Carbohydrate Binding Mechanism of the Macrophage Galactose-type C-type Lectin 1 Revealed by Saturation Transfer Experiments*

Macrophage galactose-type C-type lectins 1 and 2 (MGL1/2) are expressed on the surfaces of macrophages and immature dendritic cells. Despite the high similarity between the primary sequences of MGL1 and MGL2, they display different ligand specificities. MGL1 shows high affinity for the Lewis^X^ trisaccharide, whereas MGL2 shows affinity for N-acetylgalactosamine. To elucidate the structural basis for the ligand specificities of the MGLs, we performed NMR analyses of the MGL1-Lewis^X^ complex. To identify the Lewis^X^ binding site on MGL1, a saturation transfer experiment for the MGL1-Lewis^X^ complex where sugar-CH/CH2-selective saturation was applied was carried out. To obtain sugar moiety-specific information on the interface between MGL1 and the Lewis^X^ trisaccharide, saturation transfer experiments where each of galactose-H5-, fucose-CH3-, and N-acetylgalactosamine-CH3-selective saturations was applied to the MGL1-Lewis^X^ complex were performed. Based on these results, we present a Lewis^X^ binding mode on MGL1 where the galactose moiety is bound to the primary sugar binding site, including Asp-94, Trp-96, and Asp-118, and the fucose moiety interacts with the secondary sugar binding site, including Ala-89 and Thr-111. Ala-89 and Thr-111 in MGL1 are replaced with arginine and serine in MGL2, respectively. The hydrophobic environment formed by a small side chain of Ala-89 and a methyl group of Thr-111 is a requisite for the accommodation of the fucose moiety of the Lewis^X^ trisaccharide within the sugar binding site of MGL1.
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exact roles of these residues in the sugar binding, structural information for the interaction between MGLs and their ligand carbohydrates is required.

Crystallographic studies on engineered Gal-type mutants of mannose-binding protein complexed with Gal-type monosaccharides have provided insights into the galactose binding modes of these lectins (17–19). These structures revealed the monosaccharide binding mode but not the polysaccharide binding mode. The crystal structure of human asialoglycoprotein receptor (ASGPR) has also been reported (20). In this structure, no bound sugar is visible even though the protein was co-crystallized with ligand carbohydrate. Therefore, there is no information about the interaction between the ligand and the lectin. In addition, a crystal structure of mouse scavenger receptor C-type lectin (mSRCL) has been solved with bound LewisX trisaccharide (21). This structure revealed that the LewisX binding mode is different from that on Man-type lectins, such as ASGPR. This is because mSRCL lacks the glycine-rich loop, which is a characteristic structural component of Gal-type lectins, including MGLs and ASGPRs, and plays a crucial role in fixing the correct position of the indole ring of the tryptophan residue to interact with the galactose (17). Although x-ray studies have been extensively performed, little is known about the structural basis of the difference in the ligand binding specificities between MGL1 and MGL2.

In this report, we describe the results of our structural analyses of the MGL1-LewisX complex to understand the molecular basis for its carbohydrate specificity. First we quantitatively compared the affinities between MGL1 and its sugar ligands, the LewisX trisaccharide and β-methylgalactose, by isothermal calorimetry. Second we analyzed the 13C chemical shifts originating from MGL1 to characterize its topology. Finally we performed saturation transfer experiments to determine the sugar binding site on MGL1. Based on the structural data, we discuss the differential ligand specificities between MGL1 and MGL2.

EXPERIMENTAL PROCEDURES

Sample Preparation—Recombinant MGL1 was expressed in Escherichia coli BL21-Codon Plus (DE3)-RP cells (Stratagene) using the pET-21a plasmid containing the CRD of MGL1 (3). The C5S mutation was introduced into the CRD of MGL1, according to the QuikChange site-directed mutagenesis protocol (Stratagene), to prevent intermolecular oligomerization. In the following, we refer to MGL1 with the C5S mutation as the wild-type MGL1 unless otherwise described. Uniformly 15N-labeled and 13C,15N-labeled proteins were overexpressed in M9 minimal medium containing 1 g/liter 15NH4Cl (Shoko Co., Ltd.) and 4 g/liter glucose or 2 g/liter [13C]glucose (Cambridge Isotope Laboratories, Inc.), respectively. The cells were fermented at 37 °C until the A600 reached 0.5. Expression was induced with 1 mm isopropyl-1-thio-β-D-galactopyranoside for 5 h. The uniformly 2H,15N-labeled protein was overexpressed in 99.0% 2H2O (Cambridge Isotope Laboratories, Inc.) M9 minimal medium containing 1 g/liter 15NH4Cl, 2 g/liter [2H]glu-

