Cellular localization and characterization of proteins that bind high density lipoprotein

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Abstract High density lipoprotein (HDL) stimulates excretion of excess intracellular cholesterol from cells, presumably by interacting with a cell-surface receptor. A 110 kDa membrane protein that is a candidate for the HDL receptor has been identified by ligand blot analysis. In this study we determined the cellular localization of this and other HDL-binding proteins and characterized their properties. The plasma membranes (PM) of cultured bovine aortic endothelial cells were labeled with trace amounts of [^{3}H]cholesterol, and cell homogenates were fractionated on sucrose and Percoll gradients. Ligand blot analysis of homogenates of cultured bovine aortic endothelial cells demonstrated that cells contain multiple proteins that bind HDL_3, including a major membrane protein with an apparent M_r of 110 kDa and two minor ones with M_r of 105 and 130 kDa. The gradient distribution of the 105, 110, and 130 kDa HDL-binding proteins mirrored that of labeled cholesterol and 5'-nucleotidase, both PM markers. Treatment of intact cells with the water-soluble cross-linker bis(sulfosuccinimidyl)suberate abolished the HDL binding activity of the 110 and 130 kDa proteins but not that of the 105 kDa protein. These findings suggest that the 105, 110, and 130 kDa HDL-binding proteins are localized to the PM and that at least two of these proteins are exposed to the extracellular fluid. Solubilized 110 and 130 kDa proteins were retained on wheat-germ agglutinin and abrin lectin columns, showing that they are glycoproteins. The cellular localization and physical properties of the 110 and 130 kDa proteins suggest that they may play a role in binding of HDL to the cell surface.—**Hokland, B., A. J. Mendez, and J. F. Oram.** Cellular localization and characterization of proteins that bind high density lipoprotein. *J. Lipid Res.* 1992. **33:** 1335–1342.

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High density lipoproteins (HDL) are a heterogeneous group of particles composed of a cholesteryl ester-rich core and a surface enriched with free cholesterol, phospholipids, and apolipoproteins (apo). ApoA-I, apoA-II, and apoA-IV have all been identified in HDL fractions, but the physiological significance of these apolipoproteins is still unclear. HDL particles are believed to play a role in the transport of cholesterol from peripheral tissues to the liver for ultimate excretion (1). HDL has also been suggested as a vehicle for cholesterol delivery to certain steroidogenic tissues (2–4).

Receptor-mediated binding and endocytosis of sterol-rich lipoproteins, such as low density lipoprotein (LDL), have been firmly established (5). The process is critically dependent on the specific interaction of apolipoproteins with a cell surface receptor. High-affinity binding sites for HDL on intact cells and membranes have been identified for a variety of different cells (6–12). Studies from our laboratory have described a class of high-affinity sites on the cell surface that reversibly bind HDL without internalization of particles (6). These binding sites increase in number when cells are loaded with cholesterol (6, 7, 11, 12), suggesting that they may be receptors that function to remove cholesterol from cells. Experiments with apolipoprotein-modified HDL_3 (13, 14) suggested that receptor-apolipoprotein complex formation is involved in HDL-mediated cholesterol efflux.

Ligand blot analysis of membrane proteins has been used successfully to identify candidate receptor proteins. This approach has been attempted by several laboratories to identify the putative plasma membrane receptor protein for HDL, and HDL-binding proteins have been described that differ in apparent M_r (15–23). One of these proteins has an M_r of approximately 110 kDa and appears to be abundant in a wide variety of different cell types (15). This protein preferentially binds apoA-I and apoA-II, the two major proteins in HDL, and is up-regulated in response to cholesterol loading of cells (15) and to inhibition of cell proliferation (16). These properties are consistent with the possibility that this protein is a physiologic receptor that functions to promote removal

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; BAEC, bovine aortic endothelial cells; BS^3, bis(sulfosuccinimidyl)suberate; PM, plasma membrane.

