THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 9, Issue of February 26, pp. 5271–5278, 1999

Printed in U.S.A.

Inhibition of L-selectin-mediated Leukocyte Rolling by Synthetic Glycoprotein Mimics*

(Received for publication, November 20, 1998)

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Synthetic carbohydrate and glycoprotein mimics displaying sulfated saccharide residues have been assayed for their L-selectin inhibitory properties under static and flow conditions. Polymers displaying the L-selectin recognition epitopes 3′,6-disulfo Lewis x(Glc) (3-*O*-SO_3-Galβ1α4(Fucα1α3)-6-*O*-SO_3-Glcβ-OR) and 3′,6′-disulfo Lewis x(Glc) (3,6-di-*O*-SO_3-Galβ1α4(Fucα1α3)Glcβ-OR) both inhibit L-selectin binding to heparin under static, cell-free binding conditions with similar efficacies. Under conditions of shear flow, however, only the polymer displaying 3′,6-disulfo Lewis x(Glc) inhibits the rolling of L-selectin-transfected cells on the glycoprotein ligand GlyCAM-1. Although it has been shown to more effective than sialyl Lewis x at blocking the L-selectin–GlyCAM-1 interaction in static binding studies, the corresponding monomer had no effect in the dynamic assay. These data indicate that multivalent ligands are far more effective inhibitors of L-selectin-mediated rolling than their monovalent counterparts and that the inhibitory activities are dependent on the specific sulfation pattern of the recognition epitope. Importantly, our results indicate the L-selectin specificity for one ligand over another found in static, cell-free binding assays is not necessarily retained under the conditions of shear flow. The results suggest that monovalent or polyvalent carbohydrate or glycoprotein mimetics that inhibit selectin binding in static assays may not block the more physiologically relevant process of selectin-mediated rolling.

The selectins are a family of three saccharide-binding proteins that mediate the recruitment of leukocytes from the bloodstream to sites of tissue damage and to secondary lymphoid organs (1–4). Because they play an important role in a number of disease states including chronic and acute inflammation, the selectins have been targeted for the identification of specific, high-affinity inhibitors as potential therapeutic agents (5, 6). The search for inhibitors that meet these criteria has been impeded by the complexity of the naturally occurring selectin ligands (7), which complicates characterization of the molecular determinants of the recognition process. Moreover, the selectins function by a unique mechanism; they mediate the rolling of leukocytes under conditions of shear stress (8–10). The molecular mechanisms that govern the rolling process are only beginning to be elucidated, and the majority of studies addressing selectin recognition have been conducted under static conditions. Little is known, therefore, about the structural features required for effective inhibitors of selectin-mediated rolling.

Physiological ligands for all three members of the selectin family have been identified. Structural studies suggest that glycoprotein ligands that interact with E-selectin (cutaneous lymphocyte antigen, ESL-1,[1] PSGL-1) (11–15), P-selectin (PSGL-1) (16–18), and L-selectin (GlyCAM-1, CD34, PSGL-1) (19–23) possess saccharide chains capped with the tetrasaccharide sialyl Lewis x (sLe^x **1**, Fig. 1) (24–28). One of the best characterized of the physiological selectin ligands is the L-selectin ligand GlyCAM-1. The glycoprotein GlyCAM-1, which is secreted from endothelial cells in high endothelial venules (HEV), is a mucin: a heavily *O*-glycosylated, extended polypeptide that presents saccharide recognition elements extending from core-2 oligosaccharide structures. GlyCAM-1 displays the major saccharide capping groups 6-sulfo sialyl Lewis x (6-sulfo sLe^x **2**) and 6′-sulfo sialyl Lewis x (6′-sulfo sLe^x **3**) (25, 27, 29) (Fig. 1). The identification of these tetrasaccharide capping groups combined with the known requirement for sulfation of GlyCAM-1 (30) led to the hypothesis that sulfation at the 6- or 6′-position of sLe^x might confer recognition specificity for L-selectin. Thus, the extensive characterization of the saccharide chains of this protein has provided a structural basis from which to search for high-affinity, specific L-selectin inhibitors.

The difficulties of defining the saccharide recognition epitope for a particular selectin are exemplified by studies of the interaction of L-selectin with various carbohydrate ligands. Attempts to elucidate the relevant saccharide element for L-selectin binding have produced conflicting reports that suggest either 6′-sulfation or 6-sulfation or perhaps both modifications of the sLe^x core are critical (31–37). Several different studies highlight the importance of 6-sulfation. Antibodies uniquely reactive with 6-sulfo sLe^x inhibit L-selectin binding to HEV (31, 32), and glycolipids displaying 6-sulfation but not 6′-sulfation support L-selectin binding (36). In addition, a direct comparison of the disulfated Le^x derivatives 3′,6′-disulfo Le^x(Glc) **4** and 3′,6-disulfo Le^x(Glc) **5** (Fig. 2*A*) revealed that **5** is a superior inhibitor of L-selectin binding to GlyCAM-1 (34). The conclusions from these studies, however, have been inferred from data obtained in static binding assays, which do not incorporate the conditions of shear stress under which selectin-

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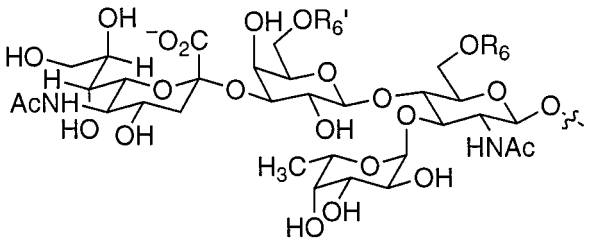
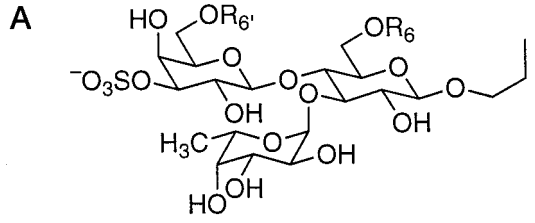
[1] The abbreviations used are: ESL-1, E-selectin ligand 1; PSGL-1, P-selectin glycoprotein ligand 1; Gal, galactose; Fuc, fucose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GlyCAM-1, glycosylation-dependent cell adhesion molecule-1; sLe^x, sialyl Lewis x; HEV, high endothelial venules; ^{1}H NMR, proton nuclear magnetic resonance; DTAB, dodecyltrimethylammonium bromide; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; ROMP, ring opening metathesis polymerization; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

