Rapid Clearance of Sialylated Glycoproteins by the Asialoglycoprotein Receptor*

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The asialoglycoprotein-receptor (ASGP-R) located on liver parenchymal cells was originally identified and characterized on the basis of its ability to bind glycoproteins bearing terminal galactose (Gal) or N-acetylgalactosamine (GalNAc); however, endogenous ligands for the ASGP-R have not to date been definitively identified. We have determined that the rat ASGP-R specifically binds oligosaccharides terminating with the sequence Siaα2,6GalNAcβ1,4GlcNAcβ1,2Man. Bovine serum albumin chemically modified with 10–15 tetrasaccharides with the sequence Siaα2,6GalNAcβ1,4GlcNAcβ1,2Man is cleared from the blood of the rat with a half-life of <1 min by a receptor located in the liver. We have isolated the receptor and identified it as the ASGP-R. Furthermore, we have determined that subunit 1 of the ASGP-R accounts for the binding of terminal Siaα2,6GalNAcβ.

Based on the newly defined specificity of the rat ASGP-R we hypothesize that the glycoproteins bearing structures that are selectively modified with terminal Siaα2,6GalNAcβ are released into the blood may be endogenous ligands for the rat ASGP-R.

The rapid clearance of glycoproteins from the blood following removal of sialic acid (Sia) residues and exposure of underlying galactose (Gal) residues was first reported by Ashwell and Morell in the early 1970s (1, 2) and led to the discovery of the asialoglycoprotein-receptor (ASGP-R). The specificity and biochemical features of this endocytic receptor have been extensively investigated since that time (3, 4). Although it is clear that the ASGP-R receptor binds oligosaccharides with terminal β-linked N-acetylgalactosamine (GalNAc) or Gal and can mediate the rapid clearance of glycoproteins bearing these terminal sugars from the circulation, endogenous ligands for this abundant receptor have not yet been identified.

We first described N-linked oligosaccharides containing β1,4-linked GalNAc on lutropin (LH) and other members of the glycoprotein hormone family of glycoproteins (5, 6). The GalNAc is found almost exclusively in the form of GalNAc-4-SO4, reflecting the sequential action of a protein-specific β1,4-N-acetylgalactosaminyltransferase (β1,4GalNAcT) and a GalNAc-4-sulfotransferase (GalNAc-4-ST) (7, 8). The terminal GalNAc-4-SO4 is recognized by a receptor in hepatic endothelial cells that regulates the circulatory half-life of LH following its stimulated release into the blood (9–12). The control of circulatory half-life is important for regulating estrogen production in vivo during implantation of the embryo (13). We subsequently described the presence of N-linked oligosaccharides terminating with Siaα2,6GalNAcβ on prolactin/growth (PLP) hormone family members that are synthesized by rat placenta spongiotrophoblasts between mid gestation and birth (14). The levels of protein-specific β1,4GalNAcT activity in rat placenta increase 150-fold between day 9 and 18 of gestation, whereas levels of a,6sialyltransferase increase 5-fold during the same period (14). We have found significant levels of PLP hormone family members bearing terminal Siaα2,6GalNAcβ in the circulation of the pregnant rat late in gestation. Furthermore, the pregnancy-specific glycoprotein glycolentin (placental protein 14) that is found in the amniotic fluid of humans also bears this structure (15).

The selective addition of β1,4-linked GalNAc to the oligosaccharides on prolactin-like protein (PLP)-A, PLP-A, PLP-B, PLP-C, PRP, and placental lactogen I variant (14) as well as human glycolentin (15) raised the possibility that a receptor specific for Siaα2,6GalNAcβ is present and may regulate the circulatory half-life of glycoproteins bearing these structures and/or direct them to specific locations such as the amniotic fluid. We have examined this possibility in the pregnant rat and report that neoglycoconjugates bearing multiple tetrasaccharides terminating with the sequence Siaα2,6GalNAcβ1,4GlcNAcβ1,2Man are rapidly removed from the circulation by the ASGP-R. Thus, the PLP hormone family members that are released into the blood during pregnancy may represent examples of endogenous ligands for the ASGP-R.

