ApoA-IV Is Secreted on Discrete HDL Particles
by the Rat Hepatoma Cell Line McA-RH7777
Transfected With ApoA-IV cDNA

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In the present study, the synthesis and secretion of transfected apolipoprotein (apo) A-IV was investigated in rat hepatoma McA-RH7777, a cell line that does not express apoA-IV mRNA or protein. An expression plasmid that contained the rat apoA-IV cDNA was transfected into the cells; five stable transformants were selected that harbor different copy numbers of the apoA-IV construct and secrete different amounts of apoA-IV. Gel filtration column chromatography and density gradient ultracentrifugation, combined with gel electrophoresis and electron microscopy techniques, demonstrated that (1) the secreted apoA-IV associated mainly with high-density lipoproteins (HDLs) and only a trace amount of apoA-IV was associated with very-low-density lipoproteins; (2) overexpression of apoA-IV resulted in an increased number of disk-shaped structures (thickness, =8.0 nm and diameter, =22 nm); and (3) the electrophoretic mobilities of the apoA-IV–containing particles differed from those of apoA-I–containing HDL. Expression of apoA-IV exerted no discernible effect on the density distribution or the secretion efficiency of apoB-100. Additionally, secretion of apoB-100 and apoA-IV exhibited opposite responses to serum: apoB-100 secretion was stimulated eightfold after addition of serum, whereas apoA-IV secretion was inhibited by 40%. These results suggest that synthesis of apoA-IV may lead to the formation of a subclass of HDL with a different metabolic fate than that of lipoproteins containing either apoA-I or apoB. (Arterioscler Thromb. 1993;13:1476-1486.)

KEY WORDS  •  apoA-IV  •  recombinant DNA  •  rat hepatoma  •  transfection  •  lipoprotein secretion  •  HDL

Mammalian apolipoprotein (apo) A-IV is a single-chain plasma glycoprotein (molecular mass about 44 000 Da) associated mainly with high-density lipoproteins (HDLs) and to a lesser degree with triglyceride-rich apoB-containing lipoproteins, such as chylomicrons and very-low-density lipoproteins (VLDLs). In humans, apoA-IV is synthesized exclusively in the small intestine. Although the precise function of apoA-IV in lipid and lipoprotein metabolism remains unclear, in vitro evidence suggests that apoA-IV is involved in activating lecithin:cholesterol acyltransferase (LCAT), in the interaction of HDL with the putative HDL receptor, and in promoting cellular cholesterol efflux and thus the process of reverse cholesterol transport. Recently, Fujimoto et al have provided evidence showing that increased apoA-IV in rat mesenteric lymph after a lipid meal acts as a physiological signal for satiation. Concentrations of apoA-IV in the plasma vary dramatically under different metabolic and pathological conditions. In humans, plasma apoA-IV levels are sensitive to changes in dietary fat content; significantly increased quantities of plasma apoA-IV (35% over baseline levels) are observed after fat feeding. Similar increases in plasma apoA-IV levels are also reported in rats after fat feeding and were associated with increased synthesis and secretion of apoA-IV by the small intestine. On the other hand, in human subjects with abetalipoproteinemia, in which apoB-containing lipoproteins are essentially absent, there is a significant reduction (to 37% of normal level) in apoA-IV concentration. Although these data might be interpreted as indicating a relation between the levels of plasma apoA-IV and plasma triglyceride-rich lipoproteins, the role of apoA-IV in the formation and secretion of lipoproteins is unknown.

In rats, apoA-IV is synthesized in both the liver and intestine; approximately 60% of the plasma apoA-IV is produced by the intestine, and the remainder is produced by the liver. The McA-RH7777 rat cell line, which is derived from a Morris hepatoma, actively synthesizes and secretes most of the rat plasma apolipoproteins. However, McA-RH7777 cells do not synthesize apoA-IV. These cells also exhibit impaired assembly of VLDL containing apoB-48. Unlike primary rat hepatocytes, which form both apoB-100– and apoB-48–containing VLDL, McA-RH7777 cells synthesize VLDL containing mainly apoB-100 and very little apoB-48. Most of the apoB-48 secreted from McA-RH7777 is associated with HDL. The inability of McA-RH7777 cells to produce apoB-48–containing VLDL is also...
reported in transfection studies using human apoB cDNA constructs.\textsuperscript{15,17} As was the case for endogenous apoB-48, the recombinant human apoB-48 expressed in stable transformants of McA-RH7777 cells associates mainly with HDL particles with a peak density at \( d = 1.10 \text{ g/mL} \).\textsuperscript{17} The reason for the failure of McA-RH7777 to produce VLDL containing apoB-48 remains to be elucidated.