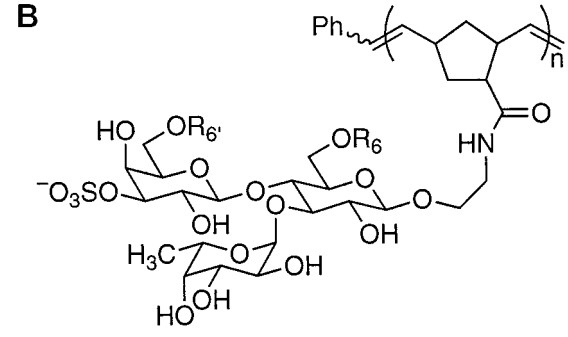


1 $R_{6'} = R_6 = H$ sialyl Lewis x
2 $R_{6'} = H$, $R_6 = SO_3^-$ 6-sulfo sialyl Lewis x
3 $R_{6'} = SO_3^-$, $R_6 = H$ 6'-sulfo sialyl Lewis x

FIG. 1. **Glycoprotein capping groups present on the L-selectin ligand GlyCAM-1.** GlyCAM-1 contains sulfated derivatives of the tetrasaccharide sialyl Lewis x at the termini of its saccharide chains.



4 $R_{6'} = SO_3^-$, $R_6 = H$ 3',6'-disulfo Le^x(Glc)
5 $R_6 = SO_3^-$, $R_{6'} = H$ 3',6-disulfo Le^x(Glc)



8 $R_{6'} = SO_3^-$, $R_6 = H$
9 $R_6 = SO_3^-$, $R_{6'} = H$

FIG. 2. ***A*, synthetic monovalent L-selectin ligands 3′,6′-disulfo Le^x(Glc)-β-OPr (4) and 3′,6-disulfo Le^x(Glc)-β-OPr (5); *B*, synthetic multivalent mimics displaying trisaccharides 3′,6′-disulfo Le^x(Glc) (8) or 3′,6-disulfo Le^x(Glc) (9).**

ligand interactions take place.

The multivalent display of saccharide-based recognition epitopes on GlyCAM-1 may provide additional features necessary for effective inhibition of L-selectin under physiological conditions. Several studies have addressed the importance of multivalent binding for high avidity interactions with L-selectin. For example, di- and tetravalent sLe^x analogs are superior inhibitors of L-selectin-mediated lymphocyte binding to HEV when compared with monovalent sLe^x (38–42). In addition, sLe^x-containing acrylamide polymers are micromolar inhibitors of L-selectin binding to 3′-sulfo Le^x ceramide (43). Again, all of these studies utilized static binding assays to assess the efficacy of the inhibitors. To determine the relevant structural characteristics of L-selectin ligands, examination of the molecular requirements for L-selectin inhibition under conditions of shear stress may be necessary (10). We set out to explore whether the apparent potencies of L-selectin inhibitors under static assay conditions were preserved under the more physiologically relevant conditions of shear stress.

Leads for bioactive inhibitors are often inspired by the structures of the physiological ligands that bind the target of interest. The naturally occurring mucin-like L-selectin ligands present multivalent displays of L-selectin recognition elements on an extended polypeptide backbone (7). To mimic critical features of these mucins, we have developed a method for the synthesis of multivalent materials, termed neoglycopolymers, that display multiple saccharide epitopes (44–47). These materials, which are generated using the ring-opening metathesis polymerization (ROMP), share critical common features with natural L-selectin ligands; they present a multivalent display of saccharide recognition elements from an extended backbone (Fig. 2*B*). The advantages of ROMP over other methods for synthesizing multivalent glycoprotein mimics are: 1) the assembly of the materials occurs in a single step from the monomer (44–47); 2) materials of defined length can be generated by varying the ratio of initiator to monomer (48–51); and 3) reporter groups can be appended to the resulting multivalent derivatives facilitating the monitoring of the biomolecular interaction. Here we report the synthesis and inhibitory properties of novel multivalent Le^x-derived L-selectin ligands **8** and **9**, synthesized by ROMP, possessing either 6-sulfation or 6′-sulfation (Fig. 2*B*). The activities of these ligands were assessed in both static and flow-based assays.

EXPERIMENTAL PROCEDURES

*Monomers **4** and **5**, and sLe^x*—Disulfated Le^x trisaccharides **4** and **5** were prepared and purified by protocols described previously (52). Sialyl Lewis x, methyl glycoside (Neu5Ac2-α-3Gal1-β-4(Fuc1-α-3)GlcNAc1-*O*-β-Me) was purchased from Toronto Research Chemicals, Inc.

