Orientation of sugars bound to the principal C-type carbohydrate-recognition domain of the macrophage mannose receptor

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The extracellular region of the macrophage mannose receptor, a protein involved in the innate immune response, contains eight C-type carbohydrate-recognition domains (CRDs). The fourth of these domains, CRD-4, is central to ligand binding by the receptor, and binds mannose, fucose and *N*-acetylglucosamine by direct ligation to Ca^{2+}. Site-directed mutagenesis combined with NMR and molecular modelling have been used to determine the orientation of monosaccharides bound to CRD-4. Two resonances in the ^1H NMR spectrum of CRD-4 that are perturbed on sugar binding are identified as a methyl proton from a leucine side chain in the core of the domain and the H-2 proton of a histidine close to the predicted sugar-binding site. The effects of mutagenesis of this histidine residue, a nearby isoleucine residue and a tyrosine residue previously shown to stack against sugars bound to CRD-4 show the absolute orientation of sugars in the binding site. *N*-Acetylglucosamine binds to CRD-4 of the mannose receptor in the orientation seen in crystal structures of the CRD of rat liver mannose-binding protein. Mannose binds to CRD-4 in the orientation seen in the CRD of rat serum mannose-binding protein and is rotated by 180 ° relative to GlcNAc bound to CRD-4. Interaction of the *O*-methyl group and C-1 of α-methyl Fuc with the tyrosine residue accounts for the strong preference of CRD-4 for this anomer of fucose. Both anomers of fucose bind to CRD-4 in the orientation seen in rat liver mannose-binding protein.

INTRODUCTION

The macrophage mannose receptor binds in a Ca^{2+}-dependent manner to terminal mannose, fucose or *N*-acetylglucosamine residues of glycoconjugates and plays a role in the innate immune system by mediating the phagocytosis of pathogens [1,2]. It is also involved in the endocytosis of endogenous glycoproteins bearing high-mannose oligosaccharides, including lysosomal enzymes [3] and tissue plasminogen activator [4]. The mannose receptor is a member of the C-type lectin family, members of which are characterized by homologous carbohydrate-recognition domains (CRDs) that mediate Ca^{2+}-dependent sugar binding [5]. Unlike most other C-type lectins, in which each polypeptide contains a single C-type CRD, the extracellular region of the mannose receptor contains eight C-type CRDs tandemly arranged in a single polypeptide, together with a fibronectin type II repeat and an N-terminal cysteine-rich domain [6]. The mannose receptor is the prototype for a new family of receptors containing multiple C-type CRDs [7].

Several of the eight CRDs of the mannose receptor, but not the cysteine-rich domain or the fibronectin type II repeat, are required for binding to carbohydrate ligands [8]. CRDs 4 and 5 form a protease-resistant ligand-binding core sufficient to bind some ligands with high affinity but CRDs 4–8 are required for high-affinity binding to natural ligands such as yeast mannan [9]. Only the fourth CRD has been shown to retain sugar-binding activity when expressed in isolation [8,9]. Thus an understanding at the molecular level of how CRD-4 interacts with sugars would be a first step towards understanding how the mannose receptor is able to select specific carbohydrate ligands that define potential pathogens or harmful glycoproteins.

CRD-4 of the mannose receptor has specificity for mannose, GlcNAc and fucose, like the CRDs of rat serum mannose-binding protein (MBP-A) and rat liver mannose-binding protein (MBP-C), which have been well characterized by crystallography, NMR and mutagenesis [10]. Ligand-binding studies on CRD-4 show that some aspects of the mode of binding of sugar and Ca^{2+} by CRD-4 are similar to those of the MBP CRDs but others are different, reflecting the fact that CRD-4 shows only 28 % and 25 % sequence identity with the CRDs of MBP-A and MBP-C respectively.

CRD-4 requires two Ca^{2+} ions for sugar binding [11]. The residues that ligate Ca^{2+} in the sugar-binding site in MBP-A and MBP-C are conserved in CRD-4 of the mannose receptor and in other C-type CRDs shown to exhibit Ca^{2+}-dependent sugar binding [5]. CRD-4 forms a ternary complex between sugar, Ca^{2+} and protein. Mutagenesis of one of the side chains at the conserved Ca^{2+} site provides evidence that the major interaction between CRD-4 and sugar is via direct ligation to Ca^{2+} bound at this site [12]. In MBP-A and MBP-C, one Ca^{2+} ligates directly to two equatorial hydroxy groups of a monosaccharide residue, whereas another Ca^{2+} is responsible for positioning the loops around the Ca^{2+} to which the sugar binds [13–15]. However, residues ligating the auxiliary Ca^{2+} in the MBPs are not conserved in CRD-4, and mutagenesis studies indicate that CRD-4 binds a second Ca^{2+} in a unique way [12]. This difference in ligation of the auxiliary Ca^{2+} is likely to cause a difference in the way in which the loops around the conserved Ca^{2+} binding site are arranged in CRD-4.

Analysis of NMR sugar titrations combined with site-directed mutagenesis revealed other unique aspects of sugar binding to CRD-4 [12]. In addition to direct ligation of sugar hydroxy

Abbreviations used: CRD, carbohydrate-recognition domain; MBP, mannose-binding protein; MBP-A, serum mannose-binding protein; MBP-C, liver mannose-binding protein.