MATERIALS AND METHODS

Timed pregnant female CD® IGS rats, E16–E18 (E1 is the day one of gestation), were obtained from Charles River Laboratories (Wilmington, MA). Bovine serum albumin (BSA) conjugated with an average of 15 trisaccharides with the sequence GalNAcβ1,4GlcNAcβ1,2Manα (GGM-BSA) or Siaα3-GalNAcβ1,4GlcNAcβ1,2Manα (SGGM-BSA) were provided by Dr. O. Hindsaul, University of Alberta (Edmonton, Canada). CMP-β-ν-sialic acid and recombinant rat α2,6-sialyltransferase (EC 2.4.99.1) were from Calbiochem-Novabiochem Corp. (La Jolla, CA). Galactose-BSA was purchased from EY Laboratories, Inc. (San Mateo, CA). GalNAc-β-BSA, CNBr-activated Sepharose, fettin (from fetal calf serum), and neuraminidase-agarase (Clostridium perfringens) were obtained from Sigma (St. Louis, MO). TRHβ® reagent and primers were purchased from Life Technologies (Grand Island, NY).

Preparation of Siaα2,6GalNAcβ1,4GlcNAcβ1,2Manα-BSA (Sia(GM-BSA)—SiaGGM-BSA) was prepared using a modification of the procedure described by van Seevent et al. (16). GGGM-BSA (700 µg)
was incubated at 37 °C in a 330-μl reaction containing 1.57 mg of CMP-Neu5Ac2en-sialic acid, 50 mM magnesium acetate, 1 mM diethylylthiol and 10% (v/v) sucrose. Homogenates were sedimented at 1,500 g for 5 min, and the resulting supernatants were layered over a 65% (v/v) sucrose cushion prior to sedimentation at 100,000 g for 45 min at 4 °C. The membrane fraction at the interphase and the soluble fraction in the upper phase were collected separately and stored at −80 °C. Protein concentrations were determined using the Brad ford method (Bio-Rad, Richmond, CA).

**Binding Assays**—Binding studies were performed in 100 μl of Buffer A containing 0.5% Triton X-100, 2 mM CaCl₂, 2-6×10⁵ cpm of ³²⁵I-Gal-GnMs-BSA, and 200 μg of membrane or soluble liver protein. Following incubation for 30 min at 25 °C, 1.5 ml of ice-cold 10% (v/v) PEG 8000 in Buffer B (25 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, pH 7.4) was added, and the reaction was vortexed for 2 s. Precipitated ligand-receptor complexes were collected after incubation for 30 min at 4 °C by vacuum filtration on Whatman GF/C filter discs that had been incubated in Buffer B containing 5 mg/ml BSA. The filters were washed three times with 1 ml of ice-cold 10% (V/V) PEG 8000 in Buffer B and counted in a MultiScreen® punch tip (Millipore) and counted in the γ-counter.

**Receptor Purification**—Rat liver membrane proteins (400 mg) prepared as described above were resuspended in 50 ml of 50 mM Tris-HCl at pH 7.4 with 150 mM NaCl, 2 mM CaCl₂, and 3% Triton X-100 using a Dounce homogenizer. Insoluble material was removed by sedimentation for 2 min at 700 × g. The supernatant was incubated with 1 ml of GalNac-β-BSA-Sepharose (5 mg of GalNac-β-BSA/ml of Sepharose) for 16 h at 4 °C. After washing five times with 5 ml of Buffer B containing 1% (v/v) Triton X-100, the bound protein was eluted with Buffer C containing 100 mM GalNAc. Fractions containing SiaGnM-BSA binding activity were pooled, dialyzed against Buffer C, and stored at −20 °C.