*Synthesis of Polymers **8** and **9***—Detailed synthetic procedures for compounds **6**–**9** will be published elsewhere. Briefly, ROMP monomers **6** and **7** were prepared by conjugation of 2-aminoethyl glycosides of the disulfated trisaccharides to (±)-bicyclo[2.2.1]hept-5-ene-*exo*-2-carboxylic acid pentafluorophenyl ester. Purification of the monomers by anion exchange chromatography (diethylaminoethyl-cellulose fast flow; 0→0.10 M $Et_3NH_2CO_3$) resulted in their isolation as bis-triethylammonium salts, which were used directly in the polymerization reactions. Monomer **6** (32.6 mg, 0.0321 mmol) or **7** (30 mg, 0.0296 mmol) and DTAB (**6**: 19.8 mg, 0.0642 mmol; **7**: 15 mg, 0.0474 mmol) were dissolved in 100 mM pH 6.0 bis-Tris buffer (**6**: 0.214 ml buffer; **7**: 0.200 ml buffer), which was deoxygenated by vigorous sparging of the solution with N_2 for 2 h, under argon. To this mixture was added a solution of ruthenium carbene **10** (**6**: 1.8 mg, 0.00214 mmol; **7**: 1.6 mg, 0.00198 mmol) in dichloroethane (**6**: 0.107 ml; **7**: 0.100 ml, deoxygenated by five freeze-pump-thaw cycles), which resulted in a bright purple emulsion. This mixture was stirred vigorously at room temperature for 1 h, after which the emulsion turned from purple to light brown. The reaction was then heated to 60 °C and stirred for 14 h. Thin layer chromatography (TLC) analysis indicated mainly baseline material at this time (no monomer was visible). The mixture was cooled to room temperature, and excess ethyl vinyl ether (100 μL) was added. The reaction was exposed to air and stirred for 2 h, and methanol (100 μL) was added to produce a homogeneous solution. Purification of the polymer was conducted by the following steps: 1) size exclusion chromatography (Sephacryl S-200; 0.5 × 1.0 cm; eluting with 0.05 M $Et_3NH_2CO_3$) followed by concentration of the relevant fractions to dryness; 2) cation exchange chromatography (SP-Sephadex C-25; 0.5 × 2.0 cm; Na^+) followed by concentration of the relevant fractions to dryness; 3) suspension of the resulting residue in methanol and centrifugation (3×); 4) dissolution of the residue in water followed by dialysis (500 molecular weight cut off in 1 liter of H_2O; 2 × 24 h). Polymers **8** (19.5 mg, 71%) and **9** (21.3 mg, 84%) were isolated as brownish, flaky solids. Analysis by ^1H NMR spectroscopy (500 MHz, D_2O) indicated that *n* values of approximately 15 were obtained for each

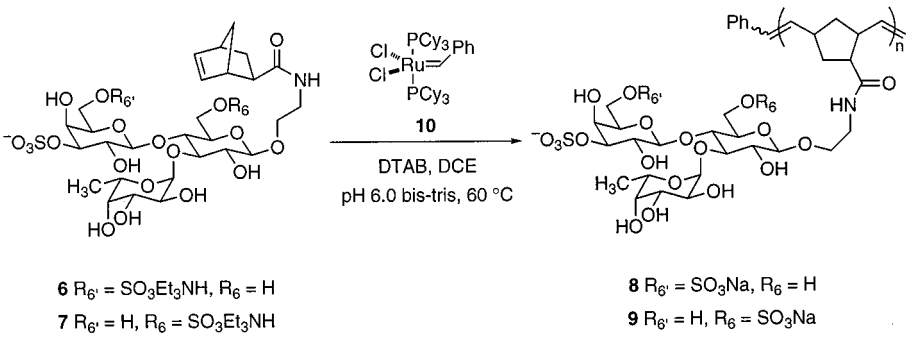


FIG. 3. **Synthesis of nonnatural glycoprotein mimics by the aqueous ROMP.** A solution of initiator **10** in deoxygenated DCE was added to a deoxygenated solution of monomer **6** or **7** and DTAB in 100 mM bis-Tris buffer, pH 6.0. The mixture was heated to 60 °C and stirred for 14 h. Purification by size-exclusion and cation exchange chromatography afforded polymers **8** and **9**.

polymer as determined by comparison of integrals of the terminal phenyl group (5H per molecule) with integrals of the olefin and Fuc-H_1 resonances (3H per monomer). These data suggest that the multivalent ligands **8** and **9** are composed of an average of 15 monomer units.

L-selectin–Heparin ELISA—Polymers **8** and **9** were assayed for their ability to inhibit the binding of an L-selectin-IgG fusion protein to heparin, as described previously (53). Briefly, serial dilutions of the test inhibitors were incubated with beads containing both goat IgG (for detection purposes) and an L-selectin-IgG chimeric protein in 96-well plates coated with heparin-albumin. The wells were sequentially washed with phosphate-buffered saline (PBS), and an anti-goat alkaline phosphatase-conjugated secondary antibody was added. The number of bound beads was determined by measuring the absorbance at 405 nm after addition of a standard alkaline phosphatase substrate.

Rolling Assay—Polymers **8** and **9** were tested for their ability to inhibit rolling of L-selectin transfected cells on substrates coated with purified GlyCAM-1. The human L-selectin-transfected mouse pre-B cell line 300.19 (54), a generous gift of Dr. G. S. Kansas (Northwestern University, Chicago, IL) was maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and 0.1 mM 2-mercaptoethanol. GlyCAM-1 purified from mouse serum was a generous gift from Dr. S. D. Rosen (University of California, San Francisco). For coating onto a substrate, GlyCAM-1 concentrate was diluted to a final concentration of 0.1 mg/ml in coating medium (PBS, supplemented with 20 mM bicarbonate, pH 8.5) and adsorbed onto a polystyrene plate at 37 °C for 2 h. Substrate was washed five times with PBS and blocked with 2% human serum albumin in PBS overnight at 4 °C. The polystyrene plate on which purified ligand was adsorbed was integrated into a parallel plate laminar flow chamber (260-mm gap), and the chamber was mounted on the stage of an inverted phase contrast microscope (Diaphot 300, Nikon Inc., Japan) as described previously (55, 56). Cells stored in H/H medium (Hank's buffered saline solution containing bovine serum albumin, 2 mg/ml, and 10 mM HEPES, pH 7.4) at 4 °C were resuspended at densities of 10^6 cells/ml in binding medium (H/H medium supplemented with 2 mM Ca^{2+}), brought to room temperature, and perfused through the flow chamber. Cellular interactions on two different fields of view (each one 0.17 mm^2 of area) were visualized with a × 10 objective. Cell images were videotaped with a long integration LIS-700 charge-coupled device video camera (Applitech, Holon, Israel) and a Sony SLV E400 video recorder. Images were manually quantitated by analysis of played-back images directly from the monitor screen. Cells were perfused through the chamber at a flow rate generated with an automated syringe pump (Harvard Apparatus, Natick, MA) attached to the outlet side of the flow chamber. Cellular attachments to and subsequent rolling on the substrate were enumerated during 60 s of cell perfusion at a shear stress of 1.75 dyne/cm^2. The attachments were expressed as a fraction of the flux of freely flowing cells that moved through the same field in close proximity with the substrate, as described previously (57). More than 99% of the rolling events scored were ligand specific, as confirmed in parallel determinations on control substrates coated with human serum albumin. For inhibition studies, cells were mixed with mono- or multivalent saccharides, preincubated for 5 min in binding medium at room temperature, and then perfused without washing into the chamber. For comparing adhesive interactions in the presence of the various L-selectin blockers, identical fields of view were used to ensure that the results reflected uniform site density and distribution of the immobilized L-selectin ligand GlyCAM-1.

