Opposing Effects of Apolipoproteins E and C on Lipoprotein Binding to Low Density Lipoprotein Receptor-related Protein*

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The low density lipoprotein receptor-related protein (LRP) from rat liver membranes binds apoprotein E (apoE)-enriched rabbit β-migrating very low density lipoproteins (β-VLDL) in a ligand blotting assay on nitrocellulose membranes. Binding was markedly activated when the β-VLDL was preincubated with recombinant human apoE-3, native human apoE-3 or E-4, or native rabbit apoE. Human apoE-2, which binds poorly (1–2% of apo E-3 binding) to low density lipoprotein receptors, was approximately 40% as effective as apoE-3 or apoE-4 in binding to LRP. Stimulation of apoE-dependent binding to LRP was blocked by the inclusion of a mixture of human apoC proteins, but not apoA-I or A-II. In the preincubation reaction, high concentrations of apoE did not overcome the apoE inhibition. The effects of apoE and apoC on the ligand blotting assay were paralleled by similar effects in the ability of β-VLDL to stimulate cholesteryl ester synthesis in mutant human fibroblasts that lack low density lipoprotein receptors. These properties of LRP are consistent with the known effects of apoE and apoC on apoE-enriched lipoproteins in the liver and raise the possibility that LRP functions as a receptor for apoE-enriched forms of these lipoproteins in intact animals.

The low density lipoprotein receptor-related protein (LRP) is a 4526-amino acid integral membrane glycoprotein that occupies the cell surface and intracellular vesicles of many animal cells (1–3). The protein was first identified when its cDNA was observed to cross-hybridize with an oligonucleotide that was complementary to a sequence encoding a cysteine-rich element that occurs multiple times in the ligand-binding domain of the low density lipoprotein (LDL) receptor and once in several proteins of the terminal complement cascade (4). The external domain of LRP contains 4400 amino acids that consist of four imperfect copies (1) of the 767-amino acid external domain of the LDL receptor (4). Each copy contains multiple cysteine-rich ligand-binding repeats followed by an epidermal growth factor precursor homology region that contains a second class of cysteine-rich sequences designated growth factor repeats. The LRP contains a total of 31 ligand binding repeats and 22 growth factor repeats as opposed to seven and three, respectively, in the LDL receptor.

Through use of different combinations of cysteine-rich ligand binding repeats, the LDL receptor is able to bind two different apolipoproteins, apoB-100 and apoE (5, 6). ApoB-100, a glycoprotein of 4536 amino acids, is the sole apoprotein of LDL (7). ApoE, a 299-amino acid protein, is found in several lipoproteins, including β-migrating very low density lipoproteins (β-VLDL), which are cholesteryl ester-rich lipoproteins that accumulate in the plasma of cholesterol-fed animals (8). Each particle of β-VLDL contains multiple molecules of apoE plus one molecule of either apoB-100 or apoB-48. Most apoB-48-containing β-VLDL are derived from the metabolism of dietary chylomicrons, whereas most apoB-100-containing β-VLDL are derived from the metabolism of endogenous VLDL (9).

The function of LRP has been studied in mutant fibroblasts from an individual with homozygous familial hypercholesterolemia (FH) who has no LDL receptors, owing to the inheritance of two null alleles at the LDL receptor locus (2). Lipoprotein uptake was estimated by measuring the rate at which the mutant cells incorporated [3H]oleate into cholesteryl [14C]oleate. This reesterification reaction is stimulated many-fold when cells have taken up a lipoprotein through receptor-mediated endocytosis and have hydrolyzed its cholesteryl esters in lysosomes (10). When the LDL receptor-negative cells were incubated with rabbit β-VLDL, there was no stimulation of cholesterol esterification (2). However, when the β-VLDL was enriched by prior incubation with recombinant human apoE, the β-VLDL acquired the ability to stimulate the synthesis of cholesteryl esters in the mutant fibroblasts. This stimulation was blocked by an antibody against LRP, indicating that LRP mediated this uptake. The stimulation was also blocked by chloroquine, suggesting that lysosomes were involved (2). These studies were consistent with the concept that LRP may serve as a receptor for the removal of apoE-enriched remnant particles derived from the metabolism of dietary chylomicrons (2). The ability of LRP to bind apoE was recently demonstrated directly by Beisiegel et al. (11), who showed that apoE contained in phospholipid vesicles could be chemically cross-linked to LRP in isolated membranes and in intact cells.

In the current studies we have sought to further characterize
the apoE-dependent binding of β-VLDL to LRP using a solid-phase in vitro ligand blotting assay. We have also used the solid-phase assay and the intact fibroblast assay to explore the ability of various polymorphic forms of apoE to interact with LRP. Human apoE exists in three common polymorphic forms (8). The most common form, designated apoE-3, has an arginine at position 158 and binds with very high affinity to LDL receptors (8, 12, 13). Another form, found in patients with familiar type III hyperlipoproteinemia, contains a cysteine substituted for the arginine at position 158. This form, designated apoE-2, binds poorly to LDL receptors. The third form, designated apoE-4, has an arginine at position 158 and an arginine substituted for the sole cysteine at position 112. It binds to LDL receptors with an affinity similar to that of apoE-3 (13). Here, we show that LRP binds all three apoE proteins, but that its binding capacity for apoE-2 is lower than that for the other two proteins.

