Replacing a Lectin Domain Residue in L-selectin Enhances Binding to P-selectin Glycoprotein Ligand-1 but Not to 6-Sulfo-sialyl Lewis x

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Selectin-ligand interactions (bonds) mediate leukocyte rolling on vascular surfaces. The molecular basis for differential ligand recognition by selectins is poorly understood. Here, we show that substituting one residue (A108H) in the lectin domain of L-selectin increased its force-free affinity for a glycosulfopeptide binding site (2-GSP-6) on P-selectin glycoprotein ligand-1 (PSGL-1) but not for a sulfated-glycan binding site (6-sulfo-sialyl Lewis x) on peripheral node addressin. The increased affinity of L-selectinA108H for 2-GSP-6 was due to a faster on-rate and to a slower off-rate that increased bond lifetimes in the absence of force. Rather than first prolonging (catching) and then shortening (slipping) bond lifetimes, increasing force monotonically shortened lifetimes of L-selectinA108H bonds with 2-GSP-6. When compared with microspheres bearing L-selectin, L-selectinA108H microspheres rolled more slowly and regularly on 2-GSP-6 at low flow rates. A reciprocal substitution in P-selectin (H108A) caused faster microsphere rolling on 2-GSP-6. These results distinguish molecular mechanisms for L-selectin to bind to PSGL-1 and peripheral node addressin and explain in part the shorter lifetimes of PSGL-1 bonds with L-selectin than P-selectin.

Leukocytes tether to and roll on the vessel wall while homing to lymphoid tissues or to sites of inflammation. This dynamic form of cell adhesion involves rapid formation and breakage of bonds between the membrane-distal lectin domains of selectins and their cell-surface glycosylated ligands (1, 2). Leukocytes express L-selectin, whereas activated platelets express P-selectin and activated endothelial cells express P-selectin and E-selectin (1, 2). All selectins bind to glycans with terminal components that include α2–3-linked sialic acid and α1–3-linked fucose, typified by the sialyl Lewis x (sLe x)6 determinant (NeuAcα2–3Galβ1–4[Fucα1–3][GlcNAcβ1–R]). However, each selectin binds better to some glycoproteins modified with sLe x-capped glycans than to others, and P- and L-selectin bind preferentially to glycoproteins that are also sulfated (3). Although the amino acid sequences of the lectin domains are >60% identical, the molecular basis for differential ligand recognition by the selectins is largely unknown.

P- and L-selectin bind to the N-terminal region of P-selectin glycoprotein ligand-1 (PSGL-1), which is expressed on leukocytes (1, 2). The interacting residues on PSGL-1 include sulfated tyrosines, other amino acids, and fucose, sialic acid, and galactose presented on an sLe x determinant that caps a core 2 O-glycan (4–7). Despite these similarities, both the solution (three-dimensional) and the solid-state (two-dimensional) affinities for PSGL-1 are 10–25-fold higher for P-selectin than for L-selectin in the absence of force (6–8), which remains unexplained. L-selectin also binds to peripheral node addressin (PNAd), a group of mucins on high endothelial venules of lymph nodes. These mucins express N- and O-glycans capped with 6-sulfo-sLe x (also called sialyl 6-sulfo Le x), a form of sLe x with a sulfate ester attached to the C-6 position of GlcNAc (3, 9, 10). L-selectin binds to the 6-sulfo-sLe x determinant on PNAd but is not known to bind to the peptide backbones of these glycoproteins.

Blood flow exerts force on the selectin-ligand bonds that anchor rolling cells. At high flow rates, increasing force shortens bond lifetimes (slip bonds), which causes more rapid and less regular rolling. At low flow rates, increasing force paradoxically prolongs lifetimes (catch bonds) (11, 12), which causes less rapid and more regular (i.e. enhanced) rolling. Catch bonds govern flow-enhanced rolling (13), whereas transport mecha-

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nisms govern flow-enhanced tethering (14). Together, these mechanisms explain the shear threshold requirement for adhesion, which is especially pronounced for L-selectin (15). Substituting a single residue in the L-selectin EGF domain to increase the flexibility of a hinge with the lectin domain reduces the shear threshold by increasing tethering through greater rotational diffusion and by strengthening rolling through augmented catch bonds with longer lifetimes at smaller forces (16). Increased hinge flexibility enhances L-selectin interactions with both PSGL-1 and 6-sulfo-sLeX despite their different molecular structures. Here, we used molecular modeling to design a single-residue substitution in the lectin domain of L-selectin that reduced its shear threshold for adhesion to PSGL-1 but not to 6-sulfo-sLeX. These data provide one molecular explanation for differential ligand recognition by selectins and illustrate how topologically remote regions of selectins cooperatively regulate leukocyte adhesion under flow.

