Closely Related Mammals Have Distinct Asialoglycoprotein Receptor Carbohydrate Specificities*

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The hepatic asialoglycoprotein receptor (ASGP-R)† identified by Van Den Hamer et al. (1) was the first mammalian lectin to be described. A characteristic of the receptor is its ability to rapidly remove glycoproteins from the circulation that have been treated with neuraminidase or mild acid (2). Rapid clearance of bovine serum albumin bearing multiple chemically coupled tetrasaccharides with the sequence Sia2,6GalNAcβ1,4GlcNAcβ1,2Man-bovine serum albumin and GalNAcβ1,4GlcNAcβ1,2Man-bovine serum albumin in ratios of 16:1.0 and 1.0:1.0, respectively. Mutagenesis was used to show that amino acids both in the immediate vicinity of the proposed binding site for terminal GalNAc and on the α2 helix that is distant from the binding site contribute to the specificity for terminal Sia2,6GalNAc. Thus, multiple amino acid sequence alterations in two key locations contribute to the difference in specificity observed for the rat and mouse ASGP-Rs. We hypothesize that the altered specificity of ASGP-R orthologues in such evolutionarily closely related species reflects rapidly changing requirements for recognition of endogenous or exogenous oligosaccharides in vivo.

The asialoglycoprotein receptor binds oligosaccharides terminating with sialic acid (Sia) α2,6GalNAc. Despite a high percentage of identical amino acids in their sequences, orthologues of the asialoglycoprotein receptor (ASGP-R) in different mammals differ in their specificity for terminal Sia2,6GalNAc. The recombinant subunit 1 of the ASGP-R from the rat (RHL-1 or rat hepatic lectin) and the mouse (MHL-1 or mouse hepatic lectin), which differ at only 12 positions in the amino acid sequence of their carbohydrate recognition domains, binds Sia2,6GalNAcβ1,4GlcNAcβ1,2Man-bovine serum albumin and GalNAcβ1,4GlcNAcβ1,2Man-bovine serum albumin in ratios of 16:1.0 and 1.0:1.0, respectively. Mutagenesis was used to show that amino acids both in the immediate vicinity of the proposed binding site for terminal GalNAc and on the α2 helix that is distant from the binding site contribute to the specificity for terminal Sia2,6GalNAc. Thus, multiple amino acid sequence alterations in two key locations contribute to the difference in specificity observed for the rat and mouse ASGP-Rs. We hypothesize that the altered specificity of ASGP-R orthologues in such evolutionarily closely related species reflects rapidly changing requirements for recognition of endogenous or exogenous oligosaccharides in vivo.

We recently reported that the rat asialoglycoprotein receptor binds oligosaccharides terminating with sialic acid (Sia) α2,6GalNAc. Despite a high percentage of identical amino acids in their sequences, orthologues of the asialoglycoprotein receptor (ASGP-R) in different mammals differ in their specificity for terminal Sia2,6GalNAc. The recombinant subunit 1 of the ASGP-R from the rat (RHL-1 or rat hepatic lectin) and the mouse (MHL-1 or mouse hepatic lectin), which differ at only 12 positions in the amino acid sequence of their carbohydrate recognition domains, binds Sia2,6GalNAcβ1,4GlcNAcβ1,2Man-bovine serum albumin and GalNAcβ1,4GlcNAcβ1,2Man-bovine serum albumin in ratios of 16:1.0 and 1.0:1.0, respectively. Mutagenesis was used to show that amino acids both in the immediate vicinity of the proposed binding site for terminal GalNAc and on the α2 helix that is distant from the binding site contribute to the specificity for terminal Sia2,6GalNAc. Thus, multiple amino acid sequence alterations in two key locations contribute to the difference in specificity observed for the rat and mouse ASGP-Rs. We hypothesize that the altered specificity of ASGP-R orthologues in such evolutionarily closely related species reflects rapidly changing requirements for recognition of endogenous or exogenous oligosaccharides in vivo.

