Mechanism of Ligand Binding to E- and P-selectin Analyzed Using Selectin/Mannose-binding Protein Chimeras*

(Received for publication, November 6, 1997, and in revised form, January 7, 1998)

Dawn Torgersen, Nicholas P. Mullin†, and Kurt Drickamer§

From the Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, United Kingdom

The mechanism of oligosaccharide binding to the selectin cell adhesion molecules has been analyzed by transferring regions of the carbohydrate-recognition domains of E- and P-selectin into corresponding sites in the homologous rat serum mannose-binding protein. Insertion of two basic regions and an adjacent glutamic acid residue leads to efficient binding of IL-60 cells and sialyl-Lewisα-conjugated serum albumin. Substitution of glycine for a histidine residue known to stabilize mannose in the binding site of wild type mannose-binding protein results in dramatic loss of affinity for mannose without decreasing binding to sialyl-Lewisα. The accumulated effect of these changes is to alter the ligand binding selectivity of the domain so that it resembles E- or P-selectin more closely than it resembles the parental mannose-binding domain. Affinity labeling using sialyl-Lewisα in which the sialic acid has been mildly oxidized has been used to verify this switch in specificity and to show that the sialic acid-containing portion of the ligand interacts near the sequence Lys-Lys-Lys corresponding to residues 111–113 of E-selectin. The binding of sialyl-Lewisα-serum albumin is inhibited dramatically at physiological and higher salt concentrations, consistent with a significant electrostatic component to the binding interaction. The binding characteristics of these gain-of-function chimeras suggest that they contain many of the selectin residues responsible for selective ligand binding.

The selectin cell adhesion molecules mediate initial binding interactions between moving leukocytes and stationary endothelia (1). The interaction of E- and P-selectins with saccharide ligands on leukocytes represents a well-characterized example of a carbohydrate-based cell-cell recognition mechanism. Among the best counter-receptors for the selectins that have been identified to date are derivatives of the Lewisα trisaccharide, Galβ1–4(Fucα1–3)GlcNAc, or of the Lewisβ trisaccharide, Galβ1–3(Fucα1–4)GlcNAc, which are sialylated and/or sulfated on the 3 position of the terminal galactose residue (2).

The sugar binding activity of each selectin has been mapped to an NH2-terminal C-type carbohydrate-recognition domain (CRD)1 that is homologous to carbohydrate-binding domains in other calcium-dependent animal lectins (3). The structures of this module and the adjacent epidermal growth factor-like domain have been determined by x-ray crystallography (4). No complex with a saccharide ligand has been reported. However, complexes of sugar ligands with CRDs from two other members of the C-type animal lectin family have been deduced by x-ray crystallography (5, 6). These studies of rat serum and liver mannose-binding proteins (MBPs) reveal that terminal mannose, N-acetylgalcosamine, or fucose residues interact directly with the protein as well as indirectly through a bound Ca2+.

Modification of a cluster of three lysine residues near the COOH terminus of the E-selectin CRD has been shown to decrease or abolish binding to leukocytic cells as well as to 3′-sialyl-Lewisα (sLeα) test ligand (4, 7–10). In previous studies, a gain-of-function mutation in the CRD of MBP was created by incorporation of this cluster of basic residues (11). The resulting chimeric CRD binds to both mannose and to sLeα on HL-60 cells and conjugated to BSA. Subsequent structural analysis (12) revealed that the fucose residue of sLeα binds to this mutant CRD in much the same way as mannose binds to the wild type domain, forming direct coordination bonds to Ca2+ and hydrogen bonds to amino acid side chains that serve as additional coordination ligands for this Ca2+. The Lewisα portion of the ligand has the same conformation as observed in solution by NMR studies and in crystals of the free oligosaccharide (for review, see Ref. 13), but the Gal and GlcNAc residues make only limited contact with the protein. The appended NeuAc residue is rotated approximately 180° from the orientation observed in solution, as predicted from previous transferred NOE analysis of ligand bound to the native E-selectin CRD (13). This residue does not make direct contact with the protein but is located in the vicinity of the incorporated cluster of lysine residues.

To probe further the mechanism of ligand binding to the selectins, additional gain-of-function mutations of MBPs have been created and analyzed. Binding and affinity labeling experiments demonstrate that a switch of preferred ligand can be achieved so that the mutants are closer to the selectins than to the parental MBPs in ligand binding specificity. The effect of changes in the lysine cluster and the sensitivity of binding to salt concentration suggests that electrostatic attractions may play an important role in initiating or stabilizing the binding interaction.

