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Asialoglycoprotein Receptor Deficiency in Mice Lacking the Major Receptor Subunit

ITS OBLIGATE REQUIREMENT FOR THE STABLE EXPRESSION OF OLIGOMERIC RECEPTOR*

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The asialoglycoprotein receptor is an abundant hetero-oligomeric endocytic receptor that is predominantly expressed on the sinusoidal surface of the hepatocytes. A number of physiological and pathophysiological functions have been ascribed to this hepatic lectin (HL), the removal of desialylated serum glycoproteins and apoptotic cells, clearance of lipoproteins, and the sites of entry for hepatotropic viruses. The assembly of two homologous subunits, HL-1 and HL-2, is required to form functional, high affinity receptors on the cell surface. However, the importance of the individual subunits for receptor transport to the cell surface is controversial. We have previously generated HL-2-deficient mice and showed that the expression of HL-1 was significantly reduced, and the functional activity as the asialoglycoprotein receptor was virtually eliminated. However, we failed to detect phenotypic abnormalities. To explore the significance of the major HL-1 subunit for receptor expression and function *in vivo*, we have disrupted the *HL-1* gene in mice. Homozygous HL-1-deficient animals are superficially normal. HL-2 expression in the liver is virtually abrogated, indicating that HL-1 is strictly required for the stable expression of HL-2. Although these mice are almost unable to clear asialo-orosomucoid, a high affinity ligand for asialoglycoprotein receptor, they do not accumulate desialylated glycoproteins or lipoproteins in the plasma.

The asialoglycoprotein receptor (ASGPR)[1] was originally identified by Ashwell and Morell as a hepatic receptor that mediates the rapid clearance of serum glycoproteins containing terminal galactose residues from the circulation (see Refs. 1–3 for review). ASGPR is abundantly expressed on the sinusoidal surface of the parenchymal cells of the liver. Its primary physiological function has been considered to be the removal and degradation of desialylated circulating proteins.

Nonreducing terminal of oligosaccharide moieties of glycoproteins are usually capped by sialic acid residues. When the terminal sialic acid residues are removed by neuraminidases, penultimate galactose residues are exposed and recognized by ASGPR. High affinity binding requires the receptor to be assembled as a hetero-oligomer consisting of two highly homologous subunits termed hepatic lectin (HL) 1 and 2 (4). Both subunits contain an N-terminal cytoplasmic domain, a single transmembrane segment, a stalk domain, and a C-terminal carbohydrate recognition domain (5). ASGPR belongs to C-type animal lectins because of the requirement of Ca^{2+} for ligand binding and disulfide bonds in carbohydrate recognition domains (6).

A number of diverse physiological roles have been proposed for ASGPR over the years. Among them, hepatic clearance of the desialylated and senascent serum proteins was most originally proposed (1). ASGPR was also postulated to account for the low density lipoprotein (LDL) receptor-independent clearance of lipoproteins including chylomicron remnants (7, 8). Recently, immunoglobulin A (9) and fibronectin (10) have emerged as likely candidates of natural ligands for ASGPR. The clearance of apoptotic cells or a subpopulation of lymphocytes in the liver has also attributed to ASGPR (See Ref. 3 for review). It is particularly noteworthy that ASGPR has also been proposed to be utilized as entry sites into hepatocytes by several hepatotropic viruses including hepatitis B virus (11), Marburg virus (12), and hepatitis A virus (13).

As an attempt to elucidate the *bona fide* functions of ASGPR, we have previously generated mice lacking a minor subunit of mouse ASGPR (MHL-2) (14). As a result of disruption of MHL-2, the expression of MHL-1 was severely reduced, and the plasma clearance of asialo-orosomucoid was almost completely abrogated in the *MHL-2−/−* mice. However, the *MHL-2−/−* mice were apparently normal and showed no detectable abnormalities even in the metabolism of remnant lipoproteins. Because *MHL-2−/−* liver expresses small but significant amounts of MHL-1 (14, 15), it is still possible that the residual MHL-1 is sufficient to execute the primary task of ASGPR as suggested by *in vitro* transfection experiments (16, 17). In the current study we have generated mice lacking the major sub-

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[1] The abbreviations used are: ASGPR, asialoglycoprotein receptor; HL, hepatic lectin; MHL, mouse hepatic lectin; ES, embryonic stem; ASOR, asialo-orosomucoid; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apolipoprotein; MAA, *Maackia amurensis* agglutinin; SNA, *Sambucus nigra* agglutinin; RCA, *Ricinus communis* agglutinin; kb, kilobase; kbp, kilobase pair.

unit (MHL-1) of ASGPR in mice and analyzed the resulting phenotypes.

