Identification of a neutral lipid core in a transiently expressed and secreted lipoprotein containing an apoB-48-like apolipoprotein

Denise J. Spring, Sun-Min Lee, Donald L. Puppione, Martin Phillips, John Elovson,* and Verne N. Schumaker[1]

Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California, Los Angeles, and the Wadsworth Veterans Administration Medical Center,* Los Angeles, CA 90024

Abstract The presence of core lipids in lipoproteins expressed and secreted by transfected HepG2 cells was demonstrated by measuring the densities of these lipoproteins before and after treatment with a bacterial lipase specific for neutral lipids. HepG2 cells were reproducibly transfected with pRSV/B48, containing a truncated human apolipoprotein B-100 (apoB-100) cDNA (nucleotides 1 to 6860, where nucleotide 129 is the start of translation). Northern blots of cellular message probed with apoB-48 showed abundant transcription of an apoB-48-sized message as well as endogenous apoB-100 message. When grown in the presence of [^{35}S]methionine, pRSV/B48-transfected cells secreted lipoproteins containing an apoB-48-like apolipoprotein. This lipoprotein banded at a density of 1.11 g/ml in isopycnic NaBr gradients. Electron microscopy of the apoB-48-containing lipoproteins demonstrated spherical particles with an average diameter of 124Å. A sedimentation rate of 8.4S was measured by sucrose gradient sedimentation. When the apoB-48-containing particles were treated with a bacterial lipase (from *Chromobacterium viscosum*), shown to hydrolyze triglycerides and cholesteryl esters but not phospholipids, their density increased to 1.18 g/ml, consistent with removal of core lipids. When the secreted lipoprotein was modeled as a spherical partical containing a single molecule of apoB-48, a triglyceride-filled core, and a surface monolayer of phospholipid and protein, the hydrodynamic properties were consistent with the observed sedimentation coefficient, buoyant densities before and after lipase treatment, and the diameter as seen with the electron microscope. These data indicate that transfected HepG2 cells assembled and secreted lipoproteins possessing the same physical structure as naturally occurring lipoproteins.—**Spring, D.J., S-M. Lee, D.L. Puppione, M. Phillips, J. Elovson, and V.N. Schumaker.** Identification of a neutral lipid core in a transiently expressed and secreted lipoprotein containing an apoB-48-like apolipoprotein. *J. Lipid Res.* 1992. **33**: 233–240.

Supplementary key words transfected HepG2 cells • bacterial lipase

Various lengths of apolipoprotein B (apoB) have been expressed recently in a variety of cell types, including COS cells, a rat hepatoma cell line (McA-RH7777), a mouse mammary cell line (C-127), and a human hepatoma cell line (HepG2) (1–3). In McA-RH7777 cells, Yao, et al. (4) have expressed a series of carboxyl-terminally truncated forms of human apoB, and have shown that these are secreted as lipoproteins; moreover, when the logarithm of the length of apoB was plotted as a function of the buoyant density of the lipoprotein, an inverse, linear function was obtained. They have also shown that the size of the secreted particles increases with apoB length. These data, however, do not define the actual shape and lipid composition of the secreted particles, leaving open the question of whether these truncated apoB peptides have been assembled into spherical lipoproteins containing a nonpolar lipid core, or whether they are disk-shaped particles containing a phospholipid bilayer rimmed with apoB, similar to the apoB disk-shaped lipoproteins reported by Hadzopoulou-Cladaras et al. (2).

In the present study, we demonstrate that transfected HepG2 cells secrete an apoB-48-containing particle of the same size and density as that found by Yao et al. (4) in transfected rat hepatoma cells. In addition, we demonstrate that this particle is spherical by electron microscopy, that it has the appropriate sedimentation coefficient for its size, and that it contains a core of hydrophobic lipids susceptible to hydrolysis by a bacterial lipase that hydrolyses triglycerides and cholesteryl esters, but not phospholipids.

Abreviations: apoB, apolipoprotein B; BHT, butylated hydroxytoluene; cDNA, complementary deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; HEPES, n-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; LDL, low density lipoprotein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; rpm, revolutions per minute; rRNA, ribosomal ribonucleic acids; SDS, sodium dodecylsulfate; Tris, tris(hydroxymethyl)aminomethane; Tris-HCl, Tris hydrochloride; VLDL, very low density lipoprotein.

[1]To whom correspondence should be addressed.

Together these data indicate that certain cell culture expression systems, utilizing transfected apoB cDNA, are capable of assembling and secreting lipoproteins that are structurally similar to naturally occurring lipoproteins.

