compare interactions mediated by the isolated I domain

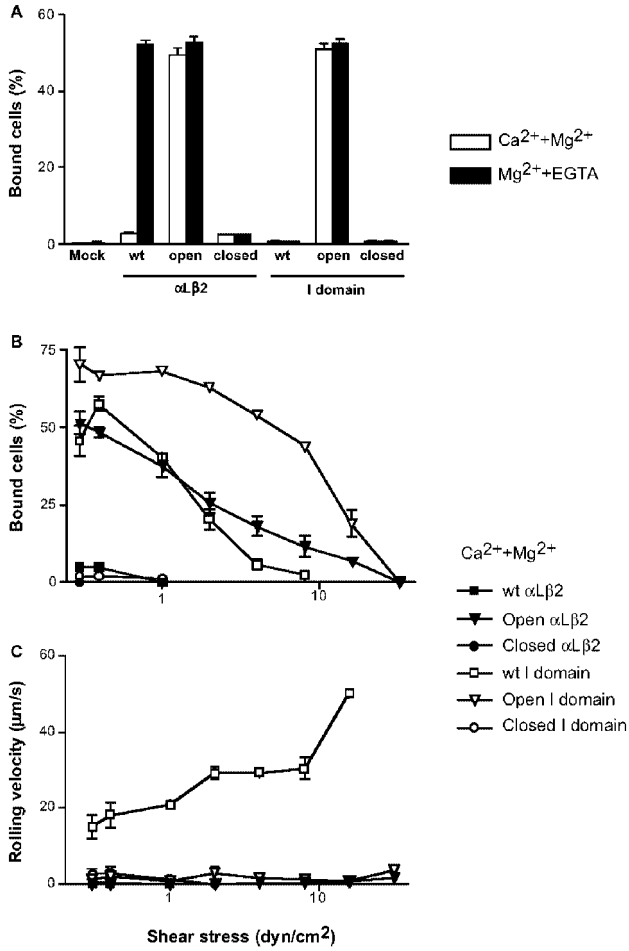

FIG. 1. **Binding of K562 transfectants to immobilized ICAM-1.** K562 transfectants expressed the indicated I domain mutants within intact $\alpha_L\beta_2$ or as isolated α_L I domains linked to the transmembrane and first 5 amino acid residues of the cytoplasmic domain of the PDGF receptor. All assays in this figure are with tonsil (native) ICAM-1 adsorbed to substrates at 6 μg/ml. *A*, static binding assays. Cells were allowed to bind in the indicated cations for 30 min at 37 °C to ICAM-1 in 96-well plates, and wells were washed by aspiration. Results are mean ± S.D. of duplicate samples from three different experiments. *B* and *C*, cells were infused into the flow chamber in medium containing 1 mM Ca^{2+} + 1 mM Mg^{2+} and allowed to settle in stasis for 5 min onto a substrate coated with ICAM-1. Flow was then initiated at a wall shear stress of 0.3 dyn/cm^2 and increased every 10 s to the indicated values. The number of cells that remained bound at the end of each interval (*B*) and the average rolling velocity of all rolling and firmly adherent cells (*C*) was measured.

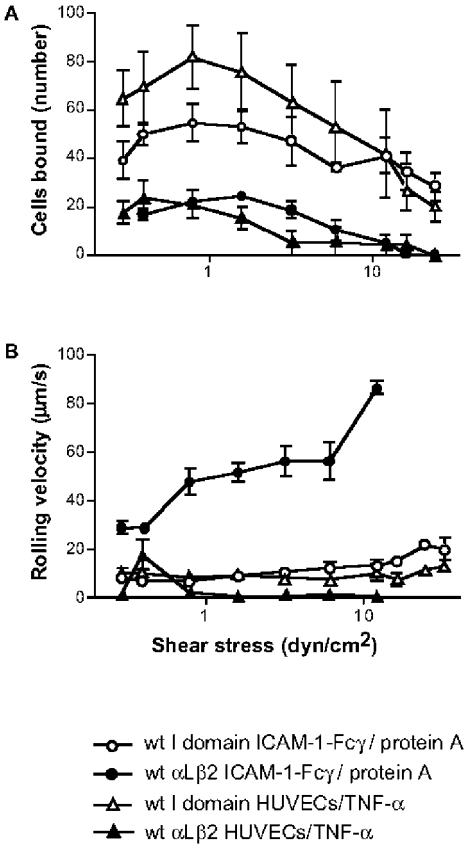

FIG. 2. **Rolling of K562 transfectants expressing wild type isolated α_L I domain or $\alpha_L\beta_2$ on ICAM-1.** Substrates consisted of ICAM-1-Fcγ/protein A coated on a plastic surface as described in "Experimental Procedures" or a monolayer of HUVECs stimulated with 100 ng/ml TNF-α for 5 h. Cells were infused into the flow chamber and allowed to accumulate for 30 s at 0.3 dyn/cm^2. Further accumulation or detachment occurred as the wall shear stress was increased in steps every 10 s. *A*, total adherent cells (rollingly and firmly adherent). *B*, average velocity of the adherent cells. Values show mean ± S.D. for three independent experiments.

