Apolipoprotein E-binding Proteins Isolated from Dog and Human Liver


Chylomicron remnant catabolism appears to be mediated by apolipoprotein (apo) E binding to hepatic lipoprotein receptors. Previously, the apo B,E(LDL) receptor and a unique apo E-binding protein (referred to as the apo E receptor) were isolated from solubilized canine and human livers. In the present study, the apo E-binding fraction was further characterized and found to contain at least three proteins, all of which bind apo E-containing lipoproteins with high affinity. The 56-kDa band was found to contain the α- and β-subunits of F₆-ATPase, presumably derived from mitochondrial membranes. In addition, an apo E-binding protein with an apparent M₆ = 59,000 was identified. The 59-kDa protein displays calcium-independent binding on ligand blots, but displays both calcium-dependent and -independent binding in assays performed with detergent-solubilized protein. The 56-kDa protein recognized lipid-free as well as lipid-bound apo E in ligand blots, and also bound apo E-2, apo E-3, and apo E-4 in a comparable way. Monoclonal antibodies produced against the 59-kDa protein did not react with the 56-kDa proteins. Normal human liver, as well as the liver of a patient lacking the apo B,E(LDL) receptor, possessed the 56-kDa and 59-kDa proteins. These data indicate that liver cells possess at least three proteins, in addition to the apo B,E(LDL) receptor, that bind apo E-containing lipoproteins with high affinity. The physiological role of these proteins in apo E metabolism remains to be determined.

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apo E-binding site has been designated the apo E receptor14,15,16 and is postulated to be the receptor responsible for the hepatic uptake of chylomicron remnants.

Recently, the protein designated as the apo E receptor was isolated from the crude membranes of dog and human liver.16 This 56-kDa protein was immunologically distinct from the apo B, E(LDL) receptor and bound to apo E HDLc, but not to LDL.18 In the current study, we demonstrate the presence of at least three proteins (two of Mr = 56000 and one of Mr = 59000) capable of binding apo E. The 56-kDa proteins represent the α- and β-subunits of F1-ATPase, whereas the 59-kDa protein has not been described previously.

Methods

Preparation of Lipoproteins and Apolipoproteins

The apo E HDLc (density < 1.060 to 1.02 g/ml) were isolated from the plasma of hypercholesterolemic dogs, purified by Peviok (Pevikon Corp., Mercer Consolidated Corp., Yonkers, NY) block electrophoresis,17 and labeled with 125I by the Bolton-Hunter procedure.18 Very low density lipoproteins (VLDL, density < 1.019 g/ml) were prepared from the plasma of normal subjects (E-3/3 and E-4/4) and a hyperlipidemic patient (E-2/2) by ultracentrifugation. The VLDL were delipidated in acetone/ethanol (1:1) at −20°C, and the apo-VLDL were used to prepare apo E. The apo E was purified on a 13% preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel as described by Neville19 by using a Desaga (Heidelberg) apparatus.

The total lipoprotein fraction (density < 1.21 g/ml) was prepared by one centrifugation at 100000 g for 20 hours at 4°C and was used after extensive dialysis against saline-EDTA. The human apo E-containing HDLc, were isolated in the density range of 1.063 to 1.125 g/ml by using a density gradient as described by Redgrave et al.20

Formation of Apolipoprotein E/Phospholipid Complexes

The procedure described by Helenius et al.21 was modified according to that described by Schneider et al.22 for apo E/phospholipid complexes. Purified apo E-3 from normal subjects (220 μg) was dissolved at room temperature in buffer A (100 mM Tris-HCl, 140 mM NaCl, 1 mM EDTA, pH 7.4). For the lipid film, 2 mg of egg lecithin (Serva) and 40 μg of cholesterol (Serva) were used. The solution of apo E and 23.4 mg of sodium cholate in buffer A was added to the lipid film, was vortexed for 1 minute, and was incubated for 1 hour at 24°C before extensive dialysis against buffer A.