In the present studies, the role of apoA-IV in the production of hepatic lipoproteins was investigated by stably transfecting McA-RH7777 cells with a rat apoA-IV cDNA expression plasmid. Effects of apoA-IV production on the synthesis and secretion of rat hepatic lipoproteins were examined in the stable transformants that expressed different amounts of the apoA-IV protein. Our data indicated that most of the lipoprotein-associated apoA-IV is present on discrete HDL-sized particles containing little, if any, apoA-I. Moreover, production of apoA-IV does not affect the production of lipoproteins containing either apoB or apoA-I. These results suggest that synthesis of apoA-IV in the rat liver may lead to the formation of a subclass of HDL with a different metabolic fate than that of apoB- or apoA-I-containing lipoproteins.

**Methods**

**Materials**

The rat hepatoma cell line McA-RH7777 was obtained from the American Type Culture Collection, Rockville, Md. Dulbecco's modified Eagle's medium (DMEM), methionine-free DMEM, and G418 were purchased from Gibco, Gaithersburg, Md. Fetal bovine serum (FBS) and horse serum were obtained from HyClone, Logan, Utah. Restriction enzymes, T4 DNA ligase, and DNA polymerase were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind, or New England Biolabs, Inc, Beverly, Mass. Reagents for polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis were obtained from Bio-Rad, Richmond, Calif. Reagents for agarose gel electrophoresis and lipoprotein staining were obtained from Baxter Diagnostics Inc, McGraw Park, Ill. Purified rat apoA-IV protein and polyclonal antibodies to rat apoA-IV, apoE, apoB, and apoA-I were gifts from Dr K. Weisgraber of the Gladstone Institute of Cardiovascular Disease, San Francisco, Calif. The \(^{125}\text{I}-\text{labelled} \) anti-rabbit immunoglobulin G antibodies, \( (\alpha-\text{P})\text{dCTP} \ (6000 \text{ Ci/mm}) \), and the ECL Western blot detecting system were obtained from Amersham, Arlington Heights, III. \( ^{35}\text{S}\) Methionine (Tran\(^{35}\text{S}-\text{label}, 1100 \text{ Ci/mmol}) was purchased from ICN Biomedicals, Inc, Costa Mesa, Calif. Enlightening and GeneScreen Plus transfer membranes were obtained from DuPont, NEN Research Products, Wilmington, Del. Nytran transfer membranes (0.45 \( \mu \text{m} \)) and nitrocellulose paper (0.45 \( \mu \text{m} \)) were obtained from Schleicher and Schuell, Inc, Keene, NH. Fumed silica was obtained from Sigma Chemical Co, St Louis, Mo.

**Construction of Rat ApoA-IV Expression Plasmids**

A previously described rat apoA-IV cDNA clone\textsuperscript{1} was modified so that \( \text{EcoRI} \) and \( \text{HindIII} \) restriction sites were created at the 5' and 3' ends, respectively, of the apoA-IV cDNA. The modified DNA was digested with \( \text{EcoRI} \) and \( \text{HindIII} \), and the apoA-IV cDNA fragment was ligated to the expression vector pCMV\textsuperscript{515} that had been cut with the same enzymes. The rat apoA-IV cDNA construct was flanked by the human cytomegalovirus promoter and enhancer regions and human growth hormone polyadenylation and termination sequences. The plasmid DNA used for transfection or sequence analysis was purified twice by cesium chloride gradient centrifugation before use.

**Cell Culture and Transfection**

McA-RH7777 cells were cultured in DMEM containing 10% FBS and 10% horse serum as described previously.\textsuperscript{17} Cotransfection of the apoA-IV expression plasmid with pSV2neo was performed by calcium phosphate coprecipitation and glycerol shock.\textsuperscript{18} Two days after the initial transfection, G418 (400 \( \mu \text{g/mL} \)) was added to the culture medium and the G418-resistant cells were selected. Production of apoA-IV in individual colonies was tested by Western blot analysis 10 to 14 days after selection (see below), and stable cell lines that showed a positive reaction were maintained for further studies.

