P-Selectin Must Extend a Sufficient Length from the Plasma Membrane to Mediate Rolling of Neutrophils

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Abstract. Under physiological shear stress, neutrophils roll on P-selectin on activated endothelial cells or platelets through interactions with P-selectin glycoprotein ligand-1 (PSGL-1). Both P-selectin and PSGL-1 are extended molecules. Human P-selectin contains an NH_2-terminal lectin domain, an EGF domain, nine consensus repeats (CRs), a transmembrane domain, and a cytoplasmic tail. To determine whether the length of P-selectin affected its interactions with PSGL-1, we examined the adhesion of neutrophils to CHO cells expressing membrane-anchored P-selectin constructs in which various numbers of CRs were deleted. Under static conditions, neutrophils attached equivalently to wild-type P-selectin and to constructs containing from 2–6 CRs. Under shear stress, neutrophils attached equivalently to wild-type and 6 CR P-selectin and nearly as well to 5 CR P-selectin. However, fewer neutrophils attached to the 4 CR construct, and those that did attach rolled faster and were more readily detached by increasing shear stress. Flowing neutrophils failed to attach to the 3 CR and 2 CR constructs. Neutrophils attached and rolled more efficiently on 4 CR P-selectin expressed on glycosylation-defective Lec8 CHO cells, which have less glycocalyx. We conclude that P-selectin must project its lectin domain well above the membrane to mediate optimal attachment of neutrophils under shear forces. The length of P-selectin may: (*a*) facilitate interactions with PSGL-1 on flowing neutrophils, and (*b*) increase the intermembrane distance where specific bonds form, minimizing contacts between the glycocalyces that result in cell–cell repulsion.

CELL–CELL adhesion is fundamental for diverse biological processes such as development, immune responses, and wound healing. Many factors influence the efficiency of cell–cell contact. These include the densities of adhesion receptors, the kinetics of association and dissociation between adhesion molecules, the topographies of the cell surfaces, the lengths and lateral mobilities of adhesion receptors, and the repulsive forces between cells (6).

The recruitment of leukocytes from the circulation into lymphatic tissues and inflammatory sites requires the coordinated expression of adhesion and signaling molecules (49). Leukocytes rapidly form rolling, transient adhesive contacts with the endothelial cell surface under the shear stresses in postcapillary venules. The cells then form more stable contacts, spread, and finally emigrate between the endothelial cells into the underlying tissues. In most cases, the initial rolling of leukocytes on the vessel wall is mediated by interactions between selectins and their cell surface carbohydrate ligands (36). This dynamic cell adhesion under shear stress requires very rapid formation and dissociation of selectin–ligand bonds that have high tensile strength (1, 23, 55).

Each of the three selectins has an NH_2-terminal carbohydrate-recognition domain characteristic of Ca^{2+}-dependent lectins, followed by an EGF-like domain, a series of consensus repeats (CRs)[1] related to those in complement-regulatory proteins, a transmembrane domain, and a cytoplasmic tail (36). L-selectin, expressed on leukocytes, binds to constitutively expressed ligands on the surface of high endothelial venules of lymph nodes and to inducible ligands on endothelium at sites of inflammation. E-selectin, expressed transiently on cytokine-activated endothelial cells, binds to ligands on myeloid cells and subsets of lymphocytes. P-selectin, stored in the membranes of secretory granules of platelets and endothelial cells, is rapidly redistributed to the cell surface in response to agonists such as

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1. *Abbreviations used in this paper*: C4BP, C4b-binding protein; CR, consensus repeat; LFA-3, lymphocyte function-associated antigen-3; PSGL-1, P-selectin glycoprotein ligand-1.

thrombin, where it binds to ligands on myeloid cells and subsets of lymphocytes.

The NH_2-terminal lectin domain of each selectin interacts with carbohydrate ligands (36), although the affinity and specificity of the interaction is modulated by the adjacent EGF domain and perhaps by one or more of the CRs (29, 34, 44). The three human selectins differ in length because they have different numbers of CRs: two in L-selectin, six in E-selectin, and nine in P-selectin (9, 26, 53). Hydrodynamic and electron microscopic analysis indicate that the longest molecule, P-selectin, is a rigid, highly extended structure that projects the lectin domain ≈38 nm from the cell surface (56). E-selectin is also a rigid, asymmetric molecule that extends ≈27 nm from the cell surface (25, 37).

The selectins bind with low affinity to small sialylated and fucosylated oligosaccharides such as the tetrasaccharide sialyl Lewis x (36, 57). However, selectins bind more avidly to carbohydrates presented on a small number of specific glycoprotein ligands, suggesting that they serve as the physiologically relevant cell surface ligands for selectins under shear forces (5, 8, 30, 33, 37, 39, 41, 45, 51, 58). This function has been established for only one of these molecules, P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is a type I membrane protein with two identical 120-kD disulfide-linked subunits (39, 45). Each subunit contains many clustered O-linked glycans, including poly-*N*-acetyllactosamine terminating in the sialyl Lewis x structure (37, 41). Purified PSGL-1 binds to E-selectin as well as to P-selectin (3, 33, 37, 45), although the binding characteristics are not identical (37, 42). Two IgG mAbs to human PSGL-1, termed PL1 and PL2, have been developed (38). PL1, but not PL2, inhibits binding of purified PSGL-1 to P-selectin. Furthermore, PL1 blocks adhesion of neutrophils to P-selectin surfaces under both static and shear conditions (38). These data indicate that PSGL-1 must interact with P-selectin in order for neutrophils to roll on P-selectin at physiologically relevant shear stress.

Based on the measured length of another sialomucin, CD43 (14), PSGL-1 can be modeled as a highly extended structure, with the extracellular portion of each subunit extending nearly 60 nm from the cell surface (36). Thus, both P-selectin and its ligand, PSGL-1, appear to be long, relatively rigid molecules. The lectin domain of P-selectin is located at the NH_2 terminus, farthest from the cell surface. The epitope on PSGL-1 for the inhibitory mAb, PL1, is located more distally from the membrane than the epitope for the noninhibitory mAb, PL2. This suggests that the critical recognition site for P-selectin also lies relatively far from the membrane (38). These data imply that the recognition domains of P-selectin and PSGL-1 project far above the cell surface to facilitate rapid interactions under shear forces. PSGL-1 is also concentrated on the tips of microvilli, the probable sites of initial cell contact (38).

