

# P- and E-Selectin Use Common Sites for Carbohydrate Ligand Recognition and Cell Adhesion

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**Abstract.** The selectins are a family of three calcium-dependent lectins that mediate adhesive interactions between leukocytes and the endothelium during normal and abnormal inflammatory episodes. Previous work has implicated the carbohydrate sialyl Lewis<sup>x</sup> (sLe<sup>x</sup>; sialic acid alpha 2-3 galactose beta 1-4 [Fucose alpha 1-3] *N*-acetyl glucosamine) as a component of the ligand recognized by E- and P-selectin. In the case of P-selectin, other components of the cell surface, including 2'6-linked sialic acid and sulfatide (galactose-4-sulfate ceramide), have also been proposed for adhesion mediated by this selectin. We have recently defined a region of the E-selectin lectin domain that appears to be directly involved with carbohydrate rec-

ognition and cell adhesion (Erbe, D. V., B. A. Wolitzky, L. G. Presta, C. R. Norton, R. J. Ramos, D. K. Burns, R. M. Rumberger, B. N. N. Rao, C. Foxall, B. K. Brandley, and L. A. Lasky. 1992. *J. Cell Biol.* 119:215-227). Here we describe a similar analysis of the P-selectin lectin domain which demonstrates that a homologous region of this glycoprotein's lectin motif is involved with carbohydrate recognition and cell binding. In addition, we present evidence that is inconsistent with a biological role for either 2'6-linked sialic acid or sulfatide in P-selectin-mediated adhesion. These results suggest that a common region of the E- and P-selectin lectin domains appears to mediate carbohydrate recognition and cell adhesion.

**P**- and E-selectin are calcium-dependent cell surface lectins that mediate leukocyte adhesion by recognition of cell-specific carbohydrate ligands. Together with L-selectin, they form a family of cell adhesion molecules (24, 25) that contain lectin, epidermal growth factor (EGF)-like, and complement binding-like domains (4, 18, 28, 36, 39). L-selectin is found on all leukocytes and appears to recognize an unknown sialylated, fucosylated, sulfated carbohydrate ligand on at least two endothelial glycoproteins (17, 42). One of these glycoproteins, which has been named GlyCAM 1, is a mucin-like endothelial glycoprotein that appears to present carbohydrate ligands to the L-selectin lectin domain (27). E-selectin is an endothelial adhesion molecule whose expression is induced by various inflammatory stimuli and which recognizes the cell surface carbohydrate, sialyl Lewis x (sLe<sup>x</sup>; sialic acid alpha 2-3 galactose beta 1-4 [fucose alpha 1-3] *N*-acetyl glucosamine) (29, 33, 40, 45). P-selectin is found stored in alpha granules of platelets as well as Weible-Palade bodies of endothelial cells (5, 30). It appears to recognize a carbohydrate that is either identical, or closely related, to sLe<sup>x</sup> (22, 23, 31, 34) in the context of perhaps a single glycoprotein (31, 32, 37).

Much evidence has accumulated to indicate similarities in the nature of the carbohydrates seen by selectins. Sialylated, fucosylated lactosaminoglycans (such as sLe<sup>x</sup>) have been shown to bind L-, E-, and P-selectin (12, 16, 29, 33, 34, 40, 45, 49). Furthermore, all three selectins require both sialic acid and fucose residues in specific linkages for adhesion (7, 8, 38, 43). However, the exact carbohydrate structures recognized by selectins are currently incompletely characterized. In addition, many studies have demonstrated clear differences in carbohydrate recognition by selectins. For example, in contrast to E-selectin, both L- and P-selectin have been shown to bind sulfatide (galactose-4-sulfate ceramide) (1, 12). Another distinction in selectin-carbohydrate interactions emerged in a recent study by Larsen and colleagues (24) in which the *Sambucus nigra* lectin, which is specific for the sialyl-2'6βGal/GalNAc linkage, blocked P-selectin binding but not E-selectin binding. This led these authors to propose that the carbohydrate recognized by P-selectin contains a terminal 2'3 sLe<sup>x</sup> core plus a second, perhaps branched terminal sialic acid linked 2'6 to a galactose, whereas the carbohydrate recognized by E-selectin may be simply 2'3 sLe<sup>x</sup> (24). In fact, P-selectin is the only selectin that binds to the 2'6 form of an sLe<sup>x</sup> glycolipid immobilized on microtitre wells, although this binding is weak and variable (12). Thus, evidence exists for the possible participation

1. *Abbreviations used in this paper:* FcγR, IgG Fc receptors; MBP, mannose binding protein; sLe<sup>x</sup>, sialyl Lewis x.

of sulfatides and/or 2'6-linked sialic acid in conferring specificity in P-selectin binding to its ligand. Specificity in selectin-carbohydrate interactions may stem from other variations on this common carbohydrate (i.e., sLe<sup>x</sup>-like) theme, such as the addition of sulfate for the L-selectin ligand (Imai, Y., L. A. Lasky, and S. Rosen, manuscript submitted for publication). Additionally, specificity may stem from variations in carbohydrate presentation (44). For instance, specific glycoproteins appear to present the L- and P-selectin cognate carbohydrate ligands whereas E-selectin appears to recognize a glycolipid (or a protease-resistant glycoprotein) (28, 32).

Because of these differences in carbohydrate recognition within the context of structural similarities, it seems reasonable to suppose that the highly homologous lectin domains of selectins may use a common recognition site for sugars, with some interspersed amino acid variation that confers specificity. Previously, we used a combination of mutagenesis and modeling to identify a discontinuous region of the E-selectin lectin domain that appears to form at least part of its binding site for 2'3 sLe<sup>x</sup> (10). This study modeled the E-selectin lectin domain using the crystal structure of the mannose binding protein (47) as a guide and demonstrated the importance of residues within a patch, formed by the antiparallel beta sheet derived from the amino and carboxy termini and two adjacent loops, in the binding of both blocking anti-E-selectin mAbs, and in the binding of sLe<sup>x</sup> itself (10). Additionally, the relatively small size of the E-selectin region identified as critical for ligand recognition was consistent with nuclear magnetic resonance solution analyses of 2'3 sLe<sup>x</sup> structure showing that the critical sialic acid and fucose residues point to one face of this carbohydrate and are separated by ~10 Å (2, 43).