EXPERIMENTAL PROCEDURES

Proteins and Glycoconjugates—L-selectin-Ig containing the lectin domain, EGF domain, and all nine consensus repeats of human L-selectin fused to the Fc portion of human IgG1 was expressed and purified as described (13). P-selectin-Ig containing the lectin domain, EGF domain, and all nine consensus repeats of human P-selectin was prepared by similar methods. The cDNA encoding L-selectin was used as template to alter the codon for Ala-108 to His (108H), and the cDNA encoding P-selectin was used as template to alter the codon for His-108 to Ala (H108A), using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). The mutations, termed L-selectinA108H and P-selectinH108A, respectively, were confirmed by DNA sequencing. L-selectinA108H-Ig, P-selectin-Ig, and P-selectinH108A-Ig were expressed and purified as described for L-selectin-Ig. cDNAs encoding L-selectin and L-selectinA108H, each comprising the lectin and EGF domains followed by a C-terminal epitope tag (17), were amplified by PCR from the plasmids encoding L-selectin-Ig or L-selectinA108H-Ig, respectively, and verified by DNA sequencing. The amplified products were inserted into the pEE14.1 expression vector (Lonza Biologics, Portsmouth, NH) and transfected into Chinese hamster ovary-lyc1 cells. Clones with high expression were selected, and the soluble selectins were purified from conditioned medium by immunoaffinity chromatography (17). To ensure that the soluble selectins were monomeric, 100-μl samples (5 mg/ml) were applied to a Superdex 200 (HR 10/30) equilibrated in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1.5 mM CaCl2, 0.005% p20. Biotinylated 2-GSP-6, a control peptide (2-GP-4, a PSGL-1-derived peptide that lacks the sulfate esters and fucose residue required for binding to L-selectin), or 6-sulfo-sLeX was captured on a research grade, streptavidin-coated sensor chip that was pretreated according to the manufacturer's instructions. Each biotinylated ligand was injected over a separate sensor surface at 5 μl/min for 7–10 min. An additional control surface was mock-treated but received no injected biotinylated ligand. Pilot experiments determined optimal conditions with the lowest ligand density that detected specific binding of analyte but minimized rebinding of analyte after it dissociated.

Increasing concentrations of soluble, monomeric L-selectin or L-selectinA108H or of dimeric P-selectin-Ig or P-selectinH108A-Ig were perfused over the captured ligands at a high flow rate (60 μl/min) for a brief period (15 s) to favor monomeric binding. The short injection period was sufficient because equilibrium binding was reached within this interval. Specific binding was measured by subtracting nonspecific binding to control surfaces, using the in-line reference subtraction feature of the Biacore 3000. In experiments performed at 5 °C, bound selectins were removed by injecting 10 mM EDTA.

Equilibrium binding data were analyzed by nonlinear curve fitting of the Langmuir binding isotherms using the Biaevaluation software. Dissociation rate measurements were performed at a flow rate of 100 μl/min, and data were collected at 0.1-s intervals.

Coupling of Selectin-Ig to Microspheres and Selectin Ligands to Flow Chambers—Each selectin-Ig was captured on polystyrene microspheres (6-μm diameter, Polysciences, Warrington, PA) coated with anti-human Fc polyclonal antibody (13). Matched densities of each selectin were confirmed by flow cytometry (13). Biotinylated 2-GSP-6 or 6-sulfo-sLeX was cap-

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tured on streptavidin (Pierce) adsorbed to flow chamber floors (13).

