Interaction of P-Selectin (CD62) and Its Cellular Ligand: Analysis of Critical Residues†

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ABSTRACT: P-Selectin (CD62, PADGEM, GMP140) is a membrane glycoprotein which is rapidly mobilized to the surface of activated platelets and endothelial cells where it mediates leukocyte–platelet and leukocyte–vascular endothelial cell adhesion, respectively. P-Selectin is a member of a family of adhesion molecules which includes the endothelial cell adhesion molecule E-selectin and the leukocyte adhesion molecule L-selectin. Selectins mediate cell–cell binding resulting from the interaction between the amino terminal lectin domains of the selectins and their respective carbohydrate ligands. Here we report on a three-dimensional model of the lectin domain of P-selectin which was derived on the basis of its structural homology to the rat mannose binding protein (MBP) whose crystal structure has recently been reported. On the basis of the model, a number of point mutants were prepared to identify the P-selectin binding site. The residues found to be important for binding are located in a shallow groove on the surface of the molecule composed of residues from the β-2, -3, and -5 strands of the P-selectin lectin domain. A number of residues within this groove, which are conserved among all selectins, were found to be critical for P-selectin binding. They include Lys113, Tyr48, and Tyr94. The single substitutions Lys113Ala, Tyr48Ala, Tyr48Phe, Tyr94Ala, and Tyr94Phe abolished P-selectin binding to myeloid cells.

P-Selectin (CD62, PADGEM, GMP140)[1] is expressed in the α-granules of platelets and the Weibel–Palade bodies of endothelial cells (Hsu-Lin et al., 1984; Stenberg et al., 1985; McEver et al., 1989; Bonfanti et al., 1989). Upon platelet and endothelial cell activation, P-selectin is rapidly redistributed from intracellular stores to the cell surface (Hsu-Lin et al., 1984; Stenberg et al., 1985; Hattori et al., 1989a,b; Patel et al., 1991) where it mediates leukocyte–platelet (Larsen et al., 1989) and leukocyte–vascular endothelial cell adhesion (Geng et al., 1990; Gamble et al., 1990). The interaction of P-selectin with leukocytes is in part responsible for mediating leukocyte rolling on the vascular cell wall (Lawrence & Springer, 1991), an early event in inflammation (Atherton & Born, 1972). Recent experiments suggest that blocking selectin function results in the prevention of neutrophil-mediated tissue damage at sites of acute inflammation (Watson et al., 1991a; Gundel et al., 1991; Mulligen et al., 1991). P-Selectin binds to leukocytes in part by interacting with carbohydrate ligand(s) on opposing cells. P-Selectin has been shown to bind to Le^x, sialyl-Le^x, the 3-sulfated galactosyl ceramide, sulfatide, and a glycoprotein of ~250 kDa (Larsen et al., 1990; Polley et al., 1991; Zhou et al., 1991; Picker et al., 1991a; Aruffo et al., 1991; Moore et al., 1992). However, the exact composition of the physiological ligand(s) of P-selectin remains to be elucidated.

P-Selectin is a member of the selectin family of adhesion molecules which includes E-selectin (ELAM-1), which mediates leukocyte binding to vascular endothelium (Bevilaqua et al., 1989; Shimizu et al., 1991) and functions as a skin-homing receptor for T lymphocytes (Picker et al., 1991b), and L-selectin (LAM-1, LECAM-1, Leu8), which mediates lymphocyte homing to peripheral lymph nodes (Camerini et al., 1989; Ord et al., 1990; Siegelman et al., 1990) and neutrophil binding to vascular endothelium at sites of inflammation (Watson et al., 1991a). The members of this family of adhesion molecules are structurally related and contain an amino terminal calcium- dependent (C-type) lectin domain, an EGF-like domain, and a variable number of short repeats similar to complement–regulatory proteins (CR domains), followed by a transmembrane and a cytoplasmic domain. Comparison of the ligand specificity of the different members of the selectin family has shown that these proteins bind to an overlapping set of carbohydrate ligands (Polley et al., 1991; Foxall et al., 1992; Aruffo, 1992).