EXPERIMENTAL PROCEDURES

Mutagenesis—Mutant cDNAs were generated by substitution of synthetic double-stranded oligonucleotides, purchased from Genosys or Applied Biosystems, for restriction fragments excised from the wild type cDNA for MBP. The mutations were created in an SacI to BamHI

* This work was supported by a grant from the Wellcome Trust. 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.

† Present address: Dept. of Chemistry, University of Edinburgh, West Mains Rd., Edinburgh EH9 3JL, U. K.

‡ Present address: Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX1 3QZ, U. K. Tel.: 44-1865-275-727; Fax: 44-1865-275-339; E-mail: kd@glycob.ox.ac.uk.

The abbreviations used are: CRD, carbohydrate-recognition domain; MBP, mannose-binding protein; sLeα, 3′-sialyl-Lewisα tetrasaccharide; BSA, bovine serum albumin.

This paper is available on line at http://www.jbc.org
Selectin/Mannose-binding Protein Chimeras

6255

fragment of the cDNA in the vector pGEM-3 (14) and were then trans-ferred into the cDNA in the expression vector pNIIINompA2 containing the wild type CRD (15). All mutations were confirmed by DNA sequence analysis using a Sequenase II kit from Amersham. Other reagents for molecular biology were purchased from New England BioLabs.

Expression and Purification—Proteins were expressed in Escherichia coli strain JA221 grown in the presence of CaCl2 to allow isolation of correctly folded and disulfide-bonded CRD directly from the periplasm. LB medium (500 ml) containing 50 μg/ml ampicillin was inoculated with 10 ml of an overnight culture and grown at 23 °C shaking at 200 rpm until the A550nm reached 0.8 (6–8 h). After the addition of 1mM isopropyl-ß-D-thiogalactopyranoside to a final concentration of 40 μM and CaCl2, to a concentration of 100 mM, incubation was continued at 23 °C for a further 16 h. Cells were harvested by centrifugation at 3,600 × g for 10 min in a Beckman JS-4.3 rotor and resuspended in 40 ml of loading buffer (1.25 mM NaCl, 2.5 mM CaCl2, 25 mM Tris-Cl, pH 7.8). Sonication was performed with the microprobe of a Branson model 250 sonifier for five bursts of 1 min interspersed with cooling on ice. The extract was centrifuged for 10 min at 11,000 × g in a Beckman JA-21 rotor and then 1 h at 100,000 × g in a T45.2 rotor. The final supernatant was passed over a 1-ml column of Man-Sepharose or invertase-Sepharose, which was washed with 5 ml of loading buffer and eluted with 5 aliquots each of 0.5 ml of eluting buffer (1.25 mM NaCl, 25 mM CaCl2, 25 mM Tris-Cl, pH 7.8). Man-Sepharose was prepared by the method of Fornstedt and Porath (16), and invertase-Sepharose was prepared following exactly the same protocol but substituting a 20% (w/v) solution of yeast invertase, obtained from Sigma Chemical Company, for the mannose.

Further purification was performed on a 4.6 × 75-mm C4 reverse phase column (Alltech series from Phenomenex). Protein in the eluting buffer was loaded directly onto the column in multiple aliquots of 100 μl under starting conditions of 10% acetonitrile and 0.1% trifluoroacetic acid. Protein was eluted with a gradient of acetonitrile increasing at a rate of 1.25%/min. Fractions were dried for 30 min in a Savant Speed-Vac concentrator to remove acetonitrile and lyophilized.

Binding Assays—Protein was adjusted to a concentration of approximately 0.1 mg/ml in loading buffer before coating into Immulon 4 Microtiter wells (Dynatech). The immobilized carbohydrate was separated from reagent by chromatography on a 2.5-ml column (0.8 × 35 cm) of Sephadex G-10 eluted with water. Fractions of 40 μl of loading buffer were loaded directly onto the column in multiple aliquots of 100 μl. Each fraction was washed with 200 μl of 0.1 M sodium acetate, pH 5.6, and 1 mM EDTA, incubated at 4 °C for 30 min, and dialyzed against two changes of 1,000 volumes of water and lyophilized. Aliquots were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described above.

