The Journal of Immunology

Talin-1 and Kindlin-3 Regulate $\alpha_4\beta_1$ Integrin-Mediated Adhesion Stabilization, but Not G Protein-Coupled Receptor-Induced Affinity Upregulation

Sharon J. Hyduk, Jacob Rullo, Adrianet Puig Cano, Haiyan Xiao, Mian Chen, Markus Moser and Myron I. Cybulsky

J Immunol 2011; 187:4360-4368; Prepublished online 12 September 2011;
doi: 10.4049/jimmunol.1003725
http://www.jimmunol.org/content/187/8/4360

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Talin-1 and Kindlin-3 Regulate $\alpha_4\beta_1$ Integrin-Mediated Adhesion Stabilization, but Not G Protein-Coupled Receptor-Induced Affinity Upregulation

Sharon J. Hyduk,*,† Jacob Rullo,*,† Adrianet Puig Cano,*,† Haiyan Xiao,*,† Mian Chen,*,† Markus Moser,‡ and Myron I. Cybulsky*,†

Chemokine/chemoattractant G protein-coupled receptors trigger an inside–out signaling network that rapidly activates integrins, a key step in inflammatory leukocyte recruitment. Integrins mediate leukocyte arrest and adhesion to endothelium through multivalent binding, and they transmit outside–in signals to stabilize adhesion and coordinate cell spreading and migration. In the present study, we used RNA interference in the U937 monocytic cell line to investigate the role of talin-1, kindlin-3, and α-actinin-1 in the fMLF- and SDF-1α–induced upregulation of $\alpha_4\beta_1$ integrin affinity and consequent adhesive events. Affinity upregulation of $\alpha_4\beta_1$ integrin was not impaired by small interfering RNA knockdown of talin-1, kindlin-3, or α-actinin-1. Only kindlin-3 knockdown increased flow-induced detachment from VCAM-1–coated surfaces in response to fluid flow, whereas knockdown of either talin-1 or kindlin-3 increased detachment from ICAM-1–coated surfaces. Biochemical analyses revealed that $\alpha_4\beta_1$ expression was highly enriched in U937 cell microridges and murine lymphocyte microvilli. Kindlin-3 was present throughout the cell, whereas talin-1 was largely excluded from microridges/microvilli. The subcellular colocalization of $\alpha_4\beta_1$ and kindlin-3 in microridges may explain why kindlin-3 rapidly associates with $\alpha_4\beta_1$ after G protein-coupled receptor signaling and contributes to adhesion strengthening. Talin-1 contributed to $\alpha_4\beta_1$-dependent chemotaxis, suggesting that it participates in a later stage of the leukocyte adhesion cascade when the leukocyte cytoskeleton undergoes dramatic rearrangement. ***The Journal of Immunology*****, 2011, 187: 4360–4368.**

Dynamic regulation of adhesive contacts with endothelium is required for the coordinated and rapid migration of leukocytes from the blood into tissues. Integrins are important mediators of these dynamic adhesive events and as such must be able to regulate their adhesive capacity. Binding of chemoattractants or chemokines to G protein-coupled receptors (GPCRs) activates integrins by rapid triggering of a complex inside–out signaling network. Integrin activation by chemokines is a key step that results in the arrest of rolling leukocytes (1) and in the recruitment of specific leukocytes from the blood. Integrin activation can occur via conformational changes that increase the intrinsic affinity of the integrin for its ligands or through increased valency (an increase in the number of cotemporaneous integrin/ligand bonds) (2–4). Rapid chemokine-induced upregulation of $\alpha_4\beta_1$ integrin affinity is required for monocyte arrest mediated by VCAM-1 (5, 6). T lymphocyte arrest through interactions with ICAM-1 is dependent on $\alpha_L\beta_2$ integrin affinity modulation and multivalent binding (7, 8). Subsequent to arrest, integrins mediate the stable adhesion of leukocytes to endothelium through multivalent binding and they transmit signals into the cell (outside–in signaling) to stabilize adhesion and coordinate cell spreading and migration. Our understanding of the molecular mechanisms that regulate these integrin activities under physiologic conditions relevant to migration is still limited.

An early step in the chemokine/chemoattractant GPCR inside–out signaling cascade is activation of phospholipase C and calcium signaling, which are required for the induction of high-affinity $\alpha_4\beta_1$ integrin and monocyte arrest following GPCR stimulation (6) and LFA-1 activation and adhesion of T lymphocytes (9). Downstream of calcium signaling, the small GTPases and their guanine nucleotide exchange factors and effectors have been shown to play an important role in β_2 integrin extension, arrest, and adhesion stabilization (9, 10), but this has not been established in $\alpha_4\beta_1$ affinity upregulation or adhesion. Interaction of integrin-binding proteins with the integrin cytoplasmic tail is thought to be the terminal event that leads to conformational change and increased ligand-binding affinity. Subsequent to ligand binding, integrin-associated proteins mediate the outside–in signaling events that contribute to stabilization of adhesion. Integrin cytoplasmic tails bind to a number of proteins, including talin, α-actinin, and members of the kindlin family.

Talin-1 is an ~240-kDa protein composed of a 47-kDa N-terminal head domain and 190-kDa C-terminal rod domain (11). The head domain contains the major integrin-binding site in the FERM-3 domain, and the rod domain contains actin-binding sites. In non-hematopoietic cells, it is increasingly evident that talin provides a key link between integrins and the actin cytoskeleton and regulates integrin adhesive and signaling functions (12). Overexpression of

*Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario M5G 1L7, Canada; †Toronto General Research Institute, University Health Network, Toronto, Ontario M5G 1L7, Canada; and ‡Department of Molecular Medicine, Max Planck Institute of Biochemistry, 82152 Martinsried, Germany

Received for publication November 9, 2010. Accepted for publication August 14, 2011.

This work was supported by Canadian Institutes of Health Research Grant MOP-89740. M.I.C. is a recipient of a Career Investigator award from the Heart and Stroke Foundation of Ontario.

Address correspondence and reprint requests to Dr. Sharon J. Hyduk, Toronto General Research Institute, 101 College Street, TMDT 3-301, Toronto, Ontario M5G 1L7, Canada. E-mail address: sharon.hyduk@utoronto.ca

The online version of this article contains supplemental material.

Abbreviations used in this article: FPR, formyl peptide receptor; GPCR, G protein-coupled receptor; LDV, 4-((*N*′-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-α-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine; PIPKIγ, phosphatidylinositol phosphate kinase type I γ; PKC, protein kinase C; PSGL-1, P-selectin glycoprotein ligand-1; siRNA, small interfering RNA.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1003725

the integrin-binding head domain of talin activates integrins (13), and short hairpin RNA against talin inhibits soluble ligand and activation reporter Ab binding to β_1 and β_3 integrins (14).

The *Drosophila* $\alpha_{PS2}\beta_{PS}$ integrin is not regulated by talin, either in its native environment or when expressed in mammalian cells, although *Drosophila* talin is able to activate mammalian $\alpha_{IIb}\beta_3$ integrin (15). A recent report showed that binding of the talin head domain to the cytoplasmic tail of $\alpha_{IIb}\beta_3$ integrin did not upregulate affinity as measured by binding of a monovalent ligand, but it did induce binding of a multivalent ligand (Pac-1 Ab), suggesting that talin may induce integrin clustering (16). However, talin head domain activated $\alpha_{IIb}\beta_3$ integrin, leading to molecular extension and Pac-1 binding in the absence of other membrane proteins in nanodiscs bearing a single lipid-embedded integrin (17).

Kindlins are FERM domain-containing proteins that interact with integrin β-chain cytoplasmic tails. Kindlins activate β_1, β_2, and β_3 integrins and have been implicated in integrin activation, leukocyte arrest, and adhesion (18–20). Recent studies have implicated kindlin-3 in leukocyte adhesion deficiency III, also known as leukocyte adhesion deficiency I variant (21–23). Kindlin-3 binds to integrin β tails (18) and has been shown to regulate integrin activation (24), integrin-dependent adhesion (20), and platelet aggregation (18). Kindlin-3 and talin associate with β tails independent of each other at membrane distal and proximal sites, respectively (18). Kindlin-3–deficient effector T cells derived from leukocyte adhesion deficiency III patients exhibited normal adhesion strengthening on VCAM-1 despite reduced basal expression of a β_1 integrin activation epitope (25).