EXPERIMENTAL PROCEDURES

Generation of MHL-1 Knockout Mice—The *MHL-1* gene was cloned from the 129/Sv mouse genomic library. The genomic organization of the *MHL-1* gene was essentially the same as recently reported by Soukharev *et al.* (18). A replacement-type targeting vector was constructed so that the genomic fragment containing exons 2–3, which encoded the ATG initiation codon and transmembrane domain, was replaced by the pol2neo cassette (19). The short arm containing a 0.8-kb *Stu*I/*Bam*HI fragment containing exon 2 and the long arm containing a 9-kb *Xho*I/*Sal*I fragment spanning exons 3–9 were inserted into the *Xho*I and *Not*I sites, respectively, of the vector pPol2short-neobpA-HSVTK as described previously (14, 20).

After linearization by digestion with *Sal*I, the vector was electroporated into JH-1 embryonic stem (ES) cells (a gift from Dr. Herz at the University of Texas Southwestern Medical Center). Targeted clones, which had been selected in the presence of G418 and 1-(2-deoxy,2-fluoro-β-D-arabinofuranosyl)-5 iodouracil, were identified by polymerase chain reaction using the following primers: 5′-CTGGTCAGGGATATTTGGAGATACGG-3′ and 5′-GATTGGGAAGACAATAGCAGGCATGC-3′ (see Fig. 1). Homologous recombination was verified by Southern blot analysis after digesting the genomic DNA with *Eco*RI using a 0.7-kbp *Stu*I fragment as a probe (see Fig. 1). Targeted ES clones were injected into the C57BL/6 blastocysts, yielding 14 lines of chimeric mice that transmitted the disrupted allele through the germline from four independent ES cell clones. All experiments reported here were performed with 129/Sv-C57BL6 hybrid descendants (F1 and subsequent generations) of these animals.

Northern Blot Analysis—Total RNA was isolated from the liver by TRIZOL™ reagent (Life Technologies, Inc.). 20 μg of total RNA was subjected to electrophoresis in an agarose gel and transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech). *MHL-1* and *MHL-2* cDNA fragments were labeled with [α-^{32}P]dCTP using a kit (Megaprime labeling kit; Amersham Pharmacia Biotech) and were used as probes for hybridization (14). Image capture and analysis were performed with BAS 2000 (Fuji Film).

Immunoblot Analysis—Liver membrane proteins were prepared as described (14). 50 μg of the proteins was separated by 5–20% SDS polyacrylamide gel electrophoresis under a nonreducing condition. Proteins were transferred to nitrocellulose membrane, and immunoblot analyses were performed using specific rabbit polyclonal anti-peptide antibodies for MHL-1 and MHL-2 (14). The antibodies were visualized by peroxidase-conjugated anti-rabbit IgG and with an ECL chemiluminescence detection kit (Amersham Pharmacia Biotech).

Plasma Turnover Experiments—Asialo-orosomucoid (ASOR) was prepared by incubating 100 mg of orosomucoid (Sigma) at 37 °C in 10 ml of sodium acetate buffer containing 2 mM $CaCl_2$, pH 5, together with 1 unit of neuraminidase-type XA (Clostridium perfringens) attached to agarose beads (Sigma). After 4 h another unit of enzyme was added, and the incubation was continued overnight. Asialofetuin (Sigma) and ASOR were labeled with ^{125}I using the IODO-GEN procedure (Pierce). Specific activities of ^{125}I-asialofetuin and ^{125}I-ASOR were 257 and 502 cpm/ng, respectively. 10 μg of iodinated protein in 200 μl of saline containing 2 mg/ml bovine serum albumin were injected intravenously into the jugular vein of anesthetized male mice (n = 3) that were wild-type and homozygous for *MHL-1* gene disruption. Blood was collected at the indicated intervals from the retroorbital venous plexus. After the labeled proteins in 20 μl of plasma were precipitated with trichloroacetic acid, their radioactivities were determined.