To address whether the length of P-selectin affected its interactions with cell surface PSGL-1, we examined the adhesion of neutrophils to CHO cells expressing membrane-anchored P-selectin constructs in which various numbers of CRs were removed. Under static conditions, neutrophils attached normally to constructs with as few as 2 CRs. Under shear forces, however, neutrophils attached poorly to P-selectin with fewer than 5 CRs. Neutrophils rolled normally on a 6 CR chimera in which 4 CRs from P-selectin were replaced with 4 CRs from another protein. Extension of the ligand-binding domain of P-selectin above the cell surface may enhance contacts with PSGL-1 on flowing neutrophils, and increase the intermembrane distance where specific bonds form, minimizing cell–cell repulsion.

Materials and Methods

Antibodies

The anti-human P-selectin mAbs S12, W40, and G1 (all $IgG_{1-\kappa}$) were prepared and characterized as previously described (20, 27, 35). G1 binds to an epitope in the lectin domain of P-selectin (21) and blocks binding of P-selectin to leukocytes (20, 22) and to PSGL-1 (37).

Cells

Normal CHO cells (CHO-K1), obtained from the American Type Culture Collection (Rockville, MD), and mutant Lec8 CHO cells, a generous gift from Dr. Richard Cummings (University of Oklahoma Health Sciences Center), were maintained in α-MEM (GIBCO BRL) supplemented with 10% FBS (GIBCO BRL), 4 mM glutamine, 200 U/ml penicillin, and 200 μg/ml of streptomycin under 5% CO_2 at 37°C.

cDNA Constructs

The cDNA clone encoding full-length P-selectin (26) was excised from the vector pIBI20 (IBI Laboratories) and cloned into the expression vector pRc/RSV (Invitrogen, San Diego, CA) at the BstX1 site. P-selectin constructs with deletions of various numbers of CRs were prepared by an overlap extension PCR protocol as previously described (17). The cDNA clone encoding human C4BP (13) was a generous gift from Dr. Björn Dahlbäck (University of Lund). Overlap extension PCR was used to construct a P-selectin/C4BP chimera in which CRs 3–6 in the 6 CR P-selectin construct were replaced with CRs 2–5 from C4BP. All constructs were inserted into the pRc/RSV expression vector. The sequences of PCR-derived cassettes and their flanking regions were confirmed in all constructs before use.

Transfections

CHO-K1 or Lec8 CHO cells were transfected with P-selectin constructs using Lipofectamine (GIBCO BRL) (38, 42). Several dozen stably-transfected clones expressing various densities of each construct at confluence were selected (38, 42). A portion of the cells from each clone was frozen. In addition, several clones expressing each construct at various matched densities were continuously maintained for use in adhesion experiments.

Western Blots

Confluent CHO cells in 35-mm dishes were washed three times with HBSS without calcium or magnesium, scraped into the same buffer, and pelleted at 300 *g* for 5 min. The cell pellets were lysed in 100 μl of 0.1% Triton X-100, 1 mM PMSF in TBS, pH 7.5, and kept on ice for 30 min. The cell nuclei were removed by centrifugation at 14,000 *g* for 10 min. The supernatants were resolved by SDS-PAGE under nonreducing conditions, transferred to an Immobilon P membrane, and probed with mAb G1. Bound antibody was detected using an HRP-conjugated goat anti-mouse antibody with enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL).

Site Density Determinations

Site densities of constructs on confluent transfected CHO cells were determined using ^{125}I-labeled mAb G1 as previously described (38, 42). Site densities for each construct were always performed on the day prior to, or the day of, each cell adhesion experiment (38, 42). These measurements were used to select confluent CHO cell monolayers that were plated under identical conditions as the cells used for site density measurements. Thus, site densities were performed in parallel for every cell adhesion experiment.

Adhesion of Neutrophils to P-Selectin Constructs under Static Conditions

Human neutrophils were isolated as previously described (60). Transfected CHO cells were grown to confluence in 24- or 48-well dishes. The monolayers were washed once with HBSS and then neutrophils (250 μl, 5×10^5 cells) in HBSS/0.5% human serum albumin were added. After 15 min at 37°C, nonadherent neutrophils were removed by gentle washing, and the number of adherent neutrophils was quantitated using a myeloperoxidase assay (20).

Adhesion of Neutrophils to P-Selectin Constructs under Flow Conditions

Fluid shear stresses present in the microvasculature were simulated in a parallel-plate flow chamber as previously described (28, 38, 42). Neutrophils (10^6/ml) in HBSS/0.5% human serum albumin were perfused through the chamber at the desired wall shear stress. Neutrophil rolling was allowed to equilibrate for 4 min prior to data acquisition. Neutrophil interactions were visualized with a 40× objective (field of view of 0.032 mm^2) using phase-contrast video microscopy. Interactions were quantified using a computer imaging system (Sun Microsystem, Mountain View, CA; Inovision, Durham, NC). The number of adherent or rolling neutrophils was measured by digitizing image frames (28). Rolling velocities were measured as previously described (28). The resistance to detachment of neutrophils was determined by allowing neutrophils to adhere to the surface under static conditions. Flow was initiated at 0.25 dyn/cm^2 for 30 s to remove loosely adherent cells, and the number of adherent cells remaining was taken to be 100% bound. The wall shear stress was then increased incrementally every 30 s and the number of neutrophils remaining adherent was determined (42). All experiments were performed at 22°C unless otherwise indicated.

Results

Neutrophils Attach to Shorter Constructs of P-Selectin under Static Conditions But Not under Shear Forces

To address whether P-selectin required a minimal length to mediate adhesion of neutrophils to cell surfaces, we made a series of membrane-anchored constructs in which various number of CRs were deleted (Fig. 1 *A*). Constructs with 2–6 CRs, designated 2 CR through 6 CR, as well as wild-type P-selectin expressing all nine CRs, were stably transfected into CHO cells. Clones expressing each construct at a variety of site densities were isolated. Western blot analysis with G1, a mAb that binds to the lectin domain, indicated that each expressed construct had an apparent molecular mass consistent with its predicted size (Fig. 1 *B*). The mAbs W40 and S12 bound only to constructs with at least 5 CRs and 6 CRs, respectively (data not shown).