Enzymatic methods for the analysis of triglycerides, cholesterol, and cholesteryl esters are widely used for the determination of the lipid composition of purified plasma lipoproteins (5). In these assays, a nonionic detergent is added to the lipoprotein preparation presumably disrupting the lipoprotein structure and exposing the core lipids to the action of the enzymes used in the analysis. Here we report that the neutral lipids in the intact lipoproteins are readily accessible to the lipase from *Chromobacterium viscosum* in the absence of detergent. It is also shown that this enzyme cleaves both triglycerides and cholesteryl esters, but no measurable phospholipid. Because the secreted lipoprotein containing the apoB-48-like apolipoprotein has a density similar to the high density lipoproteins (HDL), it is difficult to purify; therefore, colorimetric analyses performed after enzymatic hydrolysis would not distinguish between HDL neutral lipids and those present in the apoB containing particles. Here an alternate approach was used: measuring the density difference of the apoB-containing lipoproteins before and after lipase treatment demonstrated the presence of neutral lipids, which is the hallmark of the naturally occurring apoB-containing lipoproteins.

EXPERIMENTAL PROCEDURES

Reagents

Restriction enzymes were purchased from Boehringer-Mannheim (Indianapolis, IN) and BRL (Gaithersburg, MD). RSV-1 plasmid was a generous gift from Cornelia Gorman (Genentech, San Francisco, CA). HepG2 cells were obtained from American Type Culture Collection (Rockville, MD). All cell culture reagents, including reagents needed for mammalian transfection, were cell culture grade and purchased from Sigma (St. Louis, MO). [α-^{32}P]dCTP and [^{35}S] methionine (Tran^{35}S-Label) were obtained from Amersham (Arlington Heights, IL) and ICN Biomedicals (Costa Mesa, CA), respectively. Bacterial lipase (*Chromobacterium viscosum*) was purchased from Craig Bioproducts (Streamwood, IL).

Plasmid construction

To create pRSV/B48, an apoB-48 cDNA insert was placed in the RSV-1 mammalian expression vector from existing human cDNA apoB clones. Base pairs 1 to 5289 of the apoB cDNA were obtained from the plasmid pBS/A6C/ABF, where base pairs 1 through 1197, and 3146 through 5289 were obtained from ABF (6); base pairs 1198 through 3145 were from A6C (7). (The construction of pBS/A6C/ABF was necessary to correct a coding error in the central portion of ABF.) Base pairs 5289 (a SalI restriction enzyme site) through 11115 (a BamHI restriction enzyme site) were obtained from the plasmid pUC/SB9.

To construct pRSV/B48, apoB base pairs 5289 through 6860 were removed from pUC/SB9 by digestion with SalI and ScaI, and ligated to the SalI/SmaI ends in the RSV-1 vector, generating the pRSV/SB9′ intermediate. Base pairs 1 to 5289 of apoB were removed from pBS/A6C/ABF by digestion with SalI and SmaI. This fragment was ligated to the EcoRV/SalI ends of pRSV/SB9′, creating pRSV/B48.

CsCl plasmid isolation

Transfection-quality plasmids were prepared by double isolation on CsCl gradients as described (8).

Cell culture

HepG2, a human liver hepatoma cell line (9), was cultured at 37°C in 5% CO_2/95% humidified air in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, nonessential amino acids (Sigma), 1 mM sodium pyruvate, and antibiotics (penicillin/streptomycin, 100X Sigma). Cells were passaged on a 7-day cycle.

Transfection

HepG2 cells were transfected according to Wigler et al. (10), using the following modified protocol. HepG2 cells were plated on 100-mm plates the day prior to transfection at a density of ~1 × 10^6 cells/plate in order to give 30–35% confluency on the day of transfection. Twenty µg of plasmid DNA in 10 µl of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) were added to 440 µl of 2 × HEPES buffer (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.05). Four hundred fifty µl of 250 mM $CaCl_2$ was added dropwise with constant mixing to this DNA solution. Samples were incubated at room temperature for 30 min prior to the addition of the resulting precipitate to the cell culture medium. The cultures were incubated in the presence of the DNA precipitate for 14 h at 37°C in a humidified 5% CO_2/95% air incubator. Transfected cells were washed twice with Dulbecco's modified PBS (Sigma) to remove the precipitate, fed with complete medium, and allowed to grow to near confluency.

RNA isolation and Northern analysis

Total RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction from cell monolayers (11). One to five µg of total RNA was electrophoresed on a 0.8% agarose/6% formaldehyde

gel, transferred to Nytran (Schleicher & Schuell, Keene, NH) by capillary action, and crosslinked using ultraviolet light for 3 min. RNA filters were blocked for 1 h with prehybridization solution (12). Sixty ng of apoB probe (derived from the 3725 base pair PvuII fragment of apoB cDNA, and covering base pairs 2181 to 5096) was labeled with [α-^{32}P]dCTP to a specific activity of 10^9 cpm/μg, using a random hexamer priming kit (Boehringer-Mannheim). ApoB probe was hybridized to the filters in 5 ml prehybridization solution overnight at 65°C. The filters were washed twice with 2× wash solution (2× SSC, 0.1% SDS) followed by two or more washes with 0.1× wash solution (0.1× SSC, 0.1% SDS) at 65°C until the background was minimal. (20× SSC is 3 M NaCl, 0.3 M Na_3 citrate). The filters were then dried and used for autoradiography at –70°C with XAR5 film (Kodak).

