Characterization and purification of proteins which bind high-density lipoprotein

A putative cell-surface receptor

Heather M. BOND,*§ Giovanni MORRONE,† Salvatore VENUTA† and Kathryn E. HOWELL‡

*Department of Biochemistry and Medical Biotechnology, 2nd Medical School, Via Pansini 5, I-80131 Naples, Italy,

†Department of Experimental and Clinical Medicine, University Medical School, Via T. Campanella, 88100 Catanzaro, Italy,

‡Department of Cellular and Structural Biology, University of Colorado, School of Medicine, Campus Box B-111, 4200 East 9th Avenue, Denver, CO 80262, U.S.A.

High-density lipoprotein (HDL) is shown by ligand blotting to bind membrane-associated polypeptides with sizes of 60, 100 and 210 kDa. Binding was concentration-dependent and competed by excess unlabelled HDL. All the major apolipoproteins of HDL, apoA-I, apoA-II and apoA-IV, bound independently. The 100 kDa and 210 kDa HDL-binding activities were purified from membranes of Hep3B tumour cells by ion-exchange chromatography and gel filtration. The binding activities at 100 kDa and 210 kDa co-purified. After treatment with disulphide-reducing reagent, the 210 kDa band was no longer present and an increase was observed in the amount and binding ability of the 100 kDa polypeptide. The 100 kDa binding protein labelled at the cell surface with ^{125}I could be immunoprecipitated after cross-linking to cell-surface-bound HDL. It is proposed that this HDL-binding activity, a putative cell-surface receptor for HDL, exists totally or in part as a high-molecular-mass complex composed of 100 kDa subunits.

INTRODUCTION

High-density lipoprotein (HDL) is known to bind specifically to the surface of many different cell types as well as to plasma-membrane preparations. For example, HDL binds to J774 macrophage and Fao hepatocyte cell lines, with a K_d of $(2.8\text{--}3.0) \times 10^{-8}$ M ($8.0\text{--}8.6$ $\mu\text{g/ml}$) and approx. 200 000 binding sites per cell (Monaco *et al.*, 1987); this binding is mimicked by a recombinant apoA-I hybrid protein (apoA-I-Protein A).

Once HDL has bound to the cell surface, depending on metabolic state of the cell, it may either be degraded or acquire cholesterol, resulting in cholesterol efflux. Data that indicated that HDL follows a retroendocytosis pathway have been obtained by Bierman *et al.* (1974), Schmitz *et al.* (1985a,b), DeLamatre *et al.* (1989) and Alam *et al.* (1990). In this model, HDL binds to a cell-surface receptor which mediates clustering in coated pits and internalization. The HDL molecules are not degraded, but are re-secreted as cholesterol-enriched particles. Other authors propose that cholesterol is effluxed directly from the plasma membrane through an aqueous phase and then on to HDL, which is bound by a 'docking'-type receptor (DeLamatre *et al.*, 1986; Phillips *et al.*, 1987; Karlin *et al.*, 1987; Slotte *et al.*, 1987). For either of these processes to occur, cell-surface-binding receptors should exist. It is possible that more than one class of receptor is required to mediate the complex processing described for HDL in different cell types.

Here we report the identification of polypeptides of 60, 85, 100 and 210 kDa which have the ability to bind HDL *in vitro*. The 100 and 210 kDa HDL-binding proteins, purified from Hep3B human hepatoma cells, were found to co-migrate on SDS/PAGE under reducing conditions. This HDL-binding protein with an apparent subunit size of 100 kDa is present on the cell surface and can be cross-linked to HDL on intact cells; it thus represents a putative cell-surface receptor for HDL.

MATERIALS AND METHODS

Materials

HDL. This ($1.069 < d < 1.21$) was isolated from human plasma by flotation on discontinuous KBr gradients (Schumaker & Puppione, 1986). The HDL preparation contained (by weight) an average of 34% apoprotein (apo) A-I, 16% apoA-II, 4% apoC, 20% cholesteryl esters, 19% phospholipids, 4% free cholesterol and 2% triacylglycerols. Polyclonal antibodies against total HDL were raised in rabbits as described by Louvard *et al.* (1982), and were shown to recognize apoA-I and apoA-II, by immunoblotting and immunoprecipitation. ApoA-II was purchased from Sigma, and apoA-IV was a gift from A. Steinmetz (Marburg, Germany).

ApoA-I-Protein A hybrid protein (apo A-I-PA). The plasmid pLM8 was constructed from the plasmids pLM3, containing the gene for human mature apoA-I (Lorenzetti *et al.*, 1986), and pRIT2T (Nilsson *et al.*, 1985), containing the IgG-binding domains of *Staphylococcus aureus* Protein A. The fusion product, apoA-I-PA, was expressed in *Escherichia coli*, and purified by denaturation in urea and alkali, renaturation and affinity chromatography on IgG coupled to Sepharose (Monaco *et al.*, 1987).

Cell culture. Hep3B cells were grown in D-MEM medium containing 10% (v/v) fetal-calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 2 mM-glutamine and 10 mM-Hepes buffer, pH 7.4.