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of excess cholesterol from cells. However, in addition to the 110 kDa protein, most cell membranes contain several other proteins that bind HDL on ligand blots (15, 19, 22, 23). It has not been established whether these proteins are localized to the plasma membrane, a necessary requirement for a receptor molecule. The aim of the present study was to investigate the cellular localization of HDL-binding proteins in cultured bovine aortic endothelial cells and to characterize some of their physical properties. We show that, of the multiple cellular proteins identified by ligand blot analysis that bind HDL_3, only two, a major band at 110 kDa and a minor one at 130 kDa, appear to be plasma membrane glycoproteins that are exposed to the extracellular environment. These results provide further support that the 110 kDa protein and possibly the 130 kDa protein are candidates for receptor molecules that mediate cellular responses to HDL apolipoproteins.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture media was supplied by Flow Laboratories, McLean, VA, and fetal bovine serum was from Whitaker Bioproducts, Walkersville, MD. Bio-Rad protein assay was from Bio-Rad Laboratories, Richmond, CA. Uridine diphospho-D-[U-^{14}C]galactose (lithium salt, 301 Ci/mol) was from Amersham Corp., Arlington Heights, IL. [$^{1,2\text{-}3}$H (N)]cholesterol (52 Ci/mol) and ^{125}iodine were from New England Nuclear, Burbank, CA. Centricon-30 microconcentrators were supplied by Amicon, Danvers, MA. Percoll and heparin-Sepharose CL-6B were from Pharmacia LKB Biotechnology Inc., Piscataway, NJ. Nitrocellulose membranes were from Schleicher & Schuell, Keene, NH. CHAPS, leupeptin, and pepstatin were from Boehringer Mannheim Biochemicals, Indianapolis, IN.

Bis(sulfosuccinimidyl)suberate (BS^3) was from Pierce, Rockford, IL. Fatty acid-free bovine serum albumin, digitonin, Fluorinert FC-40, *Abrus precatorius* agglutinin, *Arachis hypogaea* agglutinin, concanavalin A, type III A, *Ulex europeus* I and wheat germ agglutinin (all lectins were immobilized on 4% cross-linked beaded agarose), N-acetyl- D-glucosamine, α-methyl-D-mannoside, methyl-β-D-galactoside, α-L-fucose, α-lactose, adenosine 5'-triphosphate, and trypsin inhibitor type III-O were from Sigma Chemical Company, St. Louis, MO. All other chemicals used were reagent grade or better.

Cells

Cultured bovine aortic endothelial cells (BAEC) were plated in 100 or 150 mm dishes and grown to confluence in Dulbecco's Modified Eagle's medium (DMEM) containing 10% bovine calf serum (12, 14, 15, 24, 25). Dishes were washed twice with phosphate-buffered saline (pH 7.4) containing 1.0 mg/ml bovine serum albumin (PBS-BSA) at 37°C and the cells were subsequently dislodged from the dish with a Nylon policeman into ice-cold 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 1 mM benzamidine, 0.5 mM EDTA, and 1 mM phenylmethylsulfonylfluoride (PMSF) (buffer A). The cells were collected by centrifugation at 600 *g* for 10 min at 4°C.

Lipoproteins and apolipoproteins

LDL (d 1.019–1.063 g/ml) and HDL_3 (d 1.125–1.210 g/ml) were isolated from plasma by sequential ultracentrifugation. ApoE was removed from the HDL_3 pool by heparin-Sepharose affinity chromatography (26). Lipoproteins were iodinated by a modification of the McFarlane monochloride procedure (27).

Sucrose gradient fractionation

To radiolabel the plasma membrane, cultured BAEC were incubated with [^{3}H]cholesterol (2 μCi per ml medium) for 2–3 h prior to harvesting. Collected cells were resuspended in 25 mM HEPES (pH 7.4), 0.25 M sucrose, 1 mM benzamidine, 0.5 mM EDTA, 1 mM PMSF, 1 M leupetin, and 2 M pepstatin (buffer B) and homogenized in a Douce homogenizer with a tight-fitting pestle. Digitonin treatment of cells was done according to Lange and Steck (28) with minor modifications. Digitonin was added (from a 20 mM stock solution in DMSO) prior to homogenization in buffer B. The homogenates were centrifuged at 600 *g* for 10 min or in some experiments at 10,000 *g* for 25 min. Subsequently, the supernatants were collected and layered on a 19.5–60.0% (w/v) linear sucrose gradient in 25 mM HEPES (pH 7.4), 1 mM benzamidine, 0.5 mM EDTA, and centrifuged 18–24 h at 100,000 *g* in a Beckman SW-41 Ti swinging-bucket rotor at 4°C. Fractions were collected from the top of the tubes by displacing the gradient with Fluorinert FC-40. Samples used for ligand blotting were concentrated on Centricon 30 cartridges (Amicon) according to the manufacturer's recommendations. Prior to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), samples were dispersed in buffer to a final concentration of 0.25 mM Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, 1 mM benzamidine, and 0.5 mM EDTA by aspiration through a 22-gauge needle. A constant volume (usually 90–120 μl) of each fraction was used for SDS-PAGE.