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groups at the conserved Ca^{2+}, a hydrophobic stacking interaction with a tyrosine residue contributes approx. 25 % of the total free energy of mannose binding. Stacking of mannose or GlcNAc against aromatic residues is seen in crystal structures of plant lectins, but is not seen in co-crystals of the CRDs of MBP-A or MBP-C [10]. Ring current shifts seen in the ^{1}H NMR spectra of methyl glycosides in the presence of CRD-4 also provide evidence that bound mannose must be rotated by 180° relative to bound GlcNAc because C-5 and C-6 of mannose interact with Tyr^{729}, whereas C-1 and C-2 of bound GlcNAc are closest to this residue [12]. In contrast, mannose and GlcNAc are both bound to MBP-C in the same orientation [14]. Mannose bound to MBP-A is rotated by 180° with respect to mannose and GlcNAc bound to MBP-C [13]. However, the absolute orientation of mannose bound to CRD-4 has not been determined.

This paper describes further mutagenesis studies aimed at determining the absolute orientation of monosaccharides bound by CRD-4. Additional interactions between CRD-4 and bound sugar that determine monosaccharide specificity are also defined.

EXPERIMENTAL

Materials

$Mannose_{31}$-BSA was purchased from EY Laboratories. Monosaccharides, D_2O 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 $Na^{125}I$ were obtained from Amersham.

Mutagenesis procedures

Mutagenesis was performed on a fragment corresponding to bases 2000–2467 of a cDNA for the human macrophage mannose receptor [6] in the vector pUC19. Synthetic oligonucleotides were inserted at appropriate restriction sites by using standard recombinant DNA procedures. Modified *Xho*I–*Bam*HI fragments were transferred into a CRD-4 expression plasmid derived from pINIIIompA3 [8] and transformed into *Escherichia coli* strain JA221. Mutations were confirmed by DNA sequencing.

Protein expression and purification

Growth, induction and harvesting of transformed bacteria were exactly as described previously for CRD-4 [11]. Mutant and wild-type domains were purified from bacterial lysates by affinity chromatography on mannose-Sepharose [12]. Protein for NMR experiments was further purified by reverse-phase HPLC on a C_3 column [12].

Competition binding assays

Competition assays with CRDs immobilized on polystyrene wells followed published procedures [8]. ^{125}I-Man_{31}-BSA was the test ligand. Values for K_i, corresponding to the concentration of monosaccharide giving 50 % inhibition of test ligand binding, were calculated with a non-linear, least-squares-fitting program (SIGMAPLOT; Jandel Scientific). Means ± S.D. for three independent assays performed in duplicate were used to calculate K_i values. To allow for variability in different batches of reporter ligand, each set of assays included wild-type CRD-4 and inhibition with α-methyl Man. Because K_i depends on the affinity for the reporter ligand, which can vary for mutated versions of CRD-4, relative K_i values are reported.

NMR binding assays

Titrations with methyl glycosides were performed on a Varian Unity 500 MHz spectrometer [12]. Peak shifts that occurred on the addition of sugar were analysed using the equation:

$$\Delta\delta = \Delta\delta_B \times [\text{glycoside}]/([\text{glycoside}] + K_d) \quad (1)$$

where $\Delta\delta$ 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, $\Delta\delta_B$ is the difference in peak positions between the glycoside–protein complex and the free protein, and K_d is the concentration of glycoside that gives a half-maximal change in chemical shift. $\Delta\delta_B$ and K_d were calculated with the non-linear least-squares-fitting program.

Construction of molecular models

The coordinates of MBP-C bound to α-methyl Man (1rdl), α-methyl GlcNAc (1rdn), α-methyl Fuc (1rdi) and β-methyl Fuc (1rdj) were modified with the INSIGHT II program (BioSym Technologies). After changes in key amino acid residues, rotamers of these side chains that avoid steric clashes were selected from the built-in library. No attempt was made to minimize the structure. Identical changes were introduced into all four sets of coordinates.

RESULTS AND DISCUSSION

Identification of residues perturbed on binding of monosaccharides to CRD-4

Analysis of the ^{1}H NMR spectrum of CRD-4 in the presence of increasing concentrations of methyl glycosides shows that two of the protein resonances shift consistently on the addition of sugar [12]. Dissociation constants for binding of methyl glycosides to CRD-4 can be obtained by quantifying shifts in these two resonances. The positions of the two resonances in the spectrum (Figure 1), one in the aromatic region at approx. 8.2 p.p.m. and the other the most upfield peak in the spectrum at approx. −0.4 p.p.m., allow tentative identification of the type of residues that they represent. The peak in the aromatic region moves with changing pH in the manner of a histidine proton [12], and its chemical shift is characteristic of a histidine H-2 proton [16]. The upfield resonance is in the position where methyl protons of valine, leucine or isoleucine are found [16]. Further NMR studies combined with site-directed mutagenesis were undertaken to assign these resonances to particular amino acid residues in CRD-4.

CRD-4 contains four histidine residues [6]. In a model of CRD-4, two of the histidine residues, His^{692} and His^{753}, are predicted to be in the top half of the molecule, where the sugar is thought to bind, whereas the other two histidine residues are close to the N-terminus of the domain and are thus distant from the sugar-binding site [11]. His^{692} and His^{753} were each mutated to alanine and ^{1}H NMR spectra of the mutated domains were compared with that of wild-type CRD-4. The resonance at 8.2 p.p.m. seen in the wild-type spectrum is present in the spectrum of the $His^{692} \rightarrow$ Ala mutant, but is missing from the spectrum of the $His^{753} \rightarrow$ Ala mutant (Figure 2). No other changes are apparent in the spectra of the mutated domains, indicating that the mutations do not affect the overall fold of the CRD. These results demonstrate that the resonance at 8.2 p.p.m. corresponds to the H-2 proton of His^{753}.