**Preparation of DNA and Southern Blot Analysis**

DNA was isolated after incubation of the cells with proteinase K and sodium dodecyl sulfate (SDS) according to a published method.\textsuperscript{19} For Southern blot analysis, DNA was digested with \( \text{EcoRI} \) and \( \text{HindIII} \), separated by electrophoresis on a 1.1% agarose gel, transferred to a GeneScreen Plus membrane, and hybridized with a \(^{32}\text{P}-\text{labelled} \) rat apoA-IV cDNA probe as previously described.\textsuperscript{20} The probe was a 440-bp Sac I–Sac I fragment that had been labeled by random priming using a kit obtained from Bethesda Research Laboratories Life Technologies, Inc, Gaithersburg, Md, and purified with Push Columns (Strategene, La Jolla, Calif) according to the manufacturers' instructions.

**Preparation of RNA and Northern Blot Analysis**

Total cellular RNA was isolated from cells by the guanidine isothiocyanate method.\textsuperscript{21} Northern blot analysis was carried out as described previously.\textsuperscript{22}

**Immunoblot Analysis and Quantitation of Rat ApoA-IV**

Cells (at \( \approx 60\% \) confluence) were cultured in 60-mm dishes with serum-free medium or medium containing 10% FBS and 10% horse serum. The medium was collected on ice at 30, 60, 90, and 120 minutes and supplemented with 2 mmol/L phenylmethylsulfonyl fluoride (PMSF). Apolipoproteins were concentrated with fumed silica as described previously\textsuperscript{23} and were separated on either a 12% polyacrylamide gel or a 3% to 15% gradient polyacrylamide gel in the presence of 0.1% SDS. ApoA-IV was detected by Western blot analysis using a polyclonal antibody. The apolipoproteins were visualized using \(^{125}\text{I}-\text{labelled} \) secondary antibody or the nonradioactive ECL system (Amersham). Fluorograms and autoradiograms were analyzed on an Ambis densitometer (San Diego, Calif) to quantify apoA-IV by using a standard curve generated with purified rat apoA-IV (20, 200, and 500 ng). The apoA-IV standard, a gift from Dr K. Weisgraber, was purified by S-300 chromatography from the \( d = 1.063 \) to 1.21 g/mL fraction of rat plasma.
Kinetic Studies of Secretion of ApoB and ApoA-IV

Each dish (60 mm) of 60% confluent cells was preincubated for 2 hours in methionine-free medium containing [35S]methionine (Tran35S-label, 100 µCi/mL) in either the presence or absence of 10% FBS and 10% horse serum. After labeling, the medium was replaced with nonradioactive medium and chased for up to 4 hours. The immunoprecipitation of apoB and apoA-IV and subsequent fluorography procedures were performed as described previously.4 The intensity of the labeled protein bands was quantified by densitometry or by direct counting of the bands containing apoA-IV or apoB-100.

Superose Column Chromatography and Agarose Gel Electrophoresis

Cells were incubated for 24 hours with serum-free medium (10 mL per T75 flask) containing either 0.1 mmol/L oleate complexed with 1% bovine serum albumin or albumin alone. The oleate/albumin stock solution was prepared according to a previously described procedure. The conditioned media from two culture flasks were combined, supplemented with 0.5 mmol/L EDTA and 0.015% PMSF, and dialyzed against 0.25 mmol/L EDTA and 0.15 mol/L NaCl, pH 7.4, at 4°C. After dialysis, the samples were concentrated to 200 µL by consecutive centrifugations using CentriCell filters (molecular weight cutoff, 30,000; Polysciences Inc, War- rington, Pa) and Centricon-30 filters (Amicon, Beverly, Mass). The concentrated medium was fractionated on a Superose 6 column (1x30 cm; Pharmacia, Piscataway, NJ) by using 0.15 mol/L NaCl, 10 mmol/L phosphate, and 1 mmol/L EDTA, pH 7.4.23, 24 Fractions of 1 mL were collected for analysis. Human plasma was used to calibrate the elution profile of lipoproteins on the column: fractions 17 to 21, VLDL; 22 to 25, intermediate-density lipoprotein (IDL) and small VLDL; 26 to 29, low-density lipoprotein (LDL) and large HDL; 30 to 33, main HDL; and 34 to 41, lipoprotein-free samples. A mixture of free cholesterol, esterified cholesterol, and phosphatidylcholine was used to calibrate the marker lipoprotein classes (ie, VLDL, LDL, HDL, and HDL). Each pool was applied to the gradient gel. Duplicate gels were stained with Coomassie blue, equilibrated in 33% acrylamide, and subjected to fluorography.

