The Major Subunit of the Asialoglycoprotein Receptor Is Expressed on the Hepatocellular Surface in Mice Lacking the Minor Receptor Subunit*

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Jürgen R. Braun†, Thomas E. Willnow§, Shun Ishibashi¶, Gilbert Ashwell**, and Joachim Herz‡‡

From the Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas Texas 75235, the Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan, and the **NIDDK, National Institutes of Health, Bethesda, Maryland 20892

The mammalian asialoglycoprotein receptor (ASGPR) is located on the sinusoidal membrane of hepatocytes where it binds and endocytoses galactose-terminated glycoproteins (asialoglycoproteins). ASGPR is composed of two highly homologous subunits, termed hepatic lectin 1 and 2. Despite numerous studies the contribution of both subunits to biosynthesis and functional activity of ASGPR in vivo has remained controversial. Mice lacking the murine hepatic lectin (MHL)-2 subunit are viable and fertile without obvious phenotypic abnormalities. In the absence of MHL-2, knockout mice express MHL-1 protein at reduced levels. Here, we examine the intracellular fate and function of this remaining subunit. The results show that MHL-1 reaches the hepatocellular surface in knockout mice but is unable to effectively remove any one of three different radiolabeled ligands within 30 min. A small but detectable residual ligand clearance in knockout mice at 4 h is apparently not mediated by remaining MHL-1. Serum concentrations of galactose-terminating glycoproteins are not elevated in these ASGPR-deficient mice. However, competitive in vitro degradation experiments suggest that other endogenous ASGPR ligands, the nature of which remain to be determined, accumulate in serum of knockout animals.

The asialoglycoprotein receptor (ASGPR)† is a heterooligomeric receptor that is abundantly expressed on the sinusoidal (i.e. basolateral) surface of the hepatic plasma membrane. ASGPR is an endocytic receptor that rapidly binds and internalizes galactose-terminated glycoproteins (asialoglycoproteins (ASGP)) from the circulation (1, 2). The ASGPR in the mouse is composed of two highly homologous subunits, murine hepatic lectin (MHL) 1 and 2, each consisting of a cytosolic NH₂-terminal domain, a single transmembrane segment (3), a stalk domain, and a Ca²⁺-dependent carbohydrate binding domain at the very COOH terminus (4).

Under normal circumstances, the penultimate galactose residues of glycoproteins are masked by terminal sialic acid moieties. Upon enzymatic removal of sialic acid, the now terminal galactose residues constitute the recognition determinants for ASGPR (5, 6). Binding of ligands to ASGPR depends on (i) the amount and positioning of terminal galactose residues on the ligand (7–9); (ii) the presence of Ca²⁺ in an optimal concentration of 0.1–2 mM (10); and (iii) a pH above 6.5 (11).

The fact that two largely homologous receptor subunits have been conserved in mammalian hepatocytes has led to several investigations into the roles of the respective polypeptides in intracellular transport and functional activity on the cell surface. Using cross-linking experiments on purified rat receptor and hepatocyte membrane, Halberg et al. concluded that the major and minor receptor species form independent homooligomers in the membrane (12). More recently, Bider et al. have shown that H1-overexpressing cells lacking H2 can bind iodinated asialoorosomucoid (13). Substantial binding, however, depended on the expression levels of H1 protein. High levels were required to observe ligand binding, whereas no specific binding was detected at moderate expression levels. Several other groups found that the individual ASGPR subunits have to interact with one another to form a single multicomponent receptor (14–20).

In the present experiments we used MHL-2-deficient mice generated previously in this laboratory (21). Disruption of the MHL-2 gene results in a complete absence of MHL-2 protein and a substantial reduction in the expression of the major MHL-1 subunit. The knockout mice are viable and fertile, have a normal lifespan under laboratory conditions, and display no obvious phenotypic abnormalities. The current experiments were designed to explore the fate of the remaining major MHL-1 subunit of ASGPR in vivo. In particular, we wanted to determine whether the still detectable fraction of MHL-1 is targeted through the secretory pathway in MHL-2−/− mice. Our results suggest that the major MHL-1 polypeptide is expressed on the surface of hepatocytes in knockout animals but is apparently not involved in mediating a detectable low basal rate of residual ASGPR clearance. Although ASGPR deficiency does not result in an increase in the absolute serum concentration of endogenous galactose-terminated glycoproteins, in vitro competition experiments suggest that (an)other ligand(s) accumulate(s) in their circulation. The nature of these putative alternative ASGPR ligands is currently unknown.
EXPERIMENTAL PROCEDURES

