Ligand size is a major determinant of high-affinity binding of fucose- and galactose-exposing (lipo)proteins by the hepatic fucose receptor

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Previous *in vivo* studies have demonstrated that small galactose-exposing particles are preferentially internalized by the asialoglycoprotein receptor on the parenchymal liver cell and large particles by the galactose-particle receptor on the Kupffer cell. In this study, we have investigated using *in vitro* binding studies whether the affinity for either receptor is affected by the ligand size. The asialoglycoprotein receptor appeared to bind and process lactosylated proteins irrespective of their size. In contrast, recognition of galactose-exposing proteins by the galactose-particle receptor on the Kupffer cell was strongly dependent on size. The affinity increased 3000-fold with protein sizes increasing from 5 to 15 nm, reaching its maximum at approx. 1 nM for ligands larger than 15 nm. Apparently, the preferential *in vivo* uptake of large galactose-exposing ligands by Kupffer cells does not result from an inability of the parenchymal liver cells to

internalize these ligands, but from the high affinity of large ligands for the galactose-particle receptor and the strategic anatomical localization of the Kupffer cells in the liver. In the preceding paper [Kuiper, Bakkeren, Biessen and Van Berkel (1994) *Biochem. J.* 299, 285–290] the galactose-particle receptor on the Kupffer cell was suggested to be identical with the fucose receptor. ¹²⁵I-Lac-LDL-binding studies clearly showed that the galactose-particle receptor exhibited high-affinity binding of fucose-exposing proteins also. The affinity of fucosylated proteins for the galactose-particle receptor was greatly affected by ligand size. The above data strongly support the hypothesis that the galactose-particle receptor is identical with the fucose receptor. The size of neoglycoproteins can be appreciated as a new major determinant of affinity for the fucose receptor.

INTRODUCTION

In addition to the galactose-specific asialoglycoprotein receptor on the parenchymal cell, the liver possesses a second galactose-recognizing receptor localized on the Kupffer cell, the galactose-particle receptor. The asialoglycoprotein receptor has been characterized in detail, at both the physiological and molecular level [1–3]. Binding studies using synthetic glycosides and isolated oligoantennary glycopeptides have provided insight into the structural requirements for ligand recognition by the asialoglycoprotein receptor [2,4–7]. In particular, the distance between the galactose residues within a cluster glycoside and the branching pattern of the glycoside determine the affinity for the asialoglycoprotein receptor. Optimal recognition by the receptor was attained for tetra- and tri-antennary galactosides in which the separate galactose units are spaced at least 1.5 nm [6]; the affinity decreased significantly with decreasing glycoside valency of the cluster glycoside [4]. Previous studies have demonstrated that, *in vivo*, the asialoglycoprotein receptor is responsible for uptake of small galactose-exposing particles [8–11].

The galactose-particle receptor was first described by Kempka et al. [12]. Electron-microscopic studies using gold particles coated with asialofetuin or lactosylated BSA have revealed that the galactose-particle receptor is an oligomeric membrane-associated protein. After binding to this receptor, galactose-exposing particles are internalized and subsequently processed via a lysosomal pathway [8,12–14]. Schlepper-Schäfer et al. [8] observed that the galactose-particle receptor accounted for the preferential uptake of galactose-exposing particles larger than 7.8 nm. Studies of Bijsterbosch and Van Berkel [9] and Bijsterbosch et al. [15] were in agreement with these data, in as much as

a minimal size of between 10 and 20 nm was required for uptake of galactose-exposing particles by the Kupffer cell *in vivo*. The mechanism of this preference of the asialoglycoprotein receptor for internalization of small and the galactose-particle receptor for internalization of large galactose-exposing particles has not yet been elucidated. An *in vitro* study on the effect of ligand size on their affinities for both receptor systems may provide information on the nature of this feature.

In the preceding paper [16], the galactose-particle receptor was suggested to be identical with the hepatic fucose receptor, as described by Lehrmann et al. [17]. The sugar-specificity profile and immunological reactivity of the galactose-particle receptor corresponded to that of the fucose receptor. It follows therefore that, if the two receptors are indistinguishable, the affinity of fucose-exposing ligands for the galactose-particle receptor will also depend on size. In the present study, we have investigated this issue. The results provide additional support for the galactose-particle receptor being identical with the fucose receptor.

