Secretion from cell culture of HDL and VLDL bearing apoB-33 with a large internal deletion

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Abstract Rat hepatoma McA-RH7777 cells synthesize and secrete two populations of apoB-containing lipoproteins: a larger, VLDL-sized population floating in the S_f 40–150 range and a smaller, LDL and HDL-sized population. Three permanently transfected cell lines of McA-RH7777 cells secreted (in addition to the endogenous lipoproteins) lipoproteins containing *1*) a carboxyl-terminally truncated human apoB-53 (2377 amino acids in length); *2*) a carboxyl-terminally truncated human apoB-31 (1420 amino acids in length); or *3*) an internally deleted human apoB protein, apoB-18/95, containing a total of 1490 amino acid residues, equivalent in length to an apoB-33. The apoB-18/95 protein contained amino acid residues 1–782 joined to 708 residues near the C-terminus of apoB (residues 3636–4343). All three of the apoB peptides, apoB-53, apoB-31, and apoB-18/95, were present on smaller LDL-HDL-class lipoproteins, with buoyant densities in the HDL density range. The sizes of the HDL class lipoproteins agreed with prior observations that lipoprotein core circumference is directly proportional to apoB size. As HDL containing apoB-18/95 conformed to this rule, contiguous apoB amino acid sequence is not required for the rule to be obeyed. In addition, apoB-18/95, but not apoB-31, was also present on the VLDL-sized lipoproteins even in the absence of serum or oleate supplementation. As the latter two constructs encode equally sized apoB peptides, their particular amino acid sequences rather than just overall length must determine whether they can assemble into a VLDL particle.—**Wu, M-J., L. W. Chen-Liu, Q. Xiao, M. L. Phillips, J. Elovson, M. F. Linton, S. G. Young, and V. N. Schumaker.** Secretion from cell culture of HDL and VLDL bearing apoB-33 with a large internal deletion. *J. Lipid Res.* 1997. **38:** 2473–2482.

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Electron microscope and kinetic data have suggested a two-step model for apoB-48 lipoprotein assembly in which the first step is the synthesis of a small, apoB-containing primary particle, followed by a second step in which a large amount of additional lipid is added to generate the secondary particle, the nascent apoB-48-containing VLDL (1–5).

The formation of the primary particle occurs during the cotranslational translocation of apoB into the lumen of the endoplasmic reticulum (6), and it requires the presence of the microsomal triglyceride transfer protein (7). Primary particles produced and secreted by HepG2 cells contain a single molecule of apoB and are spherical, core-containing emulsion particles (8). The sizes of the primary particles were measured for a homologous series of C-terminally truncated apoBs, and lipoprotein core circumference was found to be a linear function of apoB size, with 1 angstrom of core circumference corresponding to about 1 kDa of apoB (9). This result was compatible with a belt-like model for apoB surrounding the lipoprotein, which was previously proposed from electron microscope studies (10–12). ApoB-48 primary particles similar to those secreted by HepG2 cells were secreted by transient and stable cell lines of McArdle RH-7777 rat hepatocytes, (13, 14), and have been reported to be abundant in rat liver microsomes in vivo (3, 5).

A second step seems to be required to produce the nascent VLDL from the apoB-48-containing primary

Abbreviations: apoB, apolipoprotein B; apoB-18/95, an internally deleted human apoB protein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; SDS, sodium dodecyl sulfate.

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particle in both McArdle cells (4) and rat liver microsomes (5) during in vivo labeling kinetic experiments. In contrast to the first step in which a simple size relationship holds for the average primary particle, the circumference being approximately equal to the length of apoB, the size of the VLDL does not appear to be determined by the size of apoB. Nevertheless, a minimum length of apoB may be required for the formation of the secondary particle. Characterization of the truncated apoB-containing lipoproteins produced by human subjects with familial hypobetalipoproteinemia demonstrated that C-terminally truncated apoB-27, apoB-31, and apoB-32.5 were detected in the HDL and the normally lipoprotein-deficient fractions of plasma ($d > 1.21$ g/ml) (15–21). In contrast, apoB-46, apoB-52.8, apoB-61, and apoB-67 were present in VLDL, IDL, and LDL fractions, whereas apoB-82 and apoB-83 were present almost exclusively in the VLDL and IDL fractions (16–19).