RESULTS

Incorporation of Additional Regions of the Selectin CRDs—The effects of incorporating three different regions of E-selectin into the CRD of MBP have been investigated previously (11). The regions, two of which are shown in Fig. 1, were selected based on earlier mutagenesis studies conducted with E-selectin because changes in these regions lead to loss of binding activity (4, 7, 22). Only region 5, when tested by itself, is able to confer HL-60 cell binding activity onto MBP. In an attempt to improve the binding of MBP to these cells, further chimeras have been constructed in which both regions 4 and 5 are included. Because E- and P-selectin differ substantially in this region, sequences corresponding to both were tested. The results (Table I) indicate that when the three lysine residues of region 5 are present, E-selectin region 4 improves the HL-60 cell binding activity, whereas region 4 of P-selectin actually causes a loss of binding activity.

These results were confirmed using 125I-L-eLeBSA instead of HL-60 cells as the test ligand. Analysis of the region 5 mutant indicated measurable binding to this ligand (11). The limited availability of the test ligand prevented final analysis of binding constants for the multiple mutants created in the current study. Instead, binding was assessed at a single, relatively low concentration of the input ligand makes the value independent of fluctuations.
in test ligand concentration and gives values analogous to those obtained in the HL-60 cell assay. The relative activities of mutants containing the three different sequences in region 4 correspond reasonably well in the two assays (Table I).

Structural analysis of the original MBP mutant containing just region 5 revealed that the β-carbon of His189 contacts the fucose that is bound near Ca²⁺ site 2 much as it interacts with terminal mannose bound to wild type MBP and that the imidazole ring makes contact with the GlcNAc portion of the ligand (12). The position of the His189 side chain in this mutant is illustrated in Fig. 2. The conformation of MBP in the loop containing this residue differs substantially from the corresponding segment of E-selectin such that sugar bound to E-selectin in the observed orientation would not contact this portion of the protein at all. To eliminate the possibility that this contact affects the binding of sLe⁴, the histidine side chain was removed by mutation to glycine. In previous studies with MBP, this change was found to decrease affinity for Man-BSA and α-methyl mannoside (14). Changing this residue in the mutants containing region 5 also results in loss of mannose binding activity as shown by loss of binding to Man-Sepharose. This loss of activity makes it difficult to purify mutations effectively. A comparison of wild type and mutant behavior on Man-Sepharose and invertase-Sepharose columns is shown in Fig. 3.

CRDs prepared on invertase-Sepharose bind HL-60 cells poorly when these CRDs are coated onto polystyrene. The possibility that some immobilized ligand might be released from the affinity columns suggested that such released material could inhibit binding. Therefore, further purification of the CRDs was undertaken by reverse phase chromatography. This approach has been used previously as a final purification step to prepare active forms of other C-type CRDs (24). The repurified material does exhibit HL-60 cell binding activity. Comparison of the His189 and Gly189 proteins containing the wild type MBP sequence in region 4 shows that removal of the histidine side chain results in a roughly 2-fold increase in binding of both HL-60 cells and sLe⁴-BSA (Table I). This finding suggests that some portion of the histidine side chain at position 189 might hinder sLe⁴ binding.

In the presence of Gly189, region 4 of E-selectin does not provide significant enhancement of HL-60 cell binding and actually decreases sLe⁴-BSA binding (Table I). Region 4 of P-selectin supports less binding than the wild type MBP sequence in both assays, although the presence of Gly189 improves binding compared with His189. These results suggest that the ability of E- and P-selectin regions 4 to assume an optimal sLe⁴ binding conformation in the MBP background is influenced differentially by the residue at position 189. Additional changes that appear to enhance formation of the correct conformations of the selectin-like parts of the CRD in the presence of Gly189 are discussed below.

Affinity Labeling—The failure of the mutants containing glycine at position 189 to bind to Man-Sepharose suggested that the combination of this change with the insertion of lysine residues in region 5 results in a change in the preferred ligand for the CRD of MBP from mannose to sLe⁴. The limited quantities of sLe⁴ and related ligands available make competition binding assays in the usual format impractical, so an alternative approach using affinity labeling to compare affinities was developed. Previous studies have demonstrated that the interaction between L-selectin and its ligand can be stabilized by mild oxidation of the ligand resulting in removal of C-8 and C-9 of NeuAc and generation an of aldehyde group at C-7 (25, 26). Upon binding, this group forms a Schiff’s base with lysine residues in the selectins, which can then be stabilized by reduction with cyanoborohydride. To create an affinity label, this approach was applied to sLe⁴ oligosaccharide labeled reductively in the GlcNAc moiety with [3H]NaBH₄. The results of incubating oxidized sLe⁴ with mutant CRD containing Gly189 plus regions 4 and 5 of E-selectin are shown in Fig. 4. Concentration-dependent labeling can be detected after gel electrophoresis and fluorography. Controls in the