MATERIALS AND METHODS

Materials

Sodium cyanoborohydride was purchased from Aldrich Chemical Co. (Brussels, Belgium). Na¹²⁵I (carrier-free) in NaOH was obtained from Amersham International (Amersham, Bucks., U.K.). Collagenase type IV, was from Boehringer-Mannheim (Mannheim, Germany). BSA (type V), agarose-bound neuraminidase (from *Clostridium perfringens*, type IV-A) and ferritin (type I from horse spleen) were purchased from Sigma (St. Louis, MO, U.S.A.). Dulbecco's modified essential medium (DMEM)

Abbreviations used: ASOR, asialo-orosomucoid; Lac, lactosylated; Fuc, derivatized with phenyl-(1-β-L-fucopyranosyl)-4-isothiocyanate; LDL, low-density lipoprotein; HDL, high-density lipoprotein; DMEM, Dulbecco's modified essential medium.

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was obtained from Flow Laboratories (Irvine, Scotland, U.K.). Thyroglobulin (from bovine pituitary gland) was from Fluka (Basel, Switzerland), and γ -globulin (chicken IgG) was from Nordic (Tilburg, The Netherlands). All other chemicals were reagent grade.

Isolation of Kupffer cells and parenchymal cells

Male Wistar rats of approx. 250 g were anaesthetized by intraperitoneal injection of 20 mg of sodium pentobarbital. Parenchymal liver cells were isolated after a 20 min perfusion of the liver with collagenase (type IV, 0.05 %) at 37 °C, by the method of Seglen [18], modified as previously described [19]. After perfusion, parenchymal and Kupffer cells were purified by differential centrifugation and counterflow elutriation as described in detail elsewhere [20]. The purity of the Kupffer cells was more than 95 % as judged by peroxidase staining [0.1 % 3,3'-diaminobenzidine in 0.05 M Tris/HCl buffer, pH 7.4, containing 7 % sucrose, 0.1 % (v/v) H_2O_2 in water (30 %, v/v) and 30 % H_2O_2 ; 20 min at 37 °C].

Isolation and radiolodination of asialo-orosomucoid (ASOR), low-density lipoprotein (LDL) and apoE-free HDL

Human orosomucoid was isolated and subsequently desialylated enzymatically as described [20]. Human LDL ($1.024 < d < 1.063$) and high-density lipoprotein (HDL) ($1.063 < d < 1.21$) were isolated by differential ultracentrifugation, as described by Redgrave et al. [21]. Apolipoprotein E-free HDL was prepared by the procedure of Weisgraber and Mahley [22]. The (lipo)proteins were radiolabelled with carrier-free Na^{125}I by the ICI method of McFarlane as modified by Bilheimer et al. [23].

Modification of (lipo)proteins

Lactosylation of the proteins (i.e. BSA, β -globulin, ferritin and thyroglobulin), LDL and apoE-free HDL was performed by reductive alkylation of the lysines. Protein (1–5 mg/ml) in 20 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA was incubated for 2 days at 37 °C in the presence of sodium cyanoborohydride (50 mg/ml) and lactose (100 mg/ml). Lipo-proteins were lactosylated for 5 days at room temperature. After lactosylation, the reaction mixture was diluted twofold with PBS and dialysed thoroughly against the same buffer.

Fucosylation of (lipo)proteins was performed by the procedure of Jansen et al. [24]. In short, phenyl-(1- β -L-fucopyranosyl)-4-isothiocyanate was synthesized from 4-aminophenyl- β -L-fucopyranose in 84 % yield using thiophosgene [25]. Subsequently, protein (2 mg/ml) dissolved in 1 ml of 0.2 M Na_2CO_3 (pH 9.0) was allowed to react overnight at ambient temperature with 4 mg of phenyl-(1- β -L-fucopyranosyl)-4-isothiocyanate (14 μmol) dissolved in 400 μl of the above buffer. After the reaction had taken place, the suspension was dialysed exhaustively against PBS. The particle sizes of both native and modified (lipo)proteins were determined by native PAGE on 5–15 % and 8–22 % gradient polyacrylamide gels at pH 8.8 using calibration proteins with established particle sizes (Phast System, Pharmacia) [26] and by photon-correlation spectroscopy using the Malvern 4700C submicron particle-size analyser at 25 °C and a protein content of 1 mg/ml of PBS (scattering angle 120°; viscosity 0.8905; refractive index 1.330; particle refractive index 1.500; sampling time 240 s [27]). The spectroscopic data were analysed according to the Raleigh–Gans–Debye theory [27] to evaluate the apparent mass-weighted particle size. The following apparent protein sizes were measured: thyroglobulin (18.7 and 18.3 nm for

the lactosylated and fucosylated protein compared with 18.9 nm for the native protein), ferritin (14.8 and 13.3 nm compared with 14.3 nm), γ -globulin (8.9 and 7.9 nm compared with 9.3 nm), BSA (4.8 and 4.1 nm compared with 4.2 nm), HDL (12.9 and 10.6 nm compared with 10.9 nm) and LDL (22.2 and 20.6 nm compared with 20.9 nm).