CS7BL/6 × 129Sv hybrid mice (age, 6 weeks to 6 months) used in the experiments were bred in house and fed ad libitum during the course of the studies (Teklad 6% Mouse/Rat Diet 7001, Harlan Teklad Premier Laboratories Diets). Animal care and experimental procedures involving mice were conducted in accordance with institutional guidelines. Biochemicals were obtained from Sigma unless indicated otherwise. Human α2-macroglobulin activated with methanolamine was a gift from Dudley K. Strickland (American Red Cross, Rockville, MD). All 125I-labeled ligands were radioabeled using the IODO-GEN Iodination Reagent (Pierce) according to the manufacturer’s recommendations.

Preparation and Immunoblot Analysis of Mouse Liver Membrane Proteins—Membrane proteins were prepared using the Coomassie Plus Protein Assay Reagent (Pierce), 50 μg of protein/lane were separated by 9% SDS-PAGE under reducing conditions. Proteins were transferred to nitrocellulose paper at 4 °C and incubated with polyclonal rabbit antipeptide antibodies (5 μg/ml). Bound IgG was detected using the enhanced chemiluminescence (ECL) system (Amersham Corp.).

Subfractional Sedimentation of Mouse Liver Membranes—Mouse liver membranes were fractionated using a modified version of a previously published method (23). Briefly, livers of mice of the indicated genotypes were perfused via the portal vein with 20 ml of ice-cold 0.15 M NaCl, dissected out, and homogenized in 5 volumes of a 0.25 M sucrose buffer containing 3 mM imidazole, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 20 μg/ml aprotinin. The homogenate was centrifuged at 12,000 × g for 10 min at 4 °C, and the supernatant was collected and subsequently centrifuged at 150,000 × g for 90 min at 4 °C. Membranes were resuspended in 1.9 ml of the 0.25 M sucrose buffer/g of wet liver weight. 3–5 ml of the resuspended microsomal membranes were layered on top of 32-ml linear sucrose gradients (density 1.10–1.25 g/ml) buffered with 3 mM imidazole, pH 7.4, and centrifuged for 18 h at 83,000 × g in a swinging bucket rotor. Thirty fractions (1.2 ml each) were collected from the gradient and subjected to 9% SDS-PAGE under nonreducing conditions. Immunoblot analysis of separated proteins was done as described under "Preparation and Immunoblot Analysis of Mouse Liver Membrane Proteins." Measurement of Circulating Galactose-terminated Glycoproteins—Galactose-terminated glycoproteins—Lyophilized pellets were obtained from ~100 μl of mouse serum, dissolved in 400 μl of distilled water, and stored frozen. 100-μl aliquots were diluted to 500 μl and centrifuged for 20 min at 5,000 × g on a Centricon filter with a 10,000 mol wt cut-off (Amicon Co., Beverly, MA). After an additional wash with 500 μl of distilled water, the protein pellet was recovered by centrifugation for 2 min at 1,000 × g and diluted to the final volume of 300 μl. The protein content of this material was unchanged from that of the original solution as determined by the BCA protein assay (Pierce). Enzymatic hydrolysis of terminal galactose residues was carried out by adding 5 μl of 5 M ammonium acetate, pH 5.0, and 5 μl of Streptococcus pneumoniae β-galactosidase (1 unit/ml, Boehringer Mannheim) to 300 μl of the filtered serum. Incubation was carried out at 37 °C for 23 h. Released galactose was determined in duplicates (1.2 ml each) using a Dionex high performance liquid chromatography (Dionex Corp., Sunnyvale, CA) equipped with a pulsed amperometric detector and pellicular anion exchange column (Carboxap PA-1,4 × 250 mm). Elution was carried out isocratically with 20 mM NaOH at a flow rate of 0.8 ml/min, and detector sensitivity was set at 300 nA. Data were analyzed using the Dionex Glycostream Software.