We have also studied the effect of the C apoproteins of the apo E-dependent binding of β-VLDL to LRP. ApoCs are a mixture of three small polypeptides (designated C-I, C-II, and C-III) that are found in VLDL and high density lipoproteins (9). Previous studies by Windler and Havel (14) and Shefiburne et al. (15) showed that addition of apoC to lipoprotein particles or lipid emulsions enhances uptake by the liver and that this effect can be overcome by adding excess C apoproteins. The parallel effects of apoE and apoC on β-VLDL binding to LRP observed in the current study further supports the notion that LRP may serve as a receptor in the liver for apoE-enriched lipoproteins, such as β-VLDL and chylomicron remnants.

**Experimental Procedures**

**Materials—**Recombinant human apoE (isoform R3), obtained from Escherichia coli (batch H5-29) (16), was kindly provided by Tivka Vogel (Biotechnology General, Rehovot, Israel). The apoE monoclonal antibodies 3H1 and 3B7 (17, 18) were kindly provided by R. W. Milne and Y. L. Marcel, Montreal, (Clinical Research Institute, Canada). Rabbit polyclonal antibodies against purified rat LRP (2) and against a synthetic peptide corresponding to the COOH-terminal 15 amino acids of human LRP (1) were prepared as described in the legend for Figure 1.

**Lipoproteins—**β-VLDL (d < 1.006 g/ml) was prepared from the plasma of male New Zealand White rabbits (2-3 kg). The rabbits were sequentially fasted for 48 h, fed for 4 days with a chow diet containing 2% (w/w) cholesterol and 10% (v/v) coconut oil, and then fasted for 12 h prior to venipuncture. Blood was collected in heparin-agarose (Affi-Gel Heparin, Bio-Rad) equilibrated in buffer containing 2% (w/v) bovine serum albumin, 0.2 mM dithiothreitol, 80 mg/ml gentamycin, and 1 mM EDTA (pH 8), 80 mg/ml bovine serum albumin (Sigma) and centrifuged at 100,000 g for 1 h at 4 °C, and the floating lipoprotein fraction was passed through several Millipore filters (0.45 μm, 0.2 μm, 0.45 μm), stored at 4 °C, and used within 10 days. The ratio of total cholesterol to protein ranged from 15 to 22 in 17 preparations used during the course of this study.

**Apoproteins—**Rabbit apoE was isolated from the β-VLDL-prepared from the plasma of male New Zealand White rabbits using heparin-agarose (Affi-Gel Heparin, Bio-Rad) equilibrated in buffer containing fractions containing apoE were identified by 12% SDS-PAGE, pooled, and dialyzed against buffer A (0.2 M Tris-HCl, 50 mM NaCl, 1 mM urea at pH 8). The apoproteins were then bound to 10 ml of heparin-agarose (Affi-Gel Heparin, Bio-Rad) equilibrated in buffer A by incubation on a rotating wheel for 3 h at 4 °C. The resin was loaded into a column of 2.5-cm diameter, the flow-through collected and the column washed with 10 volumes of buffer A. ApoE was eluted with 2 mM Tris-HCl, 1 mM NaCl, and 3 M urea at pH 8. The apoE-containing fractions were pooled and dialyzed extensively against 150 mM NaCl and 0.2 mM EDTA at pH 8. ApoE was analyzed on SDS-PAGE and found to be homogeneous by Coomassie staining. Approximately 10 μg of purified apoE were obtained from ~100 ml of plasma from one cholesterol/coconut oil-fed rabbit.

**Human apoE-2, E-3, and E-4 were isolated from the d < 1.02 lipoprotein fraction obtained from subjects with the homozygous phenotypes E-2/E-2, E-3/E-3, and E-4/E-4, respectively, as previously described (20).** Briefly, the lipoproteins were collected in the presence of 6 mM sodium EDTA (pH 8), solubilized in 0.5 M sodium chloride containing 0.1 M Tris-HCl, 1 mM EDTA, and 140 mM β-mercaptoethanol at pH 7.4, and the apoproteins were separated on S-300 Sephacryl (Pharmacia LKB Biotechnology Inc.) using the above buffer supplemented with 4 M guanidine.

**Human apoA-I and A-II were isolated from high density lipoproteins (d = 1.063 - 1.21 g/ml) by Sephacryl S-300 chromatography as previously described (21). Human C apoproteins were prepared as described (22).**

**Prior to use in experiments, all apoproteins (native and recombinant) were dialyzed into buffer containing 10 mM sodium bicarbonate and 0.15 M sodium chloride at pH 7.5.**

**Fibroblasts—**Diploid human fibroblasts derived from skin biopsies (normal and FH 808) were grown in monolayer culture at 37 °C in a 5% CO₂ incubator (23). FH 808 is a French Canadian subject who is homozygous for a >21-kilobase deletion that removes the promoter region of the LDL receptor gene (24). Normal rat kidney fibroblasts (NKH, clone SA6) were obtained from J. E. DeLarco (National Cancer Institute, Bethesda, MD).