Although it is clear that the lectin domain of the selectins plays a dominant role in the adhesion function of these proteins (Imai et al., 1990; Walz et al., 1990; Geng et al., 1991; Moore et al., 1991; Watson et al., 1991b), little is known about the lectin domain binding site. Recently, the first crystal structure of a C-type lectin, the rat mannose binding protein (MBP), was reported (Weis et al., 1992). The recognition that its protein fold may be common to all C-type animal lectins has provided a basis for the investigation of the structure of the selectin lectin domains and the analysis of their binding sites. In this study, the structural homology between the P-selectin and MBP lectin domains was used to derive a three-dimensional model of the lectin domain of P-selectin. On the basis of the model, a number of residues potentially important in the binding of P-selectin to its ligand were identified and subjected to site-directed mutagenesis, and the impact on P-selectin binding was evaluated.

MATERIALS AND METHODS

Modeling of the Three-Dimensional Structure of P-Selectin. The model of the lectin domain of P-selectin was

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[1] Abbreviations: GMP140-Rg, P-selectin immunoglobulin fusion protein; Leu8-Rg, L-selectin immunoglobulin fusion protein; MBP, mannose binding protein; C-type lectin, calcium-dependent lectin.

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constructed on the basis of structural homology (Blundell et al., 1987) to the crystal structure of the rat MBP (Weis et al., 1992). The structural alignment of MBP and P-selectin suggested the conservation of the two hydrophobic core regions in MBP and of one Ca^{2+}-binding site. Relative insertions and deletions were only observed in loop regions and at the termini of the domain. Initially, these regions were deleted from the MBP crystal structure, thereby providing the backbone template for model building of P-selectin. The loop regions, with insertions and deletions, were reconstructed by systematic conformational searches following a loop-building protocol essentially as described (Bruccoleri et al., 1988) with CONGEN (R. E. Bruccoleri and Bristol-Myers Squibb Company). Conservative side-chain replacements were carried out in similar positions using interactive computer graphics (InsightII, Biosym Technologies, San Diego, CA), and nonconservative side-chain replacements were modeled using an iterative conformational search protocol (Novotny et al., 1988). The model structure was subjected to 100 steps of energy minimization with explicit hydrogen atom representation (Discover, Biosym Technologies) using a distance-dependent dielectric constant. Harmonic constraints of 20 kcal/mol/Å^2 were applied on all non-hydrogen atoms, and all atoms of the conserved Ca^{2+} coordination sphere were held fixed. Root mean square (rms) deviations of ~0.3 Å were observed for the corresponding backbone segments in MBP and P-selectin.

Construction of Mutants. The GMP140-Rg is a modified form of the GMP140-Rg Fc fusion protein described previously (Aruffo et al., 1991) containing three point mutations in the Fc region designed to reduce binding to Fc receptors (Hollenbaugh et al., 1992). Mutants were constructed with *Pfu* polymerase using an overlap extension PCR protocol (Ho et al., 1989), with the exception of the Y94F and Y94A mutants. Oligonucleotides complimentary to both strands of the GMP140-Rg gene were synthesized with the desired mutation. These primers were used in separate PCR reactions with primers outside the GMP140-Rg lectin domain. Small aliquots of these PCR products were then mixed and subjected to further rounds of PCR amplification using the external primers. The complete PCR product was digested with the appropriate restriction enzymes and ligated into the GMP140-Rg fusion gene in the plasmid CDM7B$^-$. Tyr94Ala and Tyr94Phe were constructed by ligating two PCR products, generated in the same manner as above, in a three-way ligation. The sequence of each mutant was verified by DNA sequencing. Three secondary mutations, Lys225Arg, Ile233Thr, and Ser237Phe, were found in the Tyr94Ala and Tyr94Phe mutants. As these mutations are located in the CR domains, distant from the expected binding domain, the effect is likely to be negligible. The mutations were corrected and the assays were repeated, yielding nearly identical results.