Percoll gradient fractionation

BAEC were collected and resuspended in a lysis buffer containing 25 mM Tris (pH 9.6), 125 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mM benzamidine, 0.5 mM EDTA, 1 mM PMSF, 1 M leupeptin, and 2 M pepstatin. After homogenization with a Dounce homogenizer and centrifugation (600 *g* for 10 min), 4 ml of the post-nuclear

supernatant was layered on a 18-ml Percoll gradient and centrifuged in a Beckman 60 Ti rotor as described by Payrastre et al. (29). Ten 2-ml fractions were collected from the top of the gradient. The fractions were immediately neutralized with 400 μl 1 M HEPES (pH 7.15) and diluted with 4 ml 25 mM HEPES (pH 7.4), 1 mM benzamidine, 0.5 mM EDTA. Percoll and membrane vesicles were pelleted by centrifugation (130,000 *g* for 30 min), and the membranous material overlaying the Percoll was collected by a syringe. The membranous fractions were used for enzyme assays and SDS-PAGE/ligand blots as described below and above.

Ligand blot analysis

Cellular and membrane proteins were isolated by SDS-PAGE under reducing conditions in a 7.5% slab gel according to Laemmli (30). Electroblotting of proteins onto nitrocellulose membranes and ligand binding was performed as previously described (15). Briefly, after protein transfer, nitrocellulose strips were incubated for 2 h at room temperature with blocking buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1 mM benzamidine, 0.5 mM EDTA, 10% (w/v) nonfat dried milk, 0.01% (v/v) antifoam A, 50 μg/ml LDL) and then incubated for 2 h at room temperature in the same buffer containing 5 μg/ml of apoE-free ^{125}I-labeled HDL_3. Nitrocellulose membranes were washed at room temperature once, rapidly followed by five 10-min washes with LDL-free blocking buffer. Protein bands were visualized by autoradiography.

Cross-linking of cell surface proteins

Dishes (100 mm) containing BAEC were washed once with PBS/BSA, three times with PBS, and subsequently incubated with 0, 0.2, 0.4, 0.6, 1.0 or 2.0 mM bis(sulfosuccinimidyl)suberate (BS^3) in PBS for 15 min at room temperature. The reaction was stopped by the addition of 100 μl 1 M glycine for 10 min, the dishes were washed 2 times with ice-cold PBS, and the cells were harvested into buffer A. After centrifugation (600 *g*, 10 min), the pellets were solubilized in SDS-PAGE sample buffer, and 300 μg of membrane protein was subjected to SDS-PAGE and to ligand blotting with ^{125}I-labeled HDL_3 as described above.

Lectin chromatography

Collected BAEC were harvested and homogenized in buffer A by a Polytron homogenizer (Tekmar). The homogenate was centrifuged (600 *g* at 4°C for 10 min), and membranes were prepared from the post-nuclear supernatant by centrifugation at 100,000 *g* for 60 min (4°C) in a Beckman 60 Ti rotor. The pellet was solubilized in 10 mM HEPES (pH 7.4), 30 mM CHAPS, 250 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1 M leupeptin, 2 M pepstatin (buffer C) or in 10 mM HEPES (pH 5.7), 30 mM CHAPS, 250 mM NaCl, 0.5 mM $CaCl_2$, 1 M leupeptin, 2 M pepstatin (buffer D) by aspiration through a 22-gauge needle. Undissolved material was removed by centrifugation in a Beckman 50.3 rotor (100,000 *g* for 30 min). The solubilized membranes were blended with a slurry of the following agarose bead-coupled lectins: *Tritium vulgaris* (wheat germ), concanavalin A (Con A), *Arachis hypogaea* (peanut), *Ulex europaeus* I, *Abrus precatorius* (abrin). The membrane preparation applied to the Con A column was in buffer D, while all the others were in buffer C. After 2 h of shaking at 4°C, the beads were loaded onto 0.7 × 1.5 cm columns and washed with 2 column volumes of buffer for subsequent analysis of unbound proteins. After extensive washing, 2 ml of the following sugars (all at 0.5 M) were used for eluting the columns: N-acetyl-D-glucosamine (wheat germ), α-methyl-D-mannoside (Con A), methyl-β-D-galactoside (peanut), α-L-fucose (ulex europaeus I), and α-lactose (abrin). The samples were concentrated on Centricon-30 filters and diluted with SDS-PAGE buffer. The unbound protein (300 μg) and all the eluted material were subjected to SDS-PAGE, and ligand blotting with ^{125}I-labeled HDL_3 was performed as described above.

Enzymatic analysis

5'-Nucleotidase was assayed according to Lange and Steck (28) with some modifications. Briefly, samples (< 70 μg protein) were incubated for 30 min at 37°C in assay buffer, and the reaction was stopped by adding 0.25 ml 0.25 M $ZnSO_4$ and 0.25 ml saturated $Ba(OH)_2$. After 10 min on ice, the samples were centrifuged (10,000 *g* for 10 min), and the absorbance (260 nm) was measured.

