α4β1-dependent adhesion strengthening under mechanical strain is regulated by paxillin association with the α4-cytoplasmic domain

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The capacity of integrins to mediate adhesiveness is modulated by their cytoplasmic associations. In this study, we describe a novel mechanism by which α4-integrin adhesiveness is regulated by the cytoskeletal adaptor paxillin. A mutation of the α4 tail that disrupts paxillin binding, α4(Y991A), reduced talin association to the αβ heterodimer, impaired integrin anchorage to the cytoskeleton, and suppressed α4β1-dependent capture and adhesion strengthening of Jurkat T cells to VCAM-1 under shear stress. The mutant retained intrinsic avidity to soluble or bead-immobilized VCAM-1, supported normal cell spreading at short-lived contacts, had normal α4-microvillar distribution, and responded to inside-out signals. This is the first demonstration that cytoskeletal anchorage of an integrin enhances the mechanical stability of its adhesive bonds under strain and, thereby, promotes its ability to mediate leukocyte adhesion under physiological shear stress conditions.

Introduction

Circulating leukocytes rapidly develop firm adhesion to vessel wall ligands through their various integrin receptors αβ7, αβ1 (VLA-4), αβ2 (LFA-1), and αβ3 (Mac-1; Alon and Feigel- son, 2002). Integrins bind their respective endothelial ligands under shear flow at lower efficiency than selectins (Springer, 1994). Adhesive tethers form over a fraction of a second and depend on the ability of the nascent adhesive bond to withstand disruptive shear force. In contrast to selectins, all leukocyte integrins can undergo instantaneous up-regulation of their affinity or avidity to endothelial ligands upon exposure to endothelial chemokines (Kinashi, 2005). In addition, integrins can undergo conformational changes upon ligand binding (Hynes, 2002). Cytoskeletal constraints of integrins may also control integrin adhesiveness (van Kooyk and Figdor, 2000). Previous studies on leukocyte (L)-selectin function regulation have shown that preformed cytoskeletal associations of L-selectin with the actin cytoskeleton control the ability of ligand-occupied selectin to stabilize nascent tethers under shear flow and capture leukocytes under physiological shear stresses (Kansas et al., 1993; Dwir et al., 2001). This raised the possibility that specialized subsets capable of interacting with their respective endothelial ligands under physiological shear flow may also need to properly anchor to the cytoskeleton. Although selectins and integrins are structurally distinct, we hypothesized that α4 integrin bonds forming under disruptive shear stresses may share a common regulatory mechanism with L-selectin bonds. However, as alterations in cytoskeletal constraints of integrins can modify affinity, clustering, and ligand-induced conformational rearrangements (Carman and Springer, 2003), the direct contribution of integrin anchorage to adhesive outcome has been difficult to dissect.

In this study, we unraveled novel adhesive properties of an α4-tail mutant with disrupted association with the cytoskeletal adaptor paxillin (Liu et al., 1999). We found that blocking the α4-paxillin interaction markedly impaired the integrin’s ability to anchor to the cytoskeleton in Jurkat T cells. Although not essential for α4β1 affinity, ligand-induced conformational
changes, surface clustering and topography, or redistribution at short static contacts, paxillin association with $\alpha_4\beta_1$ was crucial for $\alpha_4\beta_1$-VCAM-1 bonds to resist mechanical stress. These results suggest that subsecond stabilization of $\alpha_4$ tethers depends on the ability of ligand-occupied $\alpha_4\beta_1$ integrins to properly anchor to the cytoskeleton. This work also highlights the key role of the $\alpha$ subunit of $\alpha_4\beta_1$ in postligand binding adhesion strengthening of the integrin under mechanical strain.

### Results

**Paxillin association with the $\alpha_4$-cytoplasmic domain is required for cell resistance to detachment by shear stress**