Antibody Production

Monoclonal antibodies to the 59-kDa protein were produced by standard procedures.23,24,25 The BALB/c mouse was immunized with human liver membrane protein that had been solubilized with CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) in buffer B (50 mM Tris-HCl, pH 9, 2 mM CaCl2, 50 mM NaCl, 30 mM CHAPS, and 30% glycerol) and precipitated with phosphatidylcholine and acetone. Immunization was performed by peritoneal injection of 0.2 mg of protein with Freund's adjuvant at 3-week intervals. Complete Freund's adjuvant was used for the first injection, and incomplete Freund's adjuvant was used for all subsequent injections. The fourth injection (0.1 mg of protein) was given intravenously into the tail vein. A blood sample was taken for testing 3 days after the last injection. Positive reactions were detected at 1:106 dilution with human liver membrane protein coated on 96-well microtiter plates by an enzyme-linked immunosorbent assay (ELISA) technique. Spleen cells were fused to mouse myeloma cells (X63-Ag 8-653) with polyethylene glycol. The media from the clones were tested by an ELISA on 96-well plates coated with purified human apo E-binding protein or proteins that had been prepared on a canine HDLc-Sepharose column.

Final tests on blots with purified apo E-binding protein(s) resulted in two positive clones (14D11 and 23B10). Both of the clones produced antibodies of the IgG1 subclass. The studies reported here were performed with the antibody from the 14D11 clone, termed anti-59 on the basis of the molecular weight of the antigen. The monoclonal antibodies against apo E were prepared as described above using apo E-3 as an antigen (Beisiegel U et al., unpublished observations). The Escherichia coli anti-F1-ATPase was a gift from Mathias Lübben from the Medizinische Universität of Lübeck, Federal Republic of Germany.

The polyclonal antibodies against the canine 56-kDa apo E-binding protein were prepared as described earlier.16

Solubilization of Membrane Proteins

Livers from cholesterol-fed dogs were prepared as described.18 Human liver samples were washed in 0.15 M NaCl/0.01% EDTA before preparation of the membranes. The isolation of liver membranes (from 40 to 50 g of liver) was achieved by a technique similar to that described by Kovanen et al.26 but modified by the addition of phenylmethylsulfonyl fluoride (PMSF) and Trasylol to the homogenization buffer. All steps in the purification of the apo E-binding proteins were performed at 4°C. Membrane proteins were solubilized in buffer B (50 mM Tris-HCl, pH 9, 2 mM CaCl2, 50 mM NaCl, 30 mM CHAPS, and 30% glycerol) at a concentration of ~1 mg of membrane protein/ml and then they were mixed for 1 hour. The insoluble material was pelleted by centrifugation at 200000 g for 1 hour at 4°C. The clear supernatant was applied to a DEAE-cellulose column.18

DEAE-Anion Exchange Chromatography

Proteins from detergent-solubilized dog liver membranes were applied to a DEAE 52-cellulose column (2.5 x 7 cm) equilibrated in buffer B at a flow rate of 150 ml/hour. The column was washed with 100 ml of buffer B, and the bound protein was eluted with a 100-ml salt gradient composed of 50 to 300 mM NaCl in buffer B. Samples were collected in 3-ml fractions, and apo E binding activity in each fraction was analyzed by a binding assay described elsewhere.16,27,28 The apo E-binding fractions containing maximal binding activity were pooled and applied to a canine HDLc-Sepharose column.
The detergent-solubilized human protein sample was applied to a DEAE 52-cellulose column (2.5 x 7 cm), which was equilibrated in buffer B. Whereas the DEAE-bound fraction was used to isolate the 56-kDa proteins, the fraction that did not bind to the DEAE was used to isolate the 59-kDa apo E-binding protein. These apo E-binding fractions from human liver were applied to an apo E affinity column as described below.

HDL₃-Sepharose Chromatography of Canine Liver Apolipoprotein E-binding Proteins

After DEAE chromatography, the apo E-binding fraction was applied to an HDL₃-Sepharose column (1 x 4 cm) and was recycled through the column 10 times at a flow rate of 10 ml/hour. The HDL₃ column was washed in sequence with 300 ml of buffer B, 100 ml of buffer B without CHAPS, and 100 ml of buffer B. Proteins bound to the column were eluted with 0.5 M NH₄OH. The 56-kDa protein was further purified for protein sequencing as described below.

Apolipoprotein E Affinity Chromatography of Human Apolipoprotein E-binding Proteins

The solubilized proteins or fractions from the DEAE column were applied to a human apo E affinity column (2 mg of apo E bound) in buffer B and were recycled five times. The column was washed with 500 ml of buffer B, and the bound proteins were eluted with 0.5 M NH₄OH. The 56-kDa protein was further purified for protein sequencing as described below.