Determination of the degree of glycosylation

The degree of glycosylation was determined colorimetrically using the anthron and phenol/sulphuric acid method [28,29]. Protein content was determined by the Lowry assay [30] and was corrected for non-specific staining of the fucose residues of the neoglycoproteins.

Binding of ^{125}I -ASOR or ^{125}I -Lac-LDL and ^{125}I -Lac-HDL to parenchymal liver cells

Saturation binding studies of ^{125}I -Lac-LDL or ^{125}I -Lac-HDL to parenchymal liver cells were performed as follows. Parenchymal liver cells (1×10^6 – 1.5×10^6 cells in 1 ml of DMEM containing 2 % BSA; viability > 90 %) were incubated for 2 h at 4 °C with radiolabelled ligand at eight concentrations ranging from 0.7 to 25 nM. After incubation, the suspension was centrifuged (50 g for 1 min) and the cells were washed twice with 1 ml of DMEM containing 0.2 % BSA and once with 1 ml of DMEM. The radioactivity in the cell pellet was counted and expressed with respect to cellular protein. Non-specific binding was determined in the presence of 100 mM *N*-acetylgalactosamine. Displacement curves were produced analogously in the presence of a fixed concentration of radioligand and a variable concentration of displacer, ranging from 10^{-12} to 2×10^{-4} M. Saturation binding parameters and inhibition constants were calculated by non-linear regression analysis of the corresponding binding data using a computer program (GraphPAD, ISI Software, Philadelphia, PA, U.S.A.).

Uptake of ^{125}I -Lac-LDL and ^{125}I -Lac-HDL by parenchymal liver cells at 37 °C

Initial uptake of ^{125}I -Lac-LDL and ^{125}I -Lac-HDL by parenchymal liver cells at 37 °C was determined as follows. Parenchymal liver cells (1×10^6 – 1.5×10^6 cells, suspended in 1 ml of DMEM containing 2 % BSA; viability > 90 %) were incubated with gentle shaking for 10 min at 37 °C with radiolabelled ligand at eight concentrations ranging from 0.7 to 30 nM, in the presence or absence of 100 mM of *N*-acetylgalactosamine. After incubation, Mg/EGTA (pH 7.5) in DMEM was added to a final concentration of 10 mM and the suspension was incubated for 45 min at 4 °C, in order to remove the membrane-bound ligand (as specific binding by the asialoglycoprotein receptor is Ca^{2+} -dependent, all membrane-bound ligand is released on incubation with EGTA [16]). Subsequently, the suspension was centrifuged (50 g for 1 min) and the cells were washed twice with 1 ml of DMEM containing 0.2 % BSA and once with 1 ml of DMEM alone. The radioactivity in the cell pellet was counted and expressed on a cellular protein basis. Non-specific uptake was determined in the presence of 100 mM *N*-acetylgalactosamine. The substrate curves were used to calculate the apparent K_m and V_{max} using a computerized non-linear regression procedure (GraphPAD).

Binding of ^{125}I -Lac-LDL to Kupffer cells

^{125}I -Lac-LDL binding to Kupffer cells (0.5×10^6 – 1×10^6 cells/ml) was determined analogously to the procedure for ^{125}I -Lac-LDL binding to parenchymal liver cells except that the incubation

Figure 1 Inhibition of ^{125}I -ASOR binding (a) to parenchymal liver cells and ^{125}I -Lac-LDL binding (b) to Kupffer cells by lactosylated neoglycoproteins

Freshly isolated rat parenchymal liver cells or Kupffer cells (1×10^6 – 1.5×10^6 cells/ml) were incubated for 2 h at 4 °C with a fixed concentration of ^{125}I -ASOR (5.5 nM) and ^{125}I -Lac-LDL (5.9 nM) respectively in the absence or presence of unlabelled neoglycoprotein at eight concentrations, ranging from 10^{-11} to 2×10^{-4} M. Binding is plotted as percentage of specific binding, which was defined as the difference in ligand binding in the absence and presence of 100 mM *N*-acetylgalactosamine. The following neoglycoproteins were tested: Lac-BSA (□), Lac-globulin (●), Lac-HDL (▲), Lac-ferritin (■), Lac-thyroglobulin (△) and Lac-LDL (○).

medium was supplied with 5 mM CaCl_2 . This was required to achieve an optimal level of ^{125}I -Lac-LDL binding. After incubation, the suspension was centrifuged at 500 *g* for 1 min and the cells were washed twice with 1 ml of DMEM containing 0.2% BSA and 5 mM CaCl_2 and once with 1 ml of DMEM, containing 5 mM CaCl_2 . The radioactivity in the cell pellet was counted and expressed on a cellular protein basis. Non-specific binding was determined in the presence of 100 mM *N*-acetylgalactosamine.