Analyses of Mouse Plasma Lipoprotein Profile—Plasma lipoprotein analyses were performed as previously described (21). Briefly, after mice were bled from the retroorbital venous plexus, the blood was collected into tubes containing EDTA. Total cholesterol and triglyceride levels in the plasma were determined enzymatically using kits (Determiner TC555 and Determiner TG555; Kyowa Medex). 5 μl of plasma was diluted to 100 μl with saline and subjected to high performance liquid chromatography (HPLC) analyses using four columns of TSK gel Lipopropak XL (TOSOH, Tokyo, Japan) connected in tandem.

Lectin Blot Analyses of Plasma Protein—1 μl of plasma was separated by 3–15% SDS polyacrylamide gel electrophoresis under a reducing condition and transferred to polyvinylidene difluoride membranes. Lectin blotting was done using *Maackia amurensis* agglutinin (MAA;

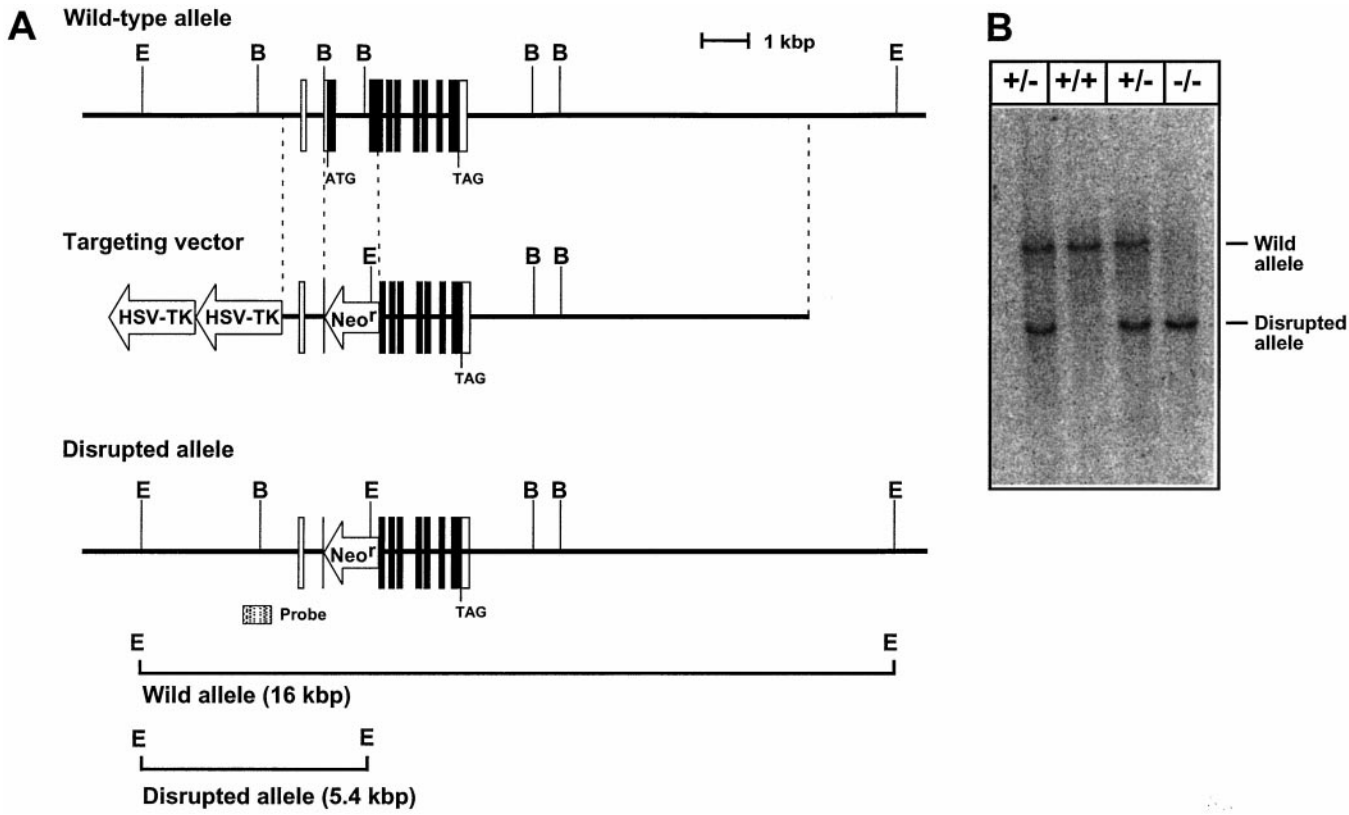

FIG. 1. **Targeted disruption of the MHL-1 gene.** *A*, a targeting vector of the replacement type was constructed by replacing the exons encoding the ATG initiation codon and transmembrane domain by the pol2neo cassette (*Neo*^{r}). Two copies of herpes simplex virus thymidine kinase (*HSV-TK*) were attached to the 5′ side of the vector. *E*, *Eco*RI; *B*, *Bam*HI. The