Introduction of Selectin-like Binding Specificity into a Homologous Mannose-binding Protein*

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The structures of the ligand-binding C-type carbohydrate-recognition domains of selectin cell adhesion molecules and of mannose-binding proteins (MBPs) are similar to each other even though these proteins bind very different carbohydrate ligands. Our current understanding of ligand binding by E-selectin is based on structural studies of unliganded E-selectin and of MBP-carbohydrate complexes, combined with results from mutagenesis of E-selectin. Five regions of E-selectin that differ in sequence from the corresponding regions of MBP have been introduced into the carbohydrate-recognition domain of MBP. Four of the changes have little effect on ligand binding. Insertion of one stretch of positively charged amino acids alters the sugar binding selectivity of the domain so that it now binds HL-60 cells and serum albumin derivatized with sialyl-LewisX tetrasaccharide, thus mimicking the properties of E-selectin.

One of the most striking properties of animal proteins that contain Ca$^{2+}$-dependent carbohydrate-recognition domains (C-type CRDs) is the diversity of sugar ligands that bind to different members of this family (1). In broad terms, these CRDs fall into two classes. CRDs in one class bind ligands containing galactose or N-acetylgalactosamine with varying degrees of selectivity and affinity. CRDs in the second class bind to mannose and/or N-acetylgalactosamine. The distinguishing feature of the ligands for these two classes is the disposition of the 3- and 4-hydroxyl groups. The results of structural and mutagenesis studies suggest that in all C-type lectins these hydroxyl groups form part of the coordination sphere of a bound Ca$^{2+}$ (2–4).

Preferential binding of sugars with an equatorial-axial or equatorial-equatorial arrangement at positions 3 and 4 is determined by the arrangement of side chains from the protein that form both coordination bonds with the Ca$^{2+}$ and hydrogen bonds with the sugar hydroxyl groups. L-Fucose binds to CRDs in the second class through analogous interactions of the equatorial 2- and 3-hydroxyl groups. Increased affinity and selectivity for certain sugars within each of the two classes of C-type CRDs results from other contacts between the protein and the sugar. For example, selective binding of N-acetylgalactosamine over galactose by the asialoglycoprotein receptor requires the presence of additional amino acid residues that are probably positioned near the 2-acetamido group (5).

High affinity binding of C-type animal lectins to complex sugar ligands results from at least two additional effects. Clustering of multiple CRDs, each with a single sugar-binding site displayed in an appropriate geometrical arrangement, results in high affinity binding of multiantennary sugars (6). In mannose-binding proteins (MBPs), such clustering results from oligomerization of polypeptides containing single CRDs (7), while there are duplicated CRDs in the single polypeptide of the macrophage mannose receptor (8). Alternatively, additional affinity and selectivity can reflect the presence of secondary or extended binding sites within single CRDs. In these cases, a single CRD can interact with more than a single sugar in an oligosaccharide. Liver MBP (MBP-C) but not serum MBP (MBP-A) appears to contain such a secondary site.

Selectin cell adhesion molecules mediate the interaction of circulating leukocytes with vascular endothelial cells, initiating a transient rolling that eventually leads to firmer attachment and extravasation (9,10). E- and P-selectins are found at the surface of epithelial cells and interact with monocytes and neutrophils, while L-selectin is expressed on circulating leukocytes and selectively recognizes the surface of high endothelial venules of the peripheral lymph nodes. Each of the selectins consists of an NH$_2$-terminal C-type CRD adjacent to an epidermal growth factor-like domain and a series of complement homology modules (9,10). The natural ligands for the selectins are not known, but fucose-containing oligosaccharides are the most effective low molecular weight ligands. The tetrasaccharide sialyl-LewisX (9) and various sulfated derivatives (11) have been studied most extensively. Recent evidence suggests that, in addition to clusters of such oligosaccharide ligands, high affinity glycoprotein ligands for P-selectin bear sulfated tyrosine residues (12–14).

The structure of a fragment of human E-selectin consisting of the CRD and epidermal growth factor-like domain has been established by X-ray crystallography (15). This structure reveals a single Ca$^{2+}$-binding site, in contrast to the two sites observed in the MBP-A and MBP-C CRDs (2,3). Evidence from binding studies suggests first order dependence on Ca$^{2+}$ concentration, consistent with the presence of a single Ca$^{2+}$-binding site (16). In the absence of structural information about the sugar-binding sites in the selectins, mutagenesis studies and modeling based on the MBP-A-oligosaccharide and MBP-C-mannosaccharide structures have been combined to suggest possible modes of interaction of E-selectin (16–18) and P-selectin (19–20).
with sialyl-Lewis\textsuperscript{x}. The fucose moiety of the ligand probably binds directly to the single Ca\textsuperscript{2+} and surrounding amino acid residues in the E-selectin CRD in a manner analogous to the interactions of mannos with Ca\textsuperscript{2+} site 2 of MBP-A (2) and of fucose and other sugars with Ca\textsuperscript{2+} site 2 of MBP-C (3), while negatively charged portions of the ligands are believed to interact with one or more regions of positive potential on adjacent portions of the CRD surface (16–22).