In vivo labeling

At 48 h posttransfection (≥80% confluency), cell monolayers were washed twice with methionine-deficient, serum-free medium followed by incubation with 50 μCi[^{35}S]methionine in 8 ml of methionine-deficient, serum-free medium for 16 h at 37°C.

Lipoprotein isolation

Labeled culture medium was collected and mixed with preservatives to the following concentrations: 30 mM Tris-HCl (pH 7.4), 20 μg/ml soybean tryspin inhibitor, 1 mM EDTA, 1 mM PMSF, 0.05 mM BHT, and 0.05% NaN_3. The density of this labeled culture medium was adjusted to 1.21 g/ml with solid NaBr. After ultracentrifugation (Type 70.1 Ti rotor, 40,000 rpm, 30 h, 15°C), the upper milliliter of medium was collected and dialyzed at 4°C against 10 mM Tris-HCl (pH 7.4), containing 1 mM EDTA, 1 mM PMSF, 0.05 mM BHT, and 0.05% NaN_3. This preparation is referred to as the "lipoprotein float."

Immunoprecipitation

To the isolated lipoprotein float Triton X-100 was added to a final concentration of 1%, and precleared with pre-immune rabbit serum followed by precipitation of the immune complexes with a 10%(w/v) suspension of fixed *Staphylococcus aureus* cells (Sigma). ApoB protein was immunoprecipitated from this precleared lipoprotein float with affinity-purified polyclonal rabbit antibody made against human apoB-100, followed by *S. aureus* cells. Immunoprecipitates were washed once with dialysis buffer containing 1% Triton X-100.

SDS-PAGE and fluorography

Immunoprecipitates were resuspended in SDS-PAGE sample buffer (0.1 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 2% β-mercaptoethanol) and heated to 95°C for 5 min. Samples were microfuged briefly, loaded on 5% polyacrylamide gels, and resolved by electrophoresis at 80 volts (13). The gels were stained with Coomassie brilliant blue and destained to visualize the molecular weight markers. The gels were treated with Enhance (ICN) according to the manufacturer's recommendations, and dried. Fluorography of the dried gels was carried out at -70°C with XAR5 film. Films were scanned using a Zenith soft laser densitometer (Biomed Instruments, Inc., Fullerton, CA).

Density determination

Dialyzed lipoprotein float was mixed into the bottom half of a step gradient composed of a 1.21 g/ml NaBr solution overlaid with an equal volume of 1.06 g/ml NaBr solution. The gradients were ultracentrifuged for 48 h in an SW41 rotor (38,000 rpm, 15°C) to create a linear salt gradient and to bring the lipoproteins toward their equilibrium density. The densities of the 1-ml fractions were determined gravimetrically or by refractometry. ApoB was then immunoprecipitated from the fractions, and analyzed by SDS-PAGE followed by fluorography (as described above).

Sedimentation rate

Dialyzed lipoprotein float was layered on a 10–20% linear sucrose gradient. After ultracentrifugation (SW41 rotor, 39,000 rpm, 20 h, 20°C), 1-ml fractions were collected and analyzed for apoB by immunoprecipitation followed by SDS-PAGE and fluorography. The distance traveled by the apoB-48-containing particle in the specified time was used to calculate the sedimentation rate using a computer program that performed a point-by-point integration over the measured density and viscosity gradient.

Lipase treatment

The lipoprotein float was dialyzed against 50 mM Tris-HCl (pH 7.4). Bacterial lipase (*C. viscosum*) (14, 15), was added to a final concentration of 100 units/ml, and the mixture was incubated at 37°C for 30 min in the absence of detergent.

In order to demonstrate that the lipase from *C. viscosum* could hydrolyze lipoprotein triglycerides and cholesteryl esters in the absence of detergents, the lipase was added to a standard human serum sample of known triglyceride and cholesteryl ester composition. Neutral lipids were analyzed as described (5); the procedure for triglycerides was modified by preparing the appropriate reagents (Craig Bioproducts) without Triton X-100. The procedure for cholesteryl esters was modified by dissolving the appropriate reagents in 50

mM PIPES, pH 6.9. The final reagent solution contained neither Triton X-100 nor sodium cholate, and the cholesteryl esterase was replaced with the *C. viscosum* lipase in varying amounts, as described in Results.

Electron microscopy

A 10-µl drop of sample was placed on a freshly glow-discharged carbon-parlodion-covered copper grid and macromolecules were allowed to adhere for 1 min. The drop was then removed by touching a filter paper wedge (Whatman #4) to the edge of the grid. The still wet grid was washed with five successive drops of 1% aqueous uranyl acetate. A sixth drop of uranyl acetate was left on the grid for 45 sec, then removed by touching a wet filter paper wedge to the edge of the grid. Samples were examined in a Hitachi H-7000 electron microscope, using a 200 µm condenser aperture and a 50 µm objective aperture, at magnifications of 30–40 thousand. The microscope magnification was calibrated using a diffraction grating replica (54,800 lines/inch).

