The apoE isoform binding properties of the VLDL receptor reveal marked differences from LRP and the LDL receptor

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Abstract Apolipoprotein E (apoE) associates with lipoproteins and mediates their interaction with members of the LDL receptor family. ApoE exists as three common isoforms that have important distinct functional and biological properties. Two apoE isoforms, apoE3 and apoE4, are recognized by the LDL receptor, whereas apoE2 binds poorly to this receptor and is associated with type III hyperlipidemia. In addition, the apoE4 isoform is associated with the common late-onset familial and sporadic forms of Alzheimer's disease. Although the interaction of apoE with the LDL receptor is well characterized, the specificity of other members of this receptor family for apoE is poorly understood. In the current investigation, we have characterized the binding of apoE to the VLDL receptor and the LDL receptor-related protein (LRP). Our results indicate that like the LDL receptor, LRP prefers lipid-bound forms of apoE, but in contrast to the LDL receptor, both LRP and the VLDL receptor recognize all apoE isoforms. Interestingly, the VLDL receptor does not require the association of apoE with lipid for optimal recognition and avidly binds lipid-free apoE. It is likely that this receptor-dependent specificity for various apoE isoforms and for lipid-free versus lipid-bound forms of apoE is physiologically significant and is connected to distinct functions for these receptors.—Ruiz, J., D. Kouiavskaia, M. Migliorini, S. Robinson, E. L. Saenko, N. Gorlatova, D. Li, D. Lawrence, B. T. Hyman, K. H. Weisgraber, and D. K. Strickland. **The apoE isoform binding properties of the VLDL receptor reveal marked differences from LRP and the LDL receptor. *J. Lipid Res.* 2005.** 46: **1721–1731.**

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Apolipoprotein E (apoE) is a 34 kDa protein that plays an important role in lipoprotein metabolism by association with lipoprotein particles and with members of the LDL receptor family (1, 2). ApoE contains a 22 kDa N-terminal domain (residues 1–191) that is recognized by receptors and a 10 kDa C-terminal domain (residues 222–299) that has high affinity for lipid and is responsible for the association of apoE with lipoproteins (3, 4). Three major isoforms of apoE exist in the population and differ by cysteine and arginine at residues 112 and 158. The most common isoform, apoE3, contains cysteine and arginine at these positions, respectively, whereas apoE2 contains cysteine at both positions and apoE4 contains arginine at both positions (5). These substitutions have important biological consequences. First, the various apoE isoforms are differentially recognized by the LDL receptor. Thus, apoE3 and apoE4 readily bind to the LDL receptor, whereas apoE2 binds poorly to the LDL receptor and is associated with type III hyperlipidemia (6). Second, the APOE-ε4 allele is associated with the common late-onset familial and sporadic forms of Alzheimer's disease (AD) (7, 8). The biochemical mechanism by which the APOE-ε4 allele increases the risk of AD is unknown, but several possibilities have been proposed (9–11), including differential functions of apoE isoforms upon interaction with members of the LDL receptor family (9).

The LDL receptor family includes the LDL receptor, the LDL receptor-related protein (LRP), LRP1b, megalin (or LRP-2), the VLDL receptor, and apoE receptor 2 (for

Abbreviations: AD, Alzheimer's disease; apoE, apolipoprotein E; LRP, low density lipoprotein receptor-related protein; RAP, receptor-associated protein; Rmax, maximal change in response units; RU, resonance units; SPR, surface plasmon resonance; sVLDLr1–8, soluble very low density lipoprotein receptor fragment containing ligand binding repeats 1–8.

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review, see 12). The LDL receptor recognizes apoE and apoB-100 and plays a critical role in cholesterol homeostasis (13), whereas the structurally related VLDL receptor recognizes apoE, but not apoB-100, and plays an important role in triglyceride metabolism (14). In addition, the VLDL receptor also participates in the reelin signaling pathway, which is important for correct cortical neuron migration during development (15, 16).

LRP is widely expressed in a variety of tissues and cells and is a major neuronal apoE receptor that has been implicated in the development of AD by virtue of its ability to affect the metabolism of amyloid precursor protein (17–20). In the liver, LRP plays an important role in chylomicron remnant metabolism (21), whereas in the vasculature, LRP plays an atheroprotective role by suppressing platelet-derived growth factor signaling pathways, thereby inhibiting vascular smooth muscle cell proliferation and migration (22). Like the VLDL receptor, LRP recognizes numerous ligands, many of which are proteinases and complexes of these proteinases with their target inhibitors.

It is well established that association of apoE with lipid is required for its high-affinity binding to the LDL receptor (23) and that the LDL receptor preferentially recognizes apoE3 and apoE4 isoforms. However, the binding of apoE to other receptor family members, such as LRP and the VLDL receptor, is not completely characterized. Data to date suggest that binding of apoE to these two receptors may differ from that of the LDL receptor. Thus, Takahashi et al. (24) found that the VLDL receptor readily recognizes apoE2 containing VLDL particles, whereas Narita et al. (25) found that cells can catabolize lipid-poor apoE forms via an LRP-mediated process. In the current investigation, we sought to characterize the interactions between apoE, the VLDL receptor, and LRP to gain understanding of the role of these receptors in a variety of physiological processes. Our results indicate that the apoE binding properties of the VLDL receptor differ markedly from those of the LDL receptor.

EXPERIMENTAL PROCEDURES

Proteins and antibodies

The soluble VLDL receptor fragment containing ligand binding repeats 1–8 (sVLDLr1–8) was prepared and characterized as described (26). In some experiments, we used a soluble form of the human VLDL receptor termed sVLDLr that contains the entire ectodomain. This receptor was prepared using the *Drosophila* Expression System (Invitrogen) using the inducible/secreted kit according to the manufacturer's protocol. The secreted sVLDLr was purified by first removing Cu^{2+} ions from the media by passage over a Chelex-100 (Bio-Rad) column and then by affinity chromatography over receptor-associated protein (RAP)-Sepharose as described (26). The apoE binding properties of the two soluble forms of the VLDL receptor were similar. Soluble forms of the LDL receptor were prepared in *Escherichia coli* (27). LRP was purified from human placenta (28), whereas RAP was expressed in *E. coli* and prepared as described (29). ApoE2, apoE3, and apoE4 were prepared as described (30). Because of the presence of cysteine in apoE2 and apoE3, they are prone to form intermolecular disulfide-linked forms that were visualized by SDS-PAGE under nonreducing conditions. When present, the disulfide-linked aggregates were removed by dialyzing the protein into 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS buffer) containing 20 mM DTT for 1 h at room temperature, followed by dialysis overnight against nitrogenated HBS buffer. SDS-PAGE under nonreducing conditions and fast-protein liquid chromatography analysis confirmed that apoE2 and apoE3 preparations were free of disulfide-linked structures after treatment. ApoE monoclonal antibodies 3H1 (31) and 1D7 (32) have been described. Mouse monoclonal anti-VLDL receptor antibodies 5F3, 1H5, and 1H10 were generated by immunizing VLDL receptor knockout mice with recombinant sVLDLr1–8 and prepared as described (33). Screening was performed using microtiter wells coated with sVLDLr1–8. Antibodies were purified using protein G-Sepharose (Amersham Pharmacia Biotech). Purified mouse IgGs from Sigma-Aldrich, Inc. (St. Louis, MO), were used as controls for mouse anti-VLDL receptor antibodies. For assays involving cells, IgG samples were heat-inactivated for 30 min at 50°C before use. BSA was purchased from Sigma-Aldrich, Inc.

