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Research Article

The role of the low density lipoprotein receptor (LDLR) in the clearance of apo-B48-containing lipoproteins and the role of the LDLR-related protein (LRP) in the removal of apo-B100-containing lipoproteins have not been clearly defined. To address these issues, we characterized LDLR-deficient mice homozygous for an "apo-B48-only" allele, an "apo-B100-only" allele, or a wild-type apo-B allele (Ldlr-/- Apob48/48, Ldlr-/-Apob100/100, and Ldlr-/-Apob+/+, respectively). The plasma apo-B48 and LDL cholesterol levels were higher in Ldlr-/-Apob48/48 mice than in Apob48/48 mice, indicating that the LDL receptor plays a significant role in the removal of apo-B48-containing lipoproteins. To examine the role of the LRP in the clearance of apo-B100-containing lipoproteins, we blocked hepatic LRP function in Ldlr-/-Apob100/100 mice by adenoviral-mediated expression of the receptor-associated protein (RAP). RAP expression did not change apo-B100 levels in Ldlr-/-Apob100/100 mice. In contrast, RAP expression caused a striking increase in plasma apo-B48 levels in Apob48/48 and Ldlr-/-Apob48/48 mice. These data imply that LRP is important for the clearance of apo-B48-containing lipoproteins but plays no significant role in the clearance of apo-B100-containing lipoproteins.

Lipoprotein Clearance Mechanisms in LDL Receptor–Deficient "Apo-B48-only" and "Apo-B100-only" Mice

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Abstract

The role of the low density lipoprotein receptor (LDLR) in the clearance of apo-B48–containing lipoproteins and the role of the LDLR-related protein (LRP) in the removal of apo-B100–containing lipoproteins have not been clearly defined. To address these issues, we characterized LDLR-deficient mice homozygous for an "apo-B48-only" allele, an "apo-B100-only" allele, or a wild-type apo-B allele ($Ldlr^{-/-}Apob^{48/48}$, $Ldlr^{-/-}Apob^{100/100}$, and $Ldlr^{-/-}Apob^{+/+}$, respectively). The plasma apo-B48 and LDL cholesterol levels were higher in $Ldlr^{-/-}Apob^{48/48}$ mice than in $Apob^{48/48}$ mice, indicating that the LDL receptor plays a significant role in the removal of apo-B48–containing lipoproteins. To examine the role of the LRP in the clearance of apo-B100–containing lipoproteins, we blocked hepatic LRP function in $Ldlr^{-/-}Apob^{100/100}$ mice by adenoviral-mediated expression of the receptor-associated protein (RAP). RAP expression did not change apo-B100 levels in $Ldlr^{-/-}Apob^{100/100}$ mice. In contrast, RAP expression caused a striking increase in plasma apo-B48 levels in $Apob^{48/48}$ and $Ldlr^{-/-}Apob^{48/48}$ mice. These data imply that LRP is important for the clearance of apo-B48–containing lipoproteins but plays no significant role in the clearance of apo-B100–containing lipoproteins. (*J. Clin. Invest.* 1998. 102:1559–1568.) Key words: low density lipoproteins • LDL receptor–related protein • mouse apo-B radioimmunoassay • LDL receptor deficiency • adenovirus-mediated gene transfer

Introduction

The B apolipoproteins (apo-B100 and apo-B48) have been studied extensively because they play central roles in lipoprotein assembly, plasma lipid metabolism, and atherogenesis (1, 2). Apo-B100 is essential for the assembly of very low density lipoproteins (VLDL)[1] in the human liver and is virtually the only protein component of cholesterol-rich low density lipoproteins (LDL) (3). Apo-B48, a truncated apo-B containing the amino-terminal portion of apo-B100, is required for the assembly of chylomicrons in the intestine (2, 3). Apo-B48 is synthesized as a result of apo-B mRNA editing, which changes codon 2153 (CAA, specifying Gln) to a translational stop codon (4). In some mammals, including mice and rats, but not humans, the liver expresses apo-B mRNA editing activity and therefore synthesizes apo-B48 in addition to apo-B100 (5). In mice, approximately 70% of the hepatic apo-B transcripts code for apo-B48 (6).

Apo-B100–containing LDL bind to and are taken up by the LDL receptor (LDLR), in both hepatic and extrahepatic tissues. When the LDLR is absent or defective, as in familial hypercholesterolemia, the plasma levels of apo-B100 and LDL cholesterol are markedly elevated (7). Apo-B48 lacks the portion of the apo-B molecule that interacts with the LDLR and therefore cannot directly mediate the uptake of lipoproteins by the LDLR (8). However, apo-B48–containing lipoproteins accommodate a large amount of apo-E (9), which is a ligand for both the LDLR and the LDLR-related protein (LRP) (10, 11). Several recent studies have suggested that the LRP plays a role in the removal of apo-B48–containing lipoproteins from the plasma (12, 13), but the relative importance of the LRP and the LDLR in the uptake of apo-B48–containing lipoproteins has never been defined precisely.

In this study, we investigated the mechanisms for the uptake of apo-B48– and apo-B100–containing lipoproteins by the LDLR and by the LRP. Specifically, we sought to understand whether the LDLR has a significant role in the clearance of apo-B48–containing lipoproteins from the plasma. We also sought to determine whether the LRP, from the perspective of lipoprotein metabolism, was simply a receptor for the apo-B48–containing lipoproteins, or whether it also has a measurable effect on the metabolism of apo-B100–containing lipoproteins. To address these issues, we have characterized gene-targeted mice that synthesize exclusively apo-B48 ("apo-B48-only" mice) or exclusively apo-B100 ("apo-B100-only" mice), both in the presence and absence of LDLR expression and in the presence and absence of receptor-associated protein (RAP) expression. Our studies have shown that the LDLR does play a significant role in the clearance of apo-B48–containing lipoproteins, but that the LRP also has a major role. Interestingly, the LRP has no apparent role in the metabolism of apo-B100–

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1. *Abbreviations used in this paper:* Ad-β-Gal, adenovirus containing the cDNA for *E. coli* β-galactosidase; Ad-RAP, adenovirus containing the cDNA for rat receptor-associated protein; apo, apolipoprotein; FPLC, fast-performance liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; pfu, plaque-forming unit; RAP, receptor-associated protein; RIA, radioimmunoassay; VLDL, very low density lipoprotein.

containing lipoproteins, even when LDLR activity is absent. Thus, from the perspective of lipoprotein metabolism, the LRP is an "apo-B48–containing lipoprotein" receptor.

Methods

Generation of LDLR-deficient mice that synthesize exclusively apo-B48 or apo-B100. Mice that synthesize exclusively apo-B48 ($Apob^{48/48}$ mice) or apo-B100 ($Apob^{100/100}$ mice) (14) were bred with LDLR-deficient mice ($Ldlr^{-/-}$) (15) to generate $Ldlr^{-/-}Apob^{48/48}$ and $Ldlr^{-/-}Apob^{100/100}$ mice. ($Ldlr^{-/-}Apob^{48/48}$, $Ldlr^{-/-}Apob^{100/100}$, $Apoe^{-/-}Apob^{48/48}$, and $Apoe^{-/-}Apob^{100/100}$ mice are currently available from The Jackson Laboratory [Bar Harbor, ME; http://www.jax.org]). We also generated a third group of mice, LDLR-deficient mice homozygous for a wild-type apo-B allele ($Ldlr^{-/-}Apob^{+/+}$ mice). All three groups of mice had a similar genetic background (~ 75% C57BL/6 and 25% 129/sv). For some experiments, we also used apo-E–deficient mice with the targeted apo-B gene mutations ($Apoe^{-/-}Apob^{48/48}$ and $Apoe^{-/-}Apob^{100/100}$ mice), as well as apo-E–deficient mice homozygous for a wild-type allele ($Apoe^{-/-}Apob^{+/+}$ mice) (14, 16). The apo-E–deficient mice also had a mixed genetic background (~ 75% C57BL/6 and 25% 129/sv). All mice were weaned at 21 d, fed a chow diet, and housed in a full-barrier facility with a 12-h light/dark cycle.

