CD62/P-Selectin Binding Sites for Myeloid Cells and Sulfatides Are Overlapping

Jürgen Bajorath,* Diane Hollenbaugh, Gordon King, William Harte, Jr.,‡ David C. Eustice,‡ Richard P. Darveau, and Alejandro Aruffo

Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, Washington 98121, and Bristol-Myers Squibb, 5 Research Parkway, Wallingford, Connecticut 06492

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ABSTRACT: P-Selectin (CD62/GMP140/PADGEM) is an inducible cell-surface glycoprotein expressed by endothelial cells and platelets following stimulation by inflammatory mediators such as thrombin, histamine, or peroxides. P-Selectin mediates the binding of leukocytes to activated vascular endothelium at sites of inflammation and plays a role in mediating the binding of activated platelets to leukocytes and the vascular cell wall. The adhesive function of P-selectin is mediated by its calcium-dependent (or C-type) lectin domain, which is known to bind to carbohydrate ligands including fucosyl-*N*-acetyllactosamine (Le^x, CD15), sialyl-Le^x, and 3-sulfated galactosylceramides (sulfatides). Sulfatides can efficiently block P-selectin/myeloid cell binding *in vitro* and are excreted at high levels by activated granulocytes. These observations led to the hypothesis that sulfatide may play a role in facilitating the disengagement of CD62, allowing the efficient exit of granulocytes from the blood stream at sites of inflammation. In this report, we extend our previous mutagenesis analysis of the P-selectin binding site [Hollenbaugh, D., Bajorath, J., Stenkamp, R., & Aruffo, A. (1993) *Biochemistry 32,* 2960] and show that replacement of Tyr48 with Ser or Lys113 with Arg results in P-selectin mutants that, although correctly folded, do not bind to HL60 cells. These results suggest that the conservation of charged and hydrogen-bonding side chains is not sufficient to maintain the P-selectin function and that the exact stereochemistry provided by the side chains of residues lining the P-selectin binding pocket is critical for P-selectin binding. We also describe the preparation of the P-selectin mutant Arg85Ala and show that Arg85 contributes to the P-selectin binding site. In addition, using computer modeling techniques, site-specific mutagenesis, and ligand and cell binding assays, we show here that P-selectin binds to both myeloid cells and sulfatides *via* an overlapping but not identical set of residues located in the region of P-selectin proximal to a functional calcium binding site. These results provide a molecular rationale for the displacement of the leukocyte carbohydrate ligands of P-selectin by sulfatide.

P-Selectin (CD62, GMP140, PADGEM) is a membrane glycoprotein of ~140 kDa expressed by activated platelets and vascular endothelial cells. In resting platelets and vascular endothelial cells, P-selectin is sequestered in α-granules and Weibel–Palade bodies, respectively (Johnston et al., 1989; Larsen et al., 1989). Following activation, P-selectin is rapidly mobilized from these intracellular stores to the cell surface (Patel et al., 1991; Sugama et al., 1992), where it mediates the initial binding interactions of activated platelets with leukocytes and the vascular wall and the interactions of leukocytes with activated vascular endothelial cells (Larsen et al., 1989; Geng et al., 1990). P-Selectin is a member of a family of adhesion molecules that includes E-selectin (ELAM-1), which is expressed by activated vascular endothelial cells, and L-selectin (Leu8, LAM-1, LECAM), which is expressed by leukocytes [for reviews, see Bevilacqua (1993), Springer (1990), Lasky (1992), and Bevilacqua and Nelson (1993)]. These proteins are type I membrane proteins and are composed of an amino-terminal lectin domain followed by an epidermal growth factor (EGF) like domain, a variable number of complement receptor related repeats (CR), a hydrophobic membrane spanning region, and a cytoplasmic domain. As indicated by their high sequence homology, these proteins are not only structurally but also functionally related, modulating the trafficking of peripheral blood leukocytes by permitting adhesive interactions between leukocytes and endothelial cells. These binding interactions are mediated predominantly by contacts between the lectin domain of the selectin and various carbohydrate ligands.

Although it is now widely accepted that a lectin domain/carbohydrate interaction is primarily responsible for mediating P-selectin/myeloid cell binding (Lasky, 1992; Bevilacqua, 1993; Bevilacqua & Nelson, 1993), the exact molecular nature of the P-selectin ligand is not known. The binding of P-selectin to myeloid cells is Ca^{2+}-dependent (Larsen et al., 1989; Geng et al., 1990, 1991; Moore et al., 1991), as well as neuraminidase- (Corral et al., 1990; Moore et al., 1991) and protease-sensitive (Moore et al., 1991). The binding of P-selectin to myeloid cell lines can be inhibited by growing the cells in the presence of sodium selenate, an inhibitor of sulfation (Aruffo et al., 1991). P-Selectin has been shown to bind to the carbohydrate Le^x (CD15) (Larsen et al., 1990) and its sialylated form, sialyl-Le^x (sLe^x)[1] (Zhou et al., 1991; Polley et al., 1991; Foxall et al., 1992), and there is evidence that these carbohydrates and/or others like them are presented to P-selectin by a discrete number of cell-surface proteins, including L-selectin (Picker et al., 1991) and a recently characterized protein of ~250 kDa (Moore et al., 1992; Norgard et al., 1993). In addition, P-selectin binds to sulfatides

* Address correspondence to this author at Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, WA 98121. Fax: (206) 727-3604. Telephone: (206) 727-3612.

‡ Bristol-Myers Squibb, Wallingford, CT.

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[1] Abbreviations: C-type lectin, calcium-dependent lectin; FC, flow cytometry; Le^x, Lewis X; mAb, monoclonal antibody; P-selectin–Rg, P-selectin–immunoglobulin fusion protein; RMS, root mean square; sLe^x, sialylated Lewis X.

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(Aruffo et al., 1991). Although the physiological relevance of this interaction remains to be elucidated, it is known that myeloid cells can excrete large quantities of sulfatides on activation. This suggests that sulfatides might participate in leukocyte extravasation at sites of inflammation by displacing the adhesion-mediating leukocyte surface ligand(s).