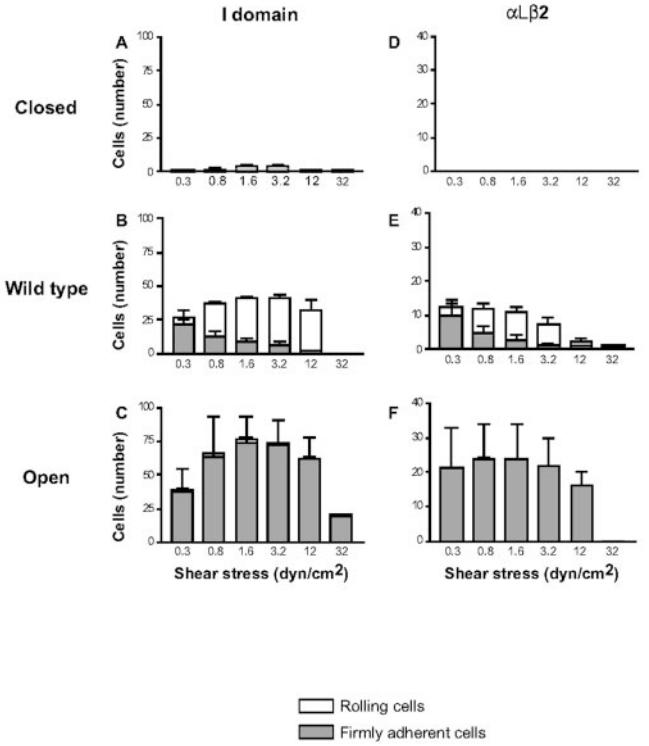

FIG. 3. **Effect of locking the I domain in a closed or an open conformation on interactions under shear stress.** ICAM-1-Fcγ was coated at 100 μg/ml (*A* and *D*) or 10 μg/ml (*B*, *C*, *E*, and *F*). K562 transfectants expressing the indicated wild type or mutant isolated α_L I domains or $\alpha_L\beta_2$ heterodimers were infused into the flow chamber in medium containing 1 mM Ca^{2+} + 1 mM Mg^{2+} and allowed to accumulate for 30 s at a wall shear stress of 0.3 dyn/cm^2 on a substrate coated with ICAM-1-Fcγ/protein A. Thereafter, shear was increased every 10 s, and the number of rollingly adherent cells (*white bars*) or firmly adherent cells (*gray bars*) was enumerated at each shear interval. Only cells interacting for ≥ 3 s were counted.

and $\alpha_L\beta_2$ using a more physiologically relevant presentation and density of ICAM-1, we used a monolayer of HUVECs either in basal conditions or after stimulation with TNF-α for 5 or 24 h. In basal conditions, HUVECs express low amounts of ICAM-1 on their surface (6.6 M.F.I. measured with IC1/12 monoclonal antibody directly conjugated with Alexa488) and support no interaction in shear flow with K562 cells expressing the isolated I domain or $\alpha_L\beta_2$ (not shown). After incubation with 100 ng/ml TNF-α, ICAM-1 expression increased in a time-dependent manner (57.3 mean fluorescence intensity at 5 h and 238.9 M.F.I. at 24 h) as previously shown (36).

The isolated I domain expressed on K562 cells efficiently interacted with TNF-α-stimulated endothelial cells (Fig. 2). The number and velocity of cells rolling on HUVEC stimulated 5 h with TNF-α was comparable with the number and velocity of rolling cells observed in 10 μg/ml ICAM-1-Fcγ/protein A-coated substrates (Fig. 2).

Compared with cells expressing the isolated I domain, K562 cells expressing $\alpha_L\beta_2$ bound less efficiently to stimulated HUVEC monolayers. The number of adherent $\alpha_L\beta_2$-K562 cells on HUVEC and ICAM-1-Fcγ/protein A-coated substrates was comparable (Fig. 2*A*). Most cells adhered to the substrate during flow at 0.3 dyn/cm^2 because there was little additional adhesion after the flow rate was increased (Fig. 2*A*). The K562 cells expressing $\alpha_L\beta_2$ rolled when flow was incremented to 0.4 dyn/cm^2 but then quickly became firmly adherent (Fig. 2*B*). This reflects a difference in behavior on HUVEC compared with purified substrates that may reflect the ability of TNF-stimulated HUVEC to activate adhesion of $\alpha_L\beta_2$ on K562 transfectants. However, the main point for this study is that adhesion and rolling of I domain transfectants is very similar on ICAM-1-Fcγ/protein A and TNF-stimulated HUVEC substrates.