Nondenaturing Gel Electrophoresis

Electrophoresis of medium lipoproteins on 4% to 30% gradient polyacrylamide gel under nondenaturing conditions was performed as described previously.17 The Superose column fractions containing LDL and HDL (fractions 26 to 33) were pooled, and 10 µL of each pool was applied to the gradient gel. Duplicate gels were used for Coomassie blue staining and for Western blot analysis using anti-rat apoA-IV and anti-rat apoA-I antibodies.

Electron Microscopy Studies of Secreted Lipoproteins

Conditioned media (20 mL) obtained from two T75 flasks of cells were collected and supplemented with EDTA (0.5 mmol/L) and PMSF (0.015%). After adjusting to the desired density with potassium bromide, the media were centrifuged at 40,000 rpm at 4°C in an SW41 rotor. To obtain the d<1.06 fraction, centrifugation was performed for 24 hours; to obtain the d=1.06 to 1.210 fraction, centrifugation was performed for 48 hours. After each centrifugation, lipoproteins were carefully collected from the top of the tubes, dialyzed, and further concentrated to 100 µL in Centricon-30 filters. The samples were stored at 4°C before negative staining with 1% phosphotungstic acid as described previously.27

The images of negatively stained lipoproteins were captured from 8 x 10 prints at a magnification of 140,000 via an XC-77 CCD video camera (SONY Corp, Japan) by an image-IAT image analysis system (Universal Imaging Corp, West Chester, Pa). The particles were counted and sized (by diameter) by automated pixel analysis. The longest and shortest chords were measured for each individual particle. For nonspherical particles, the shortest chord was its thickness, and the longest was its diameter. Disk-shaped lipoprotein profiles were selected from the general particle populations by a standardized combination of shape factor and size-cutoff filters within the image-I environment.

Metabolic Labeling With [3H]Acetate and [35S]Methionine

Cells (=70% confluence) were incubated with methionine-free DMEM containing [3H]acetate (10 µCi/mL) and [35S]methionine (Tran35S-label, 100 µCi/mL) in the presence or absence of 0.1 mmol/L oleate. After 24 hours' incubation, the media were collected, adjusted to d=1.06 g/mL with potassium bromide, and subjected to ultracentrifugation at 100,000 rpm for 2.5 hours in a Beckman TL100 ultracentrifuge. The top d<1.06 g/mL fraction was collected and divided into two 100-µL aliquots. In one aliquot, the 35S-labeled proteins were concentrated with fumed silica and then resolved by electrophoresis on a 5% to 20% polyacrylamide gel. Lipids were extracted from the second aliquot with chloroform/methanol (2:1, vol/vol), dried under a nitrogen stream, and separated by thin-layer chromatography.4 A mixture of free cholesterol, esterified cholesterol, and triglycerides was used to identify the component lipids. The bands containing the corre-
Results

Generation of Stable Transformants of McA-RH7777 Cells Expressing Recombinant Rat ApoA-IV

Unlike primary rat hepatocytes, the McA-RH7777 hepatoma cells do not produce apoA-IV. We confirmed that there was no detectable endogenous apoA-IV mRNA or apoA-IV protein in these cells (see below). We prepared a rat apoA-IV expression plasmid (Fig 1A) and stably transformed McA-RH7777 cells by using this plasmid. Southern blot analysis (Fig 1B) revealed that five clones (AIV-3, -8, -12, -15, and -18) exhibited positive reactions with a cDNA probe (1.35-kb bands). Clone AIV-15 contained the highest copy number (lane 5) among the transformants. Southern blot analysis showed that the rat apoA-IV gene was present in both the parental nontransfected cells and the stable transformants (arrow, 3.8-kb fragments). Northern (Fig 1C) and Western (Fig 1D) blot analyses demonstrated that the stable cell lines harboring the apoA-IV expression construct contained apoA-IV mRNA and secreted apoA-IV protein into the media. Endogenous apoA-IV mRNA and protein were absent from the nontransfected parental cells (lane M7777 of Fig 1C and 1D). The level of apoA-IV secretion from AIV-15 (high-expressing cell line) was estimated by densitometry of Western blots by using purified rat apoA-IV as a control (data not shown). In the absence of serum, secretion of apoA-IV was 0.6 μg/mg cell protein per hour. Stable cell lines AIV-15 and AIV-8,
representing high and low expressors, respectively, were chosen for further studies. Clone AIV-18 was not chosen to represent the low expressor because its apoA-IV production was too low to be reliably quantified.

