Interactions of the Cytoplasmic Domain of P-Selectin with Clathrin-coated Pits Enhance Leukocyte Adhesion under Flow

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Abstract. Flowing leukocytes tether to and roll on P-selectin, a receptor on endothelial cells that is rapidly internalized in clathrin-coated pits. We asked whether the association of P-selectin with clathrin-coated pits contributes to its adhesive function. Under flow, rolling neutrophils accumulated efficiently on CHO cells expressing wild-type P-selectin or a P-selectin construct with a substitution in the cytoplasmic domain that caused even faster internalization than that of the wild-type protein. By contrast, far fewer rolling neutrophils accumulated on CHO cells expressing P-selectin constructs with a deletion or a substitution in the cytoplasmic domain that impaired internalization. Neutrophils rolled on the internalization-competent constructs with greater adhesive strength, slower velocity, and more uniform motion. Flowing neutrophils tethered equivalently to internalization-competent or internalization-defective P-selectin, but after tethering, they rolled further on internalization-competent P-selectin. Confocal microscopy demonstrated colocalization of α-adaptin, a component of clathrin-coated pits, with wild-type P-selectin, but not with P-selectin lacking the cytoplasmic domain. Treatment of CHO cells or endothelial cells with hypertonic medium reversibly impaired the clathrin-mediated internalization of P-selectin and its ability to support neutrophil rolling. Interactions of the cytoplasmic domain of P-selectin with clathrin-coated pits provide a novel mechanism to enhance leukocyte adhesion under flow.

Key words: endocytosis • selectins • adhesion • clathrin • leukocytes

Regulated expression of adhesion and signaling molecules controls the recruitment of leukocytes into lymphatic tissues and sites of inflammation (Springer, 1995; Butcher and Picker, 1996). Under conditions of hydrodynamic flow, binding of selectins to cell-surface carbohydrate ligands initiates the tethering and rolling of leukocytes on the vessel wall (McEver et al., 1995; Kansas, 1996; McEver, 1998). Each of the selectins has an NH₂-terminal C-type lectin domain followed by an EGF-like domain, a series of consensus repeats, a transmembrane domain, and a short cytoplasmic tail. L-selectin expressed on leukocytes binds to constitutively expressed ligands on endothelial cells at sites of inflammation, and to ligands on other leukocytes. E-selectin expressed on activated endothelial cells, and P-selectin expressed on activated platelets and endothelial cells, bind to ligands on myeloid cells and subsets of lymphocytes.

The selectins bind preferentially to a limited number of appropriately modified glycoproteins on leukocytes or endothelial cells. Among these is P-selectin glycoprotein ligand-1 (PSGL-1), a homodimeric sialomucin on leukocytes that interacts with all three selectins (McEver and Cummings, 1997). P- and L-selectin bind to an NH₂-terminal region of PSGL-1 that must be modified by both specific O-glycosylation and tyrosine sulfation. mAbs against this region block tethering and rolling of leukocytes on P-selectin (Moore et al., 1995; Norman et al., 1995), and partially inhibit L-selectin–dependent interactions of leukocytes with other leukocytes under shear stress (Walcheck et al., 1996; Guyer et al., 1996; Patel and McEver, 1997).

The intrinsic biochemical and biophysical properties of selectin–glycoconjugate interactions direct the tethering...
and rolling of leukocytes under hydrodynamic flow. Selectin ligand bonds must be rapidly formed and broken, and they must also resist dissociation by tensile forces (Lawrence and Springer, 1991; Alon et al., 1995; Alon et al., 1997). The cell-surface organization of selectins and their ligands also contributes to the efficiency of cell–cell interactions under shear stress. For example, at least five of the nine consensus repeats of P-selectin are required to extend the lectin domain sufficiently above the cell surface to mediate optimal attachment and rolling of flowing neutrophils (Patel et al., 1995b). Concentration of L-selectin on microvilli augments the initial tethering, but not the subsequent rolling, of leukocytes on L-selectin ligands under flow (Von Andrian et al., 1995). PSGL-1 is both highly extended and localized to microvillous tips, both of which may contribute to its interactions with selectins under shear stress (Moore et al., 1995; Li et al., 1996; Bruehl et al., 1997).

Clustering of selectins or their ligands through cytoskeletal connections may also regulate cell adhesion under flow. Indeed, it has been suggested that cytoskeletal interactions are required to prevent forced extraction of selectins or their ligands from the lipid bilayer (Alon et al., 1995). The cytoplasmic tail of L-selectin binds to α-actinin (Pavalko et al., 1995), and treatment of cells expressing L-selectin with cytochalasin D abrogates adhesion to L-selectin ligands (Kansas et al., 1993). Deletion of the cytoplasmic domain does not impair the targeting of L-selectin to microvilli of transfected leukocytes (Pavalko et al., 1995). However, cells expressing tail-less L-selectin adhere much less well to L-selectin ligands than do cells expressing wild-type L-selectin (Kansas et al., 1995). PSGL-1 may also interact with the cytoskeleton, as it is redistributed from microvilli to the uropods of activated leukocytes (Lorrant et al., 1995; Bruehl et al., 1997).