**Cholesterol Esterification Assay—**Fibroblasts were set up according to a standard format unless otherwise indicated in the legends. On day 0, 4 x 10⁵ cells were seeded into each 60-mm Petri dish containing 3 ml of Dulbecco’s modified Eagle medium (DMEM) supplemented with 4 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 10% (v/v) fetal calf serum. On days 2 and 3, and the medium was replaced with 5 ml of fresh DMEM containing glutamine, antibiotics, and 10% fetal calf serum. On day 7, each monolayer received a final volume of 2 ml of DMEM (without glutamine) containing 1 mM 2-mercaptoethanol, either 6% fetal calf serum (medium A) or 2 mg/ml bovine serum albumin (medium B), and a mixture of β-VLDL and apoE (with or without apoC, A-I, or A-II as indicated in the legends). The β-VLDL/apoprotein mixture was preincubated together for 1 h at 37 °C in 0.4 ml of medium A or B before being added to the culture medium. Unless otherwise indicated, the preincubation was carried out in the presence of 4-α β-mercaptoethanol. After incubation at 37 °C for the indicated time, the cells were radiolabeled with 0.2 mM [14C]oleate (8,485 to 10,960 dpm/nmol) complexed to albumin and were then harvested for measurement of their content of 2-mercaptoethanol [14C]oleate by a modification of the methods of Wade et al. (26) and O’Shannessy et al. (27). Briefly, P-VLDL (2.5 mg/ml) was labeled with biotin by a modification of the methods of Gao et al. (28) and O’Shannessy et al. (27). Briefly, β-VLDL (2.5 mg of protein) was incubated with 4 mM NaI04, in a final volume of 2 ml of 0.15 M NaCl for 30 min at 4 °C in darkness. The reaction was quenched with the addition of 0.5 M Na2SO3 to a final concentration of 0.8% (w/v) cholesterol and 10% (v/v) coconut oil, and then}
of 5 mM. After 5 min at room temperature, the solution was mixed with 2 mg of biotin hydrazide (dissolved in 0.1 ml of 0.1 M NaOAc, pH 5.0) and incubated at room temperature for 30 min. The reaction was quenched by the addition of 0.1 volume of 1 M sodium glycine (pH 8.5) for 1 h at 4 °C, and the biotinylated β-VLDL was dialyzed overnight against buffer containing 20 mM Tris-HCl, 150 mM NaCl, and 0.25 mM EDTA at pH 7.4.

Streptavidin (Pierce) was radiolabeled with 125I by the Iodogen (Pierce) method (28). The free 125I was separated from 125I-streptavidin by gel filtration on a PD-10 column (Pharmacia) equilibrated in 10 mM Tris-HCl, 150 mM NaCl, and 2% bovine serum albumin at pH 7.5.

Other Assays—The content of protein in cell extracts and lipoproteins was determined by the Lowry method (29). Immunoblot analysis was carried out as previously described (30).

RESULTS

Fig. 1 shows the time course of [14C]oleate incorporation into cholesteryl [14C]oleate in homozygous FH 808 fibroblasts incubated with β-VLDL either in the absence or presence of recombinant apoE-3. To study this process over a prolonged period, we maintained the β-VLDL in the culture medium for 48 h, periodically changing the medium to add fresh [14C]oleate as indicated by the arrows. In the absence of apoE-3, there was very little synthesis of cholesteryl [14C]oleate. In the presence of apoE-3, this synthesis was markedly stimulated. After 24 h, a plateau was reached in which there was no further net incorporation of [14C]oleate. At this point the cells contained 160 nmol of [14C]oleate/mg protein, which is equal to 64 μg of esterified cholesterol/mg protein. Since fibroblasts generally contain less than 1 μg of esterified cholesterol/mg cell protein (31), the data indicate that the fibroblasts accumulated very elevated levels of esterified cholesterol as compared with their normal content. This degree of cholesteryl ester accumulation is usually observed only in cholesterol-loaded foam cells (32).

Fig. 2 shows a ligand blot performed with a partially purified fraction of rat liver membrane proteins. This fraction, which was obtained after DEAE-cellulose chromatography, contains the LDL receptor and LRP. The proteins were subjected to SDS-electrophoresis, blotted onto nitrocellulose, and incubated with [125I]-streptavidin followed by autoradiography. In the absence of apoE supplementation, the β-VLDL bound to the LDL receptor, which migrates at approximately 130 kDa on these nonreduced gels (25). When the β-VLDL was preincubated with recombinant human apoE-3, it acquired the ability to bind to LRP, which migrated at approximately 35 kDa on SDS-PAGE (linear gradient) and transferred to nitrocellulose paper. The ability of recombinant human apoE-3 to bind to LRP and LDL receptors (1 mg of protein) was subjected to electrophoresis on a 12-cm preparative 3-8% SDS-PAGE (linear gradient) and transferred to nitrocellulose paper. The ability of recombinant human apoE-3 to bind to LRP and LDL receptors was verified by immunoblotting of nitrocellulose strips with antibodies specific for either LRP or the LDL receptor (data not shown).