Paxillin binding to the $\alpha_4$-cytoplasmic domain is important for integrin $\alpha_4\beta_1$ signaling but not for adhesion developed in shear-free conditions (Rose et al., 2003). To examine the role of paxillin binding in $\alpha_4\beta_1$-mediated adhesion under shear stress, we analyzed the resistance to shear-induced detachment from the $\alpha_4\beta_1$-ligand VCAM-1 of $\alpha_4$-deficient JB4 Jurkat T cells transfected with either wild-type (wt) $\alpha_4$ (JB4-wt) or the paxillin binding–defective $\alpha_4(Y991A)$ mutant (JB4-$\alpha_4(Y991A)$) (Rose et al., 2003). JB4-$\alpha_4(Y991A)$ cells were less resistant to shear-induced detachment than their JB4-wt counterparts (Fig. 1 A). Notably, bivalent VCAM-1 (VCAM-1–Fc) was much more potent than monovalent soluble VCAM-1 (sVCAM-1) in supporting $\alpha_4\beta_1$-specific adhesion (Fig. 1 A), but it still could not rescue the adhesive defect of the $\alpha_4(Y991A)$ mutant. These results were confirmed with multiple clones expressing similar levels of $\alpha_4$ and $\beta_1$ subunits as well as the $\beta_1$ activation neoepitope 15/7 (Fig. 1 B and not depicted). Nevertheless, resistance to detachment from different densities of either ICAM-1–Fc or
ICAM-1 was comparable between wt- and mutant $\alpha_4\beta_1$-expressing cells (Fig. 1 C). In agreement with these results, VLA-4–dependent adhesion to TNFα-stimulated human umbilical vein endothelial cells (HUVECs) was reduced in Jurkat cells expressing the $\alpha_4(Y991A)$ mutant (Fig. 1 D), in particular at shear stresses $\geq 5$ dyn/cm$^2$, within the upper range of shear stresses prevailing in postcapillary venules where the majority of lymphocyte extravasation takes place (Firrell and Lipowsky, 1989). Whereas most cells expressing the wt $\alpha_4$ firmly arrested on the stimulated HUVEC via their VLA-4, a significant fraction of $\alpha_4(Y991A)$ mutant–expressing cells failed to arrest and established endothelial (E)-selectin–dependent rolling on the HUVEC (Fig. 1 D). In the absence of functional E-selectin, the shear resistance of cells expressing the $\alpha_4(Y991A)$ mutant was much lower than the shear resistance of cells expressing wt $\alpha_4$ (Fig. 1 D). Because the contribution of LFA-1 to Jurkat arrest was minimal, these data suggest that the Y991A $\alpha_4$ mutant is deficient in establishing $\alpha_4\beta_1$-mediated shear resistance on endothelial cells expressing VCAM-1 as well as on substrates coated with isolated VCAM-1.

Notably, preformed clustering of $\alpha_4$ subunits on JB4 cells was essentially identical (Fig. 2 A). Real time imaging of JB4 cells that adhered on VCAM-1 also showed identical cell spreading as well as the distribution of both mutant and wt $\alpha_4$ during 1-min cellular contacts before shear application (WT: $n = 44$, 16% round, 54% polarized with uniform $\alpha_4$; Y991A: $n = 27$, 18% round, 52% polarized with uniform $\alpha_4$, 30% polarized with patched $\alpha_4$; Fig. 2 B). Notably, the strength of resistance to detachment developed by wt $\alpha_4$ did not correlate with the degree of patching (Fig. 2 B) in contrast to reports on LFA-1–dependent systems (Constantin et al., 2000; Kim et al., 2004).

Thus, a mutation of the $\alpha_4$ tail defective in paxillin binding prevents $\alpha_4\beta_1$-mediated resistance to shear-induced cell detachment independent of cell spreading and $\alpha_4$ patching on VCAM-1.

The $\alpha_4(Y991A)$ mutation blocks paxillin association with the $\alpha_4$ tail selectively (Liu et al., 1999). As an alternative test of the role of the $\alpha_4$–paxillin interaction, we exploited a recently identified small molecule inhibitor of this interaction. The compound, designated A7B7C7, blocks the $\alpha_4$–paxillin interaction and interferes with $\alpha_4\beta_1$–dependent cell migration (Ambroise et al., 2002). This inhibitor, but not a control compound (A6B6C6), attenuated the shear resistance of wt $\alpha_4\beta_1$–mediated Jurkat cell adhesion to VCAM-1 (Fig. 3 A, left) but had no effect on the residual shear resistance developed by the JB4-$\alpha_4(Y991A)$ cells (Fig. 3 A, right). Adhesion mediated by the $\alpha_4\beta_2$–ICAM-1 interaction was also insensitive to the inhibitor (not depicted). Knocking down paxillin expression by up to 75% using transient short inhibitory RNA (siRNA) silencing (Fig. 3 B) resulted in reduced adhesiveness of wt $\alpha_4\beta_1$–mediated Jurkat cell adhesion to VCAM-1 (Fig. 3 C), with no inhibition of adhesiveness mediated by the $\alpha_4(Y991A)$ mutant (Fig. 3 C). Notably, LFA-1–dependent adhesion to ICAM-1 was also insensitive to identical paxillin silencing (not depicted). Thus, both genetic and pharmacological approaches indicate that the $\alpha_4$–paxillin interaction increases the resistance of $\alpha_4\beta_1$–VCAM-1 contacts to detachment by disruptive shear stresses.

