

Cytoplasmic anchorage of L-selectin controls leukocyte capture and rolling by increasing the mechanical stability of the selectin tether

Oren Dwir,¹ Geoffrey S. Kansas,² and Ronen Alon¹

¹Department of Immunology, Weizmann Institute of Science, Rehovot, 76100 Israel

²Department of Microbiology-Immunology, Northwestern Medical School, Chicago, IL 60611

L-selectin is a leukocyte lectin that mediates leukocyte capture and rolling in the vasculature. The cytoplasmic domain of L-selectin has been shown to regulate leukocyte rolling. In this study, the regulatory mechanisms by which this domain controls L-selectin adhesiveness were investigated. We report that an L-selectin mutant generated by truncation of the COOH-terminal 11 residues of L-selectin tail, which impairs association with the cytoskeletal protein α -actinin, could capture leukocytes to glycoprotein L-selectin ligands under physiological shear flow. However, the conversion of initial tethers into rolling was impaired by this partial tail truncation, and was completely abolished by a further four-residue truncation of the L-selectin tail. Physical anchorage of both cell-free tail-truncated mutants

within a substrate fully rescued their adhesive deficiencies. Microkinetic analysis of full-length and truncated L-selectin-mediated rolling at millisecond temporal resolution suggests that the lifetime of unstressed L-selectin tethers is unaffected by cytoplasmic tail truncation. However, cytoskeletal anchorage of L-selectin stabilizes the selectin tether by reducing the sensitivity of its dissociation rate to increasing shear forces. Low force sensitivity (reactive compliance) of tether lifetime is crucial for selectins to mediate leukocyte rolling under physiological shear stresses. This is the first demonstration that reduced reactive compliance of L-selectin tethers is regulated by cytoskeletal anchorage, in addition to intrinsic mechanical properties of the selectin-carbohydrate bond.

Introduction

L-selectin is a leukocyte member of the three-member selectin family which mediates initial capture (tethering) to and rolling of circulating leukocytes on high endothelial venules (HEVs)* of peripheral lymph nodes and inflamed endothelia (Rosen and Bertozzi, 1994; Kansas, 1996). These adhesive interactions, although reversible, are required for subsequent activation of the tethered leukocyte, which result in firm vascular adhesion (Springer, 1994). Binding of endothelial selectins (P- and E-selectin) and the leukocyte selectin, L-selectin to cell-surface carbohydrate ligands involves a unique tolerance of bonds to applied shear forces (Alon et al., 1995a; Chen et al., 1997). Ligand recognition by selectins is mediated by their lectin domains, yet the translation of ligand

binding into productive adhesive interactions under physiological conditions of shear flow is tightly regulated by both extracellular and cytoplasmic selectin domains (Kansas et al., 1993; Patel et al., 1995; Setiadi et al., 1998; Dwir et al., 2000). The cytoplasmic domains of many adhesion molecules regulate their affinity and adhesive activity through interactions with the cytoskeleton or inner membrane-associated proteins (Angres et al., 1996; Goldstein and Wofsy, 1996; Kucik et al., 1996; Yauch et al., 1997; Setiadi et al., 1998). The cytoplasmic tails of the three selectins are structurally distinct although highly conserved among species for any given selectin (Kansas, 1996), suggesting specialized functions for this domain. L-selectin is constitutively associated with the actin-binding protein, α -actinin (Pavalko et al., 1995), as well as with the regulatory cytoplasmic protein calmodulin (Kahn et al., 1998). Truncation of the COOH-terminal 11 residues of the cytoplasmic tail of L-selectin (see Fig. 1 A) or disruption of the actin cytoskeleton were shown to disrupt L-selectin association with α -actinin and abolish L-selectin rolling on inflamed venules under physiological shear flow, without altering carbohydrate recognition by the selectin (Kansas et al., 1993), or microvillar localization of

The online version of this article contains supplemental material.

Address correspondence to Dr. Ronen Alon, Dept. of Immunology, Weizmann Institute of Science, Rehovot, 76100 Israel. Tel.: 972-8-9342482. Fax: 972-8-9344141. E-mail: ronalon@wicc.weizmann.ac.il

*Abbreviations used in this paper: CB, cytochalasin B; GlyCAM-1, glycoprotein cell adhesion molecule 1; HEV, high endothelial venule; PNA_d, peripheral node addressin; sLe^x, sialyl Lewis^x.

Key words: selectins; inflammation; rolling; leukocyte; cytoskeleton

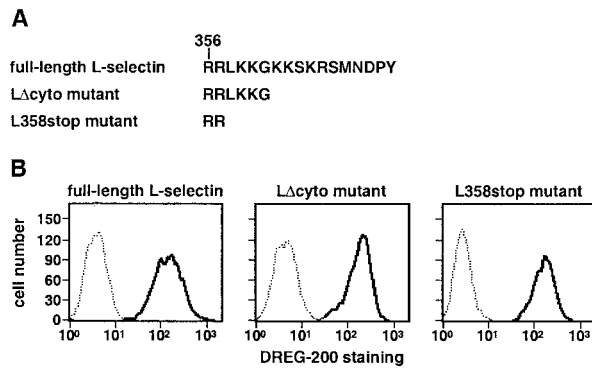


Figure 1. Amino acid sequence and surface expression of full-length L-selectin and its cytoplasmic domain mutations. (A) Amino acid sequences of the cytoplasmic domain of L-selectin and the L-selectin mutants characterized in this study. Sequences are aligned at their membrane proximal residue, R356. (B) Immunofluorescence flow cytometry of 300.19 clones stably transfected with cDNA encoding L-selectin, L Δ cyto mutant lacking the 11 C' residues of its cytoplasmic domain and L358stop mutant lacking the 15 C' residues of the cytoplasmic domain. Transfectants were stained with the anti-L-selectin mAb DREG-200 (filled line) or control mAb (dotted line) followed by FITC-labeled goat anti-mouse mAb.

L-selectin (Pavalko et al., 1995). These early data suggested that the L-selectin tail controls L-selectin function through an α -actinin-facilitated association with the actin cytoskeleton.

At least three mechanisms could account for the role of the cytoplasmic tail in L-selectin-mediated adhesion under physiological shear flow. First, the tail may prevent L-selectin from being uprooted from the membrane by its ligand before a functional tether has formed. Second, cytoskeletal association of L-selectin could modulate L-selectin clustering and facilitate formation of multivalent tethers. Finally, cytoskeletal association could directly stabilize the L-selectin tether bond. To differentiate between these possibilities, we have taken a quantitative approach to analyze at high temporal resolution the kinetic properties of cell capture events and rolling motions mediated by full-length L-selectin and two cytoplasmic tail truncation mutants on purified L-selectin ligands. This approach provides further evidence for the functional importance of association of L-selectin with the actin cytoskeleton mediated by its cytoplasmic domain. Tail truncation increased the sensitivity of the L-selectin tether k_{off} to increased shear force, and thereby dramatically lowered the lifetime of tethers forming at physiological shear stresses and restricted leukocyte capture and rolling. Cytoskeletal association of L-selectin appears therefore critical for millisecond stabilization of L-selectin bonds at reversible adhesive contacts.

Results

A tail-truncated L-selectin mutant supports reduced rolling and partial tethering to L-selectin ligands

To delineate how the cytoskeletal interactions of L-selectin control its adhesiveness, the ability of full-length and tail-truncated (L Δ cyto) L-selectin transfectants to interact with purified L-selectin glycoprotein ligands was examined under shear flow. Full-length or truncated L-selectin (L Δ cyto,

lacking the COOH-terminal 11 residues of the cytoplasmic domain) were stably expressed in the murine pre-B-lymphocyte line 300.19 (Kansas et al., 1993) (Fig. 1 A). Clones of L-selectin and L Δ cyto expressed at comparable surface levels (Fig. 1 B) were perfused under low physiological shear flow on adhesive substrates coated with glycoprotein cell adhesion molecule 1 (GlyCAM-1) or with peripheral node addressin (PNAd), the major HEV L-selectin ligands (Berg et al., 1991). Although previously shown to lack significant adhesive activity in the HEV frozen section assay or in rolling through rat mesentery venules, L Δ cyto-expressing cells established persistent rolling on high density GlyCAM-1 in vitro (Fig. 2 A). Furthermore, the ability of L Δ cyto-transfected cells to initiate tethers to GlyCAM-1 (i.e., either transient or followed by rolling) was not impaired even at low GlyCAM-1 densities (Fig. 2 A). However, the ability of the L Δ cyto to convert initial cell tethering to persistent rolling was markedly reduced compared with L-selectin, concomitant with faster rolling, especially at lower GlyCAM-1 densities or elevated shear stresses (Fig. 2, A and B). Furthermore, the ability of initial L Δ cyto-mediated tethers to form and convert to rolling adhesion on PNAd was markedly reduced relative to full-length L-selectin (Fig. 2, A and B). The ability of the tail-truncated mutant to support L-selectin-dependent neutrophil rolling on monolayers of substrate-bound selectin transfectants was also significantly impaired (Fig. 2 C). However, individual L Δ cyto transfectants were nearly as efficient as their full-length L-selectin counterparts in promoting singular neutrophil tethers (Fig. 2 D), albeit the duration of L Δ cyto-mediated tethers was significantly shorter than L-selectin-mediated tethers.

