Selective Sugar Binding to the Carbohydrate Recognition Domains of the Rat Hepatic and Macrophage Asialoglycoprotein Receptors*

Susanne T. Iobst‡ and Kurt Drickamer§

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

Asialoglycoprotein receptors on the surfaces of both hepatocytes and peritoneal macrophages bind terminal galactose residues of desialylated glycoproteins and mediate endocytosis and eventual degradation of these ligands. The hepatic receptor binds oligosaccharides with terminal N-acetylgalactosamine residues more tightly than ligands with terminal galactose residues, but the macrophage receptor shows no such differential binding affinity. Carbohydrate recognition domains from the macrophage receptor and the major subunit of the hepatic receptor have been expressed in a bacterial system and have been shown to retain the distinct binding selectivities of the receptors from which they derive. Binding of a series of N-acyl derivatives of galactosamine suggests that the 2-substituent of these sugars interacts with the surface of the hepatic receptor with highest affinity binding observed for the N-propionyl derivative. Chimeric sugar-binding domains have been used to identify three regions of the hepatic receptor that are essential for establishing selectivity for N-acetylgalactosamine over galactose. Based on these results and the orientation of N-acetylgalactosamine when bound to an homologous galactose-binding mutant of rat serum mannose-binding protein, a fourth region likely to interact with N-acetylgalactosamine has been identified and probed by site-directed mutagenesis. The results of these studies define a binding pocket for the 2-substituent of N-acetylgalactosamine in the hepatic asialoglycoprotein receptor.

Asialoglycoprotein receptors have been identified in both mammalian liver and peritoneal macrophages (1, 2). The rat hepatic receptor, also known as the rat hepatic lectin RHL, is made up of three subunits: RHL-1 (41.5 kDa), RHL-2 (49 kDa), and RHL-3 (54 kDa) (3). Each subunit is a type II transmembrane protein, with a short NH2-terminal cytoplasmic domain, an internal signal membrane anchor, and a COOH-terminal glycan-rich inserted sequence. The major subunit, RHL-1, represents about 70–80% of the total mass of the receptor. The minor subunits, RHL-2 and RHL-3, are differentially glycosylated forms of a second, homologous polypeptide. The purified receptor is a hexamer in detergent solution, although the stoichiometry of the different polypeptides is still unclear (4, 5). In contrast, the macrophage galactose receptor (MGR) contains a single type of subunit (42 kDa) that forms homooligomers (2). The CRDs of MGR and RHL-1 show 77% sequence identity, whereas there is 54% identity between RHL-2/3 and MGR. Although the CRDs of MGR and RHL-1 are quite similar to each other, the macrophage receptor has a shorter cytoplasmic tail and an inserted segment of 24 amino acids between the CRD and the membrane-spanning domain (2).

Despite the similarities in the sequences of the hepatic and macrophage receptors, they have distinct sugar-binding properties. The hepatic receptor binds GalNAc with higher selectivity than Gal, whereas the macrophage receptor binds Gal and GalNAc with roughly equal affinity. For example, in binding competition assays GalNAc competes for binding to the hepatic receptor from rabbits between 8- and 20-fold more effectively than Gal (6, 7). In contrast, an assay measuring 125I-asialoglycoprotein uptake by macrophages in the presence of monosaccharide inhibitors shows that GalNAc and Gal bind equally well (8). Sugar binding studies using the mouse macrophage receptor show a similar pattern, although the exact results depend on what type of assay is used. In a sugar inhibition assay, Gal and GalNAc bind with approximately equal affinity, whereas a competitive enzyme-linked immunoabsorption assay suggests that Gal binds with 3-fold higher selectivity than GalNAc (9). In spite of small variations in the different assay protocols, all of the studies indicate that the hepatic receptor binds GalNAc far more effectively than does the macrophage receptor.

A Gal-binding site closely similar to that of the rat hepatic asialoglycoprotein receptor has been created by modification of the CRD of rat serum mannose-binding protein (MBP-A) (10, 11). For high affinity Gal binding, residues Gln185, Asp187, and Trp189 must be inserted into the Man-binding framework. A glycine-rich inserted sequence is necessary for the ability to exclude Man. The crystal structure of this mutant, QPDWG, has been solved alone and in complex with Gal and GalNAc (12). Although these studies provide insight into the mechanism of Gal and GalNAc binding to the asialoglycoprotein receptor CRDs, they do not explain differential binding of these two sugars to the hepatic receptor.

