Affinity purification of the hepatic high-density lipoprotein receptor identifies two acidic glycoproteins and enables further characterization of their binding properties

Hiroya HIDAOKA and Noel H. FIDGE*

Protein Chemistry and Molecular Biology Unit, Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181, Australia

Several high-density lipoprotein (HDL)-binding proteins, candidates for the putative HDL receptor, have recently been identified, including two membrane proteins: HB₁ of 120 kDa and HB₂ of 100 kDa, present in rat and human liver plasma membranes respectively. Further insights into their function however, have been hampered by poor recoveries of these hydrophobic peptides, and the present work was undertaken to improve yields and enable a more detailed investigation of their properties. A significant improvement has been achieved using two affinity chromatographic procedures, one exploiting the glycoprotein nature of the proteins and the other exploiting their ligand properties, which in combination resulted in considerable enrichment of HB₁ and HB₂. Thus DEAE-Sephacel fractionation (0.05–0.2 M-NaCl) of CHAPS-solubilized plasma membranes yielded active HDL-binding proteins which bound to concanavalin A–Sephacel or wheat-germ-lectin–Sephacel columns and retained their binding activity after eluting with methyl- α -D-mannoside or N-acetylglucosamine respectively. These glycoproteins were further purified by affinity chromatography using apo-HDL–Sephacel columns. Final purification required preparative SDS/PAGE. Investigation of the carbohydrate moieties of the proteins using glycosidases and two-dimensional gel electrophoresis revealed pI values ranging from 4.6 to 4.9 and from 4.5 to 4.7 for HB₁ and HB₂ respectively, which after treatment with neuraminidase shifted towards basic pH (5.4–5.7 and 5.3–5.5 respectively). The molecular masses were decreased to 115 kDa and 95 kDa respectively, demonstrating that sialic acid residues contributed significantly to the negative charge of the glycosylated peptides. Treatment with the enzyme peptide N-glycosidase F (N-glycanase) resulted in a decrease in molecular mass of HB₁ and HB₂ to 105 kDa and 80 kDa respectively, but endo- α -N-acetylgalactosaminidase (O-glycanase) treatment was not effective. Neither neuraminidase nor N-glycanase treatment destroyed activity, suggesting that sialic acids or N-linked oligosaccharides are not important determinants of HDL binding. Digestion of plasma membranes with trypsin or Pronase resulted in a loss of activity of both HB₁ and HB₂ that was not influenced by prior treatment with neuraminidase, suggesting that sialic acid residues play no protective role against proteolytic cleavage of HDL receptor proteins.

INTRODUCTION

High-affinity high-density lipoprotein (HDL)-binding sites have been detected in a variety of tissues and cells of many species [1–10], particularly the liver, which plays a major role in processing HDL [11–13]. Further ¹²⁵I-labelled HDL₃-binding sites in rat liver are predominantly located in the plasma membrane fraction [14–16].

Although an isolated plasma membrane preparation from rat liver has previously been used to characterize HDL-binding sites, the properties of purified detergent-solubilized HDL-binding proteins have not been fully investigated. Our group reported the isolation of two HDL-binding proteins from rat liver plasma membranes following solubilization with CHAPS, DEAE-Sephacel chromatography, and then preparative SDS/PAGE [17]. These HDL-binding proteins, termed HB₁ and HB₂, showed apparent molecular masses of 120 and 100 kDa respectively on SDS/PAGE, and in ligand blots bound apolipoproteins AI and AII as well as HDL. Furthermore, we recently demonstrated that both proteins could be physiologically regulated with drugs that influence lipoprotein metabolism, supporting their role as participants in HDL transport [18].

Further attempts to characterize the properties of these candidate HDL receptors have been hampered by their low abun-

dance, with yields estimated to be lower than 0.05% of total plasma membrane protein. The present experiments were undertaken to improve recovery of receptor protein by exploiting affinity chromatographic procedures that facilitate purification, enabling new information to be obtained about the properties of HB₁ and HB₂.

MATERIALS AND METHODS

Materials

DEAE-Sephacel, concanavalin A (ConA)–Sephacel 4B, wheat-germ-lectin–Sephacel 6MB, CNBr–Sephacel and isoelectric focusing (i.e.f.) agarose were obtained from Pharmacia Fine Chemicals. Na-¹²⁵I was from Amersham. Neuraminidase (EC 3.2.1.18), peptide N-glycosidase F (N-Glycanase; EC 3.2.2.18) and endo- α -N-acetylgalactosaminidase (O-Glycanase; EC 3.2.1.97) were from Genzyme. Ampholine was from LKB and CHAPS was from Sigma. Molecular mass standards for SDS/polyacrylamide gels were purchased from Bio-Rad and Pharmacia Fine Chemicals. Nitrocellulose membrane was from Schleicher & Schell. Tos-Phe-CH₂Cl (TPCK)-treated trypsin and Pronase (from *Streptococcus griseus*) were from Sigma and Calbiochem–Behring Corp. respectively.