RESULTS

Physical characterization of glycosylated (lipo)proteins

Globular proteins (BSA, γ -globulin, ferritin and thyroglobulin) and lipoproteins (LDL and HDL) were extensively glycosylated. The protein size remained essentially unaltered on modification, thus establishing the integrity of the (lipo)proteins on glycosylation. The degree of lactosylation and fucosylation of the various (lipo)proteins was as follows: Lac-thyroglobulin contained 215, Fuc-thyroglobulin 465, Lac-ferritin 126, Fuc-ferritin 185, Lac-BSA 46, Fuc-BSA 48, Lac-globulin 67, Fuc-globulin 105, Lac-HDL 56, Fuc-HDL 149, Lac-LDL 390 and Fuc-LDL

1215 mol of glycosyl residue per mol of protein. This corresponded with an average density of 0.31 ± 0.1 lactosyl units/nm² for lactosylated and 0.57 ± 0.13 fucosyl units/nm² for fucosylated

Table 1 Inhibition constants of lactosylated (lipo)proteins for displacement of ^{125}I -Lac-LDL from Kupffer cells (KC) and ^{125}I -ASOR binding from parenchymal liver cells (PC)

Inhibition constants (pK_i) were calculated from inhibition-curve data (Figure 3) using a computerized non-linear regression procedure (Graph-Pad). Values are means of two experiments. The internal standard deviation is estimated from the mean of the two regression analyses. The genuine variation was of the same order of magnitude.

Compound	Size (nm)	KC		PC	
		pK_i	K_i (nM)	pK_i	K_i (nM)
Lac-BSA	4.8	5.22 ± 0.01	6020	7.68 ± 0.15	21
Lac-globulin	8.9	6.95 ± 0.01	110	7.90 ± 0.04	12
Lac-HDL	11.9	7.01 ± 0.05	98	7.82 ± 0.09	15
Lac-ferritin	14.8	8.68 ± 0.01	2	7.60 ± 0.04	25
Lac-thyroglobulin	18.7	9.09 ± 0.01	0.8	8.65 ± 0.05	3
Lac-LDL	22.2	8.94 ± 0.01	1	8.04 ± 0.03	9

Figure 2 Effect of ligand size of lactosylated neoglycoproteins on the affinity for the asialoglycoprotein receptor on the parenchymal cells (a) and the galactose-particle receptor on the Kupffer cell (b)

Ligand sizes were determined by particle-size analysis by photon-electron spectroscopy using the Malvern 4700C. 1, BSA (4.8 nm); 2, γ -globulin (8.9 nm); 3, HDL (11.9 nm); 4, ferritin (14.8 nm); 5, thyroglobulin (18.7 nm); 6, LDL (22.2 nm).

Figure 3 Substrate curves of initial uptake at 37 °C of ^{125}I -Lac-LDL (■) and ^{125}I -Lac-HDL (●) by parenchymal liver cells

Parenchymal liver cells (1×10^6 – 1.5×10^6 cells/ml) were incubated for 10 min at 37 °C in the absence or presence of 100 mM *N*-acetylgalactosamine, with a variable concentration of ^{125}I -Lac-LDL or ^{125}I -Lac-HDL ranging from 0.7 to 25 nM. After incubation, the cell suspension was put on ice and incubated for 10 min with 10 mM EGTA to release the membrane-associated ligand. Then the cells were washed thoroughly and the internalized radioactivity was determined. The specific initial uptake, defined as the difference between uptake in the absence and presence of 100 mM *N*-acetylgalactosamine, was plotted against the concentration of ligand.

proteins. Hence, both the lactose and fucose densities were more or less constant for the various globular proteins. This density corresponds to an average distance of 1.8 ± 0.3 nm for lactosylated and 1.3 ± 0.1 nm for fucosylated proteins between the vicinal glycosyl units.

Effect of ligand size of lactosylated proteins on the affinity for the asialoglycoprotein receptor and the galactose-particle receptor

The potency of lactosylated proteins of various sizes to displace ^{125}I -Lac-LDL binding from Kupffer cells and ^{125}I -ASOR binding from parenchymal liver cells was determined in order to clarify whether the affinity of a galactose-exposing ligand for either receptor is affected by its size. Figure 1 shows that all lactosylated (lipo)proteins exhibited monophasic displacement of ^{125}I -Lac-LDL binding to Kupffer cells and ^{125}I -ASOR binding to parenchymal cells. The inhibition constants (K_i) are given in Table 1. With sizes increasing from 4.8 to 22.2 nm, the affinity for the galactose-particle receptor ($1/K_i$) increased from $6.0 \mu\text{M}$ to 1.0 nM (Figure 2). This increase levelled off at 15 nm, reaching a maximal value of approx. 1 nM. The affinity for the asialoglycoprotein receptor, in contrast, was not affected by ligand size (Table 1; Figure 2).