In this study we extend understanding of selectin structure and function by demonstrating that the site identified as important for E-selectin binding also serves an important role in P-selectin binding. Mutagenesis of this site in P-selectin also provides clues to some of the structural basis for the similarities and differences in carbohydrate recognition by selectins.

## Materials and Methods

### Flow Cytometric Assay for P-selectin Ligand

The interaction of P-selectin and its cellular ligand was studied using a flow cytometric assay. Cells used in this assay were either HL60 cells (maintained in high glucose DME plus 10% Hyclone FBS) or fresh human neutrophils. Human neutrophils were purified from heparinized peripheral blood by a Ficoll-Hypaque gradient to remove mononuclear cells, followed by treatment with 3% dextran sulfate to remove red blood cells. The resulting cells were >90% neutrophils. Before staining with P-selectin-IgG both cell types were preincubated in Dulbecco's PBS/1% BSA/0.1% sodium azide/1% normal rabbit serum (staining medium) for 30–60 mins on ice. After this initial incubation, 1 µg of P-selectin-IgG was added to 100-µl aliquots of 10<sup>6</sup> cells and incubated for 30–60 mins on ice. The cells were then washed with staining medium and resuspended in 100 µl of staining medium to which was added 2 µl of a phycoerythrin-conjugated F(ab)<sub>2</sub> goat anti-human IgG (Fc specific). The cells were incubated for 15–30 mins on ice, washed twice with staining medium, and resuspended in 0.5 ml of staining medium before flow cytometric analysis on a FACScan (Becton Dickinson & Co., Mountain View, CA). To determine that the staining was an interaction of P-selectin with its ligand, the staining was also done in the presence of 10 mM EGTA. To determine the protease sensitivity and the requirement for sialic acid of this interaction, HL-60 cells in D-PBS and 1% BSA were incubated with either trypsin or *Arthrobacter* or *Clostridium*

sialidases at 37°C before resuspending in staining medium. To determine the effect of activation on the expression of the ligand, human neutrophils were incubated at 37°C with 50 ng/ml phorbol myristate acetate for 10 min before resuspending in staining medium. To examine the ability of various carbohydrates to inhibit staining, 50 µg/ml fucoidin (Sigma Immunochemicals, St. Louis, MO), 50 µg/ml dextran sulfate (Pharmacia Fine Chemicals, Piscataway, NJ), 10 mg/ml mannose-1-phosphate (Sigma Immunochemicals), or 10 mg/ml mannose-6-phosphate (Sigma Immunochemicals) was added to cells immediately before the addition of the P-selectin chimera. Each carbohydrate was then present until the cells were washed before the addition of the second stage antibody. A potential complication of this FACS assay arose from the use of selectin-IgG chimeras to stain cells (HL60 cells and neutrophils) which bear human IgG Fc receptors (FcγR; see reference 11 for review). Adding rabbit IgG (in the form of normal rabbit serum) to the assay medium blocked this binding in most cases. However, in some experiments with human neutrophils, it was necessary to add murine mAbs to human FcγR (Medarex, Inc., West Lebanon, NH) to the assay medium to completely block this interaction.

### Anti-selectin mAbs

The following anti-human P-selectin mAbs were purchased to characterize the mutant chimeras: mAbs AK-6 (CLB-thromb/6) and CRC 81 from Bio-Design International (Kennebunkport, ME), and mAb AC 1.2 from Becton Dickinson & Co. The anti-E-selectin mAbs 9A1, 7E10, 3B7, and 9H9 have been described (10). The anti-L-selectin mAb Leu 8 was purchased from Becton Dickinson & Co. and used in the FACS assay (a registered trademark of Becton Dickinson & Co.) according to the manufacturer's instructions.

### Construction and Expression of Wild Type and Mutant Chimeras

Production and characterization of the P- and E-selectin-IgG chimeras has been previously described (12). The PE-1 chimera was constructed in two steps. First, an EcoRI-XhoI fragment encoding the signal peptide, lectin domain, and part of the EGF domain of P-selectin was removed from a pRK5/P-selectin-IgG plasmid. This fragment was inserted into a pRK5/E-selectin-IgG plasmid which had been digested with EcoRI and BglII to remove the E-selectin signal peptide and most of the E-selectin lectin domain. Second, the P-selectin lectin domain was joined in frame to the E-selectin EGF domain via oligonucleotide-directed deletion mutagenesis using the method of Kunkel (21) as described (10). The expressed PE-1 construct consisted of the signal peptide and lectin domain from P-selectin, followed by the EGF, CR1 and CR2 domains of E-selectin, and the IgG1 hinge, CH2 and CH3 domains common to both the P-selectin-IgG and E-selectin-IgG constructs.

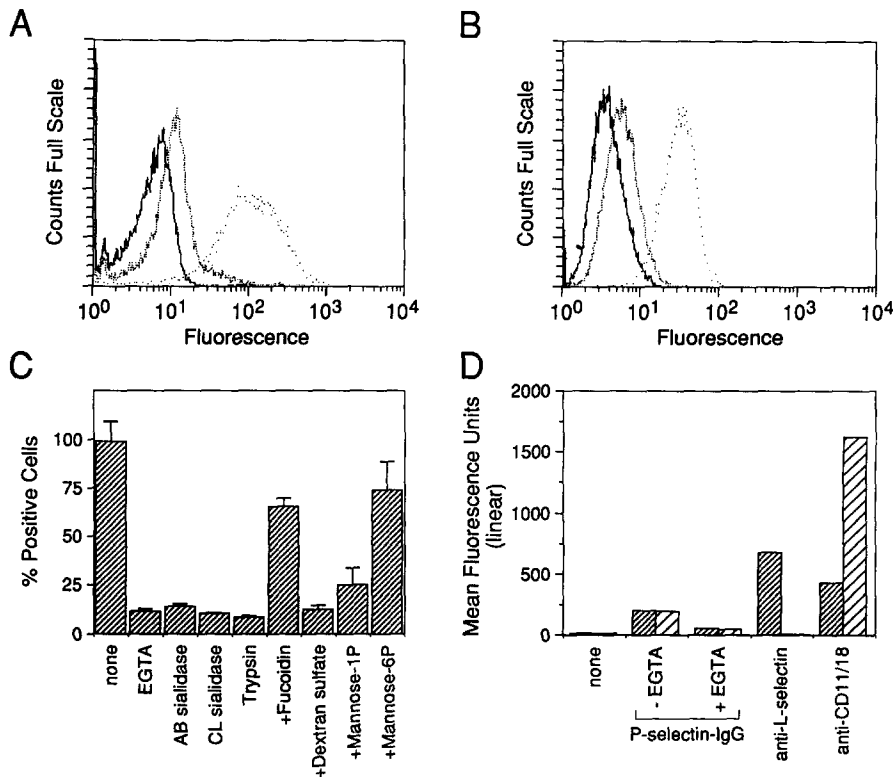
Amino acid substitutions were introduced into the lectin domain of the P-selectin-IgG chimera as previously described (10). Wild type and mutant chimeras were expressed and secreted by 293 cells, quantified and tested for anti-selectin mAb reactivity also as described (10). Mutant chimeras are defined using the nomenclature: K113A is a mutant where the lysine (K) at position 113 is changed to an alanine (A).

