Distinct kinetic and mechanical properties govern selectin-leukocyte interactions

William D. Hanley[1], Denis Wirtz[1,2,*] and Konstantinos Konstantopoulos[1,*]

[1]Department of Chemical and Biomolecular Engineering, The Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218, USA
[2]Department of Materials Science and Engineering, The Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218, USA
*Authors for correspondence (e-mails: kkonsta1@jhu.edu; wirtz@jhu.edu)

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Summary

Leukocytes are recruited from the bloodstream to sites of inflammation by the selectin family of adhesion receptors. In vivo and in vitro studies reveal distinctive rolling velocities of polymorphonuclear leukocytes over E-, P- and L-selectin substrates. The kinetic and mechanical properties of the selectin-ligand bonds responsible for these differences at the single-molecule level are not well understood. Using single-molecule force spectroscopy, we probe in situ the rupture force, unstressed off-rate and reactive compliance of single selectin receptors to single ligands on whole human polymorphonuclear leukocytes (PMNs) under conditions that preserve the proper orientation and post-translational modifications of the selectin ligands. Single L-selectin bonds to PMNs were more labile than either E- or P-selectin in the presence of an applied force. This outcome, along with a higher unstressed off-rate and a higher reactive compliance, explain the faster L-selectin-mediated rolling. By quantifying binding frequency in the presence of a specific blocking monoclonal antibody or following enzyme treatment, we determined that P-selectin glycoprotein ligand-1 is a high-affinity ligand for E-selectin on PMNs under force. The rupture force spectra and corresponding unstressed off-rate and reactive compliance of selectin-ligand bonds provide mechanistic insights that might help to explain the variable rolling of leukocytes over different selectin substrates.

Supplemental figure available online

Key words: Force spectroscopy, Leukocyte, Selectin, PSGL-1, Off-rate

Introduction

Central to all cell-cell communications are the adhesive interactions between biological receptors and their respective ligands. The kinetics regulating receptor-ligand binding impart unique properties that allow specialized cells to interact with one other amidst the challenge of physiological and competitive stresses. This phenomenon is perhaps best represented by the remarkable ability of the selectins (E-, P- and L-selectin) to mediate the initial adhesion events during leukocyte recruitment to sites of inflammation. The distinctiveness of selectin binding is attributed to their fast association/dissociation rates, as well as their ability to form high-strength tethers under rapid loading. These properties allow polymorphonuclear leukocytes (PMNs) first to tether and roll on activated vascular endothelium under hydrodynamic shear, before firm adhesion and extravasation into the tissue space (Konstantopoulos et al., 1998; McEver, 2002; Springer, 1995). In vivo (Jung et al., 1996; Kunkel and Ley, 1996) and in vitro (Alon et al., 1997; Alon et al., 1998; Puri et al., 1997) studies both reveal that E-, P- and L-selectin-mediated interactions display characteristic leukocyte rolling velocities. Structurally similar, the selectins consist of an N-terminal, calcium-dependent lectin domain, an epidermal-growth-factor-like (EGF) domain, a variable number of consensus repeat units, a transmembrane domain (TM) and an intracellular cytoplasmic tail (cyto) (Fig. 1A) (Konstantopoulos et al., 1998; McEver et al., 1995). They also share a relatively high degree of homology within each region (McEver et al., 1995). The primary module for adhesion is the lectin domain but other features (including the EGF region and the number of consensus repeat units) might confer unique biophysical and mechanical properties on each selectin when binding to its ligand (McEver et al., 1995). Additionally, selectin ligands recognize a stereochemically precise arrangement of sialic acid and fucose, termed sialyl Lewis^{x} (McEver, 2002; Varki, 1997). Although they share many common features, the selectins possess differences in their expression kinetics, tissue distributions and ability to promote leukocyte attachment, perhaps reflecting their unique roles in diverse immunological processes. E-selectin, expressed on the vascular endothelium, is transcriptionally regulated and induced in response to inflammatory stimuli (Konstantopoulos et al., 1998). P-selectin expression on both the vascular endothelium and on platelets is also inducible, but its storage in the Weibel-Palade bodies of endothelial cells and the α-granules of platelets permits rapid mobilization (within seconds to minutes) to the plasma membrane upon cell activation (Konstantopoulos et al., 1998). L-selectin, by contrast, is constitutively expressed on most leukocyte subpopulations and is responsible for amplifying the inflammatory response through leukocyte-leukocyte interactions or so-called secondary tethers (Alon et al., 1996; Konstantopoulos et al., 1998).

P-selectin glycoprotein ligand 1 (PSGL-1) is the best-characterized ligand capable of binding to all three selectins.

