Determination of the Upper Size Limit for Uptake and Processing of Ligands by the Asialoglycoprotein Receptor on Hepatocytes In Vitro and In Vivo

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Running title:
Ligand size governs ASGPr affinity

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SUMMARY

The asialoglycoprotein receptor (ASGPr) on hepatocytes plays a role in the clearance of desialylated proteins from the serum. Although its sugar preference (N-acetylgalactosamine (GalNAc) >> galactose), and the effects of ligand valency (tetra- > tri- >> di- >> monoantennary) and sugar spacing (20 Å >> 10 Å >> 4 Å) are well-documented, the effect of particle size on recognition and uptake of ligands by the receptor is poorly defined. In the present study, we assessed the maximum ligand size that still allows effective processing by the ASGPr of mouse hepatocytes in vivo and in vitro. Hereto, we synthesized a novel glycolipid, which possesses a highly hydrophobic steroid moiety for stable incorporation into liposomes, and a triantennary GalNAc₃-terminated cluster glycoside with a high nanomolar affinity (2 nM) for the ASGPr. Incorporation of the glycolipid into small (30 nm) [³H]cholesteryl oleate-labeled long-circulating liposomes (1-50%, w/w) caused a concentration-dependent increase in particle clearance, which was liver-specific (reaching 85±7% of the injected dose at 30 min after injection) and mediated by the ASGPr on hepatocytes, as shown by competition studies with asialoorosomucoid in vivo. By using glycolipid-laden liposomes of various sizes between 30 and 90 nm, it was demonstrated that particles with a diameter > 70 nm could no longer be recognized and processed by the ASGPr in vivo. This threshold size for effective uptake was not related to the physical barrier raised by the fenestrated sinusoidal endothelium, which shields hepatocytes from the circulation, as similar results were obtained by studying the uptake of fluorescently (DiO and Dil)-labeled and [³H]cholesteryl oleate-labeled liposomes on isolated mouse hepatocytes in vitro. From these data we conclude that, in addition to the species, valency, and orientation of sugar residues, size is also an important determinant for effective recognition and processing of substrates by the ASGPr. Therefore, these data have important implications for the design of ASGPr-specific carriers that are aimed at hepatocyte-directed delivery of drugs and genes.
INTRODUCTION

The hepatic asialoglycoprotein receptor (ASGPr) is a C-type (Ca^{2+}-dependent) lectin that is expressed on the surface of hepatocytes (1), and plays a role in the clearance (endocytosis and lysosomal degradation) of desialylated proteins from the serum (2, 3) as has been shown for cellular fibronectin (4) and all IgA2 allotypes (5). The human functional receptor is a non-covalent heterotetramer composed of two homologous type II membrane polypeptides with 55% sequence identity, generally called HL-1 (hepatic lectin 1) and HL-2, at a 2:2 stoichiometry (6). The ASGPr binds glycoproteins with either non-reducing terminal β-D-galactose (Gal) or N-acetylgalactosamine (GalNAc) residues, at which the affinity for GalNAc is approx. 50-fold higher than for Gal (7-9). From studies using mice that are deficient in either the subunit HL-1 (10) or HL-2 (11), it is evident that both polypeptides are necessary for efficient clearance of asialoglycopeptides. In addition to the ASGPr on hepatocytes, a homologous Ca^{2+}-dependent Gal-recognizing receptor that also recognizes GalNAc and fucose (Fuc) is present in the liver on Kupffer cells (galactose particle receptor (GPr), Gal/Fuc receptor) (12, 13) and is absent from all other types of macrophages (14, 15).

Each polypeptide subunit of the ASGPr can bind at least a single terminal Gal or GalNAc residue (16), and the affinity of ligands for the ASGPr appears to be governed by the valency of sugar residues and their appropriate spacing. Studies using asialoglycopeptides from naturally occurring glycopeptides (7, 17) and synthetic cluster glycosides (8, 18) have demonstrated that clustering of glycosides greatly enhances the affinity for the receptor through simultaneous occupation of the receptor sites of the polypeptide subunits, at the following binding hierarchy: tetra- > tri- >> bi- >> monoantennary galactosides. This effect is dependent on the structural organisation of the receptor on the cell membrane, as it is not observed on the isolated receptor (8, 18). In addition to this so-called “cluster effect”, Lee et al. (19) and Biessen et al. (20) have
shown that optimal receptor recognition of synthetic cluster glycosides is also determined by appropriate spacing (at least 15 Å) of the sugar residues.

Although the effects of sugar type and valency on the affinity of ligands for the ASGPr are now well-established, the effects of ligand size on the binding characteristics to the receptor have still not been fully mapped. Early in vivo studies suggested that the ASGPr is mainly responsible for the uptake of small (≤ 15 nm) particles exposing galactose at relatively low density, such as high density lipoproteins (HDL) that are lactosylated (21) or provided with galactose-terminated monoantennary (mono-gal-chol) (22, 23) and triantennary glycolipids (tris-gal-chol) (24), and galactose-exposing gold particles (25). In contrast, the GPr predominantly recognizes larger galactose-exposing particles (> 15 nm) (26-28), such as desialylated rat erythrocytes (29, 30), low density lipoproteins (LDL) that are lactosylated (26) or provided with mono-gal-chol (22, 23) and tris-gal-chol (31), and tris-gal-chol-exposing liposomes (31). The affinity of glycosides for the GPr was shown to increase with particle size to reach a maximum at 15 nm (27). Furthermore, it has been shown that the GPr preferentially recognizes a high density of either fucose or galactose on either proteins (13, 15) and particles (26, 32).

