Structural Requirements for High Affinity Binding of Complex Ligands by the Macrophage Mannose Receptor*

Maureen E. Taylor‡ and Kurt Drickamer§

From the Department of Biochemistry and Molecular Biophysics, Columbia University, New York, New York 10032

The mannose receptor of macrophage and hepatic endothelial cells discriminates between endogenous and exogenous sugar-bearing structures. Previous competition studies have indicated that the receptor binds the monosaccharides mannose, fucose, and N-acetylgalactosamine but displays much higher affinity for multivalent oligosaccharides, such as those found on the surface of potentially pathogenic microorganisms. The hydrodynamic properties of the receptor have been examined, revealing that the receptor is a monomer. This result suggests that multiple carbohydrate recognition domains (CRDs) in the extracellular domain of a single receptor polypeptide cooperate to achieve high affinity binding of complex ligands. In order to determine the importance of individual CRDs, properties of receptor segments containing groups of CRDs expressed in insect cells have been examined. The results indicate that two of the CRDs (4 and 5) form a protease-resistant, ligand-binding core but that five CRDs in tandem (4–8) are required to match the affinity of the intact receptor for yeast mannann. A consequence of the organization of the receptor is that both valency and geometry of glycoconjugates are important determinants of binding affinity.

The mannose receptor of macrophages and hepatic endothelial cells mediates endocytosis of glycoproteins with terminal mannose, fucose, N-acetylgalactosamine, or glucose residues (1). The mannose receptor may also mediate phagocytosis of yeast (2, 3) and other pathogenic microorganisms (4, 5). The receptor can be considered to have a role in the discrimination of self from non-self, since terminal mannose or N-acetylgalactosamine residues are rarely found in mammalian cell-surface or serum glycoproteins but are a common component of the cell-surfaces of bacteria, yeasts, and parasites. The mannose receptor also mediates clearance of endogenous glycoproteins that bear appropriate high mannose oligosaccharides. Such ligands, including lysosomal enzymes (6) and tissue plasminogen activator (7), are often released from cells in response to pathological events.

The mannose receptor of macrophages is a type I transmembrane protein with an extracellular portion consisting of several cysteine-rich domains (8). These include a fibronectin type II repeat (9) and eight domains that are related in sequence to the C-type carbohydrate-recognition domains (C-type CRDs) of other animal lectins (10). C-type CRDs are approximately 130 amino acids long and are characterized by a pattern of 14 invariant residues and 18 residues that are conserved in character (11, 12). C-type CRDs have been found in a large number of proteins, in association with a variety of effector domains (13, 14). They have specificities for various different saccharides. Examples of proteins containing C-type CRDs are the asialoglycoprotein receptor, which mediates endocytosis of serum glycoproteins (15), serum mannose-binding protein, which mediates antibody-independent defense against pathogens (16), and the selectin cell adhesion molecules (17). C-type CRDs can be found at either end of a polypeptide or at internal positions. The mannose receptor is the only protein known to contain more than one CRD within a single polypeptide (8).

Experiments in which truncated forms of the mannose receptor were expressed in fibroblasts have shown that the NH₂-terminal cysteine-rich domain, the fibronectin type II repeat, and the first three CRDs are not essential for binding and endocytosis of glycoprotein ligands (18). A truncated receptor with an extracellular portion consisting of CRDs 4–8 is able to endocytose mannose-terminated glycoproteins as efficiently as the intact receptor and has the same affinity as the intact receptor for three different ligands. In an in vitro translation assay, CRD 4 is the only domain that has carbohydrate binding activity when expressed alone (18). This domain, produced in a bacterial expression system, exhibits multispecificity and mimics the monosaccharide binding activity of the whole receptor. However, CRD 4 binds only poorly to natural glycoproteins and thus cannot account for binding of the receptor to such ligands (18).

Although CRD 5 has no demonstrable saccharide binding activity when expressed alone, a segment of the receptor consisting of CRDs 5–7 does show binding activity in the in vitro translation assay, and a receptor consisting of CRDs 5–8 mediates endocytosis of glycoproteins, albeit at a much slower rate than the intact receptor. Since a segment consisting of CRDs 6–8 is not active in similar assays, it has been concluded that CRDs 4, 5, and at least one additional CRD nearer the membrane anchor are necessary for binding and endocytosis of multivalent ligands (18). Clustering of CRDs, each with weak affinity for single sugars, to achieve high affinity binding to oligosaccharides is a feature of other C-type lectins (16, 19, 20). However, in the mannose receptor, multiple weak interactions appear to be achieved through

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‡ Present address: Glycobiology Institute, Dept. of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom.

