Cytoskeletal interactions regulate inducible L-selectin clustering

Polly E. Mattila, Chad E. Green, Ulrich Schaff, Scott I. Simon, and Bruce Walcheck

Department of Veterinary and Biomedical Sciences, University of Minnesota, St. Paul, Minnesota; and Department of Biomedical Engineering, University of California, Davis, California

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L-selectin (CD62L) amplifies neutrophil capture within the microvasculature at sites of inflammation. Activation by G protein-coupled stimuli or through ligation of L-selectin promotes clustering of L-selectin and serves to increase its adhesiveness, signaling, and colocalization with β2-integrins. Currently, little is known about the molecular process regulating the lateral mobility of L-selectin. On neutrophil stimulation, a progressive change takes place in the organization of its plasma membrane, resulting in membrane domains that are characteristically enriched in glycosyl phosphatidylinositol (GPI)-anchored proteins and exclude the transmembrane protein CD45. Clustering of L-selectin, facilitated by E-selectin engagement or antibody cross-linking, resulted in its colocalization with GPI-anchored CD55, but not with CD45 or CD11c. Disrupting microfilaments in neutrophils or removing a conserved cationic motif in the cytoplasmic domain of L-selectin increased its mobility and membrane domain localization in the plasma membrane. In addition, the conserved element was critical for L-selectin-dependent tethering under shear flow. Our data indicate that L-selectin’s lateral mobility is regulated by interactions with the actin cytoskeleton that in turn fortifies leukocyte tethering. We hypothesize that both membrane mobility and stabilization augment L-selectin’s effector functions and are regulated by dynamic associations with membrane domains and the actin cytoskeleton.

MEMBRANE DOMAINS; ADHESION; LEUKOCYTE; INFLAMMATION

Neutrophil recruitment from the bloodstream to sites of inflammation requires overcoming tremendous shear forces to initially tether to the vascular wall. Participation by the selectin adhesion protein family (E-, L-, and P-selectin) is critical for this process. The selectins are type 1 integral membrane proteins that contain a C-type lectin domain and recognize specific glycans moieties. L-selectin (CD62L) is expressed constitutively by leukocytes of myeloid and lymphoid origin; E- and P-selectin (CD62E and CD62P) are expressed by activated endothelial cells and by activated platelets (P-selectin). Neutrophils attach and roll along the vascular endothelium via the selectins, and then, if properly stimulated, integrins on the surface of neutrophils become activated and participate in adhesion strengthening and transmigration (18, 40, 41, 78).

Neutrophils that have accumulated within the microvasculature capture free-flowing leukocytes (13, 69). This process of indirect leukocyte tethering accelerates neutrophil accumulation and is facilitated by L-selectin (2, 4, 74, 85). Consistent with its role in amplifying the extent and rate of neutrophil accumulation, L-selectin is tightly regulated, which involves rapid and transient increases in its binding activity (26, 70). C-type lectins achieve high-affinity binding through the presentation of multiple carbohydrate recognition domains in a single polypeptide or by clustering (reviewed in Ref. 86). L-selectin, which contains a single carbohydrate recognition domain, undergoes clustering on neutrophil stimulation (21). Considering that all physiological ligands of L-selectin present multiple low-affinity oligosaccharide binding sites, inducible L-selectin clustering would likely provide a highly efficient means of regulating its binding activity. Indeed, L-selectin when clustered greatly enhances leukocyte tethering (12, 48).

This process also results in signaling and the induction of various postadhesion events, including oxidative burst, degranulation, cytokine expression, actin polymerization, and CD18 integrin activation (9, 21, 22, 30, 43, 64, 67, 71, 76, 80, 81).

Lateral heterogeneity of lipids and proteins occurs in the plasma membrane of neutrophils on their stimulation. This process results in the formation of membrane domains within the surrounding bilayer that are organized by interactions between cholesterol-enriched lipids and by the association of particular transmembrane proteins with the actin cytoskeleton (54, 55, 62). Such components progress from a uniform distribution in the plasma membrane to small patches and eventually to segregated caps at the uropod and lamellipodium of polarized neutrophils (55, 62), which has been observed with living cells (17, 38). Leukocyte membrane domains are identified by various approaches, including detergent resistance, lipid fluidity indicators, and resident glycosyl phosphatidylinositol (GPI)-anchored proteins (17, 20, 38, 42, 46, 51, 55, 58, 62). In this study, we examined L-selectin partitioning in the plasma membrane of neutrophils on inducible clustering and the role of cytoskeletal interactions in regulating its mobility.