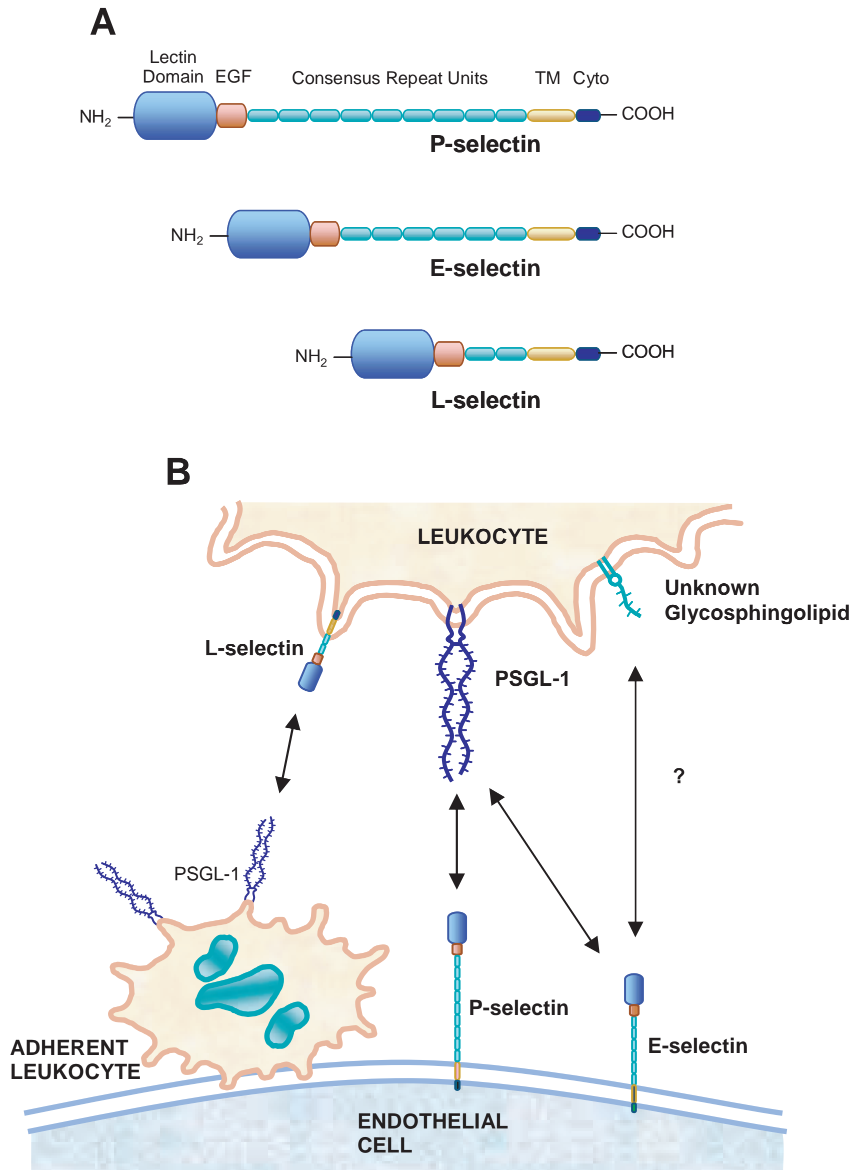

Fig. 1. (A) Structural diagrams representing P-, E- and L-selectin. The selectins are rigid, asymmetric molecules that share structural similarities including the presence of the C-type lectin domain, followed by an epidermal-growth-factor-like (EGF) motif, a variable series of short consensus repeats (nine, six and two for P-, E- and L-selectins, respectively), a transmembrane domain (TM) and a cytoplasmic tail (cyto). There is also a high degree of homology between the three proteins within each region. (B) Selectin-mediated leukocyte recruitment. PSGL-1 on free-flowing leukocytes tethers to E- and/or P-selectin on activated endothelial cells, allowing leukocytes to roll to sites of infection or inflammation. E-Selectin might also bind to an as-yet-unidentified leukocyte glycosphingolipid. PSGL-1 on adherent leukocytes participates in secondary tethering by interacting with L-selectin on free-flowing leukocytes.

Concentrated on leukocyte microvilli, PSGL-1 is a mucin-like homodimeric glycoprotein that is crucial for immune functioning. PSGL-1 serves as the primary counter-receptor for P- and L-selectin, in that it selectively binds with relatively high affinity and removal or absence of PSGL-1 essentially abrogates P- or L-selectin-mediated rolling (Varki, 1997). E-selectin also binds effectively to PSGL-1 (Goetz et al., 1997; Li et al., 1996), but accumulating evidence supports the concept that glycosphingolipids might also be physiologically relevant E-selectin ligands (Alon et al., 1995a; Bochner et al., 1994; Burdick et al., 2001; Kobzdej et al., 2002). Dynamic rolling assays conducted in the presence of a blocking anti-PSGL-1 mAb (KPL-1) indicated that PSGL-1-coated microspheres are still capable of tethering on E- but not P-selectin substrates (Goetz et al., 1997), supporting the notion that PSGL-1 has binding sites for E-selectin other than the crucial 19 amino acid sequence recognized by KPL-1. Along these lines, E-selectin appears to be capable of binding to *N*-linked oligosaccharides, including those found on PSGL-1 (Aeed et al., 2001; Patel et al., 1994; Vestweber, 1996). Taken altogether, E-selectin mediated tethering and rolling appears to involve additional as-yet-unidentified endogenous ligands distinct from PSGL-1.

Single-molecule force spectroscopy has previously been used to characterize P-selectin (Hanley et al., 2003; Marshall et al., 2003) and L-selectin (Evans et al., 2001; Sarangapani et al., 2003) binding interactions to human PMNs or purified PSGL-1 molecules. Using a modified version of the in situ force spectroscopy technique originally pioneered by Benoit et al. (Benoit et al., 2000), we now characterize E-selectin/PMN binding to assess the hypothesis that PSGL-1 serves as a high-affinity counter-receptor for E-selectin. Additionally, we present all three selectin-PMN force spectra simultaneously over a physiological range of loading rates and compare the corresponding biophysical parameters that describe the mechanics of selectin binding. Previous values for the biophysical parameters of selectin-PMN interactions have been obtained in vitro by quantifying leukocyte tethering lifetime in dynamic flow chamber experiments (Alon et al., 1998; Alon et al., 1997; Alon et al., 1995b; Smith et al., 1999). The relative trends for both the unstressed off-rate and the reactive compliance, however, show marked differences in these previous investigations. In addition, these techniques might not effectively differentiate avidity from the adhesion of a single receptor-ligand pair and, most importantly, rely on broad assumptions to estimate the forces on receptor-ligand bonds. We therefore sought to examine selectin-PMN binding cumulatively at the single molecule level to provide a mechanistic basis for the differential abilities of PMNs to interact with selectin substrates. We propose explanations based on the tensile strength of single selectin-ligand bonds, the intrinsic kinetics of bond dissociation (k^0_{off}) and the reactive compliance (x_β), which is a measure of the susceptibility for bond rupture under applied force (Alon et al., 1997). Results obtained from single-molecule measurements help to ensure that conclusions are derived from the binding of single receptors binding to single ligands and that

any avidity contribution (e.g. receptor-ligand redistribution or clustering) is minimized. Moreover, the use of whole cells rather than purified proteins ensures the proper orientation and preserves any post-translational modifications of the selectin ligands.

Materials and Methods

Reagents

The chimeric forms of E-selectin/IgG-Fc (E-selectin) and L-selectin/IgG-Fc (L-selectin), consisting of the lectin, EGF and consensus repeat domains for human E- and L-selectin, respectively, linked to each arm of human IgG_1, were generous gifts from R. Camphausen (Wyeth External Research, Cambridge, MA) (Somers et al., 2000). Unless otherwise stated, all other reagents were purchased from Sigma (St Louis, MO).

