Mechanism of Ca$^{2+}$ and Monosaccharide Binding to a C-type Carbohydrate-recognition Domain of the Macrophage Mannose Receptor*

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Site-directed mutagenesis has been used to identify residues that ligate Ca$^{2+}$ and sugar to the fourth C-type carbohydrate-recognition domain (CRD) of the macrophage mannose receptor. CRD-4 is the only one of the eight CRDs of the mannose receptor to exhibit detectable monosaccharide binding when expressed in isolation, and it is central to ligand binding by the receptor. CRD-4 requires two Ca$^{2+}$ for sugar binding, like the CRD of rat serum mannose-binding protein (MBP-A). Sequence comparisons between the two CRDs suggest that the binding site for one Ca$^{2+}$, which ligates directly to the bound sugar in MBP-A, is conserved in CRD-4 but that the auxiliary Ca$^{2+}$ binding site is not. Mutation of the four residues at positions in CRD-4 equivalent to the auxiliary Ca$^{2+}$ binding site in MBP-A indicates that only one, Asn$^{728}$, is involved in ligation of Ca$^{2+}$. Alanine-scanning mutagenesis was used to identify two other asparagine residues and one glutamic acid residue that are probably involved in ligation of the auxiliary Ca$^{2+}$ to CRD-4. Sequence comparisons with other C-type CRDs suggest that the proposed binding site for the auxiliary Ca$^{2+}$ in CRD-4 of the mannose receptor is unique. Evidence that the conserved Ca$^{2+}$ in CRD-4 bridges between the protein and bound sugar in a manner analogous to MBP-A was obtained by mutation of one of the amino acid side chains at this site. Ring current shifts seen in the $^1$H NMR spectra of methyl glycosides of mannose, GlcNAc, and fucose in the presence of CRD-4 and site-directed mutagenesis indicate that a stacking interaction with Tyr$^{729}$ is also involved in binding sugars to CRD-4. This interaction contributes about 25% of the total free energy of binding to mannose. C-5 and C-6 of mannose interact with Tyr$^{729}$, whereas C-2 of GlcNAc is closest to this residue, indicating that these two sugars bind to CRD-4 in opposite orientations. Sequence comparisons with other mannose/GlcNAc-specific C-type CRDs suggest that use of a stacking interaction in the binding of these sugars is probably unique to CRD-4 of the mannose receptor.

Many proteins of both plants and animals are involved in recognition of complex carbohydrates attached to proteins or lipids (1, 2). One such protein, the macrophage mannose receptor, binds terminal mannose, fucose, or N-acetylgalactosamine residues of glycoconjugates in a Ca$^{2+}$-dependent manner. The receptor acts as a molecular scavenger by clearing endogenous glycoproteins bearing high mannose oligosaccharides as well as pathogenic microorganisms (1). The extracellular region of the mannose receptor contains an N-terminal cysteine-rich domain and a fibronectin type II repeat as well as eight C-type carbohydrate-recognition domains (CRDs),$^1$ which make it a member of the C-type lectin family (3). The C-type lectin family is a large and diverse group of proteins characterized by homologous CRDs that generally mediate Ca$^{2+}$-dependent sugar recognition (4). Well characterized members of the C-type lectin family include the mammalian mannose-binding proteins (MBPs), the hepatic asialoglycoprotein receptor, and the selectins (1, 2).

Details of how different animal and plant proteins recognize carbohydrates are becoming clearer, due to the availability of crystal structures of the proteins in complex with sugar ligands (2). Crystal structures of two C-type CRDs, those of rat serum MBP (MBP-A), and rat liver MBP (MBP-C) have been solved in complex with sugar ligands (5, 6). The C-type CRD of E-selectin has also been crystallized, but without bound sugar (7). Examination of these crystal structures, combined with other physical techniques and mutagenesis have established some of the molecular mechanisms involved in sugar recognition by C-type CRDs (2). However, little is known about the molecular mechanisms of carbohydrate recognition by the C-type CRDs of the mannose receptor.

Analysis of sugar recognition by the mannose receptor is complicated by the presence of eight different C-type CRDs in a single polypeptide. Apart from the mannose receptor, two other proteins, a phospholipase A$_2$ receptor of muscle and an endocytic receptor called DEC-205, located on dendritic cells, have multiple CRDs in a single polypeptide (8–10). These two proteins are very divergent from other groups of C-type lectins and probably do not bind carbohydrates. The CRDs of the mannose receptor are also quite divergent from the prototypes of the C-type lectin family, but most of them contain at least some of the residues shown to be important for sugar recognition. Of the eight CRDs, only CRD-4 has been shown to bind monosaccharides when expressed in isolation, although other CRDs must contribute to binding of oligosaccharides, since CRDs 4–8 must be present to achieve the affinity of the intact receptor for natural ligands (11, 12).

Since CRD-4 is the smallest piece of the receptor that retains the ability to interact with sugars, an understanding of the molecular mechanism of sugar binding by this CRD would be a first step toward understanding how the whole receptor recog-

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1 The abbreviations used are: CRD, carbohydrate-recognition domain; MBP, mannose-binding protein; MBP-A, serum mannose-binding protein; MBP-C, liver mannose-binding protein; BSA, bovine serum albumin.

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nizes its natural ligands. Ligand binding studies with expressed CRD-4 have shown that the domain requires two Ca\(^{2+}\) for sugar binding and that a ternary complex is formed between protein, sugar, and Ca\(^{2+}\) (13). Ca\(^{2+}\) binding is pH-dependent, and a conformational change in CRD-4 due to loss of Ca\(^{2+}\) binding at low pH probably contributes to release of glycoconjugates by the mannose receptor in endosomes (13).

The fact that CRD-4 requires two Ca\(^{2+}\) for sugar binding is surprising, since sequence comparisons with other C-type CRDs suggest that it might only bind one Ca\(^{2+}\). The most informative comparison is with the extensively studied CRD of rat serum mannose-binding protein (MBP-A). CRD-4 and the CRD of MBP-A show very similar monosaccharide specificities. Although they share only 28% sequence identity overall, it is likely that they interact with sugars in similar ways (13). In the crystal structure of the CRD of MBP-A in complex with an oligosaccharide ligand, one Ca\(^{2+}\) (designated Ca\(^{2+}\) 2) ligation directly to the sugar, while the other (designated Ca\(^{2+}\) 1) is thought to be necessary for the correct positioning of the loops forming the sugar binding site (5). Alignment of the sequences of CRD-4 and MBP-A shows that all of the residues ligating Ca\(^{2+}\) 2 in MBP-A are present in CRD-4, suggesting that one of the two Ca\(^{2+}\) bound to CRD-4 is ligated at a conserved site (Fig. 1).

At this conserved Ca\(^{2+}\) binding site in MBP-A (Fig. 1), each side-chain forms a hydrogen bond to hydroxyl group 3 or 4 of mannose, as well as ligating Ca\(^{2+}\) 2, which also ligation directly to hydroxyl group 3 and 4 of mannose (5). Thus, a ternary complex between protein, sugar, and Ca\(^{2+}\) is formed. The same type of ternary complex is seen in the crystal structure of the CRD of rat liver mannose-binding protein (MBP-C) (6). The conservation of these Ca\(^{2+}\) ligands in CRD-4, combined with the fact that a ternary complex is also formed in CRD-4, suggests that the interaction of protein, Ca\(^{2+}\), and sugar at this site in CRD-4 is very similar to that seen in the mannose-binding proteins (13).

Only one of the four residues ligation the auxiliary Ca\(^{2+}\) in MBP-A is present in CRD-4 (Fig. 1). In crystals of the CRD of MBP-C, two Ca\(^{2+}\) are ligated at the same sites as in MBP-A (6). In contrast, the crystal structure of the CRD of E-selectin reveals bound Ca\(^{2+}\) only at the site corresponding to the conserved Ca\(^{2+}\) in MBP-A, and binding studies in solution confirm that there is a single Ca\(^{2+}\) binding site in this CRD (7, 14). Like CRD-4, the selectin CRD lacks the residues involved in Ca\(^{2+}\) coordination at the auxiliary site in MBP-A. Thus, the mode of binding of the second Ca\(^{2+}\) to CRD-4 must be different from that of many other C-type CRDs.

