THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 271, No. 8, Issue of February 23, pp. 4289–4297, 1996

Printed in U.S.A.

Structure-Function Analysis of P-selectin-Sialyl Lewis^X Binding Interactions

MUTAGENIC ALTERATION OF LIGAND BINDING SPECIFICITY*

(Received for publication, September 14, 1995, and in revised form, December 7, 1995)

B. Mitch Revelle‡, Dee Scott‡, Timothy P. Kogan§¶, Jianhua Zheng§, and Pamela J. Beck‡¶‖

From the Departments of ‡Molecular Biology and §Medicinal Chemistry, Texas Biotechnology Corporation and the ¶Department of Internal Medicine, University of Texas Medical School, Houston, Texas 77030

P-selectin is a vascular cell adhesion molecule that is expressed on the surface of platelets and endothelial cells in response to inflammatory stimuli. It is believed to aid in the binding and recruitment of leukocytes to inflamed tissue. P-selectin adhesion to leukocytes is mediated by the amino-terminal lectin domain that binds the sialyl Lewis^X (sLe^X) carbohydrate (Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc). Neither the three-dimensional structure of P-selectin nor the protein-carbohydrate interactions that mediate the binding of P-selectin to the sLe^X carbohydrate have been determined. The most closely related protein for which a ligand-bound three-dimensional structure has been resolved is the rat mannose-binding protein (Weis, W. I., Drickamer, K., and Hendrickson, W. A. (1992) *Nature* 360, 127–134). Using the known binding interactions that occur between the rat mannose-binding protein and its ligand (oligomannose) as a template, we have used site-directed mutagenesis to substitute Ala-77 with lysine. This substitution changed P-selectin-carbohydrate binding specificity from sLe^X to oligomannose. Further substitution altered the binding preference from mannose to galactose in a predictable manner. These results indicate that P-selectin binds sLe^X in a shallow cleft that is similar to the mannose-binding protein saccharide-binding cleft. Additionally, we present an extensive mutagenic analysis of P-selectin Lys-113, a residue that has previously been implicated in P-selectin binding to both sLe^X and 3-sulfated galactosylceramide (sulfatide). Our analysis demonstrates that Lys-113 is probably not involved in P-selectin binding to either sulfatide or sLe^X. Functionally, it appears that P-selectin has retained a conserved carbohydrate and calcium coordination site that enables it to bind carbohydrate in a manner that is quite similar to that which has been determined for the rat mannose-binding protein.

P-selectin (CD62-P, PADGEM, GMP-140) is a 140-kDa glycoprotein that is typically stored in the secretory granules of platelets and endothelial cells. Various inflammatory stimuli including thrombin and histamine induce the fusion of these storage vesicles with the cell plasma membrane and result in the immediate surface expression of P-selectin (1, 2). Once present on the cell surface, P-selectin is able to bind to carbohydrate ligands on the surface of circulating leukocytes and aid in the adhesion and subsequent recruitment of leukocytes to areas of injury or inflammation.

The nucleotide sequence of human P-selectin was reported by Johnston *et al.* (3), who also noted amino acid sequence homology to several other proteins. Following the signal sequence at the amino terminus, the protein is composed of a 120-amino acid lectin-like (lectin or carbohydrate recognition domain), an epidermal growth factor-like domain, nine short consensus or complement receptor-like repeats, a membrane-spanning domain, and a cytoplasmic tail containing ~35 amino acids.

The amino acid sequence of the P-selectin lectin domain is similar to a variety of calcium-dependent carbohydrate-binding proteins known as C-type lectins (4). E-, P-, and L-selectins, the asialoglycoprotein receptor, the low affinity IgE receptor (CD23), the pulmonary surfactant apoprotein SP-A, and the rat mannose-binding protein (rMBP)[1] are some of the members of the C-type lectin family. The lectin domains of each of these proteins are related through amino acid sequence similarity that includes 14 invariant and 17 highly conserved residues (5, 6). The conserved amino acids are thought to be essential for the establishment of a hydrophobic core or scaffold that is a structural characteristic of each of the members of this protein class (7).

E-, P-, and L-selectins are vascular cell adhesion molecules that share 60–70% identity between the amino acid sequences of their lectin domains. Each selectin has been demonstrated to bind the sLe^X carbohydrate (8–11). However, the binding characteristics of each of the selectins are not comparable, and many differences in their binding specificities have been described (12–17) and extensively reviewed (18). One important binding distinction is that P-selectin is able to bind a second ligand, 3-sulfated galactosylceramide (sulfatide), while E-selectin does not (19, 20). Similarly, the adherence of both P- and L-selectins to the sLe^X carbohydrate can be competitively inhibited by heparin, while E-selectin-sLe^X binding appears to be heparin-insensitive (14). The individual molecular components and interactions that are responsible for most of the observed differences in selectin-carbohydrate adherence remain undefined. Moreover, the mechanisms that allow each selectin to discriminate between distinct biological protein ligands have in most cases not been determined (21, 22). However, it is apparent that the physical ligand binding characteristics of one selectin should not be attributed to another member of the protein family without definitive experimental evidence.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‖ To whom correspondence should be addressed: Dept. of Molecular Biology, Texas Biotechnology Corp., 7000 Fannin Suite 1920, Houston, TX 77030. Tel.: 713-796-8822; Fax: 713-796-8232.

[1] The abbreviations used are: rMBP, rat mannose-binding protein; sLe^X, sialyl Lewis^X; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; BSA, bovine serum albumin.

We previously predicted that the C-2 and C-3 hydroxyls of the sLe^X α1–3-linked fucose coordinate in a calcium-dependent manner with E-selectin residues Glu-80, Asn-82, Asn-105, and Asp-106 and that the remainder of the sLe^X carbohydrate is retained in a shallow pocket that is defined on one side by the E-selectin β5-strand, on a second side by loop 5 (IKREK, residues 95–99), and on a third side by loop 3 (NWAPGE, residues 75–80) (23). This positioning is analogous to the site that oligomannose occupies upon binding to rMBP (24, 25). We wished to determine if P-selectin binds sLe^X in a similar manner. All previously published reports that have attempted to identify the P-selectin sLe^X-binding site have predicted that it does not. Instead, these reports have proposed that the bound sLe^X carbohydrate extends in the opposite direction and that P-selectin binding to sLe^X (20, 27) occurs through the formation of a charge-paired interaction between P-selectin Lys-113 and the sLe^X sialic acid carboxylate. It has further been hypothesized that P-selectin Lys-113 also directly coordinates with the sulfatide sulfate moiety (27) and that sulfatide binding is therefore able to displace or block sLe^X-dependent cellular adherence (27) because the sulfatide- and sLe^X-binding sites are overlapping.

To determine which of these two proposed P-selectin-sLe^X binding models is correct, we attempted to mutagenically change P-selectin-ligand recognition by altering the same amino acids that we had previously determined were essential for E-selectin-ligand recognition and discrimination. We predicted that if P-selectin mutant binding proved to be phenotypically identical to that of the E-selectin mutants, it would serve to confirm the structural conformation of the P-selectin calcium site. Additionally, if P-selectin-carbohydrate adherence was similarly affected by these same amino acid substitutions, then the mutant binding data would support our hypothesis that the sLe^X-binding site is defined by the same shallow grove that is occupied by oligomannose upon binding to MBP. Alternatively, if the P-selectin mutants did not display similar binding characteristics, it would indicate that P-selectin-carbohydrate ligation is fundamentally different from E-selectin binding and that the noted differences in sulfatide and heparin affinity merely reflect that basic difference.

