Contribution to Ligand Binding by Multiple Carbohydrate-recognition Domains in the Macrophage Mannose Receptor*

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The extracellular portion of the macrophage mannose receptor is composed of several cysteine-rich domains, including a fibronectin type II repeat and eight segments related in sequence to Ca**+-dependent carbohydrate-recognition domains (CRDs) of animal lectins. Expression of portions of the receptor in vitro, in fibroblasts and in bacteria, has been used to determine which of the extracellular domains are involved in binding and endocytosis of ligand. The NH**-terminal cysteine-rich domain and the fibronectin type II repeat are not necessary for endocytosis of mannose-terminated glycoproteins. CRDs 1–3 have at most very weak affinity for carbohydrate, so the carbohydrate binding activity of the receptor resides in CRDs 4–8. CRD 4 shows the highest affinity binding and has multispecificity for a variety of monosaccharides. However, CRD 4 alone cannot account for the binding of the receptor to glycoproteins. At least 3 CRDs (4, 5, and 7) are required for high affinity binding and endocytosis of multivalent glycoconjugates. In this respect, the mannose receptor is like other carbohydrate-binding proteins, in which several CRDs, each with weak affinity for single sugars, are clustered to achieve high affinity binding to oligosaccharides. In the mannose receptor, these multiple weak interactions are achieved through several active CRDs in a single polypeptide chain rather than by oligomerization of polypeptides each containing a single CRD.

The mannose receptor of macrophages and hepatic sinusoidal cells is one of a group of cell surface receptors which mediate endocytosis of glycoproteins (1, 2). The mannose receptor recognizes glycoproteins or sialoglycoproteins with terminal mannose, fucose, N-acetylgalactosamine, or glucose residues (3, 4). It has been proposed that this receptor has a scavenging function, acting to clear potentially harmful molecules from the bloodstream. Possible ligands include secreted lysosomal enzymes from which the mannose 6-phosphate recognition marker has been removed (5), tissue plasminogen activator (6) and the propeptide of type I procollagen (7). The mannose receptor may also mediate phagocytosis of yeast (8, 9) and other pathogenic microorganisms (10, 11), which have a high density of mannose at their cell surfaces.

The primary structure of the human mannose receptor has recently been determined (12). The receptor appears to be oriented as a type I transmembrane protein (COOH terminus on the cytoplasmic side of the membrane), since the mature polypeptide is preceded by a signal sequence, and a hydrophobic stop transfer sequence is located close to the COOH terminus. The extracellular portion of the receptor consists of three types of domains (Fig. 1). The first domain is cysteine-rich and bears no resemblance to other known sequences. There follows a domain which resembles the type II repeats of fibronectin (13). The rest of the extracellular part of the receptor consists of eight domains which are related in sequence to the Ca**+-dependent carbohydrate-recognition domains (C-type CRDs) of other animal lectins (14, 15).

C-type CRDs have been found in a large number of proteins, in association with a variety of other domains (16). The prototype of this family is the asialoglycoprotein receptor, which, like the mannose receptor, mediates endocytosis of glycoproteins (2). This receptor is a type II transmembrane protein (cytoplasmic NH**-terminal) and has a single CRD at its COOH terminus (17). COOH-terminal CRDs are found in association with collagenous domains in the soluble mannose-binding proteins (18) and pulmonary surfactant SP-A (19). In contrast, the lymphocyte homing receptor and related molecules are type I transmembrane proteins which contain NH**-terminal CRDs (20). In these selectins, epidermal growth factor and complement-regulatory domains lie between the CRD and the membrane, whereas in the core protein of cartilage proteoglycan, epidermal growth factor and complement-regulatory domains flank the CRD (21). Thus CRDs can be found at either end of a polypeptide or at an internal position. The mannose receptor is the first protein found to contain more than one CRD within a single polypeptide (12).