Although it is probable that the major interaction between sugars and C-type CRDs, including CRD-4 of the mannose receptor, is via direct ligation to a conserved Ca\(^{2+}\), there are likely to be other contacts between the protein and sugar. In MBP-A, interaction between bound sugar and the β-carbon of a histidine residue, His189, contributes significantly to the overall binding energy (15). It is probable that additional contacts between CRD-4 and bound sugar will be different from those seen in MBP-A. In MBP-A, binding of the auxiliary Ca\(^{2+}\) is responsible for correct positioning of loops around the other Ca\(^{2+}\) to which the sugar ligates (5). If, as suggested by the sequence comparisons, the mode of ligation of the auxiliary Ca\(^{2+}\) in CRD-4 is different from that seen in MBP-A, the position of the loops near the sugar binding site is likely to be different.

In order to clarify how CRD-4 of the mannose receptor binds two Ca\(^{2+}\) in the absence of amino acid residues corresponding to one of the sites in MBP-A, site-directed mutagenesis experiments have been undertaken to identify residues involved in ligating each of the two Ca\(^{2+}\) to CRD-4. NMR studies designed to probe the nature of the molecular interactions between CRD-4 and monosaccharides have also been undertaken. The results suggest that one Ca\(^{2+}\) is ligated to CRD-4 in the same way as in MBP-A but that CRD-4 has a unique binding site for the auxiliary Ca\(^{2+}\). In addition to direct ligation of sugar at the conserved Ca\(^{2+}\) site, a hydrophobic stacking interaction between the bound sugar and a tyrosine residue contributes about 25% of the overall binding energy.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine serum albumin, N-tosylphenylethyl chloromethyl ketone-treated trypsin, monosaccharides, D\(_2\)O, and deuterated buffer components were from Sigma. Deuterated imidazole was from Cambridge Isotope Laboratories. Restriction enzymes and other DNA modification enzymes were from New England Biolabs. The Sequenase II kit for DNA sequencing and Na\(^{22}\)I were obtained from Amersham Corp. Mannose-BSA was purchased from EY Laboratories. Volumetric CaCl\(_2\) solution was from BDH, and Immulon 96-well microtiter plates were from Dynatech. Mannose-Sepharose was prepared using the divinylsulfone method (16).

**Mutagenesis Procedures**—Mutagenesis was performed on a fragment corresponding to bases 2000–2467 of a cDNA for the human macrophage mannose receptor (3) inserted into the vector pUC19 grown in Escherichia coli strain HB101. Synthetic oligonucleotides were inserted at appropriate restriction sites using standard recombinant DNA procedures. Modified XhoI-BamHI fragments were transferred into a CRD-4 expression plasmid derived from pNIIlompA3 (11) and transformed into E. coli strain JA221. Plasmids were sequenced to verify the mutations made.

**Protein Expression and Purification**—Mutant proteins were prepared using the procedure developed for wild type CRD-4 (13). Growth, induction, and harvesting of transformed bacteria were exactly as described for wild type CRD-4 (13). The procedure for purification of expressed mutant domains from the bacterial cell lysates was modified slightly to allow for any decrease in sugar affinity due to the mutations. Pelleted bacteria from 1 liter of culture were lysed by sonication in 50 ml of loading buffer (25 mM Tris-HCl, pH 7.8, 1.25 mM NaCl, 3 mM CaCl\(_2\)). The lysate was spun at 45,000 rpm for 1 h in a Beckman 55.2Ti rotor. The supernatant (50 ml) was passed over a 10-ml column of mannose-Sepharose equilibrated with loading buffer. The column was washed with 10 ml of wash buffer (25 mM Tris-HCl, pH 7.8, 1.25 mM NaCl, 20 mM CaCl\(_2\)), followed by a further 15 fractions (2 ml) of wash buffer. The column was eluted with 10 aliquots (2 ml) of elution buffer (25 mM Tris-HCl (pH 7.8), 1.25 mM NaCl, 2.5 mM EDTA). Both wash fractions and elution fractions were analyzed by SDS-polyacrylamide gel electrophoresis, and fractions containing pure domains were pooled for analysis. Under these conditions, wild type CRD-4 binds to the column and only elutes upon the addition of EDTA-containing buffer. Some mutant domains eluted from the column during the wash after the addition of EDTA. Mutant CRDs were classified into four groups with phenotypes characterized by their behavior on the mannose-Sepharose column: (a) wild type or tight binding proteins eluted only after 10–12 ml of elution buffer; (b) intermediate binding proteins started to elute after 24–30 ml of wash buffer; (c) weak binding proteins were seen in all wash fractions; and (d) nonbinding proteins were only detected in the flowthrough and the first 10 ml of wash buffer.

Protein for NMR experiments was further purified by reverse phase high performance liquid chromatography on a C3 column, as described previously (13).

**Proteolytic Digestion**—Bacterial lysates containing expressed proteins were prepared as described above. Lysates were dialyzed extensively against water and freeze-dried. Dried lysates were resuspended to a concentration of 85 mg/ml in 25 mM Tris-HCl (pH 7.8), 1.25 mM NaCl, and either 1 or 25 mM CaCl\(_2\). Trypsin was added to give a concentration of 1 mg/ml, and reactions were incubated at 37°C. Reactions were stopped at various time intervals by the addition of an equal volume of 2 × SDS gel sample buffer containing 20 mM EDTA and 2% 2-mercaptoethanol followed by boiling at 100°C for 5 min. The products of the reaction were analyzed by SDS-gel electrophoresis followed by immunoblotting. Protease-resistant CRD-4 was detected by autoradiography after incubation of the blot with a rabbit polyclonal anti-mannose receptor antibody, raised against a fragment of the receptor consisting of CRDs 4–7 produced in insect cells (12), followed by \(^{125}\)I-labeled Protein A. The resistant material was quantified using the Macintosh Image 1.48 application.

**Ca\(^{2+}\) Binding Assay**—Ca\(^{2+}\)-dependence of mutant CRD-4 binding to...
125I-Man-BSA was determined using a solid phase binding assay described previously for wild type CRD-4 (13). Serial 1:1.5 dilutions of CaCl₂ in 2 × blocking buffer were prepared starting at 150 mM or 50 mM. To these, 125I-Man-BSA was added to give a final ligand concentration of 0.75 μg/ml. Aliquots (75 μl) of these mixtures were then added to duplicate wells of CRD-4-coated plates. At least two assays were performed for each mutant protein. The resulting data were fitted to equations for both first order (Equation 1) and second order (Equation 2) processes using a nonlinear least squares fitting program (SigmaPlot, Jandel Scientific).

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\text{Fraction maximal binding} = \frac{[\text{Ca}^{2+}]}{K_m} + \frac{[\text{Ca}^{2+}]}{K_d}
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\end{equation}

Closeness of fit to each equation was measured by comparing the summed squared residuals.

**Competition Binding Assays**—Competition assays using 125I-Man₃⁻-BSA as test ligand were performed as described previously (11). Values for \(K_d\), corresponding to the concentration of monoasaccharide that gives 50% inhibition of test ligand binding, were calculated using the nonlinear, least squares fitting program. Mean ± S.D. values of three independent assays performed in duplicate were used to calculate \(K_d\) values relative to the \(K_d\) for mannose.