Characterization of Lipoproteins Secreted by Stably Transfected and Nontransfected McA-RH7777 Cells

The association of apoA-IV with secreted lipoproteins was determined by Superose 6 column chromatography. Immunoblot analysis of the individual fractions shown in Fig 2A demonstrated that approximately 60% of the apoA-IV was lipoprotein associated (fractions 17 to 33) and the remainder was lipoprotein free (fractions 34 to 41). Most of the lipoprotein-associated apoA-IV appeared in fractions corresponding to human HDL (fractions 29 to 33), and a smaller proportion was found in the fractions corresponding to human LDL/LDL (fractions 23 to 27). A minute amount of apoA-IV was associated with VLDL (fractions 17 to 21) at both the high (AIV-15) and low (AIV-8) expression levels (Fig 2A).

Oleate stimulates VLDL synthesis and secretion. To determine if these conditions affect apoA-IV synthesis and secretion, cells were cultured in the presence of 0.1 mmol/L oleate, and the conditioned media were subjected to column chromatography. The relatively low concentration of oleate (0.1 mmol/L) was chosen because in McA-RH7777 cells this concentration of oleate is sufficient to stimulate triglyceride synthesis without impairing triglyceride secretion. Supplementation with oleate had no effect on the association of apoA-IV with lipoproteins; the pattern of apoA-IV distribution (Fig 2A) was the same in the presence or absence of oleate (data not shown). The effect of 0.1 mmol/L oleate on VLDL production is discussed below.

We then compared the density distribution of endogenous apolipoproteins in the medium of cells secreting high levels of apoA-IV (AIV-15) with that of nontransfected cells. Secretory proteins were labeled by incubating the cells with [35S]methionine for 24 hours, and lipoproteins were fractionated in a density gradient (from d=1.03 to d=1.23 g/mL). The apolipoprotein...
distribution in the gradient is shown in Fig 2B (top, nontransfected cells; bottom, transfected cell line AIV-15). Transfected apoA-IV was mainly distributed to the most dense fractions (lipoprotein-free and HDL), although its presence was evident throughout the lipoprotein interval. Overexpression of apoA-IV did not affect the distribution of any of the endogenous apolipoproteins; apoA-I was characteristically associated with HDL, whereas apoB-100 was found in VLDL/LDL (d<1.06 g/mL). Notably, apoB-48 appeared mainly in HDL (fractions 3 to 13) in both the absence and presence of apoA-IV (Fig 2B). The increased levels of apoB-48 seen in the media of cell line AIV-15 (Fig 2B, bottom panel) were not observed in all experiments. The identity of the band above apoB-48 (see the d=1.03 fraction of Fig 2B) was not determined; this band did not react with anti-apoB antibodies on Western blots (data not shown). Additional bands (≈97 kD) seen in the media of cell line AIV-15 (Fig 2B, bottom panel) were also not identified. These 97-kD proteins did not react with anti-apoA-IV antisera and were not seen in the intermediate-expressing (AIV-12) or low-expressing (AIV-8) cell lines (data not shown).

To further characterize the secreted lipoproteins, we pooled the Superose column fractions into four major lipoprotein classes and analyzed them by agarose gel electrophoresis and immunoblotting. Fig 3A shows the lipid-staining pattern of the pooled lipoproteins secreted from stable cell line AIV-8 (the low apoA-IV expressor). Lipoproteins with pre-β- or β-electrophoretic mobilities occurred mainly in the VLDL pool (lane 1). Lipoproteins with α-mobility were localized exclusively in the LDL/large HDL (lane 3) and the main HDL (lane 4) pools. The same lipid-staining patterns were observed in the cell line AIV-15 (the high apoA-IV expressor) and in nontransfected McA-RH7777 cells (data not shown). The apolipoprotein composition of each of the lipid-containing components was characterized by immunoblot analysis using antibodies against apoB (Fig 3B), apoA-I (Fig 3C), and apoA-IV (Fig 3D). As expected, apoB was mainly associated with the pre-β- and β-migrating lipoproteins (Fig 3B), whereas apoA-I was associated primarily with the α-migrating lipoproteins (Fig 3C). The same pattern was observed in medium fractions from nontransfected cells (data not shown). Again, most of the apoA-IV was found in the large and main HDL pools in which the α-migrating particles were present (Fig 3D, lanes 3 and 4). Interestingly, the apoA-IV–containing particles had electrophoretic mobilities distinct from those of the apoA-I–containing α-migrating lipoproteins. Trace quantities of apoA-IV were detected in the VLDL pool (Fig 3D, lane 1), where apoA-IV comigrated with the apoB-containing components on the agarose gel. By comparing the lipid-staining pattern of lipoproteins in the HDL pool (Fig 3A, lane 4) with the Western blots for apoA-I (Fig 3C, lane 4) and apoA-IV (Fig 3D, lane 4), we concluded that most of the apoA-IV was associated with discrete particles that were deficient in neutral lipids and apoA-I.
The conditioned media (with or without 0.1 mmol/L oleate) were analyzed by electron microscopy after negative staining. In nontransfected cells, most of the HDL particles were spherical, and a minor portion of the HDL was disk shaped (Fig 4, d=1.06 to 1.21 of McA-RH7777). Among 444 particles analyzed by using an IMAGE-1/AT image analysis system, 30 disk-shaped profiles were observed in the conditioned medium of nontransfected control cells (6.8%). In contrast, medium from cell line AIV-15 exhibited a striking increase in the proportion of disk-shaped particles (Fig 4, d=1.06 to 1.21 of AIV-15). Of 539 particles analyzed, 202 were disks (37.3%). The average sizes of these disk structures from the control and transfected cells were nearly identical; McA-RH7777 diameter was 21.7±4.7 nm and thickness, 7.9±1.9 nm; AIV-15 diameter was 22.2±5.4 nm and thickness, 7.2±2.8 nm. The diameter (=22 nm) of these disks resembled that of the discoidal bilayer structures of nascent apoA-I-containing HDL (=19 nm) from perfused rat liver.25 The thickness of these disks (=8.0 nm) may correspond to that of a double-bilayer structure deficient in core lipids. The diameter of the spherical particles in the HDL range (d=1.06 to 1.21) was larger in the transfected cells than in the control cells (19.6±6.4 nm versus 14.5±5.6 nm, P<.001).

