ABSTRACT In single-molecule mechanics experiments the molecular elasticity is usually measured from the deformation in response to a controlled applied force, e.g., via an atomic force microscope cantilever. We have tested the validity of an alternative method based on a recently developed theory. The concept is to measure the change in thermal fluctuations of the cantilever tip with and without its coupling to a rigid surface via the molecule. The new method was demonstrated by its application to the elasticity measurements of L- and P-selectin complexed with P-selectin glycoprotein ligand-1 or their respective antibodies, which showed values comparable to those measured from the slope of the force-extension curve. L- and P-selectin were found to behave as nearly linear springs capable of sustaining large forces and strains without sudden unfolding. The measured spring constants of ~4 and ~1 pN/nm for L- and P-selectin, respectively, suggest that a physiological force of ~100 pN would result in an ~200% strain for the respective selectins.

INTRODUCTION

Biomechanical measurements at the level of single molecules provide insights into their inner workings that complement information obtained from conventional biochemical and biophysical measurements on ensembles of large numbers of molecules (1). In the past decade, there have been many measurements of mechanical properties of single DNA, RNA, and protein molecules (2–7). In these experiments, ultrasensitive force techniques, e.g., atomic force microscopy (AFM) (8) and optical tweezers (9,10), were used to stretch the molecules to measure their force-extension curves. Typically, the applied forces and molecular extensions are in the ranges of tens to hundreds of piconewtons and a few to tens of nanometers, respectively, due to the extremely small size and softness of biomolecules. Consequently, these experiments may be susceptible to thermal excitations, which manifest as force and displacement fluctuations that reduce measurement accuracy. On the other hand, the responses to thermal excitations of small and soft mechanical systems are related to their elastic properties.
P-selectin is rapidly redistributed to the cell surface, where it binds to ligands on leukocytes. P-selectin glycoprotein ligand-1 (PSGL-1) is a sialomucin on leukocytes that binds to all three selectins. In particular, its binding to L- and P-selectin can be blocked by the same monoclonal antibody (mAb) to the N-terminal region of PSGL-1. Interactions of selectins with cell-surface glycoconjugates such as PSGL-1 mediate tethering and rolling of leukocytes on activated endothelial cells or activated platelets or other leukocytes that have previously adhered to vascular surfaces. This process initiates the multistep adhesion and signaling cascade of leukocyte recruitment to sites of inflammation and injury. The hydrodynamic forces acting on the leukocytes have to be balanced by adhesive forces on the selectin-ligand bonds, which stretch these molecules. Therefore, the molecular elasticities of the selectins may be pertinent to their functions in this mechanically stressful environment.

MATERIALS AND METHODS

Proteins and antibodies

P-selectin and L-selectin were purified from human platelets (17) and human tonsils (18), respectively, as previously described. Native dimeric PSGL-1 was purified from human neutrophils (17). Recombinant monomeric soluble PSGL-1 (sPSGL-1) was purified from Chinese hamster ovary cell transfectants (19). The blocking anti-P-selectin mAb G1 (20), the blocking anti-L-selectin mAb DREG56 (21), and the nonblocking anti-PSGL-1 mAb PL2 (22) have been described.

Forming selectin-reconstituted bilayers

Selectin-incorporated lipid vesicle solutions were prepared following the method of McConnell et al. (23). Briefly, egg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) was dissolved in chloroform and dried on a Teflon surface with argon. Vesicles were formed by rehydrating the dried lipid film with 250 μl of 2% octyl β-glucopyranoside (OG) (Fisher Scientific, Pittsburgh, PA) Tris saline solution, creating a 0.8 mM lipid solution. The 2% OG egg phosphatidylcholine solution was combined with 250 μl of 1% OG solution, containing 7 μg of P- or L-selectin. The resulting 0.4 mM lipid solution was dialyzed with three 1-liter changes of Tris saline buffer (25 mM Tris-HCl, 150 mM NaCl, pH 7.4) in 12-h increments. The resulting lipid vesicle solution was stored under argon at 4°C and used within several months.

