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Purification and characterization of two high-density-lipoprotein-binding proteins from rat and human liver

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The liver plays a major role in the metabolism of plasma high-density lipoprotein (HDL). Several groups have postulated, but others refuted, the existence of a classical membrane receptor which recognizes HDL. In the present study, we identified and purified two liver HDL-binding proteins of 120 kDa (HB_1) and 100 kDa (HB_2), with apparent specificity for HDL_3 devoid of E apolipoprotein. The plasma membrane was the richest source of the HDL-binding protein. Both proteins bound A-I and A-II apolipoproteins and retained HDL-binding activity after final purification. HB_1 activity, but not that of HB_2, was lost after treatment with β-mercaptoethanol, but reduction did not change the apparent molecular mass of either protein. Antibodies against HB_1 or HB_2 did not cross-react, and preliminary structural investigations provide evidence to suggest that HB_1 and HB_2 are not structurally related. We thus provide evidence for at least two liver plasma-membrane proteins which bind HDL apolipoproteins, suggesting that protein–protein interaction participates to some degree in the mechanism of HDL recognition by cells.

INTRODUCTION

The mode of interaction between HDL and cells remains unsolved. Several laboratories have reported on the binding of radiolabelled HDL to cells in recent years [1–8], and the processing of HDL *in vivo* has also been the subject of many recent investigations [9–12]. One of the outstanding questions still not satisfactorily resolved is whether or not a conventional receptor–ligand interaction is responsible for the cellular processing and consequent regulation of HDL metabolism in the body. Indeed, there is evidence which both supports [13–15] and refutes [16,17] the existence of the putative HDL receptor, and the acceptance of such an entity will ultimately depend on the isolation and characterization of a cellular component or complex which satisfies the physiological criteria characteristic of the role of a cell receptor.

Several groups involved in a search for the HDL receptor have identified membrane proteins from various tissues which bind HDL. Our group reported an HDL-binding protein present in adrenal-cortical cells [18], and Graham & Oram [19] found evidence of HDL-binding proteins in a number of cell types and which could be influenced by altering the cellular cholesterol content. Keso *et al.* [20] also recently identified a protein in human placenta which binds apo A-I, and Barbaras *et al.* [21] found evidence of two HDL-binding bands in cultured mouse Ob 1771 cells.

In order to characterize HDL–cell binding events further, we have focused on the liver membrane, because evidence suggests that this organ plays a major role in processing HDL [11,12,22]. We reasoned that the concentration of a putative receptor would be highest in the plasma-membrane fraction, and this paper describes the identification and purification of two HDL-binding proteins isolated from rat liver plasma membrane.

METHODS AND MATERIALS

Isolation of lipoproteins

HDL_3 (d 1.12–1.21) and LDL (d 1.019–1.055) were isolated from human serum by ultracentrifugation as described previously [23]. Lipoproteins were washed by re-centrifugation at the appropriate densities until homogeneous by electrophoresis and after establishing the apolipoprotein pattern by SDS/polyacrylamide-gel electrophoresis. Apo A-I and apo A-II were isolated from delipidated HDL after gel-filtration chromatography on Sephadex G-150 columns (5 cm × 250 cm) equilibrated in 6 M-urea/0.05 M-Tris/HCl, pH 8.0.

Lipoproteins and apoproteins were [^{125}I]iodinated by the iodine monochloride method [24] as described previously [25]. The specific radioactivity ranged from 180 to 380 c.p.m./ng of protein.

