10-1-2001

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Reprinted from *Biophysical Journal*, Volume 81, Issue 4, October 2001, pages 2001-2009.
Publisher URL: http://www.biophysj.org/cgi/reprint/81/4/2001

Tyrosine Sulfation Enhances but Is Not Required for PSGL-1 Rolling Adhesion on P-Selectin

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ABSTRACT P-selectin glycoprotein ligand-1 (PSGL-1) is a large (240 kDa) glycoprotein found on the surface of nearly all leukocytes. The mature molecule is decorated with multiple N- and O-linked glycans and displays copies of the tetrasaccharide sialyl-Lewis^x (sLe^X), as well as a cluster of three tyrosine sulfate (tyr-SO_3) groups near the N-terminus of the processed protein. Previous studies have suggested that PSGL-1 needs to be tyrosine-sulfated, in addition to glycosylated with sLe^X, to successfully interact with P-selectin. To better understand how biochemical features of the PSGL-1 ligand are related to its adhesion phenotype, we have measured the dynamics of adhesion under flow of a series of well-defined PSGL-1 variants that differ in their biochemical modification, to both P- and E-selectin-coated substrates. These variants are distinct PSGL-1 peptides: one that possesses sLe^X in conjunction with three N-terminal tyr-SO_3 groups (SGP3), one that possesses sLe^X without tyrosine sulfation (GP1), and one that lacks sLe^X but has three N-terminal tyr-SO_3 groups (SP3). Although all peptides expressing sLe^X, tyr-SO_3, or both supported some form of rolling adhesion on P-selectin, only peptides expressing sLe^X groups showed rolling adhesion on E-selectin. On P-selectin, the PSGL-1 peptides demonstrated a decreasing strength of adhesion in the following order: SGP3 $>$ GP1 $>$ SP3. Robust, rolling adhesion on P-selectin was mediated by the GP1 peptide, despite its lack of tyrosine sulfation. However, the addition of tyrosine sulfation to glycosylated peptides (SGP3) creates a super ligand for P-selectin that supports slower rolling adhesion at all shear rates and supports rolling adhesion at much higher shear rates. Tyrosine sulfation has no similar effect on PSGL-1 rolling on E-selectin. Such functional distinctions in rolling dynamics are uniquely realized with a cell-free system, which permits precise, unambiguous identification of the functional activity of adhesive ligands. These findings are consistent with structural and functional characterizations of the interactions between these peptides and E- and P-selectin published recently.

INTRODUCTION

The rolling adhesion of circulating leukocytes on vascular endothelium is one of the earliest manifestations of the inflammatory response. Such interactions are known to be due to selectin adhesion molecules and their ligands, which are expressed on the surface of endothelium and on circulating leukocytes. Stimulatory signals that a rolling cell experiences (either through internal signal generation or cytokine binding) allows for the efficient transition to firm arrest, before diapedesis (Springer, 1994; Jung et al., 1998). Rolling adhesion results from a force balance on the cell such that adhesion bonds between the cell and underlying surface are formed and broken at approximately equal rates. Such behavior requires appropriate kinetic and mechanical properties of the adhesion molecules involved, which are ultimately coded by their biochemical composition and structure (Hammer and Apte, 1992; Chen et al., 1997; Merkel et al., 1999; Evans et al., 2001). The selectin family of cell adhesion molecules (E-, L-, and P-selectin) has evolved to possess an optimal combination of these features, which support rolling adhesion both in vivo and in vitro (Chen et al., 1997; Alon et al., 1995; Smith et al., 1999).

In static binding assays, all three selectins are known to recognize and bind with low affinity (approximately millimolar) to the fucosylated, sialylated, tetrasaccharide sialyl Lewis X (sLe^X) and related structures (Berg et al., 1991, 1992; Foxall et al., 1992; Handa et al., 1991; Polley et al., 1991). Despite the low affinity of interaction, all three selectins can recognize and bind sLe^X under conditions of shear stress similar to those found in the post-capillary venules (Brunk et al., 1997; Greenberg et al., 2000; Rodgers et al., 2000). As such, sLe^X represents the minimal recognition unit necessary to enable adhesion under flow for all selectin molecules. Though sLe^X is likely a physiological ligand for E-selectin, higher-affinity, more complex, mucin-type ligands for L- and P-selectin have been characterized and represent the physiological ligands for these molecules (Rosen and Bertozzi, 1996).

The P-selectin ligand P-selectin glycoprotein ligand-1 (PSGL-1) is a 240-kDa transmembrane homodimer that is constitutively expressed on almost all circulating leukocytes, where it is localized to surface microvilli (Bruel et al., 1997). Biochemical characterization of this molecule shows it to be heavily sialylated and decorated with both N- and O-linked glycans (Moore, 1998). PSGL-1 displays sLe^X structures on branched, core-2 O-linked structures (Kumar et al., 1996). Deletional analysis of PSGL-1 clones indicates that the N-terminal 19-amino-acid segment (starting after the signal peptide region) of

Received for publication 12 September 2000 and in final form 18 July 2001.