P-selectin or L-selectin was reconstituted into glass-supported polyethyleneimine (PEI)-cushioned lipid bilayers using the method of vesicle fusion as previously described (24–26) (cf. Fig. 1). Briefly, a dry coverslip precoated with Piranha solution (70% 12 N sulfuric acid and 30% hydrogen peroxide) at 100°C for 45 min was immersed in a 100-ppm PEI (molecular weight = 1800 g/mol, 95% purity; Polysciences, Warrington, PA) solution of 0.5 mM KNO₃ (Fisher Scientific) in deionized water (pH 7.0) for 20 min, rinsed, dried by argon, and placed in a desiccator for 10 min. A 3- to 5-μl drop of P- or L-selectin-incorporated lipid vesicle solution was placed on the PEI-coated coverslip, placed in a Petri dish, and covered with a damp paper towel. After 20 min incubation, the Petri dish was filled with 10 ml Hank’s balanced salt solution with 1% Ig-free bovine serum albumin. The P-selectin and L-selectin bilayers so formed had molecular densities of a few hundred sites/μm² that resulted in infrequent binding (15–20%) to the (s)PSGL-1-, G1-, or DREG56-coated cantilever tips, as required for measuring single-bond interactions (26). The bilayers were immediately used in AFM experiments.

AFM system and cantilever functionalization

Our in-house-built AFM system and its functionalization with ligands and mAbs have been described previously (24–26). Briefly, a piezoelectric translator (PZT) (Poly Physik Instrument, Boston, MA) was used to actuate the cantilever (unsharpened gold-coated half-wafer cantilevers, Veeco Instruments, Woodbury, NY). The cantilever tip inclination was measured by bouncing a laser beam (Oz optics, Ontario, Canada) off the back of the cantilever onto a photodiode (Hamamatsu, Bridgewater, NJ). A personal computer with data acquisition boards (analog output board and multifunction I/O board, National Instruments, Austin, TX) was used to control the movement of the PZT and to collect the signal from the photodiode. Lab View (National Instruments) was used as the interface between the user and the data acquisition boards.

Cantilevers were incubated overnight at 4°C with a mAb (10 μg/ml) and followed by 30–60 min incubation at room temperature with 1% bovine serum albumin in Hank’s balanced salt solution. The cantilevers were used immediately in the AFM experiments. During each experiment, cantilevers precoated with capture mAb PL2 were functionalized by incubation with (s)PSGL-1 (PSGL-1 or sPSGL-1, 100 ng/ml, 20 min at room temperature); cantilevers coated with anti-P-selectin mAb G1 or anti-L-selectin mAb DREG56 were used directly without further modifications. The molecular systems used in this study are depicted in Fig. 1.

Calibrating cantilever spring constant

Accurate in situ calibration of each cantilever spring constant k is crucial for measuring molecular spring constants. A previous method of thermal fluctuation analysis (27) was modified, based on the theory described in Wu et al. (13). Applying the equipartition theory from statistical mechanics to the AFM cantilever, it has been shown that

$$\frac{1}{2}k_s\langle z^2 \rangle = \frac{1}{2}k_bT,$$  \hspace{1cm} (1)

where $k_b$ is the Boltzmann constant, $T$ is the absolute temperature, $z$ is the cantilever tip deflection, and the brackets denote ensemble averaging. Thus,
can be determined from the mean square of fluctuating deflections of the cantilever tip under thermal excitations.

Two corrections were made to further improve the accuracy of the cantilever spring constant estimation. The first has to do with the fact that the photodiode monitors laser light reflected from the back of the cantilever tip, which measures the cantilever tip inclination, $|\partial z/\partial t|_{t=L}$, rather than the cantilever tip deflection, $z(L)$, where $L$ is the distance from the built-in end ($x=0$) to the tip ($x=L$) along the long axis ($x$) of the cantilever. Under static loading, the two are related by $z(L) = aL|\partial z/\partial t|_{t=L}$, where the proportionality constant $a$ is the area (divided by 2) divided by the cantilever geometry (13). For each cantilever, this relationship was determined in situ by the sensitivity measurement in which the PZT bent the cantilever against a coverslip to produce a range of known static tip deflections and the corresponding photodiode voltage readings were recorded. When the cantilever fluctuates under thermal excitations with waveforms that contain many vibration modes, the real inclination at the tip is expressed in terms of the virtual tip deflection, $z' (L, t) = aL|\partial z/\partial t|_{t=L}$, where the proportionality constant $b$ is the spring constant of the molecular spring constant at that force via the thermal method (below). The mean and standard deviation of the virtual deflections were calculated from the power spectral density plot to no more than three, thereby truncating the infinite series on the right-hand side of Eq. 3 to a sum of just a few terms. Correction to this error has been made by using the following approximation:

$$k_c \approx b_N k_b T / \sum_{n=1}^{N} \langle z_n^2 \rangle,$$

where $b_N$ is a constant that depends only on the cantilever geometry. The calculated values for $b_N$ for the commercial Veeco cantilevers are presented in Supplementary Material.