**Western and Ligand Blots**—Proteins were separated in the presence of SDS without reduction on NuPAGE® Bio-Trol gels and electrophoretically transferred to Immobilon-P (Millipore). Duplicate strips were stained with Coomassie Blue or silver. For two-dimensional electrophoresis, isoelectric focusing was performed with PROTEAN® IEF system (Bio-Rad) using pH 3–10 ReadyStrip IPG strips. Separation in the second dimension was performed without reduction on 10% Crite- rion® Tris-HCl gels as recommended by the manufacturer. Rabbit and mouse anti-2,6GalNAc-IgG (1:10,000) and mouse anti-Anti-2,3GalNAc-IgG (1:10,000) were used for Western analysis. For ligand blots, the Immobilon-P membranes were blocked with 5% milk protein for 30 min, washed once with Buffer A containing 25 mM EDTA, and washed three times with Buffer A containing 0.5% Triton X-100 and 2 mM CaCl₂. The washed Immobilon-P membrane was incubated overnight at 4 °C in the presence of 2×10⁶ cpm of SiaGnM-BSA in Buffer A containing 0.5% Triton X-100 and 2 mM CaCl₂, washed three times with Buffer A containing 0.5% Triton X-100 and 2 mM CaCl₂, and exposed to film for 7–15 days. Proteins corresponding to the region reactive with SiaGnM-³²⁵I[BSA were excised and examined by MALDI-TOF at the Johns Hopkins University Mass Spectrometry Facility.

**Cloning and Expression of Rat ASGP-R Subunit 1 and Subunit 2**—Total RNA was isolated from rat liver with TRIZOL® reagent per the manufacturer’s instructions. First-strand cDNA was synthesized using oligo(T₁₄) and Superscript™ II RNase − reverse transcriptase (Life Technologies). Rat ASGP-R subunits 1 and 2 were amplified using KlenTag LA DNA polymerase mix (Sigma Chemical Co., St. Louis, MO) and the gene specific primers RHL1-F (CGG CAT CCC ATC ATG GAG AAG AAG GAG TAT CAA GAT TAT TTC C) and RHL1-AR (5’-GGAT GCT CTT GCC CAA CTC TG) and RHL2-F (CGG CAT CCC ATG GAG AAG GAC TTT CAA GAT ATC TC) and RHL2-AR (5’-GTA GGT GAT GTC CGC CTT G), respectively. Amplified products were subcloned into pcDNAs 1.5/VhHis/TOPO® and sequenced. The cDNAs were designated as RHL1VhHis and RHL2VhHis, respectively.

CHO/Tag cells grown on a 100-mm plate were transfected with 13 and 6 μg, respectively, of RHL1VhHis and RHL2VhHis using 35 μg of LipofectAMINE (Life Technologies) in serum-free medium for 5 h according to the manufacturer’s protocol. The pcDNAs 1.5/VhHis/TOPO® cloning vector was used as a control for negative expression. Forty-eight hours after transfection, the cells were washed with PBS (8 g of NaCl, 0.2 g of KCl, 14.4 g of NaHPO₄, and 0.24 g of KH₂PO₄, pH 7.4) and incubated for 10 min at −20 °C. Cells were thawed and scraped off in 1 ml of Buffer A containing 50 mM EDTA. Cells were pelleted by centrifugation for 2 min at 12,000 × g and washed three times with 1 ml of Buffer A. Cells were incubated with 250 μl of Buffer A containing 1% (v/v) Triton X-100 per 100-mm diameter culture plate for 30 min at 4 °C with mixing. The mixture was centrifuged at 12,000 × g for 10 min at 4 °C. The supernatant was collected and stored at −20 °C until needed.

**RESULTS**

*Sia2,6GalNAcβ1,4GalNAcβ1,2Manα-BSA Is Rapidly Re-moved from the Circulation by the Liver—*We had previously observed that PLP hormones bearing oligosaccharides terminating with the unique sequence Sia2,6GalNAcβ are present in the blood of pregnant rats between mid gestation and birth. We introduced SiaGnM-³²⁵I-BSA into the blood of pregnant rats between mid gestation and birth. We observed that PLP hormones bearing oligosaccharides terminating with the unique sequence Sia2,6GalNAcβ are present in the blood of pregnant rats between mid gestation and birth. We therefore studied the clearance of SiaGnM-³²⁵I-BSA in pregnancy (Fig. 1). In pregnant rats, 90% of the SiaGnM-³²⁵I-BSA was present in the liver when rats were sacrificed 10 min after injection of the SiaGnM-³²⁵I-BSA (Table I). The cpm/mg of tissue in the liver, kidneys, and lungs was 47–6, and 5-fold greater (not shown), respectively, than that in the large intestine, indicating that the SiaGnM-³²⁵I-BSA was concentrated in the liver as compared with other highly vascular tissues. Although the amount of label in the liver declined by 45 min as compared with 10 min following injection, the amount of label in the kidneys had increased. This suggested that SiaGnM-³²⁵I-BSA was taken up exclusively by receptors in the liver and subsequently degraded, resulting in the release of radiolabel into the blood and its appearance in the kidneys and urine. There was no evidence of binding to or uptake by the uterus, placenta, or pups.

