THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 278, No. 16, Issue of April 18, pp. 14370–14378, 2003

Printed in U.S.A.

Recycling of Apoprotein E Is Associated with Cholesterol Efflux and High Density Lipoprotein Internalization*

Received for publication, September 4, 2002, and in revised form, February 10, 2003
Published, JBC Papers in Press, February 12, 2003, DOI 10.1074/jbc.M209006200

Joerg Heeren‡§, Thomas Grewal‡, Alexander Laatsch‡¶, Daniel Rottke‡‖, Franz Rinninger, Carlos Enrich‡‡, and Ulrike Beisiegel‡**

*From the ‡Institute for Medical Biochemistry and Molecular Biology, the Department of Molecular Cell Biology and the **Department of Internal Medicine, University Hospital Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany and ‡‡Departament de Biologia Cellular, Institut d'Investigacions Biomèdiques August Pi i Sunyer, Facultat de Medicina, Universitat de Barcelona, 08036 Barcelona, Spain*

After receptor-mediated endocytosis of triglyceride-rich lipoproteins (TRL) into the liver, TRL particles are immediately disintegrated in peripheral endosomal compartments. Whereas core lipids and apoprotein B are delivered for degradation into lysosomes, TRL-derived apoE is efficiently recycled back to the plasma membrane. This is followed by apoE re-secretion and association of apoE with high density lipoproteins (HDL). Because HDL and apoE can independently promote cholesterol efflux, we investigated whether recycling of TRL-derived apoE in human hepatoma cells and fibroblasts could be linked to intracellular cholesterol transport. In this study we demonstrate that HDL_3 does not only act as an extracellular acceptor for recycled apoE but also stimulates the recycling of internalized TRL-derived apoE. Furthermore, radioactive pulse-chase experiments indicate that apoE recycling is accompanied by cholesterol efflux. Confocal imaging reveals co-localization of apoE and cholesterol in early endosome antigen 1-positive endosomes. During apoE re-secretion, HDL_3-derived apoA-I is found in these early endosome antigen 1, cholesterol-containing endosomes. As shown by time-lapse fluorescence microscopy, apoE recycling involves the intracellular trafficking of apoA-I to pre-existing and TRL-derived apoE/cholesterol-containing endosomes in the periphery. Thus, these studies provide evidence for a new intracellular link between TRL-derived apoE, cellular cholesterol transport, and HDL metabolism.

Chylomicrons (CM)[1] and very low density lipoproteins (VLDL) represent the two classes of triglyceride-rich lipoproteins (TRL) that are responsible for the transport of lipids to various cells of the body. After assembly in the intestine, CM mediate the transport of dietary lipids, whereas VLDL are synthesized in the liver to deliver endogenous lipids to peripheral tissues. In the bloodstream CM are hydrolyzed by lipoprotein lipase (LPL), resulting in the formation of chylomicron remnants (CR) (for review see Refs. 1–3). During lipolysis these remnants become enriched with high density lipoproteins (HDL)-derived apoprotein E (apoE) and are rapidly cleared from the plasma in a process known to depend on apoE and LPL via the LDL receptor-related protein and the low density lipoprotein (4–7).

After receptor-mediated endocytosis, the intracellular fate of TRL constituents is far more complex than the classical degradation pathway of low density lipoproteins (LDL) described by Brown and Goldstein (8). A number of studies revealed that the intracellular processing of TRL first involves disintegration in peripheral endosomal compartments, which is followed by a differential sorting of TRL components (9). Using β-VLDL as a "TRL model particle," Maxfield and co-workers (10–12) identified β-VLDL-derived lipids in lysosomal compartments of mouse macrophages, whereas apoE was present in peripheral vesicles. Consistent with these findings, lysosomal targeting of TRL proteins and apoE was markedly reduced compared with the degradation of cholesteryl oleate in mouse hepatocytes (13). In human hepatoma cells and fibroblasts, the majority of TRL lipids are targeted to the lysosomal compartment, whereas TRL-derived apoE is found in peripheral recycling endosomes, which are distinct from the perinuclear transferrin recycling compartment (14, 15). Subsequently substantial amounts of TRL-derived apoE are recycled back to the cell surface and re-secreted (14). Recently, several studies (13, 16–18) on the hepatic TRL metabolism *in vivo* confirmed the disintegration of TRL components within the endosomal compartment and the targeting of TRL lipids to lysosomes. Most remarkably, TRL-derived apoE is recycled and found associated with newly synthesized or exogenous lipoproteins (16). Fazio and co-workers (17) observed an association of recycled apoE with nascent VLDL particles in liver Golgi fractions. Alternatively, we recently identified (14, 16) that HDL serves as a potent extracel-

* This work was supported in part by the Deutsche Forschungsgemeinschaft Grants Be 829/5-1, Ja 421/3-1, and Ri 436/8-1 and Ministerio de Ciencia y Tecnologia Grants PM99-0166, Acciones Integradas HA98-0007, and Generalitat de Catalunya BE2002 (to C. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Institute for Medical Biochemistry and Molecular Biology, Dept. of Molecular Cell Biology, University Hospital Eppendorf, Martinistrasse 52, D-20246 Hamburg, Germany. Tel.: 49-40-42803-3917; Fax: 49-40-42803-4592; E-mail: heeren@uke.uni-hamburg.de.

¶ Recipient of a fellowship from the Studienstiftung des Deutschen Volkes.

‖ Recipient of Fellowship GRK 336 from the Deutsche Forschungsgemeinschaft-financed Graduiertenkolleg.

[1] The abbreviations used are: CM, chylomicrons; AP1, adaptor protein 1; apo, apoprotein; ABCA1, ATP-binding cassette transporter A1; BSA, bovine serum albumin; Chol, cholesterol; CR, chylomicron remnants; EEA1, early endosome antigen 1; FPLC, fast performance liquid chromatography; HDL, high density lipoprotein; HuH7, human hepatoma cell line 7; LDL, low density lipoprotein; LFF, lipoprotein-free fraction; LPL, lipoprotein lipase; NBD-cholesterol, 22-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)23,24-bisnor-5-cholen-3B-ol; TRL, triglyceride-rich lipoproteins; PBS, phosphate-buffered saline; PFA, paraformaldehyde; VLDL, very low density lipoproteins; DMEM, Dulbecco's modified Eagle's medium; SR-BI, scavenger receptor class B type I; w/o, without.

lular acceptor for re-secreted apoE in hepatocytes *in vitro* and *in vivo*.

Because HDL is known to induce cholesterol efflux (19–21), we investigated whether recycling of TRL-derived apoE is accompanied by the mobilization of cellular cholesterol. In this study we demonstrate that HDL_3-inducible apoE recycling is associated with cholesterol efflux. Life cell imaging and confocal microscopy revealed that this process involves the targeting of HDL_3-derived apoA-I to EEA1-positive endosomes containing both TRL-derived apoE and cholesterol. After re-secretion, TRL-derived apoE and cholesterol are found associated with extracellular HDL particles. Taken together, these findings indicate that apoE recycling provides an efficient mechanism to re-supply plasma with apoE-containing HDL particles. This process would ultimately accelerate the enrichment of CR with apoE and stimulate the hepatic clearance of CR in the postprandial state.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Paraformaldehyde (PFA), filipin, glycine, and BSA were purchased from Sigma. Mowiol®4-88 was purchased from Calbiochem. DMEM, PBS, fetal calf serum, trypsin, penicillin, and streptomycin were from Invitrogen. Iodogen was from Pierce. ^{125}I and [^{3}H]cholesterol ([^{3}H]Chol) were from Amersham Biosciences. Heparin (Liquemin®) was purchased from Roche Molecular Biochemicals. Polyclonal antibody against human apoE was from Dako. Monoclonal antibody against early endosome antigen 1 (EEA1) was from BD Biosciences. Monoclonal antibody against adaptor protein 1 (AP1) was kindly provided by E. Ungewickell (22). Alexa 568 nm fluorescence labeling kit and NBD-cholesterol were from Molecular Probes. Cy3 and Cy5 fluorescence protein labeling kit were from Amersham Biosciences. Cy2-conjugated goat anti-rabbit $F(ab')_2$ fragments and horseradish peroxidase-conjugated goat anti-rabbit $F(ab')_2$ fragments were purchased from Jackson ImmunoResearch.

Cell Culture—Human skin fibroblasts and hepatoma cells (HuH7) were grown in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin at 37 °C in 5% CO_2.