Abbreviations used: HDL, high-density lipoprotein; ConA, concanavalin A; PMSF, phenylmethanesulphonyl fluoride; apo, apolipoprotein; i.e.f., isoelectric focusing.

* To whom correspondence should be addressed.

Preparation of HDL

Human HDL₃ (*d* 1.125–1.21) were isolated from fresh human plasma by ultracentrifugation according to the method of Havel *et al.* [19]. HDL₃ was washed once by recentrifugation at the appropriate density and dialysed against Tris/HCl buffer (20 mM, containing 0.15 M-NaCl and 1 mM-Na₂ EDTA), then characterized by SDS/PAGE.

Preparation of rat plasma membranes

Plasma membranes from rat liver were prepared as described earlier [17] with minor modifications of the method described by Fleischer & Kervina [20]. Rats, weighing 150–250 g, were fasted overnight and killed by cervical dislocation. The liver was perfused *in situ* with 150 mM-NaCl/1 mM-phenylmethanesulphonyl fluoride (PMSF), pH 7.4, and then removed and homogenized in a Potter–Elvehjem-type homogenizer in 5 vol. (v/w) of ice-cold buffer (0.25 M-sucrose in 10 mM-Hepes, pH 7.5), and the plasma membrane fraction was purified as described previously [17]. Preparations were quickly frozen in liquid N₂ and stored at –70 °C.

Solubilization of rat plasma membranes

The suspension of liver plasma membranes was diluted 3-fold with 50 mM-Tris/HCl (pH 8.0) containing 1 mM-PMSF. CHAPS was added to a final concentration of 20 mM. The mixture was stirred gently at 4 °C for 60 min, and insoluble material was removed by centrifugation at 3000 *g* for 15 min.

DEAE-Sephacel chromatography

The CHAPS-solubilized extract was applied to a DEAE-Sephacel column (1.5 cm × 27 cm), previously equilibrated with buffer A (50 mM-Tris/HCl, pH 8.0, 10 mM-CHAPS and 1 mM-PMSF). The column was first washed with 150 ml of buffer A, and proteins were eluted with 100 ml of 0.05 M-NaCl in buffer A, followed by 250 ml of 0.2 M-NaCl and then 100 ml of 0.4 M-NaCl in buffer A. Fractions (10 ml each) were collected, absorbance measured at 280 nm, and assayed for activity by ligand blotting.

ConA–Sephacel and wheat-germ-lectin–Sephacel affinity chromatography

ConA–Sephacel 4B or wheat-germ-lectin–Sephacel 6MB were equilibrated with buffer B (50 mM-Tris/HCl buffer, pH 7.4, containing 0.15 M-NaCl and 10 mM-CHAPS) and the DEAE-Sephacel fractions containing HDL-binding proteins were gently agitated with each gel overnight at 4 °C. The gel was transferred to a column and washed with 2 vol. of buffer B. After washing, proteins bound to the gels were eluted with either 0.2 M-methyl- α -D-mannoside in buffer B (ConA–Sephacel) or with 0.2 M-N-acetyl-D-glucosamine in buffer B (wheat-germ-lectin–Sephacel 6MB). Fractions (10 ml each) were collected, their absorbance was measured at 280 nm, and they were assayed for activity by ligand blotting.

Apo-HDL₃ affinity chromatography

Apo-HDL₃–Sephacel was prepared by coupling apo-HDL₃ to CNBr-activated Sepharose 4B in accordance with the manufacturer's instructions [21]. A ratio of 5 mg of apo HDL₃ to 1 g of dry gel was used.

The wheat-germ-lectin-purified protein (concentrated fraction obtained as described above) was mixed with apo-HDL₃–Sephacel gel equilibrated with Tris/HCl buffer [50 mM, pH 7.4,

containing 6 mM-CHAPS, 20 % (w/v) glycerol and 1 mM-PMSF]. The mixture was gently rocked overnight at 4 °C. After transferring the mixture on to a column (1.5 cm × 30 cm), the gel was washed with the same buffer (20 times bed volume of column). The bound fraction was eluted with 0.2 M-NH₄OH (pH 10.5) containing 10 mM-CHAPS and 1 mM-PMSF. The eluted solution was immediately adjusted to neutral pH with 0.5 M-Tris/HCl buffer. Fractions containing proteins with binding activity were pooled and transferred to an Amicon ultrafiltration cell (50 ml) in order to concentrate and exchange the solution with buffer B.

Preparative SDS/PAGE and diffusional elution

Preparative gel electrophoresis was performed, using 8 % polyacrylamide gel slabs (11 cm × 16 cm × 0.15 cm) containing 0.1 % SDS, according to the method of Laemmli [22]. After electrophoresis, the gel was stained with 0.1 % Coomassie Brilliant Blue R250 and then the active bands were excised. The proteins in the gel were isolated by diffusional elution with 50 mM-NH₄HCO₃ containing 1 % SDS, and then precipitated with chloroform/methanol (1:3, v/v) as described by Wessel & Flugge [23].