RESULTS

Synthesis of L-selectin Ligands—The monovalent 3′,6′-disulfo Le^x(Glc) and 3′,6-disulfo Le^x(Glc) trisaccharides **4** and **5** were prepared by chemical synthesis as described previously (52). These trisaccharide derivatives were designed to act as mimics of 6′-sulfo sialyl Lewis x and 6-sulfo sLe^x, the major capping groups of GlyCAM-1. In previous studies, 3′,6-disulfo Le^x(Glc), the analog of 6-sulfo sLe^x, was found to be a superior inhibitor of the L-selectin –GlyCAM-1 interaction in a static binding assay (34). To determine the influence of multivalent presentation on L-selectin recognition, we prepared displays of the trisaccharide epitopes of **4** and **5** to mimic the mode of saccharide residue presentation exhibited by naturally occurring L-selectin ligands, such as GlyCAM-1. These glycoprotein-inspired materials were assembled by a ring-opening metathesis polymerization (ROMP).

To generate monomers that could be oligomerized in ROMP, intermediates in the synthesis of **4** and **5** were elaborated to possess anomeric substituents with free amino groups. The resulting compounds were appended to a bicyclic norbornene template through amide bond formation to yield compounds **6** and **7** as monomers for ROMP (Fig. 3). The template can be easily constructed (46, 47), and it contains a strained alkene group, which facilitates the ROMP assembly reaction. To increase their solubility in the organic solutions required for initiator dissolution, the polar anionic monomers were converted to their triethylammonium salts.

The glycoprotein analogs were prepared by oligomerization of compounds **6** and **7** using the nonpolar initiator **10** in an emulsion polymerization. The polymerization reactions were conducted in deoxygenated solutions composed of a mixture of dichloroethane, pH 6.0, bis-Tris buffer and the detergent DTAB. The reaction was found to proceed more rapidly and reliably under the slightly acidic reaction conditions, which is consistent with mechanistic data (48, 58). In these reactions, monomer-to-initiator stoichiometries of 15:1 were employed. For many ROMP reactions facilitated by defined metal carbene complexes, like **10,** the monomer to initiator ratio controls the size of the products (59). Small amounts of unreacted monomer as well as other low molecular weight impurities could be separated from the water-soluble neoglycopolymers by size-exclusion chromatography. To remove the emulsifying agent and any other charged species and to generate materials with defined counterions, ion exchange chromatography and subsequent dialysis further purified the glycoprotein analogs **8** and **9**. Analysis by ^{1}H NMR spectroscopy revealed the characteristic resonances expected for the saccharide units as well as those associated with the polymer backbone. These studies indicate that the sulfated saccharide units are stable to the polymerization conditions, as expected from our previous work (46, 47). NMR spectroscopic analysis indicated the purified products both contained an average of 15 monomer units per oligomer chain, which is the anticipated average length given the monomer to initiator ratio used.

Evaluation of Multivalent Saccharide Presentation in Static Binding Inhibition Assay—Trisaccharide **5** was previously

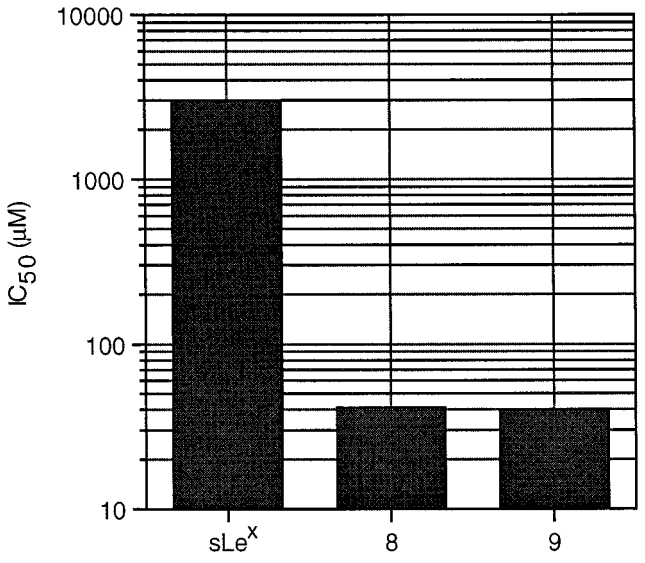


FIG. 4. **Inhibition of L-selectin binding to heparin in a static binding assay by polymers 8 and 9.** Serial dilutions of the inhibitors were incubated with magnetic beads containing both goat IgG and an L-selectin-IgG chimeric protein in 96-well plates coated with heparin-albumin. The wells were sequentially washed with PBS, and an anti-goat alkaline phosphatase-conjugated secondary antibody was added. The number of beads bound was determined by measuring the absorbance at 405 nm after addition of a standard alkaline phosphatase substrate.