Unlike L-selectin, P- and E-selectin have not been demonstrated to interact with the cytoskeleton, except under conditions where the proteins are cross-linked by antibodies or by sustained adhesive contact with leukocytes (Yoshida et al., 1996). Both proteins are normally extracted in the Triton X-100–soluble fraction of platelets and/or endothelial cells, and neither protein binds to α-actinin under conditions where L-selectin binds (Kansas and Pavalko, 1996). The absence of cytoskeletal tethering may be essential for the cellular trafficking of P- and E-selectin (McEver, 1997). After its synthesis, P-selectin is sorted into α granules of platelets and Weibel-Palade bodies of endothelial cells. Secretagogues such as thrombin or histamine induce the redistribution of P-selectin to the cell surface. On endothelial cells, P-selectin is rapidly internalized (Hattori et al., 1989), where it is targeted for delivery to lysosomes (Green et al., 1994) or recycled into new Weibel-Palade bodies (Subramaniam et al., 1993). The cytoplasmic domain of P-selectin contains signals for sorting into secretory granules (Disdier et al., 1992), for endocytosis in clathrin-coated pits (Subramaniam et al., 1993; Setiadi et al., 1995), and for delivery from endosomes to lysosomes (Green et al., 1994). The cytoplasmic domain of E-selectin does not directly sort into secretory granules, but it does contain signals for internalization (Chuang et al., 1997) and probably for sorting from endosomes to lysosomes (Kuijpers et al., 1994). An important effect of these trafficking signals is to limit the lifetime of P- or E-selectin on the cell surface.

To be internalized in clathrin-coated pits, the cytoplasmic domains of membrane proteins must interact with adaptins, which in turn bind to clathrin (Schmid, 1997). Proteins that are rapidly internalized interact particularly efficiently with adaptins, and thus cluster on the cell surface in clathrin-coated lattices or pits before the pits bud to form endocytic vesicles (Heuser and Anderson, 1989; Miller et al., 1991). We hypothesized that the cytoplasmic domain of P-selectin also binds to adaptins, leading to clustering and colocalization of both molecules in clathrin-coated pits. Under hydrodynamic flow, such clustering might be important to the ability of P-selectin to support adhesion in the absence of linkages to the cytoskeleton. To test this hypothesis, we compared the tethering and rolling of flowing neutrophils on CHO cells expressing P-selectin constructs with alterations in the cytoplasmic domain that affect the rate of endocytosis. We also asked whether an inhibitor of clathrin-mediated endocytosis affected the ability of P-selectin to support neutrophil rolling. Our data suggest that interactions of the cytoplasmic domain of P-selectin with clathrin-coated pits provide a novel mechanism for regulating the adhesive function of the extracytoplasmic domain.

Materials and Methods

Cells and Antibodies

CHO-K1 cells and human umbilical vein endothelial cells (HUVEC) were cultured as described (Setiadi et al., 1995; Yao et al., 1996). The anti-human P-selectin mAbs S12 and G1 (both IgG) were prepared as described (McEver and Martin, 1984; Geng et al., 1990). G1, but not S12, blocks binding of P-selectin to leukocytes and to PSGL-1. The anti-human PSGL-1 mAbs PL1 and PL2 (both IgG) were prepared as described (Moore et al., 1995). PL1, but not PL2, blocks binding of PSGL-1 to P-selectin. Goat polyclonal IgG antibodies to human P-selectin (Green et al., 1994) were biotinylated as described (Setiadi et al., 1995). mAbs to α-adaptin and caveolin were obtained from Transduction Laboratories (Lexington, KY).

cDNA Constructs, Transfections, and Site Density Measurements

cDNAs encoding wild-type P-selectin or P-selectin constructs with alterations in the cytoplasmic domain were prepared as described (Setiadi et al., 1995). The constructs were ligated into the expression vector pRe/RSV (Invitrogen Corp., Carlsbad, CA) and permanently transfected into CHO-K1 cells (Setiadi et al., 1995). Clones expressing each construct at different densities were selected, and portions of each clone were frozen. Site densities of constructs on confluent CHO cells were determined using 125I-labeled mAb G1 as described (Moore et al., 1995; Patel et al., 1995b). Although site densities for each construct remained stable for at least 2 wk in culture, they were always measured within 2 wk of each cell adhesion experiment.

Internalization Assay

The rate of internalization of P-selectin in transfected CHO cells or HUVEC was measured by the ability of an acidic buffer to remove 125I-labeled mAb G1, prebound to the cell surface at 4°C, after warming the cells to 37°C for various intervals (Setiadi et al., 1995).

Adhesion of Neutrophils to P-Selectin Constructs Under Flow Conditions

Human neutrophils were isolated from healthy human donors as described (Zimmerman et al., 1985). Transfected CHO cells were grown to
confluence in 35-mm culture dishes. Fluid shear stresses present in the microvasculature were simulated in a dual-chamber parallel-plate flow chamber (Moore et al., 1995; Patel et al., 1995a). All experiments were performed at 22°C. Neutrophils (10^6/ml in HBBS/0.5% human serum albumin) were perfused through the CHO cell monolayer in the chamber at the desired wall shear stress. After 4 min of perfusion, the number of neutrophils accumulated on the monolayer had reached equilibrium. The adherent neutrophils were visualized using phase-contrast video microscopy with a 40× objective of view of 0.032 mm^2. The interactions were quantified using a computer imaging system (Sun Microsystems, Mountain View, CA; Inovision Corp., Durham, NC). The number of accumulated neutrophils per unit area varied less than twofold from the proximal end to the distal end of the flow chamber. Observations were routinely made in the middle portion of the chamber. Digitization of image frames was used to distinguish rolling from firmly adherent cells. Under the experimental conditions used, virtually all adherent cells rolled on the monolayer.

