An Activated L-selectin Mutant with Conserved Equilibrium Binding Properties but Enhanced Ligand Recognition under Shear Flow

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Selectins mediate the initial tethering and rolling of leukocytes on vessel walls. Adhesion by selectins is a function of both ligand recognition at equilibrium and mechanical properties of the selectin-ligand bond under applied force. We describe an EGF domain mutant of L-selectin with profoundly augmented adhesiveness over that of native L-selectin but conserved ligand specificity. This mutant, termed LPL, was derived by a substitution of the EGF-like domain of L-selectin with the homologous domain from P-selectin. The mutant bound soluble carbohydrate L-selectin ligand with affinity comparable with that of native L-selectin but interacted with all surface-bound ligands much more readily than native L-selectin, in particular under elevated shear flow. Tethers mediated by both native and mutant L-selectin exhibited similar lifetimes under a range of shear stresses, but the rate of bond formation by the mutant was at least 10-fold higher than that of native L-selectin toward distinct L-selectin ligands. Enhanced rate of bond formation by the mutant was associated with profoundly stronger rolling interactions and reduced dependence of rolling on a threshold of shear stress. This is the first demonstration that the EGF domain can modulate the binding of the lectin domain of a selectin to surface-immobilized ligands under shear flow without affecting the equilibrium properties of the selectin toward soluble ligands.

Selectins mediate the tethering of flowing leukocytes to the vessel wall and the propagation of tethers into rolling adhesions in the direction of flow (1–4). Leukocyte rolling is the first of several sequential steps that control the entry of leukocyte subsets into lymphoid organs and inflamed sites (5, 6). Binding of the two endothelial selectins, P- and E-selectins, and the leukocyte selectin, L-selectin, to cell surface carbohydrate ligands under shear flow is labile and occurs in fractions of seconds (7, 8). L-selectin, expressed on almost all circulating leukocytes, binds to sulfated sialyl Lewis*-related glyco-
to mediate rolling on all L-selectin ligands tested, both as a cell-associated or cell-free form. This enhanced adhesion is also associated with abolishment of the shear threshold requirement of adhesion, an unprecedented property of selectin-dependent rolling (38, 39). This study is a first demonstration that the EGF domain can regulate the adhesive activity of L-selectin by altering the kinetics of ligand recognition under shear flow without affecting affinity to soluble ligand in the absence of flow.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**

The anti-L-selectin mAbs, DREG-200 and DREG-56 (40), and mAb CA21, directed against the C terminus of the cytoplasmic tail of L-selectin (41) were kindly provided by Dr. T. K. Kishimoto (Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT). The rabbit polyclonal antibody CAM02, derived against an internal peptide of GlyCAM-1 (42) was a gift from Dr. Steven Rosen (University of California, San Francisco). LAM1–101, directed against the SCR domain of L-selectin (43), was a generous gift of Dr. T. Tedder (Duke University, Durham, NC). The anti-VCAM-1 mAb, 4B9, was a gift from Dr. R. Lobb (Biogen Inc., Cambridge, MA). All mAbs were used as purified Ig. Rabbit anti-murine IgG, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, and preimmune mouse IgG were obtained from Zymed Laboratories Inc. (South San Francisco, CA). DREG-200 Fab fragments were generated by papain digestion, followed by the removal of undigested IgG on protein-G agarose (Amersham Pharmacia Biotech) (44). Fab purity was confirmed by SDS-polyacrylamide gel electrophoresis. GlyCAM-1, purified from mouse serum by immunoaffinity chromatography, was a generous gift from Dr. S. Rosen, and was stored frozen in PBS (10). Peripheral node addressin (PNAd), purified from human tonsil lysates by MECA-79 mAb affinity chromatography (45), was a generous gift from Dr. Ellen Berg (Protein Design Labs, Mountain View, CA). The glycoprotein mixture was stored in 1% octyl glucoside/PBS/1 mM CaCl2. The chemical cross-linked neonolipid 3′,6′-disulfo Leo-6-disulfo Lex glycolipid was dissolved by radioimmunoassay using125I-labeled CA21 mAb. To assure maximal binding of CA21 mAb to ligands of interest, substrates were washed three times with binding medium containing 125I-labeled CA21 mAb. Unbound IgG was removed by multiple washing, and anti-L-selectin cytoplasmic tail mAb CA21, diluted in PBS/0.1% BSA, was then adsorbed at different concentrations (1–20 ng/ml) onto the immobilized rabbit anti-mouse IgG. The site density of CA21 on the various substrates was determined by radiolmmunoassay using 125I-labeled CA21 mAb. To assure maximal interaction of solubilized native or chimeric L-selectin with immobilized CA21 mAb spot was overlaid three times with lysate aliquots, each time for 4 h at 4 °C. Following the last lysate adsorption, substrates were washed with PBS supplemented with 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 0.02% sodium azide and subjected to extensive washing with cell binding medium (Hanks’ balanced salt solution/10 mM HEPES, pH 7.4, supplemented with 2 mM CaCl2 and 2 mg/ml bovine serum albumin) in the presence of 1–200 nM 125I-labeled DREG-200 mAb for 10 min at 24 °C and for an additional 30 min at 4 °C. Cells were washed twice with binding medium and then incubated for 4 °C, and indirect fluorescence activity was determined. FITC-PFMME binding to the various transfectants (1 × 105/ml) was determined by incubating cells in 0.1 ml of binding medium with the polysaccharide ligand for 10 min at 24 °C. Cells were diluted into 2 ml of binding medium and immediately analyzed for FITC staining by fluorescence-activated cell sorter. PPME staining was completely blocked by CA21 mAb at ligand saturating concentrations (1 × 105/ml). The L-selectin chimera, LPL, in the mouse pre-B cell line 300.19 was a generous gift from Dr. Ellen Berg (Protein Design Labs, Mountain View, CA). DREG-200 Fab fragments were generated by papain digestion, followed by the removal of undigested IgG on protein-G agarose (Amersham Pharmacia Biotech) (44). Fab purity was confirmed by SDS-polyacrylamide gel electrophoresis. GlyCAM-1, purified from mouse serum by immunoaffinity chromatography, was a generous gift from Dr. S. Rosen, and was stored frozen in PBS (10). 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**Preparation of Ligand-coated Substrates**