The CRDs of RHL-1 and MGR have been produced in a bacterial expression system and the sugar binding properties of these CRDs have been characterized. Amino acid residues likely to be involved in the selective binding of GalNAc to RHL-1 have been identified by analysis of chimeric and mutagenized versions of the CRDs.

EXPERIMENTAL PROCEDURES

Materials—Monosaccharides, glycosides, acid anhydrides, and grade V BSA were products of Sigma. Gal-BSA was obtained from E-Y Laboratories (San Mateo, CA). Na125I was purchased from Amersham Corp.

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‡ Supported by Unilever Research US Inc. Present address: Unilever Research US Inc., 45 River Rd., Edgewater, NJ 07020.

§ To whom correspondence should be addressed: Dept. of Biochemistry, University of Oxford, South Parks Rd., Oxford OX1 3QU, UK. Tel.: 44-1865-275727; Fax: 44-1865-275339; E-mail: kd@glycob.ox.ac.uk.

The abbreviations used are: RHL, rat hepatic lectin; MGR, macrophage galactose receptor; CRD, carbohydrate recognition domain; BSA, bovine serum albumin; MBP-A, rat serum mannose-binding protein.
FIG. 1. Schematic diagram of expression plasmids for CRDs of RHL-1 and MGR along with predicted protein products. The pTST expression vector was modified with synthetic oligonucleotides introduced at the BamHI site in the polylinker. The resulting sequence directs termination of the T7 Gene 10 product followed by reinitiation to make the CRD. The polypeptide produced is extended at the NH2 terminus by the residues in italics. The terminal methionine residue is removed in the bacteria, and the first cysteine residue of each CRD is changed to serine (underlined).

Polystyrene removawel microtiter plates (Immulon 4) were a product of Dynatech. Boehringer Mannheim was the source for isopropyl-$\beta$-thiogalactoside. Triton X-100 was purchased from Bio-Rad Laboratories. Restriction and DNA modification enzymes were obtained from New England Biolabs. Reagents for the polymerase chain reaction were purchased from Perkin-Elmer. DNA sequencing was performed using the Sequenase II kit from U. S. Biochemicals. Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer. Gal-Sepharose was prepared by the divinyl sulphone procedure (13).

Synthesis of Sugar Derivatives—Derivatives of galactosamine were prepared using acid anhydrides available commercially following the method of Horton et al. (14) except for the formyl derivative, which was prepared with mixed acetic-formic anhydride (15).

Expression of RHL-1 and MGR—The gene for the rat MGR was amplified using 5' and 3' primers based on the previously published cDNA sequence (2). The oligonucleotides used for the liver receptor and the macrophage receptor were identical. The polypeptide produced in each case is extended at the NH2 terminus by several residues. The first cysteine residue has been changed to serine because it would normally be paired with a cysteine residue further toward the NH2 terminus of the intact receptor polypeptide. The preceding alanine in the connective region is changed to isoleucine as well. Chimeric and mutant constructs were created and sequenced in vector pGEM-3 and transferred to the pTST vector by moving a BamHI–HindIII fragment. The expression construct was transformed into Escherichia coli strain BL21(DE3), in which the T7 RNA polymerase gene is under control of the lac promoter and operator (19).

Mutagenesis—Mutagenesis was performed on a BamHI–HindIII fragment corresponding to bases 508–980 of a cDNA for RHL-1 (20) and a BamHI–HindIII fragment corresponding to bases 694-1256 of the cDNA for MGR inserted into the vector, pgEM-3 grown in E. coli strain HB101. Synthetic oligonucleotides or restriction fragments were introduced at appropriate restriction sites (Fig. 2) using standard recombinant DNA techniques (21).