PMN isolation, monolayer formation and enzyme treatment

Human PMNs were isolated from citrate phosphate dextrose anticoagulated venous blood of healthy volunteers as previously described (Hanley et al., 2003; Jadhav et al., 2001), resuspended at 5×10^6 cells ml^{-1} in Ca^{2+}- and Mg^{2+}-free D-PBS [0.1% bovine serum albumin (BSA)] and stored at 4°C for no more than 3 hours before use in all experiments. To immobilize PMNs, 200 µl of the isolated PMN cell suspension was allowed to incubate on a 35-mm tissue culture dish for 5 minutes at room temperature (RT) (Hanley et al., 2003). To prevent further activation, a 1% formalin solution in D-PBS was added to the cell culture dish and maintained at RT for 10 minutes (Hanley et al., 2003). The PMN monolayer was rinsed and refilled with D-PBS containing Ca^{2+} and Mg^{2+}. This procedure resulted in a PMN monolayer of about 40% confluence. In select experiments, PMN cell suspensions at 5×10^6 cells ml^{-1} were incubated with 120 µg ml^{-1} of *O*-sialoglycoprotein endopeptidase (OSGE; Accurate Chemical & Scientific, Westbury, NY) specifically to cleave proteins that are *O*-glycosylated on serine and threonine residues (Sutherland et al., 1992). OSGE-treated cell monolayers were prepared as described above.

Cantilever functionalization

To provide a surface that readily binds soluble proteins, molecular force probe (MFP) cantilevers (TM Microscopes, Sunnyvale, CA) were silanized with 2% 3-aminopropyltriethoxysilane (Hanley et al., 2003). The cantilevers were then incubated in a 30 µg ml^{-1} solution of anti-human-IgG-Fc mAb in D-PBS containing 50-fold molar excess of the crosslinker *bis*(sulfosuccinimidyl) suberate (BS^3; Pierce, Rockford, IL) for 30 minutes followed by quenching with Tris buffer. Cantilevers were subsequently incubated with 1.25 µg ml^{-1} L-selectin/IgG-Fc or 0.90 µg ml^{-1} E-selectin/IgG-Fc chimera protein in D-PBS for 2 hours at RT, followed by immersion in 1% BSA to block non-specific interactions. Binding the IgG Fc portion of the selectin chimera to the immobilized anti-IgG-Fc mAb on the cantilever maintains its proper functional orientation. Concentrations of the anti-human-IgG-Fc and E- and L-selectin chimera solutions were optimized to result in a low proportion of binding events during force-spectroscopy experiments (~30 binding events per 100 cell contacts).

Single-molecule force spectroscopy

Experiments were conducted using an MFP (Asylum Research, Santa Barbara, CA) (Hanley et al., 2003). The softest triangular cantilever, with a nominal spring constant of 10 pN nm^{-1} and a radius of curvature of 50 nm, was calibrated using thermal noise amplitude and its deflection measured by laser reflection onto a split photodetector. The 35-mm culture dish containing the adherent cellular monolayer immersed in D-PBS containing Ca^{2+}/Mg^{2+} was placed on the MFP stage and positioned so that the cantilever was directly above a single cell. The distance between the cantilever and the cell was adjusted so that each approach cycle resulted in a slight depression force on the cell (~100-300 pN) before reproach. This amplitude of depression force was used by others who used whole cells to make in situ force spectroscopy measurements (Li et al., 2003; Wojcikiewicz et al., 2003). The reproach velocity was varied from 0.5 µm $second^{-1}$ to 60 µm $second^{-1}$, and the dwell time between the cantilever and the cell was set to 0.001 seconds so as to minimize the occurrence of multiple events and maintain 30% binding frequency (Benoit et al., 2000; Hanley et al., 2003). The exception was in the case of L-selectin/PMN binding at low velocities (<10 µm $second^{-1}$), in which a low frequency of binding dictated that a dwell time of 0.02 seconds be used to maintain 30% binding. Rupture forces and corresponding loading rates were derived from force/time traces using IgorPro 3.11 software (Wavemetrics, Lake Oswego, OR). Histograms representing about 200 approach-reproach cycles were compiled for each reproach velocity and the mean rupture force of a selectin-ligand interaction was evaluated for successful events. Prior work has shown that the mean and mode of the rupture force approach one another over the higher loading rate regime, and the mean can accurately be used to estimate Bell model parameters (Tees et al., 2001). Force spectra for selectin-PMN interactions comprise at least eight experiments conducted on separate days and represent about 2000 successful events for each selectin.

Estimation of Bell model parameters and Monte Carlo analysis

The Bell model parameters (Bell, 1978) – the unstressed off-rate k^0_{off} and the reactive compliance x_β were first tabulated by a least-squares fit to the linear region of a graph of rupture force against the logarithm of loading rate (Hanley et al., 2003; Schwesinger et al., 2000; Strunz et al., 1999; Tees et al., 2001) coupled to the bootstrap method with replacement (Efron and Tibshirani, 1993) and, second, by modelling the experimental data over the entire range of loading rates using the probability density function for bond rupture (Evans and Ritchie, 1997; Hanley et al., 2003; Tees et al., 2001) (Eqn 1),

$$p(t,f) = k^0_{off}\exp\left(\frac{x_\beta r_f t}{k_b T}\right)\exp\left\{-\frac{k^0_{off}k_b T}{x_\beta r_f}\left[\exp\left(\frac{x_\beta r_f t}{k_b T}\right)-1\right]\right\}, \quad (1)$$

where $p(t, f)$ is the probability of bond rupture at time t, r_f is the loading rate, k_b is Boltzmann's constant and T is the absolute temperature. The mean rupture force, $\langle f_b \rangle$, from this distribution is given by (Evans and Ritchie, 1997; Hanley et al., 2003; Tees et al., 2001) (Eqn 2).

$$\langle f_b \rangle = \frac{k_b T}{x_\beta}\exp\left(\frac{k^0_{off}k_b T}{x_\beta r_f}\right)\int_1^\infty \frac{\exp\left(\frac{-k^0_{off}k_b T}{x_\beta r_f}\,t\right)}{t}\,dt\,. \quad (2)$$

The Bell model parameters, k^0_{off} and x_β, were estimated by a non-linear least-squares fit of the above equation to the experimental data over the entire range of loading rates.