Concentrates of GlyCAM-1, fucoidin-HSA, or DREG-200 (intact or Fab fragment) were diluted in coating medium (PBS supplemented with 20 mM bicarbonate, pH 8.5) and adsorbed onto the plates for 2 h at 37 °C. PNAd aliquots were diluted to concentrations of 10 ng/ml to 100 μg/ml and immediately adsorbed onto the polystyrene plate for 15 h at 4 °C. The 3′,6′ disulfo Leo-6 glycolipid was dissolved by radioimmunoassay using125I-labeled CA21 mAb. The site density of CA21 on the various substrates was determined by radiolmmunoassay using 125I-labeled CA21 mAb. To assure maximal interaction of solubilized native or chimeric L-selectin with immobilized CA21 mAb spot was overlaid three times with lysate aliquots, each time for 4 h at 4 °C. Following the last lysate adsorption, substrates were washed with PBS supplemented with 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 0.02% sodium azide and subjected to extensive washing with cell binding medium (Hanks’ balanced salt solution/10 mM HEPES, pH 7.4, supplemented with 2 mM CaCl2 and 2 mg/ml bovine serum albumin).

**Cell Lining Flow Assays**

**Cell Tethering and Rolling Measurements**—The polystyrene plate, on which purified ligand was adsorbed, was assembled in a parallel plate laminar flow chamber as described previously (1, 52). Transfected cells were washed in I/H medium containing 5 mM EDTA, resuspended in I/H medium at 107 cells/ml and stored at 4 °C up to 1 h before each flow experiment. Stored cells were resuspended in cell binding medium at 107 cells/ml and perfused at room temperature through the flow chamber at desired flow rates, generated with an automated syringe pump (Harvard Apparatus, Natick, MA). Cellular interactions were visualized at two different fields of view (each one 0.17 mm2 in area) using a 10× objective of an inverted phase contrast microscope (Diaphot 300, Nikon Inc., Tokyo, Japan). Tethering events were defined as adhesive interactions of those freely flowing cells (herein termed cell flux) moving away from the substrate and then reattaching themselves to the substrate and that are therefore the only population potentially capable of interacting with the substrate. This flux was visualized by their brighter images and slower motions than cells moving at more distant layers of the perfusate. Two types of initial cell tethers to the substrate were determined: transient tethers, cells that attached briefly to the substrate without initiating rolling motions, and rolling tethers, cells...
that remained rolling on the substrate, i.e. moving at a mean velocity
not more than a fourth the hydrodynamic velocity for at least 3 s after
initial tethering. The number of each type of tethered cells was divided
by the cell flux to yield the frequency of initial tethers. For cell inhibi-
tion studies, cells were preincubated for 5 min at 4 °C in H/H medium
with 5 µg/ml of the L-selectin blocking mAb, DREG-200, or preimmune
mouse IgG, diluted 1:10 with the binding medium, and perfused into
the chamber. For cell-free selectin or LPL mutant blocking, cells were
perfused into the flow chamber in the presence of 50 µg/ml of the
L-selectin ligand, fucoidin, or 5 mM EGTA. More than 98% of all tran-
sient cell tethers to the various glycoprotein ligands were L-selectin-
specific, because they were blocked by fucoidin. Rolling velocities were
determined by following the cell displacements over 2–3 s as described
previously (29).

To follow the kinetics of leukocyte release by a shear drop, cells were
allowed to roll on high density ligands at 1.75 dyn/cm² for at least 20 s
before the flow rate was dropped to 0.2 dyn/cm². To estimate the time
lapse required for the flow system to reach a shear stress of 0.2 dyn/cm²,
given that at this shear value, a noninteracting cell traveled at a ve-
clocity of 60 µm/s, the time point at which leukocytes freely moving at
1.75 dyn/cm² reached this lower velocity, after being subjected to the
same shear drop was determined. The number of originally rolling cells
remaining bound to the substrate after this time point was determined
by frame-by-frame analysis.

Dissociation Kinetics of Transient Tethers—Transient tethers to low
density ligands were defined as temporary pauses when separated by at
least 50 µm of motion at the hydrodynamic velocity and when no cell
motion (<1 µm displacement) occurred while the cell was tethered to
the substrate (8). The duration of transient tethers was determined at
a resolution of 0.02 s by manually counting the number of frames during
which the tethered cell was motionless (53). Tethers lasting less than
0.04 s were reanalyzed at a resolution of 0.02 s on high fidelity video
(Panasonic AG-7355, Osaka, Japan) using a digital still playback mode.
Sufficient videotape was analyzed (60–120 s) to obtain 30–40 tethering
events, and the natural log of the number of cells that remained bound
was plotted as a function of the tether duration time. The slope of the
curve represents $-k_{\text{det}}$.