MATERIALS AND METHODS

Antibodies and other reagents. The anti-L-selectin MAbs DREG-55 and DREG-200 were described previously (39, 52). Biotinylation of DREG-200 was performed with NHS-SS-LC-biotin (Pierce, Rockford, IL), per the manufacturer’s instructions. Phycoerythrin-conjugated and biotin-conjugated anti-L-selectin (LAM1–116) as well as unconjugated and biotin-conjugated anti-CD55 (Mab 67) were purchased from Ancell (Bayport, MN). FITC-conjugated anti-CD55 was purchased from BD Biosciences Pharmingen (San Diego, CA). Phycoerythrin (PE)-conjugated anti-CD11c and anti-L-selectin were purchased from Caltag Laboratories (Burlingame, CA). Recombinant chimeric human E-selectin (E-selectin/Fc) was purchased from R&D Systems (Minneapolis, MN). Rabbit polyclonal sera to Src family kinases was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-actin MAb (Ab-1) was purchased from Oncogene Research Products (San Diego, CA). FITC- and PE-conju-
gated as well as unconjugated F(ab')2 goat anti-mouse IgG secondary antibodies and Cy3-conjugated streptavidin were purchased from Jackson Immunoresearch (West Grove, PA). Horseradish peroxidase conjugated to goat anti-mouse IgG, goat anti-rabbit IgG, and streptavidin was purchased from Pierce. Latrunculin A and methyl-
-poly-cyclodextrin (MβCD) were purchased from Sigma (St. Louis, MO). RPMI, Ca"2+- and Mg"2+-free HBSS, and Ca"2+ - and Mg"2+-free PBS were purchased from Mediatech (Herndon, VA). Ultrapure grade sucrose was purchased from Amresco (Solon, OH). BSA (fraction V) was purchased from Fisher Scientific (Fairlawn, NJ). Complete Protease Inhibitor Cocktail was obtained from Roche Diagnostics (Mannheim, Germany) and suspended in HBSS plus 0.1% Triton X-100 (stock concentration 20×, working concentration 1×).

Cells. Venous blood was collected in sodium heparin from normal, healthy donors upon informed consent. These procedures were performed in accordance with protocols approved by the Institutional Review Board: Human Subjects Committee at the Universities of Minnesota and California, Davis. Total leukocytes and red blood cells were isolated by dextran sedimentation, and neutrophils were isolated by an additional Ficoll-Hypaque centrifugation, as previously described (74, 83, 85). Transduced K562 cells (erythroblast) expressing human wild-type L-selectin, 8-residue-truncated (Δ8) L-selectin, or 16-residue-truncated (Δ16) L-selectin (see Fig. 3) were maintained in modified RPMI 1640 medium as previously described (52).

Hydrodynamic shear flow assay. The adhesion behavior of transduced K562 cells under hydrodynamic shear stress was examined with a parallel plate flow chamber obtained from Glycotech as previously described (74, 83, 85). Recombinant chimeric human P-selectin glycoprotein ligand-1/Fc (PSGL-1/Fc; generously provided by the Genetics Institute, Cambridge, MA) was adsorbed on the base of the parallel plate chamber at various concentrations for 3 h at 37°C and then blocked with 2.5% BSA in PBS for a minimum of 1 h at room temperature. The PSGL-1/Fc concentration was based on a functional site density determination. Neutrophils uniformly express L-selectin, and in our hands ~90% attach to PSGL-1 on induction of a shear stress of 2.0 dyn/cm². A dose curve was examined to determine a minimum concentration of PSGL-1/Fc that resulted in a functional site density that supported the attachment of ~90% of neutrophils under a shear stress of 2 dyn/cm². A functional site density determination assesses the availability of PSGL-1 molecules in the correct functional orientation for leukocyte binding, whereas conventional site density determination does not. A minimum concentration of PSGL-1/Fc was determined to be 1.0 μg/ml. Transduced cells suspended in HBSS containing 2 mM CaCl₂ were perfused into the flow chamber and allowed to arrest on the adsorbed substrate for 1 min before shear flow was initiated.

