The asialoglycoprotein receptor clears glycoconjugates terminating with sialic acidα2,6GalNAc

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Endogenous ligands have not, to date, been identified for the asialoglycoprotein receptor (ASGP-R), which is abundantly expressed by parenchymal cells in the liver of mammals. On the basis of the rapid clearance of BSA bearing multiple chemically coupled sialic acid (Sia)α2,6GalNAcβ1,4GlcNAcβ1,2Man tetrasaccharides (SiaGGnM-BSA) from the circulation, and the ability of the ASGP-R hepatic lectin-1 subunit to bind SiaGGnM-BSA, we previously proposed that glycoproteins modified with structures terminating with Siaα2,6GalNAc may represent previously unrecognized examples of endogenous ligands for this receptor. Here, we have taken a genetic approach using wild-type and ASGP-R-deficient mice to determine that the ASGP-R *in vivo* does indeed account for the rapid clearance of glycoconjugates terminating with Siaα2,6GalNAc. We have also determined that the ASGP-R is able to bind core-substituted oligosaccharides with the terminal sequence Siaα2,6Galβ1,4GlcNAc but not those with the terminal Siaα2,3Galβ1,4GlcNAc. We propose that glycoproteins bearing terminals Siaα2,6GalNAc and Siaα2,6Gal are endogenous ligands for the ASGP-R, and that the ASGP-R helps to regulate the relative concentration of serum glycoproteins bearing α2,6-linked Sia.

clearance | galactose | *N*-acetylgalactosamine | hepatic lectin | serum glycoproteins

It is well established that the hepatic asialoglycoprotein receptor (ASGP-R) identified by Ashwell and Morell (1) has a high affinity for terminal β-linked galactose (Gal) or *N*-acetylgalactosamine (GalNAc) residues (2). However, oligosaccharides on the vast majority of serum glycoproteins terminate with sialic acid (Sia) that is linked to the C3 or C6 hydroxyl of penultimate Gal or GalNAc residues. Enzymatic removal of terminal α2,3- or α2,6-linked Sia exposes the underlying Gal or GalNAc on these glycoproteins and results in their rapid clearance from the circulation by means of the ASGP-R. Thus, it has been widely anticipated that *in vivo* ligands for the ASGP-R would be circulating asialoglycoproteins that either have had their terminal Sia removed or had not had Sia added during synthesis. However, mice deficient inASGP-R binding activity because of genetic ablation of either of its two subunits do not accumulate asialoglycoproteins in their blood (3, 4). This lack of asialoglycoprotein accumulation raises the question of whether asialoglycoproteins are the primary *in vivo* ligands for the ASGP-R.

We recently observed that BSA chemically modified with 15 trisaccharides, each with the sequence Siaα2,6GalNAcβ1,4GlcNAcβ1,2Manα- (SiaGGnM-BSA; Man, mannose), is rapidly removed from the circulation after injection into the rat (5). Furthermore, we demonstrated that the native ASGP-R binds SiaGGnM-BSA as does the recombinant hepatic lectin-1 (HL-1) subunit of the ASGP-R (5). N-linked oligosaccharides terminating with the sequence Siaα2,6GalNAcβ1,4GlcNAcβ1,2Manα- occur naturally on a number of circulating glycoproteins including human glycodelin (6), and the following select members of the rat prolactin-like protein (PLP) hormone family: PLP-A, PLP-B, PLP-C, prolactin-related protein, and placental lactogen I variant (7). We therefore proposed that these glycoproteins terminating with Sia may actually represent previously unrecognized examples of endogenous ligands for the ASGP-R (5).

The ASGP-R consists of two distinct subunits, HL-1 and HL-2, that are required for the expression of functional receptor *in vivo* (8–12). Oligosaccharide binding activity is predominantly associated with the HL-1 subunit which is expressed in all mammals that have been examined to date and retains a high percentage of identical residues in its amino acid sequence when compared across species. For example, mouse HL-1 and human HL-1 are, respectively, 89% and 80% identical to rat HL-1. All of the ASGP-Rs in mammals share their specificity for terminal Gal or GalNAc; however, the activity per gram of liver for terminal Gal or GalNAc varies markedly among mammals (13). The specificity for terminal Siaα2,6GalNAc is also retained among mammals. The ratio of Siaα2,6GalNAc to GalNAc binding activity is greater in rat liver membranes than in mouse liver membranes (13), a difference that is retained by recombinant rat and mouse HL-1 expressed in human embryonic kidney 293 cells. This variation in specificity can be mapped to differences in the amino acid sequence of the rat and mouse HL-1 that are both near the proposed GalNAc binding site and distant from the site (13).