Under static conditions, neutrophils attached equivalently to all constructs even at low densities (24–73 sites/μm^2) (Fig. 2). Adhesion to each construct was completely inhibited by the anti-P-selectin mAb G1. Thus, each construct retained its ligand-binding function, consistent with previous observations that soluble P-selectin-Ig chimeras with only one or 2 CRs bound to myeloid cells (2, 18).

Under shear stresses characteristic of postcapillary venules, neutrophils attached and rolled on CHO cells expressing wild-type P-selectin (Fig. 3 *A*). Attachment and rolling were abolished by inhibitory mAbs to P-selectin or to PSGL-1, as previously demonstrated (38). At comparable site densities, neutrophils attached equivalently to 6 CR and wild-type P-selectin at shear stresses of 2 dyn/cm^2 (Fig. 3 *A*) or 4 dyn/cm^2 (data not shown). Equivalent numbers of neutrophils also attached to the 5 CR construct at 2 dyn/cm^2 (Fig. 3 *A*), but slightly fewer cells attached at 4 dyn/cm^2 (data not shown). In marked contrast, neutrophils attached and rolled on 4 CR P-selectin only at high site densities (Fig. 3 *B*). Neutrophils failed to attach to the 3 CR and 2 CR constructs (Fig. 3 *C*), even at high site densities (see Fig. 7 *A*). A similar hierarchy of attachment efficiencies was observed when the experiments were performed at 4°C (data not shown). These results indicate that P-selectin requires a minimal number of CRs to mediate optimal attachment of neutrophils to the CHO cell surface under shear stresses, but not under static conditions.

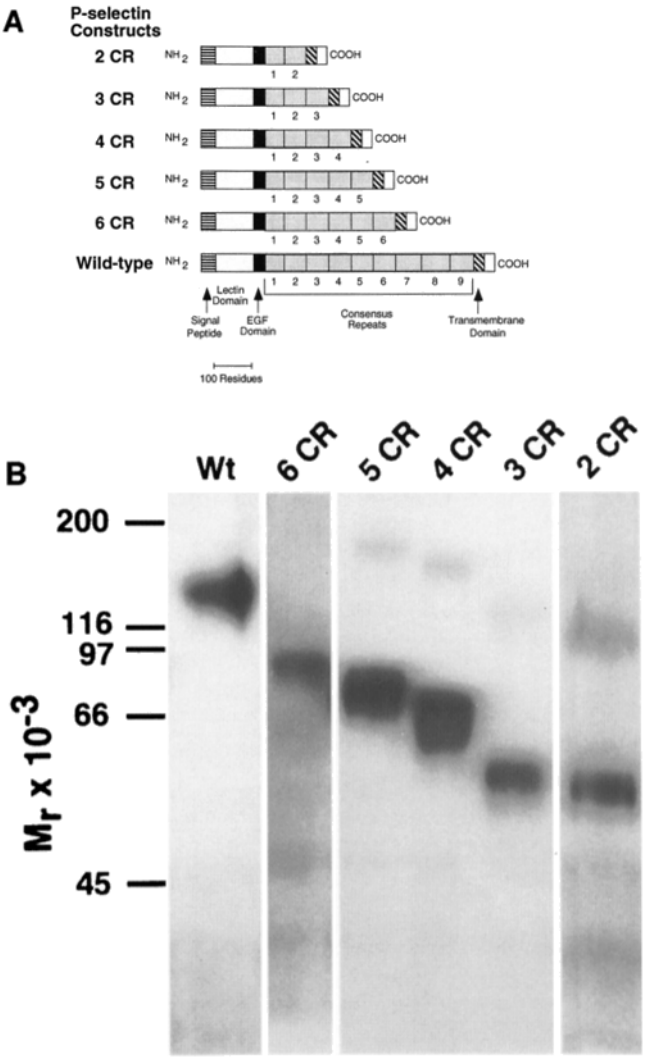


Figure 1. Human P-selectin constructs. (*A*) Schematic diagrams of wild-type P-selectin, which has 9 CRs, and deletion constructs containing from 2–6 CRs. In each construct, the most COOH-terminal CRs were deleted. (*B*) Western blot analysis of wild-type P-selectin and the deletion constructs. Lysates of transfected CHO cells expressing each construct were resolved by SDS-PAGE under nonreducing conditions and then probed with G1, a mAb that binds to an epitope in the lectin domain of P-selectin. The minor slower migrating bands in each lane are dimers of the expressed proteins. Dimerization of P-selectin has been previously observed following SDS-PAGE (35, 39, 56); it is most obvious with membrane-anchored forms of P-selectin that are electrophoresed under nonreducing conditions.

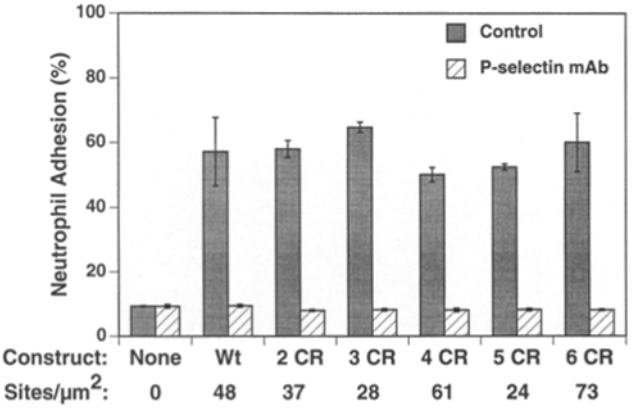


Figure 2. Neutrophils attach to both long and short constructs of P-selectin under static conditions. CHO cells expressing each construct at the indicated site density were grown to confluence in 24-well dishes. The indicated wells were preincubated with 10 μg/ml of the blocking anti-P-selectin mAb G1, and the same concentration of G1 was present in the neutrophil suspension during the adhesion assay. Static adhesion of neutrophils was measured after a 15-min incubation at 37°C. The number of adherent cells is presented as a percentage of the cells added to each well. Each point represents the mean ± range of duplicate determinations and is representative of at least three experiments.