Fig. 2. ApoE-dependent ligand blotting of β-VLDL to partially purified LRP and LDL receptors from rat liver. A DEAE-cellulose fraction of solubilized rat liver membranes containing both LRP and LDL receptors (1 mg of protein) was subjected to electrophoresis on a 12-cm preparative 3-8% SDS-PAGE (linear gradient) and transferred to nitrocellulose paper. The ability of apoE to bind to LDL receptors was determined by the Lowry method (29). Immunoblot analysis was carried out as previously described (30).

Fig. 5 compares the action of recombinant apoE-3 and the
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**Fig. 3.** Calcium-dependent binding of apoE-enriched β-VLDL to partially purified LRP and LDL receptors. Nitrocellulose strips containing a DEAE-cellulose fraction of rat liver membranes were prepared as described in the legend to Fig. 2. Replicate strips were blotted in a buffer containing 50 mM Tris-HCl (pH 8.0), 15 mM NaCl, 1 mM EDTA, 50 mg/ml bovine serum albumin, and the indicated amount of CaCl$_2$. Biotinylated β-VLDL (5 μg/ml protein) was preincubated in the presence of 10 μg/ml of r-apoE-3 as described under "Experimental Procedures." Bound β-VLDL was detected by incubation with $^{125}$I-streptavidin (10$^6$ cpm/ml; 5700 cpm/ng) after which the strips were exposed to XAR-1 film for 9 h at -70 °C with an intensifying screen. The gels were calibrated with molecular weight markers as described in the legend to Fig. 2.

**Fig. 4.** Comparison of the ability of recombinant human (A) and native rabbit (B) apoE to stimulate cholesteryl $[^{14}$C]$\text{oleate}$ formation in FH 808 fibroblasts in the absence (○, △) or presence (●, ▼) of β-mercaptoethanol. On day 7, each monolayer received 2 ml of medium A containing 30 μg of protein/ml of β-VLDL that had been preincubated with the indicated concentration of either recombinant human apoE-3 (○, △) or native rabbit apoE-3 (●, ▼) in the absence (○, △) or presence (●, ▼) of β-mercaptoethanol as described under "Experimental Procedures." After 5 h at 37 °C, the cells were pulse-labeled for 2 h with 0.2 mM $[^{14}$C]$\text{oleate}$, and their content of cholesteryl $[^{14}$C]$\text{oleate}$ was measured. Each value is derived from a single culture dish. The amount of cholesteryl $[^{14}$C]$\text{oleate}$ formed in the absence of β-VLDL and apoE was 0.08 nmol·h$^{-1}$·mg protein$^{-1}$.

**Fig. 5.** Stimulation of cholesteryl $[^{14}$C]$\text{oleate}$ formation in FH 808 fibroblasts by β-VLDL incubated with different isoforms of native human apoE. On day 7, each monolayer received 2 ml of medium B containing 20 μg of protein/ml of β-VLDL plus the indicated concentration of apoE. The β-VLDL and apoE had been preincubated as described under "Experimental Procedures." After 5 h at 37 °C, the cells were pulse-labeled for 2 h with 0.2 mM $[^{14}$C]$\text{oleate}$, and their content of cholesteryl $[^{14}$C]$\text{oleate}$ was measured. Each value is derived from a single culture dish. The amount of cholesteryl $[^{14}$C]$\text{oleate}$ formed in the absence of β-VLDL and apoE was 0.06 nmol·h$^{-1}$·mg protein$^{-1}$. The nonrecombinant forms of apoE (E-2, E-3, and E-4) were reduced and alkylated as described under "Experimental Procedures," whereas the recombinant apoE (r-E-3) was not so treated.

**Fig. 6.** Ligand blotting of β-VLDL to partially purified LRP and LDL receptors enriched with different isoforms of native human apoE. Nitrocellulose strips containing a DEAE-cellulose fraction of rat liver membranes were prepared as described in the legend to Fig. 2. Replicate strips were blotted in the presence of biotinylated β-VLDL (10 μg of protein/ml) preincubated with the indicated apoE preparation as described under "Experimental Procedures." Bound β-VLDL was detected by incubation with $^{125}$I-streptavidin (10$^6$ cpm/ml; 20,000 cpm/ng) after which the strips were exposed to XRP-1 film for 13 h at -70 °C with an intensifying screen. The gels were calibrated with molecular weight markers as described in the legend to Fig. 2.

The two apoE-3 preparations. However, in other experiments, apoE-4 was equally as effective as E-3. We also noted that high concentrations of apoE-3 and apoE-4 inhibited the β-VLDL-stimulated enhancement of cholesteryl ester synthesis, which was a consistent finding.