**Paxillin association with the $\alpha_4$ subunit promotes $\alpha_4\beta_1$ anchorage to the cytoskeleton**

Paxillin binds a number of actin-binding proteins such as talin and vinculin (Brown and Turner, 2004) and does so at sites distinct from the $\alpha_4$-binding site (Liu and Ginsberg,
We next quantified the fraction of detergent-resistant wt α4 or α4(Y991A) retained on NP-40–solubilized cells using fluorescence-tagged integrin-bound α4 mAb (Fig. 4 A). Retention of intact wt and α4(Y991A) was similar and low (20% of the total surface α4; Fig. 4 A). However, the addition of anti–mouse Ig to cluster the mAb-bound wt α4 markedly increased the association of α4β1 surface integrin with the detergent-insoluble cytoskeleton (Fig. 4 B). In contrast, the same treatment produced a negligible increase in the cytoskeletal association of α4(Y991A)β1 (Fig. 4 B). Thus, the α4(Y991A) mutant fails to anchor properly to the actin cytoskeleton in Jurkat T cells.

**The α4(Y991A) mutant poorly associates with talin and does not respond to talin suppression**

In light of this poor cytoskeletal anchorage of α4(Y991A)β1, we next compared the level of talin associated with the wt or mutant α4β1 complex in nonadherent Jurkat cells. Notably, constitutive talin binding to the α4(Y991A)β1 complex was significantly reduced compared with the wt integrin, as was evident from coprecipitation analysis (Fig. 5 A). Knocking down up to 65% of the total talin content in wt α4β1–expressing Jurkat cells (Fig. 5 B) retained integrin expression (not depicted) but resulted in significant reduction in their α4β1–mediated shear resistance on both sVCAM-1 and VCAM-1–Fc (Fig. 5 C, left and right insets, respectively). Notably, identical suppression of talin expression in the α4(Y991A)β1–expressing Jurkat cells (Fig. 5 B) had no effect on their low shear resistant adhesion to identical VCAM-1 substrates (Fig. 5 C, right). These results collectively suggest that paxillin association with α4β1 also recruits talin to the α4–paxillin complex and may enhance talin association with the β1 subunit tail. Thus, both paxillin and talin associations promote α4β1-dependent cell resistance to detachment from VCAM-1 under shear stress.

**α4-Paxillin association is not required for α4β1 avidity for VCAM-1 but increases α4 bond stiffness**

Although the affinity of integrin α4(Y991A)β1 to soluble VCAM-1–Fc is retained (Rose et al., 2003), we considered that Jurkat cells expressing the α4(Y991A)β1 mutant might fail to develop shear resistant adhesion as a result of reduced avidity for surface-bound VCAM-1. Comparing wt α4β1 with α4(Y991A)β1, adhesiveness to VCAM-1–coated beads in the absence of applied shear stress, we found that JB4-wt and JB4-
α4(Y991A) cells bound identically to magnetic beads coated with increasing site densities of VCAM-1–Fc (Fig. 6 A), which is supportive of the normal adhesion of α4(Y991A)β1-expressing cells under static conditions. Nevertheless, when VCAM-1–coated beads that bound to JB4-wt cells were exposed to abrupt mechanical stress, these beads were displaced significantly less than beads prebound to JB4-α4(Y991A) cells (Fig. 6 B and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200503155/DC1). α4 resistance to displacement required an intact actin cytoskeleton, as JB4-wt cells pretreated with the F-actin–severing drug cytochalasin D exhibited even greater displacement in response to abrupt magnetic stress (Fig. 6 C). These findings, together with the shear-based detachment assays (Fig. 1), collectively suggest that paxillin association with the α4β1 heterodimer and an intact actin cytoskeleton are both required for ligand-occupied α4β1 to develop stress-resistant adhesive bonds.

Paxillin association with the α4 tail augments α4-mediated T cell capture on VCAM-1 and MadCAM-1 under shear flow

The α4β1 and α4β7 integrins mediate leukocyte capture under physiological shear flow (Alon et al., 1995; Berlin et al., 1995). Therefore, we compared the ability of mutant α4(Y991A) versus wt α4 to support α4β1-dependent T cell capture by monovalent and bivalent VCAM-1 under continuous shear flow. Consistent with its defective resistance to shear force, α4(Y991A)β1-mediated reduced cell capture and arrest on a large range of densities of either monovalent (sVCAM) or bivalent (VCAM-Fc) VCAM-1 (Fig. 7, A and B). This differential behavior was also manifested at different levels of shear stress that were tested (Fig. 7 B, first two panels). Notably, whereas disruption of the actin cytoskeleton by cytochalasin D resulted in marked inhibition of both cell capture and arrest mediated by wt α4β1, cytochalasin D had no effect on the residual adhesions mediated by the α4(Y991A)β1 mutant (Fig. 7 A). Interestingly, the duration of individual α4β1 tethers, which is a measure of integrin affinity to the ligand (Feigelson et al., 2001), was not altered upon the loss of paxillin binding (Fig. 7 A). Thus, for optimal cell capture under shear flow, the α4 tail of α4β1 requires associations with the intact actin cytoskeleton.