Ligand Preparation—Human serum, LDL, and apoE-deprived HDL_3 (d = 1.125–1.21 g/ml) from normal healthy donors (23, 24) and TRL from an apoCII-deficient patient were isolated as described (25). ApoE3 was isolated by preparative SDS-PAGE (14). TRL were radiolabeled by the iodine monochloride method, whereas apoE was radiolabeled with Iodogen (16). 100 μg of unlabeled or ^{125}I-labeled apoE were associated with TRL (0.5 mg of protein) to prepare apoE-TRL (final concentration 0.28 mg of protein/ml) or ^{125}I-apoE-TRL as described (16). The protein concentrations of the ^{125}I-TRL and the ^{125}I-apoE-TRL preparations were 0.2 ± 0.05 mg/ml, and the specific radioactivity was 100–180 cpm/ng protein, respectively. ^{125}I-TRL and the ^{125}I-apoE-TRL preparations were routinely separated by 10% SDS-PAGE, and radiolabeling of TRL apoproteins was confirmed by autoradiography. For the labeling of lipoproteins with [^{3}H]Chol, 100 μl of [^{3}H]Chol (3.7 MBq) was dried under liquid nitrogen, resuspended in 100 μl of DMEM + 2% BSA, and incubated overnight with 0.5–1 mg of ^{125}I-apoE-TRL ([^{3}H]Chol/^{125}I-apoE-TRL) or 3–5 mg of LDL ([^{3}H]Chol-LDL) in PBS + 10 mM EDTA at 37 °C. Non-incorporated [^{3}H]Chol was removed by PD10 gel chromatography (Amersham Biosciences). For immunofluorescence experiments, apoE-TRL (0.2 mg) were labeled with Cy3 (Cy3-apoE-TRL), and HDL_3 apoproteins (1 mg) were labeled with Alexa 568 nm (Alexa-HDL_3) or Cy5 (Cy5-HDL_3) according to the instructions of the manufacturers. Fluorescent label was found predominantly in apoE of Cy3-apoE-TRL (~80–90%) and apoA-I of Alexa-HDL_3 or Cy5-HDL_3 (~90–95%) as determined by SDS-PAGE, respectively (data not shown). To label Cy3-apoE-TRL with NBD-cholesterol, 0.2 mg of Cy3-apoE-TRL was incubated with 250 μg of NBD-cholesterol in PBS, 1 mM EDTA at 37 °C overnight. Non-incorporated NBD-cholesterol was removed by PD10 gel chromatography. For electron microscopy, HDL_3 (600 μg; pI 5.5) and apoE3 (100 μg; pI 5.78) were labeled with colloidal gold of 5 (gold-HDL) and 12 nm gold, respectively, according to Handley *et al.* (26). Then apoE3 was associated with TRL (gold-apoE-TRL) as described above. Conjugations of gold-HDL_3 and gold-labeled apoE3 were confirmed after staining with sodium phosphotungstate by electron microscopy (data not shown).

Uptake and Recycling Assays—For radioactive pulse-chase experiments, cells were washed with PBS and incubated with ^{125}I-TRL (2.5 μg/ml) for 60 min at 37 °C in 1 ml of DMEM containing 5% BSA (pH 7.4). The cells were then washed with ice-cold PBS, and surface-bound ligands were released with 770 units/ml heparin (14, 23). To promote recycling, ^{125}I-TRL labeled cells were incubated for 60 min at 37 °C with DMEM + 0.1% BSA supplemented with 50 μg/ml HDL_3, 100 μg/ml LDL, or 10% human serum. Then the content of trichloroacetic acid-precipitable, recycled radioactivity in the harvested media was determined. Cells were solubilized in 0.1 N NaOH to calculate the amount of internalized and recycled radioactivity (14). In one set of experiments, recycled and radiolabeled TRL apoproteins from cell culture media were subjected to density gradient ultracentrifugation (KBr density from 1.006 to 1.21 g/ml) (23). After gradient fractionation, the radioactivity in each fraction was determined. The positions of lipoproteins were identified according to their density and the cholesterol profile of the gradient (data not shown). In another set of experiments, the recycled radiolabeled TRL apoproteins were separated by FPLC analysis with a Superdex 200 column (Amersham Biosciences). Cholesterol and protein profiles from standards (TRL, LDL, HDL, albumin) allowed the identification of the fractions containing lipoproteins and albumin (lipoprotein-free fraction (LFF)).

Immunofluorescence and Electron Microscopy—For immunofluorescence experiments, fibroblasts and hepatoma cells were incubated with apoE-TRL (1 μg/ml) in DMEM + 2% BSA for 60 min at 37 °C. Cells were washed in DMEM and treated with heparin at 4 °C for 15 min (see above). Then cells were incubated for an additional 0, 15, or 60 min at 37 °C in DMEM (0.1% BSA) ± 50 μg/ml unlabeled or fluorescent-labeled HDL_3 (Alexa-HDL_3, Cy5-HDL_3). Cells were washed in PBS and fixed in 4% PFA, and indirect immunofluorescence against apoE, AP1, and EEA1 was performed (14). Cellular cholesterol was visualized using 50 μg/ml filipin (27). Full cell images were taken with an Axiovert 100 microscope equipped with a Zeiss Axiocam. Confocal images were collected using a Zeiss LSM 510 (version 3.0) equipped with an UV laser to detect filipin-stained cholesterol. For living cell microscopy, fibroblasts were incubated with double-labeled NBD-cholesterol/Cy3-apoE-TRL (1 μg/ml) in DMEM + 2% BSA for 0–30 min at 37 °C. Cells were washed in DMEM and incubated for an additional 10 min with 50 μg/ml Cy5-HDL_3. Confocal images were taken every minute in the multitrack mode using optimized pinhole adjustment for each fluorochrome.

For electron microscopy, a pre-embedding procedure was performed. Hepatoma cells were incubated with 1 μg/ml gold-apoE-TRL (12 nm) in DMEM + 2% BSA for 60 min at 37 °C. Cells were washed, treated with heparin (see above), and incubated with 5 μg/ml gold-HDL_3 (5 nm) at 37 °C for 15 min. Cells were rinsed with PBS and fixed in 2% PFA, 2.5% glutaraldehyde in PBS for 60 min at room temperature. Then fixed cells were scraped and post-fixed in 2% PFA in PBS for 2 days at 4 °C. Samples were washed, treated with 1% OsO_4, 0.8% potassium ferricyanide (Sigma) in PBS, dehydrated with acetone, and embedded in Spurr resin (Sigma). Ultrathin sections were obtained and counterstained with uranyl acetate and lead citrate as described previously (28).

Western Blotting—Fibroblasts and hepatoma cells were incubated with apoE-TRL (1 μg/ml) for 60 min at 37 °C, washed, and incubated for an additional 0 or 60 min at 37 °C ± 50 μg/ml HDL_3 in DMEM + 0.1% BSA. The media were harvested, filtered (inner diameter, 0.45 μm), and cleared by centrifugation at 14,000 × g for 10 min. The supernatants were subjected to 10% SDS-PAGE and immunoblotted against apoE. After incubation with peroxidase-conjugated secondary antibodies, the reaction product was detected using the ECL system (Amersham Biosciences).

Cholesterol Efflux Experiments—In the first set of experiments, cells were incubated with double-labeled [^{3}H]Chol/^{125}I-apoE-TRL (1 μg/ml) for 60 min at 37 °C and then washed with ice-cold PBS and heparin to remove surface-bound ligands (see above). [^{3}H]Chol/^{125}I-apoE-TRL labeled cells were incubated for additional 60 min at 37 °C in DMEM + 0.1% BSA in the presence or absence of 50 μg/ml HDL_3. The media were harvested to quantify ^{125}I-apoE recycling after trichloroacetic acid precipitation (see above), whereas [^{3}H]Chol efflux was determined after Dole extraction (29). In the second set of experiments, intracellular cholesterol pools were radiolabeled overnight with [^{3}H]Chol-LDL (100,000 cpm/ml) at 37 °C. Non-internalized [^{3}H]Chol-LDL was removed by heparin treatment (see above). Then cells were incubated with or without apoE-TRL for 60 min at 37 °C. After heparin treatment (see above), cells were incubated in DMEM + 0.1% BSA in the presence or absence of 50 μg/ml HDL_3 for an additional 60 min at 37 °C. The media were harvested, and cells were lysed in 0.1 N NaOH. Dole extraction of media and cell lysates was performed to determine [^{3}H]Chol efflux.