Since the mannose receptor binds carbohydrate in a Ca**+-dependent manner, it is reasonable to postulate that the carbohydrate binding activity of the receptor is located within the repeated CRD-like segments of the molecule. It is still necessary to ask: 1) are the NH**-terminal cysteine-rich domain and the fibronectin type II repeats necessary for endocytosis of glycoproteins? 2) Which of the CRDs of the mannose receptor are capable of binding to carbohydrates? 3) Does each CRD have specificity for more than one sugar or does the multispecificity of the receptor arise from each CRD having specificity for different saccharides? This paper describes studies carried out to address these questions.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes, linkers, polymerase, and ligase were from New England Biolabs. Sepharose 6B, D-mannose, D-glucose, bovine serum albumin; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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† The abbreviations used are: CRD, carbohydrate-recognition domain; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
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cose, N-acetyl-D-glucosamine, D-galactose, L-fucose, invertase grade VII, and mannan were obtained from Sigma and Man$_{23}$-BSA was from E. Y. Labs. Rabbit reticulocyte lysate, dog pancreas microsomes, $[^{135}$S]methionine, $[^{35}$S]cysteine, Na$_{25}$EDTA, and Amplify fluorography solution were purchased from Amersham Corp. Immulon 96-well microtiter plates were obtained from Dynatech. An antibody against the plasmid was a derivative of pSP64 containing the 5'-untranslated region of E. coli rRNA. Rabbit reticulocyte lysate, dog pancreas microsomes, [35S]methionine, [3H]cysteine, Na$_{25}$EDTA, and Amplify fluorography solution were purchased from Amersham Corp. Immulon 96-well microtiter plates were obtained from Dynatech. An antibody against the human placental mannan receptor was the kind gift of Dr. P. D. Stahl, Washington University, St. Louis, MO. An antibody against bacterial mannan receptor CRD 4 was produced in New Zealand White rabbits, following a standard protocol for subcutaneous injection with Freund's adjuvant (22).

Expression of CRDs in Vitro—Standard recombinant DNA techniques were used in the construction of plasmids (23). The starting plasmid was a derivative of pSP64 containing the 5'-untranslated region of Xenopus laevis $\beta$-globin mRNA and the region of the dog preproinsulin gene encoding the signal sequence (24). Portions of the mannose receptor gene encoding the signal sequence (24) were cloned into this plasmid after the region encoding the insulin signal sequence was linearized and used as templates for in vitro translation reactions containing rabbit reticulocyte lysate, dog pancreas microsomes, [35S]methionine, glutathione (0.2 mM final concentration), and approximately 1 μg of mRNA were incubated for 1 h at 30 °C. After incubation, the reaction products were separated by centrifugation through sucrose (25). Microsome-associated translation products were solubilized in Triton X-100, diluted into loading buffer (25 mM Tris-HCl (pH 7.8), 1.25 M NaCl, 25 mM CaCl$_2$), and loaded onto 1 mL columns of mannose-4-BSA, fucose-4-BSA, GlcNAc-4-BSA, glucose-4-BSA, or galactose-Sepharose, prepared by the method of Forsnstedt and Porath (26). The columns were washed with loading buffer and eluted with 25 mM Tris-HCl (pH 7.8), 1.25 M NaCl, 25 mM EDTA (eluting buffer). Flow-through, wash, and eluted fractions were precipitated with trichloroacetic acid and analyzed by SDS-PAGE.

FIG. 1. Summary of portions of the mannose receptor expressed in various systems. Portions of the mannose receptor expressed in fibroblasts, in vitro or in bacteria, are shown diagramatically. The structure of the intact mannose receptor is summarized at the top with the CRDs labeled 1-8. The filled box represents the mannose receptor signal sequence, and the dotted boxes represent the stop transfer sequence. Amino acids present in each construct are indicated by the residue numbers from the intact receptor (small numerals). Open boxes represent the dog preproinsulin signal sequence, and the hatched box represents the bacterial ompA signal sequence. Block letters Y and I represent potential or actual sugar attachment sites to asparagine and threonine, respectively (12).

FIG. 2. Pulse labeling of truncated receptors expressed in fibroblasts. Cells were labeled with radioactive cysteine and methionine for 1 h and then chased in medium containing a 20-fold excess of unlabeled cysteine and methionine for 30 min. Following solubilization and immunoprecipitation with an antibody against CRD 4 (lanes 1 and 2) or an antibody against the purified placental mannan receptor (lanes 3 and 4), labeled receptors were resolved on a 10% SDS-polyacrylamide gel and detected by fluorography. Lane 1, CRD 1-8; lane 2, CRD 4-8; lane 3, CRD 5-8; lane 4, CRD 6-8.