Neutrophils Roll Faster on Shorter Constructs of P-Selectin

We next examined the rolling velocities of neutrophils on the P-selectin constructs as a measure of their adhesive strength. The velocities of 50 randomly selected neutrophils rolling on each construct at comparable site densities were determined. As previously observed (42), neutrophils rolled at heterogeneous velocities on CHO cells expressing wild-type P-selectin, and rolled more rapidly when P-selectin was expressed at lower density (Fig. 4, compare *A* and *B*). At matched densities, neutrophils rolled at a similar range of velocities on wild-type and 6 CR P-selectin (Fig. 4 *A*), but rolled more rapidly on 5 CR P-selectin than on wild-type P-selectin (Fig. 4 *B*). The few neutrophils that attached to the 4 CR construct rolled much faster than on the longer constructs (Fig. 4 *A*).

Neutrophils Attached to Shorter Constructs of P-Selectin Are Readily Detached by Shear Stress

As an alternative measure of adhesive strength, we allowed neutrophils to settle on the transfected CHO cells in the absence of flow, then subjected the adherent neutrophils to increasing wall shear stresses. Similar numbers of neutrophils attached to all constructs at both 22° and 4°C (data not shown). Upon initiation of flow, the neutrophils began to roll, and the percentage of rolling cells that remained adherent was measured as a function of wall shear stress. Neutrophils adherent to wild-type and 6 CR P-selectin at comparable site densities exhibited similar resistance to detachment by shear stress (Fig. 5 *A*). Neutrophils adherent to 5 CR P-selectin detached at slightly lower shear forces. In contrast, significantly lower shear stresses detached neutrophils from the 4 CR construct, and shear stresses as low as 0.5 dyn/cm^2 rapidly detached neutrophils from the 3 CR and 2 CR constructs (Fig. 5 *B*). At each wall shear stress, the average rolling velocities of those cells still attached to the 4 CR, 3 CR, and 2 CR constructs were much more rapid than those of the longer constructs (data not shown). Thus, neutrophils attached with much less adhesive strength to the shorter constructs.

Neutrophils Attach and Roll Normally on a 6 CR Construct That Replaces CRs 3–6 of P-Selectin with 4 CRs from C4BP

The above results indicate that 5 CR P-selectin is slightly less capable of mediating neutrophil attachment and rolling than wild-type and 6 CR P-selectin. The 4 CR has significant functional defects, and the 3 CR and 2 CR constructs cannot mediate neutrophil attachment and rolling even at high densities. To determine whether CRs 3–6 are specifically required for optimal neutrophil attachment under shear forces, we prepared the 6 CR chimera construct, in which CRs 3–6 in 6 CR P-selectin were replaced with CRs 2–5 from human C4BP (Fig. 6 *A*). We chose C4BP because its CRs are structurally related to those in the selectins (13, 26). Furthermore, rotary-shadowed electron micrographs indicate that the CRs of C4BP assemble as rigid, elongated structures like those in P-selectin (15). Western blot analysis with mAb G1 indicated that CHO cells permanently transfected with the chimeric construct expressed a protein consistent with its predicted size (Fig. 6 *B*).

Neutrophils attached and rolled equivalently on the 6 CR chimera, the 6 CR construct, and wild-type P-selectin at comparable site densities (Fig. 7 *A*). In contrast, neutrophils failed to attach to the 2 CR construct. Similar shear stresses detached neutrophils that adhered under static conditions to the 6 CR chimera, the 6 CR construct, and wild-type P-selectin (Fig. 7 *B*). Neutrophils also rolled at similar velocities on all three constructs (data not shown). Equivalent attachment and velocity profiles were observed at lower matched site densities of all three constructs (data not shown). These results indicate that CRs 3–6 are not specifically required for neutrophils to attach and roll on P-selectin under shear stresses. Instead, the CRs of P-selectin, or of another protein, appear to function by projecting the lectin domain farther from the cell surface.

More Neutrophils Attach and Roll on 4 CR P-Selectin When It is Expressed on Lec8 CHO Cells That Have Less Glycocalyx

Cell–cell contact requires that specific interactions between adhesion molecules overcome nonspecific repulsion between cells (7). Electrostatic repulsive forces are generated by contacts between the glycocalyces on apposing cells (7). If the CHO cells had less glycocalyx, shortened P-selectin might more effectively mediate neutrophil attachment and rolling. To test this hypothesis, we examined the adhesive function of wild-type and 4 CR P-selectin expressed on Lec8 CHO cells, a glycosylation-defective cell line. Lec8 cells lack the UDP-galactose Golgi translocase that transfers galactose into the lumen of the Golgi complex, where it is added to both N- and O-linked glycans (16, 50). Consequently, the oligosaccharides of Lec8 cells have only truncated core structures that lack galactose and terminal sialic acid. Therefore, the glycocalyx of these cells has less