Monte Carlo simulations of receptor-ligand bond rupture under constant loading rates were performed as previously described (Hanley et al., 2003). Briefly, given a k^0_{off} and x_β in each simulation, we calculate the rupture force ($F_{rup}=r_f\times n\Delta t$) for which the probability of bond rupture, P_{rup}, is greater than P_{ran}, a random number between 0 and 1,

$$P_{rup} = 1-\exp\left[k^0_{off}\exp\left(\frac{-x_\beta r_f n\Delta t}{k_b T}\right)\Delta t\right] \text{ where } n = 1, 2, 3 \ldots, \quad (3)$$

where n=1, 2, 3…, Δt is the interval and $n\Delta t$ is the time step.

Of note, the aforementioned equations reported previously (Hanley et al., 2003) contained minor typographical errors but are presented in the correct forms here.

Statistical analysis

Data are expressed as the mean±SEM unless otherwise stated. Statistical significance between the differences in the slopes of the linear regions of selectin-PMN force spectra were verified by pairwise comparison using Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant. Mean values of the Bell model parameters and corresponding standard deviations were tabulated by a least-squares fit to the linear region of the force versus logarithm of loading rate coupled with the bootstrap method with replacement (Efron and Tibshirani, 1993).

Results

Single-molecule force spectroscopy measurements

An MFP was used to resolve force spectra for selectin-ligand binding at the single-molecule level and to estimate the corresponding Bell model parameters, which describe the dependence of bond lifetime on the applied force (Bell, 1978). We present E- and L-selectin binding to ligands on whole human PMNs along with the previously characterized P-selectin/PMN force spectra (Hanley et al., 2003) to examine cumulatively the mechanics of selectin-mediated binding. Force-time traces (Fig. 2) were generated by lowering the selectin-functionalized cantilever to the immobilized PMN monolayer, maintaining contact for a short time (0.001 seconds) to allow establishment of receptor-ligand binding and subsequently retracting the cantilever from the cell surface at a constant, prescribed velocity. Upon retraction of the cantilever, the force was recorded as a function of the time until dissociation of selectin-ligand bonds occurred. Rupture-force measurements were reproducible for hundreds of approach-reproach cycles over the duration of the experiment, indicating that the integrity of the cell structure and its adhesion molecules were preserved.

To ensure that most binding events were mediated by a single receptor-ligand pair, a low frequency of binding events was sought (an average of 30 events per 100 contacts) by decorating cantilevers with sufficiently dilute selectin chimera solutions (Hanley et al., 2003). Based on Poisson statistics, minimizing the frequency of successful adhesions increases the likelihood that rupture events represent the unbinding of single bonds (Benoit et al., 2000; Hanley et al., 2003). The unbinding of single selectin-ligand pairs was also shown by the emergence of predominantly single rather than multiple steps in the force-time traces (Fig. 2) (Benoit et al., 2000). Moreover, rupture-force histograms, representing the most probable rupture force at a given reproach velocity, always developed a single peak as opposed to regularly spaced force distributions, which would be indicative of the unbinding of oligomeric adhesions (Benoit et al., 2000). The observance of a single significant force histogram for E-selectin/PMN binding is particularly noteworthy, considering the evidence supporting E-selectin binding to glycosphingolipids on the leukocyte cell surface (Alon et al., 1995b; Burdick et al., 2001) as well as multiple regions on PSGL-1 (Goetz et al., 1997). Several distinct E-selectin ligands or simultaneous multiple binding regions might be revealed as a broadened histogram

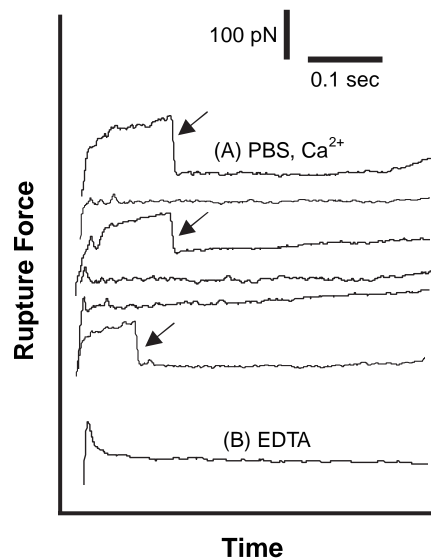

Fig. 2. Force versus time traces for E-selectin binding to PMNs in the absence (A) and presence (B) of EDTA. Experimental conditions, including selectin concentration on the cantilever, contact force and dwell time, were optimized for both E-selectin and L-selectin experiments to result in ~30% binding events (30 adhesion events per 100 contacts). The arrows indicate rupture events with magnitudes of about 100 pN taken from an experiment with a reproach velocity of 15 μm second^{-1}. The presence of EDTA was consistently found to eliminate receptor-ligand binding. The linear increase in slope just before each rupture event is indicative of the loading rate exerted on the receptor-ligand bond (Benoit et al., 2000; Li et al., 2003; Wojcikiewicz et al., 2003).

distribution or histograms with multiple peaks (Benoit et al., 2000). Our findings here, however, suggest that primarily one high-affinity ligand for E-selectin is probed by single-molecule force spectroscopy measurements at very short contact duration (0.001 seconds) and low depression force.