**Image Analysis**

An imaging system was developed for quantitative analysis of in-
stantaneous velocities of cell rolling on different adhesive substrates.
Video frame images consisting of 768 × 574 pixels (with a pixel size of
1.15 µm using a 10× objective), were digitized using a Matrox Pulsar
frame grabber (Matrox Graphics Inc., Dorval, Quebec, Canada), and
images were scanned and processed by the WSCAN-Array-3 imaging
software (Galai, Migdal-Ha’emek, Israel), running on an Atlas pentium
MMX work station. Cell motions were identified from images
tracked at 0.02 s intervals. The program output provided the coordi-
nates of the center point of each cell in successive interlaced fields 0.02 s
apart.

A computer program for cell motion analysis was developed in col-
aboration with the lab of Prof. David Malah (Electric Engineering
Faculty, Technion, Haifa, Israel). The software runs under Matlab 5.2
and compares instantaneous positions of individual cells at successive
video images over a period of up to 5 s. Tethers of individual cells rolling
persistently on the ligand-coated field or moving through it in a jerky
motion were determined according to changes in instantaneous cell
velocities in the flow direction. A rolling pause was defined as an
instantaneous velocity drop to below 29 µm/s at shear stresses of 1–1.75
dyn/cm². This threshold velocity value gave optimal correlation be-
tween pause analysis performed on representative cells by the comput-
erized system and manually, directly from the video monitor. The step
distances between successive pauses of an individual rolling cell were
averaged to yield the mean step distance of a given rolling cell.

**RESULTS**

**LPL Supports Stronger Adhesion than Native L-selectin on Different L-selectin Ligands—**Native L-selectin and the EGF
domain mutant LPL were stably expressed in the pre-B cell
line 300.19 (37), and clones with comparable surface expression
levels of each molecule were isolated (Fig. 1A). As on other
leukocytes, L-selectin preferentially localizes on the microvilli
surface projections of 300.19 pre-B cells (54). Because selectin
localization to tips of microvilli has been argued to augment its
ability to support initial cell tethering in shear flow (55, 56), we
compared the surface distribution of the native and mutated

![Fig. 1. Surface expression and distribution of L-selectin and LPL on transfected pre-B cells.](image)

**FIG. 1.** Surface expression and distribution of L-selectin and LPL on transfected pre-B cells. A, immunofluorescence flow cytometry of 300.19 clones stably transfected with cDNA encoding full-length L-selectin (native L-selectin) or an EGF domain mutant of L-selectin, which substitutes a P-selectin-derived EGF domain for the EGF domain of L-selectin (37). Transfectants were stained with the anti-L-selectin mAb DREG-200, followed by FITC-labeled goat anti-mouse mAbs (filled histograms). Background staining with nonbinding preimmune mouse IgG is shown in the open histograms. B, localization of L-selectin (I and II) and LPL (III and IV) expressed by transfected pre-B cells using immunoelectron microscopy. Transfectants were prefixed, incubated with the lectin domain-specific mAb, DREG-200, followed by a secondary
rabbit IgG. Cells were then immunogold-labeled with 5-nm gold-protein A conjugates. Gold staining is marked by arrows. The photomicrographs shown for each cell type are representative of 40–60 cells examined for each experimental group.

**L-selectin on the 300.19 clones by immunogold labeling.** Transmission electron microscopy of sectioned cells confirmed that both transfectants have comparable microvilli both in number and dimensions (data not shown). Immunogold localization of L-selectin indicated similar localization of both L-selectin and LPL to microvilli with a comparable distribution between the tips and other regions of the microvilli (Fig. 1B). Variable clusters of L-selectin and LPL were identified on both cell transfectants, and their mean size was similar. These results suggest that the surface distribution and degree of constitutive clustering of the EGF domain mutant LPL are similar to those of native L-selectin in 300.19 cells.

**L-selectin and LPL transfected cells were first perfused over substrates coated with purified GlyCAM-1, a prototypic L-selectin ligand that supports efficient rolling of L-selectin-expressing leukocytes under physiological shear flow (29, 57).** Surprisingly, LPL-expressing cells established rolling on different GlyCAM-1-coated substrates much more readily than L-selectin expressing cells, (Fig. 2A). Moreover, the rolling of LPL-expressing cells on GlyCAM-1 was 5–7-fold slower than L-selectin-mediated rolling under identical experimental con-
ditions (Fig. 2A) and remained much more adhesive than L-
The adhesiveness of EGF domain mutant of L-selectin expressed on murine 300.19 pre-B cells is stronger than that of native L-selectin. A, frequency of L-selectin- or LPL-expressing pre-B cells capable of initiating rolling adhesions over substrates coated with different densities of GlyCAM-1 or with low density PNAd under physiological shear flow. Mean velocities of 20–25 L-selectin- and LPL-expressing cells rolling on the ligand are indicated at the top of each bar. S.E. values for the mean rolling velocities were 2.2, 0.5, 5.9, 0.8, and 2.3 \( \mu \text{m/s} \), respectively, for the indicated mean values. Data are representative of six independent experiments. B, frequency of L-selectin- or LPL-expressing pre-B cells capable of initiating stable rolling adhesions over substrates coated with fucoidin-HSA coated at 5 \( \mu \text{g/ml} \), when perfused at the indicated shear stresses over the immobilized glycoconjugate. Data points represent the mean \( \pm \) range of frequency values determined on two fields of view. No shear threshold was required for the adhesion of either transfected to fucoidin-HSA (data not shown). All rolling adhesions were specifically blocked in the presence of the L-selectin blocking mAb, DREG-200, but not the control anti-VCAM-1 mAb, 4B9 (not shown). C, frequency of L-selectin- or LPL-expressing pre-B cells capable of initiating rolling adhesions or immediately arrested over immobilized glycolipid bearing the L-selectin carbohydrate ligand, 3,6-disulfated Le\( ^a \) coated at 0.5 \( \mu \text{g/ml} \) under shear flow. Data in A–C are representative of six independent experiments.