Production of RHL-1 and MGR—The CRDs of RHL-1 and MGR as well as mutants of these two proteins were prepared as described for the CRD of MBP-A (22) with minor modifications. Overnight cultures of bacteria (10 ml) were diluted to 500 ml in LB medium containing 50 μg/ml ampicillin and grown at 37°C for 2.5 h before induction with 0.125 mg/ml isopropyl-$\beta$-thiogalactoside. After 3 h of further growth at 37°C, bacteria were harvested, and denatured protein was isolated as inclusion bodies following sonication and centrifugation (22). Following solubilization of the protein with guanidine HCl and centrifugation, Triton X-100 was added to a final concentration of 0.5%. The solution was diluted 5-fold with Ca2+-containing loading buffer (1.25 mM NaCl, 25 mM Tris-Cl, pH 7.8, 25 mM CaCl2) prior to dialysis against 6 volumes of loading buffer with 3 buffer changes. After removal of insoluble material by centrifugation for 60 min at 100,000 × g in a Beckman T15.5 rotor, the protein was applied to a 1-ml Gal-Sepharose column. Five 1-ml fractions were collected during rinsing with Ca2+-containing loading buffer followed by five 0.5-ml fractions of EDTA-containing eluting buffer (1.25 mM NaCl, 25 mM Tris-Cl, pH 7.8, 2.5 mM EDTA). Fractions were examined by sodium dodecyl sulfate-polyacrylamide (17.5%) gel electrophoresis (23). Fractions containing pure CRD were pooled and adjusted to a final concentration of 25 mM CaCl2 and approximately 0.1 mg of protein for coating of wells for the binding assays.

Competition Binding Assays—Competition assays, in which wild-type and mutant CRDs were immobilized on polystyrene wells, were performed as described previously (24), using 125I-Gal34-BSA as test ligand in the presence or the absence of competing monosaccharides. Each assay was performed in duplicate. A nonlinear, least squares fitting program (SigmaPlot, Jandel Scientific) was used to obtain Kd values, corresponding to the concentration of monosaccharide giving 50% inhibition of neoglycoprotein binding according to the equation:

$$\text{Bound Radioactivity} = \frac{\text{MAX}}{\text{Ki} + [\text{monosaccharide}]}$$

where MAX is the total amount of radioactivity bound in the absence of competing monosaccharide. Mean ± standard deviation values for at least three independent assays were used to calculate Kd values.

Direct Binding Assay—Polystyrene plates were coated with CRD and blocked with BSA as for the competition assay (24). 125I-Gal34-BSA was diluted with nonradioactive Gal34-BSA to a final specific activity of 1–2 × 106 cpm/μg. Serial dilutions (2-fold) prepared in BSA-loading buffer (1.25 mM NaCl, 25 mM CaCl2, 25 mM Tris-Cl, pH 7.8, containing 5% BSA) were incubated in wells for 2 h at 4°C. Wells were emptied, rinsed three times with loading buffer at 4°C, and counted in a Packard PRIAS γ counter. The data were fitted to an equation for simple, saturable binding superimposed on a linearly increasing background of nonspecific binding:

$$\text{Man-BSA bound} = BKG + SLOPE \cdot \frac{\text{MAX} \cdot [\text{Man-BSA}]}{K_d + [\text{Man-BSA}]}$$

where BKG is the background binding, SLOPE is the linear increase in nonspecific binding, MAX is the saturation level for specific binding, and Kd is the concentration of ligand at which half-maximal specific, saturable binding is achieved. Assays were performed in duplicate. Values reported are the mean ± standard deviation for three independent assays, except in certain cases when only a single assay was performed.

Molecular Modeling—The coordinates of the Gal-binding mutant of rat serum MBP-A, QPDWG (12), with bound GaINac (Protein Data Bank 1AFB) were used as a starting point for modeling the binding site in the CRD or RHL-1 using the Insight II program (BioSym Technologies). Residues from the RHL-1 sequence were inserted at the critical positions from the standard rotamer library. No attempt was made to minimize the structure.