Immunofluorescence microscopy. Analysis of the colocalization of L-selectin with other cell surface determinants after neutrophil stimulation with E-selectin/Fc was performed as previously described (21, 22). Briefly, neutrophils (1 × 10⁹/ml) were preincubated in the presence or absence of 100 nM E-selectin/Fc and labeled with anti-L-selectin-PE and anti-CD55-FITC or anti-CD11c-FITC for 10 min at 23°C. The reaction was stopped by the addition of 100 ml of 2% paraformaldehyde in PBS and allowed to fix for 30 min at 4°C. After fixation, excess MAbs were removed by centrifugation, and the resulting neutrophil suspension was scaled between a coverslip and a slide for fluorescent imaging. No difference in binding was observed between neutrophil suspensions labeled before and after fixation. Labeled cells were imaged by fluorescence microscopy with a Nikon TE2000-S inverted microscope and a ×60 Plan Apo objective (numerical aperture = 1.4) under oil and a Sutter filter wheel (Sutter Instrument, Novato, CA) housing excitation filters appropriate for FITC and PE fluorophores. Images were captured with an ORCA digital charge-coupled device camera (Hamamatsu Photonics, Hamamatsu City, Japan) and Simple PCI acquisition software (Compix, Cranberry Township, PA). For quantitation of receptor colocalization, a cluster was defined as a localized region of the membrane with pixel intensity at least threefold greater than background fluorescent intensity. Pixel intensity values are unitless and range from 0 to 255. A threshold intensity value over the cell membrane was assigned based on that of nonimmune fluorescent mouse IgG. This value typically ranges from 80 to 140, with clusters achieving a maximum intensity value of 255 by default. After thresholding on the background fluorescence, the percent colocalization was determined. Baseline colocalization is mathematically defined as the extent of coclustering predicted for a random distribution of membrane receptors. It is defined as the average percentage of membrane area occupied by green (CD55) pixels after image thresholding based on cluster definition. This area fraction defines the level of L-selectin-CD55 coclustering expected for a random distribution of CD55 fluorescence.

Analysis of the colocalization of L-selectin with other cell surface determinants on neutrophils after antibody-mediated cross-linking was performed as previously described (21, 22). Briefly, 3 × 10⁶ neutrophils were stained on ice with a particular primary MAb that was then cross-linked with FITC-conjugated F(ab')2 goat anti-mouse IgG either on ice or at 37°C for 30 min, after which neutrophils were fixed in 2% paraformaldehyde and labeled with biotin-anti-CD55, biotin-anti-L-selectin, or biotin-anti-CD45, which was detected with streptavidin-Cy3. Fixation with paraformaldehyde was repeated. Cells were applied to poly-L-lysine-coated coverslips and mounted with Vectashield Hard-Set mounting medium (Vector Laboratories, Burlingame, CA). Analysis of fluorescence was performed on a Bio-Rad 1024 confocal laser-scanning microscope with a ×60 oil immersion objective (numerical aperture = 1.4) (Bio-Rad Laboratories, Hercules, CA). Double-channel fluorescence was analyzed at 488-nm excitation and 522 ± 16-nm emission for FITC and 568-nm excitation and 605 ± 16-nm emission for Cy3. Cell staining was analyzed by scanning horizontal sections at 1-μm vertical steps. Images were recorded (512 × 512 pixels) and processed with Adobe Photoshop software (Mountain View, CA).

Other immunooassays. Flow cytometry, immunoprecipitation, SDS-PAGE, and immunoblotting were performed as previously described (1, 52, 74, 82, 84). For flow cytometry, antibody-labeled cells were analyzed (10,000 cells/sample) or sorted on a FACScan/Calibur instrument (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

Discontinuous density gradient centrifugation. Typically, 5 × 10⁶ cells were washed in ice-cold TNE buffer (25 mM Tris-Cl, pH 7.4, 150 mM NaCl, and 5 mM EDTA) and then detergent lysed for 20 min with 2 ml of lysis buffer (1% Triton X-100 in TNE buffer containing Complete Protease Inhibitor Cocktail). After lysis, 2.5 ml of ice-cold TNE buffer containing 80% sucrose was thoroughly mixed with the lysate. This mixture was transferred to ultracentrifuge tubes and overlaid first with 6.5 ml of 35% sucrose in TNE buffer and then with 0.5 ml of 5% sucrose in TNE buffer. The gradient was centrifuged for 17 h at 100,000 g in a Beckman L8–60M ultracentrifuge at 4°C (Beckman Coulter, Fullerton, CA). Fractions were recovered from the top, starting with the interface containing the detergent-resistant domain, and then afterward in 1-ml increments.