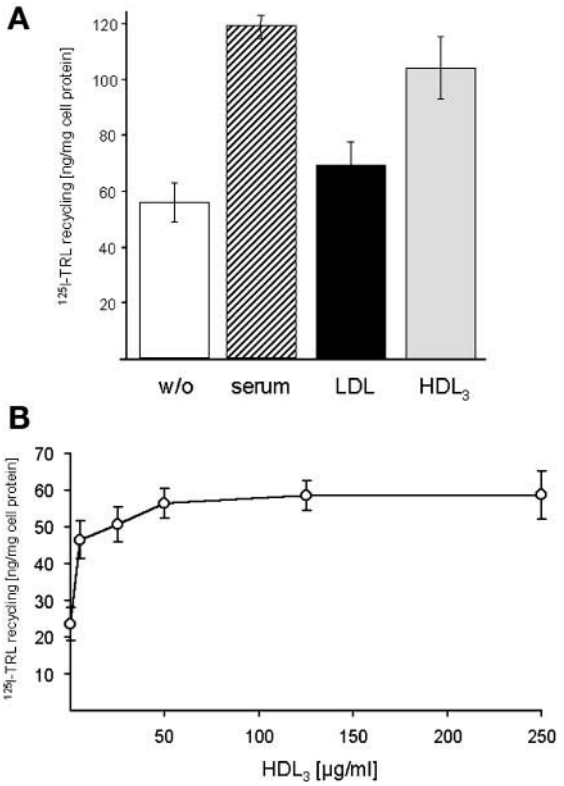

FIG. 1. **HDL-induced recycling of TRL-associated apoproteins in hepatoma cells.** Pulse-chase experiments were performed by preincubating human HuH7 hepatoma cells with ^{125}I-TRL for 60 min at 37 °C (see "Experimental Procedures"). Cells were washed at 4 °C, and cell-bound radiolabeled material was removed by heparin. After incubation for additional 60 min at 37 °C, cells were incubated with media only (0.1% BSA in DMEM; *w/o*, *white bar*) or media supplemented with 10% human serum (*hatched bar*), 100 µg/ml LDL (*black bar*), or 50 µg/ml HDL$_3$ (*gray bar*) (*A*); or with increasing amounts of HDL$_3$ (0–250 µg/ml) (*B*). The media were collected to calculate the amount of recycled, intact (trichloroacetic acid-precipitable) ^{125}I-TRL-derived apoproteins. The remaining cells were lysed, and protein content was determined. The data are given in ng of apoprotein recycling/mg of cell protein and represent the mean ± S.D. of five (*A*) and four (*B*) independent experiments with duplicate samples. Depending on the specific activity of ^{125}I-TRL preparations (100–180 cpm/ng protein, see "Experimental Procedures"), background ^{125}I-TRL recycling in the absence of HDL$_3$ was in the range of ~20–60 ng/mg cell protein.

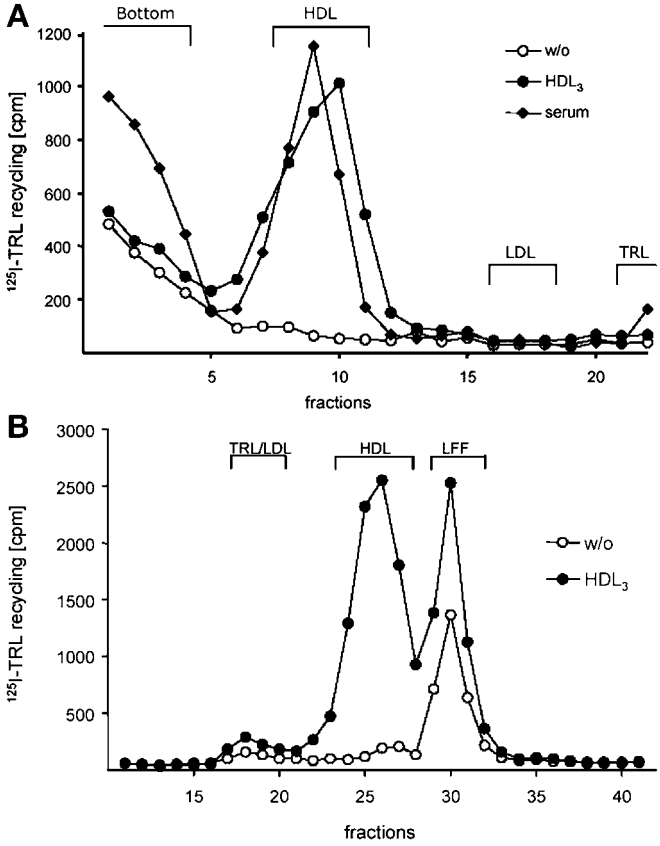

FIG. 2. **Density gradient and FPLC analysis of recycled ^{125}I-TRL-derived apoproteins.** *A*, the non-degraded radioactivity of media from cells analyzed in Fig. 1*A*, which were incubated with media alone (○, *w/o*), with 50 µg/ml HDL$_3$ (●, HDL$_3$) or 10% human serum (◆, serum) were separated by KBr density gradient (see "Experimental Procedures"). Fractions from bottom to top (500 µl) were collected, and the radioactivity was determined in each fraction. The gradient distribution (mean values of duplicate samples) of recycled ^{125}I-TRL apoproteins (counts/min) of a representative experiment is shown (n = 3). The fractions containing TRL (fractions 21 and 22), LDL (fractions 16–18), HDL (fractions 7–11), and the lipid-free bottom (fractions 1–4) are indicated and were identified by their respective density and cholesterol content. *B*, the non-degraded radioactivity of media from cells analyzed in Fig. 1*A*, which were incubated with media alone (○, *w/o*) or with 50 µg/ml HDL$_3$ (●, HDL$_3$) were separated by a Superdex 200 column (see "Experimental Procedures"); 500-µl fractions were collected, and the radioactivity was determined in each fraction. The distribution of recycled ^{125}I-TRL apoproteins (counts/min) for a representative experiment is shown (n = 3). The fractions containing TRL/LDL (fractions 17–19) and HDL (fractions 24–28) are indicated. The LFF (fractions 29–31) contain proteins in the range of 20–80 kDa. The positions of lipoproteins were identified by the comparison of cholesterol profiles from the analyzed samples with isolated preparations of TRL, LDL, and HDL, respectively.

RESULTS

HDL$_3$ Stimulates the Recycling of TRL-derived ApoE—We have demonstrated previously (9, 14, 16) that the recycling of internalized TRL-derived apoE and LPL requires the presence of an extracellular lipoprotein acceptor. To compare the ability of different lipoproteins to mediate TRL recycling, human hepatoma cells were preincubated with ^{125}I-TRL, and recycling of ^{125}I-TRL proteins was induced with human serum, LDL, or HDL$_3$ (Fig. 1*A*). Consistent with previous results (16), addition of 10% human serum stimulated recycling of internalized ^{125}I-TRL proteins 2-fold compared with the lipoprotein-free control (Fig. 1*A*, *w/o*). Similarly, in the presence of HDL$_3$ (50 µg/ml protein), a 1.8-fold increase of ^{125}I-TRL recycling was observed. This stimulatory effect of HDL$_3$ on ^{125}I-TRL recycling was already detectable at very low levels of HDL$_3$ (2.5 µg/ml), dose-dependent, and saturable in the presence of 50 µg/ml HDL$_3$ (Fig. 1*B*). In contrast, no significant stimulation of ^{125}I-TRL recycling was determined when LDL (100 µg/ml protein) was utilized (Fig. 1*A*). These findings indicated that HDL$_3$ could stimulate ^{125}I-TRL recycling and serve as an acceptor for recycled ^{125}I-TRL proteins. To analyze the association of recycled ^{125}I-TRL proteins with lipoproteins, the media containing the lipoprotein acceptors and the released ^{125}I-TRL-derived radioactivity were harvested and separated either by density gradient centrifugation (Fig. 2*A*) or gel filtration chromatography (Fig. 2*B*). Recycled ^{125}I-TRL proteins from the media of cells incubated with human serum identified ~60% of TRL radioactivity in the HDL fractions (fractions 7–11 in Fig. 2*A*), whereas 40% was found in the lipid-free bottom of the gradient (fractions 1–4). It is likely that lipid-free apoA-I in human serum is responsible for the association of ^{125}I-TRL proteins in these bottom fractions, because lipid-free apoA-I also induced recycling of ^{125}I-TRL proteins into these fractions (data not shown). When ^{125}I-TRL-loaded cells were incubated with HDL$_3$, a predominant association of recycled ^{125}I-TRL proteins with HDL was observed (fractions 7–11, Fig. 2*A*). To confirm the association of recycled ^{125}I-TRL with HDL, re-secreted

^{125}I-TRL proteins were separated by Superdex 200 gel filtration (Fig. 2*B*), which is appropriate for the resolution of small sized lipoproteins (10–600 kDa). As expected HDL_3-induced recycling of internalized ^{125}I-TRL resulted in the association of ^{125}I-labeled apoproteins with HDL (fractions 24–28; Fig. 2*B*). In addition ^{125}I-TRL proteins were found in lipid-free fractions (LFF; fractions 29–31), which were representative of recycled ^{125}I-TRL proteins when lipid-free apoA-I was used as an acceptor (data not shown). Independent of density gradient or gel filtration analysis, we never detected significant amounts of recycled ^{125}I-TRL proteins in the fractions containing LDL (fractions 16–18 in Fig. 2*A*), TRL (fractions 21 and 22 in Fig. 2*A*), or both LDL and TRL (fractions 17–19 in Fig. 2*B*). These results demonstrate that TRL or LDL do not serve as acceptors for recycled ^{125}I-TRL apoproteins. Taken together, these results indicate that HDL in human serum is capable of stimulating TRL protein recycling (Fig. 1, *A* and *B*) and serve as an acceptor for recycled TRL proteins (Fig. 2, *A* and *B*).