RESULTS

Sugar Binding Selectivity of the CRDs from RHL-1 and MGR—The gene for MGR was amplified from rat genomic DNA using primers from the 5'- and 3'-untranslated regions that flank the entire protein-coding sequence. The CRD-coding region was then assembled from genomic restriction fragments, and oligonucleotides were designed to repair the intron boundaries. The resultant partial cDNA was identical to that previously sequenced (2). cDNAs for RHL-1 have been previously described (20). The CRD-coding regions of MGR and RHL-1 were inserted after the bacteriophage T7 promoter in the pTST expression vector (18), as shown in the schematic diagram in Fig. 1. Expressed proteins were purified following a protocol slightly modified from that previously used for MBP-A. The key difference was the addition of Triton X-100 prior to renaturation by dilution and dialysis to prevent aggregation and increase the yield of protein. The CRDs were purified by affinity chromatography on Gal-Sepharose and analyzed by SDS gel electrophoresis.
electrophoresis. As shown in Fig. 3, a single species of polypeptide was obtained from each of the expression systems in a yield of 1–5 mg/l of starting bacterial culture.

Solid phase competition binding assays in which CRDs immobilized on polystyrene wells are probed with 125I-Gal34-BSA were used to characterize ligand binding by the bacterially expressed CRDs. As shown in Fig. 4, RHL-1 alone in the absence of RHL-2/3 mimics the highly GalNAc-selective binding of the intact receptor, because GalNAc competes for binding to RHL-1 60-fold more effectively than does Gal. In contrast, MGR does not show this preferential binding. The absolute affinities of the two proteins for Gal34-BSA are essentially the same (68 ± 7 nM for RHL-1 and 69 ± 11 nM for MGR), so the difference between the two CRDs in the competition assay reflects much higher affinity of RHL-1 for GalNAc. These results indicate that the different selectivities of the intact hepatic and macrophage receptors are mirrored in the properties of isolated CRDs from RHL-1 and MGR.

The nature of the interaction between GalNAc and RHL-1 was also probed using a series of galactosamine derivatives. The ability of a series of N-acyl derivatives of galactosamine to compete with 125I-Gal34-BSA for binding to the CRD of RHL-1 are compared in Table I. As the length of the side chain increases from formyl to propionyl, the affinity for RHL-1 increases. The propionyl derivative has the highest affinity for RHL-1 of any monosaccharide tested, with larger chains resulting in decreased affinity. These data suggest that there may be a hydrophobic region of the RHL-1 CRD that accommodates small alkyl chains on the 2 substituent of galactosamine.

RHL-1 and MGR Chimeras—Because RHL-1 but not MGR binds GalNAc preferentially, residues in RHL-1 that differ from those in MGR must provide the selectivity for N-acylated galactosamine. The close sequence similarity between the CRDs of RHL-1 and MGR made it possible to construct a series of stable chimeras between them. Comparison of the sugar binding selectivity of these chimeric CRDs with the character-
ics of wild-type RHL-1 and MGR CRDs allows identification of residues that determine the distinct binding specificities of the hepatic and macrophage receptors.

Chimeric cDNAs were constructed using restriction sites that are common to both RHL-1 and MGR cDNAs (Fig. 5). The chimeric proteins expressed from these hybrid cDNAs were then tested in solid phase binding competition assays. The results summarized in Table II reveal that all of the 60-fold selectivity for GalNAc can be accounted for in the COOH-terminal 81 residues of RHL-1 (amino acids 204–284). Although the COOH-terminal 81 residues of RHL-1 are necessary for 60-fold preferential binding of GalNAc, smaller effects are observed when shorter segments of RHL-1 are substituted into MGR. Stepwise substitution of portions of the COOH terminus of RHL-1 suggests that at least three regions contribute to high selectivity GalNAc binding. For example, incorporation of just the COOH-terminal 40 residues of RHL-1 into MGR results in a marginal increase in GalNAc binding selectivity. A more substantial 2-fold increase occurs when a further 25 residues of RHL-1 are included. The major, 20-fold increase in GalNAc selectivity occurs only when residues between 80 and 65 residues from the COOH terminus of RHL-1 are present.

To confirm the importance of each region individually, smaller portions of RHL-1 were used to replace corresponding parts of MGR (Fig. 5). The results of binding studies for these constructs (Table II), combined with the single cross-over data, define three regions that contribute to GalNAc binding. The major peaks eluted from Gal-Sepharose affinity columns were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lane 1, RHL-1. Lane 2, MGR. Molecular mass standards are indicated at left.