The preferential uptake of larger substrates by Kupffer cells *in vivo* might arise from an inability of the asialoglycoprotein receptor to internalize large ligands. Therefore studies were carried out to ascertain whether large galactose-exposing proteins were internalized and processed by this receptor. Binding at 4 °C and uptake at 37 °C by parenchymal liver cells were performed simultaneously using either ^{125}I -Lac-HDL or ^{125}I -Lac-LDL as radioligand. Both ^{125}I -Lac-LDL and ^{125}I -Lac-HDL bound to the asialoglycoprotein receptor with high affinity ($K_d = 8.9 \pm 1.3$ nM and $K_d = 3.15 \pm 0.45$ nM respectively; not shown). Specific ^{125}I -Lac-LDL binding to parenchymal cells was totally inhibited by ASOR ($pK_i = 8.8 \pm 0.06$) and likewise ^{125}I -ASOR binding by Lac-LDL ($pK_i = 8.04 \pm 0.05$). Hence, we may safely assume that ^{125}I -Lac-LDL binding to parenchymal liver cells involves the

Figure 4 Inhibition of ^{125}I -ASOR binding (a) to parenchymal liver cells and ^{125}I -Lac-LDL binding (b) to Kupffer cells by fucosylated proteins

Freshly isolated rat parenchymal liver cells or Kupffer cells (1×10^6 – 1.5×10^6 cells/ml) were incubated for 2 h at 4 °C with a fixed concentration of ^{125}I -ASOR (5.5 nM) or ^{125}I -Lac-LDL (5.9 nM) respectively in the absence or presence of a variable concentration of neoglycoprotein, ranging from 10^{-12} to 10^{-6} M. Binding was plotted as percentage of specific binding, which was defined as the difference between ligand binding in the absence and presence of 100 mM *N*-acetylgalactosamine. The following neoglycoproteins were tested: Fuc-BSA (□), Fuc-globulin (▼), Fuc-HDL (●), Fuc-ferritin (■), Fuc-thyroglobulin (△) and Fuc-LDL (○).

asialoglycoprotein receptor (results not shown). Uptake studies revealed that both ^{125}I -Lac-LDL and ^{125}I -Lac-HDL are indeed internalized by parenchymal liver cells (Figure 3). Uptake was of high affinity, saturable and galactose-specific. The K_m values for uptake of ^{125}I -Lac-LDL (10.8 ± 2.4 nM) and ^{125}I -Lac-HDL (2.6 ± 0.5 nM) by the parenchymal cell were essentially similar to the K_d values of the two radioligands (8.9 ± 1.3 nM and 3.1 ± 0.5 nM respectively). Further study of the kinetics of endocytosis of ^{125}I -Lac-LDL revealed that ^{125}I -Lac-LDL was not only rapidly and completely internalized but also degraded, after an 11 min lag, at a rate of 4.4 fmol/min per mg, which is comparable with the degradation rate of smaller substrates such as ^{125}I -ASOR (results not shown).

Effect of ligand size of fucosylated proteins on the affinity for the asialoglycoprotein and the galactose-particle receptor

In the preceding paper [16] it was suggested that the galactose-particle receptor might be identical with the fucose receptor. The effect of ligand size appears to be a characteristic feature of ligand recognition by the galactose-particle receptor. Therefore the ability of a series of fucosylated (lipo)proteins to displace ^{125}I -ASOR binding from the asialoglycoprotein receptor and ^{125}I -Lac-

Table 2 Inhibition constants of fucosylated (lipo)proteins for displacement of ^{125}I -Lac-LDL and ^{125}I -ASOR binding from Kupffer cells (KC) and parenchymal liver cells (PC) respectively

Inhibition constants (pK_i) were calculated from the inhibition-curve data (Figure 3) using a computerized non-linear regression procedure (Graph-Pad). Values are means of two experiments. The internal standard deviation is estimated from the mean of the two regression analyses. The genuine variation was of the same order of magnitude. n.d., No inhibition could be detected at concentrations up to 2000 nM.

Compound	Size (nm)	KC		PC pK_i
		pK_i	K_i (pM)	
Fuc-BSA	4.1	8.48 ± 0.10	3300	n.d.
Fuc-globulin	7.9	8.78 ± 0.12	1600	n.d.
Fuc-HDL	10.6	8.89 ± 0.16	1280	n.d.
Fuc-ferritin	13.3	9.81 ± 0.09	155	n.d.
Fuc-thyroglobulin	18.3	10.67 ± 0.10	21	n.d.
Fuc-LDL	20.6	11.03 ± 0.10	9	n.d.