Iodination of ligands

Ligands were iodinated by using Iodo-Gen reagent (Pierce Chemical Co.). The iodination reaction contained 100 μg of Iodo-Gen, 0.5 mg of protein in 200 μl of phosphate-buffered saline (PBS; 10 mM-potassium phosphate, pH 7.4, 145 mM-NaCl) and 1 mCi of carrier-free Na^{125}I (Amersham International;

Abbreviations used: apo, apolipoprotein; apoA-I-PA, apolipoprotein A-I-Protein A hybrid; HDL, high-density lipoprotein; LDL, low-density lipoprotein; octyl glucoside, n-octyl β -D-glucopyranoside; PBS, phosphate-buffered saline (10 mM-potassium phosphate, pH 7.4, 145 mM-NaCl); DTT, dithiothreitol; DSP, 3,3'-dithiobis(propionic acid N-succinimide ester); D-MEM, Dulbecco's minimal essential medium.

§ To whom correspondence should be addressed.

16.5 mCi/ μ g). After 10 min at 4 °C, free iodine was separated from the protein on a 10 ml Sephadex G50 column. The column was eluted with 50 mM-Tris/HCl (pH 8.0)/0.15 M-NaCl/1 mM-EDTA/0.2% BSA. Labelled proteins were extensively dialysed against PBS/0.5 mM-EDTA at 4 °C until less than 5% of the label was soluble in 10% (w/v) trichloroacetic acid. For HDL and low-density lipoprotein (LDL), less than 5% of the radioactive label was associated with lipid, as determined after lipid extraction with chloroform/methanol. The specific radioactivity usually obtained was 300–400 c.p.m./ng of protein, as measured in a Nuclear Enterprises (NE 1612) γ -radiation counter.

Ligand blotting

Membrane extracts or column fractions were precipitated with trichloroacetic acid, solubilized in SDS gel sample buffer (1% SDS, 100 mM-Tris/HCl, pH 6.8, 1 mM-EDTA, 1% Bromophenol Blue, 2 M-sucrose), heated to 95 °C for 2 min [if reduced 5 mM-dithiothreitol (DTT) was added], and the samples were subjected to SDS/PAGE on 10% or 5–15% gradient gels. The proteins were blotted on to nitrocellulose (Schleicher and Schuell; pore size 0.45 μ m) by electrotransfer at 25 V for 16 h in 0.15 M-glycine/20 mM-Tris/HCl/20% (v/v) methanol. The bands were revealed by Ponceau S (Sigma) staining, followed by washing with distilled water. At this stage a photograph was taken of the protein pattern. The nitrocellulose strips were quenched for 3 h in 50 mM-Tris/HCl (pH 7.4)/150 mM-NaCl/1 mM-EDTA containing 5% (w/v) low-fat milk powder (Frema Reform). 125 I-labelled ligands with or without competition were bound in quenching buffer at 37 °C for 4–6 h with agitation. The nitrocellulose was then washed four times in quenching buffer at 25 °C, dried and exposed to X-ray film for 24–72 h.

As a control, ligand blotting was also performed with 125 I-LDL as described by Daniel *et al.* (1983), where 50 mg of BSA/ml was used to block non-specific binding in 50 mM-Tris/HCl (pH 8.0)/90 mM-NaCl/2 mM- CaCl_2 .

Preparation of membrane, lipid-droplet and cytoplasm fractions

Hep3B cultured cells (10×10^6 grown adherent) were washed twice with PBS and detached in PBS containing 1 mM-EDTA, at 37 °C for 10 min. The cells were homogenized in buffer [10 mM-Hepes, pH 7.4, 0.25 M-sucrose, 1 mM-EDTA] containing a proteolytic-inhibitor cocktail [1 μ g of pepstatin/ml, 1 μ g of leupeptin/ml, 1 μ g of aprotinin/ml, 1 μ g of antipain/ml, 78.3 μ g (500 μ M) of benzamide/ml]. This mixture of inhibitors was present in all the purification procedures. The homogenate was centrifuged at 2000 g for 10 min and the post-nuclear supernatant centrifuged at 100 000 g for 2 h. The fractions analysed were the floating fraction containing lipid droplets with a high cholesteryl ester content, the soluble fraction (cytosol) and the 'total membrane pellet'. Each of these fractions (200 μ g) was applied to SDS/PAGE and processed for ligand blotting.

Preparation of HDL-binding proteins

Membrane preparation. Hep3B cells [$(1-2) \times 10^7$] were injected subcutaneously into the flank of 8–10-week-old female nude mice (CRL/nu, Charles River). Tumours of 0.5–1 g developed after approx. 6–9 weeks at the site of injection. Each preparation was made from 10–12 g of tumour. After removal of the tumours, they were immediately chopped with a blade in 50 ml of homogenization buffer (10 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, containing the cocktail of proteolytic inhibitors). Homogenization was carried out with a Polytron homogenizer for 4×30 s at 4 °C. The homogenate was centrifuged at 2000 g for 10 min, and the supernatant obtained was centrifuged at 100 000 g for 2 h. This 'total membrane pellet' was solubilized in 40 mM-

octyl glucoside/10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA containing proteolytic inhibitors, by resuspension with an 18-gauge needle. This material was frozen in batches and stored at –80 °C.

Ion-exchange chromatography. A DE52 DEAE-Sephadex column (5 ml) was equilibrated with 40 mM-octyl glucoside/10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA at 25 °C. Total octyl glucoside-solubilized membranes were defrozen, centrifuged at 10 000 g for 10 min, and 10 mg of protein was applied to the column. The column was washed with 10 ml of the same buffer containing proteolytic inhibitors and eluted with a linear salt gradient in this buffer up to 450 mM-NaCl. Fractions (1 ml) were collected, assayed for protein, and 50 μ l of each fraction was applied to SDS/PAGE and processed for ligand blotting.