Control and specificity experiments

Several control experiments were performed to demonstrate the specificity of selectin-ligand binding. First, addition of EDTA to the tissue culture dish at a final concentration of 0.5 mM consistently abrogated binding (Fig. 2, Fig. 3A,B), a finding that is in accordance with the calcium dependence of selectin-ligand binding (Moore et al., 1995). Second, incubating the E- or L-selectin-coated cantilever with a function-blocking anti-E-selectin mAb (50 μg ml^{-1} ENA2; BD PharMingen, San Diego, CA) or anti-L-selectin mAb (50 μg ml^{-1} LAM1-116; BD PharMingen), respectively, drastically reduced the frequency of binding events from about 32% to less than 10% (Fig. 3A,B), an observation that is consistent with previous receptor-ligand measurements at the single-molecule level blocked by mAbs (Evans et al., 2001; Hanley et al., 2003; Tees et al., 2001). Additionally, PMN monolayers incubated with a function blocking anti-PSGL-1 mAb (50 μg ml^{-1} KPL-1; BD PharMingen) also appreciably reduced the frequency of binding to about 6% for L-selectin/PMN and 12% for E-selectin/PMN binding. It is noteworthy that non-specific adhesions, evaluated with the use of selectin-free BSA-incubated cantilevers, resulted in about 6% binding events (data not shown). Thus,

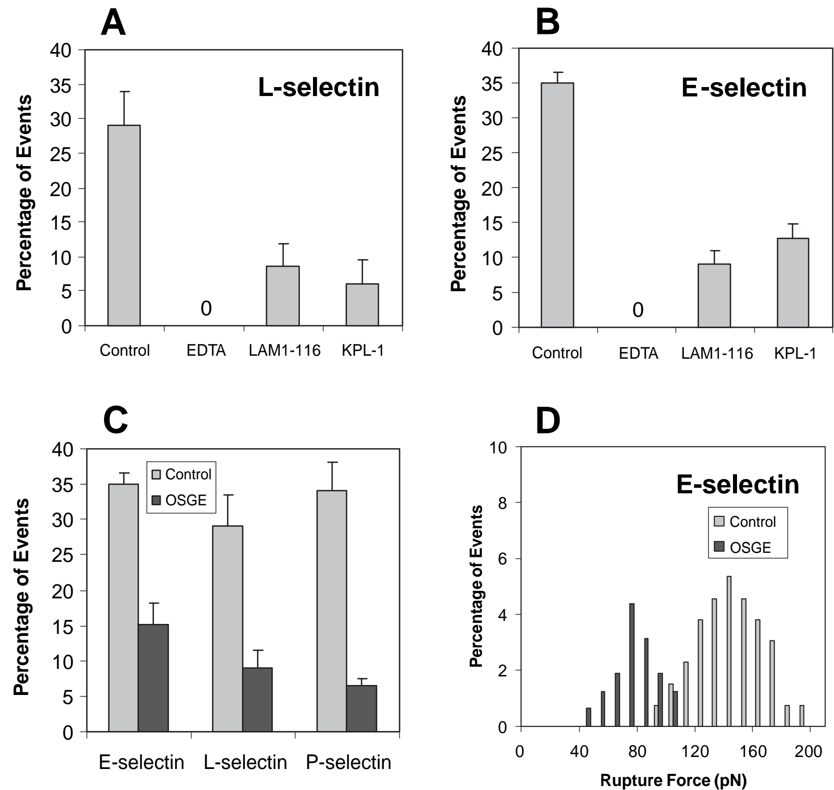

Fig. 3. Frequency of binding events for control and specificity experiments for L-selectin (A) and E-selectin (B). The presence of EDTA was found to abrogate binding. The presence of blocking antibodies for L-selectin (LAM1-116), E-selectin (ENA2) and PSGL-1 (KPL-1) dramatically reduced the frequency of binding. (C) Reduction in frequency of selectin binding to PMNs treated with OSGE. Reductions in the frequencies indicate that heavily *O*-linked glycoproteins are crucial for L- and P-selectin binding, and play a predominant role in E-selectin binding to PMNs. (D) Rupture-force histograms for E-selectin binding to control PMNs and OSGE-treated PMNs at a reproach velocity of 10 μm second^{-1}. E-selectin binding to OSGE-treated PMNs resulted in a lower frequency of rupture events with a lower overall magnitude. As a result of the lower binding frequency, the area under the force histogram for OSGE-treated PMNs is about 40% that of the control. The less rigorous requirements for E-selectin binding might allow it to bind more easily to other glycosylated ligands (*N*-linked glycoproteins or glycosphingolipids) previously shielded by the heavily *O*-linked glycoproteins.

binding frequencies in the range of 5-8% might be primarily a result of these non-specific events. This finding implies that PSGL-1, known to be the primary ligand for L- and P-selectin, probably functions as a high-affinity ligand for E-selectin in the presence of an applied force as well.

In order to assess the contribution of heavily *O*-linked glycoproteins in E-selectin binding to PMNs, PMN cell suspensions were treated with OSGE, an enzyme that selectively cleaves proteins that are *O*-glycosylated on serine and threonine residues (Sutherland et al., 1992). E-selectin/PMN binding frequency decreased to about 15% (Fig. 3C), suggesting that proteins rich in *O*-linked oligosaccharides play an important role in E-selectin/PMN binding. Moreover, the magnitude of the rupture forces between E-selectin and OSGE treated PMNs was about 60% of the control value (Fig. 3D). For comparison, L- and P-selectin-binding experiments using OSGE-treated PMNs resulted in near background binding frequencies (Fig. 3C) and illustrate that heavily *O*-linked glycoproteins play a crucial role in L- and P-selectin-mediated adhesion.

Selectin-ligand force spectra and estimation of Bell model parameters

To develop force spectra for selectin-ligand binding, rupture forces and corresponding loading rates for hundreds of events were tabulated off-line and compiled into histograms for each reproach velocity, and mean rupture forces were calculated (Hanley et al., 2003; Tees et al., 2001). Histograms from representative E- and L-selectin/PMN experiments are presented in Fig. 4. The loading rate (pN per second) for each reproach velocity was determined by evaluating the slope of the force-time trace just before each rupture event (Fig. 2). The linear increase in slope just before each rupture event, indicative of the loading rate exerted on the receptor-ligand bond, was observed by others conducting in situ force spectroscopy measurements (Benoit et al., 2000; Li et al., 2003; Wojcikiewicz et al., 2003). The Bell model parameters, namely the unstressed off-rate k^0_{off} and the reactive compliance x_β were tabulated (Table 1) by a least-squares fit to the linear region of the rupture force versus logarithm of loading rate (Fig. 5) (Schwesinger et al., 2000; Strunz et al., 1999; Tees et al., 2001) coupled to the bootstrap method with replacement (Efron and Tibshirani, 1993). Estimates for the physiological loading rates exerted on cellular adhesion molecules range from about 100 to 10,000 pN s^{-1} (Tees et al., 2001) and so we, like others, have chosen to extrapolate over the linear region spanning this regime. To validate the accuracy of the calculated Bell model parameters, the experimental data were also modelled over the entire range of loading rates (non-linear) by using the probability density function for bond rupture (Evans and Ritchie, 1997; Hanley et al., 2003). It is noteworthy that the Bell model parameters calculated by this approach showed good agreement with those obtained from linear fit over the high loading rate regime (Table 1). Furthermore, Monte Carlo simulations using these parameter values yield most frequent and mean rupture forces in accord with those observed experimentally (Fig. 4A,B).