In agreement with the fibroblast assays, apoE-2 was approximately one-third to one-half less effective than apoE-3 and E-4 in stimulating β-VLDL binding to LRP on nitrocellulose blots as quantified by densitometric scanning of the gels (Fig. 6). ApoE-3 and E-4 were approximately equivalent in this regard.
A mixture of C apoproteins isolated from human plasma abolished the ability of apoE-enriched β-VLDL to stimulate cholesteryl ester formation in FH 808 fibroblasts (Fig. 7A). Inhibition was complete at 17 μg/ml. The effect of apoC could not be overcome by increasing amounts of apoE, indicating that apoC acts in a non-competitive fashion with respect to apoE (Fig. 7B). The inhibitory effect of the apoC apoproteins was specific; similar concentrations of human apoA-I or apoA-II had markedly less inhibitory effect (Fig. 8). The apoC apoproteins did not block the ability of 25-hydroxycholesterol to stimulate cholesteryl ester synthesis, indicating that the proteins were not affecting the esterification process itself (Table I). The apoCs also did not inhibit the synthesis of triglyceride (Table I).

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**Fig. 7.** Inhibition by apoC of β-VLDL/apoE-mediated stimulation of cholesteryl $[^{14}C]$oleate formation in FH 808 fibroblasts. A, on day 7, each monolayer received 2 ml of medium A containing 20 μg of protein/ml of β-VLDL that had been preincubated with 20 μg/ml of r-apoE-3 and the indicated concentration of apoC. B, on day 7 each monolayer received 2 ml of medium A containing 20 μg of protein/ml of β-VLDL that had been preincubated with the indicated concentration of r-apoE-3 and apoC as described under "Experimental Procedures." After 5 h at 37 °C, the cells were pulse-labeled for 2 h with 0.2 mM $[^{14}C]$oleate, and their content of cholesteryl $[^{14}C]$oleate was measured. In experiment A, each value is derived from a single culture dish except for the control dish receiving no apoC, which is from duplicate dishes. In experiment B, each value is derived from duplicate dishes. The amount of cholesteryl $[^{14}C]$oleate formed in the absence of β-VLDL and apoE was 0.06 and 0.08 nmol·h$^{-1}$·mg protein$^{-1}$ for experiments A and B, respectively.

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**Fig. 8.** Lack of inhibition of apoA-I and A-II on β-VLDL/apoE-mediated stimulation of cholesteryl $[^{14}C]$oleate formation in FH 808 fibroblasts. On day 7 each monolayer received 2 ml of medium A containing 20 μg of protein/ml of β-VLDL that had been preincubated with 25 μg/ml of r-apoE-3 plus the indicated concentration of apoC (○), apoA-I (Δ), or apoA-II (■) as described under "Experimental Procedures." After 5 h at 37 °C, the cells were pulse-labeled for 2 h with 0.2 mM $[^{14}C]$oleate, and their content of cholesteryl $[^{14}C]$oleate was measured. Each value is derived from either a single culture dish (○, Δ, ■) or the average of two dishes (■). The amount of cholesteryl $[^{14}C]$oleate formed in the absence of β-VLDL and apoE was 0.04 nmol·h$^{-1}$·mg protein$^{-1}$.

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**TABLE I**

| Addition to medium | Cholesteryl $[^{14}C]$oleate \( \text{nmol·h}^{-1}·\text{mg protein}^{-1} \) | $[^{14}C]$Triglycerides \( \text{nmol·h}^{-1}·\text{mg protein}^{-1} \) |
|--------------------|---------------------------------|---------------------------------|
| None               | 0.048                           | 56.3                            |
| β-VLDL            | 0.075                           | 65.5                            |
| β-VLDL + r-apoE-3 | 11.2                            | 49.0                            |
| Sterols           | 3.8                             | 48.1                            |

**Fig. 9.** Ligand blotting of apoE-enriched β-VLDL to partially purified LRP and LDL receptors; inhibition by C apoproteins but not by A apoproteins. Nitrocellulose strips containing a DEAE-cellulose fraction of solubilized rat liver membranes were prepared as described in the legend to Fig. 2. Replicate strips were blotted with diethylaminoethyl (β-VLDL 5 μg of protein/ml) preincubated with 10 μg/ml of r-apoE-3 and the indicated concentration of apoC, apoA-I, or apoA-II as described under "Experimental Procedures." Bound β-VLDL was detected by incubation with $^{125}$I-streptavidin (10$^6$ cpm/ml; 20,000 cpm/ng) after which the strips were exposed to XRP-1 film for 13 h at room temperature with an intensifying screen. The strips were calibrated with molecular weight markers as described in the legend to Fig. 2.

