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<sup>-</sup>LRP<sup>fl</sup>LDLR<sup>−/−</sup>) 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<sup>-</sup>LRP<sup>fl</sup>LDLR<sup>−/−</sup> 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<sub>d</sub> = 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. Therefore, 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) 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 hepatic 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 LDL 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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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 ioxP sites (LRPlox/lox) 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 (LRPfln/fln), homozygous for both mutations (LRPfln/fln; LDLR−/−), homozygous for the floxed Mx1cre transgene (Mx1cre−/−LRPfln/fln), and homozygous for the floxed LRP allele, deficient for the LDL receptor, and transgenic for the Mx1cre transgene (Mx1cre−/−LRPfln/fln; LDLR−/−). Induction with polyinosinic:polycytidylic ribonucleic acid (pI:pC; Sigma) was done by intraperitoneal injection of 200 μg/mouse (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% saline in the presence of the protease inhibitor mixture, using a 21 gauge needle. The membrane fraction was stored for up to 1 week at the 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 125I-RAP either in the absence or in the presence of a 100 μg/ml excess of unlabeled RAP (final incubation volume, 250 μl; n = 4). Membrane bound 125I-RAP was separated from unbound RAP by layering 1 ml of 5% (w/v) BSA and centrifuging at 100,000 × 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 125I-RAP.

Ligand Blotting—Membranes were prepared from mouse livers as described above, and proteins separated by nondenaturing, 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 apolipoprotein.

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. The assays of Lipoprotein Lipase and Hepatic Lipase Mass in Mouse Plasma—Hepatic lipase mass was measured by ELISA developed for rat (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 His6 LPL protein was used to generate antibodies in a goat and to construct a column of mouse LPL AffiPrep 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 0.5 to 1.8 ng/ml. At 1 ng/ml 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 lipopases, all steps were conducted at 4 °C. Microtiter plates (Corning) were coated with highly purified lipases (27, 28), 10 ng of avian LPL or rat HL overnight. Confluent 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
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, 32). Highly purified LPL (75 ng) purified from chicken adipose tissue or rat HL (0.034 ng) purified from liver perfusates (27, 32). 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 13H-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 CaCl2, 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 (5 ≤ 1.006 lipoproteins) was isolated from human serum by density gradient ultracentrifugation according to Redgrave et al. (32).

Plasma Decay of 13H-Trioleate-labeled Neo-cholesterics in Hepatectomized Mice—13H-Trioleates were isolated from human serum by density gradient ultracentrifugation according to Redgrave et al. (32).

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−/−, LRPfloxFlox/LoxFlox, MX1cre, or combinations thereof were analyzed. Animals were injected three times intraperitoneally with 250 μg of pL/C at 2-day intervals. One month after the last pL/C injection, 2 × 109 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 × 109 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 MX1creLRFlox/floxLDLR−/− mice than in LDLR−/− mice and in LRPfloxFlox/LoxFloxLDLR−/− 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. 1A, compare panels d, e, and f). Plasma lipid levels in MX1creLRFlox/floxLDLR−/− mice were comparable with those of LRPfloxFlox/LoxFlox and wild type control mice, and cholesterol was contained mainly in the high density lipoprotein fraction (Fig. 1A, panels a–c). Upon Ad-RAP injection, MX1creLRPfloxFlox/ LoxFloxLDLR−/− mice showed an increase in total plasma cholesterol levels, which was significantly higher than that seen in Ad-β-Gal control mice (Table I).

