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An Extrahepatic Receptor-associated Protein-sensitive Mechanism Is Involved in the Metabolism of Triglyceride-rich Lipoproteins*

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We have used adenovirus-mediated gene transfer in mice to investigate low density lipoprotein receptor (LDLR) and LDLR-related protein (LRP)-independent mechanisms that control the metabolism of chylomicron and very low density lipoprotein (VLDL) remnants *in vivo*. Overexpression of receptor-associated protein (RAP) in mice that lack both LRP and LDLR (MX1cre^{+}LRP$^{flox/flox}$LDLR$^{-/-}$) in their livers elicited a marked hypertriglyceridemia in addition to the pre-existing hypercholesterolemia in these animals, resulting in a shift in the distribution of plasma lipids from LDL-sized lipoproteins to large VLDL-sized particles. This dramatic increase in plasma lipids was not due to a RAP-mediated inhibition of a unknown hepatic high affinity binding site involved in lipoprotein metabolism, because no RAP binding could be detected in livers of MX1cre^{+}LRP$^{flox/flox}$LDLR$^{-/-}$ mice using both membrane binding studies and ligand blotting experiments. Remarkably, RAP overexpression also resulted in a 7-fold increase (from 13.6 to 95.6 ng/ml) of circulating, but largely inactive, lipoprotein lipase (LPL). In contrast, plasma hepatic lipase levels and activity were unaffected. *In vitro* studies showed that RAP binds to LPL with high affinity (K_d = 5 nM) but does not affect its catalytic activity, *in vitro* or *in vivo*. Our findings suggest that an extrahepatic RAP-sensitive process that is independent of the LDLR or LRP is involved in metabolism of triglyceride-rich lipoproteins. There, RAP may affect the functional maturation of LPL, thus causing the accumulation of triglyceride-rich lipoproteins in the circulation.

Hypertriglyceridemia, combined with the accumulation of remnant lipoproteins in the circulation, is a major risk factor for atherosclerosis and coronary artery disease. The genetic bases of this clinically important syndrome are complex and incompletely understood. Two endocytotic receptor systems are known to remove the lipolyzed remnants of chylomicrons and very low density lipoproteins (VLDL)[1] from the circulation. They are the low density lipoprotein (LDL) receptor and the LDL receptor-related protein (LRP) (1, 2). Following lipolysis in the peripheral capillaries of muscle, heart, and adipose tissue, where chylomicrons deliver most of the triglyceride load they carry, the remnants have shrunk to a size at which they can permeate the fenestrated endothelium separating the hepatocyte surface and the space of Disse from the circulation (for review see Ref. 3). LRP and LDL receptors at the surface of hepatocytes bind and clear remnant lipoproteins from an intermediate binding site. This intermediate compartment is created by interactions of heparan sulfate proteoglycans (4), hepatic lipase (5, 6), lipoprotein lipase (7), and apoE (8) with the remnants.

Gene knockout and gene transfer experiments in mice have defined the roles of the receptors, apoproteins, and lipases in the remnant clearance process. Although the LDL receptor efficiently removes apoB100-containing LDL, as well as apoB48- containing remnants through interaction with apoE (9, 10), from the bloodstream, LRP binds B48-containing remnants exclusively through apoE (11–13).

The LRP receptor-associated protein (RAP), a specialized chaperone that is required for biosynthesis of LRP, blocks the binding function of this receptor *in vitro* and *in vivo* and has been successfully used to transiently inactivate LRP in adult mice (14). These experiments have revealed a physiological role of the LDL receptor and LRP in remnant removal. In the absence of functional LDL receptor in knockout mice, inhibition of LRP by adenovirus-mediated gene transfer and overexpression of RAP resulted in the accumulation of large, triglyceride- and cholesterol-rich apoB48-containing remnants.

In another gene knockout model generated in mice, LRP has recently been inactivated by inducible tissue-specific techniques using the Cre-lox recombination system (2). By this approach, it was possible to circumvent the early embryonic lethal phenotype caused by conventional gene disruption of LRP (15, 16). LRP inactivation was initiated in adult mice following interferon induction, which in turn led to expression of the cre recombinase from the interferon inducible MX1 promoter (17). Recombination of the loxP flanked (floxed) LRP gene was essentially complete in hepatocytes and other cell types exposed to the circulating interferons.

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[1] The abbreviations used in this paper: VLDL, very low density lipoproteins; LDL, low density lipoproteins; apo, apolipoprotein; LDLR, LDL receptor; LRP, LDL receptor-related protein; RAP, receptor-associated protein; Ad, adenoviral vector; LPL, lipoprotein lipase; HL, hepatic lipase; PFU, plaque-forming units; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; β-Gal, β-galactosidase.

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As in the RAP overexpression experiments (14), LRP gene disruption in LDL receptor-deficient mice did cause the accumulation of cholesterol-rich, apoB48-containing remnants. However, these remnants were smaller and contained significantly less triglyceride than those that accumulated in the RAP overexpressing animals. These observations suggest that a novel and hitherto unsuspected RAP-sensitive process is involved in the metabolism of triglyceride-rich lipoproteins. This could involve other RAP-sensitive receptors, such as the hypothetical lipolysis stimulated receptor (18), or a direct or indirect effect of RAP on lipase-mediated conversion of chylomicrons to the smaller remnants.

In this study we have addressed this question by measuring the effect of RAP overexpression on remnant removal in animals in which the LDL receptor, LRP, or both proteins had been inactivated. We have also investigated the presence of other potential RAP-binding sites in LRP and LDL receptor-deficient mouse liver membranes. Furthermore, we have determined the protein mass and catalytic activity of hepatic lipase and lipoprotein lipase in RAP overexpressing mice. Our results suggest that RAP affects the conversion of large triglyceride-rich chylomicrons to smaller remnants by interfering with LPL activation in the periphery and rule out the contribution of other major RAP-binding proteins in the liver. The biochemical basis underlying this process may play a role in some of the complex genetic traits that cause hypertriglyceridemia in man.