In this study involving the transfection of human apoB constructs, we have used a rat liver-derived cell line, McA-RH7777, which offers important advantages over HepG2 cells and primary hepatocytes. In contrast to HepG2 cells (22), McA-RH7777 cells retained the capacity to efficiently incorporate apoB-100 into VLDL ($d < 1.006$ g/ml) (23), although the process is considerably less efficient for apoB-48, the majority of which are secreted as primary particles. In contrast to primary hepatocytes or intact liver, McA-RH7777 cells are easily transfected, and stable transfectants may be readily obtained. The results of our study help to elucidate the roles played by apoB size and sequence in each of the two steps proposed for VLDL assembly in apoB-48-containing lipoproteins.

MATERIALS AND METHODS

Materials

Cell culture media and sera were purchased from Mediatech (Herndon, VA). Biotinylated protein molecular weight markers, avidin-horseradish peroxidase conjugate, reagents for polyacrylamide gel electrophoresis, and polyvinylidene difluoride (PVDF) membranes for immunoblot analysis were obtained from Bio-Rad (Hercules, CA). Peroxidase-conjugated goat anti-mouse antibodies and the ECL Western blotting detection system were purchased from Amersham (Arlington Heights, IL). Centricon tubes were purchased from Amicon (Beverly, MA). Most other chemical reagents were purchased from Sigma (St. Louis, MO). Monoclonal antibody 1D1 was a gift from R. W. Milne and Y. L. Marcel (Lipoprotein and Atherosclerosis group, University of Ottawa Heart Institute, Ottawa, Canada).

Expression plasmids

An EcoRI/BamHI cDNA fragment spanning nucleotides 20–2551 of apoB mRNA was inserted into the expression vector pCMV5 (24) to form the plasmid pB18, as described in (13). To create pB53, a HindIII/HindIII fragment (nucleotides 2279–7336) was cloned into the HindIII site of pB18. Production of the pB31 construct required inserting a fragment of a genomic apoB clone containing the apoB-31 mutation from a heterozygote into a vector, Bluescript KSII (Stratagene, La Jolla, CA) (13). Then, the AccI/SmaI fragment extending from mRNA nucleotides 4214 to the polylinker region of the vector, was inserted into pB53 cleaved with AccI and SmaI. To produce plasmid pB18/95, a wild-type BamHI-HindIII apoB gene fragment spanning from exon 26 to exon 29 and coding for amino acids 3636–4343 was ligated to the 3′ end of an apoB-18 (see Fig. 1A) (25).

Cell culture

The McA-RH7777 cells (ATCC CRL 1601) (26) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 10% horse serum. Cells were maintained at 37°C in a 5% CO_2, humidified atmosphere, and were split 1:20 in the same medium every 7 days.

Transfection

Co-transfection of the human apoB constructs with pSV2neo at a molar ratio of 20:1 yielded stable transfectants of McA-RH7777. We selected single foci of transfected cells, and maintained the stable cell lines in culture medium containing G418 (400 μg/ml) (13).

Total lipoprotein isolation from the culture medium

Stable transformants of McA-RH7777 cells were incubated in DMEM containing 10% fetal bovine serum and 10% horse serum for 2 days after passage; then the media were changed to serum-free DMEM and the cells were cultured for another 48–72 h to allow lipoprotein secretion. The media were collected into 0.05 M Tris buffer, pH 8.0, 10 mM EDTA, 0.5% sodium azide, 10 mM phenylmethylsulfonyl fluoride (PMSF), 200 μg/ml trypsin inhibitor, and 40 μg/ml aprotinin. The media were then adjusted to a density of 1.22 g/ml with solid sodium bromide (NaBr) and subjected to ultracentrifugation at 100,000 *g* (38,000 rpm) using the Ti 50 or Ti 70.1 rotor at 15°C for 30 h (8). The top 1 ml was then collected as a total lipoprotein fraction.

VLDL isolation from the culture medium

The collected culture media were subjected to ultracentrifugation at 100,000 *g* and 15°C for 30 h without the prior addition of NaBr. The top 1 ml was collected as the VLDL fraction.

Density determination

Densities of the lipoproteins were determined by isopycnic banding in NaBr gradients. For particles in the 1.05–1.20 g/ml density range, a steep gradient was formed by adjusting the collected medium to a density of 1.22 g/ml and overlaying it with an equal volume of medium adjusted to a density of 1.05 g/ml. For particles lighter than d 1.05 g/ml, a shallow gradient was formed by adjusting the medium to a density of 1.10 g/ml and then overlaying with an equal volume of medium without added NaBr.