Figure 5 Effect of ligand size of fucosylated neoglycoproteins on the affinity for the galactose-particle receptor on the Kupffer cell

Ligand sizes were determined by particle-size analysis by photon-electron spectroscopy using the Malvern 4700C and by native PAGE. 1, BSA (4.1 nm); 2, γ -globulin (7.9 nm); 3, HDL (10.6 nm); 4, ferritin (13.3 nm); 5, thyroglobulin (18.3 nm); 6, LDL (20.6 nm).

LDL binding from the galactose-particle receptor was monitored. Fucosylated proteins appeared to be unable to displace the binding of ^{125}I -ASOR from the parenchymal liver cell even at concentrations of up to $2 \mu\text{M}$, indicating that fucose-exposing proteins lack any affinity for the asialoglycoprotein receptor (Figure 4). Fucosylated proteins were on the other hand extremely potent inhibitors of ^{125}I -Lac-LDL binding to Kupffer cells, with K_i values ranging from the low nanomolar to the picomolar range (Figure 4, Table 2). Moreover, a clearcut size effect was observed: the affinity of Fuc-BSA ($K_i = 3.3 \text{ nM}$) was almost 400-fold lower than that of Fuc-LDL ($K_i = 9.3 \text{ pM}$) (Figure 5). This increase in affinity levelled off at a ligand size of 18 nm, although this was somewhat less pronounced than with lactosylated proteins.

DISCUSSION

Previous studies have demonstrated that *in vivo* the two hepatic galactose-recognizing receptors, the galactose-particle receptor on the Kupffer cell and the asialoglycoprotein receptor on the

parenchymal cell, differ in their selectivity towards galactose-exposing ligands. Small ligands are preferentially internalized via the asialoglycoprotein receptor whereas large ligands are taken up via the Kupffer cell receptor [8–10]. In the present study, we have elucidated the molecular basis of this phenomenon. The affinity of a series of lactosylated globular proteins, ranging from 4.8 to 22.2 nm in size, for both receptor systems was monitored using binding studies. A striking difference in the effect of protein size on its affinity for the galactose-particle and the asialoglycoprotein receptor was observed. Unexpectedly, the affinity of the lactosylated proteins for the asialoglycoprotein receptor was not significantly affected by size and was 12 nM, on average. On the other hand, ligand size had a significant effect on their affinity for the galactose-particle receptor on Kupffer cells. The K_i decreased 5000-fold from $6 \mu\text{M}$ to approximately 1 nM with ligand sizes increasing from 4.8 to 22.2 nm and levelled off for ligands larger than 15 nm. The observed increase in affinity does not result from a different spatial arrangement or local density of the lactosyl moieties within the various neoglycoproteins. Lac-HDL and Lac-globulin, being comparable in size, exhibit similar affinities for the galactose-particle receptor. The degree of lactosylation implies that every lactosyl residue is, on average, surrounded by four lactosyl units within a distance of 1.8 nm. In a preliminary study on the effect of galactosyl spacing of multiantennary cluster glycosides, a 3.2 nm spacing between the vicinal galactosyl units did not significantly affect the affinity for the galactose-particle receptor (E. A. L. Biessen, unpublished work). As there was no correlation between the average surface density of lactosyl moieties and the affinity for the galactose-particle receptor, we can safely assume that, at high levels of lactosylation, slight variations in galactose density did not cause the observed differences in affinity.

The finding that ligand size affects the affinity for the Kupffer cell receptor, but not the affinity for the asialoglycoprotein receptor, is not in agreement with the hypothesis of Schlepper-Schäfer, who attributed the size-dependent preference for *in vivo* uptake by either receptor to an inability of the asialoglycoprotein receptor to internalize coated gold particles larger than 7.8 nm [8]. When corrected for the volume of the protein (Lac-BSA) adsorbed to the colloidal gold particle, the actual size is approximately 16–17 nm. However, our *in vitro* binding and uptake studies with ^{125}I -Lac-HDL and ^{125}I -Lac-LDL as radioligand demonstrate that substrates up to at least 21 nm can be internalized by the asialoglycoprotein receptor. For ^{125}I -Lac-LDL and ^{125}I -Lac-HDL, the apparent turnover rate of the receptor pool, defined as the quotient of the V_{max} and B_{max} of the radioligand, did not significantly differ from the value obtained with ^{125}I -ASOR (9.7 ± 1.3 , $7.1 \pm 1.4 \text{ min}$ and $7.3 \pm 1.4 \text{ min}$ for ^{125}I -Lac-LDL, ^{125}I -Lac-HDL and ^{125}I -ASOR respectively). A study of the kinetics of synchronized endocytosis of ^{125}I -Lac-LDL showed that the ligand is degraded by the parenchymal cell at a rate of 4.4 fmol/min per mg, which is comparable with the rate of ^{125}I -ASOR degradation reported by Bridges et al. [31]. In agreement with the above results, the studies of Bijsterbosch and Van Berkel [9] and Van Berkel et al. [10] showed that the parenchymal liver cell accounted for the uptake of the major fraction of the injected Lac-HDL, when Kupffer cells were eliminated by preinjection of GdCl_3 .