We now take advantage of a genetic approach by using ASGP-R-deficient mice to provide compelling evidence that the ASGP-R does indeed account for the clearance of glycoproteins terminating with Siaα2,6GalNAc from the blood. In addition, we show that the HL-1 subunit is able to recognize core-fucosylated glycoconjugates terminating with Siaα2,6Gal but not those terminating with Siα2,3Gal and propose that clearance by the ASGP-R may account for the more rapid removal from the blood of glycoconjugates terminating with Siaα2,6Gal than those terminating with Siaα2,3Gal. We hypothesize that the ASGP-R functions to help regulate the concentration of glycoproteins bearing Siaα2,6Gal in the blood.

Experimental Procedures

Materials. The preparation of BSA modified with covalently attached oligosaccharides has been described for SiaGGnM-BSA (5), GalNAcβ1,4GlcNAcβ1,2Manα-BSA (GGnM-BSA)

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Abbreviations: ASGP-R, asialoglycoprotein receptor; Gal, galactose; Sia, sialic acid; Man, mannose; Fuc, fucose; CRD, carbohydrate recognition domain; SiaGGnM-BSA, Siaα2,6GalNAcβ1,4GlcNAcβ1,2Manα-BSA; GGnM-BSA, GalNAcβ1,4GlcNAcβ1,2Manα-BSA; BiF10-BSA, biantennary core-fucosylated decasaccharide-BSA conjugate; BiF1223-BSA and BiF1226-BSA, the α2,3- and α2,6-sialylated dodecasaccharide derivatives of BiF10-BSA; HL-1, hepatic lectin-1; PLP, prolactin-like protein.

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Table 1. Glycoconjugates utilized for binding assays and clearance studies

Conjugate	Oligosaccharide structure	Oligosaccharides per BSA, mol/mol
SiaGGnM-BSA	Siaα2,6GalNAcβ1,4GlcNAcβ1,2Man-BSA	15
GGnM-BSA	GalNAcβ1,4GlcNAcβ1,2Man-BSA	15
Gal-BSA	Gal-BSA	34
BiF10-BSA	Galβ1,4GlcNAcβ1,2Manα1,6\ Fucα1,6\ Manβ1,4GlcNAcβ1,4GlcNAc-BSA Galβ1,4GlcNAcβ1,2Manα1,3/	3.9
BiF1226-BSA	Siaα2,6Galβ1,4GlcNAcβ1,2Manα1,6\ Fucα1,6\ Manβ1,4GlcNAcβ1,4GlcNAc-BSA Siaα2,6Galβ1,4GlcNAcβ1,2Manα1,3/	2.9
BiF1223-BSA	Sialα2,3Galβ1,4GlcNAcβ1,2Manα1,6\ Fucα1,6\ Manβ1,4GlcNAcβ1,4GlcNAc-BSA Sialα2,3Galβ1,4GlcNAcβ1,2Manα1,3/	4.6

Fuc, fucose.

(14), the complex-type biantennary core-fucosylated decasaccharide BiF10-BSA, and the α2,3- and α2,6-sialylated dodecasaccharide derivatives of BiF10-BSA, BiF1223-BSA, and BiF1226-BSA (15). Galactosyl-BSA (Gal-BSA) was purchased from EY Laboratories. The structures of the oligosaccharides and number of oligosaccharides conjugated per BSA molecule (mol/mol of BSA) are summarized in Table 1.

Radiolabeling. Twenty-five micrograms of BSA conjugate was dissolved in 95 μl of 20 mM Tris·HCl/150 mM NaCl, pH 7.4, and incubated on ice for 15 min with one IodoBead (Pierce) and 500 μCi (1 Ci = 37 GBq) of Na^{125}I. Free ^{125}I was separated from the labeled conjugate by using a Bio-Spin 6 Tris Column (Bio-Rad). The ^{125}I-labeled neoglycoconjugates were stored at −20°C in buffer A (25 mM Tris·HCl/150 mM NaCl, pH 7.4) containing BSA (1 mg/ml).

Bacterial Expression of Carbohydrate Recognition Domain (CRD) Regions of Mouse and Rat ASGP-R HL-1. cDNAs encoding chimeric proteins consisting of the CRD of either the mouse or the rat ASGP-R HL-1 subunit followed by a V5 epitope and a poly(His) tag were cloned into the bacterial expression vector pET22b(+) as described in ref. 13. The CRDs were purified by Ni-chelate affinity chromatography after solubilization from inclusion bodies. The CRDs were allowed to fold, and those with GalNAc-binding activity were isolated by affinity chromatography on GalNAcβ-agarose. The isolated mouse and rat ASGP-R CRDs were designated M1-CRDV5His and R1-CRDV5His, respectively. Binding activity was examined by using 1 pmol of purified CRD in the binding assay (see below).