Gel filtration. A Bio-Gel 1.5A column (30 cm $\times$ 1 cm) was prepared, equilibrated at 25 °C with 10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA, and washed with 50 ml of 40 mM-octyl glucoside/10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA containing proteolytic inhibitors. The column was calibrated with 1 mg each of Blue Dextran (2000 kDa), thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa), from Pharmacia. The fractions from the DEAE-Sephadex column containing high binding activity (e.g. fraction no. 11; 0.5 ml) were applied directly to the gel-filtration column run at a rate of 0.2 ml/min. Fractions (1 ml) were collected, assayed for protein, and 0.2 ml of each was applied to SDS/PAGE and processed for ligand blotting.

Cell-surface iodination

Hep3B adherent cells (2×10^7) were washed twice with PBS and detached in PBS containing 1 mM-EDTA at 37 °C for 10 min. Cells were further washed twice by centrifugation at 1000 g for 5 min with PBS, resuspended in 200 μ l of PBS and transferred to a test tube coated with 100 μ g of Iodo-Gen reagent. Cells were labelled with 1 mCi of carrier-free Na^{125}I (16.5 mCi/ μ g; Amersham International) for 20 min at 4 °C. Labelled cells were washed with 4×15 ml of PBS containing 1 mg of BSA/ml. The cell pellet was then homogenized in 10 mM-Tris/HCl (pH 7.5)/1 mM-EDTA containing proteolytic inhibitors with a Dounce homogenizer. A post-nuclear supernatant was obtained by centrifugation at 2000 g for 10 min and was pelleted at 100 000 g for 2 h to obtain a 'total membrane pellet', which was then solubilized in 40 mM-octyl glucoside/10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA containing a cocktail of proteolytic inhibitors. After solubilization for 30 min at 25 °C this material was centrifuged at 10 000 g for 10 min, and the soluble fraction was applied to a DE52 ion-exchange column as described above. Fractions were counted for radioactivity and samples applied to SDS/PAGE. The gels were then exposed to X-ray film for 24–48 h. A parallel fractionation was carried out without cell-surface iodination, which enabled ligand blotting with ^{125}I -HDL to be performed on the unlabelled fractions.

Cross-linkage experiments

Hep3B cultured cells (1×10^7) were cell-surface iodinated as described above with Iodo-Gen reagent. Labelled cells were washed twice with 15 ml of PBS containing 1 mg of BSA/ml; 50 μ g of ^{125}I -HDL (2.6×10^6 c.p.m. in 1 ml of RPMI) was bound for 90 min at 4 °C. The cells were then washed twice in PBS and divided into portions for cross-linkage with DSP [3,3'-dithiobis(propionic acid *N*-hydroxysuccinimide ester); Sigma]. The stock solution of DSP was prepared in dimethyl sulphoxide at 10 mg/ml. Cells were treated with different concentrations of DSP for 10 min at 4 °C, washed twice with PBS and then solubilized with 1 ml of 1% Nonidet P-40/10 mM-Tris/HCl

(pH 7.5)/150 mM-NaCl/1 mM-EDTA containing proteolytic inhibitors, for 30 min at 25 °C. The extracts were centrifuged at 10000 *g* for 10 min and the soluble material was used for immunoprecipitation with rabbit antiserum specific for HDL (16 h, 4 °C) followed by Protein A–Sepharose (4 h, 25 °C). The immunoprecipitates were washed three times with 1 % Nonidet P-40/10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA, then three times with 10 mM-Tris/HCl (pH 7.5)/150 mM-NaCl/1 mM-EDTA, and subjected to SDS/PAGE under reducing conditions and processed for autoradiography.

RESULTS

Identification of HDL-binding proteins by ligand blotting

Ligand blotting was used as an assay to detect polypeptides which have the ability to bind HDL. In this procedure proteins are fractionated by SDS/PAGE and transferred to nitrocellulose. Non-specific binding sites are quenched with low-fat milk. The filter is subsequently incubated with radioactive ligand, washed and autoradiographed.

Fig. 1. Detection of HDL binding proteins by ligand blotting

Membranes from Hep3B cells were prepared, and 200 μ g of protein was loaded on to each lane of an SDS/5–15 % polyacrylamide gel and processed for ligand blotting. Strips of nitrocellulose were cut and incubated with increasing concentrations of 125 I-HDL (lane 1, 2 μ g/ml; lane 2, 4 μ g/ml; lane 3, 6 μ g/ml) and in lane C there was 4 μ g of 125 I-HDL/ml with 200 μ g of unlabelled HDL/ml for competition. After the binding, the nitrocellulose strips were washed and autoradiographed.

Fig. 2. Specificity of ligand blotting

Membranes from Hep3B cells were prepared, and 200 μ g of protein was loaded on to each lane of an SDS/5–15 % polyacrylamide gel and processed for ligand blotting. Strips of nitrocellulose were cut and incubated with the radioactive ligands at 5 μ g of protein/ml in 50 mM-Tris/HCl (pH 7.4)/150 mM-NaCl/1 mM-EDTA containing 5 % (w/v) low-fat milk powder (lanes 1–8). Competition was carried out in lanes 2, 4 and 6 with 500 μ g of unlabelled HDL in the binding medium. Binding in lane 9 was in 50 mM-Tris/HCl (pH 8.0)/90 mM-NaCl/2 mM-CaCl₂, and 50 mg of BSA/ml was used to block non-specific binding as described by Daniel *et al.* (1983). After binding, the nitrocellulose strips were washed and autoradiographed. Lanes: 1, 125 I-apoA-I-PA; 2, 125 I-apoA-I-PA competed with HDL; 3, 125 I-apoA-II; 4, 125 I-apoA-II competed with HDL; 5, 125 I-apoA-IV; 6, 125 I-apoA-IV competed with HDL; 7, 125 I-Protein A; 8, 125 I-LDL; 9, 125 I-LDL in Ca²⁺.