His^{753} is located close to where the sugar is predicted to bind on the model of CRD-4, so the change in environment of this residue that occurs on sugar binding could be due to a direct interaction of His^{753} with the bound sugar. However, the

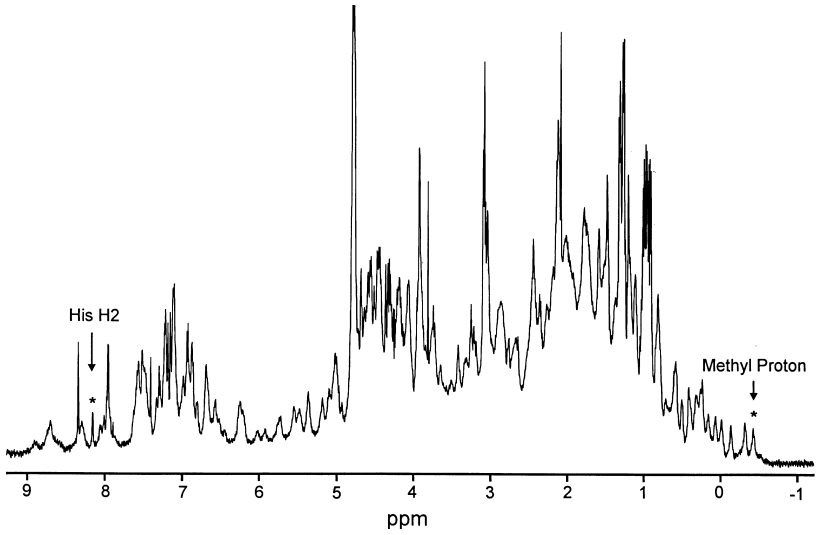

Figure 1 ^{1}H NMR spectrum of CRD-4

The asterisk denotes resonances that shift on the addition of monosaccharides bound by CRD-4.

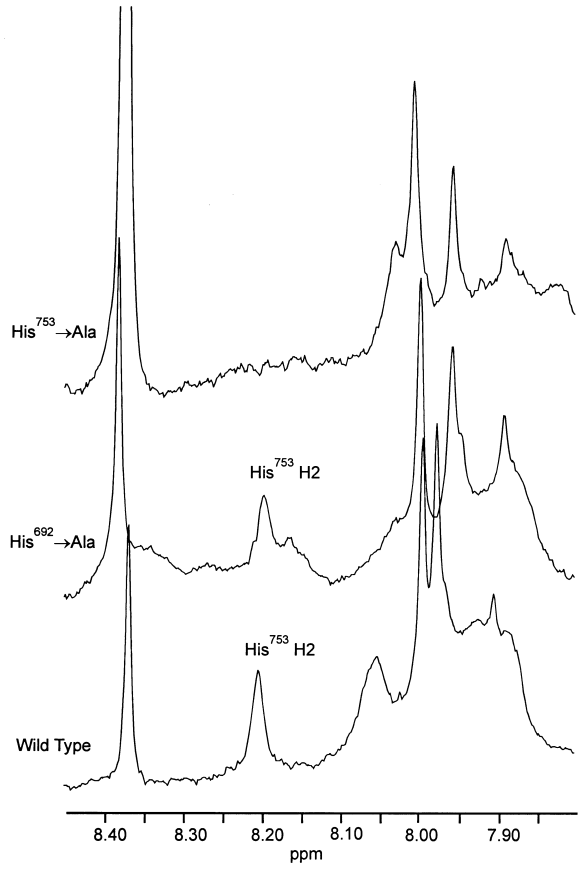

Figure 2 Part of the aromatic region of the ^{1}H NMR spectra of wild-type and mutated CRD-4

The histidine proton resonance that shifts on the addition of monosaccharides bound by CRD-4 was identified as the H-2 proton of His^{753}.

Table 1 Binding of α-methyl mannoside to wild-type and mutated CRD-4

Binding constants were determined by analysis of shifts in a methyl proton resonance and a histidine proton resonance in the ^{1}H NMR spectrum of CRD-4.

Mutant	$\Delta\delta_B$ (histidine) (p.p.m.)	$\Delta\delta_B$ (methyl) p.p.m.	$K_d^{histidine}$ (mM)	K_d^{methyl} (mM)	Average K_d (mM)
Wild type	0.039	−0.046	2.0	2.8	2.4 ± 0.4
$His^{753} \rightarrow Ala$	–	−0.046	–	3.2	3.2 ± 1.4*
$His^{692} \rightarrow Ala$	0.042	−0.049	2.4	3.9	3.1 ± 0.8
$Leu^{754} \rightarrow Ala$	0.036	−0.059	2.4	3.1	2.8 ± 0.3
$Val^{716} \rightarrow Ala$	0.029	−0.094	2.3	2.5	2.4 ± 0.1
$Val^{732} \rightarrow Ala$	0.046	−0.054	3.6	5.4	4.5 ± 0.9
$Ile^{749} \rightarrow Leu$	0.032	−0.027	1.7	1.4	1.6 ± 0.1
$Ile^{749} \rightarrow Val$	0.021	−0.055	4.2	4.2	4.2 ± 0.0
$Ile^{749} \rightarrow Ala$	–	−0.067	–	3.2	3.2 ± 0.1*

* Average K_d of two separate determinations of K_d^{methyl}, because the His^{753} proton resonance was absent from the spectrum of the $His^{753} \rightarrow Ala$ mutant and too close to the large peak from imidazole to allow shifts to be measured on sugar binding in the spectrum of the $Ile^{749} \rightarrow Ala$ mutant.

dissociation constant for α-methyl mannoside binding to the $His^{753} \rightarrow Ala$ mutant, determined by quantifying shifts in the methyl proton resonance, is not significantly different from that of the wild-type domain (Table 1). This result does not rule out the possibility of a direct interaction between His^{753} and the bound sugar, but indicates that such an interaction does not contribute significantly to the net energy of α-methyl Man binding. The change $His^{692} \rightarrow Ala$ also does not affect α-methyl Man binding. For the $His^{692} \rightarrow Ala$ mutant, the resonances at 8.2 and −0.4 p.p.m. shift to the same extent as in the wild-type domain, and the dissociation constant for α-methyl Man is not significantly different from that of the wild-type domain (Table 1).