### RESULTS

| Genotype  | Adenovirus | n | Immediately after adenovirus injection | Day 5 after adenovirus injection |
|-----------|------------|---|-------------------------------------|---------------------------------|
|           |            |   | Cholesterol | Triglycerides | Cholesterol | Triglycerides |
| MX1creLRFlox/Lox | Ad-β-Gal | 3 | 114 ± 15 | 91 ± 5 | 114 ± 18 | 244 ± 99 |
| MX1creLRFlox/Lox | Ad-RAP | 6 | 109 ± 30 | 89 ± 15 | 239 ± 132 | 296 ± 117 |
| LRFlox/Lox | Ad-β-Gal | 2 | 164 ± 24 | 114 ± 28 | 238 ± 71 | 262 ± 107 |
| LRFlox/Lox | 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 |
| MX1creLRFlox/LoxLDLR−/− | Ad-β-Gal | 3 | 1113 ± 220 | 516 ± 202 | 1098 ± 99 | 606 ± 189 |
| MX1creLRFlox/LoxLDLR−/− | Ad-RAP | 7 | 927 ± 242 | 350 ± 110 | 1359 ± 88 | 1350 ± 581 |
| LRFlox/LoxLDLR−/− | Ad-β-Gal | 2 | 284 ± 23 | 81 ± 9 | 308 ± 57 | 188 ± 61 |
| LRFlox/LoxLDLR−/− | Ad-RAP | 2 | 281 ± 1 | 72 ± 10 | 1557 ± 116 | 1054 ± 552 |
| LDLR−/− | Ad-β-Gal | 3 | 223 ± 34 | 106 ± 5 | 393 ± 117 | 212 ± 58 |
| LDLR−/− | Ad-RAP | 3 | 242 ± 44 | 109 ± 25 | 1585 ± 291 | 1074 ± 279 |

* p < 0.05, significantly different from Ad-β-Gal injected mice, using nonparametric Mann-Whitney tests.
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^Lox^/LoxLDLr^−/− 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. 1B, panel f). Total plasma lipid levels and the lipoprotein profile of Ad-RAP-injected MX1cre^Lrp^Lox^/LoxLDLr^−/− mice (Fig. 1B, panel f) closely resembled that of Ad-RAP-injected Lrp^Lox^/LoxLDLr^−/− or LDLr^−/− mice (Fig. 1B, panels d and e).

Total plasma cholesterol and triglyceride concentrations were only slightly elevated in Ad-RAP-injected MX1cre^Lrp^Lox^/Lox mice compared with Ad-β-gal-injected mice of the same genotype (Table I). However, plasma lipid levels in Ad-RAP-injected MX1cre^Lrp^Lox^/Lox mice were not different from Ad-RAP-injected Lrp^Lox^/Lox 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. 1B, 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 A1 in the pI:pC-induced wild type, Lrp^Lox^/Lox LDLr^−/−, Lrp^Lox^/Lox LDLr^−/−, and MX1cre^Lrp^Lox^/LoxLDLr^−/− mice is shown in Fig. 2. Before adenovirus injections, MX1cre^Lrp^Lox^/LoxLDLr^−/− mice had elevated levels of apoB (100+48) and apoE as compared with nontransgenic controls (Lrp^Lox^/LoxLDLr^−/−) and LDLr^−/− mice (Fig. 2A, lanes 4–6). Plasma apolipoprotein levels in MX1cre^Lrp^Lox^/Lox mice were comparable with those of Lrp^Lox^/Lox and wild type controls (Fig. 2A, lanes 1–3).

Upon Ad-RAP injection, plasma apoB48 and apoE levels were elevated in MX1cre^Lrp^Lox^/LoxLDLr^−/− mice (Fig. 2B, right panel, lane 6) but were not different from apolipoprotein levels of Ad-β-gal-injected mice of the same genotype (Fig. 2B, left panel, lane 6). In addition, plasma apolipoprotein levels in Ad-RAP-injected MX1cre^Lrp^Lox^/LoxLDLr^−/− mice were almost identical to those of Ad-RAP-injected Lrp^Lox^/LoxLDLr^−/− and LDLr^−/− mice (Fig. 2B, right panel, lanes 4–6). Consistent with the decrease in high density lipoprotein cholesterol (Fig. 1B, panels D–F), plasma apoA-1 levels were also decreased in LDLr-deficient mice injected with Ad-RAP (Fig. 2B, right panel, lanes 4–6).

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

**Binding of 125I-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^Lox^/LoxLDLr^−/− 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 125I-labeled RAP to liver membranes from pI:pC-induced adult Lrp^Lox^/Lox, MX1cre^Lrp^Lox^/Lox, Lrp^Lox^/LoxLDLr^−/−, and MX1cre^Lrp^Lox^/LoxLDLr^−/− mice. As shown in Fig. 3, liver membranes from mice expressing LRP (i.e. Lrp^Lox^/Lox and LDLr^−/−) 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<sup>−/−</sup> (lane 2), MX1cre<sup>−/−</sup>LRP<sup>−/−</sup> (lane 3), LDLR<sup>−/−</sup> (lane 4), LRP<sup>−/−</sup>LDLR<sup>−/−</sup> (lane 5), and MX1cre<sup>−/−</sup>LRP<sup>−/−</sup>LDLR<sup>−/−</sup> mice (lane 6) were injected (three times, intraperitoneally) with 250 µg of plpC in 2-day intervals. 4 weeks 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<sup>I</sup>-labeled RAP to liver membranes was measured upon incubation of the membranes with indicated amounts of 125<sup>I</sup>-labeled RAP to liver membranes as described under Experimental Procedures. Values represent the means ± S.D. of four measurements.