shown to be 3-fold more effective than **4** at inhibiting L-selectin in a static, cell-free binding assay (34). To determine whether the multivalent, glycoprotein analogs based on these saccharide epitopes were effective inhibitors of L-selectin, neoglycopolymers **8** and **9** were tested for their ability to inhibit the binding of L-selectin to immobilized heparin. Heparin is a polysulfated oligosaccharide that is known to bind to L-selectin in a calcium-dependent manner (60, 61). It inhibits L-selectin function and is likely to occupy a similar binding site to other anionic ligands such as the neoglycopolymers. An enzyme-linked immunosorbent assay (ELISA) developed for monitoring selectin–heparin interactions was employed to evaluate the inhibitory potencies of the multivalent molecules toward L-selectin (53). This assay is conducted under static conditions, and binding interactions are established between the inhibitors, the L-selectin fusion protein, and the immobilized heparin over the course of one h. The time of incubation provides an opportunity for the system to equilibrate.

Results from the L-selectin–heparin ELISA reveal that both synthetic glycoprotein mimics inhibit L-selectin with similar activities (Fig. 4). The reference compound, sLe^x, has an IC_{50} of approximately 3 mM against L-selectin in this assay, consistent with values obtained in similar assays (62, 63). In contrast, neoglycopolymers **8** and **9** exhibit IC_{50} values of approximately 39 μM (580 μM saccharide residue concentration) and 35 μM (520 μM saccharide residue concentration), respectively. The increase in inhibitory potency over sLe^x for either multivalent derivative corresponds to approximately 80-fold. This result indicates that the multivalent analogs are superior when compared with similar monovalent compounds, an observation in accord with other investigations that have identified multivalent inhibitors of L-selectin (38–43). The increase in inhibitory potency for the multidentate ligands is significant. Still, the lack of a distinguishable difference in IC_{50} values between oligomers **8** and **9** would seem to indicate that the specific sulfated epitope is less important for the inhibition of L-selectin binding to heparin than is multivalent presentation of an anionic saccharide residue.

TABLE I

*Tabulation of IC_{50} values for the inhibition of L-selectin-mediated cell rolling by compounds **4**, **5**, **8**, and **9***

Inhibitors were preincubated with L-selectin-transfected cells before perfusion through a flow chamber containing immobilized GlyCAM-1. IC_{50} values were obtained by determining the concentration of saccharide residues required to inhibit 50% of the rolling interactions relative to control experiments. For compounds **4**, **5**, and **8**, which had negligible inhibitory effects on L-selectin-mediated rolling, the percent inhibition is provided at the highest saccharide residue concentration tested.

Compound	IC_{50}
	mM
sLe^x	2.5
3′,6′-disulfo Le^x(Glc) **4**	6%, 5 mM
3′,6′-disulfo Le^x(Glc) **5**	7%, 5 mM
3′,6′-disulfo Le^x(Glc) oligomer **8**	0%, 5 mM
3′,6′-disulfo Le^x(Glc) oligomer **9**	0.015

Cell Rolling Inhibition Assay—Prior studies reveal that L-selectin can mediate cell rolling only under conditions of shear stress (64–67). Given the importance of this parameter for L-selectin function, we sought to compare directly the inhibitory activities of our synthetic substrates in an assay conducted under shear flow. We therefore evaluated compounds **4** and **5** and their multivalent counterparts in an *in vitro* rolling assay on immobilized GlyCAM-1. This glycoprotein has been found to support rolling adhesions of leukocytes displaying L-selectin in a manner similar to other endothelial L-selectin ligands, such as the peripheral node addressin (55).

Soluble inhibitors can be added to the assay to assess the ability of ligands to disrupt cell rolling. To this end, L-selectin-transfected murine 300.19 cells were perfused through a flow chamber containing immobilized GlyCAM-1 at a density of 30 sites/μm^2. The fluid shear applied in the flow chamber was 1.75 dyne/cm^2, which is within the physiological range of venular shear stresses. In the absence of inhibitors, approximately 20% of the L-selectin transfectants established rolling on GlyCAM-1. To measure the ability of the inhibitors to prevent cell rolling, cells were preincubated with the inhibitors, and the resulting percentage of rolling cells was determined. IC_{50} values obtained by this method indicate the concentration of inhibitor necessary to reduce the fraction of cells capable of rolling on immobilized L-selectin ligand (GlyCAM-1) by 50%.

Monovalent compounds **4** and **5** were tested for their ability to block the rolling of L-selectin-transfected cells on GlyCAM-1 (68), and the results were compared with those obtained with the control selectin ligand sLe^x (Table I). SLe^x was found to inhibit the rolling interaction with an IC_{50} of 2.5 mM. This value corresponds well to those measured in static cell adhesion assays (62, 63), and it is similar to the IC_{50} of 4 mM, which was determined for the interaction of L-selectin with GlyCAM-1 in an ELISA (69). In contrast, the evaluation of trisaccharides **4** and **5** in the rolling assay did not afford results consistent with those obtained in the ELISA (34). Previously, monomers **4** and **5** were found to inhibit recombinant L-selectin binding to GlyCAM-1 with IC_{50} values of 3 and 1 mM, respectively; yet these compounds did not interfere with L-selectin-mediated cell rolling on GlyCAM-1, even at concentrations up to 5 mM. The mimetics also failed to exhibit a detectable effect on the dynamics of cell rolling on GlyCAM-1, in contrast to that observed when sLe^x was present at concentrations higher than 0.5 mM (Table I). Despite the efficacies of trisaccharides **4** and **5** in the static cell-free assay (34, 70), they are significantly less potent than sLe^x in the rolling assay. These results reveal for the first time that the inhibitory activities of molecules determined in static assays do not necessarily correlate with their potencies under dynamic flow conditions (71).