FIG. 3. Uptake and degradation of ligand by cells expressing truncated mannose receptors. Cells grown on 35-mm dishes were preincubated with $^{125}$I-Man$_{23}$-BSA (1 μg/ml) for 30 min at 4 °C. Following incubation at 37 °C for the times indicated, radioactivity associated with cells (open symbols) and with acid-soluble fragments in the medium (closed symbols) was analyzed as described (29). Triangles represent values at 3 h when a 50-fold excess of ligand was present throughout the experiment.

(Fig. 1). Stop codons were created with XbaI linkers. The resulting plasmids were linearized and used as templates for in vitro transcription (28). In vitro translation reactions containing rabbit reticulocyte lysate, dog pancreas microsomes, [35S]methionine, glutathione (0.2 mM final concentration), and approximately 1 μg of mRNA were incubated for 1 h at 30 °C. After incubation, the reaction products were separated by centrifugation through sucrose (25). Microsome-associated translation products were solubilized in Triton X-100, diluted into loading buffer (25 mM Tris-HCl (pH 7.8), 1.25 M NaCl, 25 mM CaCl$_2$), and loaded onto 1 mL columns of mannose-4-BSA, fucose-4-BSA, GlcNAc-4-BSA, glucose-4-BSA, or galactose-Sepharose, prepared by the method of Forsnstedt and Porath (26). The columns were washed with loading buffer and eluted with 25 mM Tris-HCl (pH 7.8), 1.25 M NaCl, 25 mM EDTA (eluting buffer). Flow-through, wash, and eluted fractions were precipitated with trichloroacetic acid and analyzed by SDS-PAGE.
translated in vitro and the translation products were passed over a 1-
mll column of mannose-Sepharose as described under "Experimental
Procedures." Flow-through fractions (F), wash fractions (W), and
fractions eluted with EDTA (E) were precipitated with trichloroacetic
acid and analyzed on SDS-polyacrylamide gels (12.5%) followed by
fluorography.

polyacrylamide gel electrophoresis, in the buffer system of Laemmli
(27), followed by fluorography.

Expression of Truncated Receptors in Fibroblasts—Portions of
DNA coding for CRDs was inserted into the expression vector pIN-
lac operator. LB medium (500 ml) was inoculated with 10 ml of
an overnight culture of Escherichia coli strain JA221 containing the
CRD expression plasmid and incubated with shaking for 2 h at
37 °C. CaCl2 and isopropyl-β-D-thiogalactoside were then added to
final concentrations of 100 and 1 mM, respectively. After incubation
at 37 °C for a further 2 h, the cells were harvested by centrifugation
for 10 min at 5,000 rpm in a Sorvall GSA rotor. The cells were
resuspended in 35 ml of 25 mM Tris-HCl (pH 7.8), 1.25 mM NaCl, 3
mM CaCl2, and lysed by sonication (six bursts of 30-s duration).
Insoluble material was removed by centrifugation for 30 min at 15,000
rpm in a Sorvall SS-34 rotor. The supernatant was passed through a
1 ml column of mannose-Sepharose equilibrated with loading buffer.
The column was washed with loading buffer (20 ml) and eluted with
eluting buffer (5 x 1 ml). Eluted fractions were analyzed by SDS-
polyacrylamide gel electrophoresis.

CED 4 Binding Assay—Plastic microtiter plates were coated with
CRD 4 by adding 100 μl of CRD 4 solution (approximately 100 μg/
ml in loading buffer) to each well and incubating at 4 °C overnight.
To determine nonspecific binding, a duplicate set of wells was incu-
bated with loading buffer. After coating, the wells were washed three
times with loading buffer and blocked with 5% (w/v) BSA in loading
buffer for 2 h at 4 °C. The wells were again washed three times with
loading buffer and 125I-Man3-BSA (approximately 106 cpm in 100 μl
of 5% BSA in loading buffer; specific activity approximately 107 cpm/
μg) was added to each well. After incubation for 2 h at 4 °C, the wells
were washed rapidly three times with cold loading buffer, shaken dry
and counted in a γ-counter. Data were analyzed using a nonlinear
least squares fitting program (Sigmaplot; Jandel Scientific).