A two-dimensional NMR experiment was performed on wild-type CRD-4 to determine whether the methyl proton resonance at −0.4 p.p.m. is from a valine, an isoleucine or a leucine residue. Cross peaks in a TOCSY spectrum indicate that the

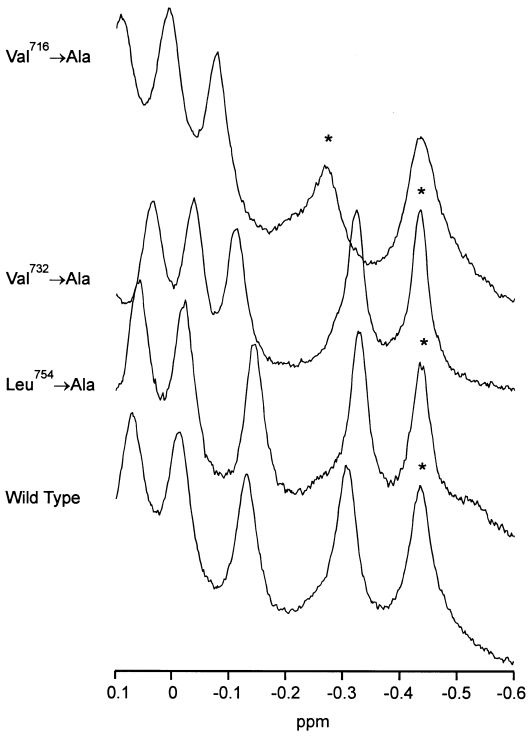

Figure 3 Upfield region of ^{1}H NMR spectra of wild-type and mutated CRD-4

The asterisks denote the methyl proton resonances that shift on the addition of monosaccharides bound to CRD-4.

resonance at −0.4 p.p.m. and the adjacent peak at approx. −0.3 p.p.m. are both from a single residue, which could be either leucine or valine but not isoleucine (results not shown). CRD-4 contains only two valine residues, Val^{716} and Val^{732}, each of which was mutated to alanine. The resonances at −0.4 and −0.3 p.p.m. are present in the ^{1}H NMR spectrum of each valine-to-alanine mutant (Figure 3), indicating that these resonances must come from a leucine residue rather than from one of the valine side chains. Interestingly, the change Val^{716} → Ala must alter the position of the leucine residue affected by sugar binding, because in this mutant domain the resonance at −0.3 p.p.m., but not that at −0.4 p.p.m., shifts during the α-methyl Man titration. Altering Val^{716} does not affect the affinity of the domain for α-methyl Man. In contrast, mutation of Val^{732} causes a small decrease in affinity for α-methyl Man (Table 1), which is probably due to perturbation of residues in the binding site because this side chain is located close to residues previously identified as Ca^{2+} ligands [12].

Methyl protons appear in the upfield regions of spectra when they are in close proximity to aromatic rings of phenylalanine, tryptophan or tyrosine residues [17]. Thus when sugar binds to CRD-4, the methyl proton of a leucine residue, which gives the resonance at −0.4 p.p.m., must move with respect to an aromatic ring so that it shifts further upfield. Because the resonance at −0.3 p.p.m. is due to the other methyl proton of the same leucine residue, and this resonance shows only a very small shift on the addition of sugar, the two terminal methyl groups of this leucine residue must be fixed in different positions with respect to the aromatic ring. Taken together, these results indicate that this leucine residue is likely to be in the core of the protein. The shift in the resonance of the methyl group is most probably an indirect effect due to a small conformational change in the domain upon binding of sugar, and probably does not reflect a direct interaction of a leucine side chain with the sugar.

Because the mutation of a residue in the core would be likely to result in disruption of the protein fold, mutagenesis of most of the nine leucine residues in CRD-4 was not practicable. However, Leu^{754} is next to His^{753} and is predicted to be close to the sugar-binding site at the surface of the protein, so the mutant Leu^{754} → Ala was created and tested. This change does not alter the upfield region of the spectrum (Figure 3) or the affinity of the domain for α-methyl Man (Table 1), confirming that the leucine residue affected by sugar binding must be in the core of the protein.

Although the two-dimensional NMR experiments indicate that the methyl proton that shifts on sugar binding to CRD-4 is not an isoleucine side chain, one of the five isoleucine residues in CRD-4 is predicted to be near the sugar-binding site. This isoleucine residue, Ile^{749}, is equivalent to Ile^{207} of MBP-A, which is seen to contact C-6 of mannose [13]. As expected, mutation of Ile^{749} to leucine, valine or alanine had no effect on the upfield methyl proton resonances (results not shown), confirming that the methyl group that shifts on sugar binding is not a side chain of this residue. However, mutation of Ile^{749} to either alanine or valine did cause a small decrease in absolute affinity for α-methyl Man (Table 1). Mutation of Ile^{749} to alanine or valine also caused a downfield shift in the H-2 proton resonance of His^{753} that is used to quantify sugar binding (results not shown). This result confirms that Ile^{749} and His^{753} are close to each other.