Jurkat cells express low levels of the β1 integrin subunit; thus, >95% of their α4β1-integrin subunits are found in α4β1 heterodimers. Nevertheless, JB4-wt cell capture on high density of the bivalent α4β1-integrin ligand MadCAM-1–Fc (Berlin et al., 1993) was inhibited by the anti-α4β1 antibody Act-1 (unpublished data). The JB4-α4(Y991A) cells formed fivefold fewer tethers on MadCAM-1 than JB4-wt cells, with a diminished fraction of tethers followed by immediate arrests (Fig. 7 B, right). Thus, paxillin association with the α4 subunit enhances the ability of α4 to promote adhesive tethers in the context of both β1 and β7 integrins under continuously applied disruptive shear stress.

Preferential localization of receptors to microvilli increases their availability for interactions with counter ligands under shear flow (von Andrian et al., 1995). Electron microscopic analysis of wt α4 and the α4(Y991A)-tail mutant revealed identical distribution of these variants to microvillar compartments (82 ± 4% for wt α4, n = 222; 80 ± 6% for the α4(Y991A) mutant, n = 196; Fig. 5 C). Furthermore, a higher ratio of the α4(Y991A)-tail mutant localized on microvillar tips than wt α4 (a 4.5 tip/base ratio for the mutant vs. only 1.8 tip/base ratio for wt α4). The number and size of microvillar projections in JB4-wt and JB4-α4(Y991A) cells were also comparable (Fig. 7 C). Thus, the enhanced ability of wt α4β1 to...
The \( \alpha_4(Y991A) \beta_1 \) mutant fails to generate productive adhesive bonds with VCAM-1 under disruptive forces

To examine the effects of disrupting the \( \alpha_4 \)-paxillin interaction at a single molecule level and in the presence of an external force other than shear stress, we next measured the force of unitary adhesive interactions between wt \( \alpha_3 \beta_1 \) or the \( \alpha_4(Y991A) \beta_1 \) mutant and immobilized VCAM-1 by atomic force microscopy (AFM). JB4-wt or JB4-\( \alpha_4(Y991A) \) cells were coupled to the end of an AFM cantilever (Fig. 8 A) and lowered onto a VCAM-1–Fc coated surface. After a 0.5-s contact, the frequency of productive adhesive events and their strength were analyzed by the degree of deflection experienced by the cantilever during its retraction from the adhesive substrate. Both cell types detached from the VCAM-1 substrate through single jumps, suggesting the breakage of individual bonds during cantilever retraction (Fig. 8 B). The adhesion frequencies of all experiments were maintained below 30%, a level assumed to reflect single \( \alpha_3 \beta_1 \)–VCAM-1 interactions (Zhang et al., 2004). The specificity of the adhesive events detected in this system were confirmed by a similar 70% reduction in total binding events by the blockade of wt \( \alpha_3 \beta_1 \) with Bio1211 (Lin et al., 1999) or by omission of VCAM-1 from the substrate (Fig. 8 C and not depicted). As indicated by the force histograms derived for JB4-wt or JB4-\( \alpha_4(Y991A) \) cells (Fig. 8 C), the frequency of productive adhesive events developed by \( \alpha_4(Y991A) \beta_1 \) was up to 10-fold lower than those developed by \( \alpha_3 \beta_1 \) after background subtraction. The distribution of unbinding (rupture) forces measured for the two integrin variants was, however, similar (Fig. 8 C). Thus, paxillin association with the \( \alpha_4 \) subunit dramatically augments the ability of \( \alpha_3 \beta_1 \) to form adhesive tethers that resist disruptive forces irrespective to whether these forces are applied during a vertical force loading (AFM) or during cell rotation (shear stress).

Paxillin association with \( \alpha_4 \) augments shear resistance of integrin tethers independent of ligand-induced conformational changes

The aforementioned data suggest that paxillin binding to \( \alpha_4 \) is required for mechanical stabilization of cell attachments rather than for cytoplasmic induction of high affinity integrin conformations. Ligand binding to integrins can induce conformational changes in the integrin, resulting in high affinity conformations (Du et al., 1991; Shimaoka et al., 2002). Therefore, we considered the possibility that the reduced tether formation by the \( \alpha_4(Y991A) \) mutant could reflect defective, instantaneous ligand-induced conformational changes in the integrin under shear stress. We first verified that the \( \alpha_3 \beta_1 \) ligand Bio1211 provoked similar conformational changes in \( \alpha_4 \beta_1 \) and \( \alpha_4(Y991A) \beta_1 \) under shear-free conditions, as indicated by the identical induction of the \( \beta_1 \) ligand–induced binding site reporter 15/7 epitope by increasing doses of the monovalent \( \alpha_4 \beta_1 \)-specific ligand Bio1211 (Fig. 9 A; Lin et al., 1999). We next tested the intrinsic attachment efficacy of either wt \( \alpha_4 \) or the \( \alpha_4(Y991A) \) mutant to surface-immobilized \( \alpha_4 \) mAb in the absence of ligand occupancy of the integrin. Notably, the HP1/2 mAb binding to \( \alpha_4 \) integrins is not sensitive to their affin-