probe for Southern blot analysis is indicated by the *shaded box*. *B*, genotypes of offspring from matings of MHL-1+/− mice. Tail DNA was digested with *Eco*RI. After the transfer, the membranes were hybridized with ^{32}P-labeled probe (a 0.7-kbp *Stu*I fragment). The positions of migration of disrupted (5.4 kb) and of wild-type alleles (16 kb) are indicated.

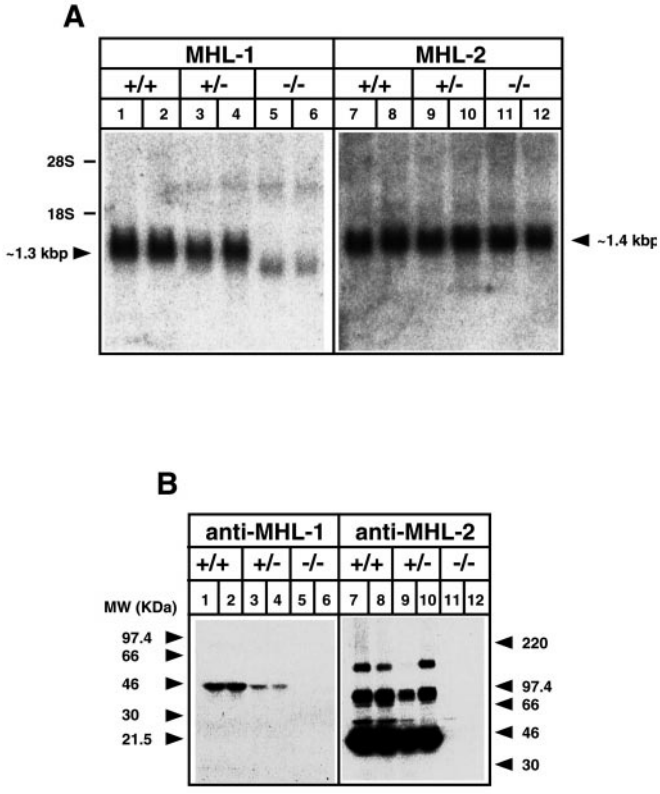

FIG. 2. **Northern and Western blot analysis of ASGPR subunits in mouse liver.** *A*, Northern blot analysis of MHL-1 (*lanes 1–6*) and MHL-2 (*lanes 7–12*) in the liver of 6-week-old mice wild-type (+/+; *lanes 1*, *2*, *7*, and *8*), heterozygous (+/−; *lanes 3*, *4*, *9*, and *10*), and homozygous (−/−; *lanes 5*, *6*, *11*, and *12*) for *MHL-1* gene disruption. 15 μg of total RNA was used for Northern blot analysis. 1.3-kb *MHL-1* and 1.4-kb *MHL-2* wild-type transcripts are indicated. *B*, Western blot analysis of MHL-1 (*lanes 1–6*) and MHL-2 (*lanes 7–12*) in the liver of 6-week-old mice wild-type (+/+; *lanes 1*, *2*, *7*, and *8*), heterozygous (+/−; *lanes 3*, *4*, *9*, and *10*), and homozygous (−/−; *lanes 5*, *6*, *11*, and *12*) for the *MHL-1* gene disruption. The size of the molecular mass markers are indicated.