In contrast to these findings, providing LDL with lactosaminated Fab fragments of anti-apoB100 antibodies induces a high uptake of LDL by the ASGPr in vivo (33). We have also recently shown that even larger (30 nm-sized) liposomes may also be specifically taken up by the ASGPr in vivo, when provided with a relatively low amount (< 10% w/w) of a non-exchangeable Gal-terminated triantennary glycolipid, with an intrinsic affinity for the ASGPr of 100 nM (32). In addition, in vitro studies have suggested that the ASGPr may represent a potential pathway of entry for 28 nm-sized hepatitis A virions (34) and 42 nm-sized hepatitis B virions (35) into hepatocytes. These data indicate that particles larger than 15 nm with their sugars presented at a high local surface density (33), low overall surface density (26) or at an appropriate spatial orientation (32) can also be taken up by the ASGPr in vivo.
The aim of the present study was to assess the intrinsic upper size limit for binding, uptake, and processing of ligands by the ASGPr. For this purpose, we synthesized a novel triantennary glycolipid that shows stable association with lipidic particles due to a highly lipophilic lithocholic oleate (LCO) structure (32, 36), and a predicted high affinity for the ASGPr by virtue of a triantennary GalNAc-terminated glycoside with 20Å spacing of the GalNAc residues (37, 38). Subsequently, we determined the effect of this glycolipid (LCO-Tyr-GalNAc₃) on the ASGPr-mediated uptake of differently-sized stable unilamellar liposomes (32, 39) in vivo and in vitro. The data indicate that the novel glycoside displays a high intrinsic affinity for the ASGPr (2 nM). Moreover, we show that the glycolipid can induce effective recognition and uptake of liposomes with a diameter as large as 70 nm by the ASGPr on hepatocytes in vitro and in vivo, whereas larger particles do not bind to the ASGPr. These findings not only add to the further characterization of the structural requirements of ligands for proper recognition by the ASGPr, but also have important implications for the design of particulate systems that are widely exploited for ASGPr-mediated targeting of drugs and genes to hepatocytes (40-42).
MATERIALS AND METHODS

Animals – 10-12-Week-old male C57Bl/6KH mice weighing 24-28 g and Wistar rats weighing 250-300 g (from Broekman Instituut BV, Someren, The Netherlands) fed ad libitum with regular chow were used for the in vivo experiments.

Chemicals - [1α,2α-3H]Cholesteryl oleate ([3H]CO) and 125I (carrier-free) in NaOH were purchased from Amersham Pharmacia Biotech. Egg yolk phosphatidylcholine (Lipoid E PC; 98%) was from Lipoid, Ludwigshafen, Germany. Galactose oxidase (EC 1.1.3.9) from Dactylium dendroides (crude) and collagenase (EC 3.4.24.3) from Clostridium histolyticum (type IV) were from Sigma. Cholesteryl oleate (CO; 97%) was from Janssen, Beersse, Belgium, and Percoll® was from Fluuka, Buchs, Switzerland. 2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt (ABTS), horse radish peroxidase type II (200 units/mg), Precipath® L, EDTA, and collagen S (type I) from calf skin were from Roche Molecular Biochemicals. Ketamine (HCl salt, 100 mg/ml) was from Eurovet (Bladel, The Netherlands). Hypnorm (0.315 mg/ml of fentanyl citrate and 10 mg/ml of fluanisone) and Thalamonal (0.05 mg/ml of fentanyl and 2.5 mg/ml of droperidol) were from Janssen-Cilag Ltd., Saunderton, UK. 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanide perchlorate (DiI) and 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) were from Molecular Probes, Leiden, The Netherlands. Asialoorosomucoid (ASOR) was prepared by enzymatic desialylation (approx. 70%, as judged by the extent of sialic acid release) of human α₁-acid glycoprotein (orosomucoid) from Cohn Fraction VI (99%) from Sigma as described (43). Multiwell cell culture dishes were from Costar, Cambridge, MA. Dulbecco’s modified Eagle’s medium (DMEM) and fetal calf serum (FCS) were obtained from Flow Laboratories, Irvine, UK. All other chemicals were of analytical grade.
Synthesis and Characterisation of Glycolipids - The synthesis of the ether-linked triantennary galactoside Z-Gly-TRIS(Gal)₃ (Gal₃; Mₘ 1484) and its γ-aminobutyric acid (GABA)-mediated coupling product with the steroid structure 3α-oleooyloxy cholenic acid, leading to the bifunctional glycolipid (3α(oleooyloxy)-5β-cholanoyl)-GABA-Gly-TRIS(Gal)₃ (LCO-Gal₃; Mₘ 2058) (Fig. 1A) has been recently reported in full detail (32). A novel triantennary N-acetylgalactosamine-terminated cluster (Z-TRIS(GalNAc)₃; Mₘ 1532) has been synthesized and conjugated with a nearly identical steroid structure via a tyrosine residue, to allow for trace labeling with ¹²⁵I, yielding (3β(oleoylamido)-5β-cholanoyl)-Tyr-Gly-TRIS(GalNAc)₃ (LCO-Tyr-GalNAc₃; Mₘ 2182) (Fig. 1B). The synthesis of this glycolipid will be described in full detail elsewhere. The homogeneity and identity of both glycolipids has been fully established by HPLC, NMR spectroscopy, and mass spectroscopy. The freeze-dried glycolipids were dissolved in PBS at a final concentration of 25-50 µg/µl and stored at -80°C under argon before use. Their stability (which exceeded 12 months) was routinely checked by thin-layer chromatography (n-butanol: n-propanol: 25% NH₄OH: H₂O = 15: 40: 30: 15, v/v/v/v, or isopropanol: 25% NH₄OH = 1: 1, v/v) and subsequent visualisation of carbohydrate and cholesterol moieties by charring with H₂SO₄/ethanol (1: 4, v/v) and MnCl₂ (44), respectively.

Radiolabeling of LCO-Tyr-GalNAc₃ and ASOR – LCO-Tyr-GalNAc₃ was radioiodinated with carrier-free ¹²⁵I at pH 7.4 using a Iodogen (10 µg)-coated reaction tube, and ASOR at pH 10.0 according to the ICl method (45), respectively. Free ¹²⁵I was removed by Sephadex G-50 medium gel filtration. The radioiodinated glycolipid migrated as a single band on TLC (n-butanol: n-propanol: 25% NH₄OH: H₂O = 15: 40: 30: 15, v/v/v/v) as determined by imaging, and more than 98% of the radiolabel in ASOR was 10% trichloroacetic acid (TCA) precipitable. The specific activities of LCO-Tyr-GalNAc₃ and ASOR were 1300-4300 dpm/ng of glycolipid and 260 dpm/ng of protein, respectively.
Protein Assay – Protein concentrations were determined according to Lowry et al. (46) using BSA as a standard.