§ Recipient of a Faculty Salary Award from the American Cancer Society. To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biophysics, Columbia University, 630 W. 168th St., New York, NY 10032. Tel.: 212-305-6453; Fax: 212-305-4131.

1 The abbreviations used are: CRDs, carbohydrate-recognition domains; BSA, bovine serum albumin.
Several active CRDs in a single polypeptide rather than by oligomerization of multiple polypeptides each with a single CRD.

This paper describes experiments designed to determine how CRDs in the mannose receptor are clustered to achieve high affinity binding to glycoproteins. It is demonstrated that a core consisting of CRDs 4 and 5, combined with accessory CRDs 6 to 8 in a single polypeptide, mediate binding of a natural glycoprotein ligand.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes, linkers, polymerase, and ligase were from New England Biolabs. Sepharose 4B, d-mannose, invertase grade VII, mannan, and molecular weight markers were obtained from Sigma. Man$_6$-BSA was a product of E. Y. Laboratories. Amersham Corp. was the source for Na$^{25}$I and Amplify fluorography solution. Immulon 96-well microtiter plates were obtained from Dynatech. Subtilisin was from Boehringer Mannheim. Immobilon membranes were obtained from Millipore Corporation.

**Hydrometric Experiments**—Fac 6 fibroblasts (four 60-mm plates at half-confluence) expressing full-length receptor (18) were labeled for 4 h in methionine-free medium supplemented with [35S]methionine-cysteine mix at a concentration of 125 $\mu$Ci/ml (2 ml/plate). Cells were harvested in phosphate-buffered saline by scraping and collected by low speed centrifugation. The cell pellet (0.25 ml) was solubilized in phosphate-buffered saline at 4°C by sonication for 30 s. The sample was again sonicated for 5 s and centrifuged at 150,000 $\times$ g for 30 min in a Beckman TLA100.3 rotor. The supernatant was applied directly to a 1-ml column of Sepharose 6B, D-mannose, and molecular weight markers were obtained from New England Biolabs. Sepharose 6B, D-mannose, and molecular weight markers were obtained from New England Biolabs.

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**Expression of Portions of the Mannose Receptor in a Baculovirus Expression System**—The procedures used for the baculovirus expression system have been described in detail by Summers and Smith (24). Standard recombinant DNA techniques were used in the construction of plasmids (25). The starting plasmid was a derivative of pBlueScript containing the 5’ untranslated segment of Xenopus autographin (26). DNA coding for portions of the mannose receptor was expressed in bacteria (18), followed by gel electrophoresis, and fluorography. Subsequent oligomerization of multiple polypeptides each with a single CRD.

**RESULTS**

**Oligomeric State of the Mannose Receptor**—Since oligomerization of the mannose receptor could potentially result in clustering of very large number of CRDs, it was important to determine the native size of the receptor. This analysis was made possible by the availability of rat fibroblasts that express the intact receptor (18). Cells were labeled with $^{35}$S-containing methionine and cysteine, following which receptor was solubilized in Triton X-100 and isolated by a single step of affinity chromatography on mannose-Sepharose. Freshly isolated receptor was subjected to velocity gradient centrifugation on Triton X-100-containing gradients. As shown in Fig. 1, the protein sediments as a single peak, with an $s_{20,w}$ value of 6.6 S. The presence of receptor from fractions sedimenting lower in the gradient (i.e. at higher s values) indicates that this is the largest form of the solubilized protein. When compared with antisera raised against intact receptor (30), anti-CRD 4 serum precipitates similar amounts of receptor from labeled cells. Therefore, it is unlikely that an oligomeric form, in which CRD 4 is inaccessible to the antibodies used, is undetectable in this experiment.

**In order to derive an estimate of the molecular weight of the protein portion of the receptor-detergent complex, the partial specific volume of the complex and its diffusion coefficient were determined by equilibrium density gradient centrifugation and gel filtration chromatography, respectively. In each case, a single peak of receptor was detected by immunoprecipitation, gel electrophoresis, and fluorography. Substitution of the resulting values ($\vec{V} = 0.844$ cm$^3$/g and $\bar{D}_{20,w} = 2.33 \times 10^{-7}$ cm$^2$/s) along with the sedimentation coefficient into the Svedberg equation yielded a molecular weight of 410,000 for the complex. The measured partial specific volume was combined with the partial specific volume of the glycosylated receptor polypeptide ($0.718$ cm$^3$/g) and Triton X-100 ($0.94$ cm$^3$/g) to estimate that the fraction of receptor in the complex is 43%. The result indicates that the non-detergent portion of the complex has a molecular weight of 180,000.