Notably, the ability of the L Δ cyto-expressing cells to form both transient and rolling tethers to GlyCAM-1 required an intact actin cytoskeleton. Disruption of actin microfilaments in both the full-length and L Δ cyto transfectants by cell pretreatment with cytochalasin B (CB) abolished all tethers to either GlyCAM-1 or PNAd (Fig. 2 A and unpublished data). Therefore, these results indicate that L Δ cyto retained considerable adhesive capacity, which depends on an intact actin cytoskeleton. Thus, the membrane proximal residues of the cytoplasmic domain of L-selectin (Fig. 1) mediate considerable tethering and rolling adhesions on physiological L-selectin ligands, albeit with reduced stability. Consistent with this notion, the ability of the L Δ cyto transfectant, but not of the L-selectin transfectant to sustain rolling on high density GlyCAM-1, rapidly declined at progressively increasing shear stresses (Fig. 3 A) and reduced GlyCAM-1 density (Fig. 3 B). Notably, dimerization of cell surface L-selectin by a non-blocking dimerizing mAb (Li et al., 1998) failed to augment tethering or rolling mediated by the L Δ cyto mutant (Fig. 3 B), although it augmented the capacity of full-length L-selectin to support rolling adhesion. Although the dimerizing mAb binds its antigenic determinant on both full-length and tail mutated L-selectin with similar efficiency (unpublished data), this mAb could not rescue the adhesive defects of the L Δ cyto detected on GlyCAM-1 (Fig. 3 B). Thus, the weaker adhesive activity of the tail truncated mutant could not be caused by impaired dimerization of this mutant. L-selectin occupancy during rolling also did not appear to modify in situ its adhesive activity, since L-selectin-expressing cells

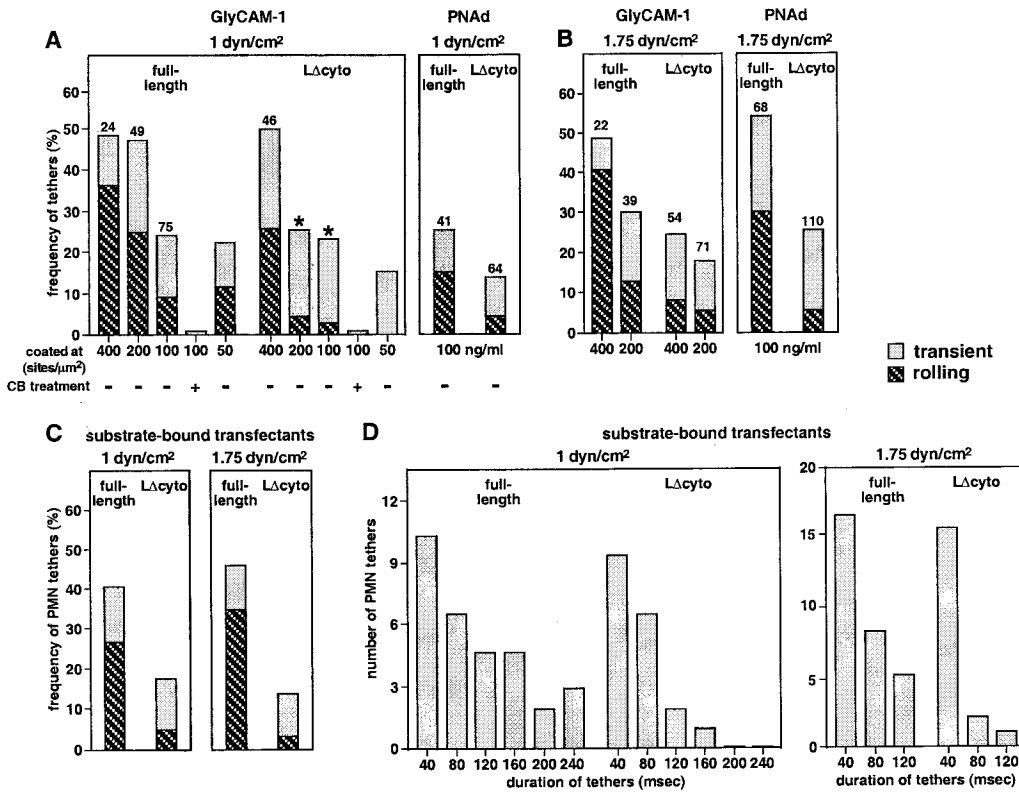


Figure 2. Rolling mediated by ΔCyto mutant on endothelial and neutrophil ligands is impaired. (A) Tethering and rolling mediated by full-length L-selectin and ΔCyto mutant at low physiological shear stress (1 dyn/cm²) on substrates coated with different densities of purified GlyCAM-1 or PNAd. Frequency values of tethering and rolling are depicted. Mean velocities of all rolling cells are indicated above the bars. (*) Rolling velocities not determined. (B) Tethering and rolling mediated by L-selectin and ΔCyto mutant interacting at moderate physiological shear stress (1.75 dyn/cm²) on substrates coated with different densities of GlyCAM-1, PNAd. (C) Tethering and rolling of neutrophils on a monolayer of substrate-adherent L-selectin and ΔCyto mutant-transfected cells at different shear stresses. Values are the mean ± range of two experiments. (D) Tethering of neutrophils to single substrate-adherent transfectants expressing either L-selectin or ΔCyto measured at 1 or 1.75 dyn/cm². Number and duration of tethers formed during 10-s perfusion of neutrophils (10⁶/ml) over six representative substrate-bound transfectant cells are shown. All tethers were blocked in the presence of EGTA or the soluble L-selectin ligand, fucoidin.

rolled at identical speeds both at upstream and downstream areas of GlyCAM-1-coated field (unpublished data).

Carbohydrate ligand clustering rescues the adhesive defects of the ΔCyto mutant

The impaired rolling adhesion mediated by the ΔCyto-expressing cells could reflect a defect in the ability of individual ΔCyto molecules to generate sufficient avidity for ligand. In light of the failure to rescue the adhesive defects of ΔCyto-expressing cells by L-selectin dimerization, we next compared the adhesiveness of ΔCyto and L-selectin towards two nonphysiological polyvalent carbohydrate ligands for L-selectin. The first ligand, the sulfated polysaccharide fucoidin, consists of closely spaced L-selectin binding sulfated fucose repeats (Mulloy et al., 1994), and the second ligand, sialyl Lewis^x (sLe^x)-decorated neoglycolipid, forms clusters when immobilized on polystyrene surfaces (Galustian et al., 1997). Strikingly, the ability of ΔCyto to tether to and roll on immobilized fucoidin or on sLe^x-glycolipid was indistinguishable from that of L-selectin, irrespective of the shear stress tested and even when ligands were present at subsaturating densities (Fig. 4, A–C). Disruption of actin microfilaments with CB inhibited rolling and tethering on immobilized fucoidin in both L-selectin and ΔCyto transfectants

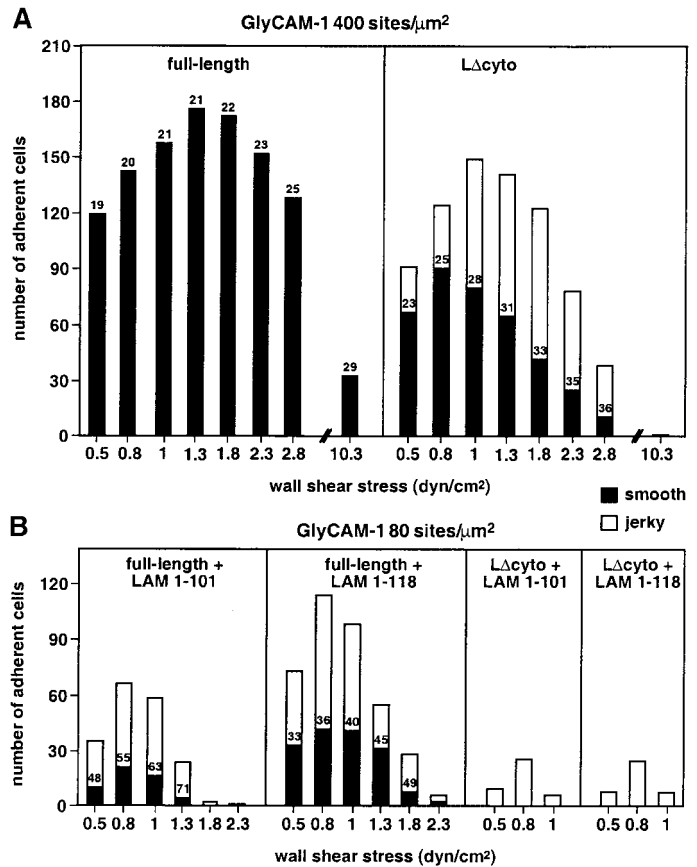
(Fig. 4 B), suggesting that the ΔCyto required cytoskeletal associations for its adhesiveness to the polysaccharide L-selectin ligand. Thus, high local valency of carbohydrate L-selectin ligand completely rescued the adhesive deficiency of the ΔCyto mutant, even under limiting ligand conditions (Fig. 4 D). Highly clustered carbohydrate ligands may therefore bind multiple ΔCyto molecules at microvillar contacts with comparable efficiency to full-length L-selectin.

Microkinetics of full-length L-selectin and ΔCyto-mediated rolling on GlyCAM-1

Displacement motions of leukocytes rolling on glycoprotein L-selectin ligands are comprised of discrete steps separated by short pauses with characteristic duration, reflective of bond stability at defined adhesive contact zones (Alon et al., 1997; Chen and Springer, 1999; Smith et al., 1999). Under conditions of ligand densities which favor the engagement of the rolling cell through singular tethers, the duration of the tether becomes progressively shorter (Chen and Springer, 1999). To gain insight into the dynamic differences between rolling tethers mediated by L-selectin or ΔCyto mutant, microkinetics of rolling motions mediated by the variants on GlyCAM-1 was next analyzed at high temporal resolution (Fig. 5 A). Pauses >0.004 s could be attributed to specific

Figure 3. Destabilization of rolling adhesions on GlyCAM-1 by tail truncation is not rescued by selectin dimerization.

(A) Accumulation of smooth rolling or jerky rolling L-selectin- or Δ Cyto-expressing cells perfused on high density (400 sites/ μm^2) GlyCAM-1 and subjected to incremented shear stress increase. Equal number of cells were perfused at a shear flow of 0.25 dyn/cm² over the GlyCAM-1 substrates, and then were subjected to step-wise increments of shear stress. The number of cells remaining adherent at each shear stress was determined as described in Materials and methods. At each indicated shear stress, the mean velocity of smoothly rolling cells, i.e., with a mean velocity < 90 $\mu\text{m/s}$ is labeled on top of the corresponding bar. (B) Effect of mAb-induced L-selectin dimerization on accumulation of L-selectin- or Δ Cyto mutant-expressing cells on medium density (80 sites/ μm^2) GlyCAM-1. Cells ($2 \times 10^6/\text{ml}$) were preincubated with the dimerizing mAb LAM1-118 or the control anti L-selectin mAb, LAM1-101 (each at 10 $\mu\text{g/ml}$), and perfused unwashed over GlyCAM-1 identically as in A. Cells incubated in mAb-free binding medium exhibited similar accumulation and resistance to detachment to cells pretreated with the control mAb. Smooth rolling was defined as cells rolling with a mean velocity < 90 $\mu\text{m/s}$. Figures shown are representative of five independent experiments.