Cell lines

293 cells were cultured in Dulbecco's modified eagle's medium (DMEM), 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. 293 cells were transfected with a plasmid expressing the human VLDL receptor, and clones were selected by growing in EMEM, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml hygromycin. All cells were passaged at subconfluence using enzyme-free cell dissociation buffer (Sigma-Aldrich, Inc.). Tissue culture plates, including 6-well and 12-well plates, were from Fisher Scientific (Pittsburgh, PA).

Solid-phase binding assay

ApoE isoforms were immobilized on microtiter wells (IMMULON 2HB plates from Fisher Scientific) at a concentration of 4 μg/ml. The microtiter wells were then blocked with 3% BSA. LRP and sVLDLr1–8 were added at the concentrations indicated, and binding was allowed to occur for 16 h at 4°C. After binding, wells were washed three times. Bound LRP was detected with monoclonal antibody 11H4, and bound sVLDLr1–8 was detected with mouse polyclonal antibodies against sVLDLr1–8. To determine specificity, the binding of LRP and sVLDLr1–8 to BSA-coated wells was also measured. Bound monoclonal antibodies were detected with anti-mouse IgG-alkaline phosphatase-conjugated antibodies (Bio-Rad). After incubation with phosphatase substrate (Sigma number 104) in 0.1 M glycine, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$, pH 10.4, the absorbance for each sample was measured at 405 nm. Data were analyzed by nonlinear regression analysis using SigmaPlot.

To measure the binding of monoclonal antibodies to the VLDL receptor, sVLDLr1–8 was first immobilized onto microtiter wells. After blocking with BSA, increasing amounts of antibodies were added. After binding and washing, bound monoclonal antibodies were detected with anti-mouse IgG-alkaline phosphatase-conjugated antibodies (Bio-Rad). After incubation with phosphatase substrate (Sigma number 104) in 0.1M glycine, 1 mM $MgCl_2$, and 1 mM $ZnCl_2$, pH 10.4, the absorbance for each sample was measured at 405 nm. Data were analyzed using nonlinear regression analysis using SigmaPlot.

Surface plasmon resonance measurements

To evaluate the affinity of lipid-free apoE isoforms for VLDL receptor and LRP, we used surface plasmon resonance (SPR) with a BIAcore 3000 biosensor (BIAcore AB, Uppsala, Sweden). Purified sVLDL1–8 and LRP were immobilized onto a CM5 sen-

sor chip surface at densities of 3.5 fmol/mm^2 [120 resonance units (RU)] and 5.8 fmol/mm^2 (3,500 RU), respectively, by amine coupling in accordance with the manufacturer's instructions (BIAcore AB). One flow cell was activated and blocked with 1 M ethanolamine without any protein and was used as a control surface to normalize SPR signal from receptors immobilized with flow cells. Most of the binding experiments were conducted in standard HBS-P buffer, pH 7.4 (BIAcore AB), containing 0.005% Tween 20 at a flow rate of 30 μl/min and temperature of 25°C. Some direct binding experiments with the LRP and sVLDLr1–8 immobilized receptors were carried out in the presence of 2 mM $CaCl_2$ in HBS-P buffer at a flow rate of 10 μl/min. Sensor chip surfaces were regenerated by 30 s pulses of 100 mM H_3PO_4. All injections used the Application Wizard in the automated method. Data were analyzed with BIA evaluation 3.0 software (BIAcore AB) using the equilibrium analysis model. The maximum change in response units (Rmax) from this analysis was replotted versus apoE concentration, and the data were fit to a single class of sites by nonlinear regression analysis using SigmaPlot 9.0 software.

To test the binding of wild-type and mutant apoE3 with monoclonal antibodies 3H1 and 1D7, we used a capture assay in which monoclonal antibodies 3H1 and 1D7 (100 nM) were first captured on goat anti-mouse Fc-γ-specific IgG for 3 min in QUICKINJECT mode. After capture, 100 nM wild-type or mutant apoE3 or running buffer was injected using KINJECT mode. Because the concentration of goat anti-mouse Fc-γ-specific IgG::monoclonal antibody complexes was constant in each cycle, the changes in Rmax value reflect the differences in affinity for wild-type and mutant forms of apoE3. Chip surfaces were regenerated by a 1 min pulse of 10 mM glycine buffer, pH 1.5, or 20 mM HCl followed by a 2 min equilibration with running buffer. The absolute SPR responses for wild-type and mutant apoE were compared and expressed as relative values (percentages) to wild-type apo E.

To measure the binding of apoE3 and mutant proteins to the VLDL receptor, 100 nM of each protein was injected directly over the CM5 chip surface in which sVLDLr was immobilized at a density of 3,000 RU. As a control for the experiment, a flow cell with immobilized ovalbumin (500 RU) was used. All injections were done in KINJECT mode, and Rmax reflected the SPR response of apoE3 or mutant protein binding to the VLDL receptor.