Since the calculated molecular weight of the glycosylated receptor polypeptide is estimated to be 183,000, the sedimentation results show that the receptor most likely is monomeric in detergent solution. Consistent with this conclusion, no

**Solid-phase Binding Assay**—This assay has been described previously (18). Plastic microtiter plates were coated overnight at 4°C with 100 $\mu$l of each baculovirus-expressed portion of the mannose receptor (approximately 100 ng/ml in loading buffer). After coating, the wells were washed three times with 25 mM Tris-HCl, pH 7.8, 1.25 M NaCl, 25 mM CaCl$_2$ (loading buffer) and blocked with 5% (w/v) BSA in the same buffer for 2 h at 4°C. The wells were again washed three times and $^{35}$P-Man$_2$-BSA (approximately 10$^6$ cpm in 100 $\mu$l of washing buffer containing 5% BSA; specific activity approximately 10$^5$ cpm/µg) with varying amounts of inhibitors was added to each well. After incubation for 2 h at 4°C, the wells were washed rapidly three times with cold washing buffer and shaken dry. Individual wells were counted in a Packard TRIAS gamma-counter. Data were analyzed using a nonlinear least squares fitting program (Sigmaplot, Jandel Scientific).
Evidence of oligomers was obtained when the solubilized protein was treated with the homobifunctional reagent bis(sulfosuccinimidy1)suberate (data not shown) under conditions that result in efficient cross-linking of the mannose-binding proteins (27). Similarly, treatment of cells with 1,5-difluoro-2,4-dinitrobenzene produced no evidence of oligomer formation, although oligomers of the chicken hepatic lectin could be readily detected under the same conditions (19). These results suggest that the receptor is probably a monomer in the membrane as well as in detergent solution.

Expression of Internal Fragments of the Receptor—Since the mannose receptor polypeptide appears to be a monomer, the clustering of CRDs necessary to achieve high affinity binding of complex ligands must result solely from the activity of multiple CRDs within a single polypeptide. In previous studies designed to probe the role of various CRDs in the mannose receptor, truncated polypeptides lacking one or more NH2-terminal CRDs were expressed in rat fibroblasts and analyzed for ligand binding. Because it was necessary to retain the COOH-terminal membrane anchor in these constructs, it was not possible to analyze COOH-terminal truncations (18).

In this study, the baculovirus system has been used to produce internal fragments of the receptor. These fragments are water soluble and can be purified and analyzed more readily than those retained in the membrane. Portions of the receptor cDNA coding for CRDs 4–5, 4–6, and 4–7 were fused to codons specifying the dog insulin signal sequence (Fig. 2). The insulin signal sequence directs sequestration of the receptor into the endoplasmic reticulum (18), leading to their eventual secretion. The fragments were purified from the medium by one step of affinity chromatography on mannose-Sepharose. In each case, gel electrophoresis revealed the presence of a single polypeptide band of the expected molecular weight (Fig. 3). The purified receptor fragments were used in proteolysis experiments and in assays to determine affinities for various ligands.

Domain Organization—A common property of C-type CRDs is resistance to proteolysis in the presence of Ca2+ (19, 27), reflecting their tightly folded structures. Therefore, it was of interest to determine whether the CRDs of the mannose receptor are similarly resistant to proteolysis. The receptor fragment consisting of CRDs 4–7 was subjected to limited proteolysis with subtilisin in the presence of Ca2+. As shown in Fig. 4, the amount of the CRD 4–7 fragment decreases, and three subfragments appear as the concentration of subtilisin is increased. The molecular weights of the protease-resistant subfragments, combined with NH2-terminal sequence analysis, indicate that they correspond to CRDs 4–6, 4–5, and 6. Similar digestion of the expressed fragment containing CRDs 4–6 also yielded subfragments corresponding to CRDs 4–5 and 6 (data not shown).

The proteolysis experiments provide information about the
structures of the individual CRDs and their arrangement in clusters. CRD 7 is the most susceptible to proteolysis and is removed from the 4–7 fragment at the lowest concentration of subtilisin. No released CRD 7 was detected, indicating that it is probably degraded by subtilisin. Although the sequence of CRD 7 indicates that it is evolutionarily divergent from the other CRDs (31), its apparent susceptibility to proteolysis in the presence of Ca\(^{2+}\) is not correlated in a simple way with the absence of residues predicted from the structure of the mannos-binding protein to be involved in Ca\(^{2+}\) ligation (12).