The morphology of VLDL/LDL (d<1.06 g/mL) secreted from the cells was also examined. In both nontransfected McA-RH7777 cells and the overexpressing cell line AIV-15, spherical particles 30 to 50 nm in diameter, representing mainly IDL and small VLDL, were observed (Fig 4, d<1.06). The near absence of particles with diameters greater than 50 nm indicated that the cells did not produce triglyceride-rich VLDL under serum-free conditions. Supplementation of the media with oleate induced the secretion of large VLDL particles (60 to 80 nm) in both the nontransfected cells and the high-expressing cell line AIV-15 (Fig 4, d<1.06, +oleate). The identical response of cells in producing large VLDL on supplementation with oleate further suggests that overexpression of apoA-IV does not influence the formation of triglyceride-rich lipoproteins by McA-RH7777 cells. The increased secretion of neutral lipids in VLDL/LDL on the supplementation of the media with 0.1 mmol/L oleate was confirmed by metabolic labeling experiments using [14C]acetate. Incubation with 0.1 mmol/L oleate for 24 hours resulted in a 3.5-fold increase in 14C-labeled triglycerides and cholesteryl esters in the d<1.06 g/mL lipoprotein fractions of the media of both nontransfected or apoA-IV-transfected cells (data not shown). Additionally, secretion of apoB-100 in the d<1.06 g/mL lipoproteins was also stimulated by the oleate treatment (Fig 5, lanes 3 and 4 versus lanes 1 and 2). The radioactivity associated with apolipoprotein bands on the polyacrylamide gel was quantified, and the rate of apolipoprotein secretion (counts per minute per milligram cell protein per 2 hours) was determined. In control medium (bovine serum albumin, BSA of Fig 5), apoB-100 was 2600; apoB-48, 1600; apoA-IV, 40 300; apoE, 90 800; and apoA-I, 21 300. In oleate-containing medium (BSA/OA of Fig 5), apoB-100 was 8900; apoB-48, 2100; apoA-IV, 25 800; apoE, 80 800; and apoA-I, 19 500. Supplementation of the media with 0.1 mmol/L oleate did not alter...
the morphology of HDL secreted from either nontransfected or cell line AIV-15 (data not shown). Taken together, these data suggested that supplementation of the McA-RH7777 medium with oleate only stimulated the secretion of apoB-containing lipoproteins and had no effect on the other endogenous apolipoproteins such as apoE or apoA-I. The reason for the decrease in secretion of the recombinant apoA-IV on supplementation of oleate was not immediately evident, especially if considering that, in the rat, fat feeding is associated with an increase in apoA-IV secretion from intestinal cells. However, to our knowledge, the response of apoA-IV production by rat hepatocytes in vivo or in vitro after fat feeding has never been studied. It is possible that apoA-IV regulation in liver is different from that in intestine.