Preparation of plasma membrane from rat and human liver

Plasma membranes were prepared with minor modifications of the method described by Fleischer & Kervina [26]. Rats (150–220 g) were fasted overnight and killed by cervical dislocation. Briefly, the rat livers were perfused *in situ* with 150 mM-NaCl/1 mM-phenylmethanesulphonyl fluoride, pH 7.4, then removed and immersed in ice-cold 10 mM-Hepes (pH 7.5)/0.25 M-sucrose (homogenization buffer). Human liver sections (1–3 g) were washed in homogenization buffer. The liver was diced with a pair of scissors and placed into 5 vol. (v/w) of the same buffer and homogenized in a Potter–Elvehjem-type homogenizer. Three strokes with a pestle of 0.026 in (0.66 mm) clearance were followed by three strokes with a pestle of 0.012 in (0.30 mm) clearance. The homogenate was filtered through a nylon mesh and centrifuged at 1000 *g* for 10 min. The pellet was

Abbreviations used: HDL, high-density lipoprotein; LDL, low-density lipoprotein; apo, apolipoprotein; PBS, phosphate-buffered saline [10 mM-sodium phosphate buffer (pH 7.2)/150 mM-NaCl].

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resuspended in the homogenization buffer with a Dounce homogenizer, and high-density sucrose (2.4 M-sucrose/10 mM-Hepes, pH 7.5) was added to give a final concentration of 1.45 M. The mixture was transferred into the centrifuge tubes, overlayered with homogenization buffer, and centrifuged at 80000 *g* for 70 min. The button pellet at the interface was removed, resuspended in homogenization buffer and washed twice by centrifugation at 2000 *g* for 10 min. The pellet was finally resuspended in homogenization buffer, high-density sucrose added to a final concentration of 1.35 M, and after being overlayered with homogenization buffer, centrifuged at 250000 *g* for 70 min. The button pellet was again washed and resuspended in the homogenization buffer, and designated as the plasma-membrane-rich fraction, which was quickly frozen in liquid N_2, and stored at −70 °C.

Electron-microscopic examination of the plasma membranes revealed typical vesicles with diameters of 200–500 nm and less than 2% contamination with mitochondria and other subcellular fractions. In addition, purity of the plasma membranes was assessed by using the marker enzyme alkaline phosphatase [27]. Specific activity of this enzyme was 10-fold higher in the plasma-membrane fraction compared with the whole homogenate.

Purification of HDL-binding protein

Solubilization of membrane proteins. The suspension of plasma membranes (approx. 500 mg in 10–20 ml) was diluted with 20 mM-Tris/HCl (pH 8.0) to a final volume of 50 ml. CHAPS was then added to a final concentration of 20 mM. The mixture was stirred at 4 °C for 15 min, and insoluble material was removed by centrifugation at 5000 *g* for 15 min.

DEAE-Sephacel chromatography. The CHAPS-solubilized sample (approx. 500 mg of protein) was applied to a DEAE-Sephacel column (1.5 cm × 27 cm), previously equilibrated with running buffer (20 mM-Tris/HCl/10 mM-CHAPS, pH 8.0). The column was first washed with 50 ml of running buffer, and proteins were eluted with 75 ml of 150 mM-NaCl in running buffer, followed by 50 ml each of first 200 mM- and then 400 mM-NaCl in running buffer at a flow rate of 28.4 ml/h. Fractions (7.1 ml each) were collected and assayed for HDL_3-binding activity by ligand blotting as described below.

Preparative SDS/polyacrylamide-gel electrophoresis. The fractions containing HDL_3-binding activity were pooled, and saturated $(NH_4)_2SO_4$ (pH 7.4) was added to a final concentration of 60% saturation. The mixture was centrifuged at 5000 *g* for 20 min. The pellet was dissolved in 0.1 M-Tris/HCl, pH 6.8, containing 4% SDS, and centrifuged to remove insoluble material. The supernatant was loaded into a wide sample well (15 cm) of a 10%-acrylamide slab gel (16 cm × 14 cm × 0.3 cm). After electrophoresis, a slice of gel was removed for ligand-blotting assay, and the remainder was stained with Coomassie Brilliant Blue R-250. Bands containing proteins which demonstrated HDL_3-binding activity were cut out, and isolated by electroelution [28]. The electroeluates were again subjected to preparative SDS/polyacrylamide-gel electrophoresis, and HDL-binding proteins were detected and eluted by the same process as described above.