Immunoreactivity with seven monoclonal antibodies (mAb) against P-selectin was assayed. mAb used were AC1.2 (Becton Dickinson, Mountain View, CA), CLB-throm/6 and CRC 81 (Biodesign, Kennebunkport, ME), and 3G8, 4C10, 1G4, and 1B11 (the generous gift of Dr. R. Darveau, Bristol-Myers Squibb). Two assays were used. In one assay, mutant genes were transfected into COS cells. Cells were fixed in 2% formaldehyde, permeabilized with 2% formaldehyde plus 0.1% Triton X-100, and incubated with mAb followed by incubation with FITC-conjugated goat anti-mouse Fc (TAGO, Burlingame, CA, 1:500 dilution). Cells were analyzed by fluorescence microscopy. In a second assay, immunoreactivity was assayed by ELISA. The wells of 96-well plates (Immunolon-2, Dynatech) were coated with goat anti-human antibody (Cappell, 10 μg/mL, 1 h) and blocked with 1× Specimen Diluent (Genetic Systems, Seattle, WA, 1 h), and fusion proteins were added (5 μg/mL in PBS containing 1 mM CaCl and $MgCl_2$ (PBS/Ca/Mg)). Wells were washed and mAb was added at the following concentrations: AC1.2, 5 μg/mL; CLB-throm/6, 10 μg/mL; supernatants diluted 1:1. The wells were washed (PBS/Ca/Mg), and peroxidase-conjugated goat $F(ab')_2$ anti-human IgG was added to each well (TAGO, Burlingame CA, 1:5000 dilution in 1× Specimen Diluent). Wells were washed (PBS/Ca/Mg) and chromogenic substrate was added (Genetic Systems chromagen diluted 1:100 in EIA buffered substrate, Genetic Systems). The reaction was stopped after 10 min with the addition of 3 N sulfuric acid, and the absorbance was measured on an ELISA reader at dual wavelengths, 450 and 630 nm. The fusion proteins were purified from COS cell supernatants by protein A–Sepharose affinity chromatography as described (Aruffo et al., 1991). Protein concentrations were determined using the Bio-Rad protein assay.

Assays of P-Selectin Binding Activity. The binding of WT and mutant GMP140-Rg to HL-60 cells was assayed by staining and by the ability of the fusion protein to mediate adhesion to plastic. In the first assay, HL-60 cells were incubated with WT or mutant GMP140-Rg or control fusion protein (25 μg/mL) in the presence of excess murine IgG_{2a} to block Fc receptors, followed by FITC-conjugated goat anti-human Fc (TAGO, Burlingame, CA, 1:500 dilution). Cells were fixed with 1% paraformaldehyde and analyzed with an Epics C flow cytometer. To assay adhesion, fusion proteins were added to COSTAR 96-well plates that had been coated with goat anti-human Fc antibody (5 μg/mL, 12–18 h, 4 °C). After 2 h, wells were washed and blocked with 1% BLOTTO. HL-60 cells were labeled with BCECF-AM (10 μM, 15 min 37 °C), washed, added to the wells (2×10^5 cells/well), and allowed to adhere with gentle shaking for 30 min. Wells were washed three times with PBS containing 1 mM each $CaCl_2$ and $MgCl_2$. Cells were lysed by the addition of 1% SDS and 50 mM Tris (pH 8) to each well, and fluorescence was quantitated on a microplate fluorescence reader.

RESULTS AND DISCUSSION

The identification of the novel protein fold of the MBP suggested a possible protein fold for the distantly related lectin domains of the selectins (Weis et al., 1991). Though the sequence homology between these C-type lectins is limited (Figure 1A), the residues that appear to be the critical determinants of the MBP protein fold are essentially conserved. For example, the residues located in the two hydrophobic cores described by Weis et al. (1991) are maintained, and differences, including insertions and deletions, map to surface loops of the MBP structure. This comparison of the sequences, which includes the relation to the three-dimensional structure, suggests that the C-type lectin domains of MBP and the selectins have a high degree of structural homology in the absence of overall sequence homology. Such relationships are not unique since structural motifs in proteins are, in general, much more conserved than their sequences (for an example, see Blundell and Doolittle (1992)), as exemplified by the existence of structural superfamilies such as the immunoglobulin superfamily. Although the structural homology between MBP and the selectins provides the rationale for the calculation of a three-dimensional model of the selectin lectin domains, the exact three-dimensional structure of these selectin domains awaits NMR and/or X-ray crystallographic analysis.