selectin transfectants did when subjected to elevated shear stresses (data not shown), collectively suggesting that the LPL mutant is more adhesive toward GlyCAM-1 than native L-selectin. Consistent with their enhanced adhesiveness to GlyCAM-1, LPL-expressing cells also rolled more readily than L-selectin expressing cells on substrates containing distinct L-selectin ligands, including human PNAd (Fig. 2A), the neo-glycoconjugate fucoidin-HSA (Fig. 2B) or the lipid-linked 3,6-disulfated Le\( ^a \) (Fig. 2C). The higher adhesiveness of the LPL mutant toward these ligands was more marked at higher shear stresses and at lower ligand coating densities (Fig. 2, A and B, and data not shown). The similarity of the results obtained with molecularly dissimilar ligands with both native L-selectin and LPL indicates that rolling on each of these ligands was mediated exclusively by the lectin domain, making unlikely any direct contribution from direct interactions between the foreign EGF domain in LPL and the different L-selectin ligands.

Displacement motions of leukocytes rolling on selectins or selectin ligands are comprised of discrete steps or jerks separated by transient pauses (53, 58). Because the microkinetics of these rolling motions provide insights into the dynamic parameters of tether formation and breakage, we used computerized image analysis to closely follow the microdynamics of rolling mediated by L-selectin and LPL on medium density GlyCAM-1. Microkinetic analysis of representative cells within the fraction of LPL or L-selectin-expressing cell rolling on GlyCAM-1 at 40 sites/\( \mu \text{m}^2 \) revealed that pauses of LPL-expressing cells were 4-fold longer than pauses of L-selectin expressing cells (mean duration of 0.14 ± 0.01 s versus mean duration of 0.03 ± 0.01 s, respectively; Fig. 3, n = 10). This dramatically increased pause duration of LPL cells is consistent with either a greater number of bonds formed, a longer lifetime of these bonds, or a combination of both.

To distinguish between these possibilities, we analyzed the step distance between successive rolling pauses, i.e. the forward travel distance between rolling pauses (53, 58). Shorter step distances of a cell rolling under a given shear stress correspond to increased rate of bond formation (58). Remarkably, the mean step distance of LPL cells rolling on GlyCAM-1 at 40 sites/\( \mu \text{m}^2 \) was 5-fold smaller than the mean step distance of L-selectin cells measured under identical conditions cells (mean step distance of 1.74 ± 0.09 \( \mu \text{m} \) versus mean step distance of 8.86 ± 0.71 \( \mu \text{m} \), respectively; Fig. 3, n = 10). Similar differences were observed on PNAd-coated surfaces: LPL-mediated rolling was associated with 3-fold longer pauses and 6-fold smaller mean step distance than the corresponding microkinetic parameters of L-selectin expressing cells rolling on identical PNAd-coated substrates (data not shown). Taken together, these results suggest a much higher rate of bond formation by LPL than by L-selectin during rolling on GlyCAM-1 and PNAd, manifested as significantly increased numbers of
stable tethers, slower rolling velocities, longer durations of rolling pauses, and closer spacing of these pauses (i.e. smaller step distance).

The Cellular k_{on} of LPL Is Higher than That of L-selectin, but the Cellular k_{off} Is Similar—The enhanced adhesiveness of the LPL EGF domain mutant was suggestive of enhanced bond formation and/or stability. The most direct measure of tether bond formation and duration is the analysis of transient tethers on densities of ligands too low to support stable tethers, i.e. rolling (53). These tethers are supported by the smallest number of bonds formed by individual selectin molecules interacting with their immobilized ligands under shear flow (53). The dependence of cell tethering on site density of extremely diluted GlyCAM-1 was determined at a representative shear stress (1 dyn/cm²), a value permissive for both L-selectin- and LPL-mediated tethering to the ligand. Consistent with the dynamic behavior of the mutant on high density GlyCAM-1, the frequency of transient tethering mediated by LPL was markedly higher than that of native L-selectin on all GlyCAM-1 densities ≤ 1 site/μm² (Fig. 4A). The tethering frequency of L-selectin-expressing cells diminished rapidly when the coating density of GlyCAM-1 dropped below 0.08 sites/μm². At that GlyCAM-1 density, tethering of LPL-expressing cells was equivalent to that supported by L-selectin expressing cells on substrates coated with 20–30-fold higher site density of GlyCAM-1 (Fig. 4A and data not shown). Therefore, the cellular k_{on} of LPL tethers to GlyCAM-1 is ~20–30-fold higher than that of native L-selectin tethers.