Detergent-resistant membrane and actin cytoskeleton disruption. MβCD treatment was performed to deplete cholesterol from the detergent-resistant membrane. Typically 2 × 10⁷ cells were washed twice in buffered saline solution (BSS; in mM: 20 HEPS, 135 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂, and 5.6 glucose) and then resuspended at 4 × 10⁹ cells/ml in BSS containing 10 mM MβCD and BSA (1 mg/ml). An additional 12.5 μl of RPMI containing 10 mM MβCD plus BSA was then added to the cells. Cells were incubated at 37°C for various time points, after which the cells were washed twice with BSS. Latrunculin A treatment was performed to block the polymerization of monomeric G-actin to F-actin (3, 8, 53, 87). Typically 3 × 10⁶ cells were incubated with 2 μM latrunculin A in HBSS for 10 min at 37°C. Latrunculin A is a reversible inhibitor and thus was not removed before L-selectin or CD55 patching for confocal microscopy.
Facilitated L-selectin association with detergent-insoluble cytoskeleton. Typically, 1 × 10⁶ cells were treated with DREG-55 MAb on ice for 30 min, washed with HBSS, and treated with F(ab′)₂ goat anti-mouse IgG on ice for 30 min. After washing, the cells were detergent extracted (1% Triton X-100 in HBSS containing 5 mM Na₂S, 5 mM HEPES, and Complete Protease Inhibitor Cocktail) on ice for 1 h. Next either the lysate was centrifuged at 20,800 g for 15 min, with the resulting pellet, which contains the detergent-resistant membrane and detergent-insoluble cytoskeleton, solubilized in cytoskeletal pellet solubilization buffer (20 mM NaH₂PO₄, pH 7.0, 0.15 M NaCl, 2 mM EDTA, 2 mM PMSF, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) for 1 h, or the lysate was subjected to density gradient centrifugation (see below) to separate the detergent-resistant membrane and detergent-insoluble cytoskeleton. The resulting cytoskeletal pellet was solubilized with cytoskeletal pellet solubilization buffer. In some cases, cells were initially cell surface biotinylated to label proteins in the plasma membrane. This was performed with EZ-Link sulfo-NHS-LC biotin.

RESULTS

Colocalization of clustered L-selectin and membrane domains on neutrophils. E-selectin recognizes sialyl Lewis^*-modified L-selectin on human neutrophils (88) and induces its clustering and signaling during neutrophil tethering or treatment in suspension with a full-length IgG chimera of E-selectin (E-selectin/Fc) (21). We treated human neutrophils with E-selectin/Fc and assessed the distribution of L-selectin in relationship to membrane domain components. Cell membrane components enriched in membrane domains include GPI-anchored proteins (7, 16). GPI-anchored CD55 (decay-accelerating factor), for instance, is a well-validated component of membrane domains on stimulated leukocytes (17, 42, 46, 58).

We observed that, on initial L-selectin patching, it coclustered with CD55 (Fig. 1A). Indeed, more than half of the membrane L-selectin colocalized with CD55, which represents a fivefold increase in CD55 expression. We treated the L-selectin MAAbb DREG-200 and FITC-conjugated F(ab′)₂ goat anti-mouse IgG were incubated on ice or at 37°C for 30 min, after which the neutrophils were stained for CD55 (Cy3). Neutrophils incubated on ice (middle left) demonstrated L-selectin patching (left), whereas the distribution of CD55 was uniform both at 37°C (middle) and on ice (right). F: coalescence of CD55 does not promote L-selectin patching. Neutrophils treated with a CD55 antibody and FITC-conjugated F(ab′)₂ goat anti-mouse IgG were incubated at 37°C for 30 min, after which the neutrophils were stained with DREG-200 to detect L-selectin (Cy3). Treatment of neutrophils with a nonstaining isotype-matched primary MAb and FITC-conjugated F(ab′)₂ goat anti-mouse IgG at 37°C for 30 min did not result in the patching of L-selectin or CD55 (data not shown). Confocal micrographs (C–F) are representative of >25 cells from 3 independent experiments performed with different donors.
increase over that due to colocalization of L-selectin with a random distribution of CD55 (Fig. 1B). On neutrophil polarization, L-selectin and CD11c demonstrate a segregated distribution in which L-selectin migrates to the uropod (21) and CD11c migrates to the lamellipodium (36). Interestingly, we found that even during initial L-selectin clustering by E-selectin/Fc treatment, L-selectin and CD11c did not undergo colocalization (Fig. 1B). Moreover, CD45, which has been noted in several reports to be excluded from membrane domains (17, 60, 62, 66), did not colocalize with L-selectin on its inducible clustering (21).

Antibody cross-linking has been used extensively for specific and precise L-selectin clustering (9, 22, 43, 64, 67, 76, 80, 81). As with E-selectin binding, antibody-mediated clustering of L-selectin promoted patching and colocalization of CD55, but not CD45 (Fig. 1, C–E). In contrast, antibody-mediated clustering of CD55 did not promote patching and colocalization of L-selectin (Fig. 1F), indicating that L-selectin is not physically associated with CD55. Altogether, the above data suggest that initial L-selectin clustering on neutrophils occurs in a membrane domain.