Glucose-6-phosphatase was measured according to Kitcher, Siddle, and Luzio (31) except that the release of inorganic phosphorus was measured according to Lanzetta et al. (32). UDP-galactosyltransferase was assayed according to Oliver, Dromy, and Hart (33) except for the suggested substitution of antitrypsin III for asialoorosomucoid as protein acceptor (Oliver, C., personal communication). Acid phosphatase was assayed by the *p*-nitrophenylphosphate hydrolysis method described by Moss (34). All enzymatic determinations were normalized for sample volume.

Other methods

Protein was assayed according to Lowry et al. (35) or by Bio-Rad protein assay (Bio-Rad Laboratories). Cholesterol was assayed with a kit from Boehringer Mannheim Biochemicals.

Densitometic scanning of autoradiographies was performed on a Quick Scan R&D densitometer (Helena Laboratories), equipped with a GS 365 data system (Hoefer Scientific Instruments).

RESULTS

Subcellular localization of binding proteins

To identify subcellular localization of HDL-binding proteins, density gradient fractionation studies were performed, and HDL-binding was assessed by ligand blot analysis. **Fig. 1A** shows that apoE-free ^{125}I-labeled HDL_3 binds to a 110 kDa protein, in agreement with earlier reports (14, 15). In most of the fractions, the 110 kDa band appeared as a doublet with a strong 110 kDa signal and a faint 105 kDa band. The fractions most abundant in HDL-binding proteins were fractions 6–10 and 15 and 16, corresponding to densities of 1.10–1.15 g/ml and 1.21 g/ml, respectively. In the former density range, HDL_3 also interacted with a protein of 130 kDa that displayed a similar distribution as the 105–110 kDa doublet. Large HDL-binding proteins with apparent M_r of 150–200 kDa were present in fraction 6 (Fig. 1A). None of the organelle markers assayed codistributed with these large proteins, suggesting that they reside in the soluble part of the cell.

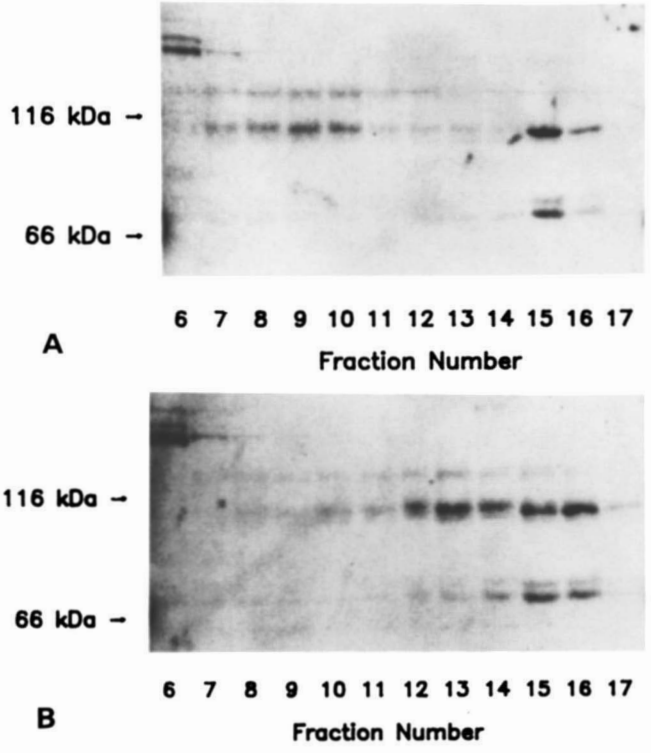

Fig. 1. Ligand blot analysis of sucrose gradient-fractionated endothelial cell homogenates. Panel A: Cultured bovine aortic endothelial cells were pretreated with [^{3}H]cholesterol to radiolabel plasma membranes, harvested, and homogenized. The homogenates were applied on a 19.5–60.0% sucrose gradient and centrifuged to equilibrium. Aliquots (350 μl) of fractions 6–13 (Fraction 1 top) were concentrated, and proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Ligand blots were performed with ^{125}I-labeled HDL_3 (5 μg/ml) as described in Experimental Procedures. Radioactive bands were visualized by autoradiography. Panel B: Same conditions as in panel A except that the cells were treated with digitonin as described in Experimental Procedures.