The need for the above two forms of corrections can be seen in the following example. For an experiment using cantilever D (nominal spring constant of 30 pN/nm as provided by the manufacturer), the cantilever spring constants estimated using Eq. 5 with one and two terms were 16.8 and 15.2 pN/nm, respectively. Had we used only a single term (similar to Eq. 4) and not corrected for virtual deflection (using Eq. 1 directly), the value would have been 24.0 pN/nm, which overestimated the cantilever spring constant by $\sim 50\%$. It should be noted that the hydrodynamic interactions of the cantilever with the wall play no role in the thermal method for determining the cantilever spring constant. These interactions manifest as viscous effects and have been accounted for by the quality factor $Q = \omega_b / 2\sqrt{\xi}$, cf. Eq. 4, in the power spectrum density function. However, the standard deviation of the measured virtual deflections is determined by the area under the power spectrum density curve, not by how broadly distributed the spectrum is. In fact, the cantilever spring constants determined in air, where hydrodynamic interactions of the cantilever with the wall are much smaller (and hence, the power spectrum density distribution is much narrower with a much higher $Q$ value), were found to be in good agreement with that determined in liquid (data not shown).

**Determining molecular spring constant**

The AFM experiments were similar to those designed for measuring lifetimes of single molecular bonds, as previously described (24–26). Briefly, binding was enabled by actuating the ligand- or antibody-coated cantilever tip into contact with the selectin reconstituted bilayer. The cantilever was retracted a predetermined distance (20–100 nm) at a predetermined speed (250 nm/s) and then held stationary. When the tip was linked to the bilayer by a molecular bond, the retraction phase yielded a force-extension curve that allowed determination of molecular elasticity via the stretch method (below). After the PZT stopped retracting and was held stationary, the cantilever fluctuated about a fixed position with a mean force applied to the selectin and ligand (or mAb) if they remained bound. This mean force dropped to zero when the bond ruptured; and the cantilever continued to fluctuate but with increased amplitudes (Fig. 2). Binding was kept infrequent ($\sim 15–20\%$) by lowering the molecular densities. Binding resulted in clearly visible discrete rupture events from the force-time scan curves that were distinct from null events. The frequencies of null, single, double, and triple rupture events followed Poisson distribution in accordance with small number statistics (data not shown), suggesting that the elasticity values measured from single rupture events represented properties of single molecules (28,29). The virtual deflections of the fluctuating cantilever were continuously monitored by the photodiode at data acquisition rates of 600 and 5000 Hz for P-selectin and L-selectin, respectively, which are much faster than the respective off-rates of P-selectin-sPSGL-1 (0.6–10 s$^{-1}$; 25) and L-selectin-PSGL-1 (10–50 s$^{-1}$; 25) interactions under the forces tested.

The mean and standard deviation of the virtual deflections were calculated from $\sim$100 consecutive data points. The mean value was used to determine the mean applied force. The standard deviation was used to determine the molecular spring constant at that force via the thermal method (below). Some of the data were acquired at a much higher rate of 80 kHz for frequency domain analysis, which allowed us to compare them with results obtained from the time domain analysis using data acquired at lower acquisition rates.

The thermal method is based on a recently developed theory (13). A key result takes the form of Eq. 1, except that $k_c$ is now replaced by $k_c + k$, i.e.,

$$1/2(k_c + k) \langle z^2 \rangle = 1/2k_b T,$$

where $k$ is the spring constant of the molecular complex. In other words, as far as the mean square tip deflection under thermal excitations is concerned, the coupled system behaves as if the cantilever spring and the molecular spring are in parallel. Thus, the added stiffness reduces the cantilever thermal fluctuations. The mean-square virtual deflections could be calculated in a fashion similar to the free cantilever case.

The validity of the thermal method and accuracy of the molecular spring constant so measured depend on whether the fluctuations recorded in the photodiode are thermally driven or contain significant contributions from
environmental noise. To address this issue, we measured the photodiode signals when the laser was reflected from the wafer where the cantilever base was mounted, which should contain virtually no thermal fluctuations but include all environmental noise. Comparison of these signals with those when the laser was reflected from the cantilever tip showed that the former were much smaller than the latter, such that the variance of the former signals is only 4% of that of the latter (Fig. 3, A and B). Significantly, the power spectrum density of the latter signals near the resonant circular frequency (1240 Hz) was about five orders of magnitude greater than that of the former signals (Fig. 3, C and D). Given the large damping in the aqueous environment, it is not possible for such a small excitation from environmental noise to be amplified by this magnitude even at the resonant frequency. It can therefore be concluded that the cantilever fluctuations are predominantly the result of purely thermal excitations.