The carbohydrate recognition domain of the mannose binding protein from rat was the first C-type lectin domain whose three-dimensional structure was elucidated crystallographically (Weis et al., 1991). The structure revealed that the C-type lectin domains display a previously undescribed protein fold characterized by a relatively high content of nonclassical secondary structure. We have previously reported the generation and analysis of a three-dimensional model of the P-selectin C-type lectin domain (Hollenbaugh et al., 1993). The P-selectin model was constructed on the basis of the proposed structural similarity of the selectins with the crystal structure of the C-type lectin domain of rat mannose binding protein (Weis et al., 1991), as suggested by the conservation of key residues in the core regions and one of the calcium binding sites of the mannose binding protein (Weis et al., 1991; Hollenbaugh et al., 1993). On the basis of the same hypothesis, model structures for E-selectin (Erbe et al., 1992) and P-selectin (Erbe et al., 1993) were also generated by others. Our P-selectin model structure revealed the presence of a shallow groove proximal to the conserved Ca^{2+} position, which we considered a potential ligand binding site.

Site-directed mutagenesis of amino acids in this region allowed the identification of a number of residues that are critical to P-selectin/myeloid cell binding (Hollenbaugh et al., 1993). The corresponding region in E-selectin, and later the same region in P-selectin, was identified independently by Erbe et al. (1992, 1993) as containing ligand binding sites. Although these early studies established the region of the selectin lectin domain and individual residues within this region that are critical for selectin binding, additional experiments are required to better define the contribution of these residues to the interaction between the selectins and their ligands. Previous work established that the residues Tyr48, Tyr94, and Lys113 play a particularly significant role in the binding of P-selectin to HL60 cells (Hollenbaugh et al., 1993; Erbe et al., 1993) and in the binding of E-selectin to immobilized sLe^x glycolipid (Erbe et al., 1992). These reports also showed that the replacement of either of these two tyrosine residues with phenylalanine was sufficient to abolish the binding of P-selectin to HL60 cells, suggesting the importance of a single hydrogen donor/acceptor functionality for the interaction between P-selectin and HL60 cells.

We now report on the results of experiments designed to establish whether a hydrogen-bonding functionality alone at position 48 (Tyr48→Ser) and a conserved positive charge at position 113 (Lys 113→Arg) would still allow binding and extend our mutagenesis analysis to another residue in the P-selectin binding site region and assess its contribution to the binding to HL60 cells. Secondly, we developed a hypothesis using computer modeling that the ligand on the HL60 cells and a sulfatide (galactosyl-β1-ceramide 3-sulfate) bind to an overlapping, but not identical, set of residues in the P-selectin binding site and tested this hypothesis using our panel of P-selectin mutants.

MATERIALS AND METHODS

Site-Specific Mutagenesis. P-Selectin–Rg is a fusion protein consisting of the lectin domain, the EGF domain, and two complement receptor related domains of P-selectin fused to a modified human Fc domain described previously (Aruffo et al., 1991; Hollenbaugh et al., 1993). Mutants were generated using *Pfu* polymerase in an overlap extension PCR protocol as previously described (Hollenbaugh et al., 1993; Ho et al., 1989). The sequence of each mutant was verified by DNA sequencing. Fusion proteins were produced by transient transfection of COS cells and purified from COS cell supernatants by protein A–Sepharose affinity chromatography, as described (Aruffo et al., 1991). The immunoreactivity of the mutants with five different monoclonal antibodies (mAb) against P-selectin was assayed by ELISA as described previously. The mAb used were AC1.2 (Becton Dickinson, Mountain View, CA), CLB-throm/6 (Biodesign, Kennebunkport, ME), 1G4, 3D3, and 2A2.

Model Building. Computer graphics manipulations and energy minimization calculations were carried out using the InsightII/Discover program suite (1993, Biosym Technologies, Inc., San Diego) on a SGI Crimson VGXB workstation. A minimum-energy conformation was generated for sLe^x [Neu-Acα(2–3)Galβ(1–3)(Fucα(1–4)GlcNAc)] on the basis of the solution structure of Le^x (Miller et al., 1992). The sulfatide (galactosyl-β1-ceramide 3-sulfate) and sLe^x ligands were constructed using the Builder module of Insight and energy-optimized using Discover. Starting from our previously derived model structure of the P-selectin lectin domain, plausible hypothetical complexes of P-selectin and sLe^x were generated interactively using Insight's Docking module and refined by constrained conjugate gradient energy minimization with explicit hydrogen atom representation. These conformational refinement calculations were carried out with harmonic constraints of 20 kcal/Å^2 on the protein backbone atoms. All non-hydrogen atoms of the conserved calcium coordination sphere were held fixed. All atoms of the protein side chains and of the entire ligand were free to move during the calculations. A cutoff distance of 14 Å was used for nonbonded interactions, and a distance-dependent constant of $4r$ was used to account for some solvation effects. Energy minimization was continued until the RMS derivative of the energy function was less than 0.2 kcal/mol. A molecular mimic of sLe^x and sulfatide was generated by superposition of the common galactose moiety in these compounds followed by analogous energy refinement. No defined conformation was generated for the long aliphatic methylene chains of the ceramide moiety in sulfatide due to their great intrinsic flexibility, and no speculations were made with regard to the eventual interactions of this moiety with the protein.

Adhesion Assays. To assess the cell binding ability of P-selectin mutants, previously described adhesion assays were used (Hollenbaugh et al., 1993). Briefly, P-selectin–immunoglobulin fusion proteins (P-selectin–Rg) were prepared with the respective P-selectin mutations. The ability of these mutants to bind to HL60 cells was tested in two assays. In the first assay the binding of the mutant P-selectin–Rg to HL60 cells was measured by indirect immunofluorescence using flow cytometry (FC). In the second assay, the ability of the mutant P-selectin–Rg to mediate cell adhesion was examined by measuring the adhesion of HL60 cells to P-selectin immobilized on anti-Rg-coated plastic. This two-assay system provides for a "low"-avidity and a "high"-avidity binding system. Examination of the ability of the P-selectin mutants to bind HL60 cells in these two assays provides for the detection of P-selectin mutants whose abilities to bind to HL60 cells have been reduced but not completely abrogated.