**FIG. 3.** Purification of the CRDs from RHL-1 and MGR expressed in bacteria. The major peaks eluted from Gal-Sepharose affinity columns were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie Blue. Lane 1, RHL-1. Lane 2, MGR. Molecular mass standards are indicated at left.

**FIG. 4.** Solid phase binding competition assays of the CRDs of RHL-1 and MGR. Experimental data (filled circles) are shown for $^{125}$I-Gal$_4$-BSA binding in the presence of either Gal or GalNAc along with theoretical curves (continuous lines) fitted to the data. Left, results for RHL-1. Right, results for MGR.
p4 of the CRD as shown in Fig. 6A.

The large effect on GalNAc binding resulting from changes in region 1 is surprising because of its probable position in the CRD. In the model shown in Fig. 6A, the shortest distance

| Sugar Derivative          | Acyl Chain | K_i/K_i,GalNAc |
|---------------------------|------------|----------------|
| N-Formylgalactosamine     | O-CH       | 13. ± 1        |
| N-Acetylgalactosamine     | O-CCH_3    | 1.             |
| N-Propionylgalactosamine  | O-CCH_2CH_3| 0.13 ± 0.04    |
| N-n-Butanoylgalactosamine| O-CCH_2CH_3| 0.29 ± 0.06    |
| N-iso-Butanoylgalactosamine| O-CCH_3     | 0.82 ± 0.06    |

between the side chain of Asn^{208} of RHL-1 and the N-acetyl group of GalNAc is approximately 7 Å. This observation, together with the fact that many amino acids at this position support high selectivity GalNAc binding, suggests that preferential binding to GalNAc by mutant MGR/N is unlikely to result from direct contact with the sugar, but the effect of the residue at position 230 could be mediated through interactions with other amino acids in the protein.

Definition of an Additional Region Involved in GalNAc Binding—Based on the crystal structure model, residues were identified that could be within contact distance of both Asn^{208} of RHL-1 and the sugar ligand. Because the 20-fold increase in GalNAc selectivity is observed following substitution of a single asparagine residue from RHL-1 in the context of MGR, any residue that mediates interaction with GalNAc is likely to be the same in both RHL-1 and MGR. Based on these two criteria, residues His^{256}, Phe^{257}, and Thr^{258} in RHL-1, corresponding to residues His^{278}, Phe^{279}, and Thr^{280} in MGR, were identified as the most likely candidates (region 4, Fig. 6A). To test the importance of these residues, His^{278} and Thr^{280} of MGR were replaced with alanine, whereas Phe^{279}, which is likely to be buried in the hydrophobic core, was replaced with isoleucine to preserve its hydrophobic character. This position is occupied by isoleucine in the RHL-2/3 polypeptide, which, like MGR, lacks preferential binding to GalNAc.<ref>2 N. I. Ruiz and K. Drickamer, manuscript in preparation.</ref>

As might be expected from the predicted orientation of Phe^{279} toward the interior of the CRD, substitution with isoleucine (mutant MGR/N/4F→I) results in only a slight loss of

Fig. 5. Sequences of RHL-1/MGR chimeras and mutants of MGR in regions 1, 2, and 3. The amino acid sequence of the CRD of MGR is shown at the top. For RHL-1 and all chimeric constructs, only the amino acids that differ from MGR are indicated. Approximate locations of restriction sites in the corresponding cDNAs used in making chimeric constructs are indicated by arrows.
Sugar Binding to Asialoglycoprotein Receptors

TABLE II

Binding constants for chimeric CRDs

Sequences of wild-type MGR and RHL-1 along with chimeric constructs are shown in Fig. 5. Dissociation constants and relative inhibition constants were determined using the solid phase binding assay. \( K_i \) values for GalNAc relative to the \( K_i \) for Gal are reported along with dissociation constants for Gal\( \alpha \)-BSA.