Discussion

Of central interest to immunology are the adhesive interactions responsible for leukocyte tethering and rolling on activated endothelium. The visually distinct rolling patterns of leukocytes over E-, P- and L-selectin substrates are presumably a

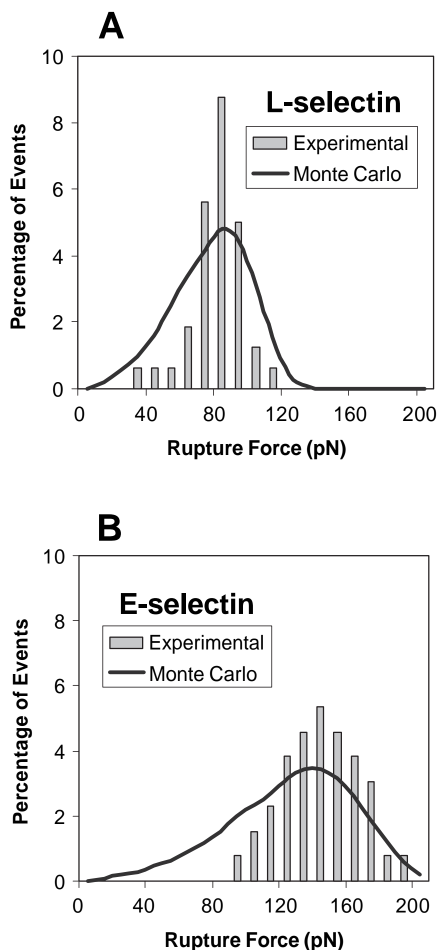

Fig. 4. Representative force histogram and Monte Carlo simulations for L-selectin (A) and E-selectin (B) binding to human PMNs at a reproach velocity of 10 μm second^{-1}. Simulations were conducted using the Bell model parameters extracted from the non-linear estimation and yielded most frequent and mean rupture forces in accord with those observed experimentally. The area under the force histograms represents the proportion of successful binding events (~30% in these cases).

Table 1: Bell model parameters for selectin/PMN interactions

	Linear (100-10,000 pN/s)		Non-linear (all)	
Receptor	x_β (Å)	k^0_{off} (s^{-1})	x_β (Å)	k^0_{off} (s^{-1})
L-selectin	1.66±0.06	0.85±0.10	1.51	0.83
E-selectin	1.23±0.08	0.31±0.06	1.11	0.24
P-selectin (Hanley et al., 2003)	1.45±0.08	0.22±0.06	1.35	0.18

Estimations for the Bell model parameters were tabulated by a least-squares fit to the linear region of the rupture force versus the logarithm of loading rate coupled to the bootstrap method with replacement (Efron and Tibshirani, 1993). In this case, the linear region represents the higher, physiologically relevant (Tees et al., 2001) loading rate regime of 100-10,000 pN/second. Data are expressed as mean±s.d. Bell model parameters were also estimated by a non-linear analysis over the entire range of loading rates and using the probability density function for bond rupture (Evans and Ritchie, 1997; Hanley et al., 2003; Tees et al., 2001). Bell model parameters for P-selectin/PMN binding have been updated to reflect the acquisition of additional points.

consequence of the kinetic and mechanical properties of participating adhesive groups. Using single-molecule force spectroscopy, we simultaneously defined the force spectra for E- and L-selectin binding to whole PMNs, along with the previously characterized P-selectin/PMN interaction (Hanley et al., 2003), so that the kinetic and mechanical parameters could be directly compared (Table 1, Fig. 5). By carefully controlling the concentration and orientation of immobilized selectin chimeras on the surface of an MFP cantilever and regulating its contact time and depression force on the immobilized cells, we probed the binding strength of single selectin-ligand interactions under conditions that preserve all appropriate post-translational ligand modifications.

Previous in vitro studies using dynamic rolling assays have found that L-selectin-mediated rolling of PMNs was five- to tenfold faster than on P-selectin substrates, and 8- to 11.5-fold faster than on E-selectin substrates (Alon et al., 1998; Alon et al., 1997; Puri et al., 1997). These trends were also observed by in vivo studies that used gene-targeted mice with homozygous deficiencies for E-, L- or P-selectin ($E^{-/-}$, $L^{-/-}$ or $P^{-/-}$, respectively) (Jung et al., 1996; Kunkel and Ley, 1996). From the single-molecule results here, the biophysical basis for the fast rolling of PMNs over L-selectin relative to E- or P-selectin becomes clear. L-selectin/PSGL-1 bonds have an intrinsically higher off-rate, as well as a higher reactive compliance, both of which result in faster rolling. It is also clear from the force spectra that the tensile strength of an L-selectin/PSGL-1 bond is lower than that of E- or P-selectin binding to PSGL-1. Although the faster L-selectin-mediated rolling was previously explained solely on the basis of a higher unstressed off-rate (Alon et al., 1997), we find that individual L-selectin/PSGL-1 bonds are more prone to rupture under an applied force and have a shorter lifetime and a lower overall tensile strength.