Roche Molecular Biochemicals) and *Sambucus nigra* agglutinin (SNA; Roche Molecular Biochemicals), which were conjugated with digoxigenin, and *Ricinus communis* agglutinin (RCA120; Sigma), which was conjugated with biotin. Digoxigenin and biotin were detected by alkaline phosphatase-labeled anti-digoxigenin antibody (Digoxigenin detection kit; Roche Molecular Biochemicals) and alkaline phosphatase-labeled anti-biotin antibody (Roche Molecular Biochemicals), respectively.

RESULTS

The *MHL-1* gene was cloned by hybridization screening of a mouse genomic library using a mouse cDNA probe. A gene replacement vector was constructed so that the initiation codon and transmembrane domain were interrupted by the pol2neo cassette (Fig. 1*A*). Following electroporation of the linearized targeting vector into JH-1 ES cells, targeted clones were obtained. Chimeric mice were generated from six independently targeted clones using a standard procedure. Four independent ES cell cloned yielded total of 14 germ line chimeric males. They were bred to wild-type female C57BL/6 mice. Heterozygous offspring (F1 generation) were crossed with each other and gave rise to mice wild-type (+/+), heterozygous (±), or homozygous (−/−) for the disrupted *MHL-1* allele in accordance with Mendelian law (+/+:±:−/− = 29:65:43; $\chi 2 = 1.51$; $p = 0.47$) (Fig. 1*B*). Homozygous *MHL-1*-deficient mice were viable and displayed no obvious phenotype under laboratory housing conditions. The animals appeared to have a normal life span.

Northern blot analysis showed that *MHL-1*−/− mice lacked 1.3-kb wild-type *MHL-1* transcript completely (Fig. 2*A*). *MHL-1*+/− mice expressed an intermediate amount of the *MHL-1* transcript. A reduced amount of truncated *MHL-1* transcript was expressed in mice having the disrupted *MHL-1* allele (*MHL-1*+/− or *MHL-1*−/−). On the other hand, there were no differences in the size and amounts of *MHL-2* mRNA. The disruption of the *MHL-1* gene resulted in complete absence of the 40-kDa-encoded protein in *MHL-1*−/− animals (Fig. 2*B*). *MHL-1*+/− mice showed reduced expression of MHL-1 protein as compared with the wild-type mice, indicating the gene dosage effect on the expressed protein levels of MHL-1. In wild-type and *MHL-1*+/− mice, four distinct bands that were immunoreactive with the anti-MHL-2 antibody were observed (36, 47, 80, and 192 kDa). Because the bands with higher molecular mass were not observed under a reducing condition (data not shown), three of these multiple bands may represent oligomers of MHL-2 protein in different degrees (monomer for 36 kDa, dimer for 80 kDa, and tetramer for 192 kDa). No bands immunoreactive with the anti-MHL-2 antibody were visible in *MHL-1*−/− mice.

To examine whether the disruption of the *MHL-1* gene resulted in the impairment of the hepatic clearance of asialoglycoproteins, we injected the iodinated asialofetuin or ASOR into +/+, +/−, and −/− mice and compared the radioactivities remaining in the plasma (Fig. 3). The plasma clearance of ^{125}I-asialofetuin was severely, albeit not completely, impaired in *MHL-1*−/− mice (Fig. 3*A*). At 30 min after the injection, plasma ^{125}I-asialofetuin was decreased to 4, 8, and 40% of the initial dose in +/+, +/−, and −/− mice, respectively. Heterozygous mice cleared ^{125}I-asialofertuin with only partially reduced efficiency. The plasma clearance of ^{125}I-ASOR was more severely impaired in *MHL-1*−/− mice (Fig. 3*B*). At 30 min after the injection, plasma ^{125}I-ASOR was decreased to 3, 24, and 65% of the initial dose in +/+, +/−, and −/− mice, respectively. Heterozygous mice cleared ^{125}I-ASOR with intermediate efficiency.