EXPERIMENTAL PROCEDURES

Transgenic Animals—Mice in which the LRP alleles have been altered by introduction of loxP sites ($LRP^{flox/flox}$) were generated by homologous recombination of the LRP allele in embryonic stem cells and have been described previously (19). Mice transgenic for the MX1cre expression construct were generated by pronuclear injection of hybrid (SJLxC57BL/6J) mice (2). LDL receptor-deficient ($LDLR^{-/-}$) mice were generated by homologous recombination of the LDLR allele in embryonic stem cells and have been described previously (10). Six genetically distinct strains of animals were used: mice that were wild type at both LRP loci, deficient for the LDL receptor ($LDLR^{-/-}$), homozygous for the floxed LRP allele ($LRP^{flox/flox}$), homozygous for both mutations ($LRP^{flox/}{}_{flox}LDLR^{-/-}$), homozygous for the floxed LRP and transgenic for the MX1cre transgene ($MX1cre^{+}LRP^{flox/flox}$), and homozygous for the floxed LRP allele, deficient for the LDL receptor, and transgenic for the MX1cre transgene ($MX1cre^{+}LRP^{flox/flox}LDLR^{-/-}$). Induction with polyinosinic:polycytidylic ribonucleic acid (pI:pC; Sigma) was done by intraperitoneal injection of 250 μg of a 1 mg/ml solution of pI:pC in water. Injections were repeated three times at 2-day intervals. For experiments, mice 10–16 weeks of age were included. Mice were housed under standard conditions in conventional cages and given free access to food (*i.e.* regular rodent chow containing 6% fat (Teklad Premier laboratory Diets, Madison, WI) and water.

Adenovirus Transfections—Recombinant adenoviruses containing the rat RAP cDNA (Ad-RAP) and β-galactosidase cDNA (Ad-β-Gal) driven by the cytomegalovirus promoter were generated, grown, and purified as described previously (14). For *in vivo* adenovirus transfection, four weeks after the last pI:pC injection, 2.0×10^9 plaque-forming units (PFU) in a total volume of 200 μl (diluted with PBS) were injected into the tail vein. Blood samples were drawn from the retro-orbital plexus before and 5 days after virus injection.

Lipid and (Apo)lipoprotein Measurements—From each individual adenovirus-injected mouse, approximately 150 μl of blood was obtained through retro-orbital bleeding. Total plasma cholesterol and triglyceride levels were measured enzymatically using assay kits from Roche Molecular Biochemicals and Sigma, respectively.

For determination of the plasma lipoprotein distribution, 60 μl of pooled plasma was analyzed by fast performance liquid chromatography on a Superose 6 column (Sigma), and the cholesterol content of each fraction was determined spectrofluorometrically as described previously (10). Pooled plasma before and 5 days after virus infection was analyzed by immunoblotting with polyclonal antibodies against mouse apoB, apoE, and apoAI (14, 20). Bound IgG was detected using enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

Binding of ^{125}I-Labeled RAP to Membranes—Glutathione *S*-transferase-RAP was grown and purified as described (21). Approximately 100 μg of RAP was radiolabeled with ^{125}I using the Iodogen method as described previously (22). Preparation of mouse liver membranes and membrane binding studies were performed as described (12). Briefly, livers of individual mice were removed and quickly transferred to 5 ml of an ice-cold solution of 0.25 M sucrose, 0.1 M Tris, and protease inhibitors (Roche Molecular Biochemicals), pH 7.4. The livers were homogenized five times using a tight fitting pestle and then centrifuged at 500 × *g* for 10 min to pellet cell debris. The supernatant was respun at 10,000 × *g* for 15 min. to remove nuclear material. The second supernatant was centrifuged at 100,000 × *g* for 30 min. The resulting membrane containing pellet was resuspended in 0.5 ml of Tris-buffered saline in the presence of the protease inhibitor mixture, using a 21 gauge needle. The membrane fraction was stored for up to 1 week in the dark at 4 °C.

For measurement of ^{125}I-RAP binding, membranes were diluted to a concentration of 1 mg of protein/ml with incubation buffer (Tris-buffered saline, containing 2 mg/ml albumin and protease inhibitor mixture). Immediately prior to use, this fraction was sonicated (Bioblock Scientific Vibracell, 30 s, power 2.5, 25% pulse). 100 μg of membranes was incubated overnight at 4 °C with increasing amounts of ^{125}I-RAP either in the presence or in the absence of a 100 μg/ml excess of unlabeled RAP (final incubation volume, 250 μl; $n = 4$). Membrane bound ^{125}I-RAP was separated from unbound RAP by layering a 200-μl aliquot over 600 μl of 5% (w/v) BSA and centrifuging at 14,000 × *g* for 25 min at 4 °C. The supernatant was carefully removed, and the bottoms of the vials were cut and counted to measure the amount of membrane bound ^{125}I-RAP.

Ligand Blotting—Membranes were prepared from mouse livers as described above, and proteins separated by nondenaturating, nonreducing SDS gel electrophoresis on 4–15% polyacrylamide gels (50 μg protein/lane). After separation, the proteins were transferred to nitrocellulose. The nitrocellulose membranes were blocked for 30 min at room temperature in PBS containing, 0.5% Tween, 2% BSA, and 5% powdered milk, pH 7.4, followed by incubation for 60 min at room temperature with 10 μg/ml peroxidase-conjugated RAP in PBS in blocking buffer either in absence or presence of an excess of nonconjugated RAP (100 μg/ml). The nitrocellulose membranes were washed three times with PBS containing 0.5% Tween and 2% deoxycholic acid with buffer changes each 5 min. Bound peroxidase-labeled RAP was detected using the ECL system.

The presence of hepatic LRP was detected using a similar method and by incubating nitrocellulose membranes with a polyclonal rabbit antibody against LRP. Bound IgG was detected as described above for apolipoproteins.

In Vivo Hepatic VLDL-Triglyceride Production—After a 5 h fasting period, mice were anesthetized by intraperitoneal injection of Nembutal (80 μg/g body weight). Mice were injected intravenously with Triton WR1339 (500 mg/kg body weight) using 15% (w/v) Triton solution in 0.9% NaCl (23). At 1, 15, and 30 min after injection, blood samples were drawn from the tail vein and analyzed for triglycerides as described above.

Assay of Lipoprotein Lipase and Hepatic Lipase Mass in Mouse Plasma—Hepatic lipase mass was measured by ELISA developed for rat HL (24). Mouse LPL was also measured by a sandwich ELISA. A full-length mouse LPL cDNA kindly provided by Michael Schotz (25) was subcloned into pQE32 vector for expression in bacteria. The His_6 LPL protein was used to generate antibodies in a goat and to construct a column of mouse LPL Affi-Prep 10 for affinity purification of the antibodies. The conditions for the assay were the same as those described for chicken LPL ELISA (26) with the following exceptions. The initial incubation of samples with the capture antibodies coated on microtiter plates was conducted at 4 °C in 0.8 M NaCl, 1% bovine serum albumin, 0.05% Tween-20, 10 mM sodium phosphate, pH 7.4. The standard curves ranged from 0.05 to 1.8 ng/well. At 1 ng/well the reading was 0.230 optical density units, and the correlation coefficient was larger than 0.99. Catalytic activity of LPL and HL in post-heparin plasma were determined as described below on 20 μl of plasma.