Controls in all experiments showed that rolling and firm adhesion were dependent on the I domain, as demonstrated by complete abrogation by two different antibodies to the I domain, TS2/6 and MHM24 (data not shown). Furthermore, both rolling and firm adhesion were completely abolished by EDTA (data not shown).

Effect of I Domain Conformation on Adhesion in Shear Flow—To examine in the context of both $\alpha_L\beta_2$ and the isolated α_L I domain the effect of I domain conformation on adhesive behavior in shear flow, transfectants were allowed to accumulate on ICAM-1-Fcγ/protein A substrates for 30 s at 0.3 dyn/cm^2 and as the wall shear stress was increased every 10 s (Fig. 3). The overall behavior in shear flow was similar for the isolated I domain and $\alpha_L\beta_2$ transfectants, except the number of cells that tethered to the substrate in shear flow was greater for I domain than $\alpha_L\beta_2$ transfectants (note difference in scale between Fig. 3, *A–C* and *D–F*).

Cells expressing the locked closed low affinity conformation of the I domain showed very little tethering to the ICAM-1 substrate. At the standard coating concentration of 10 μg/ml ICAM-1-Fcγ used elsewhere in this manuscript and in Fig. 3, *B–C* and *E–F*, no tethering of the closed I domain was seen. 100

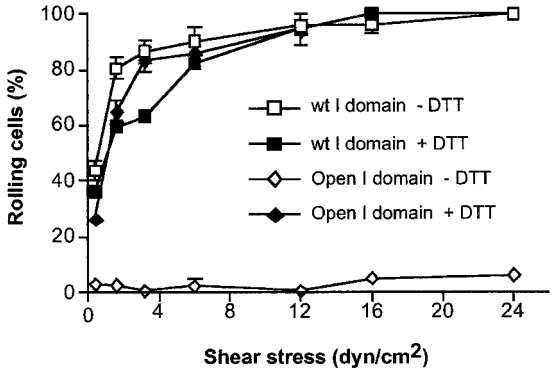

FIG. 4. **Effect of disulfide bond reduction on adhesive behavior in shear flow mediated by the open mutant isolated I domain.** K562 transfectants expressing wild type or open mutant I domains (K287C/K294C) were incubated with or without 10 mM DTT for 20 min at 37 °C. Cells were infused into the flow chamber at a wall shear stress of 0.3 dyn/cm^2, and the shear stress was incremented every 10 s. The percentage of the total adherent cells that were rollingly adherent was determined.

μg/ml was required to detect tethering of the locked closed I domain (Fig. 3*A*), and even this concentration did not support tethering of locked closed $\alpha_L\beta_2$ (Fig. 3*D*). The few cells that tethered showed rolling interactions, and the rolling cells were detached at higher shears (Fig. 3*A*).

Transfectants expressing wild type $\alpha_L\beta_2$ or the α_L I domain tethered in shear flow and the majority of adherent cells rolled (Fig. 3, *B* and *E*). The percentage of rolling cells increased with shear, so that the vast majority of cells were rolling at ≥ 0.8 dyn/cm^2, both for I domain and $\alpha_L\beta_2$ transfectants.

Cells expressing the high affinity, open mutation of the I domain tethered ~2-fold more efficiently compared with wild type (Fig. 3, *C* and *F*). However, in marked contrast to wild type, > 95% of the tethered cells were firmly adherent even at the highest shears tested. Furthermore, the open mutant transfectants were more resistant to detachment at higher wall shear stresses. The behavior of cells bearing the open mutation in the isolated I domain or in $\alpha_L\beta_2$ was qualitatively similar.

We examined the requirement of the disulfide bond in the open mutant I domain for firm adhesion in shear flow. Previous studies showed that treatment of the open mutant I domain with a reducing agent abolished its increased affinity for ICAM-1 (14) and also abolished adhesion of isolated I domain transfectants to ICAM-1-coated plates under static conditions (13). Thus the disulfide bridge is required to lock the mutant I domain in the high affinity, open conformation. Very few transfectants expressing the open mutant I domain rolled on ICAM-1 over a range of shear stresses (Fig. 4), confirming the results in Fig. 3*C*. However, after treatment with the reducing agent DTT for 20 min at 37 °C, K562 cells expressing the open mutant I domain rolled as efficiently as cells expressing the wild type I domain (Fig. 4). Furthermore, DTT treatment of K562 cells expressing the wild type I domain had no effect on rolling. Thus, the effect of locking the I domain in the open conformation with a disulfide bond is reversible with DTT treatment, and the effect of DTT treatment on adhesive behavior in shear flow, *i.e.* conversion of firm adhesion to rolling, mirrors its effect on I domain affinity for ICAM-1.