Binding Assays. Binding studies were performed as described in ref. 5. Each reaction mixture contained 75 μl of buffer B (25 mM Tris·HCl, pH 7.4/500 mM NaCl/10 mM $CaCl_2$) containing 0.5% (wt/vol) Triton X-100, 2×10^4 to 5×10^4 cpm ^{125}I-labeled BSA glycoconjugate, and 1 pmol of either M1-CRDV5His or R1-CRDV5His. After incubation for 60 min at 25°C, ligand–receptor complexes were precipitated by adding an equal volume of ice-cold 20% (wt/vol) polyethylene glycol 8000 in 25 mM Tris·HCl, pH 7.4/500 mM NaCl and incubating for 30 min at 4°C. Precipitated ligand–receptor complexes were collected by vacuum filtration on glass-fiber membranes (Millipore, multiscreen-FC, clear plates). The filtrates were washed three times with 150 μl of ice-cold 10% (wt/vol) polyethylene glycol 8000 in buffer B. Individual filters were counted in a γ counter. Inhibition curves were generated by adding incremental amounts of Gal, GalNAc, or Sia. Analyses were performed by using PRISM 4.0 software (GraphPad, San Diego).

Clearance Studies. Clearance studies were performed by using C57BL/6 (wild-type) and ASGP-R HL-2-deficient (knockout) mice (The Jackson Laboratory, B6;129SV7-ASGR2$^{tm1/Her}$) that have been shown to be deficient in ASGP-R expression (16). Mice were anesthetized by Metofane inhalation during the study. One-half of a microgram of ^{125}I-labeled BSA glycoconjugate was introduced by retroorbital injection, and blood samples were collected from the opposite orbital venous sinus at designated times. The amount of ^{125}I glycoconjugate in 20 μl of blood was determined by using a γ counter. Blood collected immediately after injection was designated as 100%, and this value was used in calculating the serum half-life of glycoconjugates. Mice were killed by cervical dislocation at the termination of the clearance study.

Results

Monosaccharide Specificity of the Rat and Mouse ASGP-R HL-1 CRDs. R1-CRDV5His and M1-CRDV5His are both able to bind SiaGGnM-^{125}I-BSA and Gal-^{125}I-BSA. K_i values for the inhibition of binding by GalNAc, Gal, and Sia are summarized in Fig. 1 and Table 2. The inhibition studies were carried out in the presence of 500 mM NaCl to reduce potential nonspecific ionic interactions. Free Sia inhibits binding of SiaGGnM-^{125}I-BSA by both R1-CRDV5His and M1-CRDV5His (Fig. 1*A*) with similar K_i values of 37.0 mM and 24.6 mM, respectively (Table 2), supporting the conclusion that Sia interacts with specific residues in the binding pocket of the CRD. The K_i values obtained for inhibition of SiaGGnM-^{125}I-BSA binding by free Sia are 50- to 60-fold greater than those obtained for free Gal and 1,200-fold greater than those obtained for free GalNAc (Table 2).

Sia is a less effective inhibitor of Gal-^{125}I-BSA binding than SiaGGnM-^{125}I-BSA binding (Table 2). At 100 mM Sia, there is less than a 10% decrease in the amount of Gal-^{125}I-BSA bound by M1-CRDV5His (Fig. 1*B*). Because free Sia would be competing with the region of an extended binding site that is occupied by the α2,6-linked Sia of SiaGGnM, it is not surprising that in the absence of covalently linked terminal Sia, free Sia no longer inhibits binding to M1-CRDV5His. Free Sia is able to inhibit binding of Gal-^{125}I-BSA by R1-CRDV5His but with a K_i of 65 mM as compared with 37 mM for inhibition of SiaGGnM-^{125}I-BSA binding. This finding suggests that there are differences in the orientation of the bound Sia relative to the terminal Gal of the Gal-BSA ligand bound to R1-CRDV5His, as compared with M1-CRDV5His, that allow the Sia to interfere with occupation of the binding site by terminal Gal to a greater extent in the rat CRD than in the mouse CRD (13).