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0006-3495/01/10/2001/09 $2.00

the PSGL-1 subunit chain contains the essential information content of the molecule (Pouyani and Seed, 1995). This region contains up to two, sLe^X-presenting, O-linked structures at threonines 57 and 70 as well as a cluster of tyrosine residues at positions 46, 48, and 51. Using transfected cell lines expressing modified and/or truncated PSGL-1 molecules, other investigators have demonstrated what appears to be a requirement for the sulfation of at least one N-terminal tyrosine residue in PSGL-1 to yield rolling adhesion on P-selectin (Pouyani and Seed, 1995; Liu et al., 1998; Li et al., 1996; Sako et al., 1995). Most recently, tether duration analysis of PSGL-1-transfected cells on P- and L-selectin was completed to examine PSGL-1 bond kinetic and mechanical properties (Ramachandran et al., 1999). For this analysis, modified PSGL-1 molecules in which N-terminal tyrosine residues were replaced by phenylalanine were employed. Phenylalanine residues cannot be sulfated, and conversion of all three tyrosine residues to phenylalanine was shown to effectively eliminate PSGL-1-mediated tethering and rolling on P-selectin.

In the current work, we use an alternative approach to probe biochemical features of the PSGL-1/P-selectin interactions. Using a reconstituted system, we have sought to investigate how the precise chemical decorations of PSGL-1 (glycosylation and sulfation) are related to the functional ability of PSGL-1 to deliver rolling adhesion on P- and E-selectin. We attached biotinylated PSGL-1 peptides, consisting of the 19 N-terminal amino acids of the processed protein that have been purified and separated according to their extent of glycosylation and tyrosine sulfation, to streptavidin-coated microspheres (beads). The purification and characterization of these peptides was recently described (Somers et al., 2000). A flow chamber was then used to measure the adhesion of the beads to surfaces coated with P- or E-selectin. In contrast to observations made using PSGL-1 molecules in which all three N-terminal tyrosines have been substituted with phenylalanine, we find that PSGL-1 peptides containing the N-terminal 19 amino acids of the molecule support rolling adhesion on P-selectin both in the presence and absence of tyrosine sulfation. Interestingly, we also find that at low values of wall shear stress, PSGL-1 peptides bearing N-terminal sulfotyrosine, with no sLe^X structures, support rolling adhesion on P- but not E-selectin. The strongest adhesion to P-selectin, as defined by the slowest rolling velocities, was seen with beads coated with PSGL-1 peptides expressing both sLe^X and tyr-SO_3 groups. Comparison of rolling velocities of PSGL-1 peptides in which sLe^X and tyr-SO_3 were co-expressed on the same peptide, or presented on separate peptides, indicates that optimal recognition of these groups by P-selectin occurs when glycosylation and sulfation are presented on the same PSGL-1 molecule.

MATERIALS AND METHODS

Adhesion molecules and antibodies

Soluble, recombinant E- and P-selectin (sE-selectin and sP-selectin), containing the entire extracellular portions of the molecules, P-selectin chimera (Aruffo et al., 1991), and E-selectin chimera (Nelson et al., 1993) were expressed in CHO cells and purified by conventional chromatography. Anti-PSGL-1 monoclonal antibody (mAb) KPL1, fluorescein isothiocyanate (FITC)-labeled anti-rat immunoglobulin (Ig)M, and FITC-labeled anti-mouse IgG_1 were purchased from Pharmingen (San Diego, CA). The anti-PSGL-1 mAb PSL-275 was produced in CHO cells and purified by conventional chromatography (Kumar et al., 1996). Biotinylated, multivalent sLe^X carbohydrate was purchased from Glycotech (Rockvale, MD).

Production and characterization of PSGL-1 peptides

A complete description of the production, purification, and characterization of the PSGL-1 peptides was published recently (Somers et al., 2000). A soluble construct containing the N-terminal 19 amino acids of mature PSGL-1 fused to the Fc region of IgG_1 via an intervening linker region coding the enterokinase cleavage sequence (DDDDK, (LaVallie et al., 1993)) was described earlier (Goetz et al., 1997). In summary, the construct (termed 19.EK.Fc) was expressed in CHO cells previously transfected with fucosyltransferase-VII and core2 β-1,6-*N*-acetylglucosaminyltransferase. A CHO cell clone expressing high levels of functional 19.EK.Fc was selected for large-scale protein production. The 19.EK.Fc was purified and digested with enterokinase, and the 19.EK peptides were recovered. The 19.EK peptides were dialyzed against water and then purified to individual species by SuperQ (TosoHaas, Montgomeryville, PA) anion-exchange chromatography using a gradient of 0–500 mM NaCl. Their structures were determined by mass spectroscopy (before and after proteolytic and glycosidic digestions), NMR, and composition analyses. The full structural characterization of the 19.EK peptides was presented by Somers et al. (2000). After purification, the 19.EK peptides were biotinylated at the C-terminal Lys residue with sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL). A schematic of the peptides is shown in Fig. 1. A non-post-translationally modified control peptide containing the N-terminal 19 amino acids of PSGL-1 was synthesized by Anaspec (San Jose, CA) and biotinylated as described.