The adhesion assay was used to test the ability of anti-P-selectin mAb to block the binding of P-selectin to HL60 cells. For the assay, P-selectin–Rg (0.12 μg/mL) was first mixed 1:1 with hybridoma supernatants containing the mAb to be

tested and then added to COSTAR 96-well plates that were coated with goat anti-human Fc antibody (Cappell, Durham, NC). Following a 2-h incubation and subsequent wash, labeled HL60 cells were added to the wells and allowed to bind for 30 min. Unbound cells were removed by washing, and binding was measured as previously described (Hollenbaugh et al., 1993).

Sulfatide Binding Assay. The sLe^x glycolipid was not available for experimental ligand binding studies. The binding of the wild-type and mutant P-selectin-Rg's to sulfatide was measured in an ELISA-based assay modified from Foxall et al. (1992). Sulfatide or lysosulfatide (0.1 mL; Sigma, St. Louis, MO), each at 1 μg/mL in MeOH, were added to the wells of a 96-well ELISA plate (Falcon ProBind, Becton Dickinson, Mountain View, CA) and allowed to dry overnight at room temperature. The next day, the antigen-coated plates were blocked for 1.5 h at room temperature with 5% BSA (ICN, Costa Mesa, CA) in buffer containing 20 mM Hepes and 0.15 M NaCl (pH 8.0). Wild-type P-selectin and mutants thereof were first mixed with HRP-conjugated goat anti-human IgG (Fisher Scientific, Pittsburgh, PA), serially diluted, and then incubated for 30 min at 37 °C in buffer containing 20 mM Hepes, 0.15 M NaCl, 1% BSA, and 0.8 mM $CaCl_2$ (pH 8.0). Following the 30-min preincubation, the fusion protein–HRP conjugate immunocomplexes were incubated on the blocked antigen-coated plates for 45 min at 37 °C and then washed to remove any unbound proteins. Bound complexes were detected by the addition of substrate buffer (95 mM $NaOAc{\cdot}3H_2O$, 5 mM citric acid, and 1.4 mM urea/H_2O_2) containing 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma). Reactions were stopped by the addition of 3 N sulfuric acid, and the absorbance was read on an ELISA reader at dual wavelengths: 450 and 630 nm. To test the role of Ca^{2+} in this binding assay, P-selectin–Rg binding to immobilized sulfatide was carried out in the presence of 10 mM EDTA.

To measure the ability of the anti-P-selectin antibodies to block the binding of P-selectin to sulfatide, the same assay described above was used with the exception that serial dilutions of the purified mAb were added to the mixture prior to the 30-min preincubation step, with the P-selectin–Rg at a final concentration of 100 ng/mL and HRP-conjugated goat anti-human Fc at a final dilution of 1:10 000. The antibody–fusion protein–HRP conjugate immunocomplexes were then added to antigen-coated plates and developed as above.

RESULTS

Requirement of Hydrogen-Bonding Capacity and Positive Charge in P-Selectin for HL60 Binding. We had previously demonstrated the importance of both Tyr48 and Tyr94 in stabilizing the P-selectin/HL60 interaction (Hollenbaugh et al., 1993). It was found that the substitution of Tyr48 by Phe alone was sufficient to abolish P-selectin binding to HL60 cells, suggesting the crucial role of a single hydrogen-bonding functionality for binding. We have now replaced Tyr48 with Ser and examined the effect of this substitution on P-selectin binding to HL60 cells. As shown in Figure 1 (A and B), the Tyr48Ser P-selectin mutant was unable to bind to HL60 cells in our adhesion assays. Likewise, we and others (Hollenbaugh et al., 1993; Erbe et al., 1993) had previously shown that Lys113 played a critical role in P-selectin binding to HL60 cells and immobilized sLe^x (Erbe et al., 1993). We examined whether the replacement of Lys113 with an Arg residue would affect P-selectin binding to HL60 cells. Figure 1 (A and B) shows that the Lys113Arg P-selectin mutant was also unable to bind to HL60 cells. Both Tyr48Ser and Lys113Arg mutants were folded correctly as monitored by binding of the mutant

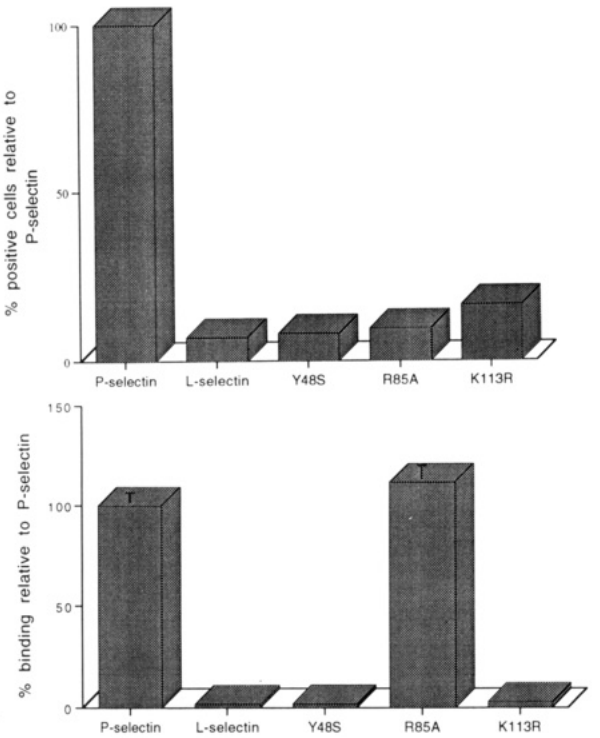

FIGURE 1: P-Selectin mutants binding to HL60 cells. Binding of wild-type (WT) and mutant P-selectin–Rg to HL60 cells as measured by flow cytometry or adhesion. (A, top) Percent positive cells stained with mutant P-selectin–Ig relative to WT in a flow cytometry assay as described in the Materials and Methods section. Values are representative of two independent experiments. (B, bottom) Percent binding of HL60 cells to immobilized mutant P-selectin–Rg relative to WT. The adhesion of HL60 cells to immobilized P-selectin–Rg is described in the Materials and Methods section. Values are the mean of three points.

protein to five anti-P-selectin monoclonal antibodies (data not shown). These results suggest that the exact stereochemistry of hydrogen bonding and the ionic interactions provided by the side chains of the residues lining the P-selectin binding pocket are critical for the binding of P-selectin to its cellular ligand. The fact that both Tyr48 and Lys113 are strictly conserved in all three selectins suggests that similar amino acid substitutions would also affect the function of E- and L-selectins.