Tethering events were visualized with a 40× or a 10× objective using frame-by-frame analysis during the first 3 min of perfusion. A transient tether was defined as a free-flowing neutrophil that tethered to the surface, but then detached before rotating at least one cell diameter on the surface. A primary tether was defined as a flowing neutrophil that tethered directly to the monolayer, and then proceeded to roll at least one cell diameter. A secondary tether was defined as a leukocyte that tethered to the monolayer after an initial interaction with an adherent leukocyte in the same field of view.

Rolling velocities were measured as described (Patel et al., 1995a; Patel et al., 1995b). The path of rolling cells were examined by a series of nine images taken every 0.5 s over a 4-s period. These paths were used to determine the percentage of rolling neutrophils that skipped at least one time on the monolayer during the 4-s interval; a neutrophil was considered to skip it if it moved more than one cell diameter during any 0.5-s interval.

The resistance to detachment of neutrophils was determined by allowing neutrophils to accumulate on the surface at a shear stress of 1 dyn/cm^2. Without stopping flow, cell-free buffer was then perfused at 1 dyn/cm^2 for 30 s, and the number of adherent cells remaining was taken to be 100%. The wall shear stress was then increased incrementally every 30 s, and the percentage of neutrophils remaining adherent was determined.

In certain experiments, CHO cells were preincubated for 10 min with 10 μg/ml of anti-PSGL-1 mAbs. Neutrophils were then perfused through the chamber in the continued presence of the mAb. These experiments revealed that anti-PSGL-1 mAb G1 or anti-PSGL-1 mAb PL1 blocked tethering and rolling of neutrophils on CHO cells expressing the P-selectin constructs, confirming previous observations that the adhesive events studied represented specific interactions between P-selectin and PSGL-1 (Moore et al., 1995; Patel et al., 1995b; Patel and McEver, 1997).

Effect of Hypertonic Medium on Internalization of P-Selectin and on the Adhesive Function of P-Selectin

Hypertonic medium containing sucrose was used to block clathrin-mediated endocytosis reversibly (Heuser and Anderson, 1989; Setiadi et al., 1995). Confluent CHO cells in 24-well plates or 35-mm dishes were incubated for 15 min at 37°C in HBBS/0.5% human serum albumin in the presence or absence of 0.45 M sucrose. Scanning EM revealed no morphological differences between cells incubated with hypertonic or isotonic buffer. After the 15-min incubation, all cells were switched to buffer lacking sucrose. In initial experiments, the cells were subjected to an internalization assay to determine the time required for the cells to recover the ability to internalize P-selectin. For this purpose, the cells were placed on ice and a saturating amount of ^[35]S]G1 in chilled isotonic buffer was added. After 30 min, the cells were washed and then subjected to the internalization assay at 37°C. These experiments showed that internalization remained impaired within the first min after return to isotonic medium at 37°C. Thus, in subsequent experiments the ability of neutrophils to roll on the CHO cells was measured within this lag period. After the cells were treated with hypertonic or isotonic medium, they were immediately placed into the flow chamber, and neutrophils in isotonic medium were rapidly introduced by perfusion for 10 s at 20 dyn/cm^2. The wall shear stress was then reduced to 1 dyn/cm^2, and the number of rolling cells after return to isotonic medium. Because all measurements were made at the same time point, valid comparisons could be made even though the number of rolling neutrophils had not reached equilibrium.

Results

Expression of P-Selectin Constructs with Different Internalization Rates on CHO Cells

P-selectin is rapidly internalized from the surfaces of activated endothelial cells (Hattori et al., 1989) and of transfected CHO cells (Setiadi et al., 1995). Because CHO cells lack regulated secretion, newly synthesized P-selectin molecules are delivered directly to the plasma membrane. At steady state, P-selectin is in equilibrium between the cell surface and the endosomal compartment, with a fraction continuously delivered from endosomes to lysosomes to be degraded (Setiadi et al., 1995; Green et al., 1994).

The 35-residue cytoplasmic domain of P-selectin has no well-defined short internalization motifs. However, some deletions or substitutions of this domain either increase or
decrease the internalization rate of P-selectin (Setiadi et al., 1995). Fig. 1 A depicts the sequences of the cytoplasmic domains of three such constructs, aligned with that of wild-type P-selectin. Fig. 1 B shows the internalization rates of these constructs in stably transfected CHO cells, confirming previous observations (Setiadi et al., 1995). Unlike the rapid endocytosis of wild-type P-selectin, the tailless construct was internalized no faster than bulk membrane flow. The Y777A construct had a moderate reduction in the internalization rate. In contrast, the G778A construct was endocytosed even more rapidly than was wild-type P-selectin. The internalization rates of the constructs remained constant on CHO cell clones with surface densities ranging from 10–250 molecules/\(\mu\)m\(^2\) (Setiadi et al., 1995).

To determine whether the internalization rate of P-selectin might affect its adhesive capability under flow, we compared the rolling of human neutrophils on CHO cell monolayers expressing the internalization-competent or internalization-incompetent forms of P-selectin. We studied four complementary measures of rolling behavior: the accumulated number of rolling cells, the resistance to detachment from the surface, the mean rolling velocity, and the uniformity of rolling. These were measured under controlled conditions with systematic variation of the wall shear stress or the P-selectin site density. We also compared the tethering of free-flowing neutrophils to the selectin constructs and the subsequent behavior of the tethered cells. The use of several different assays allowed a more detailed comparison of potential differences in adhesive function among the P-selectin constructs.