To examine the expression of the sialic acid- and galactose-terminated serum glycoproteins, plasma from the wild-type and *MHL-1*−/− mice were subjected to lectin blot analysis (Fig. 4). The following lectins were used to detect terminal sugars: MAA, a lectin specific for NeuNAc(α2–3)Gal on *N*-linked carbohydrate; SNA, a lectin specific for NeuNAc(α2–6)Gal on *N*-linked carbohydrate; and RCA120, a lectin specific for β-galactose. There were no consistent differences either in the pattern of the bands or in their intensities between *MHL-1*+/+ and *MHL-1*−/− mice.

To test whether ASGPR is involved in chylomicron remnant clearance by the liver, we analyzed the plasma lipid levels of *MHL-1*−/− mice and *MHL-1*−/− mice that also lacked the LDL receptor (Table I). The plasma lipid levels of the *MHL-1*−/− mice were indistinguishable from those of control animals expressing wild-type MHL-1, in the absence or presence of functional LDL receptors. The lipoprotein profiles evaluated by HPLC analyses failed to reveal any differences in lipoprotein fractions between wild-type and *MHL-1* −/− mice (Table II).

DISCUSSION

We have generated mice lacking functional asialoglycoprotein receptors by disrupting the gene for the major MHL-1 receptor subunit by homologous recombination in embryonic stem cells. Homozygous *MHL-1*-deficient mice displayed no obvious phenotype as long as they were maintained under the standard laboratory housing condition.

Although *MHL-1*−/− liver expressed a reduced amount of a truncated transcript, no band immunoreactive with the anti-MHL-1 antibody was detected, indicating that *MHL-1*−/−

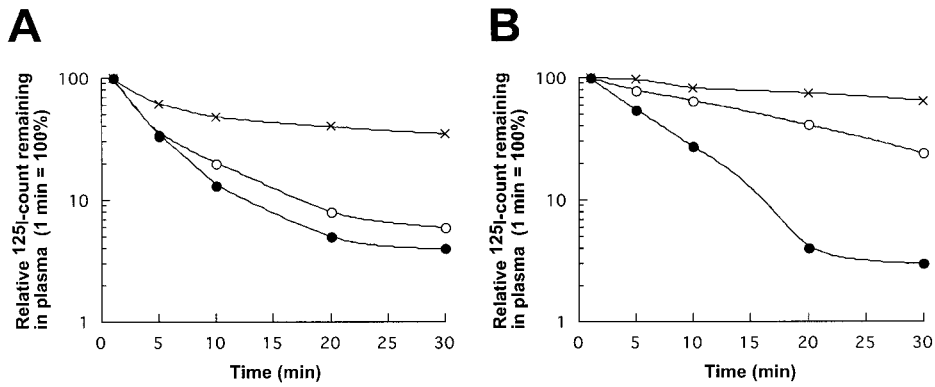

FIG. 3. **Disappearance of ^{125}I-labeled glycoproteins from the plasma.** ^{125}I-labeled asialofetuin (*Panel A*) or asialo-orosomucoid (*Panel B*) was injected into 12-week-old female mice wild-type (●), heterozygous (○), and homozygous (*X*) for the *MHL-1* gene disruption. Values shown are means of triplicates of trichloroacetic acid-insoluble radioactivity remaining in plasma at the indicated time points and are calculated as a percentage of radioactivity present 1 min after injection of the label.