Using the crystal coordinates of the MBP C-type lectin domain, a three-dimensional model of the lectin domain of

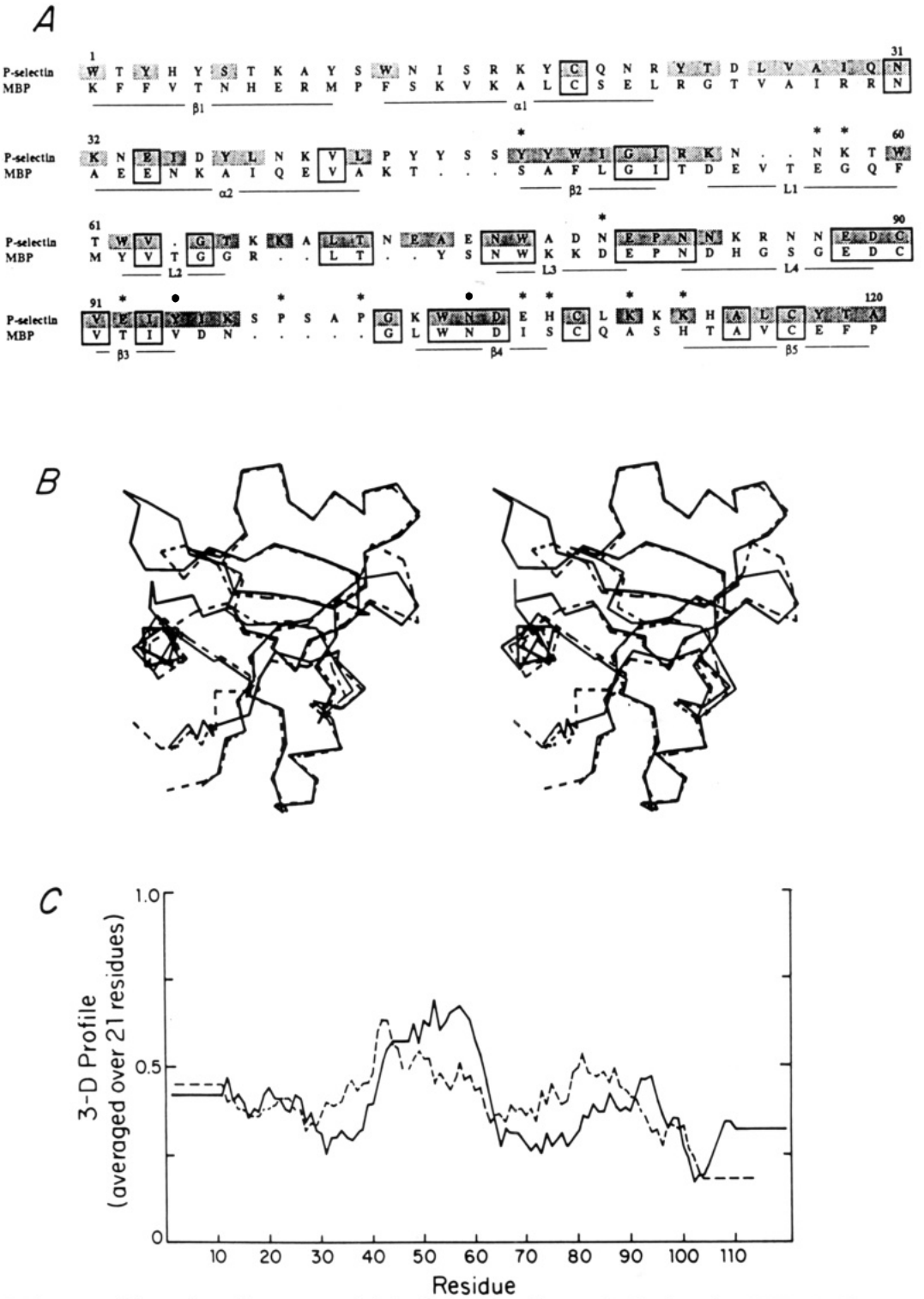

FIGURE 1: (A) Alignment of the amino acid sequences of the lectin domains of human P-selectin and rat MBP. Residues conserved among P-selectin and MBP are shown boxed and those conserved among all selectins are shown shaded. The secondary structure assignments for the P-selectin and the MBP lectin domains are shown below each row. Residue numbering is based on the P-selectin mature protein sequence (Johnston et al., 1989) and is shown above each row to the right and left. P-Selectin residues targeted for substitution are denoted by an asterisk. (B) Superposition of α-carbon stereoplots of the carbohydrate recognition domain of the P-selectin model (—) and the rat MBP crystal structure (- - -). (C) Three-dimensional profiles (Bowie et al., 1991; Lüthy et al., 1992) for the ligand-binding domain of the P-selectin model (—) and the rat MBP crystal structure (- - -). Negative profile values, which are not observed, can be used as a diagnostic of incorrect structures (Lüthy et al., 1992).