| Chimeric CRD | \( K_{i, \text{Gal}}/K_{i, \text{GalNAc}} \) | \( K_D \) |
|--------------|----------------------------------|-------|
| MGR          | 1.2 ± 0.4                        | 90 ± 40 |
| RHL-1        | 60 ± 8                           | 70 ± 10 |
| Crosses at Scal | MGR/RHL                        | 78 ± 14 | 160 ± 50 |
|               | RHL/MGR                         | 0.7 ± 0.1 | 150 ± 30 |
| Crosses at NlaII [regions 1, 2, and 3] | MGR/RHL | 68 ± 3 | 220 ± 90 |
|               | RHL/MGR                         | 0.98 ± 0.03 | 240 ± 70 |
| Crosses at NlaV [regions 2 and 3] | MGR/RHL | 3.3 ± 0.2 | 100 ± 30 |
|               | RHL/MGR                         | 23 ± 2 | 130 ± 40 |
| Crosses at Bbv [region 3] | MGR/RHL | 1.5 ± 0.2 | 90 ± 30 |
|               | RHL/MGR                         | 48 ± 5 | 170 |
| Crosses at Scal and NlaV | MGR/RHL/MGR | 22 ± 2 | 90 ± 50 |
|               | RHL/MGR/RHL                     | 1.3 ± 0.2 | 90 ± 10 |
| Crosses at NlaII and NlaV [region 1] | MGR/RHL/MGR | 20 ± 0.5 | 120 ± 40 |
|               | RHL/MGR/RHL                     | 1.2 ± 0.1 | 120 ± 80 |
| Crosses at NlaII, NlaV, and Bbv [regions 1 and 3] | MGR/RHL/MGR/RHL | 24 ± 3 | 90 ± 40 |
| Crosses at NlaIII and Bbv [regions 1 and 2] | MGR/RHL/MGR | 56 ± 12 | 150 ± 80 |

TABLE III

Binding constants for MGR CRD altered in region 1

Binding constants were determined as in Table II. Residues not listed are identical to those of the macrophage galactose receptor.

| CRD | Sequence in region 1 (residues 225-240) | \( K_{i, \text{Gal}}/K_{i, \text{GalNAc}} \) | \( K_D \text{Gal-BSA} \) |
|------|------------------------------------------|----------------------------------|-----------------|
| MGR  | HMGSVTVWGLTDQNG                          | 1.2 ± 0.4                        | 68 ± 7 |
| RHL-1| PLN                                      | 60 ± 8                           | 69 ± 11 |
| MGR/1LN | PLN                                 | 20 ± 4                           | 100 ± 10 |
| MGR/1LN | LN                                   | 23 ± 4                           | 90 ± 30 |
| MGR/1PN | P N                                  | 24 ± 6                           | 130 ± 40 |
| MGR/1PL | PL                                    | 2.6 ± 0.1                        | 90 ± 30 |
| MGR/IN | N                                      | 22 ± 5                           | 120 ± 30 |
| MGR/IV→A | A                                   | 21 ± 3                           | 100 ± 50 |
| MGR/IV→D | D                                   | 15 ± 6                           | 100 ± 50 |
| MGR/IV→S | S                                   | 13 ± 3                           | 100 ± 40 |
| MGR/IV→Q | Q                                   | 9 ± 3                            | 150 ± 40 |
| MGR/IV→H | H                                   | 38 ± 4                           | 80 ± 10 |

selectivity for GalNAc (Table IV). Replacement of Thr\(^{280}\) with alanine (mutant MGR/1N/4T→A) has only a 2-fold effect on GalNAc binding. However, substitution of His\(^{278}\) with alanine results in an almost total loss of GalNAc selectivity. Given the apparent importance of histidine at position 278, it can be suggested that the decrease in GalNAc selectivity in the alanine mutant at positions 280 may result from changes in the position of the histidine residue. To probe the nature of the interaction of His\(^{278}\) with GalNAc, individual changes were made at this position. None of the amino acids substituted support GalNAc-binding selectivity comparable with that seen when His\(^{278}\) is present (Table IV).