L-selectin binding events, as they could be completely inhibited by mAb blocking, EGTA, or soluble fucoidin (Fig. 5 B and unpublished data). The duration of the vast majority of the rolling pauses mediated by L-selectin at a shear stress of

1.75 dyn/cm² could be fit into an homogenous group with a single first order dissociation rate constant of 64 s⁻¹ (Fig. 5 C, Table I), independent of GlyCAM-1 density (unpublished data). Under identical experimental conditions, all tethers mediated by Δ Cyto could be fit into a single homogenous group that dissociated with a much faster k_{off} (Fig. 5 C, Table I). A similar yet somewhat smaller difference between L-selectin and Δ Cyto tether stability was observed at 1 dyn/cm² (Fig. 5 D, Table I). Thus, quantal adhe-

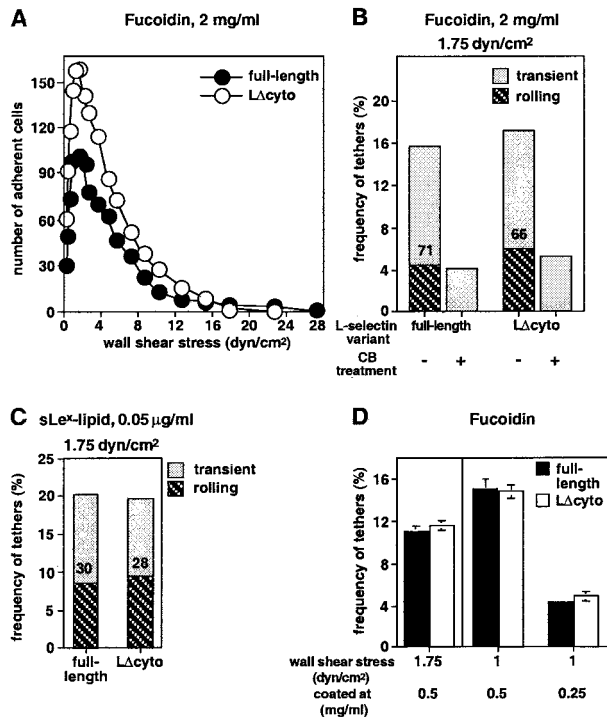


Figure 4. Tethering and rolling on polymeric L-selectin ligands are not compromised with the Δ Cyto mutant.

(A) Accumulation under flow and resistance to detachment from immobilized fucoidin by elevated shear of cells expressing full-length L-selectin or Δ Cyto mutant was determined as in Fig. 3. Data in A and B are representative of six independent experiments. (B) Frequency of tethering and rolling of intact or CB-treated 300.19 cells expressing either L-selectin or Δ Cyto mutant interacting with immobilized fucoidin at 1.75 dyn/cm². The mean velocity of all rolling cells is indicated on top of bars. Tethers of both intact and CB-treated cells were blocked in the presence of the lectin-function blocking mAb, DREG-200. (C) Frequency of tethering, fraction of rolling and mean rolling velocity of 300.19 cells expressing L-selectin or Δ Cyto mutant interacting with immobilized sLe^x-bearing glycolipid. (D) Frequency of tethers to low density fucoidin mediated by L-selectin or Δ Cyto mutant at different high shear stresses. All tethers were blocked in the presence of the L-selectin blocking mAb DREG-200. >95% of tethers were transient and duration analysis of tethers dissociated from fucoidin at 0.25 mg/ml yielded dissociation rate constants (k_{off}) of 8.9 ± 0.2 and 9.9 ± 0.1 s⁻¹ for full-length L-selectin or Δ Cyto mutant-mediated tethers, respectively. Data in C and D are representative of three independent experiments.

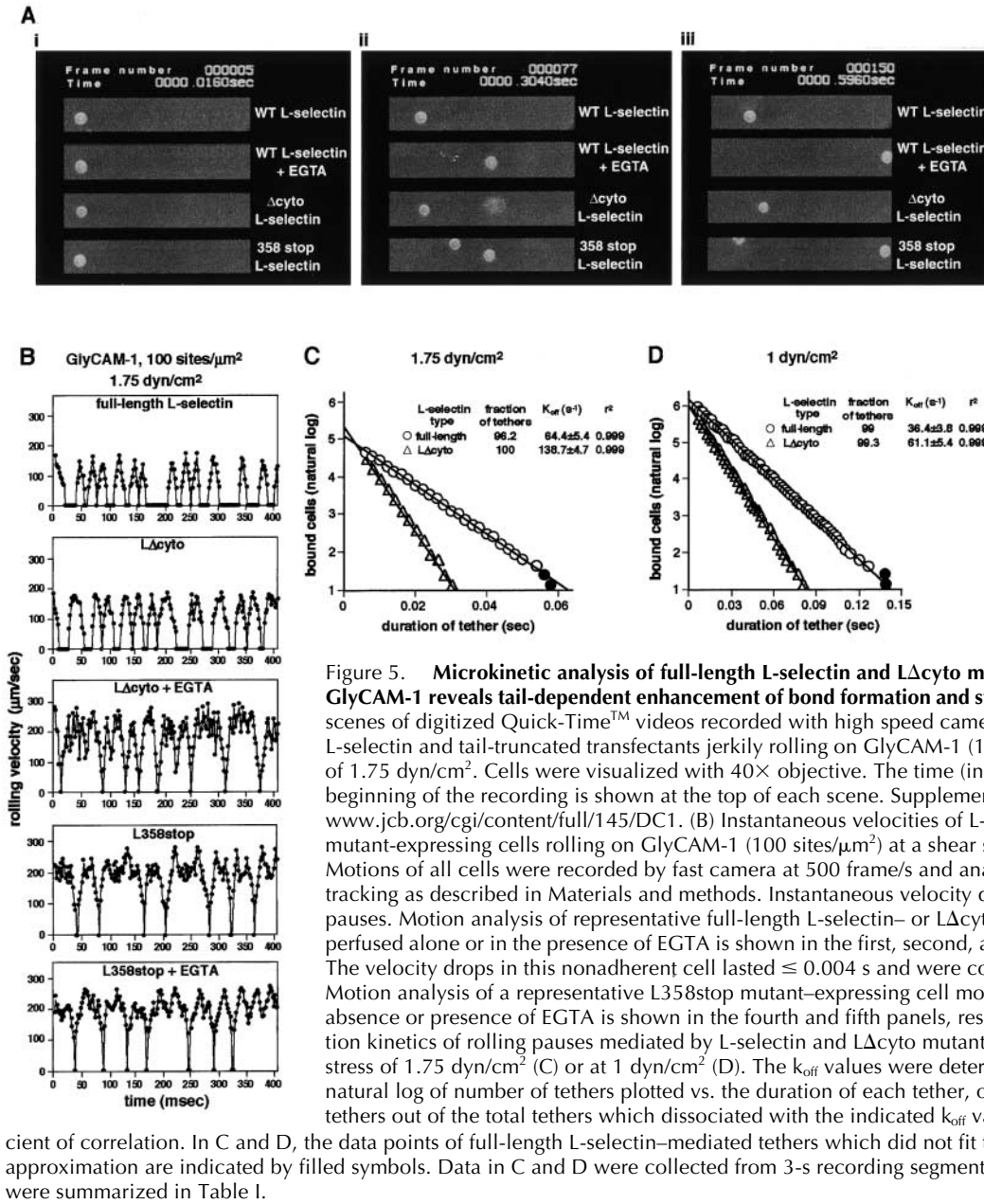


Figure 5. Microkinetic analysis of full-length L-selectin and Δ cyto mutant-mediated rolling on GlyCAM-1 reveals tail-dependent enhancement of bond formation and stabilization. (A) Characteristic scenes of digitized Quick-Time™ videos recorded with high speed camera of representative WT L-selectin and tail-truncated transfectants jerkily rolling on GlyCAM-1 (100 sites/ μm^2) at a shear stress of 1.75 dyn/cm². Cells were visualized with 40 \times objective. The time (in seconds) elapsed from the beginning of the recording is shown at the top of each scene. Supplemental Video 1 is available at www.jcb.org/cgi/content/full/145/DC1. (B) Instantaneous velocities of L-selectin- and Δ cyto mutant-expressing cells rolling on GlyCAM-1 (100 sites/ μm^2) at a shear stress of 1.75 dyn/cm². Motions of all cells were recorded by fast camera at 500 frame/s and analyzed by computerized cell tracking as described in Materials and methods. Instantaneous velocity drops were defined as rolling pauses. Motion analysis of representative full-length L-selectin- or Δ cyto mutant-expressing cells perfused alone or in the presence of EGTA is shown in the first, second, and third panels, respectively. The velocity drops in this nonadherent cell lasted ≤ 0.004 s and were considered nonspecific pauses. Motion analysis of a representative L358stop mutant-expressing cell moving on identical field in the absence or presence of EGTA is shown in the fourth and fifth panels, respectively. (C and D) Dissociation kinetics of rolling pauses mediated by L-selectin and Δ cyto mutant on GlyCAM-1 at a shear stress of 1.75 dyn/cm² (C) or at 1 dyn/cm² (D). The k_{off} values were determined from the slope of the natural log of number of tethers plotted vs. the duration of each tether, or pause. The fractions of tethers out of the total tethers which dissociated with the indicated k_{off} values are indicated. r, coefficient of correlation. In C and D, the data points of full-length L-selectin-mediated tethers which did not fit the first order dissociation approximation are indicated by filled symbols. Data in C and D were collected from 3-s recording segments and the kinetic results were summarized in Table 1.