Cell binding, internalization, and degradation assays

Anti-VLDL receptor IgG 5F3 and anti-apoE IgG 3H1 were radiolabeled with ^{125}I (Amersham Pharmacia Biotech, Piscataway, NJ) to a specific activity ranging from 2 to 10 μCi/μg protein using Iodogen (Pierce Chemical Co., Rockford, IL). For these assays, wild-type 293 cells, 293/VLDLR transfected cells, LRP-expressing mouse embryonic fibroblasts, or PEA-13 cells (LRP-deficient) were seeded onto 6-well plates (precoated with 0.1% gelatin) as indicated and grown overnight at 37°C in 5% CO_2. Cell media were removed, and cells were washed once with assay medium (DMEM, 20 mM HEPES, pH 7.4, and 1.5% BSA) and incubated in this medium for 20–30 min. Cells were then incubated with assay medium containing radiolabeled proteins in the absence or presence of excess unlabeled competitors as indicated at 37°C. In those experiments measuring the internalization of apoE using radiolabeled antibody 3H1, we confirmed that the uptake of this monoclonal antibody was totally dependent upon the addition of exogenous apoE. After incubation, cell media were removed for the determination of degraded counts, and cells were detached with trypsin/proteinase K, then pelleted by centrifugation. Bound counts were measured by determining the counts in the superna-

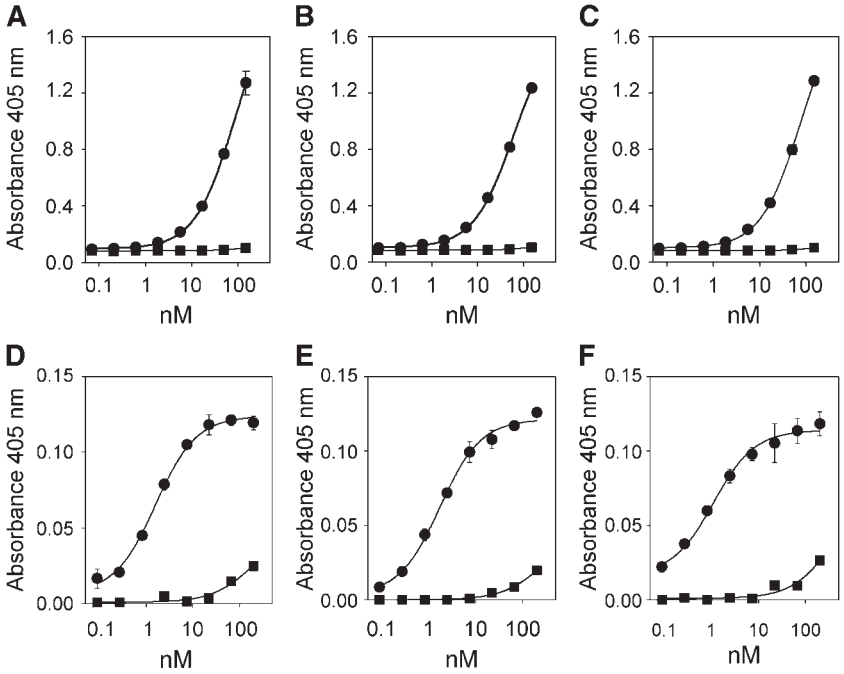

Fig. 1. Binding of LDL receptor-related protein (LRP) or soluble VLDL receptor fragment-containing ligand binding repeats 1–8 (sVLDLr1–8) to apolipoprotein E (apoE) isoforms immobilized in microtiter wells. Microtiter wells were coated with apoE2 (A, D), apoE3 (B, E), or apoE4 (C, F) at a concentration of 4 μg/ml overnight at 4°C. After blocking with 3% BSA, the wells were incubated with the indicated increasing concentrations of sVLDLr1–8 (A–C) or LRP (D–F) for 16 h at 4°C. Bound LRP and sVLDLr1–8 were detected using mouse anti-LRP or anti-VLDL receptor antibodies and anti-mouse IgG conjugated to alkaline phosphatase. Each data point represents the average of duplicate determinations. Closed circles, apoE coating; closed squares, BSA coating. Error bars represent SEM.

tant of pelleted cells. Internalized counts were those associated with the cell pellet, whereas degraded counts were measured in the trichloroacetic acid-soluble fraction of the culture supernatant. Radioactivity was measured in a γ-counter.

Expression of soluble VLDL receptor fragments

Soluble fragments of the VLDL receptor ligand binding domain were transiently expressed in Cos-1 cells using calcium phosphate precipitation as described (34). Briefly, a 100 mm dish at ~60% confluence was cotransfected with 10 μg of pSecTagB containing the cDNA for soluble human VLDL receptor fragments and 5 μg of pcDNA3RAP. Cells were washed 18 h after transfection and kept in serum-containing medium for another 24 h. Then, the medium was replaced with DMEM containing 1% Nutridoma. This medium was harvested after 48 h of incubation, subjected to immunoblot analysis using anti-*myc* antibody to detect recombinant proteins, and used in the binding assays.

RESULTS

Binding of apoE isoforms to LRP and the VLDL receptor

Normally, apoE only binds to the LDL receptor when incorporated into lipoprotein particles. However, Simmons et al. (27) demonstrated that the LDL receptor recognition site on apoE is exposed when lipid-free apoE is immobilized on microtiter wells. Using this assay, they demonstrated that a soluble LDL receptor bound poorly to immobilized apoE2 but bound with high affinity to immobilized apoE3 and apoE4, confirming the known specificity of the LDL receptor for apoE isoforms (27). To gain insight into the apoE binding properties of LRP and the VLDL receptor, we used this assay and measured the binding of purified LRP and VLDL receptor to various apoE isoforms immobilized on microtiter wells. The results of these experiments reveal that both the VLDL receptor (**Fig. 1A–C**) and LRP (Fig. 1D–F) bind to all isoforms of apoE when immobilized on microtiter wells. The apparent K_d values for the interaction of the VLDL receptor with immobilized apoE2, apoE3, and apoE4 are 86, 59, and 77 nM, respectively. The interaction of LRP with immobilized apoE was considerably stronger, with apparent K_d values of 1.6, 1.7, and 1.1 nM for the apoE2, apoE3, and apoE4 isoforms, respectively. Thus, the results from this solid-phase assay suggest that both the VLDL receptor and LRP do not discriminate between apoE isoforms.

The VLDL receptor recognizes lipid-free apoE isoforms

We next investigated the ability of the VLDL receptor and LRP to bind lipid-free forms of apoE. Previously, LRP has been immobilized on SPR surfaces and successfully used to investigate its binding of various ligands (35–37). To determine if either LRP or the VLDL receptor is capable of also recognizing lipid-free forms of apoE in solution, we immobilized purified forms of these receptors on CM5 chips and monitored the real-time binding of different lipid-free apoE isoforms injected over the chip surfaces as analytes using biosensor BIAcore 3000. The data reveal that all apoE isoforms readily bound to the immobilized sVLDLr1–8 (**Fig. 2A–C**). Equilibrium analysis of observed sensorgrams revealed a high-affinity interaction between the VLDL receptor and all apoE isoforms, with no

