

# Recycling of Apolipoprotein E and Lipoprotein Lipase through Endosomal Compartments *in Vivo*\*

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We have recently described a novel recycling pathway of triglyceride-rich lipoprotein (TRL)-associated apolipoprotein (apo) E in human hepatoma cells. We now demonstrate that not only TRL-derived apoE but also lipoprotein lipase (LPL) is efficiently recycled *in vitro* and *in vivo*. Similar recycling kinetics of apoE and LPL in normal and low density lipoprotein receptor-negative human fibroblasts also indicate that the low density lipoprotein receptor-related protein seems to be involved. Intracellular sorting mechanisms are responsible for reduced lysosomal degradation of both ligands after receptor-mediated internalization. Immediately after internalization in rat liver, TRLs are disintegrated, and apoE and LPL are found in endosomal compartments, whereas TRL-derived phospholipids accumulate in the perinuclear region of hepatocytes. Subsequently, substantial amounts of both proteins can be found in purified recycling endosomes, indicating a potential resecretion of these TRL components. Pulse-chase experiments of perfused rat livers with radiolabeled TRLs demonstrated a serum-induced release of internalized apoE and LPL into the perfusate. Analysis of the secreted proteins identified ~80% of the recycled TRL-derived proteins in the high density lipoprotein fractions. These results provide the first evidence that recycling of TRL-derived apoE and LPL could play an important role in the modulation of lipoproteins *in vivo*.

Triglycerides are transported mainly by two distinct classes of triglyceride-rich lipoproteins (TRLs),<sup>1</sup> the chylomicrons and the very low density lipoproteins (VLDLs). After assembly in the intestine, chylomicrons are transported via lymph into the bloodstream, where they are converted at the endothelial surface to remnant lipoproteins through the catalytic action of

lipoprotein lipase (LPL) (for review, see Refs. 1 and 2). After lipolysis, LPL remains associated with the chylomicron remnants and, in concert with apolipoprotein (apo) E (3–5), facilitates their clearance into hepatocytes (6) via LDL receptor (LDLR) and the LDLR-related protein (LRP) (7–10). The essential role for both receptors in TRL removal *in vivo* has been demonstrated in gene knockout and gene transfer experiments (Refs. 11 and 12; for a recent review, see Ref. 13).

Several studies have used different “model particles” to investigate the intracellular processing of TRL constituents. In contrast to the lysosomal degradation of LDL-derived apoB (14),  $\beta$ -VLDL-derived apoE was identified in widely distributed vesicles and showed a slow protein degradation in mouse macrophages (15, 16). However, in the same cells,  $\beta$ -VLDL-derived lipids were delivered to perinuclear, lysosomal compartments (17). Delayed transport and degradation of TRL proteins were also observed in hepatoma cells (18, 19). In recent studies, we have been able to demonstrate that the altered transport and retarded degradation of internalized TRLs is due to intracellular disintegration and sorting of TRL components in a peripheral cellular compartment. Whereas lipids are directed to lysosomal compartments in human hepatoma cells and fibroblasts, TRL-derived apoE and apoC are recycled back to the cell surface, where resecretion can occur (20). Accumulating evidence indicates that the complex intracellular processing of TRL constituents also exists *in vivo*. An increased intracellular resistance to lysosomal degradation of apoE compared with cholesteryl oleate was demonstrated in C57Bl/6 mice after hepatic uptake of triglyceride-rich emulsion particles (21). Furthermore, Fazio and co-workers (22, 23) identified significant amounts of internalized apoE derived from  $\beta$ -VLDL in Golgi-enriched fractions of mouse liver. These findings indicate that processing of internalized apoE might occur through distinct endosomal compartments.

In this study, we addressed the question of whether recycling of TRL-derived apoE and LPL could play a role in hepatic lipoprotein metabolism *in vivo*. The disintegration of TRL particles within sorting endosomes could be demonstrated in rat liver, where the TRL lipids can be detected in lysosomal compartments, whereas TRL-derived apoE and LPL are found in a peripheral endosomal compartment. Mobilization and subsequent resecretion of TRL-derived apoE and LPL are induced in the presence of serum. These data suggest that apoE and LPL recycling plays an important role in apoE enrichment of HDL precursors and reutilization of LPL in the space of Disse.

## MATERIALS AND METHODS

**Antibodies and Reagents**—1,1'-Dioctadecyl-3,3',3'-tetramethylindocarbocyanine (DiI) was purchased from Molecular Probes (Leiden, the Netherlands). 17- $\alpha$ -Ethinyl estradiol, paraformaldehyde, nucleus stain Hoechst 33342, glycine, and bovine serum albumin were purchased from Sigma. Mowiol®4-88 was purchased from Calbiochem. Dulbecco's modified Eagle's medium, phosphate-buffered saline, fetal

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<sup>1</sup> The abbreviations used are: TRL, triglyceride-rich lipoprotein; apo, apolipoprotein; CURL, compartment of uncoupling of receptors and ligands; FPLC, fast performance liquid chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; LRP, low density lipoprotein-related protein; MVB, multivesicular body; RRC, receptor recycling compartment; VLDL, very low density lipoprotein; DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; FH, familial hypercholesterolemia; RAP, receptor-associated protein.