---

**TABLE I**

Effect of selectin region 4 and residue 189 on binding activity of MBP

| Position 189 | Region 4 | HL-60 cell binding | 125I-S-Le⁴-BSA binding |
|-------------|----------|---------------------|------------------------|
| H           | MBP      | 15 ± 5              | 12 ± 1                 |
| H           | E-selectin | 30 ± 8              | 21 ± 5                 |
| H           | P-selectin | 6 ± 4              | 1 ± 0.2                |
| G           | MBP      | 28 ± 7              | 20 ± 1                 |
| G           | E-selectin | 29 ± 9              | 12 ± 2                 |
| G           | P-selectin | 20 ± 8              | 8 ± 1                  |

---

*2 K. Drickamer, unpublished observations.*
confirming that the binding is Ca\(^{2+}\) chromato-
graphy. The NH\(_2\)-terminal sequence of this fragment
isolated by a combination of gel filtration and reverse phase
residues must be labeled.

The three lysine residues inserted in region 5, so one or more of
the microsomal reaction retains bound radioactivity.

Stability of the label under cleavage conditions is evident from
mock cleavages and the fact that remaining intact polypep-
dide 20-residue segment, which migrates off the end of the gel.

indicating that the label must be attached in the COOH-termi-
nal 20-residue fragment that runs off the bottom of the gel.

The affinity labeling approach was used to obtain semiquan-
titative information about the relative affinity of this mutant
for different saccharide ligands. Radioactive labeling in the
presence of competing sugars was quantified and normalized
to the protein recovered after gel electrophoresis. The small vol-
ues used for the labeling reaction made it practical to test
sLe\(^x\) and 3-sulfo-Lewis\(^x\) oligosaccharides and compare them
with mannose (Fig. 5A). The results confirm that the Lewis\(^x\)
derivatives are substantially better inhibitors of binding than
mannose. The results shown in Fig. 5B demonstrate that the
absence of either the terminal Fuc or NeuAc acid residues of
sLe\(^x\) results in loss in inhibitory potency. These experiments
provide further evidence that the modified CRD mimics the
ligand binding behavior of authentic selectins.

Region 5 Interactions—The importance of the three lysine
residues in region 5 was explored further in two ways. First,
the roles of individual lysine residues in this region were ex-
plained by treatment of the labeled protein with hydroxyl-
amine. Samples mock-treated at pH 10 at 0 °C (C) or
45 °C (M) are shown along with hydroxylamine-treated sample (N). Gel
was stained with Coomassie Blue, and radioactivity was localized by
fluorography after a 3-day exposure. Under the conditions employed,
approximately 50% of the protein is cleaved between residues 201 and
202 to yield a major band just below the intact polypeptide and a
20-residue COOH-terminal fragment that runs off the bottom of the gel.
three lysine residues in each possible combination were created in the presence of region 4 (Table II). The results indicate a cumulative effect of the lysine residues, with particularly the first and third residues capable of supporting some binding. The very limited effect of the middle residue may correlate with the disposition of this side chain furthest from the sialic acid portion of the bound ligand (Fig. 2). The HL-60 cell binding data suggest that the only combination of two residues which supports maximal binding is the second and third. However, the results using 125I-sLex-BSA as test ligand, which has relatively narrower error margins, suggest that any combination of two lysines supports full binding. The data indicate that no one residue is essential for binding activity and reveal a partial correlation of binding with total positive charge in this region. These findings are consistent with the structural results showing that none of the lysine side chains makes a specific, direct contact with the NeuAc portion of the ligand. Moreover, the effective binding achieved in the absence of Lys211 suggests that the hydrogen bond between this residue and the galactose moiety is not an essential component of the binding interaction.

A second approach to understanding the role of the region 5 lysine residues was based on the observation that two of the corresponding side chains in E-selectin form hydrogen bonds with glutamic acid residues. A glutamic acid side chain at the position corresponding to Ile\textsuperscript{207} in MBP hydrogen bonds to the lysine residue in region 5 corresponding to Lys\textsuperscript{211}, whereas a glutamic acid side chain at the position of Thr\textsuperscript{197} hydrogen bonds to the lysine residue corresponding to Lys\textsuperscript{213} (Fig. 2). Because these bonds might stabilize the conformation of the region, the two glutamic acid residues were incorporated into the CRD in the presence of the region 5 lysine residues, glycine at position 189, and MBP, E-selectin, or P-selectin sequences in region 4. Incorporation of glutamic acid at position 197 results in a 2–3-fold increase in the binding of both HL-60 cells and sLex\textsuperscript{4}BSA (comparing the results in Tables I and III). Further incorporation of glutamic acid at position 207 actually has a deleterious effect. This last effect suggests distortion of the binding site by virtue of introduction of a negative charge into a position of MBP normally occupied by a hydrophobic isoleucine side chain.