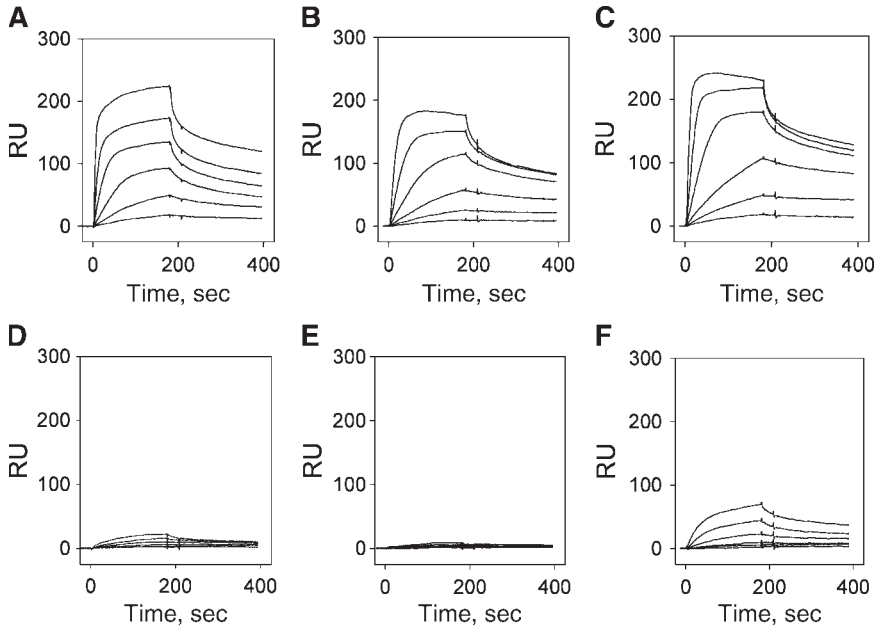

Fig. 2. Binding of apoE isoforms to immobilized sVLDLr1–8 (A–C) or LRP (D–F) assessed by surface plasmon resonance (SPR). Increasing concentrations of apoE2 (A, D), apoE3 (B, E), and apoE4 (C, F) in HBS-P buffer (pH 7.4) were injected on CM5 sensor chip surfaces to which sVLDr1–8 or LRP was coupled. Binding was measured at a flow rate of 10 μl/min for 3 min at 25°C. Dissociation was initiated upon replacement of the analyte solution with buffer. Response is indicated as resonance units (RU) and is corrected for nonspecific binding to a blank chip. The concentrations of apoE used were 6.25, 12.5, 25, 50, 100, and 200 nM for A–F, respectively.

TABLE 1. Binding constants measured for the binding of lipid-free apoE isoforms to the VLDL receptor and to LRP

Receptor	apoE2	apoE3	apoE4
		nM	
VLDL receptor	20 ± 5	25 ± 10	17 ± 9
LRP	nb	nb	weak binding
LDL receptor	nb	nb	nb

apoE, apolipoprotein E; LRP, LDL receptor-related protein; nb, no binding detected. K_d values were determined using an equilibrium method and replotting maximal change in response units (Rmax) versus apoE concentration. The data were fit to a single binding site using SigmaPlot software.

distinction between them (**Table 1**). In contrast, LRP failed to recognize lipid-free apoE2 and apoE3 and bound weakly to lipid-free apoE4 in solution (Fig. 2D–F). In some experiments, we noted weak binding of lipid-free apoE2 and apoE3 isoforms to LRP, and this was attributed to the presence of disulfide-linked oligomers that these proteins are known to form (38). Upon removal of these oligomers, no binding was detected (data not shown). The weak interaction of LRP with lipid-free apoE4 might result from the tendency of this apoE isoform to undergo aggregation (39). As expected, the LDL receptor did not bind lipid-free apoE isoforms in SPR experiments (data not shown). We conclude from these experiments that the VLDL receptor readily recognizes lipid-free forms of apoE in solution, whereas LRP does not recognize lipid-free forms of apoE2 and apoE3 and only weakly binds to lipid-free forms of apoE4.

Lipid-poor apoE isoforms are readily internalized by the VLDL receptor

We next determined whether the VLDL receptor is capable of mediating the cellular internalization of apoE isoforms when added to cells in a lipid-free state. Very likely, this apoE remains in a lipid-poor state, as Narita et al. (25) found no detectable lipid associated with recombinant apoE after incubation with cultured cells at 37°C for 30 min. Although previous studies relied on an adenovirus system to study VLDL receptor-ligand interactions (40, 41), we developed human embryonic kidney 293 cell lines stably transfected with a plasmid expressing the VLDL receptor (293/VLDLR cells). To determine whether the cells expressing the VLDL receptor are able to mediate the uptake of ligands, we first prepared specific monoclonal antibodies against the VLDL receptor by immunizing VLDL receptor-deficient mice with sVLDLr1–8. This resulted in the generation of several monoclonal antibodies, 5F3, 1H5, and 1H10, that recognize the human VLDL receptor by ELISA (**Fig. 3A–C**). We also examined the ability of these antibodies to recognize the VLDL receptor in cell extracts prepared from a rat smooth muscle cell line by using immunoblot analysis (Fig. 3A–C, insets). The results reveal that antibody 5F3 recognized the VLDL receptor under both nonreducing and reducing conditions (Fig. 3A, inset), whereas 1H10 only recognized the VLDL receptor under nonreducing conditions (Fig. 3C, inset). Monoclonal antibody 1H5 failed to recognize the VLDL receptor upon immunoblot analysis (Fig. 3B, inset).

The ability of parental and transfected cells to bind and internalize ^{125}I-labeled monoclonal antibody 5F3 was examined (Fig. 3D, E), confirming the expression and function of the VLDL receptor-transfected cell and revealing that these transfected cells are a good model system to investigate VLDL receptor-mediated uptake of lipid-poor forms of apoE2, apoE3, and apoE4. To eliminate the problem of apoE oxidation that can occur during its iodination, we measured the apoE-dependent internalization of an iodinated apoE monoclonal antibody (3H1) that binds to the C-terminal domain of all apoE isoforms. The internalization of ^{125}I-labeled 3H1 was totally dependent upon the addition of exogenously added apoE in these experiments (**Fig. 4**), confirming the validity of the assay. The results reveal that all three apoE isoforms are readily internalized in VLDL receptor-transfected cells in a process that is inhibited by RAP (Fig. 4). In contrast, the parental cell internalized very little apoE. Thus, these data demonstrate that the VLDL receptor readily mediates the internalization of lipid-poor apoE isoforms.