Solid Phase Assay of Interaction of RAP and LPL or HL—To preserve the integrity of the lipases, all steps were conducted at 4 °C. Microtiter plates (Corning) were coated with highly purified lipases (27, 28), 10 ng/well of avian LPL or rat HL overnight. Control wells were coated with nothing or an irrelevant protein (carbonic anhydrase (Sigma)). After washing three times with PBS/0.05% Tween-20 (Sigma), plates were blocked overnight with 3% BSA/PBS/0.05% Tween-20. After three washes, 200-μl aliquots containing 0–500 ng of RAP in 1% BSA/PBS/0.05% Tween-20 were added to each well in triplicate and incubated overnight. All subsequent steps were essentially as described by

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TABLE I

Plasma cholesterol and triglycerides levels after adenovirus-mediated overexpression of receptor-associated protein

Adult $LRP^{flox/flox}$ and $LRP^{flox/flox}LDLR^{-/-}$ mice either transgenic or nontransgenic for the MX1cre transgene were injected (three times, intraperitoneally) with 250 μg of pI:pC at 2-day intervals. Similarly, regular wild type and $LDLR^{-/-}$ mice were treated and included as extra controls. Four weeks after the last pI:pC injection, the mice were injected intravenously with 2×10^9 PFU of Ad-RAP. As a control similar mice were injected with 2×10^9 PFU of Ad-βGal. Before and 5 days after adenovirus injection plasma was obtained and analyzed for cholesterol and triglycerides. Values are represented as the means ± S.D. (± range, *n* = 2).

Genotype	Adenovirus	*n*	Immediately after adenovirus injection		Day 5 after adenovirus injection	
			Cholesterol	Triglycerides	Cholesterol	Triglycerides
			mg/dl		*mg/dl*	
$MX1cre^+LRP^{flox/flox}$	Ad-β-Gal	3	114 ± 15	91 ± 5	114 ± 18	244 ± 99
$MX1cre^+LRP^{flox/flox}$	Ad-RAP	6	109 ± 30	89 ± 15	239 ± 132	296 ± 117
$LRP^{flox/flox}$	Ad-β-Gal	2	164 ± 24	114 ± 28	238 ± 71	262 ± 107
$LRP^{flox/flox}$	Ad-RAP	3	133 ± 43	119 ± 23	282 ± 152	329 ± 176
Wild type	Ad-β-Gal	2	153 ± 1	90 ± 6	110 ± 33	172 ± 13
Wild type	Ad-RAP	3	155 ± 20	107 ± 6	266 ± 89	276 ± 78
$MX1cre^+LRP^{flox/flox}LDLR^{-/-}$	Ad-β-Gal	3	1113 ± 220	516 ± 202	1098 ± 99	606 ± 189
$MX1cre^+LRP^{flox/flox}LDLR^{-/-}$	Ad-RAP	7	927 ± 242	350 ± 110	1359 ± 88[a]	1350 ± 581[a]
$LRP^{flox/flox}LDLR^{-/-}$	Ad-β-Gal	2	284 ± 23	81 ± 9	308 ± 57	188 ± 61
$LRP^{flox/flox}LDLR^{-/-}$	Ad-RAP	2	281 ± 1	72 ± 10	1557 ± 116	1054 ± 552
$LDLR^{-/-}$	Ad-β-Gal	3	222 ± 34	106 ± 5	393 ± 117	212 ± 58
$LDLR^{-/-}$	Ad-RAP	3	242 ± 44	109 ± 25	1585 ± 291[a]	1074 ± 279[a]

[a] $p < 0.05$, significantly different from Ad-β-Gal injected mice, using nonparametric Mann-Whitney tests.

Sendak *et al.* (29). After washing the plate six times, an HRP-conjugated rabbit anti-rat-RAP was then added to the wells for 4 h. After six washes, binding was detected by reaction of HRP with *o*-phenylenediamine substrate solution. The optical density at 490 nm (OD490) was measured after a 30-min incubation in the dark.

Effect of RAP on Lipoprotein Lipase and Hepatic Lipase Enzyme Activity in Vitro—Highly purified LPL (75 ng) purified from chicken adipose tissue or rat HL (0.034 ng) purified from liver perfusates (27, 28) was preincubated at 4 °C for 30 min in assay tubes with 0, 10, 25, 50, or 100 μg of recombinant glutathione *S*-transferase-RAP (21). The reaction was started with the addition of triolein emulsion stabilized with gum arabic in 400 μl. The reaction mixture in 500 μl contained for the LPL assay: 1.25 μmol of ^{13}H-labeled triolein with a specific activity of 500,000 cpm/μmol of fatty acid, 0.02 ml of heat-inactivated rat serum, 2.5 mg of gum arabic, 5 mg of crystalline bovine serum albumin, 0.05 mmol of NaCl, 5 μmol of $CaCl_2$, and 0.1 mmol of Tris-HCl, pH 8.6. For the HL assay, the reaction mixture was the same with the exceptions that the rat serum was omitted and the NaCl molarity was increased to one molar. For both assays, the free fatty acids were extracted by a liquid/liquid partition system (30) and assayed for radioactivity by scintillation counting.

The effect of RAP on heparan sulfate proteoglycan-bound lipoprotein lipase *in vitro* was performed exactly as described by De Man *et al.* (31). The assay was performed using human VLDL-triglycerides as a substrate. VLDL ($d < 1.006$ lipoproteins) were isolated from human serum by density gradient ultracentrifugation according to Redgrave *et al.* (32).

Plasma Decay of [^{3}H]Trioleate-labeled Neo-chylomicrons in Hepatectomized Mice—[^{3}H]Trioleate neo-chylomicrons (size, 80 nm) were prepared by the sonication and ultracentrifugation procedure exactly as described by Rensen and van Berkel (33). Mice were anesthetized by intraperitoneal injection of Nembutal (80 μg/g body weight) and functionally hepatectomized by ligating the hepatic portal vein and the hepatic artery. Liver was incised to ensure that the liver was excluded from the circulation. Subsequently, mice were injected via the vena cava inferior with 100 μl of 500,000 dpm of [^{3}H]trioleate neo-chylomicrons either with or without an excess of RAP-glutathione *S*-transferase (1 mg/mouse). At indicated time points after injection, 40-μl blood samples were withdrawn from the vena cava inferior, and the total radioactivity in 10 μl of serum was estimated.