Figure 1. P-selectin constructs expressed in transfected CHO cells. (A) Schematic diagram of wild-type P-selectin and of P-selectin constructs with substitutions or deletions in the cytoplasmic domain. (B) Internalization rates of P-selectin constructs in transfected CHO cells. The rates were determined by the ability of acidic buffer to remove surface-bound \(^{125}\)I-labeled anti-P-selectin mAb G1 as described in Materials and Methods. The cell-bound radioactivity remaining at each time point represents the amount of internalized P-selectin, and is plotted as a percentage of the initial cell-bound radioactivity. The data represent the mean ± SEM of six experiments for G778A, 59 experiments for wild-type P-selectin, five experiments for Y777A, and 29 experiments for tail-less P-selectin.

Figure 2. More rolling neutrophils accumulate on CHO cells expressing wild-type than tail-less P-selectin. Neutrophils (10\(^6\)/ml) were perfused over CHO cells expressing wild-type or tail-less P-selectin at the specified site density. The wall shear stress was 0.05, 1, or 2 dyn/cm\(^2\) in A or 1 dyn/cm\(^2\) in B and C. (A and B) After 4 min of perfusion, the accumulated number of rolling cells was quantified by counting all cells in each of four randomly selected 40× fields. The data represent the mean ± SEM of four experiments for A and at least ten experiments for B. (C) The velocities of neutrophils rolling on CHO cells expressing wild-type or tail-less P-selectin at the indicated site density were measured. Each data point represents the mean ± SEM of the velocities of 63–218 cells.
More Rolling Neutrophils Accumulate on CHO Cells Expressing Wild-type than Tail-less P-Selectin

We first compared the accumulation of rolling neutrophils on CHO cells expressing wild-type or tail-less P-selectin at matched densities. At wall shear stresses of 0.5, 1, or 2 dyn/cm², more rolling neutrophils accumulated on cells expressing wild-type than tail-less P-selectin at densities of 50–74 molecules/µm² (Fig. 2A). At 1 dyn/cm², more neutrophils rolled on wild-type than tail-less P-selectin at densities as low as 10–24 sites/µm² (Fig. 2B). However, the accumulation of rolling neutrophils on wild-type P-selectin plateaued as site densities increased. Neutrophil accumulation on tail-less P-selectin reached that observed on wild-type P-selectin at 75–100 sites/µm² (Fig. 2B). Thus, higher site densities helped to compensate for the impaired adhesive function of tail-less P-selectin in this assay.

Lowering the site density of wild-type P-selectin enabled neutrophils to roll faster (Fig. 2C). In sharp contrast, lowering the site density of tail-less P-selectin did not enable neutrophils to roll faster. This observation suggests that the relatively few neutrophils that rolled on tail-less P-selectin at lower densities may represent a selected sub-population. Thus, most cells either failed to attach to tail-less P-selectin at these densities, or attached but then detached more rapidly from the surface. An important implication of this result is that an adhesion assay may compare different populations of neutrophils, depending on the specific conditions being examined. To avoid the selection of subpopulations of cells that might yield misleading results, we compared rolling velocities and the uniformity of rolling only under conditions where the number of rolling neutrophils and the resistance to detachment were similar for any pair of P-selectin constructs.

Neutrophils Roll with Greater Adhesive Strength, at Lower Velocities, and with More Uniform Motion on CHO Cells Expressing Wild-type than Tail-less P-Selectin

Neutrophils detached much less readily from wild-type than from tail-less P-selectin in response to increasing wall shear stress (Fig. 3). Resistance to detachment increased on both forms of P-selectin at higher site densities, presumably reflecting a greater probability of forming receptor–ligand bonds. However, the increased detachment resistance of wild-type over tail-less P-selectin persisted at all densities, even at the higher 75–100 sites/µm² (Fig. 3D) at which neutrophils accumulated in similar numbers (see Fig. 2B).

We compared the rolling velocities of neutrophils on wild-type and tail-less P-selectin at densities (75–100 sites/µm²) and shear stresses (1–8 dyn/cm²) where higher shear stresses increased rolling velocities on both forms of P-selectin, but resistance to detachment was still equivalent (see Fig. 3D). Under these conditions, neutrophils rolled more slowly on wild-type than on tail-less P-selectin at each shear stress examined (Fig. 4A). Furthermore, neutrophils rolled more uniformly on wild-type than on tail-less P-selectin (Fig. 4B and Table I). Neutrophils rolling velocities were equivalent for cells perfused at 1 dyn/cm² on tail-less P-selectin and at 4 dyn/cm² on wild-type P-selectin (Fig. 4A). However, neutrophils skipped less on wild-type than on tail-less P-selectin even though shear stress was fourfold higher on wild-type P-selectin (Table I). Thus, under conditions where the rolling neutrophil populations appeared to be similar, the cells rolled faster and skipped more on wild-type than on tail-less P-selectin.

Neutrophils Roll Differently on CHO Cells Expressing P-Selectin Constructs with Different Internalization Rates

The lessened adhesive function of tail-less P-selectin was not due simply to deletion of the cytoplasmic domain, because rolling neutrophils also accumulated poorly on the internalization-defective Y777A construct at 25–49 sites/µm². By contrast, equivalent numbers of neutrophils rolled on the G778A construct and wild-type P-selectin at these densities (Fig. 5A). Therefore, more neutrophils rolled on CHO cells expressing internalization-competent than internalization-incompetent P-selectin.