RESULTS

Plasmid construction

pRSV/B48 contained the first 6860 base pairs of human apoB cDNA (**Fig.1**). This included the 5′ noncoding region, the signal sequence, and the apoB coding region extending 65 amino acids past the natural C-terminus of apoB-48, followed by a short vector-coded non-apoB sequence coding for five C-terminal residues: Gly-Tyr-Arg-Ile-Arg. Transcription was under the control of the strong Rous sarcoma virus promoter (16).

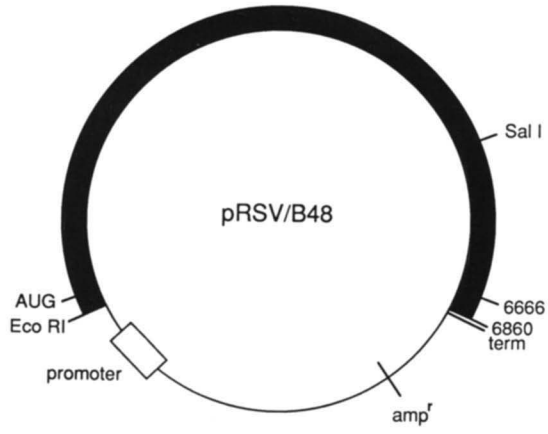

Fig. 1. Construction of pRSV/B48. pRSV/B48 was constructed in the RSV-1 vector from existing human cDNA apoB clones. Base pairs 5289 through 6860 of apoB were transferred, using a SalI/ScaI restriction enzyme digest, from pUC/SB9 to the SalI, SmaI ends of RSV-1 (pRSV/SB9′). Base pairs 1 through 5289 of apoB were removed from pBS/A6C/ABF with a SmaI, SalI digest, and directionally cloned to the EcoRV/SalI ends of pRSV/SB9′, creating pRSV/B48.

ApoB-48 expression

As seen in **Fig. 2**, HepG2 cells transiently transfected with the RSV-1 plasmid (negative control) expressed only the endogenous ≈14 kilobase apoB-100 mRNA. However, cells transfected with pRSV/B48 expressed an additional apoB mRNA, of the size expected for a construct containing approximately one-half of the apoB-100 sequence. Furthermore, cells transfected with the pRSV/B48 plasmid secreted a lipoprotein containing a B-48-sized apoB peptide. Thus, as seen in **Fig. 3**, anti-apoB immunoprecipitates of the $d < 1.21$ g/ml fraction of culture medium from [^{35}S]methionine-labeled HepG2 cells transfected with the RSV-1 control plasmid contained labeled apoB-100, while those from pRSV/B48-transfected cells contained an additional apoB-48-sized peptide. This result has now been repeated in seven independent transfections.

Characterization of the apoB-48-containing lipoprotein particle

The HepG2 cell line, derived from a human liver hepatoma, is convenient for studies involving the syn-

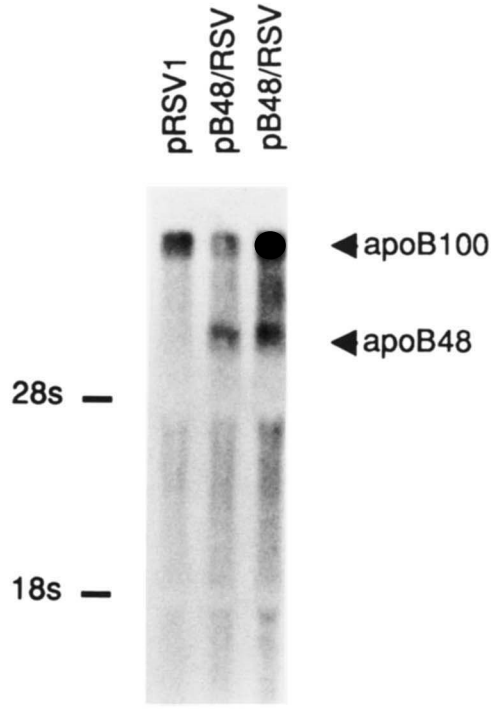

Fig. 2. Northern blot analysis of total RNA isolated from transfected HepG2 cells. 1-4 µg of total RNA from RSV-1 transfected cells (lane 1) or pRSV/B48 transfected cells (lanes 2, 3) were electrophoresed through a 0.8% agarose/formaldehyde gel, transferred to Nytran, and probed with [^{32}P]-labeled apoB cDNA. Arrows indicate the positions of apoB-100 and apoB-48 mRNA. The positions of 18S rRNA and 28S rRNA are indicated.