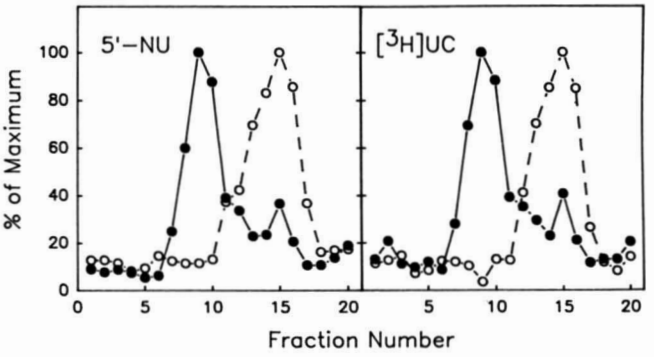

Fig. 2. Distribution of 5'-nucleotidase and [^{3}H]cholesterol in sucrose density gradients. Distribution of 5'-nucleotidase activity (5'-NU) and [^{3}H]cholesterol ([^{3}H]UC) in fractions 1–20 from the same experiment shown in Fig. 1. Measurements were done in non-digitonin-treated homogenates (closed symbols) and in digitonin-treated homogenates (open symbols).

In fractions 12–16, a doublet band of approximately 75 kDa was present. Fractions 15 and 16 also contained an intense band with an apparent M_r of 110 kDa that was not observed in all cell preparations (see Fig. 4). It is likely that this band is associated with unhomogenized cells which appear in this density region.

The distribution of HDL-binding proteins was compared to the distribution of organelle markers assayed in the sucrose gradient fractions. **Fig. 2** shows that the distribution of 5'-nucleotidase activity and [^{3}H]cholesterol, both plasma membrane markers (28, 36), corresponds to the 105, 110, and 130 kDa band profiles in fractions 7–10. The highest binding activity for these three proteins was found in homogenate fractions 9 and 10, which corresponds to the fractions with the highest 5'-nucleotidase activity and [^{3}H]cholesterol content. These HDL-binding proteins also colocalized with 5'-nucleotidase when dense organelles were removed from cell homogenates by centrifugation at 10,000 *g* prior to gradient fractionation (data not shown), indicating that these proteins were not localized to mitochondria, lysosomes, and peroxisomes (36).

To selectively perturb the density distribution of the plasma membrane in the sucrose gradient, cells were homogenized and incubated in the presence of digitonin. Digitonin intercalates in cholesterol-containing membranes making them less buoyant (28, 37). This causes a greater density shift for organelles with cholesterol-rich membranes, such as plasma membrane vesicles, than for intracellular organelles that have a relatively low cholesterol content (37). Digitonin treatment of BAEC homogenates caused a significant shift of the plasma membrane 5'-nucleotidase and [^{3}H]cholesterol to higher densities (Fig. 2). In contrast, density shifts for glucose-6-phosphatase, UDP-galctosyltransferase, and acid phosphatase (marker enzymes for the endoplasmic reticulum,

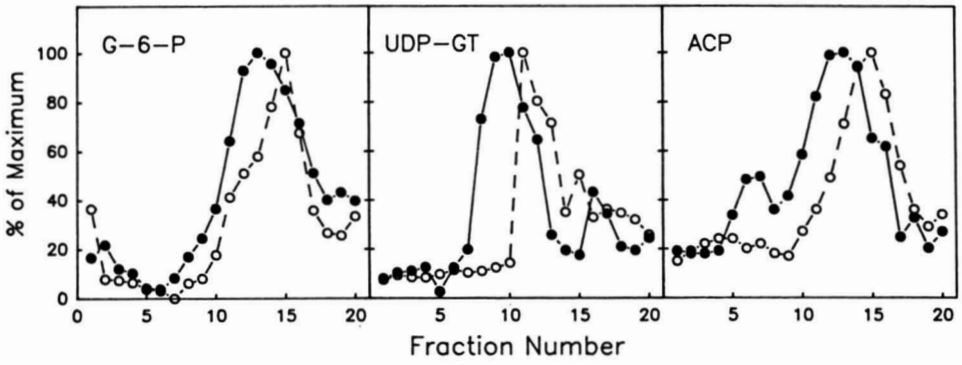

Fig. 3. Distribution of glucose-6-phosphatase, UDP-galactosyltransferase, and acid phosphatase in sucrose density gradients. Distribution of glucose-6-phosphatase (G-6-P), UDP galactosyltransferase (UDP-GT), and acid phosphatase (ACP) activity in fractions 1–20 from the same experiment shown in Fig. 1. Measurements were done in non-digitonin-treated homogenates (closed symbols), and in digitonin-treated homogenates (open symbols).