I.e.f. and two-dimensional electrophoresis

I.e.f. was carried out using an i.e.f. agarose gel according to the manufacturer's instructions (Pharmacia). I.e.f. agarose (1 %) with 10 mM-CHAPS in distilled water was solubilized (in a microwave oven) and cooled to 75 °C, and 2.4 % Ampholine (pH 3.5–9.5, or pH 3.5–5/pH 4–6, 1:1, v/v) was added. The gel was poured into a cassette (9.5 cm × 9 cm × 0.75 cm), and placed in the cold room (4 °C) for at least 1 h. The gel was removed from the cassette and excess water was removed by placing filter paper on the gel for 1 min. The gel was positioned on the cooling unit and electrode strips were attached on both sides of the gel; the anode electrolyte was 0.5 M-NaOH and the cathode buffer was 0.5 M-acetic acid. A 10–30 μ l portion of the sample was applied 2 cm from the anode electrode strip and electrofocusing was performed at 400 V for 4 h. After focusing, a control strip of gel was sliced into 2.5 mm sections and the gel pieces were soaked in 2.0 ml of water for 1 h. The pH was measured to determine the pH gradient.

Two-dimensional electrophoresis was performed with i.e.f. in the first dimension and SDS/PAGE in the second dimension. After i.e.f., a 5 mm gel strip was cut out and incubated in the SDS/PAGE buffer for 5 s, and then placed on top of an SDS/polyacrylamide gel (8 cm × 5 cm × 0.075 cm). The SDS/polyacrylamide gel was then run at 10 mA/gel for 2 h.

Ligand blotting

After electrophoresis on SDS/polyacrylamide, proteins were electrophoretically transferred on to nitrocellulose membranes [24]. The nitrocellulose membrane was incubated with blocking buffer (50 mM-Tris/HCl, pH 7.4, containing 150 mM-NaCl and 2 % skim milk powder) for 1 h, followed by incubation for 1 h in the same buffer containing 10 μ g of ¹²⁵I-labelled HDL₃/ml in 2 mM-CHAPS, pH 7.4. After washing for 5 × 5 min with Tris buffer (20 mM-Tris/HCl containing 0.15 M-NaCl and 1 mM-Na₂EDTA), the nitrocellulose membrane was exposed to Hyperfilm-MP for 48–72 h at –70 °C in a cassette containing Cronex Lightning Plus intensifying screens.

For highly sensitive detection, ligand blotting was carried out with unlabelled HDL₃, anti-apo-AI IgG and horseradish peroxidase conjugated IgG, and then staining with 20 ml of 0.3 % dianosidine in methanol/100 ml of 0.01 M-phosphate-buffered saline, pH 7.4/0.1 ml of H₂O₂ in buffer B. Nitrocellulose membrane was incubated with blocking buffer for 1 h, followed by incubation for 1 h in the same buffer containing 50 μ g of

HDL₃/ml in 2 mM-CHAPS, pH 7.4. After washing with Tris buffer, the nitrocellulose membrane was incubated with the blocking buffer containing anti human-apo-AI IgG (from rabbit) for 1 h, washed, and then incubated with HRPO-conjugated anti-(rabbit IgG) (from goat) in 2 mM-CHAPS for 1 h. After treatment, the nitrocellulose membrane was stained with buffer B containing 30 mg of dianosidine and 50 μ l of H₂O₂ per 100 ml. This method was compared with the procedure using ¹²⁵I-HDL₃ as ligand (above), and the results corresponded exactly in terms of specificity for ligand and identification of HB₁ and HB₂ bands. In addition, control strips incubated with first and second antibody alone (no ligand) were negative.

Enzymic deglycosylation of HDL-binding proteins

The stored plasma membranes (50 μ g of membrane protein) were thawed, centrifuged (10000 *g*, 1 min) and then treated as follows with deglycosylation enzymes. Desialylation of HDL-binding protein was carried out by adding 50 munits of neuraminidase in 50 μ l of 0.1 M-sodium acetate (pH 6.5) to the pellet. Tubes without enzyme were included as controls. The mixtures were incubated for 2 h at room temperature.

N-Glycanase treatment was carried out after denaturation with 10 μ l of 0.5 % SDS at room temperature for 20 h. Samples were finally adjusted to 30 μ l of reaction volume containing (final concentrations) 100 mM-sodium phosphate, pH 8.6, 2.0 % Nonidet P-40, 0.33 % SDS and 0.3 unit of *N*-Glycanase, and incubated at room temperature for a further 16 h.