Received for publication, November 1, 2007, and in revised form, November 20, 2007, published, November 28, 2008
Published 2008. This article is a U.S. Government work and is in the public domain in the U.S. 33666 J. Biol. Chem. 2008, 283(48) 28050–28058

VOLUME 283 • NUMBER 48 • NOVEMBER 28, 2008
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To characterize the carbohydrate binding activity of MGL1 quantitatively, the association constants for the interactions between wild-type MGL1 and the Lewis\textsuperscript{x} trisaccharide, wild-type MGL1 and \(\beta\)-methylgalactose, the A89L mutant of MGL1 and the Lewis\textsuperscript{x} trisaccharide, the A89L mutant of MGL1 and the Lewis\textsuperscript{x} trisaccharide, and the T111S mutant of MGL1 and the Lewis\textsuperscript{x} trisaccharide were determined by ITC. Fig. 1 shows the titration curves for the Lewis\textsuperscript{x} (A) and \(\beta\)-methylgalactose (B) binding to wild-type MGL1. The results obtained from the experiments are given in Table 1. Wild-type MGL1 showed higher affinity to the Lewis\textsuperscript{x} trisaccharide compared to the Lewis\textsuperscript{x} trisaccharide. The results are consistent with the carbohydrate binding activity of MGL1, indicating that MGL1 is a specific binding protein for the Lewis\textsuperscript{x} trisaccharide.
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FIGURE 3. Secondary structural elements, topology, and modeled structure of MGL1. A, ΔCαβ values obtained for the MGL1-LewisX complex plotted against the residue number. The regions with four or more consecutive >1.4 values and those with <−1.4 were predicted as α helices and β strands, respectively. *1 and *2 show the regions that are not likely to form β strands because no interstrand NOE between main chain protons was observed. Putative secondary structural elements labeled with residue numbers are shown on the top of the diagram. Helices and strands are represented by cylinders and arrows, respectively. B, topology diagram of the MGL1-LewisX complex predicted from the chemical shift deviations and the NOEs. The β sheets, I, II, and III are colored blue, orange, and green, respectively. Red lines indicate interstrand NOEs. C, ternary structural model of MGL1 prepared by SWISS-MODEL.

with that to β-methylgalactose. The A89L, A89R, and T111S mutants showed decreased affinity to the LewisX trisaccharide compared with that of the wild-type MGL1. It was also found that the sugar binding site within the CRD of MGL1 was single. The association constants obtained from ITC in the present study are different from those from SPR in the previous study (16). The difference is because of the immobilization effects in the SPR experiments as described previously (34, 35). In the previous SPR experiments, the rebinding effects were not negligible because the total amount of the sugar ligand immobilized on the sensor chip via polyacrylamide was high. However, what occurs in the SPR experiments under the condition with the high contents of the sugar ligands is quite similar to that on cell surfaces. In this sense, the data from the SPR experiments is meaningful. In this study, to prepare NMR samples of the MGL1-LewisX complex in a proper stoichiometry, the association constants were determined by ITC under the same condition as that for the NMR experiments.

Secondary Structural Elements and Topology of MGL1—The 66% sequence homology is high for MGL1 assuming the similar CRD folding. However, there are still possibilities of subtle structural differences between MGL1 and its model in partial unfolding of α helices, the positions of short helices, and the length of β strands. To rule out these possibilities, we analyzed the secondary structural elements and topology of the MGL1-LewisX complex using NMR. Fig. 2 shows the 1H-15N HSQC spectrum of the MGL1-LewisX complex. The backbone resonance assignments for the complex were accomplished by analyzing a set of the standard triple resonance NMR spectra (HNCA, HN(CO)CA, HN(CA)CB, and CBCA(CO)NH). Of 131 possible resonances originating from the backbone amide groups within the CRD of MGL1, 128 resonances (98%) could be assigned. Complete (137 of 137) assignments were obtained for the 13Cα and 13Cβ resonances. Using the deviations of the 13Cα and 13Cβ chemical shifts from the random coil values (Δ13Cα,β), the secondary structural elements within MGL1 were analyzed (Fig. 3A). The configurations of the β sheets were determined by analyzing the interstrand NOEs among the main chain protons. The resonances originating from the Hα were assigned by analyzing the HBHA(CO)NH spectrum. The predicted topology of the secondary structure of MGL1 is shown in Fig. 3B. MGL1 consists of two α helices (residues 27–36 and 48–59) and three β sheets (sheets I, II, and III). Sheet I consists of three β strands (β1, residues 16–19; β3, residues 39–43; and β9, residues 129–133), whereas sheet II has two β strands (β2, residues 22–26; and β7, residues 124–126), and sheet III has four β strands (β4, residues 61–64; β5,
residues 105–111; β6, residues 116–120; and β8, residues 126–129). As for the two regions comprising residues 2–5 and 85–88, no interstrand NOEs were observed, suggesting that no β sheet structure exists in these regions, although the chemical shift predicted the β strand-like conformation. Based on the similarities in the amino acid sequence and the topology of the secondary structure, the structural model of MGL1 was constructed by SWISS-MODEL (Fig. 3C).