Further Analysis of the P-Selectin Ligand Binding Site. We investigated the role of an additional residue in the P-selectin binding site region. Residue Arg85 is located on the side opposite from Tyr48 and Tyr94 (Figure 2) and is the only residue of a "belt" of positively charged residues, consisting of Arg85, His108, Lys111, and Lys113, that we have not previously subjected to mutagenesis. We, therefore, changed Arg85 to Ala. The Arg85Ala mutant bound very poorly to HL60 cells in the FC assays (Figure 1A), but was able to support HL60 binding in the cell adhesion assay (Figure 1B). These data suggest that this residue influences the adhesion interaction between P-selectin and its cellular ligand, but is not crucial to mediate this interaction. The Arg85Ala mutant bound to all of the anti-P-selectin mAb tested (data not shown).

Computer Models of Ligands in the P-Selectin Binding Site. Since sLe^x was shown to bind to the same site in P-selectin (Erbe et al., 1993) as the cellular ligand (Hollenbaugh et al., 1993; Erbe et al., 1993) we have, *via* computer modeling, addressed the question of how the sLe^x carbohydrate may bind to P-selectin. This is relevant since it is now generally accepted that P-selectin binds to sLe^x or sLe^x-like structures on target cells (Hughes, 1992; Lasky, 1992). It is strongly felt by us, as has been proposed by others (Bundle & Young,

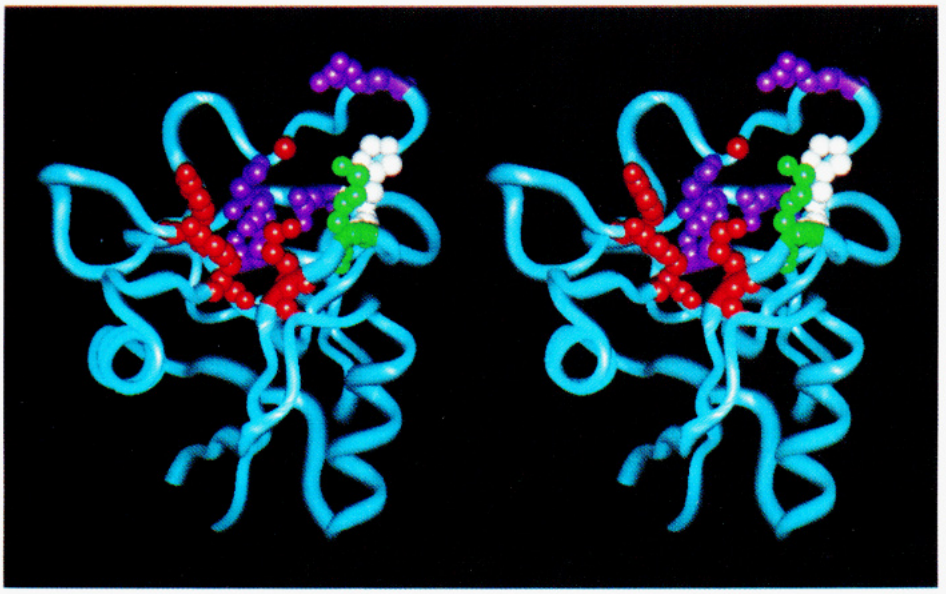

FIGURE 2: Stereo representation of the P-selectin binding site for myeloid cells. A three-dimensional model of the carbohydrate recognition domain of P-selectin is shown in solid ribbon representation. The view is along helix $\alpha 2$ (on the left). Residues in the binding site region of P-selectin for HL60 cells are depicted in a space-filling representation and categorized (color-coded) according to their importance for binding: red, mutation of these residues abolishes binding; purple, mutation of these residues significantly reduces binding (50% of wild-type binding or less) but does not abolish binding; green, mutation of these residues only slightly reduces binding; white, changes in these residues have no detectable effect on binding. The red and purple residues in the vicinity of the conserved calcium (which is shown as a red "ball" since the binding of P-selectin to HL60 cells is calcium-dependent) form the binding site for the cellular ligand of P-selectin. The following residues are shown: green, Lys111; white, His 108; red (in counterclockwise direction from the top), Tyr94, Tyr48, and Lys113; purple (in counterclockwise direction from the top), Arg85, Asn105, Glu92, and Glu107.

1992), that protein–carbohydrate complexes are not precisely predictable at the atomic level of detail. We have, therefore, only aimed at a rationalization of the gross features of carbohydrate binding to P-selectin. Using these more schematic models, we hoped to recognize the potential role of certain residues in carbohydrate binding to P-selectin.

The crystal structure of the rat mannose binding protein in a complex with high mannose (Weis et al., 1992) showed that D-mannose is directly involved in the binding of the conserved calcium in the mannose binding protein. Essentially, the equatorial C3- and C4-hydroxyl groups in D-mannose replace a water molecule, which acts as a calcium ligand. The fact that the residues forming this calcium binding site are strictly conserved in the selectins, and furthermore that L-fucose with the equatorial C2- and C3-hydroxyls is able to mimic the observed binding mode of D-mannose, has suggested that L-fucose may bind to the selectins in a manner similar to the binding of D-mannose to the mannose binding protein (Weis et al., 1992). Starting from this interaction as a "molecular anchor point", we have studied possible P-selectin–sLe^x interactions using computer modeling. Figure 3 shows a P-selectin/sLe^x model complex. In the proposed complex, a prominent second "anchor point" for P-selectin–sLe^x interactions is an ionic interaction between the carboxyl moiety of sialic acid and residue Lys113. This residue was found to be crucial for ligand binding in all previous studies. Using these interactions between P-selectin and sLe^x as the primary contacts or "anchor points", it is possible to dock sLe^x, in a conformation analogous to the solution structure of Le^x (Miller et al., 1992), into the shallow depression in the vicinity of the conserved calcium binding site in P-selectin. The two putative anchor points are separated by approximately 10 Å. We suggest that the spatially distant sialic acid carboxyl and the fucose are involved in key interactions of sLe^x binding to P-selectin, consistent with previous mutagenesis experiments (Hollenbaugh et al., 1993; Erbe et al., 1993).