Preparation of substrates

Interior divisions of one-half of eight-well, Flexiperm gaskets (Heraeus Instruments, South Plainfield, NJ) were removed to create single wells. Modified gaskets were placed on microscope slides cut from 100-cm-diameter, bacteriological polystyrene dishes (Becton Dickinson, Bedford, MA). Selectin adhesion molecules were diluted to 10 μg/ml in binding buffer (0.1 M $NaHCO_3$, pH 9.2) and incubated on slides for 2 h at room temperature. Surfaces were then washed once with Dulbecco's phosphate-buffered saline (DPBS, pH 7.4) containing calcium and magnesium ions (Sigma, St. Louis, MO) and blocked with 2% bovine serum albumin (BSA) for a minimum of 30 min. Before use in laminar flow assays, slides were incubated for 2 min with 1% Tween 20 in DPBS. A coating concentration of 10 μg/ml sP-selectin was previously shown to yield a site density of 181 sites/μm^2 using radiolabel techniques (Rodgers et al., 2000). E-selectin surfaces were prepared by incubating slides with 10 μg/ml E-selectin-Fc (E-selectin, chimera). Because of differences in the molecular weight and physisorption characteristics of sP-selectin and E-selectin-Fc, we do not know the absolute E-selectin density, although previous work with a different E-selectin-Fc indicated a solution concentration of 10 μg/ml yielded a surface concentration of 3600 molecules/μm^2 (Brunk and Hammer, 1997). All slides were prepared the same day as the experiments in

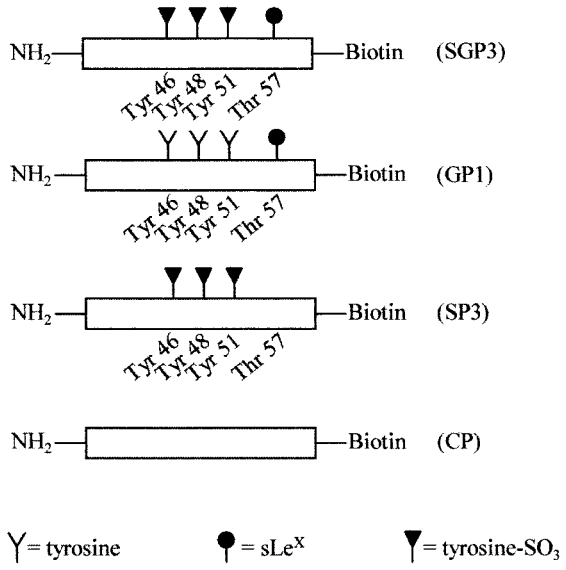

FIGURE 1 Structure of PSGL-1 ligands. Filled and open Y-shaped symbols indicate tyrosine residues (Tyr-46, Tyr-48, and Tyr-51) with and without sulfation, respectively. Bars with filled circles indicate sLe^X moieties, displayed via an O-linked glycosylation site at Thr-57. These residues translate to Tyr-5, Tyr-7, Tyr-10 and Thr-16 in the peptides used in this paper. Peptides range in molecular mass from ~3500 to 5000 Da and are biotinylated at the C-terminus.

which they were used. Control surfaces were prepared using BSA blocking only.

Preparation of microspheres

The 10.9-μm-diameter, Superavidin-coated microspheres (beads) were purchased from Bangs Laboratories (Fishers, IN). Biotinylated PSGL-1 peptides or sLe^X carbohydrate were diluted to the desired coating concentration in $DPBS^+$ for attachment to Superavidin beads. For laminar flow assays, 10^6 beads were incubated for 45 min at room temperature, with occasional vortexing. Beads were then washed and resuspended in 2 ml of $DPBS^+$ until ready for use in adhesion experiments.

Flow cytometry

All steps were performed at room temperature using $DPBS^+$ for bead washing and dilution of antibodies. Before analysis of expression levels, saturating concentrations of all primary and secondary antibodies were determined by titrating antibody coating concentrations, binding to ligand-coated beads, and examining peak fluorescent values obtained from fluorescence histograms. For analysis of peptide groups on the surfaces of the beads, 10^5 beads were incubated for 45 min with anti-PSGL-1 antibody PSL-275 (10 μg/ml). Beads were then washed and incubated for 30 min with secondary, FITC-labeled anti-mouse IgG_1. Following the secondary incubation, beads were washed three times and fixed with 1% formaldehyde in DPBS. Bead controls were performed by incubating primary and secondary antibodies, as described, under conditions where no peptide or carbohydrate was immobilized on the bead surface. Antibody binding was analyzed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA), and histogram peak analysis was completed using WinMDI software (freeware from Scripps Research Institute FACS Core Facility, La Jolla, CA).

Determination of PSGL-1 peptide site density

Quantum 26 and Quantum 24 (Flow Cytometry Standards Corp., San Juan, PR) beads were used to create a calibration curve to be used to relate peak fluorescence intensity channel number to molecules of equivalent soluble fluorescence (MESF). Given the fluorescen/protein ratio of the FITC-labeled secondary antibodies and assuming one-to-one binding between secondary and primary antibody, as well as between primary antibody and antigen, the number of peptides on the surface of the beads could be calculated. Due to multivalency of the antibodies, such methods provide only a relative estimate of the amount of molecule on the bead surface.

Laminar flow assays

A tapered-channel, parallel-plate flow chamber (Usami et al., 1993), mounted on the stage of a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) was used for laminar flow assays. Flow was initiated using a syringe pump (Harvard Apparatus, South Natick, MA), and positions in the flow chamber were monitored using Nikon stage calipers (Nikon, Melville, NY). Experiments were recorded using a Cohu black and white CCD camera (Cohu, San Diego, CA) and Sony SVO-9500MD S-VHS recorder (Sony Medical Systems, Montvale, NJ). For each experiment, chamber height (gap width) and flow rate were measured to calculate the range of wall shear stresses obtained. Bead/cell interactions with the surface were observed along the center axis of the flow chamber and recorded for later analysis.