Flow Assays—Microspheres (2 × 10⁶/ml in Hanks’ balanced salt solution containing 0.5% human serum albumin) were perfused at various flow rates over 2-GSP-6 or 6-sulfo-s-Le⁴ in a parallel-plate flow chamber. Images were captured with a Fastcam digital video camera (Super 10 K, Photron, Nashua, NH) at 250 frames/second (13). Instantaneous rolling velocities were measured by tracking individual microspheres frame-by-frame. Mean rolling velocities were calculated by averaging the instantaneous rolling velocities over 1 s. Rolling step analysis was performed on instantaneous rolling velocities with custom-designed macros prepared in Excel (Microsoft, Redmond, WA) (13). In some experiments, microspheres were perfused in medium containing 20 μg/ml DREG-56 or PL1 or 10 mM EDTA. All rolling events were specific because they were eliminated by inclusion of monoclonal antibody or EDTA in the medium.

Transient tether lifetimes were measured on low densities of 2-GSP-6 or 6-sulfo-s-Le⁴ that did not support rolling or skipping (13). Images captured at 250 frames/second were replayed in slow motion, and durations of transient tethers were measured using frame-by-frame analysis. For each mean lifetime curve, five sets of lifetimes at each wall shear stress (~100 tethering events in each set) were measured. At each wall shear stress, the exponentially distributed transient tether lifetimes in each set were averaged. The data are presented as mean ± S.D. of the five sets of average lifetimes.

AFM—Atomic force microscopy (AFM) was used to measure lifetimes of selectin-ligand bonds as described (11, 12), except that each selectin-Ig was captured on anti-human Fc antibody adsorbed on the Petri dish, and biotinylated 2-GSP-6 or 6-sulfo-s-Le⁴ was captured by adsorbed streptavidin on the cantilever tip. In our previous experiments, membrane P-selectin or L-selectin was reconstituted into a glass-supported lipid bilayer, which greatly reduced the nonspecific adhesion normally observed when the cantilever tip directly contacted glass or plastic surfaces (11, 12). To reduce nonspecific binding in the current experiments, the cantilever tip was allowed to contact the Petri dish for 0.02 s and then retracted 13 nm and held for 1 s to allow bond formation. The cantilever was then further retracted a predetermined distance (to set the constant force on the bond) for lifetime measurements. Suspending the cantilever tip 13 nm above the surface in the bond formation phase reduced nonspecific binding to <2% of the tests, whereas specific L-selectin-ligand binding was ~20% of the tests.

RESULTS

Using the crystal structure of a human P-selectin-PSGL-1 complex as template Protein Data Bank ID 1GIS (4), we docked an N-terminal glycosulfopeptide from human PSGL-1 to the lectin domain of human L-selectin. In the model of the L-selectin-PSGL-1 complex, the fucose of the O-glycan binds to the Ca²⁺ coordination site of L-selectin as it does to P-selectin, and the orientations of the bound glycan, sulfated tyrosines, and other peptide components of PSGL-1 are similar, although not identical, for P- and L-selectin (Fig. 1A). One notable difference is that P-selectin forms more atomic level interactions with a peptide segment of PSGL-1. Specifically, His-108 of P-selectin stacks through van der Waals contacts against Leu-8, Leu-13, and Pro-14 of PSGL-1 (Fig. 1A). In L-selectin, the short side chain of the corresponding Ala-108 is not predicted to make similar contacts with PSGL-1. The model predicts that a mutant L-selectin that replaces Ala-108 with His (L-selectinA108H) would bind better to this peptide region of PSGL-1 (Fig. 1, A and B) but would not bind differently to PNAd. To test the model, we expressed soluble recombinant forms of L-selectin and L-selectinA108H. As selectin ligands, we used the 6-sulfo-s-Le⁴ binding determinant from PNAd and a glycosulfopeptide termed 2-GSP-6, modeled after the N-terminal region of PSGL-1 (19) (Fig. 1C).