It seemed probable that if P-selectin binds sLe^X in a manner similar to that which we proposed for E-selectin, many of the residues predicted to be essential for E-selectin-sLe^X binding would be conserved between E- and P-selectins. In this regard, it is notable that the E-selectin calcium-coordinating ions (Glu-80, Asn-82, Asn-105, and Asp-106) as determined from the x-ray crystallographic structure (23) are also present in the P-selectin primary sequence. For clarification and comparison purposes, a structural alignment of E-selectin, rMBP, and P-selectin that was adapted from the work of Graves *et al.* (24) is shown in Fig. 1*A*, and an illustrative model of P-selectin that was constructed from the E-selectin structural coordinates (Brookhaven Protein Data Bank accession number 1ESL) is shown in Fig. 1*B*.

As can be seen from the amino acid sequence alignment (see Fig. 1*A*), substantial homology is shared between E- and P-selectins, yet the two proteins still contain a large number of amino acids that are not identical (~30%). Some of these nonidentical residues comprise the proposed sLe^X-binding site and include two amino acid substitutions in loop 3 (residues 75–80) and three of the five amino acids in loop 5 (residues 95–99; E-selectin residues IKREK as compared with P-selectin residues IKSPS).

Since the three-dimensional structure of the P-selectin lectin domain has not been determined, we do not know how these differing amino acids affect the conformation of the protein or how the individual amino acid side chains alter or influence P-selectin-ligand binding. In this regard, it is notable that extensive evidence derived from mutagenic structure-function analyses of P-selectin that were performed by Hollenbaugh *et al.* (26), Bajorath *et al.* (27) and Erbe *et al.* (20) indicate that the P-selectin amino acids in loop 5 may influence P-selectin binding to sLe^X. Additionally, each of these groups noted the importance of P-selectin Lys-113, which when changed to an alanine or arginine, obliterates P-selectin binding to both sLe^X and sulfatide.

To further our understanding of the protein-carbohydrate interactions that occur when P-selectin binds to either the sLe^X oligosaccharide or sulfatide, we have used site-directed mutagenesis to complete a more extensive mutational analysis of P-selectin-ligand binding interactions. Unlike the results previously reported by others (27), our data indicate that Lys-113 does not directly contact either the sLe^X sialic acid carboxylate or bound sulfatide in a charge-dependent manner. Furthermore, in an effort to elucidate the critical contacts that are made between P-selectin and its carbohydrate ligands, we have used the same mutagenesis scheme that we described previously for E-selectin (23) and have altered P-selectin amino acids that would be predicted from homology to the primary amino acid sequences of E-selectin and rMBP to influence the adherence of P-selectin to sLe^X. The presented evidence suggests that it is likely that P-selectin binds sLe^X in a manner analogous to that in which the rat mannose-binding protein binds oligomannose and that sulfatide is bound at an entirely different location.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, dialyzed fetal calf serum, phosphate-buffered saline (PBS), and antibiotics were obtained from Life Technologies, Inc., and fetal calf serum was from Hyclone Laboratories. The anti-P-selectin antibody (AC1.2) was purchased from Becton-Dickinson. Magnetic beads conjugated to goat anti-mouse IgG and magnetic separators were obtained from Dynal, Inc. (Great Neck, NY). Unless specifically stated, other immunochemicals were purchased from Calbiochem. Sulfatides, yeast invertase, and yeast (*Saccharomyces cerevisiae*) were purchased from Sigma. Flexible 96-well assay plates and Probind 96-well ELISA plates were purchased from Falcon. The sLe^X tetrasaccharide was purchased from Oxford Glycosystems. Synthetic oligonucleotides were purchased from Oligo Therapeutics Inc. Restriction enzymes and T4 DNA ligase were from Life Technologies, Inc. and New England Biolabs Inc., and *Taq* DNA polymerase was obtained from Perkin-Elmer.

Cell Culture and Transfection—HL-60 cells were grown at 37 °C with 5% CO_2 in RPMI 1640 medium supplemented with 10% fetal calf serum. COS-1 cells were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Prior to electroporation, COS-1 cells were trypsinized, harvested, and washed twice with PBS, and 10^6 cells (0.8 μl) were mixed with 10 μg (1 μg/μl) of plasmid DNA in a 0.4-cm electrode gap cuvette. Transfections were performed using a Bio-Rad electroporator following the manufacturer's recommendations and using 0.22 mV and 960 microfarads. After transfection, the cells were grown in 100-mm dishes for 72 h, at which time the culture medium was collected, buffered to a final concentration of 10 mM Tris-HCl (pH 7.3), and centrifuged for 5 min at 600 $\times$ *g* to remove cell debris.

Construction and Expression of P-selectin Mutants—Recombinant DNA techniques and mutagenesis were performed as described (28). Plasmid DNA for transfection or sequencing was purified as per the manufacturer's recommendation using QIAGEN reagents. All DNAs amplified by polymerase chain reaction (PCR) or subjected to site-directed mutagenesis procedures were sequenced by the Sequenase method using reagents supplied by United States Biochemical Corp. The P-selectin cDNA was amplified from human placental RNA using PCR. A P-selectin expression cassette was made by ligating the PCR-amplified P-selectin cDNA to the human vascular cell adhesion molecule (VCAM) signal sequence cDNA at the *Hin*dIII site positioned at the VCAM signal cleavage site using the 5′-oligonucleotide primer GTGGAAGCTTGGACTTATCATTACAGC and the 3′-primer GCTGAGATCTCACAGCTTTA (end of second complement receptor repeat).

The PCR-generated ~920-base pair fragment was ligated into M13.

To aid in protein stabilization, detection, quantitation, and purification, this P-selectin cassette was fused to the hinge region of the mouse IgG_{2A} heavy chain coding sequence to create a soluble, secreted protein expressed from a construct similar to that previously described (10). The mouse IgG_{2A} constant region coding sequence containing one intron was PCR-cloned from cDNA made from total RNA isolated from the hybridoma cell line 402C10 (29) (kindly provided by Dr. R. J. Bjercke) using oligonucleotides GAAAGGTACCAGAGGGCCCACAATC and GAGCAAGCTTACCCGGAGTCCG. The P-selectin-mouse IgG fusion gene was constructed in the mammalian expression vector pJB20, the pCMV plasmid described in Ref. 30 with several restriction sites removed. This construct allowed specific mutations in the P-selectin CRD to be made using site-directed mutagenesis on single-stranded M13 templates. Each mutant was plaque-purified, sequenced, and subsequently cassetted into the fusion gene as an ~700-base pair *Hin*dIII/*Kpn*I fragment. The E-selectin-IgG construct and mutant proteins were made and expressed as described previously (23).

Protein Purification and Cell Binding Assays—Recombinant protein levels in the cell supernatants were monitored by ELISA using alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Calbiochem). To affinity purify the recombinant proteins, 10 μl (4×10^8 beads/ml) of goat anti-mouse IgG-coated Dynabeads (Dynal, Inc.) were added to 10 ml of culture medium harvested from transfected cells and incubated overnight with rocking at 4 °C. The beads were concentrated by centrifugation at 600 × *g* for 5 min, and the culture medium was removed after the tubes were placed on a magnetic separator. The beads were resuspended in 1 ml of PBS (final concentration of 4×10^6 beads/ml) and stored at either 0 or 4 °C.

The amount of recombinant protein recovered on the beads was monitored by ELISA using anti-P-selectin antibody AC1.2 or anti-E-selectin antibody BBA8 (R&D Systems). ELISAs were performed as follows. Beads (8×10^4) were added to duplicate wells of 96-well flexible assay plates that had been previously blocked with PBS supplemented with 3% BSA; equivalent amounts of wild-type E-selectin- or P-selectin-coated beads together with beads prepared from mock-transfected cell lysates were tested at the same time on the same plate as positive and negative controls. Using a magnetic separator to retain the beads inside the wells, the wells were washed once with 50 μl of PBS and then incubated in a solution of 6% formaldehyde in PBS for 20 min at room temperature. This was followed by two sequential PBS washes and a second 20-min incubation in PBS supplemented with 100 mM ammonium chloride. The beads and wells were then blocked overnight in PBS supplemented with 3% BSA and 1% rabbit serum.