**NMR Binding Assays**—NMR binding assays were performed basically as described for the CRD of MBP-A (15), except that the protein was not exchanged into D₂O. Protein was dissolved in D₂O and adjusted to 25 mM deuterated imidazole–HCl, pH 7.2, 0.5 M NaCl, and 5 mM CaCl₂ using concentrated stock solutions. The final volume was 0.55 ml at a protein concentration of approximately 6–10 mg/ml. Titrations were performed by adding concentrated stock solutions of sugars (0.137 M and 1.1 M) in D₂O. One-dimensional ¹H NMR spectra were recorded on a Varian Unity 500 MHz spectrometer at a temperature of 30 °C. Chemical shifts were measured relative to an internal HDO standard, referenced 4.78 ppm from sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Peak shifts that occurred upon the addition of sugar were analyzed using the following equation.

\[
\Delta \delta = \Delta \delta_0 \times \left( [\text{glycoside}] / ([\text{glycoside}] + K_p) \right)
\]  
\begin{equation}
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\end{equation}

\(\Delta \delta\) is the shift in the peak position at a given glycoside concentration relative to the peak position in the protein spectrum in the absence of sugar, \(\Delta \delta_0\) is the difference in peak positions between the glycoside–protein complex and the free protein, and \(K_p\) is the concentration of glycoside that gives half-maximal change in chemical shift. \(\Delta \delta_0\) and \(K_p\) were calculated using the nonlinear least squares fitting program.

**RESULTS**

**Localization of Ca²⁺ Binding Sites in CRD-4**

**Interaction of CRD-4 with Ca²⁺ and Mannose at the Conserved Site**—To confirm the proposed role of the conserved Ca²⁺ binding site in CRD-4 (Fig. 1), site-directed mutagenesis was used to assess the role of Glu₁₆⁵ in sugar and Ca²⁺ binding. If the pattern of ligation in CRD-4 is the same as in MBP-A, the side chain of Glu₁₆⁵ should form a coordinate bond to Ca²⁺ and a hydrogen bond to hydroxyl 3 or 4 of mannose. Mutation of Glu₁₆⁵ to Gln should produce a domain that does not bind sugar, since the hydrogen bond donor for the sugar hydroxyl has been removed. However, the carboxyl oxygen of Gln can form a coordinate bond with Ca²⁺, so Ca²⁺ binding should be unaffected. A similar phenotype has been seen in MBP-A when Asn₁₈⁷ is mutated to Asp or Glu₁₅⁵ is changed to Gln (5, 15). However, mutation of Glu₁₆⁵ to Ala would perturb Ca²⁺ binding as well as sugar binding.

**Mutation of Glu₁₆⁵ in CRD-4 to either Glu or Ala resulted in domains that do not bind to mannosel-Sepharose, indicating that sugar binding activity is lost. The Ca²⁺ binding properties of these domains were investigated using proteolysis (Fig. 2). Since the lack of sugar binding activity made it difficult to isolate the two mutant domains, protease resistance experiments were carried out on bacterial lysates containing the expressed domains. CRD-4, like MBP-A, is resistant to proteolysis in the presence of Ca²⁺, and two Ca²⁺ must bind to the CRD to render it fully resistant to protease (13). Thus, a mutation affecting Ca²⁺ binding would be expected to produce a domain that is less resistant to proteolysis.

In the presence of 25 mM Ca²⁺, both wild type CRD-4 and the Glu₁₆⁵ → Gln mutant are resistant to proteolysis by trypsin (Fig. 2A). However, the Glu₁₆⁵ → Ala mutant shows susceptibility to proteolysis, with approximately 50% of the protein being digested over the time course of the experiment. In the presence of 1 mM Ca²⁺, all of the domains show susceptibility to proteolysis, but the degree of susceptibility differs (Fig. 2B). For wild type CRD-4 and the Glu₁₆⁵ → Gln mutant, approximately 60% of the protein is still intact after 80 min, but for the Glu₁₆⁵ → Ala mutant the amount of CRD-4 remaining drops to below 50% in only 20 min. Since the Glu₁₆⁵ → Gln mutant shows the same degree of resistance to proteolysis as the wild type CRD-4, at both Ca²⁺ concentrations, this domain must still bind two Ca²⁺. Loss of resistance to protease in the presence of Ca²⁺ by the Glu₁₆⁵ → Ala mutant indicates that this domain does not bind two Ca²⁺.

The phenotypes produced by the two mutations at position 733 suggest that the pattern of ligation at the conserved Ca²⁺
site in CRD-4 is like that of MBP-A rather than E-selectin, in which the equivalent glutamate (Glu88) is replaced as a Ca\(^{2+}\) ligand by a water molecule ligated to an adjacent asparagine side chain (7). Glu733 in CRD-4 must act as a ligand for both Ca\(^{2+}\) and a hydroxyl group of mannose.

Interaction with Ca\(^{2+}\) at the Auxiliary Site—Residues ligating Ca\(^{2+}\) 1 in the crystal structure of MBP-A and residues at the equivalent positions in a molecular model of CRD-4 are shown in Fig. 1. Only the glutamate residue of MBP-A is absolutely conserved in CRD-4. The two tyrosine residues at this site in CRD-4 would not be predicted to be involved in Ca\(^{2+}\) binding, because OH groups of tyrosines have not been seen to ligate Ca\(^{2+}\) in any other Ca\(^{2+}\)-binding proteins (17, 18). Substitution of Asp188 of MBP-A by asparagine in CRD-4 is conservative, because the carboxyl oxygen atom of asparagine can ligate Ca\(^{2+}\). The auxiliary Ca\(^{2+}\) binding site in MBP-C is identical to the site in MBP-A, except that in this case Asp188 is also replaced by asparagine (6). Thus, at the site equivalent to the Ca\(^{2+}\) 1 binding site in MBP-A, the only residues of CRD-4 with side chains that could ligate Ca\(^{2+}\) are Asn728 and Glu706.

To identify amino acid residues involved in ligation of Ca\(^{2+}\) at the auxiliary site in CRD-4, potential Ca\(^{2+}\) ligands were mutated either to alanine or phenylalanine. An initial assessment of whether a mutation alters the Ca\(^{2+}\) binding properties of the domain was made by observing the behavior of the protein on the mannose-Sepharose column used to purify the domains, as described under “Experimental Procedures.” Mutation of Glu706 to Ala or mutation of either Tyr701 or Tyr734 to Phe had no effect on the ability of CRD-4 to bind to mannose-Sepharose; each of these three mutant domains was eluted from the column only in the presence of EDTA. If, as in MBP-A, the role of Ca\(^{2+}\) at the auxiliary site of CRD-4 is in orientation of the loops forming the sugar binding site rather than in direct ligation of sugar, mutations leading to loss of Ca\(^{2+}\) at this site should not abolish sugar binding altogether. Loss of Ca\(^{2+}\) from the auxiliary site would, however, be expected to decrease the affinity of the CRD for Ca\(^{2+}\) at the conserved site, so sugar binding would be affected at low Ca\(^{2+}\) concentrations. Since these three mutants do not show reduced binding to mannose-Sepharose under the conditions used, it seems unlikely that these residues play a role in Ca\(^{2+}\) binding.

In order to confirm this conclusion, the Ca\(^{2+}\) binding properties of the domains were further investigated using a solid phase binding assay that measured Ca\(^{2+}\)-dependent binding of 125I-Man-BSA. By fitting the data to equations for either first or second order dependence on Ca\(^{2+}\), it was possible to determine whether the mutant CRDs bound one or two Ca\(^{2+}\) and to obtain values for the apparent $K_{Ca}$. A mutation that eliminated the auxiliary Ca\(^{2+}\) binding site would be predicted to change the dependence on Ca\(^{2+}\) from second order to first order and to increase the apparent $K_{Ca}$. Ca\(^{2+}\) dependence of binding to 125I-Man-BSA is shown in Fig. 3. Like wild type CRD-4 (Fig. 3A), each of these domains show second order dependence on Ca\(^{2+}\) for binding to 125I-Man-BSA, with values for $K_{Ca}$ almost identical to wild type (Table I). The results indicate that Glu706, Tyr701, and Tyr734 are not involved in...
ligation of Ca$^{2+}$ to CRD-4.