Recycling of ApoE Is Associated with Cholesterol Efflux—It is well known that HDL can stimulate cellular cholesterol efflux (19–21). Because HDL-dependent re-secretion of ^{125}I-TRL (see above) mainly involves recycling of apoE (14), we investigated whether recycling of TRL-derived apoE could be linked to intracellular cholesterol transport. Therefore, the distribution of TRL-derived apoE and cholesterol in the presence or absence of HDL_3 was first studied by immunocytochemistry. In initial experiments with human hepatoma cells, immunostaining against TRL-derived apoE interfered with endogenous apoE expression (data not shown). Thus, human fibroblasts with no detectable amounts of endogenous apoE were analyzed. These cells, similar to the HuH7 hepatoma cells described above, efficiently recycled TRL proteins through peripheral endosomal compartments (14). In the following set of experiments, fibroblasts were incubated with apoE-enriched TRL (apoE-TRL), which was followed by a 60-min chase ± HDL_3 (Fig. 3). The cells were then fixed, immunostained against apoE to detect TRL-derived apoE, and incubated with filipin to visualize cellular cholesterol. In the absence of HDL_3 (Fig. 3, *A* and *B*), significant amounts of internalized TRL-derived apoE remained intracellularly (Fig. 3*A*). In these cells large amounts of cholesterol were found in peripheral and perinuclear compartments (Fig. 3*B*), which appeared to co-localize with apoE mainly in the periphery. In contrast, when cells were incubated with HDL_3, only residual amounts of internalized TRL-derived apoE were detected intracellularly (Fig. 3*A*, *c*). These findings suggested HDL_3-induced apoE recycling, which was confirmed by apoE Western blot analysis of the chase media (Fig. 3*B*, compare *lanes 3* and *4* which correspond to media from cells shown in Fig. 3*A*, *a* and *b*, and *c* and *d*, respectively). In addition, apoE recycling was accompanied by a strong reduction of filipin staining mainly in peripheral, most likely endosomal compartments (Fig. 3*A*, *d*).

Although filipin stains endosomal cholesterol, it is well known that large amounts of cholesterol are found in perinuclear Golgi and lysosomal compartments (30). In addition, analysis of full thickness cell images could lead to the overlap of apoE and cholesterol signals by random chance. Thus, confocal microscopy was performed to characterize apoE and cholesterol-containing vesicles in detail (Fig. 4). First, human fibroblasts were incubated with apoE-TRL, fixed, and stained against apoE together with AP1, a protein predominantly found in the Golgi apparatus (22) (Fig. 4*a*), or EEA1, a marker for early endosomes (31) (Fig. 4*b*). These experiments identified large amounts of internalized TRL-derived apoE in EEA1-positive endosomes that did not co-localize with AP1. As shown in Fig. 4*c*, these apoE-containing and EEA1-positive endosomes contain cholesterol. In contrast, cholesterol in the perinuclear region that was not stained with EEA1 did not contain any TRL-derived apoE. These findings indicated that HDL_3-induced apoE recycling is linked to cholesterol efflux from endosomal compartments. However, filipin staining is not a reliable method to estimate changes in endosomal cholesterol. Therefore, we first tested whether HDL_3-induced recycling of apoE is accompanied by the mobilization of radiolabeled TRL-derived cholesterol from endosomes. Because internalization and disintegration of TRL particles could be associated with a substantial redistribution of TRL-derived cholesterol to multiple cellular compartments, control experiments with NBD-cholesterol-labeled TRL were performed. In these studies TRL-derived NBD-cholesterol and apoE co-localized in EEA1-positive endosomes even after 60 min at 37 °C (data not shown, see also Fig. 11). Then cells were incubated with double-labeled [^{3}H]Chol/^{125}I-apoE-TRL (Fig. 5). In these experiments HDL_3 simultaneously stimulated recycling of internalized TRL-derived ^{125}I-apoE (1.8–2.4-fold) and efflux of TRL-derived [^{3}H]Chol (2.8–3.2-fold). Thus HDL_3 stimulates apoE recycling and cholesterol efflux from internalized TRL particles in endosomal compartments.

To determine whether apoE recycling is associated with cholesterol efflux from intracellular cholesterol pools in the absence of HDL_3, hepatoma cells were pre-labeled overnight with

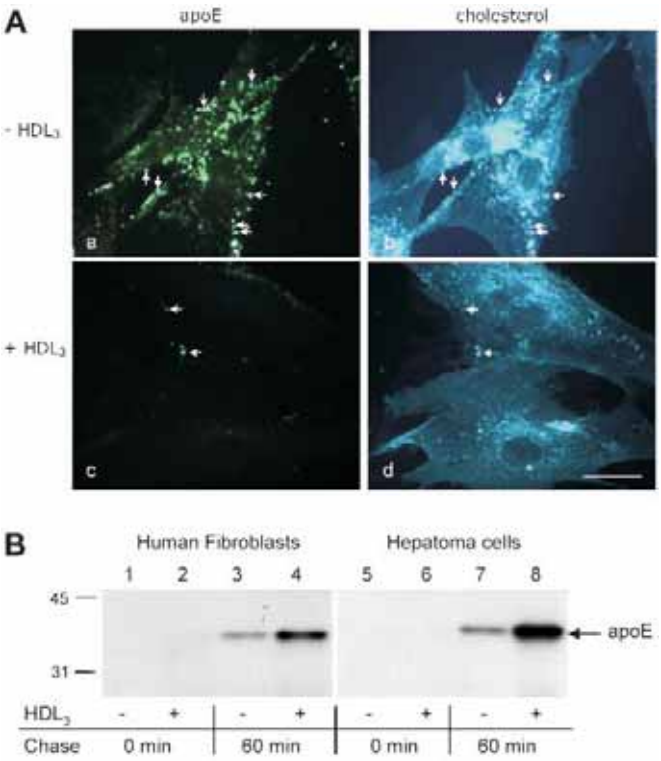

FIG. 3. **Recycling of TRL-derived apoE from cholesterol-containing endosomes.** *A*, human fibroblasts were preincubated with apoE-TRL for 60 min at 37 °C. After removal of cell-bound material with heparin, cells were incubated ± HDL_3 (50 μg/ml) for an additional 60 min at 37 °C as indicated. Media were collected to identify recycled apoE (see *B*). Cells were fixed and analyzed by fluorescence microscopy for apoE (*green*, *a* and *c*) and cholesterol (*blue*, *b* and *d*). *Arrows* point to apoE staining in cholesterol-rich endosomes labeled with filipin (see "Experimental Procedures"). *Bar* is 10 μm. *B*, pulse-chase experiments were performed by preincubating human fibroblasts (*lanes 1–4*) or hepatoma cells (*lanes 5–8*) with apoE-TRL for 60 min at 37 °C. Cell-bound material was removed with heparin, and cells were incubated ± HDL_3 (50 μg/ml) for 0 or 60 min at 37 °C as indicated. Then cell culture media were harvested, and the presence of recycled, intact apoE was determined by Western blot analysis (see "Experimental Procedures"). The position of apoE is indicated. Molecular mass is given in kDa.