The hepatic asialoglycoprotein receptor (ASGP-R)† identified by Van Den Hamer et al. (1) was the first mammalian lectin to be described. A characteristic of the receptor is its ability to rapidly remove glycoproteins from the circulation that have been treated with neuraminidase or mild acid (2). Rapid clearance reflects the specificity of the ASGP-R for terminal Sia2,6GalNAc. Thus, multiple amino acid sequence alterations in two key locations contribute to the difference in specificity observed for the rat and mouse ASGP-Rs. We hypothesize that the altered specificity of ASGP-R orthologues in such evolutionarily closely related species reflects rapidly changing requirements for recognition of endogenous or exogenous oligosaccharides in vivo.

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‡ The abbreviations used are: ASGP-R, asialoglycoprotein receptor; BSA, bovine serum albumin; Sia, sialic acid; Gal, galactose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; GGnM-BSA, GalNAcβ1,4GlcNAcβ1,2Man-BSA; HL, hepatic lectin; MHL, mouse hepatic lectin; HHL, human hepatic lectin; Sia2,6GalNAc-BSA, sialic acid 2,6α-GalNAc β1,4GlcNAcβ1,2Man-BSA; CRD, carbohydrate recognition domain.

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The ASGP-R was subsequently shown to be a hetero-oligomer consisting of two highly homologous subunits, hepatic lectin subunits 1 and 2 (HL-1 and HL-2, respectively) (3–5). Whereas both subunits are required for the endocytosis of ligands, the carbohydrate binding activity is associated predominantly with HL-1 (6, 7). Prior structural and functional studies of the ASGP-R have included the crystallization of human HL-1 (8) and the generation of mice with genetically ablated HL-2 (9) and HL-1 (10). These studies have been informative but have not revealed the identity of endogenous ligands that may be recognized by the ASGP-R in vivo.

We recently reported that, in the rat, the ASGP-R mediates the rapid clearance of bovine serum albumin bearing multiple chemically coupled tetrasaccharides with the sequence Sia2,6GalNAcβ1,4GlcNAcβ1,2Man (Sia2,6GnM-bovine serum albumin (BSA)) (11). Because the prolactin-like hormones bearing N-linked oligosaccharides terminating with the sequence Sia2,6GalNAcβ1,4GlcNAc are synthesized during the last third of pregnancy in the placenta of the rat (12), they may represent the first examples of endogenous ligands for the ASGP-R. Although rare, the terminal sequence Sia2,6GalNAcβ1,4GlcNAc has been found on a number of glycoproteins in addition to the prolactin-like hormones (12) including glycofolin from human amniotic fluid (13), pituitary glycoprotein hormones (14, 15), and recombinant human protein C (16). The presence of these structures on multiple glycoproteins raises the possibility that the ASGP-R in other mammalian species may also recognize oligosaccharides terminating with the sequence Sia2,6GalNAcβ1,4GlcNAc.

The ASGP-R is expressed by all of the mammals that have been examined (17). The amino acid sequences of HL-1 and HL-2 have high percentages of identical residues across species. For example, mouse HL-1 (MHL-1) and human HL-1 (HHL-1) are 89 and 80% identical to rat HL-1 (RHL-1), respectively, whereas MHL-2 and HHL-2 are 80 and 62% identical to RHL-2, respectively. Since all of the known mammalian HLs share a specificity for terminal Gal or GalNAc (17), the conservation of amino acid sequence among mammalian HL orthologues suggested that, like RHL-1, HLs from other species may also bind terminal Sia2,6GalNAc.

The carbohydrate recognition domain (CRD) of HL-1 is the prototypal example of a Ca²⁺-dependent CRD (18). A comparison of the binding sites of HL-1 and the CRD domain of the mannose (Man)-binding protein allowed Dickamer and co-workers (19–21) to propose a model for GalNAc binding to HL-1. Based on this model, residues that determine the specificity for GalNAc versus Man were identified and a form of the Man-binding protein that binds GalNAc was engineered. In this model, the C6 hydroxyl of the bound GalNAc projects away from the protein. This model has not yet been confirmed by the crystallization of HL-1 with a carbohydrate in the binding site; however, it suggests that the binding site of HL-1 could potentially accommodate a sialic acid linked to the C6 hydroxyl of bound Gal or GalNAc.