The change Asn$^{728} \rightarrow$ Ala did affect the ligand binding properties of CRD-4. A domain with this mutation shows weak binding to mannose-Sepharose, with protein appearing in Ca$^{2+}$-containing wash fractions before the addition of EDTA. However, the domain retains sufficient sugar-binding activity to allow purification and assaying of binding to 125I-Man-BSA. This domain shows first order dependence on Ca$^{2+}$ for binding to 125I-Man-BSA (Fig. 4B) with an apparent $K_{Ca}$ approximately 10-fold higher than that of wild type (Table I). Since $K_{Ca}$ is measured indirectly, through formation of a ternary complex of Ca$^{2+}$, protein, and sugar ligand, the concentration of the sugar ligand or the affinity of the protein for sugar could affect the apparent $K_{Ca}$ (13). However, the concentration of neoglycoprotein reporter ligand is constant, and a change in a direct interaction between the sugar and protein would not be expected to change the second order dependence on Ca$^{2+}$ and can thus be distinguished from a change in a Ca$^{2+}$ ligand. Therefore, the results in Fig. 4B, which indicate that the Asn$^{728} \rightarrow$ Ala mutant binds only one Ca$^{2+}$, suggest that Asn$^{728}$ directly ligates the auxiliary Ca$^{2+}$ in CRD-4. Thus, only one of the four residues of CRD-4 at positions equivalent to the Ca$^{2+}$ ligands in MBP-A is involved in ligation of Ca$^{2+}$ to CRD-4.

**Identification of Other Potential Ca$^{2+}$ Ligands**—Further mutagenesis was carried out to identify other amino acid residues involved in formation of the auxiliary Ca$^{2+}$ binding site in CRD-4. Since examination of crystal structures of Ca$^{2+}$-binding proteins shows that over 98% of oxygen atoms ligated to Ca$^{2+}$ come from carboxyl and carbonyl groups or water molecules (17, 18), only amino acids with side chain carboxyl or amide groups were investigated as potential Ca$^{2+}$ ligands. The position of residues with acid or amide side chains was also taken into account. Since CRD-4 shows second order dependence on Ca$^{2+}$ for sugar binding, binding of one Ca$^{2+}$ must be influenced by binding of the other. Thus, it is reasonable to suppose that the two Ca$^{2+}$ binding sites in CRD-4 will be close together. For instance, the two Ca$^{2+}$ bound to the CRD of MBP-A are 8.45 Å apart (5). Furthermore, identification of Asn$^{728}$ as a ligand for the auxiliary Ca$^{2+}$ means that other ligands must be close to Asn$^{728}$. The molecular model of CRD-4 predicts Asn$^{728}$ to be 9 Å from the conserved Ca$^{2+}$.

In the molecular model of CRD-4, seven residues with acid or amide side chains are found within 15 Å of the conserved Ca$^{2+}$. These seven residues, together with another two located 18 Å from the conserved Ca$^{2+}$, were selected for mutagenesis. Apart from Asp$^{741}$, all of these residues are also within 18 Å of Asn$^{728}$. Residues as far from the conserved Ca$^{2+}$ as 18 Å were considered to make allowances for possible differences between the structure predicted by the model and the actual structure. Although the β-strands and α-helices predicted by the molecular modeling are likely to be present in the real structure, the positions of loops cannot be predicted with the same level of confidence.

Each of the nine residues described above was mutated to alanine. The results obtained from the solid phase binding assay for the mutated domains are summarized in Table I. Of the nine mutations, only one, Glu$^{737} \rightarrow$ Ala, gives rise to a phenotype similar to that of the Asn$^{728}$ mutant. Like the Asn$^{728}$ mutant, the Glu$^{737}$ mutant shows weak binding to mannose-Sepharose. This domain also shows first order dependence on Ca$^{2+}$ for binding to 125I-Man-BSA (Fig. 4C), indicating that it binds only one Ca$^{2+}$. The apparent $K_{Ca}$ is increased approximately 10-fold compared with that of wild type CRD-4 (Table I). These results suggest that as Asn$^{728}$, Glu$^{737}$ is involved in ligation of the auxiliary Ca$^{2+}$.

**Table I**

| Mutation          | Order of dependence on Ca$^{2+}$ for sugar binding | Apparent $K_{Ca}$ (mM) |
|-------------------|-----------------------------------------------|-----------------------|
| Wild type         | Second                                        | 0.26 ± 0.01           |
| Tyr$^{707}$ → Phe | Second                                        | 0.22 ± 0.09           |
| Glu$^{736}$ → Ala | Second                                        | 0.27 ± 0.02           |
| Tyr$^{734}$ → Phe | Second                                        | 0.25 ± 0.04           |
| Asn$^{728}$ → Ala | First                                         | 2.53 ± 0.34           |
| Glu$^{737}$ → Ala | First                                         | 2.04 ± 0.30           |
| Glu$^{719}$ → Ala | Second                                        | 0.24 ± 0.0             |
| Asn$^{720}$ → Ala | Second                                        | 0.20 ± 0.0             |
| Glu$^{736}$ → Ala | Second                                        | 0.16 ± 0.02            |
| Asp$^{711}$ → Ala | Second                                        | 0.11 ± 0.01            |
| Glu$^{737}$ → Ala | Second                                        | 0.11 ± 0.01            |
| Asn$^{735}$ → Ala | Second                                        | 0.23 ± 0.08            |
| Asn$^{731}$ → Ala | Second                                        | 0.63 ± 0.03            |
| Asn$^{750}$ → Ala | Second                                        | 0.47 ± 0.02            |
| Asn$^{728}$ → Ala/Asn$^{750}$ → Ala | First | 1.74 ± 0.16 |
| Asn$^{728}$ → Ala/Asn$^{750}$ → Ala | Second | 1.05 ± 0.18 |
Mutation of six residues (Glu719, Asn720, Gln730, Asp741, Glu752, and Asn755) has no effect on binding of Ca$^{2+}$ or sugar. Domains with these residues mutated bind tightly to mannose-Sepharose and show second order dependence on Ca$^{2+}$ for binding to $^{125}$I-Man-BSA, with values for apparent $K_Ca$ very similar to wild type CRD-4 (Table I). The results indicate that side chains of these six residues are not acting as ligands for Ca$^{2+}$.

Mutation of the remaining two potential ligands for the auxiliary Ca$^{2+}$, Asn731 and Asn750, gave rise to domains that still show second order dependence on Ca$^{2+}$ for binding to $^{125}$I-Man-BSA, with values for apparent $K_Ca$ very similar to wild type CRD-4 (Table I). The results indicate that side chains of these six residues are not acting as ligands for Ca$^{2+}$.

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The hypothesis that Asn$^{731}$ and Asn$^{750}$ contribute to binding the auxiliary Ca$^{2+}$ was investigated further by making a double mutant in which both of these residues were changed to alanine. Ca$^{2+}$ dependence of binding to $^{125}$I-Man-BSA by this domain is shown in Fig. 5C. The double mutation causes a phenotype very similar to that of the Asn$^{728}$ or Glu$^{737}$ single mutations. Thus, the double mutant shows first order dependence on Ca$^{2+}$ for sugar binding, with a greatly increased apparent $K_Ca$ (Table I) and shows weak binding to mannose-Sepharose. The binding curve for a double mutant containing Asn$^{731}$ and Asn$^{750}$ both changed to alanine, prepared as a control, is also shown in Fig. 5D. This double mutant still binds to the mannose-Sepharose column in the same way as the domain with the Asn$^{731}$ → Ala single mutation and displays second order dependence on Ca$^{2+}$ for binding to $^{125}$I-Man-BSA, although there is a small rise in apparent $K_Ca$ when compared with to the Asn$^{731}$ → Ala mutant (Table I). These results support the hypothesis that side chains of Asn$^{731}$ and Asn$^{750}$ are involved in ligation of the auxiliary Ca$^{2+}$ but that they contribute less to the overall binding energy than the side chains of Asn$^{728}$ and Glu$^{737}$.

