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8. For cloning of PuF, cDNA was synthesized from HeLa cell, and polyadenylated RNA was size-fractionated and ligated into the Not I–Sal I–restricted plasmid expression vector pSPORT (SuperScript Plasmid System, BRL) and introduced into *Escherichia coli* by electroporation. The resulting cDNA library (L7) contains 2.5×10^5 independent recombinants with an average insert size of ~3 kb. For screening, 1×10^6 cells of the amplified library were plated onto nitrocellulose filters (150 mm, Triton-free, Millipore) laid on agar plates containing ampicillin (100 μg/ml), and incubated overnight at 37°C (*19*). Filters were replicated and transferred to fresh plates containing ampicillin and 1 mM isopropylthio-β-D-galactoside (IPTG) and incubated for 3 hours at 37°C to induce expression of fusion proteins. Colonies were lysed in situ in a chloroform-saturated chamber (*20*) and then transferred to lysis buffer [100 mM tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1.5% bovine serum albumin (BSA), lysozyme (40 μg/ml), and deoxyribonuclease I (2 U/ml)] for 2 hours at room temperature. After being washed in lysis buffer, filters were incubated for 1 hour each in Blotto (*21*) and then in binding buffer [10 mM tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.25% nonfat milk powder (Carnation), and heat-denatured sonicated salmon sperm DNA (5 μg/ml)] containing ^{32}P-labeled 105-bp DNA probe [100 ng/ml (6×10^6 cpm per 150-mm filter)] comprising nucleotides −200 to −96 of the c-*myc* P1 promoter. This probe contains NHE, including the PuF recognition sites GGGTGGG between nucleotides −142 to −115 (*3*). Filters were washed in binding buffer and exposed to X-AR film (Kodak).
9. Plasmids of two independent subclones of c19 were sequenced (*22*) with T7 and T3 primers and Sequenase version 2.0 kit (USB). Computer analysis was done with the GCG (Wisconsin) sequence analysis package.
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11. The nucleotide sequence of PuF cDNA has been deposited with GenBank (accession number L16785).
12. For purification of *E. coli*–expressed PuF, the DNA sequence adjacent to the initiator methionine of c19pSPORT cDNA was mutated by polymerase chain reaction to introduce an Nde I restriction site. The resulting 625-bp fragment of the protein coding sequence and the 3′ untranslated region of c19 were subcloned into the Nde I–Bam HI site of the vector PET3c (Novogen). p19PET3c was transformed into *E. coli* BL21 (DE3), expressed, and the soluble protein purified as follows: Cells from a 1-liter culture harvested 3 hours after IPTG induction were resuspended in 50 ml of 50 mM tris (pH 8)–2 mM EDTA and lysed by sonication and treatment with lysozyme (100 μg/ml). After dialysis into 20 mM tris (pH 7.4), 1 mM EDTA, 1 mM DTT, the 60 to 90% $NH_4(SO_4)_4$ fraction was applied to a hydroxylapatite column (HTP, Bio-Rad), which was developed with a 0 to 300 mM potassium phosphate gradient (*16*). HTP fractions containing c19 nm23-H2 were identified by SDS-PAGE, pooled, concentrated (Centricon), and equilibrated in 0.1 HM buffer [20 mM Hepes (pH 7.9), 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 20% glycerol, 0.1 M KCl] (*3*), and portions were frozen at −80°C. D.4 fractions containing hPuF were obtained by sequential chromatography of nuclear extracts on heparin-agarose and DEAE-Sepharose (*3*).
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Calcium-Dependent Heparin-Like Ligands for L-Selectin in Nonlymphoid Endothelial Cells

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L-Selectin is a calcium-dependent mammalian lectin that mediates lymphocyte trafficking by recognizing sialylated ligands on high endothelial venules in lymph nodes. Although L-selectin probably mediates neutrophil extravasation into nonlymphoid tissues, no corresponding ligand has been characterized. Staining of cultured endothelial cells with an L-selectin chimera (LS-Rg) showed an internal pool of ligands. Metabolic labeling with sulfur-35–labeled sulfate revealed heparin lyase–sensitive ligands that bound LS-Rg in a calcium-dependent, sialic acid–independent manner. A fraction of commercial heparin bound to LS-Rg and LS-Rg bound to heparin-agarose, both in a calcium-dependent manner. Thus, L-selectin recognizes endothelial heparin-like chains, which could be physiological ligands mediating leucocyte trafficking.