Golgi vesicles, and lysosomes, respectively) were less pronounced (**Fig. 3**). The 105, 110, and 130 kDa proteins were shifted in exactly the same way as the plasma membrane markers (Fig. 1B). These results indicate that these HDL-binding proteins are associated with cholesterol-rich membranes.

Digitonin treatment caused insignificant density shifts in the large HDL-binding proteins in the most buoyant fractions (fractions 6 and 7) and the 75 kDa doublet in the denser fractions (fractions 12–15) (Fig. 1A and B). Hence, these bands are likely to represent HDL-binding proteins in cholesterol-deficient compartments of the cell.

Homogenization of cells at a high pH (pH 9.6) has been reported to substantially improve the separation of plasma membrane vesicles from other organelles when subjected to Percoll-gradient centrifugation (29). Under these conditions, the 110 and 130 kDa HDL-binding proteins (**Fig. 4**) closely codistributed with 5' nucleotidase (**Fig. 5**), with the highest activities in gradient fraction 3. Because the 110 kDa band was broad in these ligand blots, the 105 kDa band could not be detected. The absence of the 150–200 kDa proteins observed in the sucrose gradients (fractions 6 and 7 in Fig. 1) further supports the conclusion that those are soluble proteins, for only membrane-associated proteins are isolated by the Percoll-gradient procedure. Glucose 6-phosphatase and acid phosphatase displayed a bimodal distribution, with a minor fraction codistributing with the 5'-nucleotidase activity and a major fraction in the bottom part of the gradient (Fig. 5). Golgi vesicles (UDP-galactosyltransferase) were also localized to the bottom of the gradient. The endoplasmic reticulum and lysosomal material present in fractions 3–5 is unlikely to be responsible for the binding of HDL, since the 110 and 130 kDa binding proteins were absent in fractions 7–10 where most of these organelles were found. These findings further support the conclusion that the 110 and 130 kDa proteins are localized to the plasma membrane.

Effect of cross-linking of cell surface proteins on the activities of HDL binding proteins

As the fractionation studies suggested that the 105, 110, and 130 kDa proteins were localized to the plasma membrane, we investigated whether treatment of intact cells with a chemical cross-linker abolishes HDL binding to these proteins and/or results in the formation of larger HDL binding protein complexes. Cultured cells were treated with BS^3, an amino-reactive, water-soluble cross-linker that does not penetrate the plasma membrane. Cross-linking of cell-surface proteins abolished the binding activity of the 110 and 130 kDa with increasing concentrations of the cross-linker (**Fig. 6**). At 0.2 mM BS^3, most of the binding activity was lost. Cross-linking of cell-surface proteins had no effect on HDL binding to the 105 kDa protein, even though this protein codistributed with the 110 kDa and 130 kDa bands and plasma membrane markers in the sucrose gradient fractionation experiments. The 75 kDa binding doublet was also unaltered by

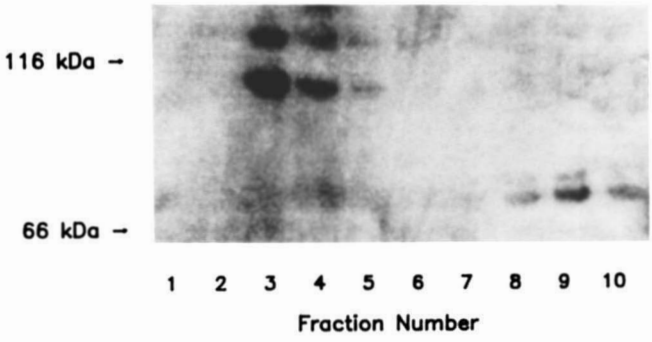

Fig. 4. Ligand blot analysis of Percoll gradient-fractionated endothelial cell homogenates. Homogenates of cultured bovine aortic endothelial cells were applied to a 50% (v/v) self-forming Percoll gradient (pH 9.6), and 2-ml fractions (Fraction 1 top) were collected after centrifugation. Ligand blot analysis of membrane proteins with ^{125}I-labeled HDL_3 was performed as described in Experimental Procedures.