Binding of HDL was more evident after incubation at 37 °C than at 25 °C or 4 °C. However, differently from LDL ligand blotting (Daniel *et al.*, 1983), the binding of HDL did not require Ca²⁺ and did occur in the presence of EDTA. Fig. 1 shows increasing concentrations of 125 I-HDL binding to total Hep3B membranes. The binding polypeptides detected had apparent sizes of 60, 100 and 210 kDa. Higher concentrations of 125 I-HDL resulted in increased amounts of binding to these three bands, accompanied by an increase in the non-specific background on the filter. Competition with 50-fold excess of unlabelled HDL resulted in a considerable decrease in binding. The small amount of binding seen after competition of the ligand blots is comparable with that found when competition is performed for HDL binding to the cell surface at 4 °C. The binding polypeptides of 100 and 60 kDa have been detected previously (Monaco *et al.*, 1987). The 210 kDa binding protein was not observed in our earlier experiments, probably owing to inefficient transfer of high-molecular-mass proteins with the gradient gel system used.

The same polypeptide bands which bind HDL were also found to bind apoA-I-PA, a recombinant hybrid protein which exhibits similar cell-surface binding properties to HDL (Fig. 2, lane 1). No binding was detected with Protein A alone (lane 7). ApoA-I-PA had a greater stability after iodination than HDL, and was therefore used for several of the ligand blots to monitor the purification of the binding proteins. Binding to the 60, 100 and 210 kDa bands was also observed with apoA-II (lane 3) and apoA-IV (lane 5). HDL was able to compete for the binding of apoA-I-PA (lane 2), apoA-II (lane 4) and apoA-IV (lane 6).

Fig. 3. Detection of HDL-binding polypeptides in membrane, cytoplasm and lipid-droplet fractions

Membranes (M), cytoplasm (C) and lipid-droplet (LD) fractions were prepared from Hep3B cells as described in the Materials and methods section. Each fraction (200 μ g) was applied per lane to an SDS/5–15% polyacrylamide gel and processed for ligand blotting, which was performed with 5 μ g of 125 I-apoA-I-PA/ml.

Thus all of the individual apolipoprotein components of HDL proved able to bind independently to these three polypeptides. These labelled preparations of apolipoproteins were also used for binding to the cell surface of Hep3B cells. Binding to the cell surface at 4 °C was observed with 125 I-HDL, 125 I-apoA-I-PA, 125 I-apoA-II and 125 I-apoA-IV, and in all cases it was competed by (50-fold excess of) unlabelled HDL (results not shown). The binding was specific for HDL; under the conditions described, binding was not observed with 125 I-labelled LDL (Fig. 2, lane 8), Protein A (lane 7), BSA (result not shown) or transferrin (result not shown). However, if the conditions described for LDL ligand blotting were used (Daniel *et al.*, 1983), binding to the 140 kDa LDL receptor in the membrane preparations could be detected (lane 9).

Subcellular localization of the HDL-binding proteins

Subcellular fractionation showed that the binding activities are present in the membrane fraction. As shown in Fig. 3, the 60, 100 and 210 kDa binding polypeptides were only detected in the membrane fraction, but not in the cytoplasm or in a lipid-droplet-enriched fraction. The membrane fraction could be washed with 50 mM-Tris/HCl (pH 7.5)/150 mM-NaCl without apparent loss of binding activity. All these three binding proteins

were soluble in the mild non-ionic detergent 40 mM-octyl glucoside. In the cytoplasm and lipid-droplet fractions an enrichment was observed of a different binding polypeptide of 85 kDa, which has not been further characterized.

Purification of HDL-binding proteins

The material used for purification of the binding proteins was tumours of Hep3B cells developed in nude mice. This was selected as a convenient source of material for several reasons. (i) Hep3B cells exhibit a high number of apoA-I-binding sites (approx. 300 000 per cell). (ii) They develop as tumours in nude mice, thereby providing large quantities of cells otherwise impractical to obtain by tissue culture. These tumours consist of over 80% hepatocyte-like cells as assessed by histochemical staining. (iii) It was appropriate to use a human material, since the HDL and apoA-I-PA ligands used for detection were also of human origin. The characterization of a human HDL receptor will be of considerable medical interest.

Membranes were prepared from tumours as described in the Materials and methods section. To prevent possible proteinase digestion during the procedure, a cocktail of proteolytic inhibitors was added throughout. The membranes were solubilized in 40 mM-octyl glucoside and applied to a DEAE-Sephadex ion-exchange column which was eluted with a linear salt gradient. Fig. 4 shows the protein profile of each fraction (Fig. 4a) and the corresponding ligand blot (Fig. 4b). The first lane contained 200 μ g of the total membrane fraction loaded (L). The 60 kDa binding polypeptide was essentially not bound by the column. The 100 kDa and 210 kDa binding polypeptides were eluted together at approx. 200 mM-NaCl.