Here we report that mammalian ASGP-Rs differ in their ability to bind terminal GalNAc and Sia2,6GalNAc. Furthermore, multiple changes in the amino acid sequence of HL-1 both in the immediate region of the binding pocket and at more distant sites contribute to the difference in specificity. Taken together, the changes in the amino acid sequence and ligand...
Protein concentrations were determined using the Bradford method reported that bovine serum albumin bearing multiple chemi-pmol of each purified CRD.

EXPERIMENTAL PROCEDURES

Materials—Bovine, chicken, mouse, porcine, rat, and rabbit livers were purchased from Pel-Freeze® Biologicals (Rogers, AK). BSA conjugated with an average of 15 tri saccharides with the sequence Sia2,6GnGM-BSA and the desialylated GnGM-BSA were prepared as previously described (11). Trisol reagent and primers were purchased from Invitrogen. Radiolabeling of proteins with [125I], isolation of total liver membrane proteins, and receptor:ligand binding assays were performed as previously described (11). However, the concentration of Ca2+ was increased from 2 to 10 mM in the binding assays.

Preparation of Total Liver Membrane Proteins—Livers were homogenized with a Polytron homogenizer (Brinkmann Instruments) in 5 volumes of buffer containing 25 mM HEPES, 50 mM KCl, 2 mM magnesium acetate, 1 mM dithiothreitol, and 10% (w/v) sucrose. Homogenates were sedimented at 1,500 × g for 5 min, and the resulting supernatants were layered over a 65% (w/v) sucrose cushion prior to sedimentation at 100,000 × g at 4 °C for 75 min. The interphase fraction, which contained the total membrane proteins, was collected and stored at -80 °C. Protein concentrations were determined using the Bradford method (Bio-Rad) or the Non-Interfering Protein Assay™ (Geno Technologies, St. Louis, MO).

Expression of Mouse, Rat, and Human Lectin Subunits 1 and 2—MHL-1 and MHL-2 were cloned and expressed in 293T cells as described for BHL-1 and RHL-2 (11). MHL-1 and MHL-2 were amplified using the gene-specific primers MHL-1-F (5'-CGG GAT CCC ATC ATG ACA AAG GAT TAT CAA GAT TTC C-3') and MHL-1-R (5'-CGG TGC CCC ATT AGC CTT ATC CAA CTT TGT CTC-3'). MHL-2-F (5'-CGG GAT CCC ATC ATG GAG AAG GAC TTT CAA GAT ATC C-3') and MHL-2-R (5'-CGG TGC ACC CTA GGG GAT CTT CCG TCT CTT C-3'), respectively. Amplified products were subcloned into pcDNA3.1/V5His-TOPO and sequenced. The resulting cDNAs of subunits 1 and 2 were designated MHL-1V5His and MHL-2V5His, respectively. To express the subunits with the V5 epitope and six histidines, the stop codon was changed to encode for a Gly residue followed by a SalI restriction enzyme site.

Hepatic Lectin Subunit 1 Amino Acid Changes—Standard molecular techniques were used to make HL-1 amino acid changes. The XcmI site at amino acid residues 156–157 was used to swap CRD-containing regions. Codon-specific mutageneses were performed using Inverse PCR. Changes were confirmed by sequencing. These constructs were expressed in HEK-293T cells, solubilized in Triton X-100, separated on NuPAGE® Bis-Tris gels, and electrophoretically transferred to Immobilon P (Millipore). Quantitative Western blots were performed using mouse anti-V5 IgG and rabbit anti-mouse IgG-hors eradish peroxidase to determine the expression levels by comparison to known amounts of V5-tagged proteins.

Bacterial Expression of CRD Regions—Constructs for bacterial expression of the CRD region of mouse and rat HL-1 were similar to those described by Iobst and Drakehammer (22) in which the amino acid residues from 1 to 149 were deleted. In our constructs, the termination codon was replaced with a glycine-encoding triplet to fuse the CRD with V5 tag at the amino terminus.