Plasma lipid and lipoprotein measurements. Total plasma cholesterol and triglyceride concentrations were measured on fresh plasma samples with colorimetric assays (16). Total cholesterol and triglyceride levels were measured at 5 mo of age, as well as immediately before and 5 d after intravenous injection of adenoviral preparations. The distribution of lipids within the plasma lipoprotein fractions was assessed by fast phase liquid chromatography (FPLC), using plasma pooled from 5 mice (14).

Analysis of plasma lipoprotein sizes. Three lipoprotein fractions (VLDL [$d < 1.006$ g/ml], LDL [$d = 1.020$–1.063 g/ml], and HDL [$d = 1.063$–1.20 g/ml]) were prepared from plasma by ultracentrifugation (17). For these studies, plasma was pooled from 5–10 female mice of each genotype. VLDL diameters were determined by dynamic light scattering (17) and are reported as the mean±SD (nm). LDL and HDL sizes were assessed by nondenaturing PAGE (17).

Radioimmunoassays (RIAs) for mouse apo-B100. To measure the concentration of mouse apo-B100 in mouse plasma, we used two solid-phase, mAb-based RIAs. The first was a competitive RIA that measured the ability of the apo-B100 in mouse plasma samples to compete with immobilized mouse LDL for binding to a ^{125}I-labeled mAb against mouse apo-B100. (Mouse mAbs specific for mouse apo-B100 were generated in $Apob^{48/48}$ mice that had been immunized with mouse apo-B100 [C.H. Zlot, L. Flynn, M.M. Véniant, E. Kim, M. Raabe, S.P.A. McCormick, P. Ambroziak, L.M. McEvoy, and S. Young, manuscript submitted for publication]. All mice are available from Jackson Labs.) Plasma apo-B100 levels in different groups of mice are presented as the percentage of the amount of apo-B100 in wild-type mice. Mouse apo-B100 concentrations were also measured with a direct-binding "sandwich" RIA (18, 19), using two different mouse apo-B100–specific mAbs.

Analysis of apo-B in the plasma by Western blotting. To compare the amounts of apo-B48 and apo-B100 in different plasma samples and to judge the relative amounts of apo-B48 and apo-B100 in mouse plasma, we performed Western blots of polyacrylamide/SDS gel as previously described (16). The intensity of the bands was quantified with a GS300 transmittance/reflectance scanning densitometer and the GS-365 data system for densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Large-scale preparation of the recombinant adenovirus. A recombinant adenovirus containing the cDNA for rat receptor-associated protein (Ad-RAP) has been described previously by Willnow and coworkers (12). As a control, we used an adenovirus containing the cDNA for *Escherichia coli* β-galactosidase (Ad-β-Gal) (20). Recombinant adenoviruses were prepared on 293 cells as previously described (12). Plaque-forming units (pfu) were compared to viral particle numbers and were similar for both Ad-RAP and Ad-β-Gal (1.1×10^{12} particles $\approx 10^{10}$ pfu).

Injection of adenoviral preparations into mice. Mice were anesthetized with an intraperitoneal injection of avertin (0.02 mg/g of body weight). Blood samples (50–100 μl) were obtained from the retroorbital sinus of each mouse with heparinized collecting tubes. An external jugular vein was exposed, and Ad-β-Gal or Ad-RAP (2×10^{11} particles diluted into 140 μl of Tris-buffered saline) was injected intravenously with a 30-gauge needle.

Statistical analysis. Mean lipid levels for each group of mice are reported, along with SEM. Differences in triglyceride levels, cholesterol levels, and apo-B100 levels were modeled by ANOVA with genotype being an intergroup factor. Differences in triglyceride and cholesterol levels measured before and after adenovirus injections were tested with a paired *t* test.

Results

Plasma lipids and lipoproteins in LDLR-deficient mice. We measured plasma lipid levels in each group of LDLR-deficient mice (Fig. 1). LDLR deficiency increased plasma cholesterol levels significantly, regardless of *Apob* genotype. However, the magnitude of this effect was greatest in the setting of the $Apob^{100}$ allele. The plasma cholesterol levels were lower in

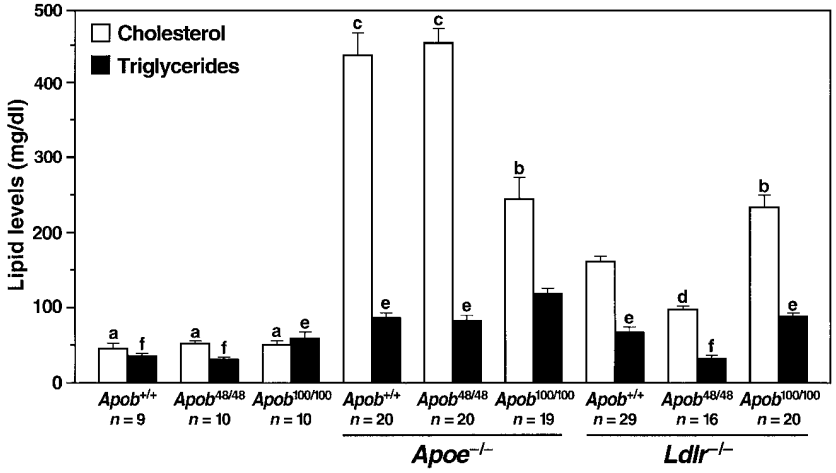

Figure 1. Bar graph comparing the plasma lipid levels from nine groups of mice. Mean plasma cholesterol and triglyceride levels were measured in chow-fed male mice after a 4-h fast. Error bars represent SEM. *n* represents the number of animals in each group. Statistical analyses were performed on both the cholesterol (*a–d*) and triglyceride (*e*, *f*) measurements. Bars not sharing the same letter are significantly different ($P < 0.001$).

$Ldlr^{-/-}Apob^{48/48}$ mice (101±5 mg/dl) and higher in $Ldlr^{-/-}Apob^{100/100}$ mice (229±13 mg/dl) than in $Ldlr^{-/-}Apob^{+/+}$ mice (159±8 mg/dl). The $Ldlr^{-/-}Apob^{100/100}$ mice had significantly higher levels of β-migrating lipoproteins and lower levels of pre-β-migrating and α-migrating lipoproteins than $Ldlr^{-/-}Apob^{48/48}$ and $Ldlr^{-/-}Apob^{+/+}$ mice (data not shown). Plasma triglyceride levels were significantly lower in the $Ldlr^{-/-}Apob^{48/48}$ mice than in $Ldlr^{-/-}Apob^{+/+}$ mice.

The plasma cholesterol levels in $Apoe^{-/-}Apob^{48/48}$ and $Apoe^{-/-}Apob^{+/+}$ mice were much higher than in any of the groups of LDLR-deficient mice (Fig. 1). However, the total plasma cholesterol levels were nearly identical in the $Apoe^{-/-}Apob^{100/100}$ and $Ldlr^{-/-}Apob^{100/100}$ mice (~ 230±15 mg/dl).