Sialoconjugate Binding Activities of the Rat and Mouse ASGP-R HL-1 CRDs. Because the rat and mouse HL-1 CRDs both bind ligands terminating with Siaα2,6GalNAcβ1,4GlcNAc, we examined the

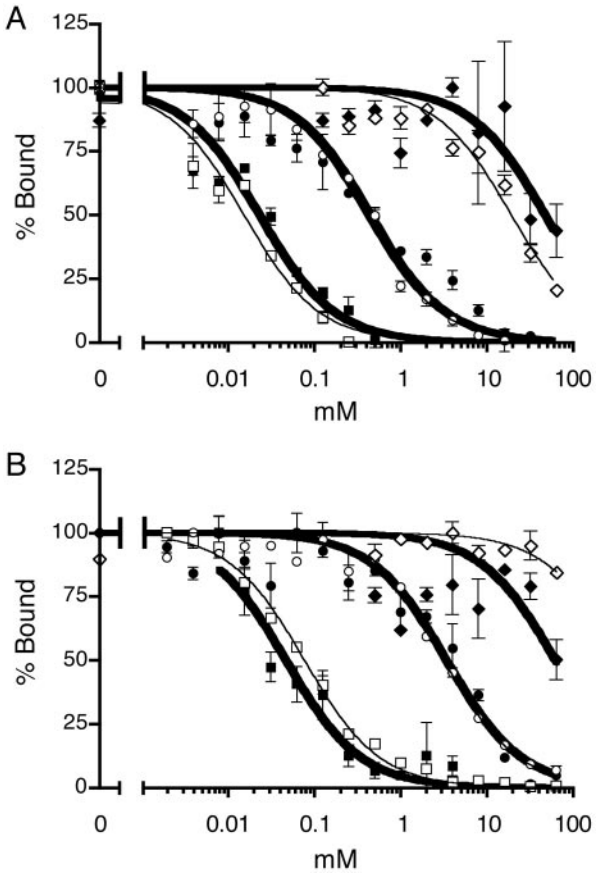

Fig. 1. Monosaccharide inhibition curves for rat and mouse ASGP-R HL-1 CRDs. The amount of SiaGGnM-^{125}I-BSA (*A*) or Gal-^{125}I-BSA (*B*) bound by 1 pmol of R1-CRDV5His (thick lines, filled symbols) or M1-CRDV5His (thin lines, open symbols) in the absence of monosaccharide inhibitor was set to 100%. The amount of SiaGGnM-^{125}I-BSA or Gal-^{125}I-BSA bound in the presence of increasing amounts of GalNAc (squares), Gal (circles), or Sia (diamonds) was then determined.

relative binding capacity of these CRDs for ligands that terminate with Siaα2,6Galβ1,4GlcNAc. Each of the BSA neoglycoconjugates was examined by using increasing amounts of R1-CRDV5His and M1-CRDV5His as shown in Fig. 2. With the exception of BiF1223-^{125}I-BSA, which has terminal α2,3-linked Sia, each of the BSA glycoconjugates was bound and precipitated by R1-CRDV5His and M1-CRDV5His. SiaGGnM-^{125}I-BSA was bound with a K_d of 1.28 μM and 1.26 μM by rat and mouse CRDV5His, respectively, whereas Gal-^{125}I-BSA was bound with a K_d of 0.19 μM and 0.22 μM by rat and mouse CRDV5His, respectively. BiF10-^{125}I-BSA was bound with K_d values of 0.16 μM and 0.45 μM that are similar to those for binding Gal-^{125}I-BSA, even though there are only a total of eight terminal Gal residues per BSA on BiF10-^{125}I-BSA as compared with 34 on Gal-^{125}I-BSA. BiF1226-^{125}I-BSA was bound with a K_d of 2.5 μM by the rat CRDV5His and 3.6 μM by mouse CRDV5His. Saturation curves obtained by using increasing amounts of Gal-^{125}I-BSA and BiF10-^{125}I-BSA confirmed the K_d values obtained for these compounds by using increasing concentrations of R1-CRDV5His and M1-CRDV5His (data not shown). Thus, even though there is an 8- to 16-fold reduction in the affinity of the rat and mouse HL-1 CRDs for glycoconjugates terminating with Siaα2,6Galβ1,4GlcNAc compared with Galβ1,4GlcNAc, binding of the Sia-containing structures is detectable and highly specific for α2,6-linked Sia relative to α2,3-linked Sia.