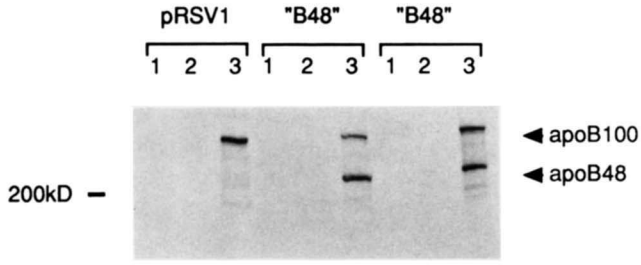

Fig. 3. Immunoprecipitation of apoB from lipoprotein floats from the cell culture media of transfected cells. Lipoprotein floats were isolated from metabolically labeled culture media and precleared. "pRSV 1" indicates samples from cells transfected with RSV-1 plasmid; "pRSV/B48" indicates samples from cells transfected with pRSV/B48. Lipoprotein floats were immunoprecipitated with 7 µl pre-immune rabbit serum (lanes 1), 7 µg rabbit anti-mouse IgG (lanes 2), or 7 µg affinity-purified rabbit polyclonal antibody against human apoB-100 (lanes 3). Pre-immune rabbit serum and rabbit anti-mouse IgG were used as negative controls. The position of myosin (200 K) is given as a molecular weight marker.

thesis of truncation products of human apoB-100, since these cells secrete apoB-100 as the sole protein on a lipoprotein particle having a density of less than 1.063 g/ml (17). This apoB-100-containing lipoprotein has a core consisting primarily of triglyceride (17). As seen in **Fig. 4**, isopycnic density gradient analysis of the d < 1.21 g/ml fraction of culture medium from [^{35}S]methionine-labeled HepG2 cells transfected with either pRSV/B48 or RSV-1 (control) plasmids showed the endogenous apoB-100-containing lipoproteins in the top fraction (d < 1.06 g/ml), as expected for particles previously shown to resemble human plasma LDL in size and density. In contrast, the apoB-48-containing particle found in the medium of pRSV/B48-transfected cells banded at a density of 1.11 g/ml, indicating a considerably lower lipid/protein ratio than for the apoB-100-containing particle, consistent with its being a smaller, denser entity. The sedimentation rate of the apoB-48-containing particle was measured on a 10–20% linear sucrose gradient, from which an $s_{20,w}$ of 8.4 Svedbergs was calculated (**Fig.5**). The sedimentation coefficient was determined in three independent experiments, as summarized in **Table 1.**

Composition of the apoB-48-containing particle

Inasmuch as the amount of material recovered from transiently transfected cells was insufficient for direct compositional analysis, we chose an indirect approach to determine the presence or absence of a neutral lipid core in the apoB-48-containing particles. Lipase from *C. viscosum* was known to hydrolyze triglycerides; we have demonstrated that the enzyme is also effective in the absence of detergent. Moreover, we have demonstrated that it completely hydrolyzes cholesteryl esters as well as triglycerides (**Fig.6**). These experiments, performed by colorimetric assay of the released glycerol and cholesterol, have been confirmed by thin-layer chromatography, demonstrating complete hydrolysis of cholesteryl esters and triglycerides with no trace of phospholipid hydrolysis (data not shown).

Treatment of lipoprotein float with bacterial lipase in the absence of detergents altered the density of the apoB-48-containing particles, shifting the peak on isopycnic density gradients from 1.108 to 1.176 g/ml (**Fig. 7**, Table 1). The density of the endogenous apoB-100 particles secreted by HepG2 cells was also increased by the bacterial lipase treatment, as expected for particles of LDL size with a triglyceride-rich core (17) (Table 1).

Electron micrographs

Negatively stained apoB-48-containing density fractions showed circular particles in projection, compatible with spherical lipoproteins (**Fig. 8**) with an average diameter of 124 Å ± 2Å (SEM, n = 46).

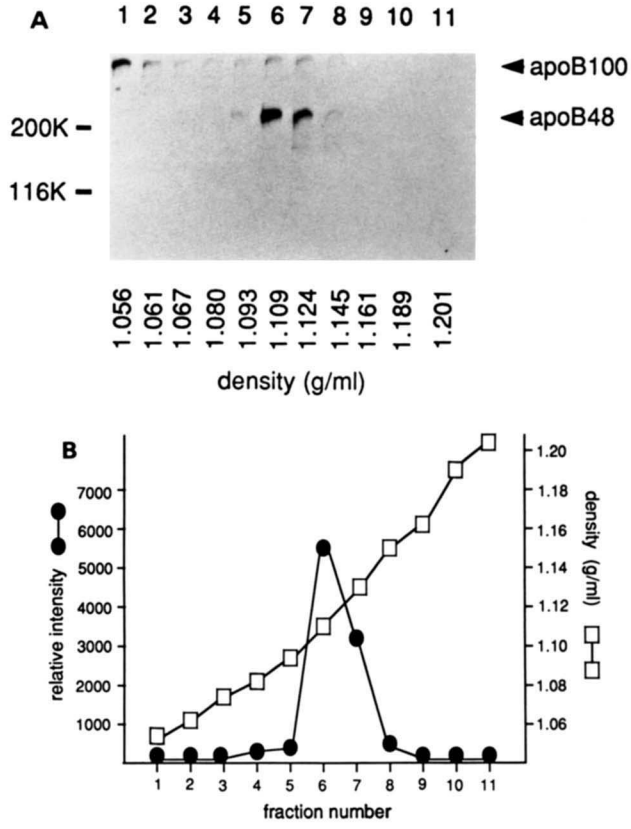

Fig. 4. Density determination of the apoB-48-containing lipoprotein. A: Metabolically labeled apoB from isopycnic gradient fractions was analyzed by SDS-PAGE and fluorography. Lanes 1–11 represent fractions 1 (top) to 11 (bottom) of the gradient. The positions of myosin (200 K) and β-galactosidase (116 K) are given as molecular weight markers. B: Density of each fraction and the intensity of the apoB-48 signal is plotted as a function of fraction number. Fraction numbers correspond to A.