Determinants of specificity and orientation of monosaccharides bound to CRD-4

Mannose, GlcNAc and fucose are predicted to bind at a common site on CRD-4. Methyl glycosides of mannose, fucose and GlcNAc all produce shifts in the same two resonances when their binding to CRD-4 is analysed in NMR titrations [12]. However, there are differences in the way in which each sugar interacts with CRD-4. NMR studies showing ring current effects on specific sugar proton resonances due to interaction with Tyr^{729} indicate that mannose bound to CRD-4 must be rotated by 180° relative to bound GlcNAc [12]. Each sugar is predicted to ligate to the conserved Ca^{2+} ion via two equatorial hydroxy groups, but other interactions of the sugar with the protein must determine the orientation of sugars in the binding site as well as the relative affinities of CRD-4 for different sugars. Dissociation constants determined for binding of methyl glycosides indicate that CRD-4 binds α-methyl Man approximately twice as tightly as β-methyl Fuc and α-methyl GlcNAc, but has a strong preference for α-methyl Fuc, which is bound approx. 10-fold more tightly than β-methyl Fuc [12]. Further experiments were undertaken to ascertain the absolute orientation of mannose and GlcNAc bound to CRD-4, and to determine the basis for selective binding of α-methyl Fuc.

Table 2 shows the results of an extended analysis of the specificity of CRD-4 for monosaccharides. Inhibition constants were determined by using a solid-phase assay in which the inhibition of binding of CRD-4 to mannose-BSA is measured. The results are reported relative to the K_i for α-methyl Man. These ratios are very similar to those obtained by comparing dissociation constants determined by NMR titration, indicating that the competition assay is valid for determining specificity and for testing effects of mutations. The results confirm that CRD-4 shows the greatest affinity for α-methyl Fuc, which binds approx. 5-fold more tightly than α-methyl Man. The results also confirm

Table 2 Specificity of CRD-4 determined by NMR titration and competition binding

Inhibition constants were determined with the competition binding assay. Binding constants were determined by analysis of shifts as in Table 1. NMR titration results for α-methyl Man, α-methyl Fuc, β-methyl Fuc, α-methyl GlcNAc and GlcNAc were reported previously [12]. K_i for α-methyl Man was 3.4 ± 1.1 mM; K_d for α-methyl Man was 2.4 ± 0.4 mM.

Sugar	$K_i^{sugar}/K_i^{\alpha\text{-methyl Man}}$	$K_d^{sugar}/K_d^{\alpha\text{-methyl Man}}$
α-Methyl Fuc	0.13 ± 0.07	0.22 ± 0.10
ManNAc	1.4 ± 0.8	–
α-Methyl GlcNAc	2.1 ± 1.1	2.2 ± 0.4
β-Methyl Fuc	2.2 ± 0.9	2.2 ± 0.5
β-Methyl Man	–	2.5 ± 0.6*
GlcNAc	–	3.4 ± 1.2
2-Deoxy Glc	3.9 ± 2.3	–
α-Methyl Glc	4.4 ± 3.0	7.8 ± 3.3*
β-Methyl Glc	6.5 ± 3.7	–

* Results of NMR titrations with α-methyl Glc and β-methyl Man were: α-methyl Glc $\Delta\delta_B$ (histidine) = 0.031, $\Delta\delta_B$ (methyl) = −0.071, $K_d^{histidine}$ = 14.38 mM, K_d^{methyl} = 23.8 mM; β-methyl Man $\Delta\delta_B$ (histidine) = 0.033, $\Delta\delta_B$ (methyl) = −0.072, $K_d^{histidine}$ = 5.73 mM, K_d^{methyl} = 6.51 mM.

that CRD-4 binds α-methyl Fuc almost 20-fold more tightly than β-methyl Fuc, and indicate that CRD-4 shows the greatest discrimination between different anomers for fucose, because the preference for the α anomer of mannosyl and glucosyl glycosides is only 2–3-fold.

Absolute orientation of *N*-acetylglucosamine bound to CRD-4

Because the exact position of Tyr[729], the residue that causes ring current effects on C-5 and C-6 of mannose and on C-1 and C-2 of GlcNAc, cannot be predicted, information from the ring current shifts alone is not sufficient to determine whether the absolute orientation of mannose bound to CRD-4 is the same as mannose bound to MBP-A (orientation A) or to MBP-C (orientation C). However, the identification of His[753] as one of the two residues perturbed on sugar binding provides additional evidence that makes it possible to predict the absolute orientation of GlcNAc and mannose bound to CRD-4.

Evidence from NMR titrations suggests that the 2-acetamido group of GlcNAc bound to CRD-4 is close to His[753]. The binding of α-methyl Man, α-methyl Fuc or β-methyl Fuc causes quantitatively similar downfield shifts in the H-2 proton res-