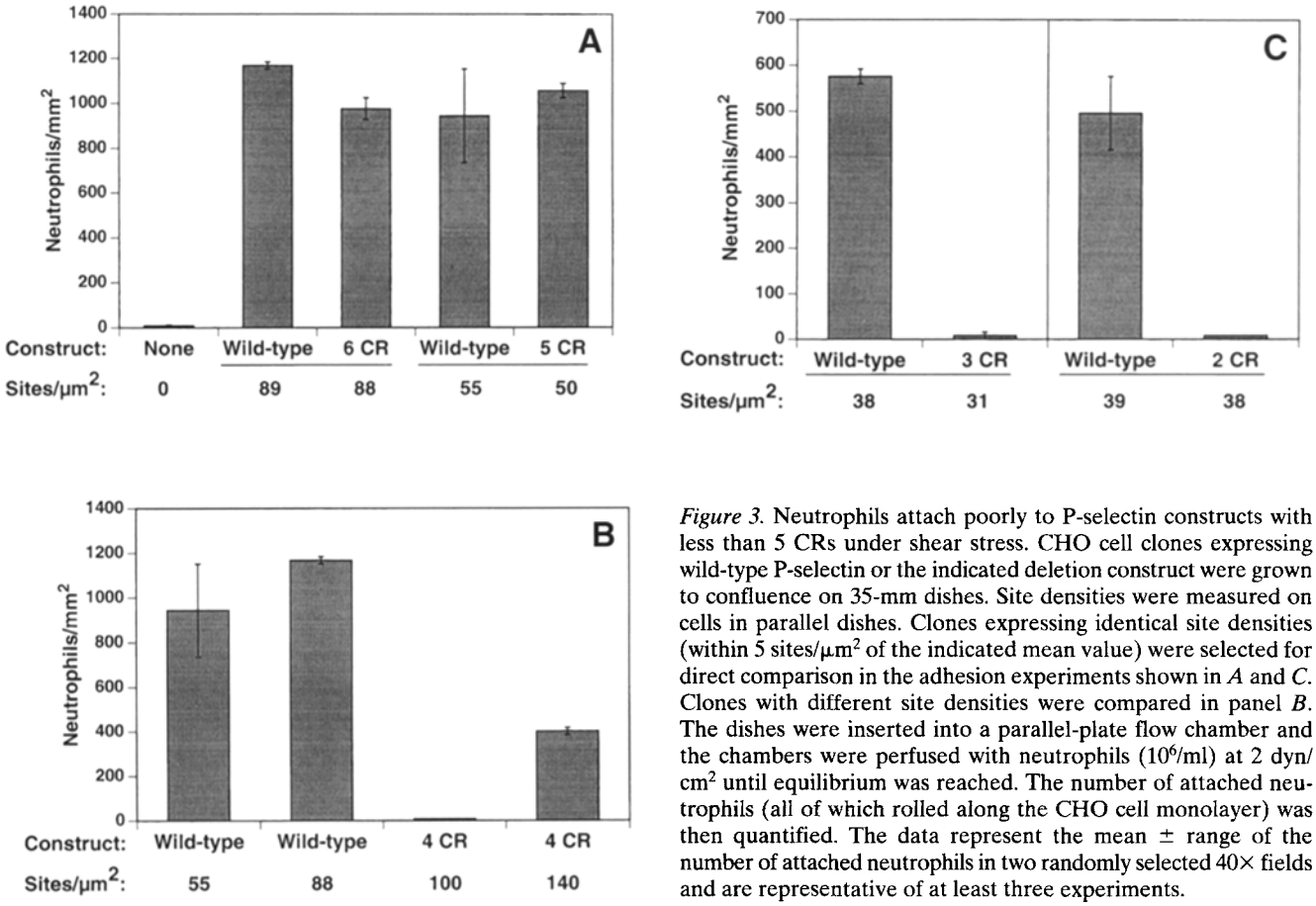


Figure 3. Neutrophils attach poorly to P-selectin constructs with less than 5 CRs under shear stress. CHO cell clones expressing wild-type P-selectin or the indicated deletion construct were grown to confluence on 35-mm dishes. Site densities were measured on cells in parallel dishes. Clones expressing identical site densities (within 5 sites/μm^2 of the indicated mean value) were selected for direct comparison in the adhesion experiments shown in *A* and *C*. Clones with different site densities were compared in panel *B*. The dishes were inserted into a parallel-plate flow chamber and the chambers were perfused with neutrophils (10^6/ml) at 2 dyn/cm^2 until equilibrium was reached. The number of attached neutrophils (all of which rolled along the CHO cell monolayer) was then quantified. The data represent the mean ± range of the number of attached neutrophils in two randomly selected 40× fields and are representative of at least three experiments.

negative charge and fewer hydrated polymers, two major contributors to its repulsive properties (7). Flowing neutrophils attached equivalently to wild-type P-selectin expressed at matched densities on Lec8 CHO cells and normal CHO cells (Fig. 8 *A*). This finding indicates that P-selectin does not require addition of complex oligosaccharides in order to function. As described earlier, flowing neutrophils attached much less well to 4 CR P-selectin than to wild-type P-selectin when expressed on normal CHO cells (Fig. 8 *B*). In contrast, significantly more neutrophils attached and rolled on 4 CR P-selectin expressed by Lec8 CHO cells. Higher shear forces were also required to detach neutrophils bound to 4 CR P-selectin on Lec8 CHO cells than on normal CHO cells (Fig. 8 *C*). At matched densities, the 4 CR construct functioned better on Lec8 cells than on normal CHO cells in all experiments. Even on Lec8 CHO cells, however, the 4 CR construct functioned less well than wild-type P-selectin (Fig. 8, *B* and *C*). These results suggest that flowing neutrophils attach and roll poorly on 4 CR P-selectin, in part, because the decreased distance between apposing cells increases their repulsive forces.

Discussion

Attachment and rolling of leukocytes on the vessel wall under shear stresses require the rapid formation and then the rapid dissociation of selectin-ligand bonds (1, 23, 55). P-selectin must interact with its mucin-like ligand, PSGL-1, in order for flowing neutrophils to attach and roll on P-selectin (38). P-selectin is a long, asymmetric molecule (56), and PSGL-1 is predicted to have a similar extended structure (36). We found that P-selectin must project its ligand-binding domain a significant distance above the membrane of CHO cells to mediate optimal attachment of neutrophils under shear forces. Fewer neutrophils attached to shorter constructs of P-selectin, and those that did had less adhesive strength, as reflected by their faster rolling velocities and their sensitivity to detachment by relatively low shear stresses.

There are three principal mechanisms by which lengthening P-selectin might facilitate attachment of flowing leukocytes. First, the additional CRs might affect the conformation of the lectin domain, improving its ligand-binding properties. Although this is a formal possibility, we found that the 6 CR form of P-selectin had equivalent adhesive function when four of its endogenous CRs were replaced by 4 CRs from another protein. Thus, any conformational effects on the lectin domain do not specifically require any of the CRs of P-selectin except possibly for CRs 1 and 2, which were present in all constructs. Second, extending the lectin domain above the membrane should allow it to rotate through a larger radius, enhancing its interactions with ligands. In detergent solutions, P-selectin oligomerizes through its transmembrane domain (56). If such oligomers form in the membrane, the effective volume of the ligand-binding domain(s) would be further increased. Third, extending P-selectin should promote interactions with