Statistical Analysis—Data were analyzed using nonparametric Mann-Whitney rank sum tests. *p* values less than 0.05 were regarded as significant.

RESULTS

Plasma Lipid and Lipoprotein Levels after Adenovirus-mediated Gene Transfer of RAP in LDL Receptor and/or LRP-deficient Mice—We have previously reported the use of the Cre/loxP recombination system to achieve inducible disruption of the LRP gene in adult mice. Transgenic mice that were homozygous for a loxP-flanked (floxed) LRP gene and that expressed Cre recombinase under the control of the interferon-inducible MX1 promoter were used to quantitatively inactivate the LRP gene in the liver of these animals. Inactivation of LRP in the livers of mice that were also LDL receptor-deficient resulted in the accumulation of cholesterol-rich remnant lipoproteins in the circulation. These findings provided unequivocal *in vivo* evidence for a physiological role of LRP in the clearance of cholesterol-rich remnant particles from the circulation, in concert with the LDL receptor.

In the present study we have used mice lacking both LRP and LDL receptor in their livers to investigate whether another RAP-sensitive pathway, independent of the LDLR and LRP, might contribute to the clearance of chylomicron and VLDL remnant lipoproteins. To this end, we employed adenovirus-mediated gene transfer to overexpress RAP in mice lacking both receptors in their livers. Mice that were wild type, $LDLR^{-/-}$, $LRP^{flox/flox}$, MX1cre, or combinations thereof were analyzed. Animals were injected three times intraperitoneally with 250 μg of pI:pC at 2-day intervals. One month after the last pI:pC injection, 2×10^9 PFU of an adenovirus containing the rat RAP cDNA driven by the cytomegalovirus promoter (Ad-RAP) were injected into the tail vein of the different mice. As a control, similar groups of mice were injected with 2×10^9 PFU of an adenovirus encoding the β-galactosidase gene encoding driven by the cytomegalovirus promoter (Ad-β-Gal). Animals were analyzed within 5 days following virus administration.

Before adenovirus injection, plasma cholesterol and triglyceride levels were approximately 3-fold higher in $MX1cre^+LRP^{flox/flox}LDLR^{-/-}$ mice than in $LDLR^{-/-}$ mice and in $LRP^{flox/flox}LDLR^{-/-}$ mice lacking the cre transgene (Table I). Fast performance liquid chromatography revealed that the increase in total plasma cholesterol was mainly due to an increase in the chylomicron remnant/VLDL and LDL lipoprotein fractions (Fig. 1*A*, compare *panels d*, *e*, and *f*). Plasma lipid levels in $MX1cre^+LRP^{flox/flox}$ mice were comparable with those of $LRP^{flox/flox}$ and wild type control mice, and cholesterol was contained mainly in the high density lipoprotein fraction (Fig. 1*A*, *panels a–c*). Upon Ad-RAP injection, $MX1cre^+LRP^{flox/flox}$-$LDLR^{-/-}$ mice showed an increase in total plasma cholesterol

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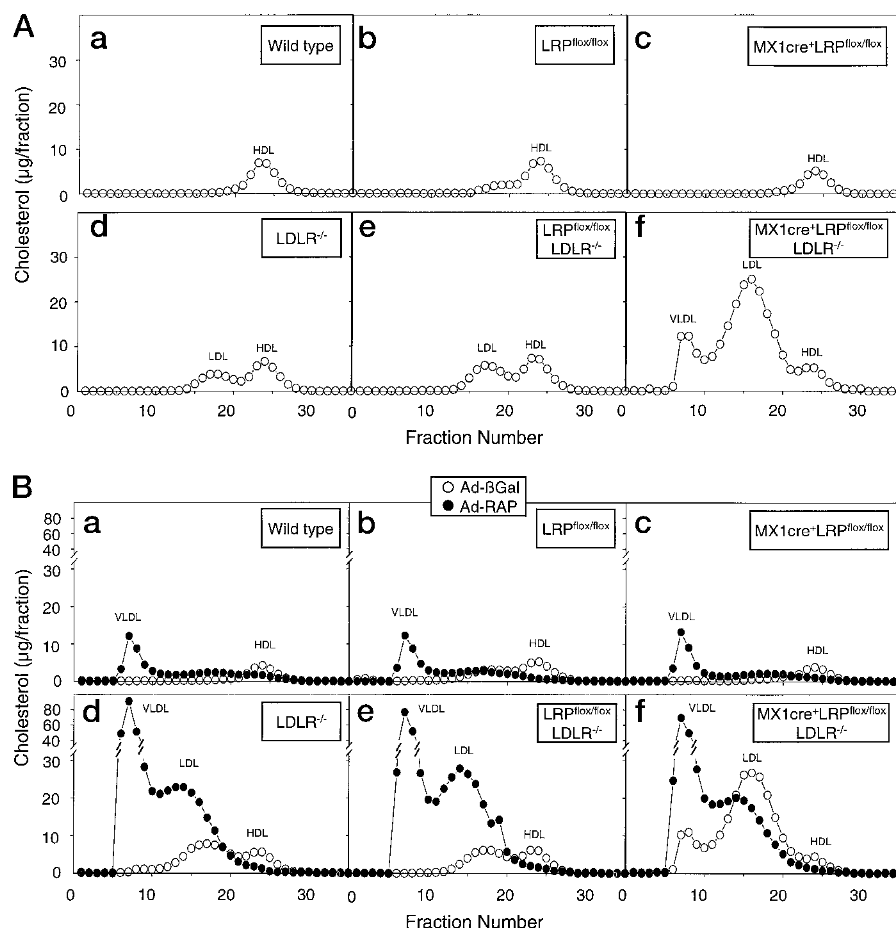

FIG. 1. **Distribution of serum cholesterol among lipoprotein fractions before and 5 days after adenovirus-mediated RAP gene transfer.** Adult wild type (*panel a*), LRP$^{flox/flox}$ (*panel b*), MX1cre$^+$LRP$^{flox/flox}$ (*panel c*), LDLR$^{-/-}$ (*panel d*), LRP$^{flox/flox}$LDLR$^{-/-}$ (*panel e*), and MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice (*panel f*) were injected (three times, intraperitoneally) with 250 μg of pI:pC at 2-day intervals. 4 weeks after the last pI:pC injection, the mice were injected intravenously with 2×10^9 PFU of Ad-RAP (*black circles*) or Ad-β-Gal as a control (*open circles*). Before (*A*) and 5 days after (*B*) adenovirus injection plasma was obtained from the animals, and lipoproteins were size-fractionated on a Superose 6 fast performance liquid chromatography column. Cholesterol content of the individual fractions was determined spectrofluorometrically. The relative positions of VLDL, LDL, and high density lipoprotein-sized lipoproteins are indicated. Shown profiles are the average of profiles obtained from 2–7 individual animals of the indicated genotype.