*Cation-dependent Binding of SiaGnM-BSA by a Receptor in Rat Liver—*The rapid and efficient clearance of SiaGnM-BSA by the liver indicated that a receptor specific for terminal Sia2,6GalNAcβ is present in either hepatocytes or hepatic
endothelial cells. The membrane fraction from liver, but not the soluble fraction, displayed cation-dependent binding of SiaGGnM-[125I]BSA (Fig. 2). Binding was pH-dependent, reaching a maximum between pH 5.5 and 7.0, and declining markedly below pH 5.0 (Fig. 3A). Binding did not occur in the absence of cations (Fig. 3B). Whereas Cd2+ or Co2+ enhanced SiaGGnM-[125I]BSA binding as compared with Ca2+, other cations with the exception of Ni2+ and Mn2+ were significantly less effective.

A range of monosaccharides were tested as inhibitors of SiaGGnM-[125I]BSA binding (Fig. 3C). At a concentration of 20 mM, GalNAc was the most potent inhibitor of binding (3% of control). Lactose (Galβ1,4Glc) and Gal both were good inhibitors, although not as potent as GalNAc, indicating the binding site can accommodate either Gal or GalNAc. Free sialic acid was also an inhibitor (36% of control) suggesting that the binding site accommodated both the terminal sialic acid and the β-linked GalNAc. The lack of inhibition by Man, GlcNAc, and α-methyl-Man indicated the receptor was not likely the site can accommodate either Gal or GalNAc. Free sialic acid indicated that the rat ASGP-R is capable of binding saccharides with terminal Sia2,6GalNAcβ.

Isolation of a Sia2,6GalNAcβ-specific Receptor—The marked inhibition of binding seen with GalNAc (Fig. 3C) suggested that an affinity matrix containing β-linked GalNAc could be utilized for purification of the Sia2,6GalNAcβ-specific receptor. Rat membrane proteins were solubilized using Triton X-100 and incubated with GalNAcβ-BSA-Sepharose. The SiaGGnM-[125I]BSA specific binding activity was retained by the affinity matrix and was eluted with 100 mM GalNAc. When examined by SDS-PAGE on a 10% gel under non-reducing conditions, the GalNAc-eluted material contained two major protein species with mobilities equivalent to 33 and 25 kDa when stained with Coomassie Blue (Fig. 4, lane 3). Because we expected the ASGP-R to be present in the material that was bound by GalNAcβ-BSA-Sepharose and eluted with 100 mM GalNAc, Western blot analysis with rabbit anti-rat ASGP-R antibody was used to identify the ASGP-R-derived proteins. Proteins with mobilities equivalent to 33, 70, 130, and >170 kDa reacted with anti-ASGP-R antibody in both the unfractinated Triton X-100 solubilized membrane fraction (Fig. 4, lane 4) and the affinity purified fraction (Fig. 4, lane 5). The same protein species bound SiaGGnM-[125I]BSA when a ligand blot was performed on the affinity-purified fraction (Fig. 4, lane 6). We also examined the proteins in the affinity-purified fraction by two-dimensional electrophoresis (Fig. 5). A ligand blot using SiaGGnM-[125I]BSA (Fig. 5B) indicated that proteins migrating with pI values between 4.6 and 5.6 and molecular masses of ~33, 75, and 150 kDa (Fig. 5A) accounted for the Sia2,6GalNAcβ-specific binding activity. The material migrating at 33 kDa was visualized by silver staining (Fig. 5A) and identified as the ASGP-R by MALDI-TOF analysis of tryptic fragments. Six out of a total of 31 potential tryptic peptides for subunit 1 of the ASGP-R were identified, accounting for 17% of the subunit 1 sequence. The ASGP-R represents the highest non-trivial protein detected in the sample with a molecular weight search (MOWSE) score of 197 for subunit 1. The only other non-trivial protein detected was keratin. Because the SiaGGnM-[125I]BSA used for the binding assays and the ligand blots does not contain any terminal GalNAc, the results indicated that the rat ASGP-R is capable of binding saccharides with terminal Sia2,6GalNAcβ.