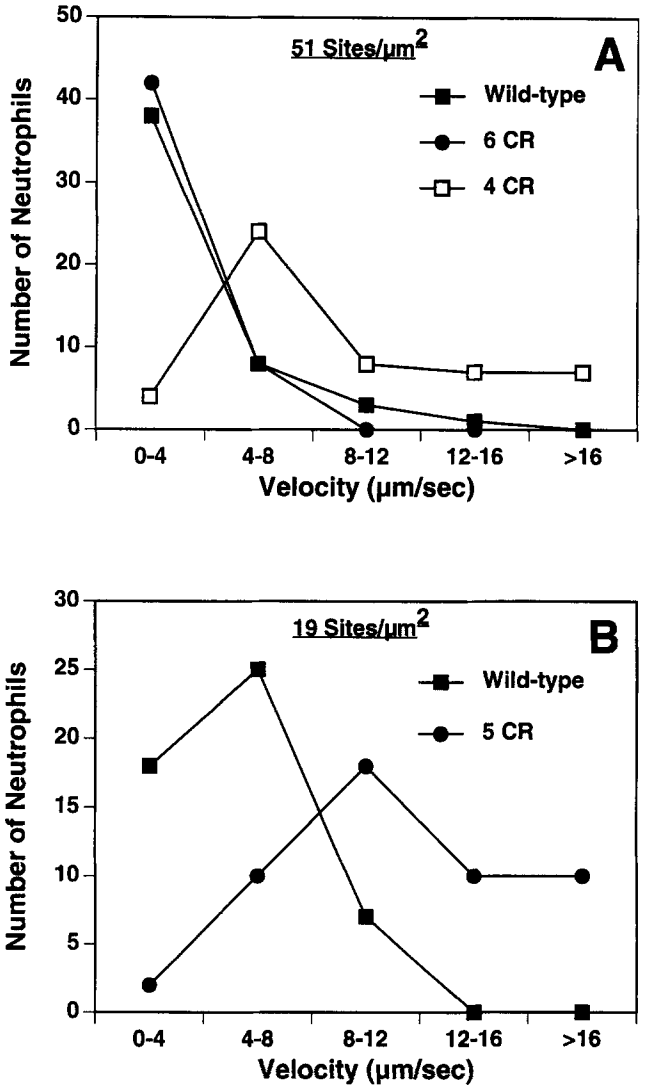


Figure 4. Neutrophils roll more rapidly on shorter constructs of P-selectin. Neutrophils were perfused at 2 dyn/cm^2 over confluent CHO cells expressing the indicated construct until equilibrium was reached. For each construct, the velocities of 50 rolling neutrophils were determined. Each panel represents parallel comparisons of neutrophil rolling velocities on CHO cells expressing equivalent densities of the indicated construct. (*A*) The site densities of wild-type, 6 CR, and 4 CR P-selectin were 51 sites/μm^2. (*B*) The site densities of wild-type and 5 CR P-selectin were 18 and 19 sites/μm^2, respectively. The data are representative of at least three experiments.

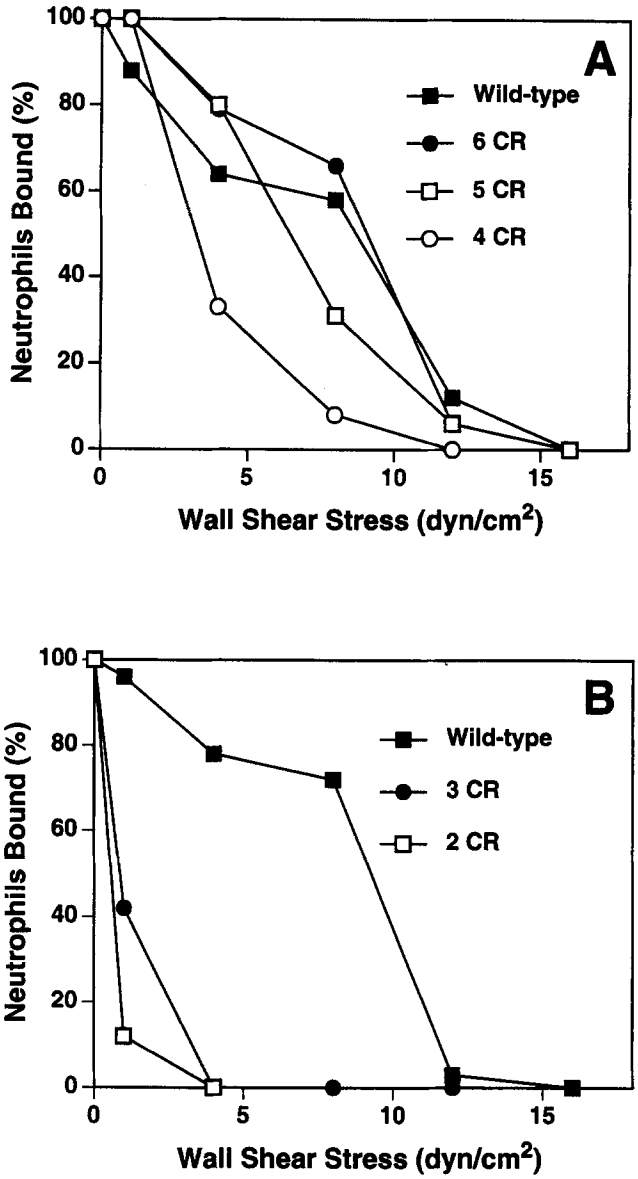


Figure 5. Neutrophils attached to shorter constructs of P-selectin are detached more readily by shear stress. CHO cells expressing the indicated construct were grown to confluence. Neutrophils (10^6/ml) were allowed to bind to the selectin surface for 5 min under static conditions. Flow was initiated at 0.25 dyn/cm^2 for 30 s to remove loosely adherent cells, and the number of adherent cells remaining was taken to be 100% bound. Each panel compares constructs at matched densities; similar numbers of neutrophils statically adhered to each construct at the matched densities. Shear was increased every 30 s; the number of neutrophils bound was quantified and the percent remaining was determined. (*A*) The site densities for wild-type, 6 CR, 5 CR, and 4 CR P-selectin were between 48 and 55 sites/μm^2. (*B*) The site densities for wild-type, 3 CR, and 2 CR P-selectin were between 31 and 38 sites/μm^2. The data are representative of at least three experiments.

PSGL-1 at a greater intermembrane distance, minimizing repulsive forces between the glycocalyces. These repulsive forces increase markedly as the glycocalyces of apposing cells come into contact (7). The 4 CR and 3 CR constructs functioned much less well than the 5 CR construct, which had adhesive properties approaching those of wild-type P-selectin. The dramatic decrease in function effected by only a small reduction in length suggests that constructs with less than 5 CRs do not extend above the glycocalyx of CHO cells. The 4 CR construct functioned much better on Lec8 CHO cells, which are significantly less glycosylated than wild-type CHO cells. This further suggests that P-selectin must extend above the glycocalyx to function optimally. Even on Lec8 CHO cells, the 4 CR construct was less effective than wild-type P-selectin in mediating attachment and rolling of neutrophils. Therefore, lengthening P-selectin may facilitate contacts with PSGL-1 as well as increase the intermembrane distance where specific bonds form, reducing repulsive forces between cells.