RESULTS

Localization of Mannose-binding Activity to CRDs 4-8—In
order to determine which of the extracellular domains of the
mannose receptor are necessary for binding and endocytosis
of glycopolymers, DNA coding for truncated receptors fused to
the insulin signal sequence was expressed in fibroblasts and
in vitro (Fig. 1). A transfected cell line expressing the intact
mannose receptor has been described previously (12). A simi-
lar expression system was used to express truncated recep-
tors. Cell lines expressing truncated receptors were charac-
terized by pulse labeling. In each case, proteins of the expected
size were immunoprecipitated by antibodies to the mannose
receptor (Fig. 2). The cell lines were tested for their ability to
endocytose and degrade ligand. It was first demonstrated that

Fig. 6. Expression of CRD 4 in bacteria. Left, induction of
expression by isopropyl-β-D-thiogalactoside. Aliquots of cells contain-
ing the expression plasmid were grown without induction (−) or
induced with isopropyl-β-D-thiogalactoside (+) and analyzed on a
17.5% SDS-polyacrylamide gel which was stained with Coomassie
Blue. Right, purification of the expressed domain. The domain was
purified on a column of mannose-Sepharose and analyzed by SDS-
polyacrylamide gel electrophoresis as described above.
signal sequence in an SP64 expression vector (Fig. 1). The insulin signal sequence directs the translation product into the microsomes and is cleaved when the polypeptide is translocated into the lumen of the microsomes. Disulfide bond formation, which is necessary for production of an active CRD, can occur in the luminal environment. Translation products were extracted from the microsomes with detergent prior to testing for binding activity.

The distribution of restriction sites in the mannose receptor cDNA made it most straightforward to create two constructs consisting of CRDs 1–3 and 4–8. The results for these two segments are shown in Fig. 4. For CRDs 1–3, a band of the expected molecular weight is seen in the flow-through fraction but nothing is seen in the eluted fractions, indicating that these domains do not bind to mannose-Sepharose. In contrast, although some of the translation product from the CRD 4–8 construct is seen in the flow-through fraction, a significant amount binds to mannose-Sepharose and can be eluted by EDTA.

The in vitro translation results suggested that the carbohydrate-binding activity of the receptor is located in CRDs 4–8. These results were confirmed by further expression studies in fibroblasts. Cells expressing a truncated receptor consisting of domains 4–8 (Fig. 3C) are able to endocytose 125I-Man23-BSA as efficiently as cells expressing either the intact receptor or the truncated receptor consisting of CRDs 1–8.

Saccharide Binding Activity of CRD 4 Alone—Further division of CRDs 4–8 showed that CRD 4 alone can bind to mannose-Sepharose following in vitro translation (Fig. 5, left panel) and that a segment consisting of CRDs 5–8 also has carbohydrate-binding activity (Fig. 5, right panel). CRD 4 was produced in a bacterial expression system so that it could be characterized further (Fig. 6). The expression vector used, pINIompA2, is under the control of the ompA promoter and the lac operator (30). The ompA signal sequence directs the expressed protein to the periplasmic space of the cells and is then cleaved. This system has been used to produce large amounts of the CRDs of rat mannose-binding proteins A (32, 33) and C (34). For these two domains, a denaturation-renaturation method was developed for extraction and purification (33). For CRD 4 of the mannose receptor, it was found that very little of the protein extracted by denaturation can renature to form an active domain. However, if CaCl2 is added to the medium at the time of induction, some of the protein secreted into the periplasmic space folds into an active domain and can be released from the cells by mechanical
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![Fig. 9. Binding of COOH-terminal CRDs to mannose-Sepharose following in vitro translation. DNA coding for CRDs was translated in vitro and the translation products were passed over a 1-ml column of mannose-Sepharose as described under “Experimental Procedures.” Flow-through fractions (F), wash fractions (W), and fractions eluted with EDTA (E) were precipitated with trichloroacetic acid and analyzed on SDS-polyacrylamide gels (12.5, 15, or 17.5%) followed by fluorography.](image)