### Binding of Selectin-IgG Chimeras to Sialyl Lewis x and Sulfatides

Assays for binding of the different selectin-IgG chimeras to immobilized sLe<sup>x</sup> glycolipids or sulfatides were performed as described (12). Briefly, 2'3 sLe<sup>x</sup> glycolipids, 2'6 sLe<sup>x</sup> glycolipids, or bovine brain sulfatides (Sigma Immunochemicals) were dried onto microtitre wells, washed with distilled water, and then blocked with BSA. Biotinylated goat anti-human IgG Fc and alkaline phosphatase-streptavidin (Caltag Labs, South San Francisco, CA) were each diluted 1:1,000 into 293 cell supernatants containing equal concentrations of wild type or mutant chimeras and allowed to form a complex before the addition to the wells. These supernatants were then incubated on the sLe<sup>x</sup> glycolipid or sulfatide-coated surfaces, followed by washing, addition of substrate (*p*-nitrophenyl phosphate), and measurement of the OD at 405 nm.

### Generation of a P-selectin Lectin Domain Model

A model of the P-selectin lectin domain was generated based on the crystal structure of the rat mannose-binding protein (MBP) (47) as previously described for an E-selectin lectin domain model (10). Briefly, MBP residues were changed to the P-selectin sequence with the sidechain conformations



**Figure 1.** Staining of HL60 cells and neutrophils by selectin-IgG chimeras. Selectin-IgG chimeras were tested by flow cytometry for staining of either HL60 cells (A and C) or human neutrophils (B and D) as described in Materials and Methods. In A and B, dotted lines represent P-selectin-IgG staining, dashed lines represent P-selectin-IgG staining in the presence of 10 mM EGTA, and solid lines represent E-selectin-IgG staining. Staining with no chimera (secondary antibody only) was identical to E-selectin-IgG staining for both cell types. (C) HL60 cells were treated as indicated (see Materials and Methods) and stained with P-selectin-IgG as in A. Results are expressed as percentage of cells staining positively ( $\pm$ SD of duplicates) based on staining with secondary antibody alone. (D) Human neutrophils before ( $\text{⊘}$ ) or after ( $\text{⊚}$ ) activation with PMA were stained with the indicated reagents and evaluated by flow cytometry as described in Materials and Methods. Results are shown as the linear mean fluorescence.

kept similar to those of MBP where possible. Otherwise sidechain conformations were based on rotamer libraries (35), packing, and hydrogen-bonding considerations. Possible loop structures for the 11 insertions and two deletions in P-selectin relative to MBP were gleaned from a search of crystal structures in the Protein Data Bank (3). Finally, the P-selectin model was subjected to repetitive cycles of energy minimization using the method described for E-selectin (10).

## Results

As a starting point for evaluating the residues in P-selectin responsible for binding ligand, we developed a flow cytometric assay using the P-selectin-IgG chimera to stain HL60 cells and neutrophils. Whereas E-selectin-IgG did not bind HL60 cells or neutrophils in this assay, P-selectin-IgG staining resulted in a strong fluorescence shift for both cell types (Fig. 1, A and B). This binding was inhibited by EGTA, reflecting the calcium requirement for P-selectin's interaction with its ligand. Further controls indicated that this assay using the P-selectin-IgG chimera reflects the published characteristics of P-selectin-ligand binding (8, 24, 31, 32, 37, 49). In particular, treatment of HL60 cells with either trypsin or sialidase abolished staining (Fig. 1 C). Furthermore, P-selectin-IgG staining was inhibited by dextran sulfate and mannose-1-phosphate, but not by fucoidin or mannose-6-phosphate (Fig. 1 C). After activation of human neutrophils with PMA, although surface expression of L-selectin decreased and surface expression of CD11/18 increased, surface expression of the P-selectin ligand did not change (Fig. 1 D). In addition to neutrophils, monocytes and NK/LGL cells were positive when stained with P-selectin-IgG (data not shown), which is consistent with the expression of the P-selectin ligand on these cells.

As noted above, the E-selectin-IgG chimera did not bind

HL60 cells or neutrophils in the soluble FACS assay. We exploited this finding to aid in mapping the region of P-selectin necessary for conferring this high affinity binding. Since our previous study with E-selectin (10) had localized its ligand binding site to a region within its lectin domain, we sought to determine if the apparent differences in E- and P-selectin binding could be attributed to differences in their lectin domains. Consequently, we constructed a chimera (PE-1) which consisted of E-selectin-IgG with the E-selectin lectin domain replaced with the lectin domain from P-selectin. To see if this chimera was folded correctly, we tested its binding to antibodies specific for the various domains of E- and P-selectin. The PE-1 chimera reacted well with antibodies to the CR1 and CR2 domains of E-selectin (mAbs 9A1 and 7E10, Table I) but not with antibodies to the lectin domain of E-selectin (mAbs 3B7 and 9H9, Table I). PE-1 bound to the blocking antibody to P-selectin (9) (AK-6, Table I), consistent with the localization of the epitope recognized by this mAb to the lectin domain of P-selectin. By contrast, the non-blocking antibodies to P-selectin, AC 1.2 and CRC 81, did not recognize PE-1 (Table I). This latter result is consistent with earlier studies which indicated a contribution of residues within the EGF and/or CR domains of P-selectin in AC 1.2 binding (19). These results are consistent with the PE-1 chimera being correctly folded, and indicate that at least part of the epitope recognized by the blocking mAb AK-6 is localized to the lectin domain of P-selectin.