α-Actinins are actin-binding proteins that bundle actin filaments in cytoskeleton frameworks. They are found at adhesion sites, where they associate with integrins and a number of cytoskeletal and signaling molecules. α-Actinins associate directly with the cytoplasmic tail of β_1- and β_2-chains (26, 27), but they may also appear in integrin/cytoskeletal complexes as a binding partner of actin and regulate lymphocyte migration (28, 29). The presence of α-actinin at adhesion sites has been shown to correlate with adhesion strengthening (30, 31). In fibroblasts, α-actinin-1 is not found in newly formed adhesions or small integrin clusters, but it is found in mature focal adhesions (30, 31). Knockdown of α-actinin in T cells increased initial attachment and decreased migration on ICAM-1, but it did not alter LFA-1 activation (extension). Coimmunoprecipitation studies revealed that α-actinin associates with low- and/or intermediate-affinity, but not high-affinity, LFA-1 (29). Pavalko and colleagues (28) proposed that following fMLF stimulation of neutrophils the α-actinin binding site on β_2 integrin is revealed following a conformational change, suggesting that α-actinin binding occurs secondary to integrin activation.

Earlier reports indicate that integrin functions can be modulated by talin, kindlin, and α-actinin; however, their role in GPCR-induced upregulation of $\alpha_4\beta_1$ integrin affinity in leukocytes has not been established. Most of these studies focused on LFA-1, and much less is known about $\alpha_4\beta_1$. The $\alpha_4\beta_1$ integrin has a different subcellular distribution than does LFA-1, and it therefore may be regulated by distinct cytoskeletal proteins (32, 33). In the present study, we investigated the role of talin-1, α-actinins, and kindlin-3 in key steps of the leukocyte adhesion cascade that are mediated by $\alpha_4\beta_1$ integrin binding to VCAM-1. We found that these molecules were involved in $\alpha_4\beta_1$ integrin-dependent adhesion or chemotactic migration, but not upregulation of affinity.

Materials and Methods

Leukocytes

The myelomonocytic U937 cell line transfected with human formyl peptide receptor (U937-FPR) was cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS and 500 μg/ml Geneticin (5). U937-FPR cells were used since they are readily transfectable and mirror $\alpha_4\beta_1$ integrin biology of peripheral blood monocytes, including expression of $\alpha_4\beta_1$ with relatively low basal affinity that is rapidly and transiently upregulated by GPCR signaling (5, 6).

Lymphocytes were harvested from the spleens of mice by passage through a 0.117-mm wire mesh. Lymphocytes were concentrated by centrifugation at 300 × *g* at 4°C for 5 min and resuspended in PBS containing 0.5% FBS (PBS-FBS). Mice were bred and housed under pathogen-free conditions at the University Health Network Animal Facility. All experiments were performed according to institutional guidelines, as well as Canadian federal and provincial laws for animal protection.

Small interfering RNA-mediated gene silencing and transfection of DNA constructs

Gene silencing was achieved by nucleofection of small interfering RNA (siRNA) oligonucleotides using the Amaxa Nucleofector device and cell line Nucleofector kit V (Lonza, Walkersville, MD). Twenty-one–nucleotide siRNAs targeting exon 6 of talin-1 (ID no. 5552), exon 3 of α-actinin-1 (ID no. 9416), exon 9 of kindlin-3 (ID no. s38130), and a negative control (no. 1) were obtained from Ambion (Austin, TX). Transfected U937-FPR cells were maintained in RPMI 1640/10% FBS for 48–72 h prior to use in functional assays. Gene expression was monitored by real-time PCR and/or Western blotting of cell lysates and normalized to actin expression. Cells were transfected with 2 μg phosphatidylinositol phosphate kinase type I γ (PIPKIγ-90)-GFP construct (obtained from Dr. P. De Camilli, Yale University School of Medicine) or a control GFP construct (Lonza) as described for siRNA silencing. Expression of transfected constructs was determined by flow cytometry.

LDV-FITC binding assays

The binding of a monomeric soluble ligand is dependent on individual integrin/ligand interactions and can be used to assess integrin affinity (34). FITC-conjugated peptide 4-((*N*′-2-methylphenyl)ureido)-phenylacetyl-L-leucyl-L-α-aspartyl-L-valyl-L-prolyl-L-alanyl-L-alanyl-L-lysine (LDV-FITC) (35) was synthesized by Commonwealth Biotechnologies (Richmond, VA) and used as a ligand for $\alpha_4\beta_1$ integrin. The LDV-FITC binding assay was performed as previously described (6). Briefly, U937-FPR cells (0.5×10^6 cells in 50 μl) suspended in HBSS (with 1 mM $Ca^{2+}Mg^{2+}$) supplemented with 20 mM HEPES and 0.5% FBS (assay buffer) were incubated with LDV-FITC (3 nM) for 30 min at 37°C. Cells were stimulated at 37°C with fMLF (100 nM, 2 min), SDF-1α (500 ng/ml, 2 min), PMA (50 nM, 30 min), or $MnCl_2$ (1 mM, 5 min), diluted with 1 ml HBSS, and immediately fixed by adding 1 ml 2% paraformaldehyde at 22°C to fix bound LDV-FITC. Cells were washed with HBSS and bound LDV-FITC was detected using biotin-conjugated anti-FITC followed by streptavidin-PE (or allophycocyanin) (Jackson ImmunoResearch Laboratories, West Grove, PA). The FITC signal from the bound LDV-FITC was quenched by the anti-FITC Ab. Cells were analyzed by flow cytometry using a Cytomics FC500 (Beckman Coulter, Fullerton, CA). Consistent with previous studies (5, 6, 35), U937-FPR cells had low $\alpha_4\beta_1$ integrin affinity, and treatment with 1 mM $MnCl_2$, a positive control that increases $\alpha_4\beta_1$ integrin affinity for ligand by association with divalent cation binding sites (36), induced maximal LDV-FITC binding. Affinity was upregulated by fMLF, SDF-1α (GPCR-dependent), or PMA (GPCR-independent), and the data are expressed as percentage of maximal stimulation by Mn^{2+} (Fig. 1). In our experience, the LDV-FITC assay is more robust than binding of Abs that detect conformational changes in the β_1-chain. Furthermore, these Abs are difficult to use in the rapid time frame of GPCR-induced $\alpha_4\beta_1$ integrin affinity, and they may detect occupancy of the ligand-binding pocket (37). The specificity of LDV-FITC for α_4 integrin was shown previously (35).

Parallel plate flow chamber assays

Leukocyte arrest and detachment assays were performed using parallel plate flow chambers (Glycotech, Rockville, MD) as described previously (5, 6). Human VCAM-1/Fc or ICAM-1/Fc chimeric proteins (R&D Systems, Minneapolis, MN) and SDF-1α (PeproTech, Rocky Hill, NJ) were immobilized where indicated to 35-mm polystyrene tissue culture dishes as previously described (5). Briefly, goat anti-human IgG $F(ab')_2$ was incubated for 60 min at 22°C (10 μg/ml for VCAM-1/Fc or 100 μg /ml for ICAM-1/Fc) followed by SDF-1α at 20 μg/ml (90 min at 22°C). Nonspecific binding sites were blocked with 5% FBS, and the coated area was incubated with VCAM-1/Fc (5 μg/ml) or ICAM-1/Fc (20 μg/ml) for 60 min. The coating density using this method was determined to be 544 VCAM-1 molecules or 2400 ICAM-1 molecules/mm^2 (6). Adhesion molecule-coated dishes served as the bottom surface, and a silicone gasket formed a 0.254-mm-high and 2.5-mm-wide flow path. Flow chambers

were maintained at 37°C with a stage heater. Cells were observed with a Diaphot 300 inverted phase contrast microscope (Nikon, Melville, NY), recorded with a Sony DXC-151A video camera and Sony SVT-S3100 time-lapse video cassette recorder, and analyzed manually offline.

For arrest assays, U937-FPR cells in assay buffer were infused with a programmable syringe pump (Kent Scientific) at a wall shear stress of 0.8 dyne/cm^2. The number of accumulated (stationary) leukocytes was determined in six nonoverlapping high-power fields after 2 min of flow. Individual adhesive events were further classified in a single field by the duration and type of adhesive interactions. Tethers were defined as adhesions lasting <1 s, and arrest events were subdivided into adhesions of 1–5 s or >5 s. It was unusual for cells that were adherent for >5 s to detach subsequently.

For detachment assays, U937-FPR cells were injected via the outflow port into inverted flow chambers and allowed to settle for 2 min under static conditions once chambers were overturned. Shear stress of 1, 2, 4, 10, and 20 dynes/cm^2 at 20-s intervals was applied by pulling assay buffer through chambers with a programmable syringe pump. The number of U937 cells remaining attached after each interval was determined and expressed as a percentage of originally settled cells. Cells were pretreated with 100 nM fMLF (2 min at 22°C) or 50 nM PMA (30 min at 37°C) prior to introduction into the flow chamber.

Cell lysis, isolation of microridges/microvilli, and immunoprecipitation

An equal volume of 2× ice-cold lysis buffer was added to a suspension of U937 cells. Cell lysis buffer (2×) contained 40 mM Tris, 300 mM NaCl, 20 mM EDTA, 2 mM sodium orthovanadate, 2 mM NaF, 2% Triton X-100, 0.1% Tween 20, 1 mM PMSF, and other protease inhibitors (Complete Mini protease inhibitor mixture; Roche, Laval, QC, Canada). Phalloidin (10 μM) was included in the lysis buffer to stabilize actin and actin-containing complexes. Lysates were centrifuged at 12,000 × *g* for 15 min at 4°C to remove nuclei and insoluble cytoskeleton.