Precipitation of HDL_3-binding protein. Electroeluates containing binding protein, SDS and Coomassie Blue were extracted with 10 vol. of cold methanol or acetone, placed in a freezer at −20 °C for 16 h and then centrifuged at 3000 *g* for 15 min to precipitate the protein. The precipitations were washed twice with cold methanol or acetone.

Ligand-blotting assay for HDL-binding protein

Samples of plasma membrane (250 μg) or of DEAE-Sephacel fractions (50–100 μg) were loaded on 10%-polyacrylamide gels (containing SDS) and electrophoretically separated by the method of Laemmli [29]. Separated proteins were electrophoretically transferred on to nitrocellulose membranes (Schleicher and Schuell) [30], which were then incubated with blocking buffer [50 mM-Tris/HCl (pH 7.4) containing 90 mM-NaCl and 3% (w/v) casein] for 1 h, followed by incubation for 1 h in the same buffer containing 10 μg of ^{125}I-labelled HDL_3/ml, ^{125}I-labelled apoA-I or ^{125}I-labelled apoA-II. After washing twice for 5 min with PBS (pH 7.4) containing 0.1% Tween 20 and then for 3 × 5 min with PBS, the membrane was exposed to Hyperfilm-MP (Amersham) for 48–60 h at −70 °C in a cassette containing Cronex Lightning Plus intensifying screens (DuPont).

Antisera

Antibodies to human apolipoproteins were prepared by injecting rabbits as described previously [31]. Antibodies against rat HDL_3-binding proteins were also produced in rabbits. To electroeluate solutions containing 5–10 μg of HDL_3-binding protein (see above), 50 μl of rabbit serum was added as carrier, mixed, and 10 vol. of acetone was added to precipitate proteins as described above. After washing once with acetone, the protein was dissolved in 0.5 ml of 0.15 M-NaCl, pH 7.4, and 0.5 ml of Freund's complete adjuvant was added to prepare emulsions for subcutaneous injection into multiple sites of rabbits. Booster injections were given twice more every 3 weeks before antibodies were detected by Western blotting [30].

Protein methods

Amino acid analysis was performed on acetone-precipitated proteins electroeluted from SDS/polyacrylamide gels. They were dissolved in 70% (v/v) formic acid, transferred to hydrolysis tubes, dried under vacuum and then hydrolysed in 6 M-HCl after evacuating and sealing the tubes. After hydrolysis for 22 h at 110 °C, the HCl was removed by rotary evaporation and the amino acid composition determined by using the Beckman 6300 amino acid analyser.

RESULTS

Plasma membranes from rat liver and human liver were solubilized with electrophoresis buffer, and separated by SDS/polyacrylamide-gel electrophoresis, transferred on to nitrocellulose membranes, and incubated with ^{125}I-labelled HDL_3. Two major HDL_3-binding proteins (HB)

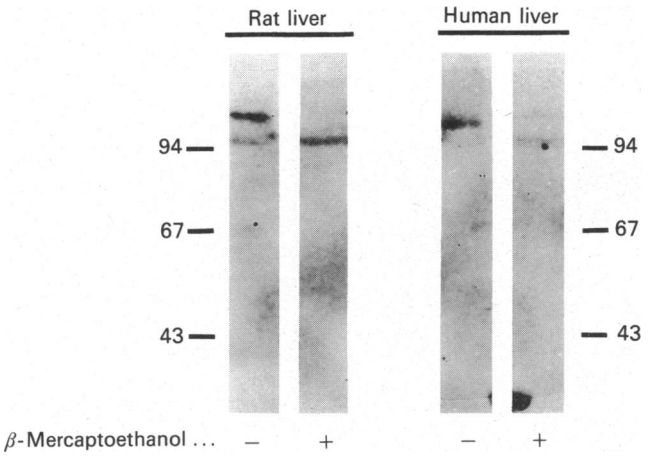

Fig. 1. Ligand blotting of the HDL_3-binding proteins

Samples (250 μg) of proteins from rat and human liver plasma membranes were incubated with 62.5 mM-Tris/HCl (pH 6.8)/2% SDS in the absence (−) or presence (+) of 5% (v/v) β-mercaptoethanol. The samples were applied to SDS/10%-polyacrylamide gels, and after electrophoresis the proteins were transferred to a nitrocellulose membrane, which was then incubated with 10 μg of ^{125}I-labelled HDL_3/ml for 1 h. The binding proteins were detected by autoradiography. Molecular masses of proteins (kDa) were determined by comparison with standards as indicated.