To assess the distribution of lipids within the plasma lipoproteins, we fractionated the plasma on an FPLC column (Fig. 2). The $Ldlr^{-/-}Apob^{100/100}$ mice had higher LDL cholesterol levels and lower HDL cholesterol levels than $Ldlr^{-/-}Apob^{+/+}$ mice. The LDL cholesterol levels were lower in $Ldlr^{-/-}Apob^{48/48}$ than in $Ldlr^{-/-}Apob^{+/+}$ mice. Interestingly, the height of the LDL cholesterol peak in the $Ldlr^{-/-}Apob^{48/48}$ mice was virtually identical to that in $Apob^{100/100}$ mice, which had normal LDLR activity. However, it is important to note that both the total plasma cholesterol levels (Fig. 1) and the height of the LDL cholesterol peak (Fig. 2 *A*) were significantly higher in $Ldlr^{-/-}Apob^{48/48}$ than in $Apob^{48/48}$ mice, indicating that LDLR deficiency raises LDL cholesterol levels even when apo-B100 synthesis is absent. In both $Ldlr^{-/-}Apob^{100/100}$ and $Ldlr^{-/-}Apob^{+/+}$ mice, as in human apo-B transgenic mice (6, 21, 22), a large fraction of the triglycerides was contained in the LDL fraction (Fig. 2 *B*).

None of the LDLR-deficient mice had large amounts of cholesterol or triglycerides in the VLDL fraction. Indeed, the VLDL cholesterol and triglyceride peaks in the LDLR-deficient mice did not differ significantly from those in mice that

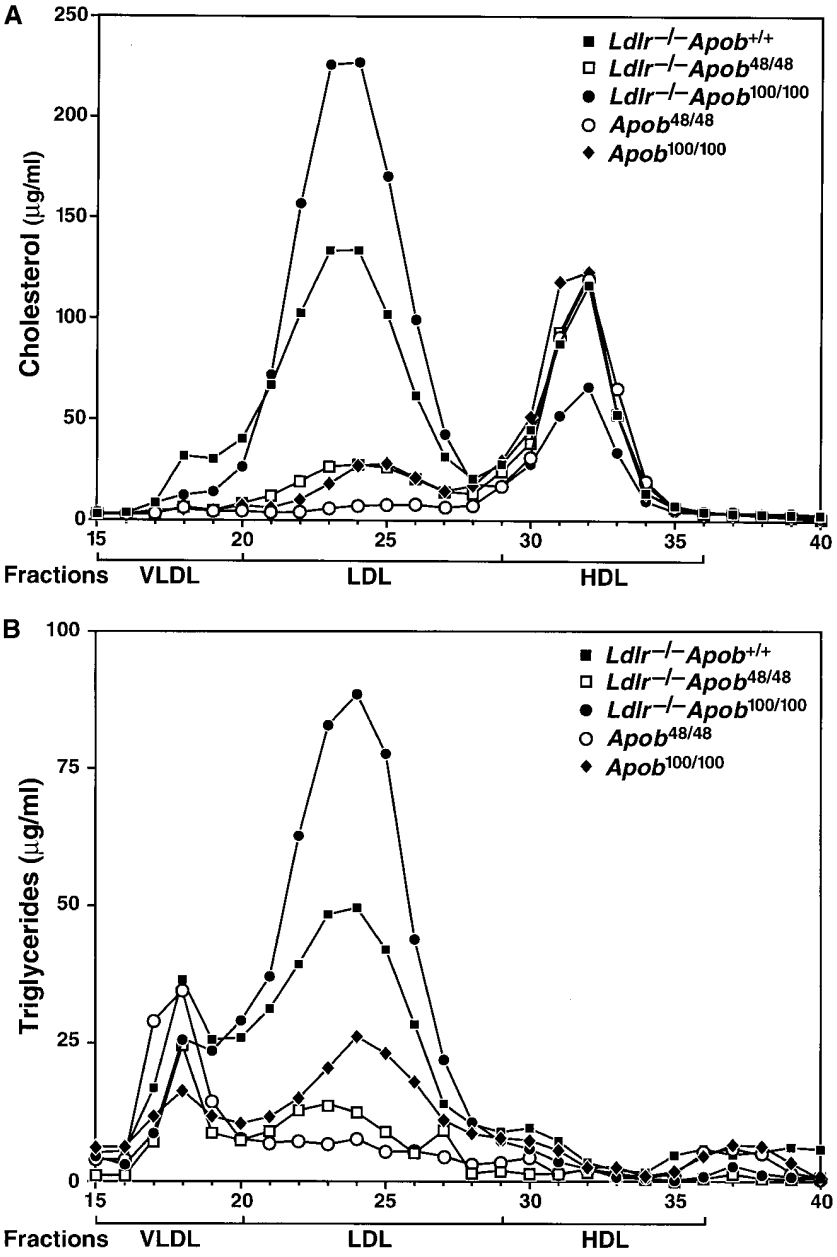

Figure 2. Distribution of lipids within different lipoprotein fractions, as assessed by FPLC. A total of 150 μl of mouse plasma (pooled from five male mice after a 4-h fast) was size-fractionated on an FPLC Superose 6 column, and the cholesterol and triglyceride contents of each fraction were measured with an enzymatic assay. *A* shows the distribution of cholesterol; *B* shows the distribution of triglycerides.

had normal levels of LDLR expression (Fig. 2). The relatively low levels of VLDL lipids is quite different from the situation in *Apoe*$^{-/-}$*Apob*$^{100/100}$, *Apoe*$^{-/-}$*Apob*$^{+/+}$, or *Apoe*$^{-/-}$*Apob*$^{48/48}$ mice. In the setting of apo-E deficiency, most of the lipids are within the VLDL, regardless of *Apob* genotype (14, 16).

Analysis of lipoprotein particle sizes. There were significant differences in lipoprotein size in the different groups of mice, particularly with respect to VLDL size. The VLDL from *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice were smaller than those from *Ldlr*$^{-/-}$*Apob*$^{+/+}$ and *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice (Table I). In the setting of apo-E deficiency, the results were quite different. The VLDL of *Apoe*$^{-/-}$*Apob*$^{+/+}$ and *Apoe*$^{-/-}$*Apob*$^{48/48}$ mice were smaller than those in *Apoe*$^{-/-}$*Apob*$^{100/100}$ mice. The LDL were larger in *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice than in *Ldlr*$^{-/-}$*Apob*$^{48/48}$ (24.4 versus 23.0 nm). No differences in HDL size were observed.

Apo-B levels in apo-E- and LDLR-deficient mice. To quantify the amount of apo-B100 in the plasma, we used mAb-based RIAs (Fig. 3 *A*) as well as Western blot analysis (Fig. 3 *B*). Relative levels of apo-B48 in different mouse plasma samples were assessed by Western blots. Relative to *Apob*$^{+/+}$ mice, the plasma apo-B100 levels in *Ldlr*$^{-/-}$*Apob*$^{+/+}$ and *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice were increased by five- and eightfold, respectively (Fig. 3 *A*). LDLR deficiency resulted in a significant increase in the plasma levels of apo-B48, although the increase was much smaller than that caused by apo-E deficiency (Fig. 3 *B*). Densitometric analysis of Western blots revealed that the plasma apo-B48 levels in *Ldlr*$^{-/-}$*Apob*$^{48/48}$ and *Apoe*$^{-/-}$*Apob*$^{48/48}$ mice were increased by 2.5-fold and fivefold, respectively, compared with those in *Apob*$^{48/48}$ mice.