To purify the microridge/microvillus-enriched fraction, cells were passed six times through a 27-gauge needle (22°C) to shear cell surface processes. Low-speed centrifugation (2,500 × *g*, 5 min, 4°C) was used to pellet cells, and sheared microvilli and microridges from the supernatant were collected by high-speed centrifugation (16,000 × *g*, 40 min, 4°C). Microvilli were washed with PBS and lysed in 1× lysis buffer. Trypan blue exclusion determined that cells subjected to shearing remained viable. They were washed, lysed, and the nuclei and insoluble cytoskeleton were removed by centrifugation.

Protein concentration in lysates was determined using a DC protein assay kit (Bio-Rad). For precipitations, 2 mg total lysate was precleared with mouse IgG and protein G-agarose beads for 30 min at 4°C. Anti-α_4 integrin (HP2/1; Serotec, Raleigh, NC) was incubated with the precleared lysates for 2 h followed by protein G-agarose (Invitrogen, Burlington, ON, Canada) overnight (4°C with rocking). Beads were washed three times in PBS containing protease inhibitors and boiled in SDS-PAGE sample buffer. Equal amounts of each fraction were subjected to electrophoresis.

SDS-PAGE and Western blotting

SDS-PAGE was carried out using 8% acrylamide gels (100 V, 22°C), and proteins were transferred to polyvinylidene difluoride membrane (22 V, 4°C, 18 h). Membranes were probed with primary Abs, HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories) and developed using the ECL system (Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada). Abs against the following proteins were used for Western blotting: talin (Chemicon), α_4 integrin (Santa Cruz Biotechnology, Santa Cruz, CA), P-selectin glycoprotein ligand-1 (PSGL-1; R&D Systems), kindlin-3 (M. Moser, Max Planck Institute of Biochemistry), α-actinin (clone BM 75.2; Sigma-Aldrich), and actin (Sigma-Aldrich). Blots were stripped in 100 mM 2-ME, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) prior to reprobing.

Scanning electron microscopy

For electron microscopic analysis, U937-FPR cells (10 μl at a concentration of 1.0 × 10^6 cells/ml) were allowed to adhere for 15 min to 40-mm glass coverslips coated with poly-L-lysine (150,000–300,000 kDa molecular mass). Cells were fixed with 2.5% glutaraldehyde and 2.0% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 h at 22°C. After washing, coverslips were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h. Samples were dehydrated through an ascending graded ethanol series, infiltrated with a graded hexamethyldisilizane (Sigma-Aldrich) in absolute ethanol, and allowed to dry overnight in a fume hood. Coverslips were mounted on aluminum stubs and sputter coated with gold-palladium (Bal-Tec SCD 050) and examined using a Hitachi S-2500 scanning electron microscope operated at 15 kV. Digital images were acquired using a frame grabber and Quartz PCI software.

Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). A Student *t* test was used to compare two groups, and an ANOVA with the Bonferroni post hoc test was used for comparisons of multiple groups. Significant differences ($p < 0.05$) were indicated.

Results

Talin-1 and kindlin-3 are not involved in chemokine-induced $\alpha_4\beta_1$ integrin affinity upregulation

In nonhematopoietic cells, talin is an important regulator of integrin adhesive and signaling functions. To investigate the role of talin in $\alpha_4\beta_1$ integrin affinity upregulation we used siRNA-mediated gene silencing to suppress talin-1 expression in U937-FPR cells. Maximal knockdown of talin protein expression was achieved with 2 μg siRNA (Supplemental Fig. 1*A*). Relative to negative control siRNA, suppression of talin protein expression ranged from 60 to 95%, with an average of 76%. The Ab used to detect talin levels by Western blotting (TD77) detects both talin-1 and talin-2 and therefore detects all remaining talin expression by the cells. Expression of $\alpha_4\beta_1$ integrin on the cell surface was not altered by siRNA knockdown of talin-1 (Supplemental Fig. 1*C*).

Talin-1 siRNA had no effect on basal or Mn^{2+}-induced LDV-FITC binding (data not shown). Significant differences in the induction of LDV-FITC binding following stimulation with fMLF, SDF-1α, or PMA were also not observed when comparing untransfected (data not shown), control siRNA-treated (Fig. 1, Fig. 2*A*), or talin-1 siRNA-treated cells (Fig. 2*A*). The relationship between the extent of talin knockdown and inhibition of fMLF-induced affinity change did not reveal a positive correlation (Fig. 2*B*). These results suggest that talin does not regulate basal, fMLF-, SDF-1α–, or PMA-induced $\alpha_4\beta_1$ integrin affinity.

An alternative approach was used to assess talin function. U937-FPR cells were transfected with a GFP-tagged fragment of PIPKIγ-90, which binds to the F3 domain of talin in a region that overlaps with the integrin binding site and consequently competes with integrins for talin binding (38). No differences in LDV-FITC binding were observed in unstimulated, Mn^{2+}-, fMLF-, or PMA-stimulated cells gated for high, low, or negative PIPKIγ-90-GFP expression, and the induction of LDV-FITC binding following stimulation with fMLF or PMA was similar (Fig. 2*C*). Similar results were obtained with cells transfected with GFP alone (not shown).

A recent study demonstrated that kindlin-3 is critical for LFA-1 but not $\alpha_4\beta_1$ integrin adhesive functions in human lymphocytes (25). Therefore, we investigated the role of kindlin-3 in the regulation of α_4 integrin affinity. siRNA knockdown of kindlin-3 reduced mRNA levels by 84% and resulted in undetectable residual kindlin-3 protein levels (Supplemental Fig. 1*B*). Expression of $\alpha_4\beta_1$ integrin on the cell surface was not altered by siRNA knockdown of kindlin-3 (Supplemental Fig. 1*C*). Kindlin-3 knockdown did not affect fMLF- or SDF-1α–induced α_4 integrin affinity upregulation (GPCR-induced signaling); however, PMA-induced affinity upregulation was inhibited by 50% (Fig. 2*D*).

Talin-1 and kindlin-3 differentially regulate postadhesion strengthening on VCAM-1

Chemokine-induced $\alpha_4\beta_1$ integrin-mediated leukocyte arrest is dependent on integrin affinity upregulation (5, 6). Consistent with these and the above data that talin is not involved in fMLF-induced upregulation of integrin affinity, the accumulation of infused U937-FPR cells on a surface with coimmobilized VCAM-1

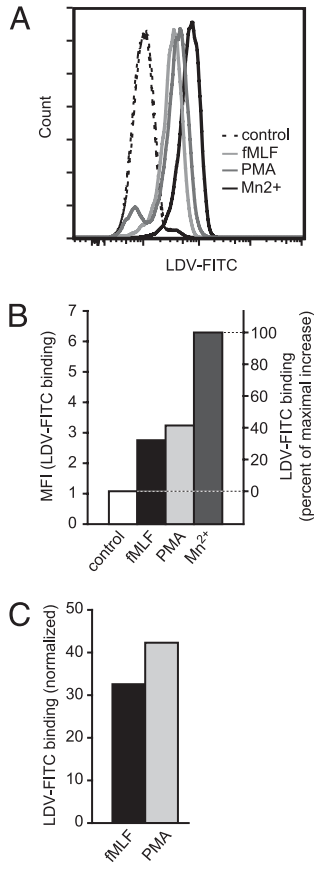

FIGURE 1. Measurement of $\alpha_4\beta_1$ integrin affinity upregulation with LDV-FITC. *A*, U937-FPR cells were stimulated with fMLF (2 min, 100 nM), PMA (30 min, 50 nM), or $MnCl_2$ (5 min, 1 mM) in the presence of LDV-FITC peptide. After fixation with paraformaldehyde, bound LDV-FITC was detected with biotin-anti–FITC and streptavidin-PE by flow cytometry. *B* and C, To standardize for variability between multiple experiments, fMLF- and PMA-induced LDV-FITC binding was normalized to untreated and $MnCl_2$-treated cells.

and SDF-1α was not significantly inhibited by siRNA suppression of talin-1 or kindlin-3 expression (Fig. 3*A*). A closer examination of the adhesive contacts made by talin-1– or kindlin-3–deficient cells revealed that contacts tended to be of shorter duration. A significant increase in the number of tethers of <1 s and a trend toward fewer arrest events were observed (Fig. 3*B*).