Protein Sequencing of 56-kDa Protein

Protein samples eluted from the HDL₃-Sepharose column were further purified by preparative SDS-polyacrylamide gels. The samples were eluted from the affinity column with 0.5 M NH₄OH, were lyophilized, and were resuspended in sample application buffer (62.5 mM Tris-Cl, pH 8.8, 3% SDS, 0.1 mM sodium thioglycolate, 100 mM dithiothreitol, and 10% glycerol). The solubilized proteins were then electrophoresed on a 7.5% polyacrylamide gel as described. Sodium thioglycolate (1 mM) was included in the cathode buffer reservoir to minimize free radical-induced protein degradation in the gel. The protein band(s) were stained with Coomassie Brilliant Blue and rapidly destained. The major protein band (56-kDa) was excised from the slab gel and was electro-eluted according to the method of Hunkapiller et al. The eluted protein was separated from the SDS and Coomassie stain by extraction with chloroform/methanol (1:1 vol/vol) and was precipitated by methanol as described. Protein recovery, usually in excess of 85%, was quantitated by comparing the staining intensity with known standards on an SDS-polyacrylamide gel. Partial protein hydrolysis was performed by incubation of the purified protein with 126l-apo E HDL₃. Radiolabeled lipoproteins bound to the proteins were separated from the unbound lipoproteins by filtration on cellulose acetate membrane filters.

Isolation of 59-kDa Protein

Immunoaffinity chromatography was used to isolate the 59-kDa protein. Monoclonal antibodies against the 59-kDa protein were immobilized on CNBr-activated Sepharose using the procedure suggested by the manufacturer. A ratio of 2 mg of IgG to 0.3 g of Sepharose beads was used. In a typical experiment, 120 mg of CHAPS-solubilized dog liver membranes was applied to the IgG-Sepharose column. The sample was recycled 7 to 10 times at a flow rate of 5 to 20 ml/hour. The column was extensively washed, and the bound 59-kDa protein was eluted with 0.5 M NH₄OH. The eluted sample was lyophilized, stored at −20°C, and used within a week.

Determination of Binding Activity of 56-kDa and 59-kDa Proteins and Purified F,ATPase

To test the lipoprotein-binding ability of the purified proteins, the lyophilized sample was resuspended in buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM CaCl₂, and 50 mM NaCl, and precipitated with cold acetone in the presence of phosphatidylcholine as described. The purified proteins, complexed to the phospholipid, were assayed for binding activity by incubation for 1 hour at 4°C with [125I]-apo E HDL₃. Radiolabeled lipoproteins bound to the proteins were separated from the unbound lipoproteins by filtration on cellulose acetate membrane filters.

Gel Electrophoresis and Blotting Procedures

Proteins were analyzed on SDS-polyacrylamide gel electrophoresis either by the procedure of Laemmli or by the method of Neville. Immunoblotting was carried out according to the procedures described by Beisiegel et al. and Hui et al. The procedure for each experiment is indicated in the respective figure legend. The ligand blotting experiments were done according to the method of Daniel et al. Modifications included omitting NaCl from the incubation buffer and lowering the pH to 6. Antibodies against apo E were labeled by Bolton-Hunter reagent. When human apo E was used as the ligand, it was identified on the ligand blot by a monoclonal antibody produced against apo E-3 (Beisiegel U et al., unpublished data). The IgG fraction of this monoclonal antibody was conjugated to peroxidase and used at a dilution of 1:20 (0.1 mg of protein/ml). The unlabeled antibody was used at a dilution of 1:50 (0.4 mg of protein/ml). Peroxidase-conjugated goat antimouse IgG was used as a second antibody at 1:1000 dilution to visualize the apo E/anti-apo E complex.

Isoelectric focusing was performed on a flat bed system containing Sephadex G75 and ampholytes (pH of 4 to 7).