To determine if transferring the P-selectin lectin domain onto E-selectin-IgG transferred carbohydrate specificity, we examined binding of PE-1 to various immobilized glycolipids. This binding was compared to that seen with either P-selectin-IgG or E-selectin-IgG. As shown in Fig. 2, the PE-1 chimera appeared to closely mimic P-selectin-IgG in

**Table I. PE-1 Binding to Anti-selectin Antibodies**

mAb Class	Clone	P-selectin-IgG	OD 450 PE-1	E-selectin-IgG
Blocking mAb to P-selectin	AK-6	1.3 ± 0.1	1.7 ± 0.1	0.0 ± 0.0
Nonblocking mAb to P-selectin	AC 1.2	2.8 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
	CRC 81	3.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0
mAb to CR1 and CR2 of E-selectin	9A1	0.1 ± 0.0	1.1 ± 0.2	0.9 ± 0.1
	7E10	0.0 ± 0.0	1.6 ± 0.0	1.2 ± 0.2
mAb to lectin domain of E-selectin	3B7	0.1 ± 0.1	0.0 ± 0.0	1.9 ± 0.0
	9H9	0.0 ± 0.0	0.0 ± 0.0	2.2 ± 0.1

The P-selectin-IgG, E-selectin-IgG, and PE-1 chimeras were tested for capture by the antibodies indicated using the ELISA format described previously (10). Results shown are the mean optical density ±SD of duplicate determinations.

binding to all three glycolipids tested: 2'3 sLe<sup>x</sup> (Fig. 2 A), 2'6 sLe<sup>x</sup> (Fig. 2 B) and sulfatides (Fig. 2 C). Therefore, the lectin domain of P-selectin appears to be sufficient for transferring specificity in binding to these purified glycolipids.

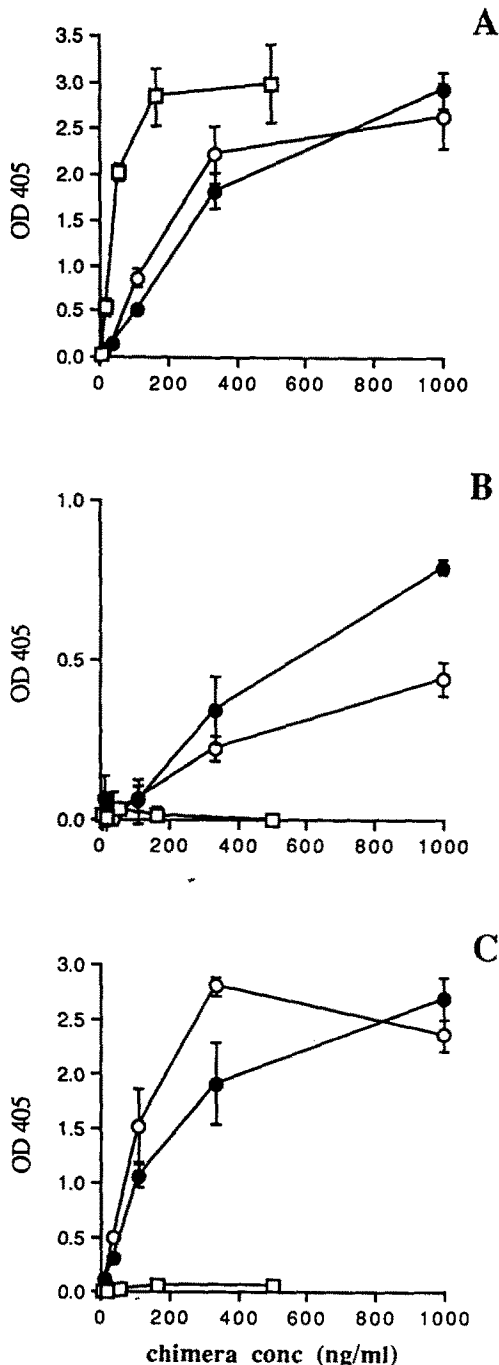
We then tested the PE-1 chimera for cell staining to see if the P-selectin lectin domain could also confer the high affinity binding to the P-selectin ligand on HL60 cells. As seen in Fig. 3, the PE-1 chimera did bind HL60 cells. However, the shift in fluorescence seen with PE-1 staining was not as great as that seen with P-selectin-IgG (Fig. 3). Therefore, although the lectin domain of P-selectin did appear to clearly confer HL60 cell staining, some contribution of the EGF and/or CR1 domain of P-selectin may be required for full, high-affinity binding to these cells. Similar results were seen when neutrophils were stained with these three chimeras (data not shown).

The above results using the PE-1 chimera indicated that the lectin domain of P-selectin contained elements responsible for the differences in binding of E- and P-selectin to immobilized glycolipids and cells. Therefore, we performed mutagenesis of the P-selectin lectin domain to further localize the residues responsible for the interaction of P-selectin with its ligand. P-selectin mutagenesis was focused on those sites which in our previous study (10) proved to be important for E-selectin binding to its ligand. This strategy was followed for two reasons. First, as mentioned above, a wealth of experimental evidence exists indicating similarities in recognition of sugars by E- and P-selectin. Thus, it is reasonable to suppose that a site important for E-selectin-mediated adhesion would also participate in P-selectin-mediated binding. The second reason derived from an experimental consideration. In the E-selectin study we were able to generate an entire panel of antibodies to serve as structural controls for the effects of point mutations on lectin domain structure (10). This allowed the elimination of amino acid substitutions which grossly affected folding of the E-selectin lectin domain from consideration. In this study, we were limited to just three anti-P-selectin mAbs (AK-6, AC 1.2, and CRC 81), only one of which (AK-6) was clearly shown to bind a determinant in the lectin domain (see above). To avoid the generation and analysis of mutants which do not bind ligand due to a gross conformational effect rather than a specific side chain substitution, we restricted our analysis to only those mutations which had resulted in correctly folded proteins in the E-selectin analysis (10).