O-Glycanase digestion was carried out after denaturation in 0.5 % SDS and then desialylation by neuraminidase as described above. Samples were adjusted to a 50 μ l reaction volume containing (final concentrations) 10 mM-acetate buffer (pH 6.5), 2.0 % Nonidet P-40, 0.33 % SDS and 7.5 munit of *O*-Glycanase, and incubated for 16 h at room temperature.

Digestion of HDL-binding proteins

The stored plasma membranes were thawed, centrifuged (10000 *g*, 1 min) and then treated as follows with proteolytic enzymes. Plasma membranes (50 μ g of protein) were resuspended in PBS (0.05 M-phosphate buffer, pH 7.5, containing 0.15 M-NaCl) and then treated with Pronase (10–100 ng of protein) or trypsin (1.25–25 μ g of protein) at 37 °C for 30 min.

Analytical procedures

Proteins were analysed by SDS/PAGE as described by Laemmli [22]. Polyacrylamide gels were stained with silver nitrate and Coomassie Blue R-250 for protein. Staining with silver nitrate was carried out as described before [25].

The protein content of samples were determined by a modified version [26] of the Lowry procedure.

RESULTS

DEAE-Sephacel fractions enriched with HDL-binding proteins were eluted with 0.2 M-NaCl in the presence of 10 mM-CHAPS. Binding activity was established with a ligand blot assay [17], as described in Materials and methods section.

Active fractions were applied to ConA–Sephacrose 4B or wheat-germ-lectin–Sephacrose 6MB columns. Proteins which bound to the ConA–Sephacrose 4B or wheat-germ-lectin–Sephacrose were eluted with 0.2 M-methyl- α -D-mannoside in buffer B (from ConA–Sephacrose) or with 0.2 M-*N*-acetyl-D-glucosamine in buffer B (from wheat-germ-lectin–Sephacrose 6MB) (Fig. 1). By ligand blotting it was established that all HDL-binding proteins were recovered in the fraction bound to either the ConA or the wheat-germ lectin column (Fig. 2).

Active fractions from wheat-germ lectin affinity chromatography were applied to apo-HDL₃–Sephacrose. Proteins which bound to the apo-HDL₃–Sephacrose column were eluted with 0.2 M-NH₄OH (pH 10.5) (Fig. 3). Ligand blotting revealed that all HDL-binding protein activity was recovered in the fraction bound to apo-HDL₃–Sephacrose (Fig. 4). Although this procedure produced a significant enrichment of HB₁ and HB₂ proteins (weakly silver stained in Fig. 4a, lane 4), further purification of HDL-binding proteins from apo-HDL₃ affinity chromatography was required; this was performed by preparative SDS/PAGE [17] and extraction with chloroform/methanol/water as described previously [23]. The active bands were identified after staining the gels, elution of bands and assay by the highly sensitive ligand blot assay described in the Materials and methods section. Purification to apparent homogeneity as assessed by silver staining, ConA staining and ligand blotting is shown in Fig. 6 (see below). Following all purification steps, the recovery (not accounting for unknown losses) of HB₁ and HB₂ was approx. 2.0 μ g and 3.9 μ g per liver respectively.

Enzymic deglycosylation of HDL-binding proteins

To identify the carbohydrate moieties of the HDL-binding proteins, we treated plasma membranes with various deglycosyl-

Fig. 1. ConA–Sephacrose (a) and wheat-germ-lectin–Sephacrose (b) column chromatography of the DEAE-Sephacel-eluted protein

The fractions eluted from DEAE-Sephacel with 0.2 M-NaCl were gently agitated with 23 ml of ConA–Sephacrose or 7 ml of wheat-germ-lectin–Sephacrose equilibrated with 50 mM-Tris/HCl, pH 7.4, containing 10 mM-CHAPS and 0.15 M-NaCl overnight at 4 °C. The gels were then washed with the same buffer, and ConA–Sephacrose-bound proteins were eluted with buffer containing 0.2 M-methyl- α -D-mannoside, while wheat-germ-lectin–Sephacrose-bound proteins were eluted with the buffer containing 0.2 M-*N*-acetyl-D-glucosamine.

Fig. 2. Silver staining and ligand blots of HDL-binding proteins after ConA-Sepharose and wheat-germ-lectin-Sepharose chromatography

Samples were subjected to SDS/polyacrylamide gel (8%) electrophoresis. After electrophoresis, half of the gel was stained with silver nitrate (lanes 1-4) and the remainder was transferred to a nitrocellulose membrane and then incubated with ^{125}I -labelled HDL₃ as described in the Materials and methods section (lanes 5-8). Lane 1, molecular mass markers; lane 2, DEAE-Sepharose fraction (0.05-0.2 M-NaCl). Lanes 5 and 7 represent unbound fractions from ConA and wheat-germ lectin columns respectively. Lanes 3 and 6 are ConA-bound fractions, and lanes 4 and 8 are wheat-germ-lectin-bound fractions.