The importance of histidine in region 4 was further substantiated by incorporating the change His\(^{276}\) → Ala in the CRD of RHL-1 rather than in the MGR background. As shown in Table IV, this change results in 25-fold loss of relative affinity for GalNAc without detectable change in the absolute affinity for Gal\( \alpha \)-BSA. This finding confirms the importance of His\(^{276}\) in RHL-1 and verifies that the high affinity GalNAc-binding site created in the context of MGR accurately models the natural site in RHL-1. In all cases, the dramatic effects of changing the histidine residue in region 4 on binding of GalNAc compared with Gal are not accompanied by substantial changes in the absolute affinity for Gal\( \beta \)-BSA, demonstrating that although this histidine plays a critical role in establishing the high affinity of RHL-1 for GalNAc, it does not form part of the binding site for Gal.

Importance of Residues in Region 2—RHL-1 and MGR differ at seven positions within region 2. Based on the relative location of these amino acids predicted from the three-dimensional structure of the QPDWG mutant of MBP-A (Fig. 6A), two of the seven amino acids, Arg\(^{236}\) and Gly\(^{238}\) in RHL-1, were judged likely to be located near the sugar-binding site. These residues correspond to Ala\(^{236}\) and Lys\(^{236}\) in MGR. To test their importance, Ala\(^{236}\) and Lys\(^{236}\) were changed pairwise and individually in mutant MGR/1N. Competition binding assay data (Table V) show that both arginine at position 258 and glycine at position 260 of MGR (mutant MGR/1N/2A→R, 2K→G) are necessary to increase selectivity for GalNAc 2-fold. The side chain of arginine has the potential for hydrogen bonding to the acetyl group of the sugar, but substitution of this residue alone confers only a marginal increase in selectivity for GalNAc, whereas substitution of glycine at position 260 has no effect on its own. It is possible that glycine at position 260 plays a role in the correct positioning of arginine at position 258, because the presence of glycine may alter the structure of the loop in which these two residues are located (Fig. 6A).

Residue His\(^{278}\) was changed to alanine in mutant MGR/1N/2A→R, 2K→G, resulting in the loss of high selectivity GalNAc binding as expected. However, the effect on GalNAc binding seen in the presence of the region 2 changes alone is still evident, because mutant MGR/1N/2A→R, 2K→G/4H→A binds GalNAc with at least 2-fold higher selectivity than does wild-type MGR CRD (Table V). These data suggest that the effects of the residues in region 2 are independent of the interactions of His\(^{278}\) in RHL-1.

Importance of Residues in Region 3—Within region 3, five amino acid residues differ between RHL-1 and MGR. Based on
placed with region 3 of RHL-1, suggesting that the presence of amino acids from region 2 as well as region 1 might be necessary to demonstrate an effect of region 3. Therefore, mutant MGR/1N/2A was selected to correspond with residues of RHL-1 in these regions. Sequences of mutant constructs are summarized in Table IV, no significant effect of the relative binding of GalNAc to RHL-1 mutant MGR/1N and RHL-1 were determined using solid phase binding assays as in Table II. Residues not listed are identical to those of the mutant MGR/IN or RHL-1 parent.

### Table IV

| CRD | Sequence in region 4 | \( K_{D,Gal} \) | \( K_{D,GalNAc} \) | \( \text{nM} \) |
|-----|---------------------|----------------|-----------------|--------|
| MGR Mutants | Residues 278–280 | 22 ± 5 | 90 ± 30 | |
| 1N | HFT | | |
| 1N/AF → I | I | 18 ± 2 | 80 | |
| 1N/4T → A | A | 12 ± 2 | 150 ± 40 | |
| 1N/4H → A | A | 1.7 ± 0.2 | 90 ± 10 | |
| 1N/4H → E | E | 4 ± 1 | | |
| 1N/4H → Q | Q | 1.6 ± 0.1 | | |
| 1N/4H → Y | Y | 0.4 ± 0.1 | 90 | |
| 1N/4H → D | D | 0.11 ± 0.02 | | |
| 1N/4H → K | K | 0.03 ± 0.01 | | |
| RHL-1 Mutant | Residue 256 | | | |
| Wild type | H | 60 ± 8 | 69 ± 11 | |
| 4H → A | A | 2.4 ± 0.5 | 110 ± 40 | |