As a starting point for P-selectin mutagenesis we gener-

ated a three-dimensional model of the P-selectin lectin domain in the same manner that the E-selectin model was generated (see Fig. 4). Comparison of the two models revealed that of the residues that appeared most important for E-selectin binding to 2'3 sLe<sup>x</sup>, three are conserved in P-selectin: Y48, K111, and K113. In E-selectin, the substitutions Y48F, K111A, and K113A each profoundly decreased sLe<sup>x</sup> binding (10). Mutation of position 84 from R to A did not affect sLe<sup>x</sup> binding by E-selectin, and mutation of position 8 from E to A increased sLe<sup>x</sup> binding by E-selectin (10). Fig. 5 shows the effect of complimentary substitutions at these positions in P-selectin on the binding of the anti-P-selectin mAbs. Whereas none of these substitutions significantly affected capture by the nonblocking antibodies (AC 1.2 and CRC 81), each of the substitutions K8A, K111A, and K113A partially decreased binding of the blocking antibody AK-6 (Fig. 5). These results are consistent with the PE-1 chimera results above which localized part of the AK-6 epitope to the lectin domain of P-selectin. These results are also consistent with the relatively close alignment of these three positions along the same face of the P-selectin lectin domain, as predicted by the model (Fig. 4). Furthermore, the complimentary substitutions E8A and K113A in E-selectin completely abolished binding of a number of blocking mAbs to E-selectin (10). Also like E-selectin, mutation of the residues at positions 48 and 84 in P-selectin did not affect mAb binding (Fig. 5).

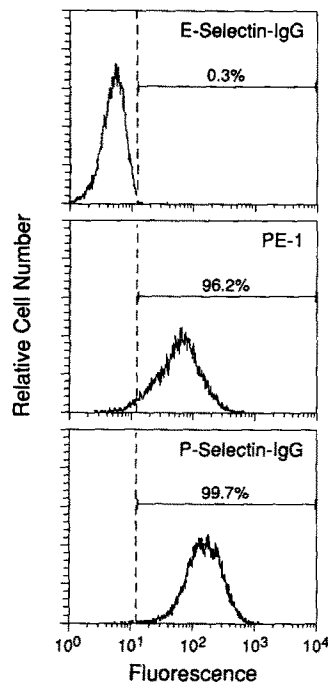
Next, we evaluated these P-selectin mutants for binding to immobilized glycolipids and cells (Fig. 6). Measurement of the binding of this panel of mutants to the 2'3 sLe<sup>x</sup> glycolipid indicated that P-selectin appears to use some of the same residues as E-selectin in binding this carbohydrate (Fig. 6 A). Whereas P-selectin mutants with the substitutions K8A and K84A still bound 2'3 sLe<sup>x</sup>, the mutants Y48F and K113A were completely negative. In E-selectin the mutant K111A did not bind 2'3 sLe<sup>x</sup> at all (10). Here, however, the P-selectin mutant K111A mediated partial binding to 2'3 sLe<sup>x</sup>, perhaps indicating a subtle difference in recognition of this sugar by E- and P-selectin. A different set of residues appeared to be important for binding of P-selectin to the 2'6 form of sLe<sup>x</sup> (Fig. 6 B). The substitutions K8A, K111A, and K113A ablated binding, while Y48F had no effect. The mutant K84A also still bound 2'6 sLe<sup>x</sup> (Fig. 6 B). When sulfatide binding was evaluated, a third pattern emerged (Fig. 6 C). Only the mutation K113A significantly decreased sulfatide binding by P-selectin. These results indicate that the



**Figure 2.** Binding of the PE-1 chimera to immobilized sLe<sup>x</sup> glycolipids and sulfatides. P-selectin-IgG (○), E-selectin-IgG (□), or PE-1 (●) were tested at the indicated concentrations for binding to immobilized 2'3 sLe<sup>x</sup> glycolipid (A), 2'6 sLe<sup>x</sup> glycolipid (B), or sulfatides (C) by the ELISA procedure described in Materials and Methods. Results shown represent the mean ± SD of triplicate determinations.

same face of P-selectin appears to participate in binding these three glycolipids, with subtle differences in the residues used to bind each sugar.

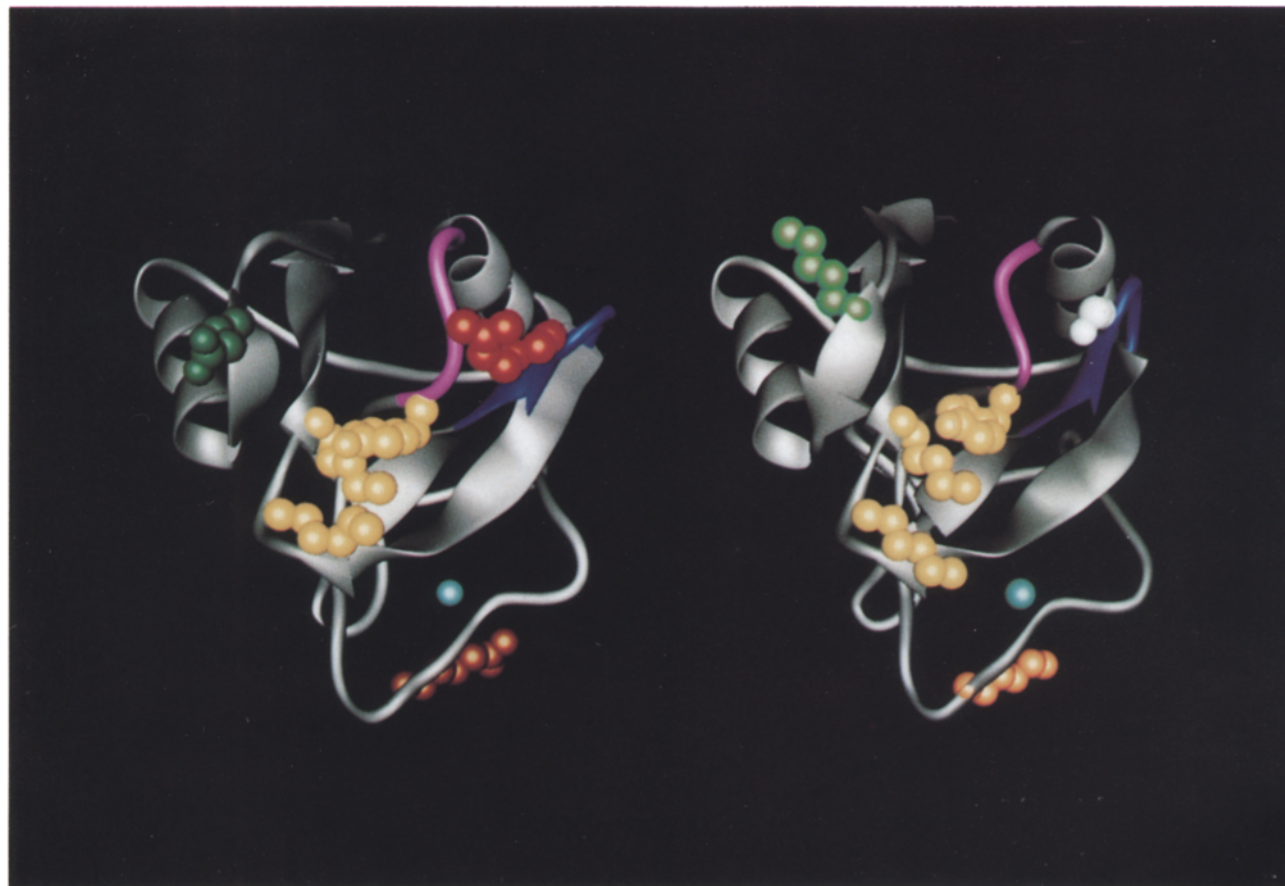
Since a more relevant assay for measuring P-selectin interactions with its ligand is the cell binding assay, the panel of mutants was evaluated by flow cytometry for staining of