Differential Effect of Divalent Cations on $\alpha_L\beta_2$ and α_L I Domain Interactions in Shear Flow with ICAM-1—The presence of Mg^{2+} and absence of Ca^{2+}, *i.e.* Mg^{2+}/EGTA, activates adhesion through wild type $\alpha_L\beta_2$. Interactions mediated by $\alpha_L\beta_2$ with a locked open I domain were efficient in Ca^{2+}/Mg^{2+} as shown above, and were not affected by removal of calcium (data not shown). We compared the effect of removal of Ca^{2+} on

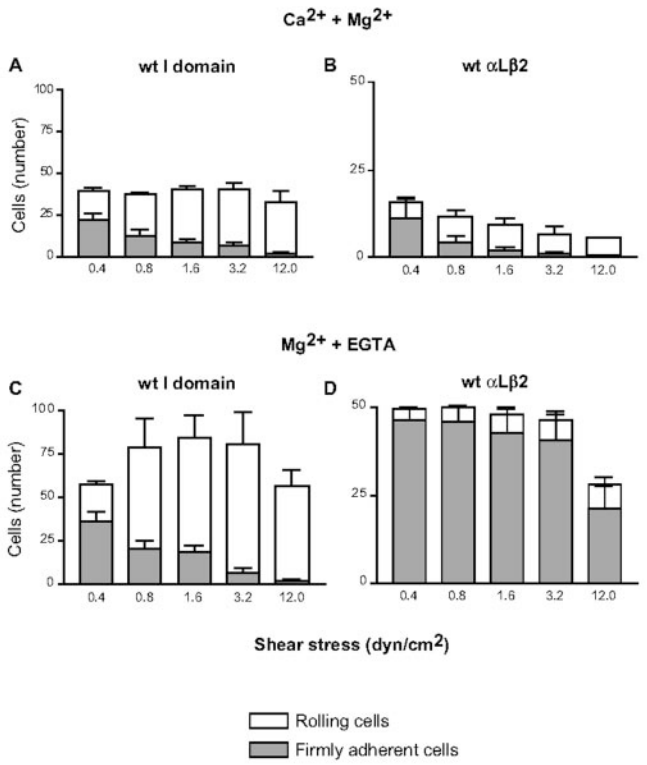

FIG. 5. **Effect of divalent cations on adhesive behavior in shear flow mediated by wild type isolated I domains or $\alpha_L\beta_2$ heterodimers.** K562 transfectants expressing the wild type isolated α_L I domain or $\alpha_L\beta_2$ heterodimer in medium containing either 1 mM Ca^{2+} + 1 mM Mg^{2+} (*A* and *B*) or 2 mM Mg^{2+} + 1 mM EGTA (*C* and *D*) were infused for 30 s at 0.3 dyn/cm^2 on a substrate coated with ICAM-1-Fcγ/protein A. Thereafter, shear was increased every 10 s and the numbers of rollingly adherent cells (*white bars*) and firmly adherent cells (*gray bars*) was determined.

adhesive behavior in shear flow of wild type $\alpha_L\beta_2$ and wild type α_L I domain transfectants (Fig. 5). In the presence of Ca^{2+}/Mg^{2+}, interactions through wild type $\alpha_L\beta_2$ were low in number (Fig. 5*B*); chelation of Ca^{2+} by EGTA greatly increased the number of cells that tethered and accumulated in shear flow (Fig. 5*D*). Furthermore, removal of Ca^{2+} changed the character of the interactions, because all of the increase was accounted for by cells that were firmly adherent. By contrast, for cells that expressed isolated I domains, removal of Ca^{2+} did not affect the percentage of rolling *versus* firmly adherent cells (Fig. 5, *A* and *C*). Nonetheless, the number of isolated I domain-expressing cells that accumulated and remained interacting was higher in Mg^{2+}/EGTA than in Ca^{2+}/Mg^{2+} (Fig. 5, *A* and *C*). These results suggest that activation of firm adhesion through $\alpha_L\beta_2$ by removal of Ca^{2+} requires a domain of the integrin other than the I domain, such as the β_2 I-like domain (15).