We then investigated the role of talin-1 and kindlin-3 in integrin-mediated adhesive functions that occur after the initial binding to VCAM-1 and performed detachment assays that model the adhesion strengthening step of the leukocyte adhesion cascade. U937-FPR cells were allowed to adhere for 2 min to VCAM-1/Fc–coated surfaces in parallel plate flow chambers prior to introduction of fluid flow with incremental increases in shear stress. Unstimulated U937-FPR cells adhered very weakly to VCAM-1, in that <1% of cells remained adherent after very low shear stress (1 dyne/cm^2) was induced (data not shown). siRNA knockdown of talin-1 did not affect this weak basal adhesion to VCAM-1. Stimulation of cells in solution with fMLF or through surface coimmobilized SDF-1α induced significant adhesion of U937-FPR cells that resisted detachment even at high fluid shear stresses. Cells transfected with control or talin-1 siRNA showed a similar resistance to detachment (Fig. 4*A*). Even when talin expression was suppressed by 90%, no inhibition of adhesion was observed. In contrast, siRNA-mediated knockdown of talin-1 significantly attenuated fMLF-induced adhesion strengthening on ICAM-1 (Fig. 4*C*).

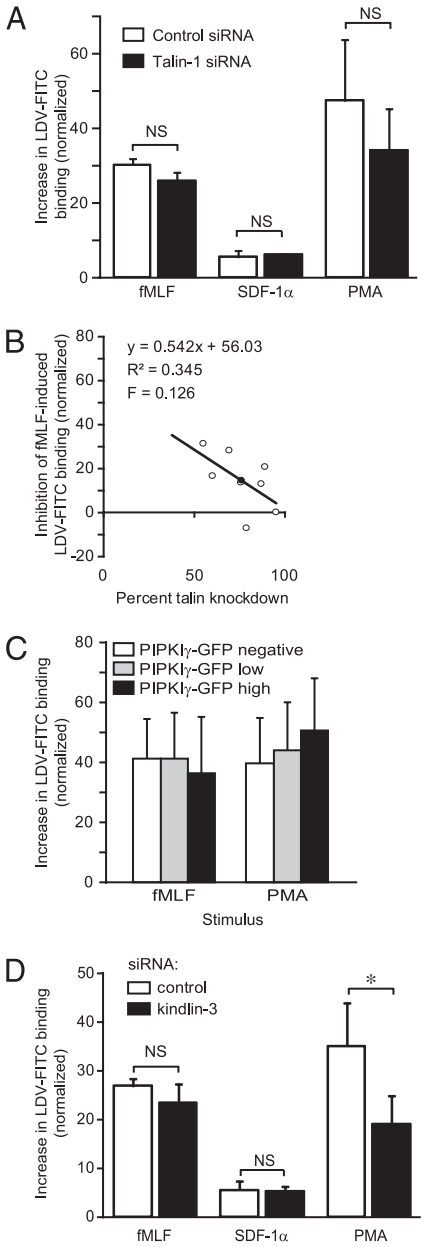

FIGURE 2. Talin-1 and kindlin-3 do not mediate fMLF- or PMA-induced $\alpha_4\beta_1$ integrin affinity upregulation. *A*, siRNA silencing of talin-1 did not inhibit fMLF-, SDF-1α–, or PMA-induced binding of LDV-FITC peptide (mean ± SEM; $n = 8$ for fMLF, $n = 4$ for SDF-1α and PMA). *B*, The relationship between the extent of talin knockdown and fMLF-induced affinity change is plotted. Each of eight individual experiments (○) and the calculated mean (●) are shown. *C*, U937-FPR cells were transfected with a GFP-tagged fragment of PIPKIγ90, which binds to the F3 domain of talin and competes with integrins for talin binding. LDV-FITC binding (detected with anti–FITC-biotin and streptavidin-allophycocyanin) was analyzed on cells gated for high, low, or negative PIPKIγ90-GFP expression. Differences in stimulated LDV-FITC binding were not observed (mean ± SEM, $n = 3$). *D*, siRNA silencing of kindlin-3 did not inhibit fMLF- or SDF-1α–induced LDV-FITC binding to U937-FPR cells, but inhibited PMA-induced binding by ~50% (mean ± SEM, $n = 5$). *$p < 0.05$. NS, $p \geq 0.05$.

Kindlin-3 knockdown diminished the ability of U937-FPR cells to resist detachment from both VCAM-1– and ICAM-1–coated surfaces after stimulation of GPCRs (Fig. 4*B*, 4*D*). Both talin-1 and kindlin-3 contribute to PMA-induced resistance to detachment on VCAM-1 (Fig. 4*A*, 4*B*).

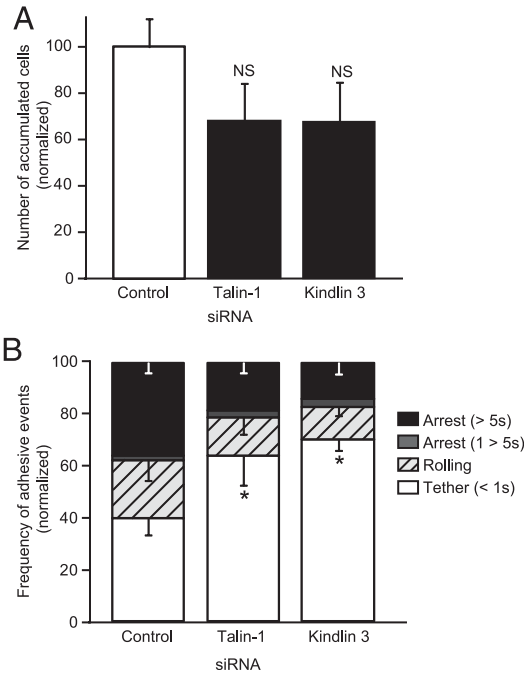

FIGURE 3. Silencing of talin-1 or kindlin-3 reduces the duration of adhesive contacts with VCAM-1 under shear flow. *A*, Cells were infused at 0.8 dyne/cm^2 into parallel plate flow chambers in which the adhesion surface was cocoated with VCAM-1/Fc and SDF-1α. The mean ± SEM of cells accumulated during 2 min of flow in six independent microscopic fields is shown. siRNA to talin-1 or kindlin-3 did not significantly reduce the total number of accumulated cells. *B*, The adhesive events observed during 1 min of flow in a single microscopic field were categorized by duration and type of contact. Silencing of talin-1 and kindlin-3 significantly increased the proportion of cells that made tethers of <1 s ($p < 0.05$, $n = 7$ assays in three independent experiments). The absolute number of adhesive events was not significantly different between groups (mean ± SEM; control siRNA, 16.3 ± 2.7; talin-1 siRNA, 13.1 ± 2.5; kindlin-3 siRNA, 11.1 ± 2.5; ANOVA, $p > 0.05$).

Talin-1 is required for $\alpha_4\beta_1$ integrin-dependent migration

Following adhesion to inflamed endothelium, leukocytes undergo intravascular crawling, diapedesis across the vascular endothelium, and they migrate into extravascular tissues. We therefore examined the role of talin-1 in α_4 integrin-dependent chemotaxis through VCAM-1–coated filters using fMLF as a chemoattractant. fMLF induced a 10-fold increase in migration compared with basal (unstimulated) migration (Supplemental Fig. 2). Migration was dependent on α_4 integrins, as was demonstrated by preincubating cells with a function-blocking anti-α_4 integrin Ab (Supplemental Fig. 2). fMLF-induced migration of U937-FPR cells transfected with talin-1 siRNA was significantly impaired (Supplemental Fig. 2).

Membrane microridges of U937-FPR cells and mouse splenocytes contain $\alpha_4\beta_1$ integrin, but not talin

Leukocyte microvilli and microridges mediate the initial adhesive interactions with vascular endothelial cells during emigration from blood into tissues. PSGL-1 and α_4 integrins are predominantly expressed on leukocyte microvilli, whereas β_2 integrins are not (32, 33). These distinct cell surface patterns of α_4 and β_2 integrin expression could account for their differential regulation. We used scanning electron microscopy to evaluate the cell surface topology of U937-FPR cells and observed abundant microridges (Fig. 5*A*). Thus, we adapted a method to isolate lymphocyte microvilli by subjecting cells to high fluid shear stress (39), and we isolated membrane microridges from U937-FPR cells and freshly isolated mouse splenocytes. Protein expression profiles in the microridge-enriched fraction were compared with lysates from sheared cells. Western blotting revealed a relative abundance of α_4 integrin in the microridge fraction (Fig. 5*B*), consistent with expression on microvilli. In contrast, talin was not detected in this fraction but was abundant in lysates of cells that were subjected to shearing. Fluid shear stress removed only a fraction of microridges (determined by scanning electron microscopy), and the protein expression profile of these cells was identical to untreated U937 cells (data not shown). Kindlin-3 was detected in the microridge fraction but was relatively more abundant in the cell lysate. PSGL-1 was highly abundant in the microridge fraction, whereas actin and α-actinin-1 were present in both fractions. A similar distribution was observed in microridges isolated from wild-type U937 cells (data not shown).