LRP does not appear to mediate the cellular catabolism of lipid-poor apoE isoforms

The apparent inability of LRP to bind lipid-poor forms of apoE in solution (Fig. 2) seems in conflict with cell-based studies in which LRP was shown to mediate the catabolism of lipid-poor apoE2, apoE3, and apoE4 isoforms (25). Thus, we also performed studies to determine whether LRP is capable of mediating the catabolism of lipid-poor apoE isoforms. For these experiments, we used LRP-expressing mouse embryonic fibroblasts and LRP-deficient forms of these cells (PEA-13), the same cell system used by Narita et al. (25). Once again, to prevent the oxidation of apoE that can occur during iodination procedures, we measured the apoE-dependent uptake of radiolabeled monoclonal antibody 3H1. The results of this experiment reveal no significant difference in the RAP-sensitive uptake of apoE isoforms in LRP-deficient versus LRP-expressing cells (**Fig. 5A–C**). To confirm that LRP is functional in these cells, we examined the uptake of domain 3 of RAP, a known LRP ligand, and the results reveal that LRP-expressing cells mediate the uptake of this ligand, whereas the LRP-deficient cells fail to internalize the ligand (Fig. 5D). Thus, these experiments reveal that LRP does not appear capable of mediating the uptake of lipid-free forms of apoE. At this time, it is not clear why the results in the current study differ from previously published results (25). In the current study, we have taken steps to minimize the formation of multimeric forms (i.e., disulfide-linked aggregates) of apoE, and the different results might reflect the different apoE preparations used in the two studies.

The VLDL receptor recognizes the same region in apoE that binds to the LDL receptor

The LDL receptor binding site for apoE has been localized to the N-terminal portion of apoE within a helical region spanning residues 136–150 (42). Because the VLDL

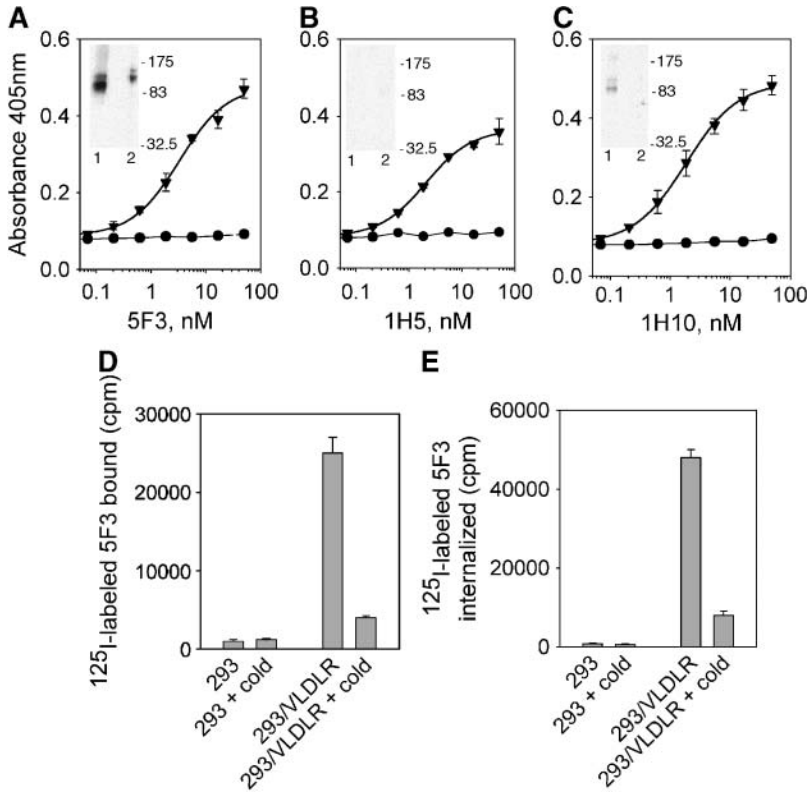

Fig. 3. Characterization of VLDL receptor monoclonal antibodies 5F3, 1H5, and 1H10 and 293/VLDLR transfected cells. A–C: Microtiter wells were coated with sVLDLr1–8 (4 μg/ml) or BSA overnight at 4°C. After blocking and washing, wells were incubated with 5F3 (A), 1H5 (B), or 1H10 (C) at the indicated concentrations. Wells were then washed, and bound monoclonal antibodies were detected using anti-mouse IgG conjugated to alkaline phosphatase. Insets: Lysates of human vascular smooth muscle cells were subjected to SDS-PAGE under nonreducing (lane 1) or reducing (lane 2) conditions. Proteins were then transferred to nitrocellulose and subjected to immunoblot analysis with 5F3 (A), 1H5 (B), or 1H10 (C). D, E: Wild-type 293 or 293/VLDLR cells were plated onto 12-well plates (1×10^5 cells/well) and incubated with 5 nM ^{125}I-labeled 5F3 IgG in the presence or absence of 200 nM unlabeled 5F3 IgG. After 2.5 h, the radioactivity associated with the cell surface (D) and internalized (E) was determined as described in Experimental Procedures. Each bar represents the average of duplicate determinations. Error bars represent SEM.

receptor readily recognizes lipid-poor forms of apoE and the LDL receptor does not, it seems that different regions of apoE might be responsible for recognition by these two receptors. To investigate this, competition experiments were performed using two apoE monoclonal antibodies, 1D7 and 2E8, that are known to block the binding of apoE to the LDL receptor (43). These experiments revealed that 1D7 effectively blocked the binding of all apoE isoforms

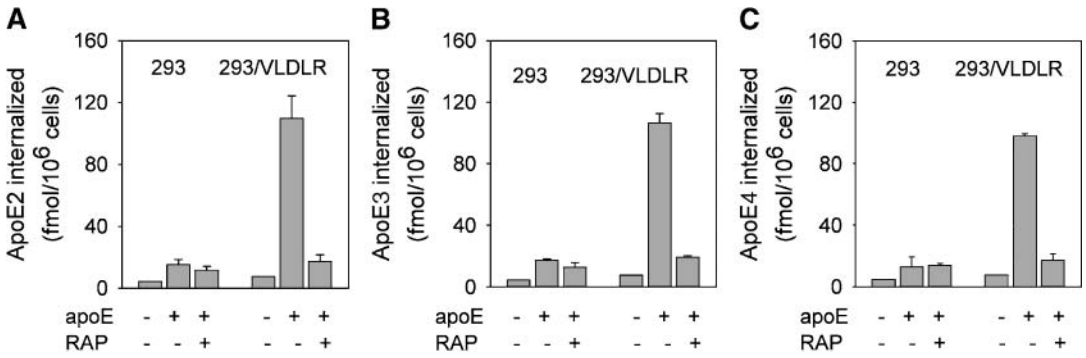

Fig. 4. VLDL receptor readily internalizes lipid-poor forms of apoE2 (A), apoE3 (B), and apoE4 (C). Wild-type 293 and 293/VLDLR cells were plated onto six-well plates (2.5×10^5 cells/well). Then, 10 nM ^{125}I-3H1 in the absence or presence of 10 nM apoE2, apoE3, or apoE4 was added in the presence or absence of 500 nM unlabeled receptor-associated protein (RAP; as indicated). After 1.5 h, the extent of internalized ligand was determined as described in Experimental Procedures. Each bar represents the average of triplicate determinations. Error bars represent SEM.