Subunit 1 Accounts for Sia2,6GalNAcβ-specific Binding by the Rat ASGP-R—cDNAs encoding subunit 1 (RHL1) and subunit 2 (RHL2) of the rat ASGP-R were amplified from rat liver mRNA. The stop codons were mutated to permit expression as chimeric proteins containing the V5 epitope followed by six His residues at the carboxyl terminus. CHO-Tag and 293-Tag cells

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**TABLE I**

| SiaGGnM-[125I]BSA cpm × 10^3/organ | Rat #1 | Rat #2 | Rat #3 | Rat #4 |
|------------------------------------|-------|-------|-------|-------|
| Liver                              | 3,421 | 1,875 | 2,174 |       |
| Kidney                             | 114   | 186   | 281   |       |
| Lung                               | 36    | 197   | 118   |       |
| Spleen                             | 12    | 13    | 17    |       |
| Heart                              | 9     | 44    | 41    |       |
| Small intestine                    | 9     | 43    | 72    |       |
| Large intestine                    | 3     | 9     | 15    |       |
| Placenta                           | 3     | 24    | 22    |       |
| Ovary                              | 2     | 11    | 19    |       |
| Uterus                             | 2     | 23    | 19    |       |
| Pup                                | <1    | 4     | 3     |       |

* Clearance was terminated at 10 min (Rat #1) or 45 min (Rat #2 and #3).
were transfected with pcDNA3.1-RHL1V5His or pcDNA3.1-RHL2V5His. Equal fractions of the transfected cells were examined by Western blot analysis with anti-V5 antibody following SDS-PAGE on a 10% gel under reducing conditions. RHL1V5His is expressed at higher levels than RHL2V5His in both cell lines. In addition, RHL1V5His is more heterogeneous with major bands migrating with molecular masses of 52 and 57 kDa, whereas RHL2V5His has a single band migrating with a molecular mass of 57 kDa (Fig. 6). Small amounts of what are likely homodimeric species of RHL1V5His and RHL2V5His were detected at 105 and 115 kDa, respectively.

RHL1V5His and RHL2V5His were solubilized from CHO-Tag and 293-Tag cells using Triton X-100, and their ability to bind SiaGGnM-[125I]BSA and Gal-[125I]BSA was compared as shown in Fig. 6. RHL1V5His was able to bind and precipitate both SiaGGnM-[125I]BSA and Gal-[125I]BSA when expressed in either CHO-Tag or 293-Tag cells. No evidence of binding activity was seen in extracts from cells transfected with the pcDNA3.1V5His vector alone. In addition, no binding activity for either SiaGGnM-[125I]BSA or Gal-[125I]BSA was associated with RHL2V5His. This is consistent with the observations of other groups that lectin activity is primarily associated with RHL1 (17–19). Nonetheless, it is clear that recombinant RHL1V5His is able to bind both SiaGGnM-BSA and Gal-BSA as was seen with the affinity-purified ASGP-R prepared from rat liver above.

**DISCUSSION**

The ASGP-R was originally identified on the basis of its ability to mediate the rapid clearance of glycoproteins such as ceruloplasmin from the blood following removal of sialic acid and exposure of underlying Gal moieties. Numerous studies have supported this conclusion, and sialylated glycoproteins are typically used to demonstrate the specificity of the ASGP-R for terminal Gal and GalNAc moieties. Our observation that the rat ASGP-R is able to bind saccharides with the terminal sequence Sia\(^2\)Gal\(^1,4\)GlcNAc\(^1\) is both unexpected and remarkable. The binding activity we have observed does not reflect the presence of terminal GalNAc on the SiaGGnM-BSA glycoconjugate, because less than 1% of the SiaGGnM-[125I]BSA preparation was bound by Wistaria floribunda agglutinin-agarose (not shown), a lectin that would detect the presence of saccharides with a single terminal β-linked GalNAc (20). Furthermore, the same amount of SiaGGnM-[125I]BSA was precipitated by the ASGP-R before and after neuraminidase digestion to remove the sialic acid (not shown), indicating that removal of the sialic acid did not result in a significant increase in binding by the ASGP-R.