Table 1. Microkinetics of full-length L-selectin-, Δ cyto- and L358stop-expressing 300.19 cells interacting with GlyCAM-1 100 sites/ μm^2 at 0.5, 1, and 1.75 dyn/cm²

	Wall shear stress ^a						
	1.75 dyn/cm ²		1 dyn/cm ²		0.5 dyn/cm ^d		
L-selectin variant	Full-length	Δ cyto	Full-length	Δ cyto	Full-length	Δ cyto	L358stop5
Rolling fraction (%)	40	16	54	37	0	0	0
Tethering fraction (%)	ND	ND	ND	ND	32	26	7
Mean pause duration (ms)	20 ± 1	12 ± 1	31 ± 1	20 ± 1	61 ± 6	47 ± 4	23 ± 2
	(n = 104)	(n = 88)	(n = 393)	(n = 286)	(n = 76)	(n = 65)	(n = 39)
k _{off} (s ⁻¹)	64.4 ± 5.4	138.7 ± 4.7	34.1 ± 2.3	61.1 ± 5.4	16.2 ± 1.6	20.9 ± 1.5	44.6 ± 3.8
Mean step distance (μm) ^b	4 ± 0.1	10.2 ± 0.3	2.5 ± 0.1	4.6 ± 0.1	NA ^c	N.A.	N.A.

^aAll measurements were performed on cell motion segments of at least 20 cells recorded at 500 frames/s.

^bStep distance was defined as the distance traveled by a rolling cell between successive tethers.

^cNot applicable, as no rolling could be detected at low shear stress.

^dThe 358stop mutant did not support any cell tethering to GlyCAM-1 at shear stresses >1 dyn/cm².

sive units mediated by full-length L-selectin dissociated from GlyCAM-1 at significantly slower rates than quantal tethers mediated by $L\Delta$ cyto dissociating from GlyCAM-1. Impaired rolling of the $L\Delta$ cyto-expressing cells resulted also from a reduced rate of tether formation downstream of initial tethers, i.e., increased step distance between two successive tethers during the rolling motion. The mean step distance between $L\Delta$ cyto-mediated rolling pauses formed on GlyCAM-1 at 1.75 dyn/cm² was 2.5-fold longer than the mean step distance between L-selectin-mediated tethers formed on identical substrates (Table I). Smaller differences in mean step distances between L-selectin and $L\Delta$ cyto tethers were observed at 1 dyn/cm² (Table I), consistent with smaller differences in rolling dynamics mediated by the two variants at reduced shear stresses (Fig. 3). Thus, the weaker and faster rolling of $L\Delta$ cyto was the result of the shorter duration and increased spacing of successive tethers mediated by this mutant.

Tethering and rolling supported by $L\Delta$ cyto are mediated by the residual cytoskeletal association of its membrane-proximal region

The susceptibility of the weak adhesive activity of $L\Delta$ cyto-expressing cells to disruption of the actin cytoskeleton (Figs. 2 A and 4 B) suggested that its RRLKKG segment maintains residual cytoskeletal association sufficient to mediate leukocyte capture and weak rolling. Indeed, further truncation of the $L\Delta$ cyto mutant by four additional residues, (designated L358stop) (Fig. 1 A), abolished all rolling adhesion even on high density GlyCAM-1 (Fig. 6 A). Furthermore, only a minor fraction of L358stop transfected cells could establish transient tethering to fucoidin, (Fig. 6 B), despite the fact that fucoidin completely rescued the adhesion deficiency of the $L\Delta$ cyto mutant (Figs. 4 and 6 B). Moreover, the residual tethering activity of the L358stop on fucoidin was insensitive to CB treatment (Fig. 6 B), in contrast to the sensitivity of L-selectin and of $L\Delta$ cyto to the cytoskeletal drug (Fig. 4 B). Microkinetic analysis of L358stop transfectant adhesion to medium density GlyCAM-1 at millisecond temporal resolution revealed that this tail mutant could not support any L-selectin specific adhesive tethers to GlyCAM-1 at a shear stresses of 1.75 dyn/cm² (Fig. 5 B). However, analysis of cell capture to GlyCAM-1 at drastically reduced shear (0.5 dyn/cm²) revealed low frequency of short-lived L-selectin specific tethers supported by the L358stop mutant (Fig. 6 C). The duration of these tethers could be fit into an homogenous group with a single first order dissociation rate constant (k_{off}) independent of GlyCAM-1 density (unpublished data), but threefold faster than the corresponding k_{off} of L-selectin-mediated tethers (Fig. 6 C). Reminiscent of the tethering results observed on fucoidin (Fig. 6 B), the tethering ability of the L358stop to GlyCAM-1 was completely insensitive to CB treatment of the transfectants (tether frequency of 7.3% vs. 7.1%). Furthermore, the dissociation rate constant of L358stop-mediated tethers from GlyCAM-1 was not affected by CB treatment ($k_{off} = 44.6 \text{ s}^{-1}$ vs. 45.3 s^{-1} , respectively). Thus, very low residual adhesive activity of the L358stop towards GlyCAM-1 or fucoidin which did not depend on association of the mutant with the actin cytoskele-

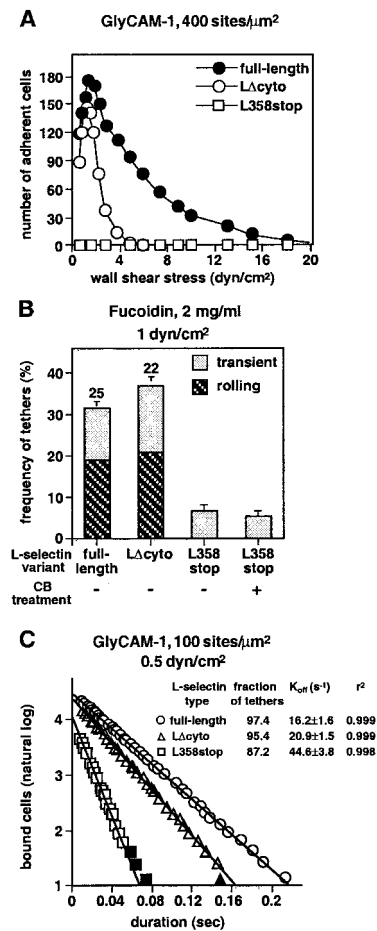


Figure 6. Further truncation of the $L\Delta$ cyto mutant abolishes rolling and impairs tether formation and millisecond stabilization under physiological shear flow. (A) Lack of accumulation under flow of L358stop mutant-expressing 300.19 cells on high density GlyCAM-1 (400 sites/μm²). Accumulation and resistance to clearance of cells expressing full-length L-selectin and $L\Delta$ cyto mutant by incremented shear stresses measured under identical conditions is shown for comparison. The experiment shown is a representative of three independent experiments. (B) Frequency of tethering and rolling of 300.19 cells expressing L-selectin, $L\Delta$ cyto mutant or L358stop mutant interacting with immobilized fucoidin at a shear stress of 1 dyn/cm². Mean values ± range of overall tether frequencies determined in several fields are shown. Mean velocities of rolling cells are shown on top of the bars. Both rolling and tethering were blocked in the presence of L-selectin blocking mAbs (unpublished data). (C) Dissociation kinetics of transient tethers mediated by L-selectin-, $L\Delta$ cyto mutant- or L358stop mutant-expressing cells interacting with medium density GlyCAM-1 (100 sites/μm²) at 0.5 dyn/cm² determined from high speed camera recordings performed as in Fig. 5. Pauses ≤ 0.006 s were excluded from this analysis. The kinetic results are summarized in Table I.

ton could be detected at low shear stresses, but was eliminated at higher stresses.

The response of tether k_{off} to elevated shear forces is augmented by progressive truncation of L-selectin tail
The duration of individual transient tethers to medium density GlyCAM-1 mediated by the various mutants was next measured over a range of wall shear stresses. As observed

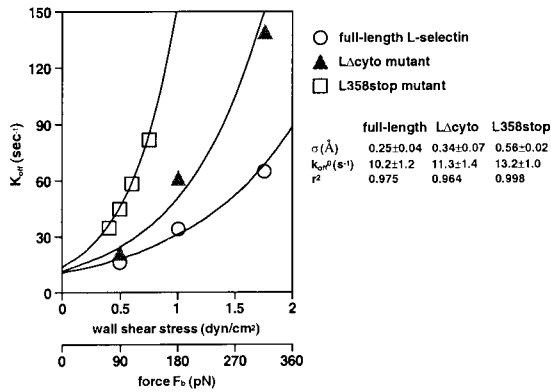


Figure 7. Shear sensitivity of full-length L-selectin tethers is increased by progressive truncation of the cytoplasmic tail of the selectin. Effect of increased wall shear stress and force applied on the tether bond on dissociation kinetics of L-selectin-, LΔcyto mutant-, and L358stop mutant-expressing cells interacting with medium density GlyCAM-1 (100 sites/μm²). The lines connecting the determined k_{off} values for all three transfectants at the indicated shear stresses were fit to the Bell expression $k_{off} = k_{off}^0 \exp(\sigma F_s/kT)$ where k_{off} and k_{off}⁰ are the stressed and unstressed dissociation rate constants, respectively, σ is the reactive compliance of the tether, k is Boltzmann’s constant, and T is the absolute temperature.

with L-selectin and the LΔcyto mutant, the dissociation rate constants of L358stop-mediated tethers increased with increasing shear stresses in an exponential manner (Fig. 7). This was in agreement with a relationship previously found between quantal L-selectin tether dissociation rates and applied force (Alon et al., 1997; Ramachandran et al., 1999). Strikingly, all three exponential curves established for the various tethers predict a similar k_{off}⁰ i.e., the k_{off} of the unstressed tether bond in the absence of shear force for all three L-selectin variants (Fig. 7). However, the reactive compliance of L358stop tethers, i.e., the increase in k_{off} of the stressed tether with applied shear force, was twofold higher than the reactive compliance of L-selectin tethers. As the reactive compliance exponentially decreases tether bond stability, this difference in reactive compliance was sufficient to destabilize tethers mediated by the L358stop on GlyCAM-1 to an extent they became essentially undetectable at physiological shear stresses >1 dyn/cm² (k_{off} > 300 s⁻¹; Figs. 5 B and 7). In contrast, the reactive compliance of LΔcyto tethers was 1.4-fold higher than that of full-length L-selectin tethers (Fig. 7). This milder adhesive deficiency abrogated rolling adhesions at elevated shear forces, while cell capture ability of this mutant was still largely conserved (Figs. 2, 3, 5, and 6). Consequently, even at high shear stresses, the k_{off} of tethers mediated by the LΔcyto mutant was only twofold faster than the k_{off} of L-selectin tethers (Fig. 7).