Bell model parameters extracted from the force spectra of selectin-PMN interactions indicate that E-selectin/PSGL-1 binding has a modestly higher unstressed off-rate, k^0_{off}, but a lower reactive compliance, x_β, relative to P-selectin/PSGL-1 (Hanley et al., 2003). A higher unstressed off-rate would suggest that E-selectin/PSGL-1 bonds have a shorter lifetime in the absence of force, whereas a lower reactive compliance and higher tensile strength would suggest that E-selectin/PSGL-1 bonds are more resistant to rupture in the presence of force. Although strikingly similar, statistical pairwise comparison of the slopes of the linear regions of E- and P-selectin/PMN force spectra verified their difference. E-selectin/PSGL-1 bonds have a higher tensile strength at loading rates in excess of about 1000 pN second^{-1}, which results in a lower reactive compliance. Because dynamic rolling assays of leukocytes are conducted in the presence of hydrodynamic shear, the lower reactive compliance and additional tensile strength of E-selectin/PSGL-1 tethers might allow PMNs to roll more slowly relative to experiments using P-selectin substrates. Nevertheless, flow-based adhesion assays indicate that the differences in the rolling velocities of PMNs on E- versus P-selectin substrates are subtle compared to those on L-selectin (Alon et al., 1998; Alon et al., 1997; Puri et al., 1997; Smith et al., 1999).

The simultaneous estimation and comparison of selectin/PMN Bell model parameters were previously reported by Smith et al., using neutrophil tethering lifetimes as assessed in dynamic flow chamber experiments (Smith et al., 1999).

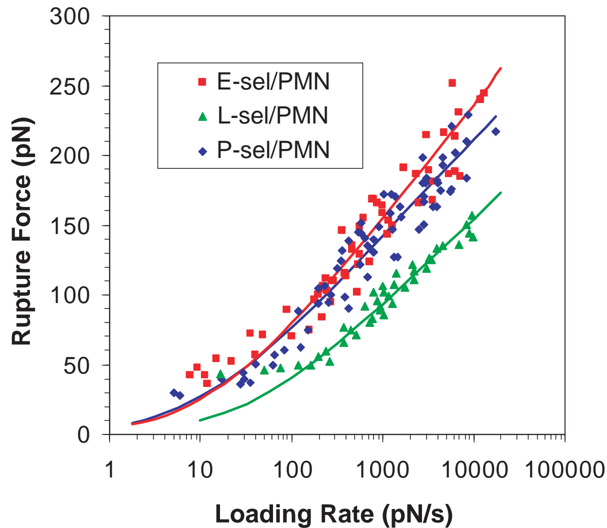

Fig. 5. Rupture force plotted against the natural logarithm of loading rate for E-, P- and L-selectin binding to PMNs. Data comprise at least eight independent experiments and a range of reproach velocities for each selectin, conducted on separate days to validate reproducibility. Each force spectrum represents at least 2000 successful events. The superimposed solid lines indicate the non-linear least-squares fit over the entire range of experimental loading rates, found from the probability density function for bond rupture and corresponding Bell model parameters.

Although the actual values for the unstressed off-rates found here from single-molecule force spectroscopy are lower and the values for the reactive compliance are higher, the trends for both parameters are the same:

unstressed off-rate, k^0_{off}: P < E < L

reactive compliance, x_β: E < P < L

In accordance with the results presented here, previous determinations for the unstressed off-rates assessed by force spectroscopy (Evans et al., 2001; Fritz et al., 1998; Hanley et al., 2003) typically yielded lower values than those found through flow-chamber studies (Alon et al., 1998; Alon et al., 1997; Alon et al., 1995b; Smith et al., 1999) for both L- and P-selectin binding to human PMNs or purified PSGL-1. In addition, values for reactive compliance in these same studies were higher in force-spectroscopy than in flow-chamber experiments. Because multivalent binding is expected to decrease the reactive compliance, we speculate that force-spectroscopy experiments are more likely to isolate single-molecule interactions than flow-chamber assays. Despite the low site densities of adhesion molecules in flow-chamber experiments, the flattening of leukocytes during their tethering and the resulting increase in contact surface area increases the likelihood of multiple selectin-ligand bonds forming, especially at higher shear stresses (Evans et al., 2001). Moreover, the inability directly to quantify the force on the selectin-ligand bond in flow-chamber assays might contribute to the differences in the Bell model parameters determined by force spectroscopy. It is also noteworthy that significant differences in the estimation of the aforementioned parameters (in both magnitude and relative trends) have been reported using flow-chamber assays (Alon et al., 1995b; Alon et al., 1997; Alon et al., 1998; Smith et al., 1999). Although these variations could be attributed to the higher temporal and spatial resolution analysis that enabled the acquisition of previously undetected events of much shorter duration (Smith et al., 2002), differences in the biological preparations or other technical issues also cannot be excluded (Springer et al., 2002).

A key advantage of the in situ single-molecule measurements described here is the ability to isolate high-affinity ligands by evaluating binding frequency at short contact duration (0.001 seconds) and low depression force in the presence or absence of highly specific blocking antibodies. Here, we found that the frequency of binding events for E-selectin to KPL-1-blocked PMNs was dramatically reduced to about 12% (Fig. 3B), so we presume that the crucial first 19 amino acids of PSGL-1 (which are known to be the target of KPL-1 and essential for P- and L-selectin binding) probably serve as a primary binding region for E-selectin during single-molecule experiments as well. Treating PMNs with OSGE also dramatically reduced the frequency of E-selectin binding events to about 15% (Fig. 3B,C). Although this frequency is slightly above the 5-8% threshold for non-specific events, the magnitude of rupture forces was significantly lower (Fig. 3D). Control E-selectin/PMN binding experiments, however, only developed single force histograms at each reproach velocity. Thus, the less rigorous requirements for E-selectin binding might allow it to bind more easily to other glycosylated ligands previously shielded by the heavily *O*-linked glycoproteins. These findings do not necessarily imply that E-selectin-mediated leukocyte rolling exclusively involves E-selectin/PSGL-1 binding events but rather that PSGL-1 is primarily probed by single-molecule force spectroscopy under the conditions of this study and that it probably serves as an important ligand on leukocytes for E-selectin in the presence of an applied force. The presentation of PSGL-1 as a highly extended glycoprotein on PMN microvilli might facilitate its selective binding to E-selectin during the single-molecule measurements made here. However, the extended contact time between PMNs and the E-selectin substrate during rolling adhesion assays, compared with those used here, could more readily allow E-selectin/glycosphingolipid bonds to form.