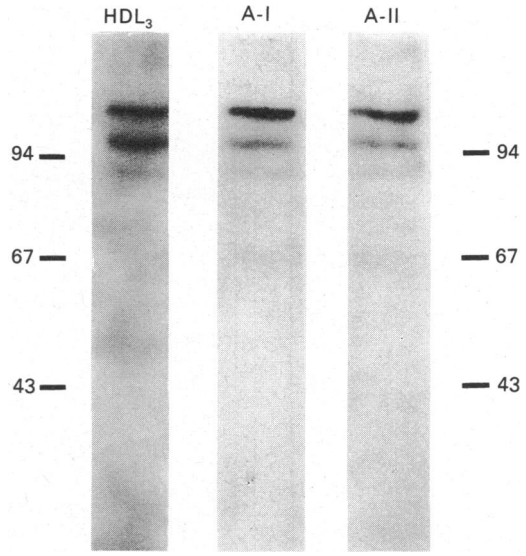

Fig. 3. Apolipoprotein specificity

Rat liver plasma membranes (250 μg of protein/lane) were fractionated on SDS/10%-polyacrylamide gels, and transferred to nitrocellulose membranes. These strips were incubated with 10 μg of ^{125}I-labelled HDL_3/ml (left lane), 5 μg of ^{125}I-labelled apoA-I/ml (middle lane) or 5 μg of ^{125}I-labelled apoA-II (right lane)/ml. Positions of molecular-mass standards (kDa) are indicated.

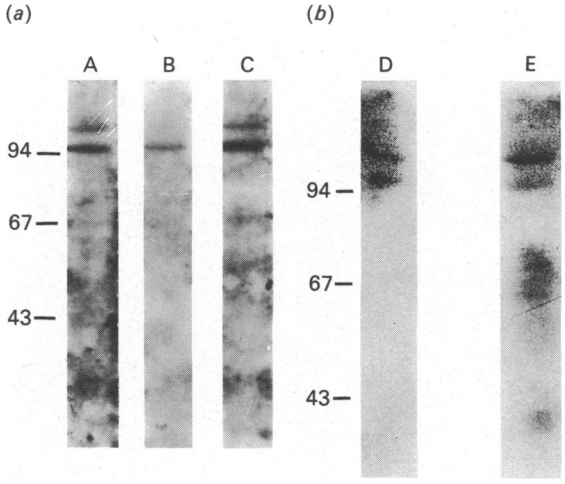

Fig. 2. Competitive binding study (*a*) and effect of EDTA on the binding activity of HDL_3-binding proteins (*b*)

(*a*) Rat liver plasma membranes (250 μg of protein/lane) were fractionated by electrophoresis on SDS/10%-polyacrylamide gels, and then transferred to a nitrocellulose membrane. Nitrocellulose strips were incubated with 10 μg of ^{125}I-labelled HDL_3/ml with no lipoprotein (lane A), 500 μg of HDL_3/ml (lane B) or 500 μg of LDL/ml (lane C). Molecular-mass standards (kDa) are indicated. (*b*) Rat liver plasma membranes (250 μg of protein/lane) were applied to SDS/10%-polyacrylamide gels, and transferred to a nitrocellulose membrane. Nitrocellulose strips were incubated with 10 μg of ^{125}I-labelled HDL_3/ml in the absence (D) or presence (E) of 10 mM-EDTA (disodium salt). Positions of molecular-mass standards (kDa) are indicated.

were identified, with molecular masses of approx. 120 kDa (HB_1) and 100 kDa (HB_2) (Fig. 1). Most enrichment of HDL-binding proteins was found in the plasma-membrane fraction, and was practically not detectable in the mitochondrial fraction (results not shown). After reduction with 5% β-mercaptoethanol, binding of ^{125}I-labelled HDL_3 to the 120 kDa protein was markedly decreased, but the 100 kDa binding protein retained binding activity (Fig. 1).