P-selectin was derived as described in the Materials and Methods section (Figure 1B). To assess the P-selectin model, the three-dimensional profile method of Eisenberg and coworkers (Bowie et al., 1991; Lüthy et al., 1992) was used. This process evaluates the compatibility of a window of amino acids within a protein sequence with their environment in a given three-dimensional structure using the criteria of surface accessibility, polarity, and local secondary structure. We have calculated and compared the Z scores and three-dimensional profiles of the MBP sequence relative to its three-dimensional structure and of the P-selectin sequence relative to its model structure (Figure 1C). The Z score (Bowie et al., 1991) of

FIGURE 2: Ligand binding site in the P-selectin lectin domain model. The stereoview is approximately along helix α-2 and is similar to the view in Figure 1. The structure of P-selectin is traced, and the position of the conserved calcium is indicated with an asterisk. The side chains of the conserved residues which form the center of the ligand-binding site are displayed and color-coded according to their importance for ligand binding to P-selectin (purple, mutation of the residue abolishes binding; green, mutation of the residue significantly reduces but does not abolish binding; blue, mutation of the residue slightly reduces binding). Residues crucial for ligand binding (purple) are (from the top in a counterclockwise direction) Tyr94, Tyr48, and Lys113. Residues important for ligand binding (green) are (from the top in a counterclockwise direction) Asn105, a conserved calcium ligand, Glu92, and Glu107. Residue Lys111 is shown in blue. Residues found to be crucial for ligand binding to P-selectin are rigorously conserved among all selectins.

the P-selectin sequence and model (34.9) is comparable to that of the MBP sequence and crystal structure (30.8). Z scores greater than 7 are thought to indicate amino acid sequence compatibility with a given structure (Bowie et al., 1991). The three-dimensional profiles of the MBP crystal and P-selectin model structure are very similar, suggesting that the P-selectin model is compatible with the P-selectin sequence and does not contain local structural errors. This analysis further supports the proposed structural homology between the MBP and P-selectin and suggests that the P-selectin lectin domain adopts the basic MBP fold.

The nature of one of the two calcium-binding sites found in the crystal structure of MBP appears to be a major difference in the structures of MBP and the selectins (Weis et al., 1991). Calcium-binding site 2 (Weis et al., 1991) in MPB appears to be fully conserved in the selectins. In contrast, two calcium-coordinating residues in calcium-binding site 1 in MBP are absent in the selectins. In addition, in the P-selectin model, the positively charged residue Lys55, which is conserved in all selectins, partially occupies calcium-binding site 1. Together, these observations suggest that only one of the two calcium-binding sites found in MBP is conserved in the selectins. This, however, does not exclude the possibility that another calcium may be bound elsewhere in the selectins, as proposed by Geng et al. (1991). This possibility is exemplified by the recently described crystal structure of the rat MBP complexed with an oligosaccharide (Weis et al., 1992) which contains an additional Ca^{2+} site which is absent in the rat MBP structure (Weis et al., 1991).

Examination of the P-selectin model and its solvent-accessible surface suggested a shallow depression in the vicinity of the conserved calcium as a potential ligand-binding site (Figure 2). This region is mainly formed by polar and charged residues contributed by the β-2, -3, and -5 strands of the P-selectin lectin domain and includes residues such as Lys113, Tyr48, and Tyr94. To investigate the importance of residues in this groove in P-selectin-mediated cell binding, site-specific mutagenesis of a soluble P-selectin immunoglobulin fusion protein (GMP140-Rg) (Aruffo et al., 1991) was performed. GMP140-Rg consists of the extracellular domain of P-selectin fused to the hinge and constant regions of human IgG_1 and has been shown previously to mediate leukocyte binding in a manner similar to that of membrane-bound P-selectin (Aruffo et al., 1991). Mutations were introduced in the GMP140-Rg by overlap extension PCR.