The ASGP-R Accounts for Clearance of Neoglycoconjugates with Terminal Gal and Siaα2,6GalNAc in Mice. We previously reported that SiaGGnM-^{125}I-BSA is rapidly cleared from the circulation of the rat (5). We also determined that HL-1 of the ASGP-R of the rat binds SiaGGnM-^{125}I-BSA and could therefore account for the clearance of glycoproteins bearing this structure. We examined the clearance of the glycoconjugates described in Table 1 in wild-type mice and mice genetically deficient in HL-2 of the ASGP-R. Expression of the ASGP-R at the cell surface of hepatocytes is deficient in these knockout mice (16). Clearance of BiF10-BSA, which bears terminal β1,4-linked Gal, is markedly reduced in ASGP-R-deficient mice as compared with wild-type mice (Fig. 3*C*). Similarly, clearance of SiaGGnM-BSA is reduced in ASGP-R-deficient mice as compared with wild-type mice (Fig. 3*A*). In contrast with BiF10-BSA with terminal Gal and SiaGGnM-BSA, the rapid clearance of GGnM-BSA with its terminal β1,4-linked GalNAc is not altered in HL-2-deficient mice (Fig. 3*B*). BiF1226-BSA and BiF1223-BSA were cleared at rates too slow to detect any difference between wild-type and ASGP-R-deficient mice at 10 min. (Fig. 3 *D* and *E*).

Discussion

This study demonstrates that the ASGP-R accounts for the *in vivo* clearance of glycoconjugates terminating with Siaα2,6GalNAc from the circulation. Glycoproteins bearing oligosaccharides that terminate with Siaα2,6GalNAc therefore represent examples of endogenous ligands for the ASGP-R. Oligosaccharides terminating with Siaα2,6GalNAc have been described on a number of glycoproteins, including human glycodelin isolated from amniotic fluid (6), urokinase (17), tissue plasminogen activator produced by Bowes melanoma cells (18), recombinant protein C (19), glycoprotein hormones (20), bovine lactotransferrin (21), bovine CD36 (22), bovine butyrophilin (23), and members of the PLP family of hormones produced by spongiotrophoblasts in the rat placenta (7). β1,4-Linked GalNAc is transferred to Asn-linked oligosaccharides on pituitary glycoprotein hormones (24) and the PLP family of hormones (7), as well as a number of other glycoproteins, by one or more protein-specific β1,4-*N*-acetylgalactosamine-transferases (β1,4GalNAcT) that are expressed in a wide range of tissues and cells (25). Oligosaccharides terminating with the disaccharide sequence GalNAcβ1,4GlcNAcβ- can be further modified with Sia, SO_4, or Fuc to produce the unique structures Siaα2,6GalNAcβ1,4GlcNAcβ-, SO_4-4-GalNAcβ1,4GlcNAcβ-,

Table 2. K_i values for inhibition by monosaccharides

Monosaccharide inhibitor	K_i for complex inhibition, mM			
	CRD·SiaGGnM-BSA		CRD·Gal-BSA	
	R1-CRDV5His	M1-CRDV5His	R1-CRDV5His	M1-CRDV5His
GalNAc	0.03 ± 0.003	0.02 ± 0.001	0.05 ± 0.013	0.10 ± 0.003
Gal	0.6 ± 0.1	0.5 ± 0.02	2.7 ± 0.8	3.8 ± 0.5
Sia	37.0 ± 11.2	24.6 ± 1.2	65.1 ± 28.8	330.5 ± 30.7