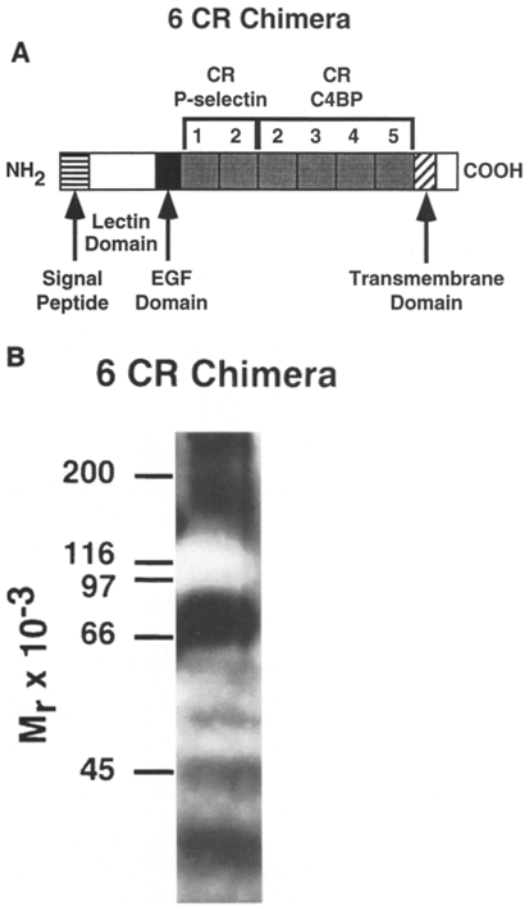


Figure 6. Human 6 CR chimera construct. (*A*) Schematic diagram of the chimera, indicating where CRs 3–6 of human P-selectin were replaced by CRs 2–5 of C4BP. (*B*) Western blot analysis of 6 CR P-selectin. SDS-PAGE and immunoblotting with mAb G1 were performed as in Fig. 1 *B*. The slower migrating band identified by G1 represents a dimer of the construct that is sometimes observed followed SDS-PAGE under nonreducing conditions (see also legend to Fig. 1 *B*).

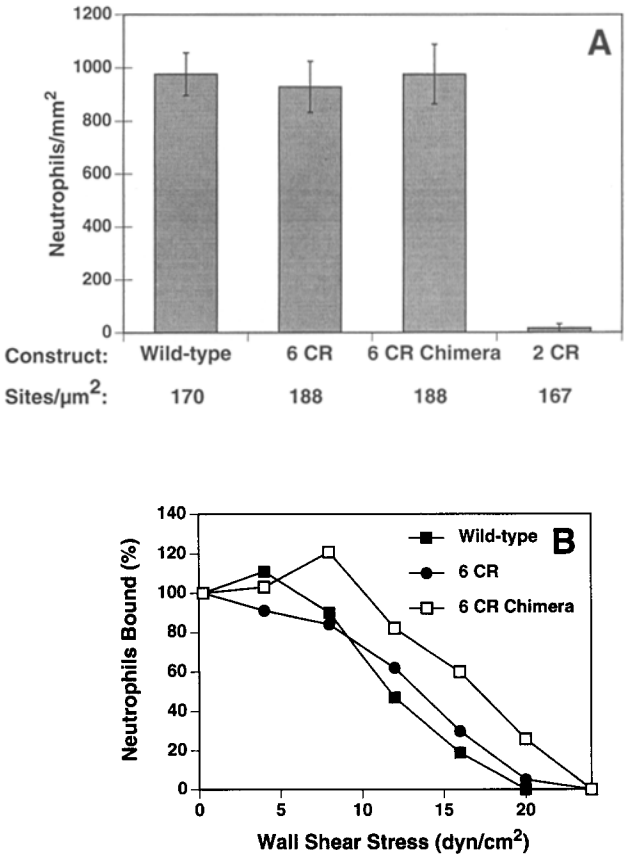
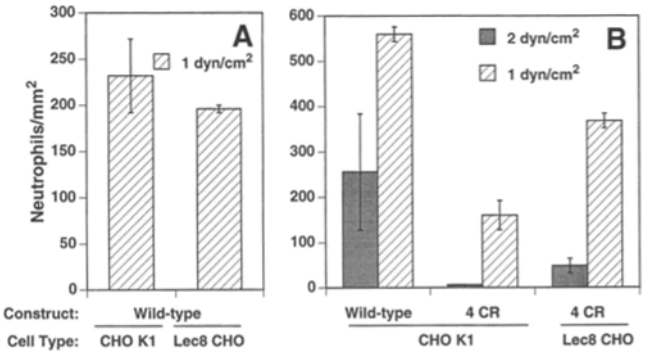


Figure 7. Neutrophils attach and roll normally on the 6 CR chimera construct. (*A*) Neutrophils were perfused at 2 dyn/cm^2 over confluent CHO cells expressing the indicated P-selectin construct at matched densities. The number of attached neutrophils was measured as in Fig. 3. (*B*) Neutrophils were allowed to attach statically to the indicated constructs at the same densities as in *A*. The number of cells remaining attached as a function of increasing shear stress was determined as in Fig. 5. Each point represents the mean ± range of duplicate determinations and is representative of at least three experiments.

P-selectin must also extend significantly above the membrane for flowing neutrophils to attach with optimal adhesive strength, as measured by their slower rolling velocities and by their resistance to shear-induced detachment. The bonds between P-selectin and PSGL-1 appear to have rapid off rates that are not significantly increased by hydrodynamic forces (1). However, bonds between P-selectin and PSGL-1 on the trailing edge of a rolling leukocyte are likely to stretch and dissociate much faster if P-selectin is shortened. Shortened P-selectin may also be less able to form new bonds to replace those that have dissociated, in part because of the repulsive forces of the glycocalyx. This latter defect is suggested by the lower shear stresses required to detach neutrophils from 4 CR P-selectin when it is expressed on normal CHO cells instead of on Lec8 CHO cells.