**Figure 3.** Staining of HL60 cells with the PE-1 chimera. The P-selectin-IgG, E-selectin-IgG, and PE-1 chimeras were tested for binding to HL60 cells by flow cytometry as in Fig. 1. The percentage of cells staining positively (based on staining with the secondary antibody alone) with each chimera is indicated.

HL60 cells (Fig. 5 D). Interestingly, the binding pattern seen with cells closely mimics that seen with the immobilized glycolipid 2'3 sLe<sup>x</sup>. K8A and K84A both bound to HL60 cells, Y48F and K113A did not, and K111A bound HL60 cells only partially. Similar reactivities were seen when neutrophils were stained (data not shown). So, mutation of residues within this pocket of P-selectin also affected binding to its cognate ligand on cells. Furthermore, comparison of the reactivity of this panel of mutants with purified glycolipids provided some potential insights into the nature of the carbohydrate seen by P-selectin (see Discussion).

In E-selectin the arginine at position 97 was also important for sugar recognition. Mutation of this residue to alanine completely abolished E-selectin/2'3 sLe<sup>x</sup> binding (10). The residue at position 97 in P-selectin is a serine and the above results indicated that P-selectin appears to use the same region as E-selectin in binding to its ligand. Therefore, we tested if this difference in residues at position 97 could account for the differences in ligand binding by E- and P-selectin. Examination of the three-dimensional models of the E- and P-selectin lectin domains (Fig. 4) reveals that amino acid 97 falls within a loop formed by residues 94-100, which is an insertion in selectins relative to the mannose binding protein. The sequence of these two selectins is quite different through this stretch -YIKREKDV for E-selectin vs. YIKSP-SAP for P-selectin - so these loops would be expected to have different conformations. To test the importance of the residue at position 97 in conferring specificity to selectins, we made a P-selectin-IgG mutant with the 94-100 loop replaced with the corresponding residues from E-selectin: S97R, P98E, S99K, A100D, and P101V. We then tested this mutant (abbreviated REKDV) for binding to antibodies, glycolipids, and cells. Binding of the P-selectin-IgG REKDV mutant to each of the three anti-P-selectin mAbs (AK-6, AC 1.2, and CRC 81) was ~70% of control P-selectin-IgG binding. This would seem to indicate that although folding of this mutant



E-selectin	1	WSYNTSTEA	MTYDEASA	YCGQRYTH	LVAIQNK	EIEV	LNS
P-selectin	1	WTYHYS	TKAYS	WNISRK	YCGNRYT	D	LVAIQNKNEIDV
E-selectin	41	LSVSP	SYWIGIRK	VNNVW	VWGTQK	PLTE	EAKNWA
P-selectin	41	LPVYS	SYWIGIRK	NKKT	WTWVGT	KJAL	TNEAENWADNE
E-selectin	81	PNNRQK	EDCVEIYIK	REKDV	GMWNDER	CGSKK	KLALC
P-selectin	81	PNNKRNN	EDCVEIYIK	SPSAP	GKWNDE	HCL	KKKHALC

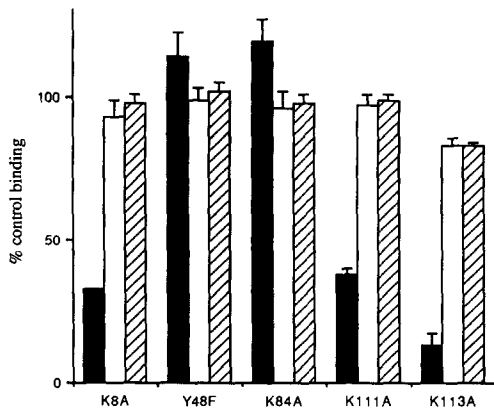
(left) E8-dark green, R84-dark brown, R97-red, and top-to-bottom Y48-K113-K111-yellow. The purple loop (residues 43-48) and the dark blue loop (residues 94-100) in each model denote two insertions near the proposed carbohydrate binding sites that are not found in the mannose binding protein. In each model the single bound calcium is depicted as a light blue ball. (Bottom) Sequence alignment of the human E- and P-selectin lectin domains.

**Figure 4.** A model of the lectin domain of P-selectin. (Top) Shown is a ribbon model of the P-selectin lectin domain (right) alongside a ribbon model of the E-selectin lectin domain (10) (left). Each model is similarly derived from the published coordinates of the related type C lectin, the mannose binding protein (47) as described (10 and Materials and Methods). Residues shown are colored as follows: for P-selectin (right) K8-light green, K84-light brown, S97-white, and top-to-bottom Y48-K113-K111-yellow, for E-selectin

is largely correct, some subtle structural perturbations may be present. Accordingly, this mutant did not bind any of the purified glycolipids in the solid phase ELISA (data not shown). However, the REKDV mutant did stain HL60 cells in the FACS assay, although its binding was significantly less than that seen with control P-selectin-IgG (70% cells positive, MFI 290 for REKDV mutant vs. 97% cells positive, MFI 416 for control P-selectin-IgG). Thus, transferring this loop (containing residue 97) from E- to P-selectin did not completely disrupt the ability of the resultant P-selectin mutant to recognize its cellular ligand. This may indicate that the binding of P-selectin-IgG to its cellular ligand is of higher affinity than binding of P-selectin-IgG to the immobilized glycolipids. Also, these results would seem to imply that at least some of the differences in binding between E- and P-selectin must be due to differences outside of this region (see Discussion).