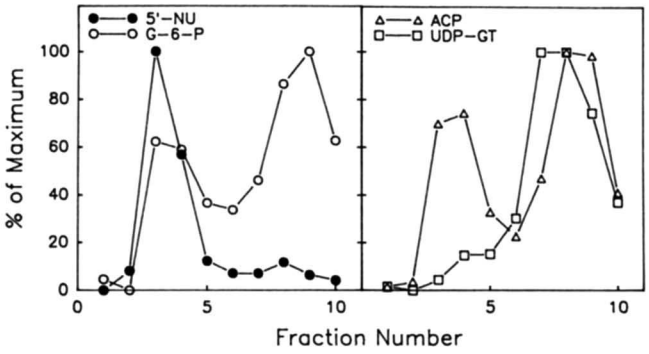

Fig. 5. Distribution of 5'-nucleotidase, glucose-6-phosphatase, UDP-galactosyltransferase, and acid phosphatase in Percoll gradients. The marker enzymes 5'-nucleotidase (5'-NU), glucose-6-phosphatase (G-6-P), acid phosphatase (ACP), and UDP-galactosyltransferase (UDP-G) were assayed in fractions 1-20 from the same experiment shown in Fig. 4.

cross-linking (not shown), consistent with an intracellular localization of these proteins. No larger HDL-binding proteins were observed by ligand blot analysis after cross-linking.

Lectin chromatography of HDL binding proteins

Since most plasma membrane receptors are glycoproteins, we investigated the affinity of the 110 and 130 kDa proteins and other membrane HDL-binding proteins for binding to lectins. Detergent-solubilized membranes were applied to five lectin columns with different glycoprotein affinity, and the unbound material was collected and analyzed for HDL-binding proteins by ligand blotting. Bound material was analyzed in the same way after elution from the columns with specific sugars. Only the wheat germ agglutinin (**Fig. 7**) and abrin (not shown) columns retained HDL-binding proteins to a significant degree. With the other lectin columns tested (Con A, peanut lectin, *Ulex europaeus* I), most of the applied HDL-binding proteins were recovered in the unbound fraction based on densitometric scanning of the ligand blots (data not shown). The wheat germ agglutinin column bound more than 80% of the applied 110 and 130 kDa proteins (Fig. 7). In the unbound fraction (Fig. 7, left lane), the 75 kDa doublet accounted for more than 90% of the HDL-binding protein recovered. Although 105 kDa and 110 kDa doublets were not resolved in these experiments, the 105 kDa protein did not appear to be present in the unbound fraction, suggesting that it also is a glycoprotein. These results indicate that the 110 kDa, the 130 kDa, and probably the 105 kDa HDL binding proteins are glycosylated, consistent with their plasma membrane localization.

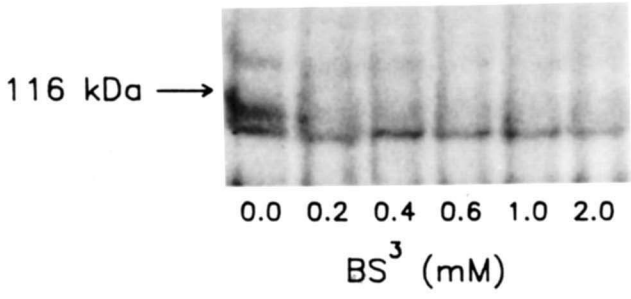

Fig. 6. Effect of treatment of intact cells with water-soluble cross-linker on HDL_3 binding activity of membrane proteins. Bovine aortic endothelial cells were washed and incubated with the indicated concentrations of bis(sulfosuccinimidyl)suberate (BS^3) for 15 min at room temperature. The reaction was quenched by the addition of 100 μl 1M glycine, cells were washed, and membrane proteins were isolated and assayed for ^{125}I-labeled HDL_3 binding activity by ligand blot analysis as described in Experimental Procedures. Three hundred μg of protein was applied to each lane.

DISCUSSION

In the current study we used ligand blot analysis to characterize the cellular distribution of HDL binding proteins in cultured bovine aortic endothelial cells. Our results show that multiple membrane and soluble proteins bind ^{125}I-labeled HDL_3 on ligand blots. Membrane proteins with molecular masses of 105, 110, and 130 kDa codistributed with the plasma membrane markers [^3H]cholesterol and 5'-nucleotidase in sucrose and Percoll gradients. Of these three proteins, the 110 kDa band was either the most abundant or had the highest HDL bind-

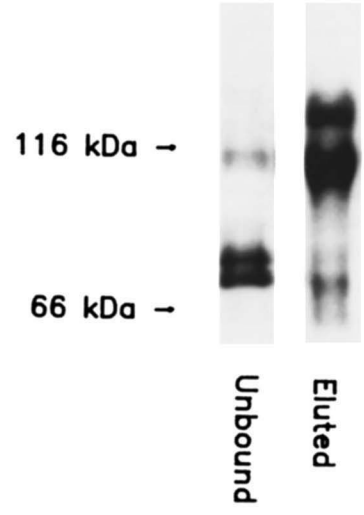

Fig. 7. Wheat germ agglutinin chromatography of HDL-binding proteins. Membranes from bovine aortic endothelial cells were obtained and solubilized in 30 mM CHAPS as described in Experimental Procedures. Nonsolubilized material was removed by centrifugation, and the supernatant was applied to a wheat germ agglutinin column. After washing, bound glycoproteins were eluted with 0.5 M N-acetylglucosamine. Aliquots of the unbound material (left lane) and of the material eluted with the sugar (right lane) were concentrated and solubilized in SDS, and proteins were separated by SDS-PAGE. Ligand blot analysis with ^{125}I-labeled HDL_3 was performed as described in Experimental Procedures.