Anchorage of cell-free L-selectin tail mutants rescues their adhesive defects

We previously described an adhesion assay to quantify the adhesive properties of cell-free L-selectin variants under shear flow (Dwir et al., 2000). In this assay, L-selectin mutants are captured in a functional state on a substrate coated with nonfunction blocking L-selectin specific mAbs, and their lectin-dependent adhesive capacities to ligands ex-

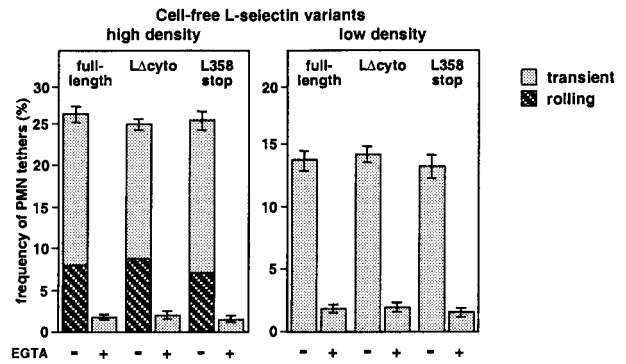


Figure 8. Artificial surface-anchorage of cell-free L-selectin tail mutants fully rescues their adhesive deficiencies under shear flow. The figure shows the frequency of neutrophil tethering and rolling on L-selectin mutants captured on immobilized anti-L-selectin mAb. Total neutrophil tethers to cell-free full-length L-selectin or the indicated truncated mutants were determined at a shear stress of 1 dyn/cm². Each L-selectin variant was anchored on the substrate at identical density, by binding to surface-bound anti-SCR-specific mAb, LAM1-118, coated at either 20 or 10 μg/ml (high or low density, respectively). Neutrophil tethering to anchored L-selectin variants performed in Ca²⁺-free medium in the presence of 2 mM EGTA is also shown. No neutrophil tethering was observed on substrates coated with anti-actin mAb. Results represent mean ± range of data collected in two fields of view.

pressed on perfused neutrophils are compared under physiological shear flow. Selectin mutants differing in their intrinsic adhesiveness exhibited large adhesive differences in this assay (Dwir et al., 2000). However, when the full-length and the two tail-truncated L-selectin mutants were captured at similar densities on a substrate coated with the anti-SCR domain specific mAb, LAM1-118 (Fig. 8), or other SCR mAbs (unpublished data), essentially identical capacities to support neutrophil tethering or rolling were detected for all L-selectin mutants. Thus, anchorage of both tail truncated L-selectin mutants to a solid surface appears to have fully rescued their adhesive defects.

Discussion

The ability of selectin tethers to successfully support persistent rolling depends on a proper balance of tether formation and breakage at the cell front and rear, respectively. Leukocyte rolling mediated by L-selectin is supported by single microvillus tethers per adhesive contact zone lasting between 10⁻² and 10⁰ s (Alon et al., 1997; Chen and Springer, 1999). We find that L-selectin tethers shorter than 20 ms fail to support persistent rolling adhesion, suggesting that the probability of successive tether formation collapses when tether duration drops below this critical duration. Microkinetic analysis of L-selectin tethers, the building units of leukocyte rolling, mediated by structural tail variants of L-selectin on the prototypic L-selectin ligand, GlyCAM-1, provided key insights into how the cytoplasmic domain of L-selectin controls its ability to mediate leukocyte rolling. The first order dissociation kinetics of tethers formed by all structural L-selectin variants was found to approach a constant value near zero shear stress, suggesting that the k_{off} of unstressed L-selectin tethers is independent of the cytoplas-

mic tail (Fig. 7). Nevertheless, the reactive compliance of tethers, i.e., the sensitivity of the tethers to shear force, was found to be considerably lower for full-length L-selectin than for its respective Δ Cyto or L358stop mutants. Low compliance of L-selectin tethers to applied shear forces results in prolonged tether duration at elevated shear stresses and permits L-selectin to support both leukocyte capture and rolling under a wide range of shear stresses. In contrast, the Δ Cyto, and to a greater degree the L358stop mutant, exhibit impaired abilities to support leukocyte rolling or capture. The analysis of L-selectin tether kinetics, performed here for the first time on tail mutants of L-selectin at millisecond temporal resolution, strongly suggests that translation of bonds to adhesive tethers involves millisecond stabilization events, critically regulated by the cytoplasmic tail of L-selectin and an intact cytoskeleton.

What could be the molecular basis for the impaired stabilization of tail-mutated L-selectin-mediated tethers? The exceptional ability of L-selectin to mediate cell capture under highly disruptive shear flow, even in its cell-free state (Alon et al., 1998; Dwir et al., 2000; Greenberg et al., 2000) has been attributed to intrinsically low reactive compliance of its carbohydrate bonds (Alon et al., 1997; Ramachandran et al., 1999). The present results suggest that, in addition to intrinsic molecular properties of L-selectin bonding with ligand, the association of L-selectin with the actin cytoskeleton reduces the effective reactive compliance of its cellular tethers under flow. How could cytoskeletal association of L-selectin contribute to dynamic stabilization of its tethers? Our observation that immobilization of cell-free tail-truncated L-selectin mutants fully rescues their adhesive defects, and the earlier finding that tethers mediated by cell-free L-selectin retain kinetic properties of tethers mediated by leukocyte-expressed L-selectin (Alon et al., 1998; Greenberg et al., 2000), suggests that mere anchorage of the selectin to a solid support can confer on L-selectin tethers their characteristic low reactive compliance. It is therefore possible that restriction of L-selectin's lateral mobility within the adhesive contact zone is both required and sufficient for the L-selectin bond to undergo rapid stabilization at microvillar contact sites (von Andrian et al., 1995).

Recent measurements on single L-selectin carbohydrate bonds ruptured by a biomembrane force microsphere probe (Evans et al., 2001) reveals a k_{off} value of unstressed L-selectin: carbohydrate bond ($k_{\text{off}}^0 = 3 \text{ s}^{-1}$) similar to that of unstressed tethers measured previously ($k_{\text{off}}^0 = 7\text{--}9 \text{ s}^{-1}$) (Alon et al., 1997, 1998; Ramachandran et al., 1999; Dwir et al., 2000), as well as those measured here at temporal resolution comparable to that in the biomembrane force probe experiments (Evans et al., 2001). However, under forces predicted to be applied to individual leukocyte microvilli at physiological shear stresses, i.e., $> 100 \text{ pN}$ (Alon et al., 1997), the bond between a single L-selectin and carbohydrate ligand forms and dissociates within a single millisecond period (Evans et al., 2001). Bond rupture by a force probe escaping instantaneously from the contact zone does not allow any rebinding of the dissociated receptor to ligand on the counter surface effects (Evans et al., 2001). Thus, dissociation kinetics of the receptor ligand pair studied in this system may in fact reflect the inherent reactive compliance of single L-selectin: ligand

bonds dissociating in the absence of any stabilizing effects (Evans et al., 2001). Notably, single L-selectin bonds exhibit sharp response to applied force, i.e., a 1,000-fold increase in bond k_{off} between zero force and force applied on a single microvillus at a shear stress of 1 dyn/cm^2 (Evans et al., 2001). Remarkably, this high force sensitivity (reactive compliance) is reminiscent of the high response of tethers mediated by the L358stop mutant in our experimental system (Fig. 7). Thus, it appears that the k_{off}^0 and the force sensitivity of the L358stop tethers are similar to the corresponding values measured for singular L-selectin bonds ruptured by force probes (Evans et al., 2001). Therefore, it is possible that once dissociated from the ligand, tail-truncated L-selectin variants are unable to instantaneously rebind it and thereby stabilize the tether at its original contact (Fig. 9 A).

We hypothesize that constitutive association of L-selectin with the actin cytoskeleton anchors the selectin within the plasma membrane, reduces its effective escape from the original ligand binding site, and thus facilitates ligand rebinding and tether stabilization. The escape radius of full-length L-selectin is predicted to be 7–8-fold smaller than that of the tailless mutant based on differences in lateral diffusion coefficients reported for anchored and unanchored proteins of similar dimensions to L-selectin (10^{-11} and $5 \times 10^{-10} \text{ cm}^2/\text{s}$, respectively) (Chan et al., 1991; Kucik et al., 1996). The degree to which ligand rebinding by L-selectin may take place would therefore be considerably higher for L-selectin than for the L358stop mutant, with an intermediate value for the tail truncated Δ Cyto mutant. Preliminary mathematical simulations of L-selectin rebinding kinetics suggests that this event takes place over a range of $10 \text{ }\mu\text{s}$ (unpublished data). This mechanism of ligand rebinding would become more pronounced if the density of glycoprotein ligands for L-selectin at the microvillar site of contact is low, such that the probability of L-selectin rebinding to a neighboring ligand molecule on the adhesive surface is low (Fig. 9). Indeed, at GlyCAM-1 densities $\leq 100 \text{ sites}/\mu\text{m}^2$, the contact area encountered by a single tip of a microvillus contains, on average, only one GlyCAM-1 molecule (Bruehl et al., 1996). In contrast, when a highly clustered ligand like fucoidin or sLe^x-decorated lipid serves as the leukocyte tethering ligands, this contact contains 10–100 carbohydrate sites (Alon et al., 1995b). The adhesive defects of the tail mutants could be therefore partially or fully rescued on these ligands through rebinding to a neighboring ligand molecule within the microvillar contact.