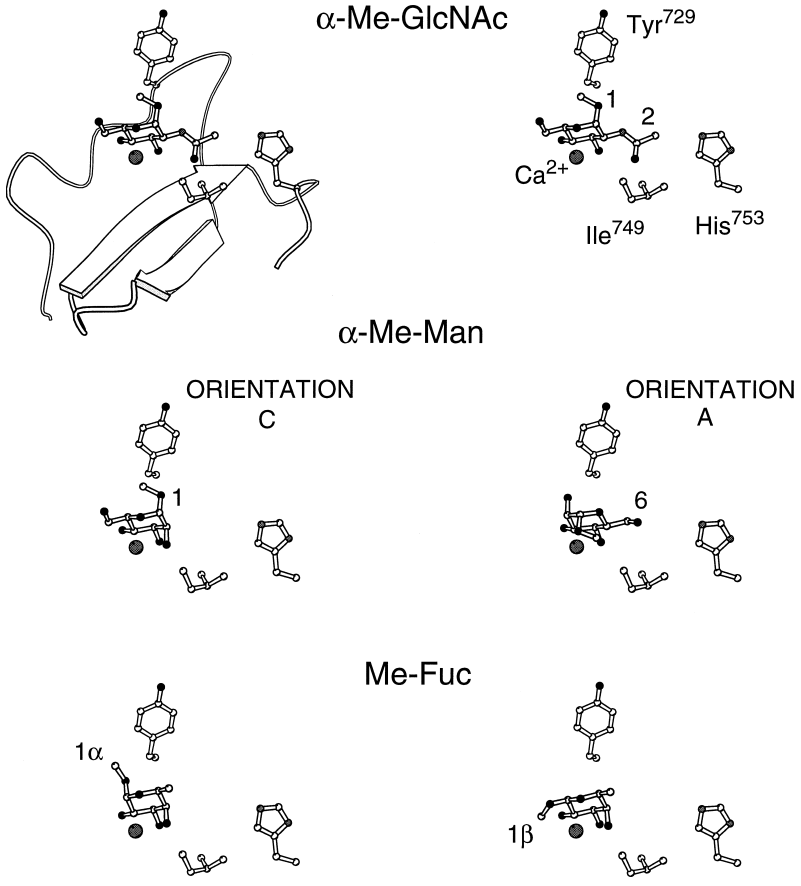

Figure 4 Orientation of monosaccharides bound to CRD-4

A model of methyl glycosides bound to CRD-4 based on the crystal structures of the CRD of rat MBP-C in complex with methyl glycosides of mannose, fucose and GlcNAc [14] was developed by introducing changes to CRD-4 residues Tyr[729], Ile[749] and His[753] at the equivalent positions in MBP-C. Top panels: α-methyl GlcNAc bound in the orientation seen in the MBP-C crystals, and predicted to be the orientation in CRD-4. In the middle panels, α-methyl Man is shown in the orientation seen in MBP-C (orientation C) and in the orientation seen in MBP-A, which is predicted to be the orientation in CRD-4. Bottom panels: the predicted orientations of α- and β-methyl Fuc bound to CRD-4. Carbon atoms are white, nitrogen atoms are grey and oxygen atoms are black. This figure was prepared with MOLSCRIPT [22].

Table 3 CRD-4 residues and their equivalents in other C-type CRDs

The CRD-4 residues mutated in this study and residues at equivalent positions in the CRDs of MBP-A [13], MBP-C [14] and the chicken hepatic lectin [18] are shown.

CRD-4	MBP-A	MBP-C	Chicken hepatic lectin
Tyr^{729}	His^{189}	Val^{194}	Arg^{173}
Ile^{749}	Ile^{207}	Val^{212}	Val^{191}
His^{753}	Ala^{211}	Asp^{216}	Tyr^{195}

Table 4 Effects of mutation of His^{753} and Ile^{749} on specificity of CRD-4

Inhibition constants were determined with the competition binding assay. Results are shown relative to the K_i for α-methyl Man.

	$K_i^{sugar}/K_i^{\alpha\text{-methyl Man}}$		
Mutant	α-methyl GlcNAc	α-Methyl Fuc	β-Methyl Fuc
Wild type	1.9 ± 0.4	0.13 ± 0.07	2.2 ± 0.9
$His^{753} \rightarrow Ala$	1.9 ± 0.6	0.15 ± 0.05	2.4 ± 0.9
$Ile^{749} \rightarrow Leu$	2.3 ± 0.7	0.14 ± 0.03	2.0 ± 0.4
$Ile^{749} \rightarrow Val$	2.5 ± 0.5	0.07 ± 0.03	1.5 ± 0.4
$Ile^{749} \rightarrow Ala$	5.3 ± 1.2	0.13 ± 0.04	1.9 ± 0.6

Table 5 Effect of mutation of Tyr^{729} on specificity of CRD-4 for methyl fucosides

Inhibition constants were determined with the competition binding assay. Results for GlcNAc [12] are shown in the last row for comparison.

Inhibition constant ratio	Wild type	$Tyr^{729} \rightarrow Ala$
$K_i^{\beta\text{-methyl Fuc}}/K_i^{\alpha\text{-methyl Fuc}}$	18.0 ± 3.4	1.2 ± 0.6
$K_i^{\alpha\text{-methyl Fuc}}/K_i^{\alpha\text{-methyl Man}}$	0.13 ± 0.02	0.6 ± 0.4
$K_i^{\beta\text{-methyl Fuc}}/K_i^{\alpha\text{-methyl Man}}$	2.1 ± 0.7	1.0 ± 0.6
$K_i^{GlcNAc}/K_i^{mannose}$	1.7 ± 0.4	1.6 ± 0.4

onance of His^{753} at 8.2 p.p.m. In contrast, GlcNAc binding causes an upfield shift in this resonance [12]. The binding of two additional sugars, α-methyl Glc and β-methyl Man, was analysed by NMR titration and these both caused the His^{753} proton resonance to shift downfield (Table 2). These results indicate that GlcNAc binding has a different effect on the environment of His^{753} than the binding of sugars that do not contain the 2-acetamido group. The most likely explanation for this difference is that GlcNAc is orientated in the binding site such that the 2-acetamido group is close to His^{753}.

Siting the 2-acetamido group of GlcNAc close to His^{753} makes it possible to propose that the absolute orientation of GlcNAc bound to CRD-4 is the same as in MBP-C. Starting from the crystal structure of the CRD of rat MBP-C in a complex with α-methyl GlcNAc [14], models for methyl glycosides bound to CRD-4 were created. Figure 4 (top left panel) shows the backbone of the top part of the MBP-C CRD with α-methyl GlcNAc bound through hydroxy groups 3 and 4 at Ca^{2+}-2, with three MBP-C residues (shown in Table 3) replaced by CRD-4 residues Tyr^{729}, Ile^{749} and His^{753}. Ring current effects seen on protons from the *O*-methyl group and C-2 during titrations with α-methyl GlcNAc indicate that these two groups must be closest to Tyr^{729} [12], so the side chain of Tyr^{729} was rotated slightly to achieve optimal alignment with the *O*-methyl group.