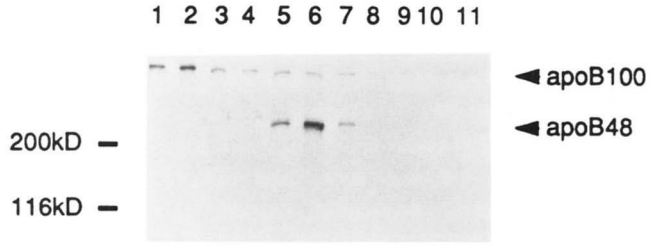

Fig. 5. Sedimentation rate determination of the apoB-48-containing lipoprotein. Metabolically labeled lipoprotein float obtained from the medium of pRSV/B48-transfected cells was layered on top of a linear 10–20% sucrose gradient, and ultracentrifuged for 20 h at 37,000 rpm. One-ml fractions were immunoprecipitated with anti-apoB-100 antibody, and visualized by SDS-PAGE followed by fluorography. Lanes 1–11 represent fractions 1 (top) to 11 (bottom) of the gradient. The positions of myosin (200 K) and β-galactosidase (116 K) are given as molecular weight markers.

We were unable to obtain satisfactory images of the lipase-treated apoB-48-containing particles, but in control experiments, bacterial lipase treatment of human LDL converted the spherical LDL into discs; this will be the subject of a future communication.

DISCUSSION

Our data show that HepG2 cells, transiently transfected with pRSV/B48, transcribed mRNA and synthesized an apoB-48-like protein. This apolipoprotein was secreted into the cell culture medium together with lipids as a spherical lipoprotein having a density of 1.11 g/ml, an $s_{20,w}$ of 8.4 S, and an average diameter of 124 Å as measured on electron micrographs. The internal consistency of the electron microscope and hydrodynamic data can be checked by calculating the size and molecular weight of the secreted apoB-48-containing lipoprotein from the observed density (1.108 g/ml) and sedimentation coefficient (8.43 S), assuming that the frictional ratio was the same as the frictional ratio observed for LDL (i.e., $f/f_o = 1.11$) (18). This calculation yielded a particle diameter of 124 Å, in agreement with the electron microscope results.

It may also be asked whether these values for density and molecular weight are compatible with the standard model for a spherical lipoprotein which assumes a surface monolayer of phospholipid in which the apoB is embedded, and central core of neutral lipid. In order to model the secreted lipoprotein, the monolayer was assumed to be a shell with a thickness of 21.5 Å (19), composed of phospholipid with a partial specific volume of 0.984 ml/g (20), in which was embedded a single apoB-48-like glycoprotein with an estimated molecular weight of 264,000, assuming 6% carbohydrate. This surface monolayer of phospholipid and apoB protein was assumed to be the particle remaining after lipase treatment. From the observed density of the surface [1.176 g/ml (Table 1)] and the shell volume, it was then possible to calculate a partial specific volume of 0.725 ml/g for the protein. From the dimensions and density of the lipoprotein and the

TABLE 1. Hydrodynamic parameters of expressed and secreted lipoproteins

Observed Parameters: Preparation Number	$s_{20,w}$	Density: Before Lipase	Density: After Lipase
	Svedbergs	*g/ml*	
1	8.4	1.11	
2	8.3	1.114	1.165
3	8.6	1.112	1.188
4		1.105	
5		1.098	
Average	8.43	1.108	1.176

Calculated Parameters[a]: Radius	Mol Wt	$s_{20,w}$	Density: Of Lipoprotein	Density: Of Surface
Å		*Svedbergs*	*g/ml*	
60	610,800	8.85	1.121	1.190
61	638,000	8.64	1.114	1.183
62	666,100	8.43	1.108	1.176
63	695,000	8.22	1.102	1.170
64	724,900	8.02	1.096	1.165
65	755,700	7.82	1.091	1.159

[a]The secreted, apoB-48-containing lipoprotein was modeled as a spherical particle containing a surface monolayer of phospholipid of thickness 21.5 Å in which was embedded a single molecule of apoB-48, surrounding an apolar core containing a mixture of triglyceride and cholesteryl esters. For these calculations, partial specific volumes for protein, phospholipid, and core density were 0.725 ml/g, 0.984 ml/g, and 0.930 g/ml, respectively. Glycoprotein molecular mass was assumed to be 264,000 g/ml.