Results

Antibody Preparation

Polyclonal antibodies were prepared in rabbits against the dog liver apo E-binding fraction purified by apo E HDL₃-Sepharose column chromatography as described. The antibodies primarily reacted with the 56-kDa protein that has been postulated to be an apo E receptor.
attempts to produce monoclonal antibodies against this protein, an antibody (anti-59) against a 59-kDa protein was obtained. This 59-kDa protein was a minor component in the bound fraction eluted from the HDLc-Sepharose affinity column and was detected by immunoblotting. The monoclonal anti-59 did not cross-react with the 56-kDa protein, and the anti-56-kDa did not cross-react with the 59-kDa protein (Figure 1). Furthermore, neither antibody reacted with the apo B,E(LDL) receptor (data not shown). The presence of 56-kDa and 59-kDa proteins in the HDLc-Sepharose-purified fraction indicated that apo E may interact with more than one liver protein. The availability of polyclonal antibodies against the 56-kDa protein and monoclonal antibodies against the 59-kDa protein provided a convenient way to monitor the purification of the apo E-binding proteins.

**Purification of Apolipoprotein E-binding Proteins**

The initial step in the isolation of the apo E-binding proteins was the solubilization of the liver membranes with the zwitterionic detergent CHAPS. This detergent is known to be the most effective in solubilizing apo E binding activity from dog and human liver membranes. The 56-kDa and the 59-kDa proteins could be detected in the CHAPS-solubilized fraction of the membranes by immunoblotting (data not shown). The apo E-binding proteins from the solubilized liver membranes were partially purified by anion exchange chromatography on a DEAE-cellulose column. The 56-kDa protein bound more tightly to the DEAE column than did the 59-kDa protein. Hence, the DEAE-bound fraction was enriched with the 56-kDa protein in comparison with the 59-kDa protein (Figure 2, Lane 3). In contrast, most of the 59-kDa protein was in the DEAE-unbound fraction (data not shown). Therefore, the 56-kDa protein was isolated from the DEAE-bound fraction, whereas the 59-kDa protein was isolated from either the DEAE-bound or unbound fraction. Furthermore, a human apo E-3 affinity column (either free of lipids or with apo E-3 complexed to lipoparticles containing egg lecithin and cholesterol)

![Image of Immunoblot analysis of liver apo E-binding proteins from dog liver purified by HDLc-Sepharose affinity chromatography.](http://atvb.ahajournals.org/)

**Figure 1.** Immunoblot analysis of liver apo E-binding proteins from dog liver purified by HDLc-Sepharose affinity chromatography. Dog liver membranes were solubilized with CHAPS, and the apo E-binding proteins were isolated by an HDLc-Sepharose column as described in Methods and in reference 16. Lane A shows the identification of the major 56-kDa protein by immunoblotting with polyclonal antibodies against the entire HDLc-Sepharose-bound fraction. Lane B shows the immunoblot of the HDLc-Sepharose-bound fraction with anti-59 (1:20). Antibody interaction with the proteins was detected by a second antibody labeled with 125I as described.  

**Figure 2.** Identification of apo E-binding proteins from human liver. Samples were applied to a 13% SDS-polyacrylamide gel and electrophoresed onto nitrocellulose paper. The nitrocellulose paper was either stained with amido black (Lanes 1 and 2) or immunoblotted (Lanes 3, 4, and 5). A double Immunoblot with polyclonal antibodies against the 56-kDa protein and monoclonal anti-59 (specific for the 59-kDa protein) was used. The paper was incubated with bovine serum albumin to block nonspecific sites and was then incubated with the polyclonal anti-56 (1:500). The positive reaction was detected by incubation with peroxidase-labeled goat antirabbit IgG (1:1000). After substrate incubation and detection of the first reactive band, the nitrocellulose was then incubated with the monoclonal anti-59 (1:20 dilution of culture supernatant). This positive reaction was visualized by goat antimouse IgG labeled with peroxidase. Lane 1, molecular weight standards; Lane 2, amido black stain of the DEAE-cellulose-bound fractions (specific 56-kDa and 59-kDa proteins are not identified); Lane 3, Immunoblot identifying the 56-kDa and 59-kDa proteins in the DEAE-cellulose-bound fraction; Lane 4, Immunoblot identifying the 56-kDa and 59-kDa proteins isolated from solubilized human liver membranes on a human apo E-3/lipid complex affinity column; and Lane 5, Immunoblot identifying the 56-kDa and 59-kDa proteins isolated from solubilized human liver membranes on an apo E-3 (lipid-free) affinity column. The protein identified in Lane 5 was obtained from solubilized membranes previously passed over the apo E-3/lipid complex column.
could be used to isolate the apo E-binding proteins from the solubilized membranes. Both the 56-kDa and the 59-kDa proteins bound to the apo E/lipid affinity column, as well as to the lipid-free apo E affinity column (Figure 2, Lanes 4 and 5). The co-isolation of the human liver apo E-binding proteins necessitated a new procedure to separate the proteins for further characterization.