Data analysis

Bead/cell velocity measurements were obtained through digital image analysis of video records of adhesion experiments, using LabView software (National Instruments, Austin, TX) on a Gateway 2000 computer (Gateway 2000, Sioux City, SD). Average rolling velocities were determined by dividing bead displacement, over multiple frames, by the elapsed time. Rolling fluxes were determined by manually counting the number of rolling beads/cells within a field of view and dividing by the elapsed time, as indicated by a time stamp on video images, and viewing area. Only beads continuously rolling for at least 3 s were used in calculations of average rolling velocities. Average rolling velocities reported represent the mean velocity of at least 10 beads for every value of wall shear stress included.

RESULTS

Production and characterization of PSGL-1 peptides

For the specific purpose of producing monomeric PSGL-1 peptides, we engineered a protease site into a highly truncated, chimeric form of PSGL-1 that we previously demonstrated is capable of supporting high-affinity binding to P-selectin (Sako et al., 1995). The modified construct (19.EK.Fc) encodes the 19 amino acids of mature PSGL-1 that codes for its selectin-binding activity (Pouyani and Seed, 1995). This region includes three potential sulfation sites at Tyr-46, Tyr-48, and Tyr-51 and a potential site for sLe^X modification at Thr-57. (In the PSGL-1 peptides used here, those residues correspond to Tyr-5, Tyr-7, Tyr-10, and Thr-16, respectively.) The PSGL-1 sequence was fused to the constant region of IgG_1 via a nine-amino-acid sequence that includes the enterokinase cleavage sequence (LaVallie

et al., 1993). The 19.EK.Fc purified from CHO cells expressing α1,3-fucosyltransferase and core-2 *N*-acetylglucosaminyltransferase activities supports attachment and rolling over E- and P-selectin in an in vitro model of flow (Goetz et al., 1997).

Monomeric PSGL-1 peptides were generated by cleaving 19.EK.Fc with enterokinase. Preliminary analysis by mass spectroscopy indicated that the 19.EK peptides were heterogeneous, so these were purified to individual species by anion-exchange high-performance liquid chromatography and characterized by NMR and mass spectroscopy. Details of the characterization were published by Somers and coworkers (2000). The major species of 19.EK peptides contains sulfate on all three tyrosine residues and a sLe^X-modified core-2-type O-glycan localized to Thr-16 (termed sulfoglycopeptide-3, SGP3). This glycan is identical to one of two sLe^X-containing structures isolated from myeloid PSGL-1 (Wilkins et al., 1996). Additional species of 19.EK peptides present in smaller relative quantity include versions of SGP3 containing no carbohydrate (sulfopeptide-3, SP3) or no sulfotyrosines (glycopeptide-1, GP1). A non-post-translationally modified control peptide, consisting of the N-terminal 19 amino acids of PSGL-1, was also examined (CP).

Immobilization of PSGL-1 peptides

To examine how the chemistry of different PSGL-1 peptides influences the adhesive interactions of PSGL-1 with P- and E-selectin, we prepared beads coated with various PSGL-1 peptides for use in adhesion assays. Biotinylated peptides were attached to the surface of 10.9-μm, streptavidin-coated, polystyrene microspheres, serving as model cells with defined protein composition. Expression of PSGL-1 peptides was verified by flow cytometry. Fig. 2 shows resultant fluorescence histograms for beads stained with the anti-PSGL-1 mAb PSL-275 and a secondary species-matched fluorescent antibody. At a peptide coating concentration of 0.05 μg/ml, which yields $\sim$172 sites/μm^2, we were able to achieve nearly equal surface coverage for all peptides.

PSGL-1-coated beads roll on P- and E-selectin

Previous work using beads coated with heterogeneously decorated PSGL-1 ligands showed dynamic adhesion to P- and E-selectin-coated surfaces (Goetz et al., 1997; Rodgers et al., 2000). Here, we have used biochemically well-characterized PSGL-1 ligands to interrogate how molecular features of PSGL-1 modulate its adhesive phenotype. Fig. 3 shows bead flux measurements for peptide-coated beads on surfaces coated with P-selectin, E-selectin, or BSA. As shown, all but control-peptide-coated beads displayed rolling adhesive interactions on P-selectin surfaces. SGP3- and

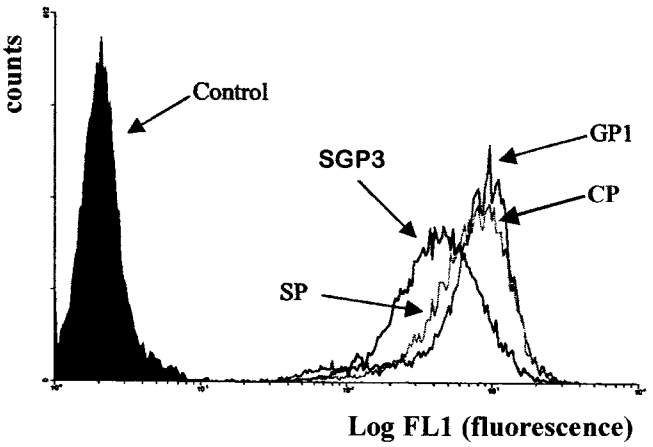

FIGURE 2 Flow cytometry of PSGL-1-coated beads. The 10.9-μm superavidin-coated beads were coated with 0.05 μg/ml (172 sites/μm^2) of PSGL-1 peptides. Beads were labeled with PSL-275, an anti-PSGL-1 mAb, followed by an FITC-labeled secondary antibody. Control beads were labeled with antibodies but not coated with any peptides.