When the blocking buffer was removed, three sequential PBS washes were performed, and the primary anti-selectin antibodies were added at final concentrations of 0.2 μg/ml in PBS supplemented with 3% BSA and incubated for a minimum of 2 h at 4 °C. The sample wells were then washed three times with PBS and, in the case of BBA8, incubated with a 1:2000 dilution of alkaline phosphatase-conjugated streptavidin (Calbiochem, catalog No. 189732). For AC1.2, a 1:5000 dilution of alkaline phosphatase-conjugated goat anti-mouse κ light chain (Caltag Laboratories, catalog No. M33008) or, for some experiments, a 1:5000 dilution of alkaline phosphatase-conjugated rabbit anti-mouse IgG_1 (Cappel, catalog No. 59569) was added and incubated in a solution of PBS supplemented with 3% BSA for a minimum of 2 h at 4 °C. The sample wells were then washed two times with PBS and once with alkaline phosphatase ELISA buffer solution (pH 9.4) of 10 mM diethanolamine, 0.5 mM $MgCl_2$, and 10 mM NaCl. The alkaline phosphatase colorimetric assays were typically developed overnight at 4 °C using standard buffers and substrates (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium) (31). The absorbance was monitored with a 415-nm filter in a Bio-Rad model 450 microplate reader.

All of the mutant proteins discussed in this work were readily detectable using these mouse monoclonal antibodies, and the background absorbance generated by the mock control beads was negligible and typically undetectable. Furthermore, the ELISA results (data not shown) indicate that the proteins were synthesized and secreted in similar quantities and that they each have similar stabilities when purified and stored for extended periods of time at 4 °C.

P- and E-selectin-HL-60 cell binding assays were performed in Falcon 96-well flexible assay plates. The wells were first blocked by incubating briefly with PBS supplemented with 3% BSA. After aspirating the blocking buffer, 10 μl of HL-60 cells (10^7 cells/ml of RPMI 1640 medium supplement with 10% FCS) that had been fluorescently labeled with calcein AMC-3099 (Molecular Probes, Inc.) were added to each well, followed by 10 μl of beads (4×10^6/ml). Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. The cells and beads were incubated together at room temperature for 10 min. If sLe^X tetrasaccharide inhibition was being tested, the tetrasaccharide was added immediately prior to this incubation. Following incubation, the assay plate was then placed on a magnetic separator and incubated for an additional 2 min. While the plate remained on the separator, excess unbound HL-60 cells were removed, and the wells were washed twice with PBS to remove any remaining unbound cells. The HL-60 cells remaining bound to the beads were inspected by microscopy and then lysed by adding 50 μl of a 1% solution of Nonidet P-40 in PBS. Binding was quantitated fluorometrically using a Millipore Cytofluor 2350 fluorometer.

Invertase Filter Binding Assay—Invertase was coated onto a nitrocellulose filter, and the binding of P-selectin-IgG proteins conjugated to beads was assessed using a slot-blot apparatus without vacuum using the methods described by others (7) with the following exceptions. P-selectin proteins bound to Dynabeads were incubated in the wells of the slot-blot apparatus (Hoefer Scientific Instruments). Beads (10^6) were incubated in each well in 50 μl of buffer. It was not necessary to use an enzyme-linked second antibody to detect the bound protein, so this step was omitted. The dark brown areas shown in Fig. 3*A* are the actual protein-bound beads retained on the filter after washing. P-selectin mutants were expressed and purified as described (see Fig. 2*A*).

P-selectin Mutant-Oligomannose Binding Assay—Stock solutions containing 2.5 mg/ml (lyophilized weight/volume) yeast invertase, *S. cerevisiae* (yeast), and glycated BSA in 25 mM Tris (pH 7.5), 1 mM 2-mercaptoethanol, 0.5% SDS, and 5% glycerol were boiled for 10 min, cleared by brief centrifugation, chilled to 0 °C, diluted to final concentrations of 25 μg/ml in PBS, and coated onto 96-well flexible assay plates. Plates were subsequently blocked with 3% BSA in PBS. Beads conjugated with various P-selectin-IgG proteins (2×10^4 beads) were added to each well in a 55-μl volume of PBS supplemented with 0.5 mM $MgCl_2$ and 2.5 mM $CaCl_2$ and incubated for 1 h. Wells were then washed sequentially with PBS until unbound beads were removed. The number of beads retained in the wells was determined by ELISA using an alkaline phosphatase-conjugated rabbit anti-goat antibody. The ELISA absorbance readings at 415 nm were curve-fit to serially diluted mock and P-selectin-IgG-coated bead standards, both of which had essentially identical amounts of goat IgG. The binding of the P-selectin A77K mutant to invertase in the presence of increasing concentrations of free mannose or galactose was performed as described above, except that serial dilutions of free mannose or galactose were included during the 1-h incubation.

Glycolipid and Sulfatide Binding Assays—sLe^X-containing glycolipids were prepared from HL-60 cells as described (32). Glycolipid or sulfatide (0.1 ml of a 1 μg/ml stock solution) was coated onto 96-well plates as described (11, 27). Beads conjugated with various P-selectin-IgG proteins (2×10^4 beads) were added to each well in a 55-μl volume of PBS and incubated for 1 h. The plate was then inverted, and unbound beads were blotted away. The plate was then washed once with PBS, and unbound beads were removed by vacuum aspiration. The number of beads retained in the wells was determined by ELISA using an alkaline phosphatase-conjugated rabbit anti-goat antibody. The ELISA absorbance readings at 415 nm were curve-fit to serially diluted mock and P-selectin-IgG-coated bead standards, both of which had essentially identical amounts of goat IgG.

RESULTS

If P-selectin-sLe^X binding is indeed similar to that which we have proposed for E-selectin (23), the P-selectin sLe^X tetrasaccharide-binding site would be defined by a cleft located between loop 3 (NWADNE, residues 75–80)), loop 5 (IKSPS, residues 95–99), and the β_5-strand (WNDE, residues 104–107) as determined from homology to E-selectin and rMBP (23–25). Lys-113 would not be expected to be directly involved in adherence to the sLe^X tetrasaccharide (Fig. 1*B*). Since this did not agree with previous proposals (20, 26, 27), it was important to analyze the precise involvement of this residue by completing a more extensive mutagenic analysis.