In a PSGL-1-deficient (PSGL-$1^{-/-}$) murine model, Xia et al. (Xia et al., 2002) found that the number of leukocytes that tether to E-selectin expressing tissues is much lower than in wild-type controls. Once tethered, however, they roll with the same velocity, suggesting that other significant E-selectin ligands on leukocytes might contribute greatly to the overall rolling velocity in vivo. It should be realized that selectin ligands on mice leukocytes are remarkably different from those on humans (Kobzdej et al., 2002) and therefore definitive conclusions must be made with caution. This does suggest, however, that E-selectin/PSGL-1 binding in vivo mediates the initial tethering and rolling of leukocytes before other ligands become involved. It is also noteworthy that in vitro experiments involving completely reconstituted cell-free systems consisting of sialyl-Lewis^x-coated microspheres rolling on P-selectin (Rodgers et al., 2000) or E-selectin (Brunk and Hammer, 1997) substrates also found that E-selectin-mediated rolling is slower. Taken together, whereas several diverse mechanisms might explain the subtly different rolling characteristics of PMNs on E- and P-selectin, the distinct kinetic and mechanical properties of participating adhesive groups are likely to be crucial determinants of this phenomenon.

In order to evaluate selectin-ligand bond lifetimes, flow-

chamber assays quantify transient neutrophil tether duration to selectin substrates with low site density. The first-order kinetics of transient tether dissociation and independence of these kinetics from selectin or ligand density suggest, but do not prove, that they represent single receptor-ligand bonds (Alon et al., 1998; Alon et al., 1997). Similarly, limiting the binding frequency in single-molecule force measurements implies by Poisson statistics that bond ruptures primarily result from single receptor-ligand pairs (Benoit et al., 2000; Hanley et al., 2003). Although we cannot exclude the possibility that bond ruptures were the result of multiple adhesions, only one type of de-adhesion event was observed, as evidenced by the emergence of a single histogram or most probable rupture force for every reproach velocity. As noted by Benoit and Gaub (Benoit and Gaub, 2002), low receptor concentration on the cantilever and decreasing the contact duration and depression force help to minimize multiple events and other problems associated with in situ cell adhesion measurements, such as membrane tether formation.

It is also possible that primarily dimeric de-adhesions were observed, a result of each arm of the dimeric form of the selectin chimeras simultaneously binding to in situ dimeric PSGL-1. If dimeric adhesions did occur, they behaved as a single event, indistinguishable from monomeric binding. Thus, we did not see the development of force histograms with one and two times the quantal unbinding force, as would be expected if we had primarily monomeric and occasional dimeric adhesions. In addition, others have concluded that the flexibility of the Fab arms of the dimeric selectin chimeras should allow each to behave independently, equivalent to separate selectin molecules (Tees et al., 2001). Although experimental comparisons between monomeric and dimeric selectin chimeras were beyond the scope of this study, the use of selectin chimeras of identical forms and the careful regulation of binding frequencies ensures that the direct comparisons between E-, P- and L-selectin are valid.

Using PMNs in an MFP experiment requires that they be firmly immobilized on a substrate (Hanley et al., 2003). By using fixed PMNs, we avoid the complications that would arise during PMN immobilization and subsequent activation. The continuous morphological changes that PMNs undergo during this process would prevent meaningful single-molecule measurements. Fixation of PMNs has been shown to abrogate microvillus elasticity, causing them to behave like PSGL-1-coated rigid microbeads, without impairing the molecular adhesive interaction (Park et al., 2002). In addition, at low shear stresses, PSGL-1-coated microbeads and live PMNs roll over P-selectin substrates with similar velocities (Yago et al., 2002). Cumulatively, these data suggest that fixation does not impair the adhesive interaction of selectin/PSGL-1 binding at the molecular level, although the mechanical properties of the cell are affected. Moreover, the protein-specific nature of fixation is much more likely to affect globular proteins than linear ones (such as PSGL-1) or glycosphingolipids. Although heavy fixation might obscure glycosphingolipids, presumably by burying them in a heavily cross-linked protein matrix, it is unlikely that the mild fixation procedure (1% formalin) would have this effect. Rather, the short contact duration and low depression force used here, as well as the presentation of PSGL-1 on PMN microvilli has allowed its selective binding to E-selectin in our assay.

Previously, we found that fixation did not appreciably affect the rupture-force spectra or the Bell model parameters for P-selectin binding to its ligand on LS174T colon carcinomas (Hanley et al., 2003) [see supplemental data Fig. S1 (http://jcs.biologists.com/supplemental/)], a finding that might be attributed at least in part to the absence of actin cytoskeletal involvement with this ligand. PSGL-1, however, is connected to the actin cytoskeleton and previous studies have reported on the integral role of the cytoskeleton during P-selectin adhesion to PSGL-1 on PMNs and other leukocyte subpopulations (McCarty et al., 2003; Sheikh and Nash, 1998; Snapp et al., 2002). This cytoskeletal interaction is known to regulate the strength of leukocyte adhesion, as shown by the resistance to shear-induced detachment of PMNs treated with cytochalasin B, a compound that caps the growing end of actin filaments (McCarty et al., 2003; Sheikh and Nash, 1998). The process of fixing PMNs decreases the likelihood that the applied load on the selectin/PSGL-1 complex will be partially dissipated by viscous deformation during microvilli extension. Thus, rupture forces for fixed PMNs may be higher at a given loading rate than for unfixed PMNs, because nearly the entire applied load is placed on the receptor-ligand pair and any cytoskeletal contributions have been eliminated. Under the conditions of this study, all PMNs were immobilized and fixed according to the same experimental protocol, allowing direct comparisons.

In conclusion, the force spectra for E-, P- and L-selectin binding to human PMNs reveal the kinetic and mechanical properties responsible for the differences in leukocyte rolling over selectin substrates. Single L-selectin bonds to PSGL-1 were found to be more labile than either E- or P-selectin in the presence of an applied force. This outcome, as well as a higher unstressed off-rate and a higher reactive compliance, explain the faster L-selectin-mediated rolling. By quantifying the binding frequencies of E-selectin to PMNs in the presence of a highly specific blocking mAb or following enzyme treatment, the primary ligand probed by E-selectin during single-molecule experiments was determined to be PSGL-1. Also, although the requirements for E-selectin binding to PSGL-1 are less rigorous than those for L- or P-selectin, the primary epitope is likely to be *O*-linked oligosaccharides on the first 19 amino acids of PSGL-1, as is seen with P-selectin experiments (Goetz et al., 1997).

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