Rolling neutrophils detached readily from both the Y777A and tail-less constructs in response to increasing shear stress. In contrast, rolling neutrophils resisted detachment equivalently on wild-type P-selectin and on the G778A construct, which is internalized even more rapidly than wild-type (Fig. 5B). Comparisons of rolling velocity...
and rolling motion were made only between Y777A and tail-less P-selectin, or between G778A and wild-type P-selectin. For these paired comparisons, conditions could be selected where the number of rolling cells and the detachment resistance were equivalent. These conditions could not be met for other paired comparisons, where the presence of different populations of rolling neutrophils might provide misleading information as to the relative adhesive properties of the P-selectin constructs.

Neutrophils rolled more slowly on Y777A than on tail-less P-selectin, consistent with the less severely impaired internalization rate of Y777A compared with that of tail-less P-selectin (Fig. 5C). Moreover, fewer neutrophils skipped on Y777A than on tail-less P-selectin, even at the same rolling velocity (compare rolling at 1 dyn/cm² on tail-less P-selectin with rolling at 4 dyn/cm² on Y777A; Fig. 5C and Table I).

Neutrophils rolled more slowly on G778A than on wild-type P-selectin, consistent with the faster internalization rate of G778A compared with that of the wild-type (Fig. 5D). Fewer neutrophils skipped on G778A than on wild-type P-selectin, even at the same rolling velocity (compare rolling at 1 dyn/cm² on wild-type P-selectin with rolling at 4 dyn/cm² on G778A; Fig. 5D and Table I).

Collectively, these data indicate that neutrophils roll with greater adhesive strength, at slower velocities, and with more uniform motion on P-selectin as its internalization rate is increased. These properties should lessen the rate of detachment of rolling neutrophils from the cell monolayer. Therefore, more rolling neutrophils accumulate on internalization-competent than internalization-incompetent forms of P-selectin.

### Flowing Neutrophils Tether Equivalently to CHO Cells Expressing Internalization-competent or Internalization-incompetent P-Selectin

Under hydrodynamic flow, the accumulated number of rolling leukocytes is a function of both the rates of tethering to and detachment from the substrate (Puri et al., 1997). A free-flowing neutrophil can form a primary tether with the substrate, and then either rapidly detach back into the fluid stream or develop rolling adhesion as it forms new bonds at the leading edge of the cell to replace those broken at the trailing edge. A flowing neutrophil may also tether to an adherent neutrophil and then translate onto the substrate to form a secondary tether (Alon et al., 1996; Walcheck et al., 1996).

We found that neutrophils flowing at a shear stress of 1 dyn/cm² tethered equivalently to internalization-competent and -incompetent forms of P-selectin at densities of...
25–49 sites/μm² (Fig. 6 A). For all constructs, most neutrophils formed direct primary tethers to the CHO cell monolayer that converted to rolling adhesion. A lesser number of neutrophils transiently tethered to the monolayer. Some of the neutrophils also first interacted with an adherent rolling neutrophil, and then tethered secondarily to the monolayer. L-selectin–dependent neutrophil–neutrophil interactions can cause the continued accumulation of rolling neutrophils on immobilized P-selectin (Alon et al., 1996; Walcheck et al., 1996). However, the number of neutrophils rolling on the CHO cell monolayers reached equilibrium after 3–4 min of perfusion, suggesting that secondary tethers were not a major contributor to neutrophil accumulation in this experimental system.

The tethering rates or types of tethers did not differ significantly among the constructs under the conditions shown in Fig. 6 A, or at other site densities or wall shear stresses (data not shown). After the cells tethered, however, they rolled longer distances on internalization-competent than on internalization-incompetent P-selectin before they detached into the fluid stream (Fig. 6 B). These data demonstrate that differences in tethering do not explain why more rolling neutrophils accumulate on internalization-competent than on internalization-incompetent P-selectin. Instead, the increased accumulation reflects the enhanced ability of the cells to roll on internalization-competent P-selectin after they tether to the surface.
Treatment of CHO Cells or Endothelial Cells with Hypertonic Medium Reversibly Impairs Both the Internalization and the Adhesive Function of P-Selectin

The preceding data demonstrated that substitutions or deletions in the cytoplasmic domain of P-selectin that inhibited its endocytosis also diminished its adhesive function under shear stress. We next asked whether an inhibitor of endocytosis would impair the ability of wild-type P-selectin to support neutrophil rolling. Exposing cells to hypertonic medium reversibly inhibits endocytosis in clathrin-coated pits by blocking assembly of the clathrin lattice (Heuser and Anderson, 1989). Treatment of transfected CHO cells with hypertonic medium blocks endocytosis of wild-type, G778A, and Y777A P-selectin (Setiadi et al., 1995). Thus, treatment of cells with hypertonic medium dissociates P-selectin from the clathrin-mediated endocytic pathway. We pretreated CHO cells expressing wild-type or tail-less P-selectin with hypertonic or isotonic medium. We then rapidly exchanged all cells into isotonic medium to avoid the potential effects of hypertonic medium on the cell adhesion assay. Preliminary experiments revealed that internalization of wild-type P-selectin remained completely impaired during the first minute after return to isotonic medium (Fig. 7A). After this lag period, the internalization rate of wild-type P-selectin began to approach that of cells maintained in isotonic medium. In contrast, tail-less P-selectin was not internalized in cells pretreated with either medium (Fig. 7A).