Both gradients were ultracentrifuged for 48 h at 168,000 *g* (37000 rpm in an SW 41 rotor) and 15°C to form an approximately linear gradient and bring the lipoproteins to their equilibrium densities. The gradients were fractionated into 1-ml samples, and the density of each fraction was determined by refractometry. Lipoproteins in each of the fractions were concentrated by adsorption onto 125 μg of fumed silica (Cab-O-Sil, Sigma) at 4°C for 1 h (27). Apolipoproteins were recovered by elution from the fumed silica using 35 μl of SDS-gel sample buffer containing 4% SDS and 2% beta-mercaptoethanol. To determine the density distribution of the apoB-containing lipoproteins, the proteins were resolved on 5% SDS-polyacrylamide gels and subjected to immunoblotting as described below.

Determination of lipoprotein radius from apoB molecular weight and lipoprotein density

The radius of a lipoprotein can be determined when the lipoprotein density and the molecular weight of the protein are known, as well as the partial specific volumes of the lipid and protein.

$$R = \left[\frac{3M_p(\bar{v}_p - \bar{v}_l)}{4\pi N(1 - \bar{v}_l d_{lp})}\right]^{1/3} \qquad \textit{Eq. 1)}$$

where R is the lipoprotein radius, M_p is molecular weight of the protein, $\bar{v}_p$ and $\bar{v}_l$ are the partial specific volumes of protein and lipid, respectively, and d_{lp} is the lipoprotein density.

It is assumed that all of the protein is contributed by a single molecule of truncated human apoB with a $\bar{v}_p$ = 0.73 ml/g (8). For the apoB-31, apoB-18/95, apoB-48, and apoB-53-containing lipoproteins used to generate the data of Fig. 3, $\bar{v}_l$ is a function of lipid composition and varies with particle radius over a range from 1.025 to 1.035 ml/g. Radius values calculated from the last equation were not significantly affected by using an average value of $\bar{v}_l$ = 1.030 ml/g.

Flotation velocity centrifugation

Isolated total lipoproteins were concentrated 3-fold by Centricon-30 centrifugation (Amicon, Beverly, MA); the concentrated lipoproteins were then adjusted to a density of 1.32 g/ml by adding solid NaBr. This solution was then placed beneath a linear 1.25–1.30 g/ml NaBr gradient and ultracentrifuged in an SW 41 rotor at 168,000 *g* and 20°C, to move the lipoproteins into the center of the tube (9). The gradients were fractionated into 1-ml samples. The density and apolipoprotein content of each fraction were analyzed by refractometry and SDS-gel electrophoresis and immunoblotting.

Sedimentation coefficients (which were negative numbers) were determined by integration over the viscosity and density gradient, as described in (9).

Immunoblotting of apolipoprotein B proteins

The size-fractionated proteins in the 5% SDS-polyacrylamide gels were transferred onto PVDF membranes in a transfer buffer that contained 10 mM 3-cyclohexylamino-1-propane sulfonic acid (CAPS) and 10% methanol, pH 11.0 (28), at 150 mA and 4°C overnight. The high pH was essential for efficient transfer of apoB. The truncated human apoB variants were probed with the human apoB-specific monoclonal antibody, 1D1 (29), overnight at 4°C. Peroxidase-conjugated goat anti-mouse antibodies were used as secondary antibodies. Biotinylated high-molecular-weight protein markers were also included in the same SDS-polyacrylamide gel and transferred onto the same PVDF membrane, then probed with the avidin-horseradish peroxidase conjugate. The molecular weight markers and apoB were visualized using the ECL chemiluminescent system (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The midpoint of a cumulative plot of band intensities in consecutive fractions, as estimated by a scanning densitometer, was used to determine the average density of the corresponding lipoproteins in a density gradient.

Preparation of acrylic beads coupled to monoclonal antibody, 1D1, and immunoprecipitation of VLDL

One hundred mg of Eupergit C (oxirane acrylic beads, Sigma, St. Louis, MO) was added to 1 mg of monoclonal antibody 1D1 in 1 ml of 0.1 M phosphate buffer, pH 8.0, and the resulting suspension was incubated for 24 h at room temperature, resulting in the binding of essentially all of the antibody to the beads. The antibody–bead complex was separated in a microfuge, and the remaining oxirane groups were blocked by incubation

with 10% ethanolamine, pH 9.0 (1 ml), for a further 24 h at room temperature. The immobilized antibody preparation was washed repeatedly with distilled water and finally suspended in 1 ml of phosphate-buffered saline and 5% BSA.

For selective immunoprecipitation of human apoB-containing lipoproteins, VLDL (1 ml) isolated by ultracentrifugation of cell culture media at d 1.006 g/ml, were adjusted to 1 M NaCl and 5% BSA. Then 6 μl of 1D1-coated beads was added, and the solution was rocked overnight at 4°C. The beads were isolated in the microfuge, washed once with distilled water, and boiled with sample buffer for 5 min prior to SDS-PAGE and Western blotting.