LewisX Binding Site on MGL1 Determined by Saturation Transfer Experiments—To determine the binding site of the LewisX trisaccharide on MGL1, saturation transfer experiments (36–38) were performed on MGL1 complexed with the LewisX trisaccharide. We first checked the effect of the saturation on MGL1 in the free state. Each of the saturations at 3.7, 1.1, 0.81, and 1.7 ppm caused no obvious signal intensity reductions for the amide resonances because the protein had been fully deuterated. Exceptionally signal intensity reduction ratios from 0.1 to 0.8 were observed for Glu-37, Asn-38, Ser-39, and Val-44 by the saturation at 3.7 ppm probably because of the spin diffusion from nearby hydroxyl protons, such as those of Tyr-13 and Ser-39.

The LewisX-selective saturation was achieved by applying an rf irradiation centered at 3.7 ppm, which corresponds to most of the CH/CH2 protons within the sugar in the MGL1-bound state. Fig. 4 shows an expended region of the 1H-15N HSQC spectra of the MGL1-LewisX complex without and with saturation. Residue-selective signal intensity reductions were observed at 15 °C. Few signal intensity reductions were observed at 25 °C because of the short rotational correlation time of the MGL1-LewisX complex. It might be effective to lower the experimental temperature for the saturation transfer experiment on a complex with a molecular weight of ~20,000. Of 127 analyzed signals originating from the main chain amide groups of MGL1, the signals from Gln-92, Asp-94, Trp-96, and Asp-118 exhibited intensity reduction ratios of more than 0.3; the signals from Ala-89, His-109, Trp-116, and Asn-117 showed intensity reduction ratios from 0.15 to 0.3; and the signals from Phe-97, Gly-98, Asp-106, Cys-107, Thr-111, and Asp-119 showed intensity reduction ratios from 0.1 to 0.15 (Fig. 5A).

The residues affected by the saturation were mapped on the model structure of MGL1 (Fig. 6A). The affected residues formed a contiguous surface on MGL1, indicating that these residues form the LewisX binding site (Fig. 6D).

To obtain the sugar moiety-specific information on the interface between MGL1 and the LewisX trisaccharide, saturation transfer experiments, where the H5 proton of the Gal moiety (Gal-H5), the methyl protons of the Fuc moiety (Fuc-CH3), and the methyl protons of the GlcNAc moiety (GlcNAc-CH3) were saturated, were performed. The selective saturations of Gal-H5, Fuc-CH3, and GlcNAc-CH3 in the MGL1-bound state were achieved by applying rf irradiations centered at 1.1, 0.81, and 1.7 ppm, respectively. The resonances originating from the LewisX trisaccharide complexed with 2H,15N-labeled MGL1. By the Gal-H5 saturation, the signals from Asp-94, Trp-96, and Asp-118 showed intensity reduction ratios from 0.15 to 0.3, and the signals from Ala-89, Gln-92, His-109, and Asn-117 showed intensity reduction ratios from 0.1 to 0.15 (Fig. 5B). The affected residues were mapped on the model structure of MGL1 (Fig. 6B). By the Fuc-CH3 saturation, the signals from Ala-89, Gln-92, Asp-94, Trp-116, Asn-117, and Asp-118 showed intensity reduction ratios from 0.15 to 0.3, and the signals from Trp-96, His-109, and Thr-111 showed intensity reduction ratios from 0.1 to 0.15 (Fig. 5C). The affected residues were also mapped on the model structure of MGL1 (Fig. 6C). The GlcNAc-CH3 saturation slightly affected the intensity of the signal from Trp-96 (Fig. 5D).