On the basis of the postulated orientation of sLe^x in the P-selectin binding site, we next sought to address the binding of sulfatides to P-selectin. The ability of P-selectin to recognize sulfatide compounds has been described (Aruffo et al., 1991; Yuen et al., 1992; Erbe et al., 1993). The galactose moieties in sLe^x and sulfatide that are common to both ligands were superimposed in the molecular model. This superposition, shown in Figure 3, is not intended to suggest that the binding of the galactose moieties is identical in both ligands, but to assess potential contacts between sulfatide and P-selectin. In this superposition, the galactose 3-sulfate moiety in the sulfatide spatially corresponds to the galactosylsialyl moiety in sLe^x. This allows the negatively charged carboxyl group in sLe^x and the negatively charged sulfate group in sulfatide to bind to the same subsite in P-selectin. The sulfate group in sulfatide, which is larger and more negatively charged than the carboxyl group in sLe^x, would be more suitable to form simultaneous electrostatic interactions with both positively charged residues, Lys113 and Lys111, in P-selectin (whose side chains are 5–6 Å apart). These two residues differ in that Lys113 was found to be critical for the binding of P-selectin to HL60 cells, while Lys111 was not. The molecular models do not suggest a specific importance of Tyr48 and Tyr94 for the binding of P-selectin to sulfatide or sLe^x.

Binding of P-Selectin Mutants to Sulfatide. On the basis of the developed molecular model of sulfatide binding to P-selectin, we first assessed the binding of the Lys111Ala, Lys113Ala, and Lys113Arg mutants to sulfatide (Figure 4A). The studies with the P-selectin mutants binding to sulfatide immobilized on plastic plates showed that Lys111 and Lys113 are critical for P-selectin binding to sulfatide, since changing either one of these residues to Ala or Lys113 to Arg abolished binding. We then examined the ability of the Tyr48Ala, Tyr48Phe, Tyr48Ser, Tyr94Ala, and Tyr94Phe P-selectin mutants to bind to immobilized sulfatides. These five selectin mutants all bound to sulfatide (Figure 4B and data not shown). In contrast to the interactions of P-selectin with its cellular ligand, residues Tyr48 and Tyr94 are not critical for sulfatide binding.

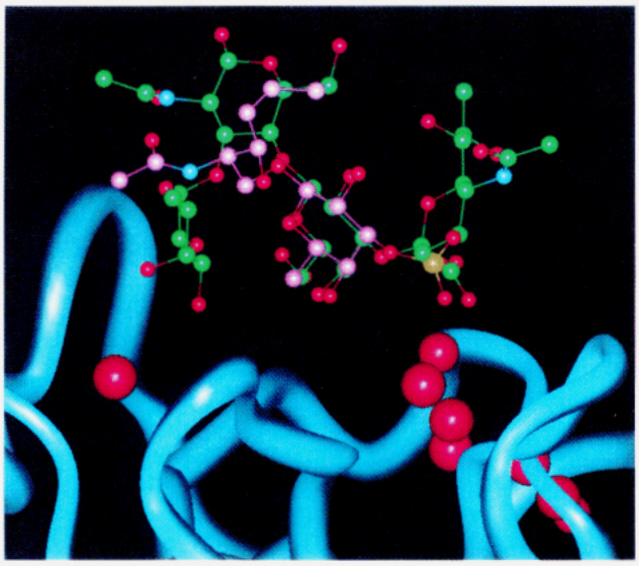

FIGURE 3: Schematic P-selectin–sLe^x model complex and a molecular mimic of sLe^x, sulfatide. The side view is obtained from Figure 2 by approximately a 90-deg rotation around the *y*-axis followed by approximately 30-deg rotation around the *x*-axis. P-Selectin is shown in blue solid ribbon representation. The proposed molecular anchor points for binding of sLe^x, the conserved Ca^{2+}, and Lys113 are colored in red. sLe^x and sulfatide are shown with atoms represented as "balls" and in atom coloring (sLe^x: carbon, green; nitrogen, blue; oxygen, red. sulfatide: carbon, pink; nitrogen, blue; oxygen, red; sulfur, yellow). The fucose moiety in sLe^x is proposed to interact with the calcium coordination sphere, and the negatively charged sialic acid is proposed to interact with the positively charged Lys113 residue. The molecular anchor points that are thought to determine the orientation of the sLe^x carbohydrate in the P-selectin binding site are spatially separated by approximately 10 Å in the P-selectin model. The orientation of sulfatide in the binding site was obtained by superposition of the central galactose moiety, which is common to both ligands. In this superposition, the sulfate group in sulfatide spatially corresponds to the negatively charged sialic acid moiety.

We have previously reported that Glu107, Glu92, and Asn105 contributed to the P-selectin–HL60 interaction. This is now extended to show that Arg85 also contributes to the P-selectin–HL60 interaction. Therefore, we next addressed the question of whether these residues in P-selectin contribute to the binding to sulfatide. It was found that only Asn105 contributes to the P-selectin–sulfatide interaction (Figure 4C). Asn105 is a calcium ligand of the conserved calcium. Residues of this calcium coordination sphere are surrounded by structural elements of nonclassical secondary structure (Weis et al., 1991) and are thought to stabilize the structure of the C-type lectin domain. Mutation of calcium-coordinating residues in P-selectin may, therefore, lead to a distortion of the ligand binding site geometry in P-selectin. This is consistent with our finding that the binding of HL60 cells and of sulfatide is strongly dependent on the presence of calcium (Figure 5). Furthermore, we had previously shown that the substitution of other similar residues, Asn57, Lys58, and His108, with Ala had no effect on P-selectin binding to HL60 cells. Sulfatide binding assays with these P-selectin mutants showed that these residues, located outside the P-selectin ligand binding site, do not contribute to the P-selectin–sulfatide interaction (Figure 4). These results are summarized in Table 1 and represented in the model shown in Figure 6.