The effect of apo-E deficiency on plasma apo-B100 levels was quite distinct from the changes caused by LDLR deficiency. Apo-B100 levels were 65% lower in *Apoe*$^{-/-}$*Apob*$^{+/+}$ mice than in *Apob*$^{+/+}$ mice and were consistently higher in *Apoe*$^{-/-}$*Apob*$^{100/100}$ than in *Apob*$^{100/100}$ mice. (Why apo-E deficiency produces a sharp ($\sim$ 65%) decrease in mouse apo-B100 levels in the setting of the *Apob*$^{+}$ allele but an increase ($\sim$ 165%) in apo-B100 levels in the setting of the *Apob*^{100} allele is not clear. We speculate that this difference may relate to the fact that the apo-B100 is produced by both the intestine and liver in the setting of the *Apob*^{100} allele but apo-B100 is produced only by the liver in the setting of the *Apob*$^{+}$ allele. In the setting of apo-E deficiency, it is possible that intestinal apo-B100–containing lipoproteins are cleared somewhat less efficiently by the LDLR than apo-B100–containing lipoproteins derived from the liver.)

Table I. Mean VLDL Particle Diameters in Nine Different Genotypes of Mice

Genotype	VLDL
Apob$^{+/+}$	62.3±19.0
Apob$^{48/48}$	62.7±22.8
Apob$^{100/100}$	64.3±24.5
Apoe$^{-/-}$*Apob*$^{+/+}$	41.1±11.0
Apoe$^{-/-}$*Apob*$^{48/48}$	39.2±11.0
Apoe$^{-/-}$*Apob*$^{100/100}$	61.7±18.5
Ldlr$^{-/-}$*Apob*$^{+/+}$	56.3±18.4
Ldlr$^{-/-}$*Apob*$^{48/48}$	59.4±21.1
Ldlr$^{-/-}$*Apob*$^{100/100}$	35.7±11.6

VLDL samples were prepared by ultracentrifugation from plasma pooled from five female mice from each group. The table shows population mean VLDL particle diameter (nm)±SD.

Metabolic effects of adenoviral-mediated overexpression of RAP in the liver. To assess the effect of the LRP on the metabolism of apo-B100–containing lipoproteins, we blocked hepatic LRP function in LDLR-deficient mice (both in *Ldlr*$^{-/-}$*Apob*$^{+/+}$ and *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice) and then determined whether the plasma apo-B100 levels were perturbed. To block LRP function, we used a recombinant adenovirus to overexpress RAP in the liver. We also defined the impact of RAP overexpression on plasma apo-B48 levels in both *Ldlr*$^{-/-}$*Apob*$^{+/+}$ and *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice. To be sure that RAP overexpression inhibited LRP function, we assessed the turnover of ^{125}I–α_2-macroglobulin in wild-type and *Ldlr*$^{-/-}$*Apob*$^{+/+}$ mice 5 d after the injection of Ad-RAP or Ad-β-Gal. Ad-RAP, but not Ad-β-Gal, dramatically retarded the clearance of α_2-macroglobulin in both groups of mice (data not shown).

To assess the impact of RAP overexpression on apo-B metabolism, we measured plasma apo-B levels in *Ldlr*$^{-/-}$*Apob*$^{+/+}$, *Ldlr*$^{-/-}$*Apob*$^{48/48}$, and *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice before and 5 d after the injection of the adenoviral preparations. Of note, overexpression of RAP had no effect on plasma apo-B100 levels in *Ldlr*$^{-/-}$*Apob*$^{+/+}$ or *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice, as assessed by a mAb-based RIA (Fig. 4 *A*) or by Western blot analysis (Fig. 4 *B*). These results in LDLR-deficient mice strongly suggest that blocking LRP function has little or no effect on the uptake and clearance of apo-B100–containing lipoproteins. The absence of a measurable effect of RAP overexpression on plasma apo-B100 levels stands in contrast to the effects on plasma apo-B48 levels. In both *Ldlr*$^{-/-}$*Apob*$^{+/+}$ and *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice, RAP overexpression caused a four- to fivefold increase in plasma apo-B48 levels (Fig. 4 *B*).

The plasma lipids and the distribution of lipids within the lipoprotein fractions were also assessed 5 d after the injection of Ad-β-Gal and Ad-RAP. Plasma lipid levels increased after the injection of both of the adenoviruses, although Ad-RAP had a much greater effect (Fig. 5, *A* and *B*). After the injection of Ad-β-Gal, the plasma cholesterol and triglyceride levels increased by $\sim$ 30% and $\sim$ 100%, respectively, and the magnitude of the changes was similar in *Ldlr*$^{-/-}$*Apob*$^{+/+}$, *Ldlr*$^{-/-}$*Apob*$^{48/48}$, and *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice (Fig. 5, *A* and *B*). After injection of Ad-RAP, plasma cholesterol levels increased by 800% in the *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice, 420% in the *Ldlr*$^{-/-}$*Apob*$^{+/+}$ mice, and only 210% in the *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice (Fig. 5 *A*). Triglyceride levels increased by 1500% in the *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice, 659% in the *Ldlr*$^{-/-}$*Apob*$^{+/+}$ mice, and 545% in the *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice (Fig. 5 *B*). Although the percentage of changes in plasma lipid levels in *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice and *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice were quite different, it is important to remember that plasma apo-B levels increased by more than fourfold in the *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice while they were unchanged in *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice. Thus, relative to the number of apo-B–containing lipoproteins in the plasma (see Fig. 4), the hyperlipidemic effect of RAP expression was likely no greater in *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice than in *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice.

The fact that overexpression of RAP increased plasma lipid levels in the *Ldlr*$^{-/-}$*Apob*$^{100/100}$ mice in the absence of any effect on plasma apo-B100 levels strongly suggested that RAP had the effect of increasing lipoprotein particle size. To gauge the size of lipoproteins in the plasma, we used FPLC fractionation studies to assess the distribution of lipids in the plasma