Inherent in attempts to evaluate the function of specific residues in such experiments is the possibility that mutation of a certain residue may perturb the local structure or the global fold of the protein, leading to the misinterpretation of results. However, previous mutagenesis studies (Matthews, 1991; Cunningham & Wells, 1989; Cunningham et al., 1989) suggest that single-site mutations of surface residues may be introduced without significantly perturbing a protein structure. These experiments have shown that the most significant effects on ligand binding are found when ligand-contact residues are changed (Cunningham & Wells, 1989; Cunningham et al., 1989). Conversely, mutating residues in the core region of a protein may lead to misfolded structures (Lim & Sauer, 1991). In our experiments, only proposed surface residues located outside the two hydrophobic core regions of P-selectin were subjected to mutagenesis. The structural integrity of each mutant was monitored by examining its reactivity with a panel of seven anti-P-selectin mAb (Table I and data not shown), including one mAb (CLB-throm/6) known to block the interaction between P-selectin and HL-60 cells and two mAb (CLB-throm/6 and 4C10) which recognize epitopes sensitive to formaldehyde.

The ability of the mutant proteins to mediate leukocyte adhesion was measured in two different assays. In the first assay, the soluble P-selectin mutants, the wild-type P-selectin, or the control immunoglobulin fusion proteins were incubated with cells of the human myeloid line HL-60, and their binding was examined by fluorescence-activated flow cytometry (FC) (Figure 3A,B). The second was an adhesion assay in which dilutions of the mutant proteins were immobilized in the wells of a microtiter plate, and their ability to mediate the adhesion of the HL-60 cells was compared to that of wild-type GMP140-Rg or a negative control fusion protein, L-selectin-Rg (an immunoglobulin fusion protein of L-selectin which does not bind to HL-60 cells) (Figure 3C,D). This provided a high-avidity binding assay, due to the multiple binding sites of P-selectin immobilized on the plastic. Comparison of the

Table I: Binding of Anti-P-Selectin mAb to WT and Mutant GMP140-Rg and the L-Selectin-Rg (Negative Control)[a]

	mAb					
mutant	AC1.2	CLB-throm/6	3G8	4C10	1G4	1B11
Y48A	102	94	100	101	100	88
Y48F	107	106	92	103	99	96
N57G	104	100	90	101	96	92
K58A	104	89	98	101	96	97
N79A	98	99	96	95	94	96
E92A	100	89	98	99	97	98
Y94A	109	104	90	157	98	103
Y94F	113	102	107	155	99	109
P98, 101A	91	99	92	93	84	95
N105D	99	100	99	93	98	98
E107A	98	89	91	93	102	90
H108A	92	91	102	109	101	97
K111A	111	102	107	113	104	101
K113A	105	102	100	108	102	95
L-selectin	5	4	4	4	3	5

[a] The values are given as percent of WT absorbance.

results of these two assays allowed the identification of mutants with intermediate binding affinities.

Initially, charged residues within the putative shallow binding groove of P-selectin were targeted for substitution (Figure 2). Changing Lys111 to Ala resulted in a protein which showed reduced binding in the FC assay but had almost wild-type binding in the adhesion assay (Figure 3), indicating a slight effect that is not detectable in the high-avidity assay. In contrast, the corresponding change of Lys113 completely abolished the binding of P-selectin to HL-60 cells in both the FC and adhesion assays (Figure 3). Substitution of the two negatively charged residues Glu92 or Glu107 to Ala abolished the binding of P-slectin to HL-60 cells in the FC assay and diminished binding in the adhesion assay (Figure 3). These results suggest that Lys113, Glu92, and Glu107 are critical for ligand binding to P-selectin.

Residues Tyr48 and Tyr94 form the side of the binding groove distal to the Ca^{2+} coordination sphere with their hydroxyl groups protruding outward toward the solvent (Figure 2). Changing residue Tyr48 or Tyr94 to Ala abolished the binding of P-selectin to HL-60 cells in both the FC and adhesion assays (Figure 3). To investigate possible hydrogen-bonding interactions of these two Tyr residues, they were individually changed to Phe. These changes also resulted in complete loss of P-selectin/HL-60 binding as measured in the two assays (Figure 3). Thus, hydrogen-bonding interactions formed by the hydroxyl groups of Tyr48 and Tyr94 contribute significantly to P-selectin–ligand binding. As described in the Materials and Methods section, the initial construction of the Tyr94Ala and Tyr94Phe led to PCR-induced secondary mutations at positions 225, 233, and 237. When these mutations were removed, the results were essentially unchanged.