In the presence of a glutamic acid residue at position 197, incorporation of region 4 of E-selectin (but not P-selectin) enhances binding to both HL-60 cells and sLe\textsuperscript{a}-BSA in the presence of His\textsuperscript{189} but not Glu\textsuperscript{189}. In the presence of glutamic acid residues at both positions 197 and 207, a similar enhancement by E-selectin region 4 is observed, although overall binding is much less in the Glu\textsuperscript{207}-containing proteins. These effects suggest that region 4 of selectin can make a positive contribution to ligand binding but that this contribution is only evident when the binding site is in other respects suboptimal. Structural analysis will be necessary to determine if residues in

| Region 5 | HL-60 cell binding | 125I-S-Lex\textsuperscript{4}-BSA binding |
|----------|-------------------|---------------------------------|
| ASH      | 2 ± 1             | 2 ± 1                           |
| KSH      | 5 ± 3             | 7 ± 1                           |
| AKH      | 3 ± 3             | 2 ± 0.1                         |
| ASK      | 8 ± 4             | 8 ± 1                           |
| KKH      | 14 ± 6            | 19 ± 1                          |
| RSK      | 13 ± 8            | 22 ± 1                          |
| AKK      | 28 ± 8            | 24 ± 2                          |
| KKK      | 30 ± 8            | 21 ± 5                          |

| Position 189 | Region 4 | Position 197 | Position 207 | HL-60 cell binding | 125I-S-Le\textsuperscript{a}-BSA binding |
|--------------|----------|--------------|--------------|-------------------|---------------------------------|
| H            | MBP      | E            | I            | 3 ± 1             | 1.3 ± 0                         |
| H            | E-selectin | E            | I            | 46 ± 4            | 18 ± 3                          |
| H            | P-selectin | E            | I            | 4 ± 2             | 0.4 ± 0.1                       |
| G            | MBP      | E            | I            | 71 ± 18           | 32 ± 2                          |
| G            | E-selectin | E            | I            | 63 ± 17           | 39 ± 1                          |
| G            | P-selectin | E            | I            | 51 ± 15           | 33 ± 1                          |
| G            | MBP      | E            | E            | 1 ± 1             | 0.2 ± 0.1                       |
| G            | E-selectin | E            | E            | 21 ± 4            | 14.3 ± 0.4                     |
| G            | P-selectin | E            | E            | 2 ± 1             | 5.1 ± 0.2                      |

Fig. 5. Inhibition of affinity labeling of mutant MBP. Labeling of the mutant containing regions 4 and 5 of E-selectin plus Gly\textsuperscript{189} was conducted as described under “Experimental Procedures” in the presence of increasing concentrations of potential inhibitors. Radioactivity and protein were quantified by densitometry, and the specific activity of the labeling was determined from the ratio of these two values. The results reported are normalized to the specific activity achieved in the absence of inhibitor.

TABLE II
Role of lysine residues in region 5 of MBP mutants
All mutants contain the sequence YIKREK from region 4 of E-selectin in place of Val\textsuperscript{199} of wild type MBP. The histidine residue at position 189 of wild type MBP is retained.

TABLE III
Effect of glutamic acid substitutions on binding activity
All mutants contain the sequence KKK (selectin region 5) in place of residues 211–213 of mannose-binding protein. Region 4 corresponds to a single valine residue at position 199 in wild type MBP. This residue is replaced by the sequence YIKREK in E-selectin and YIKSPS in P-selectin.
region 4 interact directly with the ligand or if the effect of this region is a result of stabilization of other key interacting residues. The fact that either removal of His189 or insertion of Glu189 is as effective as incorporation of region 4 might favor the latter interpretation.