Mammals Differ in Their Capacity to Bind Oligosaccharides Terminating with Sia2,6GalNAc and Sia2,6GalNAc. The binding capacity of liver membrane proteins from the mammals shown was determined using 200 μg of Triton X-100 solubilized liver membrane proteins per receptor-ligand complex precipitation assay (11). Panel A, GGnM-[125I]BSA. Panel B, Sia2,6GnGM-[125I]BSA. Panel C, ratio of Sia2,6GnGM-[125I]BSA:GGnGM-[125I]BSA bound/μg.

Since we previously determined that the ASGP-R could account for the Sia2,6GalNAc-specific binding activity in rat liver, the difference in the ratio of Sia2,6GnGM-BSA to GGnGM-BSA binding activity seen in livers from different mammals is universal. In contrast, bovine, mouse, and pig liver extracts displayed equal or better activity for Sia2,6GnGM-BSA than for GGnGM-BSA (Fig. 1B). In contrast, bovine, mouse, and pig liver extracts displayed little binding activity for Sia2,6GnGM-BSA.
forms of the rat ASGP-R compared with the native and recombinant forms of the mouse ASGP-R (Fig. 2). Similar to MHL-1, recombinant HHL-1 did not exhibit enhanced binding of Sia\(^2\)H\(^2\)2,6GGnM-BSA compared with GGnM-BSA (data not shown), indicating that the specificity of the human receptor more closely resembles that of the mouse than that of the rat.

**Identification of the Amino Acids Required for Recognition of** Sia\(^2\)H\(^2\)2,6GalNAc—When aligned with MHL-1 and HHL-1 (Fig. 2A), the amino acid sequence of RHL-1 is 88.7% identical to MHL-1 and 78.4% identical to HHL-1 (data not shown), indicating that a limited number of alterations in their amino acid sequences account for the difference in specificity. Chimeric HL-1 subunits were used to establish that changes within the CRD of rat and mouse HL-1 rather than within the cytosolic, transmembrane, and stem regions predominantly account for the difference in specificity. The amino acid sequences of the CRD domains of RHL-1 and MHL-1 differ at 12 positions. We have divided these positions into three groups (Fig. 2A): Group I includes positions 152–284, which is a region in the CRD that is 100% identical in RHL-1 and MHL-1; Group II includes positions 1–151, which is a region in the CRD that is 100% identical in MHL-1 and RHL-1; and Group III includes positions 1–151, which is a region in the CRD that is 100% identical in MHL-1 and RHL-1.

**Fig. 2. Specificities of RHL-1 and MHL-1 chimeras and mutants.** A, the sequence of the CRD of RHL-1 (residues 152–284) is shown in boldface letters. The amino acids that differ in MHL-1 are indicated by non-boldface letters. Residues 1–151 are indicated by --- for RHL-1 and --- for MHL-1. Mut-A and Mut-B are chimeras in which the residues 1–151 of the MHL-1 and RHL-1 have been fused with the CRDs of RHL-1 and MHL-1, respectively. The 12 amino acid differences are indicated by the boldface (rat) and non-boldface (mouse) letters. The amino acids from RHL-1 that are indicated by the boldface letters have been introduced into MHL-1 in Mut-E through Mut-L. The amino acid changes were introduced in the combinations indicated as Groups I, II, and III. In Mut-M and Mut-N, only Glu and Gly have been introduced into RHL-1 and MHL-1, respectively. All of the constructs have a V5 epitope followed by six His residues at their C terminus. B, the results of binding assays performed with Sia\(^2\)H\(^2\)2,6GGnM-[\(^{125}\)I]BSA and GGnM-[\(^{125}\)I]BSA are summarized. *, Sia\(^2\)H\(^2\)2,6GGnM-[\(^{125}\)I]BSA (1 × 10\(^{5}\) cpm/ng) was digested with neuraminidase to generate GGnM-[\(^{125}\)I]BSA with the identical specific activity. Equal quantities of the HL-1 constructs based on quantitative Western blots using anti-V5 were utilized for the binding assays. \(^{\dagger}\), only the ratios are given for Mut-M and Mut-N as they were analyzed using Sia\(^2\)H\(^2\)2,6GGnM-[\(^{125}\)I]BSA and GGnM-[\(^{125}\)I]BSA that had a significantly different specific activity. All of the assays were performed in triplicate, and the mean ± S.D. is presented in the table.