Fig. 3. Apo-HDL₃-Sepharose column chromatography of wheat-germ-lectin-Sepharose-eluted proteins

The wheat-germ-lectin-Sepharose fractions were gently agitated with 25 ml of apo-HDL₃-Sepharose equilibrated with 50 mM-Tris/HCl, pH 7.4, containing 6 mM-CHAPS, 20% glycerol, 0.5 M-NaCl, 1 mM-PMSF and 2 mM-CaCl₂ overnight at 4 °C. The gels were then washed with the same buffer, and apo-HDL₃-Sepharose-bound proteins were eluted with 0.2 M-NH₄OH containing 10 mM-CHAPS and 1 mM-PMSF (approx. pH 10.5).

ation enzymes, and then analysed the fractions by ligand blotting. Treatment of plasma membranes with neuraminidase produced a decrease in apparent molecular mass of 10-15 kDa for HB₁ and of 15-20 kDa for HB₂ (Fig. 5). The activities of HB₁ and HB₂ were not destroyed by desialylation.

O-Glycanase treatment of denatured and desialylated plasma membrane proteins removes O-linked oligosaccharide chains at threonine or serine residues. However, treatment of the denatured and desialylated plasma membranes with O-Glycanase had little effect on HB₁ and HB₂, both of which retained binding activity.

Fig. 4. Silver staining and ligand blots of HDL-binding proteins after apo-HDL₃-Sepharose chromatography

Samples were subjected to SDS/polyacrylamide-gel (8%) electrophoresis; after electrophoresis, half of the gel was stained with silver nitrate (a) and the remainder was transferred to nitrocellulose membrane and then incubated with ^{125}I -labelled HDL₃ containing 2 mM-CHAPS as described in the Materials and methods section (b). Lane M of (a) shows molecular mass calibration markers. Lanes 1, the wheat-germ-lectin-Sepharose-bound fraction; lanes 2, apo-HDL₃-Sepharose-unbound fractions; lanes 3, apo-HDL₃-Sepharose-bound fractions.

Fig. 5. Glycosidase treatment of HDL₃-binding proteins of rat liver plasma membranes

Rat liver plasma membranes (50 µg of membrane protein/tube) were centrifuged to remove stored buffer solutions. The plasma membrane pellets in five tubes were resuspended in 10 µl of 0.5% SDS. The denatured plasma membranes in three of the tubes (lanes 1-3) were adjusted for the absence (lane 1) or presence of 40 munits of neuraminidase (lanes 2 and 3) as described in the Materials and methods section. Reaction mixtures were incubated at room temperature for 2 h. After neuraminidase treatment, the solutions were further incubated either without (lane 2) or with (lane 3) 7.5 munits of O-Glycanase at room temperature for 16 h. The denatured plasma membranes in the remaining two tubes (lane 4 and 5) were adjusted to conditions for N-Glycanase treatment as described in the Materials and methods section, and then incubated without (lane 4) or with (lane 5) 0.3 unit of N-Glycanase at room temperature for 16 h.

N-Glycanase treatment of denatured plasma membrane proteins removes N-linked oligosaccharide chains at asparagine, converting that residue to aspartic acid. Treatment of the denatured plasma membrane with N-Glycanase resulted in a

Fig. 6. *N*-Glycanase treatment of purified HDL₃-binding proteins

Purified HDL₃-binding proteins obtained by preparative SDS/PAGE of apo-HDL₃-Sepharose-bound fractions were resuspended in 0.2% SDS and distributed (15 ng of protein/tube) into six tubes for each of HB₁ and HB₂. Tubes were then adjusted for the absence [lane 1 (HB₁) and lane 3 (HB₂)] or presence [lane 2 (HB₁) and lane 4 (HB₂)] of 0.1 unit of *N*-Glycanase as described in the Materials and methods section. Reaction mixtures were incubated at room temperature for 16 h. After *N*-Glycanase treatment, HDL-binding proteins were subjected to SDS/PAGE; following electrophoresis, one-third of the gel was stained with silver nitrate (a) and the remainder was transferred to a nitrocellulose membrane by electroblotting. Half of this membrane was incubated with ConA solution and then peroxidase solution at room temperature for 1 h (b). The remainder was subjected to highly sensitive ligand blotting (c).

Fig. 7. Effects of Pronase and trypsin

Rat liver plasma membranes (50 μ g of membrane protein) were pretreated without (lanes 1–4) or with (lanes 5–8) neuraminidase. (a) Membranes were then incubated in the absence (lanes 4 and 5) or presence of various concentrations of Pronase (a; lanes 1 and 8, 100 μ g/ml; lanes 2 and 7, 10 μ g/ml; lanes 3 and 6, 4 μ g/ml). (b) Membranes were incubated in the absence (lanes 4 and 5) or presence of different concentrations of trypsin (lanes 1 and 8, 5 mg/ml; lanes 2 and 7, 2 μ g/ml; lanes 3 and 6, 1 mg/ml). After incubation, membranes were solubilized with SDS buffer and applied to an SDS/polyacrylamide gel. After transfer to nitrocellulose, ligand blotting was performed with ¹²⁵I-HDL₃ as described in the Materials and methods section.

decrease in apparent molecular mass of both HB₁ and HB₂ of 5 kDa (Fig. 5).