Monosaccharide-Protein Interactions

Determination of Sugar Dissociation Constants by NMR—An NMR-based assay has been used to study the interaction of CRD-4 with monosaccharides. This type of assay allows the determination of direct binding constants for relatively weak interactions and has been used previously to study sugar binding by the CRD of MBP-A (15) as well as by various plant lectins (19) and influenza hemagglutinin (20). Inhibition assays, which measured inhibition of binding to CRD-4 by monosaccharides in a solid phase assay, indicate that the affinity of CRD-4 for monosaccharides is in the 2–8 mM range (11), but this type of assay cannot be used to determine actual dissociation constants. In the NMR assay, binding of a sugar to the protein causes a change in the chemical environment of some protons in both the sugar and the protein, and the resultant changes in chemical shifts of these resonances can be quantified and used to derive binding constants for the interaction in solution. Since sufficient amounts of both wild type and mutant CRD-4 could be produced to give samples of 0.2–1 mM, shifts in the protein spectrum were used to derive binding constants in this study.

The $^1$H NMR spectrum of CRD-4 has good dispersion, characteristic of a folded protein (not shown). When $\alpha$-methyl mannoside ($\alpha$-Me-Man) was titrated in, shifts of several peaks were observed, but the greatest change occurred in a resonance in the aromatic region of the spectrum at about 8.2 ppm and in the

![Graphical representation of Ca$^{2+}$ concentration vs. binding fraction for different mutants](image-url)
Ca\textsuperscript{2+} and Sugar Binding by the Macrophage Mannose Receptor

The dissociation constants obtained for the methyl glycosides show that CRD-4 binds α-Me-Man more tightly than α-Me-GlcNAc and β-Me-Fuc. These results are in good agreement with inhibition studies with free monocarbohydrates in a solid phase assay, where mannose and fucose showed approximately equal inhibition of neoglycoprotein binding to CRD-4, with GlcNAc being a less potent inhibitor (11). The results are also similar to the dissociation constants obtained by NMR for binding of the same methyl glycosides to the CRD of MBP-A (15). However, CRD-4 shows a strong preference for α-Me-Fuc, which binds 10 times more tightly than β-Me-Fuc.

**Effects of Binding on Sugar Spectra**—Although shifts in the protein spectrum were used to derive dissociation constants, analysis of the sugar spectra during the titrations also proved to be informative about the interaction between sugar and CRD-4. Changes occurring in the spectrum of α-Me-Man in the presence of CRD-4 are shown in Fig. 8A. At low concentrations of sugar, when a high proportion of the sugar is bound by the protein, large shifts are seen in the resonances for some protons. The resonances for H-6, H-6′, and H-5 are broadened and suppressed. The resonance broadening is due to the difference in chemical shift between the protons in the bound and free state. The decrease in the height of the peaks for H-6, H-6′, and H-5 relative to those for H-2, H-3, and H-4 indicates that the first set of protons are shifting much more in the bound state. The effect on the H-6 resonances is quite obvious, because these peaks are well resolved. The resonances for H-3 and H-6′ overlap, but at low concentrations of sugar only the two double peaks of H-3 are present, with the two double peaks representing H-6′ gradually reappearing as the concentration of unbound sugar increases. Similarly, the triplet representing H-4 overlaps with the complex of peaks (eight in total in a well resolved spectrum) representing H-5, but at low concentrations of sugar only the H-4 triplet is seen, with the H-5 resonances gradually reappearing with increasing concentration of sugar. Such large shifts in resonances are characteristic of the ring current effect induced by proximity to an aromatic ring (22). The data suggest that when bound to CRD-4, C-5 and C-6 of mannose are located close to an aromatic residue.

The ring current effect is also seen in the spectra of the other glycosides tested. When α-Me-GlcNAc interacts with CRD-4, the most marked effect is on the H-2 resonances, which are suppressed relative to H-6, H-6′, and H-3 (Fig. 8D). The O-methyl group resonance is also suppressed when α-Me-GlcNAc binds to CRD-4 (not shown). Therefore, α-Me-GlcNAc bound to CRD-4 must be orientated such that C-2 and the O-methyl group are close to an aromatic residue. In the spectrum of α-Me-Fuc, the greatest ring current effect is seen on the O-methyl resonance, with smaller effects on H-1 and H-5, whereas the ring current effect is not apparent in the spectrum of β-Me-Fuc (data not shown).

**Role of Tyr\textsuperscript{729} in Sugar Binding by CRD-4**—Comparison of the sequence of CRD-4 and MBP-A suggests that the residue most likely to be causing the ring current effect is Tyr\textsuperscript{729}. This residue is at the position corresponding to His\textsuperscript{189} of MBP-A (Fig. 1). In the crystal structure of MBP-A, both the β-carbon and the imidazole ring of His\textsuperscript{189} are in van der Waals contact.

Fig. 6. Shifts in resonances in the spectrum of CRD-4 occurring upon the addition of α-Me-Man. A, histidine proton. B, Methyl proton. Downfield shifts in the histidine proton and upfield shifts in the methyl proton marked with asterisks were used to quantify sugar binding.
with mannose bound to Ca$^{2+}$, but mutagenesis combined with NMR have shown that only the β-carbon contributes significantly to the net binding energy (15). No ring current shifts are seen in the spectrum of α-Me-Man in the presence of MBP-A (15). However, in most galactose-binding C-type CRDs, tryptophan is present at the position corresponding to His$^{189}$ of MBP-A (4). NMR analysis of a version of MBP-A that has been mutated to incorporate tryptophan at position 189 so that it binds galactose does show a ring current shift in the spectrum of galactose, indicative of an interaction between galactose and this tryptophan (23). Sequence comparisons and molecular modeling show that the Ca$^{2+}$ binding site of MBP-A is conserved in CRD-4, and data presented above provide evidence that mannose binds at this site. Thus, it is likely that Tyr$^{729}$ will be close enough to interact with sugars bound at the conserved Ca$^{2+}$ site.

Site-directed mutagenesis was used to determine the role of Tyr$^{729}$ in sugar binding by CRD-4. Domains with Tyr$^{729}$ changed to alanine, leucine, or histidine showed reduced binding to mannose-Sepharose but bound well enough that they could be purified for analysis by NMR. In contrast, a domain with glycine at position 729 did not bind to mannose-Sepharose, so it could not be purified for further analysis. CRD-4 with phenylalanine at position 729 showed wild type binding to mannose-Sepharose and was also analyzed by NMR.

Binding curves obtained from titrations of mutated domains with α-Me-Man are shown in Fig. 9. The binding constants derived from these data are summarized in Table III. For each mutant domain, the maximum shifts in the histidine resonance and in the methyl proton resonance were similar to those for wild type CRD-4, except that the methyl proton resonance shifted less in the Tyr$^{729}$ → Leu mutant. These data suggest that the sugar binding site is not grossly rearranged by any of the mutations.

The change Tyr$^{729}$ → Ala decreases the affinity of CRD-4 for α-Me-Man by a factor of 4 (Table III), indicating that interactions with the side chain of Tyr$^{729}$ must contribute significantly to the binding energy. Mutation of Tyr$^{729}$ to Phe has no effect on α-Me-Man binding, indicating that the hydroxyl group of Tyr$^{729}$ does not play a role in sugar binding. Mutation of Tyr$^{729}$ to His causes less than 2-fold decrease in affinity for α-Me-Man, indicating that the imidazole ring of histidine can interact with bound sugar in a similar manner to the aromatic ring of tyrosine and phenylalanine. With leucine at position 729, the affinity for α-Me-Man is decreased somewhat more, indicating that a hydrophobic, nonaromatic side chain can also partially compensate for the loss of the aromatic ring of tyrosine.

Inspection of the spectrum of α-Me-Man in the presence of the mutated domains confirms that interaction of the sugar with Tyr$^{729}$ causes the ring current shifts observed in the
presence of wild type CRD-4. No suppression of resonances for the H-6', H-6, and H-5 protons of α-Me-Man or of any other resonances is seen in the presence of the Tyr$^{729}$ → Ala or Tyr$^{729}$ → Leu mutants (Fig. 10, A and B). In contrast, in the presence of CRD-4 with histidine at position 729, suppression of peaks for the H-6, H-6', and H-5 resonances of α-Me-Man is observed, indicating that histidine at this position causes a similar ring current effect to tyrosine (Fig. 10C). The ring current effect is also seen in the spectrum of α-Me-Man in the presence of CRD-4 with mutation of Tyr$^{729}$ to Phe (data not shown).