The selectins are a family of calcium-dependent (C-type) mammalian lectins found on cells of the vascular system (*1*). P- and E-selectin are inducible receptors that recognize certain sialylated fucosylated ligands on leukocytes. They participate in the trafficking of these cells to areas of inflammation and injury (*1*). In contrast, L-selectin is expressed on leukocytes and recognizes specific carbohydrate ligands on endothelial cells. L-Selectin mediates the trafficking of lymphocytes by binding to specific sialylated sulfated ligands on the high endothelial venules (HEVs) of lymph nodes (*2*, *3*). L-Selectin is also expressed on neutrophils and monocytes and participates in the emigration of these cells through the endothelium of other organs (*1*, *4*). Although human umbilical vein endothelial cells (HUVECs) have a cytokine-inducible ligand for L-selectin (*5*), no candidate molecule has been positively identified in any endothelial cells from nonlymphoid tissues.

To search for L-selectin ligands, we used a chimeric probe (LS-Rg) (*6*–*8*) consisting of the entire extracellular domain of the L-selectin molecule attached to the Fc domain of an immunoglobulin G2 (IgG2) COOH-terminus. Ligands were not detectable in the postcapillary endothelium of normal rat tissues other than in the lymph nodes (*6*–*8*). However, this probe detected ligands in cultured calf pulmonary artery endothelial (CPAE) cells. When the cells were fixed under specific conditions (*9*), we observed divalent cation–dependent intracellular staining (Fig. 1). In contrast with previous findings with lymph node HEV (*8*), this staining was not enhanced by mild periodate oxidation (*10*). Cells grown on glass slides instead of plastic slides had reduced staining (*11*). This, together with the intracellular location of the ligands, may account for the previous inability to detect them.

The L-selectin ligands isolated from lymph node HEV are mucin-like and carry sialylated, fucosylated, sulfated, O-linked oligosaccharides that are best labeled with $^{35}SO_4$ (*2*, *3*). We therefore metabolically labeled CPAE cells, HUVECs, and AGO8132 cells (a fetal bovine aortic endothelial cell line) with $^{35}SO_4$ and looked for radioactive macromolecules recognized by LS-Rg. Similar to $^{35}SO_4$-labeled material from lymph nodes (*2*, *3*), a portion of the radioactivity from each cell type bound to

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an LS-Rg column in the presence of calcium (*12*) and was eluted by EDTA (Fig. 2). Furthermore, most of the material from CPAE cells could rebind in the presence of excess calcium (Table 1). When analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions, the ligands from all three cell types were different from the HEV material, which appeared, as expected, as a major 50-kD band (*2, 3, 13*). The CPAE ligands migrated as a broad smear between 50 to 100 kD and a second band at >200 kD, whereas both AGO8132 and HUVEC showed broadly smeared material of >200 kD (*11*). These SDS-PAGE patterns are reminiscent of either large sulfated mucins (*14*) or proteoglycans with sulfated glycosaminoglycan chains (*15*).

To characterize the oligosaccharides of the $^{35}SO_4$-labeled ligands from CPAE cells, we compared them to the HEV ligand by a variety of enzyme treatments (Fig. 3A). The HEV ligand behaved as expected for a sialomucin, being susceptible to sialidase and to O-sialoglycoprotease, an enzyme that specifically cleaves O-linked sialoglycoproteins (*16*). In contrast, the ligand from CPAE cells was unaffected by either of these enzymes (*17*). Instead, it was completely degraded by a mixture of heparin lyases and chondroitinases (Fig. 3A) indicating that the label was in glycosaminoglycan chains (heparin, heparan sulfate, or chondroitin sulfate). Studies with individual glycosaminoglycan-degrading enzymes showed that the CPAE ligand was primarily degraded by heparin lyases and, to a very limited extent, by chondroitinases (Fig. 3B).