In Vitro Binding to Hepatocytes - Hepatocytes were isolated from anesthetized rats or mice by perfusion of the liver with collagenase (type IV, 0.05% (w/v)) for 10 min at 37°C according to the method of Seglen (47) as detailed earlier (27). The cells were ≥ 99% pure as judged by light microscopy and their viabilities were ≥ 95% (rat) and ≥ 80% (mouse) as determined by 0.2% trypan blue exclusion. Hepatocytes were incubated (2 h at 4°C) in DMEM containing 2% BSA (1 x 10^6 cells/ml) with 5 nM 125I-ASOR in the presence of increasing amounts of unlabeled galactose (0.2-200 mM), Z-Gly-TRIS(Gal)_3 (1-1000 nM), or Z-Tyr-Gly-TRIS(GalNAc)_3 (0.2-200 nM) under gentle shaking in a circulating lab shaker (Adolf Kühner AG, Basel, Switzerland) at 150 rpm. After incubation, cells were pelleted by centrifugation (1 min at 50 g), and unbound 125I-ASOR was removed by washing twice with ice-cold 50 mM Tris.HCl, 150 mM NaCl, 5 mM CaCl_2 (TBS), pH 7.4, containing 0.2% BSA, and once with TBS without BSA. The cell pellet was lysed in 0.1 N NaOH, the radioactivity and protein content was measured, and 125I-ASOR binding was calculated (dpm/mg of cell protein). Non-specific binding was determined in the presence of 100 mM N-acetylgalactosamine (GalNAc). Displacement binding data were analyzed according to a single-site binding model. Inhibition curves were calculated by non-linear regression analysis (GraphPad, ISI Software, Philadelphia, PA, USA).

Preparation and Characterization of Liposomes – Liposomes (mean diameter 30, 50, and 70 nm) were prepared by sonication as described (39). In short, egg yolk phosphatidylcholine (EYPC, 25 mg), cholesteryl oleate (CO, 1 mg), and [3H]CO (50-100 µCi) were hydrated in 10 ml of 0.1 M KCl, 10 mM Tris.HCl, pH 8.0, and subsequently sonicated at 54°C using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK) at 18 µm output. Alternatively, liposomes (mean
diameter 50 and 90 nm) were prepared after hydration of the lipids in 2.0 ml of buffer and multiple extrusion (11 times) at 54°C through 50 nm and 100 nm Whatman Nuclepore® (Pleasanton, CA, USA) polycarbonate filters, respectively, using a Liposofast-Pneumatic (Avestin Inc., Ottawa, ON, Canada) (48). All liposomes were purified and concentrated (1.014 g/ml) by density gradient ultracentrifugation according to Redgrave et al. (49) using NaCl/KBr/EDTA density solutions in a Beckman SW 40 Ti rotor at 40,000 rpm for 18-22 h at 4°C. Particle sizes were determined by photon correlation spectroscopy (Malvern 4700 C System, Malvern Instruments, Malvern, UK) at 27°C and a 90° angle between laser and detector. Sonication for 60, 15, and 10 min resulted in liposomes with mean particle diameters of 29.4±2.2, 55.7±0.9, and 72.3±3.6 nm (mean ± S.D.; n = 3, 2, and 3) that were homogeneous with respect to size (polydispersities of 0.14-0.17, 0.28-0.29, and 0.26-0.27). Extrusion led to liposomes of 48.3 nm (50 nm filter; n = 1) and 90.3±6.1 nm (100 nm filter; mean ± S.D.; n = 4) with polydispersities of 0.15 and 0.11-0.15, respectively. When indicated, liposomes were labeled with 1% (w/w) DiO or Dil by adding 0.25 mg from 10 mg/ml stock solutions in CHCl₃:CH₃OH (1:1, v/v) before hydration of lipids. The phosphatidylcholine and cholesterol ester contents were determined with the Roche Molecular Biochemicals enzymatic kits for phospholipid and cholesterol, respectively. Precipath® L was used as an internal standard. The particles were stored at 4°C under argon and used for characterization and metabolic studies within 7 days following preparation, in which period no physicochemical changes occurred.

Association of LCO-Tyr-GalNAc₃ with Liposomes – Liposomes (100 µg of phospholipid) were incubated (30 min at 37°C) with (radioiodinated) glycolipid in PBS pH 7.4. The mixtures were subjected to 0.75% (w/w) agarose gel electrophoresis at pH 8.8, and the resulting gels were stained for lipid using Sudan Black. Radioactivity was visualized by imaging using a Packard Instant Imager (Hewlett-Packard Co., Palo Alto, CA). The electrophoretic mobility (Rf) of the Coomassie Brilliant blue-stained liposomes (0.18±0.01) was determined relative to the front...
marker bromophenol blue. Alternatively, incubation mixtures (50 µl) were subjected to fast protein liquid chromatography (SMART System; Amersham Pharmacia Biotech) using a Superose® 6 (PC 3.2/30) column at a flow rate of 50 µl/min and with PBS, 1 mM EDTA, 0.02% NaN₃, pH 7.4, as eluent. The galactose content of the collected fractions was determined using a galactose oxidase assay (recovery 85-100%). In short, samples were incubated in the dark (30 min at Tr) with 0.9 mM ABTS, 66.5 mU/ml peroxidase, 2.2 U/ml galactose oxidase, 0.1 M KP₂ buffer, pH 7.0, and the absorbance was measured at 405 nm. LCO-Tyr-GalNAc₃ was used as a standard. The number of associated glycolipid molecules per 30 nm-sized particle was calculated assuming 7.62x10¹³ liposomes per mg of phospholipid (39).