calf serum, trypsin, penicillin, and streptomycin were purchased from Life Technologies, Inc. [ $^{125}$ I]Iodine was from Amersham Pharmacia Biotech. Heparin (Liquemin®) and tetrahydrolipstatin (Orlistat®) were purchased from Roche Molecular Biochemicals. Bovine dimeric LPL and  $^{125}$ I-LPL were obtained from Dr. G. Olivecrona (Umea, Sweden). Recombinant RAP was provided by Dr. S. K. Moestrup (Aarhus, Denmark). Polyclonal antibody against LDL receptor was obtained from J. Herz (Dallas, TX). The affinity-purified sheep anti-LRP antibody (AB104-97) was raised against a synthetic peptide corresponding to the final 14 amino acids of the human LRP C terminus (CGRG-PEDEIGDPLA) (24). Polyclonal antibody against human apoE was from Dako. Horseradish peroxidase and dichlorotriazinyl-fluorescein-conjugated goat anti-rabbit and donkey anti-sheep F(ab')<sub>2</sub> fragments were purchased from Jackson Immuno Research (Dianova, Hamburg, Germany).

**Cell Culture**—Human HuH7 hepatoma cells and human fibroblasts were plated in 6-well plates (Nunc) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and penicillin/streptomycin at 37 °C in 5% CO<sub>2</sub>. Cells were used after 2 days in culture.

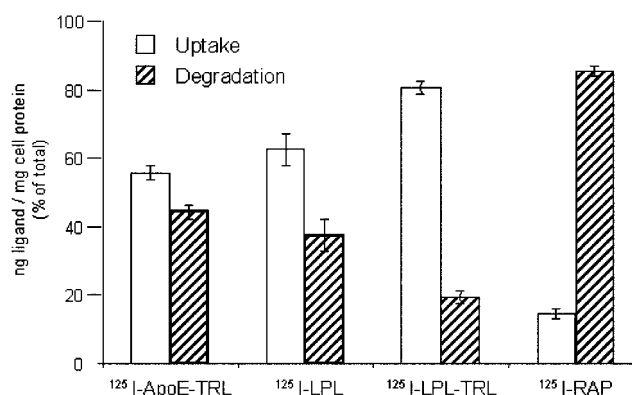
**Ligand Preparation**—TRLs from an apoCII-deficient patient were isolated and associated with 100 μg of labeled or unlabeled LPL (LPL-TRL) and/or apoE (apoE/LPL-TRL, apoE-TRL) at 37 °C for 30 min as indicated. Unbound apoE and LPL proteins were removed by ultracentrifugation as described previously (20). Radiolabeled LDL, TRLs, and fluorescence-labeled DiI-LPL-TRLs were prepared by the iodine monochloride method and incorporation of DiI as described previously (20). The protein content of the different radiolabeled TRL preparations was 0.21 ± 0.05 mg/ml, and the specific radioactivity in the protein was 80–170 cpm/ng. The protein content of  $^{125}$ I-LDL was 1.4 mg/ml, and the specific radioactivity was 90 cpm/ng. Protein content for DiI-LPL-TRL was 0.25 mg/ml. apoE 3/3, prepared by preparative SDS-PAGE (20), and RAP were iodinated by the IODOGEN method according to the manufacturer's instructions (Pierce). The specific radioactivity of different TRL preparations was 200 cpm/ng for  $^{125}$ I-apoE-TRL, 450 cpm/ng for  $^{125}$ I-LPL-TRL, and 320 cpm/ng for  $^{125}$ I-apoE/ $^{125}$ I-LPL-TRL, respectively. All radiolabeled ligands were separated by 10% SDS-PAGE and checked by autoradiography.

**Uptake, Degradation, and Recycling Assays**—Cultured cells were incubated with the different radiolabeled ligands as described previously (25, 26). After removal of surface-bound ligands with 770 units/ml heparin, cells were incubated at 37 °C for various time points in Dulbecco's modified Eagle's medium with 10% human plasma. The radioactivity of internalized, degraded, and recycled radiolabeled proteins was determined as described previously (26).

**Indirect Immunofluorescence**—Male Sprague-Dawley rats were injected with 200 μl of DiI-labeled LPL-TRL (0.5 μg/μl protein). Rats were sacrificed 20 min after injection, and cryosections (8 μm) from liver were prepared. For immunofluorescence labeling, sections were blocked with 2% bovine serum albumin and incubated overnight at 4 °C with an antibody against human apoE. To visualize the primary antibody, we used immune-adsorbed dichlorotriazinyl-fluorescein-conjugated F(ab')<sub>2</sub> fragments goat anti-rabbit. Finally, sections were washed with phosphate-buffered saline containing nucleus stain Hoechst 33342 and subjected to phase-contrast and confocal laser scanning microscopy using a Zeiss axiovert 100 and Leica TCS (Leica Lasertechnik, Heidelberg, Germany), respectively.