To characterize the specificity of these binding proteins, competitive binding studies were carried out. A 50-fold excess of unlabelled HDL_3 markedly decreased binding of ^{125}I-labelled HDL_3 to both binding proteins, whereas a 50-fold excess of unlabelled LDL resulted in minimal competition of ^{125}I-HDL_3 binding (Fig. 2, lanes A–C). However, 10 mM-EDTA failed to inhibit the binding of ^{125}I-labelled HDL_3 to both binding proteins (Fig. 2, lanes D and E). The same proteins also bound ^{125}I-labelled apoA-I or ^{125}I-labelled apoA-II (Fig. 3).

Anion-exchange chromatography on DEAE-Sephacel produced significant enrichment of fractions containing HDL_3-binding proteins. A typical separation is shown in Fig. 4. After fractions were analysed for ^{125}I-HDL_3-binding activity by ligand-blotting assay, the major activity of the 100 kDa binding protein was found to be eluted at approx. 200 mM-NaCl, whereas activity of the 120 kDa binding protein was eluted in a broader salt concentration in the range 200–400 mM-NaCl. When the 120 kDa binding protein was eluted with smaller stepwise concentrations of NaCl (200–400 mM at 50 mM intervals), the ^{125}I-HDL_3-binding activities were detected in all fractions (results not shown). No activity was found in the unbound protein fraction.

Preparative SDS/polyacrylamide-gel electrophoresis

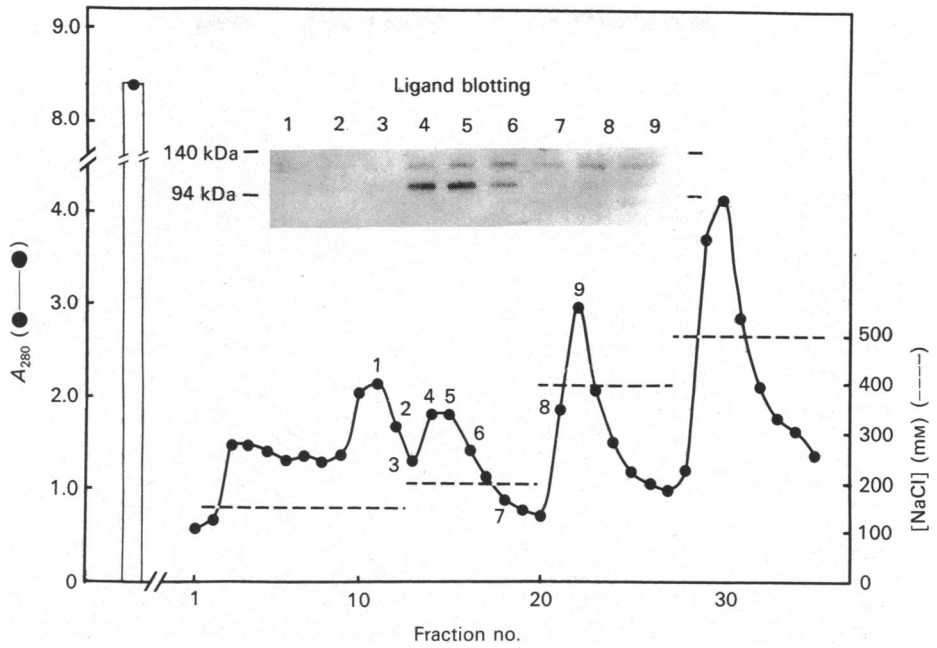

Fig. 4. DEAE-Sephacel chromatography of solubilized rat liver plasma membrane

Solubilized rat liver plasma membrane (500 mg) was applied to a DEAE-Sephacel column (1.5 cm × 27 cm) equilibrated with 20 mM-Tris/HCl, pH 8.0, as described in the text. Unbound protein was collected as a single fraction, and the protein was then eluted with stepwise concentrations of NaCl, shown by broken lines, as described in the Methods and materials section. The inset shows the ligand-blotting assay of DEAE-Sephacel fractions, and the number of each lane corresponds to the fraction number in the elution profile.