**Table:** Specificities of RHL-1 and MHL-1 chimeras and mutants. A, the sequence of the CRD of RHL-1 (residues 152–284) is shown in boldface letters. The amino acids that differ in MHL-1 are indicated by non-boldface letters. Residues 1–151 are indicated by --- for RHL-1 and --- for MHL-1. Mut-A and Mut-B are chimeras in which the residues 1–151 of the MHL-1 and RHL-1 have been fused with the CRDs of RHL-1 and MHL-1, respectively. The 12 amino acid differences are indicated by the boldface (rat) and non-boldface (mouse) letters. The amino acids from RHL-1 that are indicated by the boldface letters have been introduced into MHL-1 in Mut-E through Mut-L. The amino acid changes were introduced in the combinations indicated as Groups I, II, and III.

| Construct | Sia\(^2\)H\(^2\)2,6GGnM-BSA | GGnM-BSA | Sia\(^2\)H\(^2\)2,6GGnM-BSA:GGnM-BSA Ratio |
|-----------|-----------------|----------|-------------------------------------|
| RHL-1     | 15.7            | 1.0      | 15.7                                |
| MHL-1     | 1.0             | 1.0      | 1.0                                 |
| Mut-A     | 6.6             | 0.7      | 9.2                                 |
| Mut-B     | 0.3             | 0.5      | 0.6                                 |
| Mut-E     | 0.5             | 0.5      | 1.0                                 |
| Mut-F     | 18.3            | 1.5      | 11.9                                |
| Mut-G     | 2.2             | 0.6      | 3.6                                 |
| Mut-H     | 3.4             | 0.6      | 5.9                                 |
| Mut-J     | 1.3             | 1.1      | 1.2                                 |
| Mut-K     | 21.4            | 3.4      | 6.3                                 |
| Mut-L     | 2.0             | 0.8      | 2.5                                 |
| Mut-M     | 6.7\(^{*}\)     |          |                                     |
| Mut-N     | 2.5\(^{*}\)     |          |                                     |
into three groups as shown in Fig. 2A. Mutagenesis was used to systematically convert amino acids at these positions in MHL-1V5His to those present in RHL-1. Mut-E (Fig. 2), which introduced the C-terminal most three amino acid differences (Group III) of RHL-1 into MHL-1, bound equal amounts of Siaα2,6GalMN-BSA and GGnM-BSA (a ratio of 1:1) similar to MHL-1. Mutating the Group II amino acids, Glu195, Gly238, and Asp242 (Mut-F), resulted in an HL-1 subunit that bound more Siaα2,6GalMN-BSA than GGnM-BSA (a ratio of 11.9), effectively converting the specificity of MHL-1 to that of RHL-1.

Other differences in the amino acid sequence of RHL-1 and MHL-1 also contribute to the specificity of RHL-1 for Siaα2,6GalMN-BSA. For example, the introduction of Arg198, Val200, and Gln202 of Group I in the rat in place of Asn198, Leu200, and Arg202 in MHL-1 reduced the ratio of Siaα2,6GalMN-BSA to GGnM-BSA binding from 11.9 (Mut-F) to 3.6 for Mut-G. The additional introduction of Trp194 and Glu195 also in Group I in place of Arg194 and Asp195 (Mut-H) increased the Siaα2,6GalMN-BSA:GGnM-BSA binding ratio to 5.9, still significantly less than that seen for either RHL-1 or Mut-F. Whereas the replacement of Arg172 with Lys172 in Mut-J did not markedly enhance the binding of either Siaα2,6GalMN-BSA or GGnM-BSA, the introduction of Lys172 in combination with the Group II sequence (Lys237, Gly238, and Asp242) enhanced the binding of both Siaα2,6GalMN-BSA and GGnM-BSA (Mut-K). As a result, the ratio of Siaα2,6GalMN-BSA to GGnM-BSA was reduced even though both ligands were bound more avidly. As was seen for Mut-G, the replacement of Asn198, Leu200, and Arg202 of Group I with Arg198, Val200, and Gln202 reduced the binding of both Siaα2,6GalMN-BSA and GGnM-BSA (Mut-L) as well as the Siaα2,6GalMN-BSA to GGnM-BSA ratio.