A sample of fraction no. 11, which contained the above binding activity, was treated with the disulphide-reducing agent dithiothreitol (DTT). The protein profile of this fraction (Fig. 5a) and the corresponding ligand blot (Fig. 5b: reduced, R, and non-reduced, N) shows that after treatment the 210 kDa band disappeared both in the protein profile and in its ability to bind HDL. In the reduced samples the 100 kDa band was increased in amount, consistent with the decrease in the 210 kDa protein. However, in ligand blotting the 100 kDa protein bound more than the combination of the two, suggesting an increased binding affinity of the 100 kDa protein. Binding of 125 I-HDL to the DEAE-Sephadex-purified 210 and 100 kDa bands was competed by excess unlabelled HDL (results not shown).

The peak of binding activity from the DEAE-Sephadex ion-exchange column was applied directly to a gel-filtration column (Bio-Gel 1.5A), which was also run in octyl glucoside. Fractions from the protein peaks were analysed by SDS/PAGE and ligand blotting (Fig. 6), either DTT-treated (right panel) or non-reduced (left panel). The 100 and 210 kDa binding polypeptides are visible as major protein bands (see in particular fractions 12 and 13, left panel). These fractions represent highly purified preparations of the binding proteins. The peak of elution of binding-activity fractions 13/14 is at approx. 400–450 kDa, similar to the peak of ferritin (440 kDa) in octyl glucoside. In these conditions, an estimate of molecular mass is only approximate, owing to the presence of varying amounts of detergent associated with the proteins.

From 12 g of Hep3B tumour, 80 mg of octyl glucoside-solubilized protein was obtained and fractionated by ion-exchange chromatography. The peak of binding activity (for the 100 and 210 kDa bands) contained a total of 7 mg of protein. After the second column, gel-filtration-purified fractions (11 + 12) contained 20 μ g of the 100 and 210 kDa binding proteins (Table 1). These binding proteins were eluted in a relatively broad peak and part of them was discarded owing to the presence of other contaminating polypeptide bands, thus decreasing the yield.

Fig. 4. Purification of HDL-binding proteins; ion-exchange chromatography

DEAE-Sephadex ion-exchange chromatography was performed in octyl glucoside as described in the Materials and methods section; a sample of each fraction (50 μl) was precipitated with trichloroacetic acid, applied to SDS/PAGE (5–15 % gels) and processed for ligand blotting. The fraction (L) is 200 μg of the material loaded to the column, the fractions 1–6 were not bound by the ion-exchange column, and the subsequent fractions (7–24) were eluted by a salt gradient. The ion-exchange chromatography shown is representative of four different preparations where ligand blotting was performed with labelled apoA-I-PA, apoA-II or HDL. A mixture of unlabelled protein standards and radioactive [^{14}C]methylated protein markers (Amersham; 14.3, 30, 46, 69, 97.4 and 200 kDa) were used, and are visible in the photograph of the Ponceau S-stained filter (a) and in the autoradiograph of the ligand blot (b) respectively. As no fluorography was used on the blots, the latter markers show only weakly (Fig. 4b). (a) Protein profile of fractions (Ponceau S stain of nitrocellulose filter). (b) Ligand blotting performed with 6 μg of ^{125}I -apoA-I-PA/ml.

When the purified fractions (11 + 12) were labelled with ^{125}I and re-run on SDS/PAGE, only very minor additional bands could be detected (results not shown). The binding activity was assayed by ligand blotting and specific activity estimated based on densitometry of the autoradiographs (Table 1). An apparent 240-fold increase in specific binding activity was obtained (from 0.05

to 12 units/ μg), indicating a loss of binding activity during the purification procedure.

Cell-surface labelling of the binding proteins

If the purified HDL-binding protein represents a physiologically important receptor for HDL, involved in receptor-

Fig. 5. Effect of treating HDL-binding proteins with the disulphide-reducing agent DTT

A sample (20 μ g) of fraction 11 from the ion-exchange column shown in Fig. 4 was either reduced with 5 mM-DTT (R) or not treated (N) before application to SDS/PAGE (5–15% gels) and processing for ligand blotting. A combination of unlabelled protein standards and radioactive [14 C]methylated protein markers were used as described in the legend to Fig. 4. As no fluorography was used on the blots, the radioactive markers show only weakly (Fig. 5b). (a) Protein profile of fraction 11 (Ponceau S stain of the nitrocellulose filter). (b) Ligand blotting of the nitrocellulose filter with 4 μ g of 125 I-HDL/ml. Note the strong binding to the 100 kDa protein in the reduced fraction, whereas in the non-reduced lane the 210 kDa protein binds relatively weakly.

mediated endocytosis, it would be necessary that this protein be accessible at the cell surface. To verify that this was the case, cell-surface proteins of cultured Hep3B cells were labelled with 125 I at 4 °C and purified as described above. Fig. 7 shows the labelled proteins eluted from the DEAE-Sephadex column. In the 200 mM-NaCl-eluted fractions, a highly labelled 100 kDa protein was present. In a parallel unlabelled preparation from Hep3B cells, the same fraction was positive for the 100 kDa HDL-binding activity detected by ligand blotting (lane LB):

More direct evidence that HDL has the ability to bind to a 100 kDa protein at the cell surface was obtained from cross-linking studies. Hep3B cell-surface proteins were labelled with 125 I at 4 °C. 125 I-HDL was bound to the cell surface at 4 °C for 2 h. The cross-linking reagent DSP was added, which cross-links adjacent proteins via lysine residues. The linkage can subsequently be broken by reduction of disulphide bonds. After cross-linking, the cells were lysed with detergent, and HDL together with associated proteins were immunoprecipitated with anti-

bodies specific for HDL, treated with DTT and analysed by SDS/PAGE. Fig. 8 shows that, in addition to apoA-I (the large band at 28 kDa), a radioactive band at 100 kDa was present when 120 μ g of DSP was used. There was also a minor band at approx. 140 kDa. In the absence of DSP or at lower concentrations, these bands were not detectable. If samples were not reduced, only complexes too large to enter the gel were found (results not shown). The 210 kDa band was therefore not detectable.