Mobility and partitioning of L-selectin in plasma membrane are regulated by its association with actin cytoskeleton. L-selectin has been reported by various groups to associate with the actin cytoskeleton (15, 27, 28, 45, 59). We assessed the effects of disrupting this association on the lateral mobility of L-selectin in the plasma membrane. In an initial series of experiments, the actin cytoskeleton of neutrophils was disrupted with latrunculin A, which blocks the polymerization of monomeric G-actin to F-actin (3, 8, 53, 87). We observed that L-selectin’s association with the actin cytoskeleton on antibody cross-linking was reduced by ~44% after latrunculin A treatment, as determined by density gradient centrifugation and examination of the cytoskeletal pellet (data not shown). In the presence of latrunculin A, L-selectin coalesced into fewer and, on average, larger patches after antibody cross-linking (Fig. 2A). Image analysis of micrographs revealed that for sham-treated neutrophils (Fig. 2A1), the average number of L-selectin patches per cell and their average size were 13.48 ± 4.51 and 0.71 ± 0.32 μm, respectively, whereas for latrunculin A-treated neutrophils (Fig. 2A2) the average number of L-selectin patches per cell and their average size were 2.72 ± 1.46 and 3.04 ± 2.20 μm, respectively (means ± SD, n = 25 cells per treatment group). The number of patches per cell differed significantly between treatment groups (P < 0.001; data are representative of 3 separate experiments involving different blood donors). The large L-selectin patches occurring on latrunculin A-treated neutrophils also contained colocalized CD55 (Fig. 2A). Of interest is that antibody cross-linking of CD55 on latrunculin A-treated neutrophils resulted in patching and coclustering of L-selectin (Fig. 2B), which was not as apparent in the absence of latrunculin A (Fig. 1F). One explanation for this finding is that by disrupting L-selectin’s association with the actin cytoskeleton it was free to be “swept up” with CD55 on its patching. Alternatively, Leitinger and Hogg (46) reported a similar effect on leukocyte function-associated antigen-I (LFA-1) after antibody-mediated patching of CD55 on human T cells treated with either cytochalasin D or latrunculin A and proposed that disrupting cytoskeletal anchorage by LFA-1 results in its lateral migration into microdomains occurring on resting cells.

In an additional series of experiments, we examined the effects of directly preventing cytoskeletal anchorage by L-selectin on its partitioning in the plasma membrane. Several molecules linked to cytoskeletal organization associate with the cytoplasmic region of L-selectin (27, 28, 32, 52, 59). To disrupt these various interactions, the 17-amino acid cytoplasmic region of L-selectin was truncated by 16 residues (Δ16 L-selectin; Fig. 3). The human myeloid cell line K562 does not express endogenous L-selectin and was transduced to express

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**Fig. 2.** Disruption of actin microfilaments increases L-selectin clustering. A: latrunculin A treatment enhances antibody-mediated L-selectin clustering. Neutrophils in the absence or presence of latrunculin A (Δlat A), as indicated, were treated with the L-selectin MAb DREG-200 and FITC-conjugated F(ab’)2 goat antimouse IgG and then incubated at 37°C for 30 min (L-selectin staining, 1 and 2), after which the latrunculin A-treated neutrophils were stained for CD55 (3). A merged image of the regions in 2 and 3 indicated by boxes is shown in 4. B: latrunculin A treatment increases colocalization of L-selectin with clustered CD55. Human neutrophils were treated with a CD55 antibody and FITC-conjugated F(ab’)2 goat antimouse IgG in the presence of latrunculin A and incubated at 37°C for 30 min (left), after which the neutrophils were stained for L-selectin (Cy3; middle). A merged image is shown at right, which represents the region indicated in the boxes. Yellow areas in A and B (some of which are indicated by arrows) signify the colocalization of L-selectin and CD55 patches (perimeters of neutrophils are outlined with dotted line). Confocal micrographs are representative of >25 cells from 3 independent experiments performed with different donors.
either wild-type or Δ16 L-selectin (52). Resistance of integral membrane proteins to mild nonionic detergent extraction after antibody cross-linking is a well-documented approach for assessing cytoskeletal binding (15, 19, 45, 56, 57). To this end, the transductants were sorted to ensure equivalent expression levels of the constructs (Fig. 4A) and biotinylated for subsequent detection of cell surface L-selectin. Equal numbers of each transductant were subjected to antibody cross-linking of L-selectin followed by Triton X-100 extraction. The detergent-insoluble fraction was then subjected to density gradient centrifugation to separate the insoluble microfilament bundles from the detergent-resistant membrane. Immunoblot analysis of the cytoskeleton-enriched fraction revealed much greater levels of associated wild-type L-selectin than Δ16 L-selectin (Fig. 4A). Actin levels were found to be similar for all samples, indicating that equivalent amounts of cell lysates were analyzed (Fig. 4A).