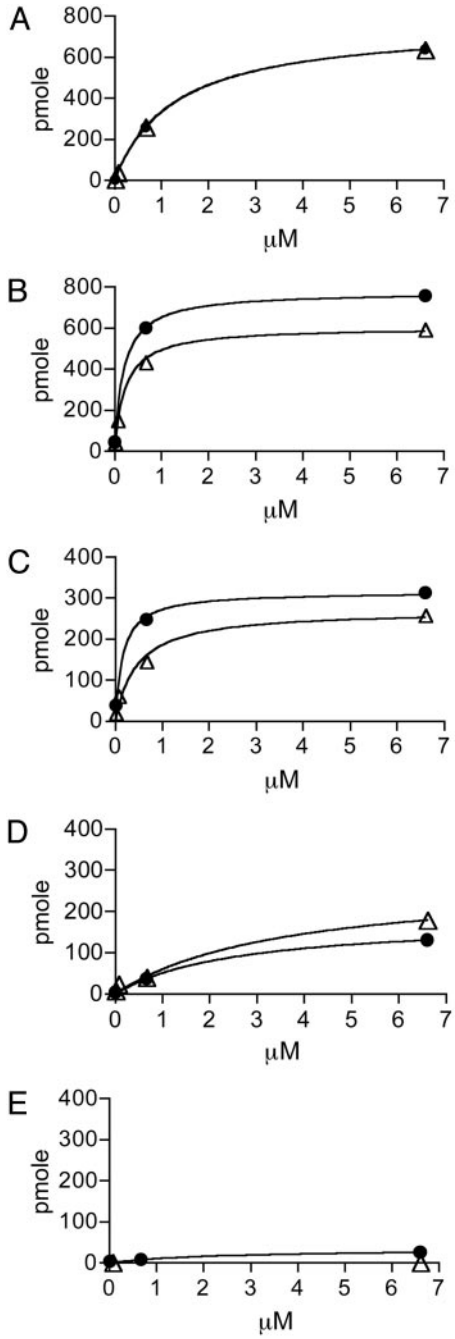

Fig. 2. Binding curves for BSA glycoconjugates. Increasing concentrations of R1-CRDV5His (●) and M1-CRDV5His (△) were incubated with 1 nmol of each neoglycoprotein: SiaGGnM-^{125}I-BSA (*A*), Gal-^{125}I-BSA (*B*), BiF10-^{125}I-BSA (*C*), BiF1226-^{125}I-BSA (*D*), or BiF1223-^{125}I-BSA (*E*). The amount of bound ^{125}I-BSA glycoconjugate was determined by precipitation with polyethylene glycol 8000 as described in *Experimental Procedures*.

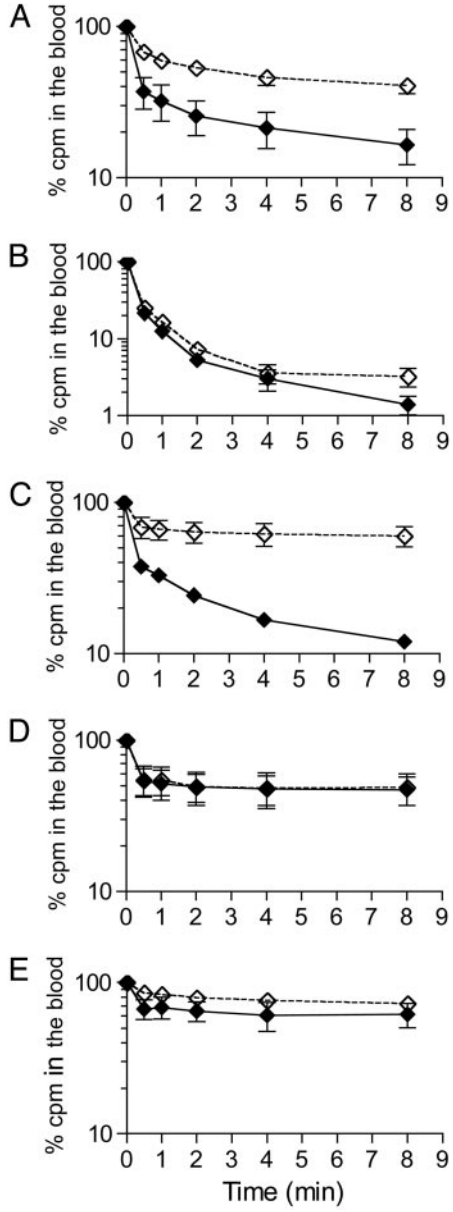

Fig. 3. Comparison of clearance rates in wild-type and ASGP-R-deficient mice. Wild-type (◆) and HL-2-knockout (◇) mice were injected with ^{125}I-labeled BSA glycoconjugates, and the amount of label remaining per 20 μl of blood was determined at the times indicated for SiaGGnM-BSA (*A*), GGnM-BSA (*B*), BiF10-BSA (*C*), BiF1226-BSA (*D*), and BiF1223-BSA (*E*).

or GalNAcβ1,4[Fucα1,3]GlcNAcβ-, respectively (24). Thus, the synthesis of oligosaccharides terminating with Siaα2,6GalNAcβ1,4GlcNAcβ- that will be recognized by the ASGP-R is a highly regulated and protein-specific event *in vivo*.

SiaGGnM-BSA is cleared from the circulation of both the mouse and the rat, although relatively little binding activity for SiaGGnM-BSA is detected in liver membrane extracts from the mouse. This reflects differences in the specificity of the mouse and rat HL-1 subunit for terminal Siaα2,6GalNAc that is reflected in a 35-fold greater ratio of SiaGGnM-BSA to GGnM-BSA binding activity in rat liver membrane extracts than in mouse liver membrane extracts (13). Nonetheless, the amount of Siaα2,6GalNAc-specific binding activity is adequate in the mouse to mediate the rapid clearance of SiaGGnM-BSA from the blood. Genetic ablation of the HL-2 subunit results in loss of ASGP-R function and reduces the rate of clearance for SiaGGnM-BSA and BiF10-BSA, which contains terminal β1,4-linked Gal, as compared with wild-type mice, indicating that the ASGP-R represents the major, if the not only, mechanism for rapid clearance for SiaGGnM-BSA and BiF10-BSA. In contrast with SiaGGnM-BSA, the rapid clearance of GGnM-BSA is not altered by ablation of HL-2 of the ASGP-R. The rapid clearance of GGnM-BSA suggests that another receptor that recognizes terminal GalNAc but not Siaα2,6GalNAc or terminal β1,4-

linked Gal mediates the clearance of GGnM-BSA in the absence of the functional ASGP-R.