The terminal sequence Sia\(^2\)Gal\(^1,4\)GlcNAc\(^1\)2Man that we described on PLP hormones produced by rat spongiotrophoblasts from mid gestation to birth is a unique structure that is present on only a limited number of glycoproteins (14, 15, 21). It is not yet clear if the terminal sequence Sia\(^2\)Gal\(^1\), 4GlcNAc\(^1\),2Man that is present on large numbers of glycoproteins will also be recognized by the ASGP-R. Our observation that SiaGGnM-[125I]BSA binding by the ASGP-R is partially inhibited by 20 mM sialic acid suggests that the sialic
acid may actively participate in binding to the RHL1 subunit. The crystal structure of the Gal/GalNAc binding site of subunit 1 of the human ASGP-R has been resolved (22). It will be of interest to determine how Sia\textsubscript{2,6}GalNAc\textbeta\textsubscript{1,4}GlcNAc\textbeta is bound as compared with either GalNAc\textbeta\textsubscript{1,4}GlcNAc\textbeta or Gal\textbeta\textsubscript{1,4}GlcNAc\textbeta. Because the pH and calcium dependence for binding are similar for binding Sia\textsubscript{2,6}GalNAc\textbeta\textsubscript{1,4}GlcNAc\textbeta and Gal/GalNAc terminal ligands, it is likely that GalNAc coordinates with the same calcium (site 2) and other contact residues in the presence or absence of the α2,6-linked sialic acid.

The rapid clearance of the neoglycoconjugate SiaGGnM-BSA by the ASGP-R was unanticipated, because the ASGP-R has long been assumed to exist for the clearance of ligands whose terminal sialic acid has been removed to expose underlying Gal or GalNAc moieties. Because SiaGGnM-BSA is multivalent, we have not yet been able to establish the affinity of RHL1 for Sia\textsubscript{2,6}GalNAc\textbeta\textsubscript{1,4}GlcNAc\textbeta as compared with GalNAc\textbeta\textsubscript{1,4}GlcNAc\textbeta or Gal\textbeta\textsubscript{1,4}GlcNAc\textbeta. The \( K_d \) values for glycoproteins bearing 1, 2, 3, or more terminal Sia\textsubscript{2,6}GalNAc\textbeta\textsubscript{1,4}GlcNAc\textbeta sequences will have to be determined to establish how rapidly such glycoproteins would be cleared from the circulation. The results we have obtained with the Man/GalNAc-4-SO\textsubscript{4}\textsubscript{-} receptor are, however, instructive.

The Man/GalNAc-4-SO\textsubscript{4}\textsubscript{-}receptor is found predominantly in the form of a dimer at the surface of hepatic endothelial cells (9). Each cysteine-rich domain at the amino terminus of the receptor is able to engage a single terminal GalNAc-4-SO\textsubscript{4}\textsubscript{2} (23), and two terminal GalNAc-4-SO\textsubscript{4}\textsubscript{-} moieties on separate N-linked oligosaccharides must be engaged simultaneously to form a stable complex with the affinity seen for binding bovine LH to isolated endothelial cells through its sulfated carbohydrate chains. The neoglycoconjugate SO\textsubscript{4}\textsubscript{2}-GalNAc\textbeta\textsubscript{1,4}GlcNAc\textbeta, 2Mano-BSA, which like SiaGGnM-BSA has a high density of conjugated saccharides, is cleared from the circulation of the rat in less than 2–3 min, whereas LH with three N-linked oligosaccharides bearing terminal GalNAc-4-SO\textsubscript{4}\textsubscript{-} moieties is cleared with a half-life of 7.5 min (11, 12). The clearance rate is consistent with the \( K_d \) of 1.6 \( \times \) 10\textsuperscript{-7} \( M \) for binding to the Man/GalNAc-4-SO\textsubscript{4}\textsubscript{-} receptor; however, LH in the circulation never exceeds a concentration of 1 \( \times \) 10\textsuperscript{-9} \( M \) (12).