Role of the Actin Cytoskeleton in the Interactions Mediated by $\alpha_L\beta_2$ and Isolated I Domain Transfectants—Could the actin cytoskeleton be involved in regulating the transition between rolling and firm adhesion by wild type $\alpha_L\beta_2$? The isolated I domains are expressed using a PDGFR transmembrane and a truncated PDGFR cytoplasmic domain, which are not expected to interact with the actin cytoskeleton. $\alpha_L\beta_2$ and I domain transfectants were treated with latrunculin A, which associates specifically with actin monomers, preventing them from polymerizing into filaments (37). Disruption of actin filaments did not significantly affect the percentage of cells that mediated rolling *versus* firmly adherent interactions for wild type or open $\alpha_L\beta_2$ or wild type or open α_L I domain transfectants (Fig. 6). However, treatment with latrunculin A did significantly in-

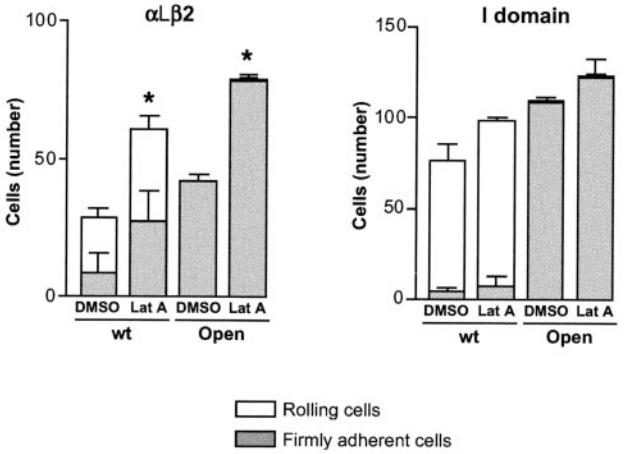

FIG. 6. **Role of the actin cytoskeleton in rolling interactions.** Cells expressing the wild type or open $\alpha_L\beta_2$ or isolated I domain were treated with 1 μM latrunculin A or an equivalent volume of Me_2SO for 20 min at RT. Cells were resuspended in buffer A containing 2 mM Mg^{2+}, infused into the flow chamber, and allowed to accumulate for 30 s at 0.3 dyn/cm^2 over ICAM-1-Fcγ/protein A. Shear stress was increased every 10 s, and the number of rollingly adherent cells (*white bars*) and firmly adherent cells (*gray bars*) was determined. *Bars* represent the average ±standard deviation. *, $p < 0.05$ *versus* Me_2SO treatment.

crease the total number of $\alpha_L\beta_2$ transfectants that interacted with the ICAM-1 substrates, both for the wild type and the open mutant. This is consistent with the generally observed enhancing effect of actin disruption on adhesion through $\alpha_L\beta_2$.

A Small Molecule Allosteric Antagonist of $\alpha_L\beta_2$ Inhibits Interactions in Shear Flow—As an independent method of examining the effect of I domain conformation on adhesive interactions in shear flow, we took advantage of a small molecule antagonist of $\alpha_L\beta_2$. LFA703 is a statin-like analogue that is 10–36 times more potent than lovastatin, a previously described $\alpha_L\beta_2$ inhibitor (21, 35). Lovastatin was previously shown to inhibit adhesion under static conditions to ICAM-1 through $\alpha_L\beta_2$ stimulated by Mn^{2+} or CBR LFA-1/2 (an activating antibody to the β_2 subunit). However, lovastatin did not inhibit adhesion through locked open $\alpha_L\beta_2$, confirming that the mode of action of lovastatin is to stabilize the I domain in the closed conformation (13). We first examined cells that were allowed to adhere to ICAM-1 in stasis and then subjected to increasing wall shear stress (Fig. 7). Adhesion through wild type $\alpha_L\beta_2$ was activated with either Mg^{2+}/EGTA or Mn^{2+}. Under these conditions essentially all of the cells were firmly adherent, *i.e.* there was no rolling. Resistance to detachment in shear was marked in the presence of Mg^{2+}/EGTA and even greater in Mn^{2+} (Fig. 7*A*). The shear resistance of Mn^{2+}-activated wild type $\alpha_L\beta_2$ was dramatically decreased by incubation with 10 μM LFA703, and no adhesion whatsoever was demonstrable for Mg^{2+}/EGTA-activated $\alpha_L\beta_2$ in the presence of LFA703 (Fig. 7*A*). Under the same divalent cation conditions, adhesion through locked open $\alpha_L\beta_2$ was markedly resistant to shear, with greater shear resistance in Mn^{2+} than in Mg^{2+}/EGTA (Fig. 6*B*). However, in contrast to firm adhesion through wild type $\alpha_L\beta_2$, firm adhesion mediated by open $\alpha_L\beta_2$ was not susceptible to inhibition by LFA703 as shown by the lack of effect on resistance to detachment (Fig. 7*B*, *closed squares* and *circles*).