We measured the number of neutrophils rolling on CHO cells within 30 s after returning to isotonic medium during the lag period in which endocytosis remained completely inhibited. To do this, we rapidly introduced neutrophils into the flow chamber at a high flow rate, and then immediately reduced the shear stress to 1 dyn/cm². Significantly fewer neutrophils rolled on CHO cells expressing wild-type P-selectin after exposure to hypertonic medium than on the same cells maintained in isotonic medium. The reduction in rolling neutrophils was similar to that observed on CHO cells expressing tail-less P-selectin (Fig. 7B). The ability of neutrophils to roll on wild-type P-selectin normalized within 2–5 min after exchange from hypertonic to isotonic medium (data not shown). These data reveal a direct correlation between the ability of hypertonic medium to inhibit reversibly the endocytosis and the adhesive function of wild-type P-selectin on transfected CHO cells.

We also exposed HUVEC to hypertonic or isotonic medium and then treated the cells with histamine for 4 min to redistribute P-selectin from Weibel-Palade bodies to the cell surface. The cells were then exchanged into isotonic medium before performing the internalization or cell adhesion assays. P-selectin was rapidly internalized from the surface of HUVEC maintained in isotonic medium. In contrast, cells exposed to hypertonic medium had markedly impaired endocytosis of P-selectin during the first 1–2 min after return to isotonic medium (Fig. 7C). During this lag period, fewer neutrophils rolled on HUVEC pretreated with hypertonic medium than on HUVEC maintained in isotonic medium (Fig. 7D). The number of rolling neutrophils normalized within 2–5 min after returning to isotonic medium (data not shown). This result demonstrates that blockade of endocytosis impairs the adhesive capability of P-selectin on activated endothelial cells as well as on transfected CHO cells.

Wild-type but Not Tail-less P-Selectin Colocalizes with α-Adaptin, a Component of Clathrin-coated Pits

The enhanced adhesive capability of internalization-competent P-selectin might be due to association of a subset of the molecules with clathrin-coated pits before they are internalized (Heuser and Anderson, 1989; Miller et al., 1991). We used dual-label immunofluorescence confocal microscopy to determine whether wild-type or tail-less P-selectin colocalized with α-adaptin in fixed permeabilized CHO cells. α-Adaptin is a subunit of the adaptor protein-2 complex found in clathrin-coated pits of the plasma membrane. The adaptor protein-2 complex binds to puta-
tive docking proteins at the inner face of the plasma membrane, to clathrin, and to the cytoplasmic domains of membrane proteins that are rapidly internalized (Schmid, 1997). After the clathrin-coated pit buds to form a vesicle, α-adaptin dissociates as the vesicle loses its coat. Thus, the total pool of α-adaptin is distributed between clathrin-coated pits and the cytoplasm.

Fig. 8 depicts confocal images of CHO cells expressing wild-type or tail-less P-selectin that were pretreated with isotonic or hypertonic medium before fixation. Green or red staining illustrates the respective distribution of P-selectin or α-adaptin. For cells in isotonic medium, there was partial colocalization of α-adaptin with wild-type P-selectin, as manifested by the punctate yellow staining in the merged images. For cells in hypertonic medium, there was little or no colocalization of α-adaptin with wild-type P-selectin, consistent with the ability of hypertonic medium to disassemble clathrin-coated pits (Fig. 8 A). There was virtually no colocalization of α-adaptin with tail-less P-selectin in cells treated with either isotonic or hypertonic medium (Fig. 8 B). Quantification of the percentage of P-selectin pixels that colocalized with α-adaptin pixels confirmed the conclusions from the visual images (Fig. 8 C). Neither wild-type nor tail-less P-selectin colocalized with caveolin, a component of the noncoated caveolae of the plasma membrane that mediates internalization of some proteins (Parton and Simons, 1995; data not shown). These results demonstrate that a subset of wild-type, but not of tail-less, P-selectin colocalizes with α-adaptin in clathrin-coated pits. They also confirm that hypertonic medium disrupts the association of wild-type P-selectin with α-adaptin in coated pits.

**Discussion**

The selectins have specialized features that enable flowing leukocytes to tether to and roll on the vessel wall. Selectin–ligand bonds form and break rapidly, and they resist premature dissociation by applied forces (Lawrence and Springer, 1991; Alon et al., 1995; Alon et al., 1997; Chen et al., 1997). Leukocyte adhesion under flow also requires the appropriate presentation of selectins or their ligands on the cell surface (Von Andrian et al., 1995; Patel et al., 1995b). The cytoplasmic domains of many adhesion receptors regulate their properties through interactions with the cytoskeleton (Gumbiner, 1996; Lauffenburger and Horwitz, 1996; Briehr et al., 1996; Angres et al., 1996; Kucik et al., 1996; Lub et al., 1997; Stewart et al., 1998). The cytoplasmic domain of L-selectin also binds to the cytoskeleton (Pavalko et al., 1995), and this interaction is required for L-selectin to mediate optimal leukocyte adhesion under flow (Kansas et al., 1993). Here we show that associa-
tion of the cytoplasmic domain of P-selectin with clathrin-coated pits enhances neutrophil rolling adhesion under flow. Thus, the initial stages of clathrin-mediated endocytosis can also regulate the function of an adhesion receptor.

P-selectin is rapidly internalized from the surfaces of activated endothelial cells (Hattori et al., 1989) and of transfected CHO cells (Setiadi et al., 1995). Some alterations in the cytoplasmic domain of P-selectin either increase or decrease the internalization rate in transfected CHO cells (Setiadi et al., 1995). Rolling neutrophils accumulated efficiently on CHO cells expressing wild-type P-selectin or the G778A construct, which is internalized even faster than the wild-type. However, significantly fewer rolling neutrophils accumulated on the internalization-defective tail-less or Y777A constructs. The ability of neutrophils to roll well on G778A indicates that mutation of the cytoplasmic domain per se does not impair leukocyte adhesion. Furthermore, treatment of CHO cells with hypertonic medium reversibly impaired both the clathrin-dependent internalization and the adhesive function of wild-type P-selectin. The concordant results from both mutational and chemical strategies that block endocytosis strongly suggest that the association of P-selectin with clathrin-coated pits improves its adhesive capacity under flow. The enhancement of adhesion most likely reflects an altered distribution of P-selectin on the cell surface rather than the actual process of internalization.