To preserve the hydrophilic nature of the side chain, K113E and K113Q mutants together with a K113A control mutant were generated by site-directed mutagenesis. These mutants were analyzed for sLe^X binding, which was assessed using an sLe^X-dependent HL-60 cell assay (see "Materials and Methods" for details) (20, 24, 26, 27). The results in Fig. 2*A*, together with the previously reported inability of the K113R mutant to bind

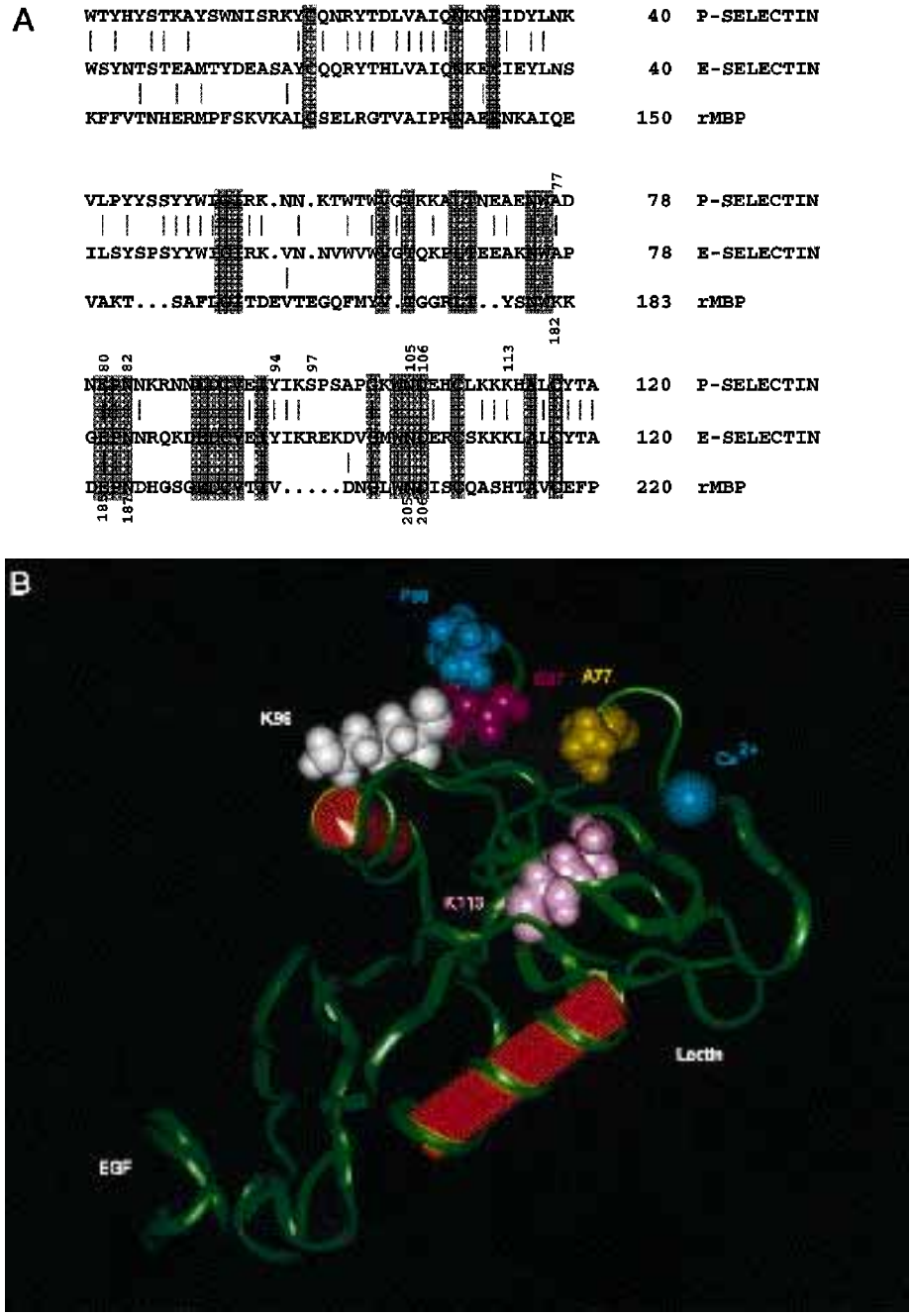

FIG. 1. *A*, structural alignment of the P-selectin lectin domain with E-selectin and the rat mannose-binding protein. Shown is a protein sequence alignment of human P- and E-selectin and rat mannose-binding protein lectin domains as deduced from a comparison of the respective crystal structures (24). Specific residues discussed in the text have been labeled with the appropriate amino acid sequence numbers. *Shaded* residues denote residues conserved among all three proteins. *B*, illustration of the approximate positioning of pertinent P-selectin amino acids. A model of P-selectin was constructed for illustrative purposes only using the three-dimensional crystal structure of E-selectin as a template. The amino acid backbone is shown in *green*, with the α-helices pictured as *red cylinders*. The bound calcium is a *turquoise sphere*. Pertinent amino acid side chains described in the text are illustrated as space-filling spheres as follows: Lys-96 is *white*, Ser-97 is *magenta*, Pro-98 is *turquoise*, Ala-77 is *gold*, and Lys-113 is *pink*.

sLe^X (27), indicate that the side chain length of the amino acid at this position may be critical for sLe^X binding. However, replacement of this lysine residue with an uncharged or oppositely charged amino acid does not alter P-selectin adherence to HL-60 cells, to sLe^X glycolipid, or to sulfatide coated onto polystyrene dishes (Fig. 2, *B* and *C*). Therefore, it appears that the Lys-113 side chain probably does not form a charge-paired electrostatic interaction with either the negatively charged sialic acid carboxylate of sLe^X or the galactose 3-sulfate group of sulfatide as previously hypothesized (27). Instead, it seems likely that the precise positioning of this amino acid is essential for local secondary structure or otherwise affects ligand recognition in some as yet undetermined manner.

As described for Lys-113, the simple substitution of many amino acid residues with alanine or a seemingly closely related amino acid (arginine in the case of Lys-113) does not always yield conclusive data that are easy to interpret. Consequently, we attempted to mutagenically alter P-selectin-ligand binding specificity to that of a different carbohydrate to identify structural or functional features of the protein that are essential for ligand recognition and adherence. Since it seemed very possible that P-selectin might bind sLe^X in the same shallow pocket that is analogous to the rMBP oligosaccharide-binding cleft (25, 33), we attempted to alter P-selectin-ligand binding specificity to oligomannose as described previously for E-selectin (23).

The binding of rMBP to the terminal mannose residue of the *N*-linked oligosaccharide chain is very specific and appears to be coordinated through several interactions with the protein and the bound calcium atom. However, rMBP interactions with other mannose units in the *N*-linked oligosaccharide chain are primarily mediated through water molecules, with the exception of rMBP Lys-182, which appears to directly contact the mannose-6 residue of the oligosaccharide (25).

A major difference between the putative P-selectin binding cleft and that of rMBP is the presence of a five-amino acid insertion loop (residues 95–99) that is present in P-selectin. An