**Immunooaffinity Chromatography of Apolipoprotein E-binding Proteins**

The apo E-binding proteins were separated by immunooaffinity column techniques with antibodies specific for each protein. Characterization of the 56-kDa protein isolated by immunooaffinity chromatography was reported in a previous study. An immunooaffinity column containing the monoclonal antibody anti-59 was used to isolate the 59-kDa protein (Figure 3). Dog liver membranes were solubilized with detergent and applied immediately to the IgG-Sepharose column. The 59-kDa protein bound to the affinity column and was eluted with 0.5 M NH₄OH. The 59-kDa protein isolated by immunooaffinity chromatography reacted on an immunoblot with the anti-59 (Figure 3, Lane 3), and the same protein reacted with apo E on a ligand blot (Figure 3, Lane 4).

**Characterization of 56-kDa Apolipoprotein E-binding Protein**

The 56-kDa protein was isolated from the crude membrane fraction of dog liver by HDLc-Sepharose affinity chromatography and was further purified on SDS-polyacrylamide gels. The major protein band (56-kDa) was eluted from the gel and was subjected to microsequence analysis. Initial analysis of the intact protein revealed a blocked amino terminus. In subsequent studies, the isolated 56-kDa protein was subjected to partial proteolysis by either S. aureus V8 protease digestion or by chemical hydrolysis with CNBr. The digested peptides were separated by HPLC, and sequence analysis of the peptides revealed high homology with both the α- and β-subunits of bovine F₆₄ATPase (Table 1). The homology between the 56-kDa protein band and the α- and β-subunits included regions at both the amino and carboxyl termini of F₆₄ATPase. These results, together with immunological data demonstrating the enrichment of the 56-kDa protein(s) in the mitochondria and the cross-reactivity of the protein band with anti-F₆₄ATPase (data not shown), strongly suggested that the 56-kDa band is composed of the α- and β-subunits of mitochondrial F₆₄ATPase.

The interaction of apo E HDLc with F₆₄ATPase was investigated directly by using purified bovine heart mitochondrial F₆₄ATPase (a gift from Harvey Penefsky, Public Health Research Institute, New York, NY). This purified F₆₄ATPase, prepared by alkaline pH fractionation of sonicated submitochondrial particles followed by isoelectric and ammonium sulfate precipitation steps as described, was precipitated with cold acetone as a complex with phosphatidylcholine for assaying ¹²⁵I-apo E HDLc binding. The purified F₆₄ATPase displayed specific high-affinity binding with ¹²⁵I-apo E HDLc (Figure 4). The binding was similar in affinity (Kᵦ = 2.1 x 10⁻⁹ M) to the previously

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### Table 1. Comparison of Protein Sequence of 56-kDa Apolipoprotein E-binding Protein and α- and β-Subunits of Mitochondrial F₆₄ATPase

<table>
<thead>
<tr>
<th>S. aureus V8 proteolytic products</th>
<th>Canine 56-kDa protein</th>
<th>Bovine α-F₄₅ATPase (49-58)</th>
<th>Bovine β-F₄₅ATPase (450-455)</th>
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<tr>
<td><strong>Bovine α-F₄₅ATPase (49-58)</strong></td>
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<tr>
<td><strong>Bovine β-F₄₅ATPase (450-455)</strong></td>
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**CNBr products**

| Bovine α-F₄₅ATPase (383-369) | M-K-Q-V-A-G-T |

*The sequence of bovine heart mitochondrial F₆₄ATPase was reported in references 37 and 38.
†Indicates mismatches between the two sequences.*
observed binding of apo E HDLc to the 56-kDa protein fraction. The binding of the apo E HDLc was calcium-independent (Figure 4). Furthermore, apo E HDLc binding to purified F1-ATPase was significantly inhibited by the apo E monoclonal antibody 1D7 (Table 2). The 1D7 monoclonal antibody has been shown to interact with residues 140 to 150 of apo E and to inhibit apo E binding to the apo B,E(LDL) receptor. These data indicate that the same domain in apo E is involved in the binding to both F1-ATPase and to the apo B,E(LDL) receptor. In addition, the binding of apo E HDLc to F1-ATPase also was abolished by 20 mM suramin (Table 2). Previously, it has been shown that suramin blocks the binding of apo E-containing lipoproteins to the apo B,E(LDL) receptor and to the hepatic 56-kDa proteins.