Given this difference between L-selectin and LPL in cellular on rates, the dissociation kinetics of these transient tethers was next compared. More than 95% of the transient tethers, mediated by either L-selectin or LPL, dissociated from GlyCAM-1 with first order kinetics (Fig. 4B). The dissociation data of each transfectant fit a single, straight line, which corresponded to a single k_{off}, and the homogeneity of the lifetime of these tethers indicated that the transient tethers represented quantal adhesive units. Notably, the k_{off} of these quantal tethers mediated by either L-selectin or LPL was quite similar between 0.5 and 1 dyn/cm² (Fig. 4B and data not shown). These results suggest that interactions between either L-selectin or LPL and GlyCAM-1 exhibit similar cellular k_{off} and comparable response of the k_{off} to increasing shear force. Similar results were obtained on low density PNAd substrates (data not shown). The higher kinetic stability of LPL-mediated rolling pauses at higher GlyCAM-1 or PNAd concentrations described above is therefore due to a higher number of bonds formed by the mutant than by L-selectin rather than to an increase in individual bond lifetime or decrease in the cellular dissociation rate. These kinetic results demonstrate that the effective avidity of LPL for surface-bound ligand under shear flow is significantly higher than that of L-selectin. However, despite this marked difference between LPL and L-selectin avidity toward surface-immobilized ligands, both molecules bind the soluble L-selectin carbohydrate ligand PPME with identical avidity in saturation binding assays performed in the absence of shear flow (Fig. 5). The rate of PPME binding to either L-selectin or LPL appeared also similar under these conditions (Fig. 5). These results indicate that the EGF domain mutant exhibits augmented binding solely to surface-immobilized L-selectin ligands under shear flow rather than to a soluble L-selectin ligand in the absence of shear flow.

The EGF Mutant Exhibits a Greatly Reduced Shear Threshold for Tethering to L-selectin Ligands—Because adhesion through L-selectin requires shear stress above a threshold value to form and persist (38, 39), we compared the shear threshold required for L-selectin and LPL to promote and sus-tain stable rolling on GlyCAM-1. L-selectin-expressing cells required a threshold of 0.5 dyn/cm² to establish stable tethers on GlyCAM-1 coated at 80 sites/μm² (Fig. 6A); below this shear stress, cells could tether only transiently to the ligand. Importantly, the transient tethering efficiency also dropped sharply when the shear stresses was reduced to 0.3 dyn/cm², indicating that shear not only enhanced the conversion of L-selectin tran-
sient tethering into stable tethering leading to rolling adhesion but also directly enhanced the frequency of transient L-selectin tethers, as previously reported for PMN tethered to low density PNAd (53). In contrast, LPL-expressing cells tethered and rolled on identical GlyCAM-1 substrates below this shear stress, and the efficiency of LPL-expressing cells capable of rolling on GlyCAM-1 was nearly shear-independent (Fig. 6A). This greatly reduced shear threshold requirement for LPL tethering was observed on GlyCAM-1 as low as 1 site/\(\mu\text{m}^2\) (data not shown). Interestingly, L-selectin failed to support any tethering to GlyCAM-1 coated at 40 sites/\(\mu\text{m}^2\), at shear stresses below 0.5 dyn/cm\(^2\), whereas LPL both tethered and rolled on this ligand under identical shear conditions (data not shown).

Shear threshold is required not only to initiate L-selectin rolling but also to maintain it (38). Indeed, cells rolling through L-selectin at permissive shear stress were rapidly released to the medium when the shear was dropped to subthreshold levels and subsequently traveled at their hydrodynamic velocity (Fig. 6B). By contrast, LPL transfectants, rolling on identical substrates, remained adherent to the substrate when subjected to an identical drop in shear stress (Fig. 6B) and dissociated slowly from the substrate only at much later periods (data not shown). The release of L-selectin expressing cells from the substrate in response to a drop in shear force to subthreshold levels has been recently suggested to be a function of the number of bonds formed between a rolling cell and the ligand at the adhesive contact zone of the cell and substrate (58). The slower release of LPL-expressing cells rolling on GlyCAM-1 compared with L-selectin expressing cells in response to a shear drop to subthreshold values is consistent with a higher number of bonds simultaneously formed by the mutant than by L-selectin at the adhesive contact zone, in agreement with the results of the microkinetic analysis (Fig. 3).