Monoclonal Antibody Blocking Experiments. If the sulfatide binds to the same site as the cellular ligand, it would be expected that the mAb able to block the binding of P-selectin to cells would also block the binding to sulfatide. To address this, the ability of mAb to block the binding of P-selectin to sulfatide was assayed. Two mAb that bind to P-selectin–Rg and block binding to HL60 cells (2A2 and 3D3, Figure 7A) were also found to block the binding of P-selectin to sulfatide (Figure 7B), while a nonblocking anti-P-selectin mAb (1G4) did not.

DISCUSSION

Although sLe^x is now generally accepted to be recognized by P-selectin (Lasky, 1992; Bevilacqua, 1993; Springer, 1990; Bevilacqua & Nelson, 1993), the exact nature of the P-selectin ligands remains unclear at present. The selectins display distinct structural similarity in their binding site regions. Most of the P-selectin residues found to be responsible for mediating the binding to HL60 cells and to immobilized sLe^x are conserved in all selectins. Using a simple molecular model, we have attempted to recognize some features of P-selectin–ligand interactions. It is worth noting that the mode of carbohydrate binding proposed here for sLe^x is quite distinct from previously described protein–carbohydrate interactions [see, for example, Vyas (1991) and Bundle and Young (1992)], in that the carbohydrate is not bound in a distinct cavity or groove. Instead, only one side of sLe^x appears to be involved in binding to P-selectin, whereas the other remains essentially exposed to solvent. This mode of binding is consistent with a shallow depression as the carbohydrate binding site in P-selectin. On the basis of this model, the *N*-acetyl and glycerol moieties of the sialic acid are not expected to contribute to the binding in a significant way. The picture of a "one-phasic" carbohydrate binding on the P-selectin protein surface over at least 10 Å is consistent with conclusions regarding carbohydrate binding to E-selectin drawn from ligand binding studies in the absence of any structural information (Tyrrell et al., 1991).

The molecular models have also suggested that the same region in P-selectin may be responsible for the binding of sLe^x and sulfatide and suggested a role for specific residues in the interaction. In the model, Lys113 anchors the negatively charged carboxyl group of the sLe^x and the sulfate group of the sulfatide. The sulfate group of the sulfatide, because of its size, symmetry, and greater negative charge, appears to interact electrostatically with Lys111 to a greater extent than the carboxyl group of the sLe^x. In this report, we have experimentally confirmed the critical contribution of Lys111 and Lys113 to the ability of P-selectin to bind sulfatide.

The conserved calcium coordination sphere is likely to interact directly with the fucose moiety, which is absent in sulfatide. Yet the binding of sulfatide to P-selectin is calcium-dependent (Larsen et al., 1989; Geng et al., 1990; Moore et al., 1991; Geng et al., 1991). This is likely to be due to a more indirect structural effect. Because of the limited resolution of the models and the approximations inherent in the modeling procedure, a detailed role for Tyr94 and Tyr48 in the binding to sLe^x or sulfatide was not apparent. Although specific contacts cannot be proposed, the modeled binding of sLe^x to P-selectin places the sialic acid moiety of sLe^x proximal to Tyr48. This sialic acid is absent in sulfatide and represents significant structural differences between the binding moieties of the two ligands. Erbe and colleagues (1993) prepared five P-selectin mutants, Lys8Ala, Lys84Ala, Lys111Ala, Lys113Ala, and Tyr48Phe, and examined their abilities to bind to HL60 cells, 2′,3-sLe^x, 2′,6-sLe^x, and sulfatides to elucidate the nature of the cell-surface ligand for P-selectin. They reported that the Lys111Ala, Lys113Ala, and Tyr48Phe substitutions reduced the binding of P-selectin to HL60 cells, while only the Lys113Ala substitution affected P-selectin binding to sulfatides. In our study, Tyr48Phe, Tyr48Ala, Tyr94Phe, and Tyr94Ala P-selectin mutants bound sulfatide, while Lys111Ala did not. These results are consistent with