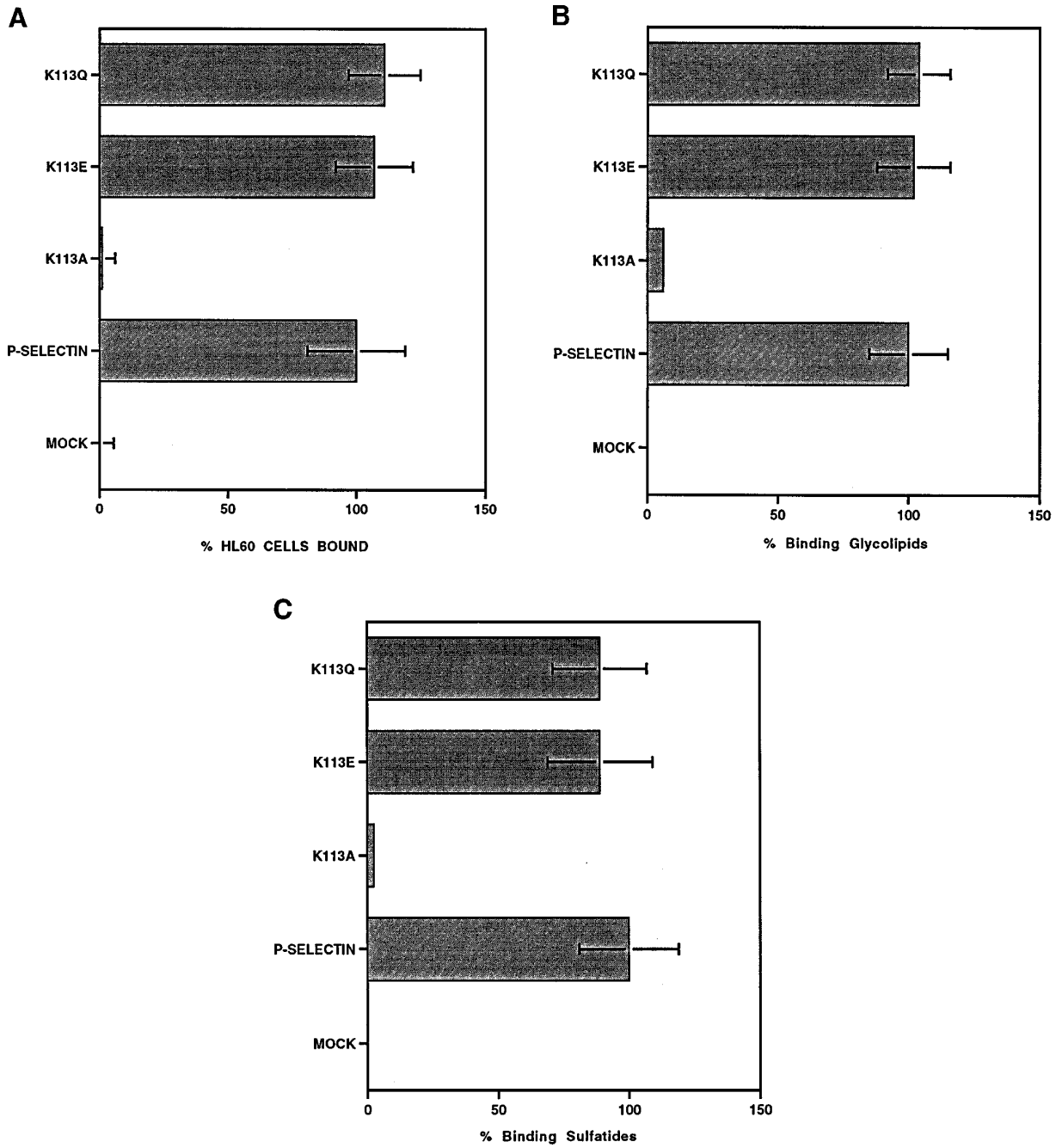

FIG. 2. *A*, lysine substitution mutants are able to bind HL-60 cells. P-selectin mutant proteins were immunoprecipitated using goat anti-mouse IgG-conjugated magnetic beads. After ELISA quantitation to ensure that the same amount of recombinant P-selectin protein was attached to the beads, 4×10^4 P-selectin-IgG-adsorbed beads were mixed with 10^5 fluorescently labeled HL-60 cells and incubated together at room temperature for 10 min. A magnetic separator was used to separate the bead-bound HL-60 cells. Unbound cells were removed by sequential PBS washes. The number of cells bound by the wild-type P-selectin-IgG fusion protein is defined as 100% and that for the mock control wells is designated 0%. All assays were performed in duplicate, and the results shown are the average of two independent experiments. Beads used in the mock experiments were prepared from COS-1 supernatants that had been transfected with vector alone. Binding was quantitated fluorometrically using a Millipore Cytofluor 2350 fluorometer. *B*, shown is P-selectin mutant glycolipid-binding ability. P-selectin-IgG fusion proteins were assayed for adherence to sLe^x glycolipid. Stock solutions containing sLe^x glycolipid were prepared and coated onto 96-well flexible assay plates as described (32). Beads conjugated with various P-selectin-IgG proteins (2×10^4 beads) were added to each well. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate. *C*, P-selectin mutant sulfatide-binding ability. P-selectin-IgG fusion proteins were assessed for the ability to adhere to sulfatide-coated 96-well flexible assay plates. Beads conjugated with various P-selectin mutants (2×10^4 beads) were added to each well. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate.

adjacent residue, P-selectin Tyr-94, is a valine in rMBP (Val-199). Additionally, three amino acids located between residues 75 and 79 (rMBP loop 3) are also not conserved between rMBP and P-selectin. These include rMBP Lys-182, Lys-183, and Asp-184 and P-selectin Ala-77, Asp-78, and Asn-79. One of these unconserved amino acids, Lys-182, is known to coordinate with the 4-hydroxyl of the 6-mannose residue on the Man9α1–2Man6α1–3Man4 branch of the *N*-linked chain (25). It seemed possible that replacing P-selectin Ala-77 with lysine might enable P-selectin to bind oligomannose in a similar manner.

