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 Mr = 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 receptor\textsuperscript{14, 15, 18} 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.\textsuperscript{16} This 56-kDa protein was immunologically distinct from the apo B,E(LDL) receptor and bound to apo E HDL\textsubscript{c}, but not to LDL.\textsuperscript{18} In the current study, we demonstrate the presence of at least three proteins (two of $M_r = 50000$ and one of $M_r = 59000$) capable of binding apo E. The 56-kDa proteins represent the $\alpha$- and $\beta$-subunits of F\textsubscript{1}-ATPase, whereas the 59-kDa protein has not been described previously.

## Methods

### Preparation of Lipoproteins and Apolipoproteins

The apo E HDL\textsubscript{c} ($d = 1.006$ to $1.02$ g/ml) were isolated from the plasma of hypercholesterolemic dogs, purified by Pevikon (Pevikon Corp., Mercer Consolidated Corp., Yonkers, NY) block electrophoresis,\textsuperscript{17} and labeled with \textsuperscript{125}I by the Bolton-Hunter procedure.\textsuperscript{18} Very low density lipoproteins (VLDL, $d < 1.019$ 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 $-20^\circ$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 Neville\textsuperscript{19} by using a Desaga (Heidelberg) apparatus.

The total lipoprotein fraction ($d < 1.21$ g/ml) was prepared by one centrifugation at 100000 $g$ for 20 hours at 4$^\circ$C and was used after extensive dialysis against saline-EDTA. The human apo E-containing HDL, were isolated from the crude membranes of dog and human liver.\textsuperscript{16} This 56-kDa protein was immunologically distinct from the 59-kDa protein has not been described previously.

### Formation of Apolipoprotein E/Phospholipid Complexes

The procedure described by Helenius et al.\textsuperscript{21} was modified according to that described by Schneider et al.\textsuperscript{22} for apo E/ phospholipid complexes. Purified apo E-3 from normal subjects ($200 \mu g$) was dissolved at room temperature in buffer A ($100 \text{ mM Tris-HCl}, 140 \text{ mM NaCl}, 1 \text{ mM EDTA}, pH 7.4$). For the lipid film, 2 mg of egg lecithin (Serva) and 40 $\mu$g 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$^\circ$C before extensive dialysis against buffer A.

### Antibody Production

Monoclonal antibodies to the 59-kDa protein were produced by standard procedures.\textsuperscript{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 ($50 \text{ mM Tris-HCl}, pH 9, 2 \text{ mM CaCl}_2, 50 \text{ mM NaCl}, 30 \text{ mM CHAPS}, and 30\% glycerol) and precipitated with phosphatidylincholine and acetone. Immune responses were performed by peritoneal injection of 0.2 mg of protein with Freund's adjuvant at 3-week intervals. Complete Freund's adjuvant was used for the first injection, and incomplete Freund's adjuvant was used for all subsequent injections. The fourth injection (0.1 mg of protein) was given intravenously into the tail vein. A blood sample was taken for testing 3 days after the last injection. Positive reactions were detected at 1:10$^4$ 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 HDL\textsubscript{c}-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 IgG\textsubscript{1} 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 \textit{Escherichia coli} anti-F\textsubscript{1}-ATPase was a gift from Mathias Lübben from the Medizinische Universität von Lübeck, Federal Republic of Germany.

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

### Solubilization of Membrane Proteins

Livers from cholesterol-fed dogs were prepared as described.\textsuperscript{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.\textsuperscript{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$^\circ$C. Membrane proteins were solubilized in buffer B ($50 \text{ mM Tris-HCl}, pH 9, 2 \text{ mM CaCl}_2, 50 \text{ mM NaCl}, 30 \text{ mM CHAPS}, and 30\% glycerol) at a concentration of $\sim$1 mg of membrane protein/ml and then they were mixed for 1 hour. The insoluble material was pelleted by centrifugation at 200 000 $g$ for 1 hour at 4$^\circ$C. The clear supernatant was applied to a DEAE-cellulose column.\textsuperscript{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.\textsuperscript{16, 27, 28} The apo E-binding fractions containing maximal binding activity were pooled and applied to a canine HDL\textsubscript{c}-Sepharose column.
The detergent-solubilized human protein sample was applied to a DEAE 52-cellulose column (2.5 × 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.

**HDL2-Sepharose Chromatography of Canine Liver Apolipoprotein E-binding Proteins**

After DEAE chromatography, the apo E-binding fraction was applied to an HDL2-Sepharose column (1 × 4 cm) and was recycled through the column 10 times at a flow rate of 10 ml/hour. The HDL2 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.

**Protein Sequencing of 56-kDa Protein**

Protein samples eluted from the HDL2-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 6.8, 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 Daniel et al. The eluted protein was separated from the unbound lipoproteins containing Sephadex G75 and ampholytes (pH of 4 to 7). 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 F1-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 HDLc. 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/ant apo E complex.