One residue within the proposed binding site, Asn105, is a conserved Ca^{2+} ligand (Figures 1 and 2). A mutant in which this residue was changed to Asp, thereby preserving its potential to ligate Ca^{2+} but introducing an additional negative charge, resulted in impaired HL-60 binding in both the FC and the adhesion assay (Figure 3). This observation suggests that Asn105 may be involved in both Ca^{2+} and ligand binding. Although the mutation of Asn105 to Asp is isosteric and is likely to preserve the full calcium coordination sphere, it cannot be excluded that subtle structural changes occur around the calcium-binding site which, in turn, may indirectly influence ligand binding to P-selectin.

The loop connecting the β-3 and β-4 strands is located at the end of the binding groove and contains two Pro residues (Pro98 and Pro101) (Figure 1B). The proximity of this loop to the proposed binding site and its apparent rigidity raises the possibility that the loop may be of importance to the structural integrity of this region in P-selectin. In order to test this hypothesis, a double mutant was prepared in which both Pro residues were replaced with Ala. This change is thought to increase the flexibility of the loop and may change its conformation. The double mutant displayed significantly reduced binding activity in the FC assay and slightly reduced activity in the adhesion assay (Figure 3).

Four residues distant from the proposed P-selectin-binding site (Asn57, Lys58, Asn79, and His108) were also mutagenized (Figure 1). These mutants displayed HL-60 binding properties which were indistinguishable from wild-type P-selectin in the two binding assays (Figure 3). Residue Asn57 is part of an N-linked carbohydrate addition recognition sequence, and although substituting it with Gly did not affect the binding of P-selectin to HL-60 cells, the mobility in SDS–PAGE of this protein was found to be increased compared to the WT and other mutants (data not shown). This result suggests that P-selectin undergoes post-translational modification which includes the addition of a carbohydrate moiety at this site. The lack of this carbohydrate moiety, however, does not perturb the binding properties of the protein.

Our analysis shows that both aromatic and charged residues within a solvent-accessible depression proximal to the conserved Ca^{2+}-binding site contribute substantially to the interaction between P-selectin and its ligand. Of particular interest are residues Tyr48 and Tyr94, which are strictly conserved among all selectins. Substitution of either one of them with Phe completely abolished the interaction between P-selectin and HL-60 cells in our two binding assays. Interestingly, a recent analysis of the binding cleft of an MHC class I molecule (HLA-A2) revealed that changing a single conserved Tyr to Phe greatly reduces HLA-A2 peptide binding presumably by eliminating a critical buried hydrogen-bonding interaction between the NH_3^+ amino terminus of the peptide antigen and HLA-A2 (Latron et al., 1992). Our results parallel those found in the analysis of the HLA-A2 binding site and suggest that ligand binding to P-selectin is sensitive to the removal of a single hydrogen-bonding functional group.

Residues which were found to be crucial for P-selectin ligand binding are rigorously conserved among all selectin family members, while residues which, when substituted, resulted in proteins with wild-type binding properties are not rigorously conserved. This suggests that some residues, such as Tyr48, Tyr94, and Lys113, play a common functional role in all selectin-binding sites, whereas residues such as those found in the loop connecting β-3 and β-4 may contribute to the individual specificity of binding, either as contact residues or by modulating the architecture of the binding site. While this report was under review, an analysis of residues within the lectin domain of E-selectin responsible for binding to sialyl-Le^x was published (Erbe et al., 1992). This study identified the analogous region of that protein as important in mediating E-selectin binding to sialyl-Le^x. In particular these two studies have identified Tyr48, Lys111, and Lys113 as critical in selectin-mediated cell adhesion. These two studies show that replacement of Tyr48 with a Phe, effectively removing a single hydrogen-bonding functional group, results in the loss of selectin function. In addition to the study of Lasky and his colleagues on the E-selectin-binding site (Erbe et al., 1992), Geng et al. (1992) have observed that peptides derived from