The introduction of Gln at position 238 in RHL-1 (Mut-M) did not reduce the ratio of Siaα2,6GalMN-BSA to GGnM-BSA to that of MHL-1, nor did the introduction of a Gly at position 238 in MHL-1 increase the ratio to that of RHL-1. Thus, a single change within the Group II residues that are in closest proximity to the probable contact residues of HL-1 is not sufficient to account for the difference in specificity seen in RHL-1 and MHL-1.

**Rat and Mouse HL-1 CRDs Expressed in Bacteria Retain Their Specificity for Terminal Siaα2,6GalNAc—**Rat and mouse HL-1 CRDs consisting of residues 152–284 of the V5His sequence at the C terminus were expressed in bacteria and isolated by affinity chromatography on GalNAcβ-agarose columns. The affinity-purified products were homogeneous when analyzed by SDS-PAGE (data not shown) and were active as demonstrated by their ability to bind GGnM-[125I]BSA in the polyethylene glycol precipitation assay (Fig. 3). The bacterially expressed and affinity-purified rat and mouse HL-1 CRDs retain the ability to bind Siaα2,6GalMN-BSA (Fig. 3); however, the rat HL-1 CRD (R1-CRDV5His) binds 3–5-fold more Siaα2,6GalMN-[125I]BSA per picomole of CRD than the mouse HL-1 CRD (M1-CRDV5His). The difference in ratio of Siaα2,6GalMN-[125I]BSA to GGnM-[125I]BSA seen with the bacterially expressed HL-1 CRDs and those expressed in HEK-293T cells may reflect a number of possibilities related to the absence of cytosolic, transmembrane, and stem regions and post-translational modifications such as glycosylation.

**DISCUSSION**

We previously reported that the HL-1 subunit of the rat ASGP-R binds terminal Siaα2,6GalNAc and can account for the rapid clearance of Siaα2,6GalMN-BSA from the blood following injection (11). The studies we have presented here indicate that orthologues of the ASGP-R display marked differences in their specificity for terminal Siaα2,6GalNAc. For example, recombinant HL-1 subunits of the rat and mouse ASGP-R differ by 16-fold in their capacity to bind terminal Siaα2,6GalNAc compared with terminal GalNAc. This is the case, even though 87% of the amino acids in rat and mouse HL-1 is identical. The differential capacity for binding terminal Siaα2,6GalNAc displayed by extracts prepared from rat, rabbit, mouse, bovine, and porcine liver (Fig. 1) suggests that orthologues of the ASGP-R do not have identical specificities.

The 16-fold difference in the ratio of Siaα2,6GalMN-BSA:GGnM-BSA binding exhibited by RHL-1 and MHL-1 is of particular interest, because the subunits differ at only 12 positions within the CRD (residues 152–284). An exchange of the cytosolic, transmembrane, and stem regions of rat and mouse HL-1 confirmed that differences within the CRD account for the respective specificities of the rat and mouse ASGP-R for Siaα2,6GalNAc. Our systematic mutation of the MHL-1 to convert the amino acids at these 12 positions to those of the rat revealed that multiple changes in the sequence of the CRD contribute to the difference in specificity. The introduction of the Group II residues (Figs. 2A and 4, red-colored residues) produces a receptor that has the specificity of RHL-1 (Fig. 2B, Mut-F). Modeling studies based on the crystal structure of the HLH-1 CRD suggest that bound GalNAc would be stacked against Trp243 (colored white in Fig. 4) and that the three and four hydroxyls of the GalNAc would be coordinated by the Ca2+ at site 2 (colored green in Fig. 4) (8). The side chain of either a Gly or Glu at position 238 would be located adjacent to the side chain of Glu239 that is proposed to coordinate the C4 hydroxyl of the GalNAc (22). However, substitution of Gly238 in RHL-1 with Glu reduces but does not completely abolish Siaα2,6GalNAc binding (a ratio of 6.7) and the substitution of Glu238 in MHL-1 with Gly increases the level of Siaα2,6GalNAc binding (a ratio of 2.5) but not to the level seen for RHL-1. The presence of a Gly at position 238 is not sufficient to increase Siaα2,6GalNAc binding by MHL-1 to that seen for RHL-1. Asp242, which coordinates the Ca2+ (colored blue in Fig. 4) at site 1 of HL-1, may have an impact on interaction of the sialic acid with the peptide.