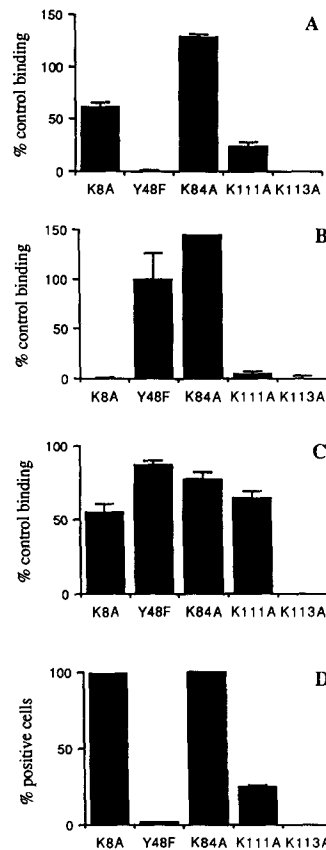
## Discussion

Research on selectin-carbohydrate interactions continues to be hampered by a lack of detailed understanding of the sugar structures seen by each adhesion molecule. However, results from a number of approaches, including direct-binding studies, soluble carbohydrate inhibition studies, and structural and conformational analyses of purified potential ligands, have indicated commonalities in selectin recognition. Many of these findings have centered around the sLe<sup>x</sup> core structure. However, many of these proposed similarities may be artifacts of forced binding under experimentally manipulated circumstances (see reference 44 for discussion). In vitro assays with solid phase carbohydrate ligands and transfected, over-expressed selectins can be misleading due to the unnaturally high densities of both receptors and ligands (44). Furthermore, unrelated sugars can inhibit the



**Figure 5.** Reactivity of anti-P-selectin mAbs with mutant chimeras. P-selectin-IgG chimeras with the substitutions indicated were tested for capture by the mAbs AK-6 (■), AC 1.2 (□), and CRC 81 (▨) as in Table I. Results shown represent the mean ± SD of duplicate determinations and are expressed as percentage of control P-selectin-IgG binding.

same lectin interaction due to structural mimicry (44). The flow cytometric assay used here to measure P-selectin's interactions with its cellular ligand should avoid most of these limitations while still being sensitive and convenient. The experiments presented here indicate that the measured binding observed using the P-selectin-IgG chimera to stain cells accurately represents the interaction of P-selectin with its ligand. Studies to date have shown that P-selectin binds a single, possibly unique, major glycoprotein of 120 kD (32). The same glycoprotein has been isolated from both neutrophils and HL60 cells (32) and the number of such binding sites for P-selectin is estimated at 10,000–20,000 per cell (31, 32, 37). sLe<sup>x</sup> may form some component of this glycoprotein ligand, and sLe<sup>x</sup> is sufficient to confer some P-selectin binding. However, sLe<sup>x</sup> is not sufficient to confer the saturable, high-affinity binding characteristic of P-selectin adhesion (31). Therefore, the P-selectin ligand must have structural features in addition to sLe<sup>x</sup> that confer specificity and affinity (32, 49). The protein portion of the P-selectin ligand may contribute to this specificity and affinity by: (a) presenting the sugar in the correct configuration; (b) presenting multivalent sugars to enhance binding avidity; and/or (c) participating in a protein-protein contact with P-selectin (32). In fact, a role for presentation of polyvalent ligands to L-selectin by the GlyCAM 1 ligand has already been proposed (27). In the assay described here, P-selectin-IgG binding was ablated by protease treatment of cells, consistent with a requirement for this glycoprotein (24). As noted above, sialic acid is crucial to P-selectin binding and sialidase treatment also abolished binding. Most importantly, removal of calcium chelation by EGTA also led to a loss of binding, a result that is a signature of the interactions mediated by all C-type lectins (47). A surprising finding was that the E-selectin-IgG chimera did not bind HL60 cells or neutrophils in this fluid phase staining assay. This is despite the fact that the E-selectin carbohydrate ligand, sLe<sup>x</sup>, is clearly expressed by these cells (33, 45). Furthermore, we (10) and others (41) have found that E-selectin-IgG is capable of binding HL60 cells and neutrophils when the chimera is



**Figure 6.** Binding of P-selectin-IgG mutants to immobilized glycolipids and cells. P-selectin-IgG chimeras with the substitutions indicated were tested for binding to immobilized 2'3 sLe<sup>x</sup> glycolipid (A), 2'6 sLe<sup>x</sup> glycolipid (B), or sulfatides (C) as in Fig. 2, and for staining of HL60 cells (D) as in Fig. 1.

presented on a solid substrate, suggesting that the lack of binding in the fluid phase may be due to lower affinity of E-selectin for its cognate cell surface ligand. Thus, E- and P-selectin are clearly distinct in binding to cells both as soluble Ig chimeras, as well as when they are expressed on endothelial/platelet cell surfaces.

At least part of this difference between E- and P-selectin must be due to differences in their lectin domains. Transferring the P-selectin lectin domain onto the E-selectin-IgG construct resulted in a molecule (PE-1) which stained cells, albeit at a lower intensity than P-selectin-IgG. Carbohydrate reactivity was completely transferred with the relevant lectin domain. Thus, PE-1 reacted with the purified glycolipids in a manner that was indistinguishable from P-selectin-IgG and quite distinct from E-selectin-IgG. Therefore, the lectin domain of each selectin appears sufficient for determining the differences in reactivities with these relatively small sugars. This result is consistent with a study by Kansas et al. (20) in which domains of L- and P-selectin were exchanged to show that PPME and fucoidin binding, both L-selectin-specific carbohydrate ligands, as well as the epitope defined by blocking mAb LAMI-3, map at least in part to the COOH-terminal 67 amino acid residues of the L-selectin lectin domain. These authors also demonstrated that the CR domains are not important for conferring PPME or fucoidin specificity (20). The EGF and CR domains of selectins have clearly been shown to perform vital structural roles for these receptors (6, 19, 45, 46). Whether these domains in P-selectin also participate in making crucial contacts with its glycoprotein ligand cannot be answered here. However, the results of this study do place limitations on the nature of any such contacts.