We used surface plasmon resonance to compare the three-dimensional kinetics and affinity of L-selectin and L-selectinA108H binding in the absence of force. Monomeric L-selectin or L-selectinA108H was flowed over 2-GSP-6 or 6-sulfo-s-Le⁴ captured on a sensor chip. Specific, saturable binding to both ligands was observed, as determined by comparison with binding to a control sensor surface. Fig. 2 illustrates the specific binding isotherms for a representative experiment. When compared with L-selectin, L-selectinA108H bound with 2.5-fold higher affinity to 2-GSP-6 (Fig. 2A and Table 1). In contrast, L-selectin and L-selectinA108H bind with indistinguishable and lower affinity to 6-sulfo-s-Le⁴ (Fig. 2B and Table 1). At 25 °C, L-selectin dissociated from 2-GSP-6 and 6-sulfo-s-Le⁴ with a faster off-rate (k.off) than the Biacore instrument can accurately measure (up to ~10 s⁻¹), in agreement with the previously observed fast dissociation of L-selectin from the PNAd mucin GlyCAM-1 (21). To obtain measurable off-rates for L-selectin, we reduced the temperature in the instrument to 5 °C, which slows on- and off-rates but does not change the equilibrium affinity. In a representative experiment, the off-rate for dissociating from 2-GSP-6 was slower for L-selectinA108H than for L-selectin (5.0 versus 7.0 s⁻¹) (Fig. 2C). The differences in these values were reproducible and statistically significant (Table 1). Taking the experimentally measured Kₘ and off-rate values at 5 °C, the calculated on-rate (k.on) for binding to 2-GSP-6 was 1.5 × 10⁷ M⁻¹ s⁻¹ for L-selectin and 2.7 × 10⁵ M⁻¹ s⁻¹ for L-selectinA108H at 5 °C (Table 1). Thus, when compared with L-selectin, L-selectinA108H binds with higher affinity to 2-GSP-6 because of a slower off-rate and a faster on-rate. The Biacore instrument can accurately measure the slower rate that P-selectin dissociates from PSGL-1 at 25 °C (22). To determine the extent that temperature affects bond kinetics, we measured binding of P-selectin to PSGL-1 at both 25 °C and 5 °C. Lowering the temperature reduced the off-rate by ~40% and the on-rate by ~10% (Table 1). At 5 °C, P-selectin had a faster on-rate, slower off-rate, and higher affinity for 2-GSP-6 than either L-selectin or L-selectinA108H (Table 1). Although the absolute on- and off-rates will increase at physiological temperature, the relative rates will not change. The data therefore provide accurate comparisons of the binding kinetics of each selectin for the same ligand.

We used two independent methods, flow chamber experiments and AFM, to measure how force affected the lifetimes of bonds between L-selectin or L-selectinA108H and their ligands. In the flow chamber experiments, microspheres bear-
The lifetimes of transient tethers were measured by high speed video microscopy. In the AFM experiments, bonds between L-selectin or L-selectinA108H on a Petri dish and 2-GSP-6 or 6-sulfo-sLex on a cantilever tip were stressed by lifting the cantilever to allow lifetime measurements at various levels of constant force. A large number of lifetime measurements were used to derive the mean lifetime (which equals the reciprocal off-rate $1/k_{off}$ for first-order dissociation of single bonds) for each interaction at each wall shear stress (for the flow chamber) or each tensile force level (for AFM). As observed previously for L-selectin–PSGL-1 bonds (12, 13, 16), the lifetimes of L-selectin–2-GSP-6 bonds exhibited a biphasic pattern characteristic of transitions from catch to slip bonds (Fig. 3, A and C). As force increased, mean lifetimes increased until an optimal value was reached; further increases in force progressively shortened lifetimes. The effects of force on lifetimes of bonds between

![Model of L-selectin binding to PSGL-1](image)