Liver Uptake and Serum Decay of Liposomes in Mice - Mice were anesthetized by subcutaneous injection of a mixture of ketamine (120 mg/kg body weight), Thalamonal (0.03 mg/kg fentanyl and 1.7 mg/kg droperidol), and Hypnorm (1.2 mg/kg fluanisone and 0.04 mg/kg fentanyl citrate), and the abdomens were opened. [³H]CO-labeled liposomes (100 µg of phospholipid) were injected via the inferior vena cava, after previous incubation (30 min at 37°C) with PBS or the indicated amounts of glycolipid. When indicated, mice received a preinjection of ASOR (25 mg/kg) at 1 min before injection of the particles. At the indicated times, blood samples (< 50 µl) and liver lobules were taken and processed as described in detail (50). At 30 min after injection, the mice were sacrificed, and their livers and spleens were excised and weighed. Radioactivity in duplicate serum samples of 10 µl was counted in 2.5 ml of Emulsifier Safe (Packard Instrument Co.). The total serum volume of C57Bl/6KH mice was 1.068±0.066 ml (50). Radioactivity in liver samples and spleens was counted in 15 ml of Hionic Fluor (Packard Instrument Co.) after solubilization of the organs in 500 µl of Soluene®-350 (Packard) for 5 h at 65°C. Radioactivity values are corrected for the serum radioactivity (liver: 84.7 and spleen: 64.6 µl/g wet weight) present at the time of sampling (50).
In Vitro Uptake of Fluorescently Labeled Liposomes by Mouse Hepatocytes – Mouse hepatocytes were isolated from anesthetized mice as described above, and subjected to Percoll® gradient centrifugation to discard non-viable cells. The cells (viability >99% as judged from 0.2% trypan blue exclusion) were attached to collagen S (3.87 µg/cm²)-coated 2.5-cm glass cover slips in 9.6-cm² 6-well dishes (1x10⁶ cells per well) by culturing in DMEM + 10% FCS (3-4 h at 37°C). Cover slips were washed to remove unbound cells and transferred to a Zeiss IM-35 inverted microscope (Oberkochen, Germany) with a Zeiss plan-apochromatic 63x/1.4 NA oil objective and fitted with a temperature-controlled incubation chamber, which was equipped with a Bio-Rad 600 MRC confocal laser scanning microscopy (CLSM) system. Cells were incubated (20 min at 37°C) in DMEM + 2% BSA with DiO-labeled 30 nm-sized liposomes and/or Dil-labeled 90 nm-sized liposomes (200 µg of phospholipid/ml), after previous incubation with 5% (w/w) LCO-Tyr-GalNAc₃ or PBS, in the absence or presence of 100 mM GalNAc. Subsequently, the cells were washed twice with DMEM + 2% BSA to remove unbound particles, and (intra)cellular localisation of DiO and Dil was visualized during further incubation at 37°C.

In Vitro Association of Radioactively Labeled Liposomes with Mouse Hepatocytes – Mouse hepatocytes were isolated from anesthetized mice as described above and viable cells were harvested by Percoll® gradient centrifugation. The cells (1 x 10⁶) were incubated at 37°C in 0.5 ml of DMEM + 2% BSA with [³H]CO-labeled 30 nm (sonicated), 50 nm (extruded), 70 nm (sonicated), and 90 nm (extruded) liposomes (240 µg of phospholipid/ml) with or without previous incubation (30 min at 37°C) with LCO-Tyr-GalNAc₃ (5% w/w), in the absence or presence of 100 mM GalNAc. The incubations were performed in plastic containers (Kartell, Milan, Italy) in a circulating lab shaker (Adolf Kühner AG, Basel, Switzerland) at 150 rpm, with brief oxygenation every 60 min. After incubation, the cells were cooled to 0°C, pelleted (1 min at 50 g), and unbound particles were removed by washing twice with ice-cold TBS pH 7.4, containing 0.2% BSA, and once with TBS without BSA. The cell pellet was lysed in 0.1 N NaOH,
the radioactivity and protein content were measured, and association of liposomes was calculated as µmol phospholipid/mg of cell protein.
RESULTS

Affinity of GalNAc-Terminated Triantennary Cluster for the Hepatic ASGPr – We determined the affinity of the newly synthesized triantennary Gal-terminated cluster glycoside Z-Gly-TRIS(Gal)$_3$ (Fig. 1A) and the GalNAc-terminated cluster glycoside Z-Gly-TRIS(GalNAc)$_3$ (Fig. 1B) for the rat and murine ASGPr, by determining the ability of the cluster glycosides to compete for the binding of the high-affinity ligand $^{125}$I-ASOR to isolated hepatocytes in vitro (Fig. 2). Both galactosides displayed competitive inhibition of ASOR binding, as shown by monophasic inhibition curves with a Hill coefficient close to unity. In agreement with earlier studies (8, 51), galactose was only marginally capable of inhibiting $^{125}$I-ASOR binding to rat ($K_i$ 4.3±0.8 mM) and mouse ($K_i$ 3.6±1.0 mM) hepatocytes. The clustered presentation of Gal residues in Z-Gly-TRIS(Gal)$_3$ increased the potency approx. 40-fold ($K_i$ 100±1 nM; mean ± SEM; $n$ = 3). Replacement of Gal by GalNAc in Z-Gly-TRIS(GalNAc)$_3$ caused a further 50-fold increased affinity (2.1±0.3 nM and 2.7±1.0 nM towards rat and mouse hepatocytes, respectively), which is in agreement with observations from Lee et al. (37, 38).