The impact of genetic ablation of HL-2 of the ASGP-R on clearance of SiaGGnM-BSA suggests that endogenous glycoproteins bearing oligosaccharides terminating with Siaα2,6Gal are removed from the circulation by the ASGP-R. In support of this possibility, we found that monomeric, recombinant forms of the CRD of HL-1 bind BiF10-BSA with an affinity that is 3- to 5-fold greater than SiaGGnM-BSA and 8- to 16-fold greater than BiF1226-BSA. BiF1226-BSA has a reduced serum half-life relative to BiF1223-BSA as determined by examining blood levels 1 h and 6 h after injection into wild-type mice (15). Complex-type biantennary N-glycans with a bisecting GlcNAc bearing α2,6-linked Sia also have a shorter serum half-life than the identical biantennary N-glycans bearing α2,3-linked Sia. However, the difference in serum half-life is only slight for these sialylated forms in the absence of either of the core Fuc or bisecting GlcNAc (15, 26, 27), suggesting these core substituents promote recognition of the Siaα2,6Gal termini. We did not observe a difference in the clearance rate for BiF1226-BSA in wild-type as compared with ASGP-R-deficient mice (Fig. 3*E*). The lack of a difference in clearance rate may reflect the short duration of the present studies ($<$10 min) and the slow rate of clearance.

There is no doubt that the ASGP-R rapidly removes glycoproteins bearing either β1,4-linked Gal or GalNAc from the circulation; however, glycoproteins with such termini have not been identified in either normal individuals or mice that are deficient in ASGP-R activity because of ablation of either HL-1 (4) or HL-2 (3). The lack of asialoglycoprotein accumulation in the blood of receptor-deficient mice is remarkable in light of the abundance of the ASGP-R on hepatocytes, with reports of 150,000–500,000 binding sites at the cell surface of isolated hepatocytes (28–30); the large number of hepatocytes present in the liver, $\approx 2 \times 10^8$ per mouse (31, 32); and the rapid rate of ASGP-R internalization, 0.1 pmol/min per 10^6 cells (28, 33). On the basis of such estimates, the ASGP-R is capable of removing $\approx$1.2 nmol of ligand per h or the equivalent of 90 μg/h of a glycoprotein with a molecular weight of 75,000. This rate of clearance would represent 1–3% of the total complement of glycoproteins in the serum every 24 h. If glycoproteins with terminal Gal or GalNAc are the physiologic targets for the ASGP-R, they should be present in the blood of ASGP-R-deficient mice. Their absence suggests that other mechanisms are able to remove these glycoproteins in the ASGP-R-deficient mice and/or that few glycoproteins with terminal Gal or GalNAc are actually present in the circulation because of incomplete synthesis or removal of terminal Sia as had been presumed.

Asn-linked oligosaccharides terminating with Siaα2,6GalNAc are recent examples of oligosaccharide structures that are cleared from the circulation by the ASGP-R without necessitating additional modification such as removal of the α2,6-linked Sia. However, the number of glycoproteins known to terminate with Siaα2,6GalNAc is small. In contrast, the number of glycoproteins present in the blood bearing oligosaccharides that terminate with Siaα2,6Gal is large. It is possible that serum glycoproteins that have a sufficient number of Siaα2,6Gal termini are removed from the circulation at a slow but significant rate by the ASGP-R. The more rapid clearance of the neoglycoproteins with α2,6-sialylated complex-type biantennary N-glycans than those with α2,3-sialylated complex-type biantennary N-glycans is consistent with this hypothesis.

Our results suggest that ASGP-R-deficient mice should have alterations in the relative concentrations of Siaα2,6GalNAc- and Siaα2,6Gal-bearing glycoproteins in their blood rather than an increase in glycoproteins with terminal Gal or GalNAc. The next step will be to investigate this possibility by comparing the serum protein profiles of wild-type and HL-2-deficient mice.