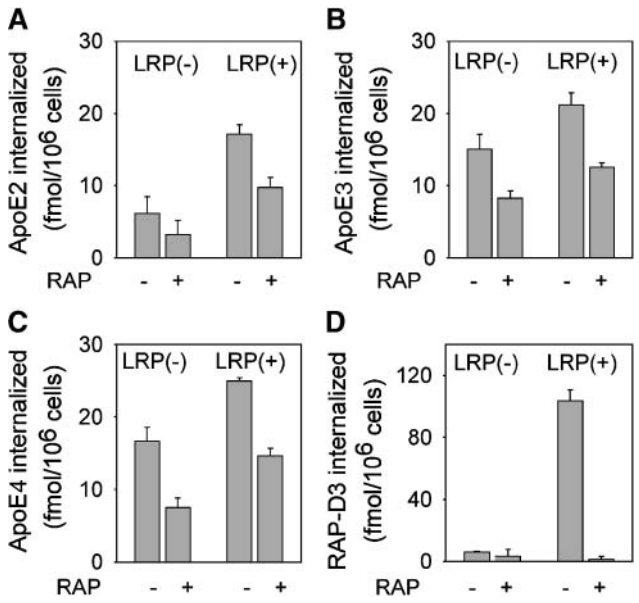

Fig. 5. LRP fails to mediate the internalization of lipid-poor forms of apoE. LRP-expressing or LRP-deficient mouse embryonic fibroblasts were incubated with 10 nM ^{125}I-3H1 in the absence or presence of 10 nM apoE2 (A), apoE3 (B), or apoE4 (C). RAP was used to inhibit the LRP-mediated internalization at a concentration of 500 nM (as indicated). After 1.5 h, the extent of internalized ligand was determined as described in Experimental Procedures. Each bar represents the average of triplicate determinations. D: A concentration of 5 nM ^{125}I-labeled RAP domain 3 was incubated with the cells in the presence or absence of 500 nM unlabeled RAP (as indicated). After 1.5 h, the extent of internalized ligand was determined as described in Experimental Procedures. Each bar represents the average of triplicate determinations. Error bars represent SEM.

to immobilized VLDL receptor, whereas monoclonal antibody 2E8 partially blocked the binding of apoE isoforms to the immobilized VLDL receptor (**Fig. 6**). Because these two antibodies are potent inhibitors for the binding of apoE to the LDL receptor, these data suggest that the binding site on apoE that is recognized by the LDL receptor might overlap the binding site for the VLDL receptor as well.

To gain further insight into the region on apoE that is recognized by the VLDL receptor, we used well-characterized apoE mutants whose binding to the LDL receptor has been characterized previously. To measure binding, sV-LDLr was first coupled to SPR surfaces. We also coupled the two apoE antibodies, 1D7 and 3H1, to serve as controls for this experiment. Antibody 3H1, which binds to a determinant within the C-terminal domain of the molecule, was used as a control to confirm that the concentrations of each mutant protein were accurate. **Figure 7** shows the results of the binding of various mutant apoE molecules to 3H1, 1D7, and VLDL receptor-coupled surfaces. The data are normalized to the amount of binding detected for wild-type apoE3. A mutation at residue 142 (R142C) of apoE generates a molecule that is defective in binding to the LDL receptor and to monoclonal antibody 1D7 (44). Our data reveal that this apoE mutant also failed to bind to the VLDL receptor but was readily recognized by 3H1 antibody. Likewise, a double mutation (R142A/K143S) also failed to bind to either the VLDL receptor or monoclonal antibody 1D7. A mutation at arginine 145 (R145C) partially inhibited the binding of the apoE molecule to the VLDL receptor but completely prevented monoclonal antibody 1D7 from binding. The apoE2 (R145C) mutation is associated with type III hyperlipidemia (45, 46) and, interestingly, seems to increase the binding of apoE2 containing dimyristoylphosphatidylcholine vesicles to the LDL receptor (46). A forth mutant that is defective in binding to the LDL receptor is apoE(R172A) (47). We also tested this mutant for VLDL receptor binding and found a significant reduction in the binding of this receptor to this mutant apoE. In contrast, binding of monoclonal antibody 1D7 was not affected. Overall, these studies reveal that amino acid residues that impair the interaction of apoE with the LDL receptor also impair its interaction with the VLDL receptor.

Identifying regions on the VLDL receptor that bind apoE

We next examined the ability of various monoclonal antibodies prepared against the VLDL receptor to block the binding of ^{125}I-labeled apoE4 to microtiter wells coated with the VLDL receptor. The results reveal that monoclonal antibodies 1H5 and 1H10 are both very effective at blocking the binding of ^{125}I-apoE4 to immobilized sV-LDLr, whereas antibody 5F3 failed to inhibit apoE binding (**Fig. 8A**). Because monoclonal antibodies 1H5 and 1H10 effectively block the binding of apoE to the VLDL receptor, we wished to map out the sites on the VLDL receptor to which these antibodies bind. To accomplish this, Cos-1 cells were transfected with constructs encoding various repeats of the VLDL receptor ligand binding domain, each containing both a polyhistidine and a myc tag (Fig. 8B). Media of Cos-1 cells transfected with cDNAs coding for the indicated regions of the VLDL receptor ligand binding domain were used as a source of soluble receptor fragments and were captured on microtiter wells coated with anti-*myc* IgG. Binding of ^{125}I-labeled anti-histidine IgG confirmed that equivalent amounts of each fragment were captured to the microtiter wells (data not shown). Both antibodies (1H5 and 1H10) readily recognized fragments containing repeats 1–8, 3–6, and 5–8. Antibody 1H10 failed to recognize a fragment containing repeats 1–4, whereas antibody 1H5 showed reduced binding to this fragment (Fig. 8C). These results indicate that both antibodies prefer repeats located within the C-terminal region of the VLDL ligand binding domain, suggesting that this is the region that recognizes apoE as well. In contrast, using similar assays, RAP and urokinase:plasminogen activator inhibitor type I complexes were found to bind to the N-terminal region of the VLDL receptor ligand binding domain (repeats 1–4) (34).