**LPL Binds a Surface-immobilized Lectin Domain-specific mAb More Efficiently than Native L-selectin**—The above data show that an L-selectin EGF domain mutant can stimulate the adhesive reactivity of L-selectin to immobilized ligands under flow. To test whether the EGF mutation characterized here enhanced the intrinsic adhesiveness of the lectin domain, we next analyzed the tethering of L-selectin and LPL to domain-specific mAbs under defined conditions of shear flow at anti-body concentrations too low for stable cell capture from the flow. When perfused over substrates coated with low density of the L-selectin lectin domain-specific mAb, DREG-200, L-selectin-expressing cells transiently tethered to this mAb much less efficiently than LPL-expressing cells did (Fig. 7A). In contrast, cells expressing either L-selectin or LPL tethered at lower but similar efficiency to the L-selectin-SCR domain-specific mAb, LAM-1–101 (Fig. 7A). Higher efficiency of LPL tethering than of L-selectin tethering was also observed on immobilized Fab fragments of DREG-200, in particular at higher shear stresses (Fig. 7B). However, both L-selectin and LPL bound soluble DREG-200 with identical equilibrium affinity (\(K_d\) of 1.3–1.8 \(\times 10^{-9}\) M). This is a further direct demonstration that the EGF domain mutation augments the intrinsic adhesive reactivity of the lectin domain toward surface-immobilized ligand, independently of the equilibrium binding properties of this domain to the same ligand in solution.

**Cell-free LPL Supports Higher Frequency of Tethering and Rolling than Native L-selectin**—The augmented adhesive activity of the mutant toward different ligands could in principle depend on the cellular background on which LPL was expressed. We therefore tested whether, in a cell-free state, LPL
could still interact more efficiently than L-selectin with cell surface L-selectin ligands. A single-step procedure to immobilize solubilized full-length L-selectin in a uniform functional orientation on a solid substrate was developed. Native L-selectin or LPL was extracted from their respective transfectants and adsorbed onto a surface coated with a capturing antibody, mAb CA21 (41), directed against the C terminus of the cytoplasmic tail, which is shared between L-selectin and the LPL chimera.

Neutrophils, known to express high levels of L-selectin ligands (18, 21), established stable rolling adhesion in physiological shear flow on immobilized cell-free L-selectin or LPL (Fig. 8A), and all adhesion was completely abolished by L-selectin blocking with fucoidin or EGTA (Fig. 8A and data not shown). However, the fraction of neutrophils capable of rolling was quite low on L-selectin captured at site densities lower than 200 sites/µm² (Fig. 8). In contrast, cell-free LPL, identically immobilized, supported up to 50-fold higher frequency of neutrophil rolling than the native cell-free L-selectin or LPL. Frequencies of total tethers of neutrophils at a shear stress of 1 dyn/cm² were measured on L-selectin or LPL, each adsorbed on the indicated densities of immobilized CA21, a mAb specific for the cytoplasmic-tail of both L-selectin molecules. Saturation binding of each L-selectin isoform to the immobilized tail-specific mAb was verified by multi-cycle adsorptions of lysates containing either L-selectin or LPL. In control experiments, neutrophils were perfused on the same substrates in the presence of soluble fucoidin at 50 µg/ml. Inclusion of EGTA in the perfusate also fully blocked tethering (data not shown). No tethering was observed on substrates coated with CA21 and then with lysates of either L-selectin or LPL-transfected pre-B cells that had been depleted on an anti-L-selectin mAb affinity column (data not shown). At representative densities of immobilized CA21, the fractions of tethered cells that established rolling after tethering to the substrates are indicated. Results represent the means ± range of data collected in two fields of view. Ranges smaller than 5% of the mean are not shown. B, effect of shear stress on tethering followed by rolling of neutrophils perfused over immunoadsorbed L-selectin or LPL. LPL and L-selectin were adsorbed on substrates coated with the indicated site densities of the anti-cytoplasmic tail antibody CA21. These substrates were found to support comparable levels of rolling adhesions at optimal conditions of shear flow. The mean velocities of neutrophils rolling on L-selectin or on LPL at 2 dyn/cm² are indicated on top of the stack bars. Results in A and B are representative of three independent experiments.
contrast, neutrophils established rolling on immobilized cell-free LPL at a 3-fold lower shear stress (Fig. 8B). A marked difference in the shear requirement between L-selectin and LPL was also observed in the response of neutrophils to a sharp drop in shear stress. Neutrophils rolling on L-selectin at 1.8 dyn/cm² rapidly released upon a drop of shear to 0.25 dyn/cm², whereas neutrophils rolling on LPL at 1.8 dyn/cm² remained adherent for at least 10 s after being subjected to an identical shear drop (data not shown). This indicates that cell-free LPL, like the cell surface expressed molecule, is much less dependent on a threshold of shear stress to promote or maintain rolling adhesions with neutrophil-based ligands than native L-selectin. The properties of these two molecules observed when they were expressed on cell surfaces were therefore precisely recapitulated when they were expressed in cell-free form. The strikingly higher binding activity of cell-free LPL toward neutrophil L-selectin ligands therefore demonstrates that the stronger adhesiveness of LPL seen above was an intrinsic rather than a cellular property of the L-selectin EGF domain mutant.