Having established that wild-type and Δ16 L-selectin differentially associate with the actin cytoskeleton, we then examined their constitutive partitioning in the plasma membrane. This was done by density gradient centrifugation of K562 detergent extracts, by which the detergent-resistant (cholesterol enriched) and detergent-soluble membrane lipids sediment at different densities (54, 65). Immunoblotting of collected fractions from the density gradients revealed that wild-type L-selectin partitioned in the high-density fractions (detergent-soluble membrane), whereas considerably higher levels of Δ16 L-selectin partitioned in the low-density fractions (detergent-resistant membrane) (Fig. 4B). Particular members of the Src family of kinases can localize in the detergent-resistant membrane through an association with the cytoplasmic leaflet of the lipid bilayer of leukocytes (50), as well as K562 cells (58). As expected, Src kinases were detected in the low-density fractions of the detergent-extracted K562 transductants. Src kinase levels for wild-type and Δ16 L-selectin transductants were similar, indicating that equivalent amounts of detergent extracts were analyzed (Fig. 4B). L-selectin is heavily glycosylated and a less decorated biosynthetic precursor is typically detected by SDS-PAGE and immunoblotting (31). Detection of this precursor was consistently more apparent for Δ16 L-selectin than for wild-type L-selectin (Fig. 4B), which suggests that the absence of L-selectin’s cytoplasmic region may impede its biosynthesis.

**Fig. 3.** The cytoplasmic region of L-selectin contains a conserved cationic motif. Highly basic conserved motif in the cytoplasmic region of L-selectin (boxed region) is shown at top. Engineered L-selectin constructs in which the cytoplasmic region was truncated by 16 (Δ16) or 8 (Δ8) residues are shown at bottom.

**Fig. 4.** Cytoskeletal association and plasma membrane partitioning by L-selectin are altered on removal of its cytoplasmic region. A: wild-type (WT) but not Δ16 L-selectin associates with the detergent-insoluble cytoskeleton on antibody cross-linking. Equal numbers of K562 transductants expressing WT and Δ16 L-selectin at equivalent levels, as determined by flow cytometry (see histogram, inset), were biotinylated to label cell surface proteins and then treated with DREG-55. Both transductants were split into equal numbers and treated with or without F(ab′)2 goat anti-mouse IgG (GAM) to cross-link L-selectin, as indicated. All samples were detergent extracted and subjected to density gradient centrifugation. Cytoskeletal pellets from each sample were solubilized, and equivalent volumes of each sample were used for immunoprecipitation (i.e., agarose beads conjugated to the L-selectin MAb DREG-200 or an isotype negative control MAb) or were directly subjected to SDS-PAGE for the detection of actin levels. DREG-200 and DREG-55 recognize distinct epitopes on L-selectin (52, 84). After SDS-PAGE, biotinylated L-selectin was detected by streptavidin-horseradish peroxidase and actin was detected with a MAb. Immunoprecipitations performed with isotype control MAb-conjugated agarose beads revealed no detectable antigens that migrated similar to L-selectin (data not shown). Data are representative of 3 independent experiments. B: Δ16 L-selectin, but not WT L-selectin, partitions into the low-density detergent-resistant membrane (DRM). K562 transductants expressing equivalent levels of either WT or Δ16 L-selectin (see A) were detergent extracted and subjected to density gradient centrifugation. The DRM and other fractions were collected and analyzed by SDS-PAGE and immunoblotting (DRM, fractions 1–4 and 8 and 9 are shown), using antibodies to L-selectin (DREG-200) and Src kinases. L, mature form of L-selectin; L’, biosynthetic precursor of L-selectin. Data are representative of 3 independent experiments. C: methyl-β-cyclodextrin (MβCD) treatment decreases the presence of Δ16 L-selectin in the DRM. Equal numbers of K562 transductants expressing Δ16 L-selectin were treated in the presence or absence of MβCD at 37°C for the indicated time points, after which the samples were detergent extracted and the insoluble portion of each DRM + detergent-insoluble cytoskeleton was subjected to SDS-PAGE and immunoblotting with antibodies to L-selectin, Src kinases, and actin.