The size of apoA-IV- and apoA-I-containing lipoprotein particles was further analyzed by immunoblot analysis of nondenaturing gradient gels (Fig 6). Two apoA-IV-containing populations were detected in the HDL pool of cell line AIV-15 (Fig 2, fractions 26 to 33). The larger particles were approximately 8.0 nm in Stokes’ diameter, whereas the smaller were approximately 7.0 nm (Fig 6, lanes 3 and 4). In contrast, the endogenous apoA-I-containing particles in this HDL pool were smaller than the apoA-IV-containing population (less than 7.0 nm in Stokes’ diameter) (Fig 6, lanes 1 and 2). The relatively decreased levels of apoA-I in the media of the transfected cell line shown in the immunoblot (Fig 6, lane 2) were attributable to unequal amounts of proteins applied to the gels and did not reflect an impairment in apoA-I secretion. In general, the levels of endogenous apoA-I secretion were not significantly altered in any of the transfected cell lines compared with the nontransfected McA-RH7777 cells (Fig 2B). Whether apoA-IV is also associated with the large VLDL/IDL particles (Fig 2, fractions 17 to 25) in the nondenaturing gel system was not examined.

### Kinetic Studies of Apolipoprotein Secretion

To further investigate the possible role that apoA-IV plays in the production of apoB-containing lipoproteins, we compared the rate of apoB and of apoA-IV secretion after stimulation of lipogenesis. Because apoA-IV transcription in the transfected cells was driven by the powerful human cytomegalovirus promoter, expression of the transgene was constitutive and was not regulated by hormones or other metabolic conditions (eg, fat). The reason for the decrease in secretion of apoA-IV is presumably attributable to stimulated triglyceride synthesis and enhanced assembly and secretion of apoB-containing lipoproteins. However, as was the case for the oleate-supplementation experiments (Fig 5), secretation and enhanced assembly and secretion of apoB-containing lipoproteins.
ApoA-IV and ApoB-100 in cell lines AIV-15 and AIV-8 were preincubated with \[^{35}S\]methionine for 2 hours and then chased for up to 4 hours in a serum-free medium. Cellular and medium apoB-100 (A) and apoA-IV (B) were immunoprecipitated with corresponding antibodies. Radioactivity associated with apoB-100 and apoA-IV was quantified after resolution of the proteins on polyacrylamide gels. C and D, Effect of serum on the secretion of apoB (C) and apoA-IV (D). Cell line AIV-15 was prelabeled for 2 hours and then chased for 1 and 2 hours in the presence or absence of serum (10% fetal bovine serum and 10% horse serum). The media were collected, apoA-IV was precipitated with anti-apoA-IV antibody, and apoB-100 was recovered from the d<1.06-g/mL fraction by ultracentrifugation. ApoB-100 and apoA-IV were resolved by electrophoresis and visualized by fluorography. Bands containing apoB-100 and apoA-IV were analyzed by densitometry.

Discussion

Although apoA-IV is a major apolipoprotein component of plasma HDL, its involvement in the formation of lipoproteins is poorly understood. High-level production of recombinant rat apoA-IV was achieved using McA-RH7777 cells stably transfected with a rat apoA-IV cDNA construct. Determination of association of the recombinant apoA-IV with lipoproteins and the analysis of their density distribution suggested that the majority of apoA-IV, secreted at either low or high levels, formed discrete disk-shaped particles the size of HDL. Overexpression of apoA-IV did not affect the rate or efficiency of apoB secretion, nor did it alter the buoyant densities of the apoB-containing lipoproteins: apoB-100 was in VLDL/LDL, whereas apoB-48 appeared mainly in HDL.