The characteristics of apo E HDLc binding to F1-ATPase differed from the binding to the 56-kDa protein fraction reported in the previous study in two respects: the apo E HDLc interaction with F1-ATPase had lower capacity than previously reported for the 56-kDa protein fraction, and the binding of apo E HDLc to the purified F1-ATPase was resistant to inhibition by 30 mM EDTA. The reasons for these discrepancies remain unclear; however, the differences in the procedures used to isolate the hepatic 56-kDa protein and the cardiac F1-ATPase probably have an impact on specific properties of the proteins. Nevertheless, data presented in this paper clearly demonstrate the ability of mitochondrial F1-ATPase to interact with apo E HDLc and indicate that the 56-kDa protein described in the previous study was primarily, or exclusively, the α- and β-subunits of mitochondrial F1-ATPase.

Characterization of 59-kDa Apolipoprotein E-binding Protein

Ligand blotting studies were performed to analyze the binding characteristics of the 59-kDa protein. Either the solubilized human liver membrane fraction, the DEAE-unbound fraction, or the apo E affinity column-purified fraction was used in these studies. As shown in Figure 5, the binding of the apo E to the 59-kDa protein on ligand blots was not entirely dependent on calcium. However, apo E HDLc binding to the detergent-solubilized 59-kDa protein displayed both a calcium-dependent and -independent component (Figure 6). Scatchard analysis of the calcium-dependent binding of apo E HDLc indicated a high-affinity binding to the 59-kDa protein (10⁻⁹ M).

The ability of the 59-kDa protein to interact with different apo E isoforms was tested on ligand blots. As shown in Figure 7A, the 59-kDa protein was capable of binding to apo E-2, apo E-3, and apo E-4 when the apolipoproteins were used at a concentration of 5 or 10 μg/ml. The binding

Table 2. Inhibition of 125I-Apolipoprotein E HDLc Interaction with F1-ATPase by Monoclonal Antipolipoprotein E and Suramin

<table>
<thead>
<tr>
<th>Additions (ng/assay)</th>
<th>Specific binding (ng/assay)</th>
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<tbody>
<tr>
<td>125I-apo E HDLc (0.7 μg/ml)</td>
<td>3.0</td>
</tr>
<tr>
<td>+ 1D7 (102 μg/ml)</td>
<td>0.6</td>
</tr>
<tr>
<td>+ suramin (20 mM)</td>
<td>0</td>
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Figure 4. Binding of 125I-apo E HDLc to F1-ATPase. Purified bovine heart mitochondrial F1-ATPase was provided by Harvey Penefsky. The purified enzyme was precipitated by acetone in the presence of phosphatidylcholine. The lipid-protein complex was resuspended in buffer and tested for binding activity with 125I-apo E HDLc in the presence (A) or absence (C) of 30 mM EDTA. Nonspecific binding of 125I-apo E HDLc, determined by inclusion of 20-fold excess unlabeled HDLc or with 30 mM suramin, was subtracted from the total binding data to obtain the specific binding.

Figure 5. Ligand binding of lipid-complexed apo E-3 to human liver 59-kDa protein in the absence (Lane 1) or presence (Lane 2) of calcium. Lane 1, 20 mM EDTA; Lane 2, 2 mM Ca²⁺ (no EDTA). The ligand is recognized by peroxidase-labeled monoclonal antibody directed against apo E. Lane 3 is an immunoblot with anti-59-kDa as a control.
Figure 6. Binding of $^{125}$I-apo E HDL$_C$ to the 59-kDa apo E-binding protein. The 59-kDa protein was isolated from solubilized dog liver membranes by immunoaffinity chromatography on an anti-59-Sepharose column. The isolated protein was precipitated with acetone and phosphatidylcholine and was used for the binding assay as described in Methods. Specific binding of $^{125}$I-apo E HDL$_C$ (●) was determined by subtracting the amount of binding observed in the presence of excess unlabeled lipoproteins from the total binding observed in the absence of unlabeled lipoproteins. Calcium-dependent binding (○) was calculated by subtracting the binding observed in the presence of 30 mM EDTA from the total binding data.

