Evidence in liver for a disulphide-linked scavenger receptor containing a binding site for acetylated low-density lipoprotein and maleylated bovine serum albumin*

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Membranes from rat liver were analysed under reducing conditions. The components of the soluble membranes responsible for the binding of acetylated low density lipoprotein (acetyl-LDL) and maleylated bovine serum albumin (Mal-BSA) were chromatographed on a polyethyleneimine–cellulose column and subsequently separated by gel electrophoresis. For both ligands a major binding protein ($M_r = 35000$) was revealed by ligand blotting. A minor protein ($M_r > 67000$) exhibited little binding. The Scatchard plot of the ^{125}I -Mal-BSA binding data of the 35 kDa protein was linear, with a K_d of 17.3 nM. High concentrations of acetyl-LDL competed for half of the ^{125}I -Mal-BSA binding. Excessive Mal-BSA competed for all the visible acetyl-LDL binding. The findings indicate the existence, in the reduced hepatic membrane, of a 35 kDa protein that has two binding sites for ^{125}I -Mal-BSA and one binding site for acetyl-LDL.

INTRODUCTION

Like macrophages, the sinusoidal endothelial cells of the liver express a 'scavenger receptor' (Goldstein *et al.*, 1979; Nagelkerke *et al.*, 1983; Blomhoff *et al.*, 1984; Pitas *et al.*, 1985), which binds acetylated low-density lipoprotein (acetyl-LDL), maleylated serum albumin (Mal-BSA) and several polyanions (Brown *et al.*, 1980). It has been hypothesized that the hepatic scavenger receptor of the sinusoidal cells has an important physiological role in removing modified LDLs from the circulation (Nagelkerke *et al.*, 1983; Brown & Goldstein, 1983; Dresel *et al.*, 1985a).

We have previously investigated the rat hepatic scavenger receptor *in vivo* and *in vitro*. The liver is the organ with the highest specific-uptake capacity for intravenously injected acetyl-LDL and Mal-BSA. The liver was therefore used as a tissue source for biochemical characterization of the scavenger receptor.

Under non-reducing conditions, a predominant 250 kDa acetyl-LDL-binding protein with an apparent Stokes radius of 8.5 nm (85 Å) could be solubilized and partially purified from rat liver membranes (Dresel *et al.*, 1985b). This 250 kDa protein was identified *in vitro* as an active scavenger receptor: (1) by inhibition of radioligand binding with poly(vinyl sulphate), (2) by demonstration of Mal-BSA and acetyl-LDL binding to a single common binding site, and (3) by competition experiments, which, comparable with *in vivo* results, demonstrated 20% inhibition of Mal-BSA binding by excess of acetyl-LDL antagonist and 90% inhibition of acetyl-LDL binding by Mal-BSA (Dresel *et al.*, 1987). A 260 kDa protein with high affinity for acetyl-LDL and

Mal-BSA was also detected in a macrophage-derived cell line (Via *et al.*, 1985).

The purpose of the present investigation was to study the scavenger-receptor structure and its ligand-binding sites under reducing conditions. We reasoned that the high-molecular-mass scavenger receptor could be a complex with multiple binding sites and disulphide-linked subunits in an oxidized state, and anticipated that we could demonstrate single binding subunits under reducing conditions. To test this hypothesis, solubilized membrane proteins containing scavenger receptors were incubated with β -mercaptoethanol, separated by gel electrophoresis under reducing conditions, and analysed by ligand blotting with ^{125}I -Mal-BSA and acetyl-LDL.