With their multivalent display of saccharide epitopes, neo-

glycopolymers **8** and **9** resemble the glycoprotein ligands to which L-selectin binds. To ascertain whether these molecules would possess enhanced inhibitory potencies under shear flow, glycoprotein analogs **8** and **9** were tested for their ability to block the L-selectin-mediated rolling on GlyCAM-1 (Table I). We found that oligomer **9,** which presents 3′,6-disulfo Le^x(Glc) epitopes that are designed to mimic 6-sulfo sLe^x, exhibits an IC_{50} of 1.0 μM (15 μM saccharide residue concentration). Thus, this glycoprotein analog is approximately 1000-fold more potent than sLe^x at inhibiting L-selectin-mediated rolling. The dramatic increase in activity for compound **9** suggests that the multidentate display of saccharides exhibited by natural L-selectin ligands is critical for their function.

By synthesizing the two polyanionic oligomers **8** and **9** that differ only in the placement of a sulfate group within the saccharide epitope, the L-selectin specificity for multivalent ligands could be assessed in the rolling assay. Strikingly, compound **8,** which displays 3′,6′-disulfo Le^x(Glc), a mimic of 6′-sulfo sLe^x, showed no inhibition of rolling, even when tested at saccharide residue concentrations up to 5 mM. This result contrasts sharply with the potency of this mimic in the static, cell-free binding assay (Fig. 4), in which multivalent derivatives **8** and **9** were found to have similar activities. The selective inhibition of rolling by polymer **9,** but not **8,** indicates that L-selectin interactions with ligand under shear flow are very sensitive to the sulfation pattern of the glycoprotein mimetics. Our data suggest that static interactions of L-selectin with ligand, however, are less sensitive to the sulfation pattern of the multivalent ligands although the inhibition does depend on the presence of clustered sulfate groups. Thus, the assay conducted under shear flow augments the differences in inhibitory potencies of two highly related multidentate, anionic ligands.

DISCUSSION

Despite the important roles of the selectins in the inflammatory response, an understanding of their recognition properties remains elusive. We have developed methods to chemically synthesize structurally defined monovalent and multivalent saccharide derivatives in a directed effort to illuminate selectin recognition properties. The binding properties of the selectins have been explored using a variety of saccharide analogs and glycomimetics (5, 72, 73), with most studies focusing on E-selectin recognition under static binding conditions. The synthetic compounds examined in this study were devised to evaluate the contributions of two characteristics inherent in the structure of physiological L-selectin ligands: the sulfation pattern present on the saccharide epitope and the importance of multivalent display of an L-selectin binding epitope. Our objective is to provide insight into the properties of L-selectin ligands that can affect L-selectin function.

The ligands that we designed and characterized are based on the discovery that sulfation of the L-selectin-binding glycoprotein GlyCAM-1 is important for L-selectin interaction (30). The identification of the major GlyCAM-1 capping groups 6′-sulfo sLe^x and 6-sulfo sLe^x prompted us to synthesize and evaluate sulfated Le^x derivatives based on these core compounds. Sulfated derivatives **4** and **5** have been used to investigate the impact of 6′- *versus* 6-sulfation on L-selectin binding in a static, cell-free assay (34), but their activities under shear stress had not been determined previously.

Because most of the naturally occurring L-selectin ligands are highly *O*-glycosylated, mucin-like proteins that present multiple copies of sulfated saccharide epitopes on an extended backbone, multidentate counterparts to trisaccharides **4** and **5** were produced. The multivalent derivatives **8** and **9** were assembled through a polymerization process, termed ROMP, which provides the means to control the oligomer size. In this reaction, a ruthenium carbene initiator is used, and by varying the ratio of this initiator to monomer unit, the length of the resulting polymer can be controlled (48, 50). For this study, relatively short oligomers, composed of an average of 15 monomer units, were generated. Analysis suggests that both **8** and **9** are of the same molecular weight and possess the same molecular mass distribution. The charged, polar nature of these materials precludes determination of the exact molecular weight distribution, but related multivalent saccharide derivatives have narrow polydispersity indices of approximately 1.1–1.2 (50). Molecular modeling of the polymer backbone suggests that materials **8** and **9** can adopt extended structures that span distances of $\geq$70–80 Å (50). Oligomers **8** and **9,** therefore, have structural features that allow them to bind to multiple L-selectin molecules at the cell surface or to occupy a secondary binding site on a single L-selectin molecule that extends beyond the saccharide binding pocket. Access to the two closely related multivalent materials **8** and **9** provides an opportunity to address whether defined multivalent arrays of saccharide epitopes exhibit differences in their abilities to bind L-selectin.

Monovalent Saccharide Epitopes in Static Binding Versus Dynamic Rolling Assays—Several groups have evaluated different sulfated saccharide epitopes in L-selectin binding assays, as such compounds could provide insight into L-selectin recognition and serve as leads for the design of L-selectin inhibitors (34, 36, 70, 74–77). Our previous investigation of L-selectin recognition focused on comparing the abilities of two mimics of the GlyCAM-1 determinants, trisaccharides 3′,6′-disulfo Le^x(Glc) **4** and 3′,6-disulfo Le^x(Glc) **5,** to inhibit the interaction of L-selectin with GlyCAM-1 in an ELISA (34). The monovalent compounds, **4** and **5,** were designed to display anionic substituents at sites that correspond to those found in the natural capping groups of GlyCAM-1. The trisaccharide derivatives **4** and **5** differ from the GlyCAM-1 capping groups in that they possess a sulfate group rather than a sialic acid residue at the 3-position of the Le^x core. Both the sulfate substituent and the carboxylic acid group of the sialic acid residue are negatively charged at physiological pH values, suggesting 3′-sulfo Le^x and sLe^x share structural features. In addition, 3′-sulfo Le^x inhibits L-selectin–ligand interactions with IC_{50} values similar to those of sLe^x(34) in static, cell-free binding assays. Moreover, several studies comparing the ability of L-selectin to bind to glycolipid derivatives of 3′-sulfo Le^x and sLe^x indicate that the sulfated derivatives are superior to the corresponding compounds bearing the sialic acid residue (74, 75, 78), and 3′-sulfo Le^x-substituted lipids can support L-selectin-mediated rolling (79). Together, these studies suggest that the 3′-sulfate substituent can replace the sialic acid residue to afford a mimetic with binding properties similar to sLe^x. In the ELISA involving GlyCAM-1 as the L-selectin ligand, the disulfated analog of 6′-sulfo sLe^x, 3′6′-disulfo Le^x(Glc) **4,** was found to exhibit similar IC_{50} values as sLe^x, but 3′,6-disulfo Le^x(Glc) **5** was found to be 3-fold more active. The enhancement afforded by 6-sulfation documented in our study is comparable with that observed for monovalent 6-sulfo sLe^x observed by others (35, 36). Most of the competition binding assays, however, were performed under static, cell-free conditions. We found that the static ELISA does not reproduce the results observed for the monovalent sLe^x when it is compared with 3′,6′disulfo Le^x(Glc) and 3′,6-disulfo Le^x(Glc) in a rolling assay. Specifically, sLe^x, although a weaker inhibitor of L-selectin binding to GlyCAM-1 than 3′,6-disulfo Le^x(Glc) in the static assay, is a more effective inhibitor of L-selectin-mediated rolling on GlyCAM-1. The differences in activity among the monovalent ligands in the static and rolling assays are not predicted by current models of selectin–ligand recognition. Our