Experiments were also performed with purified receptor proteins. Treatment of purified HB₁ and HB₂ with neuraminidase or *N*-Glycanase resulted in a decrease in apparent molecular mass of 5 kDa for both HB₁ and HB₂, and of 15 kDa for HB₁ and 10 kDa for HB₂ respectively (Fig. 6a). However, there was no apparent loss of binding activity, as observed using the sensitive ligand blot assay (Fig. 6c). This retention of binding activity occurred despite apparent removal of all carbohydrate; as shown

Fig. 8. Two-dimensional PAGE of HDL-binding proteins

CHAPS-solubilized rat plasma membranes (100 μ g of membrane protein) were applied at a position 2 cm from the anode side of the i.e.f. agarose gel, and electrophoresis was run as described in the Materials and methods section. After electrophoresis, gel strips from sample and control (C) lanes were cut out and the sample strip was soaked in SDS buffer for 5 s. This strip was then placed on to the SDS/polyacrylamide gel; after electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were incubated with 10 μ g of ¹²⁵I-labelled HDL₃/ml and the binding proteins were detected by autoradiography. Another control strip was sliced into 2.5 mm-wide sections and soaked in 2 ml of distilled water for 1 h, and the pH gradient was measured in a pH meter.

in Fig. 6(b), treatment with *N*-Glycanase resulted in loss of all ConA-binding material.

The differences in changes in molecular mass observed in these experiments are probably due to the digestion of pure HB₁ and HB₂ compared with the plasma membrane preparations used in experiments shown in Fig. 5.

After preincubation with or without neuraminidase, HDL-binding proteins were then treated with Pronase or trypsin (Fig. 7). Trypsin treatment of the plasma membranes significantly decreased the activities of HB₁ and HB₂. Pronase treatment also lowered the activity of both HB₁ and HB₂, although HB₁ activity

Fig. 9. Two-dimensional PAGE of HDL-binding proteins from CHAPS-solubilized plasma membrane before and after treatment with neuraminidase

Plasma membranes were incubated in sodium acetate buffer (pH 6.5) containing either no neuraminidase (a) or 1.0 unit of neuraminidase/ml (b) at room temperature for 2 h. Lanes C and Cn show ligand blots from SDS/PAGE only. Panels (a) and (b) are ligand blots of ^{125}I -HDL₃ from two-dimensional gel experiments.

was decreased at lower concentrations than that of HB₂. Before protease treatment, intact plasma membrane fractions were incubated with neuraminidase as described in the Materials and methods section. Deglycosylation had little influence on the susceptibility of either HB₁ or HB₂ to proteolysis. The presence of a doublet at the HB₂ position in Fig. 7, lane 5, probably reflects the incomplete removal of all sialic acid residues from HB₂ in this preparation; this does not however alter the conclusion of the subsequent proteolysis experiments.

Ligand blots from two-dimensional electrophoretic fractionation of plasma membranes are shown in Fig. 8. Location of active spots, corresponding to HB₁ and HB₂, were identified by reference to autoradiographs of marker lanes containing active HB₁ and HB₂ separated in the second (PAGE) dimension only. The autoradiographs show that ^{125}I -labelled HDL bound to at least two proteins in the HB₁ position, with pI values ranging from 4.6 from 4.9, and to another two in the HB₂ position, with pI values of 4.5–4.7. After treatment with neuraminidase, the active binding proteins shifted to a lower molecular mass position and a more basic isoelectric point, with HB₁ and HB₂ now appearing in the pI 5.4–5.7 and 5.3–5.5 ranges respectively (Fig. 9).

DISCUSSION

As reported earlier by our laboratory [17], two HDL-binding proteins (HB₁ and HB₂) were purified from CHAPS-solubilized preparations of rat liver plasma membranes, and the present study describes a significant improvement in the purification of these proteins, subsequently enabling further characterization of their structural properties. This new work extends isolation beyond the ion-exchange and preparative SDS/PAGE procedures described previously [17], thus improving the chances of isolating pure proteins, a suggestion supported by the apparent homogeneity observed on silver-stained SDS/polyacrylamide gels and after two-dimensional gel electrophoresis. A preliminary observation by us identifying the glycoprotein nature of HB₁ and HB₂ suggested the potential benefits of exploiting affinity chromatography on lectin columns. This resulted in significant enrichment of both active HB₁ and active HB₂ in wheat-germ-

lectin-bound fractions; this enriched fraction was then successfully further purified on Sepharose columns to which the ligand (apo-HDL) had been linked, using a basic buffer (NH_4OH) to elute the proteins. This procedure was adopted following our observation of the highly acidic nature of the proteins on two-dimensional gel electrophoresis; previous desorption systems employing acidic, high-salt or chaotropic buffers had been unsuccessful in yielding active HB₁ or HB₂ from affinity columns (H. Hidaka & N. H. Fidge, unpublished work).