DISCUSSION

A cell-surface receptor for HDL is considered to play a crucial role in HDL-mediated cholesterol efflux and its metabolism, and is very likely to be involved in the pathogenesis of atherosclerosis. We have previously demonstrated that an apoA-I-PA hybrid protein was able to mimic both the binding of HDL to the cell surface and the binding *in vitro* to a protein of approx. 100 kDa. In this present study, by using the technique of ligand blotting, polypeptides at 60, 85, 100 and 210 kDa are identified which have the ability to bind HDL and its apolipoproteins. All of the major HDL apolipoproteins, apoA-I, apoA-II and apoA-IV, are able to bind independently. The binding of the apolipoproteins was competable by unlabelled HDL. These HDL apolipoproteins share structural similarities (Boguski *et al.*, 1986), and have been found to compete with each other for binding to cell surface sites (Rifici & Eder, 1984; Havekes *et al.*, 1984; Fidge & Nestel, 1985; Hwang & Menon, 1985; Stein *et al.*, 1986; our results not shown).

In preliminary experiments, recombinant apoA-I-PA bound to IgG-Sephacrose was used as an affinity system for the purification of HDL-binding proteins. When the affinity matrix was treated with SDS and the eluted material was analysed by SDS/PAGE, a 100 kDa protein was detected, and proved positive by ligand blotting (results not shown). However, the yield of 'receptor' bound to the matrix was relatively low. There was a loss of 80% of binding activity in the unbound fractions. Elution conditions milder than SDS (e.g. high salt or low pH) were ineffective for elution. For these reasons affinity chromatography was not used in the purification procedure.

Sequential application of ion-exchange chromatography and gel filtration allowed the purification of two HDL-binding polypeptides, of 100 kDa and 210 kDa respectively. With gel filtration in octyl glucoside, the 'receptor complex' was eluted at an apparent molecular mass of 400–450 kDa. When fractions were treated with DTT, only a 100 kDa binding protein was detected. In the absence of reduction, a 210 kDa binding polypeptide was present in addition to the 100 kDa binding band. It is thus probable that at least some of the 100 kDa binding polypeptide exists as high-molecular-mass complexes. According to Driel *et al.* (1987), LDL-receptor dimers and monomers are in dynamic equilibrium in the plasma membrane. During homogenization, disulphide-bond formation may occur within the dimers. In our case it is unlikely that dimerization occurred during homogenization: in some experiments where whole cells were directly solubilized in 1% SDS and electrophoresed, binding to both 100 kDa and 210 kDa bands was still observed, in a similar ratio to that found with membrane preparations (results not shown). Although ligand blotting suggests that monomers efficiently bind ligand, oligomerization might play an important role for clustering in coated pits and internalization *in vivo*.

Direct evidence that the 100 kDa binding polypeptide is present at the cell surface and interacts with HDL was obtained by cell-surface labelling and cross-linking studies. A polypeptide of 100 kDa which co-purified with the 100 kDa binding activity was cross-linked to cell-surface-bound HDL by the bifunctional

Fig. 6. Purification of HDL-binding proteins; gel filtration

The fractions from the peak of binding activity from the DEAE-Sephadex ion-exchange column were applied to a gel-filtration column (Bio-Gel 1.5A) in octyl glucoside as described in the Materials and methods section. Fractions 11–20 of the protein peak (200 μ l) were precipitated with trichloroacetic acid, applied to SDS/PAGE (5–15 % gels) and processed for ligand blotting. Fractions were either non-treated (left-hand panel) or treated with 5 mM-DTT (right-hand panel). (a) Protein profile of fractions (Ponceau S stain of nitrocellulose filter). (b) Ligand blotting was performed with 6 μ g of 125 I-apoA-I-PA/ml.

reagent DSP. Since the spacer-arm distance for DSP is 1.2 nm, the HDL must be bound to or in close proximity to this 100 kDa polypeptide. This interaction is probably similar to those reported for HDL cross-linked to proteins at 110 kDa (Vadiveloo & Fidge, 1990) or at 100 and 130 kDa (Barbaras *et al.*, 1987).

Other authors have reported high-molecular-mass proteins which have the ability to bind HDL. A 110 kDa binding activity was detected by ligand blotting (Graham & Oram, 1987; Ghiselli

et al., 1990), whose amount increased when cells were fed on cholesterol or treated with γ -interferon (Oppenheimer *et al.*, 1988), and in parallel to this phenomenon up-regulation of cell-surface HDL binding was detected. Tozuka & Fidge (1989) purified 120 kDa (HB1) and 100 kDa (HB2) HDL-binding activities from liver by ion-exchange chromatography and repeated preparative SDS/PAGE. After β -mercaptoethanol treatment the 120 kDa (HB1) binding activity was lost, whereas the

Table 1. Purification of HDL-binding proteins

The starting material used was 12 g of tumours raised in nude mice derived from Hep3B cells. Membrane extracts were prepared as described in the Materials and methods section (80 mg) and were fractionated by DEAE-Sephadex ion exchange; the peak of binding activity (fractions 10 + 11) eluted with a salt gradient contained 7 mg of protein. This peak was applied to a gel-filtration column and the fractions (11 + 12) with binding activity were pooled (20 µg). The relative amounts of binding activities are estimated from scanning the autoradiographs after ligand blotting with ^{125}I -apoA-I-PA (6 µg/ml), under non-reducing conditions. The arbitrary units indicated are relative to the binding for the 100 kDa band in the membrane fraction (10 units). The amount bound is also calculated per µg loaded in each lane of the SDS/polyacrylamide gel and is indicated in parentheses. There was a 240-fold increase in specific binding activity for the 100 kDa band, from 0.05 to 12 units/µg.