We next examined the effect of LFA703 in shear flow under conditions where cells roll on ICAM-1, *i.e.* with cells expressing wild type $\alpha_L\beta_2$ in Ca^{2+} + Mg^{2+} or with cells expressing the wild type I domain (Fig. 8). Use of a range of concentrations of LFA703 with wild type $\alpha_L\beta_2$ transfectants demonstrated a dose-dependent decrease in the number of rolling cells at each

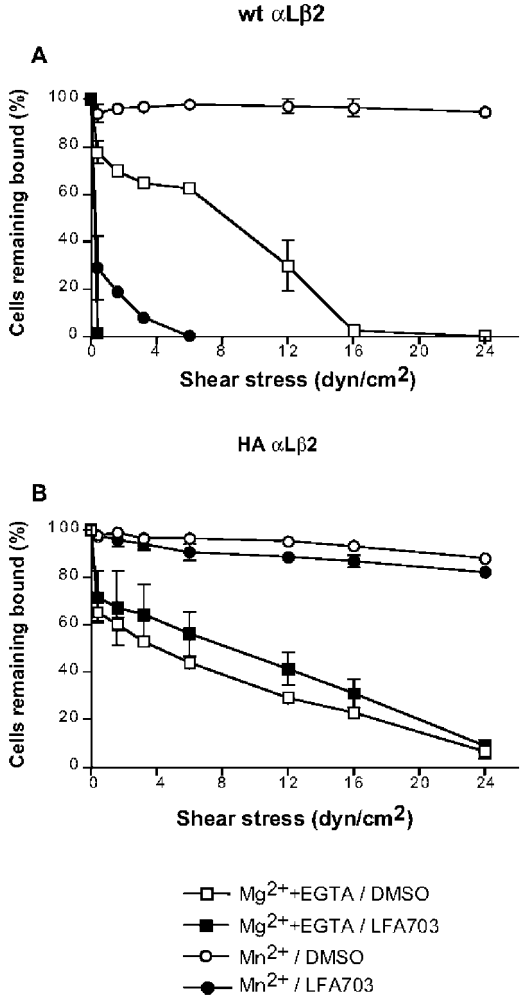

FIG. 7. **Effect of the small molecule antagonist LFA703 on interactions of wild type or locked open $\alpha_L\beta_2$ with ICAM-1.** Cells were incubated at room temperature for 15 min with 10 μM LFA703 or an equivalent amount of Me_2SO, infused in the flow chamber in medium containing the indicated divalent cations, and allowed to settle on the ICAM-1-Fcγ/protein A substrate for 2 min. Shear flow was then initiated, and the wall shear stress was increased every 10 s. At the end of each shear stress interval the number of cells that remained bound to the substrate was counted and expressed as a percentage of the cells present during the 2 min incubation at stasis.

shear stress (Fig. 8*A*). The IC_{50} was shear-dependent, with an IC_{50} of about 3 μM at 0.8 dyn/cm^2, about 1 μM at 1.6 and 3.2 dyn/cm^2, and about 0.5 μM at 6 dyn/cm^2 (Fig. 8*A*). LFA703 also inhibited rolling mediated by the wild type isolated α_L I domain (Fig. 8*B*). The IC_{50} was consistently higher for the isolated α_L I domain than $\alpha_L\beta_2$ and again was shear-dependent. The IC_{50} was about 200 μM at 0.8 dyn/cm^2, about 75 μM at 1.6 dyn/cm^2, about 30 μM at 3.2 dyn/cm^2, and about 20 μM at 6 dyn/cm^2 (Fig. 8*B*). The 50–100-fold lower IC_{50} for $\alpha_L\beta_2$ than the α_L I domain is likely to reflect the finding that the C-terminal α-helix under which LFA703 binds has marked segmental mobility in isolated I domains (38), whereas when this helix is connected to the β-propeller domain in intact $\alpha_L\beta_2$, it is likely to be much more ordered and provide a higher affinity binding pocket. The more intimate association of the C-terminal α-helix with the side of the I domain in $\alpha_L\beta_2$ is corroborated by the activating effect of mutations in this helix in $\alpha_L\beta_2$ but not isolated α_L I domains (17).