GP1-coated beads showed nearly equivalent rolling fluxes, whereas SP3-coated beads showed a much lower rolling flux, indicative of a weaker interaction. A comparison of rolling fluxes between beads expressing sLe^X and tyr-SO_3 groups, on separate and identical peptides, was also completed. In this study, one set of beads was incubated with equal concentrations (0.05 μg/ml) of a mixture of GP1 and SP3 peptides, and another set of beads was incubated with

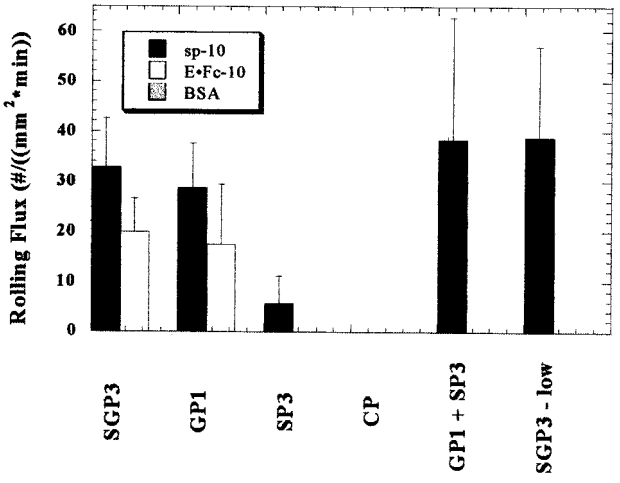

FIGURE 3 Rolling flux on P-selectin, E-selectin, and BSA. Rolling flux measurements are shown for an average wall shear stress of 0.65 dynes/cm^2 on surfaces physisorbed with either sP-selectin surface concentration made using a bulk concentration of 10 μg/ml (equivalent to 181 sites/μm^2 (Rodgers et al., 2000) (*open bars*), E-selectin-Fc chimera made using a bulk concentration of 10 μg/ml (*solid bars*), or BSA (*gray shaded bars*). The ordinate describes different bead coatings. GP1 + SP3 refers to beads coated with a mixture of these peptides, and SGP3-low refers to beads coated with only 0.025 μg/ml of SGP3 peptide. None of the peptides showed rolling adhesion on control surfaces coated with BSA.

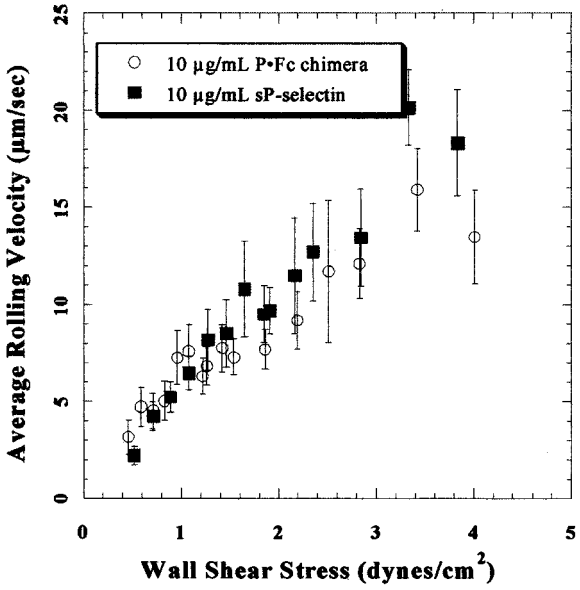

FIGURE 4 Comparison of rolling velocities on sP-selectin and P-selectin chimera. The 10.9-μm beads coated with 0.5 μg/ml biotinylated sLe^X carbohydrate were perfused over surfaces coated with 10 μg/ml (181 sites/μm^2) sP-selectin or P-selectin chimera. Average rolling velocities were determined for several values of wall shear stress.

half the concentration (0.025 μg/ml) of SGP3 peptide. This approach permitted examination of adhesion mediated by equal numbers of sLe^X and tyr-SO_3 groups, though presented to the P-selectin substrate in a different manner. Rolling flux measurements from these experiments are also shown in Fig. 3. Beads presenting sLe^X and tyr-SO_3 groups together, whether located on the same or separate peptides, showed similar rolling fluxes. Rolling flux also appeared to be insensitive to the coating of peptide for the concentrations studied, as beads coated with either a higher (0.05 μg/ml) or lower concentration of SGP3 peptide (0.025 μg/ml) showed similar rolling fluxes.

In contrast to results seen on sP-selectin surfaces, only beads coated with SGP3 or GP1 peptides, both of which display sLe^X groups, showed rolling interactions on E-selectin surfaces. Before completion of adhesion assays with the E-selectin chimera, the adhesion of peptide-coated beads on surfaces coated with a soluble form of the E-selectin molecule (sE-selectin) was examined. At surface coatings of up to 20 μg/ml sE-selectin, no adhesion was seen (data not shown). Such behavior may be attributable to insufficient length of the sE-selectin molecule, which is shorter than sP-selectin by three complement-binding domains, or due to vagaries of adsorption of sE-selectin on plastic surfaces. That the Fc portion of the chimeric adhesion molecules does not influence adhesion dynamics is shown in Fig. 4, which shows essentially no difference in rolling velocities of beads coated with sLe^X carbohydrate on 10 μg/ml of either sP-selectin or P-selectin chimera-coated surfaces.