First, the P-selectin-IgG chimera used here only contains the lectin, EGF, and CR1 domains of P-selectin (12). Thus, CR2-CR9 must not form necessary contacts for the high affinity binding between P-selectin and its ligand and it is interesting to note that mouse P-selectin lacks the CR2 domain (48). In addition, because the PE-1 chimera did bind cells, any potential protein-protein contact sites may map to the lectin domain of P-selectin. The difference in staining between PE-1 and P-selectin-IgG might reflect subtle conformational effects of the P- or E-selectin EGF domains interacting with the common lectin domain. However, it is important to stress that protein-protein contacts mediated by the EGF or CR1 domains cannot be ruled out.

Two recent studies have identified regions of the P-selectin lectin domain that may be important for cell adhesion (14, 15). Geng and co-workers showed that a mAb capable of inhibiting neutrophil binding to P-selectin mapped to residues 19-34 of this molecule, and that a peptide corresponding to this stretch also inhibited neutrophil binding to P-selectin (14). This group described other peptides from the lectin domain of P-selectin (corresponding to residues 23-30, 54-63, and 70-79) which blocked P-selectin-mediated adhesion (15). In the model of P-selectin, these residues fall on the opposite side of the lectin domain from the site that we have identified as important for selectin-carbohydrate binding and cell adhesion (see Fig. 4 and reference 10). The residues characterized by Geng and coworkers may represent a second site in P-selectin which may bind the carbohydrate and/or protein component of its ligand. In light of this, it is important to remember that the results with the REKDV mutant indicated that not all of the differences in specificity between E- and P-selectin can be explained by the region identified in this study. Therefore, the possible co-operation of this site with those described by Geng et al. (14, 15) in conferring P-selectin binding specificity warrants exploration.

The results presented here establish that the site previously identified as crucial for E-selectin binding to 2'3 sLe<sup>x</sup> is also crucial to P-selectin binding to this ligand. Mutations in two of the conserved residues within this site, Y48 and K113, completely abolished 2'3 sLe<sup>x</sup> binding and cell adhesion by both E- and P-selectin. The anti-P-selectin blocking mAb AK-6 mapped to this same site, as did all of the anti-E-selectin blocking mAbs (10). Furthermore, Mel-14, a mAb that blocks L-selectin-mediated adhesion in vitro and in vivo, maps to this region (6, 10). The fact that adhesion blocking mAbs to all three selectins bind to residues within this site emphasizes its importance to the adhesive functions of these proteins.

By comparing the binding of the panel of P-selectin mutants to 2'3 sLe<sup>x</sup>, 2'6 sLe<sup>x</sup>, and sulfatides with their ability to bind cells, some insight as to the nature of the carbohydrate component of the P-selectin ligand can be gained. As noted above, one study has shown that E- and P-selectin have related but distinct carbohydrate specificities (24). For example, these authors found that the interaction of E-selectin with the sLe<sup>x</sup> component of the P-selectin ligand precludes P-selectin binding (24). Using a 2'6 sialyl-specific lectin to block P-selectin binding, they also proposed that the P-selectin ligand may contain a bidentate carbohydrate structure with one arm containing 2'3 sLe<sup>x</sup> and the other a terminal sialyl-2'6 beta Gal (13, 24). However, our results with

the P-selectin mutants would seem to question a role for 2'6-linked sialic acid in cell adhesion. The mutant K8A did not bind the 2'6 form of sLe<sup>x</sup> at all, but still bound the P-selectin ligand on cells. Furthermore, the Y48F mutant did not bind cells at all, but still bound 2'6 sLe<sup>x</sup>. Consequently, 2'6 sLe<sup>x</sup> binding did not correlate with ligand binding. However, the binding to 2'6 sLe<sup>x</sup> in the solid phase assays used here is weak compared to 2'3 sLe<sup>x</sup> and sulfatide binding, so caution is warranted in interpreting these results. Thus, it is conceivable that presentation of the 2'6 sialylated carbohydrate to P-selectin provides a critical parameter of ligand recognition that is not replicated in our solid phase assay (27).

A second binding activity of P-selectin whose biological relevance has been recently questioned is its interaction with sulfatides. Sulfatide binding by P-selectin is probably not relevant in vivo due to the observation that this interaction would be protease resistant, and that cells expressing sulfatides (erythrocytes and platelets) do not necessarily bind P-selectin (32). Also, sulfatide binding by the P-selectin mutants studied here did not correlate with cell binding. For example, mutant Y48F bound sulfatides well but did not adhere to cells at all. Cell binding was only correlated with binding to 2'3 sLe<sup>x</sup>. Each mutant which bound 2'3 sLe<sup>x</sup> bound cells (K8A and K84A), while those which did not bind 2'3 sLe<sup>x</sup> (Y48F and K113A) did not bind cells, and one mutant (K111A) showed partial binding to both 2'3 sLe<sup>x</sup> and cells. This is interesting given a recent study demonstrating that expression of 2'3 sLe<sup>x</sup> correlated with a cell's ability to bind activated platelets via P-selectin (9), and it is consistent with the mAb and carbohydrate blocking studies of Polley et al. (34).

Although one cannot rule out the involvement of 2'6-linked sialic acid or sulfatides in P-selectin's interactions with its ligand, the data presented here clearly questions the role they may play. Specificity of binding between E- and P-selectin may derive from the manner in which 2'3 sLe<sup>x</sup> is presented (i.e., glycolipid vs. glycoprotein). However, it must be allowed that 2'3 sLe<sup>x</sup> may not be the naturally occurring carbohydrate ligand recognized by either selectin, and that these differences in selectin binding could be accounted for by subtle changes in the saccharide itself (44). Sulfatides, as well as the sulfated glycans heparin, fucoidin, and dextran sulfate, may inhibit P-selectin function by mimicking its ligand (32). sLe<sup>x</sup>, sulfated glycans, and sulfatides all have a negative charge which may play a role in the interaction of P-selectin with its ligand (9, 10) and these sugars may inhibit selectin-mediated adhesion by binding to a common site (for example, at K113) which is important for P-selectin-ligand interactions.

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