Restriction of L-selectin's lateral mobility within its contact zone may be required but insufficient for rapid bond stabilization. Anchorage of surface proteins to the cytoskeleton has also been suggested as means to protect these molecules from being uprooted from the membrane by receptors on counter surfaces (Evans et al., 1991; Shao and Hochmuth, 1999). However, uprooting of L-selectin from the plasma membrane of neutrophils takes 0.5–1 s (Shao and Hochmuth, 1999), much more slowly than L-selectin occupancy by ligand at reversible adhesive contacts. Even after disruption of cytoskeletal associations of membrane glycoproteins, uprooting of unanchored glycoproteins from the plasma membrane takes 0.3–0.5 s (Shao and Hochmuth, 1999), two orders of magnitude longer than the duration of

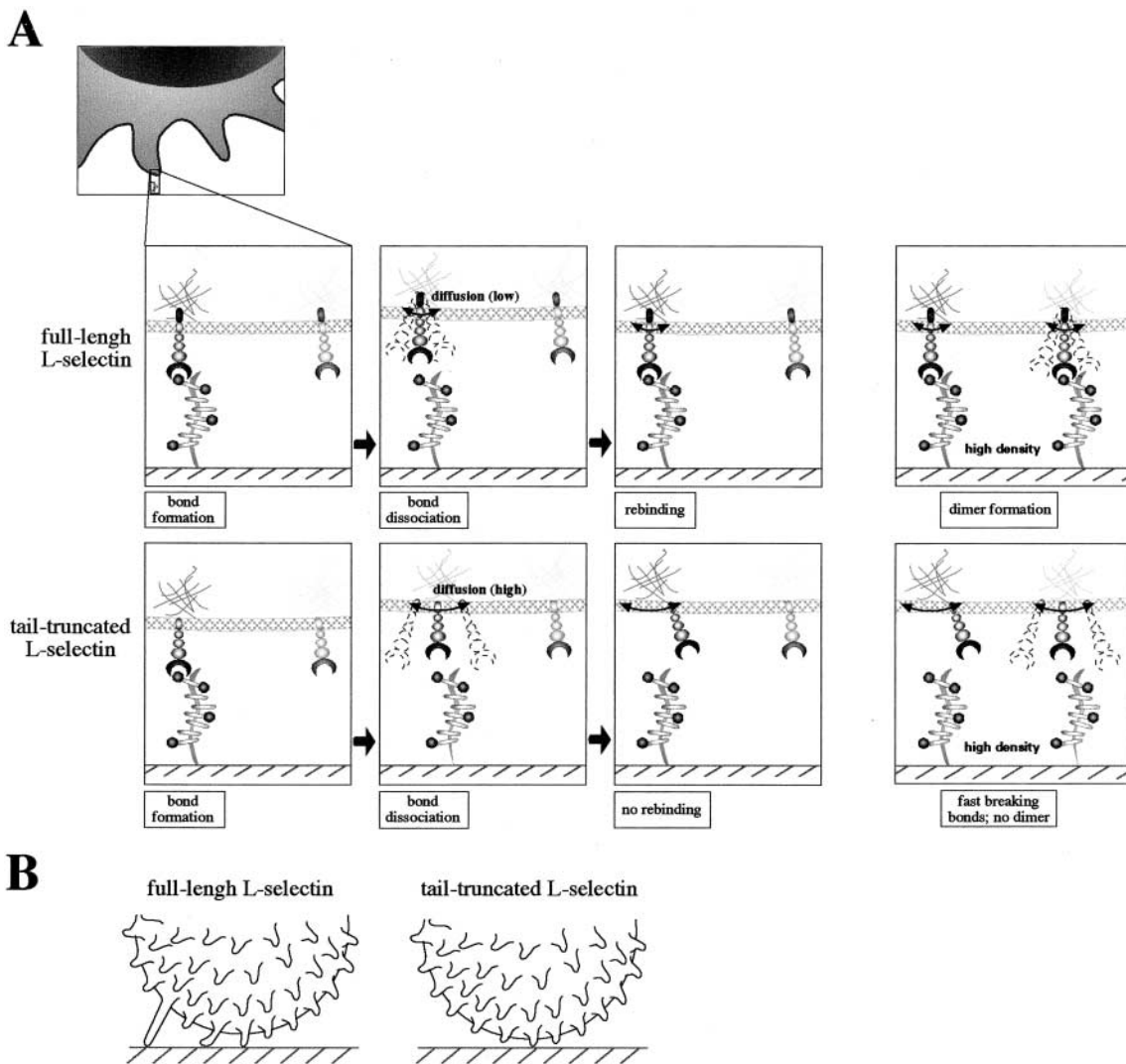


Figure 9. Scheme showing postulated mechanisms for the role of L-selectin anchorage in tether stabilization. (A) A postulated sequence of bond formation, dissociation and rebinding events potentially mediated by L-selectin or tail-truncated mutant at a single microvillus contact site bearing GlyCAM-1 under shear flow ≥ 1 dyn/cm². At 100 sites/ μ m², GlyCAM-1 supports rolling through a single GlyCAM-1 molecule at each microvillar contact site. The bond between L-selectin and GlyCAM-1 forms and dissociates within a millisecond irrespective of the selectin tail (left panels). Once dissociated from the ligand, full-length L-selectin or tail-truncated mutant is either restricted or mobile within the microvillus membrane, respectively (center panels). Reduced mobility of L-selectin limits its escape from the original ligand and increases the probability of its rebinding to the same GlyCAM-1 molecule (upper row, third panel). No rebinding takes place for the highly mobile mutant (lower row, third panel). Successful ligand rebinding by anchored L-selectin retards the leukocyte sufficiently to allow subsequent stabilization steps. The neighboring L-selectin (shown as a faded image) is too distant from the GlyCAM-1 site to participate in this interaction, unless GlyCAM-1 density is higher (right panel). For simplicity, only a single microvillus contact site is depicted. (B) Engagement of multiple microvillar sites facilitate cell capture and rolling.

tethers mediated by unanchored L358stop mutant (Fig. 6). Bond stabilization through the L-selectin tail could also conceivably involve enhanced L-selectin clustering in microvilli. However, constitutive L-selectin clustering is apparently unaffected by tail truncation (Pavalko et al., 1995). Moreover, artificially induced dimerization of L-selectin ectodomains could not rescue the adhesive defects of either the Δ cyto or L358stop mutant on any ligand system tested (Fig. 3 B and unpublished data). Thus, the adhesive defect caused by tail truncation is unlikely to be the result of impaired dimerization of the Δ cyto or L358stop mutants on the leukocyte surface. Furthermore, whereas the adhesive differences between full-length L-selectin and the Δ cyto or L358stop

mutants increased as the density of GlyCAM-1 decreased, the contribution of L-selectin dimerization to L-selectin adhesiveness in fact diminished as ligand density was reduced (Dwir et al., in preparation). Even when ligand was present at density below that sensitive to L-selectin dimerization, tail-dependent association of L-selectin still augmented selectin adhesiveness. Therefore, tether stabilization through the L-selectin tail appears to primarily depend on intrinsic stabilization of singular bonds, even though at high ligand densities, individual microvillar contacts may stabilize through more than one bond (Chen and Springer, 1999) (Fig. 9 A, right panel). Rapid stabilization of the first microvillus contact would favor subsequent formation of mul-

multiple microvilli tethers at the cell-substrate contact zone (Fig. 9 B). Upon temporary capture, leukocytes are flattened by downward forces that increase their contact area to several μm^2 within a few milliseconds (Evans and Leung, 1989; Evans et al., 2001), which potentially contain several microvillar sites (Chen and Springer, 1999). Multimicrovillar contacts would be important for dispersing the force applied on each selectin bond, slow down its dissociation, and further prolong the lifetime of the tether. Such avidity enhancement may underlie the shear-dependent increase in the number of L-selectin bonds formed at adhesive contact zones during leukocyte capture and rolling (Finger et al., 1996; Lawrence et al., 1997; Chen and Springer, 1999).

L-selectin is the only selectin constitutively associated with the actin cytoskeleton (Pavalko et al., 1995). P-selectin regulates its adhesiveness through constitutive clustering on the cell surface via an association with α -adaptin, a component of clathrin-coated pits (Setiadi et al., 1998; Ramachandran et al., 2001). Interestingly, interference with the P-selectin α -adaptin association does not abolish the selectin's ability to support leukocyte capture and subsequent rolling, although it impairs the mechanical strength of P-selectin tethers (Setiadi et al., 1998; Ramachandran et al., 2001). It is striking that although L-selectin is the shortest selectin, it exhibits the highest efficiency of cell capture at elevated shear stresses among the three selectins (Arbones et al., 1994; Bosse and Vestweber, 1994; Alon et al., 1997) and the highest shear tolerance (Alon et al., 1997; Ramachandran et al., 1999). Thus, the ligand-rebinding mechanism postulated here may provide specialized means for cytoskeletally anchored L-selectin to mediate cell capture over a wider range of shear conditions than P- or E-selectin. The residual cytoplasmic associations of the Δcyto mutant gave rise to intermediate tether stabilization levels between those of the L358stop mutant and those of full-length L-selectin. Although the RRLKKG segment retained by the Δcyto mutant was insufficient for L-selectin association with α -actinin under in vitro conditions of detergent cell lysis (Pavalko et al., 1995), this segment appears to mediate considerable anchorage to the cytoskeleton based on the much higher tether stabilization generated by this mutant relative to the L358stop mutant lacking the LKKG segment. These four COOH-terminal cytoplasmic residues may confer residual cytoskeletal anchorage through binding of the regulatory protein calmodulin, also implicated in L-selectin shedding (Kahn et al., 1998). However, PMA-induced or spontaneous shedding of L-selectin were fully conserved upon L-selectin tail truncation (unpublished data), suggesting that differences in L-selectin shedding were unlikely to account for the adhesive differences among L-selectin and its tail mutants. Indeed, inhibition of L-selectin shedding did not rescue the adhesive defects of the tail-truncated mutants (unpublished data).