ing activity. When cell homogenates were treated with digitonin, which causes a density shift of cholesterol-rich vesicles (28, 37), a pronounced shift of all three of these proteins in the gradients was observed in association with an identical shift of the plasma membrane markers. These findings suggest that the 105, 110, and 130 kDa proteins reside in a cholesterol-rich cellular compartment, most likely the plasma membrane.

Further evidence that the 110 and 130 kDa proteins are localized to the plasma membrane of intact cells was provided by studies showing that the ability of both the 110 and 130 kDa proteins to bind HDL was reduced when cells were treated with the crosslinker BS^3. This was probably because intra- or intermolecular cross-linking destroyed their HDL-binding activity. These results suggest that these proteins are at least partially exposed on the cell surface, since BS^3 is hydrophilic and does not penetrate the plasma membrane. HDL-binding to the 105 kDa protein was not changed by cross-linking, even though this protein co-localized with plasma membrane markers in the sucrose gradients. It may be more resistant to cross-linking than the other plasma membrane HDL-binding proteins because of its structural properties or intramembranous location.

We were unable to determine whether the different HDL-binding proteins were capable of forming complexes with themselves or other membrane proteins similar to what has been described for some receptors (38, 39) and hepatic HDL binding proteins (23). Interaction through disulfide linkages does not appear to occur, as the electrophoretic mobility of the HDL-binding proteins was similar under reducing and nonreducing conditions (data not shown). Treatment of cells with cross-linker did not result in the appearance of larger molecular weight bands on ligand blots, suggesting that if complexes of HDL-binding proteins are formed, they have lost their ability to bind HDL under the conditions of our ligand blot assay.

When solubilized membrane-derived HDL-binding proteins were subjected to lectin chromatography, the 110 and 130 kDa proteins bound to wheat germ agglutinin, showing that they are glycoproteins. Both proteins also bound to abrin, suggesting the presence of β-galactose- or N-acetylgalactosamine-containing terminal glycosyl residues. The two membrane-associated HDL-binding proteins with M_r of 75 to 80 kDa did not bind to lectin columns, implying that they are nonglycosylated.

The cellular distribution and physical properties of the 110 kDa protein are consistent with the previous hypotheses that this protein is a candidate for the putative HDL receptor (14, 15). HDL-binding proteins of similar size have been identified in membranes of a variety of different cell types (15–17, 19, 22, 23). Expression of these proteins is regulated as predicted for receptors that function to rid cells of excess cholesterol, in that their membrane content increases when cells are cholesterol-loaded (15) or growth-inhibited (16). Plasma membranes from bovine aortic endothelial cells also contain additional proteins of 130 and 105 kDa that bind HDL_3 on ligand blots, although to a lesser extent than the 110 kDa protein. Previous studies (15) suggested that expression of the 130 kDa protein also increases upon cholesterol loading of cells. Inasmuch as the 105 kDa protein was undetected in these earlier studies, the effect of cholesterol on its expression has not been determined.

Recently, a membrane protein was cloned from a human fibroblast cDNA expression library using an antibody raised against the 110 kDa HDL binding protein partially purified from cultured bovine aortic endothelial cells (40). The cellular content of its mRNA was shown to increase several-fold when macrophages were loaded with cholesterol. This protein is mostly comprised of 14 tandem repeats of approximately 70 amino acids in length, with each repeat containing two amphipathic helixes. When overexpressed in baby hamster kidney cells, a significant fraction of the protein was found to be associated with the plasma membrane (40). As it does not contain a classic transmembrane spanning domain, the protein appears to interact with membranes through its amphipathic regions. It is not known yet if the different plasma membrane-associated HDL binding proteins are processed forms of this cloned protein. However, immunoblot analysis suggested that the antibody used for the expression cloning cross-reacted with both the 110 kDa and 130 kDa proteins, raising the possibility that they share common epitopes. Results from the current and previous studies indicate that several related or unrelated proteins have the potential to interact with HDL particles on the cell surface. The functional significance of this cell surface binding remains to be determined.

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