DISCUSSION

ApoE is a component of plasma lipoproteins that mediates their binding to members of the LDL receptor family

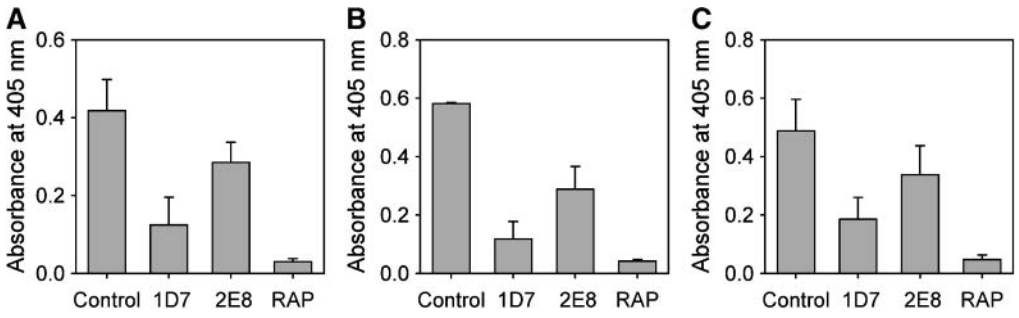

Fig. 6. Inhibition of apoE binding to immobilized sVLDLr1–8 by RAP and monoclonal antibodies 1D7 and 2E8. Microtiter wells were coated with sVLDLr1–8 (4 μg/ml) overnight at 4°C. After blocking and washing, wells were incubated with 1 nM apoE2 (A), apoE3 (B), or apoE4 (C) in the absence or presence of 1D7 (1 μM), 2E8 (1 μM), and RAP (1 μM). After incubation, the wells were washed, and bound apoE was detected using rabbit polyclonal antibodies and anti-rabbit IgG conjugated to alkaline phosphatase. Each data point represents the average of duplicate determinations. Error bars represent SEM.

and to heparin sulfate proteoglycans. The structural features of apoE that are recognized by the LDL receptor have been investigated extensively (1, 2, 48–51), and these experiments reveal an absolute requirement for lipid association with apoE for this ligand to be recognized by this receptor. Furthermore, the LDL receptor shows a marked preference for the apoE3 and apoE4 isoforms and binds the apoE2 isoform poorly. In contrast to the LDL receptor, the interaction of apoE isoforms with other members of the LDL receptor family have not been studied extensively. Thus, it has been generally assumed that LRP and the VLDL receptor, two important members of the LDL receptor family that are expressed in the brain, display a similar specificity for apoE as does the LDL receptor. However, the VLDL receptor has been reported to bind VLDL particles composed of apoE2 isoforms (24), and LRP has been reported to mediate the cellular uptake of lipid-free forms of apoE (25). These two studies suggest that the apoE binding properties of LRP and the VLDL receptor might differ from those of the LDL receptor, and in the current study we investigated the binding of apoE isoforms to the VLDL receptor and to LRP.

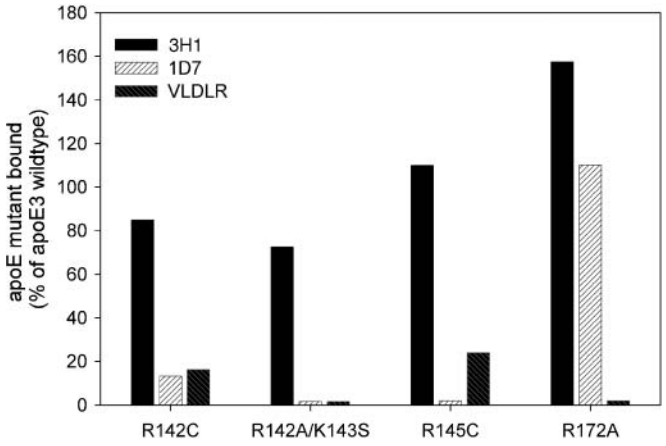

Fig. 7. The VLDL receptor recognizes the same region on apoE recognized by the LDL receptor. In these experiments, 3H1 and 1D7 monoclonal antibodies (100 nM) were first captured on the CM5 chip surface immobilized with goat anti-mouse Fc-γ-specific immunoglobulins, then 100 nM wild-type or mutant apoE [apoE3(R142C), apoE3 (R142A/K143S), apoE3(R145C), or apoE3(R172A)] or buffer was injected. In each cycle, maximal change in response units (Rmax) was measured by SPR with the BIAcore 3000. The data are expressed as relative values in which the mutant apoE binding relative to that of wild-type apoE is calculated. The binding of wild-type and mutant apoE to sVLDLr was tested with another chip in which wild-type and mutant forms of apoE (100 nM) were injected directly over the VLDL receptor-immobilized flow cell surface. All binding experiments were conducted at a flow rate of 30 μl/min and a temperature of 25°C.

Our studies reveal that the interaction of apoE with the VLDL receptor differs markedly from its interaction with the LDL receptor. First, we found that apoE free of lipid bound avidly to the VLDL receptor. This binding was assessed by SPR experiments and by demonstrating that cells transfected with the VLDL receptor effectively internalized lipid-poor forms of apoE. Second, our experiments confirmed that the VLDL receptor recognizes all apoE isoforms with equal affinity. Thus, the VLDL receptor failed to discriminate between the apoE isoforms by in vitro binding assays and cell uptake experiments. Despite the differences in specificity for apoE between these two receptors, the same regions of apoE appear to be involved in receptor recognition. Thus, antibodies known to block the binding of apoE to the LDL receptor also prevented its binding to the VLDL receptor. Furthermore, mutations in the apoE site that are known to affect LDL receptor binding also were found to impair VLDL receptor binding.

The apoE binding properties of LRP in our studies seemed more similar to those of the LDL receptor. Early work with LRP suggested a requirement for apoE-enriched remnant particles or β-migrating VLDL particles (52). However, these early findings were recently challenged by Narita et al. (25), who found that LRP-expressing mouse embryonic fibroblasts, but not LRP-deficient fibroblasts, could mediate the uptake of lipid-poor apoE. In contrast, our experiments failed to detect the uptake of lipid-poor forms of apoE by these same cells and are more in agreement with earlier work (52). Although the reason for this discrepancy is not clear at this time, we suspect that one difference between our work and that of Narita et al. (25) might result from differences in the apoE preparations. We found that apoE2 and apoE3 tend to form disulfide-