Talin and kindlin-3 associate with $\alpha_4\beta_1$ integrin following stimulation of chemoattractant receptors

Because siRNA suppression revealed that talin-1 and kindlin-3 are involved in migration and adhesion, we evaluated their association with $\alpha_4\beta_1$ integrin before and after stimulation with fMLF. In unstimulated U937-FPR cells, the basal association of talin with α_4 integrin was low, and stimulation with fMLF induced a time-dependent association that was maximal at 3 min and persisted at 20 min (Fig. 5*C*). The predominant talin band that coimmunoprecipated with $\alpha_4\beta_1$ integrin had a high molecular mass (>200 kDa), suggesting that full-length talin (240 kDa) was associated with $\alpha_4\beta_1$ integrin in both basal and stimulated conditions. The amount of talin that associated with the $\alpha_4\beta_1$ integrin was only a very small fraction relative to the total amount of talin present in the cell lysates. Kindlin-3 association with $\alpha_4\beta_1$ integrins in unstimulated U937-FPR cells was minimal, but it increased rapidly and transiently upon stimulation of U937-FPR cells with fMLF (Fig. 5*C*). Evaluation of the kinetics suggested that the maximal association of kindlin-3 preceded talin. These data are consistent with the participation of these proteins in $\alpha_4\beta_1$ integrin-mediated adhesion and migration, respectively.

α-Actinin is not involved in $\alpha_4\beta_1$ integrin affinity upregulation or adhesion to VCAM-1

The cytoskeletal protein α-actinin can associate with the cytoplasmic tails of β_1 and β_2 integrins, and this association is induced by stimulating cells with a chemoattractant (28). T cell migration, but not adhesion to ICAM-1, is dependent on the association of α-actinin with both the actin cytoskeleton and the integrin cytoplasmic tails (29). U937 cells express α-actinin isoforms 1 and 4 (determined by real-time PCR; data not shown). We investigated the role of α-actinin-1 by transfecting U937-FPR cells with siRNA. This resulted in 86% (±3.5%) knockdown of α-actinin-1 at 48 h (Supplemental Fig. 1). siRNA targeting of α-actinin-1 also resulted in 40–60% knockdown of α-actinin-4 mRNA levels (data not shown). Knockdown of α-actinin-1 did not inhibit fMLF- or PMA-induced α_4 integrin affinity upregulation (Fig. 6*A*). In detachment assays, α-actinin knockdown did not have significant effects on adhesion to VCAM-1 induced by fMLF, SDF-1α, or PMA, or adhesion to ICAM-1 induced by fMLF (Fig. 6*B*). siRNA targeting of α-actinin-4 resulted in a 70% selective knockdown of α-actinin-4 mRNA levels, but no inhibition of fMLF-induced LDV-FITC binding (data not shown).

Discussion

Integrin activation is critical for a variety of pathological events such as thrombosis, inflammation, angiogenesis, and tumor meta-

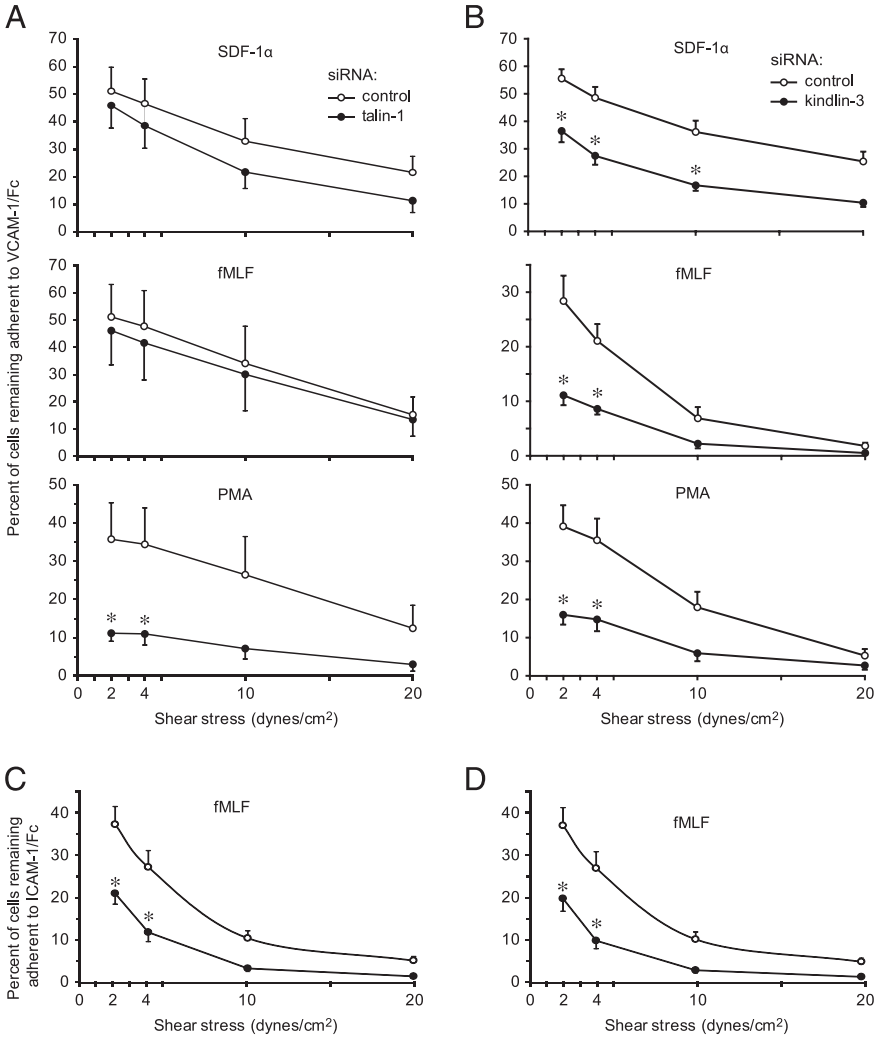

FIGURE 4. siRNA knockdown of kindlin-3, but not talin-1, impairs SDF-1α– and fMLF-induced $\alpha_4\beta_1$ integrin-mediated resistance to detachment. Detachment assays using adhesion surfaces coated with VCAM-1/Fc (*A*, *B*) or ICAM-1/Fc (*C*, *D*) were performed with U937-FPR cells transfected with control (*A–D*), talin-1 (*A*, *C*), or kindlin-3 (*B*, *D*) siRNA. Cells were allowed to adhere for 2 min under static conditions in a parallel plate flow chamber prior to the introduction of flow with incrementally increasing shear rates, and the percentage of cells remaining attached at each shear rate was determined. SDF-1α was coimmobilized with VCAM-1/Fc or, alternatively, cells were pretreated with PMA (50 nM) for 30 min or stimulated in suspension with fMLF (100 nM) immediately prior to infusion into flow chambers. Means ± SEM are plotted. $*p < 0.05$. For *A*, $n = 9$ for SDF-1α, $n = 7$ for fMLF and PMA; for *B*, $n = 8$ for SDF-1α, $n = 6$ for fMLF and PMA; for *C* and *D*, $n = 8$. (The data in *C* and *D* are from the same series of experiments and the same control siRNA data series is presented.)

stasis. During leukocyte recruitment from the blood to extravascular sites of inflammation, modulation of leukocyte integrin adhesive functions is essential for the coordinated arrest, adhesion stabilization, and migration on and through the endothelial cell monolayer. This modulation of integrin functions is complex and involves chemokine GPCR inside–out signaling that triggers rapid