was used for final purification of HDL-binding proteins. Before electrophoresis, active DEAE-Sephacel fractions were precipitated with 60%-satd. $(NH_4)_2SO_4$ which resulted in full recovery of binding activity. Binding proteins, which were identified by the ligand-blot assay, were cut out, electroeluted and subjected to repetitive SDS/polyacrylamide-gel electrophoresis. This resulted in purification to apparent homogeneity, as shown after analysis by silver staining of SDS/polyacrylamide gels (Fig. 5). The apparent loss of mass from HB_1 after reduction and silver staining was never accompanied by the appearance of smaller subunits. We have concluded that treatment of HB_1 with β-mercaptoethanol before electrophoresis decreases its silver-staining properties, since little decrease in intensity was observed after staining with Coomassie Blue (results not shown). Both proteins retained ^{125}I-HDL_3-binding activity (Fig. 6). However, after reduction with β-mercaptoethanol, losses of binding activity were observed with the purified 120 kDa binding protein, but not the 100 kDa binding protein (Fig. 6).

The amino acid compositions of the two HDL-binding proteins, shown in Table 1, reveal differences between the HDL-binding proteins of 120 kDa (HB_1) and of 100 kDa (HB_2) in serine, glutamic acid and valine residues, suggesting that these two proteins are different polypeptides. This suggestion is strengthened by the results of Western blotting shown in Fig. 7. Antibodies raised against HB_1 reacted only against the HB_1 antigen, and conversely antibodies prepared against HB_2 only reacted with HB_2 and not with HB_1.

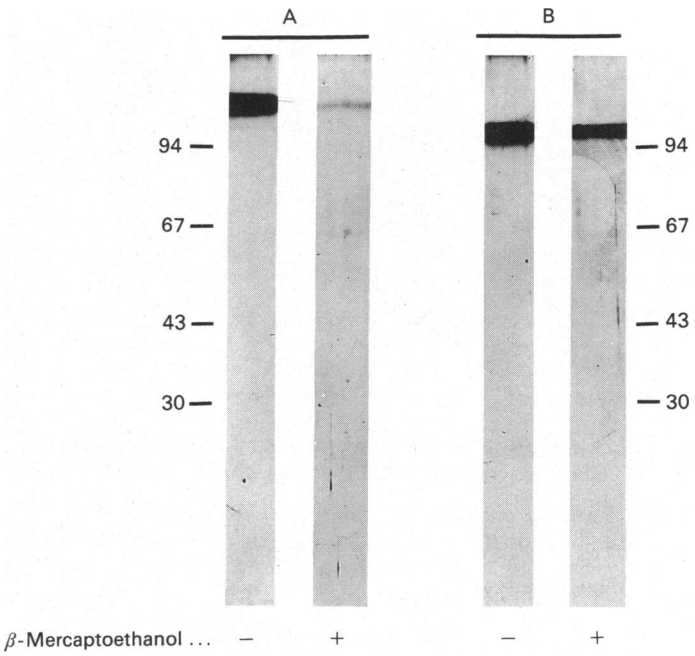

Fig. 5. Silver stain of SDS/polyacrylamide gels of the purified rat liver HDL_3-binding proteins

Purified HB_1 (A) and HB_2 (B) were incubated with 62.5 mM-Tris/HCl (pH 6.8)/2% SDS in the absence (−) or presence (+) of 5% β-mercaptoethanol, and applied to SDS/10%-polyacrylamide gels. Positions of molecular-mass standards (kDa) are indicated.