Most reports have indicated that calcium-dependent recognition by selectins requires sialic acids (*18, 19*). Thus, it is possible that the CPAE ligand has sialyloligosaccharides responsible for binding, and that it only incidentally carries sulfated glycosaminoglycan chains as well. However, sialidase digestion of the CPAE ligand had no effect on its rebinding to LS-Rg under conditions where binding of the HEV ligand was reduced (Table 1). To address this issue further, we treated the CPAE and HEV ligands with either proteinase K (which converts intact proteoglycans or mucins into glycopeptides) or beta-elimination and reduction (which releases both xylose-linked glycosaminoglycans and O-GalNAc–linked oligosaccharides as free sugar chains) (Table 1) (*20*). Proteinase K treatment reduced rebinding of the HEV ligand to LS-Rg, and beta-elimination abolished rebinding. These results indicate that the intact sialomucin is required for optimal binding and that the free oligosaccharides do not interact well with L-selectin. In contrast, a substantial fraction of the radioactivity from the CPAE ligand continued to bind to LS-Rg after either proteinase K treatment or beta-elimination (Table 1). These data indicate that the glycosaminoglycan chains on the CPAE ligand are sufficient for this natural ligand to bind to L-selectin. Furthermore, the CPAE ligand was eluted from LS-Rg by commercial heparin (10 mg/ml).

Because heparin and heparan sulfates consist of a polydisperse, complex family of

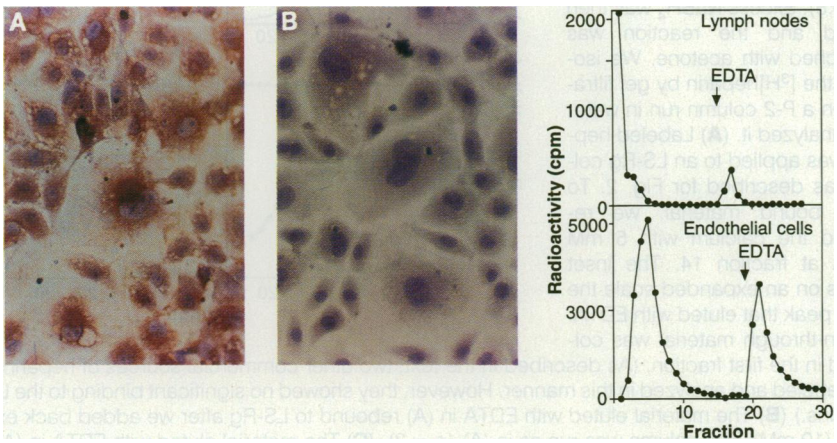

Fig. 1 (left). CPAE cells (American Type Culture Collection CCL 209, passages 7 to 13) were grown in alpha–minimal essential medium (MEM) with 10% heat-inactivated fetal calf serum in eight-well plastic slides. The cells shown in the left two micrographs were split at confluency at a ratio of 1:10 with EDTA and trypsin. For staining, confluent monolayers were washed and fixed in 3.7% formalin and 0.1 M sodium cacodylate in phosphate-buffered saline (PBS) for 1 hour, followed by permeabilization in methanol for 1 hour. After being washed with Hanks' balanced salt solution (HBSS), slides were incubated for 30 min in a solution containing LS-Rg (10 μg/ml) in HBSS, 1 mM Ca^{2+}, 1 mM Mg^{2+}, 10% goat serum, and 1% bovine serum albumin, without (**A**) or with (**B**) the addition of 10 mM EDTA. LS-Rg binding was detected with biotinylated goat antibody to human immunoglobulin and peroxidase-conjugated avidin, developed with 3-amino-9-ethylcarbazole. This is a representative example from more than 20 similar experiments. **Fig. 2 (right).** To look for macromolecules recognized by LS-Rg, murine lymph nodes or CPAE cells in P-100 tissue culture dishes were labeled with [^{35}S]Na_2SO_4 in modified alpha-MEM (*34*) with 0.2 × normal glucose, 15 μM SO_4, and no methionine or cysteine. The lymph node organ culture media was enriched for sialoglycoconjugates by passage over a wheat germ aglutinin–agarose column as previously described (*2, 3*). The CPAE cells were washed in PBS and extracted into PBS with 2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pepstatin (10 μg/ml), and 0.02% sodium azide (*2*). Wheat germ agglutinin–enriched lymph node material or total CPAE cell lysate was applied to LS-Rg protein A–Sepharose columns (*8*) in 100 mM NaCl, 20 mM MOPS (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% Triton X-100, and 0.02% sodium azide. For eluting bound material, we replaced the $CaCl_2$ and $MgCl_2$ in the loading buffer with 5 mM EDTA. Radioactivity was measured in counts per minute (cpm). Similar results have been seen in more than ten such experiments.