levels of approximately 30%, and total plasma triglyceride levels increased approximately 2-fold as compared with Ad-β-Gal-injected mice of the same genotype (Table I). Although cholesterol levels were only slightly elevated, Ad-RAP-injected MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice showed a dramatic shift in cholesterol distribution from LDL-sized lipoprotein fractions to large VLDL-sized lipoproteins, with a concomitant reduction in high density lipoprotein cholesterol (Fig. 1*B*, *panel f*). Total plasma lipid levels and the lipoprotein profile of Ad-RAP-injected MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice (Fig. 1*B*, *panel f*) closely resembled that of Ad-RAP-injected LRP$^{flox/flox}$LDLR$^{-/-}$ or LDLR$^{-/-}$ mice (Fig. 1*B*, *panels d* and *e*).

Total plasma cholesterol and triglyceride concentrations were only slightly elevated in Ad-RAP-injected MX1cre$^+$LRP$^{flox/flox}$ mice compared with Ad-β-Gal-injected mice of the same genotype (Table I). However, plasma lipid levels in Ad-RAP-injected MX1cre$^+$LRP$^{flox/flox}$ mice were not different from Ad-RAP-injected LRP$^{flox/flox}$ or wild type mice. In these mice, the slight elevation in plasma cholesterol was caused mainly by the accumulation of large, remnant-sized lipoproteins (Fig. 1*B*, *panels a–c*).

Immunoblot Analysis of Plasma Apoproteins after Adenovirus-mediated RAP Gene Transfer—The effect of RAP overexpression on the plasma concentrations of apolipoproteins B100, B48, E, and AI in the pI:pC-induced wild type, LRP$^{flox/flox}$, MX1cre$^+$LRP$^{flox/flox}$, LDLR$^{-/-}$, LRP$^{flox/flox}$LDLR$^{-/-}$ and MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice is shown in Fig. 2. Before adenovirus injections, MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice had elevated levels of apoB (100+48) and apoE as compared with nontransgenic controls (LRP$^{flox/flox}$LDLR$^{-/-}$) and LDLR$^{-/-}$ mice (Fig. 2*A*, *lanes 4–6*). Plasma apolipoprotein levels in MX1cre$^+$LRP$^{flox/flox}$ mice were comparable with those of LRP$^{flox/flox}$ and wild type controls (Fig. 2*A*, *lanes 1–3*).

Upon Ad-RAP injection, plasma apoB48 and apoE levels were elevated in MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice (Fig. 2*B*, *right panel*, *lane 6*) but were not different from apolipoprotein levels of Ad-β-Gal-injected mice of the same genotype (Fig. 2*B*, *left panel*, *lane 6*). In addition, plasma apolipoprotein levels in Ad-RAP-injected MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice were almost identical to those of Ad-RAP-injected LRP$^{flox/flox}$LDLR$^{-/-}$ and LDLR$^{-/-}$ mice (Fig. 2*B*, *right panel*, *lanes 4–6*). Consistent with the decrease in high density lipoprotein cholesterol (Fig. 1*b*, *panels D–F*), plasma apoA-I levels were also decreased in LDLR-deficient mice injected with Ad-RAP (Fig. 2*B*, *right panel*, *lanes 4–6*).

Ad-RAP-injected MX1cre$^+$LRP$^{flox/flox}$ showed only a slight elevation in plasma apoB48 and apoE levels as compared with Ad-β-Gal-injected mice of the same genotype (Fig. 2*B*, *lanes 3*, *right* and *left panels*, respectively). Plasma apolipoprotein levels were also not different from Ad-RAP-injected LRP$^{flox/flox}$ or wild type mice (Fig. 2*B*, *right panel*, *lanes 1* and *2*).

Binding of ^{125}I-Labeled RAP and Peroxidase-labeled RAP to Liver Membranes—The striking increase in plasma lipids and shift in lipoprotein profile in Ad-RAP-injected MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice indicates that RAP acts on another process besides the LDL receptor and LRP that is also involved in the metabolism of triglyceride-rich lipoproteins. To investigate whether another RAP-binding protein might exist on liver membranes, we determined the binding of ^{125}I-labeled RAP to liver membranes from pI:pC-induced adult LRP$^{flox/flox}$, MX1cre$^+$LRP$^{flox/flox}$, LRP$^{flox/flox}$LDLR$^{-/-}$, and MX1cre$^+$LRP$^{flox/flox}$-LDLR$^{-/-}$ mice. As shown in Fig. 3, liver membranes from mice expressing LRP (*i.e.* LRP$^{flox/flox}$ and LDLR$^{-/-}$LRP$^{flox/flox}$; Fig. 3, *A* and *C*) bound RAP with high affinity. In contrast, membranes from

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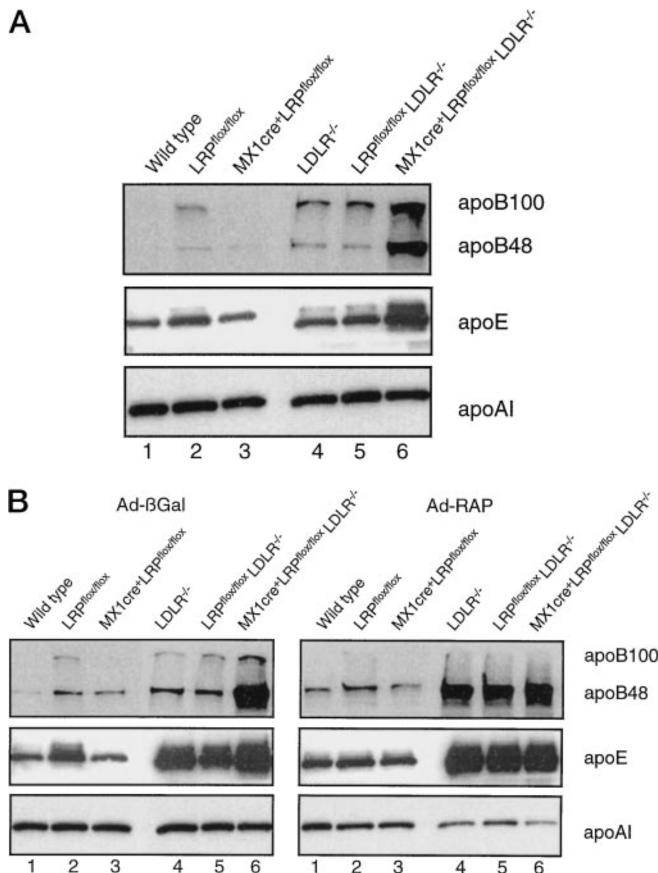