Fraction tested	Protein loaded (µg/lane)	Binding to each band (units/µg):		
		60 kDa	100 kDa	210 kDa
Membrane (80 mg)	200	8 (0.04)	10 (0.05)	8 (0.04)
DEAE-Sephadex peak (7 mg)	70	0.1 (0.001)	180 (2.57)	10 (0.14)
Gel filtration (20 µg)	1	0.0 (0)	12 (12)	2 (2)

Fig. 7. Cell-surface labelling of Hep3B cells

Hep3B cells were cell-surface labelled with ^{125}I as described in the Materials and methods section, membranes were prepared and the octyl glucoside-soluble material was fractionated by ion-exchange chromatography; fractions were treated with 5 mM-DTT, applied to SDS/PAGE (5–15 % gels) and autoradiographed. The figure shows the material loaded on to the column (lane L), the unbound eluted material (lane E) and the fractions eluted with the salt gradient (lanes 8–15). A ligand blot (lane LB) with ^{125}I -HDL was performed on a parallel unlabelled Hep3B preparation of fraction no. 12.

100 kDa (HB2) remained active. Keso *et al.* (1987) describe purification of a binding protein from human placenta at 120 kDa, which when treated with β -mercaptoethanol gave 50

Fig. 8. Cross-linkage of cell-surface proteins to HDL bound at the cell surface

Hep3B cells were cell-surface labelled with ^{125}I and then incubated with 50 µg of ^{125}I -HDL/ml at 4 °C. Cells were washed and cross-linkage was performed with increasing concentrations of DSP (0, 20, 120 µg/ml) at 4 °C. Cells were detergent solubilized, immunoprecipitated with antiserum specific for HDL followed by Protein A-Sepharose, separated by SDS/PAGE (10 % gel) and autoradiographed. The broad band at 28 kDa is apoA-I, and at least two bands can be seen when cross-linking was carried out with 120 µg of DSP/ml: a clear band at 100 kDa and another, less prominent, at approx. 140 kDa. The band smaller than 46 kDa is probably due to distortion by IgG and was present with or without cross-linking.

and 20 kDa polypeptides no longer able to bind HDL. Barbaras *et al.* (1990) have purified from adipose cells HDL-binding polypeptides of 80 and 92 kDa. In contrast, radiation-inactivation studies (Mendel *et al.*, 1988) suggested that the binding site for HDL on the cell surface is much smaller (16 kDa). Until these HDL-binding activities are further characterized, it is unclear if they represent different or related HDL 'receptors'.

In addition to the 210 and 100 kDa binding polypeptides purified, we identified binding activities at 85 and 60 kDa. It is possible that the 85 kDa band, which was not associated with the membrane fraction, is similar to that initially detected by Fidge *et al.* (1985) at 78 kDa. The 60 kDa protein which is found associated with the membranes could be the same as the 56 kDa protein originally detected by its ability to bind apoE (Hui *et al.*, 1986) and subsequently identified a subunit of the F1-ATPase (Beisiegel *et al.*, 1988) or as the enzyme protein disulphide-isomerase (58 kDa) (Beisiegel *et al.*, 1989). It is interesting that protein disulphide-isomerase has recently been identified as a component of the microsomal triacylglycerol-transfer-protein complex (Wetterau *et al.*, 1990). It is conceivable that protein

disulphide-isomerase does associate physiologically with HDL in the process of lipoprotein assembly.

Proteins which specifically bind HDL may be required at different points of HDL synthesis and metabolism. For example, in addition to a cell-surface receptor, various intracellular binding proteins may also exist. These could function in the biosynthesis and assembly of the HDL particles or in the sorting between targeting to the lysosomes for degradation and, after acquisition of cholesterol, recycling to the cell surface. The signals which determine where HDL is degraded, recycled or acquires cholesterol are not known, but may involve oligomerization of binding proteins and/or interaction with other proteins. It is important to establish which of these functions are associated with the HDL-binding protein(s) characterized in this paper. The approach underway is aimed at the cloning of the corresponding gene(s) and expression of their products in prokaryotic and eukaryotic systems.

Part of this work was carried out at the European Molecular Biology Laboratory, Heidelberg, Germany. H.M.B. was supported by fellowships from Boehringer Mannheim and an EEC medical research fellowship. Support by American Heart Association Grand-in-Aid 98-0653 to K.E.H. is acknowledged. We thank H. A. Dresel and E. von Hodenberg for providing HDL, A. Steinmetz for the apoA-IV and L. Monaco and R. Cortese for the recombinant apoA-I-PA. This work was supported in part by funds from the AIRC and MURST 40%.