Effect of Salt on Binding—The fact that the NeuAc residue in sLe\(^{\alpha}\) does not make direct contact with lysine residues in region 5 and the apparent role of overall charge in this region of the CRD surface suggest that there may be a strong electrostatic component to the interaction between the selectin-like binding site and its ligand. To assess this possibility, the effect of salt on binding activity was evaluated. As shown in Fig. 6, the interaction of wild type CRD with Man-BSA is relatively insensitive to salt, whereas the interaction of a selectin-like mutant with sLe\(^{\alpha}\)-BSA is weakened drastically as the ionic strength is increased. At physiological salt concentration, roughly 40% of the activity in the absence of added NaCl is observed. Previous studies with L-selectin on cell surfaces revealed a very similar inhibition of binding, with roughly 40% of activity retained under physiological conditions (27). Similar effects, over a more limited concentration range, have been observed for the other selectins (28). Thus, in addition to providing evidence for the electrostatic contribution to binding of the selectin-like mutant, these studies also demonstrate further parallels between the activity of the mutant and natural selectins.

Role of Loop 3—Studies using the extracellular portion of E-selectin have suggested that residues in loop 3 of the CRD might affect ligand binding activity. Substitution of a lysine residue from this region of MBP into the E-selectin CRD has been proposed to introduce mannose binding activity into the domain (22). Therefore, it was of interest to examine the effect of changing the residues in this loop to resemble selectin sequences to see if they affect the relative affinity for Man-BSA and sLe\(^{\alpha}\)-BSA. In the case of mutants designed to resemble E-selectin either at the single position tested previously (residue 182 in MBP, corresponding to position 77 in E-selectin) or for all three residues in this loop which differ between MBP and E-selectin (positions 182–184), there was no detectable effect on relative binding to these two ligands (Table IV). It should be noted that the absolute value of the binding ratio has no direct meaning, as the ligands are not used at precisely the same concentration, and, more importantly, the degree of substitution with sugars is substantially different. Nevertheless, the results do not support a role for loop 3 residues in ligand discrimination.

A similar set of mutations designed to mimic P-selectin revealed an increase in relative affinity for Man-BSA when the entire loop was changed from the MBP to the P-selectin sequence (Table IV). This effect is the opposite of what would have been predicted from the previous E-selectin studies and is associated with the changes at positions 183 and 184, not 182. Although there is no simple explanation for these observations, they again do not support a role for the residues in this loop in determining ligand binding selectivity for P-selectin.

### DISCUSSION

Mutational analysis of the selectins has revealed that changes at roughly 15 positions affect their ligand binding activity (4, 7–9, 22, 29). Several of the critical residues are Ca\(^{2+}\) ligands, whereas many of the rest have been included in the current studies, which extend the analysis of loss-of-function mutations in the selectins. The changes introduced into the MBP framework have been analyzed for increased binding to selectin ligands and thus represent gain-of-function mutations. It is often easier to interpret such effects rather than loss of function because they are less likely to result from indirect alterations in CRD structure and stability. Similarly, the use of purified CRDs rather than proteins expressed on cell surfaces or as Fe chimeras, combined with use of sLe\(^{\alpha}\)-BSA ligand as well as HL-60 cells as target ligands, eliminates possible effects on cell surface expression and other confounding factors.

The fact that all of the mutants analyzed show Ca\(^{2+}\)-dependent binding to either Man-Sephose or invertase-Sephose argues that they have achieved the correct overall conformation. Differential binding to invertase, sLe\(^{\alpha}\)-BSA, and HL-60 cells by the various mutants investigated therefore presumably reflects conformational differences near the modified binding sites. The lack of any detectable retardation of the glycine-containing mutants of MBP on Man-Sephose suggests that the affinity for invertase-Sephose is not just a result of higher concentrations of mannose residues on these columns. Previous studies indicate that mannose phosphate residues in yeast polysaccharides are ligands for L-selectin (30), so similar structures in the invertase preparation might provide binding sites for the selectin-like mutants of MBP.

The phenotypes of mutant CRDs containing various portions of the E- and P-selectin CRDs suggest specific roles for several of these regions in ligand discrimination and stabilization of ligand-CRD complexes. Affinity for sLe\(^{\alpha}\) seems to be dependent primarily upon the presence of a sufficient number of lysine residues.
residues in region 5. Selectin mutagenesis data in this region are not entirely consistent with each other, but several studies have suggested that neither Lys11 nor Lys11 is essential for ligand binding (4, 7–9, 29). These results are consistent with the findings reported in Table II.