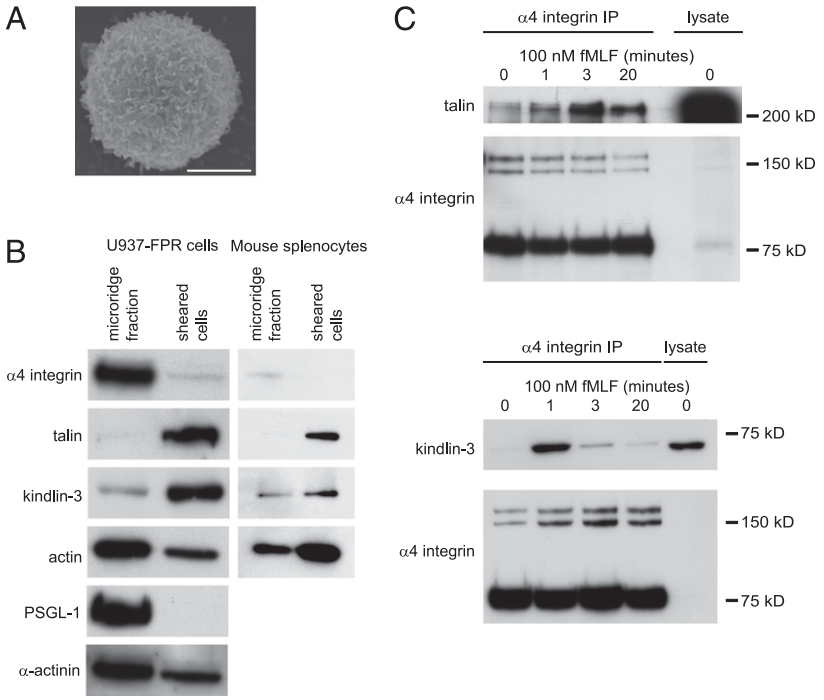

FIGURE 5. Biochemical studies showing distinct subcellular localization of $\alpha_4\beta_1$ integrin, kindlin-3, and talin, as well as rapid association of kindlin-3 with $\alpha_4\beta_1$ after stimulation with fMLF. *A*, A representative scanning electron micrograph of a U937-FPR cell reveals the presence of abundant membrane microridges and microvilli. Scale bar, 4.3 μm. *B*, Protein lysates (10 μg protein per lane) isolated from microridges and sheared cells were resolved by PAGE and subjected to repeated Western blotting with the indicated Abs. Representative blots (one of three independent experiments) are shown. *C*, fMLF stimulation induces the association of kindlin-3 and talin with $\alpha_4\beta_1$ integrin. U937-FPR cells were stimulated with fMLF (100 nM) for the indicated period of time prior to lysis and immunoprecipitation (IP) of cell lysates with anti-α_4 integrin (HP2/1). Shown are representative Western blots (one of three independent experiments) probed for talin and α_4 integrin (*upper panels*) or for kindlin-3 and α_4 integrin (*lower panels*). In parallel, the relative abundance of each protein in whole cells was determined by Western blotting (*right lane*, 10 μg protein). Talin was very abundant in the whole cell lysate relative to the α_4 integrin IP lanes, whereas α4 integrin was barely detectable.

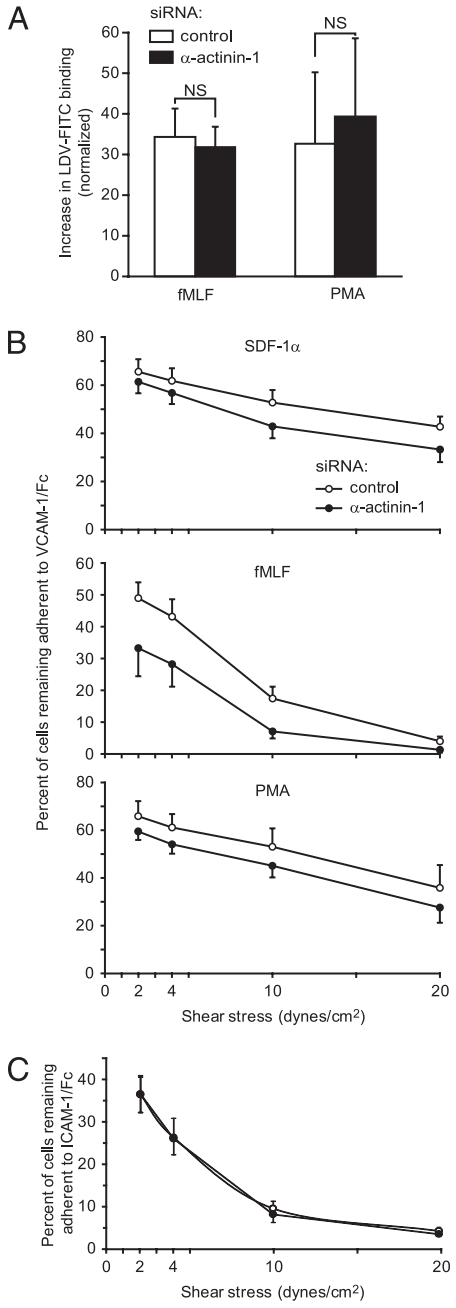

FIGURE 6. siRNA knockdown of α-actinin-1 does not inhibit fMLF- or PMA-induced $\alpha_4\beta_1$ integrin affinity upregulation or $\alpha_4\beta_1$ integrin-mediated resistance to detachment induced by SDF-1α, fMLF, or PMA. U937-FPR cells were transfected with control or α-actinin-1 siRNA. The means ± SEM are plotted. *A*, LDV-FITC binding assays (n = 5, NS indicates $p \geq 0.05$). *B* and *C*, Detachment assays performed with adhesion surfaces coated with VCAM-1/Fc (*B*) or ICAM-1/Fc (*C*). For VCAM-1: SDF-1α, eight assays from three independent experiments; fMLF, six assays from two independent experiments; PMA, eight assays from three independent experiments. For ICAM-1: eight assays from three independent experiments. Significant differences were not observed between cells transfected with control and α-actinin-1 siRNA.

and transient upregulation of integrin affinity, as well as outside–in signaling upon ligand binding. Thus, distinct inside–out and outside–in signaling pathways are interrelated in the context of integrin-mediated cell adhesion. We studied inside–out GPCR signaling by directly measuring $\alpha_4\beta_1$ integrin affinity using a soluble monomeric ligand (LDV-FITC) binding assay. Cell adhesion and chemotactic migration were also studied, in which outside–in signaling undoubtedly contributes to $\alpha_4\beta_1$ integrin functions.

Microvilli and microridges are actin-rich cell surface protrusions whose molecular structure is organized to provide a favorable platform for adhesion of leukocytes in flowing blood. Expression of L-selectin on tips of microvilli is required for efficient tethering of leukocytes (40). Consistent with previous reports (39), we found enrichment of the selectin ligand PSGL-1 in microridges isolated from U937-FPR cells. $\alpha_4\beta_1$ integrin was enriched in microridges/microvilli from U937 cells and mouse splenocytes. Microvilli isolated from peripheral blood T cells contain relatively abundant cytoskeletal proteins such as actin and ERM family members (ezrin, radixin, moesin), but not talin (39). We also demonstrated an enrichment of actin and α-actinins in U937 microridges. Despite the colocalization of $\alpha_4\beta_1$ and α-actinins in microridges, knockdown of α-actinins had no effect on GPCR-induced $\alpha_4\beta_1$ affinity upregulation or adhesion to VCAM-1 or ICAM-1. These data are consistent with reports that α-actinin participates in migration of T cells on ICAM-1 but not LFA-1 activation (29). We found that kindlin-3 was present in microridges from U937 cells and mouse splenocytes, but talin was essentially excluded. Consistent with these data, kindlin-3 associated rapidly and transiently with $\alpha_4\beta_1$ integrin upon fMLF stimulation, whereas maximal talin association appeared at later time points and to a lesser extent. Perhaps this explains why kindlin-3, but not talin-1, functions to stabilize $\alpha_4\beta_1$-mediated adhesion in detachment assays.

Despite associating with $\alpha_4\beta_1$ integrin upon GPCR signaling, neither kindlin-3 nor talin-1 was required for affinity upregulation. This suggests that kindlin-3 and talin-1 function primarily in adhesion strengthening, cell spreading, and motility, events that occur subsequent to rapid chemokine-induced $\alpha_4\beta_1$ integrin affinity changes and arrest. In this case, their role may be to promote outside–in signaling though $\alpha_4\beta_1$ integrin clustering and to enhance or stabilize the linkage of integrins to the actin cytoskeleton, which is critical for integrin function, as was demonstrated by observations that deletion of the β-chain cytoplasmic tail disrupts normal integrin–ligand interactions (41).

Both kindlin-3 and talin-1 contribute to fMLF-induced adhesion to ICAM-1. This suggests that the regulation of $\alpha_4\beta_1$ and $\alpha_L\beta 2$ integrin functions is not identical. Regulation of the same integrin in different cell types may also be distinct. For example, kindlin-3–deficient effector T cells exhibited impaired adhesion to ICAM-1, but normal adhesion to VCAM-1 (25).

The rapid upregulation of integrin affinity triggered by GPCR signaling may involve mechanisms distinct from those that regulate integrin functions in cells with prolonged integrin activation, such as Ag-activated T cells and PMA-treated cells, as well as cells that express integrins with constitutively high affinity, such as the Jurkat T cell line. Knockdown of talin-1 in Jurkat cells reduced basal $\alpha_4\beta_1$ integrin affinity and inhibited unstimulated and chemokine-induced arrest and adhesion (42). However, $\alpha_4\beta_1$ integrins on unstimulated Jurkat cells mediate arrest on VCAM-1-coated surfaces without a requirement for GPCR signaling. Thus, Jurkat cells do not model circulating leukocytes that express low-affinity $\alpha_4\beta_1$ integrins. Talin-1 also regulates T cell adhesion to ICAM-1 (43) but not LFA-1 affinity (43). Overexpression of the talin-1 head domain results in separation of LFA-1 cytoplasmic tails, which is also observed following chemokine stimulation and correlates with integrin conformational change and activation (44). However, studies suggest that talin induces an intermediate-, but not a high-, affinity LFA-1 conformation (45). Talin-1 knockdown in Jurkat and peripheral blood T cells greatly reduced adhesion and LFA-1 clustering induced by TCR crosslinking or PMA stimulation, but did not inhibit LFA-1 affinity changes induced by TCR crosslinking

and had a small but significant inhibitory effect on PMA-induced affinity (46). Although the above reports suggest that talin can activate leukocyte integrins and may play a role in prolonged integrin activation and adhesion, our data show that talin is not essential for the rapid and transient GPCR-induced activation of $\alpha_4\beta_1$ integrin. However, we confirm that talin-1 is involved in mediating GPCR-induced adhesion to ICAM-1.