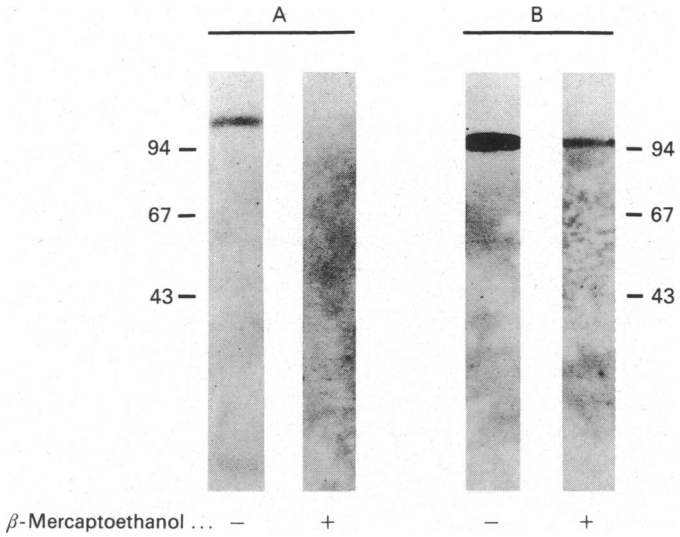

Fig. 6. Effect of reducing agent on the binding activity of the purified rat liver HDL_3-binding proteins

The purified HB_1 (A) and HB_2 (B) were incubated with 62.5 mM-Tris/HCl (pH 6.8)/2% SDS in the absence (–) or presence (+) of 5% β-mercaptoethanol. After electrophoresis on SDS/10%-polyacrylamide gels, proteins were transferred to a nitrocellulose membrane. The membrane was incubated with 10 μg of ^{125}I-labelled HDL_3/ml for 1 h and the binding proteins were detected by autoradiography. Molecular-mass standards (kDa) are indicated.

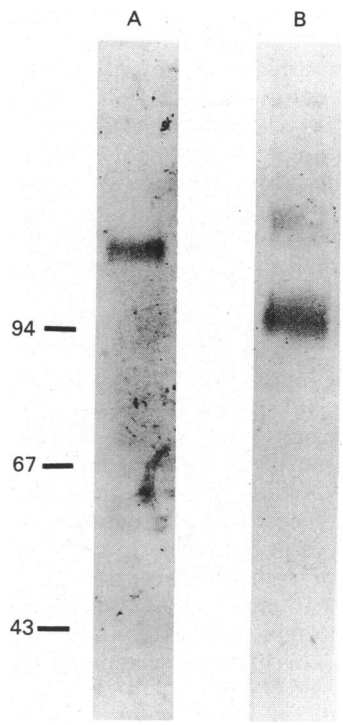

Fig. 7. Immunoblotting of the HDL_3-binding proteins

Rat liver plasma membranes (75 μg of protein/lane) were applied to SDS/10%-polyacrylamide gels, and after electrophoresis were transferred to a nitrocellulose membrane. These were incubated with anti-HB_1 (lane A) or anti-HB_2 (lane B) antibodies raised against purified HDL_3-binding proteins from rat liver. After incubation, the strips were incubated with peroxidase-conjugated anti-rabbit IgG. Molecular-mass standards (kDa) are indicated.

Table 1. Amino acid composition of the rat liver membrane HDL_3-binding proteins

Two different preparations of HB_1 were studied. Abbreviation: ND, not determined.

Amino acid	Composition (mol%)		
	HB_1	HB_1	HB_2
Asp	11.8	12.2	12.8
Thr	6.7	7.0	5.9
Ser	9.1	8.3	14.7
Glu	16.5	16.2	19.9
Pro	5.6	5.4	4.3
Gly*	–	–	–
Ala	10.2	10.1	10.3
Cys†	–	–	–
Val	8.3	9.0	5.7
Met†	–	–	–
Ile	5.5	5.8	4.1
Leu	8.9	9.3	7.5
Tyr†	–	–	–
Phe	3.5	3.4	2.8
Lys	7.4	7.4	6.2
His	2.0	1.7	1.8
Arg	3.8	3.5	2.9
Trp	ND	ND	ND

* Values for glycine are omitted, because glycine present in the electrophoresis buffers led to overestimates of this residue.
† Values too low for accurate determination.