**TABLE I**

<table>
<thead>
<tr>
<th>Immobilized ligand</th>
<th>Relative binding</th>
<th>CRDs 1–3</th>
<th>CRDs 4–5</th>
<th>CRDs 6–8</th>
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</thead>
<tbody>
<tr>
<td>Man</td>
<td>&lt;0.02</td>
<td>1.00</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Fuc</td>
<td>&lt;0.04</td>
<td>1.42</td>
<td>&lt;0.02</td>
<td></td>
</tr>
<tr>
<td>GlcNAc</td>
<td>0.08</td>
<td>1.14</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Glc</td>
<td>0.16</td>
<td>1.05</td>
<td>&lt;0.04</td>
<td></td>
</tr>
<tr>
<td>Gal</td>
<td>0.16</td>
<td>0.17</td>
<td>0.04</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Domains</th>
<th>Man&lt;sub&gt;-BSA&lt;/sub&gt;</th>
<th>Invertase</th>
<th>Mannan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>Intact MMR</td>
<td>50 ± 20</td>
<td>25 ± 8</td>
<td>110 ± 13</td>
</tr>
<tr>
<td></td>
<td>CRDs 1–8</td>
<td>42 ± 20</td>
<td>18 ± 6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CRDs 4–8</td>
<td>50 ± 19</td>
<td>20 ± 8</td>
<td>110 ± 82</td>
</tr>
<tr>
<td></td>
<td>CRDs 5–8</td>
<td>93 ± 56</td>
<td>248</td>
<td>ND</td>
</tr>
<tr>
<td>Plates</td>
<td>CED 4</td>
<td>203 ± 24</td>
<td>920 ± 370</td>
<td>10,600 ± 2,200</td>
</tr>
</tbody>
</table>

*ND*, not determined.

disruption. The active domain was purified by affinity chromatography on mannose-Sepharose (Fig. 6).

Ligand-binding activity of purified CRD 4 was demonstrated in a solid phase assay in which <sup>125</sup>I-Man<sub>-BSA</sub> was bound in a saturable fashion to CRD 4 immobilized on a microtiter plate (data not shown). This assay was used to measure the ability of the domain for monosaccharides based on their ability to compete with the radiolabeled ligand (Fig. 7). CRD 4 was found to have specificities for fucose, N-acetylglucosamine, and glucose as well as for mannose (Fig. 7), with dissociation constants in the millimolar range. The order of potency, Man ≈ fucose > GlcNAc > Glc, and the affinity constants are similar to those seen for the intact receptor (3, 4). These results indicate that a single CRD of the receptor can reproduce the multispecificity of the intact receptor for a variety of monosaccharides, and it is not necessary to envision each of the mannose receptor CRDs having specificities for different sugars.

**Saccharide-binding Activity of CRDs 5–8**—As noted above, CRDs 5–8 display mannose-binding activity when tested in vitro (Fig. 5, right panel). This result was confirmed by fibroblast expression studies. Cells expressing CRDs 5–8 are able to endocytose and degrade ligand (Fig. 3D) although at a much slower rate than cells expressing larger portions of receptor. The slower endocytosis observed in cells expressing CRDs 5–8 indicates that CRD 4 is essential for efficient binding and endocytosis of ligand. Cells expressing CRDs 6–8 are not able to endocytose <sup>125</sup>I-Man<sub>-BSA</sub> (data not shown).

In order to determine that lack of endocytosis by CRDs 6–8 was not due to aberrant cell-surface expression, a surface labeling experiment was performed (Fig. 8). Cell-surface expression of CRDs 6–8 was compared with cell-surface expression of CRDs 4–8. The cells expressing CRDs 4–8 endocytose ligand efficiently, indicating that this truncated receptor must be expressed at the cell surface. After surface labeling with <sup>125</sup>I using lactoperoxidase and glucose oxidase, labeled proteins of the expected size were immunoprecipitated by antibodies to the receptor in the cell line expressing CRDs 6–8 (Fig. 8, lanes 2) as well as in the cell line expressing CRDs 4–8 (Fig. 8, lane 5). No proteins were immunoprecipitated from untransfected cells (Fig. 8, lanes 3 and 4) or by an irrelevant antibody (Fig. 8, lanes 1 and 6).