The inhibitory effect of apoC was due to its inhibition of the binding of apoE-enriched β-VLDL to LRP. Fig. 9 shows that increasing concentrations of apoC abolished this binding on nitrocellulose blots, whereas apoA-I and apoA-II had no effect. At a concentration of apoC (5 μg/ml) that markedly inhibited β-VLDL binding to LRP (70% inhibition by scanning densitometry), apoC did not inhibit β-VLDL binding to the LDL receptor (Fig. 9), nor did it inhibit the β-VLDL-mediated enhancement in cholesteryl ester synthesis in normal human fibroblasts (Table II), which take up β-VLDL predominantly through the LDL receptor (2). In two ligand blotting experiments with different apoprotein preparations, apoC was at least 8-fold more potent in inhibiting β-VLDL binding to LRP than to the LDL receptor (data not shown). The apoC concentration that gave 50% inhibition averaged <5 μg/ml for LRP compared with >40 μg/ml for the LDL receptor.
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Antibody 3H1 reacts with both human and rabbit apoE, as demonstrated under "Experimental Procedures" except that on day 5 the medium was replaced with 2 ml of fresh DMEM containing 10% human lipoprotein-deficient serum (23), glutamine, and antibiotics. On day 7, each monolayer received 2 ml of medium B containing one of the following additions in the presence of 0-33 μg/ml apoC as indicated: none, 20 μg/ml of β-VLDL, or 20 μg/ml protein/ml of β-VLDL that had been preincubated with 20 μg/ml of r-apoE. All preincubations were carried out in the presence of β-mercaptoethanol as described under "Experimental Procedures." After 5 h at 37°C, the cells were pulse-labeled for 2 h with 0.2 mM [3H]oleate, and their content of cholesteryl [3H]oleate was measured. Each value is derived from duplicate dishes except for the dishes receiving r-apoE, which are from single dishes.

The experiment of Fig. 10 was performed to determine whether rabbit β-VLDL and human apoE form a stable complex after preincubation. For this purpose we employed two monoclonal antibodies prepared against human apoE (17, 18). Antibody 3H1 reacts with both human and rabbit apoE, whereas antibody 3B7 reacts only with human apoE. As shown in Fig. 10, recombinant human apoE reacted with both antibodies (lanes 1 and 4). Rabbit β-VLDL reacted with 3H1 (lane 2), but not with 3B7 (lane 5). When β-VLDL had been incubated with recombinant human apoE and was then reisolated by gel filtration, the β-VLDL in the void fraction contained human apoE as indicated by its equal reactivity with the 3H1 antibody (lane 3) and the 3B7 antibody (lane 6). The apoE-enriched β-VLDL that was isolated in the void fraction of the gel filtration column bound to LRP on nitrocellulose blots (Fig. 11).

We previously described a polyclonal rabbit antibody that was prepared against LRP purified from rat liver (2). When incubated with FH 908 fibroblasts, this antibody prevented the stimulation of cholesteryl esterification by apoE-enriched β-VLDL. Fig. 12 shows that this antibody did not directly block the binding of apoE-enriched β-VLDL to rat LRP on nitrocellulose blots. The antibody did, however, cause the rapid degradation of LRP in intact cells. Fig. 13 shows immunoprecipitates of NRK cells that were pulse-labeled with [35S]cysteine and then incubated for varying times with anti-LRP. In the absence of antibody treatment (lane 1), three immunoprecipitable forms of LRP were observed: a 600-kDa band that represents the intact protein and a 515-kDa band and 85-kDa band that result from a specific proteolytic cleavage. After incubation of the intact cells for 3 h with anti-

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\text{Addition to medium} \quad \text{Conc. of apoC in medium} \quad [\text{[^3]C]Oleate incorporation}} \quad ([\text{[^3]C]Oleate incorporation}} \quad \text{cholesteryl [[^3]C]oleate} \\
\begin{array}{|c|c|c|}
\hline
\text{None} & 0 & 0.04 \\
\hline
\beta-VLDL & 0 & 12.8 \\
\beta-VLDL + r-apoE-3 & 0 & 13.7 \\
\beta-VLDL + r-apoE-3 & 1.7 & 15.5 \\
\beta-VLDL + r-apoE-3 & 5 & 17.2 \\
\beta-VLDL + r-apoE-3 & 17 & 13.4 \\
\beta-VLDL + r-apoE-3 & 35 & 11.8 \\
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\end{array}
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FIG. 10. Association of exogenous apoE with β-VLDL after gel filtration: detection by immunoblot analysis. A DEAE-cellulose fraction of solubilized rat liver membranes containing both LRP and LDL receptors (1 mg of protein) was subjected to electrophoresis and transferred to nitrocellulose paper as described in the legend to Fig. 2. Replicate strips (5-6 mm wide) containing ~50 μg of protein/strip were cut and blotted with one of the following: lane 1, 7 μg protein/ml of biotinylated β-VLDL; lane 2, 7 μg protein/ml of biotinylated β-VLDL preincubated with 7 μg/ml of r-apoE-3 in the presence of 5 mM β-mercaptoethanol; lane 3, void fraction containing 7 μg protein/ml of biotinylated β-VLDL that had been incubated with r-apoE and β-mercaptoethanol prior to gel filtration; the gel-filtered biotinylated β-VLDL/apoE complex was prepared exactly as described in the legend to Fig. 10 except that biotinylated β-VLDL rather than untreated β-VLDL was preincubated with r-apoE prior to gel filtration. Bound β-VLDL was detected by incubation with [125I] streptavidin (10⁶ cpm/ml; 30,000 cpm/ng) after which the strips were exposed to XRP film for 12 h at ~70°C with an intensifying screen. The position of migration of LRP and LDL receptors (LDLR) was verified as described in the legend to Fig. 2.