Free-flowing neutrophils tethered equivalently to the internalization-competent and -incompetent P-selectin constructs. Thus, the decreased accumulation of rolling neutrophils on internalization-defective P-selectin was not due to an impairment in the initial tethering event. Instead, there was a defect in the subsequent rolling on these constructs. Neutrophils rolled on internalization-defective P-selectin with less adhesive strength, as revealed by their greater susceptibility to detachment by increasing shear stress. Neutrophils also rolled faster and with less uniform motion on the internalization-defective constructs. The consequence of these alterations was more frequent detachment of rolling neutrophils back into the fluid stream.

The decreased accumulation of neutrophils on internalization-defective P-selectin was most evident at densities below 75 sites/μm². Similar numbers of rolling neutrophils accumulated on wild-type and tail-less P-selectin at densities of 75–100 sites/μm² (Fig. 2 B). However, neutrophils still rolled faster on tail-less P-selectin under these conditions (Fig. 4 A), and they detached more readily at higher shear stresses (Fig. 3 D). In general, neutrophils rolled with greater strength, at slower velocities, and with more uniform motion on P-selectin as its internalization rate increased. To avoid selection of subpopulations of neutrophils with different adhesive function, we always compared rolling velocities and rolling motion under conditions where equivalent numbers of neutrophils rolled on any two paired P-selectin constructs. Differences in rolling velocity among constructs could be detected even when shear stresses were adjusted so that resistance to detachment was identical. Differences in rolling motion could be detected even when shear stresses were adjusted so that rolling velocities were equivalent. In a previous study, equivalent numbers of neutrophils were observed to adhere to COS cells overexpressing wild-type or tail-less P-selectin under conditions of rotational shear at 4°C (Kansai and Pavalko, 1996). The inability to detect a difference in adhesion to these constructs may have been due to the use of lower shear stresses and/or higher site densities in the assay. The low temperature would also slow the k_{off} of selectin-ligand bonds, enhancing adhesion (Mason and Williams, 1986; Lawrence and Springer, 1993).

Resistance of cells to detachment by increasing shear stress is a measure of adhesive strength. This fact may reflect the number of receptor–ligand bonds, which is determined by the ratio of the association rate, k_{on}, to the dissociation rate, k_{off}, of these bonds (Puri et al., 1997). Because the cells are rollingly adherent, other important factors may include the collision frequency, the cell contact duration, the contact area, and the surface densities of receptor and ligand. The rolling velocity is primarily limited by k_{off}, but is also partially dependent on k_{on}, because the rate of bond formation must keep pace with the rate of bond dissociation for the cell to continue to roll. The uniformity or jerkiness of rolling depends on the distribution of receptors or ligands, and the probability that new bonds will form before old bonds dissociate (Alon et al., 1997).

The normal tethering but defective rolling of neutrophils on internalization-incompetent P-selectin suggests a normal association rate, but an accelerated dissociation rate for P-selectin-PSGL-1 bonds. The tethering rate primarily reflects the k_{on}. A faster k_{off} but constant k_{on} would reduce resistance to detachment. The higher rolling velocities on internalization-defective P-selectin also suggest a faster k_{off}. A transient tether may require only one P-selectin-PSGL-1 bond, whereas rolling adhesion probably requires at least two bonds (Alon et al., 1995). A slower k_{off} should increase the probability of more bonds and ensure more uniform rolling motion. A faster k_{off} should increase the probability that a cell would disengage all its bonds before a new bond formed, resulting in detachment of the cell into the fluid stream. This could account for the increased percentage of neutrophils that skipped on CHO cells expressing internalization-defective P-selectin.

A common mechanism for slowing the k_{off} of a biochemical reaction is the use of multivalent binding to increase avidity (Mason and Williams, 1986). In the fluid phase, multimeric P-selectin binds to PSGL-1 on leukocytes with higher avidity than does monomeric P-selectin (Ushiyama et al., 1993). Rapidly internalized membrane proteins cluster in clathrin-coated pits and lattices (Heuser and Anderson, 1989; Miller et al., 1991), and our immunofluorescence data show that a subset of wild-type, but not tail-less, P-selectin colocalizes with α-adaptin, a component of these structures. Thus, some internalization-competent P-selectin molecules are probably clustered in clathrin-coated pits, whereas internalization-incompetent P-selectin molecules are more likely to be diffusely distributed on the cell surface. If P-selectin were clustered on the cell surface, a PSGL-1 molecule on the neutrophil might dissociate from one P-selectin molecule but then rapidly reassoclate with an adjacent P-selectin molecule. Under shear forces, reassociation would be favored by site densities sufficient for rolling, i.e., the presence of at least two P-selectin-PSGL-1 bonds. Reassociation might not be observed at the very low P-selectin densities that favor transient tethers; after dissociation of a single bond the cell would flow
away too rapidly to rebind even to an adjacent molecule in a cluster. Conversely, at high site densities, more bonds should ensure multivalent attachment even in the absence of clustering.