Figure 7. Apolipoprotein E binding to the 59-kDa protein in human liver membranes. The proteins were electrophoresed in 13% SDS-polyacrylamide gels and transferred to nitrocellulose as described in Methods. A. Lane 1, an immunoblot using monoclonal anti-59 (1:20 dilution of culture hybrid supernatant); Lanes 2, 3, and 4 are ligand blots with lipoprotein-free apo E-2, E-3, and E-4, respectively. Ligand binding (10 μg of apo E/ml) was detected with a monoclonal anti-apo E and peroxidase-labeled goat antimouse IgG. Lane 5 is a ligand blot with human apo E-containing lipoproteins (d < 1.019 g/ml), detected as described above. B and C. Ligand blots of the 59-kDa protein performed with increasing concentrations of lipoprotein-free apo E-3 or apo E-2, respectively. The concentrations of apo E used in this study were 20 μg/ml (Lane 1), 10 μg/ml (Lane 2), 5 μg/ml (Lane 3), 2.5 μg/ml (Lane 4), 1.25 μg/ml (Lane 5), and 0.625 μg/ml (Lane 6).

Immunoblotting experiments with liver membranes prepared from different species revealed cross-reactivity of anti-59 IgG with the 59-kDa protein in human, dog, and rabbit livers. In addition, the 59-kDa protein was present in the liver membranes of a homozygous FH patient (Figure 8). Likewise, the 59-kDa protein was present in the WHHL rabbit liver at a level similar to that observed in normal rabbits (data not shown). No apo B,E(LDL) receptor could be detected immunologically in the liver of the FH patient.

Isoelectric Focusing of Apolipoprotein E-Binding Proteins

The human apo E-binding protein was separated by preparative isoelectric focusing. A difference in isoelectric

activities of apo E-2 and apo E-3 to the 59-kDa protein were compared at various concentrations of the apolipoproteins (Figures 7B and 7C). No significant differences in binding activities for the two apo E isoforms were observed. Significant binding activities were detectable at concentrations at least down to 1.25 μg/ml. To rule out nonspecific effects due to the second antibodies, the blotting was performed with peroxidase-labeled goat antimouse IgG without prior incubation with apo E. No positive reaction was detectable in the absence of the ligand. Furthermore, either apo E labeled directly with peroxidase or apo E together with peroxidase-labeled anti-apo E identified the 59-kDa protein.
Figure 8. Immunoblotting studies on normal human liver (A) and liver from an FH patient with defective apo B,E(LDL) receptors (B). Lane 1, amido black stain of the CHAPS-solubilized liver membrane; Lane 2, identification of the apo B,E(LDL) receptor with a polyclonal apo B,E(LDL) receptor antibody after reaction with peroxidase-labeled goat antirabbit IgG; Lane 3, identification of the 56-kDa protein with the 56-kDa polyclonal antibody following reaction with peroxidase-labeled goat antirabbit IgG; and Lane 4, identification of the 59-kDa protein with anti-59 following reaction with peroxidase-labeled goat antimouse IgG. There was no detectable apo B,E(LDL) receptor in the FH liver, as noted in B, Lane 2.

Discussion

Strong functional and genetic evidence argues for a mechanism distinct from the apo B,E(LDL) receptor in the catabolism of chylomicron remnants by the liver. The mechanism probably involves a receptor-mediated pathway for the hepatic clearance of chylomicron remnants.4–8 Based on these investigations, the involvement of a distinct receptor, the chylomicron remnant (apo E) receptor, has been postulated. Other biochemical and genetic evidence indicates that the apo E on chylomicron remnants mediates their clearance by this hepatic receptor-mediated mechanism.9–13 The conclusions derived from the current study do not affect the postulated existence of a chylomicron remnant (apo E) receptor or the evidence that apo E mediates the clearance of chylomicron remnants by the liver.