Interaction of Glycolipids with Liposomes - To investigate the interaction of LCO-Tyr-GalNAc$_3$ with the differently sized EYPC:CO liposomes, we first examined the effect of the glycolipid on the electrophoretic pattern of the liposomes (Fig. 3). Incubation of 30 nm-sized liposomes with radiiodinated glycolipid resulted in an LCO-Tyr-GalNAc$_3$ concentration-dependent reduction of the electrophoretic mobility of the liposomes ($R_f$ 0.18±0.01) (Fig. 3A). Incorporation of the glycolipid was evidenced by comigration of the radiolabeled glycolipid with the liposomes (Fig. 3B). Identical patterns were obtained using liposomes of sizes 55, 70, and 90 nm, indicating a similar extent of glycolipid incorporation (not shown). The incorporation capacity of 30 nm-sized liposomes for LCO-Tyr-GalNAc$_3$ was determined by separation of the liposome-bound glycolipid from the free glycolipid with high resolution by size exclusion chromatography on a Superose 6
column (Fig. 3C). LCO-Tyr-GalNAc$_3$ elutes at excellent yield (>95%) at an elution volume of 1.53 ml, indicating that the glycolipids form stable micelles with a size slightly larger than that of human HDL (8-10 nm; $V_e$ 1.58 ml), and can easily be separated from the relatively large liposomes ($V_e$ 0.90 ml). Incubation of liposomes with LCO-Tyr-GalNAc$_3$ (5, 10, 25, and 50%, w/w) led to incorporation of 160, 200, 360, and 415 glycolipid molecules per particle, respectively. Incorporation of the glycolipid did not substantially alter the liposomal size, as judged from Sephacryl S-1000 elution profiles (not shown).

Liver Uptake and Serum Decay of (Glycolipid-Laden) Liposomes in Mice – As both glycolipids show similar incorporation characteristics into liposomes, the glycolipid-induced liver uptake of 30 nm-sized liposomes was evaluated for LCO-Tyr-GalNAc$_3$ and LCO-Gal$_3$. Upon intravenous injection into mice, the [HCO-labeled liposomes showed a low uptake by the liver (7.7±0.4% of the injected dose at 30 min after injection) and a high remaining fraction in the serum (81.3±2.1%), as a consequence of their low affinity for the reticuloendothelial system (Fig. 4) (32, 39). In accordance with previous observations obtained with LCO-Gal$_3$ (32), incubation of the liposomes with LCO-Tyr-GalNAc$_3$ dose-dependently accelerated their serum clearance in a monophasic manner (Fig. 4B), indicating that the glycolipid firmly associates with the particles due to its highly hydrophobic moiety, and does not readily redistribute to serum lipoproteins. The increased serum clearance of the liposomes was mainly caused by uptake by the liver, which was dose-dependently enhanced via 31.2±3.0% (1%, w/w; $P < 0.05$) to 65.1±0.3% (5%, w/w; $P < 0.0001$) of the injected dose at 30 min after injection (Fig. 4).

The effect of the 50-fold higher ASGPr affinity of LCO-GalNAc$_3$ as compared to LCO-Gal$_3$ on the extent of the glycolipid-induced liver uptake of the 30 nm-sized liposomes was addressed by determining the liver uptake of liposomes after incubation with increasing amounts of both glycolipids (Fig. 5). A differential effect of the glycolipids on total liver uptake of the liposomes could predominantly be detected at low incorporation levels. Whereas at 1%
(w/w) LCO-Gal₃ did not affect the liver uptake, indicating that a threshold loading of liposomes is necessary for inducing affinity for the liver, a 4-fold increased uptake ($P < 0.05$) could already be detected using the same amount of LCO-Tyr-GalNAc₃.

Although the effect of the increased ASGPr affinity of LCO-Tyr-GalNAc₃ on the total uptake of liposomes by the liver may be limited, a large effect was observed on the ASGPr specificity of the liposomes. This was determined by a preinjection of ASOR, which specifically blocks ASGPr-mediated uptake by hepatocytes, but not GPr-mediated uptake by Kupffer cells (24, 31). At low amounts of LCO-Gal₃ ($\leq 5\%$, w/w), the glycolipid-induced liver uptake could be completely blocked by a preinjection of ASOR. However, at higher LCO-Gal₃ concentrations, the effect of ASOR on the induced uptake rapidly declined, indicating an almost complete shift in uptake from hepatocytes to Kupffer cells at 50% (w/w). This can be explained by a high surface density of galactose residues that are readily recognized by the GPr (26, 32) (Fig. 5A). In contrast, preferential uptake of the liposomes by the ASGPr was observed even at high LCO-Tyr-GalNAc₃ concentrations, since a high degree of inhibition (i.e. 62%) of the liver uptake by the ASGPr competitor could still be detected at 50% (w/w) (Fig. 5B). Apparently, the very high affinity of LCO-Tyr-GalNAc₃ for the ASGPr overrules the stimulating effect of a high glycoside surface density on the induced uptake by the GPr.

**Size-Dependent Association of Liposomes to the ASGPr in Mice** – Since it is now evident that LCO-Tyr-GalNAc₃ is superior to LCO-Gal₃ in its capacity to selectively stimulate the ASGPr-mediated uptake of liposomes *in vivo*, LCO-Tyr-GalNAc₃ (5% w/w) was used to evaluate the effect of liposomal size on the ASGPr-mediated uptake by hepatocytes (Fig. 6). As expected from the size-dependent enhanced affinity of liposomes for the reticuloendothelial system, both the hepatic and splenic uptake of the ligand-deficient liposomes increased with increasing liposomal diameter. LCO-Tyr-GalNAc₃ was able to induce the liver uptake of liposomes irrespective of their size, although the extent of liver uptake slightly decreased with increasing
particle size. Importantly, the glycolipid-induced liver uptake of 30, 55, and 70 nm-sized particles (prepared by sonication) could be almost completely blocked by preinjection of ASOR, indicating that the uptake is fully mediated by the ASGPr. In contrast, although the liver association of the 90 nm-sized liposomes (prepared by extrusion) also seemed to be enhanced by LCO-Tyr-GalNAc$_3$, ASOR was unable to compete for the liver uptake, indicating that the ASGPr is not involved (Fig. 6). The inability of ASOR to block the glycolipid-induced liver uptake of these large liposomes cannot be ascribed to an increased affinity of these liposomes for the ASGPr. At a concentration of 0.4 µM, LCO-Tyr-GalNAc$_3$ micelles inhibited the ASOR binding to hepatocytes for 80.6% (Fig. 2B), and a similar inhibition (77.3%) could be detected for glycolipid-laden 30 nm-sized liposomes (5%, w/w). In contrast, glycolipid-laden 90 nm-sized liposomes displayed a severely reduced inhibitory activity (20.0%), indicating a much lower affinity of these glycolipid-containing liposomes for the ASGPr (not shown). The liver uptake of 50 nm-sized liposomes that were prepared by extrusion (10.2±1.8% of the injected dose at 30 min after injection) was enhanced to a similar extent as compared to the 55 nm-sized sonicated liposomes by LCO-Tyr-GalNAc$_3$ (59.3±3.1%), and could also be fully inhibited by a preinjection of ASOR (13.6±1.9%). Therefore, a potentially disturbing effect of preparation method on the in vivo characteristics of the liposomes can be excluded. No effects of LCO-Tyr-GalNAc$_3$ were observed on the splenic accumulation of the liposomes, despite the presence of binding sites for galactose-terminated triantennary glycosides in the spleen (Rensen et al., unpublished observations).