**Isolation of Rat Liver Endosomes**—Male Sprague-Dawley rats (200–250 g) were treated with 17- $\alpha$ -ethynyl estradiol, and endosomes from rat liver homogenates were isolated as described previously (27). Rats were anesthetized with diethyl ether, and 1–2 mg of human  $^{125}$ I-LDL or 0.1 mg of  $^{125}$ I-apoE/ $^{125}$ I-LPL-TRL was injected into a femoral vein. 20 min after injection, the portal vein was cannulated, livers were flushed thoroughly with 150 ml of ice-cold 0.15 M NaCl and removed, and endosomes were prepared as described previously (27). Three distinct endosomal populations (MVBs, CURL, and RRC) were obtained and stored at –80 °C before further analysis.

**Rat Liver Perfusion and FPLC Analysis**—Male Sprague-Dawley rats were anesthetized with diethyl ether. After closure of the arteria hepatica, arteria mesenterica, and vena cava inferior, the vena portae was cannulated, and rat livers were washed with Krebs-Hanseleit buffer for 5 min (2 ml/5 min). Radiolabeled  $^{125}$ I-apoE/ $^{125}$ I-LPL-TRL was added, and after a single pass of the radiolabeled ligand through rat livers, the system was washed extensively with heparin (100 units/ml) containing Krebs-Hanseleit buffer to remove noninternalized  $^{125}$ I-apoE/ $^{125}$ I-LPL-TRL. After 5–10 min, 10% human serum or 25 μg/ml HDL<sub>3</sub> was added to the washing buffer and passed through rat liver (2 ml/5 min); samples were collected every 5 min after passage through the perfused



**FIG. 1. Internalization and degradation of TRL-associated proteins in hepatoma cells.** Human HuH7 hepatoma cells were incubated for 6 h at 37 °C with  $^{125}$ I-apoE-TRL,  $^{125}$ I-LPL,  $^{125}$ I-LPL associated with apoE-containing TRL ( $^{125}$ I-LPL-TRL), and  $^{125}$ I-RAP as indicated. To determine protein degradation, the media were harvested, the trichloroacetic acid-precipitable material was removed, and the content of  $^{125}$ I-tyrosine in the supernatant was determined (see "Materials and Methods"). Cells were washed and lysed in 0.1 N NaOH, and internalized radioactivity was determined. Values of specific uptake (□) and degradation (▨) are given as a percentage of total metabolized radioactivity and represent the mean ± S.D. of four separate experiments with triplicate samples.

liver, and the secreted radioactivity was determined (28).

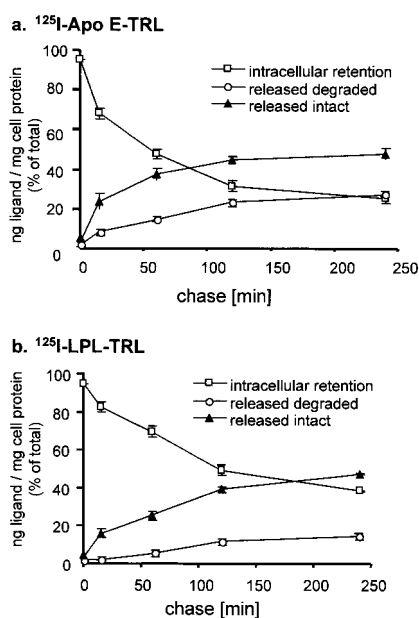
10 ml of the chase perfusate was centrifuged through a centricon filter unit (exclusion size, 10 kDa; Amicon) to separate degraded lipoproteins from the remaining intact lipoproteins. 200 μl of the intact lipoproteins was separated on a Sepharose G6 column (Amersham Pharmacia Biotech) in 100 mM NaCl and 10 mM Tris-HCl, pH 8.0. Fractions of 500 μl were collected, and the radioactivity was determined. Cholesterol was measured in each fraction (Monotest®; Roche Molecular Biochemicals). Pooled lipoprotein fractions were prepared and separated by 10% SDS-PAGE to visualize radiolabeled apolipoproteins by autoradiography.

## RESULTS

**Intracellular Processing of apoE and LPL Derived from Tri-glyceride-rich Lipoproteins**—To study the intracellular fate of apoE and LPL associated with TRLs, we compared the uptake and degradation of LPL (55 kDa) and TRL-associated LPL with TRL-associated apoE (34 kDa). Another ligand for lipoprotein receptors (12), the RAP protein (39 kDa), served as a control for the lysosomal pathway (Fig. 1). As expected ~90% of internalized RAP was targeted to lysosomes and degraded. In contrast, only 35–45% of endocytosed LPL and TRL-derived apoE was degraded, respectively. The reduced degradation was most pronounced for cells incubated with LPL and apoE-containing TRLs (20%; see Fig. 1). These results indicate that complementary properties of apoE and LPL are responsible for efficient avoidance of lysosomal degradation.