The HDL-binding proteins bound to both ConA–Sepharose 4B and wheat-germ-lectin–Sepharose 6MB. ConA–Sepharose binds glycopeptides with biantennary complex-type, high-mannose-type, and hybrid-type oligosaccharides [27], while glycopeptides with hybrid-type structures and a ‘bisecting’ *N*-acetylglucosamine bind to wheat-germ-lectin–Sepharose [28]. Thus our results suggest that the HDL-binding proteins are glycoproteins containing hybrid-type structures, i.e. a bisecting *N*-acetylglucosamine or *N*-acetylneuraminic acid. Other studies have demonstrated that many cell-surface glycoproteins contain either *N*- or *O*-linked oligosaccharides, or both, and then one or more sialic acid residues attached to the core structure of *N*- or *O*-linked oligosaccharides [29–31]. Neuraminidase cleaves the sialic acid bound at the terminal carbohydrate of the glycoprotein. When the plasma membrane was treated with neuraminidase, the molecular mass of both HDL-binding proteins was decreased, and their charge was shifted to a more basic isoelectric point. Further, the ability of both purified HDL-binding proteins to bind ^{125}I -HDL₃ was not affected by neuraminidase treatment. These results suggest that the HDL-binding proteins contain several moles of sialic acid, positioned on the surface of the plasma membrane; however, these sialic acid residues apparently do not influence the HDL-binding properties of HB₁ and HB₂. In fact, glycosylation appears to play no part in the binding activity of HB₁ and HB₂, since the apparently complete removal of carbohydrate chains from purified HB₁ and HB₂, as determined by ConA–peroxidase staining following deglycosylation with *N*-Glycanase (Fig. 6), did not affect HDL binding.

The *O*-linked oligosaccharides are mono- and di-sialylated species containing Gal–GalNAc–Ser/Thr, and the *N*-linked oligosaccharides are (Man)_n–(GlcNAc)₂–Asn. *O*-Glycanase specifically cleaves the disaccharide (Galβ1–>3GalNAc) bound to serine or threonine residues of glycoproteins, while *N*-Glycanase cleaves the oligosaccharides bound to asparagine residues in the glycoprotein. We have provided evidence that *N*-linked oligosaccharide chains are present on both of the HDL-binding proteins. The decrease in molecular mass of HB₁ was more pronounced than that of HB₂ after treatment with *N*-Glycanase. It appears that the *N*-linked oligosaccharide chain of HB₁ thus differs from that of HB₂. *O*-Glycanase did not significantly alter the molecular mass of either HDL-binding protein. The functional significance of glycosylation of the HDL-binding proteins, in view of the retention of activity after deglycosylation, remains unknown.

Both binding proteins appear to comprise isoforms with low isoelectric points (possibly two each, although not absolutely distinct in Figs. 8 and 9) that were shifted to more basic pI values after treatment with neuraminidase. The experiments show that a part of the negative charge on the HDL-binding protein is due to the sialic acid content, but that the negative charge due to the sialic acid is not important for binding to HDL. These sialic acid and oligosaccharide residues of HDL-binding protein may contribute to stability (protection from proteolysis) or assist in translocation to the cell surface during synthesis. It is possible that the function of sialic acid and oligosaccharide residues present on HDL-binding proteins is similar to that involved in the platelet-derived growth factor receptor [31] and low-density

lipoprotein receptor [30,32] activities. Thus, since carbohydrate moieties may play some protective role in preventing proteolytic cleavage *in vivo*, we investigated this aspect of the structure and function of HDL-binding proteins by comparing proteolysis before and after removal of sialic acid residues. Prior treatment of plasma membranes with neuraminidase did not affect cleavage by trypsin or Pronase, or destruction of HB₁ and HB₂ binding sites, demonstrating that carbohydrate attachment may not fulfil the binding function either. In the case of the putative HDL cell receptor complex, these findings suggest that the HB₁ and HB₂ components are homogeneous in their conformational accessibility of tryptic (or Pronase) cleavage sites; in this respect they differ from the platelet-derived growth factor receptors, which are heterogeneous in their susceptibility to tryptic cleavage [31].

The demonstration of complex, and possibly different, oligosaccharide side-chains on HB₁ and HB₂ may provide one explanation for the differences in molecular mass reported for HDL-binding proteins by several groups [33,34]. This variation may arise from different purification conditions which contribute to varying degrees of deglycosylation, or be the result of isolating proteins during different stages of glycosylation *in vivo*. Whatever physiological function is involved, the present demonstration of glycosylation and charge values contributes significant new knowledge about the properties of these liver membrane HDL-binding proteins, which mounting evidence [18] suggests are candidates for a role as an HDL receptor.