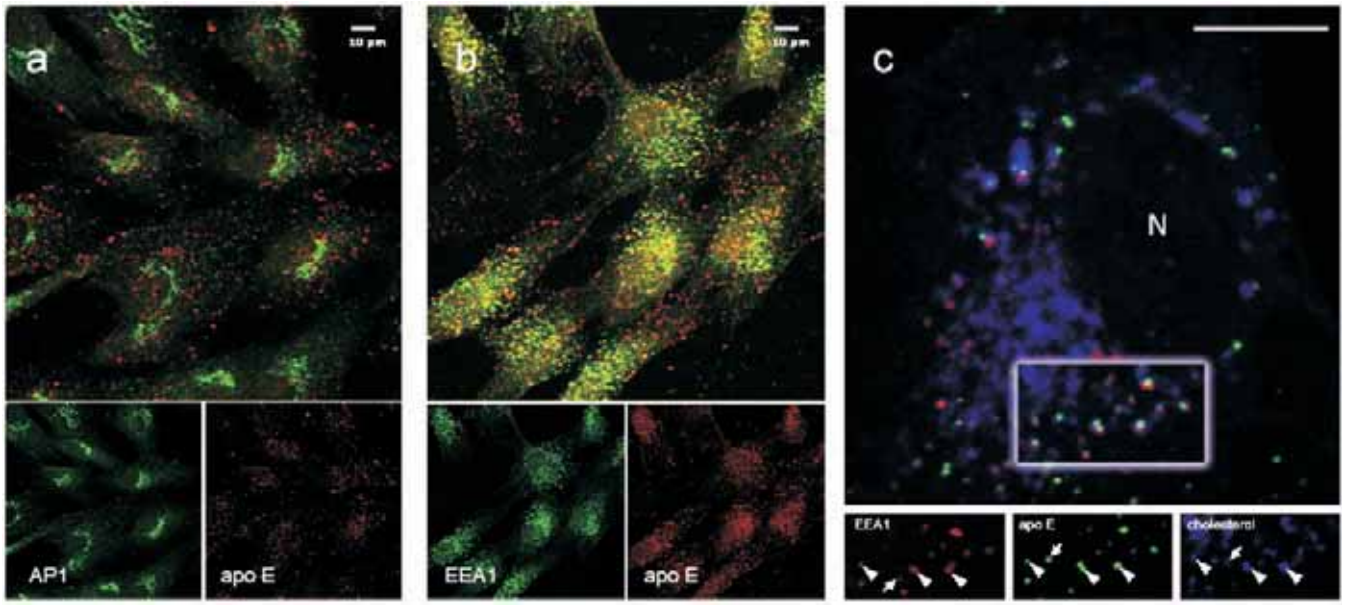

FIG. 4. **Internalized TRL-derived apoE co-localizes with cholesterol in EEA1-positive endosomes.** Human fibroblasts were incubated with apoE-TRL for 60 min at 37 °C. After removal of cell-bound material with heparin, cells were fixed, and sections (≤1 μm) were analyzed by confocal fluorescence microscopy. Fibroblasts were stained against apoE (*red*) and AP1 (*green*) in *a* or apoE (*red*) and EEA1 (*green*) in *b* as indicated in the *lower panel*. The merged images and co-localization of apoE and EEA1 (*yellow*) are shown in the *upper panels*. *c*, the merged image of fibroblasts stained against EEA1 (*red*), apoE (*green*), and cholesterol (*blue*) is shown in the *upper panel* (*N* = nucleus). In the *lower panel* the *highlighted area* is enlarged and shown separately for each channel. *Arrowheads* point to EEA1 and apoE staining in cholesterol-rich endosomes labeled with filipin. *Arrows* mark endosomes only containing EEA1, apoE or cholesterol, respectively. *Bar* is 10 μm.

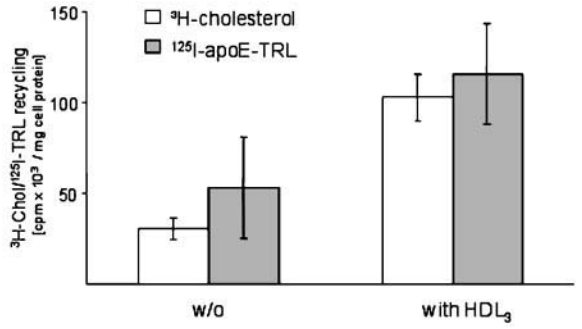

FIG. 5. **HDL$_3$-induced recycling of TRL-derived apoE and cholesterol.** Pulse-chase experiments were performed by preincubating human HuH7 hepatoma cells with [^{3}H]Chol/^{125}I-apoE-TRL for 60 min at 37 °C. Cells were washed with heparin and incubated for additional 60 min at 37 °C with media in the presence or absence of 50 μg/ml HDL$_3$ as indicated. The media were harvested, and the amount of re-secreted ^{125}I-apoE (*gray bar*) and [^{3}H]Chol (*white bar*) was determined. The remaining cells were lysed, and protein content was determined. The radioactivity is given (cpm × 10^3/mg of cell protein) and represents the mean ± S.D. of four independent experiments with triplicate samples.

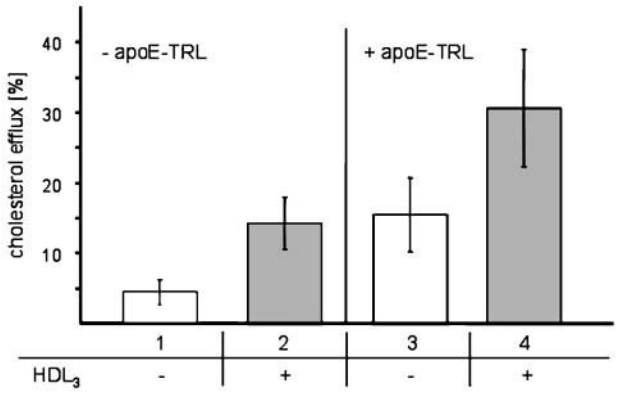

FIG. 6. **Recycling of apoE is associated with efflux of cellular cholesterol.** Human hepatoma cells were pre-loaded with [^{3}H]Chol-LDL (100,000 cpm/ml) for 24 h at 37 °C. Cell surface-bound [^{3}H]Chol-LDL was removed with heparin, and cells were incubated in the presence or absence of apoE-TRL for 60 min at 37 °C. After incubation with or without HDL$_3$ (50 μg/ml) as indicated, aliquots of the media were harvested to determine [^{3}H]Chol efflux. The remaining media were analyzed by FPLC (see Fig. 7). Cells were lysed, and the internalized amount of [^{3}H]Chol was determined. Values of [^{3}H]Chol efflux are given as a percentage of internalized radioactivity and represent the mean ± S.D. of four independent experiments with triplicates.

[^{3}H]Chol-LDL, and cell surface-bound [^{3}H]Chol-LDL was removed by heparin. Then cells were incubated ± apoE-TRL for 60 min, followed by an additional incubation ± HDL$_3$ for 60 min. Finally the amount of [^{3}H]Chol in the media was determined (Fig. 6). Similar to results obtained from hepatoma cells (32), HDL$_3$ stimulated [^{3}H]Chol efflux 3-fold (compare *columns 1* and *2*). In the absence of HDL$_3$, internalized TRL-derived apoE is concatenated by [^{3}H]Chol efflux ~3-fold compared with controls (compare *columns 1* and *3*). In the presence of HDL$_3$, a 6-fold induction of [^{3}H]Chol efflux from apoE-TRL-loaded cells was observed (compare *columns 1* and *4*). These findings indicate that apoE recycling is linked to cholesterol efflux not only from TRL-associated pools but also from other intracellular cholesterol pools (Figs. 5 and 6). To study the possible association of secreted [^{3}H]Chol with HDL, Superdex 200 gel filtration analysis of the chase media was performed (Fig. 7). The majority of released [^{3}H]Chol (~80%) was found in HDL fractions, which were shown to contain recycled TRL-derived apoE in this study (see Fig. 2, *A* and *B*) and previous studies (16). In contrast, only ~20% of [^{3}H]Chol was found in fractions smaller than HDL (LFF). Taken together, HDL$_3$-dependent and -independent apoE recycling is accompanied by the mobilization of intracellular cholesterol leading to a concomitant association of TRL-derived apoE and cholesterol with HDL particles.

Recycling of ApoE Is Associated with the Internalization of HDL$_3$-derived apoA-I—Most current models favor the localization of HDL at the plasma membrane to promote cholesterol efflux. We suggested that HDL is located at the cell surface to serve as an acceptor for recycled apoE (9, 14). Although the pulse-chase experiments described above (Figs. 2 and 7) demonstrated the association of recycled apoE and cholesterol with

HDL, these experiments did not provide information on the question whether complex formation of TRL-derived apoE and cholesterol with HDL could already occur intracellularly. To address this matter, apoE recycling in apoE-TRL-loaded cells was induced with fluorescent-labeled HDL_3 proteins (Alexa-HDL_3) containing predominantly labeled apoA-I (see "Experimental Procedures"). Cells were fixed and analyzed for the distribution of apoE, cholesterol, and Alexa-HDL_3. Similar to the results described in Fig. 3, *a* and *b*, TRL-derived apoE accumulates in peripheral compartments (Fig. 8*a*), which could also be stained with filipin (Fig. 8*c*), indicating co-localization of apoE and cholesterol in endosomes. Because complete recycling of TRL-derived apoE is observed after 60 min (Fig. 3*A*, *c* and *d*), only a 15-min induction of apoE recycling with Alexa-HDL_3 was performed (Fig. 8, *d–f*). This enabled us to detect simultaneously TRL-derived apoE, cholesterol, and Alexa-HDL_3. As expected, Alexa-HDL_3 stimulated apoE recycling and cholesterol efflux as shown by a reduced endosomal apoE and cholesterol staining (Fig. 8, *d* and *f*). In these experiments the reduction in cholesterol staining in the periphery was not as prominent as described in Fig. 3, which could in part be explained by the shorter exposure time to HDL_3. In addition, filipin staining of full cell images is not suitable to detect minimal changes in endosomal cholesterol concentrations. Most strikingly, co-localization of apoE/cholesterol and Alexa-HDL_3 was visible in peripheral compartments (compare *arrows* in Fig. 8, *d–f*) indicating the formation of endosomal apoE/cholesterol and apoA-I complexes. This observation was confirmed by confocal microscopy, which identified the co-localization of apoE and apoA-I in EEA1-positive endosomes during apoE recycling (Fig. 9).