**Table 1.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Wild-type L-selectin</th>
<th>Δ16 L-selectin</th>
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<tbody>
<tr>
<td>Human</td>
<td>RLLKGKGGSRSMNPDY</td>
<td>R-L</td>
</tr>
<tr>
<td>Baboon</td>
<td>RLLKGKGGKKSQSMNPDY</td>
<td>R-L</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>RLLKGKGGKKSQSMNPDY</td>
<td>R-L</td>
</tr>
<tr>
<td>Orangutan</td>
<td>RLLKGKGGKKSQSMNPDY</td>
<td>R-L</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>RLLKGKGGKKSQSMNPDY</td>
<td>R-L</td>
</tr>
<tr>
<td>Mouse</td>
<td>RLLKGKGGKSSQERMDPY</td>
<td>R-L</td>
</tr>
<tr>
<td>Rat</td>
<td>RLLKGKGGKSSQERMDPY</td>
<td>R-L</td>
</tr>
<tr>
<td>Rabbit</td>
<td>KGGKSSQKSDLHPL</td>
<td>K-R</td>
</tr>
<tr>
<td>Bovine</td>
<td>RLLKGKGGKVSEKHG</td>
<td>R-L</td>
</tr>
</tbody>
</table>
To verify that Δ16 L-selectin partitioning in the low-density fractions was not an artifact or anomalous process on detergent extraction and fractionation, MβCD was used to disrupt cholesterol-enriched, detergent-resistant lipid complexes. MβCD forms stable complexes with cholesterol and is used to extract these molecules from the plasma membrane (29, 61, 63). We observed that MβCD treatment of K562 transductants reduced the level of Src kinases in the detergent-resistant domain (Fig. 4C). MβCD treatment, which had no effect on the cell surface expression levels of Δ16 L-selectin on resting cells, as determined by flow cytometry (data not shown), also reduced the levels of Δ16 L-selectin partitioning in the detergent-resistant membrane (Fig. 4C). Actin levels were equivalent in untreated and treated samples, indicating that equivalent amounts of detergent extracts were analyzed (Fig. 4C). In contrast to the mature form of Δ16 L-selectin, detection of its precursor increased after treatment of the transductants with MβCD (Fig. 4C), which perhaps was due to a more efficient extraction after cholesterol depletion. Together our data demonstrate that disrupting L-selectin’s association with the actin cytoskeleton, by either latrunculin A or cytoplasmic region deletion, increases its mobility and capacity to partition in membrane domains, suggesting that constitutive cytoskeletal interactions may normally impede L-selectin clustering.

Conserved cationic motif in cytoplasmic region of L-selectin is critical for membrane domain partitioning and function. We next examined features of L-selectin’s cytoplasmic region that may be important for regulating its lateral migration in the plasma membrane. Of initial interest was a highly conserved cationic motif located proximal to the cell membrane (Fig. 3). We directly examined this segment of L-selectin by truncating the cytoplasmic region by eight residues (Δ8 L-selectin; Fig. 3). To examine the plasma membrane partitioning of Δ8 L-selectin, transduced K562 cells were detergent extracted and subjected to density gradient centrifugation, as described above. Immunoblotting of the gradient fractions revealed Δ8 L-selectin partitioned in the high-density fractions (detergent-soluble membrane; Fig. 5A), as occurred with wild-type L-selectin (Fig. 4B). Moreover, antibody cross-linking resulted in the association of wild-type and Δ8 L-selectin with the detergent-insoluble cytoskeleton of extracted cells (Fig. 5B). These data indicate that wild-type and Δ8 L-selectin are similar in their membrane domain partitioning and capacity to associate with the actin cytoskeleton.

We further examined the structural variants of L-selectin by assessing their functional capacity. This was done with an in vitro shear flow assay, which allows for the analysis of cell adhesion behavior under conditions that simulate the hydrodynamics of blood flow. To be consistent with our experiments involving neutrophils, E-selectin/Fc would have been used as an adhesive substrate, but K562 cells do not express α1,3-fucosyltransferases VII for sialyl Lewis8 biosynthesis and E-selectin binding (68). PSGL-1 is also a physiological ligand of L-selectin (23, 47, 69, 85), and thus a PSGL-1/Fc chimera was used as a substrate in the shear flow assay. As shown in Fig. 6, the K562 transductants being compared in this assay expressed equivalent levels of L-selectin. A comparison of transductants expressing wild-type or Δ16 L-selectin revealed a considerable difference in their shear resistance. In fact, Δ16 L-selectin was essentially incapable of maintaining cell attachment at a shear stress of 1.5 dyn/cm2 or higher (Fig. 6A). These findings are consistent with the studies of Dwir et al. (11), in which the duration between tether formation and breakage with the ligand GlyCAM-1 was greatly decreased for L-selectin molecules truncated by 15 residues.