study highlights the profound effect of shear flow on the L-selectin inhibitory potencies of various monovalent compounds.

Our results suggest that the kinetics of binding of the disulfated derivatives **4** and **5** to L-selectin are different from those of the sulfated sLe^x counterparts. One explanation is that the sialic acid residue, or perhaps the nature of its charged carboxylate, contributes to L-selectin recognition under physiological shear flow. Although the sulfate and the sialic acid carboxylate groups are both negatively charged under physiological conditions, the anions have different geometries. When compared with sulfate groups, carboxylic acids also differ in that they can better form hydrogen bonds. Finally, the solvation shells of each anion both in the bound and unbound states will be different. Thus, a variety of factors could influence the binding kinetics of the sialylated *versus* sulfated Le^x derivatives. Several lines of evidence indicate that sialic acid is a critical component of physiological L-selectin ligands (25, 80, 81). Our data suggest that this residue may facilitate the rapid formation of productive complexes between L-selectin and its target ligands.

Multivalent Compounds in a Static L-selectin Inhibition Assay—We found that the multivalent ligands **8** and **9** were approximately 80-fold more effective than monovalent sLe^x at inhibiting L-selectin binding to heparin in the static, cell-free binding assay. This finding is consistent with several other reports indicating that multivalent ligands have superior L-selectin inhibitory capabilities than do monovalent counterparts (38, 40–42, 46, 75, 78, 82). This enhanced functional affinity for the multivalent ligands can arise from several different mechanisms, and the similar activities of the different multivalent derivatives **8** and **9** are revealing.

Proteins can exhibit not only higher avidities but also higher specificities for certain multivalent displays (45, 46, 83–86). One mechanism that can result in large increases in functional affinities involves the formation of multiple receptor-ligand complexes. In such a system, a subtle increase in binding between two monovalent epitopes is amplified when the corresponding polyvalent arrays are compared (44, 45, 84, 86, 87). If such a mechanism were important in L-selectin recognition, it would be anticipated that monovalent ligand **5,** which was 3-fold more potent than **4** in inhibiting L-selectin binding to GlyCAM-1(34), could act as a specific L-selectin inhibitor (45, 83) when displayed in a multivalent array as in **9**. No large differences between the activities of **8** and **9** in the static assay, however, were observed. The lack of selectivity observed in this assay is consistent with many reports in the literature in which a multitude of sulfated, multivalent ligands show comparable inhibition of L-selectin in static assays (77). This lack of selectivity in static binding assays impedes the identification of specific inhibitors of selectin function (7). Thus, other factors that can contribute to the enhanced functional affinities for multivalent binding interactions must be considered.

The similarity in the abilities of polymers **8** and **9** to inhibit L-selectin binding in the static assay is consistent with coulombic interactions providing an important driving force for binding under these conditions. The features of L-selectin–ligand binding events suggest ionic interactions are important, and that these recognition processes are promoted by an entropically favorable release of ions (88). In a static binding assay, a charged ligand and its complementary receptor can form a complex that maximizes favorable ionic interactions. A hallmark of such processes is that they generally can be inhibited by a variety of ionic molecules. The binding data presented here are consistent with this mechanistic picture, as are the cumulative observations that a wide range of charged molecules block L-selectin binding interactions (7, 77). In addition to the known L-selectin ligands GlyCAM-1, CD34, and MAdCAM-1, a variety of soluble mucins can interact *in vitro* with L-selectin provided they possess sialic acid residues, sulfation, and fucose residues. Additionally, the observation that different saccharide core structures can present such recognition elements supports the idea that several sulfated saccharide-containing epitopes can support L-selectin binding (77). Thus, evaluation of different polyanionic inhibitors in static assays is complex. In physiological settings, however, L-selectin must bind its ligands under the highly dynamic conditions of shear flow.

Increased Activity of Multivalent Ligands Over Related Monovalent Ligands in Flow—Mounting evidence indicates that the dynamic conditions under which L-selectin functions can have profound effects on its activity (64–67). Our studies of monovalent inhibition suggest that the potencies of various monovalent ligands toward L-selectin inhibition determined in static assays are not reproduced under conditions of flow. These results, as well as the potential therapeutic benefits of generating effective L-selectin inhibitors, led us to compare the activities of the multivalent L-selectin ligands **8** and **9** with those of sLe^x and trisaccharides **4** and **5** under conditions of shear flow. We found that the activity of multivalent ligand **9** is significantly increased relative to its monovalent counterpart in the rolling assay.