**DISCUSSION**

The biophysical basis of adhesion by selectins and its regulation by ligand recognition and mechanical properties of bonds subjected to hydrodynamic force in shear flow is still obscure (38, 39, 53, 59). It has become increasingly evident that selectin adhesiveness under fluid shear is controlled by both cellular and mechanical properties independently of selectin binding to soluble ligands under equilibrium (36, 60). To address the contribution of equilibrium and mechanical properties of selectin bonds to selectin adhesion under flow, we have characterized the kinetic properties of adhesion mediated by L-selectin and LPL, an L-selectin EGF domain swap mutant. This L-selectin mutant has conserved the ligand binding specificity and equilibrium binding affinity to soluble ligands of native L-selectin but exhibited dramatically augmented adhesiveness to structurally distinct L-selectin ligands, including the high endothelial venule-derived ligands GlyCAM-1 and PNA. L-selectin ligands expressed on intact neutrophils, and glycoconjugate L-selectin ligands such as fucoidin and sulfated Leα. This augmented adhesion was particularly evident under limiting conditions of high shear flow or low ligand densities that minimize the duration of the cell-substrate contact. The higher adhesiveness of the mutant resulted in a dramatic enhancement of the rate of tether formation to immobilized ligands, corresponding to a higher cellular $k_{on}$, and was manifested as increased numbers of rolling cells, slower rolling velocities, and a significantly reduced shear threshold requirement for both the initiation and maintenance of rolling. Notably, these properties were each recapitulated by a cell-free immobilized form of LPL. The present study therefore constitutes the first evidence that the kinetics of L-selectin bond formation to immobilized ligand can be regulated under shear flow by the EGF-like domain independent of soluble ligand recognition, which is controlled primarily by the lectin domain of the selectin (37).

Our findings extend recent results suggesting that kinetic stability of L-selectin bonds under shear flow can be modulated by chemical modification of an L-selectin ligand, independent of equilibrium properties of the bonds (36). Analysis of the properties of the EGF mutant showed that it tethered to low density GlyCAM-1 10–30-fold faster than L-selectin but dissociated from the ligand with identical $k_{off}$ and exhibited a similar response of $k_{on}$ to increasing shear stresses. Thus, the reactive compliance (35, 53) and kinetic stability of native L-selectin bonds were conserved in bonds mediated by the EGF mutant. The longer duration of the pauses exhibited by LPL during rolling, together with the conserved $k_{off}$ of single bonds mediated by the EGF mutant, therefore suggest that the mutant can form multiple bonds with immobilized ligand much more readily than native L-selectin, because of a higher $k_{on}$ of its bonds.

What could be the molecular basis for this higher $k_{on}$? Previous epitope mapping of LPL with different mAbs directed against distinct epitopes in each of the domains of the molecule suggested that LPL retained the overall structure of native L-selectin (61). Furthermore, both L-selectin and LPL would be expected to associate equally with the cytoskeleton, because they share identical membrane proximal, transmembrane, and cytoplasmic domains (54, 60). L-selectin rolling velocity can be affected by proteolytic shedding of the selectin by a cell surface protease (62, 63). However, we found that the mutant and wild type forms were similarly susceptible to spontaneous or phorbol ester-induced shedding (data not shown), consistent with shedding being controlled by regions of the molecule near the membrane distal from the EGF-like domain (64, 65). The possibility that the EGF domain mutant has augmented spontaneous clustering on the surface of the pre-B cells appears unlikely in light of the results obtained by immunogold staining (Fig. 1). A recent study in the same pre-B cell system indicated that dimerization of L-selectin caused a modest decrease of rolling velocity with no shift in the shear threshold requirement of L-selectin (66). In contrast, LPL-mediated rolling was 5–10-fold slower than rolling mediated by L-selectin, and its shear threshold requirement was dramatically changed. Thus, enhanced dimerization of the EGF domain mutant could not have accounted for its intrinsically increased adhesiveness. Moreover, a purified cell-free form of the mutant immobilized on a substrate also exhibited dramatically augmented tethering and rolling over that of native L-selectin, even though both molecules were identically attached to the substrate through binding to a tail-specific mAb (Fig. 8). The faster bond formation and correspondingly higher cellular $k_{on}$ mediated by the EGF mutant under all experimental conditions therefore reflects intrinsic binding properties of the mutant rather than differences in surface topology, receptor clustering, shedding properties, or cytoskeletal attachment.

Previous functional analysis of the LPL mutant (37) provided the first indication of an important role for the EGF domain in cell adhesion by selectins, although the molecular mechanism(s) by which the EGF domain exerted its effect was not elucidated in that study. Under the assay conditions employed in the previous work (37), HL60 cells failed to attach to COS cells expressing L-selectin but did attach to COS cells expressing LPL. The current results make it clear that the ability of LPL but not L-selectin to mediate detectable adhesion of transfectected COS cells to HL-60 cells was due to the higher adhesiveness of LPL for L-selectin ligands per se, rather than to any newly acquired specificity. The present results argue strongly against any direct interaction between the P-selectin EGF domain in LPL and any of the L-selectin ligands tested, which might account for the enhanced adhesiveness of LPL. Increased adhesiveness of LPL was observed on entirely distinct glycoprotein L-selectin ligands of both leukocyte and endothelial origin, as well as on purely carbohydrate selectin ligands, such as fucoidin and sulfated Leα, which presumably interact exclusively with the lectin domain of L-selectin. Moreover, increased LPL adhesiveness was observed also on an immobilized L-selectin mAb that interacts exclusively with the lectin domain on the LPL mutant. In addition, P-selectin expressed in the same pre-B cells failed to detectably interact with any of the L-selectin ligands tested (data not shown). The introduction of the EGF domain of P-selectin into the L-selectin backbone in LPL therefore did not change the adhesive specificity or ligand recognition properties of the L-selectin EGF mutant. Finally,
the observation that the P-selectin EGF domain also imparts higher levels of adhesion to the P-selectin lectin domain (37) suggests that the P-selectin EGF domain has an inherent enhancing effect on the adhesive activity of both L-selectin- and P-selectin-lectin domains to which it is attached.