L-selectin is normally dispersed at the tips of leukocyte microvilli (14), which is necessary for flowing cells to tether to L-selectin ligands (72, 79). It is conceivable that L-selectin molecules devoid of a cytoplasmic region exhibit an altered topographic dynamics. Moreover, studies performed in vitro and in vivo have demonstrated that blocking L-selectin ectodomainshading affects the rolling velocity and tethering of leukocytes (24, 25, 77, 83), indicating that L-selectin cleavage occurs on ligand binding and participates in dissociating these bonds. Because of the influences of proteolysis on L-selectin function, it is also possible that the adhesion deficiency of Δ16 L-selectin may result from elevated shedding and bond dissociation on ligand binding. To control for altered topographic or shedding properties affecting Δ16 L-selectin function, our adhesion assay was designed to quantify the detachment efficiency of cells that are allowed to sediment on the substrate under gravity. This methodology was shown previously to be insensitive to topographic redistribution of L-selectin (72). We also engineered a deletion in the cleavage region of Δ16 L-selectin, which we reported previously blocks L-selectin shedding (52, 82). Despite this cleavage resistance, we found that the adhesion defect by Δ16 L-selectin was not altered (Fig. 6B). In contrast to Δ16 L-selectin, truncating L-selectin by eight residues (Δ8 L-selectin), which preserves the conserved element of the cytoplasmic region, did not drastically alter L-selectin adhesivity. As shown in Fig. 6C, K562 transductants expressing Δ8 L-
selectin demonstrated a shear resistance similar to that of wild-type L-selectin over a range of shear stresses.

DISCUSSION

On neutrophil stimulation, the plasma membrane reorganizes to form membrane domains that support cell migration (17, 38, 55, 62). During this process, L-selectin progresses from a uniform distribution to discrete clusters (21). Our data suggest that nucleated L-selectin occurs in a membrane domain, as revealed by its colocalization with CD55, but not CD45. Consistent with this, Leid et al. (44) reported that polyunsaturated fatty acid incorporation into the plasma membrane of leukocytes, which alters membrane domains (75), disrupts L-selectin function. The lateral diffusion of L-selectin on unstimulated neutrophils appears to be impeded by its association with the actin cytoskeleton, as indicated by two lines of evidence. First, L-selectin molecules devoid of a cytoplasmic region and incapable of direct cytoskeletal anchorage, partitioned to a greater extent than wild-type L-selectin in the detergent-resistant membrane. In addition, the disruption of actin microfilaments in neutrophils significantly increased L-selectin clustering and localization in a membrane domain.

Our studies involving expressed structural variants of L-selectin show that a conserved cationic motif in the cytoplasmic region of L-selectin, juxtaposed to the cell membrane (Fig. 3), is critical for regulating L-selectin’s mobility. For instance, Δ16 L-selectin did not associate with the actin cytoskeleton and partitioned in the detergent-resistant membrane, in contrast to Δ8 and wild-type L-selectin. These data are congruent with intermolecular interactions reported to take place within the conserved cationic element of L-selectin’s cytoplasmic region, including calmodulin and the ezrin-radixin-moesin proteins ezrin and moesin (27, 28, 32, 52). The latter molecules concentrate in microvilli projections, as does L-selectin (14), and link a subset of transmembrane proteins to the actin cytoskeleton (6). It is conceivable that one or more of these interactions with L-selectin may facilitate constitutive restraint by the cytoskeleton. Whether this is disrupted in an inducible manner during neutrophil stimulation to enhance L-selectin mobility and clustering has yet to be resolved. Incidentally, L-selectin is phosphorylated at serine residues juxtaposed to the conserved motif on neutrophil stimulation, and this correlates in time with transient increases in L-selectin binding activity and subsequent transmembrane signal transduction (22, 26, 35). It will be useful to determine whether this event promotes a transient release of L-selectin from cytoskeletal restraint, thus increasing the efficiency and kinetics of inducible L-selectin clustering.

Despite the increased mobility and clustering of L-selectin that occurs by molecules devoid of a cytoplasmic region or on disrupting actin microfilaments, these manipulations abrogate L-selectin-dependent cell tethering under shear flow conditions (Fig. 6; Refs. 11, 33). This suggests that an association with the actin cytoskeleton is also necessary to stabilize L-selectin. Thus L-selectin’s interactions with the actin cytoskeleton may occur in a dynamic manner that provides negative and positive regulation. In resting neutrophils, constitutive cytoskeletal interactions may confine L-selectin to prevent clustering and consequently reduce adhesiveness and signaling. After neutrophil stimulation, induced cytoskeletal anchorage may stabilize L-selectin and promote its effector functions. Consistent with the latter are studies by Ivetic et al. (27) showing that moesin interacts with L-selectin in an inducible manner. Moreover,
current published data provide several examples of temporal and dynamic associations between various adhesion proteins and the actin cytoskeleton resulting in augmented function, including CD43, LFA-1, Mac-1, VCAM-1, and ICAM-1 (S. I. Simon) and C. M. Iverson (PLSCR1) co-localize to uropod rafts in formylated Met-Leu-Phe-stimulated neutrophils. J Biol Chem 279: 2321–2329, 2004.


REGULATION OF L-SELECTIN CLUSTERING