DISCUSSION

Recent studies (8–10) *in vivo* and *in vitro* have shown that $\alpha_L\beta_2$ can contribute to tethering and rolling interactions in

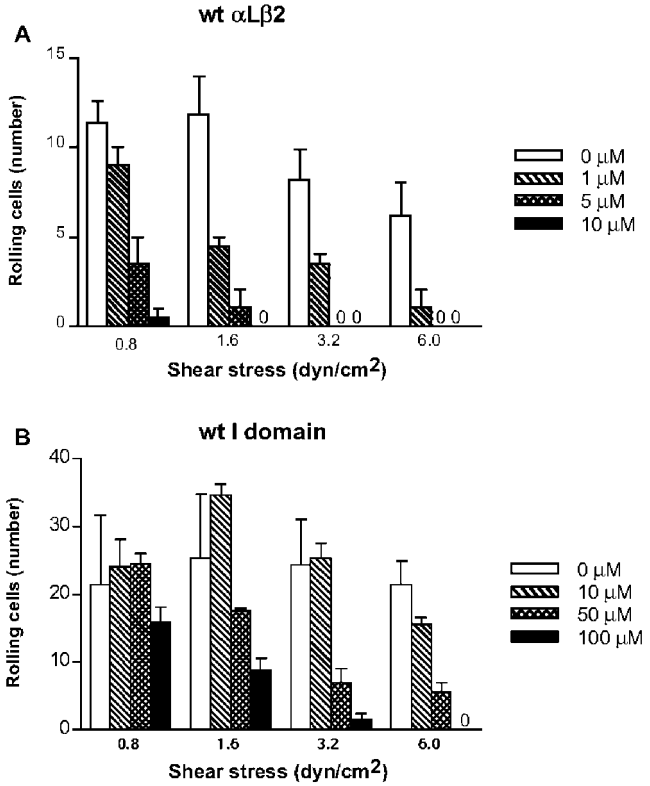

FIG. 8. **Effect of the small molecule antagonist LFA703 on rolling on ICAM-1 mediated by wild type $\alpha_L\beta_2$ or isolated I domain.** Cells expressing the wild type $\alpha_L\beta_2$ or isolated α_L I domain were incubated at room temperature with the indicated concentrations of LFA703 or Me_2SO for 15 min. Cells were infused into the flow chamber in medium containing 1 mM Ca^{2+} + 1 mM Mg^{2+} and allowed to accumulate for 30 s at 0.3 dyn/cm^2. Further accumulation or detachment occurred as wall shear stress was increased every 10 s.

shear flow, although less robustly than in supporting firm adhesion and cell migration. A question of major biological interest is how the conformation of $\alpha_L\beta_2$, which is known to be regulated by signals within the cell in inside-out signaling, affects its adhesiveness in shear flow, and in particular, the critical transition from rolling adhesion to firm adhesion. Here we show for the first time that conformational change in an adhesion receptor can alter adhesive behavior in shear flow. Although the wild type I domain and resting wild type $\alpha_L\beta_2$ mediate rolling, the locked open I domain and locked open $\alpha_L\beta_2$ mediate firm adhesion. High affinity for ICAM-1 resulted in firm adhesion that was highly resistant to detachment by increasing shear. The conversion from rolling adhesion to firm adhesion effected by the change in conformation of the I domain was mirrored by activation with Mg^{2+}/EGTA or Mn^{2+} of wild type $\alpha_L\beta_2$. Interestingly, reduction of the disulfide bond constraining the I domain in the open conformation fully restored the ability of the isolated I domain to roll and abolished its ability to mediate firm adhesion, in agreement with abolition of adhesion in static assays and abolition of high monomeric affinity (14). Therefore, conformational change with an accompanying increase in affinity is sufficient to convert $\alpha_L\beta_2$ from a receptor that mediates rolling adhesion to a receptor that mediates firm adhesion. Although "avidity regulation" has been suggested for $\alpha_L\beta_2$, results interpreted in support of avidity regulation could also be explained by an intermediate increase in the affinity of $\alpha_L\beta_2$ (14). Our results with latrunculin A-treated $\alpha_L\beta_2$ transfectants show that association with the actin cytoskeleton does not regulate the transition from rolling to firm adhesion. Actin cytoskeleton disruption did not change the ratio of rolling *versus* firmly adherent cells for wild type or open $\alpha_L\beta_2$ transfectants, or wild type or open I domain transfectants. Isolated I domains were expressed using heterologous transmembrane and cytoplasmic domains, and only five residues were present in the artificial cytoplasmic domain. This further supports the conclusion that neither inside-out signaling nor avidity changes via clustering are required for regulating the transition between the rolling and firm adhesion states.