The surface labeling experiment indicated that the truncated receptor consisting of CRDs 6–8 is expressed at the cell surface as efficiently as the truncated receptor consisting of domains 4–8, but that it cannot mediate endocytosis of ligand. CRDs 6–8 also failed to bind to mannose-Sepharose after translation in vitro (Fig. 9, 1st panel). Since removal of CRD 5 results in loss of ability to bind and endocytose ligand, this domain must contribute to the carbohydrate-binding activity of CRDs 5–8. However, CRD 5 alone does not bind to mannose-Sepharose after translation in vitro (Fig. 9, 2nd panel). CRD 8 is not essential for binding in vitro, since a segment consisting of CRDs 5–7 binds to mannose-Sepharose (Fig. 9, 4th panel). However, CRDs 5–6 do not bind to mannose-Sepharose (Fig. 5, 3rd panel), indicating that CRD 7 must also contribute to the binding activity of the CRD 5–8 segment. A segment consisting of CRDs 7 and 8 does not bind to mannose-Sepharose (data not shown).

**Alternative Monosaccharide Ligands**—The mannose receptor has specificities for fucose, N-acetylglucosamine, and glucose as well as for mannose (3, 4), so CRDs 1–3 and 6–8, which did not bind to immobilized mannose, were tested on other sugars. Galactose was used as a negative control. The results are shown in Table I, expressed as a fraction of the
total translation product appearing in the eluted fractions relative to the results obtained for CRDs 4–5 tested on the same sugars. CRDs 1–3 and 6–8 showed essentially no binding to any of the sugars tested, whereas the segment consisting of CRDs 4–5 bound to all of the sugars except galactose. Therefore, the CRDs which fail to display detectable binding to mannose do not have higher affinity for other common monosaccharides.

Role of Multiple CRDs in Establishing Affinity for Complex Ligands—Affinity constants of the intact receptor and the truncated receptors for Man$_{23}$-BSA, invertase, and mannan were determined by using unlabeled ligands as competitors of $^{125}$I-Man$_{23}$-BSA uptake (Table II). No significant difference was seen among the intact receptor, CRDs 1–8, and CRDs 4–8 in the affinities for Man$_{23}$-BSA, invertase, or mannan, confirming that CRDs 1–3 are not essential for high affinity binding. Cells expressing CRDs 5–8 showed a decreased affinity for both Man$_{23}$-BSA and invertase (mannan was not tested).

The dissociation constants of CRD 4 for Man$_{23}$-BSA, invertase, and mannan were determined, using the plate assay, and compared with the results obtained with the truncated receptor consisting of CRDs 4–8 expressed in cells (Fig. 10 and Table II). The truncated receptor shows the same affinities for these ligands as the intact receptor; however, CRD 4 alone has much lower affinities for all three ligands. The difference is particularly marked for the two natural glycoproteins, mannan and invertase, which both have high mannose type oligosaccharides. Thus CRD 4 alone cannot account for the high affinity binding to multivalent ligands shown by the intact receptor and the truncated receptor consisting of CRDs 4–8. Multiple CRDs in the CRD 4–8 segment must contribute to the binding of such ligands.

**DISCUSSION**

Role of NH$_2$-terminal Domains of the Mannose Receptor—The results described here indicate that the NH$_2$-terminal cysteine-rich domain and the fibronectin type II repeat of the
mannose receptor are not necessary for endocytosis of carbohydrate-containing ligands. The function of these domains remains unclear. It is possible that they are required for binding and internalization of particulate ligands but not for endocytosis of soluble glycoproteins. Another possibility is that they bind to a noncarbohydrate ligand. Interestingly, another receptor which contains a fibronectin type II repeat, the cation-independent mannose 6-phosphate receptor (35), binds to two types of ligand: mannose 6-phosphate and insulin-like growth factor II (36). The mannose 6-phosphate receptor also contains multiple ligand-binding domains (37), although the repeating motif is not related to the C-type CRDs.

It is difficult to reconcile our results with those obtained by Shepherd et al. (38) indicating that cleavage of a 35-kDa extracellular portion of the rat bone marrow mannose receptor by trypsin results in loss of ability to bind and endocytose horseradish peroxidase. A 35-kDa NH₂-terminal segment of the human mannose receptor would correspond to the cysteine-rich domain, the fibronectin type II repeat, and possibly part of CRD 1. Our results indicate that removal of these domains does not result in loss of ability to endocytose Man₃GlcNAc₂-BSA, invertase, or mannan. However, the portion of the rat receptor released by trypsin has not been characterized by sequence analysis, so it is difficult to compare the results. It is possible that the rat mannose receptor has a different domain structure.