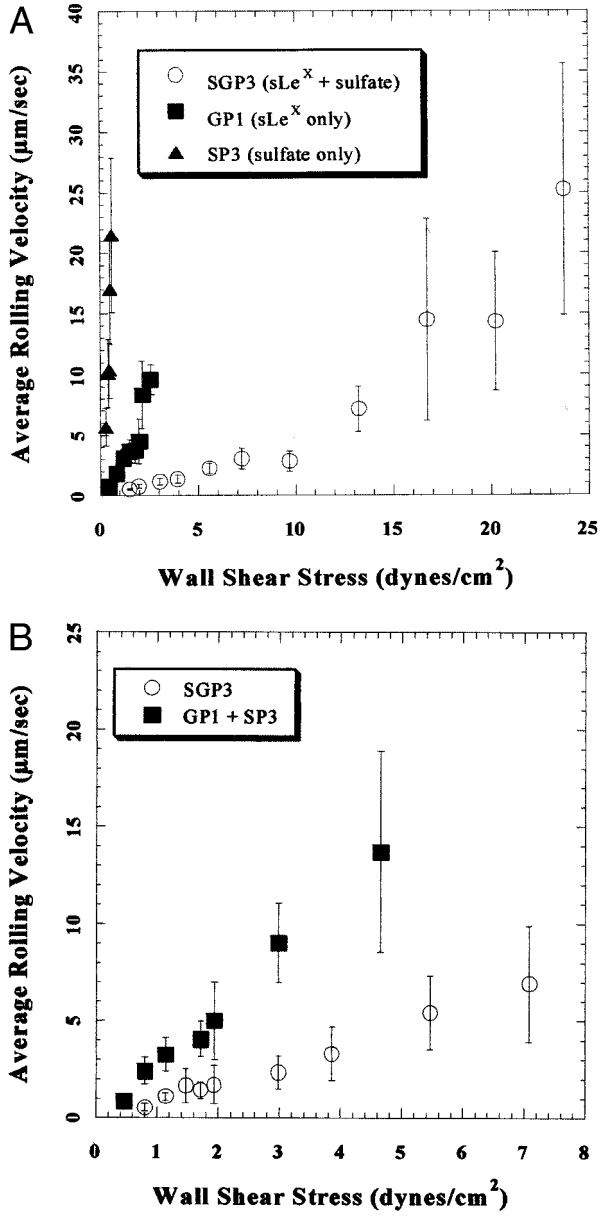

FIGURE 5 Average rolling velocity as a function of wall shear stress for PSGL-1 ligands on P-selectin. (*A*) Surfaces were coated with 10 μg/ml (181 sites/μm^2) sP-selectin, and 1×10^6 beads/ml, coated with equal amounts (0.05 μg/ml) of PSGL-1 peptides, were perfused. Data for beads coated with the control peptide are not shown, as they displayed no rolling interactions on sP-selectin-coated surfaces. (*B*) Beads were coated with an equal concentration of GP1 and SP3 peptides, 0.05 μg/ml each, or 0.025 μg/ml of SGP3 peptide and perfused over a surface coated with 10 μg/ml sP-selectin.

Peptide chemistry specifies rolling behavior

Depending on the chemistry of the PSGL-1 peptide involved, rolling adhesion was different on surfaces coated with sP-selectin. Results are shown in Fig. 5 *A*. Beads coated with PSGL-1 peptides displaying sulfotyrosine but no sLe^X (SP3) showed the fastest rolling velocities. These

beads were observed to roll up to a wall shear stress of only 0.67 dynes/cm^2. Beads coated with the SGP3 peptide, which displays both sLe^X and three sulfotyrosines, showed the strongest interaction with sP-selectin surfaces, exhibiting slower rolling velocities and ability to support rolling adhesion at wall shear stresses as high as 40 dynes/cm^2. GP1-coated beads yielded intermediate rolling velocities and were able to support rolling adhesion up to ~5 dynes/cm^2.

As shown, at nearly equal surface density, co-expression of sLe^X and tyr-SO_3 on PSGL-1 reduces rolling velocities to a level lower than that observed with beads coated with peptides expressing either sLe^X or tyr-SO_3 alone. To investigate whether it is necessary for sLe^X and tyr-SO_3 to be expressed on the same PSGL-1 molecule to achieve an increasing strength of adhesion, we prepared beads coated with an equal concentration of GP1 and SP3 peptides (0.05 μg/ml each) as well as beads coated with a half-as-concentrated (0.025 μg/ml) solution of SGP3 peptide. Assuming adsorption in proportion to concentration (supported by Fig. 2), these two populations of beads should have equal total numbers of sLe^X and tyr-SO_3 groups. Rolling velocities of the two populations of beads, on P-selectin, were then measured. Fig. 5 *B* shows that, comparing equal surface density of sLe^X and Tyr-SO_3 groups, co-expression of these two chemistries on the same peptide yields a slower rolling velocity (stronger interaction) than co-expression on separate peptides.