In this study, we observed that the cell line that expressed high levels of recombinant apoA-IV produced more disk structures exhibiting electrophoretic mobilities distinct from HDL containing apoA-I. These disk structures may be apoA-IV-containing HDL particles that are deficient in core neutral lipids. It has not been determined whether the disks are assembled within the cells or formed in the culture medium after apoA-IV secretion. One report, however, indicates that apoA-IV can associate with liposomes prepared from dioleylphosphatidylcholine to form disks in vitro. In perfused liver, nascent HDL particles appear as disks, particularly when cholesteryl ester generation by LCAT is inhibited during perfusion. Discoidal particles are thought to be precursors of the circulating spherical HDL. It has been postulated that these HDL precursors mature extracellularly through association with various exchangeable apolipoproteins and phospholipids. It is tempting to speculate that the apoA-IV disks secreted by McA-RH7777 cells were poorer substrates for LCAT reaction than apoA-I-containing disks; thus, they could retain their unique morphology in the medium after prolonged incubation. Because the thickness of the disk-like structures accumulated in the medium of
apoA-IV—transfected cells (~8.0 nm) was twice that of the phospholipid bilayer, it is equally possible that they were derived from collapsed vesicles. In the current study, LCAT activity in the McA-RH7777 media was not analyzed. The interaction of apoA-IV disks with LCAT deserves further investigation. Alternatively, the existence of stable discoidal apoA-IV particles may result from association with a subclass of HDL-like particles that have the optimal phospholipid surface pressure for apoA-IV binding.24 Cell lines that overexpress the recombinant apoA-IV would provide useful tools for these studies. The present data suggested that hepatic synthesis of apoA-IV in rodents may lead to the formation of a different subclass of HDL than that seen in other animals, such as humans, who lack heptatically derived apoA-IV. Such apoA-IV–HDL may have a different metabolic fate than apoA-I–HDL.

The two major apolipoprotein constituents in human chylomicrons are apoA-IV and apoB-48, both produced only by the intestine in humans. ApoA-IV synthesis in intestine and its secretion into mesenteric lymph are known to be enhanced by fat feeding.35,36 and this has led to the assumption that both apoB-48 and apoA-IV might be involved in the formation of chylomicrons. In contrast to humans, the rat synthesizes apoA-IV and apoB-48 in both the intestine and the liver, and apoB-48 derived from rat liver can form VLDL.37 Furthermore, recent transfection studies using rat or human hepatoma cell lines demonstrate that the length of the apoB molecule has an important effect in recruiting lipids and assembling buoyant, lipid-rich lipoproteins.16,17 In transfected hepatoma cells, the length of apoB peptides determines the buoyant density and the core circumference of lipoproteins.16,17,38 However, a question arising from these studies is why apoB-48, a truncated species that is only half the length of apoB-100, can assemble chylomicrons in intestine and VLDL in rat liver. In fact, VLDL particles synthesized by rat liver contain either one apoB-100 molecule or one apoB-48 molecule.29 This is in sharp contrast to the observation from the transection studies in which the recombinant human apoB-48 forms only lipid-poor HDL.17 It is conceivable that the inability of hepatoma cell lines to form apoB-48–VLDL results from the deficiency of factors other than apoB. We speculated that the lack of apoA-IV synthesis in McA-RH7777 cells might be responsible for the impaired apoB-48–VLDL synthesis. However, the current studies demonstrated that apoA-IV synthesis is not sufficient for the production of VLDL containing apoB-48 in McA-RH7777 cells. If apoA-IV is not involved in the formation of apoB-containing lipoproteins, then what is the real physiological function of this apolipoprotein? A most intriguing observation, reported by Fujimoto et al.,8 suggests that rat apoA-IV that is released in response to fat feeding may act as a signal for satiation. The increased apoA-IV in chylomicron lymph after fat feeding exerts an anorectic effect on 24-hour fasted rats, and this function of apoA-IV is not shared by apoA-I.8 Obviously, the hormonal effect of apoA-IV that may suppress food intake merits further investigation.

Since the small intestine is the major organ for the synthesis of apoA-IV and apoB-48 in humans, more conclusive proof that apoA-IV is not involved in the formation of apoB-48–containing lipoproteins remains to be established in intestinal cells. Among other possibilities, the lack of an effect of apoA-IV synthesis on apoB-48 lipoprotein formation in McA-RH7777 cells may be attributable to the synthesis of apoE. In newly synthesized hepatic VLDL, more than 20% of the apolipoprotein constituent is apoE.39 Synthesis of apoE in McA-RH7777 cells may have masked the requirement of apoA-IV in apoB-48–VLDL formation, whereas apoE synthesis is absent in the intestine. It is noted that in transgenic mice in which the apoE gene has been inactivated, an increased amount of apoA-IV is found in the plasma VLDL of these animals.40,41 Working with HepG2 cells, we found that association of apoE with nascent apoB-containing lipoproteins was enhanced under conditions when VLDL secretion was stimulated (S. Fazio, MD, PhD, and Z. Yao, PhD, unpublished data). However, the relationship of apoB, apoE, and apoA-IV during the formation of triglyceride-rich lipoproteins needs to be explored further. The cell lines described herein, as well as other transfected cells of hepatic and intestinal origin, will be useful in elucidating these relations.

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ApoA-IV is secreted on discrete HDL particles by the rat hepatoma cell line McA-RH7777 transfected with ApoA-IV cDNA.

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