To support these findings, electron microscopy of apoE-TRL-loaded hepatoma cells ± HDL_3 was performed. Incubation of cells with gold-labeled apoE-TRL (12 nm gold) resulted in the association of gold-apoE with endocytic structures (data not shown). To promote apoE recycling, gold-apoE-loaded cells

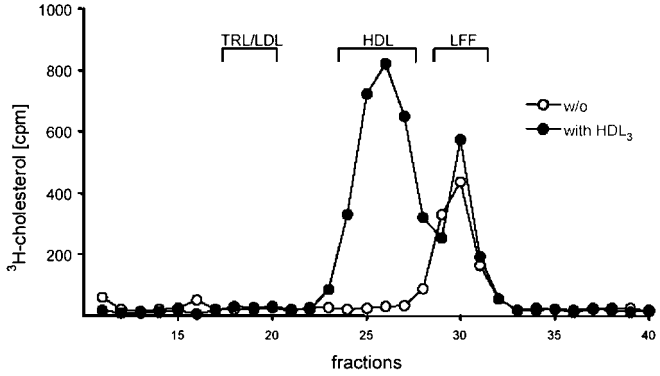

FIG. 7. **FPLC analysis of re-secreted cellular [^{3}H]cholesterol.** [^{3}H]Chol-containing media (see Fig. 5) obtained from cells incubated with (●, HDL_3) or without HDL_3 (○, *w/o*) were separated by a Superdex 200 column; 500-μl fractions were collected, and the radioactivity was determined in each fraction. The association of re-secreted [^{3}H]Chol (counts/min) with HDL and LFF for a representative experiment is shown. The fractions containing TRL/LDL (fractions 17–19), HDL (fractions 24–28) and LFF (fractions 29–31) are indicated. The positions of lipoproteins were determined as described above (Fig. 2*B*).

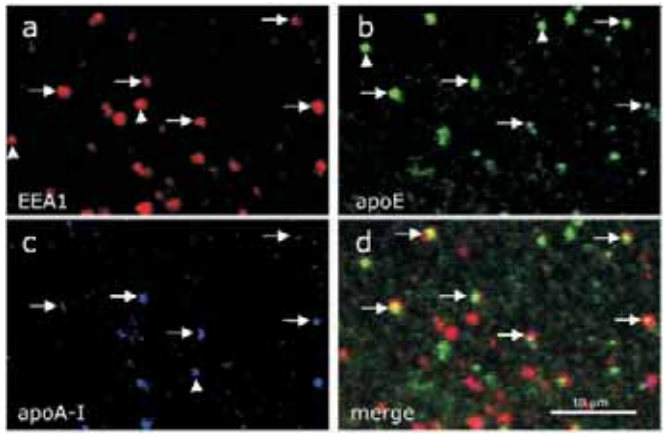

FIG. 9. **TRL-derived apoE co-localizes with apoA-I in EEA1-positive endosomes during apoE recycling.** Human fibroblasts were incubated with apoE-TRL for 60 min at 37 °C. After removal of cell-bound material with heparin, cells were incubated for 15 min with 50 μg/ml Cy5-HDL_3. Cells were fixed, and sections (≤1 μm) of a representative area in the cellular periphery were analyzed by confocal fluorescence microscopy. Fibroblasts were analyzed for EEA1 (*red*) (*a*), apoE (*green*) (*b*), and apoA-I (*blue*) (*c*). The merged image is shown in *d*. *Arrows* point to EEA1-positive endosomes containing apoE and apoA-I. *Arrowheads* mark endosomes only containing EEA1, apoE, or apoA-I, respectively. *Bar* is 10 μm.

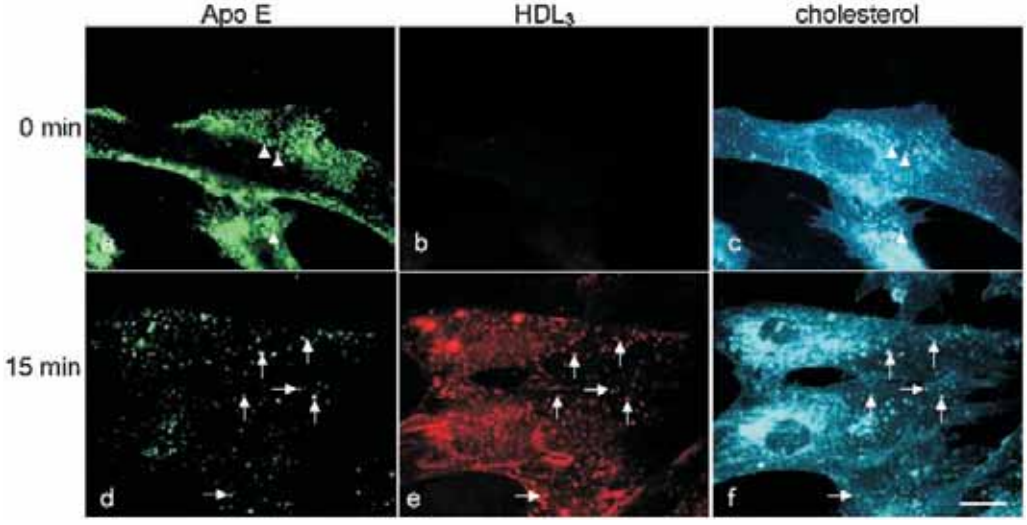

FIG. 8. **Internalized apoA-I co-localizes with TRL-derived apoE and cholesterol.** Human fibroblasts were preincubated with apoE-TRL for 60 min at 37 °C. After removal of cell-bound material with heparin, cells were incubated with HDL_3 (50 μg/ml) for 0 (*a–c*) or 15 min (*d–f*) at 37 °C as indicated. Cells were fixed and analyzed by fluorescence microscopy for apoE (*green*, *a* and *d*), HDL_3 (*red*, *b* and *e*) and cholesterol (*blue*, *c* and *f*). *Arrowheads* point to apoE and cholesterol-containing endosomes (compare *a* and *c*). In the presence of fluorescent-labeled HDL_3, HDL_3-derived apoA-I is found almost exclusively in apoE-containing and cholesterol-rich endosomes (compare *arrows*, *d–f*). *Bar* is 10 μm.

were incubated with gold-labeled HDL_3 (5 nm gold) for an additional 15 min (Fig. 10). Subsequent analysis of EM sections identified gold-labeled protein complexes consisting of apoE- and HDL_3-derived apoA-I to be associated with peripheral endosomes and membranous structures at the plasma membrane.

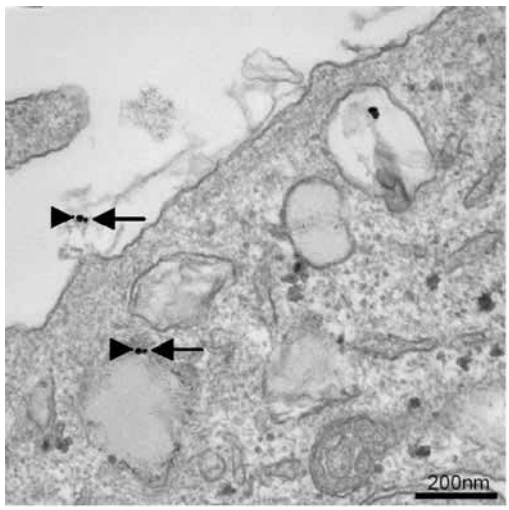

FIG. 10. **Co-localization of apoA-I with TRL-derived apoE.** Human hepatoma cells were preincubated with gold-apoE-TRL (12 nm) for 60 min at 37 °C. After removal of cell-bound material with heparin, cells were incubated with gold-HDL_3 (5 nm) for 15 min at 37 °C. Cells were fixed, prepared, and analyzed by electron microscopy for apoE (*arrowheads*) and HDL_3 (*arrows*). In the presence of HDL_3, complexes containing gold-labeled apoE and HDL_3 are found associated with endosomal structures or at the plasma membrane. *Bar* is 200 nm.

These findings suggested that HDL_3-derived apoA-I is internalized to pre-existing apoE/cholesterol-containing endosomes to promote apoE recycling and cholesterol efflux. To confirm this hypothesis we performed time-lapse confocal microscopy to study intracellular localization of apoA-I during early stages of apoE recycling (Fig. 11). Cells were first incubated for 30 min with double-labeled NBD-cholesterol/Cy3-apoE-TRL. Then Cy5-HDL_3 was added for an additional 10 min. An example of a mobile NBD-cholesterol/apoE-containing endosome (*frames 6–15*) that interacts with another mobile apoA-I-containing endosome is shown (*frames 16–20*). The merged images (*frames 1–5*) revealed co-localization of NBD-cholesterol and apoE indicating that these TRL components remained associated even 30 min after TRL internalization (*frames 2* and *3*). Most importantly, immediately after addition of Cy5-HDL_3, endosomal apoA-I is targeted to the NBD-cholesterol/apoE-containing vesicle (*frames 4* and *5*). Thus, the intracellular formation of apoE/cholesterol/apoA-I-containing complexes is involved in apoE recycling.