As previously observed, we found that beads coated with PSGL-1 ligands also allowed for attachment and rolling on surfaces coated with E-selectin (Goetz et al., 1997; Patel et al., 1995). Fig. 6 shows rolling velocities as a function of wall shear stress for PSGL-1-coated beads (GP1 and SGP3) on E-selectin-coated surfaces. In contrast to results seen on P-selectin, tyr-SO_3 does not support rolling adhesion on E-selectin, nor does it augment rolling adhesion of sLe^X-bearing PSGL-1 on E-selectin. Beads coated with the SP3 peptide, which express sulfotyrosine but no sLe^X, are not adherent to E-selectin surfaces. In addition, the magnitude of the rolling velocity observed with beads coated with SGP3 on E-selectin is very similar to that seen with beads coated with GP1 (sLe^X only) on P-selectin.

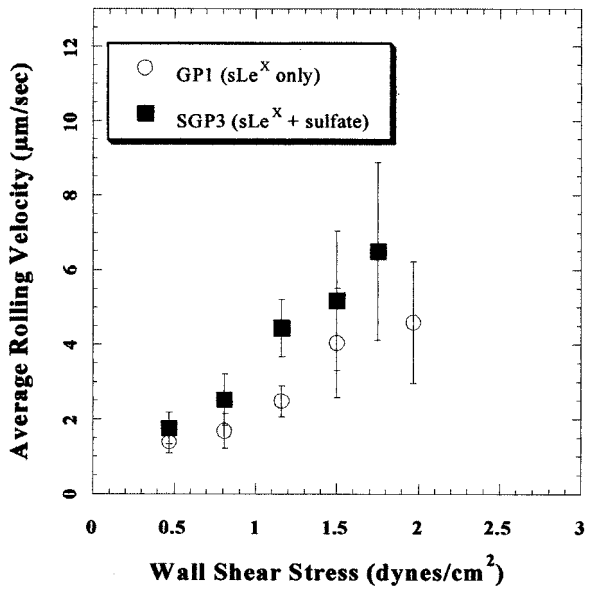

FIGURE 6 Average rolling velocity as a function of wall shear stress for PSGL-1 ligands on E-selectin. Surfaces were coated with 10 μg/ml E-selectin chimera, and 1×10^6 beads/ml, coated with 0.05 μg/ml of the PSGL-1 peptides, were perfused. Only peptides displaying sLe^X moieties, SGP3 or GP1, displayed rolling interactions on E-selectin-coated surfaces.

DISCUSSION

PSGL-1 is a high-affinity, mucin-type ligand for P-selectin. It has been proposed that high-affinity recognition of PSGL-1 is achieved though a combination sLe^X and tyrosine sulfate groups on the N-terminus of the PSGL-1 molecule (Moore, 1998; McEver and Cummings, 1997). The recent paper by Camphausen and co-workers, in which co-crystals of P- or E-selectin and the different peptides used here were examined by x-ray crystallography, shed significant light on the combined role of glycosylation and tyrosine sulfation on ligation to P-selectin and E-selectin (Somers et al., 2000). Previous approaches to this problem have included adhesion studies using modified PSGL-1 structures expressed in model cell lines (Liu et al., 1998; Ramachandran et al., 1999), attachment of purified PSGL-1 molecules to the surface of microspheres (Rodgers et al., 2000; Moore, 1998), and synthetic PSGL-1 peptides used as adhesion inhibitors (Leppanen et al., 1999).

The use of a reconstituted, cell-free system in conjunction with well-characterized PSGL-1 peptides has allowed us to probe interactions that appear to have been masked in the approaches previously described. The additive nature of the approach taken, in which specific chemistries are incorporated into the system, rather than subtraction, blocking, or mutation, permits previously unavailable control over the chemistry of adhesion. We have compared both the rolling flux and rolling velocity of beads coated with PSGL-1 peptides displaying sLe^X, sulfotyrosine, and combinations thereof. We find that though weak in nature (judging by both low rolling flux and high rolling velocity), beads coated with PSGL-1 peptides expressing sulfotyrosine groups but no sLe^X are capable of weakly supporting adhesion under flow to P-selectin. In our experiments, we used only tri-sulfated peptides, so it remains an open question as to whether mono- or di-sulfated peptides will support rolling adhesion on P-selectin.

Published reports indicate that PSGL-1 decorated with sLe^X alone cannot support rolling adhesion on P-selectin (Liu et al., 1998; Ramachandran et al., 1999). However, a recent study completed in our laboratory (Rodgers et al.,

2000), examining the rolling adhesion of sLe^X-coated beads on P-selectin surfaces, indicated that sLe^X alone, outside the context of PSGL-1, can support rolling adhesion on P-selectin. Here, we show that PSGL-1 peptides expressing sLe^X, but not sulfotyrosine, are also able to support rolling adhesion on P-selectin. A possible explanation for the disparity seen is that previous studies made use of PSGL-1 mutants in which tyrosine residues were replaced with phenylalanine. Changes in the primary amino acid structure of tyrosine to phenylalanine may affect post-translational modifications at Thr-16, rendering attachment of sLe^X groups less efficient. In addition the peptide backbone itself may contribute to adhesive binding interactions (Somers et al., 2000). Our results indicate that when sulfate groups are removed, but tyrosine residues are left in tact, the function of sLe^X is restored, and robust rolling may be seen up to 5 dynes/cm^2. Measurements of the affinity of binding of glycosylated peptides (GP1) to P-selectin using surface plasmon resonance show that there is a modest binding affinity between GP1 and P-selectin (Somers et al., 2000), consistent with the functional rolling results shown here.