Table 1. Effects of various treatments on the rebinding of $^{35}SO_4$-labeled ligands to L-selectin. The $^{35}SO_4$-labeled ligands from lymph node HEV and from CPAE cells were purified by binding to LS-Rg and elution by EDTA. We subjected aliquots of each ligand to the various treatments as indicated and re-applied them to the LS-Rg column to examine the rebinding in the presence of calcium. For the sialidase digestions we used 2 mU of sialidase from *Arthrobacter ureafaciens* in 125 mM sodium acetate (pH 5.5) at 37°C for 1 hour and then inactivated the mixture by boiling for 5 min (n = 3). For proteinase K digestions we added 0.5 to 1 mg/ml of enzyme in LS-Rg column buffer to the ligands and incubated the mixture at 37°C overnight. We inactivated the digestion by adding a 1 mM final concentration of DFP (n = 3). Reductive beta-elimination was carried out with 0.12 M NaOH and 1 M $NaBH_4$ at room temperature for 16 hours, quenched by acidified methanol, and desalted by passage over a Dowex-50 column (n = 2).

Ligand	Treatment	Radioactivity (% of total)	
		Unbound	Eluted by EDTA
HEV	Control	10	90
	Sialidase	39*	61
CPAE	Control	31†	69
	Sialidase	35	65
HEV	Control	13	88
	Proteinase K	62	38
	Beta-elimination	96	4
CPAE	Control	45	55
	Proteinase K	55	45
	Beta-elimination	53	47

*Complete abolition of HEV ligand binding with sialidase does not occur, even with prolonged digestion and high concentrations of enzyme (*33*). In this particular set of experiments, we did short incubations with moderate amounts of enzyme. Prolonged incubations of the CPAE ligand with sialidase had no effect on rebinding (*11*). †The extent of rebinding of the ligand from CPAE cells varied depending on the LS-Rg column. However, the fraction that rebound was consistent within a given experiment.

related molecules that can share common structural sequences, we hypothesized that high-affinity recognition by L-selectin might require a specific sulfation pattern present only on a minor subset of these molecules. This could explain conflicting reports concerning the ability of heparin preparations to inhibit L-selectin interactions (*6*, *21*, *22*). To explore this hypothesis, we labeled three commercial heparins from different tissue sources by [^{3}H]$NaBH_4$ reduction (*23*). One batch of porcine intestinal mucosal heparin (ungraded) and a preparation from bovine lung did not contain any ^{3}H-labeled heparin that bound to LS-Rg (*11*). However, a minor but significant fraction of the total [^{3}H]heparin from porcine intestinal mucosa (Grade I) bound to LS-Rg and was eluted with EDTA (Fig. 4A). This material rebound specifically in the presence of calcium and was re-eluted with EDTA (Fig. 4B) (*24*). This material was also partially susceptible to heparin lyases (Fig. 4C) and was almost completely cleaved by nitrous acid (Fig. 4D), which selectively cleaves heparin chains with sulfoamino or free amino groups. Thus, although the native ligands from the cells are intact proteoglycans, free heparin or heparan sulfate chains can bind directly to L-selectin.

Although most of the [^{3}H]heparin did not bind tightly to the LS-Rg column, it did show some retardation, indicating that there was a weak interaction with the LS-Rg (Fig. 3A, in which all unretarded material should have eluted in the first fraction). Therefore, this L-selectin interaction appears to be similar to that of antithrombin III with unfractionated heparin. Antithrombin III has a high affinity for a small subfraction of heparins that carry a specific pentasaccharide sequence and a weak affinity for most other types of heparin and heparan sulfate sequences (*25*). However, neither this nor any other reported glycosaminoglycan-protein interaction has yet been shown to be calcium-dependent (*15*).

The binding of the $^{35}SO_4$-labeled CPAE ligand and the [^{3}H]heparin to the LS-Rg column might also be explained by the presence of unrelated protein contaminants in the LS-Rg preparation. So, to demonstrate directly the calcium-dependent interaction of L-selectin with heparin, we passed the LS-Rg itself over a heparin-agarose column (Fig. 5). The chimera bound in a calcium-dependent manner and was eluted specifically with EDTA. Contaminants present in the preparation were either eluted from the column prior to application of EDTA or were not eluted at all. The calcium dependency indicates that the lectin domain of the LS-Rg is responsible for binding, not some