MATERIALS AND METHODS

Na^{125}I (10–20 mCi/ μg) was obtained from Amersham International (Braunschweig, Germany). Egg phosphatidylcholine, polyethyleneimine–cellulose, Triton X-114 and bovine serum albumin were from Serva, Heidelberg, Germany; phenylmethanesulphonyl fluoride (PMSF), octyl β -D-glucoside (OG) and 4-chloro-1-naphthol were from Boehringer, Mannheim, Germany. Poly(vinyl sulphate) was from Sigma, St. Louis, MO, U.S.A.; cellulose acetate filters (diameter 25 mm; pore size 45 μm ; catalogue no. HULP 025 00) were from Millipore Corp., Bedford, MA, U.S.A.; nitrocellulose paper was from Schleicher und Schüll. Electrophoresis reagents (acrylamide, methylenebisacrylamide, tetraethylmethylenediamine, ammonium persulphate) and molecular-mass standards were from BRL, Offenbach, Germany, and

Abbreviations used: acetyl-LDL, acetylated low-density lipoprotein; Mal-BSA, maleylated bovine serum albumin; OG, octyl β -D-glucoside; PMSF phenylmethanesulphonyl fluoride; NBS, *N*-bromosuccinimide; e.l.i.s.a., enzyme-linked immunosorbent assay.

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Fig. 1. Revelation of ^{125}I -Mal-BSA and acetyl-LDL binding sites to transblotted hepatic membrane proteins as a function of ligand concentration

Aliquots (100 μl) from the partially purified preparation of soluble hepatic membrane proteins (600 μg of protein) were chromatographed on Sephadex G-25 into Laemmli buffer + 0.1% β -mercaptoethanol. Samples (50 μl) were subjected to electrophoresis in a 10%-(w/v)-polyacrylamide slab gel containing 0.1% SDS. The proteins were transblotted subsequently to nitrocellulose paper. Each strip of nitrocellulose paper was incubated with 3.75, 7.5, 15 and 30 pmol of acetyl-LDL/ml (group A, lanes 1–4) and 3.6, 7.9, 22.5, and 72 pmol of ^{125}I -Mal-BSA (43800 c.p.m./pmol)/ml (group B, lanes 1–4). The ^{125}I -Mal-BSA blots were exposed to X-ray films for detection of the ligand binding. Acetyl-LDL blots were developed by the anti-LDL e.l.i.s.a. described by us previously (Dresel *et al.*, 1984). Lane O shows a probe stained with Amido Black. Molecular-mass standards are indicated.

Bio-Rad, Munich, Germany. Trasylol (100000 kallikrein-inactivating units/10 ml) was from Bayer, Leverkusen, Germany.

Iodination of Mal-BSA and acetylation of LDL

LDL was prepared from normal blood donors by sequential ultracentrifugation (Havel *et al.*, 1955). Acetyl-LDL was prepared by the method of Basu *et al.* (1976). Lipoprotein concentration was expressed in terms of protein content. BSA was maleylated as described by Imber *et al.* (1982); maleylation was measured by the method of Fields (1972). In our experiment, 50 out of 59 lysine residues of the albumin molecule were maleylated. Radioiodination of Mal-BSA and *N*-bromosuccinimide (NBS) was performed with ^{125}I as described by Sinn *et al.* (1988).

Preparation of scavenger receptor from rat liver

The preparation of the scavenger receptor from rat liver was carried out as previously described (Dresel *et al.*, 1985a,b). Two rat livers were homogenized in 50 mM-Tris/HCl (pH 8)/150 mM-NaCl/PMSF (23 μg /ml)/0.2% (v/v) β -mercaptoethanol/Trasylol (1:1000, w/w) (TN buffer; 5 ml/g of tissue) and centrifuged at 200 *g* for 5 min at 20 °C. Membranes were pelleted from the supernatant by high-speed ultracentrifugation at 100000 *g* for 1 h. The membrane pellet was solubilized in TN buffer containing 1% (v/v) Triton X-114 and

fractionated by phase separation at 37 °C (Bordier, 1981). The amphiphilic phase was chromatographed on polyethyleneimine-cellulose and eluted under reducing conditions by a high-salt step (TN buffer containing 1000 mM-NaCl and 40 mM-OG). The scavenger-receptor-dependent binding was determined in portions of the eluate fractions after reconstitution into egg phosphatidylcholine and subsequent incubation with ^{125}I -Mal-BSA (4 μg /ml), using the membrane-filter assay described by Dresel *et al.* (1987). Non-specific binding was measured in presence of poly(vinyl sulphate) (10 μg /ml). Aliquots (100 μg) were applied to Sephadex G-25 columns in small syringes and eluted by centrifugation at 2000 *g*.