Generation of Desialylated Glycoproteins—Asialoorosomucoid was generated by incubating 100 mg of orosomucoid in 10 ml of 0.1 M sodium acetate buffer containing 2 ml CaCl2, pH 5, with 1 unit of insoluble neuraminidase type X-A attached to beaded agarose for 4 h at 37 °C. After adding another unit of the enzyme, the incubation was continued overnight. The neuraminocyt (Galacto-BSA) was obtained by incubation of neuraminic acid-resistant protein (Galacto) with bovine serum albumin (BSA) according to a method derived from (24). Briefly, 1 ml of a BSA solution (2 mg/ml in 1 M sodium carbonate, pH 9.0) was incubated with 50 μl of Galacto-dimethyl sulfoxide (1 mg/ml in dimethyl sulfoxide) in the dark for 8 h at 4 °C. NH4Cl was added to 50 mM final, and the solution was incubated for 2 h. Galacto-BSA conjugate was separated from unbound Galacto on a Sephadex G-25 column (PD-10, Pharmacia Biotech) and stored in a light-tight container in a 10 mM glycine-NaOH buffer (pH 8.0) at 4 °C. Membrane proteins were prepared from mouse livers as described previously (22). Protein content of membrane preparations was determined using the Coomassie Plus Protein Assay Reagent (Pierce). 50 μg of protein/lane were incubated with polyclonal antibodies (5 μg/ml). Bound IgG was detected using the enhanced chemiluminescence (ECL) system (Amersham Corp.).

RESULTS

We have previously reported the generation of mice lacking the minor subunit (MHL-2) of the ASGPR by homologous recombination in embryonic stem cells (21). Knockout animals do not express MHL-2 as judged by Western blot analysis of liver membrane proteins using an MHL-2-specific anti-peptide antibody (Fig. 1, lanes 2 and 3) and expression of the major MHL-1 subunit is substantially reduced (Fig. 1, lanes 2 and 3). The low density lipoprotein receptor-related protein (LRP), an endocytic receptor that is not functionally or physically related to ASGPR, is not affected by the knockout of the MHL-2 subunit (Fig. 1, lanes 1–4). This result raised the question of...
whether the apparent lack of phenotypical abnormalities in the MHL-2<sup>−/−</sup> animals might be due to the fact that a fraction of the residual MHL-1 protein manages to escape intracellular degradation, reach the surface of liver cells, and mediate uptake of ASGP by forming quasi-functional galactose-binding receptors. To explore this issue we first characterized the intracellular processing of the major ASGPR subunit using subcellular fractionation by sucrose density ultracentrifugation to separate intracellular organelles of wild type (MHL-2<sup>+/+</sup>) and MHL-2-deficient (MHL-2<sup>−/−</sup>) mouse livers (Fig. 2). The distribution of the indicated proteins was determined by immunoblotting. The processed form of LRP (LRP 85), a protein whose intracellular pathway has been extensively studied and that undergoes proteolytic processing in a post Golgi compartment (28, 29), serves as a marker for the endosomal compartment and plasma membrane. Although expression levels are significantly lower in MHL-2<sup>−/−</sup> mice compared with control animals (Fig. 2, MHL-2<sup>+/+</sup>), no major differences are observed in the distribution profiles of MHL-1 in MHL-2<sup>−/−</sup> mice compared with wild type (Fig. 2, MHL-2<sup>+/+</sup>). Furthermore, MHL-1 can be localized in the same fractions as LRP 85, strongly suggesting that the major subunit of ASGPR is being delivered to the hepatocyte plasma membrane. In other subcellular fractionation experiments not shown here, MHL-1 subunits also colocalize with the low density lipoprotein receptor.

Interestingly, MHL-1 shows a shift in mobility in SDS-PAGE between fractions 18 and 20 in the knockout animals (Fig. 2, MHL-2<sup>−/−</sup>), indicating that the protein migrates at an apparently increased molecular mass in the later fractions enriched in Golgi and endosomal membranes. To investigate whether...
carbohydrate modifications indicative of trans-Golgi processing are responsible for the observed differences in MHL-1 migration, we determined the sensitivity of MHL-1 protein to neuraminidase (Fig. 3). After 1 h of neuraminidase digestion, the protein band in both wild type (Fig. 3B) and MHL-2 knockout mice (Fig. 3A) displays an increased mobility in the gel (Fig. 3, compare lanes 2 and 4 with lanes 1 and 3). Thus, MHL-1 in the later fractions was carrying N-linked carbohydrates in the neuraminidase-sensitive form, further arguing strongly in favor of the major subunit having traversed the trans-Golgi en route to the cell surface in MHL-2/−/− animals.