Size-Dependent Uptake of Liposomes by the ASGPr on Hepatocytes – The in vivo results point to the existence of a particle size limit (< 90 nm) below which liposomes still can associate with the hepatic ASGPr. Since this diameter concurs with the size of the fenestrae that are present in the sinusoidal endothelium of the liver (approx. 100 nm) (52), we subsequently evaluated whether this physiological barrier may have contributed to the observed size effects in vivo.
Therefore, liposomal uptake experiments were performed using freshly isolated mouse hepatocytes in vitro (Fig. 7 and 8).

To quantify the association of the liposomes via the ASGPr on hepatocytes in vitro, hepatocytes were incubated with [³H]CO-labeled particles (Fig. 7). The glycolipid-deficient 30, 50, and 70 nm-sized liposomes showed a time-dependent association with the hepatocytes (3.24, 5.23, and 5.04 mmol of phospholipid/ mg of cell protein after 180 min of incubation, respectively), which was substantially higher than that of the 90 nm-sized liposomes (0.89 mmol of phospholipid/mg of cell protein). The cellular association (binding and uptake) of the 30, 50, and 70 nm-sized liposomes was increased upon the addition of 5% (w/w) LCO-Tyr-GalNAc₃, and could be blocked by the presence of an excess of GalNAc. In contrast, the cellular uptake of the 90 nm-sized liposomes was not affected by the glycolipid. The observation that the 50 nm-sized liposomes (synthesized by extrusion) showed a similar glycolipid-dependent cell association as the 30 and 70 nm liposomes (prepared by sonication) excludes the possibility that the absence of an effect of the glycolipid on the cell association of the 90 nm-sized liposomes could be due to a difference in liposomal preparation method.

The observed differences in interaction of small (30 nm) and large (90 nm) liposomes with isolated hepatocytes were also demonstrated by CLSM (Fig. 8). Pulse-labeling of hepatocytes for 20 min with fluorescently labeled liposomes of 30 nm (DiO) and 90 nm (DiI) that contain 5% (w/w) LCO-Tyr-GalNAc₃ resulted in a strong fluorescent lining of the cell surface with the 30 nm-sized liposomes, but not the 90 nm-sized particles. This effect is not caused by mutual competition between the differently sized liposomes, since exclusion of the small liposomes from the incubation did not result in an enhanced binding of the 90 nm-sized liposomes (not shown). After removal of unbound particles, the surface-bound 30 nm-liposomes were rapidly taken up, with complete internalisation observed between 30 min and 60 min. Binding and uptake of the liposomes was markedly inhibited in the presence of 100 mM GalNAc or in the absence of the glycolipid (data not shown).
DISCUSSION

So far, the ligand recognition by the ASGPr has been well-characterised with respect to sugar preference (GalNAc >> Gal) (7-9), optimum ligand valency (tetra- > tri- >> di- >> monoantennary) (8, 18) and sugar spacing (20Å >> 10Å >> 4Å) (19, 20), but the effect of size on recognition and processing of ligands by the ASGPr has been subject of controversy. Early in vivo studies on galactose-terminated glycolipids (22-24, 31, 53), have suggested that small (≤ 15 nm) galactose-exposing particles are preferentially taken up by the ASGPr on hepatocytes, whereas larger particles (> 15 nm) mainly associate with the GPr on Kupffer cells. Since this hypothesis has been disputed by the findings that the hepatitis A virus (28 nm) (34), hepatitis B virus (42 nm) (35), 25 nm-sized LDL (33), and 30 nm-sized liposomes (32) may also be internalised by hepatocytes via the ASGPr, the present study was undertaken to conclusively establish the effects of size on processing of globular ligands by the ASGPr.

Taking advantage of the above-mentioned ‘affinity rules’, we have synthesized the triantennary glycoside Z-TRIS(GalNAc)₃ with a nanomolar affinity for the ASGPr (Kᵢ 2 nM). This affinity is similar to that of the triantennary glycopeptides YEE(GalNAcAH)₃ and YDD(G-ah-GalNAc)₃ that have been developed by Lee et al. (37, 38), and utilized for ASGPr-directed delivery of DNA (54) and oligodeoxynucleoside methylphosphonates (55). To establish firm association with liposomes, the glycoside was coupled to lithocholic oleate, which has already been shown to confer a stable incorporation of antisense oligodeoxynucleotides (36), anthracyclines (56), and glycosides (32) into lipidic particles. Also in this study, a tight association of the Gal₃ and GalNAc₃-terminated glycolipids with the liposomes was observed, withstanding dissociation in the blood. Unlike the previously applied cholesterol-coupled glycosides, which induced a biphasic serum decay of lipoproteins as explained by partial glycolipid-induced clearance of injected lipoproteins by the liver in the α-phase, followed by
redistribution of glycolipid over the endogenous lipoprotein pool in the β-phase (23, 24, 53), the present glycolipids induced a monophasic clearance of liposomes from the serum upon intravenous injection. The differences between both glycolipids with respect to the lipophilic moiety, the absence or presence of a Tyr moiety, and the sugar type (Gal or GalNAc) apparently do not affect the association of the glycolipid with the liposomes.