### Table V

| Mutant MGR CRD | \( K_{D,GalNAc} \) | \( K_{D,Gal} \) | \( \text{nM} \) |
|----------------|----------------|----------------|--------|
| Wild type | 1.2 ± 0.4 | 90 ± 40 | |
| 1N | 22 ± 5 | 90 ± 30 | |
| 1N/2A → R | 28 ± 3 | | |
| 1N/2K → G | 20 ± 0.6 | | |
| 1N/2A → R,2K → G | 49 ± 6 | | |
| 1N/2A → R,2K → G/4H → A | 3 ± 1 | | |
| 1N/region 3 | 24 ± 3 | 100 ± 40 | |
| 1N/2A → R,2K → G/3R → H | 52 ± 6 | | |
| 1N/2A → R,2K → G/3S → T | 67 ± 6 | | |

**DISCUSSION**

The data presented indicate that substitution of four amino acids, asparagine at position 230, arginine at position 258, glycine at position 260, and threonine at position 281 of MGR, is sufficient to give GalNAc binding that is comparable with that of native RHL-1. Using the structure of the QPDWG mutant of MBP-A as a scaffold, the relative positions of the amino acid side chains known to be required for high affinity binding of GalNAc to RHL-1 can be modeled as shown in Fig. 6. Although the conformations of the side chains are hypothetical, a binding surface can be formed around the atoms

**FIG. 6. Molecular interaction between RHL-1 and GalNAc.** A, model of GalNAc bound to RHL-1. The side chains of RHL-1 that have been shown to affect selective binding of GalNAc have been inserted at the corresponding positions of the crystallographically determined structure of the QPDWG mutant of MBP-A in complex with GalNAc. The substitutions made correspond to: Asn208 (RHL-1) replacing Thr202; Phe257 replacing Ile203; Thr258 replacing Val204; and Thr259 replacing Asp205. Regions 1–3 are shown in black, whereas region 4 is in gray. The remainder of the polypeptide is shown as an arrow, denoting fold enhancement of binding affinity for GalNAc relative to Gal.

The data presented indicate that substitution of four amino acids, asparagine at position 230, arginine at position 258, glycine at position 260, and threonine at position 281 of MGR, is sufficient to give GalNAc binding that is comparable with that of native RHL-1. Using the structure of the QPDWG mutant of MBP-A as a scaffold, the relative positions of the amino acid side chains known to be required for high affinity binding of GalNAc to RHL-1 can be modeled as shown in Fig. 6A. Although the conformations of the side chains are hypothetical, a binding surface can be formed around the atoms...
on the acetamido substituent of the sugar, with the relative importance of specific interactions shown schematically in Fig. 6B.

The most important interactions documented in the present studies are with His\textsuperscript{256} of RHL-1, which is predicted to be positioned approximately halfway between the terminal methyl group of the 2 substituent of GalNAc and the side chain of Asn\textsuperscript{208}. The proximity of Asn\textsuperscript{208} to His\textsuperscript{256} (under 4 Å in the model) is consistent with the hypothesis that the presence of valine at position 208 decreases the affinity of the CRD for GalNAc indirectly by altering the disposition of the histidine residue. The fact that many other side chains are tolerated at position 208 is also consistent with this suggestion.

The approximate distance from the imidazole ring of His\textsuperscript{256} to the terminal methyl group of the N-acetyl moiety in GalNAc is also under 4 Å in the model, suggesting that there may be a direct interaction between His\textsuperscript{256} and the 2 substituent of GalNAc. Such an interaction would be consistent with the observed importance of this amino acid side chain for the binding of GalNAc but not of Gal. From the enhancement in GalNAc binding affinity, it can be calculated that the interaction with His\textsuperscript{256} contributes approximately 8 kJ/μmol to the free energy of binding GalNAc. The exact nature of the interaction is not obvious from the data available. Because asparagine and glutamine might be expected to form hydrogen bonds similar to those made by the imidazole ring of histidine, the failure of the 2-amino group to support tight binding of GalNAc argues that this specificity has been selected in RHL-1 to serve an important biological function, although there has been no such selective pressure on RHL-2/3 or MGR. Evaluation of potential naturally occurring ligands for each of these receptors, possibly bearing distinct patterns of terminal GalNAc and Gal residues, will be of interest.

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Susanne T. Iobst and Kurt Drickamer

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