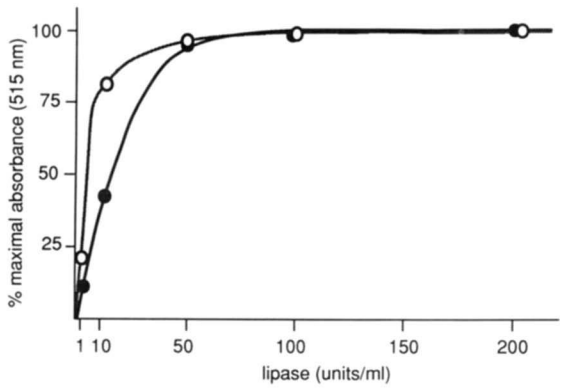

Fig. 6. Substrate specificity of bacterial lipase from *C. viscosum*. A standard human serum of known triglyceride and cholesteryl ester content was treated for 30 min at 37°C with the specified amounts of *C. viscosum* lipase in the absence of detergent, and the hydrolysis of cholesteryl esters and triglycerides was determined by the release of free cholesterol (○) and glycerol (●), respectively. Complete hydrolysis of these neutral lipids was achieved at lipase concentrations above 50 units/ml.

volume and density of the surface monolayer, it was then possible to calculate a value of 0.93 g/ml for the density of the core. Since the secreted lipoproteins appeared in several consecutive density factions, with the average values representing the center of the density distributions, a range of calculated hydrodynamic parameters based on this model is listed in Table 1.

We have also calculated the hydrodynamic properties expected for the alternate disk-shaped model composed of a phospholipid bilayer rimmed by a single apoB molecule. Assuming a single apoB-48 of 264,000 g/mol (0.725 ml/g), combined with sufficient phospholipid (0.984 ml/g) to yield a particle of the observed buoyant density of 1.108 g/ml, calculation showed that the disk would be 285 Å in diameter and have a sedimentation coefficient of 16.5 S. These

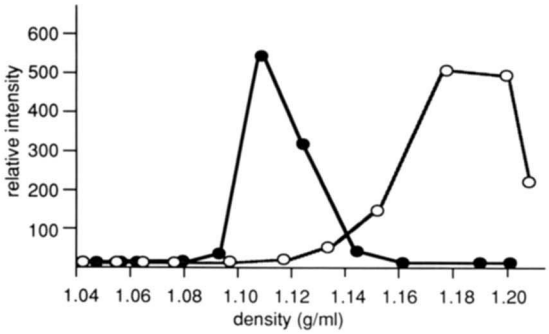

Fig. 7. Effect of bacterial lipase treatment on the density of apoB-48-containing lipoprotein in transfected HepG2 cells. Fluorograms of immunoprecipitated apoB from fractions of isopycnic gradients were scanned by densitometry. Relative intensities were plotted as a function of density. The position of the apoB-48-containing particle in isopycnic gradients was compared for samples obtained before (●) and after (○) lipase treatment.

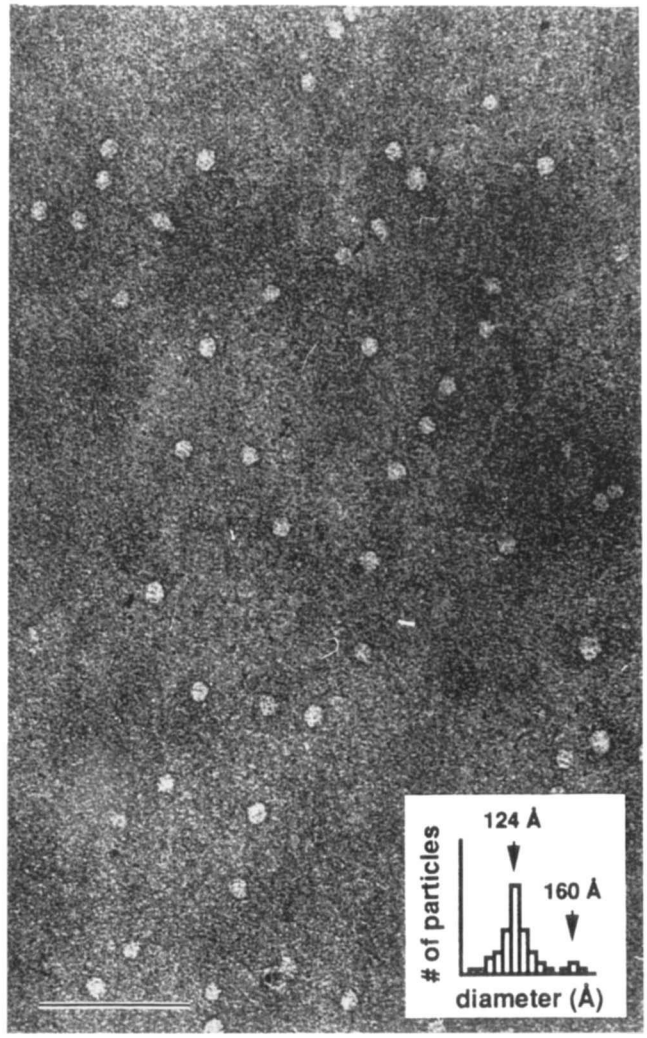

Fig. 8. Electron micrograph of negatively stained apoB-48-containing lipoprotein density fraction. A density fraction containing apoB-48-containing lipoproteins was negatively stained and examined by electron microscopy (see Materials and Methods). The diameter of these particles was 124 Å ± 2 Å (SEM, n = 46). A small subpopulation of particles had a diameter 160 Å; these are probably apoB-100-containing particles which are present in this fraction (n = 50). The bar corresponds to 1000 Å.