promote adhesive tethers under shear flow was not the result of its preferential distribution to cellular microvilli or to increased localization on microvillar tips.
Feigelson et al., 2001) and, thus, should be insensitive to intrinsic or ligand-induced affinity changes under shear stress. Notably, in the presence of shear flow, the \( \alpha_\text{v}\beta_\text{3}(Y991A) \) mutant formed adhesive tethers to immobilized HP1/2 mAb much less efficiently than wt \( \alpha_\text{v}\beta_\text{3} \) (Fig. 9B), as was observed for VCAM-1 (Fig. 1). In addition, adhesive contacts generated by the \( \alpha_\text{v}\beta_\text{3}(Y991A) \) mutant after 1 min of static contact also exhibited poor resistance to detachment by increasing shear forces relative to wt \( \alpha_\text{v}\beta_\text{3} \)–mediated contacts (Fig. 9C).

Thus, paxillin association with the \( \alpha_\text{v}\beta_\text{3} \)-integrin tail enhances the ability of the integrin subunit to generate resistance to detachment forces independently of ligand-induced conformational rearrangements under shear stress conditions.

**Figure 7.** Paxillin association with the \( \alpha_\text{v}\)-cytoplasmic tail facilitates tethering mediated by \( \alpha_\text{v}\beta_\text{1} \) and \( \alpha_\text{v}\beta_\text{7} \) under shear flow without altering \( \alpha_\text{v} \) distribution on microvilli. (A) Tethering (transient or followed by immediate arrest) of Jurkat cells expressing either wt \( \alpha_\text{v} \) (WT) or the \( \alpha_\text{v}(Y991A) \) mutant (Y991A) to immobilized VCAM-1. The mean duration of transient tethers is shown in parenthesis above bars. Where indicated, cells were pretreated with 20 \( \mu \text{M} \) cytochalasin D (cyto D) or carrier (carr). Error bars represent SD. (B) Tethering under shear flow of Jurkat cells mediated by either WT or Y991A to distinct \( \alpha_\text{v}\beta_\text{3} \)-integrin ligands. Tethers (transient or arrest) were determined under the indicated shear stresses on surfaces coated with either monomeric 7D VCAM-1 (sVCAM-1), dimeric 7D VCAM-1 (VCAM-1–Fc), or high density MadCAM-Fc. In each panel, the mean range of two experimental fields is depicted. All tethers to VCAM-1 were blocked in the presence of the \( \alpha_\text{v}\)-integrin mAb HP1/2 (not depicted). All tethers to MadCAM-1 were blocked by the anti-\( \alpha_\text{v}\beta_\text{3} \)-antibody Act-I (not depicted). Results in A and B are representative of five and four independent experiments, respectively. (C) Surface distribution of wt \( \alpha_\text{v} \) (WT) or the \( \alpha_\text{v}(Y991A) \) mutant on JB4 Jurkat cells monitored by immunoelectron microscopy. Insets show lower magnification images. The boxed areas depict the cellular areas enlarged. Prefixed cells were stained with the nonblocking \( \alpha_\text{v} \)-specific mAb B5G10.

**Figure 8.** \( \alpha_\text{v}(Y991A) \beta_\text{1} \) fails to stabilize bonds ruptured by an AFM probe.

(A) Schematic representation of the experimental system. JB4 cells were coupled to an AFM cantilever tip via an anti-CD43 mAb. VCAM-Fc was immobilized onto the substrate as in previous figures. (B) Representative AFM force–displacement curves acquired with wt \( \alpha_\text{v} \)-expressing JB4 cells (top) or \( \alpha_\text{v}(Y991A) \)-expressing cells (middle) approaching the VCAM-1–Fc-bearing substrate. A force–displacement curve of wt \( \alpha_\text{v} \)-expressing JB4 approaching a control substrate devoid of VCAM-1 is indicated in the bottom curve. (C) Force histograms of \( \alpha_\text{v}\beta_\text{3}–\text{VCAM-1} \) unbinding forces measured under a fixed loading rate of 0.33 nN/s. The number of productive adhesive interactions and their unbinding force distribution are depicted. Background binding is depicted by the dashed line. The mean unbinding force (UF) values of 10 independent experiments are indicated near each histogram. Pulling velocity was 3 \( \mu \text{m/s} \), and the cell–substrate contact time was 0.5 s. A representative result of 10 independent experiments is depicted.