CRD 6 is released intact from the CRD 4–6 fragment at higher concentrations of subtilisin. Significant amounts of CRDs 4–5 and CRD 6 remain even after digestion for 60 min with 4 \(\mu\)g/ml subtilisin. These results indicate that CRDs 4, 5, and 6 each have compactly folded conformations that probably resemble the CRD of mannos-binding protein. The data also show that CRDs 4 and 5 form a tightly linked unit, suggesting that these CRDs form extensive contacts and are not simply linked by a flexible tether. Such an arrangement would be expected to fix the relative orientation of the saccharide-binding sites in these domains. Since CRD 4 has been shown to display the highest affinity for sugar of any of the CRDs (18), the CRD 4–5 pair probably forms a core for binding of multivalent ligands. This hypothesis is supported by results described below.

Binding Affinities—A solid-phase binding assay previously used to study the binding of CRD 4 to saccharide ligands was used to measure binding to the larger fragments containing additional CRDs. In this assay, \(^{125}\)I-Man\(_{23}\)-BSA binds to portions of the receptor immobilized on microtiter plates. Affinities for Man\(_{23}\)-BSA, invertase, and mannan were determined based on their ability to compete with the labeled ligand. Typical binding curves for mannan are shown in Fig. 5, and the results for all three ligands are summarized in Table I. No differences in affinities for Man\(_{23}\)-BSA or invertase were seen between the three fragments consisting of CRDs 4–5, 4–6, and 4–7. However, the affinity for mannan increases as additional CRDs are added.

The results obtained previously for CRD 4 alone and for CRDs 4–8 expressed in fibroblasts (18) are compared to the present results in Fig. 6, which shows a plot of affinities for the three ligands tested versus the number of CRDs present. CRDs 4–8 have been shown to have the same affinities as the intact receptor for these three ligands (18). The greatest increase in affinity for all three ligands is seen when CRD 5 is combined with CRD 4. The increase is particularly marked for the two natural glycoproteins. For Man\(_{23}\)-BSA and invertase, CRDs 4 and 5 are sufficient to reproduce the affinity of the intact receptor. The graph emphasizes that the affinity for mannan continues to increase as more CRDs are added. The full affinity of the intact receptor for this ligand is only obtained when CRDs 4–8 are present.

In related studies, fragments were labeled and tested for binding to various neoglycoproteins and natural glycoproteins resolved by gel electrophoresis and immobilized on nitrocel-

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**FIG. 4.** Limited subtilisin digestion of CRD 4–7 fragment. Digestion with subtilisin was performed at a protein concentration of approximately 100 \(\mu\)g/ml in a volume of 25 \(\mu\)l of 0.5 M NaCl, 25 mM Tris-Cl, pH 7.8, and 25 mM CaCl\(_2\) for 30 min at 37 °C. Concentrations of subtilisin are indicated at the top of each lane. Digestions were stopped by addition of an equal volume of double strength sample buffer followed by immediate heating to 100 °C for 5 min. Digestion fragments were resolved on an SDS gel (17.5% polyacrylamide) that was stained with Coomassie Blue.

**FIG. 5.** Inhibition of \(^{125}\)I-Man\(_{23}\)-BSA binding to immobilized receptor fragments. Binding assays were performed as described under "Experimental Procedures" in the presence of increasing concentrations of yeast mannan. The resulting data were fitted with a nonlinear least squares program according to the equation: fraction of maximal binding = \(K_i/(K_i + [\text{mannan}])\), where \(K_i\) is the concentration at which half-maximal inhibition of binding is observed.

### TABLE I

**Inhibition of binding to mannos receptor CRDs**

| Ligand     | \(K_i\) values (nM) |
|------------|---------------------|
| CRDs 4–5   | CRDs 4–6            | CRDs 4–7            |
| Man\(_{23}\)-BSA | 70 ± 6             | 72 ± 40             | 70 ± 15             |
| Invertase  | 27 ± 17             | 19 ± 16             | 28 ± 14             |
| Mannan     | 2300 ± 1000         | 600 ± 400           | 260 ± 50            |

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ligase. As expected from the results just described, CRD-4 alone as well as CRDs 4–5, 4–6, and 4–7 all bind Man3-BSA. However, CRD 4 alone does not bind to invertase or ribonuclease B, while the three larger fragments do. Thus, as in the assay using immobilized CRDs, the binding of larger receptor fragments to natural glycoproteins such as invertase and ribonuclease B reflects an increase in affinity due to the presence of multiple CRDs. Because it does not migrate as a discrete band on SDS gels, mannan could not be tested in this assay. Since these experiments were conducted with receptor fragments in solution, the fact that the results are completely consistent with those obtained with immobilized CRDs indicates that the densities of receptor fragments on the polystyrene surface used in the solid-phase assay do not affect the way multivalent ligands are bound.