In the present studies, segments of E-selectin have been substituted into the MBP-A CRD. Replacement of a single segment of 3 amino acid residues confers upon the CRD the ability to bind HL-60 cells and sialyl-Lewis\textsuperscript{x}-BSA. While many previous experiments have relied on negative phenotypes of mutants in the E-selectin CRD to identify functionally important residues, these experiments provide positive evidence for the importance of a second portion of the CRD surface in addition to the region directly surrounding Ca\textsuperscript{2+} site 2.

**Experimental Procedures**

Materials—Materials for construction of mutated expression plasmids, protein preparation, and solid phase binding assays have been previously described (23). Man\textsubscript{2}BSA was purchased from EY Laboratories, and sialyl-Le\textsubscript{b}BSA containing 12.6 mol of tetrasaccharide/mol of BSA was purchased from Oxford Glycosystems. Cell culture medium was obtained from Life Technologies, Inc. Tritiated thymidine was purchased from Amersham Corp. Scintillation fluid was a product of Packard Instruments. The soluble extracellular domain of E-selectin produced in Chinese hamster ovary cells was a gift of Pfizer Central Research.

Mutation of MBP-A—Mutations were introduced into the wild type MBP-A DNA (24) by replacing short restriction fragments with pieces of synthetic oligonucleotides following standard recombinant DNA techniques. Mutations were created in a Sac I to BamH I fragment coding for the COOH-terminal 92 amino acids of the CRD. Within this fragment, the following substitutions were made. In region 1, the segment starting at base 80 (numbered from the 5' end of the Sac site and flanked by Avr II and NiaI sites was replaced with the sequence CCTAGGCATCAACTGCTTCTTGATG. In region 2, the segment starting at base 166 and flanked by BamI and BsdI sites was replaced with the sequence GAAGACTGTGTCACTATATACATCCTAGGCATCAAACACTGCTTCTTGATG. In region 3, the segment starting at base 53 and flanked by Xmn I and Avr II sites was replaced with the sequence CCAAGAAGTGCTAATAGGCGAGGACTGTGTGC. In region 4, the segment starting at base 190 and flanked by BsdI and Accl sites was replaced with the sequence GAAGAAGACGGCTGTCCGAGTCTCGAGTCCGACTGTGTGC. In region 5, the segment starting at base 242 and flanked by HindIII and Bsa36I sites was replaced with the sequence GACCGGCTGGCGACGGGACTGTGTGC. Additionally, a segment starting at base 80 of 100 amino acids was deleted from the MBP-A framework. Two of the regions were hypothesized to be responsible for the absence of Ca\textsuperscript{2+} binding, i.e. 3-5 affect binding to HL-60 cells. The figure was prepared using MOLSCRIPT (28).

Results and Discussion

Five segments of E-selectin were incorporated into the MBP-A framework. Two of the regions were hypothesized to be involved in Ca\textsuperscript{2+} ligation, while there is evidence that the other three may be directly associated with oligosaccharide binding.

Modification of Ca\textsuperscript{2+}-binding Sites—Comparison of the structures of the MBP-A and E-selectin CRDs reveals that the position of one of the two Ca\textsuperscript{2+} sites in MBP-A (Fig. 1, site 2) corresponds closely to the single Ca\textsuperscript{2+} site in E-selectin (15). The presence of a single functional Ca\textsuperscript{2+}-binding site in E-selectin has been confirmed by biochemical analysis (16). Although 1 of the 5 amino acid side chains that ligate this Ca\textsuperscript{2+} site differs between the 2 CRDs, the other 4 liganding amino acids are located at exactly corresponding positions in the two sequences and form sites with very similar geometry. In contrast, most of the amino acid side chains in MBP-A that form Ca\textsuperscript{2+} site 1 have been nonconservatively substituted in E-selectin so that this portion of the polypeptide is arranged in a substantially different way than in MBP-A.