To characterize the pathway of TRL-associated apoE and LPL in more detail, pulse-chase experiments were performed. Recycling and degradation of both proteins were determined after HuH7 cells were incubated for 60 min with  $^{125}$ I-apoE-TRL (Fig. 2a) and apoE-containing TRL associated with  $^{125}$ I-LPL (Fig. 2b). Consistent with the results described above (Fig. 1), only a minor proportion (30% at  $t = 240$  min) of TRL-associated apoE was degraded, whereas ~50% of intact radiolabeled apoE was secreted back into the media within 120 min. Furthermore, we observed a reduced (2-fold) degradation and similar recycling kinetics of  $^{125}$ I-LPL from apoE-containing  $^{125}$ I-LPL-TRL (Fig. 2b) compared with  $^{125}$ I-apoE-TRL (Fig. 2a). This indicates that after receptor-mediated endocytosis of TRLs, both proteins promote sorting mechanisms to escape lysosomal degradation.

**Recycling of TRL-associated LPL and apoE Occurs in FH Fibroblasts**—To study the different role of the LDL receptor

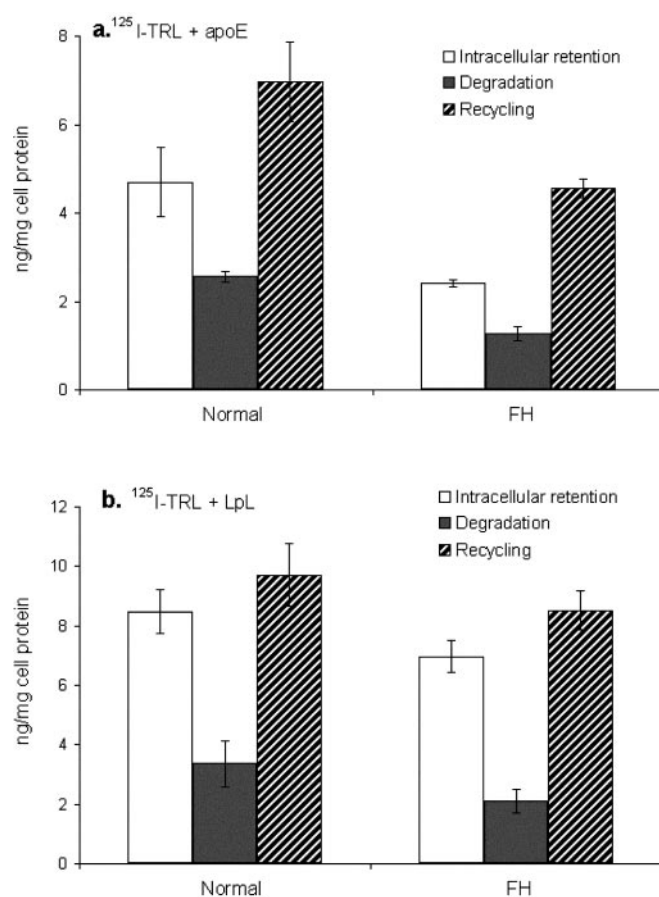


**FIG. 2. Recycling of TRL-associated proteins in hepatoma cells.** Pulse-chase experiments were performed by preincubating HuH7 hepatoma cells with <sup>125</sup>I-apoE-TRL (a) and apoE-containing TRLs associated with <sup>125</sup>I-LPL (<sup>125</sup>I-LPL-TRL; b) for 60 min at 37 °C (see “Materials and Methods”). Cells were washed at 4 °C, and cell-bound material was removed by heparin. After incubation for an additional 0–240 min at 37 °C, the media were collected to determine intact (▲) and degraded (○) <sup>125</sup>I-apoE and <sup>125</sup>I-LPL proteins. The remaining cells were lysed, and radioactivity was defined as the cell-associated fraction (□). The data are given as a percentage of total radioactivity and represent the mean ± S.D. of four independent experiments with triplicate samples for each time point.

and LRP for resecretion of TRL-associated apoE and LPL, normal fibroblasts and fibroblasts from patients with FH were analyzed. As shown in Fig. 3, normal fibroblasts efficiently internalized and recycled <sup>125</sup>I-TRL enriched with apoE or LPL, whereas degradation played only a minor role in intracellular TRL processing. In these experiments, FH fibroblasts demonstrated a significant reduction of apoE-enriched TRL internalization as compared with normal fibroblasts. However, the relative amount of recycled TRL constituents remained unaltered (Fig. 3a). Fig. 3b demonstrates that similar amounts of LPL-enriched TRLs were internalized in normal and FH fibroblasts. Both fibroblast cell lines resecreted ~50% of LPL-enriched <sup>125</sup>I-TRL radiolabeled proteins after a 90-min chase (Fig. 3b). Thus, the efficient uptake, degradation, and recycling of TRL-derived apoE and LPL in FH fibroblasts and the marginal expression of other LDLR family lipoprotein receptors in these cells suggest that LRP plays an important role for endocytosis and the intracellular processing of TRL components.

**TRL-derived apoE and LPL Are Present in Recycling Endosomes in Vivo**—To investigate the presence of TRL-associated apoE and LPL in recycling endosomes *in vivo*, male Sprague-Dawley rats were injected with DiI-TRL associated with LPL (DiI-LPL-TRL), and rat liver sections were analyzed 20 min after injection (Fig. 4). As shown in Fig. 4a, DiI-containing vesicles derived from DiI-LPL-TRL were found intracellularly, with a significant proportion located at the perinuclear region of hepatocytes that have been demonstrated to represent lysosomal-associated membrane protein-1-positive (pre-) lysosomes (20).