NMR titrations with other glycosides were not performed for the mutant domains. However, inhibition of mannose-BSA binding to wild type CRD-4 and CRD-4 with the change Tyr$^{729}$ → Ala in a solid phase assay was used to show that Tyr$^{729}$ is...
important for binding to sugars other than mannose. Values of $K_i$ obtained for mannose and GlcNAc are shown in Table IV. Comparison of the absolute values of $K_i$ obtained for wild type CRD-4 and the domain with alanine at position 729 is not meaningful, since the affinity of the mutant domain for the reporter ligand is greatly reduced. Comparison of the relative inhibitory potencies of mannose and GlcNAc, given by $K_i,\text{GlcNAc}/K_i,\text{mannose}$, gives similar values for the wild type and mutated domains. Since the NMR assay showed that mutation of Tyr729 to Ala decreases the affinity for mannose by a factor of 4 when compared with wild type CRD-4, the affinity for GlcNAc must also decrease by a factor of 4 for the value of $K_i,\text{GlcNAc}/K_i,\text{mannose}$ to be the same as that of wild type. These data, combined with the fact that ring current shifts are observed in the spectra of other sugars in the presence of CRD-4, make it possible to conclude that interaction with Tyr729 is an important factor in binding of GlcNAc and fucose as well as of mannose to CRD-4.

$\Delta G$ values for binding of $\alpha$-Me-Man to wild type and mutated CRD-4 can be calculated from the values for the dissociation constant obtained in this study. Comparison of $\Delta G$ values for wild type CRD-4 (−15.1 KJ/mol) and for the Tyr729→Ala mutant (−11.5 KJ/mol) shows that interaction of the sugar with the aromatic ring of Tyr729 provides 3.6 KJ/mol (24%) of the total free energy of binding. The contribution to free binding energy by this interaction in CRD-4 is similar to that of the $\beta$-carbon of His189 to binding of mannose by MBP-A (15). Since mutation of Tyr729 to Gly resulted in a domain that could not be purified, it is not possible to say whether the $\beta$-carbon of Tyr729 is also important for sugar binding to CRD-4. Lack of binding to mannose-Sepharose could be due to loss of affinity because interactions with the $\beta$-carbon as well as the aromatic ring contribute to sugar binding, but it could also be due to misfolding of the domain.

DISCUSSION

Localization of $Ca^{2+}$ Binding Sites in CRD-4

Conserved $Ca^{2+}$ Sites in C-type Lectins—Most other C-type CRDs for which $Ca^{2+}$-dependent sugar binding activity has been demonstrated contain the five residues (two glutamic acids, two asparagines, and an aspartic acid) present at the conserved $Ca^{2+}$ site in MBP-A, E-selectin, and CRD-4, except that in CRDs that bind galactose rather than mannose, one glutamate and one asparagine are changed to glutamine and aspartate, respectively (4, 24). Although each of the CRDs will probably have additional contacts with the bound sugar, such as that seen between the $\beta$-carbon of His189 of MBP-A and mannose (15), it is likely that in each case, the main interaction with sugar will be via direct ligation to $Ca^{2+}$ at this conserved site. Either the MBP-A pattern of ligation or the alternative ligation pattern seen in E-selectin could be utilized. However, the presence of the ligands for the conserved $Ca^{2+}$ in a CRD
does not necessarily mean that this CRD will bind sugar. The type II antifreeze protein of herring, which is homologous to C-type CRDs, contains all of the ligands for the conserved Ca\(^{2+}\) and does bind one Ca\(^{2+}\), probably at this site, but it does not bind carbohydrate (25). Equally, some of the more divergent groups of C-type lectins, including the type II transmembrane proteins found on natural killer cells, may utilize a completely different mechanism for binding sugar and/or Ca\(^{2+}\), since these proteins do not contain the ligands for the conserved Ca\(^{2+}\) (4, 26, 27).

Position of Proposed Auxiliary Ca\(^{2+}\) Binding Site in CRD-4—The positions of potential Ca\(^{2+}\) ligands mutated in this study are shown on the molecular model of CRD-4 in Fig. 11. Four residues, Asn\(^{728}\), Asn\(^{731}\), Glu\(^{737}\), and Asn\(^{750}\), are predicted, based on the mutagenesis results, to be involved in ligation of the auxiliary Ca\(^{2+}\) (Fig. 11A). Mutagenesis of nine other potential Ca\(^{2+}\) ligands (Fig. 11B), including three of the four residues at positions equivalent to the Ca\(^{2+}\) ligands in MBP-A, has no effect on Ca\(^{2+}\) binding. The results of the mutagenesis of potential Ca\(^{2+}\) ligands in CRD-4 indicate that the auxiliary Ca\(^{2+}\) of CRD-4 is not ligated at the same site as in MBP-A or in the same way.

Three of the four residues predicted to be ligands for the auxiliary Ca\(^{2+}\), Asn\(^{728}\), Asn\(^{731}\), and Asn\(^{750}\), are located close to each other in the model structure. In this orientation, it would be possible for them to form a Ca\(^{2+}\) binding site. The fourth residue, Glu\(^{737}\), located on \(\beta\)-strand 3, is too far away from the other three residues for it to be part of a Ca\(^{2+}\) binding site involving all four residues. Although Fig. 11 is only a model of CRD-4, based on the structure of a protein with which it shares 28% sequence identity, it is difficult to imagine sufficient rearrangement of \(\beta\)-strand 3 to bring Glu\(^{737}\) close enough to take part in direct Ca\(^{2+}\) ligation with the other three residues.

If Glu\(^{737}\) is not coordinated directly to the auxiliary Ca\(^{2+}\), then other possibilities must be considered to explain the phenotype caused by mutation of this residue to alanine. In the CRDs of E- and P-selectin, glutamate is also found at the position equivalent to Glu\(^{737}\). In the crystal structure of E-selectin, this glutamate residue (Glu\(^{92}\)) forms an ion pair with a lysine residue (Lys\(^{113}\)) and a hydrogen bond with the amide group of Asn\(^{105}\), which is a ligand for the conserved Ca\(^{2+}\) (7). Mutation of this glutamate to alanine in both E- and P-selectin reduces their ability to bind to sugar ligands (28, 29). It seems likely that this reduction in binding ability is due to loss of the hydrogen bond between Glu\(^{92}\) and Asn\(^{105}\) and that this affects the ability of the domain to ligate Ca\(^{2+}\) at the conserved site. By analogy, it might be proposed that the Glu\(^{737}\) \(\rightarrow\) Ala change in CRD-4 indirectly affects the ability of the domain to bind the conserved Ca\(^{2+}\) and not the auxiliary Ca\(^{2+}\). However, it is difficult to rationalize the phenotype of the Glu\(^{737}\) \(\rightarrow\) Ala mutation with an effect on binding of the conserved Ca\(^{2+}\). This mutant domain shows first order dependence on Ca\(^{2+}\) for sugar binding, indicating that it is binding only one Ca\(^{2+}\), and its

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**TABLE IV**

Comparison of inhibition by mannose and GlcNAc for wild type CRD-4 and Tyr\(^{729}\) \(\rightarrow\) Ala mutant CRD-4

| Mutant          | \(K_i\), mannose | \(K_i\), GlcNAc | \(K_i\), GlcNAc/\(K_i\), mannose |
|-----------------|------------------|----------------|---------------------------------|
| Wild type       | 7.0 ± 1.2        | 11.8 ± 0.5     | 1.7                             |
| Tyr\(^{729}\) \(\rightarrow\) Ala | 2.6 ± 0.1        | 4.1 ± 1.0      | 1.6                             |

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**Fig. 10.** Changes in resonances of \(\alpha\)-Me-Man protons in the presence of CRD-4 mutated at position 729. A, spectrum of \(\alpha\)-Me-Man in the absence and presence of CRD-4 with histidine at position 729. B, spectrum of \(\alpha\)-Me-Man in the absence and presence of CRD-4 with leucine at position 729. C, spectrum of \(\alpha\)-Me-Man in the absence and presence of CRD-4 with histidine at position 729.
sugar binding activity is reduced. Assuming that sugar binds to the conserved Ca\(^{2+}\), the fact that the domain retains the ability to bind sugar, albeit weakly, indicates that it must be the conserved Ca\(^{2+}\) that is present and the auxiliary Ca\(^{2+}\) that is absent. Thus, it is most likely that mutation of Glu\(^{737}\) affects binding of the auxiliary Ca\(^{2+}\).