Washed cells were stained with rabbit anti–mouse Ig and 5 nm gold particle–conjugated goat anti–rabbit as described in Materials and methods. Gold particles are marked by arrowheads. Photomicrographs are representative of 20–30 cells.
Figure 9. Paxillin association with α4 integrins stabilizes adhesive tethers to immobilized α4-specific mAbs independent of ligand-induced rearrangements. (A) Dose-dependent induction of the 15/7 epitope by the α4β1-specific ligand Bio1211 on wt α4 or α4(Y991A)–expressing Jurkat cells. (B) Reduced tethering and firm adhesion of the α4(Y991A) mutant to immobilized α4 mAb (1H1/2) under shear flow. Frequency of tethers and their categories were determined as in Fig. 7. (C) Strength of adhesion developed by JB4 expressing either wt or α4(Y991A) settled for 1 min on low or high density mAb. Experiments in A and B are each representative of three independent experiments. Error bars represent SD.

Discussion

This study shows that the disruption of paxillin binding to the integrin α4 tail abrogates its anchorage to the actin cytoskeleton and impairs the ability of integrin ligand bonds to withstand immediate rupture by shear stress, an AFM-pulling device, or abruptly applied magnetic force. Despite normal distribution on the cell surface and retained avidity to immobilized VCAM-1, in the presence of applied forces, this anchorage-deficient mutant poorly mediates tether formation and rapid adhesion strengthening on its ligand. Paxillin-dependent cytoskeletal anchoring of ligand-occupied α4 integrins may thus underlie their unique capacity to resist disruptive forces and support leukocyte adhesion under shear flow. Thus, although cytoskeletal constraints of integrins were predicted to restrict mobility and clustering on the cell surface and reduce cell adhesiveness (Kucik et al., 1996; Yauch et al., 1997; Kim et al., 2004), we propose that α4 integrins must retain correct cytoskeletal associations to resist immediate rupture by shear stresses exerted at leukocyte contacts with target blood vessels. Our findings indicate that α4-integrin anchorage to the cell cytoskeleton is critical for nascent adhesive contacts to resist immediate rupture by shear stress, but it is not required for integrin binding to the ligand nor for ligand-induced conformational rearrangements in the absence of external force. The anchorage deficiency of the α4-tail mutant resulted in an inability to develop...
adhesion to a high affinity mAb, which binds the integrin independently of affinity to native ligands (Feigelson et al., 2001; Kinashi et al., 2004). Altogether, these data suggest that paxillin associations with the α4 tail control (a postligand occupancy anchorage step that is critical for tether stabilization under stress), which is a mechanical property underlying the ability of lymphocytes to capture and arrest on endothelial α4-integrin ligands under shear flow. Our experiments on cytokine-activated endothelial cells also predicted an increased contribution of this α4–paxillin association to T cells interacting with endothelial beds expressing α4-integrin ligands in the absence of endothelial selectins.

Integrin affinity is controlled by β-subunit associations with the talin head domain (Tadokoro et al., 2003) and by Rap1 (Kinashi, 2005) via effectors such as RAPL (regulator of adhesion and cell polarization enriched in lymphoid tissues; Katagiri et al., 2004). The effect of RAPL requires α-tail sequences (Katagiri et al., 2004) that are distant from the paxillin-binding site on α4 (Liu and Ginsberg, 2000). The retained affinity of the α4(Y991A) mutant and its capacity to mediate static adhesion suggest that lack of paxillin association with the α4-integrin tail does not interfere with Rap1-dependent signals whether mediated through RAPL or other Rap1 effectors. The reduction in talin association with α4(Y991A)βββ did not alter basal α4ββ affinity for VCAM-1 (Rose et al., 2003), suggesting that the paxillin-mediated association of talin with α4ββ does not contribute to affinity modulation. On the other hand, the extent of these cytoskeletal associations and an intact actin cytoskeleton critically determine the mechanical strength of α4ββ VCAM-1 bonds (i.e., tether formation, adhesion strengthening, and resistance to mechanical stress). Thus, we propose that the ability of α4 integrins to translate ligand occupancy into immediate mechanical stability of subsecond adhesive contacts requires paxillin and talin-mediated linkages of α4ββ to the actin cytoskeleton. The regulation of α4ββ adhesiveness by talin has never been addressed, especially not under shear stress conditions. The finding that talin suppression impairs the strengthening of wt α4ββ bonds under strain is reminiscent of results reporting the involvement of talin1 in ligand-driven α4ββ-cytoskeletal bonds in fibroblasts (Jiang et al., 2003). Although different integrins may anchor differently to the cytoskeleton in distinct cell types, this involvement of talin in both α4- and αβ-integrin associations with the actin cytoskeleton is consistent with the notion that talin, apart from its role in integrin affinity regulation (Tadokoro et al., 2003), is a key postligand occupancy adaptor that promotes integrin bond stabilization in distinct mechanical contexts and cellular environments.