Both immunological and *in vitro* binding studies [16] have revealed that the galactose-particle receptor may be identical with the fucose receptor, first described by Lehrmann et al. [17], rather than with the C-reactive protein, as proposed by Kempka et al. [12]. The structural requirements of ligand recognition by the fucose receptor were studied in detail by Lehrmann and associates [17,32–34]. The spatial density of accessible fucose

residues appeared to be a major determinant of affinity [17]. Ligand size can also be considered an important determinant of affinity for the galactose-particle receptor. Thus the effects of sugar density and ligand size of a fucosylated protein on its ability to displace ^{125}I -Lac-LDL binding to Kupffer cells were tested in order to validate further the potential identity of the galactose-particle and the fucose receptor. The affinity for the galactose-particle receptor appeared to be dependent on the extent of fucosylation of BSA [16]. The K_i values of Fuc₁₈-BSA, Fuc₃₈-BSA and Fuc₄₈-BSA of 420 ± 140 , 48 ± 7 and 2.1 ± 0.7 nM respectively were similar to the K_i values, previously reported by Lehrmann et al. [17] (670, 7 and 0.5 nM).

The asialoglycoprotein receptor did not display any affinity towards fucosylated ligands. This is in accordance with the study of Lehrmann et al. [34], showing that fucosylated BSA was not recognized by the asialoglycoprotein receptor. The effect of ligand size on the affinity of fucosylated proteins for the galactose-particle receptor was similar to that of lactosylated proteins. The affinity increased 400-fold with sizes increasing from 4.1 to 20.6 nm. The ability to displace ^{125}I -Lac-LDL did not correlate with the surface density of exposed fucosyl units. Furthermore, the affinities of the fucosylated proteins were consistently higher than those for the lactosylated proteins. This implies that the ^{125}I -Lac-LDL-binding site involves a fucose- rather than a galactose-specific receptor, an additional indication that the galactose particle and the fucose receptor are indistinguishable.

Interestingly, the size-determined increase in affinity towards the galactose-particle receptor levelled off at 15 nm for lactosylated proteins and at 18 nm for fucosylated proteins. The fucose receptor was reported to be an oligomeric protein, consisting of covalently bound 88 kDa subunits, with a molecular mass of at least 150–200 kDa [33]. Viewed in that light, this limiting value of 15–18 nm might reflect the absolute size of the fucose receptor complex. Further study on the valency of ligand binding by the fucose receptor will conclusively clarify this issue.

In conclusion, the asialoglycoprotein receptor recognizes and processes lactosylated proteins, irrespective of their size. Recognition of galactose-exposing proteins by the galactose-particle receptor on the Kupffer cell is strongly dependent on size. The preferential *in vivo* uptake of large galactose-exposing proteins and particles by Kupffer cells is not the result of an inability of the parenchymal liver cells to internalize these ligands. Apparently, it arises from both the high affinity of large ligands for the galactose-particle receptor and the strategic anatomical localization of Kupffer cells, which favours them in monitoring the blood compartment. The affinity of fucose-terminated (neo)glycoproteins for the galactose-particle receptor was similarly size-dependent. Together, the above data strongly support the hypothesis that the galactose-particle receptor is identical with the fucose receptor. The size of fucose-terminated glycoproteins can be appreciated as one of the principal determinants of affinity for the fucose receptor, besides sugar density. The extremely high affinity of large fucosylated proteins for the galactose-particle receptor of the Kupffer cell may be used to

develop carriers for targeting of immunostimulating drugs to Kupffer cells.