It is possible that the interaction between Glu\(^{737}\) and the auxiliary Ca\(^{2+}\) is not direct but involves bridging via a water molecule as has been seen in a number of Ca\(^{2+}\)-binding proteins including E-selectin (7). A modest change in the side chain conformation predicted by the model would suffice to bring Glu\(^{737}\) into a position where it could be involved in ligation of the auxiliary Ca\(^{2+}\) through an intermediary water molecule. Alternatively, it is possible that Glu\(^{737}\) is involved in an interaction that orients a backbone carbonyl oxygen atom that interacts directly with the auxiliary Ca\(^{2+}\). In MBP-A, backbone carbonyl oxygens are seen to be involved in the ligation of both Ca\(^{2+}\).

The phenotypes caused by mutation of the other three proposed auxiliary Ca\(^{2+}\) ligands could also be explained by indirect effects. However, all other side chains with the potential to ligate Ca\(^{2+}\) located in the top half of the molecule have been mutated with no effect on Ca\(^{2+}\) binding. Thus, it seems likely that at least some of the four amino acids proposed to form the auxiliary Ca\(^{2+}\) binding site must coordinate directly to Ca\(^{2+}\).

7-Fold co-ordination of Ca\(^{2+}\) is usually seen in Ca\(^{2+}\)-protein complexes (30). In MBP-A, the shell of ligands for the auxiliary Ca\(^{2+}\) is supplied by the two carboxylate oxygens of the side chain of Glu\(^{193}\), one carboxylate oxygen from the side chains of each of the three aspartate residues, the backbone carbonyl oxygen of Glu\(^{193}\), and the oxygen of a water molecule (5). It is possible that an eighth ligand is supplied by the other carboxylate oxygen of Asp\(^{161}\). Assuming that the three asparagine residues proposed to be involved in ligation of the auxiliary Ca\(^{2+}\) to CRD-4 make direct contact with the Ca\(^{2+}\) but that Glu\(^{737}\) does not, three ligands for the Ca\(^{2+}\) would be supplied. The oxygen atom from the carbonyl group of each of the three asparagine residues could form bonds to the Ca\(^{2+}\). This means that four bonds must be supplied by either backbone carbonyl oxygens or water molecules.

*Function of the Auxiliary Ca\(^{2+}\) in CRD-4*—The phenotypes produced by mutation of residues at this site in CRD-4 suggest that, as in MBP-A, the auxiliary Ca\(^{2+}\) in CRD-4 is required for correct positioning of loops around the sugar binding site but is not involved in direct ligation of sugar. The different modes of ligation of the auxiliary Ca\(^{2+}\) seen in CRD-4 of the mannose receptor and MBP-A might be related to the different functions of the two proteins. The mannose receptor is an endocytic receptor, which must release its ligands in the acidic environment of the endosome. Since stability of Ca\(^{2+}\) binding to CRD-4 is pH-dependent, loss of Ca\(^{2+}\) binding at low pH probably contributes to release of glycoconjugates by the mannose receptor in the endosomes (13). It is interesting to note that the magnitude of the increase (approximately 10-fold) in \(K_c\) seen when binding of the auxiliary Ca\(^{2+}\) to CRD-4 is disrupted by mutation is the same as the increase in \(K_c\) seen when the pH is reduced from 7.8 to 5.0 (13). Thus, it can be imagined that a change in conformation in CRD-4 at low pH causes loss of Ca\(^{2+}\) from the auxiliary site, which leads to a decrease in affinity for Ca\(^{2+}\), so that the conserved Ca\(^{2+}\) and its bound sugar are released. In contrast, MBP-A, which is a soluble extracellular protein that initiates fixation of complement after binding to carbohydrates on the surface of pathogens, is not known to require pH-dependent release of Ca\(^{2+}\) or sugar (1).

*Comparison with Other C-type CRDs*—These studies increase to three the different modes of Ca\(^{2+}\)-dependent sugar binding to C-type CRDs. The structure of E-selectin reveals that the conserved Ca\(^{2+}\) alone can support sugar binding, while the mannose-binding proteins and CRD-4 of the mannose receptor demonstrate that there are at least two alternative ways in which an auxiliary site can be formed.

Two other CRDs, those of the chicken hepatic lectin and the rat asialoglycoprotein receptor, have been shown to bind two Ca\(^{2+}\) in solution but have not yet been crystallized (31, 32). These CRDs have the same residues as the MBPs at both Ca\(^{2+}\)-binding sites (4). Examination of the sequences of C-type CRDs for which Ca\(^{2+}\)-dependent sugar binding has been demonstrated, but the stoichiometry of the requirement for Ca\(^{2+}\) has not been determined, indicates that the ligands for the auxiliary Ca\(^{2+}\) of the MBPs are present in most of them (4). Of these CRDs, only those of the placental mannose receptor and the bovine lectin CL-43 contain more than two of the ligands for the auxiliary Ca\(^{2+}\) in CRD-4, and these only contain three (33, 34). Therefore, it is probable that if these proteins do bind a second Ca\(^{2+}\), it will be ligated in a similar manner to the auxiliary Ca\(^{2+}\) of the MBPs.

So far, only the CRDs of E-selectin and of the type II antifreeze protein of herring have been shown to bind only one Ca\(^{2+}\) (7, 25). Solution binding studies on another member of
the selectin family, P-selectin, have shown that this protein binds two Ca$^{2+}$ (35). However, this study was carried out on the whole protein, not just the CRD, so it is possible that one of the Ca$^{2+}$ is bound to the epidermal growth factor-like domain or to one of the complement-regulatory domains. Residues found at positions equivalent to the auxiliary Ca$^{2+}$ binding site of MBP-A are Lys, Asn, Asn, and Asp in E-selectin and P-selectin, and Lys, Gly, Asn, and Asp in L-selectin. Only two of the four proposed ligands for the auxiliary Ca$^{2+}$ in CRD-4 are present in each of the selectins (4). Thus, it seems unlikely that any of the selectins will bind a second Ca$^{2+}$ at either the auxiliary site of MBP-A or the proposed auxiliary Ca$^{2+}$ site of CRD-4.

Ca$^{2+}$-dependent sugar binding has not yet been demonstrated for many of the more divergent members of the C-type lectin family, including other CRDs of the mannosereceptor. Of the eight CRDs of the mannosereceptor, apart from CRD-4, only CRD-5 contains all of the conserved Ca$^{2+}$ binding site residues of MBP-A, indicating that Ca$^{2+}$ and sugar could be ligated at this site. Sugar binding activity was not detected for CRD-5 when it was expressed alone, but binding studies with different combinations of expressed mannosereceptor CRDs indicate that CRD-5, as well as CRDs 6, 7, and 8 must have the ability to interact with sugar (11, 12). Neither the auxiliary Ca$^{2+}$ binding site of the MBPs or the proposed auxiliary site of CRD-4 is conserved in CRD-5 or in the other six mannose receptor CRDs. Thus, the proposed method of ligation of the auxiliary Ca$^{2+}$ in CRD-4 is not a general mechanism for Ca$^{2+}$ ligation at this site in the other mannosereceptor CRDs. However, the residues identified here as ligands for the auxiliary Ca$^{2+}$ in CRD-4 of the human mannosereceptor are all conserved in CRD-4 of the mouse mannosereceptor (36).