The length requirements for P-selectin were evident only under conditions of hydrodynamic flow. This suggests that competition between cell repulsion and specific bond formation is particularly critical under the kinetic constraints that flowing leukocytes must overcome to attach to P-selectin. Under static conditions, neutrophils attached equivalently to both long and short P-selectin constructs during a 15-min incubation at 37°C. Thus, given time, shortened P-selectin could mediate neutrophil attachment, overcoming the repulsive forces of the glycocalyx. Neutrophils attached statically to the shorter constructs after a 5-min incubation at either 4° or 22°C. The 4°C temperature minimizes membrane fluidity, suggesting that shortened P-selectin mediates static neutrophil attachment without lateral diffusion of additional molecules into the cell contact zone. A previous study compared the static adhesion of T cells expressing CD2 to CHO cells expressing a long or short form of glycophosphatidyl inositol-anchored lymphocyte function-associated antigen 3 (LFA-3) (12). The short wild-type LFA-3 had two extracellular Ig-like domains. The longer LFA-3 was created by inserting four Ig-like domains from another protein between the membrane anchor and the two native Ig-like domains. The longer LFA-3 bound more efficiently to CD2 on T cells; the enhanced adhesion persisted when the charge repulsion of the CHO cell glycocalyx was reduced by treatment with sialidase. However, the superior adhesive function of lengthened



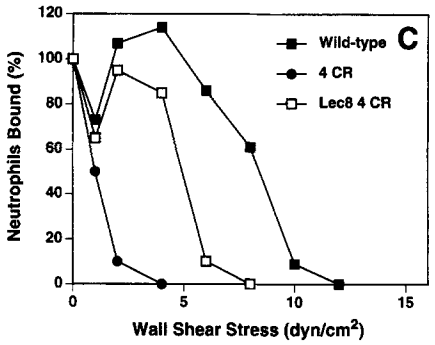


Figure 8. More neutrophils attach and roll on 4 CR P-selectin when it is expressed on Lec8 CHO cells with less glycocalyx. (*A* and *B*) Neutrophils were perfused over normal CHO cells or Lec8 CHO cells expressing wild-type or 4 CR P-selectin at the indicated shear stress. (*C*) Neutrophils were allowed to attach statically to the indicated constructs. The number of cells remaining attached as a function of increasing shear stress was determined as in Fig. 5. The site densities for all constructs were between 15 and 19 sites/μm^2. Each point represents the mean ± range of duplicate determinations and is representative of at least three experiments.

LFA-3 was evident only at 4°C. At 37°C, lateral diffusion apparently allowed sufficient numbers of short LFA-3 molecules to enter the cell contact zone. In contrast, shortened P-selectin mediated static neutrophil adhesion even at 4°C. Furthermore, extension of P-selectin served to overcome cell repulsion under shear, although extension was not required under static conditions.

Although we studied P-selectin on transfected CHO cells, the results suggest that P-selectin on activated platelets and endothelial cells must also extend a sufficient distance above the membrane to function. P-selectin has nine CRs in humans (26), eight CRs in rodents (4, 46, 59), and 6 CRs in cattle (52), implying evolutionary pressure to maintain its length. As determined by electron microscopy, the extracellular domain of human P-selectin is 38-nm long, with each CR estimated to be 3.8-nm long (56). Extrapolating from these data, the lengths of the extracellular domains of the 5 CR and 4 CR constructs are 19 and 16 nm, respectively. The depth of the glycocalyx may vary on different cell types. We hypothesize that the glycocalyx on CHO cells is ≈20-nm thick, as measured for endothelial cells (48) and some other cell types (7). If so, the 5 CR construct may extend to, or just above, the outer limits of the glycocalyx on CHO cells as well as on endothelial cells and platelets. PSGL-1 is predicted to extend 50–60 nm from the cell surface (36), significantly above the usual 20-nm glycocalyx. However, much of the negative charge on leukocytes may be contributed by the abundant sialomucin CD43 (40), which extends ≈50 nm above the membrane (14). PSGL-1 may need to project its binding site well above the cell surface to overcome the electrostatic repulsion of the long CD43 molecule.

The combined lengths of P-selectin and PSGL-1 may cooperatively enhance their interactions under conditions of hydrodynamic flow. Other selectins and selectin ligands may also extend their binding domains to facilitate attachment of flowing leukocytes. E-selectin has from 4–6 CRs in different species (9, 54, 59). Variability in the lengths of E-selectin ligands among species might affect the length requirements for E-selectin. Human neutrophils use a related site on PSGL-1 to mediate attachment under flow to both P- and E-selectin, although they also use other ligands to attach to E-selectin (32, 42, 51). Whether the lengths of these other ligands affect the length requirements for E-selectin is unknown. The extracellular domain of L-selectin, which has just 2 CRs (31, 47, 53), is probably only 10–12-nm long. Despite its short length, L-selectin may function because it is localized on the tips of microvilli (10, 19, 43), and because it may bind to extended glycoconjugates on the endothelial cell surface. Leukocytes attach and roll on one L-selectin ligand, MAdCAM-1, when it is coated in glass tubes (8). However, the putative O-linked glycans recognized by L-selectin are located just above the membrane-proximal Ig-like domain of MAdCAM-1, only 10–15 nm from the cell surface (11). If these oligosaccharides are within the glycocalyx, MAdCAM-1 on intact endothelial cells may not interact well with L-selectin on flowing leukocytes. Two other candidate ligands for L-selectin are CD34, a mucin-like protein with an estimated length of 35 nm (14), and a 200-kD mucin-like ligand that might be even longer (24). The role of these molecules in cell–cell adhesion under shear has not been confirmed.

In summary, P-selectin must extend its ligand-binding domain well above the membrane to mediate optimal attachment of neutrophils under shear forces. The long length of P-selectin appears to enhance its interactions with PSGL-1 on flowing leukocytes and to reduce cell–cell repulsion by increasing the intermembrane distance where selectin–ligand bonds form. Under shear forces, cell–cell contact may require that other adhesion molecules project their binding domains well above the membrane, particularly if they are not concentrated on the tips of microvilli.

We thank Ginger Hampton, Cindy Carter, Sheryl Christofferson, and Chris Titsworth for technical assistance. We also thank Richard Cummings for suggesting the experiment with Lec8 CHO cells and Kevin Moore for helpful discussions.

This work was supported by grant HL 45510 from the United States Public Health Service. K. D. P. is the recipient of an individual National Research Service Award (HL 09035) from the National Institutes of Health.

Received for publication 15 August 1995 and in revised form 4 October 1995.

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