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REFERENCES

- 1 Ashwell, G. and Harford, J. (1982) *Annu. Rev. Biochem.* **51**, 531–554
- 2 Breitfield, P. P., Simmons, C. F., Strous, G. J. A. M., Geuze, H. J. and Schwartz, A. L. (1985) *Int. Rev. Cytol.* **97**, 47–95
- 3 Spiess, M. *Biochemistry* **19**, 10009–10018
- 4 Connolly, D. T., Townsend, R. R., Kawaguchi, K., Bell, W. R. and Lee, Y. C. (1982) *J. Biol. Chem.* **257**, 939–945
- 5 Kawaguchi, K., Kuhlenschmidt, M., Roseman, S. and Lee, Y. C. (1980) *Arch. Biochem. Biophys.* **205**, 388–395
- 6 Lee, Y. C., Townsend, R. R., Hardy, M. R., Lönngren, J., Arnarp, J., Haraldsson, M. and Lönn, H. (1983) *J. Biol. Chem.* **258**, 199–202
- 7 Baenziger, J. A. and Maynard, Y. (1980) *J. Biol. Chem.* **255**, 4607–4613
- 8 Schlepper-Schafer, J., Hulsman, D., Djovkar, A., Meyer, H. E., Herbertz, L., Kolb, H. and Kolb-Bachofen, V. (1986) *Exp. Cell Res.* **165**, 494–506
- 9 Bijsterbosch, M. K. and Van Berkel, Th. J. C. (1990) *Biochem. J.* **270**, 233–239
- 10 Van Berkel, Th. J. C., Kruijt, J. K., Spanjer, H. H., Nagelkerke, J. F., Harkes, L. and Kempen, H. J. M. (1985) *J. Biol. Chem.* **260**, 2694–2699
- 11 Van Berkel, Th. J. C., Kruijt, J. K. and Kempen, H. J. M. (1985) *J. Biol. Chem.* **260**, 12203–12207
- 12 Kempka, G., Roos, P. H. and Kolb-Bachofen, V. (1990) *J. Immunol.* **144**, 1004–1009
- 13 Kolb-Bachofen, V., Schlepper-Schafer, J., Vogel, W. and Kolb, H. (1982) *Cell* **29**, 859–866
- 14 Kempka, G. and Kolb-Bachofen, V. (1985) *Biochim. Biophys. Acta* **847**, 108–114
- 15 Bijsterbosch, M. K., Ziere, G. J. and Van Berkel, Th. J. C. (1990) *Mol. Pharmacol.* **36**, 484–489
- 16 Kuiper, J., Bakkeren, H. F., Biessen, E. A. L. and Van Berkel, Th. J. C. (1994) *Biochem. J.* **299**, 285–290
- 17 Lehrman, M. A., Haltiwanger, R. S. and Hill, R. L. (1986) *J. Biol. Chem.* **261**, 7426–7432
- 18 Seglen, P. O. (1976) *Methods Cell. Biol.* **13**, 29–83
- 19 Nagelkerke, J. F., Barto, K. P. and Van Berkel, Th. J. C. (1983) *J. Biol. Chem.* **258**, 12221–12227
- 20 Whitehead, P. H. and Sammons, H. G. (1966) *Biochim. Biophys. Acta* **244**, 209–211
- 21 Redgrave, T. G., Roberts, D. C. K. and West, C. E. (1975) *Anal. Biochem.* **65**, 42–49
- 22 Weisgraber, K. H. and Mahley, R. W. (1980) *J. Lipid Res.* **21**, 316–325
- 23 Bilheimer, D. W., Eisenberg, S. and Levy, R. I. (1972) *Biochim. Biophys. Acta* **260**, 212–221
- 24 Jansen, R., Molema, G., Ching, T. L., Oosting R., Harms, G., Molenaar, F., Hardonk, M. J. and Meyer, D. K. F. (1991) *J. Biol. Chem.* **266**, 3343–3348
- 25 Monsigny, M., Kieda, G. and Roche, A. C. (1983) *Biol. Cell* **47**, 95–110
- 26 Blanche, P. J., Geng, E. L., Forte, T. M. and Nichols, A. V. (1981) *Biochim. Biophys. Acta* **665**, 408–419
- 27 Stack, R. S. and Ray, W. H. (1985) *J. Polymer Sci.* **23**, 1393–1447
- 28 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350–356
- 29 Spiro, R. G. (1966) *Methods Enzymol.* **8**, 3–26
- 30 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 31 Bridges, K., Harford, J., Ashwell, G. and Klausner, R. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 350–354
- 32 Lehrman, M. A., Pizzo, S. V., Imber, M. J. and Hill, R. L. (1986) *J. Biol. Chem.* **261**, 7412–7418
- 33 Haltiwanger, R. S., Lehrmann, M. A., Eckhardt, A. E. and Hill, R. L. (1986) *J. Biol. Chem.* **261**, 7433–7439
- 34 Lehrman, M. A., Haltiwanger, R. S. and Hill, R. L. (1986) *J. Biol. Chem.* **261**, 7426–7432