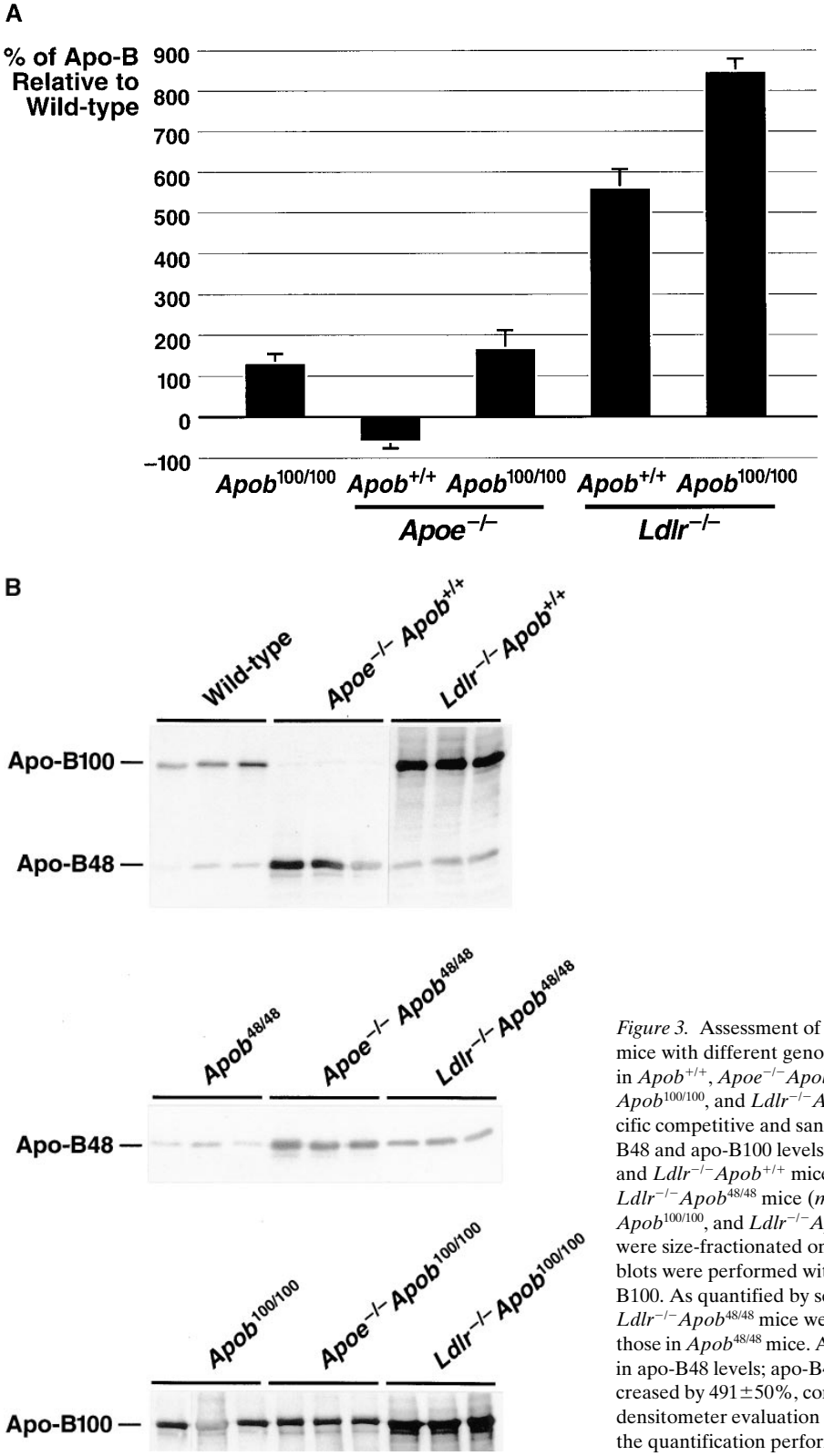

Figure 3. Assessment of apo-B48 and apo-B100 levels in the plasma of mice with different genotypes. (*A*) Quantification of apo-B100 levels in $Apob^{+/+}$, $Apoe^{-/-}Apob^{+/+}$, $Ldlr^{-/-}Apob^{+/+}$, $Apob^{100/100}$, $Apoe^{-/-}Apob^{100/100}$, and $Ldlr^{-/-}Apob^{100/100}$ mice, as judged by apo-B100–specific competitive and sandwich RIAs. (*B*) Western blot analysis of apo-B48 and apo-B100 levels in the plasma of $Apob^{+/+}$, $Apoe^{-/-}Apob^{+/+}$, and $Ldlr^{-/-}Apob^{+/+}$ mice (*top panel*); $Apob^{48/48}$, $Apoe^{-/-}Apob^{48/48}$, $Ldlr^{-/-}Apob^{48/48}$ mice (*middle panel*); and $Apob^{100/100}$, $Apoe^{-/-}Apob^{100/100}$, and $Ldlr^{-/-}Apob^{100/100}$ mice (*bottom panel*). Plasma samples were size-fractionated on a 4% polyacrylamide/SDS gel, and Western blots were performed with a rabbit antiserum specific for mouse apo-B100. As quantified by scanning densitometer, the apo-B48 levels in $Ldlr^{-/-}Apob^{48/48}$ mice were increased by 278±39% compared with those in $Apob^{48/48}$ mice. Apo-E deficiency resulted in a striking increase in apo-B48 levels; apo-B48 levels in $Apoe^{-/-}Apob^{48/48}$ mice were increased by 491±50%, compared with those in $Apob^{48/48}$ mice. Scanning densitometer evaluation of the apo-B100 bands were in agreement with the quantification performed by RIA.

5 d after Ad-RAP administration. There was a striking increase in VLDL cholesterol in the $Ldlr^{-/-}Apob^{48/48}$, $Ldlr^{-/-}Apob^{+/+}$, and $Ldlr^{-/-}Apob^{100/100}$ mice after Ad-RAP injection (Fig. 6, *C* and *D*; compare with the baseline distribution of lipids in Fig. 2). After the injection of Ad-β-Gal, there were increased lipids in the VLDL fraction (Fig. 6, *A* and *B*), although the magnitude of this effect was less than that observed after Ad-RAP.

In addition to the FPLC fractionation studies, we also assessed VLDL particle diameter in $Ldlr^{-/-}Apob^{48/48}$ and $Ldlr^{-/-}$