To compare this lipid staining pattern with DiI-LPL-TRL-derived apoE, immunofluorescence analysis of rat liver sections was performed (Fig. 4, b–d). A specific antibody against human apoE was utilized to exclude endogenous rat apoE detection. Whereas apoE was widely distributed within the cyto-

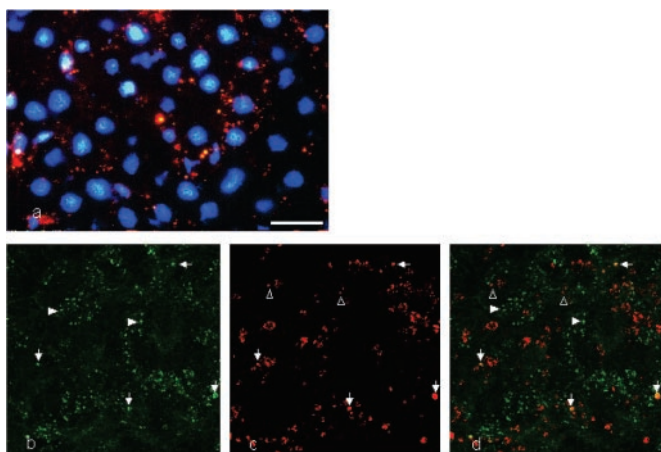


**FIG. 3. Intracellular processing of TRL-associated proteins in normal and FH fibroblasts.** Pulse-chase experiments were performed by preincubating LPDS-treated human fibroblasts (Normal) and human FH fibroblasts (FH) with <sup>125</sup>I-TRL enriched with apoE (a) and apoE-containing <sup>125</sup>I-TRL associated with LPL (b) for 60 min at 37 °C (see “Materials and Methods”). Cells were washed at 4 °C, and cell-bound material was removed by heparin. After incubation for an additional 90 min at 37 °C, the media were collected to determine degraded and recycled <sup>125</sup>I-TRL proteins. The remaining cells were lysed as described in the Fig. 2 legend. The data are given in ng/mg cell protein and represent the mean ± S.D. of three (a) and six (b) independent experiments with duplicate samples.

plasm of rat hepatocytes (Fig. 4b), DiI was again localized in part at the perinuclear region (Fig. 4c). The merged image of the confocal analysis demonstrated that the majority of apoE-containing endosomal compartments did not colocalize with phospholipid representing DiI (Fig. 4d). The rare appearance of yellow spots suggests that DiI-LPL-TRL uptake via receptor-mediated endocytosis is followed by a rapid sorting mechanism leading to a different intracellular fate of DiI-LPL-TRL-derived apoE and DiI.

To confirm these observations biochemically, hepatic recycling endosomes were isolated, and their content of TRL-derived apoE and LPL or LDL-derived apoB<sub>100</sub> was compared. The subcellular fractionation developed by Belcher *et al.* (27) separates endosomal fractions on the basis of their morphology: CURL represents the early endocytic compartment involved in the sorting of ligands, whereas MVBs correspond to late endosomes and prelysosomal structures. The third endosomal fraction, the RRC, is characterized by tubular structures that originate from membranous appendages emanating from CURL and MVBs that are enriched in recycling receptors and depleted of ligands (29, 30).

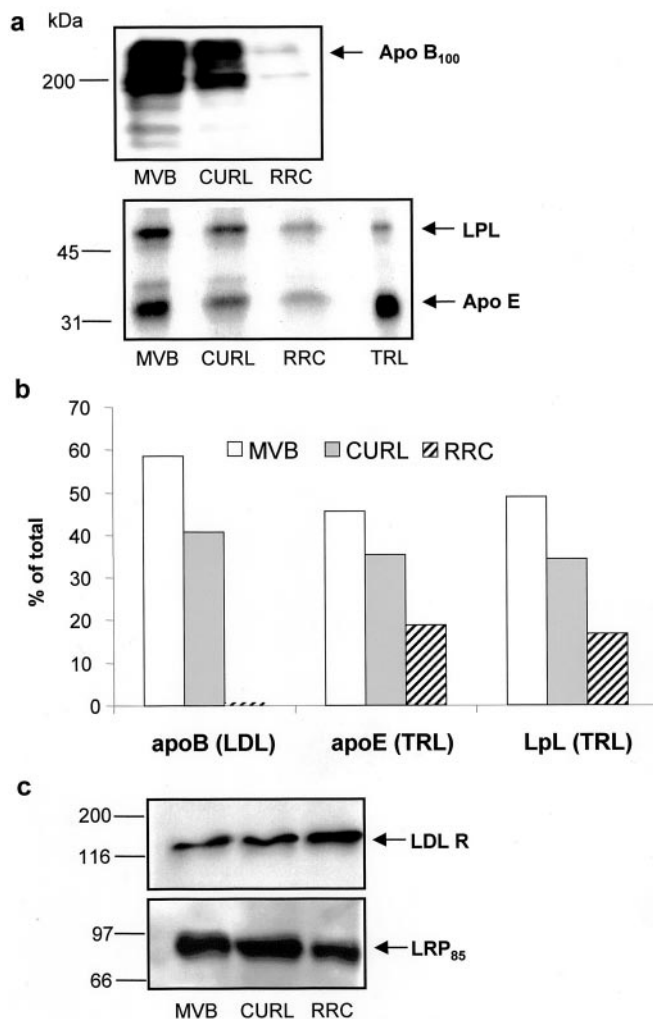
Therefore MVB, CURL, and RRC endosomes from rat liver were prepared 20 min after injection of double-labeled <sup>125</sup>I-apoE/<sup>125</sup>I-LPL-TRL and <sup>125</sup>I-LDL. As expected, radiolabeled