**FIGURE 1.** Model of L-selectin binding to PSGL-1. A, left, crystal structure of the lectin domain of P-selectin complexed with an N-terminal glycosulfopeptide generated from the sequence of PSGL-1 (from Ref. 4) (Protein Data Bank ID 1G1S). Middle, model of the lectin domain of L-selectin complexed with the glycosulfopeptide. Right, model of the lectin domain of L-selectinA108H complexed with the glycosulfopeptide. The upper row shows lower magnification views of binding of the entire glycosulfopeptide to each lectin domain. A golden sphere represents a Ca$^{2+}$ ion (or a strontium ion that was substituted for Ca$^{2+}$ in the P-selectin-PSGL-1 complex). This ion is coordinated by residues in the lectin domain and by a fucose in the O-glycan attached to the PSGL-1 peptide. The boxed regions highlight interactions of each lectin domain with selected peptide components of PSGL-1 that are magnified in the lower row. Leu-8, Leu-13, and Pro-14 are in the glycosulfopeptide. His-108 or Ala-108 is in the lectin domain. B, alignment of the amino acid sequences of the lectin domains surrounding residue 108 (boxed), where a substitution was made to generate L-selectinA108H. C, schematics of glycoconjugates used as selectin ligands. The glycosulfopeptide 2-GSP-6 was sequenced based on the N-terminal region of PSGL-1. A C-terminal cysteine was introduced to enable coupling of a single biotin by a spacer group (not shown). The 6-sulfo-sLe$^{x}$ is a glycan determinant on O-glycans attached to PNAd. A spacer group links a biotin to the glycan.
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L-selectinA108H and 2-GSP-6 differed dramatically from those between L-selectin and 2-GSP-6 (Fig. 3, A and C). Catch bonds were changed to slip bonds as lifetimes were prolonged at very low forces and monotonically decreased with increasing force. The mean lifetime versus force curve for L-selectinA108H merged with that for L-selectin as force increased above the optimal level where L-selectin catch bonds transition to slip bonds. Therefore, the residue substitution in L-selectinA108H selectively altered the catch-bond regime with 2-GSP-6. In marked contrast, interactions of L-selectin and L-selectinA108H with 6-sulfo-sLe\(^\text{x}\) exhibited equivalent transitions from catch to slip bonds as force increased (Fig. 3, B and D). These results demonstrate that the substitution in L-selectinA108H altered the force-dependent kinetics of L-selectin bonds in a ligand-specific manner as predicted by the structural model.

To determine whether prolonging bond lifetimes at low forces affected the shear threshold for L-selectin-dependent rolling, we perfused microspheres bearing each selectin over higher densities of 2-GSP-6 or 6-sulfo-sLe\(^\text{x}\). Rolling motions were monitored by high speed video microscopy as a function of wall shear stress. Rolling consists of cycles of acceleration and deceleration, with some decelerations resulting in stops in which the instantaneous velocity reduces to zero (13). On both ligands, the mean rolling velocities of L-selectin microspheres first decreased, reached a minimum, and then increased, a characteristic of the shear threshold phenomenon (Fig. 4, A and B). At very low wall shear stress, rolling motions were highly irregular. Rolling motions became more regular as shear stress reached the optimal value where mean rolling velocities were lowest, and they became irregular again as wall shear stress increased further. More regular rolling and slower mean rolling velocities were associated with longer mean stop times (Fig. 4, C and D) and higher fractions of time in the stop phase (Fig. 4, E and F). Rolling on 6-sulfo-sLe\(^\text{x}\) was indistinguishable for microspheres bearing L-selectinA108H or L-selectin at all wall shear stresses by all metrics used (Fig. 4, B, D, and F), which correlates with the indistinguishable force-dependent off-rates for each selectin dissociating from 6-sulfo-sLe\(^\text{x}\) (Fig. 3, B and D). Remarkably, there was no shear threshold for rolling of L-selectinA108H microspheres on 2-GSP-6. Instead, mean rolling velocities were lower at low wall shear stresses and gradually became higher as wall shear stress increased (Fig. 4A). At wall shear stresses that were suboptimal for rolling of L-selectin microspheres on 2-GSP-6, rolling of L-selectinA108H micro-