**DISCUSSION**

It has been shown previously that, while CRD 4 alone can mimic the binding of monosaccharides to the intact mannose receptor, additional CRDs are needed for binding to complex ligands (18). In the present study, hydrodynamic experiments and expression of internal fragments of the receptor in insect cells have been used to define further which CRDs are required for binding ligand with high affinity.

The results confirm that the mannose receptor binds oligosaccharides through several CRDs in a single polypeptide. No evidence for oligomer formation was seen. The number of CRDs needed for high affinity binding is dependent on the ligand. CRDs 4 and 5 form the basic unit required for binding multivalent ligands with submicromolar affinities. While CRD 4 alone has weak affinity for natural glycoproteins such as invertase (\(K_d \approx 1\ \mu M\)), CRDs 4 and 5 together bind to this ligand almost as tightly as the whole receptor (\(K_d \approx 20\ nm\)). Since these two domains cannot be separated by proteolysis, it is concluded that they form a tightly linked structural unit.

For other natural ligands, such as yeast mannan, CRDs 4–5 have a much higher affinity than does CRD 4 alone, but additional increases in affinity are seen as CRDs 6, 7, and 8 are added.

Clustering of CRDs has been shown to be important in several other C-type lectins. These other lectins contain only a single CRD in each polypeptide and thus must form homo- or hetero-oligomers to achieve clustering of CRDs. The chicken hepatic lectin is a trimer formed through the association of transmembrane regions of identical polypeptides. This oligomerization results in the intact receptor binding a multivalent glycoprotein ligand with 25-fold greater affinity than does the monomeric CRD (19). Two different polypeptides of the rat asialoglycoprotein receptor, each with a single CRD, form hetero-oligomers (20). The different subunits bind to different terminal galactose residues on a triantennary glycopeptide (32), indicating that clustering of CRDs determines the specificity of the interaction, as well as the affinity.

It is probable that different forms of clustering of CRDs seen in various C-type lectins reflects differences in their modes of binding to multivalent ligands. The CRDs of each protein must be arranged spatially to match the geometric configurations of their particular oligosaccharide ligands. The structure of the hepatic lectins may provide optimal binding to clusters of terminal residues, while the linear arrangement of CRDs in the mannose receptor may be more suitable for high affinity binding to repeated polymers such as yeast mannan.

The observation that the core CRD 4–5 unit binds certain saccharides, such as those found in invertase and ribonuclease B, with high affinity, suggests that the binding sites in these CRDs are suitably disposed to interact with a structural motif common to the oligosaccharide portions of these glycoproteins. The role of accessory CRDs such as 6, 7, and 8, may be to provide alternative geometrical arrangements of binding sites that can interact with differently disposed sugars, such as those in mannan. Some C-type animal lectins, such as the asialoglycoprotein receptor, bind endogenous glycoproteins with a limited set of structures. In contrast, the mannose receptor must recognize diversity of foreign oligosaccharides. The presence of multiple CRDs in addition to CRD 4–5 core may provide the geometrical flexibility needed to interact with this diversity of ligands.

Analysis of the crystal structure of the CRD of rat mannose-binding protein bound to a high mannose oligosaccharide (33) has highlighted why clustering of CRDs might be necessary for achieving high affinity binding and specificity. In the crystal, each CRD interacts with a single terminal sugar residue. Two hydroxyl residues of the sugar are ligated in a shallow indentation on the surface of the protein. The limited contact between the sugar and the CRD explains how the CRD can have specificity for several different monosaccharides, and why the interactions with monosaccharides are weak. The fact that two CRDs in the crystals simultaneously bind different terminal mannose residues in a single oligosaccharide indicates why more than one CRD may be required for recognition of branched oligosaccharides.

Each of the CRDs of the mannose receptor contains most of the conserved residues necessary for establishing the overall folded structure of the mannose-binding protein CRD. In CRDs 4 and 5, all 5 residues involved in ligation of the 2 sugar hydroxyl residues to the mannose-binding protein CRD are also conserved (33, 34). Thus, it is likely that the interaction of these two domains with monosaccharides is similar to that of the mannose-binding protein CRD. CRDs 6, 7, and 8 do

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2 M. E. Taylor, K. Bezouska, and K. Drickamer, unpublished observations.

3 F. Verrey and K. Drickamer, manuscript submitted.
not contain all of the residues involved in ligation of sugar to mannose-binding protein and thus may bind sugars in a different manner. Further structural work will be required to determine how each of the mannose receptor CRDs interacts with sugars.

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