Similar to the cantilever spring-constant calibration, correction is required to relate the mean-square virtual deflections to the mean-square real deflections, which takes the form:

\[
< Z^2 > = (a \frac{k_c}{k_c} + b) \times \frac{k_S T}{k_c + k} \\
< Z_c^2 > = b \times \left( \frac{k_S T}{k_c} \right)
\]

where \(a\) and \(b\) (1/3 and 4/3, respectively, for a rectangular cantilever) are constants that depend only on the cantilever geometry (see Supplementary Material). By comparing the thermal fluctuations of the surface-coupled cantilever with those of the free cantilever, we can determine the molecular spring constant (Fig. 2). Specifically, it follows from Eqs. 1 and 6 that

\[
k = k_c b k_{Q} T - k_c \sum_{n=1}^{N} < z_n^2 > - a k_b k_{Q} T
\]

The calculated \(a\) and \(b\) values for the V-shaped commercial Veeco cantilevers are presented in the Supplementary Material. Just like the cantilever spring-constant calibration, correction is also required to account for the bandwidth limitations, which assumes a form similar to Eq. 7 (13):

\[
k \approx c_k k_c \sum_{n=1}^{N} < z_n^2 > + a k_b k_{Q} T
\]

where the sums on the right-hand side represent the \(N\) term truncation of the power spectral density series of the square virtual deflections. The coefficients \(a_{Q}, b_{Q}\), and \(c_{Q}\) are constants that depend only on the cantilever geometry. Their calculated values for the commercial Veeco cantilevers are presented in the Supplementary Material. As noted before, the hydrodynamic interactions of the cantilever with the wall play no role in the thermal method in the determination of the molecular spring constant.

The stretch method measures the molecular spring constant directly from the force-extension curve when the selectin-ligand (or selectin-mAb) complex is stretched (Fig. 4). In contrast to the thermal method that extracts
information from the standard deviation, the stretch method utilizes the mean of the fluctuating force-scan curve. Since the PZT retracts the built-in end of the cantilever at a constant speed low enough to neglect the cantilever inertia and viscous drag, the mean photodiode signal measures the quasistatic tip inclination that is directly proportional to the quasistatic tip deflection. As depicted in Fig. 4, force is directly measured by $f = k_c \langle \Delta z \rangle$ where the quasistatic tip deflection $\langle \Delta z \rangle$ is obtained from the mean photodiode signals. Subtracting $\langle \Delta z \rangle$ from the PZT movement $z_{pzt}$ gives the molecular extension $z_m$. Unlike the thermal case, here the coupled system behaves as if the cantilever spring and the molecular spring are in series because the same mean force is applied to both the cantilever and the molecular complex.

**Statistical analysis**

Statistical significance, or the lack thereof, of differences between two measurements were assessed using the two-tailed Student’s $t$-test (assuming unequal variances) and analysis of variance. The two methods give comparable $p$-values that are indicated in the text and figures.

**RESULTS AND DISCUSSION**

**Spring constants determined from time-domain and frequency-domain analysis**

The molecular spring constants estimated from the thermal method were mostly determined by analyzing the cantilever fluctuations directly in the time domain, i.e., calculating the standard deviation of $\sim$100 consecutive points from the force-scan time course acquired at a relatively low rate of 600–5000 Hz. To assess the accuracy of spring constants so measured, some data were also acquired at a much higher scan rate of 80 kHz to allow frequency-domain analysis. Fig. 5 A compares spring constants of the same molecular complexes determined by the respective time-domain and frequency-domain analyses using separate data measured independently with the same cantilever, which show

**FIGURE 4 Stetch method for measuring molecular spring constants by AFM.** The stretch method measures the molecular spring constant from the slope of the linear ascending phase of the force-extension curve before rupture. A dead zone of zero mean force was observed between this ascending phase and another one to the left, which had negative (compressive) forces, indicating decreased indentation of the AFM tip against the bilayer. The dead zone represents the resting length of the molecular complex as it has to be picked up and fully extended before it could resist tensile force (see text). The force on the molecular complex is calculated using $f = k_c \langle \Delta z \rangle$ where the quasistatic tip deflection $\langle \Delta z \rangle$ is obtained from the mean photodiode signals. Subtracting $\langle \Delta z \rangle$ from the PZT movement $z_{pzt}$ gives the molecular extension $z_m$. Unlike the thermal case, here the coupled system behaves as if the cantilever spring and the molecular spring are in series because the same mean force is applied to both the cantilever and the molecular complex.