**FIG. 4. Localization of apoE and phospholipids (DiI) from LPL-TRL in rat liver cells.** DiI-labeled LPL-TRLs (250  $\mu$ g of protein) were injected into the femoral vein. The liver was removed 20 min after injection and stored at  $-80^{\circ}\text{C}$ . Frozen sections (5  $\mu\text{m}$  thick) were fixed in methanol, washed with phosphate-buffered saline, and analyzed by fluorescence microscopy for DiI (red) distribution and DAPI-stained nuclei (a). Confocal microscopy of apoE (b) and DiI (c) in the same liver sections was performed. Arrows demonstrate colocalization of apoE and DiI in the merged image (d; yellow). Filled (apoE) and open (LPL) arrowheads point out noncolocalized staining of apoE or DiI, respectively. Bar, 10  $\mu\text{m}$ .

LDL-derived  $^{125}\text{I}$ -apoB<sub>100</sub> was found predominantly in the prelysosomal MVB fraction as judged by SDS-PAGE analysis (Fig. 5a) and subsequent quantification (55–60%; see Fig. 5b). A substantial amount (40%) of  $^{125}\text{I}$ -apoB<sub>100</sub> was still present in sorting endosomes (CURL) that will ultimately be processed for lysosomal degradation via the MVB fraction after prolonged incubation periods (31). Only a minor proportion of  $^{125}\text{I}$ -apoB<sub>100</sub> (1–2%) was detectable in the recycling endosome fraction (RRC), which confirmed the purity of the recycling endosomal fraction analyzed. Similar experiments were performed with  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL that contained an approximately 5:1 apoE:LPL ratio, respectively (Fig. 5a, TRL). apoE and LPL were detectable in similar amounts in MVBs (45–50%) and CURL (35–40%), and ~20% of apoE and LPL was found in recycling endosomes (Fig. 5, a and b, RRC). As described previously (29), Western blot analysis of MVB, CURL, and RRC endosomal fractions identified the presence of LDLR and LRP (Fig. 5c). The presence of both lipoprotein receptors in hepatic MVB endosomes demonstrates that this fraction contains substantial amounts of recycling endosomes, which has also been demonstrated by others (32). Taken together, the presence of apoE and LPL and the lipoprotein receptors in the MVB and RRC endosomes indicate that both fractions contain significant amounts of recycling endosomes from which resecretion of apoE and LPL can occur.

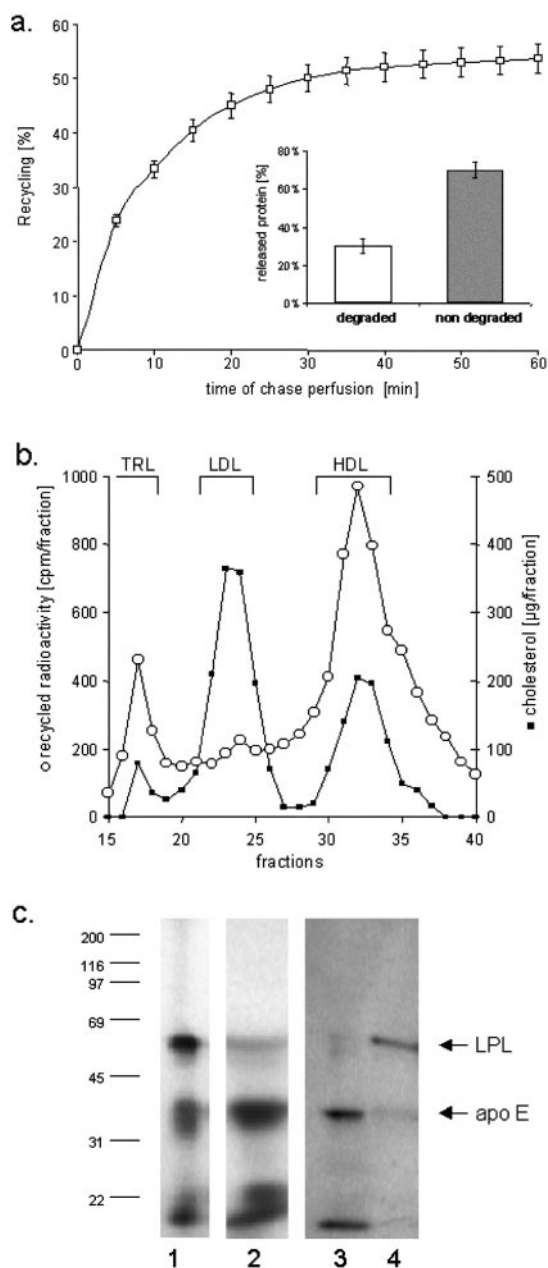
**Recycling of TRL Constituents in Vivo**—The presence of apoE and LPL in recycling endosomes indicated significant recycling of endocytosed TRL constituents *in vivo*, and therefore we analyzed the potential resecretion of internalized TRL proteins in a perfused rat liver system. After single-pass endocytosis of  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL, surface-bound material was removed with heparin. Subsequently, 10% human serum was passed through the perfused rat liver system. The released TRL-derived radioactivity was collected and shown to represent mainly intact protein (see Fig. 6a, inset). As shown in Fig. 6a, ~50% of internalized  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL was recycled after 60 min. These findings are in agreement with the results obtained from cell culture experiments (Figs. 2 and 3; see also Refs. 20 and 23). Analysis of the recycled TRL-derived apoE and LPL demonstrated a more efficient recycling of apoE com-