Although kindlin-3 was not required for fMLF- or SDF-1α–induced upregulation of $\alpha_4\beta_1$ integrin affinity, knockdown of kindlin-3 reduced PMA-induced affinity upregulation and adhesion to VCAM-1. Phorbol esters, such as PMA, activate PKC and have been shown to activate integrins (6, 7). Previously, we demonstrated that PMA induces a gradual and sustained up-regulation of α4β1 integrin affinity, with kinetics that were distinct from chemokine GPCR-induced affinity changes, and that protein kinase C (PKC) signaling was not required for GPCR-induced $\alpha_4\beta_1$ affinity changes (6). The different signaling pathways involved in PMA- and GPCR-induced affinity changes may explain why knockdown of kindlin-3 inhibited only PMA-induced and not GPCR-induced upregulation of $\alpha_4\beta_1$ integrin affinity. Knockdown of neither talin-1 nor α-actinin-1 had an effect on upregulation of $\alpha_4\beta_1$ integrin affinity by PMA. PMA induces LFA-1 affinity for ICAM-1, but chemokine-induced β_2 integrin affinity is independent of PKC activity (8), and the arrest of SDF-1–stimulated peripheral blood leukocytes on ICAM-1 is unaffected by inhibitors of PKC (43). Knockdown of talin-1 inhibited PMA- but not GPCR-induced U937 cell adhesion to VCAM-1, whereas knockdown of kindlin-3 inhibited adhesion to VCAM-1 after stimulation with either PMA or GPCR agonists. fMLF-induced adhesion to ICAM-1 was dependent on both talin-1 and kindlin-3. In contrast, kindlin-3–deficient effector T cells exhibited normal adhesion strengthening on VCAM-1, but impaired adhesion to ICAM-1 (25).

Numerous signaling events contribute to the regulation of leukocyte motility. Cell motility involves changes in integrin affinity, valency of binding, as well as major remodeling of the actin cytoskeleton, each of which may involve distinct or overlapping signaling molecules. Integrin interactions with the actin cytoskeleton regulate avidity by directing clustering and lateral diffusion in the plane of the cell membrane (47). Cytoplasmic tail deletions of α_4 integrin restrict lateral mobility and abrogate cell adhesion to VCAM-1 without changing $\alpha_4\beta_1$ integrin affinity for soluble ligand (48). Because talin was largely excluded from U937 cell microridges and its subcellular localization was distinct from $\alpha_4\beta_1$ integrin, it is not surprising that this cytoskeletal protein does not participate in the rapid affinity upregulation induced by GPCR signaling. With time, chemoattractant GPCR signaling induces leukocyte cytoskeletal reorganization (49), which may favor talin interactions with $\alpha_4\beta_1$ integrin during migration.

In summary, we showed that talin-1, kindlin-3, and α-actinins are not involved in chemokine-induced affinity upregulation of $\alpha_4\beta_1$ integrin in monocytic cells. Binding of ligands to chemokine or chemoattractant GPCRs activates numerous signal transduction cascades (50); however, only activation of phospholipase C and calcium signaling are involved in upregulation of $\alpha_4\beta_1$ integrin affinity (5, 6). The signaling cascade downstream of calcium/calmodulin (6) remains to be elucidated. Kindlin-3 and, to a lesser extent, talin-1 bind to $\alpha_4\beta_1$ integrin after GPCR signaling and function in $\alpha_4\beta_1$-mediated adhesion strengthening and/or chemotactic migration.

Acknowledgments

We thank Henry Hong from the Cell Systems and Biology Imaging Facility at the University of Toronto for assistance with electron microscopy. We are grateful to Dr. Pietro De Camilli from Yale University School of Medicine for providing the PIPKI-γ construct.

Disclosures

The authors have no financial conflicts of interest.

References

1. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76: 301–314.
2. Xiong, J. P., T. Stehle, S. L. Goodman, and M. A. Arnaout. 2003. New insights into the structural basis of integrin activation. *Blood* 102: 1155–1159.
3. Takagi, J., B. M. Petre, T. Walz, and T. A. Springer. 2002. Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. *Cell* 110: 599–511.
4. Hughes, P. E., and M. Pfaff. 1998. Integrin affinity modulation. *Trends Cell Biol.* 8: 359–364.
5. Chan, J. R., S. J. Hyduk, and M. I. Cybulsky. 2001. Chemoattractants induce a rapid and transient upregulation of monocyte α_4 integrin affinity for vascular cell adhesion molecule 1 which mediates arrest: an early step in the process of emigration. *J. Exp. Med.* 193: 1149–1158.
6. Hyduk, S. J., J. R. Chan, S. T. Duffy, M. Chen, M. D. Peterson, T. K. Waddell, G. C. Digby, K. Szaszi, A. Kapus, and M. I. Cybulsky. 2007. Phospholipase C, calcium, and calmodulin are critical for $\alpha_4\beta_1$ integrin affinity up-regulation and monocyte arrest triggered by chemoattractants. *Blood* 109: 176–184.
7. Constantin, G., M. Majeed, C. Giagulli, L. Piccio, J. Y. Kim, E. C. Butcher, and C. Laudanna. 2000. Chemokines trigger immediate β_2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13: 759–769.
8. Giagulli, C., E. Scarpini, L. Ottoboni, S. Narumiya, E. C. Butcher, G. Constantin, and C. Laudanna. 2004. RhoA and ζPKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocyte in vivo homing. *Immunity* 20: 25–35.
9. Ghandour, H., X. Cullere, A. Alvarez, F. W. Luscinskas, and T. N. Mayadas. 2007. Essential role for Rap1 GTPase and its guanine exchange factor CalDAG-GEFI in LFA-1 but not VLA-4 integrin mediated human T-cell adhesion. *Blood* 110: 3682–3690.
10. Ebisuno, Y., K. Katagiri, T. Katakai, Y. Ueda, T. Nemoto, H. Inada, J. Nabekura, T. Okada, R. Kannagi, T. Tanaka, et al. 2010. Rap1 controls lymphocyte adhesion cascade and interstitial migration within lymph nodes in RAPL-dependent and -independent manners. *Blood* 115: 804–814.
11. Rees, D. J. G., S. E. Ades, S. J. Singer, and R. O. Hynes. 1990. Sequence and domain structure of talin. *Nature* 347: 685–689.
12. Ginsberg, M. H., A. Partridge, and S. J. Shattil. 2005. Integrin regulation. *Curr. Opin. Cell Biol.* 17: 509–516.
13. Calderwood, D. A., R. Zent, R. Grant, D. J. Rees, R. O. Hynes, and M. H. Ginsberg. 1999. The talin head domain binds to integrin β subunit cytoplasmic tails and regulates integrin activation. *J. Biol. Chem.* 274: 28071–28074.
14. Tadokoro, S., S. J. Shattil, K. Eto, V. Tai, R. C. Liddington, J. M. de Pereda, M. H. Ginsberg, and D. A. Calderwood. 2003. Talin binding to integrin beta tails: a final common step in integrin activation. *Science* 302: 103–106.
15. Helsten, T. L., T. A. Bunch, H. Kato, J. Yamanouchi, S. H. Choi, A. L. Jannuzi, C. C. Féral, M. H. Ginsberg, D. L. Brower, and S. J. Shattil. 2008. Differences in regulation of *Drosophila* and vertebrate integrin affinity by talin. *Mol. Biol. Cell* 19: 3589–3598.
16. Bunch, T. A. 2010. Integrin $\alpha_{IIb}\beta_3$ activation in Chinese hamster ovary cells and platelets increases clustering rather than affinity. *J. Biol. Chem.* 285: 1841–1849.
17. Ye, F., G. Hu, D. Taylor, B. Ratnikov, A. A. Bobkov, M. A. McLean, S. G. Sligar, K. A. Taylor, and M. H. Ginsberg. 2010. Recreation of the terminal events in physiological integrin activation. *J. Cell Biol.* 188: 157–173.
18. Moser, M., B. Nieswandt, S. Ussar, M. Pozgajova, and R. Fässler. 2008. Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat. Med.* 14: 325–330.
19. Harburger, D. S., M. Bouaouina, and D. A. Calderwood. 2009. Kindlin-1 and -2 directly bind the C-terminal region of β integrin cytoplasmic tails and exert integrin-specific activation effects. *J. Biol. Chem.* 284: 11485–11497.
20. Moser, M., M. Bauer, S. Schmid, R. Ruppert, S. Schmidt, M. Sixt, H. V. Wang, M. Sperandio, and R. Fässler. 2009. Kindlin-3 is required for β_2 integrin-mediated leukocyte adhesion to endothelial cells. *Nat. Med.* 15: 300–305.
21. Svensson, L., K. Howarth, A. McDowall, I. Patzak, R. Evans, S. Ussar, M. Moser, A. Metin, M. Fried, I. Tomlinson, and N. Hogg. 2009. Leukocyte adhesion deficiency-III is caused by mutations in *KINDLIN3* affecting integrin activation. *Nat. Med.* 15: 306–312.
22. Malinin, N. L., L. Zhang, J. Choi, A. Ciocea, O. Razorenova, Y. Q. Ma, E. A. Podrez, M. Tosi, D. P. Lennon, A. I. Caplan, et al. 2009. A point mutation in *KINDLIN3* ablates activation of three integrin subfamilies in humans. *Nat. Med.* 15: 313–318.
23. Kuijpers, T. W., E. van de Vijver, M. A. J. Weterman, M. de Boer, A. T. J. Tool, T. K. van den Berg, M. Moser, M. E. Jakobs, K. Seeger, O. Sanal, et al. 2009. LAD-1/variant syndrome is caused by mutations in FERMT3. *Blood* 113: 4740–4746.
24. Montanez, E., S. Ussar, M. Schifferer, M. Bösl, R. Zent, M. Moser, and R. Fässler. 2008. Kindlin-2 controls bidirectional signaling of integrins. *Genes Dev.* 22: 1325–1330.
25. Manevich-Mendelson, E., S. W. Feigelson, R. Pasvolsky, M. Aker, V. Grabovsky, Z. Shulman, S. S. Kilic, M. A. Rosenthal-Allieri, S. Ben-Dor, A. Mory, et al. 2009. Loss of kindlin-3 in LAD-III eliminates LFA-1 but not VLA-4 adhesiveness developed under shear flow conditions. *Blood* 114: 2344–2353.