**FIGURE 5 Comparison of the time-domain and frequency-domain analyses.** (A) Molecular spring constants and (B) cantilever spring constants were both measured from time-domain analysis of low-scan-rate data and frequency-domain analysis of high-scan-rate data. Data are presented as mean ± SD of $n$ (indicated by numbers over the error bars) independent measurements.
satisfactory agreement. Additional comparisons between
time-domain analysis of low-scan-rate data and frequency-
domain analysis of high-scan-rate data were made for free
fluctuations of three uncoupled Veeco cantilevers B, C, and
D, which had different shapes, sizes, and spring constants
(Fig. 5 B). Again, no statistically significant differences ($p >
0.3$) were found between values determined from analyses of
the time data and frequency data for each cantilever. These
results have validated the time-domain analysis that was
based on standard-deviation calculations of low-scan-rate
data. Note that the nominal cantilever spring constant values
provided by the manufacturer are, respectively, 20, 10, and
30 pN/nm for cantilevers B (rectangular), C (V-shaped), and
D (V-shaped), respectively. These differ from the experi-
mentally determined values by as much as 60%, which
emphasizes the need for in situ calibration of each cantilever
used for quantitative mechanical measurements.

Molecular spring constants measured by
two methods

To test the validity and accuracy of the thermal method
for measuring molecular spring constant, we compared the
values so measured with those measured by the conventional
stretch method (Fig. 6). The measured spring constants vary
statistically significantly ($p < 0.001$) with the selectin used,
indicating the ability of our experiment to discriminate
elastic properties of P-selectin (Fig. 6 A) and L-selectin (Fig.
6 B). There were no statistically significant differences in the
$k$ values when (s)PSGL-1 was replaced by the respective
mAb for the P-selectin (G1, $p = 0.86$) and L-selectin
(DREG56, $p = 0.27$). This suggests that (s)PSGL-1 and
antibody are much less stretchable than selectins. By the
same token, the lipid bilayer and the underlying PEI layer
must be much less deformable under tensile forces, hence
having limited (if any at all) contributions to the measured
spring constants. This is also supported by the much higher
spring constant of the lipid bilayer and the underlying PEI
layer during compression (not shown). A reasonable expla-
nation for the selectin dependence of the spring constant may
be that the CRs act as a spring in series so that the spring
constants of the two selectins are inversely proportional to
their lengths. These conclusions will be demonstrated more
definitively in a separate article (K. K. Sarangapani, B. T.
data). For the same molecular complex, the spring constants
measured by two methods show no statistically significant
differences ($p$-values ranging from 0.26 to 0.70), regardless
of the particular molecules tested and their specific spring-
constant values. These data support the validity and accuracy
of both methods, which are based on very different prin-
ciples. The thermal method is based on statistical mechanics.
It analyzes the standard deviations of the force-scan curves
and views the cantilever spring and the molecular spring in
parallel. By comparison, the stretch method is based on
deterministic mechanics. It analyzes the mean of the force-
extension curves and views the two springs in series.

Molecular spring constants measured by
cantilevers of different shapes and sizes

To further test the reliability of the estimated molecular
spring constants, we compared the values measured using
Veeco cantilevers of different shapes, sizes, and spring con-
stants. Fig. 7 A shows values of the L-selectin-DREG56
spring obtained by the thermal method using rectangular
cantilever B and V-shaped cantilever C. Fig. 7 B shows
values of the same molecular spring obtained by the stretch
method using cantilevers B, C, and D. Fig. 7 C shows values
of the L-selectin-PSGL-1 spring by the stretch method using three different D cantilevers, which had different spring constants. For the data in Fig. 7, A and C, there were no statistically significant differences ($p$-values ranging from 0.38 to 0.95) in the spring-constant values for the same molecules regardless of the cantilever used in the measurement. Although very small $p$-values were obtained for the small differences (ranging from 9 to 25%) between the spring constant of the L-selectin-DREG56 complex measured by the rectangular cantilever B and those measured by V-shaped cantilevers C and D, they likely result from the unusually small standard deviations (0.5–1.1 pN/nm for data in Fig. 7 B compared to 1.0–1.8 pN/nm for data in Fig. 7 C). This conclusion is confirmed by the much larger $p$-values (0.1, 0.25, and 0.02 for C versus B, C versus D, and B versus D, respectively) obtained when standard deviations of 1.8 pN/nm are used along with the means from Fig. 7 B in the Student's $t$-test. Thus, the measured molecular spring constants do not depend on the shape, size, and spring constant of the cantilevers used, as expected. It follows from Eq. 7 that the relative change in the mean-square virtual deflections of a cantilever due to a molecular bond that elastically links its tip to the coverslip can be expressed as:

$$\frac{\langle z^2 \rangle_b - \langle z^2 \rangle_f}{\langle z^2 \rangle_f} = \frac{1 - a/b}{1 + k_c/k}$$

(9)

where the subscripts $f$ and $b$ on the left-hand side indicate the free and bound cantilevers. The left-hand side can be viewed as a measure of the signal/noise ratio, which decreases as $k_c/k$ increases according to the right-hand side. Since we are clearly able to discriminate the spring constant of P-selectin from that of L-selectin, the lack of effect on the measured molecular spring constant of the cantilever used for measurement suggests that the $k_c/k$ ratios are not too high to affect the accuracy of the molecular spring-constant values.