The clearance rate seen for LH in vivo reflects the enormous amount of Man/GalNAc-4-SO\textsubscript{4}\textsubscript{-} receptor that is expressed in the liver. With 4 \( \times \) 10\textsuperscript{8} endothelial cells in the liver, >600,000 receptors at the surface of each cell, and a rapid rate of endocytic uptake (\( t_{1/2} \) of 20 s) there is sufficient capacity to clear all of the LH from the blood even if only 0.5\% of the receptor is occupied. Furthermore, the rate of clearance will remain constant at all concentrations of LH below its \( K_d \) of 1.6 \( \times \) 10\textsuperscript{-7} \( M \) for binding to the Man/GalNAc-4-SO\textsubscript{4}\textsubscript{-} receptor. Based on the example of LH and the Man/GalNAc-4-SO\textsubscript{4}\textsubscript{-} receptor, our studies suggest that a different group of circulating glycoproteins may be the endogenous ligands for the ASGP-R in vivo. Like the Man/GalNAc-4-SO\textsubscript{4}\textsubscript{-} receptor, ASGP-R is highly expressed at the cell surface (500,000 receptors/cell) (24, 25) of an abundant cell and the hepatocyte (6–7 \( \times \) 10\textsuperscript{8} per rat liver) (26) and is rapidly endocytosed (27–29). Thus, if glycoproteins with multiple terminal Sia\textsubscript{2,6}GalNAc\textbeta moieties can be engaged with affinities in the range of 1 \( \times \) 10\textsuperscript{-7} \( M \) or below, they would likely be removed from the blood with half-
lives of 5–20 min that would not change with the changing concentration of each glycoprotein below its $K_d$. Because β1,4-linked Gal and GalNAc are both recognized by the ASGP-R, it is possible that Sia2,6Galβ may also be recognized. This raises the possibility that the ASGP-R may function to actually clear glycoproteins with terminal Sia2,6GalNAcβ or Sia2,6Galβ moieties in vivo rather than ones with terminal Gal or GalNAc.

Our current studies have revealed that glycoproteins bearing terminal Sia2,6GalNAcβ are recognized by the rat ASGP-R. Thus, the PLP hormones that bear these structures may represent examples of endogenous ligands for the ASGP-R. Notably, in mice the levels of ASGP-R have been reported to increase between mid gestation and birth and return to the levels seen in non-pregnant animals within 24 h after delivery (30). This pattern of expression further supports a potential relationship between the addition of these carbohydrate structures to PLP hormones and the regulation of their circulatory half-life by the ASGP-R.

The specificity of the ASGP-R may also provide insight into other observations. For example, terminal Sia2,6GalNAcβ is found on glycodelin, a human pregnancy-specific glycoprotein with potent immunosuppressive and contraceptive activities that is found in the amniotic fluid (15, 31). The immunosuppressive effects seen with glycodelin have been attributed to the Sia2,6GalNAcβ-bearing oligosaccharides that may specifically block adhesive and activation-related events mediated by CD22, the human B cell-associated receptor. Because the ASGP-R is present on late stage spermatids (32), the contraceptive activity associated with glycodelin may be mediated through the carbohydrate recognition via the ASGP-R subunit 1.

Our observations clearly demonstrate that the rat ASGP-R is capable of binding oligosaccharides terminating with Siaα2,6GalNAcβ as well as ones terminating with GalNAc or Gal. Furthermore, glycoproteins bearing oligosaccharides terminating with Siaα2,6GalNAcβ are rapidly removed from the blood by the ASGP-R. Although PLPs bearing 2–4 termini with the sequence Siaα2,6GalNAcβ would not likely be removed from the circulation as rapidly as SiaGGnM-BSA, their half-lives, like that of LH, may reflect clearance by the ASGP-R. Our observations raise the possibility that oligosaccharides bearing sialic acid in α2,6-linkage to GalNAc and possibly also to Gal may represent endogenous ligands for the ASGP-R.

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