Alterations in the sequence that are distant from the proposed GalNAc binding site also have an impact on binding...
Sia2,6GnM-BSA. For example, the Group I residues (Fig. 2A) that are located on the surface of the o2 helix (blue and yellow residues in Fig. 4) have an impact on binding Sia2,6GnM-BSA. Introduction of three of the five Group I residues at positions 198, 200, and 202 (residues colored blue in Fig. 4) in the presence of the Group II residues reduced the ratio of Sia2,6GnM-BSA to GnM-BSA binding from 11.9 to 3.6 (Mut-F versus Mut-G). The additional introduction of the remaining two residues at positions 194 and 195 (residues colored yellow in Fig. 4) increased the ratio to 5.9 for Mut-H. Thus, the multiple residues that are present in Group I and form the o2 helix must be introduced together to preserve Sia2,6GnM-BSA binding in the presence of the Group II residues. It is notable that, with the exception of Lys172 (colored aqua in Fig. 4) that enhances the binding of both Sia2,6GnM-BSA and GnM-BSA, all of the differences in the sequence of RHL-1 and MHL-1 that are visible in the crystal structure are located on the surface of HL-1 that contains the o2 helix. In addition, Lys253 from Group II and His260 from Group III are also on the surface of HL-1 in close proximity to the Group I residues in the o2 helix (Fig. 4). We have not determined how or whether the introduction of Group I residues alone would influence the binding of oligosaccharides. Because the Group I residues are distant from the binding site itself, it is not clear whether they alter interactions with contact residues in the binding site and/or whether they influence the formation of ASGP-R oligomers that are known to exist in vivo. The precise manner in which the HL-1 subunit oligomerizes with itself and HL-2 could have a significant impact on binding terminal Sia2,6GalNAc.

It is striking that multiple changes in the sequence of RHL-1 contribute to the increased avidity of RHL-1 for Sia2,6oGalNAc compared with MHL-1. Thus, it appears that few of the changes in the sequence of RHL-1 and MHL-1 can be viewed as silent alterations with respect to the ligand specificity of the ASGP-R. The different specificities of RHL-1 and MHL-1 may reflect exogenous and/or endogenous selection pressures. For example, the rat produces large amounts of prolactin-like glycoprotein hormones, prolactin-like hormones, that bear N-linked oligosaccharides terminating with Sia2,6oGalNAcβ1,4GlcNAcβ1,2Man in the placenta and releases them into the blood (11, 12). In contrast, we have not detected either the protein-specific GalNAc-transferase or glycoproteins bearing terminal Sia2,6oGalNAcβ1,4GlcNAcβ1,4GlcNAcβ1,2Man in the placentas of mice. The difference in the specificity of the rat versus mouse ASGP-R may reflect a requirement to recognize terminal Sia2,6oGalNAc during pregnancy in the rat. Alternatively, other selection pressures such as infectious or parasitic agents may account for changes in the specificity of the ASGP-R.

Our results indicate that orthologues of the ASGP-R in different mammals cannot be assumed to display identical specificities for carbohydrate moieties terminating with β1,4-linked Gal or GalNAc (23) despite the high percentage of identical amino acids in their sequences. Furthermore, glycoproteins bearing terminal α2,6-linked sialic acid rather than terminal Gal or GalNAc may prove to be endogenous ligands for the ASGP-R. The multiple amino acid sequence modifications required to account for the altered specificity of the ASGP-R in such evolutionarily closely related mammals may indicate that we are observing a carbohydrate-specific receptor, modulating its specificity to accommodate a need to recognize endogenous or exogenous oligosaccharide structures. Understanding the
The relationship between the specificity of the ASGP-R and the carbohydrates that are recognized may provide new insight into the role such receptors play in vivo.

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