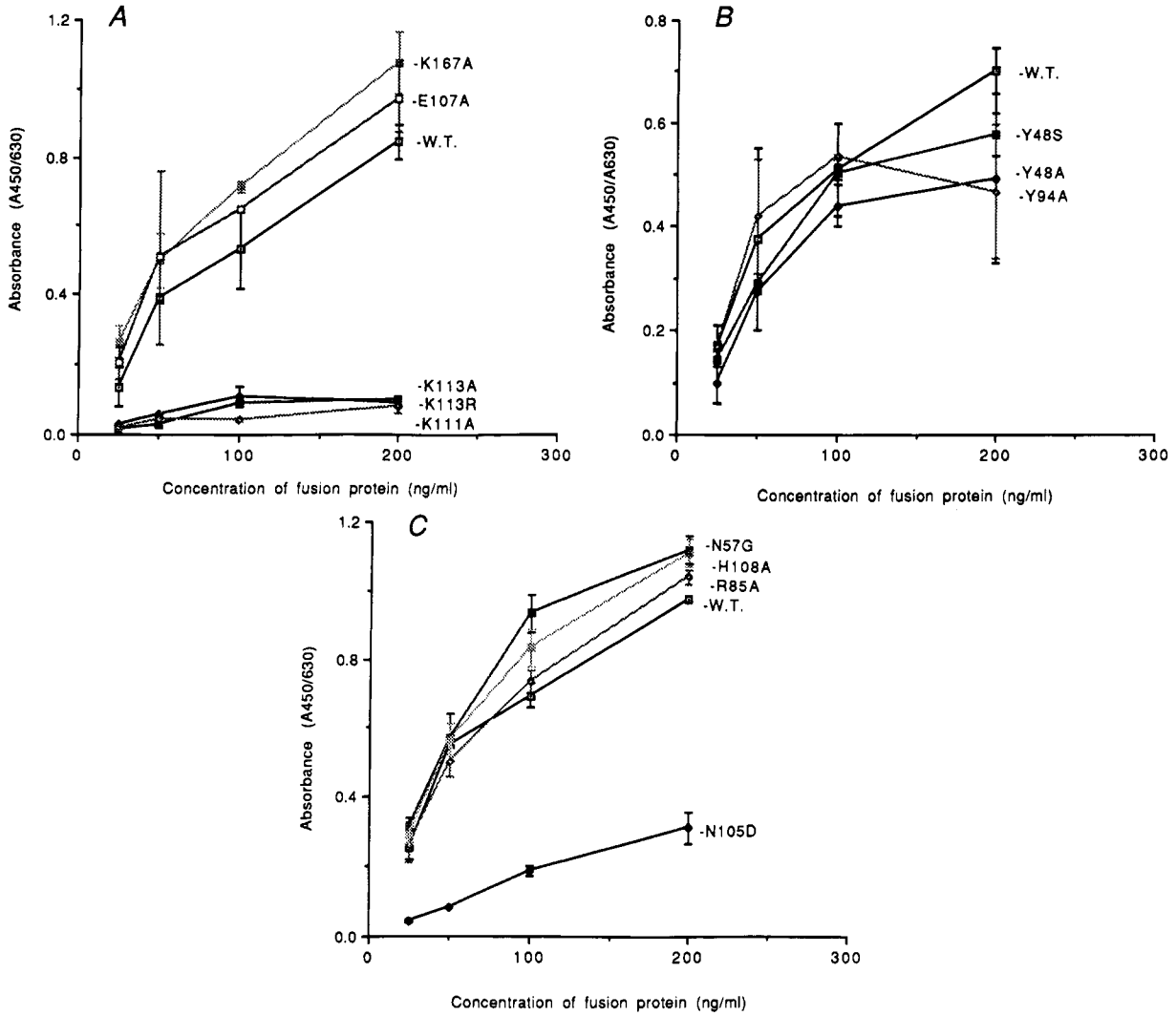

FIGURE 4: P-Selectin–Rg mutants binding to sulfatide. Binding of increasing concentrations of mutant P-selectin–Rg to immobilized sulfatide (1 μg/mL) in an ELISA assay as described in the Materials and Methods section. The binding of each P-selectin–Rg mutant to immobilized lysosulfatide (1 μg/mL) was used as a negative control. (A) Binding of increasing concentrations of the Lys111Ala, Lys113Ala, Lys113Arg, Lys167Ala, and Glu107Ala P-selectin–Rg mutants and wild-type (WT) P-selectin–Rg to immobilized sulfatide; (B) binding of increasing concentrations of the Tyr48Ala, Tyr48Ser, and Tyr94Ala P-selectin–Rg mutants and WT P-selectin–Rg to immobilized sulfatide; and (C) binding of increasing concentrations of the Asn57Gly, Asn105Asp, His108Ala, and Arg85Ala P-selectin–Rg mutants and WT P-selectin–Rg to immobilized sulfatides.

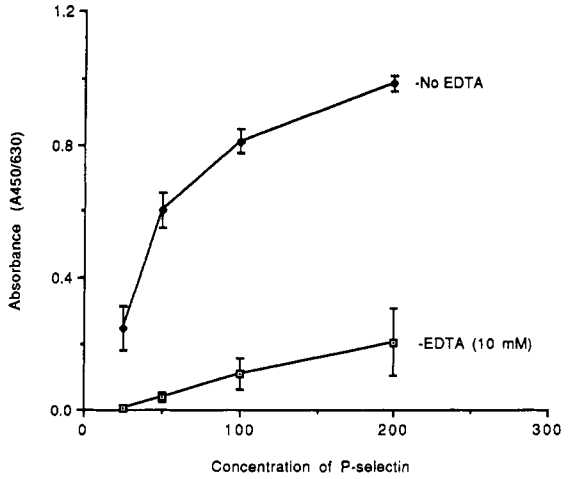

FIGURE 5: P-Selectin–Rg binding to sulfatide is Ca^{2+}-dependent. Binding of increasing concentrations of P-selectin–Rg to immobilized sulfatide (1 μg/mL) in the absence (no EDTA) or presence of 10 mM EDTA. The binding was monitored by an ELISA assay as described in the Materials and Methods section.

the models of the P-selectin–sLe^x and P-selectin–sulfatide complexes and indicate that the two ligands bind to overlapping sites of the P-selectin. The presence of a common binding site was verified in an alternate approach by demonstrating the ability of antibodies that block the binding of P-selectin to HL60 cells to also block the binding to sulfatide.

It is intriguing that we find that the majority of residues which are important for P-selectin binding are conserved in P-, E-, and L-selectins. Therefore, it appears reasonable to assume that similar interactions may mediate the binding of these Ca^{2+}-dependent lectins to their carbohydrate ligands. However, *in vivo* the three selectins appear to exhibit overlapping but not identical ligand specificities (Bevilacqua, 1993; Springer, 1990; Lasky, 1992; Bevilacqua & Nelson, 1993). These observations suggest that additional parameters contribute to ligand specificity. Examples may include the highly variable amino acid sequence of the loop located between Pro98 and Pro101, which is proximal to the binding site, the contribution of other regions of the lectin domain, and/or the contribution of other selectin domains, in particular the EGF domain, which is critical for selectin function. Initial attempts to address some of these questions have been made. These include, for example, exchange of residues Pro98 and Pro101 with Ala to increase the flexibility of the discussed loop region (Hollenbaugh et al., 1993) or the interchange of all or a portion

Table 1: Binding Properties of Mutant P-Selectins

substitution	sulfatide binding	HL60 binding	
		FC	adhesion
Y48A	+	–	–
Y48S	+	–	–
Y48F	+	–	–
N57G	+	+	+
K58A	+	+	+
N79A	ND	+	+
R85A	+	–	+
E92A	+	+/–	+/–
Y94A	+	–	–
Y94F	+	–	–
P98,101A	ND	+/–	+
N105D	–	–	–
E107A	+	–	+/–
H108A	+	+	+
K111A	–	+/–	+
K113A	–	–	–
K113R	–	–	–

[a] Summary of the effect of site-specific mutations on the binding of P-selectin–Rg mutants to HL60 cells and sulfatide. This table summarizes the data in this report and our previous report (Hollenbaugh et al., 1993) on the P-selectin binding site. Mutants whose binding is indistinguishable from wild-type P-selectin are listed as +, mutants whose binding is significantly diminished but detectable are listed as +/–, and mutants which fail to bind are listed as –. Mutants not tested in the sulfatide binding assay are noted as ND (not done).