In the crystal structure of the initial MBP mutant containing only the three lysines of region 5, the lysine side chains align closely with the arrangement seen in the natural E-selectin domain (4, 12). Thus, although the addition of a glutamic acid residue that hydrogen bonds to one of these lysine residues results in increased binding activity, its role is likely to be in stabilization of the binding site rather than orientation of the lysine side chains. Mutagenesis studies with E- and P-selectin indicating that alanine substitutions for either of these residues results in at least partial loss of binding activity (4, 9) are consistent with this suggestion.

The effects of region 4 are more subtle because the E-selectin sequence inserted in this region supports increased binding to HL-60 cells and sLeA-BSA only in the presence of a histidine residue at position 189. When present, this side chain has a potential role in the binding site because the imidazole ring makes contact with the N-acetylglucosamine portion of sLeA in the crystal structure of the region 5 mutant (31). Thus, the effect of the E-selectin sequence in the presence of His189 may be caused by a slight reorientation of the ligand resulting from interactions with either the β-carbon or the imidazole ring, or it may reflect compensating interactions with the ligand which are only evident when the binding site has an initially weaker affinity.

In addition to providing insight into common aspects of ligand binding to the selectins, these studies provide some initial suggestion of why different selectins show preferential binding to different ligands. In particular, the differing behavior of mutants containing E- and P-selectin sequences in region 4 implicates this segment of the CRD in differential ligand binding. In the most selectin-like constructs, the effect can be viewed as a relative loss of affinity for the simple test ligand, sLeA-BSA, in the presence of the P-selectin rather than the E-selectin sequence (Table III). High affinity P-selectin ligands contain a tyrosine-sulfated region in addition to a specific set of sugar structures (32–34). Additional interactions with such a region, either through the CRD or the adjacent EGF-like domain, might compensate for lower affinity for sLeA reflected in the behavior of the mutants analyzed here.

Mutagenesis studies with E-selectin identified one additional portion of the protein, region 3, which might be involved in sLeA-BSA binding, although the phenotype resulting from changes in this segment were less severe than changes in regions 4 and 5 (4, 7, 22). Previous studies showed that incorporation of region 3 alone does not confer sLeA-BSA binding activity on MBP (11). Further studies in which regions 3, 4, and 5 of E-selectin were all incorporated into MBP failed to show any enhancement of sLeA-BSA binding compared with regions 4 and 5 alone.2 These results and the relatively large distance between region 5 and sLeA in the crystal structure of the selectin-like mutant of MBP (Fig. 2) suggest that loss of binding caused by changes in region 3 of E-selectin represents an indirect effect on stability or on the arrangement of residues that are directly in the binding site.

The current results do not support a role for loop 3 of the CRD (region 6) in ligand binding selectivity suggested by previous studies with E-selectin (22). The earlier conclusions were based in part on the effect of mutations in this region on binding to yeast and to yeast invertase. The results reported here indicate that binding activity for commercial preparations of invertase is actually correlated with sLeA binding activity, a result that probably confounds the previous use of such material as a test for mannoside binding activity.

The oligomeric state of the selectin molecules has not been documented clearly. However, many previous studies have utilized chimeric constructs containing immunoglobulin Fc domain fused to the COOH terminus of CRD and epidermal growth factor domains of the selectins. Such chimeras form either dimers, when the Fc from IgG is employed, or higher oligomers in the case of the Fc domain of IgM. All mutants employed in the present studies contain the neck region from MBP and are thus almost certainly trimers stabilized by the formation of a coiled coil of β-helices as observed in the crystal structures of both wild type MBP and the initial region 5 selectin-like mutant (12, 35). Neither the Fc chimeras nor the MBP mutants are likely to reflect the relative arrangement of multiple CRDs in intact selectins at the cell surface. Therefore, the fact that the binding activities of Fc fusions and MBP chimeras closely parallel the activity of the membrane-bound molecules argues against the need for a specific geometry of interaction between an array of ligands and the multiple binding sites in oligomeric selectins. However, the way CRDs are presented may have more influence on transient interactions with ligands observed in cell rolling assays in contrast to static, equilibrium assays employed in the present studies.

Several modified versions of the CRD from MBP have been crystallized successfully in forms that are isomorphous with the wild type CRD, and the spacing in these crystals accommodates introduction of ligands by soaking (12, 36). Thus, structural analysis of some of the mutants described here may prove feasible. The evidence presented showing that the binding activity of some of these mutants closely parallels the activity of the natural selectins suggests that such studies may provide a viable route to a detailed molecular understanding of ligand binding to the selectins.

Acknowledgments—We thank Maureen Taylor, Bill Weis, and Ken Ng for comments on the manuscript.