Upon observation that MHL-1 protein is expressed at the hepatocellular surface of knockout animals, we next tested whether these remaining ASGPR subunits harbor any residual functional activity by exploring the ability of −/− animals to clear desialylated ligands from their circulation. As illustrated in Fig. 4A, asialoorosomucoid but not orosomucoid, the corresponding glycoprotein that does not exhibit terminal galactose residues and therefore is not a ligand for ASGPR, is removed from the circulation of MHL-2+/+ mice within 30 min. In contrast, the clearance rate of 125I-asialoorosomucoid in knockout animals is indistinguishable from that of iodinated orosomucoid (Fig. 4B). We used two other ligands, a synthetically derived neoglycoprotein prepared by chemically coupling BSA to a monosaccharide (galactose) derivative (Fig. 4, C and D) and asialofetuin (Fig. 4E and F). Comparison of the clearance kinetics of 125I-Galacto-BSA and unmodified 125I-BSA in knockout animals reveals a small but reproducible difference. 125I-Galacto-BSA is consistently removed at a slightly faster rate than 125I-BSA from plasma of knockout animals (Fig. 4D). To further explore the possibility of a low residual clearance activity of the remaining MHL-1 receptor subunits in MHL-2+/+ mice that was not readily detectable within the 30-min interval, we extended the turnover period to 4 h. The results of these experiments show a 75% clearance rate for 125I-asialoorosomucoid in knockout animals (Fig. 5, right panel, open triangles) compared with a 54% nonspecific disappearance of 125I-orosomucoid from the plasma of animals of either genotype after 4 h (Fig. 5, left and right panels, closed triangles).

In order to determine whether the low level ligand clearance in MHL-2−/− animals is mediated by residual MHL-1 protein, we performed degradation experiments using isolated primary mouse hepatocytes. As shown in Fig. 6A (open symbols), 125I-labeled asialoorosomucoid is efficiently degraded by isolated hepatocytes of MHL-2+/+ mice over 23 h. 125I-Orosomucoid, the corresponding sialylated ligand, is not degraded by hepatocytes of either genotype (Fig. 6, A and B, closed triangles). The results of 125I-asialoorosomucoid degradation by isolated MHL-2+/+ hepatocytes parallel those obtained by turnover studies in the MHL-2−/− animals in that again a low level rate of ligand uptake can be observed (compare Fig. 6B with Fig. 5, right panel). In control experiments we show that disruption of the MHL-2 gene locus does not result in a general dysfunction of the endocytic apparatus of the mutant cells. As demonstrated in Fig. 6D, methylamine-activated human α2-macroglobulin, one of the well-characterized ligands of LRP (30, 31), is readily degraded by hepatocytes isolated from MHL-2−/− animals.

To control for nonspecific binding of radiolabel to the membrane surface, the following experiment was performed (Fig. 7). Nonspecific degradation was determined by measuring the degradation of 125I-asialoorosomucoid over 23 h on addition of an 100-fold excess of unlabeled asialoorosomucoid and subtracting it from the total degradation of iodinated ligand. As illustrated in Fig. 7 (open triangles), no specific degradation by isolated hepatocytes from MHL-2−/− mice is detectable over 23 h. As expected, MHL-2+/+ hepatocytes specifically degrade iodinated asialoorosomucoid (Fig. 7, closed triangles). Taken together, our data clearly demonstrate that the major ASGPR subunit, although expressed on the hepatocellular surface of MHL-2−/− mice, is not able to specifically mediate degradation of ASGP in primary cultures of isolated knockout hepatocytes.