**Irrelevance of polymer elasticity models**

The use of a spring constant to describe the mechanical property of a selectin assumes that the molecule behaves as a linearly elastic material. To test the validity of this assumption, we examined a large number of force-extension curves. In the four representative force-extension curves exemplified in Fig. 8, a continuous transition from compressive to tensile forces as $z_{exp} - \langle z \rangle$ increased was seen in two (Fig. 8, A and C), whereas a “dead zone” of zero mean force between the compressive and tensile force regimes was seen in the other two (Fig. 8, B and D, cf. Fig. 4). The presence of a dead zone gives the appearance of a nonlinear force-extension relationship. This might have prompted the use of the modified free joint chain (MFJC) model in a previous study, which depicted P-selectin and PSGL-1 as chain-like polymers that required little initial force to straighten their randomly coiled shapes (30). Other studies have used the free joint chain
The FJC model (31) and the worm-like chain (WLC) model (32) to describe the elastic behaviors of DNA and proteins (7,33,34). These polymer elasticity models are given by the following equations:

- **FJC**:  
  \[ z(f) = L \left[ \coth(fl/k_B T) - k_m T/fl \right] \]

- **WLC**:  
  \[ z(z) = (k_B T/l) \left( z/L + 0.25 \left[ (1 - z/L)^{-2} - 1 \right] \right) \]

- **MFJC**:  
  \[ z(f) = \left( L + f/k_m \right) \left[ \coth(fl/k_B T) - k_m T/fl \right] \]

where \( L \) is the contour length, \( l \) is the persistence length, and \( k_m \) is an equivalent molecular spring constant. To test the applicability of these nonlinear elastic models to our data, Eq. 10 was fit to the measured force-extension curves (Fig. 8). All three models were able to fit curves with no or small dead zones, as these models all have linear regimes that can match the data by adjusting the model parameters (Fig. 8, A and C). For curves with larger and larger dead zones, the WLC model, and especially the FJC model, became less and less able to fit the force-extension curves, as they could not follow the piecewise linear trend of the data no matter how the parameters were adjusted (Fig. 8, B and D). Although the MFJC model remained capable of fitting the data and the parameters reported by Fritz et al. (30) were able to predict some P-selectin-sPSGL-1 force-extension curves we measured, the best-fit parameters varied widely with the dead-zone length. For the P-selectin-G1 complex, the best-fit values are \( L = 27.7 \) nm, \( l = 0.90 \) nm, and \( k_m = 4.55 \) pN/nm for the curve without dead zone in Fig. 8 A, but \( L = 36.2 \) nm, \( l = 0.66 \) nm, and \( k_m = 6.26 \) pN/nm for the curve with an \(~15\)-nm dead zone in Fig. 8 B. For the L-selectin-PSGL-1 complex, the parameter values are \( L = 2.74 \) nm, \( l = 0.77 \) nm, and \( k_m = 4.16 \) pN/nm for the curve without dead zone in Fig. 8 C, but \( L = 47.6 \) nm, \( l = 4.79 \) nm, and \( k_m = 5.29 \) pN/nm for the curve with an \(~40\)-nm dead zone in Fig. 8 D. Moreover, no correlations were found between the \( k_m \) values and the slopes of the linear segments of the tensile force-molecular extension curves, between the \( L \) values and the total resting lengths of the four molecular complexes (cf. Fig. 10 below), or between the \( l \) values and any characteristic lengths from the structures of these molecules. Furthermore, although it strongly affects the best-fit parameters, the dead-zone length did not correlate with the slope of the tensile force versus molecular extension curve. By contrast, similar slopes (which were taken as molecular spring constants by the stretch method) were seen for the same selectin regardless of the dead-zone length and were distinct for the two different selectins. For example, the P-selectin-G1 values estimated from the data in Fig. 8, A and B, are \( k = 1.39 \) and 1.22 pN/nm, respectively; and the L-selectin-PSGL-1 values estimated from the data in Fig. 8, C and D, are \( k = 4.53 \) and 4.71 pN/nm, respectively.