DISCUSSION

The topology of MGL1 predicted by the NMR analyses indicated that the CRD of MGL1 basically adopts the typical C-type lectin fold, which characteristically has two α helices and two antiparallel β sheets within the fold (39). In addition, the topology of MGL1 was almost identical to that of the x-ray crystal structure of the human ASGPR, which shares high (66%) amino acid sequence homology to MGL1 (20). The secondary structural elements observed within the model structure that was made using SWISS-MODEL (Fig. 3C) agreed well with those predicted by the NMR analyses, supporting the validity of the main chain configuration of the modeled structure. In the following discussion, we use the modeled structure of MGL1 to interpret the results.

The LewisX binding site on MGL1 was successfully determined by the saturation transfer experiment where the CH/CH2 protons within the LewisX trisaccharide were saturated (Fig. 6, A and D). The central region of the LewisX binding
The carbohydrate binding site of MGL1 is mainly composed of polar residues, Gln-92, Asp-94, Asn-117, and Asp-118. The polar region is sandwiched by two hydrophobic regions. One region is composed of Trp-96, Phe-97, and Gly-98, and the other region is formed by Ala-89, Thr-111, and Trp-116.

Among the residues in the Lewis\(^X\) binding site, three residues, Asp-94, Trp-96, and Asp-118, experienced signal intensity reductions of more than 0.15 by the Gal-H5-selective saturation (Fig. 6B). The duration of 2.5 s used for the selective saturation is long enough to cause intra-Gal spin diffusion. Therefore, the residues affected by the Gal-H5-selective saturation are in close proximity to the protons within the Gal moiety. By the Fuc-CH\(_3\)-selective saturation, six residues, Ala-89, Gln-92, Asp-94, Trp-116, Asn-117, and Asp-118 experienced signal intensity reductions of more than 0.15 (Fig. 6C). These residues are in close proximity to the protons within the Fuc moiety. In contrast, no residues experienced signal intensity reductions of more than 0.15 by the GlcNAc-CH\(_3\)-selective saturation. This result suggests that the GlcNAc moiety of the Lewis\(^X\) trisaccharide does not make direct interactions with MGL1. This observation is supported by the fact that the sum of the residues affected by the Gal-H5 saturation and those affected by the Fuc-CH\(_3\) saturation corresponded to that of the residues constituting the Lewis\(^X\) binding site. In addition, few chemical shift perturbations were observed for resonances originating from the GlcNAc moiety in the Lewis\(^X\) trisaccharide upon the MGL1 binding (data not shown).

Weak signal intensity reduction of Trp-96 by the saturation of GlcNAc-CH\(_3\) (1.7 ppm) will be caused by incidental saturation of the H6 proton of the Gal moiety (1.9 ppm).

In total, the Gal and Fuc moieties of the Lewis\(^X\) trisaccharide occupy the entire Lewis\(^X\) binding site on MGL1. The Gal moiety is located in close proximity to Asp-94, Trp-96, and Asp-118. The Fuc moiety binding site is located adjacent to the Gal...
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In the crystal structures of the Gal-type mannose-binding protein mutants complexed with Gal-type monosaccharides, the galactose ring is bound on the calcium ion, which is chelated by five residues, corresponding to Gln-92, Asp-94, Glu-105, Asp-118, and Asn-117 in MGL1. The tryptophan residue, corresponding to Trp-96 in MGL1, packs against the apolar face of the galactose ring, and the amide group of the aspartic acid residue, corresponding to Asp-118 in MGL1, is located near the 2-CH of the galactose (17–19). These arrangements are consistent with the present results of the saturation transfer experiments (21). Interestingly the overall LewisX binding mode on MGL1 is highly related with that on mSRCL. However, amino acid residues interacting with the Fuc moiety of the LewisX trisaccharide are not conserved between these lectins, and the orientation of the tryptophan side chain of mSRCL (Trp-698), which interacts with the galactose moiety of the LewisX trisaccharide, is different from those of typical Gal-type C-type lectins with the glycan-rich loop (17–20). These structural differences might explain the 4.3 times higher affinity of MGL1 for the LewisX trisaccharide than that of human scavenger receptor C-type lectin (40).