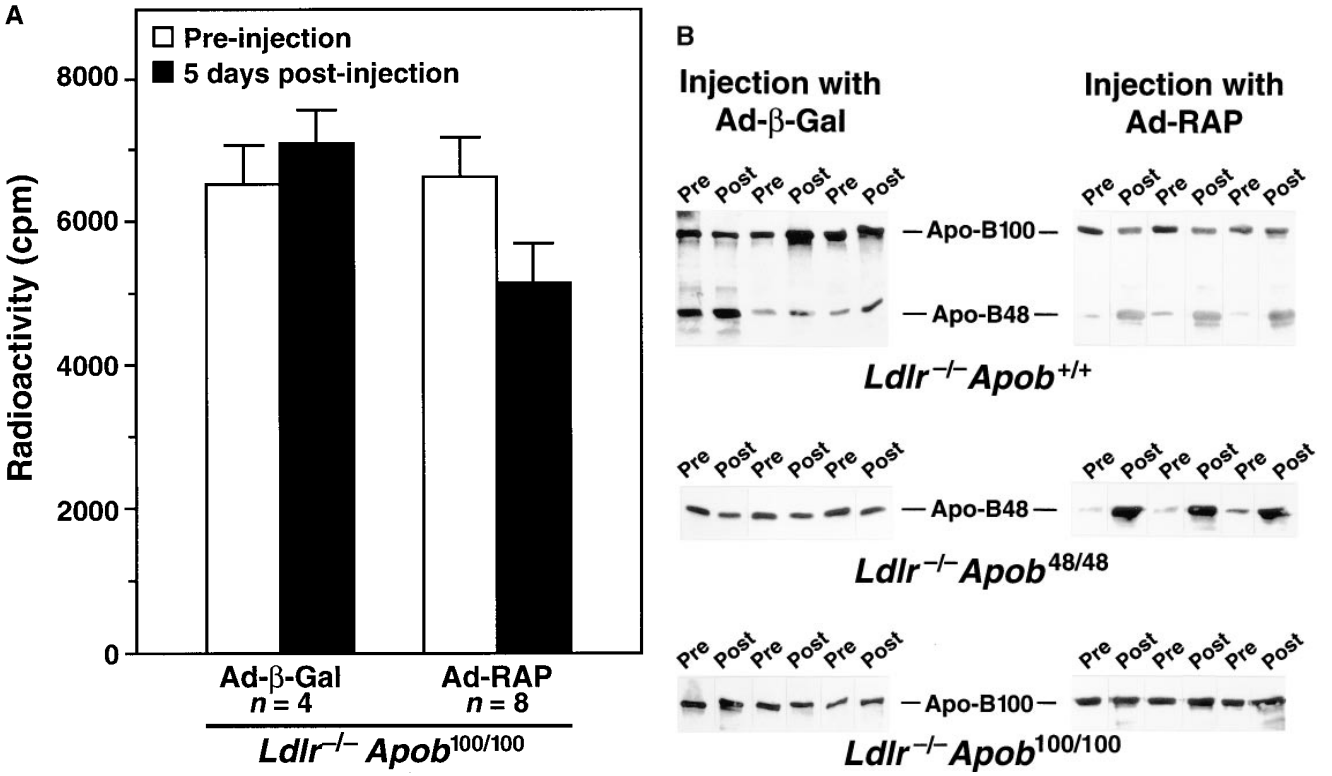

Figure 4. Comparisons of plasma apo-B levels before and 5 d after intravenous injection of either Ad-β-Gal or Ad-RAP. (*A*) Comparison of apo-B100 levels in $Ldlr^{-/-}Apob^{100/100}$ before and after injection of Ad-β-Gal or Ad-RAP as determined by a mAb-based sandwich RIA. Data are expressed as cpm bound in the RIA when 0.5 μl of plasma was added to the well. Identical findings were noted when 2.5 μl of plasma was added to each well. (*B*) Western blot analysis of plasma apo-B levels in $Ldlr^{-/-}Apob^{+/+}$, $Ldlr^{-/-}Apob^{48/48}$, and $Ldlr^{-/-}Apob^{100/100}$ mice, both before and after injection of either Ad-β-Gal (*left panel*) or Ad-RAP (*right panel*). As judged by densitometry, overexpression of RAP had no effect on plasma apo-B100 levels in $Ldlr^{-/-}Apob^{+/+}$ (9783±980 arbitrary units [AU] before injection of Ad-RAP; 9400±432 AU after injection of Ad-RAP) or $Ldlr^{-/-}Apob^{100/100}$ mice (10930±951 AU before and 11878±1133 AU after injection of Ad-RAP). In $Ldlr^{-/-}Apob^{+/+}$ mice, apo-B48 levels were 901±255 AU before and 4171±442 AU after injection; in $Ldlr^{-/-}Apob^{48/48}$ mice, apo-B48 levels were 1133±222 AU before and 5455±693 AU after injection.

$Apob^{100/100}$ mice after Ad-RAP injection. The mean VLDL diameter increased by 36% to 81 nm in $Ldlr^{-/-}Apob^{48/48}$ mice and by 34% to 50 nm in the $Ldlr^{-/-}Apob^{100/100}$ mice (baseline sizes are shown in Table I). Thus, the percentage increase in VLDL diameter with Ad-RAP was similar for apo-B48– and apo-B100–containing lipoproteins.

Apo-B48 and apo-B100 levels after expression of RAP in wild-type and $Apob^{48/48}$ mice. In addition to assessing the effects of RAP in LDLR-deficient mice, we determined its effect on apo-B100 and apo-B48 levels in wild-type mice (Fig. 7). In those mice, we suspected that RAP overexpression would result in increased plasma apo-B100 levels, since high levels of RAP expression interfere with the binding of lipoproteins to the LDLR, at least to a small extent (23, 24). This suspicion was confirmed. In wild-type mice, RAP overexpression increased plasma apo-B100 levels twofold (Fig. 7). The effects of RAP overexpression on the apo-B48 levels in wild-type mice were more intriguing because they shed light on the issue of whether the clearance of apo-B48–containing lipoproteins normally depends largely or entirely on the LDLR or depends on *both* the LDLR and the LRP. If the clearance of apo-B48–containing lipoproteins in wild-type mice were entirely or largely due to the LDLR, one would expect to observe an ~ twofold elevation in plasma apo-B48 levels, similar in magnitude to the increase in plasma apo-B100 levels. This was not the case; RAP overexpression in wild-type mice increased apo-B48 levels sixfold. Finding a much greater increase in plasma apo-B48 levels strongly suggests that the LRP has a substantial role in the clearance of apo-B48–containing lipoproteins, even in the setting of normal levels of LDLR expression. Consistent with these results, we observed a sevenfold increase in plasma apo-B48 levels in $Apob^{48/48}$ mice after the injection of Ad-RAP.

Discussion

The role of the LDLR in removing apo-B100–containing lipoproteins from the plasma is well established (25), and recent studies have implicated the LRP in the clearance of apo-B48–containing lipoproteins in animals that are deficient in the LDL receptor (12, 13, 26). In the current study, we sought to define whether the LRP can play a role in removing apo-B100–containing lipoproteins from the plasma and whether the LDLR has a significant role in the clearance of apo-B48–containing lipoproteins. To examine these issues, we used adenoviral-mediated overexpression of RAP to block LRP function in LDLR-deficient mice that synthesized exclusively apo-B48 or apo-B100. The use of "apo-B48-only" and "apo-

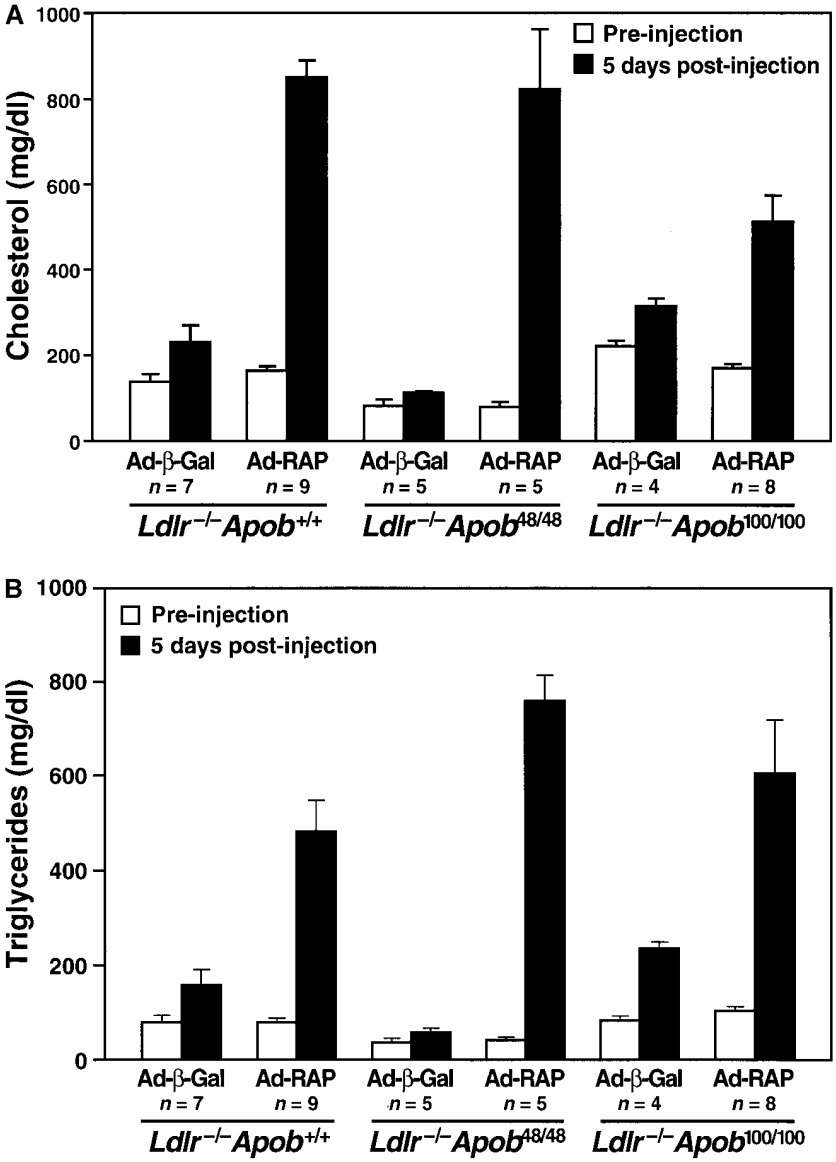

Figure 5. Total plasma (*A*) cholesterol and (*B*) triglyceride levels measured both before and 5 d after intravenous injection of Ad-β-Gal or Ad-RAP into $Ldlr^{-/-}Apob^{+/+}$, $Ldlr^{-/-}Apob^{48/48}$, and $Ldlr^{-/-}Apob^{100/100}$ mice. Error bars represent SEM. *n* indicates the number of mice in each group.