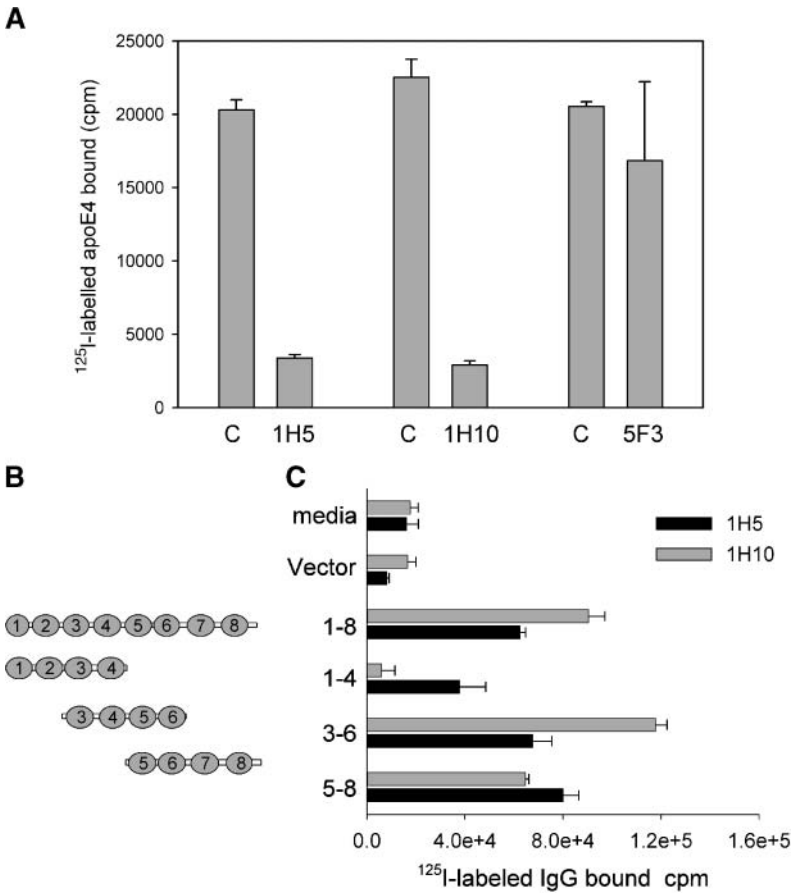

Fig. 8. VLDL receptor monoclonal antibodies 1H10 and 1H5 inhibit the binding of ^{125}I-apoE4 to the VLDL receptor. A: Microtiter wells were coated with VLDL receptor (4 μg/ml). After coating and blocking with BSA, the wells were incubated with 5 nM ^{125}I-labeled apoE4 in the absence or presence of monoclonal antibodies 1H10, 1H5, or 5F3 (1 μM). After incubation, the wells were washed and counted. C, control, no antibody added. B: Schematic diagram of the modular structure of the ligand binding domains of the VLDL receptor used in the assay. C: Solid-phase assay measuring the binding of ^{125}I-labeled 1H10 and ^{125}I-labeled 1H5 to various VLDL receptor fragments. For these assays, microtiter wells were first coated with anti-myc IgG (10 μg/ml) overnight at 4°C. After blocking for 1 h with 3% BSA, 100 μl of conditioned media expressing various fragments of the VLDL receptor was incubated with the plates for 1 h. After washing, the wells were incubated with 10 nM ^{125}I-labeled 1H10 for 2 h at room temperature. After washing, the amount of radioactivity bound to the wells was measured. To confirm that equal amounts of receptor fragment were captured in the wells, the binding of ^{125}I-labeled antihistidine IgG to wells was also measured. These data revealed that equal amounts of receptor fragment were absorbed in each case. Error bars represent SEM.

linked dimers that do indeed bind to LRP, as assessed by SPR measurements. Thus, in our study, we were careful to eliminate disulfide-linked dimers from the apoE preparations.

The finding that the VLDL receptor is able to bind lipid-poor forms of this molecule is interesting and suggests that lipid-poor forms of apoE might have physiological significance. Studies are emerging suggesting that lipid-poor forms of apoE are associated with the extracellular matrix and with cell surface proteoglycans (53) and may play an important role in lipoprotein uptake (53) or cholesterol efflux (54). Furthermore, recent studies using transgenic apoE-deficient mice reveal that low-level expression of apoE has profound effects on the development of atherosclerosis independent of its effect on plasma lipoprotein removal (55). In these mouse models, apoE was specifically expressed in the adrenal gland, and two transgenic lines that expressed too little apoE to correct their hypercholesterolemia were found to have a significant reduction in cholesteryl ester deposition in their aortas. These surprising results reveal that low levels of apoE (concentrations of 10–30 nM) can block atherogenesis in the vascular wall independent of the removal of plasma lipoproteins. In addition, a recent atherosclerosis reversal study demonstrated that apoE reduced cholesterol deposits in plaques independent of decreasing plasma cholesterol levels (56). The mechanism of this effect is not known, but it is interesting to speculate that it may involve the association of lipid-poor forms of apoE with the VLDL receptor. The VLDL receptor is known to participate in signaling pathways, and in the endothelium binding of tissue factor pathway inhibitor to the VLDL receptor it modulates endothelial cell proliferation in response to fibroblast growth factor (26, 57). Further studies are required to characterize the potential effect of apoE interaction with the VLDL receptor on the endothelium.

The recognition of apoE by various members of the LDL receptor family is of great interest because the APOE-ε4 allele is associated with the common late-onset familial and sporadic forms of AD (7, 8). The genetic studies are consistent with the conclusion that the APOE-ε4 allele is not a causative factor but rather is a genetic risk modifier for AD and decreases the age of onset of AD in a dose-dependent manner (58). The biochemical mechanism by which APOE-ε4 increases the risk of AD is still unknown, but several possibilities have been proposed. Lipid-free apoE3, but not apoE4, stimulates neurite outgrowth when added to neuronal cultures (9, 59). Curiously, the isoform-specific effects are only seen when exogenously added lipid (β-VLDL) is included. These effects have been attributed to LRP-mediated events, as revealed by the use of specific antibodies (60), and have been duplicated in cells transfected to express large amounts of apoE3 or apoE4 (61) in the absence of exogenously added β-VLDL, revealing that the minimally lipidated forms of apoE3 can also promote neurite outgrowth. The mechanism of this effect is

not yet understood, and the potential role of the VLDL receptor has not been investigated. It is possible that differential effects of apoE isoforms on the apoE-mediated transport of lipoproteins into cells, or differential effects of apoE isoforms on signaling mediated by the VLDL receptor or LRP, may contribute to these effects.

In summary, the current study reveals that the apoE binding properties of the VLDL receptor differ markedly from those of the LDL receptor. The substantial increase in the affinity of apoE upon lipid association for the LDL receptor is not observed for the VLDL receptor. Furthermore, the VLDL receptor readily recognizes all apoE forms in a lipid-poor state, whereas LRP recognizes all isoforms, but in a lipid-bound state. It is likely that this receptor-dependent specificity for various apoE forms (i.e., lipid-bound versus lipid-free) is physiologically significant and is connected to the distinct functions of these receptors. The LDL receptor in liver plays a major role in the removal of apoE- and apoB-containing lipoproteins from the plasma, whereas the VLDL receptor and LRP play important roles in the brain and in maintaining the integrity of the vasculature. The recent studies revealing that apoE also plays an important, but undefined, role in maintaining the integrity of the vasculature (55) raise the possibility that its association with the VLDL receptor or LRP may be required for this effect.

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