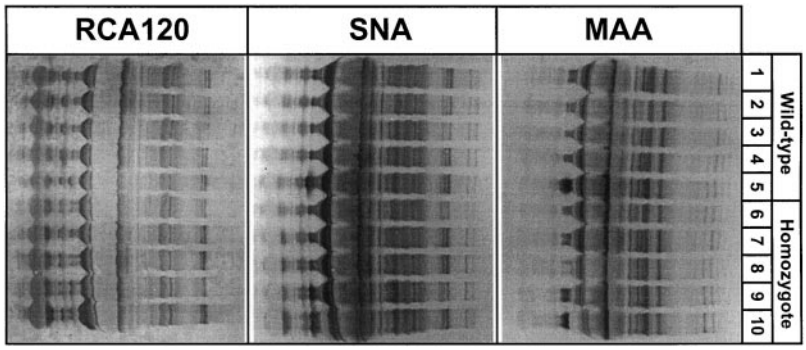

FIG. 4. **Lectin blot of plasma proteins.** Blood was collected from 32-week-old male mice wild-type (*lanes 1–5*) or homozygous (*lanes 6–10*) for the *MHL-1* disruption. Lectin blotting was done using MAA, SNA that were conjugated with digoxygenin, and RCA120 that was conjugated with biotin. Digoxigenin and biotin were detected by alkaline phosphatase-labeled anti-digoxigenin antibody and alkaline phosphatase-labeled anti-biotin antibody, respectively.

TABLE I

Plasma lipid levels in mice deficient in MHL-1

Blood was collected from mice aged 8–12 weeks after an overnight fast. Total cholesterol (TC) and triglyceride (TC) concentrations in the plasma were determined and expressed as means ± S.D. Analysis of variance was employed to compare the means between the genotypes. *LDLR* and *MHL-1* denote low density lipoprotein receptor and mouse hepatic lectin-1, respectively.

LDLR	*MHL-1*	*n*	TC	TG
			mg/dl	
+/+	+/+	4	83 ± 22	89 ± 6
+/+	+/−	4	105 ± 11	99 ± 17
+/+	−/−	4	89 ± 18	102 ± 38
−/−	+/+	5	328 ± 70	277 ± 33
−/−	+/−	9	327 ± 21	268 ± 29
−/−	−/−	7	279 ± 19	233 ± 27

TABLE II

Plasma lipoproteins in mice deficient in MHL-1

Blood was collected from 4–5 mice aged 12 weeks after an overnight fast. Plasma lipoproteins were analyzed by high performance liquid chromatography. The results were expressed as means ± S.D. Analysis of variance was employed to compare the means between the genotypes. CM, VLDL, LDL, and HDL denote chylomicron; very low density lipoproteins, low density lipoproteins, and high density lipoproteins, respectively.

Cholesterol	Wild-type	+/−	−/−
		mg/dl	
Total	85.7 ± 2.5	79.1 ± 13.0	72.2 ± 8.4
CM	0.2 ± 0.2	0.1 ± 0.1	0.3 ± 0.4
VLDL	1.9 ± 1.2	1.7 ± 1.0	1.5 ± 1.4
LDL	7.7 ± 1.3	5.4 ± 2.4	4.8 ± 2.2
HDL	78.0 ± 3.4	72.0 ± 12.0	65.5 ± 7.6

mice were virtually null for the *MHL-1* gene. In *MHL-1*+/− mice, the amounts of both mRNA and protein of MHL-1 were reduced 2-fold, indicating the gene-dosage effects of the inactivation. This reduction was accompanied by the reduction in the amounts of MHL-2 protein, even if the mRNA levels were not affected. In *MHL-1*−/− mice, MHL-2 protein was undetectable. These results strongly indicate that MHL-1 is obligatorily required for the stable expression of MHL-2. This is consistent with the *in vitro* results in transfected cells that the minor subunit is unstable in the absence of coexpression of the major subunit (22–24). Without HL-1, HL-2 may succumb to degradation within endoplasmic reticulum. It is interesting to compare this to *MHL-2*−/− mice in which substantially reduced but still significant amounts of MHL-1 were expressed. Together these results indicate that both subunits are required for the stable expression of oligomeric receptor and that HL-1 is more strictly required than HL-2.