Gel electrophoresis and ligand blotting

The soluble scavenger-receptor samples were subjected to SDS/polyacrylamide slab-gel electrophoresis by the method of Laemmli (1970). The resulting gels were equilibrated in transfer buffer, pH 8.3, containing 20 mM-Tris, 150 mM-glycine and 20% (v/v) methanol. The proteins were electroblotted on to nitrocellulose (12 h, 0.25 A) by using a Bio-Rad Transblot unit (Bio-Rad, Munich, Germany) at 4 °C. Transfers were blocked by 1 h incubation with 5% (w/v) BSA and probed for 30 min at 21 °C with acetyl-LDL or ^{125}I -Mal-BSA as described previously. In some assays, poly(vinyl sulphate) was included to control the binding specificity of the ligands. The acetyl-LDL blots were developed by sequential incubation with rabbit anti-LDL IgG (5 μg /ml) and goat anti-rabbit IgG-horseradish peroxidase conjugate (1:1000, v/v) for 60 min at 21 °C with 0.018% 4-chloro-2-naphthol and 10 μl of H_2O_2 /50 ml. The ^{125}I -Mal-BSA blots were developed by autoradiography on Kodak XR-5 film.

RESULTS

Revelation of ^{125}I -Mal-BSA and acetyl-LDL binding to electrophoretically separated hepatic membrane proteins

Hepatic membrane proteins were extracted with Triton X-114 and partially purified by ion-exchange chromatography on a polyethyleneimine-cellulose column as described by Dresel *et al.* (1985a,b). The membrane proteins eluted from the ion-exchange column by a high-salt step were assayed for acetyl-LDL- and ^{125}I -Mal-BSA-binding activity. The active fractions were pooled and reduced with 1% β -mercaptoethanol in Laemmli (1970) buffer, subjected to 0.1%-SDS/polyacrylamide-gel electrophoresis and transferred to nitrocellulose paper for ligand blotting with ^{125}I -Mal-BSA and acetyl-LDL. Saturation binding with increasing concentrations of ^{125}I -Mal-BSA was detected by autoradiography, and acetyl-LDL binding was assayed by an anti-LDL e.l.i.s.a., as described by Dresel *et al.* (1984). Binding of ^{125}I -Mal-BSA and acetyl-LDL to a 35 kDa protein was saturable, with increasing concentrations of both ligands, at approx. 72 pmol of ^{125}I -Mal-BSA/ml and 30 pmol of acetyl-LDL/ml, as judged by visual inspection of the blots (Fig. 1). The ^{125}I -Mal-BSA binding was analysed quantitatively by cutting out the 35 kDa region of the nitrocellulose strips and counted directly with a γ -radiation counter. For determining non-specific binding, the 18 kDa region of the same strip, which had no visible binding of the radioligand to a protein band, was counted for radioactivity as a reference and

Fig. 2. Binding of ^{125}I -Mal-BSA to the 35 kDa binding protein

The 35 kDa region of each ^{125}I -Mal-BSA ligand blots in Fig. 1, group B was excised to determine the total binding (●). The 18 kDa region without a visible band in the autoradiogram was cut out to measure non-specific binding (□). To determine specific binding (■), the non-specific binding was subtracted from total binding. (a) Saturation binding profile; (b) (inset) Scatchard analysis of specific binding data.

subtracted from the radioactivity of the 35 kDa band. The specific binding component and the dissociation constant, K_d , for high-affinity binding was determined by Scatchard analysis (Fig. 2). The high-affinity binding was 75 fmol of ^{125}I -Mal-BSA/mg of protein applied to the gel, with an apparent K_d of 17.3 nM.