Fig. 11. Association of exogenous apoE with β-VLDL after gel filtration: detection by ligand blot analysis. A DEAE-cellulose fraction of solubilized rat liver membranes containing both LRP and LDL receptors (1 mg of protein) was subjected to electrophoresis and transferred to nitrocellulose paper as described in the legend to Fig. 2. Replicate strips (5-6 mm wide) containing ~50 μg of protein/strip were cut and blotted with one of the following: lane 1, 7 μg protein/ml of biotinylated β-VLDL; lane 2, 7 μg protein/ml of biotinylated β-VLDL preincubated with 7 μg/ml of r-apoE-3 in the presence of 5 mM β-mercaptoethanol; lane 3, void fraction containing 7 μg protein/ml of biotinylated β-VLDL that had been incubated with r-apoE and β-mercaptoethanol prior to gel filtration; the gel-filtered biotinylated β-VLDL/apoE complex was prepared exactly as described in the legend to Fig. 10 except that biotinylated β-VLDL rather than untreated β-VLDL was preincubated with r-apoE prior to gel filtration. Bound β-VLDL was detected by incubation with [125I] streptavidin (10⁶ cpm/ml; 30,000 cpm/ng) after which the strips were exposed to XRP film for 12 h at ~70°C with an intensifying screen. The position of migration of LRP and LDL receptors (LDLR) was verified as described in the legend to Fig. 2.
FIG. 12. Ligand blotting of apoE-enriched β-VLDL to partially purified LRP and LDL receptors in the presence of anti-LRP IgG. Nitrocellulose strips containing a DEAE-cellulose fraction of solubilized rat liver membranes were prepared as described in the legend to Fig. 2. Replicate strips were incubated with blotting buffer for 30 min at room temperature. The buffer was replaced with blotting buffer containing the indicated amount of immune or nonimmune IgG, and the strips were incubated for 1 h at room temperature. Strips were blotted in the presence of the indicated IgG and biotinylated β-VLDL (5 μg of protein/ml) that had been preincubated in the absence (lane 1) or presence (lanes 2-6) of 10 μg/ml of r-apoE-3 as described under "Experimental Procedures." Bound β-VLDL was detected with 125I-streptavidin (10,000 cpm; 20,000 cpm/ng), after which the strips were exposed to XRP-1 film for 14 h at -70 °C with an intensifying screen. The strips were calibrated with molecular weight markers as described in the legend to Fig. 2.

LRP (lane 4), the amount of immunoprecipitable LRP was markedly reduced when compared with cells that had been incubated for 5 h with nonimmune serum (designated zero time in Fig. 13, lane 1), and a new degradative product migrating at ~110 kDa appeared (lane 4). This rapid degradation of LRP presumably accounted for the previously observed antibody-induced inhibition of β-VLDL/apoE-mediated stimulation of cholesteryl esterification in fibroblasts (2).

DISCUSSION

The current paper describes several properties of LRP that are consistent with its postulated role as a remnant receptor. These include 1) its ability to bind apoE-enriched β-VLDL on nitrocellulose blots, which correlates with the ability of apoE-enriched β-VLDL to stimulate cholesteryl ester formation in fibroblasts, 2) its ability to distinguish between apoE-2 and the other apoE isoforms, and 3) its sensitivity to inhibition by the C apoproteins. All of these properties correlate with known parameters for the uptake of chylomicrons and VLDL remnants in intact livers (8, 14, 15, 33, 34).

The opposing effects of apoC and E on remnant uptake by the liver have been studied most extensively by Havel and coworkers (14, 33, 34). These workers showed that freshly secreted VLDL and chylomicrons are taken up at measurable but relatively slow rates by perfused rat livers. Incubation of (515 and 85 kDa). The larger NH2-terminal subunit lacks a membrane-spanning region, but it remains attached to the membrane by noncovalent association with the smaller COOH-terminal subunit. The strong noncovalent interaction between the two subunits allows them to be isolated together and immunoprecipitated together by an anti-peptide antibody directed against the COOH-terminal tail of the 85-kDa subunit.

these lipoproteins with VLDL-free plasma produced an increase in the apoC content and a concomitant decrease in the rate of hepatic uptake, even though apoE remained on the particles. The inability of apoE to overcome the inhibitory effect of apoC was particularly evident in the case of small chylomicrons, which showed a 20-fold increase in apoE content after incubation with VLDL-free plasma. Nevertheless, this incubation decreased hepatic uptake, apparently owing to a simultaneous increase in apoC. When VLDL or chylomicrons were injected into eviscerated rats, the apoCs came off of the particle, and this was followed by rapid hepatic uptake (34). These studies and others (14, 15, 33) have led to the concept that hepatic uptake of remnants is governed by the balance of apoE and C on the particle.