FIG. 2. **Immunoblot analysis of plasma apoproteins before and 5 days after adenovirus-mediated RAP gene transfer.** Adult wild type (*lane 1*), LRP$^{flox/flox}$ (*lane 2*), MX1cre$^+$LRP$^{flox/flox}$ (*lane 3*), LDLR$^{-/-}$ (*lane 4*), LRP$^{flox/flox}$LDLR$^{-/-}$ (*lane 5*), and MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice (*lane 6*) were injected (three times, intraperitoneally) with 250 μg of pI:pC at 2-day intervals. 4 weeks after the last pI:pC injection, the mice were injected intravenously with 2 × 10^9 PFU of Ad-RAP or Ad-β-Gal as a control. Before adenovirus injection (*A*) and 5 days after injection (*B*, *left* and *right panel*, for Ad-β-Gal and Ad-RAP, respectively) plasma was obtained from the animals. 3 μl of plasma was separated by SDS gel electrophoresis and immunoblotted with the indicated polyclonal antibodies against apoB, apoE, and apoAI using the ECL system. The relative positions of migration of apoB100, B48, E, and AI are indicated.

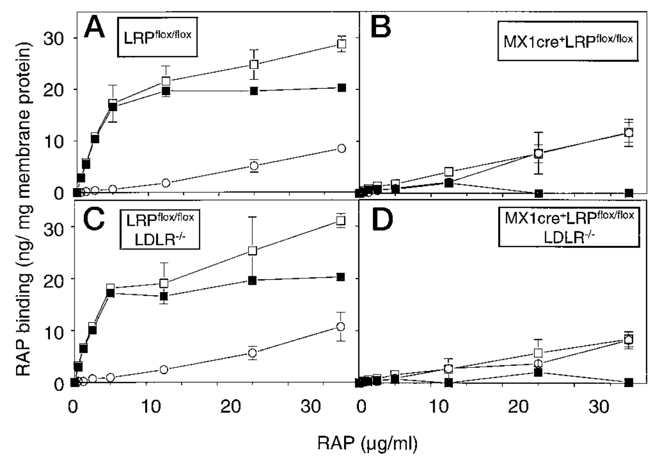

FIG. 3. **Binding of ^{125}I-labeled RAP to liver membranes.** Adult LRP$^{flox/flox}$ (*A*), MX1cre$^+$LRP$^{flox/flox}$ (*B*), LRP$^{flox/flox}$LDLR$^{-/-}$ (*C*), and MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice (*D*) were injected (three times, intraperitoneally) with 250 μg of pI:pC in 2-day intervals.10 days after the last injection, membrane fractions were prepared from livers of the animals. Total (*open squares*), nonspecific (*open circles*), and specific binding (*black squares*) binding of ^{125}I-labeled RAP to liver membranes was measured upon incubation of the membranes with indicated amounts of ^{125}I-labeled RAP overnight at 4 °C as described under "Experimental Procedures." Values represent the means ± S.D. of four measurements.

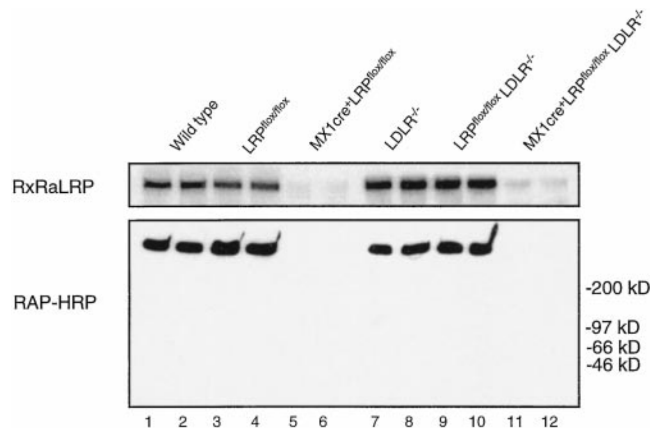

FIG. 4. **Binding of peroxidase labeled RAP to liver membranes.** Adult wild type (*lanes 1* and *2*), LRP$^{flox/flox}$ (*lanes 3* and *4*), MX1cre$^+$LRP$^{flox/flox}$ (*lanes 5* and *6*), LDLR$^{-/-}$ (*lanes 7* and *8*), LRP$^{flox/flox}$LDLR$^{-/-}$ (*lanes 9* and *10*), and MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice (*lanes 11* and *12*) were injected (three times, intraperitoneally) with 250 μg of pI:pC at 2-day intervals.10 days after the last injection, membrane fractions were prepared from livers of the animals, and 50 μg protein/lane was separated by SDS gel electrophoresis and either used for ligand blotting (*lower panel*) with peroxidase labeled RAP (RAP-HRP) or immunoblotted with an antibody that specifically recognizes the 85-kDa subunit of LRP (*RxRaLRP*; *upper panel*). Bound RAP-HRP and IgG were detected using the ECL system.

mice lacking LRP (*i.e.* MX1cre$^+$LRP$^{flox/flox}$ and MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$; Fig. 3, *B* and *D*) did not bind RAP specifically. These results show that LRP is the only liver membrane protein that binds RAP with high affinity.

A similar result was obtained when we determined the ability of RAP to bind to liver membrane proteins by ligand blotting using peroxidase-labeled RAP (Fig. 4, *lower panel*). The presence of LRP was detected by immunoblotting with antibodies directed against the 85-kDa subunit of LRP (Fig. 4, *upper panel*). A prominent band of approximately 515 kDa that bound RAP was present in the livers of mice expressing LRP and absent from livers lacking this receptor. No other RAP-binding protein was detected in these ligand blotting experiments. These findings further show that LRP is the only high affinity RAP-binding protein in liver membranes and suggest that the hyperlipidemia caused by overexpression of RAP in MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ is not due to inhibition of an LRP-independent RAP-binding protein.