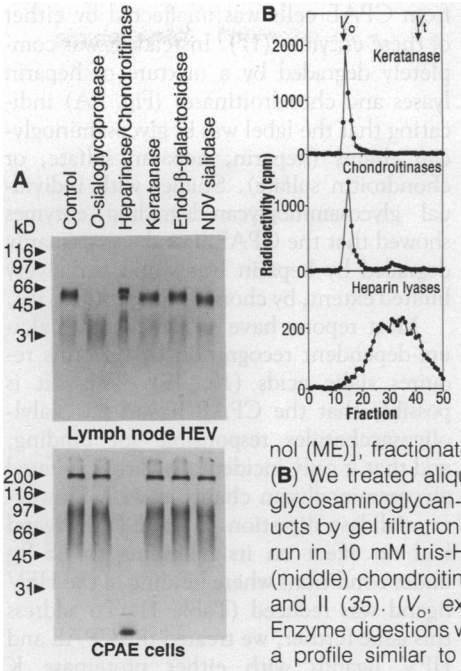

Fig. 3. (**A**) Analysis by SDS-PAGE of $^{35}SO_4$-labeled LS-Rg ligands from lymph nodes (12% acrylamide) and CPAE cells (15% acrylamide). Aliquots were treated with (i) 1 μl of *O*-sialoglycoprotease (specific activity: 1 μl cleaves 5 μg glycophorin A per hour) (*16*); (ii) 25 mU of heparin lyase I (*35*), 25 mU of heparin lyase III (*35*), 200 mU of chondroitinase ABC (Sigma), and 200 mU of chondroitinase AC (Sigma); (iii) 0.1 mU of keratanase I (Sigma); (iv) 1 mU of endo-β-galactosidase; or (v) 1 mU of New Castle Disease Virus Neuraminidase (NDV), prepared as previously described (*36*). All enzyme reactions and control incubations were done in 10 mM sodium phosphate (pH 7.4) at 37°C for 3 hours. Reactions were quenched by heating the mixture in sample buffer [with 2–mercaptoethanol (ME)], fractionated by SDS-PAGE, and subjected to fluorography. (**B**) We treated aliquots of the ligands from CPAE cells with different glycosaminoglycan-degrading enzymes and then analyzed the products by gel filtration on a column of Sephacryl S-200 equilibrated and run in 10 mM tris-HCl (pH 6.5) and 0.2% SDS. (Top) keratanase I; (middle) chondroitinases ABC and AC; and (bottom) heparin lyases I and II (*35*). (V_o, excluded volume; V_t, total volume of the column.) Enzyme digestions were carried out as in (A). A control incubation gave a profile similar to that obtained with the keratanase (not shown). Recoveries of radioactivity from all column runs were comparable.

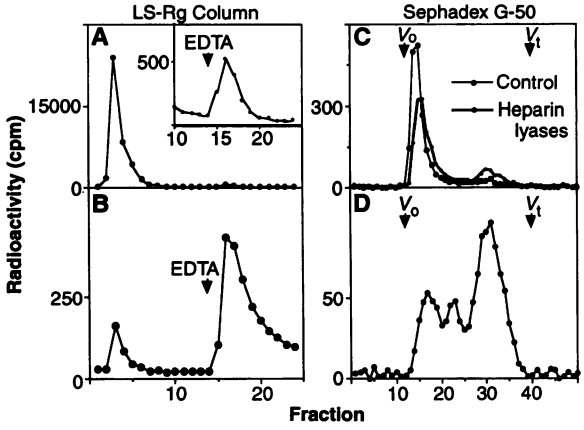

Fig. 4. We labeled porcine intestinal heparin (Grade I, Sigma) for 3.5 hours with [^{3}H]$NaBH_4$ (60 Ci/mmol) in 200 mM sodium borate (pH 9.8). Excess $NaBH_4$ was then added and the reaction was quenched with acetone. We isolated the [^{3}H]heparin by gel filtration on a P-2 column run in water and analyzed it. (**A**) Labeled heparin was applied to an LS-Rg column as described for Fig. 2. To elute bound material, we replaced the calcium with 5 mM EDTA at fraction 14. The inset shows on an expanded scale the small peak that eluted with EDTA. All run-through material was collected in the first fraction. (As described in the text, two other commercial sources of heparin were also labeled and analyzed in this manner. However, they showed no significant binding to the LS-Rg columns.) (**B**) The material eluted with EDTA in (A) rebound to LS-Rg after we added back excess Ca^{2+} (10 mM). The column was run as in (A) (n = 3). (**C**) The material eluted with EDTA in (A) was treated with heparin lyase. We analyzed control and enzyme incubations by gel filtration on a column of Sephadex G-50 equilibrated and run in water. (V_o, excluded volume; V_t, total volume of the column.) (**D**) The material eluted with EDTA in (A) was deaminated-cleaved by nitrous acid (pH 3.0). We analyzed the sample after neutralization by gel filtration as in (C).