As a result of the 50-fold higher ASGPr affinity of the GalNAc₃-terminated glycolipid over the Gal₃-terminated glycolipid, an effective targeting of 30 nm-sized liposomes to the ASGPr could already be accomplished at concentrations as low as 1% (w/w), at which the Gal₃-terminated glycolipid had no effect. This amount corresponds to only 36 glycolipid molecules per particle, assuming 7.62x10¹³ liposomes per mg of phospholipid (39). When taking into account that 60% of the phospholipids are located in the outer phospholipid layer (39), it thus appears that approx. 22 molecules of the GalNAc₃-terminated glycolipid are sufficient for inducing uptake of liposomes by the ASGPr. The most prominent effect of the higher affinity for the ASGPr, however, involves the considerably enhanced specificity of liposomes for the ASGPr as opposed to the GPr over a wide glycolipid loading range (1-50%, w/w). It can be calculated that at an incorporation of 50% (w/w) of glycolipid, the glycoside occupies only a very restricted surface area (approx. 3 nm²) (32). In this case, the conformational properties of the individual clusters of the Gal₃-terminated glycolipid, which are of vital importance for high-affinity recognition by the ASGPr, may be overruled by the high overall Gal density on the liposomal surface, that has been shown to lead to efficient uptake by the GPr on Kupffer cells (27).

Apparently, the preferential uptake of liposomes provided with an equal concentration (i.e. 50%, w/w) of the GalNAc₃-terminated glycolipid by hepatocytes indicates that, in contrast to Gal clusters, a similarly high density of GalNAc clusters on the particle surface does not impair the specificity for the ASGPr. Therefore, the GalNAc₃-exposing glycolipid is very suitable for evaluation of the effect of particle size on recognition and uptake by the ASGPr in vivo and in vitro. These findings have also important implications for research on liposome-mediated
ASGPr-directed drug delivery to hepatocytes. Although galactose is generally utilized as a recognition marker for the ASGPr (e.g. 57), the present data demonstrate that the specificity for this receptor in vivo may be greatly improved by the application of the ligand GalNAc instead.

The hepatic and splenic uptake of the glycolipid-deficient liposomes appeared to be enhanced with increasing particle size. Since an opposite effect was observed with respect to the uptake of glycolipid-deficient liposomes by isolated hepatocytes (30-70 nm versus 90 nm), and given the fact that the affinity of particulate carriers for the reticuloendothelial system increases with increasing particle size, it is likely that this size dependent (glycolipid-independent) liver uptake is exerted by Kupffer cells. At a load of 5% (w/w), the GalNAc₃-exposing glycolipid did not stimulate the uptake of liposomes by macrophages in general and splenic macrophages in particular, although C-type Gal/GalNAc-recognizing receptors have been described on extrahepatic macrophages from rats and mice (58-62) in addition to S-type (soluble) Ca²⁺-independent galactoside-binding proteins (galaptins, galectins) (63, 64). Possibly, the macrophage asialoglycoprotein binding protein displays a lower affinity towards GalNAc than Gal and lacks the cluster effect that has been observed for the ASGPr (65). Similarly, galectins have been shown to have minimal affinity for triantennary cluster galactosides as compared to C-type lectins (63, 64, 66).

Using LCO-Tyr-GalNAc₃ at a load of 5% (w/w), it appeared that liposomes with a size up to 70 nm are effectively recognized by the ASGPr on hepatocytes in vivo, whereas the 90-nm particles did not associate with the ASGPr. This phenomenon is not caused by the physical barrier raised by the fenestrated endothelium that shields hepatocytes from the circulation, as similar findings were obtained from liposome uptake experiments by isolated hepatocytes in vitro. It is unlikely that these observations are related to a restricted size limit of endosomes formed after ASGPr-mediated endocytosis via clathrin-coated pits, as the average endosome size in mammalian hepatocytes has been reported to be 100 nm (67), with a wide size distribution of 50-350 nm (68). Moreover, as compared to the 30-70 nm-sized particles, the 90
nm-sized liposomes already displayed an impaired binding to isolated hepatocytes. Taking into account that the ASGPr is predominantly diffusely, and perhaps inaccessibly, distributed within the microvilli clefts at the sinusoidal hepatocyte surface (69-71), it is tempting to assume that penetration of particles between the microvilli may be a limiting factor for ASGPr-mediated uptake, but this possibility can not be conclusively established from the current experimental set up. Regardless of the precise mechanism, our present data may explain why earlier attempts to efficiently target relatively large liposomes to the ASGPr on hepatocytes have not been successful. For example, the observation that 100 nm-sized liposomes provided with (monoantennary) polyethyleneglycol-coupled galactolipids were mainly taken up by Kupffer cells (57) may not only be explained by lack of ASGPr specificity, but also by their unfavorable dimensions.

In conclusion, in addition to the sugar preference and cluster effect, we have further elucidated the ligand recognition characteristics of the ASGPr, by demonstrating that effective binding and internalisation by the receptor is restricted to glycoside-exposing particles with a diameter \( \leq 70 \) nm, whereas larger particles are not recognized. Due to its unique localisation, abundance, and high internalisation capacity, the ASGPr is widely used as a target for the specific delivery of genes and therapeutic agents to hepatocytes. Therefore, our findings also have important implications for the design of such non-viral gene vectors and drug targeting vehicles with respect to sugar ligand (GalNAc >> Gal) and particle size (\( \leq 70 \) nm).
REFERENCES


ABBREVIATIONS

ASGPr, asialoglycoprotein receptor; ASOR, asialoorosomucoid; BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; CO, cholesteryl oleate; Dil, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate; DiO, 3,3′-dioctadecyloxacarbocyanine perchlorate; DMEM, Dulbecco’s modified Eagle medium; EYPC, egg yolk phosphatidylcholine; FCS, fetal calf serum; FPLC, fast protein liquid chromatography; Fuc, fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; GPr, galactose particle receptor; LCO-Gal₃, (3α(oleoyloxy)-5β-cholanoyl)-GABA-Gly-TRIS(Gal)₃; LCO-Tyr-GalNAc₃, (3β(oleoylamido)-5β-cholanoyl)-GABA-Tyr-Gly-TRIS(GalNAc)₃; PBS, phosphate-buffered saline.