Effect of Adenovirus-mediated RAP Gene Transfer on Triglyceride Metabolism—The predominant increase in plasma triglyceride rather than cholesterol levels in MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ following Ad-RAP-mediated gene transfer suggests that RAP may directly interfere with triglyceride metabolism. This effect of RAP overexpression on triglyceride levels may take place at the level of VLDL-triglyceride production or result from direct inhibition of triglyceride lipolysis by LPL and/or HL.

Production of VLDL-triglycerides was measured by determining the rate of triglyceride secretion in pI:pC-induced MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ and wild type control mice 5 days after injection of 2 × 10^9 PFU of Ad-RAP or Ad-β-Gal (Fig. 5). VLDL-triglyceride production rate was similar in all groups of mice, indicating that RAP overexpression did not affect hepatic VLDL-triglyceride production.

To determine whether RAP interferes with triglyceride metabolism through a direct effect on LPL and/or HL-mediated triglyceride hydrolysis, wild type mice were injected with Ad-

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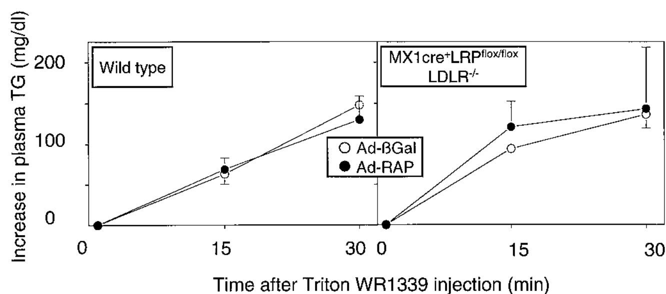

FIG. 5. **Production of VLDL-triglycerides after adenovirus-mediated RAP gene transfer.** Adult wild type (*left panel*) and MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice (*right panel*) were injected (three times, intraperitoneally) with 250 μg of pI:pC in 2-day intervals. 4 weeks after the last pI:pC injection, the mice were injected intravenously with 2×10^9 PFU of Ad-RAP (*black circles*) or Ad-β-Gal as a control (*open circles*). At 5 days after adenovirus injection, mice were fasted for 5 h and injected intravenously with Triton WR1339 (500 mg/kg body weight). At 1, 15, and 30 min after injection blood samples were drawn and analyzed for triglycerides (*TG*). The increase in serum triglyceride was normalized to the 1-min point. Values are represented as the means ± S.D of six mice.

RAP or Ad-β-Gal. Pre- and post-heparin plasma LPL and HL levels and activities were determined 5 days after adenovirus injection. As shown in Table II, plasma HL levels and activity in pre- and post-heparin plasma of Ad-RAP-injected mice were similar to those of Ad-β-Gal-injected mice. In contrast, LPL concentrations (protein mass) were increased approximately 7-fold in pre-heparin plasma of Ad-RAP-injected mice as compared with Ad-β-Gal-injected animals. Surprisingly, the accumulating LPL was almost completely enzymatically inactive. Upon heparin injection, plasma LPL levels increased by the same amount in animals injected with either virus.

We next determined, *in vitro*, whether the effect of RAP on plasma LPL, but not HL, levels and activity may result from a direct effect of RAP on the activity of these lipases. Although RAP bound with high affinity to both HL and LPL (K_d = 8 and 5 nM, respectively; data not shown), it did not affect lipolytic activity when both were assayed in solution using Triton X-100 stabilized triolein emulsions (Table III). There was also no effect of RAP on lipolysis when bovine milk LPL was bound to heparan sulfate proteoglycans, and the substrate employed was human d<1.006 lipoproteins (Table III). Thus, RAP overexpression apparently affects the biological activity of LPL *in vivo*, although probably not by direct inhibition of enzyme activity.

To study whether RAP can acutely affect triglyceride hydrolysis by LPL *in vivo*, we determined plasma triglyceride decay in functionally hepatectomized mice. As shown in Fig. 6 the plasma decay of [^{3}H]-trioleate labeled neo-chylomicrons was not different for hepatectomized mice that were co-injected with a high dose of RAP (1 mg/mouse) and control-injected mice. This indicates that the effect of hepatic RAP overexpression on LPL specific activity in the circulation could not be mimicked *in vivo* by intravenous injection of a bolus of RAP and that the effect of RAP on LPL activity requires a prolonged overexpression of this protein.

DISCUSSION

In the present study, we demonstrated that a RAP-sensitive process, independent of the LDL receptor and the LRP, is involved in the metabolism of triglyceride-rich lipoproteins. This is illustrated by the fact that adenovirus-mediated overexpression of RAP increased plasma lipid and lipoprotein levels in MX1cre$^+$LRP$^{flox/flox}$LDLR$^{-/-}$ mice. The presence of this RAP-sensitive site explains the difference in lipid levels and lipoprotein profile of LDL receptor-deficient mice in which LRP was inactivated transiently by RAP overexpression (14) and animals in which the LRP gene was disrupted by inducible Cre/loxP-mediated recombination (2).

Our binding and ligand blotting studies with LRP- and LDL-receptor-deficient mouse liver membranes have shown that the RAP-mediated effect on the metabolism of triglyceride-rich lipoproteins was not due to inhibition of an unknown RAP-sensitive hepatic lipoprotein receptor. Others have postulated that the uptake of chylomicrons and/or VLDL may also involve hepatic lipoprotein receptors other than the LDL receptor and the LRP, for instance a hypothetical lipolysis-stimulated receptor (34) and remnant receptor (35). However, our findings rule out the possibility of other major RAP-binding proteins in the liver that may participate in this process.

RAP overexpression strongly affected triglyceride metabolism. This was not due to a RAP-mediated stimulation of hepatic VLDL-triglyceride production. RAP overexpression resulted in an almost complete inactivation of (postheparin) plasma LPL. Thus, RAP has a direct or indirect effect on lipase-mediated conversion of chylomicrons to the smaller remnants, leading to the observed accumulation of large triglyceride-rich particles. This also explains the lack of an effect of RAP on apolipoprotein levels (Fig. 2*B*).

RAP binds with high affinity to LPL. Because RAP does not affect LPL activity *in vitro* (Table III), we can conclude that RAP does not bind to the domains essential for the catalytic activity of the enzyme. Because a high dose of intravenous RAP protein had no effect on liver-independent triglyceride removal (Fig. 6), we can also conclude that RAP had no direct effect on LPL activity within the vascular bed. The heparin-releasable LPL mass, that is the increment above the pre-heparin level, was not significantly different in Ad-RAP- or Ad-β-Gal-injected mice, suggesting that RAP overexpression did not affect the amount of LPL bound to the endothelium. In addition, RAP does not compete for binding of apolipoprotein CII with VLDL or LPL, because no such effect was detectable, *in vitro*, even at concentrations of 200–500 μg RAP/ml.