### TABLE 1

| Selectin        | Ligand           | Temperature | \(K_d\) (\(\mu M\)) | \(k_{\text{off}}\) (\(s^{-1}\)) | \(k_{\text{on}}\) (\(M^{-1}s^{-1}\)) |
|-----------------|------------------|-------------|----------------------|-------------------------------|-----------------------------------|
| L-selectin      | 6-sulfo-sLe\(^\text{x}\) | 25          | 109 ± 12 (\(n = 6\)) | Not measurable                |                                   |
| L-selectin      | 6-sulfo-sLe\(^\text{x}\) | 25          | 97 ± 5 (\(n = 3\))  | Not measurable                |                                   |
| L-selectin      | 6-sulfo-sLe\(^\text{x}\) | 25          | 47 ± 8 (\(n = 7\))  | Not measurable                |                                   |
| L-selectinA108H | 2-GSP-6          | 5           | 46 ± 6 (\(n = 4\))  | 7.0 ± 1.0 (\(n = 8\))         | 1.5 × 10^3                       |
| L-selectinA108H | 2-GSP-6          | 25          | 19 ± 1 (\(n = 6\))  | Not measurable                |                                   |
| L-selectin      | 2-GSP-6          | 25          | 17,20 (\(n = 2\))   | 5.0 ± 1.0 (\(n = 8\))         | 2.7 × 10^2                       |
| L-selectin      | 2-GSP-6          | 5           | 1.5 ± 0.4 (\(n = 3\))| 1.4 ± 0.1 (\(n = 3\))\(^a\) | 9.3 × 10^2                       |
| P-selectin      | 2-GSP-6          | 25          | 0.9 ± 0.2 (\(n = 3\))| 0.8 ± 0.2 (\(n = 5\))\(^a\) | 9.0 × 10^2                       |
| P-selectin      | 2-GSP-6          | 5           |                      |                               |                                   |

\(^a\) \(p < 0.0001\).

\(^b\) \(p < 0.001\).

\(^c\) \(p < 0.01\) by unpaired Student \(t\)-test.
spheres was more regular, exhibiting higher mean stop times and greater fractions of time in the stop phase that monotonically decreased with increasing wall shear stress (Fig. 4, C and E). The distinctly different rolling of L-selectin or L-selectinA108H microspheres on 2-GSP-6 also correlates with the distinctly different force-dependent off-rates for dissociation of each selectin from 2-GSP-6 (Fig. 3, A and C). Thus, the amino acid substitution in L-selectinA108H lifted both the shear threshold requirement for rolling and the force-dependent off-rate in a ligand-specific manner, further supporting the causal relationship between rolling and off-rate (13, 16).

The A108H substitution in L-selectin was designed according to crystallographic evidence that His-108 of P-selectin interacted with peptide components of PSGL-1 (4) (Fig. 1A). To further test the importance of this residue, we made a reciprocal P-selectin construct in which the natural His-108 was replaced with Ala. As measured by surface plasmon resonance, the respective affinities of P-selectin-Ig and P-selectinH108A-Ig for 2-GSP-6 were 159 ± 1 and 467 ± 30 nM (mean ± range, n = 2). These values for binding of dimeric Ig chimeras do not represent the actual affinities of monomeric selectins for 2-GSP-6. Nevertheless, they demonstrate that the H108A substitution reduced the affinity of P-selectin for PSGL-1 as predicted by the structural model. We captured P-selectin-Ig and P-selectinH108A-Ig on microspheres at matched densities and compared their ability to roll on 2-GSP-6 under flow. When compared with microspheres bearing P-selectin, those bearing P-selectinH108A rolled more rapidly on 2-GSP-6 at all wall shear stresses examined (Fig. 5). Thus, substituting histidine for alanine at residue 108 of L-selectin augments adhesion,
whereas substituting alanine for histidine at residue 108 of P-selectin impairs adhesion.