Since the thermal method allows the measurement of the “local” spring constant in the vicinity of a fixed mean force even if the molecule is highly nonlinearly elastic, we used this method to obtain collections of spring constants for the four selectin-(s)PSGL-1 (and -mAb) complexes in the corresponding ranges of forces. As exemplified for the P-selectin-G1 complex (Fig. 9 A) and L-selectin-PSGL-1 complex (Fig. 9 B), the local spring constant appears to be fairly independent of force in the respective force ranges tested, supporting the linear spring model. In fact, no statistically significant differences between spring constant values of any two neighboring data points are noted. Furthermore, the slopes of the trend lines of the data in both panels of Fig. 9 are not statistically significantly different from zero (\( p = 0.68 \)). This was also the case for the molecular spring constants obtained by the stretch method at different forces (data not shown).

To further confirm the irrelevance of the polymer elasticity models, the respective local spring constants at a given force (or extension) level for the three models were calculated from Eq. 10.
FIGURE 9 Molecular spring constants of P-selectin-G1 complex (A, 86 measurements) and L-selectin-PSGL-1 complex (B, 106 measurements) measured in a range of fixed forces by the thermal method (points; mean ± SE) are compared to fits (curves) by the three polymer elastic models (Eq. 11). The best-fit FJC model parameters are, respectively, $L = 1107$ nm and $l = 0.12$ nm (A) and $L = 90.9$ nm and $l = 0.04$ nm (B). The best-fit WLC model parameters are, respectively, $L = 292$ nm and $l = 0.022$ nm (A) and $L = 114$ nm and $l = 0.02$ nm (B). The best-fit MFJC model parameters for the P-selectin-G1 complex are: $L = 9.89$ nm, $l = 0.53$ nm, and $k_m = 1.05$ pN/nm (A). Two sets of MFJC model parameters for the L-selectin-PSGL-1 complex are presented in the text; these correspond to two very distinct curves (dark dashed and light dotted) with similar goodness of fit. Part of the light dotted curve is obscured because it overlaps with the light solid curve of the FJC fit (B). The respective spring constants (mean ± SE) for P-selectin-G1 and L-selectin-PSGL-1 averaged from all data over their respective force ranges are 0.99 ± 0.11 and 3.3 ± 0.15 pN/nm, respectively.

MFJC model fits of nearly identical goodness-of-fit ($\chi^2 = 1.8$ vs. 1.9) can have quite different shapes (Fig. 9 B, light dotted and dark dashed curves) and parameters ($L = 50.3$ nm, $l = 0.01$ nm, and $k_m = 1.76$ pN/nm vs. $L = 2.86$ nm, $l = 0.88$ nm, and $k_m = 3.9$ pN/nm). Thus, aside from its ability to fit the data by freely adjusting its parameters, it is not justified to apply to our data the MFJC model proposed for the elasticity of P-selectin in the previous study (30). Furthermore, the previous study used biotinylated P-selectin-Ig and PSGL-1-Ig chimeras (where the antibody alone on average was modified with 10 biotins) to immobilize the molecules on the coverslips and on the AFM tips (30). This most likely resulted in random length in the specimens that were stretched, which might also have contributed to the much higher P-selectin spring constant of $5.3 \pm 1.5$ pN/nm (30). By comparison, we used the bilayer and capture protocols for immobilizing the selectins and (s)PSGL-1 (cf. Fig. 1). The immobilization of P-selectin through the lipid bilayer was determined by dual color fluorescence recovery after photobleaching experiment. Both lipid (labeled by nitrobenzoxadiazole fluorescence) and P-selectin (labeled with G1) appeared uniformly distributed under confocal microscopy. However, lipid fluorescence, but not protein fluorescence, could quickly recover after photobleaching, indicating that the bilayer was continuous and that P-selectin was immobilized presumably by attaching its cytoplasmic tail to the PEI layer (26). This procedure ensures a uniform length of the extracellular domain outside the lipid bilayer for both P- and L-selectin. Although it is still possible for the (s)PSGL-1 captured (and mAbs directly coated) on the AFM tip to have variable angular rotations, they would most likely only result in small errors because the noncoaxial effect manifests primarily as variable dead-zone lengths. Taken together, our results suggest that L-selectin and P-selectin can be modeled as linear springs of respective spring constants of $-4$ and $-1$ pN/nm.