If the P-selectin A77K mutant did bind oligomannose, it

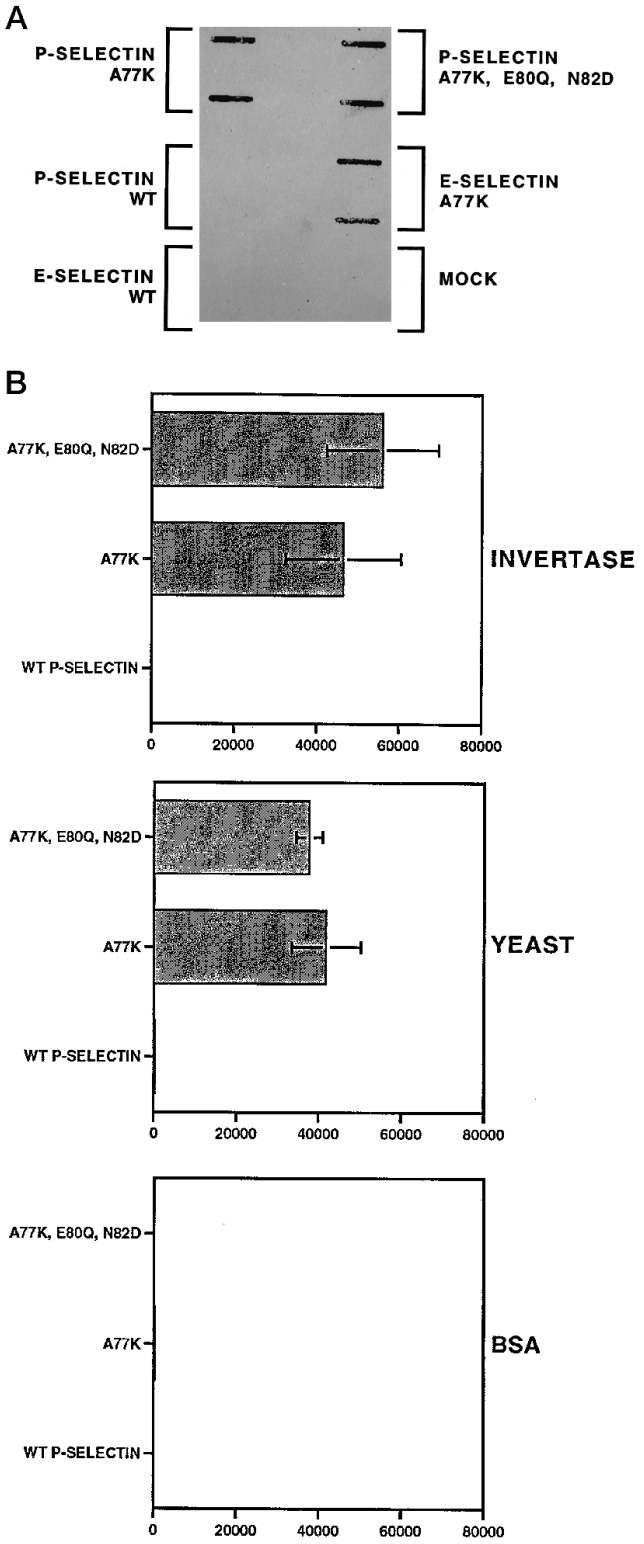

FIG. 3. *A*, invertase filter binding assay. The wild-type P-selectin-IgG fusion protein (unsubstituted) and two P-selectin mutants containing the A77K mutation bound to Dynabeads were assessed for their ability to bind to an invertase-coated nitrocellulose filter. The *dark gray* areas shown are the actual protein-bound beads retained on the filter after washing. It is apparent that each of the P-selectin A77K mutants, like the E-selectin A77K positive control, bind the oligomannose present on yeast invertase and are retained on the filter, whereas the unmutagenized wild-type (*WT*) P- and E-selectins as well as the mock IgG beads are not retained. *B*, assay of P-selectin mutant mannose-binding activity. Stock solutions containing 2.5 mg/ml (lyophilized weight/volume) yeast invertase, *S. cerevisiae* (yeast), and BSA in 25 mM Tris (pH 7.5), 1 mM 2-mercaptoethanol, 0.5% SDS, and 5% glycerol were coated onto 96-well flexible assay plates. Beads conjugated with various P-selectin-IgG proteins (2×10^4 beads) were added to each well. After washing, the number of beads retained in the wells was determined by ELISA. The results shown are the average of two independent experiments run in duplicate.

would provide evidence that the spatial orientation of the putative binding pocket and the calcium-carbohydrate coordination are conserved between rMBP and P-selectin. Additionally, to provide further confirmation that P-selectin A77K mutant binding to oligomannose occurred in the same manner as rMBP-oligomannose binding, a triple mutant with the substitutions A77K, E80Q, and N82D was also generated. The additional mutation of Glu-80 and Asn-82, two of the calcium-coordinating amino acids, was previously shown by Drickamer (34) to increase rMBP binding affinity for galactose and to reduce affinity for mannose. We believed that a similar effect upon P-selectin-saccharide binding would serve to substantiate the proposed carbohydrate binding interactions.

The ability of the P-selectin A77K mutant to bind oligomannose was assessed using an invertase-coated filter binding assay (7). As can be seen by inspection of Fig. 3*A*, the P-selectin A77K mutant and the A77K triple mutant (A77K,E80Q,N82D) bind to the filter, whereas wild-type P-selectin, wild-type E-selectin, and mock control protein do not. The E-selectin A77K mutant was included in the experiment as a positive control (23). The P-selectin A77K mutant proteins can be released from the filter by washing briefly in 5 mM EDTA or by incubating the filter with free mannose monomer. These results indicate that the binding is both Ca^{2+}-dependent and saccharide-specific, respectively.

To confirm that these mutants adhere to oligomannose, both A77K single and triple mutants were tested for binding to denatured yeast protein or denatured invertase coated onto polystyrene dishes (Fig. 3*B*). While none of the P-selectin proteins tested were retained in wells coated with glycated BSA, only the mutants possessing the A77K substitution bound to both denatured yeast protein and invertase. Like the binding to the invertase-coated filter, this adherence could be effectively inhibited by increasing concentrations of free mannose or galactose monomer (Fig. 4, *A* and *B*). The binding of the A77K mutant to oligomannose is inhibited by 20 mM mannose and 125 mM galactose monomer. As illustrated in Fig. 4 (*A* and *B*), the binding of the triple mutant (A77K,E80Q,N82D) is inhibited by much lower concentrations of galactose (25 mM) and higher concentrations of mannose (60 mM). This reversal in binding sensitivity to inhibition by these sugars is consistent with the effect of the original mutations on rMBP binding (6), evidence that supports the interpretation that changing one P-selectin amino acid residue from alanine to lysine allows P-selectin to bind oligomannose in a manner that is probably quite similar to rMBP.

The A77K mutants were analyzed further for their effect on P- and E-selectin binding to sLe^X and sulfatide using the same HL-60 cell and sulfatide assays described previously (see "Materials and Methods" and the legend to Fig. 2 (*A* and *C*)). The results of these assays are shown in Table I. While substitution of Ala-77 with lysine did not appreciably alter sulfatide binding, it did abolish binding to HL-60 cells and to sLe^X-containing glycolipids (data not shown). Taken together with the mannose binding data, these results indicate that the substituting lysine residue is essential for P-selectin-mannose binding, interferes with sialyl $Lewis^X$ binding, and does not substantially affect sulfatide binding.

We had previously hypothesized that the sLe^X sialic acid carboxylate might form a charge-paired interaction with E-selectin Arg-97 (23). Since this residue is not conserved be-

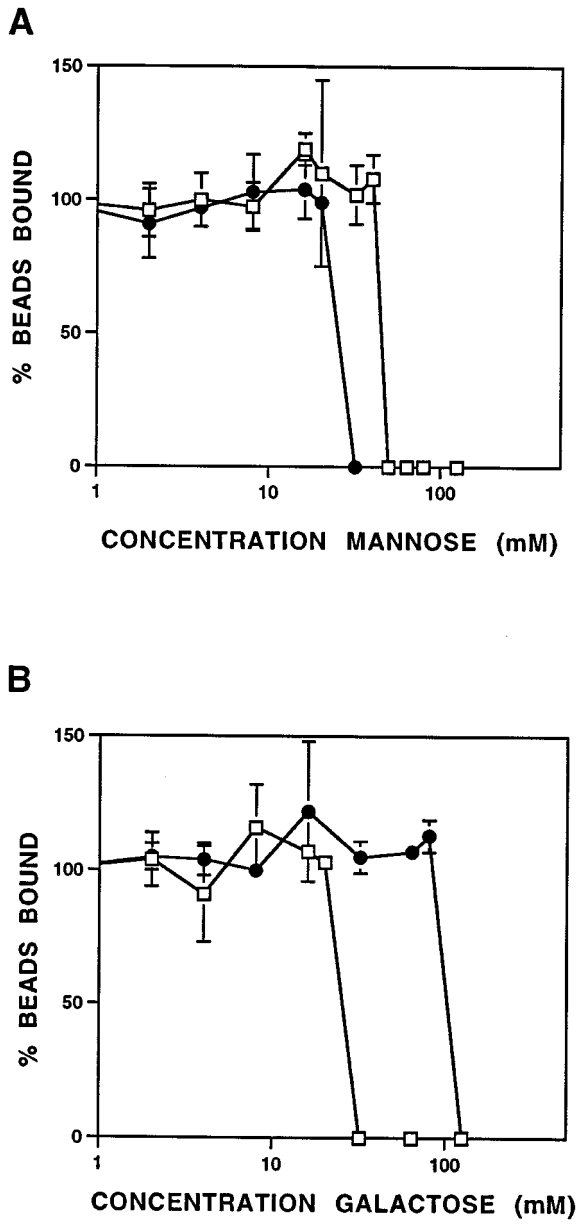

FIG. 4. *A*, effect of free mannose on mutant invertase binding. The binding of the P-selectin A77K (*closed circles*) and A77K,E80Q,N82D (*open squares*) mutants to invertase in the presence of increasing concentrations of free mannose was determined. This assay was performed as described for Fig. 3*B*, except that serial dilutions of free mannose were included during the 1-h P-selectin incubation. The concentrations of free mannose at which half-maximal binding was observed were 25 mM (A77K) and 45 mM (A77K,E80Q,N82D). The results shown are the average of two independent experiments that were completed in duplicate. Percentages were determined by dividing the number of beads bound in the presence of free mannose by the number of beads bound by each mutant in its absence. *B*, effect of free galactose on mutant invertase binding. The binding of the P-selectin A77K (*closed circles*) and A77K,E80Q,N82D (*open squares*) mutants to invertase in the presence of increasing concentrations of free galactose was determined. This assay was performed as described for Fig. 3*B*, except that serial dilutions of free galactose were included during the 1-h P-selectin incubation. The concentrations of free galactose at which half-maximal binding was observed were 100 mM (A77K) and 25 mM (A77K,E80Q,N82D). The results shown are the average of two independent experiments that were completed in duplicate. Percentages were determined by dividing the number of beads bound in the presence of galactose by the number of beads bound in its absence.

tween E- and P-selectin and both proteins are believed to require sialic acid for highest affinity binding (35–37), we also performed a more extensive mutagenic analysis of each of the positively charged amino acids located in this region of both E- and P-selectin. The results of these experiments are detailed in Table I. Substitution of several amino acid residues within the putative sLe^X-binding pocket (amino acid residues 96–99) with amino acids possessing oppositely charged side chains did not significantly influence selectin binding to HL-60 cells, nor did these mutations appear to alter the sLe^X tetrasaccharide $K_{Inhibition}$ of 3 mM. This was also true of a K112E,K113E double-charge substitution mutant.