We are grateful to the National Heart Foundation for funds supporting this research, and to Mrs. Traci Novak and Mrs. Christine Anketell for secretarial assistance.

REFERENCES

1. Nakai, T., Otto, P. S., Kennedy, D. L. & Wayne, T. F. (1976) *J. Biol. Chem.* **251**, 4914–4921
2. Ose, L., Ose, T., Norum, K. R. & Berg, T. (1979) *Biochim. Biophys. Acta* **574**, 521–536
3. Gwynne, J. T. & Hess, B. (1980) *J. Biol. Chem.* **255**, 10875–10883
4. Van Berkel, T. J. C., Kruij, J. K., van Gent, T. & van Tol, A. (1980) *Biochem. Biophys. Res. Commun.* **92**, 1002–1008
5. Van Berkel, T. J. C., Kruij, J. K., van Gent, T. & van Tol, A. (1981) *Biochim. Biophys. Acta* **655**, 22–23
6. Soltys, P. A., Portman, O. W. & O'Malley, J. P. (1982) *Biochim. Biophys. Acta* **713**, 300–314
7. Bachorik, P. S., Franklin, F. A., Virgil, D. G. & Kwiterovich, P. O., Jr. (1982) *Biochemistry* **21**, 5675–5684
8. Suzuki, N., Fidge, N. H., Nestel, P. J. & Yin, J. (1983) *J. Lipid Res.* **24**, 253–264
9. Rifci, V. A. & Eder, H. A. (1984) *J. Biol. Chem.* **259**, 13814–13818
10. Fidge, N. H. & Nestel, P. J. (1985) *J. Biol. Chem.* **260**, 3570–3575
11. Koelz, H. R., Sherrill, B. C., Turley, S. D. & Dietschy, J. M. (1982) *J. Biol. Chem.* **257**, 8061–8072
12. Glass, C. K., Pittman, R. C., Weinstein, D. B. & Steinberg, D. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5434–5439
13. Cohn, J. S., Fidge, N. H. & Nestel, P. J. (1985) *Am. J. Physiol.* **249**, G369–G376
14. Chacko, G. K. (1982) *Biochim. Biophys. Acta* **712**, 129–141
15. Chacko, G. K. (1984) *Biochim. Biophys. Acta* **795**, 417–426
16. Chacko, G. K. (1985) *J. Lipid Res.* **26**, 745–754
17. Tozuka, M. & Fidge, N. H. (1989) *Biochem. J.* **261**, 239–244
18. Matthai, D., Fidge, N. H., Tozuka, M. & Mitchell, A. (1990) *Arteriosclerosis* **10**, 1045–1050
19. Havel, R. J., Eder, H. A. & Bragdon, J. H. (1955) *J. Clin. Invest.* **34**, 1345–1353
20. Fleischer, S. & Kervina, M. (1974) *Methods Enzymol.* **31**, 6–41
21. Pharmacia-LKB Biotechnology. *Affinity Chromatography (Principles & Methods)*
22. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
23. Wessel, D. & Flugge, U. I. (1984) *Anal. Biochem.* **138**, 141–143
24. Burnette, W. N. (1981) *Anal. Biochem.* **112**, 195–203
25. Morrissey, J. H. (1981) *Anal. Biochem.* **117**, 307–310
26. Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
27. Cummings, R. D. & Kornfeld, S. (1982) *J. Biol. Chem.* **257**, 11235–11240
28. Yamamoto, K., Tsuji, T., Matsumoto, I. & Osawa, T. (1981) *Biochemistry* **20**, 5894–5899
29. Cummings, R. D., Kornfeld, S., Schneider, W. J., Hobgood, K. K., Tolleshaug, H., Brown, M. S. & Goldstein, J. L. (1983) *J. Biol. Chem.* **258**, 15261–15273
30. Davis, G. C., Elhammer, A., Russell, D. W., Schneider, W. J., Kornfeld, S., Brown, M. S. & Goldstein, J. L. (1986) *J. Biol. Chem.* **261**, 2828–2838
31. Daniel, T. O., Milfay, D. F., Escobedo, J. & Williams, L. T. (1987) *J. Biol. Chem.* **262**, 9778–9784
32. Schneider, W. J., Beisiegel, U., Goldstein, J. L. & Brown, M. S. (1982) *J. Biol. Chem.* **257**, 2664–2673
33. Barbaras, R., Puchois, P., Grimaldi, P., Barkia, A., Fruchart, J. C. & Ailhaud, G. (1987) *Biochem. Biophys. Res. Commun.* **149**, 545–554
34. Graham, D. L. & Oram, J. F. (1987) *J. Biol. Chem.* **262**, 7439–7442

Received 22 July 1991/20 November 1991; accepted 13 December 1991