26. Otey, C. A., F. M. Pavalko, and K. Burridge. 1990. An interaction between α-actinin and the β_1 integrin subunit in vitro. *J. Cell Biol.* 111: 721–729.
27. Pavalko, F. M., and S. M. LaRoche. 1993. Activation of human neutrophils induces an interaction between the integrin β_2-subunit (CD18) and the actin binding protein α-actinin. *J. Immunol.* 151: 3795–3807.
28. Sampath, R., P. J. Gallagher, and F. M. Pavalko. 1998. Cytoskeletal interactions with the leukocyte integrin β_2 cytoplasmic tail: activation-dependent regulation of associations with talin and α-actinin. *J. Biol. Chem.* 273: 33588–33594.
29. Stanley, P., A. Smith, A. McDowall, A. Nicol, D. Zicha, and N. Hogg. 2008. Intermediate-affinity LFA-1 binds α-actinin-1 to control migration at the leading edge of the T cell. *EMBO J.* 27: 62–75.
30. von Wichert, G., B. Haimovich, G.-S. Feng, and M. P. Sheetz. 2003. Force-dependent integrin-cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2. *EMBO J.* 22: 5023–5035.
31. Laukaitis, C. M., D. J. Webb, K. Donais, and A. F. Horwitz. 2001. Differential dynamics of α_5 integrin, paxillin, and α-actinin during formation and disassembly of adhesions in migrating cells. *J. Cell Biol.* 153: 1427–1440.
32. Abitorabi, M. A., R. K. Pachynski, R. E. Ferrando, M. Tidswell, and D. J. Erle. 1997. Presentation of integrins on leukocyte microvilli: a role for the extracellular domain in determining membrane localization. *J. Cell Biol.* 139: 563–571.
33. Berlin, C., R. F. Bargatze, J. J. Campbell, U. H. von Andrian, M. C. Szabo, S. R. Hasslen, R. D. Nelson, E. L. Berg, S. L. Erlandsen, and E. C. Butcher. 1995. α_4 Integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 80: 413–422.
34. Bednar, B., M. E. Cunningham, P. A. McQueney, M. S. Egbertson, B. C. Askew, R. A. Bednar, G. D. Hartman, and R. J. Gould. 1997. Flow cytometric measurement of kinetic and equilibrium binding parameters of arginine-glycine-aspartic acid ligands in binding to glycoprotein IIb/IIIa on platelets. *Cytometry* 28: 58–65.
35. Chigaev, A., A. M. Blenc, J. V. Braaten, N. Kumaraswamy, C. L. Kepley, R. P. Andrews, J. M. Oliver, B. S. Edwards, E. R. Prossnitz, R. S. Larson, and L. A. Sklar. 2001. Real time analysis of the affinity regulation of α_4-integrin: the physiologically activated receptor is intermediate in affinity between resting and Mn^{2+} or antibody activation. *J. Biol. Chem.* 276: 48670–48678.
36. Jakubowski, A., B. N. Ehrenfels, R. B. Pepinsky, and L. C. Burkly. 1995. Vascular cell adhesion molecule-Ig fusion protein selectively targets activated α_4-integrin receptors in vivo: inhibition of autoimmune diabetes in an adoptive transfer model in nonobese diabetic mice. *J. Immunol.* 155: 938–946.
37. Chigaev, A., A. Waller, O. Amit, L. Halip, C. G. Bologa, and L. A. Sklar. 2009. Real-time analysis of conformation-sensitive antibody binding provides new insights into integrin conformational regulation. *J. Biol. Chem.* 284: 14337–14346.
38. Calderwood, D. A., V. Tai, G. Di Paolo, P. De Camilli, and M. H. Ginsberg. 2004. Competition for talin results in trans-dominant inhibition of integrin activation. *J. Biol. Chem.* 279: 28889–28895.
39. Hao, J. J., G. Wang, T. Pisitkun, G. Patino-Lopez, K. Nagashima, M. A. Knepper, R. F. Shen, and S. Shaw. 2008. Enrichment of distinct microfilament-associated and GTP-binding-proteins in membrane/microvilli fractions from lymphoid cells. *J. Proteome Res.* 7: 2911–2927.
40. Stein, J. V., G. Cheng, B. M. Stockton, B. P. Fors, E. C. Butcher, and U. H. von Andrian. 1999. L-selectin-mediated leukocyte adhesion in vivo: microvillous distribution determines tethering efficiency, but not rolling velocity. *J. Exp. Med.* 189: 37–50.
41. Williams, M. J., P. E. Hughes, T. E. O'Toole, and M. H. Ginsberg. 1994. The inner world of cell adhesion: integrin cytoplasmic domains. *Trends Cell Biol.* 4: 109–112.
42. Manevich, E., V. Grabovsky, S. W. Feigelson, and R. Alon. 2007. Talin 1 and paxillin facilitate distinct steps in rapid VLA-4-mediated adhesion strengthening to vascular cell adhesion molecule 1. *J. Biol. Chem.* 282: 25338–25348.
43. Shamri, R., V. Grabovsky, J. M. Gauguet, S. Feigelson, E. Manevich, W. Kolanus, M. K. Robinson, D. E. Staunton, U. H. von Andrian, and R. Alon. 2005. Lymphocyte arrest requires instantaneous induction of an extended LFA-1 conformation mediated by endothelium-bound chemokines. *Nat. Immunol.* 6: 497–506.
44. Kim, M., C. V. Carman, and T. A. Springer. 2003. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science* 301: 1720–1725.
45. Li, Y. F., R. H. Tang, K. J. Puan, S. K. Law, and S. M. Tan. 2007. The cytosolic protein talin induces an intermediate affinity integrin $\alpha_L\beta_2$. *J. Biol. Chem.* 282: 24310–24319.
46. Simonson, W. T. N., S. J. Franco, and A. Huttenlocher. 2006. Talin1 regulates TCR-mediated LFA-1 function. *J. Immunol.* 177: 7707–7714.
47. Calderwood, D. A., S. J. Shattil, and M. H. Ginsberg. 2000. Integrins and actin filaments: reciprocal regulation of cell adhesion and signaling. *J. Biol. Chem.* 275: 22607–22610.
48. Yauch, R. L., D. P. Felsenfeld, S.-K. Kraeft, L. B. Chen, M. P. Sheetz, and M. E. Hemler. 1997. Mutational evidence for control of cell adhesion through integrin diffusion/clustering, independent of ligand binding. *J. Exp. Med.* 186: 1347–1355.
49. Vicente-Manzanares, M., and F. Sánchez-Madrid. 2004. Role of the cytoskeleton during leukocyte responses. *Nat. Rev. Immunol.* 4: 110–122.
50. Mellado, M., J. M. Rodríguez-Frade, S. Mañes, and C. Martínez-A. 2001. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annu. Rev. Immunol.* 19: 397–421.