Though possessing similar domain structures and sequence homology (Bevilacqua, 1993; Bevilacqua and Nelson, 1993), it is apparent from our findings that subtle differences in the binding regions of E- and P-selectin exist, such that the presence of negatively charged Tyr-SO_3 groups dramatically affects adhesion dynamics mediated by PSGL-1 on P- but not E-selectin. Such behavior has been previously suggested by results from affinity-capture assays (Sako et al., 1995) and in experiments using beads coated with a PSGL-1 mutant in which the anionic Tyr-SO_3 region of the N-terminal amino acids was deleted (Goetz et al., 1997). Similar behavior is seen with the current system, in which we compare adhesion on P- and E-selectin while maintaining the native amino acid sequence of the PSGL-1 peptides. On P-selectin, addition of tyrosine sulfation to glycosylation leads to a super-antigen that supports slower rolling at higher shear stresses, up to 40 dynes/cm^2, well into the physiologically relevant range of wall shear stresses. On E-selectin surfaces, no differences in rolling flux or velocity between beads coated with peptides expressing sLe^X alone or sLe^X in addition to sulfotyrosine were observed. It is also important to note that monomeric peptides of PSGL-1 are perfectly capable of supporting rolling, as has been observed elsewhere (Epperson et al., 2000), although there may be a functional role of PSGL-1 dimerization in increasing the efficiency of tethering (Somers et al., 2000; Epperson et al., 2000).

The structural origins of these differences are now clear from Somers et al. (2000). Both P- and E-selectin are able to ligate sLe^x through an electrostatically driven association between the fucose of sLe^x and the Ca^{2+} in the selectin-binding site, supported by weaker hydrogen bonding interactions between carbohydrate hydroxyls and amino acids that surround the Ca^{2+} atom. In P-selectin, this involves glutamine (at position 88) and asparagine (at position 83). There are small differences in the amino acid sequences of P- and E-selectin in the region of Ca^{2+} ligation, which lead to stronger hydrogen bonding for E-selectin and a slightly different affinity of binding. Because there are relatively minor differences in how P- and E-selectin bind sLe^x, one would expect, and indeed we found, that sLe^x-bearing GP1 would support rolling on both selectins. However, the additional role of tyrosine sulfation in aiding PSGL-1 binding to P-selectin, and not to E-selectin, is also suggested by structural information. On P-selectin, there are two separate locations where tyrosine sulfates ligate to multiple amino acids, distant from the site of sLe^x ligation to the Ca^{2+} atom. However, both interactions involving tyrosine sulfates and sLe^x can occur simultaneously (in a molecular hairpin), which likely lead to the higher affinity of interaction between SGP3 and P-selectin (as seen in Biacore measurements (Somers et al., 2000)) and here in adhesion assays (manifested in slower rolling velocities at given shear rates). Indeed, although sLe^x ligation appears in regions dominated by anionic amino acids (to ligate calcium) and hydrogen bonding to hydroxyls on fucose, tyrosine sulfation ligation occurs in cationic regions of P-selectin. We can further speculate that the tyrosine sulfates act as a molecular tailhook that allows cells and beads bearing tyrosine sulfation to tether and roll at higher shear rates on P-selectin; these initial interactions are strengthened by sLe^x ligation, which leads to sustainable rolling at high shear rates. The overall strength of binding is supported by the two ends of the molecular hairpin: one anchored by tyrosine sulfate interactions and the other anchored by sLe^x-Ca^{2+} interactions.

The physiological role of weak, PSGL-1/P-selectin bonds, such as those mediated by sLe^X or Tyr-SO_3 alone, has not been investigated. Although fully competent PSGL-1, displaying both sLe^X and Tyr-SO_3, may be largely responsible for the rolling of leukocytes, other roles for weaker P-selectin/PSGL-1 interactions may exist. For example, in a thrombogenic environment, platelet P-selectin may have the opportunity to interact with differentially glycosylated and/or sulfated forms of leukocyte PSGL-1. Due to their size and shape, platelets can experience extremely low hemodynamic shear forces, and hence even such weak PSGL-1/P-selectin interactions may contribute to adhesive events of this type.

At this time, detailed information regarding both the mechano-chemical properties of these various PSGL-1 peptides with P- or E-selectin is not available. A common approach to measurement of these parameters involves characterization of the pause times of beads or cells in flow, as mediated by the molecules of interest. As noted, Ramachandran et al. (1999) measured off-rates and reactive compliance, from pause time distributions, for mutants of PSGL-1 in which tyrosine was replaced with phenylalanine. Similar work must be done with the peptides used here to

understand the functional basis of the differences among the various peptides. However, measurement of weaker interactions, such as P-selectin/sLe^X and P-selectin/Tyr-SO_3, may require a more sensitive measurement technique, such as dynamic force spectroscopy using the biomembrane force probe (Evans et al., 1995, 2001) or atomic force microscopy. Measurements of this nature will add quantitative and perhaps mechanistic insight to the details of PSGL-1-mediated adhesion.

The Hammer lab gratefully acknowledges support from HL18208 and GM59100. The analogy of the selectins to a molecular tailhook is attributable originally to Evan Evans.

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