Fig. 8 compares the amino acid sequences of MGL1, MGL2, and the related Gal-type C-type lectins. Although most of the amino acid residues identified within the LewisX binding site on MGL1 are conserved in MGL2, interestingly Ala-89 and Thr-111 are replaced by Arg-89 and Ser-111 in MGL2, respectively. This observation suggests that the amino acid residues at positions 89 and 111 are responsible for the different ligand specificities between MGL1 and MGL2. As for MGL1, the side chain of Ala-89 is small enough to accommodate the Fuc moiety of the LewisX trisaccharide within the secondary sugar binding site around Ala-89. On the other hand, the bulky side chain of Arg-89 in MGL2 would sterically hinder the access of the Fuc moiety, leading to lower affinity for the LewisX trisaccharide. This consideration is supported by the site-directed mutagenesis study where the A89L and A89R mutants of MGL1 showed lower affinity (<10%) for the LewisX trisaccharide as compared with that of the wild type. The importance of the methyl group of Thr-111 for the binding to the LewisX trisaccharide was also confirmed by the site-directed mutagenesis study. The hydrophobic environment formed by a small side chain of Ala-89 and a methyl group of Thr-111 is a requisite for the accommodation of the Fuc moiety of the LewisX trisaccharide within the sugar binding site of MGL1.

There is a 6-fold difference in the affinity of MGL1 for the LewisX trisaccharide versus β-methylgalactose. This difference corresponds to ~1 kcal/mol at 25°C. Based on the NMR results, we suggest that MGL1 will form two more van der Waals interactions with the LewisX trisaccharide compared with β-methylgalactose: one between the methyl group of fucose and the methyl group of Ala-89 and the other between the methyl group of fucose and the methyl group of Thr-111.

Figure 5. Comparison of the reduction ratios of the signal intensities obtained in the saturation transfer experiments. The reduction ratios of the signal intensities were calculated using the formula $\frac{I_{\text{ref}} - I_{\text{sat}}}{I_{\text{ref}} + I_{\text{ref}}} = \frac{I_{\text{sat}} - I_{\text{noise(sat)}}}{I_{\text{sat}} + I_{\text{noise(ref)}}}$, where $I_{\text{sat}}$ and $I_{\text{ref}}$ represent the signal intensity of an amide proton resonance with saturation and without saturation, respectively.

Figure 6. Mapping of the results of the saturation transfer experiments on the modeled structure of MGL1. A–C correspond to Fig. 5, A–C. The residues with reduction ratios of more than 0.3, 0.15–0.3, and 0.1–0.15 are colored red, orange, and yellow, respectively. Residues without available data (prolines, Glu-22, Asn-70, Asn-87, His-99, and Met-131) are colored cyan. D, surface representation of the modeled structure of MGL1. The residues colored as the LewisX binding site on MGL1 are mapped on the surface of the modeled structure of MGL1. The colors are used in the same scheme as in A. The residues colored red, orange, and yellow form a contiguous face on MGL1.

Figure 7. LewisX binding mode on MGL1. The colors are used in the same scheme as in Fig. 5. The LewisX binding site on MGL1 are illustrated in Fig. 7. The residues colored red, orange, and yellow form a contiguous face on MGL1.
The ~1 kcal/mol binding free energy can be explained by two van der Waals interactions (41).

In MGLs and hepatic lectins, two types of amino acids can be found at the position corresponding to Ala-89 and Thr-111 of MGL1 (Fig. 6). One type has alanine residues at this position (A-type), whereas the other type has arginine/lysine residues (RK-type). The residues identified at the position corresponding to Ala-89 in MGL1 (Fig. 8).

The putative arrangement of each carbohydrate residue of the Lewis type trisaccharide on the active site of MGL1 is shown by circles. The magenta, blue, and green circles correspond to the Gal, Fuc, and GlcNAc moieties within the Lewis type trisaccharide, respectively. MGL1 is colored according to the same scheme as in Fig. 6A. The structural model of the Lewis type trisaccharide was taken from the Protein Data Bank (code 1S6L).

![FIGURE 7. Putative Lewis binding mode of MGL1. The putative arrangement of each carbohydrate residue of the Lewis type trisaccharide on the active site of MGL1 is shown by circles. The magenta, blue, and green circles correspond to the Gal, Fuc, and GlcNAc moieties within the Lewis type trisaccharide, respectively. MGL1 is colored according to the same scheme as in Fig. 6A. The structural model of the Lewis type trisaccharide was taken from the Protein Data Bank (code 1S6L).](image)

![FIGURE 8. Comparison of the selected regions of the amino acid sequences for the CRDs of Gal-type C-type lectins. The residues identified as the Lewis type binding site on MGL1 are colored according to the same scheme as in Fig. 6A. The residues corresponding to Ala-89 and Thr-111 of MGL1 are represented by blue shading, rMGL, rat MGL; hMGL, human MGL; mASGPR, human ASGPR; RHL, rat hepatic lectin; MHL, mouse hepatic lectin.](image)

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