Fig. 3. Binding of 125<sup>I</sup>-labeled RAP to liver membranes. Adult LRP<sup>−/−</sup> (A), MX1cre<sup>−/−</sup>LRP<sup>−/−</sup> (B), LRP<sup>−/−</sup>LDLR<sup>−/−</sup> (C), and MX1cre<sup>−/−</sup>LRP<sup>−/−</sup>LDLR<sup>−/−</sup> mice (D) were injected (three times, intraperitoneally) with 250 µg of plpC in 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 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.

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 plpC-induced MX1cre<sup>−/−</sup>LRP<sup>−/−</sup>LDLR<sup>−/−</sup> and wild type control mice 5 days after injection of 2 × 10<sup>9</sup> 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-
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 (Kd = 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 c~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 [14C]-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<sup>lox/lox</sup>LDLR<sup>−/−</sup> 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. 2B).

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 vivo* 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).
Wild type Ad-RAP 162
min serum sample. was determined. Values are the means (±
6
S.D.) of five animals/group.

| Genotype | Adenovirus | Hepatic lipase | Lipoprotein lipase |
|----------|------------|----------------|-------------------|
|          |            | Pre-heparin levels | Post-heparin levels | Activity |
|          |            | ng/ml | ng/ml | µmol FA/h | µmol FA/h | µmol FA/L/h | µmol FA/L/h |
| Wild type | Ad-β-Gal | 155 ± 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 | 137.5 ± 49.5 | 25 ± 2% |

"p < 0.05, significantly different from Ad-β-Gal injected mice, using nonparametric Mann-Whitney tests.

FIG. 6. The effect of intravenous RAP on plasma decay of [3H]trioleate-labeled neo-cholesteromic in hepatectomized mice. Functionally hepatectomized wild type mice were injected with 500,000 dpm of [3H]trioleate neo-cholesteromins 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 percentage 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 con-