**Results**

**Antibody Preparation**

Polyclonal antibodies were prepared in rabbits against the dog liver apo E-binding fraction purified by apo E HDL2-Sepharose column chromatography as described. The antibodies primarily reacted with the 56-kDa protein that has been postulated to be an apo E receptor. During
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 lipid vesicles containing egg lecithin and cholesterol).
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.

**Immunoadfinity Chromatography of Apolipoprotein E-Binding Proteins**

The apo E-binding proteins were separated by immunoadfinity column techniques with antibodies specific for each protein. Characterization of the 56-kDa protein isolated by immunoadfinity chromatography was reported in a previous study. An immunoadfinity 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 immunoadfinity 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 HDL₄-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 F₄-ATPase (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 F₄-ATPase. 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-F₄-ATPase (data not shown), strongly suggested that the 56-kDa band is composed of the α- and β-subunits of mitochondrial F₄-ATPase.

The interaction of apo E HDL₄ with F₄-ATPase was investigated directly by using purified bovine heart mitochondrial F₄-ATPase (a gift from Harvey Penefsky, Public Health Research Institute, New York, NY). This purified F₄-ATPase, 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 HDL₄ binding. The purified F₄-ATPase displayed specific high-affinity binding with ¹²⁵I-apo E HDL₄ (Figure 4). The binding was similar in affinity (Kᵦ = 2.1 × 10⁻⁹ M) to the previously

**Table 1. Comparison of Protein Sequence of 56-kDa Apolipoprotein E-Binding Protein and α- and β-Subunits of Mitochondrial F₄-ATPase**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canine 56-kDa protein</td>
<td>V-A-Q-G-L-G-Q-S-L-V</td>
</tr>
<tr>
<td>Bovine α-F₄-ATPase</td>
<td>V-A-Q-H-L-G-E-S-T-V</td>
</tr>
<tr>
<td>Bovine β-F₄-ATPase</td>
<td>Y-D-H-L-P-P-E</td>
</tr>
<tr>
<td>Canine 56-kDa protein</td>
<td>Y-D-H-L-P-P-E</td>
</tr>
<tr>
<td>Bovine α-F₄-ATPase</td>
<td>M-K-Q-V-A-G-T</td>
</tr>
<tr>
<td>Canine 56-kDa protein</td>
<td>A-Q-Y-K-E-V-A-A</td>
</tr>
</tbody>
</table>

*The sequence of bovine heart mitochondrial F₄-ATPase 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 F,-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 F,-ATPase and to the apo B,E(LDL) receptor. In addition, the binding of apo E HDLc to F,-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 F,-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 F,-ATPase had lower capacity than previously reported for the 56-kDa protein fraction, and the binding of apo E HDLc to the purified F,-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 F,-ATPase probably have an impact on specific properties of the proteins. Nevertheless, data presented in this paper clearly demonstrate the ability of mitochondrial F,-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 F,-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
Figure 6. Binding of \(^{125}\text{I}-\text{apo E} \text{ HDL}_c\) 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 phosphatidyicholine and was used for the binding assay as described in Methods. Specific binding of \(^{125}\text{I}-\text{apo E} \text{ HDL}_c\) (▼) was determined by subtracting the amount of binding observed in the presence of excess unlabeled HDL 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 lipid-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 lipid-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).

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 antimal mouse 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.

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 proteins were separated by preparative isoelectric focusing. A difference in isoelectric
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. 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. 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. 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 Mᵦ = 56,000 and one at Mᵦ = 59,000). 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 protein. 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-
tein from apo E HDL supported this conclusion. Furthermore, it has been shown that F_1-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 1D7 to inhibit binding suggested that the positively charged region of apo E that is responsible for the binding of this protein to the apo B,E(LDL) receptor was also responsible for its binding to the F_1-ATPase. Thus, the 59-kDa protein fraction appears to be composed of the α- and β-subunits of mitochondrial F_1-ATPase. The molecular weights of the α- and β-subunits are approximately 55,000 and 52,000, respectively.

A second apo E-binding protein was detected during the preparation of monoclonal antibodies against proteins purified from the HDL_c affinity column. This 59-kDa protein was present in the HDL_c-Sepharose-purified fraction and could be purified from solubilized liver membranes by immunoadsorption 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. In addition, a divalent metal ion such as calcium is necessary for the binding of apo E to the apo B,E(LDL) receptor. 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 HDL_c was demonstrated. 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 F_1-ATPase. These two proteins were presumably solubilized from the mitochondrial membranes and were isolated on the HDL_c-Sepharose affinity column. They represent the functional catalytic units of F_1-ATPase located on the inner mitochondrial membrane that bind ATP and ADP. 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 HDL_c-Sepharose fraction, the 59-kDa protein, requires further study before any functional role can be assigned.

**Acknowledgments**

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