Activity of Individual CRDs—The results of the in vitro translation assays suggest that CRDs 1–3 have at most very weak carbohydrate-binding activity, since this segment is not able to bind to sugars immobilized on Sepharose. Thus the carbohydrate-binding activity of the mannose receptor must reside in the segment consisting of CRDs 4–8. This observation is confirmed by the fibroblast expression studies, since removal of CRDs 1–3 does not result in any change in affinity of the receptor for three different ligands. Of CRDs 4–8, only CRD 4 has a high enough affinity for carbohydrate to bind sugars in the absence of any of the other CRDs. CRDs 5 and 7 must have weaker affinities for carbohydrate, since they are both necessary for the activity of the segment consisting of CRDs 5–8. CRDs 6 and 8 may also contribute to binding, although CRD 8 is not essential for binding in this in vitro assay. Thus it can be concluded that of the 8 CRDs, at least three (CRDs 4, 5, and 7) are involved in binding to glycoconjugates, whereas three others (CRDs 1–3) are not required for binding.

It is interesting to compare the amino acid sequences of the mannose receptor CRDs (12) with the CRDs of other C-type animal lectins. Based on the comparison of the amino acid sequences of the C-type CRDs of 22 animal lectins, 31 invariant or highly conserved residues have been identified.2 Four of these residues are cysteines which are involved in forming two disulfide bonds. Determination of the crystal structure of rat mannose-binding protein A has shown that several of the highly conserved residues are involved in ligating 2 Ca²⁺, whereas others are critical for establishing the overall folded structure.3 All of the mannose receptor CRDs contain the 2 pairs of conserved cysteine residues, and the presence of most of the conserved residues essential for maintaining the CRD fold4 make it likely that each CRD has a configuration similar to that of the rat mannose-binding protein A CRD. In the mannose receptor, there appears to be no correlation between the number of these conserved residues in a CRD and ability of that CRD to bind carbohydrate. Of the 31 invariant or highly conserved residues, CRD 3 contains the least (26/31) and CRD 5 contains the most (31/31).2 CRD 4, which was shown to have the highest affinity for carbohydrate, has 27 of the 31 highly conserved residues.

Since CRD 4 was the only CRD shown to have carbohydrate-binding activity in the absence of other CRDs, it could be argued that the contribution of the other “active” CRDs (5 and 7) to ligand binding is merely conformational. However, since the amino acid sequences indicate that these CRDs are all likely to fold in a similar manner, it is probable that domains 5 and 7 contribute to ligand binding by direct interaction with sugars.

Effect of Combining CRDs on Ligand Binding—CRD 4 exhibits multispecificity for monosaccharides and can account for the observed binding of the intact receptor to monosaccharides. Multispecificity of a single CRD for monosaccharides is also seen in the mannose-binding proteins (34, 40). However, CRD 4 alone cannot account for the high affinity binding of the receptor to glycoproteins. Multiple CRDs in the CRD 4–8 segment are required to achieve tight binding to multivalent ligands. The difference in affinity between CRD 4 and CRDs 4–8 is more marked for the two natural glycoproteins than for the neoglycoprotein, suggesting that the multiple CRDs are arranged spatially to accommodate the geometric configurations of natural oligosaccharides.

Other carbohydrate-binding proteins also rely on multiple CRDs to achieve high affinity binding of multivalent ligands. In the case of the chicken hepatic lectin, the single subunit, which has a COOH-terminal CRD, forms oligomers in the membrane (41). This arrangement produces a cluster of CRDs, each with a low affinity site for a single sugar, which together allow high affinity binding of oligosaccharides. Clustering of CRDs is also seen in the soluble mannose-binding proteins. In this case the single subunits each with a COOH-terminal CRD are held together by the association of collagen-like domains (18). In the rat asialoglycoprotein receptor, two different polypeptides, each with a single CRD, are involved in ligand binding (39). The mannose receptor appears to achieve high affinity binding of oligosaccharides by having multiple active CRDs in a single polypeptide. Whether the mannose receptor also forms oligomers in the membrane, to allow even greater clustering of CRDs, remains to be determined.

REFERENCES


2 K. Drickamer, unpublished observations.
Ligand Binding by Multiple CRDs in Mannose Receptor