B100-only" mice allowed us to examine lipoprotein metabolism in a less complex setting, one where the normal competition between apo-B48– and apo-B100–containing lipoproteins for metabolic processing and uptake (27) is nonexistent. Our experiments described here have clarified the roles of both the LRP and the LDLR by showing that the LRP has little if any capacity to remove apo-B100–containing lipoproteins from the plasma and that the LDLR plays a significant role in the clearance of apo-B48–containing lipoproteins.

If the LRP played a significant role in removing apo-B100–containing lipoproteins from the plasma, we reasoned that blocking LRP function with RAP would cause significantly increased plasma levels of the apo-B100–containing lipoproteins. Our experiments demonstrated that this did not occur. As judged by Western blots as well as mAb-based RIAs, RAP overexpression did not affect the plasma levels of apo-B100 in $Ldlr^{-/-}Apob^{100/100}$ or $Ldlr^{-/-}Apob^{+/+}$ mice. This finding is especially noteworthy, given the fact that the apo-B–containing lipoproteins in the RAP-expressing mice were large and buoyant. In contrast, RAP overexpression increased apo-B48 levels fivefold in both $Ldlr^{-/-}Apob^{48/48}$ and $Ldlr^{-/-}Apob^{+/+}$ mice. The striking effects on plasma apo-B48 levels, along with the absence of an effect on apo-B100 levels, indicate that the LRP is, from the perspective of lipoprotein metabolism, a receptor only for the apo-B48–containing lipoproteins.

The uptake of apo-B–containing remnant lipoproteins by the LRP is mediated by apo-E (28). Interestingly, the amount of apo-E on circulating remnant lipoproteins may be insufficient for uptake by the LRP; rather, these lipoproteins appear to require a "supplemental dose" of apo-E before uptake occurs (29). The laboratory of Linton and Fazio reported data suggesting that the apo-E "supplement" must be provided by hepatocytes (30). They found that apo-E–rich remnant lipoproteins were readily taken up (as a result of the LRP) by hepatocytes that were deficient in the LDLR but not by hepatocytes that lacked *both* the LDLR and apo-E. These experiments suggested that the uptake of apo-E–containing remnant lipoproteins by the LRP is largely or completely dependent on the ability of hepatocytes to synthesize and secrete apo-E (30). These data led to an intriguing hypothesis: that the apo-E se-

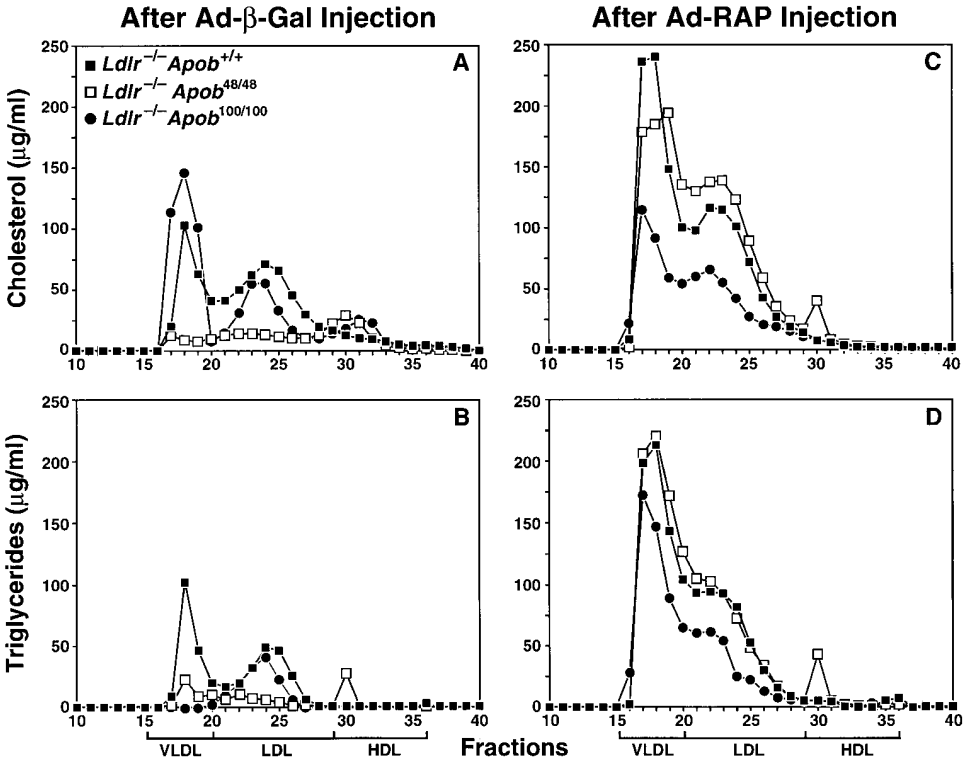

Figure 6. Distribution of plasma lipids 5 d after injection of Ad-β-Gal or Ad-RAP. A total of 150 μl of plasma (pooled from five mice in each group) was size-fractionated on an FPLC column. *A* and *B* show lipid distributions after Ad-β-Gal injection; *C* and *D* show lipid distributions after Ad-RAP injection. Cholesterol distributions are shown in *A* and *C*; triglyceride distributions are shown in *B* and *D*. As illustrated by these FPLC profiles, Ad-RAP had a more potent hyperlipidemic effect than Ad-β-Gal. In addition, Ad-RAP had a more potent HDL-lowering effect than Ad-β-Gal.

creted by hepatocytes initially binds to the LRP, and the *bound* apo-E subsequently attaches to remnant lipoproteins. Remnant lipoproteins supplemented with apo-E in this fashion would then be internalized by the LRP. Alternatively, the apo-E secreted by hepatocytes into the space of Disse may be captured by proteoglycans and then facilitate the LRP-mediated uptake of lipoproteins (31). However, regardless of how "apo-E supplementation" occurs, our data indicate that the LRP is active only in the uptake of apo-B48–containing lipoproteins. We suggest that the presence of the carboxyl terminus of apo-B100 (amino acids 2153–4536) on the surface of the lipoprotein prevents the lipoprotein particle from binding a sufficient dose of "supplemental" apo-E, thereby preventing its uptake via the LRP.