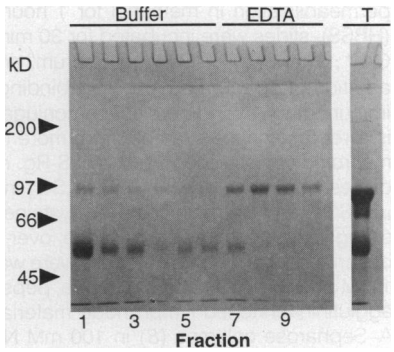

Fig. 5. We eluted LS-Rg produced by 293 cells from a protein A–Sepharose column with 4 M imidazole (pH 7.5) and dialyzed the LS-Rg against PBS (pH 7.4). A column (0.2 ml) of heparin-agarose (Sigma) was equilibrated with the same buffer used in Fig. 2 and loaded with the purified LS-Rg adjusted into the same buffer. Fractions 1 through 6 consist of the run-through and washes, and fractions 7 through 10 were eluted with 5 mM EDTA that we added in place of calcium and magnesium. Samples were collected into microdialyzer wells, dialyzed against water, lyophillized, boiled with sample buffer containing 2-ME, and analyzed by SDS-PAGE in 7.5% acrylamide. The gel was fixed and stained with Coomassie blue. Lane T shows the total amount of LS-Rg material we started with before applying it to the heparin-agarose column. The band at 96 kD is the LS-Rg chimera (reduced). The other bands are contaminants. This is a representative example of five independent experiments.

other portion of the molecule such as the Fc region of the IgG.

These data suggest that nonlymphoid endothelial cells contain heparin or heparan sulfate ligands for L-selectin that have properties different from those of the inducible ligands reported recently (*5*). The apparent intracellular location of the ligands detected here requires that they be translocated to the surface to either bind L-selectin or perhaps be secreted where they could function to modulate L-selectin–mediated binding in a negative manner. Alternatively, they may be incorporated by endothelial cells into the basement membrane (*26*) and be recognized by leukocytes during the later phases of diapedesis (*27*) or when the endothelium is damaged. However, contact with this ligand would need to occur before the L-selectin itself is shed from the surface of the activated neutrophil (*28*).

Many investigators have emphasized that biologically relevant selectin binding usually involves sialic acid–containing ligands (*18*). However, many reports have indicated that the selectins can also recognize sulfated or phosphorylated molecules, or both (*2, 3, 21, 29–31*). It is possible that some of these varied compounds act as partial surrogates for the natural ligands described here. Our data may also help to reconcile conflicting reports about heparin inhibition of selectin interactions (*6, 21, 22, 29, 30*). The manufacturing of commercial heparin is geared toward the selection of a subset of molecules that recognize antithrombin III with high affinity (*25*). Thus, each commercial lot may or may not include a specific subset of molecules with high affinity for L-selectin. Regardless of the physiological functions of the heparin-like ligands of endothelial cells, selected subfractions of crude commercial heparin may well serve as therapeutic blockers of L-selectin interactions.

This study raises some new issues. Direct competition studies with small-sized ligands will be required to determine if the calcium-dependent binding site for heparin is distinct from that for the well-recognized sialylated, fucosylated L-selectin ligands. Perhaps the effects of sulfated molecules on P-selectin–mediated binding (*30, 31*) suggest a similar recognition site for glycosaminoglycans. It also remains to be seen if calcium is involved indirectly, by altering the conformation of the lectin binding pocket, or directly, by bridging with the ligand, as in the mannose-binding lectin (*32*).

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9. Cell surface staining was not seen after short formaldehyde fixation without permeabilization. Formaldehyde fixation and subsequent methanol permeabilization revealed an internal pool of ligands. However, rapid permeabilization with saponin followed by fixation resulted in loss of the ligand.
10. Staining was also not affected by treatments with sialidase or heparin lyases. However, the negative results could be because the enzymes have poor access to the ligands. Periodate is a small molecule, so it should have had access to the ligands. The negative result with this treatment therefore suggests that sialic acids are not involved.
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12. The studies reported here included both Ca^{2+} and Mg^{2+} in the buffers used for binding because Geng *et al.* (*19*) reported that Mg^{2+} potentiated the calcium-dependent binding of a selectin. However, Mg^{2+} alone did not support binding of the CPAE heparin-like ligands to the LS-Rg (studied using Mg^{2+} in EGTA buffers).
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