In order to probe which of the amino acid substitutions might be responsible for the absence of Ca\textsuperscript{2+} site 1 in E-selectin, two segments of polypeptide that contribute ligands for this Ca\textsuperscript{2+} in MBP-A were analyzed for the corresponding regions of E-selectin. These segments are designated regions 1 and 2 in Fig. 1. The mutant CRDs containing changes in regions 1 and 2 were prepared in a bacterial expression system previously used for analysis of wild type MBP-A CRD. Interaction of the mutant CRDs with Ca\textsuperscript{2+} was analyzed by measuring the Ca\textsuperscript{2+} dependence of ligand binding in a solid phase assay (Fig. 3). As indicated by the shape of the curves, both mutant CRDs retain second order dependence on Ca\textsuperscript{2+}, indicating that two Ca\textsuperscript{2+}-binding sites are still present. The measured K\textsubscript{Ca} for the region 1 mutant (2.8 ± 0.3 mM) is substantially weaker than for the region 2 mutant (1.4 ± 0.1 mM) and wild type (1.2 ± 0.1 mM) (26).

The second order dependence on Ca\textsuperscript{2+} was confirmed for the region 2 mutant using limited proteolysis with subtilisin to detect a Ca\textsuperscript{2+}-dependent conformational change similar to that.
observed for the wild type CRD (26) (data not shown). This assay could not be utilized in the case of the region 1 mutant, because this CRD proved resistant to proteolysis even in the absence of Ca\(^{2+}\), probably because the initial protease-sensitive site detected in these assays lies within the loop that has been substituted.

As shown in Fig. 2, substitution of region 2 of \(E\text{-selectin}\) into MBP-A results in a change of one Ca\(^{2+}\) site 1 coordination ligand from aspartic acid to asparagine, while the substitution of region 1 results in more substantial changes, as a stretch of 8 amino acid residues including one aspartic acid and one glutamic acid ligand is replaced with a shorter sequence of 6 residues containing lysine and asparagine residues at the corresponding positions. These results indicate that Ca\(^{2+}\)-binding site 1 is not significantly affected by substitution of region 2, while it is preserved but weakened by substitution of region 1. The fact that the Ca\(^{2+}\) dependence of sugar binding to the region 1 mutant remains second order indicates that both sites 1 and 2 must be occupied in order to create a sugar-binding site. The absolute affinity of both region 1 and region 2 mutants for Man-BSA is unchanged from wild type (data not shown). Thus, although substitution of residues in region 1 reduces the affinity of this site for Ca\(^{2+}\) and thus increases the overall \(K_{\text{Ca}}\), the ultimate conformation achieved is not distinguishable from that of the wild type CRD.

Like the region 1 and region 2 mutants, a double mutant containing both region 1 and region 2 substitutions was produced in the bacterial expression system in yields comparable with wild type. However, unlike the single mutants, the double mutant could not be purified by affinity chromatography on Man-Sepharose. It is likely that the presence of both regions 1 and 2 in the MBP-A background reduces the affinity of site 1 for Ca\(^{2+}\) much more than does either region alone, so a conformation like the doubly Ca\(^{2+}\)-ligated form of MBP-A is not stable even at 25 mM Ca\(^{2+}\). It is possible that the presence of regions 1 and 2 from \(E\text{-selectin}\) is not sufficient to induce the alternative stable conformation seen in \(E\text{-selectin}\) in the absence of Ca\(^{2+}\) site 1, and thus the double mutant does not fold correctly. However, since \(E\text{-selectin}\) itself does not bind to Man-Sepharose, if the conformation of MBP-A containing both regions 1 and 2 from \(E\text{-selectin}\) does fold similarly to \(E\text{-selectin}\), it might still not bind to Man-Sepharose. Unfortunately, since it was not possible to purify the mutant CRD containing both region 1 and 2 substitutions in soluble form, binding to HL-60 cells or known oligosaccharide ligands for \(E\text{-selectin}\) could not be tested.

Binding to HL-60 cells—A common feature of the best ligands for \(E\text{-selectin}\) is the presence of a fucose residue (9). It is believed that the 2- and 3-hydroxyl groups of fucose may ligate the Ca\(^{2+}\) bound to the CRD in a manner analogous to the way that the 3- and 4-hydroxyl groups of mannose interact with Ca\(^{2+}\) site 2 of MBP-A (2). Results of mutagenesis targeted at residues near the Ca\(^{2+}\)-binding site of \(E\text{-selectin}\) are consistent with this suggestion (15, 18). One possible orientation of fucose ligated in this manner in a complex between the homologous MBP-C CRD and \(\alpha\)-methyl fucoside has been analyzed crystallographically (3). Extensive mutagenesis studies of the \(E\text{-selectin}\) have led to the identification of three additional regions of the CRD that might be involved in interactions with other sugar residues in the sialyl-Lewis\(^x\) ligand (regions 3, 4, and 5 in Figs. 1 and 2). Regions 4 and 5 contain several basic amino acid side chains that could interact with the charged sialic acid residue or with sulfates attached to related oligosaccharide ligands. The importance of positively charged residues in region 5 of the P-selectin CRD has recently been demonstrated by chemical modification studies, in which the first and third lysine residues in this region have been replaced by various charged and uncharged derivatives of cysteine (22).