Competition between ^{125}I -Mal-BSA and acetyl-LDL binding to the 35 kDa protein when an excess of the second ligand was used

The competition for acetyl-LDL binding to the 35 kDa protein from rat liver membrane when excess Mal-BSA was used is shown in Fig. 3. Blots on nitrocellulose paper with equal amounts of hepatic membrane protein were incubated with acetyl-LDL (30 pmol/ml) in the absence of Mal-BSA as a control and in the presence of 460, 920, 1840 pmol of Mal-BSA/ml (group A). The binding was determined by anti-LDL e.l.i.s.a. Saturation of acetyl-LDL binding was indicated by an intensive band at 35 kDa in the control (lane 1). With increasing Mal-BSA concentrations, the 35 kDa band disappeared, although the background of the blot was not significantly changed (lanes 2–4). Thus the disappearance of the 35 kDa-binding activity is due to competitive Mal-BSA binding at this site.

The competition for Mal-BSA binding to the 35 kDa protein by excess acetyl-LDL is shown in Fig. 3 (group B). Incubation of nitrocellulose strips with equal amounts of hepatic membrane protein with 58.9 pmol of ^{125}I -Mal-BSA/ml showed saturated binding of the radioligand at 35 kDa (lane 1). ^{125}I -Mal-BSA binding was only partially inhibited by excess acetyl-LDL. As judged by visual inspection, the intensity of the 35 kDa band was diminished only slightly (lanes 2–4). To obtain quantitative information, the 35 kDa area of the radioblots was cut out of the blots and counted for radioactivity directly. The relative amounts of the ^{125}I -Mal-BSA detected at each concentration of acetyl-LDL competitor in the blot were correlated with the expected occupancy of the 35 kDa area (Fig. 4). A decrease in ^{125}I -Mal-BSA binding of about 55% was caused by a 16-fold excess of

Fig. 3. Inhibition of the binding of acetyl-LDL to hepatic membrane proteins by an excess of Mal-BSA and of the ^{125}I -Mal-BSA binding by an excess of acetyl-LDL (inhibition by a second ligand)

The samples of the hepatic membrane proteins were prepared for gel electrophoresis as described in Fig. 1. Aliquots (50 μl) containing 300 μg of protein were separated by electrophoresis on 10%-(w/v)-polyacrylamide gels containing 0.1% SDS and transblotted to nitrocellulose strips. Group A: competition for acetyl-LDL binding by Mal-BSA. Nitrocellulose strips were incubated with 0, 460, 920, 1840 pmol of Mal-BSA/ml for 15 min and subsequently with 30 pmol of acetyl-LDL/ml and the same Mal-BSA concentrations for a further 45 min. Group B: competition of ^{125}I -Mal-BSA binding by acetyl-LDL. Nitrocellulose strips were incubated with 0, 120, 480, 960 pmol of acetyl-LDL/ml for 15 min and subsequently with 58.9 pmol of ^{125}I -Mal-BSA/ml (43 800 c.p.m./pmol) and the same acetyl-LDL concentrations for a further 45 min. To measure non-specific binding a nitrocellulose strip was incubated with ^{125}I -Mal-BSA in presence of a polyanionic inhibitor. Group C: binding of ^{125}I -Mal-BSA (29.4 pmol/ml) in presence of 10 μg of poly(vinyl sulphate)/ml. Autoradiography was performed with the radioblots; for the detection of the bound acetyl-LDL, the anti-LDL e.l.i.s.a (Dresel *et al.*, 1984) was used. Lane O shows a probe stained with Amido Black. Molecular-mass standards are indicated.

unlabelled acetyl-LDL. Poly(vinyl sulphate), a known polyanionic inhibitor (Brown *et al.*, 1980), inhibited visible ^{125}I -Mal-BSA binding completely at a concentration of 10 $\mu\text{g}/\text{ml}$ (Fig. 3; group C).