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1. Van Den Hamer, C. J., Morell, A. G., Scheinberg, I. H., Hickman, J. & Ashwell, G. (1970) *J. Biol. Chem.* **245,** 4397–4402.
2. Ashwell, G. & Harford, J. (1982) *Annu. Rev. Biochem.* **51,** 531–554.
3. Braun, J. R., Willnow, T. E., Ishibashi, S., Ashwell, G. & Herz, J. (1996) *J. Biol. Chem.* **271,** 21160–21166.
4. Tozawa, R., Ishibashi, S., Osuga, J., Yamamoto, K., Yagyu, H., Ohashi, K., Tamura, Y., Yahagi, N., Iizuka, Y., Okazaki, H., *et al.* (2001) *J. Biol. Chem.* **276,** 12624–12628.
5. Park, E. I., Manzella, S. M. & Baenziger, J. U. (2003) *J. Biol. Chem.* **278,** 4597–4602.
6. Dell, A., Morris, H. R., Easton, R. L., Panico, M., Patankar, M., Oehniger, S., Koistinen, R., Koistinen, H., Seppala, M. & Clark, G. F. (1995) *J. Biol. Chem.* **270,** 24116–24126.
7. Manzella, S. M., Dharmesh, S. M., Cohick, C. B., Soares, M. J. & Baenziger, J. U. (1997) *J. Biol. Chem.* **272,** 4775–4782.
8. Holland, E. C., Leung, J. O. & Drickamer, K. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7338–7342.
9. Spiess, M. & Lodish, H. F. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6465–6469.
10. McPhaul, M. & Berg, P. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8863–8867.
11. McPhaul, M. & Berg, P. (1987) *Mol. Cell. Biol.* **7,** 1841–1847.
12. Shia, M. A. & Lodish, H. F. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1158–1162.
13. Park, E. I. & Baenziger, J. U. (2004) *J. Biol. Chem.* **279,** 40954–40959.
14. Vandana, Hindsgaul, O. & Baenziger, J. U. (1987) *Can. J. Chem.* **65,** 1645–1652.
15. Unverzagt, C., Andre, S., Seifert, J., Kojima, S., Fink, C., Srikrishna, G., Freeze, H., Kayser, K. & Gabius, H. J. (2002) *J. Med. Chem.* **45,** 478–491.
16. Ishibashi, S., Hammer, R. E. & Herz, J. (1994) *J. Biol. Chem.* **269,** 27803–27806.
17. Bergwerff, A. A., Van Oostrum, J., Kamerling, J. P. & Vliegenthart, J. F. G. (1995) *Eur. J. Biochem.* **228,** 1009–1019.
18. Chan, A. L., Morris, H. R., Panico, M., Etienne, A. T., Rogers, M. E., Gaffney, P., Creighton-Kempsford, L. & Dell, A. (1991) *Glycobiology* **1,** 173–185.
19. Yan, S. B., Chao, Y. B. & Van Halbeek, H. (1993) *Glycobiology* **3,** 597–608.
20. Stockell Hartree, A. & Renwick, A. G. C. (1992) *Biochem. J.* **287,** 665–679.
21. Coddeville, B., Strecker, G., Wieruszeski, J. M., Vliegenthart, J. F. G., Van Halbeek, H., Peter-Katalinic, J., Egge, H. & Spik, G. (1992) *Carbohydr. Res.* **236,** 145–164.
22. Nakata, N., Furukawa, K., Greenwalt, D. E., Sato, T. & Kobata, A. (1993) *Biochemistry* **32,** 4369–4383.
23. Sato, T., Takio, K., Kobata, A., Greenwalt, D. E. & Furukawa, K. (1995) *J. Biochem. (Tokyo)* **117,** 147–157.
24. Manzella, S. M., Hooper, L. V. & Baenziger, J. U. (1996) *J. Biol. Chem.* **271,** 12117–12120.
25. Dharmesh, S. M., Skelton, T. P. & Baenziger, J. U. (1993) *J. Biol. Chem.* **268,** 17096–17102.
26. Andre, S., Unverzagt, C., Kojima, S., Frank, M., Seifert, J., Fink, C., Kayser, K., Von Der Lieth, C. W. & Gabius, H. J. (2004) *Eur. J. Biochem.* **271,** 118–134.
27. Andre, S., Unverzagt, C., Kojima, S., Dong, X., Fink, C., Kayser, K. & Gabius, H. J. (1997) *Bioconjugate Chem.* **8,** 845–855.
28. Schwartz, A. L., Rup, D. & Lodish, H. F. (1980) *J. Biol. Chem.* **255,** 9033–9036.
29. Weigel, P. H. (1980) *J. Biol. Chem.* **255,** 6111–6120.
30. Baenziger, J. U. & Fiete, D. (1980) *Cell* **22,** 611–620.
31. Kedderis, G. L. & Held, S. D. (1996) *Toxicol. Appl. Pharmacol.* **140,** 124–130.
32. Kawada, N. (1997) *Histol. Histopathol.* **12,** 1069–1080.
33. Tolleshaug, H., Berg, T., Nilsson, M. & Norum, K. R. (1977) *Biochim. Biophys. Acta* **499,** 73–84.