In conclusion, our study supports the hypothesis that extremely rapid bond stabilization at microvillus-endothelium contact sites is facilitated by cytoskeletal association that anchors L-selectin within the ligand contact site. Our results suggest for the first time that the low force sensitivity of L-selectin tethers may be regulated by a post ligand diffusion-controlled stabilization mechanism in addition to in-

trinsic kinetic and mechanical properties of the selectin-carbohydrate bond. The notion that cytoskeletal anchorage of L-selectin can dramatically prolong tether lifetime introduces a new regulatory mechanism for L-selectin adhesiveness under shear flow. This anchorage may vary considerably among different cell types as well as in different states of leukocyte activation (Spertini et al., 1991; Roberts et al., 1999). Hyperthermic conditions have been shown to enhance cytoplasmic tail-dependent association of L-selectin with the actin cytoskeleton and to increase L-selectin-dependent lymphocyte binding to HEV (Evans et al., 1999). Further insights into how cytoskeletal L-selectin anchorage is regulated in different leukocytes under homeostatic and inflammatory conditions will allow better understanding of its indispensable functions in immune cell recruitment to lymphoid and extralymphoid organs.

Materials and methods

Antibodies and reagents

The anti-L-selectin mAbs, LAM1-101, and LAM1-118, directed against the SCR domain of L-selectin (Steeber et al., 1997; Li et al., 1998) were a gift of Dr. T.F. Tedder (Duke University, Durham, NC). The anti-L-selectin mAb, DREG-200 (Kishimoto et al., 1990), was provided by Dr. T.K. Kishimoto (Boehringer). All mAbs were used as purified Ig. Rabbit anti-murine IgG Fc and FITC-conjugated goat anti-mouse IgG were obtained from Zymed Laboratories, Inc. GlyCAM-1, purified from mouse serum by immunoaffinity chromatography (Lasky et al., 1992), was a gift from Dr. S.D. Rosen (University of California at San Francisco, San Francisco, CA). PNA^d, purified from human tonsil lysates by MECA-79 mAb affinity chromatography (Berg et al., 1991), was a gift from Dr. E.L. Berg (Protein Design Labs). The glycoprotein mixture was stored in 1% octyl glucoside/PBS solution at 4°C. The chemically synthesized neoglycolipid 3' sLe^x (glc)-phosphatidylethanolamine dipalmitoyl (C16:0) was a gift from Dr. L.L. Kiessling (University of Wisconsin, Madison, WI). Fucoidin, a plant-derived sulfated polyfuran that saturably blocks the lectin domain of L-selectin (Rosen and Bertozzi, 1994), BSA (fraction V), protein A, Ca²⁺- and Mg²⁺-free HBSS, Ficoll-Hypaque 1077, cytochalasin B (CB), *n*-octyl- β -glucopyranoside (octyl glucoside), and poly-L-lysine were all obtained from Sigma-Aldrich. HSA (Fraction V) was obtained from Calbiochem.

Cells

The stable expression of full-length L-selectin or Δcyto in the mouse pre-B-cell line 300.19 was described elsewhere (Kansas et al., 1993). L358stop (Fig. 1) was stably expressed in 300.19 cells at comparable levels to the other two transfectants. Clones expressing identical levels of native and mutated L-selectin were maintained as described by Dwir et al. (2000). Peripheral blood granulocytes were isolated from anticoagulated blood after dextran sedimentation and density separation over Ficoll-Hypaque as described by Alon et al. (1997).

Laminar flow assays

Preparation of ligand-coated substrates has been described previously (Dwir et al., 2000). GlyCAM-1 site densities were assessed using ¹²⁵I-labeled CAM02 (Dwir et al., 2000). Cell-free L-selectin and L-selectin tail mutants were derived from lysates of the various transfected 300.19 cells, as described (Dwir et al., 2000). Polystyrene plates were overlaid with the various selectin variants using a specific capture approach designed to immobilize the selectin variants in a functional state by the use of SCR-specific mAbs. Specificity of the adsorbed L-selectin variants was confirmed by complete blockage of neutrophil adhesion in the presence of the L-selectin ligand, fucoidin.

Transfected cells ($2 \times 10^6/\text{ml}$) were perfused at room temperature in binding medium (HBSS/10 mM Hepes, pH 7.4, supplemented with 1 mg/ml BSA and 2 mM CaCl₂) through the flow chamber at desired flow rates as described (Dwir et al., 2000). Cellular interactions were visualized at two fields of view (each one 0.17 mm² in area) using a 10 \times objective of an inverted phase contrast microscope (Diaphot 300; Nikon Inc.). Cells were videotaped at 0.02-s resolution with an LIS-700 CCD camera (Applitech) and a time-lapse SVHS Video recorder (AG-6730; Panasonic). Tethering events were defined as adhesive interactions of those freely flowing cells

moving closest to the lower wall of the flow chamber coated with the test substrate. Two types of initial cell tethers to the substrate were determined: transient tethers, in which cells attached briefly to the substrate, and rolling tethers, in which tethered cells remained rolling on the substrate, i.e., moving at a mean velocity < 0.25 the hydrodynamic velocity for at least 3 s after initial tethering. Transient tethers to low density ligands was determined as described (Alon et al., 1997).

For inhibition studies, cells were perfused in Ca^{2+} -free binding medium supplemented with EGTA, or preincubated in binding medium for 5 min at 4°C with $10 \mu\text{g/ml}$ of either the L-selectin blocking mAb, DREG-200, or with $50 \mu\text{g/ml}$ of fucoidin. Dimerization of surface L-selectin on the various transfectants was induced by incubating L-selectin-expressing cells ($2 \times 10^6/\text{ml}$) with the L-selectin dimerizing mAb LAM1-118 (Li et al., 1998), or the control L-selectin SCR-specific mAb, LAM1-101, in binding medium at 25°C for 15 min. To determine the adhesiveness of the various transfectants towards L-selectin ligands expressed on neutrophils, fresh human neutrophils ($10^6/\text{ml}$) were perfused over either scattered substrate-bound transfectant cells or monolayers of closely spaced transfectants immobilized on poly-L-lysine (at 100 ng/ml).

Computerized microkinetic analysis

The imaging system developed for quantitative analysis of instantaneous velocities of cell movement, WSCAN-Array-3 (Galai), was described elsewhere (Dwir et al., 2000). Individual transfectants rolling on GlyCAM-1 were tracked for 3 s at 0.02 s resolution and their mean displacement velocities were derived. Two categories of rolling cells, jerky and smooth rolling, were identified based on their mean displacement velocities. Microkinetics of individual cells exhibiting jerky rolling on low density ligand was analyzed at high temporal resolution on video segments of rolling cells recorded with a high-speed camera (Motion Corder Analyzer, FAST-CAM-SUPER 500; Eastman Kodak). Cell movement was recorded at a rate of 500 frames/s and digital movies were recorded at a rate of 50 frames/s on SVHS Video recorder. Analogue movies (Fig. 5) were used as a template for the WSCAN-Array-3 software. Motion analysis compared the forward displacement of a moving cell at 0.02 s intervals, corresponding to 0.002 s intervals between successive frames of the original digital movie. Pauses shorter than 0.006 s could not be eliminated by blocking L-selectin. The majority of these pauses were 2 ms long. As they were observed also in freely flowing cells, they were considered as imaging noise. At a shear stress of 1.75 dyn/cm^2 , specific pauses were defined as cell displacements of $< 0.9 \mu\text{m}$ during a period of at least 0.006 s . At $0.75\text{--}1$ or $0.4\text{--}0.6 \text{ dyn/cm}^2$, pauses were defined as displacements of $< 0.6 \mu\text{m}$ or of $< 0.4 \mu\text{m}$, during this period, respectively. The natural log of the number of pauses with a given duration after pause initiation was plotted against pause duration. First-order dissociation plot yielded a straight line with the slope equal to $-k_{\text{off}}$. The dependence of k_{off} on applied force was assumed to follow the Bell equation (1978): $k_{\text{off}} = k_{\text{off}}^0 \exp(\sigma F_b/k_b T)$, where k_{off}^0 is the dissociation rate constant in the absence of applied force, σ is the reactive compliance of the tether, F_b is the force on the bond, k_b is the Boltzmann's constant and T is the absolute temperature. The force on the bond was calculated to be $180 \text{ pN/1 dyn/cm}^2$ wall shear stress using a diameter of $12 \mu\text{m}$ and assigning a bond angle of 50° . The Bell equation was fit to the k_{off} vs. F_b data using the exponential curve-fitting function in EXCEL.

Online supplemental material

Video 1 shows a playback of motions of WT and tail-truncated L-selectin transfectants with substrate bound GlyCAM-1 under physiological shear flow, recorded with a high speed camera at 500 frames/s. The actual time elapsed from the beginning of each recording is indicated at the top of each scene.

The authors thank Drs. S.D. Rosen, T.F. Tedder, E.L. Berg, T.K. Kishimoto and L.L. Kiessling for gifts of reagents. We wish to thank Drs. U. Schwarz, R. Posner, O. Gutman, and Y. Henis for insightful discussions of the manuscript, and Dr. S. Schwarzbaum for editorial assistance. R. Alon is the Incumbent of The Tauro Career Development Chair in Biomedical Research. G.S. Kansas is an Established Investigator of the American Heart Association.

Parts of this work were supported by the United States Israel Binational Science Foundation (to R. Alon and G.S. Kansas), by the Israel Science Foundation founded by the Israel Academy of Sciences and Humanities (to R. Alon), and by National Institutes of Health grants HL55647 (to G.S. Kansas).