A number of investigations suggest that L-selectin–ligand interactions benefit from multivalent binding. Several studies of L-selectin recognition suggest multivalency may contribute to both ligand recognition and L-selectin function. For example, data from static assays indicates that multivalent molecules are superior inhibitors of selectin binding when compared with related monovalent compounds (38, 40–42, 46). In addition, recent investigations suggest that clustering of L-selectin could result in increased selectivity for specific ligands (78). Such clustering could induce specific cellular responses, including the activation of signaling events (89, 90) or the proteolytic release of L-selectin from the cell surface (91, 92). The results presented here, however, provide the first evidence that multivalent presentation of a specific saccharide epitope can result in large increases in inhibitory activity under physiologic flow conditions.

Several mechanisms may be responsible for the dramatic increase in the activity of multivalent compound **9** when compared with its monovalent counterpart in an L-selectin rolling assay. One possible origin of the increased activity of the multivalent compound is that this species is capable of binding to more than one L-selectin protein at the cell surface, whereas the monovalent compound can occupy only a single site. In this model, the activity of multivalent compound **9** arises from the increased local concentration of saccharide ligands present in the multivalent compounds, which could result in occupation of an adjacent L-selectin binding site at the cell surface. Thus, compound **9** may cluster L-selectin or bind to clustered L-selectin at the cell surface. A similar mechanism can explain the observation that GlyCAM-1 binds with a high functional affinity (~34 nM) to immobilized L-selectin (68), but the binding of monovalent soluble L-selectin to soluble GlyCAM-1 is much weaker (K_d = 100 μM). Even if the multidentate **9** does not cluster L-selectin, the ligand is expected to display slower rates of dissociation from the cell surface than a monovalent counterpart; the former presents a high local concentration of saccharide residues that increase its probability of rebinding to the cell surface before dissociation. Alternatively, the multivalent ligand could exhibit enhanced binding because it can occupy a site for a polyanionic ligand, which is adjacent to the saccharide binding site (93). Finally, the multivalent ligand could display enhanced activity through steric stabilization (94,

95) in which the size and anionic character of the cell surface-bound oligomer can act as a barrier that blocks additional interactions. All of these mechanisms, which may operate in concert, are consistent with observations that multivalent **9** is a more effective inhibitor of L-selectin than monovalent **5**. Because the neoglycopolymers were designed to mimic natural L-selectin ligands, the increased activity of glycoprotein analog **9** provides new support for the involvement of multivalent interactions in selectin–ligand binding and selectin function.

Selectivity between Multivalent Ligands Manifested in Rolling Assay—The results of the rolling inhibition assay were striking in that multivalent ligand **9** displayed potent inhibitory capacity, but the related material **8** showed no ability to diminish L-selectin-mediated cell rolling. The large difference in activity between **8** and **9** under the dynamic conditions contrasts with their similarity in the static binding study. The rolling assay illuminates differences in the kinetics and/or in the mode of cell surface L-selectin interaction between these related compounds. Moreover, the data suggest that the specific site of sulfation within the saccharide epitope presented in the multivalent array has a dramatic effect on the ability of the multivalent ligand to inhibit L-selectin function under shear stress. In the static cell-free assay, however, the specific sites of saccharide sulfation, appear to be unimportant, but a cluster of anionic residues, such as that presented by both polymers, is needed. Molecules **8** and **9** have the same number of anionic groups; consequently, the potency of **9** in the rolling assay does not stem from nonspecific coulombic interactions. Our data suggest that the pattern of substituents on polymer **9,** which is created by multivalent presentation of 3′,6-disulfo Le^x moieties, results in effective interactions with cell surface L-selectin under shear flow, but the highly homologous display of 3′,6′-disulfo Le^x epitopes in material **8** does not.

Several molecular mechanisms underlying the distinctly different activities of compounds **8** and **9** under dynamic conditions can be envisioned. These mechanistic hypotheses must rationalize major differences in the binding kinetics of the effective multivalent derivative **9** *versus* that of **8**. Although highly anionic molecules can adopt conformations that maximize favorable electrostatic interactions with L-selectin in a static assay, compound **8** may have a limited opportunity to access to the necessary favorable orientations under conditions of shear flow. In contrast, glycoprotein analog **9** may present an array of functional groups that are poised to bind L-selectin with rapid association kinetics similar to those used by naturally occurring L-selectin ligands. The differences in rolling inhibition for **8** and **9** may also stem from their relative abilities to bridge L-selectin molecules at the cell surface. The localization of L-selectin to the microvilli may facilitate the formation of multiple L-selectin ligand contacts with appropriate multidentate ligands. If oligomer **9** can interact in a multivalent fashion, but substance **8** cannot, the dissociation rate of **9** from cell surface L-selectin would be expected to be much slower than that of **8**. This situation would also lead to the superior inhibitory potencies for substance **9,** especially with regard to inhibition of L-selectin-mediated rolling.

These studies reveal features of L-selectin recognition not apparent from static binding assays. Our results suggest that inhibition of L-selectin function under physiological conditions is strongly dependent upon two factors: multivalency of recognition elements combined with the specific structure of those elements. These are the two factors that may be employed by physiological L-selectin ligands, most of which display multiple copies of L-selectin binding moieties. The importance of compound **9,** however, extends beyond its potential relationship to natural selectin ligands. The ability of multidentate **9** to effectively diminish L-selectin-mediated rolling provides insight into features important for L-selectin recognition. The glycomimetics described here are useful not only for their value as therapeutic leads; they also can be used to illuminate unique structure-function relationships that occur under static and dynamic conditions. The heterogeneity of the natural L-selectin ligands precludes such analyses. Our synthetic glycoprotein mimics, however, are constructed by appending specific recognition elements to a template such that they are displayed with defined spacings. The defined structures of these molecules can provide key insights into the requirements for recognition of ligand by cellular L-selectin. Finally, our studies suggest that static assays may fail to predict the therapeutic potential of L-selectin ligands under conditions of shear flow.

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