Previous studies have shown that a protein fraction possessing apo E HDL₆ and chylomicron remnant binding activity could be isolated by HDL₆-Sepharose affinity chromatography for canine and human liver membranes.16 Further analysis of this fraction in the present study showed that it contained at least three apo E-binding proteins with slightly different molecular weights (two proteins associated with a band at Mr = 56000 and one at Mr = 59000). These proteins were not related as degradation products because there was no immunological cross-reactivity.

The major protein purified in the previous study and referred to as the apo E receptor was the 56-kDa proteins.8 In the present study, the 56-kDa protein band was characterized by partial amino acid sequence analysis and was shown to display amino acid homology with the α- and β-subunits of bovine mitochondrial F₁-ATPase. The identity of the 56-kDa band with the α- and β-subunits of F₁-ATPase was determined by immunological reactivity of the 56-kDa antibody with mitochondrial protein and with purified F₁-ATPase. The binding of apo E to F₁-ATPase observed in vitro was most likely due to charge interaction between the proteins. The ability of suramin, a highly negatively charged hydrocarbon, to dissociate the binding pro-
Proteins from apo E HDL₃ supported this conclusion. Furthermore, it has been shown that F₅₋ATPase binds to an inhibitory protein that is enriched in lysine residues. It is possible that apo E bound to the ATPase at similar sites. The ability of the apo E monoclonal antibody 1D7⁴⁰, ⁴¹ to inhibit binding suggested that the positively charged region of apo E that is responsible for binding of this protein to the apo B,E(LDL) receptor was also responsible for its binding to the F₅₋ATPase. Thus, the 59-kDa protein fraction appears to be composed of the α- and β-subunits of mitochondrial F₅₋ATPase. The molecular weights of the α- and β-subunits are approximately 55000 and 52000, respectively. ³⁷ ³⁸

A second apo E-binding protein was detected during the preparation of monoclonal antibodies against proteins purified from the HDL₃ affinity column. This 59-kDa protein was present in the HDL₃-Sepharose-purified fraction and could be purified from solubilized liver membranes by immunoaffinity chromatography techniques. The purified 59-kDa protein displayed specific binding activity to apo E by both ligand blotting and the solubilized membrane binding assay. The 59-kDa protein bound to lipid-free apo E as well as to apo E complexed with phospholipid and cholesterol. Previously, we have shown that phospholipid is required for apo E binding to apo B,E(LDL) receptors. ⁴₂ In addition, a divalent metal ion such as calcium is necessary for the binding of apo E to the apo B,E(LDL) receptor. ⁴² Binding assays performed with CHAPS-solubilized 59-kDa protein demonstrated both calcium-dependent and -independent binding components. Previously, in binding experiments performed with intact membranes, calcium-dependent and -independent binding of apo E HDL₃ was demonstrated. ¹⁴

Apolipoprotein E occurs in the plasma as apo E-2, apo E-3, and apo E-4. The apo E-2 isoform is associated with type III hyperlipoproteinemia, binds poorly to the apo B,E(LDL) receptor, and is cleared from the circulation at a much reduced rate (for review, see references ⁴⁴ and ⁴⁵). These apo E isoforms were tested for binding to the 59-kDa protein on ligand blots. Both lipid-free and lipid-bound apo E isoforms bound comparably to the 59-kDa protein. Preliminary results of immunofluorescence studies (Boyles JK, Hul DY, Innerarity TL, Mahley RW, unpublished observations) and subcellular fractionation studies (Wernette-Hammond ME, Innerarity TL, Mahley RW, unpublished observations) have indicated that the 59-kDa protein is predominantly localized in the endoplasmic reticulum. Although the function of the 59-kDa protein is not known and is currently under investigation, it is possible that this protein serves in the intracellular transport of apo E-containing lipoproteins.

In conclusion, three apo E-binding proteins from canine and human liver have been identified. These include the α- and β-subunits of mitochondrial F₅₋ATPase. These two proteins were presumably solubilized from the mitochondrial membranes and were isolated on the HDL₃-Sepharose affinity column. They represent the functional catalytic units of F₅₋ATPase located on the inner mitochondrial membrane that bind ATP and ADP. ³⁷ ³⁸ It is unclear whether apo E might have a role in mitochondrial metabolism; however, it is unlikely that these proteins have a role in chylomicron remnant metabolism. The other protein in the HDL₃-Sepharose fraction, the 59-kDa protein, requires further study before any functional role can be assigned.

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