This orientation of α-methyl GlcNAc places the 2-acetamido group close to the side chain of Ile^{749} as well as that of His^{753}. In the C-type CRD of the chicken hepatic lectin, residues equivalent to Ile^{749} and His^{753} are Val^{191} and Tyr^{195} (Table 3). These two residues in the chicken hepatic lectin are thought to form part of a binding pocket for the 2-acetamido group, which confers selective binding of GlcNAc [18]. Thus the effects of mutation of Ile^{749} and His^{753} on GlcNAc binding were examined to provide additional evidence for the proposed orientation of GlcNAc in the binding site.

As discussed above, NMR titration with the $His^{753} \rightarrow Ala$ mutant shows that this residue does not contribute to the net energy of binding of α-methyl Man. Comparison of K_i values obtained with the competition binding assay further demonstrates that this mutation also has no effect on the binding of α-methyl GlcNAc or either methyl fucoside (Table 4). Thus although the 2-acetamido group of GlcNAc is close enough to perturb the NMR spectrum of His^{753}, this interaction does not provide net binding energy. In contrast, a comparison of K_i values obtained with the competition binding assay shows that the mutation of Ile^{749} to leucine, valine or alanine has a selective effect on α-methyl GlcNAc binding. The $Ile^{749} \rightarrow Ala$ mutant binds α-methyl GlcNAc one-fifth as tightly as α-methyl Man, compared with only a two-fold difference for wild-type CRD-4 (Table 4). Mutation of Ile^{749} does not affect the affinities of either methyl fucoside relative to α-methyl Man. These results suggest that an interaction with the side chain of Ile^{749} contributes to binding of α-methyl GlcNAc but not to binding of α-methyl Man or either methyl fucoside, and confirm that α-methyl GlcNAc must bind to CRD-4 in the orientation shown in Figure 4.

Absolute orientation of mannose bound to CRD-4

Having determined that α-methyl GlcNAc must bind to CRD-4 in the orientation seen in MBP-C (orientation C), and optimized the position of Tyr^{729}, the absolute orientation of α-methyl Man can be deduced. Figure 4 (middle panels) shows α-methyl Man bound in both orientations A and C. Because ring current shifts owing to interaction with Tyr^{729} are seen on H-5, H-6 and H-6′ but not on the *O*-methyl group of α-methyl Man during NMR titrations [12], α-methyl Man must bind to CRD-4 in orientation A. If α-methyl Man were bound to CRD-4 in orientation C, strong ring current effects on the *O*-methyl group protons would be seen. Orientation A also puts C-6 close to the side chain of Ile^{749}, consistent with the small decrease in binding seen when this residue is mutated to valine or alanine (Table 1). Ring current effects were also seen on protons of C-5 and C-6 in NMR titrations with β-methyl Man (results not shown), indicating that the two anomers of mannose both bind in orientation A.

Determinants of preference of CRD-4 for α-methyl Fuc

A strong ring current effect is seen on the resonance for the *O*-methyl group in the spectrum of α-methyl Fuc in the presence of CRD-4, with smaller effects on C-5 and C-1 [12]. No ring current effect is seen for β-methyl Fuc, suggesting that interaction with Tyr^{729} could determine the preference for the α anomer. Binding of both anomers of fucose to the $Tyr^{729} \rightarrow Ala$ mutant of CRD-4 was investigated with the competition binding assay. Values of K_i obtained for α- and β-methyl Fuc are summarized in Table 5,

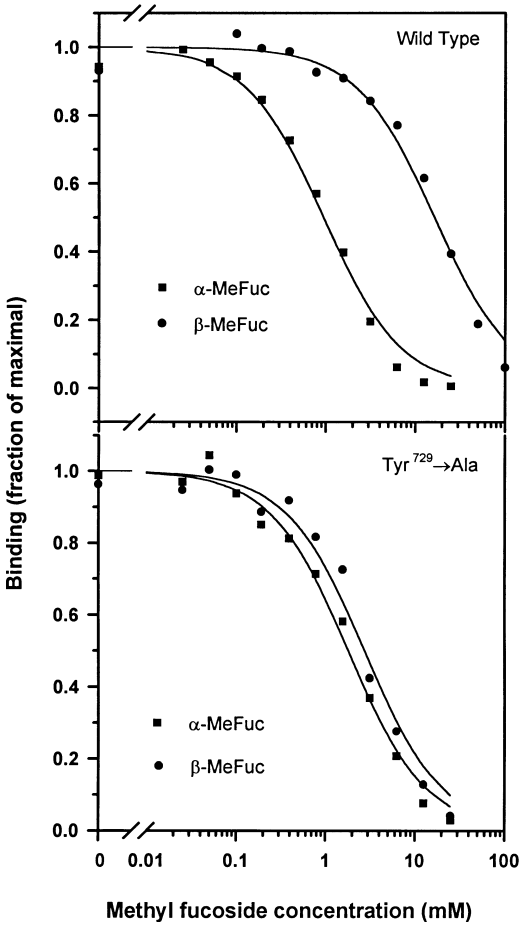

Figure 5 Inhibition of ^{125}I-mannose-BSA binding to wild-type and mutated CRD-4 by methyl fucosides

Each curve is representative of three separate experiments performed in duplicate.

with representative binding curves shown in Figure 5. Because mutation of Tyr^{729} to Ala decreases the affinity of CRD-4 for mannose [12], and thus for the mannose-BSA reporter ligand, comparison of the absolute values of K_i for the wild type and the $Tyr^{729} \rightarrow$ Ala mutant is not meaningful. However, comparison of the relative inhibitory potencies of α- and β-methyl Fuc reveals that CRD-4 with an alanine residue at position 729 shows almost no preference for α-methyl Fuc over β-methyl Fuc. These results indicate that interaction of the *O*-methyl group with Tyr^{729} contributes substantially to the binding of α-methyl Fuc.