Rates of tether bond formation measured on adhesive substrates under flow conditions reflect the effective or cellular $k_{on}$ of surface-based L-selectin associating with a surface-bound ligand, under nonequilibrium conditions (67). The cellular on rate is therefore distinct from and not necessarily predictively related to the molecular thermodynamic on rate between soluble and surface-bound counter-receptors measured at equilibrium. Cellular $k_{on}$, unlike molecular $k_{on}$, could vary with the availability and diffusivity of the receptors within the interacting surfaces and the separation distance between these surfaces (68, 69). However, as pointed out above, LPL shares identical dimensions, cytokines associations, and surface distribution with L-selectin, and thus, topographical or diffusion considerations could not account for the markedly higher cellular $k_{on}$ of this EGF mutant. Furthermore, the enhanced adhesiveness of the mutant was retained in its cell-free state, when both the mutant and the native L-selectins were identically anchored to an adhesive surface through their shared cytoplasmic domain. The identical $k_{off}$ measured here for LPL and native L-selectin, together with their similar equilibrium binding to the soluble carbohydrate ligand, PPME, indicate that the molecular thermodynamic $k_{on}$ of both variants toward soluble L-selectin ligands is quite similar. This was further supported by the similarly rapid binding of soluble PPME to both cell surface L-selectin and LPL.

The lectin domain of the LPL mutant appears to be stabilized in a conformation with higher accessibility to immobilized ligand than the corresponding domain in native L-selectin. Direct support for this conclusion was provided by the faster rates of tethering mediated by the lectin domain on LPL to an immobilized lectin-specific mAb, DREG-200, but not to an SCR-specific mAb. Increased elasticity of lectin domain regions outside the receptor-lectin interface have been proposed to regulate mechanical properties of L-selectin bonds without altering selectin affinity to soluble ligand (36). Our results suggest that subtle conformational changes of the lectin domain introduced by the EGF domain swap could dynamically facilitate the recognition by the lectin domain of an immobilized ligand. This is likely due to the fact that surface-bound ligand is dynamically restricted within the adhesive contact zone, in contrast to a soluble ligand. Although the precise nature of this conformational change is still unclear, it is evident that the EGF domain swap in LPL did not alter the conformation of the lectin domain to an extent that it could bind soluble carbohydrate ligands more efficiently than native L-selectin. This is probably because soluble ligands have diffusion rate orders of magnitude faster than those of immobilized ligands (35) and would not discriminate between subtle conformational changes of the lectin domain.

An unprecedented property of selectin-dependent rolling is that it requires shear stress above a threshold value to form and persist (38, 39), in sharp contrast to most types of adhesive interactions that are destabilized by applied shear forces (35, 70, 71). The biophysical basis of these unique properties of L-selectin is still unclear. Fluid shear may enhance rolling by the generation of torque forces subsequent to cell tethering (39). Kinetic analysis of L-selectin interactions with low density ligands has revealed specialized properties of L-selectin bonds, such as shorter lifetimes and lower susceptibility of the bond lifetime to applied force (i.e. lower reactive compliance), compared with other selectin bonds that show reduced or no requirement for a threshold shear to form and persist (53). We now show that increased cellular $k_{on}$ of selectin interaction without alteration in its bond lifetime reduces dramatically the threshold shear required by this interaction to initiate and maintain rolling adhesion. Recent studies predicted that shear flow acts to increase L-selectin bond number at adhesive contact zones (58). Binding rate of surface-bound reactants increases with the relative velocity between the adhesive surfaces because of an increase of collision rate with increasing shear (72). Shear flow may act to facilitate cellular transport along the adhesive contact zone to increase the probability of bond formation and may increase the force with which selectins contact their ligands on counter surfaces (39). Our results extend this idea and demonstrate a reciprocal relationship between the kinetics of tether bond formation and the shear threshold requirement of selectin adhesion. When L-selectin bond formation with immobilized ligand is rendered high, as with the EGF mutant, the requirement for shear-facilitated cell transport along the adhesive contact zone to increase the probability of bond formation is diminished, resulting in reduced dependence of adhesion on a threshold shear stress.

In summary, we have made use of an EGF domain mutant of L-selectin with unexpectedly high reactivity of the lectin domain toward surface-immobilized ligands to test how augmentation of L-selectin adhesiveness affects the dynamic stability and shear dependence of rolling adhesion at the cell substrate contact zone. Higher rates of bond formation by the EGF domain mutant resulted in slower rolling, a reflection of a larger number of bonds forming at any given time at the cell substrate contact zone. The LPL mutant is the first example of a specific domain of an adhesion molecule controlling a particular dynamic property of that molecule. The present results extend previous findings that linked the dynamics of selectin-mediated rolling to intrinsic bond lifetime in the presence of shear forces (36, 53). This is the first evidence that selectin adhesiveness is controlled by the binding reactivity of the lectin domain toward surface-bound ligand under shear flow. Our results suggest for the first time that mechanical properties of the lectin domain can be regulated by the EGF domain independent of the equilibrium binding properties of the molecule. Future crystallographic and spectroscopic analysis of L-selectin may highlight how the EGF domain and possibly other ectodomains of the molecule regulate selectin adhesion independent of the thermodynamic equilibrium aspects of soluble ligand recognition.

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