Submitted: 12 March 2001

Revised: 21 August 2001

Accepted: 27 August 2001

References

- Alon, R., T. Feizi, C.T. Yuen, R.C. Fuhlbrigge, and T.A. Springer. 1995a. Glycolipid ligands for selectins support leukocyte tethering and rolling under physiologic flow conditions. *J. Immunol.* 154:5356–5366.
- Alon, R., D.A. Hammer, and T.A. Springer. 1995b. Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. *Nature.* 374:539–542.
- Alon, R., S. Chen, K.D. Puri, E.B. Finger, and T.A. Springer. 1997. The kinetics of L-selectin tethers and the mechanics of selectin-mediated rolling. *J. Cell Biol.* 138:1169–1180.
- Alon, R., S. Chen, R. Fuhlbrigge, K.D. Puri, and T.A. Springer. 1998. The kinetics and shear threshold of transient and rolling interactions of L-selectin with its ligand on leukocytes. *Proc. Natl. Acad. Sci. USA.* 95:11631–11636.
- Angres, B., A. Barth, and W.J. Nelson. 1996. Mechanism for transition from initial to stable cell–cell adhesion: kinetic analysis of E-cadherin-mediated adhesion using a quantitative adhesion assay. *J. Cell Biol.* 134:549–557.
- Arbones, M.L., D.C. Ord, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D.J. Capon, and T.F. Tedder. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity.* 1:247–260.
- Berg, E.L., M.K. Robinson, R.A. Warnock, and E.C. Butcher. 1991. The human peripheral lymph node vascular addressin is a ligand for LECAM-1, the peripheral lymph node homing receptor. *J. Cell Biol.* 114:343–349.
- Bosse, R., and D. Vestweber. 1994. Only simultaneous blocking of the L- and P-selectin completely inhibits neutrophil migration into mouse peritoneum. *Eur. J. Immunol.* 24:3019–3024.
- Bruehl, R.E., T.A. Springer, and D.F. Bainton. 1996. Quantitation of L-selectin distribution on human leukocyte microvilli by immunogold labeling and electron microscopy. *J. Histochem. Cytochem.* 44:835–844.
- Chan, P.Y., M.B. Lawrence, M.L. Dustin, L.M. Ferguson, D.E. Golan, and T.A. Springer. 1991. Influence of receptor lateral mobility on adhesion strengthening between membranes containing LFA-3 and CD2. *J. Cell Biol.* 115:245–255.
- Chen, S., R. Alon, R.C. Fuhlbrigge, and T.A. Springer. 1997. Rolling and transient tethering of leukocytes on antibodies reveal specializations of selectins. *Proc. Natl. Acad. Sci. USA.* 94:3172–3177.
- Chen, S., and T.A. Springer. 1999. An automatic braking system that stabilizes leukocyte rolling by an increase in selectin bond number with shear. *J. Cell Biol.* 144:185–200.
- Dwir, O., G.S. Kansas, and R. Alon. 2000. An activated L-selectin mutant with conserved equilibrium binding properties but enhanced ligand recognition under shear flow. *J. Biol. Chem.* 275:18682–18691.
- Evans, E., and A. Leung. 1989. Apparent viscosity and cortical tension of blood granulocytes determined by micropipet aspiration. *Biophys. J.* 56:151–160.
- Evans, E., D. Berk, and A. Leung. 1991. Detachment of agglutinin-bonded red blood cells. I. Forces to rupture molecular-point attachments. *Biophys. J.* 59:838–848.
- Evans, E., A. Leung, D. Hammer, and S. Simon. 2001. Chemically-distinct transition states govern rapid dissociation of single L-selectin bonds under force. *Proc. Natl. Acad. Sci. USA.* 98:3784–3789.
- Evans, S.S., D.M. Schleider, L.A. Bowman, M.L. Francis, G.S. Kansas, and J.D. Black. 1999. Dynamic association of L-selectin with the lymphocyte cytoskeletal matrix. *J. Immunol.* 162:3615–3624.
- Finger, E.B., K.D. Puri, R. Alon, M.B. Lawrence, U.H. von Andrian, and T.A. Springer. 1996. Adhesion through L-selectin requires a threshold hydrodynamic shear. *Nature.* 379:266–269.
- Galustian, C., A.M. Lawson, S. Komba, H. Ishida, M. Kiso, and T. Feizi. 1997. Sialyl-Lewis(x) sequence 6-O-sulfated at N-acetylglucosamine rather than at galactose is the preferred ligand for L-selection and de-N-acetylation of the sialic acid enhances the binding strength. *Biochem. Biophys. Res. Commun.* 240:748–751.
- Goldstein, B., and C. Wofsy. 1996. Why is it so hard to dissociate multivalent antigens from cell-surface antibodies? *Immunol. Today.* 17:77–80.
- Greenberg, A.W., D.K. Brunk, and D.A. Hammer. 2000. Cell-free rolling mediated by L-selectin and sLe(x) reveals the shear threshold effect. *Biophys. J.* 79:2391–2402.
- Kahn, J., B. Walcheck, G.I. Migaki, M.A. Jutila, and T.K. Kishimoto. 1998. Calmodulin regulates L-selectin adhesion molecule expression and function through a protease-dependent mechanism. *Cell.* 92:809–818.
- Kansas, G.S., K. Ley, J.M. Munro, and T.F. Tedder. 1993. Regulation of leukocyte rolling and adhesion to high endothelial venules through the cytoplasmic domain of L-selectin. *J. Exp. Med.* 177:833–838.

- Kansas, G.S. 1996. Selectins and their ligands: current concepts and controversies. *Blood*. 88:3259–3287.
- Kishimoto, T.K., M.A. Jutila, and E.C. Butcher. 1990. Identification of a human peripheral lymph node homing receptor: a rapidly down-regulated adhesion molecule. *Proc. Natl. Acad. Sci. USA*. 87:2244–2248.
- Kucik, D.F., M.L. Dustin, J.M. Miller, and E.J. Brown. 1996. Adhesion-activating phorbol ester increases the mobility of leukocyte integrin LFA-1 in cultured lymphocytes. *J. Clin. Invest.* 97:2139–2144.
- Lasky, L.A., M.S. Singer, D. Dowbenko, Y. Imai, W.J. Henzel, C. Grimley, C. Fennie, N. Gillett, S.R. Watson, and S.D. Rosen. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell*. 69:927–938.
- Lawrence, M.B., G.S. Kansas, E.J. Kunkel, and K. Ley. 1997. Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E). *J. Cell Biol.* 136:717–727.
- Li, X., D.A. Steeber, M.L.K. Tang, M.A. Farrar, R.M. Perlmutter, and T.F. Tedder. 1998. Regulation of L-selectin-mediated rolling through receptor dimerization. *J. Exp. Med.* 188:1385–1390.
- Mulloy, B., A.C. Ribeiro, A.P. Alves, R.P. Vieira, and P.A. Mourao. 1994. Sulfated fucans from echinoderms have a regular tetrasaccharide repeating unit defined by specific patterns of sulfation at the 0-2 and 0-4 positions. *J. Biol. Chem.* 269:22113–22123.
- Patel, K.D., M.U. Nollert, and R.P. McEver. 1995. P-selectin must extend a sufficient length from the plasma membrane to mediate rolling of neutrophils. *J. Cell Biol.* 131:1893–1902.
- Pavalko, F.M., D.M. Walker, L. Graham, M. Goheen, C.M. Doerschuk, and G.S. Kansas. 1995. The cytoplasmic domain of L-selectin interacts with cytoskeletal proteins via α -actinin: receptor positioning in microvilli does not require interaction with α -actinin. *J. Cell Biol.* 129:1155–1164.
- Ramachandran, V., M.U. Nollert, H. Qiu, W.J. Liu, R.D. Cummings, C. Zhu, and R.P. McEver. 1999. Tyrosine replacement in P-selectin glycoprotein ligand-1 affects distinct kinetic and mechanical properties of bonds with P- and L-selectin. *Proc. Natl. Acad. Sci. USA*. 96:13771–13776.
- Ramachandran, V., T. Yago, T.K. Epperson, M.M.A. Kobzdej, M.U. Nollert, R.D. Cummings, C. Zhu, and R.P. McEver. 2001. *Proc. Natl. Acad. Sci. USA*. 98:10166–10171.
- Roberts, A.W., C. Kim, L. Zhen, J.B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J.D. Pollock, J.B. Borneo, G.B. Bradford, S.J. Atkinson, M.C. Dinauer, and D.A. Williams. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity*. 10:183–196.
- Rosen, S.D., and C.R. Bertozzi. 1994. The selectins and their ligands. *Curr. Opin. Cell Biol.* 6:663–673.
- Setiadi, H., G. Sedgewick, S.L. Erlandsen, and R.P. McEver. 1998. Interactions of the cytoplasmic domain of P-selectin with clathrin-coated pits enhance leukocyte adhesion under flow. *J. Cell Biol.* 142:859–871.
- Shao, J.Y., and R.M. Hochmuth. 1999. Mechanical anchoring strength of L-selectin, β 2 integrins, and CD45 to neutrophil cytoskeleton and membrane. *Biophys. J.* 77:587–596.
- Smith, M.J., E.L. Berg, and M.B. Lawrence. 1999. A direct comparison of selectin-mediated transient, adhesive events using high temporal resolution. *Biophys. J.* 77:3371–3383.
- Spertini, O., G.S. Kansas, J.M. Munro, J.D. Griffin, and T.F. Tedder. 1991. Regulation of leukocyte migration by activation of the leukocyte adhesion molecule-1 (LAM-1) selectin. *Nature*. 349:691–694.
- Springer, T.A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: The multistep paradigm. *Cell*. 76:301–314.
- Steeber, D.A., P. Engel, A.S. Miller, M.P. Sheetz, and T.F. Tedder. 1997. Ligation of L-selectin through conserved regions within the lectin domain activates signal transduction pathways and integrin function in human, mouse, and rat leukocytes. *J. Immunol.* 159:952–963.
- von Andrian, U.H., S.R. Hasslen, R.D. Nelson, S.L. Erlandsen, and E.C. Butcher. 1995. A central role for microvillous receptor presentation in leukocyte adhesion under flow. *Cell*. 82:989–999.
- 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 recruitment, independent of ligand binding. *J. Exp. Med.* 186:1–9.