It is also not yet clear whether the multiple CRDs of the phospholipase A$_2$ receptor and of DEC-205 are capable of Ca$^{2+}$-dependent sugar binding. No studies of ligand binding to DEC-205 have been reported. The phospholipase A$_2$ receptor has been shown to bind neoglycoprotein ligands, but this binding is not Ca$^{2+}$-dependent (8). Since the secretory phospholipases that the phospholipase A$_2$ receptor binds with high affinity are not glycosylated, the physiological relevance of sugar binding by this receptor is unclear (8, 9). Few of the ligands for either the conserved Ca$^{2+}$ or the auxiliary Ca$^{2+}$ of the MBPs or CRD-4 are present in the CRDs of the phospholipase A$_2$ receptor or DEC-205 (8–10). If the CRDs of these two proteins do bind sugar and/or Ca$^{2+}$, it must be by a different mechanism from that seen in MBP-A or CRD-4 of the mannosereceptor.

From the comparisons above, it can be seen that the proposed binding site for the auxiliary Ca$^{2+}$ in CRD-4 is not conserved in other CRDs, indicating that this is not a general mechanism for Ca$^{2+}$ binding to CRDs that do not contain the ligands for the auxiliary Ca$^{2+}$ of MBP-A. Clearly, sequence comparisons alone are insufficient for determining the Ca$^{2+}$ binding characteristics of a C-type CRD, and physical techniques will have to be applied.

Monosaccharide-Protein Interactions

Stacking Interactions in Protein-Carbohydrate Complexes—The stacking interaction between the aromatic ring of Tyr$^{729}$ and bound sugar in CRD-4 is a common feature of protein-carbohydrate complexes (2, 37). In all structures of proteins in complex with galactose, including plant lectins, bacterial toxins, animal S-type lectins, and the galactose-binding mutant of MBP-A, the nonpolar B-face of galactose stacks against tryptophan or phenylalanine residues (2). However, stacking of mannosesecondary amino acid residue, as predicted for binding of mannose to CRD-4 (38). In other mannose-specific legume lectins, C-6 and C-5 stack against a phenylalanine residue (39, 40). Stacking of mannose is not seen in the snowdrop lectin or in either MBP-A or MBP-C (5, 6, 41). MBP-C has been co-crystallized with GlcNAc and fucose as well as mannose. These sugars bind at the same site as mannose, and neither of them stack against an aromatic ring. Only one other GlcNAc-binding lectin, wheat germ agglutinin, has been crystallized with GlcNAc, and in this case stacking of GlcNAc against tyrosine is seen (42). Thus, while there is precedent for stacking of mannose and GlcNAc in plant lectins, CRD-4 of the mannosereceptor provides the first example of this type of interaction with these sugars in an animal lectin.

Sequence comparisons of C-type CRDs allows prediction of whether stacking interactions with bound sugar will be seen in those that have not yet been analyzed, either by crystallography or by NMR binding studies. All C-type CRDs shown to bind galactose have an aromatic residue at the position equivalent to Tyr$^{729}$ of CRD-4 and His$^{189}$ of MBP-A (4, 26), and tryptophan at this position is seen to stack against galactose in the crystal structure of the MBP-A galactose-binding mutant (43). Thus, stacking of galactose is likely to be a common feature of all galactose-binding C-type CRDs.

Of the mannose/fucose/GlcNAc-specific C-type CRDs, none apart from MBP-A has an aromatic residue at the position equivalent to Tyr$^{729}$ (4, 26). His$^{189}$ at this position in MBP-A, however, is splayed away from bound mannose and makes only an edgewise contact with the 2-hydroxy group (5). The only C-type lectin to show strong preference for GlcNAc rather than mannose, the chicken hepatic lectin, also lacks an aromatic residue at this position (26). It is also interesting to note the absence of aromatic residues in the corresponding position of the other CRDs of the mannosereceptor (3). Therefore, CRD-4 of the mannosereceptor appears to be the only known C-type CRD in which hydrophobic stacking against an aromatic residue is important for binding of mannose, GlcNAc, or fucose.

Position of Tyr$^{729}$ in CRD-4—The data presented here show that replacing Tyr$^{729}$ of CRD-4 with histidine has only a small effect on the ability of the domain to bind mannose and that C-5 and C-6 can interact with the imidazole ring in the same way as with the aromatic ring of tyrosine. It is therefore probable that the exact position of Tyr$^{729}$ in CRD-4 is different from that of His$^{189}$, the equivalent residue in MBP-A, bringing it closer to the bound sugar. In galactose-binding C-type CRDs, a glycine-rich loop is inserted adjacent to the tryptophan that stacks against galactose. This glycine-rich loop, which is not present in the mannose/fucose/GlcNAc-specific CRDs, including CRD-4, locks the tryptophan residue into the optimal position for stacking against galactose (23, 43). It seems that a difference between CRD-4 and MBP-A in the arrangement of the loops in this area of the molecule must be responsible for positioning Tyr$^{729}$ so that it can stack against bound sugars. This difference in the arrangement of loops around the sugar binding site could be brought about by the different modes of ligation of the auxiliary Ca$^{2+}$ in the two proteins.

Orientation of Monosaccharides Bound to CRD-4—Observation of which protons of each sugar are affected by the ring current of Tyr$^{729}$ allows some conclusions to be made about the orientation of monosaccharides bound to CRD-4. When α-Man is bound, C-5 and C-6 stack against Tyr$^{729}$, whereas it is C-2 and the O-methyl group that interact with Tyr$^{729}$ when α-Me-GlcNAc is bound. Assuming that each of these two sugars is ligated to the conserved Ca$^{2+}$ via hydroxyls 3 and 4, as is the case in the MBPs, then bound GlcNAc must be rotated 180° relative to bound mannose.
The orientations of α- and β-Me-Fuc, which would ligate to Ca²⁺ via hydroxyls 2 and 3, would also be predicted to differ by 180° with respect to each other, since C-1 and the O-methyl group of α-Me-Fuc interact with Tyr⁷²⁹, whereas no ring current effect is apparent when β-Me-Fuc binds. Thus, in bound α-Me-Man and β-Me-Fuc, C-5 and C-6 would be positioned near Tyr⁷²⁹, while C-1 and C-2 of α-Me-GlcNAc and α-Me-Fuc would be closest to the tyrosine. The difference in bound orientation of α- and β-methyl fucosides and the consequent lack of interaction of β-Me-Fuc with Tyr⁷²⁹ probably account for the 10-fold tighter binding of α-Me-Fuc.

Although both MBP-A and MBP-C bind GlcNAc and fucose as well as mannose, only MBP-C has been crystallized in complex with all three sugars (6). Unlike what appears to be the case in CRD-4, mannose and GlcNAc and the two anomers of fucose bind in the same orientation to MBP-C. However, mannose bound to MBP-A is rotated 180° with respect to mannose and GlcNAc bound to MBP-C (5). In MBP-C, C-5 and C-6 of these two sugars are closest to Val¹¹⁹⁴, the residue equivalent to Tyr⁷²⁹, whereas in MBP-A, C-1 and C-2 of mannose are closest to His¹⁸⁹. Since the position of Tyr⁷²⁹ is likely to be shifted slightly compared with that of the equivalent residue in each of the MBPs, it is not possible to predict with certainty whether the absolute orientation of mannose, with respect to the binding of hydroxyls 3 and 4, in CRD-4 is the same as in MBP-A or as in MBP-C. Nevertheless, it is clear that the relative orientations of various sugars bound to CRD-4 must be different from either MBP.

Conclusions—While the major interaction between sugars and CRD-4 is via direct ligation to a conserved Ca²⁺, as is seen in the mannose-binding proteins, other aspects of sugar and Ca²⁺ binding to CRD-4 are different. CRD-4 ligates a second Ca²⁺ in a unique way, and this difference in the mode of ligation of the auxiliary Ca²⁺ is likely to cause a difference in the position of loops around the sugar binding site when compared with other C-type CRDs. A stacking interaction with Tyr⁷²⁹ provides approximately 25% of the binding energy for mannos binding to CRD-4, and interaction with this residue is also important for binding to GlcNAc and fucose. Among C-type CRDs, CRD-4 seems to have evolved a unique way of binding to mannose, GlcNAc, and fucose.