version 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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REFERENCES
1. Goldstein, J. L., and Brown, M. S. (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 1215–1250, McGraw-Hill Publishing Co., New York
2. Rohmann, A., Gotthardt, M., Hammer, R. E., and Herz, J. (1998) J. Clin. Invest. 101, 689–695
3. Mahley, R. W., and Ji, Z.-S. (1999) J. Lipid. Res. 40, 1–16
4. Ji, Z.-S., Fazio, S., Lee, Y. L., and Mahley, R. W. (1994) J. Biol. Chem. 269, 27664–27672
5. Ji, Z.-S., Lauer, S. J., Fazio, S., Besadoun, A., Taylor, J. M., and Mahley, R. W. (1994) J. Biol. Chem. 269, 13429–13436
6. Shafi, S., Brady, S. E., Besadoun, A., and Havel, R. J. (1994) J. Lipid. Res. 35, 709–720
7. Olivecrona, G., and Olivecrona, T. (1995) Curr. Opin. Lipidol. 6, 291–305
8. Hamilton, R. L., Wong, J. S., Guo, L. S. S., Krisans, S., and Havel, R. J. (1990) J. Biol. Chem. 265, 15804–15811
9. Choi, S. Y., and Cooper, A. D. (1993) J. Biol. Chem. 268, 15854–15861
10. Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E., and Herz, J. (1993) J. Clin. Invest. 92, 883–893
11. Kowal, R. C., Herz, J., Goldstein, J. L., Eser, V., and Brown, M. S. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5810–5814
12. Kowal, R. C., Herz, J., Weisgraber, K. H., Mahley, R. W., Brown, M. S., and Goldstein, J. L. (1990) J. Biol. Chem. 265, 10771–10779
13. Veniant, M. M., Zlot, C. H., Wolzem, R. L., Pierotti, V., Driscoll, D., Dichek, D., Herz, J., and Young, S. G. (1998) J. Clin. Invest. 102, 1539–1568
14. Willnow, T. E., Sheng, Z., Ishibashi, S., and Herz, J. (1994) Science 264, 1471–1474
15. Herz, J., Christoff, D. E., and Hammer, R. E. (1992) Cell 71, 411–421
16. Herz, J., Christoff, D. E., and Hammer, R. E. (1993) Cell 73, 428–437
17. Kühn, R., Schwenk, F., Auge, M., and Rajewsky, K. (1995) Science 269, 1427–1429
18. Yen, F. T., Mann, C. J., Guo, L. S. S., Krisans, S., and Havel, R. J. (1990) J. Lipid. Res. 31, 1562–1565
19. Willnow, T. E., Armstrong, S. A., Hammer, R. E., and Herz, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4537–4541
20. Herz, J., Goldstein, J. L., Strickland, K. D., Ho, Y. K., and Brown, M. S. (1991) J. Biol. Chem. 266, 21232–21238
21. Fraker, P. J., and Speck, J. C. (1978) Biochem. Biophys. Res. Commun. 80, 849–857
22. Ottway, S., and Robinson, D. S. (1967) J. Physiol. (Lond.) 190, 321–332
23. Cisar, L., and Besadoun, A. (1985) J. Lipid. Res. 26, 380–386
24. Kirchgesser, T. G., Svenson, K. L., Lusis, A. J., and Schotz, M. C. (1987) J. Biol. Chem. 262, 8460–8466
25. Cisar, L. A., Hogerwerf, A. J., Cupp, M., Rappon, A. C., and Besadoun, A. (1989) J. Biol. Chem. 264, 1767–1774
26. Besadoun, A., Hou, J., and Hughes, B. (1988a) Methods Mol. Biol. 109, 145–159
27. Besadoun, A., Hughes, L. B., Melford, K., Hou, J., and Braeserelme, L. (1998b) Methods Mol. Biol. 109, 151–156
28. Sendak, A. A., Melford, K., Kao, A., and Besadoun, A. (1998) J. Lipid. Res. 39, 633–646
29. Belfrage, P., and Vaughan, M. (1969) J. Lipid. Res. 10, 341–344
30. de Man, F. H., de Beer, P., van der Laarse, A., Smelt, A. H., and Havelkes, L. M. (1972) J. Lipid. Res. 88, 245–2472
31. Redgrave, T. G., Roberts, D. C., and West, C. E. (1975) Anal. Biochem. 65, 42–49
32. Renssen, P. C. N., and van Berkel, T. J. C. (1996) J. Biol. Chem. 271, 14791–14799
33. Troussard, A. A., Khallou, J., Mann, C. J., André, P., Strickland, D. K., Bihain, B. E., and Yen, P. (1995) J. Biol. Chem. 270, 17668–17671
35. Van Dijk, M. C., Kruijt, J. K., Boers, W., Linthorst, C., and van Berkel, T. J. C. (1992) *J. Biol. Chem.* **267**, 17732–17737
36. Argraves, K. M., Battey, F. D., MacCalman, C. D., McCrae, K. R., Gafvels M., Kozarsky, K. F., Chappell, D. A., Strauss, J. F., III, and Strickland, D. K. (1995) *J. Biol. Chem.* **270**, 26550–26557
37. Takahashi, S., Suzuki, J., Kohno, M., Oida, K., Tamai, T., Miyake, S., Yamamoto, T., and Nakai, T. (1995) *J. Biol. Chem.* **270**, 15747–15754
38. Frykman, P. K., Brown, M. S., Yamamoto, T., Goldstein, J. L., and Herz, J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 8453–8457
39. Chappell, D., Fry, G. L., Walnitz, M. A., Iverius, P. H., Williams, S. E., and Strickland, D. K. (1992) *J. Biol. Chem.* **267**, 25764–25767
40. Beisiegel, U., Weber, W., and Bergtsson-Olivecrona, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8342–8346
41. Nielsen, M. S., Jacobsen, C., Olivecrona, G., Gliemann, J., and Petersen, C. M. (1999) *J. Biol. Chem.* **274**, 8832–8836
42. Willems van Dijk, K. W., van Vlijmen, B. J. M., van’t Hof, H. B., van der Zee, A., Santamarina-Fojo, S., van Berkel, T. J. C., Havekes, L. M., and Hofker, M. H. (1999) *J. Lipid. Res.* **40**, 336–344
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