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LEGENDS TO SCHEMES AND FIGURES

Fig. 1. **Chemical structures of glycolipids.** A, (3α(oleoyloxy)-5β-cholanoyl)-GABA-Gly-TRIS(Gal)₃ (LCO-Gal₃); B, (3β(oleoylamido)-5β-cholanoyl)-GABA-Tyr-Gly-TRIS(GalNAc)₃ (LCO-Tyr-GalNAc₃).

Fig. 2. **Inhibition of ASOR binding to hepatocytes by triantennary galactosides.** Freshly isolated rat (left) and mouse (right) hepatocytes (approx. 1 x 10⁶ cells/ml) were incubated with 5 nM ¹²⁵I-ASOR (2 h at 4°C) in the absence or presence of unlabeled galactose (Δ, 0.2-200 mM) and the galactosides Gal₃ (Ο, 1-1000 nM) or GalNAc₃ (●, 0.2-200 nM). Binding is plotted as percentage of specific binding, which is defined as the difference in ligand binding in the absence (total binding) and presence (non-specific binding) of 100 mM N-acetylgalactosamine.

Fig. 3. **Association of LCO-Tyr-GalNAc₃ with liposomes.** Liposomes (30 nm; 100 µg of phospholipid) were incubated (30 min at 37°C) with LCO-[¹²⁵I]Tyr-GalNAc₃ (0, 5, 10, and 25%, w/w phospholipid) and subjected to electrophoresis in a 0.75% agarose gel. Subsequently, the liposomes were stained for lipid (A), and glycolipid-associated ¹²⁵I-radioactivity was visualized by imaging (B). The anode and cathode are indicated by (+) and (-), respectively. Alternatively, liposomally incorporated glycolipid was separated from unincorporated glycolipid by FPLC, the galactose content of the resulting fractions was determined, and the number of glycolipid molecules per particle was calculated (C).

Fig. 4. **Effect of LCO-Tyr-GalNAc₃ on the liver uptake and serum decay of liposomes in mice.** [³H]CO-labeled 30 nm-sized liposomes (100 µg of phospholipid) were injected i.v. into anaesthetized C57Bl/6 mice, without (Ο) or with previous incubation (30 min at 37°C) with 1%
(w/w) (●) or 5% (w/w) (■) of LCO-Tyr-GalNAc₃. At the indicated times, the liver uptake (left) and serum decay (right) were determined. Liver values are corrected for entrapped serum radioactivity. Values are means ± variation of two experiments.

Fig. 5. **Involvement of ASGPr in the glycolipid-induced liver uptake of liposomes.** [³H]CO-labeled 30 nm-sized liposomes (100 µg of phospholipid) were injected i.v. into anaesthetized C57Bl/6 mice, without or with previous incubation (30 min at 37°C) with 1, 5, 10, and 50% (w/w) of LCO-Gal₃ (left) or LCO-Tyr-GalNAc₃ (right). At 30 min after injection of the liposomes, the liver uptake was determined, without (O) or with (●) previous administration of ASOR (25 mg/kg). Liver values are corrected for serum radioactivity and represent means ± variation of two experiments.

Fig. 6. **Effect of liposomal size on LCO-Tyr-GalNAc₃-induced uptake by the liver.** [³H]CO-labeled 30, 55, 70, and 90 nm-sized liposomes (100 µg of phospholipid) were injected i.v. into anaesthetized C57Bl/6 mice, without (O) or with (●, ■) previous incubation (30 min at 37°C) with 5% (w/w) of LCO-Tyr-GalNAc₃. At 30 min after injection, the mice were sacrificed, and the uptake by the liver (left) and spleen (right) were determined, without (O, ●) or with (■) previous injection of ASOR (25 mg/kg) at 1 min before injection of the liposomes. Values are corrected for serum radioactivity and represent means ± variation of two experiments.

Fig. 7. **Effect of liposomal size on LCO-Tyr-GalNAc₃-induced association with isolated mouse hepatocytes.** Freshly isolated mouse hepatocytes were incubated at 37°C with [³H]CO-labeled 30, 50, 70, or 90 nm-sized liposomes (240 µg of phospholipid/ml) without (O) or with (●, ■) 5% (w/w) of LCO-Tyr-GalNAc₃. Incubation with LCO-Tyr-GalNAc₃-laden liposomes was done in the absence (●) or presence (■) of 100 mM N-acetylgalactosamine (GalNAc). At the
indicated times, the cells were washed and lysed, and cell protein was determined. Values are means of duplicate incubations.

Fig. 8. **Effect of liposomal size on LCO-Tyr-GalNAc$_3$-induced uptake by isolated mouse hepatocytes.** Freshly isolated mouse hepatocytes were cultured (3-4 h at 37°C) in DMEM + 10% FCS and coincubated (20 min at 37°C) in DMEM + 2% BSA with fluorescently labeled 30 nm-sized DiO-labeled (*left*) and 90 nm-sized Dil-labeled (*right*) liposomes (200 µg of phospholipid/ml) (preincubated with 5% (w/w) of LCO-Tyr-GalNAc$_3$). Cells were washed to remove unbound particles, and further incubated at 37°C. After 5 min (*top*), 30 min (*middle*), and 60 min (*bottom*), localisation of DiO (excitation 488 nm, *left*) and Dil (excitation 543 nm, *right*) was determined by CLSM.
Fig. 1
Fig. 2
A. lipid

B. $^{125}$I-activity

added LCO-$[{^{125}I}]$Tyr-GalNAc$_3$ (% w/w)

C. 

- Fig. 3
Fig. 4
Fig. 5

[Diagram showing the effect of added glycolipids (% w/w) on [3H]CO (% of injected dose) for LCO-Gal$_3$ and LCO-GalNac$_3$ with and without ASOR.]
Fig. 6
Fig. 7
Fig. 8
Determination of the upper size limit for uptake and processing of ligands by the asialoglycoprotein receptor on hepatocytes in vitro and in vivo
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