**DISCUSSION**

We have demonstrated that substituting a single residue in the lectin domain of L-selectin profoundly affects its interaction with one physiologically relevant ligand but not with another. When compared with L-selectin, L-selectinA108H bound with higher three-dimensional affinity to 2-GSP-6, a glycosulfopeptide that recapitulates the binding site on PSGL-1. The force-free measurements provide support for a model in which PSGL-1 docks to a similar surface on P-selectin and L-selectin (Fig. 1A). The prolonged lifetimes of L-selectinA108H bonds with 2-GSP-6 suggest that stacking His-108 on peptide components of PSGL-1 provides additional atomic level interactions, analogous to those documented between His-108 of P-selectin and specific amino acids of PSGL-1 (4). By contrast, L-selectin and L-selectinA108H bound equivalently to 6-sulfosLe^\text{a}^, which recapitulates the binding site on PNAd. The higher affinity of L-selectin and L-selectinA108H for 2-GSP-6 than for 6-sulfosLe^\text{a}^ (Table 1) is consistent with cooperative binding to sulfated tyrosines, other amino acids, and glycan components of PSGL-1. The substitution in L-selectinA108H augmented its affinity for 2-GSP-6 by increasing on-rate as well as decreasing off-rate. P-selectin, however, bound to 2-GSP-6 with even higher affinity, faster on-rate, and slower off-rate, both in three-dimensional interactions measured by surface plasmon resonance (22) (Table 1) and in two-dimensional interactions measured by thermal fluctuations (8). Therefore, other amino acid differences between P-selectin and L-selectin must contribute to their distinct affinities for PSGL-1. Reducing the temperature only modestly slowed the on- and off-rates of P-selectin with 2-GSP-6, which could be accurately measured at both temperatures in the Biacore instrument. This suggests that the off-rate of L-selectin dissociating from 2-GSP-6 at 25 °C was only slightly faster than the measured off-rate at 5 °C (≈7 s\textsuperscript{−1}), putting it just beyond the measurable limit of ≈10 s\textsuperscript{−1}. Supporting this hypothesis, the higher temporal resolution of the thermal fluctuation method determined the off-rate for L-selectin dissociating from PSGL-1 to be ≈10 s\textsuperscript{−1} at 25 °C (8).

The A108H substitution in L-selectin dramatically altered the force-dependent kinetics of its interaction with 2-GSP-6 (but not with 6-sulfosLe^\text{a}^). Although the structural basis of catch bonds between L-selectin and PSGL-1 requires further study, our data suggest that increasing force progressively creates new atomic level contacts that substitute for and may be close to where His-108 in P-selectin and L-selectinA108H docks with PSGL-1. These putative new contacts are probably with peptide rather than glycan components of PSGL-1. In L-selectinA108H, the stronger interactions that yielded longer lifetimes at zero force obviated a requirement for force to enhance the overall interaction, turning catch bonds to slip bonds. At higher forces, lifetimes of L-selectin and L-selectinA108H bonds with 2-GSP-6 were identical, indicating that the putative new contacts for L-selectinA108H, should they still form, no longer contributed to overall bond lifetimes. In support of this hypothesis, molecular dynamics simulations revealed that His-108 of P-selectin separated from Leu-8, Leu-

13, and Pro-14 of PSGL-1 before complete dissociation of the P-selectin-PSGL-1 complex (23). Because the force-dependent off-rate was altered at low forces, L-selectinA108H microspheres rolled slowly and with regular motions on 2-GSP-6 at low wall shear stresses that supported only rapid and irregular rolling of L-selectin microspheres. By contrast, the equivalent force-dependent off-rates at higher forces resulted in similar rolling velocities and regularities for L-selectin and L-selectinA108H microspheres at higher wall shear stresses. The reciprocal substitution in P-selectinH108A accelerated rolling velocities, reinforcing the importance of His-108 for optimal interactions with PSGL-1.

Because L-selectin and L-selectinA108H formed similar catch bonds with 6-sulfosLe^\text{a}^, there must be other mechanisms by which force enhances interactions with this ligand, which has no peptide components. Interactions of L-selectin with 2-GSP-6 (and PSGL-1) might share these mechanisms. One such mechanism involves alterations in the hinge between the lectin and EGF domains. Increasing hinge flexibility augments catch bonds of L-selectin with both PSGL-1 and 6-sulfosLe^\text{a}^ by increasing lifetimes at lower forces (16). Increased hinge flexibility may reduce the force required to open the interdomain angle, allowing sliding of both ligands across the interface with L-selectin to promote rebinding events (16, 23). Alterations in the interdomain angle might also propagate conformational changes to the ligand-binding surface (24); such changes might influence binding to one ligand but not to another. Taken together, these data demonstrate that residues in topologically distinct regions of selectins influence ligand binding in the presence or absence of force. Dissecting the details of these cooperative interactions will further illuminate how selectins engage particular ligands to mediate cell adhesion under the kinetic and mechanical constraints of flowing blood.

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