As was observed in *MHL-2*−/− mice, the plasma clearance of asialoglycoproteins was severely impaired in *MHL-1*−/− mice (Fig. 3). It is interesting to note that the impairment of the clearance of ASOR appeared milder than that of asialofetuin in the heterozygotes. This is probably because some other pathway also contributes to the plasma clearance of asialofetuin but not to that of ASOR. For example, a macrophage lectin that is conceivably expressed in hepatic Kupffer cells may mediate this uptake, because it recognizes both galactose and *N*-acetylgalactosamine (25, 26). The plasma clearance curve of ASOR was indistinguishable from that of orosomucoid (data not shown), suggesting that *MHL-1*−/− mice clear ASOR via a pathway(s) that is irrelevant to the galactose-recognition system. Although ASGPR function was severely impaired in *MHL-1*−/− mice, the plasma glycoproteins levels were not significantly increased (Fig. 4). Together with our previous observations in *MHL-2* knockout mice, ASGPR is unlikely to be essential for the homeostasis of the major plasma glycoproteins as has been frequently discussed. In support of this, Kido *et al.* (27) have recently reported that serum glycoprotein levels were maintained in mice lacking β-1,4-galactosyltransferase I. Although nearly 90% of the serum glycoproteins lacked β-1,4-galactose in the knockout mice, their serum protein concentrations were similar to those in wild-type mice. This does not necessarily rule out the possible role of ASGPR in the regulation of minor serum glycoproteins (28). In this context, Rotundo *et al.* (10) have proposed that ASGPR is responsible for the disposal of cellular fibronectin from the plasma and/or in the

liver. Cellular fibronectin contains large amounts of terminal galactose residues, and intravenous infusion of excess asialofetuin caused retention of labeled cellular fibronectin in the liver (29). However, in multiple experiments not shown here, by Western blot analyses in the liver membrane we failed to find the abnormal accumulation of fibronectin in the liver of *MHL-1*−/− mice. Furthermore, we failed to find significant morphological changes in the liver of *MHL-1*−/− mice. With regard to IgA metabolism, Rifai *et al.* (9) have recently reported the impaired clearance of the IgA2 isoform in *MHL-2*−/− mice. However, its physiological relevance is currently unknown.

Windler *et al.* (7) proposed a possible function of ASGPR in the hepatic lipoproteins metabolism. ApoB-100 and apo(a) are heavily glycosylated giant proteins. Therefore it is reasonable to consider that the lipoproteins containing these apolipoproteins is cleared by ASGPR when the LDL receptor system is dysfunctional. To test this hypothesis, we crossed *MHL-1*−/− mice to the LDL receptor-deficient mice to generate mice lacking both ASGPR and the LDL receptor. As was the case in the mice lacking both MHL-2 and the LDL receptor, we failed to detect the elevation of plasma lipoproteins as compared with the LDL receptor knockout mice (Tables I and II). Accumulating evidence indicates that LDL receptor-related protein is involved in the plasma clearance of apoE-rich remnant lipoproteins (30). Therefore, the role of ASGPR in the plasma clearance of lipoproteins is minimal if present.

ASGPR is a member of animal C-type lectins (6). Because most of C-type lectins appear to be involved in host defense, ASGPR may have been evolved as a molecule to protect mammals from viral or bacterial insults (31), probably because exposed galactose residues may be harmful to the vascular system (32). Avian and reptiles have a similar hepatic lectin that binds to *N*-acetylglucosamine. The ASGPR system may protect vertebrates from pathogenic organisms that take advantage of neuraminidase to invade the hosts. A significant decrease in the expression of ASGPR accompanied by accumulation of serum asialoglycoproteins is observed in the patients with advanced liver diseases such as liver cirrhosis (33, 34). They frequently develop serious infectious diseases such as spontaneous bacterial peritonitis and sepsis. It is tempting to speculate that ASGPR is relevant to these complications associated with liver diseases. Besides hepatocytes, macrophages express a lectin specific for both galactose and *N*-acetylgalactosamine as mentioned above (25, 26). Its presence may have masked the phenotype in ASGPR-deficient animals. Further studies are absolutely needed to prove these possibilities. In this context, it is intriguing that some hepatotropic virus infects the hepatocytes through ASGPR (11–13).

In summary, ASGPR functions were more completely abrogated in *MHL-1*-deficient mice as compared with in *MHL-2*-deficient mice. Probably, requirement of HL-1 for stable expression of functional ASGPR is stricter than that of HL-2. Despite such absolute deficiency of ASGPR function, we failed to detect physiological evidence for several postulated functions that have been ascribed to ASGPR. These *MHL-1*-deficient mice should provide the basis for understanding the physiology of this receptor.

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