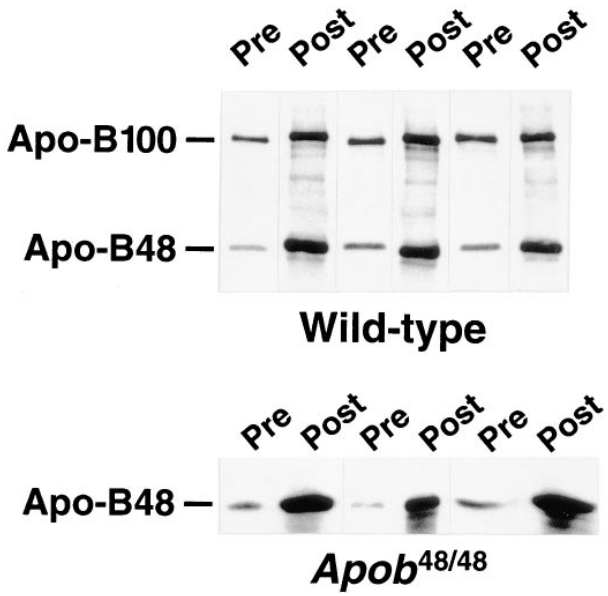

Figure 7. Western blot analysis of plasma apo-B levels in wild-type and *Apob*$^{48/48}$ mice, both before and after injection of Ad-RAP. In wild-type mice, apo-B100 levels increased approximately twofold after injection of Ad-RAP (5414±938 AU before and 12765±2062 AU after injection). In wild-type mice, apo-B48 levels increased sixfold after injection of Ad-RAP (2752±1749 AU before and 16994±3029 AU, after injection); in *Apob*$^{48/48}$ mice, apo-B48 levels increased sevenfold after injection of Ad-RAP (2601±587 AU before and 19295±6500 AU, after injection).

The studies of Willnow et al. (12), Ishibashi et al. (11, 32), and Rohlmann et al. (13) highlighted the role of the LRP in the uptake of apo-B48–containing lipoproteins in LDLR-deficient mice. However, the issue of whether the LDLR plays a significant role in the uptake of apo-B48–containing lipoproteins has never been addressed definitively in in vivo models. Our studies with *Ldlr*$^{-/-}$*Apob*$^{48/48}$ and *Apob*$^{48/48}$ mice provide clear insights into this issue. The *Ldlr*$^{-/-}$*Apob*$^{48/48}$ mice did not have severe hypercholesterolemia, but they had two- to threefold higher plasma apo-B48 levels, higher total cholesterol levels, and much higher LDL cholesterol levels than the *Apob*$^{48/48}$ mice. These observations indicate that the LDLR normally plays a significant role in the clearance of apo-B48–containing lipoproteins.

The fact that the LDLR has an easily detectable role in the clearance of apo-B48–containing lipoproteins raises the question of whether the LDLR, under normal circumstances, clears most or all of the apo-B48–containing lipoproteins from the plasma. Our data would argue that it does not; rather, we believe that the LRP has a key role in the clearance of apo-B48–containing lipoproteins, even with physiologically normal levels of LDLR expression. First, in the setting of homozygosity for the *Apob*^{48} allele, we documented that apo-E deficiency caused a far greater increase in plasma apo-B48 levels than LDLR deficiency, suggesting that a significant fraction of

apo-B48–lipoproteins is cleared from the plasma via a second receptor that recognizes apo-E (i.e., the LRP). Second, in wild-type mice, adenoviral-mediated overexpression of RAP produced a far greater increase in apo-B48 levels than in apo-B100 levels. Both observations strongly suggest that the LRP plays a fundamental role in the clearance of apo-B48–containing lipoproteins, even with normal LDLR expression, and that it is not simply a "back-up" for the LDLR. A primary role for the LRP in lipoprotein uptake is also supported by the recent studies of Rohlmann et al. (13). They inactivated the LRP gene in the liver and documented a twofold increase in hepatic LDLR expression. That observation suggests that the LRP is normally involved in lipoprotein uptake and that eliminating LRP-mediated uptake results in an upregulation of LDLR expression.

Our measurements of VLDL size provided intriguing insights into the metabolism of apo-B48 and apo-B100. The mean VLDL particle diameter was ~ 40% smaller in $Apoe^{-/-}Apob^{48/48}$ mice than in $Apob^{48/48}$ or $Apoe^{-/-}Apob^{100/100}$ mice. Similarly, the mean VLDL particle diameter was ~ 40% smaller in $Ldlr^{-/-}Apob^{100/100}$ mice than in $Apoe^{-/-}Apob^{100/100}$ mice. These reductions correspond to more than a 2.5-fold reduction in particle volume. What is the likely explanation for these findings? We believe that VLDL are small in $Apoe^{-/-}Apob^{48/48}$ mice because defective clearance by the LDLR and LRP prolongs their circulation time, leading to a more extensive hydrolysis of core lipids. Similarly, we suspect that the small size of VLDL in $Ldlr^{-/-}Apob^{100/100}$ mice is due to absent LDLR uptake and virtually nonexistent LRP uptake, leading to more complete removal of core lipids. To our knowledge, these data are the first to raise the possibility that the efficiency of receptor-mediated uptake could have striking influences on the size of circulating VLDL.

In the prior studies by Willnow and coworkers (12) and in our current studies, adenoviral expression of RAP in the setting of LDLR deficiency resulted in turbid plasma and grossly elevated plasma cholesterol and triglyceride levels. The hyperlipidemia observed after Ad-RAP injection was much more severe than that in mice lacking LRP gene expression in the liver (13). Our experiments indicate that the Ad-RAP hyperlipidemia can be resolved into two components. In the case of $Ldlr^{-/-}Apob^{48/48}$ and $Ldlr^{-/-}Apob^{+/+}$ mice, one component results from a profound accumulation of apo-B48–containing lipoproteins. However, the Ad-RAP hyperlipidemia also involves a second component, inasmuch as the $Ldlr^{-/-}Apob^{100/100}$ mice developed hypercholesterolemia and hypertriglyceridemia without an increase in apo-B100 levels. The second component leads to the appearance of larger and more buoyant lipoproteins in the plasma, perhaps as a result of altered lipolytic processing of lipoproteins. At least in part, the larger lipoproteins could relate to the viral infection itself. In our experiments, and in those of Tsukamoto (33), the injection of Ad-β-Gal led to a shift toward larger lipoproteins (more VLDL on the FPLC fractionation studies), although the magnitude of this effect was less than that observed with Ad-RAP.

In their original description of the LDLR knockout mice, Ishibashi et al. (15) noted that the hypercholesterolemia was only mild to moderate, in contrast to the severe hypercholesterolemia that occurs in LDLR-deficient humans. They reasoned that the mild phenotype in the mouse probably relates to the ability of mice to synthesize apo-B48–containing lipoproteins in the liver. In our studies, we have demonstrated that their reasoning was correct: the $Ldlr^{-/-}Apob^{100/100}$ mice had much higher LDL cholesterol levels than the $Ldlr^{-/-}Apob^{+/+}$ mice. Another difference between the $Ldlr^{-/-}Apob^{100/100}$ and $Ldlr^{-/-}Apob^{+/+}$ mice also deserves underscoring. The mild hypercholesterolemia in $Ldlr^{-/-}Apob^{+/+}$ mice prevents them from developing significant atherosclerosis when fed a low-fat, chow diet (34). Although we have not yet performed systematic studies of atherosclerosis in the three groups of LDLR-deficient mice described in this paper, we have noted very severe atherosclerosis throughout the arterial tree in a few $Ldlr^{-/-}Apob^{100/100}$ mice that were maintained on a low-fat chow diet for 10 mo (M. Véniant and S. Young, unpublished observations). Moreover, we have noted severe atherosclerosis in a large number of chow-fed $Ldlr^{-/-}Apobec1^{-/-}$ mice, which also synthesize exclusively apo-B100 (35). We suspect that the $Ldlr^{-/-}Apob^{100/100}$ and $Ldlr^{-/-}Apobec1^{-/-}$ mice will be very attractive as experimental models for atherosclerosis studies. Like most humans with atherosclerosis, these animals have high plasma levels of apo-B100–containing, cholesterol-rich LDL.

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