As an alternative approach to evaluating the role of regions 3, 4, and 5, each of these segments of \(E\text{-selectin}\) has been substituted into the MBP-A background. All three of the modified MBP-A CRDs can be purified by affinity chromatography on Man-Sepharose, suggesting that their ability to bind mannose is not substantially diminished by the substitutions. The ability to bind HL-60 cells was utilized to determine if these CRDs have \(E\text{-selectin}\)-like binding characteristics. As shown in Fig. 4, only the region 5 mutant displays Ca\(^{2+}\)-dependent binding to HL-60 cells. CRDs containing region 5 in combination with either region 3 or region 4 were purified and found to have binding characteristics indistinguishable from those of the region 5 mutant alone (data not shown). These results provide positive evidence that the cluster of basic residues in region 5 plays a role in binding of HL-60 surface ligands. Since wild type MBP-A does not bind HL-60 cells, this binding must reflect a novel activity induced by the presence of region 5.

The nature of the binding activity of the region 5 mutant was
investigated further by testing the ability of this mutant to bind to sialyl-Lewis$^\text{a}$ conjugated to BSA. The iodinated ligand was bound substantially more effectively to plates coated with the region 5 mutant than to wild type mannose-binding protein (Fig. 5). Because of the limited quantities of the neoglycoprotein ligand available, it was not possible to achieve saturation of binding, so an accurate dissociation constant could not be determined. However, the results in Fig. 5 suggest that incorporation of region 5 results in a substantial increase in affinity for the sialyl-Lewis$^\text{a}$ structure. Parallel experiments with 125I-selectin-like binding site

Selectin-like Binding Site

FIG. 4. HL-60 binding to wild type MBP-A and E-selectin and mutants of MBP-A containing E-selectin sequences. Binding experiments were performed in triplicate, with the results of five separate experiments averaged, except in the case of region 3 and 4 mutants, which are the average of two experiments. Error bars represent standard deviations. Results are shown for binding in the presence of 10 mM Ca$^{2+}$ (Ca) or in the presence of 1 mM EDTA (−Ca).

The fact that binding to sialyl-Lewis$^\text{a}$-BSA can be observed in the simple solid phase assay with the region 5 mutant, while it is difficult to demonstrate with natural E-selectin may reflect the trimeric nature of the MBP-A derivative (7). The relatively close clustering of the three binding sites in the trimer probably increases the affinity for the polyvalent test ligand. This result suggests that formation of similar clusters of the E-selectin CRD may be a useful way to increase the affinity of the natural CRD in order to simplify measurement of ligand binding.

Conclusions—In the absence of direct structural information about the mode of sialylated, fucosylated ligand binding to E-selectin, negative mutagenesis data have been the primary source of enlightenment about likely sites of interaction with the sugar ligand. Extensive studies of this type have defined three regions that appear to mediate binding to ligands such as sialyl-Lewis$^\text{a}$. The most definitive phenotypes are associated with changes in tyrosine and arginine residues in region 4 and one of the three lysine residues in region 5 (Fig. 2 (16–18)). The importance of amino groups in region 5 has been further demonstrated using chemical modification (22). A caveat of such studies is the concern that changes distant from the binding site might cause a decrease in affinity for ligand by indirectly affecting the actual binding site, so the negative phenotype cannot be taken to prove direct interaction with ligand. The present studies extend these results by providing positive evidence that inclusion of one of the regions identified by negative mutagenesis can generate specificity for the sialyl-Lewis$^\text{a}$ epitope. While it cannot be stated with certainty that the mode of ligand binding to the region 5 mutant is the same as the mode of binding to E-selectin, analysis of the region 5 mutant complexed with sialyl-Lewis$^\text{a}$ may provide insight into the possible ways that the selectins bind to their ligands.

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![FIG. 5. Solid-phase binding assays wild type and mutant MBP-A probed with $^{125}$I-sialyl-Lewis$^\text{a}$-BSA. Experimental data (filled circles) are shown along with theoretical curves (continuous lines) fitted to the data.](http://www.jbc.org/Downloadedfrom)
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