Dead-zone analysis

To identify what the dead zone may represent, its length distribution was characterized by histogram analysis (Fig. 10 A). All histograms exhibited a single peak for the four selectin-(s)PSGL-1 and -mAb complexes studied. The extracellular domain of P-selectin appears rod-like and measures 38 nm in length under electron microscopy (35), which predicts a 12-nm resting length for the L-selectin ectodomain. The linear length of an IgG is 16 nm. PSGL-1 also appears extended and measures 50 nm in length under electron microscopy (36). Since it was captured by PL2 at nearly the middle, the binding pocket of PSGL-1 should extend $\sim 41$ nm from the AFM tip (16 nm from the IgG and $\sim 25$ nm from where PL2 captured (s)PSGL-1) (cf. Fig. 1). Interestingly, the dead-zone length distribution for the longer molecular complex shifted rightward relative to that for the shorter molecular complex in both cases of P-selectin and
L-selectin (Fig. 10 A). Indeed, the mean dead-zone length (Fig. 10 B) and the most probable dead-zone length (i.e., the peak location) (Fig. 10 C) were found to correlate linearly with the total resting length of the molecular complex. Remarkably, the mean (and most probable) dead-zone lengths were nearly the same for the P-selectin-G1 complex and the L-selectin-PSGL-1 complex, which have nearly the same total resting length (54 and 53 nm, respectively, from the AFM tip to the bilayer) but are two very different systems. The maximum dead-zone length observed for any molecular system was never longer than the total resting length of that molecular complex (measured from the AFM tip to the bilayer) but are two very different systems. The maximum dead-zone length observed for any molecular system was never longer than the total resting length of that molecular complex (measured from the AFM tip to the bilayer). These combined data suggest that the dead zone arises from the fact that a molecule has to be picked up by its counter molecule, both of which have finite lengths. Further, the molecular complex has to be oriented and aligned along its long axis before it can resist tensile force that stretches it beyond its resting length. The highly variable dead-zone length (Figs. 8 and 10 A) can be explained as follows. The densities of selectins on the bilayer and (s)PSGL-1 or mAb on the AFM tip were kept low to ensure single molecular interactions. The average distance between two neighboring selectins on the bilayer was tens of nanometers, comparable to the size of the AFM tip, which on average had only an (s)PSGL-1/mAb capable of forming bonds with the selectin bilayer. As such, the experimenter could not always land an (s)PSGL-1/mAb right on top of a selectin, thereby yielding variable angular rotations of the AFM tip during noncoaxial alignment, resulting in broad distributions in the dead-zone length.

**Resistance to sudden unfolding**

For measurements with the thermal method, the P-selectin and L-selectin were subjected to respective holding forces (and elongations) as high as 50 pN (50 nm) and 150 pN (35 nm), respectively. The highest forces (and elongations) measured in the stretch method were even higher, ~200 pN (~200 nm) for P-selectin and ~250 pN (~60 nm) for L-selectin. Thus, the highest strain that P- and L-selectin experienced in our experiments was ~500%. Despite the high forces and high strains, we did not find any evidence of sudden protein unfolding, manifesting as an abrupt increase in molecular extension with a concurrent abrupt drop in force without dissociation of the selectin-(s)PSGL-1 (or -mAb) complex. By comparison, a number of studies have reported successive sudden unfolding of protein globular domains, e.g., titin (7,37,38), tenascin (5), fibronectin (39), and ubiquitin (40), manifesting as a sawtooth pattern in the force-extension curve. Such sudden unfolding was observed to
occur at comparable forces and at strains as low as 20% in both the constant-rate stretch mode and constant-force holding mode. The high level of resistance to sudden unfolding for the selectins may be due to the presence of six cysteines in each of their CR domains (41). These cysteines are predicted to form three intradomain disulfide bonds per CR domain, which have been shown to protect melanoma cell adhesion molecules from being unfolded by force (2). Additional resistance may come from the lectin domain, which has two disulfide bonds, and from the EGF domain, which has three disulfide bonds (41,42).

In summary, elasticity measurements of P- and L-selectin complexed with (s)PSGL-1 and mAbs support the validity of the theoretical analysis of mechanical responses of AFM cantilevers to thermal excitations (13), as values measured by the thermal fluctuation method that is based on this theory are comparable to those measured by the conventional stretch method. Our data suggest that selectins behave as linear springs with compliance proportional to their length, which are much greater than those of (s)PSGL-1 and IgG. They can sustain large forces and high strain and resist sudden unfolding under physiological forces. These properties may be important for the selectins, which function in a mechanically stressful environment.

**SUPPLEMENTARY MATERIAL**

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org.

*Note added in proof:* After this article was accepted, we became aware of a related article (43), which used a single-degree-of-freedom model to analyze the thermal fluctuations of an AFM cantilever coupled to a molecule. We thank Vincent Moy for providing the AFM design and training. We also thank John Slania and Jizhong Lou for assistance in the data collection and analysis.

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