**FIG. 5. Distribution (a) and quantitative analysis (b) of apoB-labeled LDL and TRL-derived proteins in rat liver endosomes.** Rat liver MVB, CURL, and RRC endosomal membranes were isolated after injection of  $^{125}\text{I}$ -LDL or double-labeled  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL, and 100  $\mu\text{g}$  of each fraction was analyzed by 10% SDS-PAGE. Arrows indicate the presence of radiolabeled LDL-derived  $^{125}\text{I}$ -apoB<sub>100</sub> or TRL-derived  $^{125}\text{I}$ -apoE and  $^{125}\text{I}$ -LPL (a). Gels were dried, and autoradiographs were obtained after overnight exposure at  $-80^{\circ}\text{C}$ . Molecular masses are given in kDa. Quantitative analysis of the radiolabeled  $^{125}\text{I}$ -apoB<sub>100</sub> from LDL,  $^{125}\text{I}$ -apoE, and  $^{125}\text{I}$ -LPL from apoE-containing TRLs in MVB (□), CURL (▒), and RRC (▨) was determined by densitometric scanning of the autoradiographs and is given as a percentage of the total protein detected (b). The data represent one of two independent experiments with two animals analyzed. c, immunoblot analysis of LDL receptor and LRP in membrane preparations of rat liver endosomes. Endosomal proteins from MVB, CURL, and RRC membranes (50  $\mu\text{g}$  each) were separated by 10% SDS-PAGE and analyzed by Western blot for LDL receptor and the 85-kDa LRP protein fragment as indicated. Molecular mass is given in kDa.

pared with LPL (Fig. 6c, compare lanes 1 and 2).

Separation of the lipoproteins by FPLC was performed to analyze the possible association of recycled TRL proteins with lipoprotein particles. Only a minor proportion of radiolabeled material was detected in fractions 16–18 representing the TRL fraction (Fig. 6b). In contrast, HDL fractions 31–34 contained ~80% recycled  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL proteins. SDS-PAGE of the radioactive material identified predominantly  $^{125}\text{I}$ -apoE (Fig. 6c, lane 3) in the HDL fractions, whereas the majority of  $^{125}\text{I}$ -LPL was detected in the TRL fractions (Fig. 6c, lane 4). These experiments provide the first demonstration that efficient recycling of internalized TRL proteins might participate in the alteration of plasma lipoprotein composition.



**FIG. 6. a.** Recovery of recycled  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL after rat liver perfusion.  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL were passed through the perfused rat liver. After 5 min (flow rate, 2 ml/5 min), noninternalized radiolabeled TRLs were removed with heparin (100 units/ml)-containing Krebs-Hanseleit buffer until the remaining radioactivity reached background levels in the perfusate. Recycling was induced with 10% human serum, and samples were collected every 5 min thereafter to determine the radioactivity resecreted from the liver. The amount of released TRL components was calculated at each time point (percentage of internalized) and represents the mean  $\pm$  S.D. of four animals analyzed. The amount of intact and degraded radioactivity in the resecreted material was determined by size exclusion ( $<10$  kDa) and is given as a percentage (see inset). **b.** FPLC analysis of recycled TRL-derived  $^{125}\text{I}$ -apoE and  $^{125}\text{I}$ -LPL. The nondegraded radioactivity from **a** (inset) was separated by a Sepharose G6 column (see "Materials and Methods"), and 500- $\mu\text{l}$  fractions were collected. Cholesterol ( $\blacksquare$ ) and radioactivity ( $\circ$ ) were determined in each fraction and show the mean of a representative experiment ( $n = 4$ ) with triplicate samples. The fractions containing TRL (fractions 16–18), LDL (fractions 23–25), and HDL (fractions 31–35) are indicated. **c.** autoradiography of recycled TRL-derived  $^{125}\text{I}$ -apoE and  $^{125}\text{I}$ -LPL. The  $^{125}\text{I}$ -apoE/ $^{125}\text{I}$ -LPL-TRL preparation before injection (lane 1) and the recycled TRL-derived  $^{125}\text{I}$ -apoE and  $^{125}\text{I}$ -LPL before FPLC separation (lane 2) are shown. After FPLC, the HDL (lane 3) and TRL (lane 4) fractions were collected, pooled, and separated by 10% SDS-PAGE. The positions of radiolabeled apoE and LPL are indicated. Molecular mass is given in kDa.