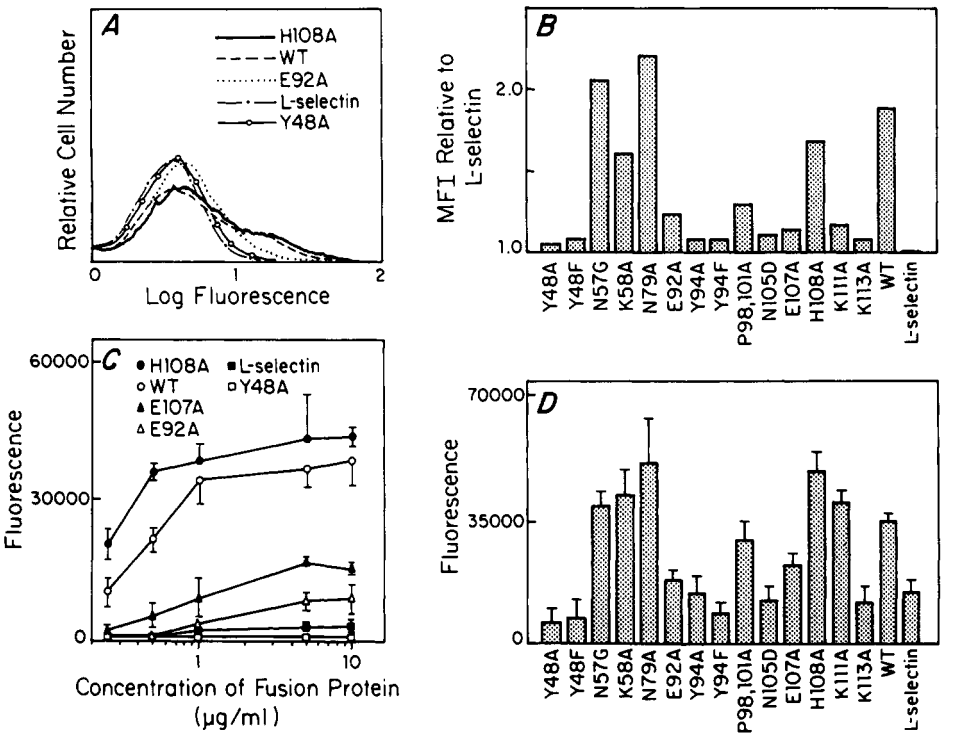

FIGURE 3: Binding of WT and mutant GMP140-Rg to HL-60 cells as assayed by flow cytometry or adhesion. (A) Representative profiles of flow cytometry analysis of HL-60 cells stained with WT or mutant GMP140-Rg or L-selectin-Rg (Aruffo et al., 1991). While staining is low, likely due to low affinity, it is highly reproducible. Mean fluorescence intensities relative to secondary antibody alone and percent cells positive are as follows: H108A, 1.6, 29%; WT, 2.2, 42%; E92A, 1.2, 11%; L-selectin, 1.0, 3%; Y48A, 1.0, 3%. (B) Mean fluorescence intensity of cells stained with each mutant and analyzed by flow cytometry relative to L-selectin (representative of three experiments). (C) Representative experiment of adhesion of HL-60 cells to increasing concentrations of WT or mutant GMP140-Rg. The value is the mean of three points. (D) Representative experiment of adhesion of HL-60 cells to WT or mutant GMP140-Rg or L-selectin-Rg at a single-coating concentration of 5 μg/mL fusion protein. The value is the mean of five points.

residues 23–30 and 54–63 of the P-selectin lectin domain are able to prevent P-selectin-mediated cell adhesion. The regions encompassed by these peptides are distinct from that identified by this study and by the work of Erbe et al. (1992).

The reduction of neutrophil-mediated damage at sites of inflammation by inhibition of selectins has been demonstrated. A soluble fusion protein of L-selectin was used to block the influx of neutrophils in a murine model of acute inflammation (Watson et al., 1991a), antibodies against E-selectin have been used to alleviate neutrophil-mediated lung damage after deposition of immune complexes in rats (Mulligan et al., 1991) and monkeys (Gundel et al., 1991), and a soluble form of P-selectin is able to block adhesion of activated neutrophils to endothelium in vitro (Gamble et al., 1990). Additionally, chronically activated T cells found in the synovial fluids of patients with rheumatoid arthritis are able to bind to P-selectin (Damle et al., 1992). Taken together these results suggest that the ability to block the adhesion of selectins to their ligands may be clinically useful in the treatment of acute and chronic inflammatory disease. The identification of the P-selectin-binding site and residues that are involved in ligand binding by all selectins may aid the design of inhibitors which could be used therapeutically to alleviate clinical complications associated with inflammation.

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