Our results highlight the role of the α4-integrin subunit rather than the β subunit in postligand binding adhesion strengthening of the α4ββ–VCAM-1 bond under mechanical strain. Previous findings suggested that a nearly complete truncation of the α4-cytoplasmic tail impairs α4ββ adhesion strengthening without altering initial cell capture to VCAM-1 under shear flow (Alon et al., 1995; Kassner et al., 1995). This truncation of the α4-integrin tail also reduced integrin mobility (Yau et al., 1997) and may have increased integrin cytoskeletal anchorage via the intact β1 subunit, although this was not experimentally demonstrated. Therefore, in these earlier studies, it was impossible to distinguish between the contributions of α4 anchorage versus mobility to rapid mechanical stabilization of α4 integrin–mediated tethers. Our present results provide the first direct evidence for a positive role of α4 anchorage for the earliest stabilization events of α4ββ–VCAM-1 bonds subjected to mechanical strain. In addition to the α4 Y991 residue, the phosphorylation level of the α4 serine 988 has been shown to control the degree of α4 association with paxillin (Han et al., 2001). A dephosphorylated serine variant mimicked by the phosphodefficient mutant α4 S988A was reported to bind paxillin at enhanced levels (Nishiya et al., 2005). Interestingly, this phosphodefficient mutant did not properly anchor to the cytoskeleton and supported reduced adhesiveness to VCAM-1 (unpublished data). These findings, together with the paxillin-silencing data of this study, suggest that paxillin binding to the α4 subunit is required but is insufficient to anchor α4 to the cytoskeleton. Thus, α4 phosphorylation, which is postulated to attenuate paxillin binding to the α4 subunit, is in fact required, at least at a basal level, for proper cytoskeletal α4 association and productive adhesiveness under shear stress. Overphosphorylation of α4, which reduces paxillin association, may, on the other hand, attenuate both anchorage and adhesiveness. Studies are ongoing to address both the positive and negative effects of serine phosphorylation on α4 anchorage and function under strain.

α4 association with paxillin enhances the activation of the focal adhesion kinases FAK and PYK-2 after α4ββ ligation (Liu et al., 1999; Rose et al., 2003). This association also restricts Rac activation at the leading edge via recruitment of the Arf GTPase-activating protein (Nishiya et al., 2005). Nevertheless, at 1-min contacts with VCAM-1, cells expressing the α4-tail mutant spread normally on VCAM-1. Suppression of tyrosine phosphorylation or inhibition of PYK-2 activity in Jurkat T cells had no effect on α4ββ-mediated adhesion strengthening developed under shear stress (unpublished data). Thus, the ability of paxillin association with α4ββ to enhance integrin anchorage to the cytoskeleton and promote mechanical stability of adhesive tethers at short-lived contacts is distinct from its roles in focal adhesion turnover and Rac deactivation during cell spreading on VCAM-1-containing substrates (Nishiya et al., 2005). Altogether, our findings suggest that modulating mechanical properties of α4 integrins by the inhibition of specific associations between α4-cytoplasmic tails and the cytoskeleton may be a selective strategy to fine tune integrin-mediated adhesion under shear stress without altering integrin affinity.

Materials and methods

Reagents and antibodies

Recombinant seven-domain human VCAM-1 (sVCAM-1) was provided by B. Pepinsky (Biogen, Cambridge, MA). VCAM-1–Fc fusion protein containing seven-domain VCAM-1 fused to IgG was generated as described previously (Rose et al., 2000). VCAM-1–Fc constructed from domains 1 and 2 of VCAM-1 fused to Fc, termed 2D VCAM-1–Fc, was provided by B. Pepinsky. Affinity-purified human full-length spleen-derived ICAM-1 was a gift from T. Springer (Harvard University, Boston, MA). ICAM-Fc and SDF-1α were purchased from R&D Systems. BSA (fraction V), poly-L-lysine, and Ca2+/Mg2+-free HBSS were obtained from Sigma-Aldrich. Human serum albumin (fraction V) and PMA were purchased from Calbiochem.
The α4-integrin function-blocking HP1/2 mAb, the E-selectin-blocking mAb BB11, the β3-specific TS2/16 mAb, the αv-specific nonblocking SBG10 mAb (all provided by B. Pepinsky), the β3-integrin subunit mAb 15/7 (provided by T. Yednock, Elan Pharmaceuticals, San Francisco, CA; Yednock et al., 1995), and the anti-αvβ3 Act-1 (a gift from M. Briggin, Millennium Pharmaceuticals, Cambridge, MA) were all used as purified lg, Antibody mabs (clone 8d4) was purchased from Sigma-Aldrich. Anti-paxillin mAb (clone 349) was purchased from BD Transduction Laboratories. Goat polyclonal anti-α4 Ab (clone C-20) was purchased from Santa Cruz Biotechnology, Inc.