It has been suggested that the VLDL receptor may play a role in peripheral triglyceride metabolism. The VLDL receptor and LPL are expressed and localized in peripheral tissues involved in triglyceride metabolism. Furthermore, the VLDL receptor binds RAP and LPL with high affinity (36, 37). RAP may affect the role of the VLDL receptor in LPL-mediated lipolysis. However, VLDL receptor-deficient mice have a normal lipoprotein profile (38) and display a normal plasma triglyceride removal rate and normal lipoprotein uptake by peripheral tissues (data not shown), suggesting that the RAP-mediated effect on LPL activity is not related to the VLDL receptor activity.

RAP gene transfer resulted in greatly elevated levels of inactive LPL in pre- and post-heparin plasma. The high concentration of inactive LPL in plasma may result from an overproduction of LPL or a defect in its removal. LPL has been shown to bind LRP both by solid phase assays with purified LRP (39) and by Western blotting of liver membranes extracts (40). In addition, in cell culture systems, LRP antibodies have been shown to inhibit LPL degradation (39). Thus, inactivation of LRP by RAP is a likely cause for the accumulation of LPL in the plasma, even in the absence of marked hypertriglyceridemia in the wild type mice that received Ad-RAP intravenously.

The effect of hepatic RAP overexpression on LPL specific activity in the circulation could not be mimicked *in vitro* by adding RAP to a VLDL lipolysis assay (Table III) or *in vivo* by intravenous injection of a bolus of RAP (Fig. 6). This suggests that RAP may have a function in LPL processing in the capillary bed, possibly by associating with Sortilin (41).

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TABLE II
Hepatic lipase and lipoprotein lipase levels and activities after adenovirus-mediated overexpression of receptor-associated protein

Adult wild type mice (n = 5–6 per group) were injected with 2×10^9 PFU of Ad-RAP or Ad-β-Gal. At 5 days after adenovirus injection, mice were injected intravenously with heparin (100 units/kg body weight). Immediately before and 15 min after heparin blood samples were drawn, and plasma was analyzed for hepatic lipase and lipoprotein lipase levels and activities (see methods). Values are represented as the means ± S.D.

Genotype	Adenovirus	Hepatic lipase			Lipoprotein lipase		
		Pre-heparin levels	Post-heparin levels	Activity	Pre-heparin levels	Post-heparin levels	Activity
		ng/ml	*ng/ml*	*μmol/h/μg*	*ng/ml*	*ng/ml*	*μmol/h/μg*
Wild type	Ad-β-Gal	158 ± 21	188 ± 33	54.8 ± 18	13.6 ± 16.1	48.3 ± 36.5	343 ± 360
Wild type	Ad-RAP	162 ± 23	198 ± 36	55.8 ± 20	95.6 ± 49.7[a]	137.5 ± 49.5[a]	25 ± 21[a]

[a] $p < 0.05$, significantly different from Ad-β-Gal injected mice, using nonparametric Mann-Whitney tests.

TABLE III
The effect of RAP on hepatic lipase and lipoprotein lipase enzyme activity in vitro

$d < 1.006$ lipoproteins were isolated from human serum by density gradient ultracentrifugation and artificial lipoprotein particles were prepared as described under "Experimental Procedures." The rate of lipolysis by HL, LPL (both in solution using the artificial lipoproteins), and HSPG-bound LPL (using the human $d < 1.006$ lipoproteins) *in vitro* in the presence of increasing amounts of RAP was determined as described in the methods. ND, not determined.

RAP	HL	LPL	HSPG-bound LPL
μg/ml	*μEq FA/h*	*μEq FA/h*	*μmol FA/L/h*
0	0.15	0.65	0.74
20	0.15	0.74	ND
50	0.16	0.74	0.78
100	0.18	0.67	0.78
200	0.16	0.72	ND
500	ND	ND	0.76

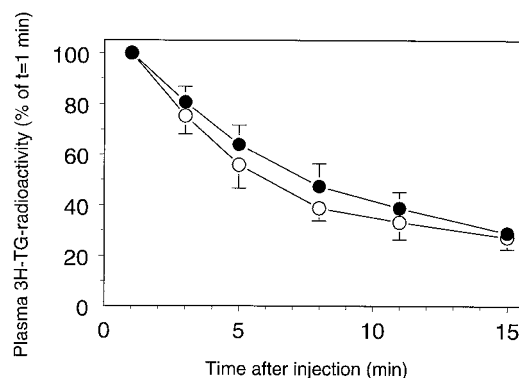

FIG. 6. **The effect of intravenous RAP on plasma decay of [^{3}H]trioleate-labeled neo-chylomicrons in hepatectomized mice.** Functionally hepatectomized wild type mice were injected with 500,000 dpm of [^{3}H]trioleate neo-chylomicrons either without (*open circles*) or with an excess of RAP (1 mg RAP/mouse; *black circles*). Blood was drawn at 1, 3, 5, 8, 11, and 15 min after injections and the radioactivity was determined. Values are the means (± S.D.) of five animals/group and are expressed as a percentages of the radioactivity present in t = 1 min serum sample.

The RAP-mediated inhibition of LPL activity resulted in massive hypertriglyceridemia in mice that lack the LDL receptor or both LDL receptor and LRP. However, wild type mice and LRP-deficient mice, both having normal LDL receptor expression, did not display hypertriglyceridemia upon inactivation of LPL. This is consistent with the observation that mice lacking both the apoE and LDL receptor genes and not mice lacking only the apoE gene display massive hypertriglyceridemia upon apoE-induced inhibition of lipolysis (42). These data demonstrate that in contrast to LDL receptor-independent pathways, the LDL receptor is capable of removing triglyceride-rich lipoproteins from the circulation, even when the lipoproteins are poorly lipolyzed.

In summary, our results suggest that RAP affects the conversion of large triglyceride-rich chylomicrons to smaller remnants by interfering with LPL activation in the periphery and rule out the contribution of other major RAP-binding proteins in the liver. This mechanism may play a role in some of the complex genetic traits that cause hypertriglyceridemic syndromes in man.

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An Extrahepatic Receptor-associated Protein-sensitive Mechanism Is Involved in the Metabolism of Triglyceride-rich Lipoproteins

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