DISCUSSION

The present study has resulted in the purification and characterization of two HDL_3-binding proteins present in rat liver plasma membranes. These proteins, which show apparent molecular masses of 120 and 100 kDa respectively, were different polypeptides according to immunochemical identification and amino acid composition, strengthening the suggestion that more than one membrane protein may be involved in the interaction between HDL_3 and cells. In a previous report from this laboratory [18] we found evidence for the existence of a binding protein in sheep adrenal-cortical membranes of approx. 78 kDa, and a similar protein was identified in human placental extracts by Graham & Oram [19]. However, this protein was barely detectable in rat liver membranes [18], and we suggested that structural differences between the liver and adrenal binding protein may have accounted for its inability to react in the assay system. The present findings, based on a more sensitive and specific ligand-blot assay utilizing radiolabelled HDL_3, instead of an antibody-detection system, revealed the bands identified as HB_1 and HB_2 in this paper and little evidence for the 78 kDa binding protein in rat liver.

The advantages of the present assay system enable the specificity of the binding proteins to be investigated. Incubations in which excess unlabelled HDL_3, LDL or EDTA were included with ^{125}I-labelled HDL_3 showed

that only HDL_3 significantly decreased radiolabelling of both HB_1 and HB_2. Although this detection system is not quantitative, it does, however, reveal involvement of a significant specific component in HDL_3 binding to these membrane proteins, which is consistent with the data obtained from cultured cell systems [1–8]. The identity of the two proteins differed according to immunochemical and peptide analysis, demonstrating that glycosylation or proteolytic cleavage of one common polypeptide is an unlikely explanation for the existence of these two HDL_3-binding proteins.

These data support previous observations from other laboratories. A protein of 110 kDa in fibroblast membranes which binds HDL_3 has been identified by Graham & Oram [19], who also showed that the same protein was expressed by a variety of cultured cells, including rat hepatocytes. Although the 110 kDa peptide was the dominant binding protein, minor HDL-binding proteins were observed with apparent molecular masses of 70–80 kDa in several cell types, and the major HDL_3-binding protein of human placenta was of approx. 80 kDa. Of further interest was the identification of an apo A-I-binding protein of 120 kDa in human placenta, by Keso *et al.* [20]. This protein was reduced to two bands of approx. 50 and 30 kDa that did not bind apoA-I, after treatment with β-mercaptoethanol. In our studies, although observing a decrease in the intensity of silver-stained HB_1 after treatment with β-mercaptoethanol, we found no evidence of disulphide-linked subunits of any size including 50 or 30 kDa proteins. In a study carried out to characterize apo A-I/A-II-binding sites in differentiated Ob 1771 cells, Barbaras *et al.* [21] used bivalent cross-linking with ^{125}I-labelled ligands and identified binding complexes of 130 and 100 kDa respectively. One of their interpretations was that two surface proteins of approx. 100 and 70 kDa (after subtraction of apo A-I) present in adipose tissue were capable of binding apo HDL. Taken together, there is strong evidence for the existence of at least two and possibly three HDL_3-binding proteins, and the present paper supports this view by reporting identification of HB_1 and HB_2, both of which retain their ability specifically to bind HDL_3 after purification to apparent homogeneity.

HB_1 and HB_2 bound apo A-I and apo A-II as well as HDL_3, and LDL was unable to compete as effectively as HDL_3 for binding of ^{125}I-labelled HDL_3 to both proteins. Antibodies to HB_1 and HB_2 were produced in rabbits, and there was no evidence of cross-reactivity between the antisera. These antibodies will provide useful tools, and one application is the search for the corresponding cDNA clones in rat liver libraries. Such work will hopefully yield more detailed information about the primary sequence and enable further structural comparisons of HB_1 and HB_2.

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