DISCUSSION

We have attempted to identify the amino acid residues involved in P-selectin-sLe^X binding by altering P-selectin-ligand specificity and by determining the effect of amino acid substitutions on the binding of the protein to its known ligands, sLe^X and sulfatide. In contrast to analyses reported previously by others, the results presented here do not support a hypothesized charge-paired interaction between P-selectin Lys-113 and sulfatide or the sialic acid residue of sLe^X. Indeed, the role of this amino acid in selectin binding appears to be entirely speculative. We present data that support an alternative binding hypothesis, that P-selectin binds sLe^X in a region that is analogous to the binding pocket that is occupied by oligomannose when bound by the closely related rat mannose-binding protein.

The relationship between L-fucose and D-mannose and its implication in selectin binding have been noted (24, 25, 38, 39). The ability of the P-selectin A77K mutant to alter P-selectin binding specificity from sLe^X to oligomannose indicates that the P-selectin calcium-coordinating ions are spatially positioned similarly to E-selectin and rMBP (23–25) and that the P-selectin site is also capable of recognizing mannose. This is further supported by the ability of the P-selectin E80Q and N82D mutations to predictably alter ligand preference from mannose to galactose, which is the same effect that the analogous mutations (E185Q and N187D) had upon rMBP binding (34).

Perhaps the most compelling argument for positioning the P-selectin sLe^X-binding cleft between loop 3 (NWADNE, residues 75–80), loop 5 (IKSPS, residues 95–99), and the β5-strand (WNDE, residues 104–107) as determined from homology to E-selectin and rMBP (23–25) is the loss of sLe^X binding that is generated by the replacement of Ala-77 with lysine. It seems most probable that this loss of binding may be attributed to steric interference generated by the lysine side chain extending into the sLe^X-binding pocket. A computer-generated modeling analysis completed using the E-selectin structural coordinates (24) supports this proposal (23). Although we do not know the precise manner in which P- and E-selectins contact sLe^X, the A77K mutation does disrupt that interaction.

It is pertinent that the P-selectin A77K mutant is able to bind sulfatide, while it has lost the ability to bind sLe^X. It is possible that the sulfatide-binding site is located in the same cleft where we believe the sLe^X-binding site to be located and that since the sulfatide molecule is actually much smaller than sLe^X, the A77K substitution does not sterically interfere with its binding. Alternatively, the sulfatide-binding site may be completely removed from the sLe^X-binding site, and therefore, the position of the Lys-77 side chain would not be critical and would not interfere with P-selectin-sulfatide binding. Although we have not modeled the proposed sulfatide interaction, one would predict from the mutagenesis data that P-selectin binding to free galactose occurs via coordination with the 3′- and 4′-equatorial hydroxyls as has been noted for the mannose-binding protein (25, 34). It is sterically difficult to rationalize this type of interaction with the presence of the 3′-sulfate residue that comprises the sulfatide molecule. Alternatively, if

TABLE I

Shown is the relative adhesion of P- and E-selectin mutants to wild-type selectin-IgG fusion protein controls. All recombinant protein was expressed in COS-1 cells. Binding assays were performed as described under "Materials and Methods." Results are the average of duplicate experiments

Selectin	Mutation	Bound HL60 cells	Bound HL60 cells + 3 mM sLe^X	Bound sulfatide
		%	%	%
P-selectin		100 ± 12	49 ± 14	100 ± 13
P-selectin	A77K	0	ND[a]	112 ± 8.5
P-selectin	A77K,E80Q,N82D	0	ND	ND
P-selectin	K84A	160 ± 15	45 ± 6	111 ± 4
P-selectin	K96Q	102 ± 13	ND	ND
P-selectin	K96R	102 ± 12	ND	ND
P-selectin	K96E	94 ± 28	50 ± 2	ND
P-selectin	S97A	104 ± 9	ND	99 ± 11
P-selectin	S97D	114 ± 18	44 ± 9	ND
P-selectin	S97R	110 ± 9	ND	ND
P-selectin	S97R,P98E,S99K	92 ± 8	42 ± 8	ND
P-selectin	K112E,K113E	111 ± 15	54 ± 15	90 ± 2.5
P-selectin	S99D	100 ± 15	ND	ND
E-selectin		100 ± 12	44 ± 5	0
E-selectin	R97D	111 ± 15	47 ± 6	ND
E-selectin	R97S	107 ± 6	ND	ND
E-selectin	K99E	99 ± 21	43 ± 7	ND

[a] ND, not determined.

the 3′-sulfated galactoceramide is bound identically to the galactose found in sLe^X, one would expect sulfatide to be excluded from the binding pocket just as sLe^X is. Since sulfatide binding by both the P-selectin A77K and A77K,E80Q,N82D mutants is not sensitive to competitive inhibition by free mannose or galactose (data not shown), the preponderance of evidence generated by this study actually supports the proposal that the sulfatide site is in fact removed from the sLe^X-binding site.

To our knowledge, with the exception of the noted report that sulfatide binding is dependent upon Lys-113 and that two anti-P-selectin antibodies are able to block both sLe^X and sulfatide binding (27), the sulfatide-binding site has not been identified. Further mutagenesis work performed in this laboratory as a result of the data described here also indicates that while the sulfatide-binding site can allosterically influence sLe^X binding, there is in fact no overlap between the amino acids involved in binding sLe^X and sulfatide.[2]

We had previously hypothesized that the sLe^X sialic acid carboxylate might form a charge-paired interaction with E-selectin Arg-97 (23). Since this residue is not conserved between E- and P-selectins and both proteins are believed to require sialic acid for highest affinity binding (35–37), we undertook a more extensive mutagenic analysis of each of the positively charged amino acids located in this region of both E- and P-selectins. It is notable that like the Lys-113 mutagenesis results, altering the charge of each of these residues appeared to have no effect on selectin binding to HL-60 cells (Table I). Thus, with the exception of Tyr-94 (20, 23, 24, 40), we have not been able to identify other amino acid residues that may directly contact the bound carbohydrate. This is perhaps not surprising since the interactions between the selectins and their carbohydrate ligands are thought to be of relatively low affinity as is evidenced by the comparably high $K_{Inhibition}$ (3 mM) for the soluble sLe^X tetrasaccharide (Table I). By analogy with rMBP-oligomannose binding, it seems probable that further contacts between the selectins and the bound sLe^X carbohydrate may be mediated through water molecules with the peptide backbone and side chains.

While it is tempting to generalize and simply ascribe the physical characteristics of one selectin to each of the other members of this protein family, this is not necessarily true. In this regard, like the oligomannose binding that we have measured for both E- and P-selectin A77K mutants, sLe^X recognition and binding could be maintained by both proteins without strict conservation of the amino acid side chains that line the saccharide-binding pocket. Indirect interactions between the protein and carbohydrate, mediated by water molecules, might well generate the diversity in ligand recognition that has already been noted. It seems probable that only ligand-bound three-dimensional structural determinations will be able to confirm these possibilities.