Cell culture and flow cytometry

Cells that deficient in α4 (8d4) were stably transfected with either wt α4 or α4(Y991A) cDNA as described previously (Liu et al., 1999). Cells were subcloned, and multiple clones expressing identical levels of α4 and β1 subunits were taken for functional analysis. Clones were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 mM nonessential amino acids, and antibiotics (Biological Industries). Primary HUVECs were established as previously described (Shamri et al., 2005). HUVECs were left intact or stimulated for 4 h with 0.1 ng/ml TNF-α and antibiotics (Biological Industries). A laser-scanning system (model 2000; Bio-Rad Laboratories).

For real-time flow cytometry, Jurkat cells deficient in paxillin expression monitored by immunoblotting was maximally sup-

sequent functional assays. Immunoprecipitation of α4 localization was assessed by immunoelectron microscopy as previously described (Chen et al., 1999). In brief, cultured Jurkat cells were washed and prefixed in 0.1 M phosphate buffer, pH 7.4, containing 2% PFA and 0.05% glutaraldehyde. Washed cells in H/H medium (HBSS containing 2 mg/ml BSA and 10 mM Hepes, pH 7.4, supplemented with 1 mM CaCl2 and 1 mM MgCl2) were incubated with 10 μg/ml anti-α4 BSG10 mAb for 40 min at 22°C. Washed cells were stained with 10 μg/ml rabbit anti–mouse Ig, washed, and incubated for 45 min with 5 nM Act-1 (a gift from M. J. Briskin, Cell Signaling Technology) and stored on ice. Cells and VCAM-1–coated beads were mixed at RT for 1 min in binding medium at a concentration of 107 cells/ml at a cell/ bead ratio of 1:8 followed by a threefold dilution in binding medium. The cellular side scatter, distinguishing between bead-bound and bead-free cells, was analyzed immediately in a FACSscan flow cytometer (Becton Dickinson). Background binding determined with protein A–coated beads was <10% of the maximal binding observed at VCAM-1 saturation and was subtracted from the total binding results.

The mechanical stiffness of α4/VCAM-1 adhesions was measured by electromagnetic pulling cytometry using VCAM-1–coated beads (Matthews et al., 2004). The detailed method is described in supplemental material (available at http://www.jcb.org/cgi/content/full/jcb.200503155/DC1).

AFM measurements

All force measurements were conducted at 35 ± 2°C using a previously described AFM apparatus (Benoit et al., 2000). In brief, a microfabricated Si3N4 cantilever tip (Parker Scientific Instruments) was coated with 0.1 mg/ml of the anti-CD43 mAb (R&D Systems). The spring constants of the cantilevers used were determined at ~4.7 ± 0.6 mN/m. A single cell was immobilized on the cantilever tip shortly before experimentation. The device was mounted with a piezo-actuator (Piezosystem Jena) on an inverted optical microscope (Carl Zeiss Microlmaging, Inc.) containing a heating stage. A diode laser beam focused on the sensor was used to measure the displacement of the cantilever by the laser beam deflection on a two-segment photodetector. The cell adhering to the cantilever was positioned above an adhesive substrate coated with 2D VCAM-1–Fc captured via human IgG Fc mAb (Jackson ImmunoResearch Laboratories). The cantilever was lowered until the sensor detected a contact force equal to a preselected value (typically 50 pN). After the contact was established for a dwelling time of 500 ms, the cell-bearing cantilever was lifted up by the piezo-actuator, and the de-adhesion force was monitored by a force–dis-

Laminar flow adhesion assays

Purified ligands or mAbs were coated on polystyrene plates as previously described (Grabovsky et al., 2000). Site densities of coated sVCAM-1 and α4/αv/VCAM-1 adhesions were previously described (Grabovsky et al., 2000; Sigal et al., 2000). The polystyrene plates were each assem-

2 s) to the substrate and as arrests if they immediately arrested and remained stationary for at least 5 s of continuous flow. Frequencies of adhesive categories within differently pretreated cells or rates of cell ac-

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Fig. S1 shows the analysis of VCAM-1-coated bead displacement during a magnetic force pulse applied on wt Jurkat cells. The supplemental Materials and methods section describes the experimental setup.

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