DISCUSSION

The aim of this study was to identify a possible intracellular link between the recycling of apoE, cholesterol efflux, and HDL metabolism. It is well documented that internalized TRL-derived apoE is efficiently recycled in liver cells (13, 14, 16–18), but relatively little is known about the intracellular events that accompany apoE recycling. Here we demonstrate that apoE recycling is linked to HDL_3-dependent and -independent cholesterol efflux. In the presence of HDL the re-secretion of apoE and cholesterol leads to the formation of apoE/cholesterol-enriched HDL particles. Detailed confocal microscopy and live cell imaging revealed that after internalization TRL-derived apoE

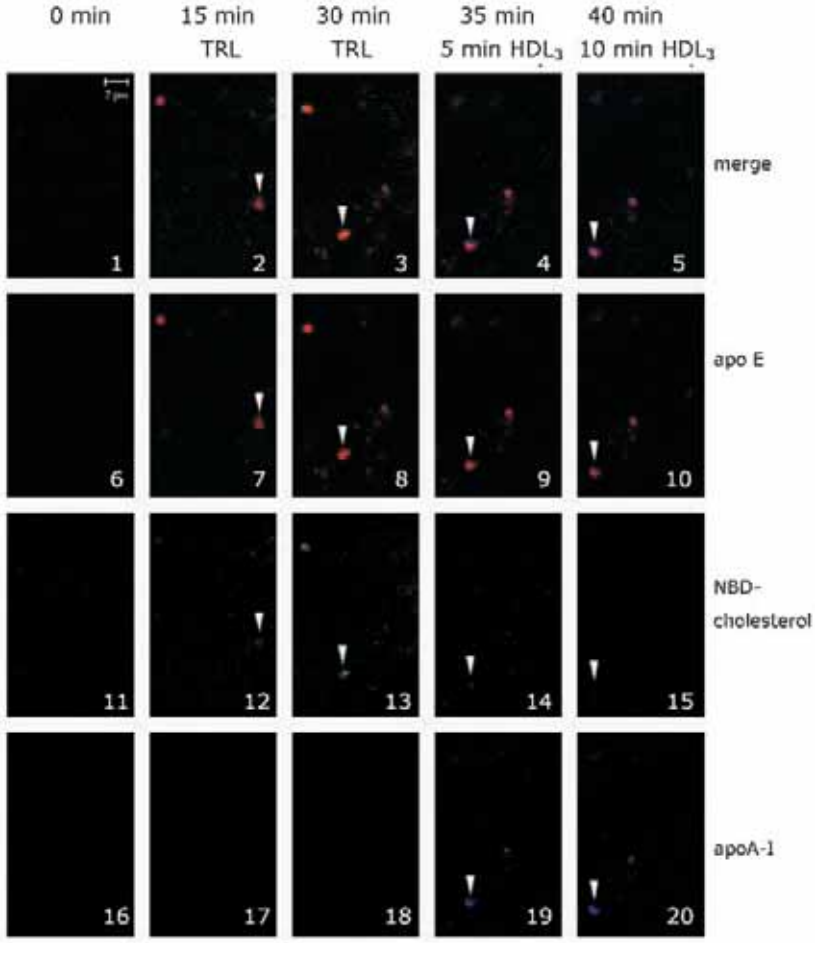

FIG. 11. **Dynamic association of apoA-I with pre-existing apoE- and cholesterol-containing complexes.** Human fibroblasts were incubated with double-labeled NBD-cholesterol/Cy3-apoE-TRL for 0–30 min at 37 °C. Cells were washed in DMEM and incubated for an additional 0–10 min with 50 μg/ml Cy5-HDL_3. Time-lapse confocal image acquisition of apoE (*red*), NBD-cholesterol (*green*), and HDL_3-protein (*blue*) in a representative area of the cellular periphery was performed at 37 °C (see "Experimental Procedures"). The merged image is shown in the *upper panel* (*image numbers 1–5*). Each channel is shown separately in the *lower panels* as indicated. *Arrowheads* mark and follow the movement of a complex in the merge (*image numbers 2–5*) and in each *lower panel*: apoE (*image numbers 7–10*), NBD-cholesterol (*image numbers 12–15*), and apoA-I (*image numbers 19* and *20*). *Bar* is 2 μm.

and cholesterol remain associated in EEA1-positive endosomes. Most importantly, apoE recycling involves the internalization of HDL_3-derived apoA-I and its targeting to apoE/cholesterol-containing endosomes. These findings indicate that endosomal HDL_3-derived apoA-I can mobilize pre-existing apoE/cholesterol complexes.

In the light of these findings, our previous model that HDL acts as an extracellular acceptor for recycled TRL-derived apoE (9) deserves to be reconsidered and extended. Concurrent cholesterol efflux and apoE recycling would be in agreement with models favoring the binding of HDL to the plasma membrane. At the cell surface, HDL could interact with ATP-binding cassette transporter A1 (ABCA1), scavenger receptor class B type I (SR-BI), or other HDL-binding proteins (for reviews see Refs. 19–21 and 33–36). However, the evidence of HDL-derived apoA-I internalization during apoE/cholesterol re-secretion (Figs. 8–11) is in agreement with a number of reports describing the intracellular localization of HDL receptors during HDL-mediated cholesterol transport (for reviews see Refs. 20, 33, and 36). Similarly, results presented in this work identified HDL-derived apoA-I in EEA1-positive early endosomes. Oram (20) recently proposed that ABCA1 and apoA-I are internalized to intracellular lipid vesicles, where ABCA1 could deliver lipids into the lumen of exocytic vesicles before re-secretion. This is based on the following observations. First, ABCA1 recycles between the plasma membrane and endosomes (37). Second, ABCA1 promotes cholesterol efflux from endosomal compartments (38). Third, apoA-I is internalized and re-secreted during cAMP-inducible and ABCA1-dependent cholesterol efflux (39–42).

Although these observations would favor a role for ABCA1 in the HDL-induced and concurrent apoE recycling and cholesterol efflux, the potential contribution of SR-BI cannot be excluded. SR-BI is localized predominantly in caveolae and not in clathrin-coated pits (43, 44). However, detailed studies revealed that a significant proportion of SR-BI proteins are rapidly internalized (45). This is accompanied by endocytosis and re-secretion of HDL (46). The concept that SR-BI could mediate uptake and recycling of HDL particles is consistent with a punctate, endosomal localization of fluorescent-labeled HDL immediately (10 min) after cell surface ligand binding in SR-BI-overexpressing cells (47) (see also Figs. 8–11). In addition, Silver *et al.* (45) observed the internalization and recycling of HDL particles through an endocytic recycling compartment in non-polarized hepatocytes. These results are in agreement with earlier studies (48–51) describing receptor-mediated endocytosis and retroendocytosis of HDL.

Co-localization of apoA-I, apoE, and cholesterol in TRL-loaded cells indicates that apoE recycling and cholesterol efflux results in the intracellular assembly of apoA-I with apoE/cholesterol-containing complexes. Similarly, a number of reports (52, 53) hypothesized an interaction between apoA-I and apoE and concluded that apoA-I concurrently stimulates secretion of endogenous apoE and cholesterol efflux from lipid-loaded macrophages. In addition secretion of apoE from macrophages is at least in part associated with cell-derived cholesterol (54, 55). This stimulatory role of apoA-I and endogenous apoE for cholesterol efflux in peripheral cells is the presupposition for efficient reverse cholesterol transport to the liver for bile secretion (33, 56). In our study we identified the concomitant re-secretion of endosomal TRL-derived and/or intracellular cholesterol with internalized TRL-derived apoE in hepatoma cells. These findings suggest that intracellular association of cholesterol with TRL-derived apoE and concurrent re-secretion could provide a mechanism allowing dietary cholesterol to escape bile secretion.

Most importantly we demonstrate that the intracellular association of apoA-I with apoE-cholesterol complexes could lead to the formation of apoE-enriched HDL particles. Once secreted, these particles would represent a pool of apoE proteins associated with HDL. In the postprandial state HDL-derived apoE is the major source for apoE enrichment of CR to ensure the rapid clearance of CR into the liver (1, 2). To avoid a rapid decrease of HDL-derived apoE during the postprandial state, the recycling of TRL-derived apoE and subsequent generation of apoE-containing HDL particles would secure the maintenance of high HDL-associated apoE levels in plasma. Therefore, recycled apoE could serve as a continuous donor for intravascular apoE transfer from HDL to CR during lipolysis and hepatic clearance (9, 16). Future *in vivo* experiments will have to clarify whether newly formed HDL particles play a physiological role in CR enrichment with apoE.

Acknowledgments—We thank D. Wendt for excellent technical assistance. We are most grateful to M. Calvo (Institut d'Investigacions Biomèdiques August Pi i Sunyer/Fons d'Investigació Sanitària) for assistance in the preparation of gold probes and to Serveis Científico-tècnics, Universitat de Barcelona, for the electron microscopy facilities.