We have previously shown that there was no accumulation of any specific galactose-terminal glycoprotein(s) in plasma of
knockout animals using two-dimensional gel electrophoresis (21). In the current experiments we have further quantitated whether the absence of normal ASGPR function in MHL-2 knockout animals might result in an increase in steady state levels of desialylated plasma glycoproteins terminating in galactose. This was done by measuring the absolute concentration of galactose-terminal proteins in the serum of knockout mice (Table I). Our data do not reveal any significant differences in the absolute levels of circulating glycoproteins terminating in galactose in MHL-2\textsuperscript{-/-} compared with wild type animals. Therefore, we wanted to determine whether ligands other than galactose-terminating glycoproteins accumulate in serum of knockout mice. If ligand(s) do accumulate in serum of knockout mice, then it is likely that degradation of a known ligand for ASGPR by wild type hepatocytes will be competitively inhibited in the presence of knockout serum. To test this, we isolated wild type hepatocytes and incubated them with degradation medium containing iodinated asialoorosomucoid (0.5–2.0 \text{mg/ml}) as well as increasing concentrations of MHL-2\textsuperscript{1/1} and MHL-2\textsuperscript{2/2} serum, respectively. The presence of MHL-2\textsuperscript{2/2} serum (Fig. 8, open squares) consistently inhibits degradation of 125\textsuperscript{I}-asialoorosomucoid to a higher extent than does MHL-2\textsuperscript{1/1} serum (Fig. 8, closed squares) over a wide range of concentrations in eight independent experiments. Statistical analysis of the data shows that this difference reaches significance at 1:10 dilution of degradation medium. Nonspecific degradation of 125\textsuperscript{I}-asialoorosomucoid has been determined in the presence of a 200-fold excess of unlabeled ligand. Thus, our results suggest the presence of as yet unidentified endogenous ASGPR ligand(s), which accumulate(s) in MHL-2\textsuperscript{-/-} serum and competitively inhibit(s) 125\textsuperscript{I}-asialoorosomucoid uptake and degradation by primary cultures of wild type mouse hepatocytes.

**DISCUSSION**

The current experiments were designed to examine the fate of the MHL-1 subunit of the heterooligomeric ASGPR in MHL-2 knockout mice and further characterize their phenotype. Our results indicate that MHL-1 receptor subunits can be transported to the hepatocellular surface in the absence of MHL-2. This conclusion is supported by two lines of evidence: (i) When hepatocytes from knockout animals were fractionated into early and late secretory pathway (Fig. 2), MHL-1 protein was detectable in the same light-density fractions as LRP85, a proteolytic maturation product of the 600-kDa precursor LRP known to be produced in the trans-Golgi or trans-Golgi network (28). (ii) The MHL-1 polypeptide exhibited maturation of carbohydrate side chains characteristic of Golgi-processing, i.e. it acquired sialic acid residues and thus became sensitive to treatment with neuraminidase (Fig. 3). In the past, several investigators have addressed the role of the individual receptor subunits in intracellular transport of ASGPR in vitro using different transfection assays (for review, see Ref. 2). Our results are in agreement with the findings of Shia and Lodish.
Biosynthesis and Function of MHL-1 in MHL-2−/− Mice

FIG. 7. Specific degradation of 125I-labeled asialoorosomucoid by primary cultures of isolated mouse hepatocytes. Wells with isolated hepatocytes from mice either wild type (closed symbols) or homozygous for the MHL-2 gene disruption (open symbols) received 500 μl of DMEM (without glutamine) containing 0.2% (w/v) BSA and the indicated concentrations of 125I-labeled asialoorosomucoid. After incubation for 23 h at 37 °C, the total amount of radiolabeled degradation products secreted into the medium during steady state conditions was measured. Non-specific degradation was determined in the presence of a 100-fold excess of unlabeled asialoorosomucoid and subtracted from total degradation to yield the specific degradation. Each symbol represents the mean of duplicate determinations of specific degradation from one animal.

TABLE I

Serum concentrations of galactose-terminating glycoproteins in normal (MHL+/+) and MHL-2 deficient (MHL-2−/−) mice

| Genotype | n | Galactose (nmol/mg protein) |
|----------|---|----------------------------|
| MHL-2−/− | 19 | 1.076 ± 0.318 |
| MHL-2+/+ | 6  | 0.808 ± 0.421 |

(17), who demonstrated that in stably transfected NIH 3T3 fibroblasts the major subunit of ASGPR is able to traverse through the Golgi complex to the cell surface in the absence of minor receptor subunit. Because proper folding and oligomerization of various membrane proteins are major prerequisites for their transport from the endoplasmic reticulum to the cis-Golgi complex (32), our data further suggest that a fraction of MHL-1 in the knockout animals might have escaped potential intracellular degradation, possibly by forming homooligomers. It has been shown previously that oligomerization of the major ASGPR subunit can occur as judged by chemical cross-linking experiments (12, 17). However, the finding that a small amount of MHL-1 reaches the hepatocellular surface in MHL-2-deficient mice does not rule out the possibility that a subfraction of the major ASGPR subunit is degraded in an early compartment of the secretory pathway. This consideration is supported by the fact that MHL-1 expression is profoundly reduced in the knockout animals, although MHL-1 mRNA levels are essentially unaffected by the gene disruption (21). Thus, MHL-2 seems to be required for post-translational stability of MHL-1.