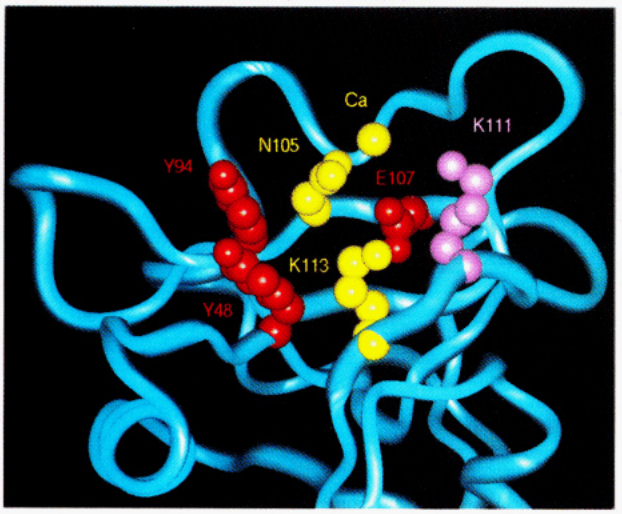

FIGURE 6: Comparison of residues important for P-selectin binding to HL60 cells and sulfatide. The view and the representation of the P-selectin are analogous to those in Figure 1. Binding site residues are color-coded according to the following classification: red, residues in P-selectin crucial only for binding to HL60 cells but not to sulfatide; pink, residues crucial for the binding only to sulfatide but not to HL60 cells; yellow, residues in P-selectin crucial for the binding to HL60 cells and to sulfatide. For clarity, residues with intermediate or weak influences on binding are omitted from the representation.

of the selectin lectin domain among the three family members to examine the contribution of other selectin domains to ligand specificity (Walz et al., 1990; Kansas et al., 1991). These experiments have not yet allowed the identification of the molecular determinants that mediate the differences in the *in vivo* specificity among the selectins. Further experiments will be required to resolve the important question of what determines the specificity of the selectins.

We previously proposed that the interaction between P-selectin and sulfatides secreted by activated leukocytes at sites of inflammation plays a role in mediating leukocyte/P-selectin disengagement during leukocyte extravasation (Aruffo et al., 1991). This would require sulfatide binding to disrupt the interaction between P-selectin and its cellular ligand. Although a multitude of mechanisms could give rise

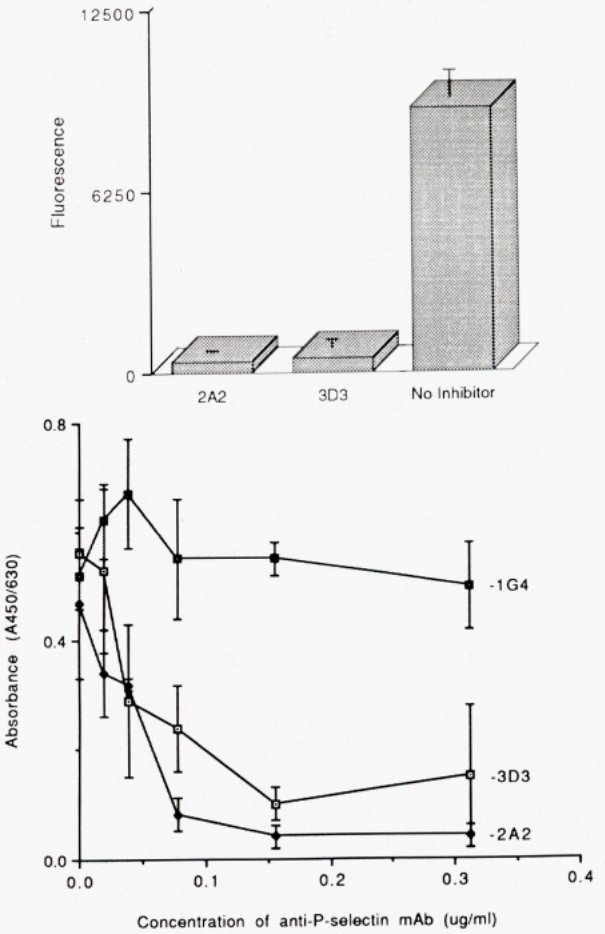

FIGURE 7: Anti-P-selectin monoclonal antibody blocking of P-selectin–Rg to HL60 cells and sulfatide. Inhibition of P-selectin–Rg binding to HL60 cells (A, top) and sulfatide (B, bottom) by anti-P-selectin mAb as described in the Materials and Methods section.

to the sulfatide-dependent disruption of leukocyte/P-selectin binding, perhaps the simplest one is that sulfatide displaces P-selectin's leukocyte ligand by binding to the same region of the protein as the cellular ligand. *In vitro* and *in vivo* studies have shown that, under conditions of flow, the selectins mediate leukocyte rolling on activated vascular endothelium (Lawrence & Springer, 1991; Ley et al., 1991; Mayadas et al., 1993; Bevilacqua & Nelson, 1993). These weak interactions account for the reversible nature of the selectin/leukocyte binding and provide a mechanism whereby sulfatide could bind to the P-selectin active site and displace the cellular ligand. Alternatively, sulfatide could block the interaction between P-selectin and leukocytes by binding distal to the selectin binding site and disrupting selectin function indirectly or by sterically blocking the interaction between P-selectin and its ligand. The results presented in this report demonstrate that the binding sites for the cellular ligand of P-selectin and sulfatides are overlapping and that sulfatides inhibit P-selectin-mediated cell adhesion by displacing its cellular ligand.

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