The CRD of rat MBP-A binds α-methyl Fuc approx. 5-fold more tightly than β-methyl Fuc and thus also discriminates between the two anomers of fucose, although less effectively than does CRD-4 [19]. The difference in affinities for α- and β-methyl Fuc is abolished in the $His^{189} \rightarrow$ Ala mutant of MBP-A [19]. His^{189} is equivalent to Tyr^{729} of CRD-4 (Table 3). In contrast, mutation of Ile^{207}, the residue seen to contact C-6 of mannose in the crystal structure of MBP-A, has no effect on the ability of the CRD to discriminate between α- and β-fucose. Ile^{749} aligns with Ile^{207} in MBP-A (Table 3). As discussed above, mutation of Ile^{749} to alanine, leucine or valine had little effect on the relative affinities of CRD-4 for α-methyl Fuc and β-methyl Fuc (Table 4). Mutation of His^{753} also did not affect the relative affinities for the two anomers of fucose. These results suggest that interaction with Tyr^{729} is the major determinant of relative affinities of CRD-4 for α-methyl Fuc and β-methyl Fuc.

Comparison of the effects of the mutation $Tyr^{729} \rightarrow$ Ala on the relative affinities of CRD-4 for α-methyl Fuc, β-methyl Fuc and α-methyl Man shows that interaction of the *O*-methyl group of α-methyl Fuc with Tyr^{729} is also a major factor in the higher affinity for this sugar relative to α-methyl Man (Table 5). Interaction of C-5 and C-6 of α-methyl Man with Tyr^{729} has been shown to contribute to the binding energy for α-methyl Man [12]. However, this interaction seems to be weaker than the interaction of α-methyl Fuc with Tyr^{729}, because the $Tyr^{729} \rightarrow$ Ala mutant shows less preference for α-methyl Fuc over α-methyl Man than does the wild-type domain (Table 5). In contrast, the mutation $Tyr^{729} \rightarrow$ Ala abolishes the preference of CRD-4 for α-methyl Man over β-methyl Fuc, confirming that the higher affinity for α-methyl Man is due to interaction with Tyr^{729}. Previous experiments have indicated that interaction with Tyr^{729} is important for the binding of GlcNAc and that mutation of this residue does not affect the relative affinities of CRD-4 for GlcNAc and mannose [12].

Absolute orientations of methyl fucosides bound to CRD-4

Mutagenesis and NMR studies described above indicate that α-methyl Fuc binds tightly to CRD-4 as a result of the interaction of the *O*-methyl group with Tyr^{729}. MBP-C binds α-methyl Fuc through hydroxy groups 2 and 3 in the same orientation as β-methyl Fuc. As shown in Figure 4, modelling of CRD-4 with α-methyl Fuc in this orientation places the *O*-methyl group close to Tyr^{729}. In contrast, the *O*-methyl group of β-methyl Fuc is pointed away from Tyr^{729}, accounting for the weaker binding of this anomer. This orientation of methyl Fuc also shows that no part of the sugar is very close to the side chain of Ile^{749}, in keeping with the observation that mutagenesis of Ile^{749} has no effect on binding of this sugar. Thus it is likely that each anomer of fucose binds to CRD-4 in the orientation seen in the MBP-C crystals.

Conclusions

Determination of the absolute orientations of sugars bound to CRD-4 of the mannose receptor shows that the relative orientations of different sugars are not the same as is seen in the CRD of MBP-C. CRD-4 binds GlcNAc in the same orientation as MBP-C, which is also the probable orientation of GlcNAc bound to the CRD of the chicken hepatic lectin [18]. CRD-4 is also predicted to bind both anomers of fucose in the same orientation as in MBP-C. However, mannose binds to CRD-4 in the orientation seen in the crystal structures of MBP-A, which is rotated by 180° with respect to mannose and GlcNAc bound to MBP-C and also to GlcNAc bound to CRD-4.

The macrophage mannose receptor and soluble MBPs represent components of alternative pathways in the innate immune response. They both recognize potential pathogens on the basis of the presence of a similar set of sugars, particularly mannose and GlcNAc, but the spectra of bacteria and fungi that interact with the two systems seem to be different [20,21]. These differences probably reflect differing affinities for particular arrays of oligosaccharides. The selection of potential ligands by these two branches of the innate immune response depends on the relative orientations of the multiple binding sites in the clusters of CRDs in each of these proteins. The binding of oligosaccharide and polysaccharide ligands depends both on the relative disposition of the binding sites and on the orientation of sugars bound in

each site. The NMR and mutagenesis data presented in this paper establish that the relative orientations of monosaccharides bound to the primary sugar-binding site in the mannose receptor and in MBP-C are different. This difference is likely to underlie some of the different functional properties of these molecules.

The different ligand orientations observed for various mannose-binding and GlcNAc-binding CRDs presumably reflect subtle differences in the binding sites. Some of the results presented here suggest contacts that are important for binding each of the different monosaccharide ligands, but it is not yet possible to define the underlying physical chemistry that determines preferred binding orientation. Further structural analysis of CRD-4 in complex with oligosaccharide ligands will be required as a basis for developing this type of understanding.

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