In summary, we have attempted to identify the P-selectin-sialyl Lewis^X binding interactions by altering ligand binding specificity. The results reported here are consistent with those previously reported for E-selectin (23). However, they are novel with regard to previously proposed models of P-selectin-carbohydrate interactions. Additionally, the mutagenesis data that we have reported for P-selectin Lys-113 indicate that this residue may not be directly involved in either sLe^X or sulfatide binding. We propose that the P-selectin sLe^X-binding pocket may be partially defined by loop 3 (NWADNE, residues 75–80) on one side, on a second side by loop 5 (IKSPS, residues 95–99), and on a third side by the β5-strand (WNDE, residues 104–107). It is hoped that this information will aid in the rational design of pharmacologically active and therapeutically effective selectin inhibitors.

Acknowledgments—We thank Peter Vanderslice, Ajay Rege, Richard Dixon, and Bill Weis for helpful discussions and critical reading of this manuscript.

REFERENCES

1. Hattori, R., Hamilton, K. K., Fugate, R. D., McEver, R. P., and Sims, P. J. (1989) *J. Biol. Chem.* **264,** 7768–7771
2. Lorant, D. E., Patel, K. D., McIntyre, T. M., McEver, R. P., Prescott, S. M., and Zimmerman, G. A. (1991) *J. Cell Biol.* **115,** 223–234
3. Johnston, G. I., Cook, R. G., and McEver, R. P. (1989) *Cell* **56,** 1033–1044
4. Drickamer, K. (1988) *J. Biol. Chem.* **263,** 9557–9560
5. Bezouska, K., Crichlow, G. V., Rose, J. M., Taylor, M. E., and Drickamer, K. (1991) *J. Biol. Chem.* **266,** 11604–11609
6. Drickamer, K. (1993) *Curr. Opin. Struct. Biol.* **3,** 393–400
7. Quesenberry, M. S., and Drickamer, K. (1992) *J. Biol. Chem.* **267,** 10831–10841
8. Lowe, J. B., Stoolman, L. M., Nair, R. P., Larsen, R. D., Berhend, T. L., and Marks, R. M. (1990) *Cell* **63,** 475–484
9. Phillips, M. L., Nudelman, E., Gaeta, F. C. A., Perez, M., Singhal, A. K., Hakomori, S.-I., and Paulson, J. C. (1990) *Science* **250,** 1130–1135
10. Walz, G., Aruffo, A., Kolanus, W., Bevilacqua, M., and Seed, B. (1990) *Science* **250,** 1132–1135
11. Foxall, C., Watson, S. R., Dowbenko, D., Fennie, C., Lasky, L. A., Kiso, M., Hasegawa, A., Asa, D., and Brandley, B. K. (1992) *J. Cell Biol.* **117,** 895–902

[2] B. M. Revelle, D. Scott, and P. J. Beck, manuscript in preparation.

12. Berg, E. L., Magnani, J., Warnock, R. A., Robinson, M. K., and Butcher, E. C. (1992) *Biochem. Biophys. Res. Commun.* **184,** 1048–1055
13. Green, P. J., Tamatani, T., Watanabe, T., Miyasaka, M., Hasegawa, A., Kiso, M., Yuen, C.-T., Stoll, M. S., and Feizi, T. (1992) *Biochem. Biophys. Res. Commun.* **188,** 244–251
14. Nelson, R. M., Cecconi, O., Roberts, W. G., Aruffo, A., Linhardt, R. J., and Bevilacqua, M. P. (1993) *Blood* **82,** 3253–3258
15. Cecconi, O., Nelson, R. M., Roberts, W. G., Hanasaki, K., Mannori, G., Schultz, C., Ulich, T. R., Aruffo, A., and Bevilacqua, M. P. (1994) *J. Biol. Chem.* **269,** 15060–15066
16. Kunzendorf, U., Kruger-Krasagakes, S., Notter, M., Hock, H., Walz, G., and Diamantstein, T. (1994) *Cancer Res.* **54,** 1109–1112
17. Rossiter, H., Van Reijsen, F., Mudde, G. C., Kalthoff, F., Bruijnzeel-Koomen, C. A. F. M., Picker, L. J., and Kupper, T. S. (1994) *Eur. J. Immunol.* **24,** 205–210
18. Varki, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7390–7397
19. Aruffo, A., Kolanus, W., Walz, G., Fredman, P., and Seed, B. (1991) *Cell* **67,** 35–44
20. Erbe, D. V., Watson, S. R., Presta, L. G., Wolitzky, B. A., Foxall, C., Brandley, B. K., and Lasky, L. A. (1993) *J. Cell Biol.* **120,** 1227–1235
21. Rosen, S. D., and Bertozzi, C. R. (1994) *Curr. Opin. Cell Biol.* **6,** 663–673
22. McEver, R. P., Moore, K. L., and Cummings, R. D. (1995) *J. Biol. Chem.* **270,** 11025–11028
23. Kogan, T. P., Revelle, B. M., Tapp, S., Scott, D., and Beck, P. J. (1995) *J. Biol. Chem.* **270,** 14047–14055
24. Graves, B. J., Crowther, R. L., Chandran, C., Rumberger, J. M., Li, S., Huang, K.-S., Presky, D. H., Familletti, P. C., Wolitzky, B. A., and Burns, D. K. (1994) *Nature* **367,** 532–538
25. Weis, W. I., Drickamer, K., and Hendrickson, W. A. (1992) *Nature* **360,** 127–134
26. Hollenbaugh, D., Bajorath, J., Stenkamp, R., and Aruffo, A. (1993) *Biochemistry* **32,** 2960–2966
27. Bajorath, J., Hollenbaugh, D., King, G., Harte, J., W., Eustice, D. C., Darveau, R. P., and Aruffo, A. (1994) *Biochemistry* **33,** 1332–1339
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, and references therein
29. Bjercke, R. J., Cook, G., Rychlik, N., Gjika, H. B., Van Vunakis, H., and Langone, J. J. (1986) *J. Immunol. Methods* **90,** 203–213
30. Beck, P. J., Orlean, P., Albright, C., Robbins, P. W., Gething, M. J., and Sambrook, J. F. (1990) *Mol. Cell. Biol.* **10,** 4612–4622
31. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
32. Tiemeyer, M., Swiedler, S. J., Ishihara, M., Moreland, M., Schweingruber, H., Hirtzer, P., and Brandley, B. K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1138–1142
33. Weis, W. I., Kahn, R., Fourme, R., Drickamer, K., and Hendrickson, W. A. (1991) *Science* **254,** 1608–1615
34. Drickamer, K. (1992) *Nature* **360,** 183–186
35. Corral, L., Singer, M. S., Macher, B. A., and Rosen, S. D. (1990) *Biochem. Biophys. Res. Commun.* **172,** 1349–1356
36. Moore, K. L., Varki, A., and McEver, R. P. (1991) *J. Cell Biol.* **112,** 491–499
37. Polley, M. J., Phillips, M. L., Wayner, E., Nudelman, E., Singhal, A. K., Hakomori, S.-I., and Paulson, J. C. (88) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6224–6228
38. Mills, A. (1993) *FEBS Lett.* **319,** 5–11
39. Weis, W. I. (1994) *Structure* **2,** 147–150
40. Erbe, D. V., Wolitzky, B. A., Presta, L. G., Norton, C. R., Ramos, R. J., Burns, D. K., Rumberger, J. M., Rao, B. N. N., Foxall, C., Brandley, B. K., and Lasky, L. A. (1992) *J. Cell Biol.* **119,** 215–227