Although the characteristics of the remnants that favor
hepatic uptake have been well defined by the studies discussed above, the putative hepatic receptor that mediates this uptake has remained obscure. In particular, it has not been clear as to whether this uptake is mediated by the LDL receptor or by another receptor with partially overlapping properties. The notion of a separate receptor emerged from observations on individuals with homzygous FH, who lack LDL receptors and have a marked delay in clearance of intermediate density lipoproteins and LDL from plasma. Despite this deficiency, there is no evidence for delayed clearance of chylomicron remnants (35). The same is true in Watanabe-heritable hyperlipidemic rabbits, whose mutation in the LDL receptor gene severely impairs its function (36). Intravenous infusion of apoE into Watanabe-heritable hyperlipidemic rabbits (37, 38) or into cholesterol-fed normal rabbits that have down-regulated their LDL receptors (37) lowers plasma cholesterol levels, further suggesting that apoE may mediate the uptake of lipoproteins through pathways independent of the LDL receptor. The initial phase of this uptake appears to involve the sequestration of lipoproteins in the space of Disse (8, 39). This sequestration is followed by uptake of the particle into hepatocytes, an event that is likely to be mediated by a receptor with the properties of LRP.

Although the current studies are consistent with the notion that LRP may function as a chylomicron remnant receptor, they leave many questions unanswered. Why is it necessary to add exogenous apoE to β-VLDL, which already contains abundant apoE? Coomassie Blue-stained electrophoresis gels of the unincubated rabbit β-VLDL used in these studies showed that apoE was the most abundant protein, staining much more darkly than either apoB-100 or apoB-48. It is possible that the endogenous apoE is inactive by virtue of an association with C apoproteins and that additional apoE is required in order to exceed the inhibitory capacity of the apoCs. Arguing against this notion is the finding that the inhibitory effect of additional apoC cannot be overcome by raising the amount of apoE (Fig. 7B).

An alternative possibility is that the endogenous apoE on circulating β-VLDL is in a configuration that is inactive for binding to LRP. When we extracted apoE from rabbit β-VLDL and then incubated it with fresh rabbit β-VLDL, the native rabbit apoE was just as effective as human recombinant apoE in stimulating cholesterol esterification in FH 808 fibroblasts (Fig. 4B). Thus, the ineffectiveness of the apoE on β-VLDL is not due to a permanent modification but rather to some modification that is reversed when the apoE is removed from the lipid/apoprotein environment of β-VLDL and then reconstituted.

It seems likely that the apoE on the β-VLDL can also be activated in vivo and that this may trigger the final endocytic uptake of a partially metabolized chylomicron or VLDL remnant. The activation might be produced by the acquisition of additional apoE, as might occur if the lipoprotein was trapped in the space of Disse where it would encounter newly secreted apoE. Such activation might involve a loss of C proteins that unmask the apoE already resident on β-VLDL. Or, it might be triggered by a change in the phospholipid or core lipid components of β-VLDL resulting from the action of hepatic lipase (40). In order to solve this problem, it will be important in the future to determine the stoichiometry of the interaction between apoC and apoE on β-VLDL. Can one apoC molecule inactivate multiple apoEs? Are all of the apoCs effective, or is there selectivity for one of the different apoCs?

Another question relates to the tissue distribution of LRP. LRP is found nearly ubiquitously in animal cells (Ref. 1). The bulk of chylomicron remnants, however, are taken up by the liver and by the bone marrow in some species (41, 42). These relative uptake rates do not correlate with the relative abundance of LRP in the different organs. This discrepancy may arise because a rate-limiting step in remnant removal is penetration across capillary endothelia. The fenestrated endothelium of the hepatic sinusoids would allow relatively rapid access to the LRP on the hepatocyte surface. Such access may be much slower in other tissues. It is also possible that in most tissues LRP is not displayed predominantly on the cell surface but functions more restrictively in intracellular lipoprotein transport.

ApoE-2 is clearly defective in its ability to bind to LRP, but the degree of reduction is less than the loss of binding to the LDL receptor (12). It has recently been shown that the arginine to cysteine substitution at position 158 in apoE-2 does not directly disrupt binding to the LDL receptor. Rather, this substitution appears to alter the conformation of the receptor-binding domain (residues ~140–150) of apoE and prevent normal receptor interaction (43, 44). Furthermore, it has been shown that the receptor-binding activity of the 158 variant of apoE-2 can be modulated under conditions from very inactive to active (44). Most subjects who are homozygous for the apoE-2 isoform do not accumulate large amounts of chylomicron and VLDL remnants in their plasma unless they have some other defect such as hypothyroidism or heterozygous FH (45). These latter events may somehow alter the activity of apoE-2, perhaps by changing the size or composition of the remnant lipoproteins (44). It is possible that the intermediate result obtained with apoE-2 binding to LRP relates to the fact that some of the apoprotein is present in an active form and some in an inactive form or that the multiple ligand-binding domains in the LRP provide additional opportunities for the binding of the defective apoE-2. Regardless of the mechanism, the defective apoE could lead to increased plasma levels of remnant lipoproteins seen in patients with type III hyperlipoproteinemia if, in fact, the LRP is involved in remnant metabolism.

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