REFERENCES

- Alam, R., Yatsu, F. M., Tsui, L. & Alam, S. (1989) *Biochim. Biophys. Acta* **1004**, 292–299
- Barbaras, R., Puchois, P., Grimaldi, P., Barkia, A., Fruchart, J. C. & Ailhaud, G. (1987) *Biochem. Biophys. Res. Commun.* **149**, 545–554
- Barbaras, R., Puchois, P., Fruchart, J. C., Pradines-Figueres, A. & Ailhaud, G. (1990) *Biochem. J.* **269**, 767–773
- Beisiegel, U., Weber, W., Havinga, J. R., Ihrke, G., Hui, D. Y., Wernette-Hammond, M. E., Truck, C. W., Innerarity, T. L. & Mahley, R. W. (1988) *Arteriosclerosis* **8**, 288–297
- Beisiegel, U., Weber, W., Ihrke, G. & Greten, H. (1989) *Atherosclerosis (Berlin)* **8**, 293–295
- Bierman, E. L., Stein, O. & Stein, Y. (1974) *Circ. Res.* **35**, 136–150
- Boguski, M. S., Elshourbragy, N. A., Taylor, J. M. & Gordon, J. I. (1986) *Methods Enzymol.* **128**, 753–772
- Daniel, T. O., Schneider, W. J., Goldstein, J. L. & Brown, M. S. (1983) *J. Biol. Chem.* **258**, 4606–4611
- DeLamatre, J., Wolfbauer, G., Phillips, M. C. & Rothblat, G. H. (1986) *Biochim. Biophys. Acta* **875**, 419–428
- DeLamatre, J. G., Sarphie, T. G., Archibold, R. C. & Hornick, C. A. (1989) *J. Lipid Res.* **31**, 191–202
- Driel, van I. R., Davis, C. G., Goldstein, J. L. & Brown, M. S. (1987) *J. Biol. Chem.* **262**, 16127–16134
- Fidge, N. H. & Nestel, P. J. (1985) *J. Biol. Chem.* **260**, 3570–3575
- Fidge, N., Kagami, A. & O'Connor, M. (1985) *Biochem. Biophys. Res. Commun.* **129**, 759–765
- Ghiselli, G., Crump, W., Musanti, R. & Gotto, A. M. (1990) *Biochim. Biophys. Acta* **1044**, 173–175
- Graham, D. L. & Oram, J. F. (1987) *J. Biol. Chem.* **262**, 7439–7442
- Havekes, L., Schouten, D., van Hinsbergh, V. & de Wit, E. (1984) *Biochem. Biophys. Res. Commun.* **122**, 785–790
- Hui, D. J., Brecht, W. J., Hall, E. A., Freidman, G., Innerarity, T. L. & Mahley, R. W. (1986) *J. Biol. Chem.* **261**, 12557–12564
- Hwang, J. & Menon, K. M. L. (1985) *J. Biol. Chem.* **260**, 5660–5668
- Karlin, J. B., Johnson, W. J., Benedict, C. R., Chacko, G. K., Phillips, M. C. & Rothblat, G. H. (1987) *J. Biol. Chem.* **262**, 12557–12564
- Keso, L., Lukka, M., Ehnholm, C., Baumann, M., Vihko, P. & Olkinuora, M. (1987) *FEBS Lett.* **215**, 105–108
- Lorenzetti, R., Sidoli, A., Palaomba, R., Monaco, L., Martineau, D., Lappi, D. A. & Soria, M. (1986) *FEBS Lett.* **194**, 343–346
- Louvard, D., Reggio, H. & Warren, G. (1982) *J. Cell Biol.* **92**, 92–107
- Mendel, C. M., Kunitake, S. T., Kane, J. P. & Kempner, E. S. (1988) *J. Biol. Chem.* **263**, 1314–1319
- Monaco, L., Bond, H. M., Howell, K. E. & Cortese, R. (1987) *EMBO J.* **6**, 3253–3260
- Nilsson, B., Abrahamssén, L. & Uhlén, M. (1985) *EMBO J.* **4**, 1075–1080
- Oppenheimer, M. J., Oram, J. F. & Bierman, E. L. (1988) *J. Biol. Chem.* **263**, 19318–19323
- Phillips, M. C., Johnson, W. J. & Rothblat, G. H. (1987) *Biochim. Biophys. Acta* **906**, 223–276
- Rifici, V. A. & Eder, H. A. (1984) *J. Biol. Chem.* **259**, 13814–13818
- Schmitz, G., Robenek, H., Lohmann, U. & Assmann, G. (1985a) *EMBO J.* **4**, 613–622
- Schmitz, G., Niemann, R., Brennhäusen, B., Krause, R. & Assmann, G. (1985b) *EMBO J.* **11**, 2773–2779
- Schumaker, V. N. & Puppione, D. L. (1986) *Methods Enzymol.* **128**, 155–170
- Slotte, J. P., Oram, J. F. & Bierman, E. L. (1987) *J. Biol. Chem.* **262**, 12904–12907
- Stein, O., Stein, Y., Lefevre, M. & Roheim, P. S. (1986) *Biochim. Biophys. Acta* **878**, 7–13
- Tozuka, M. & Fidge, N. H. (1989) *Biochem. J.* **261**, 239–244
- Vadiveloo, P. K. & Fidge, N. H. (1990) *Biochim. Biophys. Acta* **1045**, 135–141
- Wetterau, J. R., Combs, K. A., Spinner, S. N. & Joiner, B. J. (1990) *J. Biol. Chem.* **265**, 9800–9807

Received 5 February 1991/13 June 1991; accepted 24 June 1991