The resistance of adhesion molecules to extraction from the membrane may also regulate cell rolling. The forces applied to a rolling leukocyte are considered sufficient to remove a selectin or its ligand from the lipid bilayer, unless the proteins are anchored through their cytoplasmic domains to the cytoskeleton (Alon et al., 1995). Should extraction occur, the $k_{	ext{off}}$ is likely to be different from that governing dissociation of receptor-ligand bonds. Clustering of P-selectin in clathrin-coated pits may provide another mechanism to resist extraction by applied force. The cytoplasmic domains of several P-selectin molecules could bind multivalently to the adaptor protein complex, which itself attaches multivalently to the clathrin lattice (Schmid, 1997). Such a structure might resist extraction with the same strength as a group of adhesion receptors linked to a branched, cytoskeletal protein network. Distribution of the force over a larger number of bonds may also prevent extraction. Both mechanisms would enable neutrophils to roll more slowly, with greater adhesive strength, and more uniformly on internalization-competent forms of P-selectin.

That some neutrophils still rolled on internalization-defective P-selectin suggests that an adhesion receptor need not bind to a multivalent network of cytoplasmic proteins to resist extraction by applied force. Furthermore, flowing neutrophils tethered similarly to both internalization-competent and -incompetent forms of P-selectin.

The clustering of P-selectin in clathrin-coated pits may cooperate with other mechanisms that bring P-selectin and PSGL-1 into close proximity under flow. P-selectin and PSGL-1 are extended molecules (Ushiyama et al., 1993; Li et al., 1996), and PSGL-1 is located on microvillous tips (Moore et al., 1995; Bruehl et al., 1997). These orientations favor rapid contacts under shear stress. PSGL-1 is a dimer (Moore et al., 1992), and biochemical studies suggest that P-selectin also forms dimers or oligomers (Ushiyama et al., 1993). Furthermore, PSGL-1 may be clustered through cytoskeletal interactions (Lorant et al., 1995). These arrangements should delay dissociation of bonds and/or prevent extraction from the membrane.

Optimal rolling of leukocytes under shear stress may be particularly dependent on the cell-surface organizations of PSGL-1 or P-selectin if their site densities are relatively low. Blockade of internalization impaired the adhesive function of P-selectin on activated HUVEC, which express P-selectin at a density of $\sim50$ sites/$\mu m^2$ (Hattori et al., 1989). The density of P-selectin on endothelial cells in vivo is unknown. Recently, a gene knock-in strategy was used to generate mice that express P-selectin lacking the cytoplasmic domain (Hartwell et al., 1997). Leukocytes rolled on tail-less P-selectin in mesenteric venules of these mice. However, the endothelial-cell site densities of wild-type or tail-less P-selectin were not quantified, and the relative adhesive competencies of the molecules could not be determined in vivo (D.D. Wagner, personal communication).

Tail-less P-selectin was not sorted into Weibel-Palade bodies, and was presumably internalized slowly. Both alterations might increase the surface density of the molecule on endothelial cells, compensating for loss of clustering in clathrin-coated pits. Specific challenges may also reveal impaired leukocyte recruitment due to the defective adhesive capability of tail-less P-selectin. P-selectin does not appear to be rapidly internalized on the surface of activated platelets (George et al., 1986). However, these cells contain clathrin, and P-selectin might be recruited into clathrin lattices at the plasma membrane (Klinger and Klüter, 1995).

There are at least 200 P-selectin molecules/$\mu m^2$ on activated platelets (McEver and Martin, 1984; Hsu-Lin et al., 1984; Gibson et al., 1995; Yeo et al., 1994). At such high densities, P-selectin may mediate leukocyte rolling without clathrin-mediated clustering.

Like P-selectin, E-selectin may modulate its adhesive capacity through association with clathrin-coated pits. E-selectin is internalized from the surfaces of activated endothelial cells (Von Asmuth et al., 1992; Kuijpers et al., 1994; Subramaniam et al., 1993) and of transfected CHO cells (Subramaniam et al., 1993; Chuang et al., 1997). However, it is not internalized as rapidly as P-selectin (H.S. and R.P.M., unpublished observations). Hypertonic medium prevents internalization of E-selectin, suggesting that it is also endocytosed through clathrin-coated structures (H. Setiadi and R.P. McEver, unpublished observations). Because rolling leukocytes interact with any given P- or E-selectin molecule for no more than a few seconds, the cell surface distribution of these proteins at the onset of cell contact must regulate adhesion. This fact contrasts with cell attachment that develops over many minutes, where adhesion molecules and even clathrin pits containing such molecules can migrate to the site of contact (Gumbiner, 1996; Lauffenburger and Horwitz, 1996; Weigel and Oka, 1991).

Internalization of P- and E-selectin precludes their binding to the cytoskeleton, which would impair recruitment into clathrin-coated pits (Miettinen et al., 1992). Leukocytes that adhere for many minutes to E-selectin cause its linkage to the actin cytoskeleton of endothelial cells (Yoshida et al., 1996). However, this induced cytoskeletal interaction occurs over a much longer period than the rolling adhesion studied here.

Internalization of membrane proteins in clathrin-coated pits contributes to many functions, notably delivery of nutrient ligands to intracellular compartments and downregulation of ligand-bound signaling receptors (Schmid, 1997). The rapid internalization of P-selectin is an important mechanism to limit its appearance on the endothelial cell surface (McEver, 1997). Here we demonstrate that interactions of P-selectin with clathrin-coated pits enhance its capacity to support leukocyte rolling under flow. These findings extend the known functions of clathrin-coated structures to regulation of cell adhesion.

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