The observation that MHL-1 protein is expressed at the surface of liver cells in MHL-2−/− animals raised the possibility that residual functional activity of the remaining receptor subunits might account for the lack of obvious phenotypic abnormalities in the knockout mice. Earlier work has shown that individual ASGPR subunits are, in fact, expressed alone (33, 34) and can function as independent galactose binding proteins (12, 13, 35). Our evaluation of the functional status of the MHL-1 subunits in MHL-2−/− animals showed a low level clearance of iodinated ASGP (Fig. 4D and Fig. 5, right panel), which could be explained by either one or a combination of the following mechanisms: (i) residual functional activity of MHL-1 subunits in knockout mice, albeit at a markedly reduced level; (ii) existence of other mammalian galactose-specific recognition systems such as the lectin-like receptor on Kupffer cells (36) and the homologous galactose/GalNAc-specific lectin expressed on macrophages (37), which are not affected by the disruption of the MHL-2 gene locus; and (iii) nonspecific binding phenomena. In order to distinguish between these possibilities, we performed degradation experiments using isolated mouse hepatocytes from animals of either genotype. Our results clearly demonstrate that MHL-2−/− hepatocytes do not specifically degrade iodinated asialoorosomucoid (Figs. 6 and 7). Thus, normal ASGPR function is totally ablated in homozygous MHL-2-deficient mice and the observed residual clearance most likely due to nonspecific ligand binding and/or uptake by non-ASGPR members of the C-type lectin superfamily. Our data do not exclude the possibility that MHL-1 subunits per se might be able to bind ASGP in vivo. Bider et al. have shown that H1 subunits bind 125I-labeled asialoorosomucoid in vitro with high affinity in the absence of H2 (13). However, the H1-overexpressing cells were only able to bind iodinated asialoorosomucoid at very high levels of H1 expression. Thus, it is likely that the density of residual MHL-1 receptor subunits on the hepatocellular surface of MHL-2 knockout mice might be too low to allow detectable ligand binding.

The total absence of normal ASGPR function does not entail a measurable increase in the steady state concentrations of galactose-terminating glycoproteins in plasma of knockout...
mice (Table I). This finding further supports the previously stated hypothesis that ASGPR is not involved in normal turnover of serum glycoproteins (Refs. 38–40; for review, see Ref. 41). During acute surges in the plasma concentration of desialylated glycoproteins, however, the alternative galactose-specific recognition systems are not able to compensate for the loss of ASGPR function in knockout mice. Such excess levels of ASGP might occur during certain infectious diseases known to be associated with increased sialidase activity (42) or experimentally (Figs. 4 and 5). Taken together, our data are in agreement with a model in which ASGPR might function to prevent acute increases in the concentration of desialylated soluble or particulate galactose-terminating glycoproteins that might be harmful in mammals (43).

The results of our competitive degradation experiments (Fig. 8) are of particular interest considering that no endogenous ligands for ASGPR have been identified despite its discovery almost 30 years ago (44). The generation of MHL-2 knockout mice allowed us to test for the presence of an accumulation of potential ASGPR ligand(s) in their serum regardless of knowledge about ligand structure. When primary cultures of MHL-2 / Mice were incubated with medium containing different concentrations of knockout serum, we consistently observed a greater inhibition of specific 125I-asialoorosomucoid degradation compared with wild type serum (Fig. 8). These findings are consistent with a model in which cross-competing endogenous ASGPR ligands accumulate in the serum of MHL-2 / Mice. Thus, ASGPR-deficient animals will provide an important tool to further analyze the nature of the endogenous ligand(s) of ASGPR, thereby advancing our understanding of the physiological function of this major endocytic receptor system.

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