REFERENCES
1. McEver, R. P. (1994) Curr. Opin. Immunol. 6, 75–84
2. Rosen, S. D., and Bertozzi, C. R. (1994) Curr. Opin. Struct. Biol. 6, 683–673
3. Drickamer, K., and Taylor, M. E. (1993) Annu. Rev. Cell Biol. 9, 297–364
4. 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
5. Weis, W. I., Drickamer, K., and Hendrickson, W. A. (1992) Nature 360, 127–134
6. Ng, K. K.-S., Drickamer, K., and Weis, W. I. (1996) J. Biol. Chem. 271, 663–674
7. 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 Laskey, L. A. (1992) J. Cell Biol. 119, 215–227
8. Erbe, D. V., Watson, S. R., Presta, L. G., Wolitzky, B. A., Foxall, C., Brandley, B. K., and Laskey, L. A. (1993) J. Cell Biol. 120, 1227–1235
9. Hollenbaugh, D., Bajerath, J., Stenmark, R., and Aruffo, A. (1993) Biochemistry 32, 2960–2966
10. Hollenbaugh, D., Aruffo, A., and Senter, P. D. (1995) Biochemistry 34, 5678–5684
11. Blance, O., Iobst, S. T., Gabel, C., and Drickamer, K. (1996) J. Biol. Chem. 271, 7289–7292
12. Ng, K. K.-S., and Weis, W. I. (1997) Biochemistry 36, 979–988
13. Varki, A. (1997) Curr. Opin. Struct. Biol. 7, 617–623
14. Iobst, S. T., Wormald, M. R., Weis, W. I., Dwek, R. A., and Drickamer, K. (1994) J. Biol. Chem. 269, 15505–15511
15. Drickamer, K. (1989) Biochim. Soc. Trans. 17, 13–15
16. Furnstedi, N., and Forth, J. (1975) FEBS Lett. 57, 187–191
17. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) Biochem. J. 89, 114–123
18. Askford, D. A., Dwek, R. A., Welby, J. K., Amatayakul, S., Homans, S. W., Lis, H., Taylor, G. N., Sharon, N., and Rademacher, T. W. (1987) Eur. J. Biochem. 166, 311–320
19. Hansson, D. E., and Ashford, D. A. (1972) Methods Enzymol. 26, 209–211
20. Laemmli, U. K. (1970) Nature 266, 680–685
21. Bornstein, P., and Balian, G. (1977) Methods Enzymol. 47, 132–145
22. Kagan, T. P., Revelle, B. M., Tapp, S., Scott, D., and Beck, P. J. (1995) J. Biol. Chem. 270, 14047–14055
23. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
24. Weis, W. I., Chichlow, G. V., Murthy, H. M. K., Hendrickson, W. A., and Drickamer, K. (1991) J. Biol. Chem. 266, 20675–20686
25. Norgard, K. E., Han, H., Powell, L., Kriegler, M., Varki, A., and Varki, N. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1068–1072
26. Puri, K. D., and Springer, T. A. (1996) J. Biol. Chem. 271, 5404–5413
27. Stoolman, L. M., and Rosen, S. D. (1983) J. Cell Biol. 96, 722–729
28. Koenig, A., Jain, R., Vig, R., Norgard-Sumnicht, K. E., Matta, K. L., and Varki, A. (1997) Glycobiology 7, 79–93
29. Bajorath, J., Hollenbaugh, D., King, G., Harte, W., Jr., Eustice, D. C., Darveau, R. P., and Aruffo, A. (1994) Biochemistry 33, 1332–1339
30. Yednock, T. A., and Rosen, S. D. (1989) Adv. Immunol. 44, 313–378
31. Lax, I., Mitra, A. K., Ravera, C., Hurwitz, D. R., Rubinstein, M., Ullrich, A., Stroud, R. M., and Schlessinger, J. (1991) J. Biol. Chem. 266, 13828–13833
32. Wilkins, P. P., Moore, K. L., McEver, R. P., and Cummings, R. D. (1995) J. Biol. Chem. 270, 22677–22680
33. Sako, D., Comess, K. M., Barone, K. M., Camphausen, R. T., Cumming, D. A., and Shaw, G. D. (1995) Cell 83, 323–331
34. Pouyani, T., and Seed, B. (1995) Cell 83, 333–343
35. Weis, W. I., and Drickamer, K. (1994) Structure 2, 1227–1240
36. Kolatkar, A., and Weis, W. I. (1996) J. Biol. Chem. 271, 6679–6685