REFERENCES

1. Olivecrona, G., and Olivecrona, T. (1995) *Curr. Opin. Lipidol.* **6,** 291–305
2. Mahley, R. W., and Ji, Z. S. (1999) *J. Lipid Res.* **40,** 1–16
3. Goldberg, I. J., and Merkel, M. (2001) *Front. Biosci.* **6,** D388–D405
4. Beisiegel, U., Weber, W., Ihrke, G., Herz, J., and Stanley, K. K. (1989) *Nature* **341,** 162–164
5. Beisiegel, U., Weber, W., and Bengtsson-Olivecrona, G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 8342–8346
6. Rohlmann, A., Gotthardt, M., Hammer, R. E., and Herz, J. (1998) *J. Clin. Invest.* **101,** 689–695
7. Zhang, S. H., Reddick, R. L., Piedrahita, J. A., and Maeda, N. (1992) *Science* **258,** 468–471
8. Brown, M. S., and Goldstein, J. L. (1986) *Science* **232,** 34–47
9. Heeren, J., and Beisiegel, U. (2001) *Curr. Opin. Lipidol.* **12,** 255–260
10. Tabas, I., Lim, S., Xu, X. X., and Maxfield, F. R. (1990) *J. Cell Biol.* **111,** 929–940
11. Tabas, I., Myers, J. N., Innerarity, T. L., Xu, X. X., Arnold, K., Boyles, J., and Maxfield, F. R. (1991) *J. Cell Biol.* **115,** 1547–1560
12. Myers, J. N., Tabas, I., Jones, N. L., and Maxfield, F. R. (1993) *J. Cell Biol.* **123,** 1389–1402
13. Rensen, P. C., Jong, M. C., van Vark, L. C., van der Boom, H., Hendriks, W. L., van Berkel, T. J., Biessen, E. A., and Havekes, L. M. (2000) *J. Biol. Chem.* **275,** 8564–8571
14. Heeren, J., Weber, W., and Beisiegel, U. (1999) *J. Cell Sci.* **112,** 349–359
15. Enrich, C., Pol, A., Calvo, M., Pons, M., and Jäckle, S. (1999) *Hepatology* **30,** 1115–1120
16. Heeren, J., Grewal, T., Jäckle, S., and Beisiegel, U. (2001) *J. Biol. Chem.* **276,** 42333–42338
17. Fazio, S., Linton, M. F., Hasty, A. H., and Swift, L. L. (1999) *J. Biol. Chem.* **274,** 8247–8253
18. Swift, L. L., Farkas, M. H., Major, A. S., Valyi-Nagy, K., Linton, M. F., and Fazio, S. (2001) *J. Biol. Chem.* **276,** 22965–22970
19. von Eckardstein, A., Nofer, J. R., and Assmann, G. (2001) *Arterioscler. Thromb. Vasc. Biol.* **21,** 13–27
20. Oram, J. F. (2002) *Curr. Opin. Lipidol.* **13,** 373–381
21. Fielding, C. J., and Fielding, P. E. (2001) *Adv. Drug Delivery Rev.* **49,** 251–264
22. Ahle, S., Mann, A., Eichelsbacher, U., and Ungewickell, E. (1988) *EMBO J.* **7,** 919–929
23. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) *Methods Enzymol.* **98,** 241–260
24. Rinninger, F., Brundert, M., Jäckle, S., Galle, P. R., Busch, C., Izbicki, J. R., Rogiers, X., Henne-Bruns, D., Kremer, B., Broelsch, C. E., and Greten, H. (1994) *Hepatology* **19,** 1100–1114
25. Niemeier, A., Gafvels, M., Heeren, J., Meyer, N., Angelin, B., and Beisiegel, U. (1996) *J. Lipid Res.* **37,** 1733–1742
26. Handley, D. A., Arbeeny, C. M., Witte, L. D., and Chien, S. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 368–371
27. De Diego, I., Schwartz, F., Siegfried, H., Dauterstedt, P., Heeren, J., Beisiegel, U., Enrich, C., and Grewal, T. (2002) *J. Biol. Chem.* **277,** 32187–32194
28. Ortega, D., Pol, A., Biermer, M., Jäckle, S., and Enrich, C. (1998) *J. Cell Sci.* **111,** 261–269
29. Merkel, M., Heeren, J., Dudeck, W., Rinninger, F., Radner, H., Breslow, J. L., Goldberg, I. J., Zechner, R., and Greten, H. (2002) *J. Biol. Chem.* **277,** 7405–7411
30. Holtta-Vuori, M., Tanhuanpaa, K., Mobius, W., Somerharju, P., and Ikonen, E. (2002) *Mol. Biol. Cell* **13,** 3107–3122
31. Christoforidis, S., McBride, H. M., Burgoyne, R. D., and Zerial, M. (1999) *Nature* **397,** 621–625
32. Mahlberg, F. H., and Rothblat, G. H. (1992) *J. Biol. Chem.* **267,** 4541–4550
33. Silver, D. L., and Tall, A. R. (2001) *Curr. Opin. Lipidol.* **12,** 497–504
34. Krieger, M. (2001) *J. Clin. Invest.* **108,** 793–797
35. Schmitz, G., and Langmann, T. (2001) *Curr. Opin. Lipidol.* **12,** 129–140
36. Santamarina-Fojo, S., Remaley, A. T., Neufeld, E. B., and Brewer, H. B., Jr.

(2001) *J. Lipid Res.* **42,** 1339–1345
37. Neufeld, E. B., Remaley, A. T., Demosky, S. J., Stonik, J. A., Cooney, A. M., Comly, M., Dwyer, N. K., Zhang, M., Blanchette-Mackie, J., Santamarina-Fojo, S., and Brewer, H. B., Jr. (2001) *J. Biol. Chem.* **276,** 27584–27590
38. Chen, W., Sun, Y., Welch, C., Gorelik, A., Leventhal, A. R., Tabas, I., and Tall, A. R. (2001) *J. Biol. Chem.* **276,** 43564–43569
39. Takahashi, Y., and Smith, J. D. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11358–11363
40. Bortnick, A. E., Rothblat, G. H., Stoudt, G., Hoppe, K. L., Royer, L. J., McNeish, J., and Francone, O. L. (2000) *J. Biol. Chem.* **275,** 28634–28640
41. Zheng, P., Horwitz, A., Waelde, C. A., and Smith, J. D. (2001) *Biochim. Biophys. Acta* **1534,** 121–128
42. Oram, J. F., Lawn, R. M., Garvin, M. R., and Wade, D. P. (2000) *J. Biol. Chem.* **275,** 34508–34511
43. Babitt, J., Trigatti, B., Rigotti, A., Smart, E. J., Anderson, R. G., Xu, S., and Krieger, M. (1997) *J. Biol. Chem.* **272,** 13242–13249
44. Calvo, M., and Enrich, C. (2000) *Electrophoresis* **21,** 3386–3395
45. Silver, D. L., Wang, N., and Tall, A. R. (2000) *J. Clin. Invest.* **105,** 151–159
46. Silver, D. L., Wang, N., Xiao, X., and Tall, A. R. (2001) *J. Biol. Chem.* **276,** 25287–25293
47. Gu, X., Trigatti, B., Xu, S., Acton, S., Babitt, J., and Krieger, M. (1998) *J. Biol. Chem.* **273,** 26338–26348
48. Schmitz, G., Robenek, H., Lohmann, U., and Assmann, G. (1985) *EMBO J.* **4,** 613–622
49. Garcia, A., Barbaras, R., Collet, X., Bogyo, A., Chap, H., and Perret, B. (1996) *Biochemistry* **35,** 13064–13071
50. Klinger, A., Reimann, F. M., Klinger, M. H., and Stange, E. F. (1997) *Biochim. Biophys. Acta* **1345,** 65–70
51. Rogler, G., Herold, G., and Stange, E. F. (1991) *Biochim. Biophys. Acta* **1095,** 30–38
52. Rees, D., Sloane, T., Jessup, W., Dean, R. T., and Kritharides, L. (1999) *J. Biol. Chem.* **274,** 27925–27933
53. Bielicki, J. K., McCall, M. R., and Forte, T. M. (1999) *J. Lipid Res.* **40,** 85–92
54. Basu, S. K., Ho, Y. K., Brown, M. S., Bilheimer, D. W., Anderson, R. G., and Goldstein, J. L. (1982) *J. Biol. Chem.* **257,** 9788–9795
55. Herscovitz, H., Gantz, D., Tercyak, A. M., Zannis, V. I., and Small, D. M. (1992) *J. Lipid Res.* **33,** 791–803
56. Schwartz, C. C., Halloran, L. G., Vlahcevic, Z. R., Gregory, D. H., and Swell, L. (1978) *Science* **200,** 62–64