## DISCUSSION

We have recently proposed a model of intracellular TRL processing that comprises both recycling and degradation of TRL components (6, 20). In the current study, we demonstrated that significant amounts of  $^{125}\text{I}$ -apoE-TRL and even more apoE-containing  $^{125}\text{I}$ -LPL-TRL were not degraded but recycled back to the cell surface. Therefore, the association of LPL with apoE-containing TRLs not only stimulates TRL internalization but also reduces its lysosomal degradation compared with apoE-TRL (Figs. 1 and 2). Although internalization of apoE-containing lipoproteins is thought to be mediated in part by cell surface heparan sulfate proteoglycans (2), recent experiments have demonstrated that both LRP and the LDL receptor (11) are essential for hepatic uptake of TRL lipoproteins. However, the possible involvement of the two lipoprotein receptors in the recycling of apoE is a topic of disagreement (21, 22), which might be due to the different "model particles" used in these studies. In agreement with the recycling of apoE in LDLR(-/-) mice (22), our results imply that LRP probably participates in the intracellular processing of human TRL-derived apoE and LPL, leading to the recycling of both proteins (Fig. 3). Results presented here indicate that the composition of ligands seems to determine their specific intracellular fate. Thus, the high binding affinities of multivalent ligands on lipoproteins to receptors seem to play an important role in their intracellular metabolism. In support of this hypothesis, the most likely altered ligand binding affinity of apoE3 and E4-enriched  $\beta$ -VLDL has recently been shown to result in different intracellular processing after internalization (33).

The main focus of our studies was to characterize TRL processing *in vivo*. As observed in cell culture experiments (20), the disintegration of TRLs leads to a peripheral, endosomal distribution of apoE, whereas the majority of lipids seem to accumulate in the perinuclear, prelysosomal compartment (Fig. 4). These findings correlate with the relative resistance of apoE against degradation and the concomitant hydrolysis of cholesterol ester from internalized apoE-cholesterol ester-labeled liposomes in mouse liver (21). In addition, Fazio *et al.* (22) demonstrated the reutilization of internalized apoE for VLDL assembly in Golgi-enriched fractions. In support of these observations, we detected apoE and LPL in three different endosomal preparations from rat liver during analysis of the intracellular fate of internalized TRL protein components *in vivo*.

First of all, CURL represents the early endosomal sorting compartment and contains internalized radiolabeled LDL or TRL that will subsequently be directed to either RRC or MVB vesicles (30). RRC endosomal preparations contain substantial amounts of TRL-derived apoE and LPL (Fig. 5). Although RRC fractions are highly enriched for recycling proteins (*e.g.* transferrin or LDL receptor), it has been demonstrated that these preparations also contain 5'-nucleotidase and sialyl-transferase activity specific for Golgi secretory vesicles (29). These findings correlate with the presence of apoE in Golgi-enriched fractions (22) and indicate that recycling of apoE and LPL via RRC could be mediated in part by Golgi-derived secretory vesicles. In addition, significant amounts of radiolabeled apoE and LPL were found in MVBs, which represent predominantly late endosomes. However, because LRP and LDL receptor (Fig. 5c) can be found in the MVB fraction, it can be postulated that a significant portion of recycling endosomes is present in this fraction, as also observed by others (29). These vesicles, in addition to RRC endosomes, are likely to be responsible for apoE and LPL recycling.

A number of studies have recently postulated the recycling of internalized apoE in mice hepatocytes (21–23). We now provide direct evidence that TRL-derived apoE and LPL are efficiently

recycled and resecreted *in vivo* (Fig. 6a). Because we have recently described that HDL serves as an extracellular acceptor for the resecretion of apoE (20), HDL was utilized to stimulate apoE and LPL resecretion after TRL internalization in perfused rat livers (Fig. 6b). Similar to our observations *in vitro* (Ref. 20; see also Fig. 2) we determined an ~60% resecretion of internalized TRL-derived apoE and LPL. Only a minor proportion of radioactivity, representing predominantly LPL, was detected in the VLDL fraction after FPLC and SDS-PAGE analysis (Fig. 6, b and c). In contrast, the majority of resecreted apoE-derived radioactivity was found in HDL, indicating a reutilization of TRL-derived apoproteins for HDL modulation. These apoE-enriched HDL particles would provide a pool of apoE proteins in the plasma, possibly serving as an apoE donor for intravascular transfer to chylomicrons during lipolysis. Although endogenously synthesized apoE might be able to fulfill this function, recycling of apoE would provide a more readily available pool to promote apoE-mediated chylomicron remnant uptake in the postprandial state.

In conclusion, we have demonstrated that significant amounts of internalized TRL-derived apoE and LPL escape the lysosomal pathway and are targeted in a new recycling compartment for resecretion. The resecreted apoE and LPL seem to participate in the modulation of VLDL and HDL *in vivo*. Because apoE recycling depends on the presence of extracellular HDL, future experiments will have to clarify a potential regulatory role of apoE recycling in HDL-induced cholesterol efflux or HDL catabolism.

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