DISCUSSION

Previous studies *in vivo* and *in vitro* have shown that the hepatic scavenger receptor binds acetyl-LDL and Mal-BSA at the same binding site with a K_d of 0.8 nM and 15 nM respectively. Under non-reducing conditions, a predominant ~250 kDa protein with specific Mal-BSA and acetyl-LDL binding, purified 1450-fold, has been identified by ligand blotting. Binding of both ligands to this 250 kDa protein could be blocked by poly(vinyl sulphate), whereas ^{125}I -Mal-BSA binding could be only partially (<20%) diminished by excess acetyl-LDL (Dresel *et al.*, 1987). Similar results were obtained *in vivo*.

In the present paper we report that there is high-affinity binding of acetyl-LDL and ^{125}I -Mal-BSA to reduced hepatic membrane proteins, determined by

Fig. 4. Inhibition of ^{125}I -Mal-BSA binding at the 35 kDa binding protein by excess acetyl-LDL

The bands corresponding to 35 kDa from the experiment in Fig. 3, group B, were excised to determine the ^{125}I -Mal-BSA with a γ -radiation counter. Specific ^{125}I -Mal-BSA binding was determined by subtraction of the radioactivity determined at 18 kDa in the same lane from the ^{125}I -Mal-BSA in the 35 kDa band.

ligand blotting. Blot analyses by anti-LDL e.l.i.s.a. for acetyl-LDL and by autoradiography for ^{125}I -Mal-BSA demonstrate a highly specific binding of both ligands to a 35 kDa binding protein. The affinity of this 35 kDa-protein binding site on the ligand blot is remarkably similar to that reported for the high-molecular-mass form. We conclude that the reduced 35 kDa protein is a close homologue of the 250 kDa scavenger receptor with respect to ligand binding specificity and sensitivity to specific inhibitors. A band of $M_r > 67000$, to which binding of acetyl-LDL is competed for by Mal-BSA, and to which binding of ^{125}I -Mal-BSA is not (or is only partially) competed for by acetyl-LDL, shows little binding. This binding protein could correspond to a dimer of the reduced 35 kDa binding protein. The other reduced membrane proteins gave little, if any, binding.

Reduction of the sulphide bridges appears to be a necessary condition for demonstration of the 35 kDa protein and for a decrease in the binding activity of the high-molecular-mass species. Thus disulphide-bridged octamers of the 35 kDa binding protein or a heteromer containing the 35 kDa protein might constitute the high-molecular-mass scavenger receptor. We have previously proposed that the high-molecular-mass scavenger receptor has a quaternary structure, in which all the binding sites are available for Mal-BSA, but not for the larger lipoprotein ligand (Dresel *et al.*, 1987). The present experiments indicate that the high-affinity binding does not depend critically on the quaternary structure and intact disulphide bonds. The present finding that about half of the Mal-BSA binding to the 35 kDa protein could be competed for by excess acetyl-LDL demonstrates that cleavage of the disulphide bonds might change the binding stoichiometry of the scavenger receptor. The difference in binding stoichiometry for both ligands should depend on the structure of the 35 kDa protein

itself. The partial inhibition of ^{125}I -Mal-BSA binding by acetyl-LDL remains an apparent paradox, since the earlier experiments provided evidence for just a single binding site for both ligands on the high-molecular-mass scavenger receptor. On the basis of the present data, it could be that the reduced 35 kDa protein has very similar binding sites for Mal-BSA, only one of them interacting with the large acetyl-LDL particle. It is also possible that Mal-BSA is somehow processed by the 35 kDa protein to make it accessible to a second binding site. Further studies with both ligands will require affinity-purified 35 kDa and > 67 kDa binding protein.

It is of interest to note that irradiation-inactivation studies of mouse macrophage-derived tumours have suggested a molecular mass for the functional unit of the scavenger receptor of 32–35 kDa (D. P. Via, S. Vignale & E. S. Kempner, unpublished work). The existence of a similar lower-molecular-mass protein of the mouse macrophage tumour is also being explored.

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