values are very different from the diameter and sedimentation coefficient observed for the apoB-48-containing particle, effectively excluding the disk-shaped model from serious consideration.

Our work illustrates that transfected HepG2 cells secrete apoB-48 as a lipoprotein complete with core lipids, presumably a mixture of triglycerides and cholesteryl esters. Calculations indicate that the apoB-48-containing lipoprotein is composed by weight of 40% protein, 37% phospholipid, and approximately 23% core lipids. The hydrodynamic and electron microscopic analyses, in conjunction with data from bacterial lipase treatment, provide information about the composition of a lipoprotein, including the presence

of core lipids, for which direct compositional analysis is difficult due to scarcity of material. Together, these data indicate that the lipoproteins secreted by HepG2 cells in response to transfection with an apoB-containing plasmid have the same physical structure as that of naturally occurring lipoproteins.

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REFERENCES

1. Blackhart, B. D., Z. Yao, and B. J. McCarthy. 1990. An expression system for human apolipoprotein B-100 in a rat hepatoma cell line. *J. Biol. Chem.* **265**: 8358–8360.
2. Hadzopoulou-Cladaras, M., H. Herscovitz, C. Cladaras, D. Small, and V. I. Zannis. 1990. Synthesis, secretion, and flotation of human apoB-17 expressed in C-127 cells. *Arteriosclerosis.* **10**: 774a.
3. Spring, D. J., S-M. Lee, J. Elovson, and V. N. Schumaker. 1990. Transient expression and secretion of a lipoprotein containing an apoB-48-like apolipoprotein. *Arteriosclerosis.* **10**: 774a.
4. Yao, Z., B. D. Blackhart, M. F. Linton, S. M. Taylor, S. G. Young, and B. J. McCarthy. 1991. Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. *J. Biol. Chem.* **266**: 3300–3308.
5. Warnick, G. R. 1986. Enzymatic methods for the quantitation of lipoprotein lipids. *Methods Enzymol.* **129**: 101–123.
6. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complex protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323**: 734–738.
7. Mehrabian, M., V. N. Schumaker, G. C. Fareed, R. West, D. F. Johnson, T. Kirchgessner, H. C. Lin, X. Wang, Y. Ma, E. Mendiaz, and A. J. Lusis. 1985. Human apolipoprotein B: identification of cDNA clones and characterization of mRNA. *Nucleic Acids Res.* **13**: 6937–6953.
8. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA. *Proc. Natl. Acad. Sci. USA.* **57**: 1514–1521.
9. Aden, D. P., A. Fogel, I. Damjanov, S. Plotkin, and B. B. Knowles. 1979. Controlled synthesis of HBs Ag in a differentiated human liver carcinoma-derived cell line. *Nature.* **282**: 615–616.
10. Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell.* **14**: 725–731.
11. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenolchloroform extraction. *Anal. Biochem.* **162**: 156–159.
12. Church, G. M., and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA.* **81**: 1991–1995.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**: 680–685.
14. Horiuti, Y., H. Koga, and S. Gocho. 1976. Effective method for active assay of lipase from *Chromobacterium viscosum*. *J. Biochem.* **80**: 367–370.
15. Horiuti, Y., and S. Imamura. 1977. Purification of lipase from *Chromobacterium viscosum* by chromatography on palmitoyl cellulose. *J. Biochem.* **81**: 1639–1649.
16. Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard. 1982. The Rous sarcoma virus late terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. USA.* **79**: 6777–6781.
17. Thrift, R. N., T. M. Forte, B. E. Cahoon, And V. G. Shore. 1986. Characterization of lipoproteins produced by the human liver cell line, HepG2, under defined conditions. *J. Lipid Res.* **27**: 236–250.
18. Fisher, W. R., M. E. Granade, and J. L. Mauldin. 1971. Hydrodynamic studies of low density lipoproteins: evaluation of the diffusion coefficient and the preferential hydration. *Biochemistry.* **10**: 1622–1629.
19. Sata, T., R. J. Havel, and A. L. Jones. 1972. Characterization of subfractions of triglyceride-rich lipoproteins separated by gel chromatography from blood plasma of normolipemic and hyperlipemic humans. *J. Lipid Res.* **13**: 757–768.
20. Small, D. 1978. Physical chemistry of lipids. *In* Handbook of Lipid Research. D. J. Hannan, editor. Plenum Press, New York. 386.