Three properties of a receptor-ligand bond are important for its ability to mediate rolling: on-rate, off-rate, and the mechanical property (*i.e.* the susceptibility of off-rate to increase by force). So far, the mechanical property is not known for the ICAM-1—α_L I domain bond, although it has been measured for selectin and α_4 integrin bonds (7). It is intriguing that the conformation-induced change in the off-rate of the ICAM-1—α_L I domain bond appears sufficient to explain the change in state from rolling to firm adhesion. The off-rate of the wild type I domain of 5 s^{-1} is in the range of 0.5–10 s^{-1} measured for rolling interactions through selectins and the integrin $\alpha_4\beta_7$ in Ca^{2+} (reviewed in Refs. 7 and 24). This correlates with the ability of the wild type I domain to support rolling. By contrast, the off-rate of the locked open I domain of 0.025 s^{-1} (14) is well outside this range, correlating with its ability to support firm adhesion and not rolling adhesion. Longer bond lifetimes are theoretically more conducive to firm adhesion. Furthermore, the off-rate of the $\alpha_4\beta_7$—MAdCAM bond in Mg^{2+} of 0.046 s^{-1} is in the same range as the locked open α_L I domain and supports firm adhesion not rolling adhesion (7). Thus, the transition of the α_L I domain to the open conformation triggers a change in off-rate that appears to be perfectly tailored biologically to trigger a transition from rolling adhesion to firm adhesion.

We believe that the surprising effectiveness in mediating rolling of the isolated I domain is a consequence of the downward force exerted on the C-terminal α-helix by tethering in shear flow that stabilizes the open, high affinity conformation of the I domain. When the I domain on the cell surface binds to ICAM-1 on the substrate, the cell becomes tethered through the I domain. The hydrodynamic force exerted on the tethered cell is balanced by a force exerted on its connection to the substrate through ICAM-1 and the I domain. If we analyze how this force is transmitted through the I domain, it is clear that a force exerted on the I domain interface with ICAM-1 at the top face of the I domain bearing the metal ion adhesion site is balanced by an opposing force exerted on the C-terminal α-helix of the I domain, which is connected through a linker to the cell membrane. The direction of this force is roughly parallel to the axis of the C-terminal α-helix, such that it will exert a downward pull on it. Downward movement of this helix stabilizes the open, high affinity conformation of the α_L I domain (17). Therefore, whereas the wild type isolated I domain exists predominantly in the closed conformation, after binding to ICAM-1 the tether force will shift the conformational equilibrium toward the open conformation and increase the effective affinity for ICAM-1. This explains a number of our observations. 1) The wild type α_L I domain was markedly more active than the locked closed I domain in mediating cell accumulation on ICAM-1 substrates in shear flow, and after adhesion was initiated under static conditions, in mediating cell rolling and resistance to detachment with increasing shear. Rolling can be observed for the closed I domain but requires high ICAM-1 densities and low shear stresses. In the locked closed I domain the disulfide bridge prevents the force exerted on the α_7-helix from pulling it downwards and reshaping the critical β_6-α_7 loop. 2) In static assays, adhesion is not detectable with the wild type I domains, whereas it is readily detectable and indeed robust in shear flow assays. 3) LFA703 inhibits rolling through

the wild type I domain. Because this compound stabilizes the closed conformation of the I domain this provides direct evidence that the wild type I domain shifts to the open conformation during rolling and that this is crucial to stabilize rolling.

Rolling adhesion through the wild type isolated α_L I domain is far more efficient that through wild type $\alpha_L\beta_2$. This may in part reflect the connection of the I domain through both its N and C termini in $\alpha_L\beta_2$ and through only its C terminus in the isolated I domain, which would alter the effect of applied force on the equilibrium between the open and closed conformations. However, another important difference is that $\alpha_L\beta_2$ appears to assume the same bent conformation as $\alpha_V\beta_3$ in the resting state (26, 27). This places the I domain close to the cell membrane, in a less favorable orientation for interaction with ICAM-1 than in the isolated I domain.

Recently developed small molecule antagonists of $\alpha_L\beta_2$, including the statin analogue tested here, LFA703, stabilize the α_L I domain in the closed conformation (13, 21, 22, 35). This has been confirmed here by resistance of locked open $\alpha_L\beta_2$ to inhibition by LFA703 in shear flow assays. Although these allosteric antagonists have previously been shown to bind to I domains in crystal and NMR studies, they have not previously been assessed for effect on soluble ligand binding or adhesive activity by isolated I domains. We show here that rolling of cells expressing both the isolated wild type I domain and wild type $\alpha_L\beta_2$ was dramatically impaired in a dose-dependent manner by the allosteric inhibitor LFA703. Our findings show that allosteric inhibitors inhibit both the rolling and firm states of adhesion mediated by $\alpha_L\beta_2$. These findings have important implications for mode of action of this class of antagonists *in vivo*.

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