For selective immunoprecipitation of rat apoB-containing lipoproteins, the VLDL, which had been adjusted to 1 M NaCl and 5% BSA, were first precleared by incubation overnight with non-immune serum and precipitation using a 10% (w/v) suspension of fixed *Staphylococcus aureus* cells. After preclearing, the VLDL were incubated overnight with rabbit anti-rat apoB antibody, AB100-4 (30), a highly selective antibody reisolated after absorption against human LDL. Immune complexes were precipitated with *Staphylococcus aureus* cells, washed, and boiled with sample buffer for 5 min prior to SDS-PAGE and Western blotting.

RESULTS

Buoyant density of lipoproteins secreted by stably transformed McA-RH7777 cells

The three stably transformed cell lines of McA-RH7777 cells were used in this study, B18/95, B31, and B53 (13, 25). **Figure 1A** shows a cDNA fragment encoding the N-terminal end of human apoB (amino acid residues 1–782) ligated to a human apoB genomic DNA fragment containing sequences of exons 26–29 encoding a C-terminal region of apoB (amino acid residues 3636–4343) (25). Thus, this B18/95 DNA encodes a human apoB containing an internal deletion of almost 3000 amino acids, as well as a small C-terminal deletion. The total length of the human apoB (Fig. 1B) specified by B18/95 was 1490 amino acids, corresponding in size to a C-terminally truncated apoB-33 (25). The B31 and B53 cell lines produced and secreted lipoproteins (Fig. 1B) containing C-terminally truncated human apoBs of 1425 amino acids and 2377 amino acids, respectively (13).

The buoyant densities of lipoproteins secreted by these three stably transfected McArdle-RH7777 cell lines were determined by isopycnic density gradient ultracentrifugation in a gradient extending from 1.05 g/ml to 1.25 g/ml. The truncated human apoB products in the isopycnic fractions were detected by immunoblotting using monoclonal antibody 1D1, which recognizes an epitope located between residues 474 to 539 of human apoB and does not cross-react with rat apolipoproteins (29). Representative immunoblots are shown in **Fig. 2.** The B31 cells secreted a human apoB-31-containing lipoprotein in four adjacent fractions with a midpoint density of 1.176 g/ml (Fig. 2A). Immunoblots of lipoproteins secreted by the apoB-53 cells revealed that the cells expressed not only human apoB-53-containing lipoproteins but also human apoB-48-containing lipoproteins (Fig. 2B). The formation of the apoB-48-containing lipoproteins by the stably transformed B53 cells probably resulted from the post-transcriptional editing mechanism that converted the

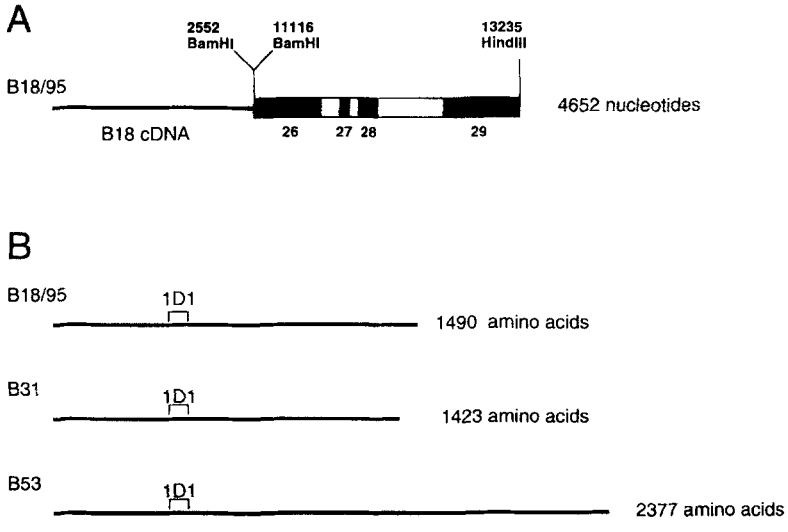

Fig. 1. Schematic diagram of a truncated human DNA construct and three human proteins expressed by stably transformed McA-RH7777. A: The apoB-18/95 construct encoding the human N-terminal end of apoB-18 (encoding amino acid residues 1–782) fused to exons 26–29 (encoding amino acid residues 3636–4343). B: Three human proteins stably expressed by the McA-RH7777 cells include apoB-18/95 composed of 1490 amino acids; apoB-31, composed of 1423 amino acids; and apo-53, composed of 2377 amino acids. The site recognized by 1D1 has been identified as residues 474–532, and appears on all three constructs.

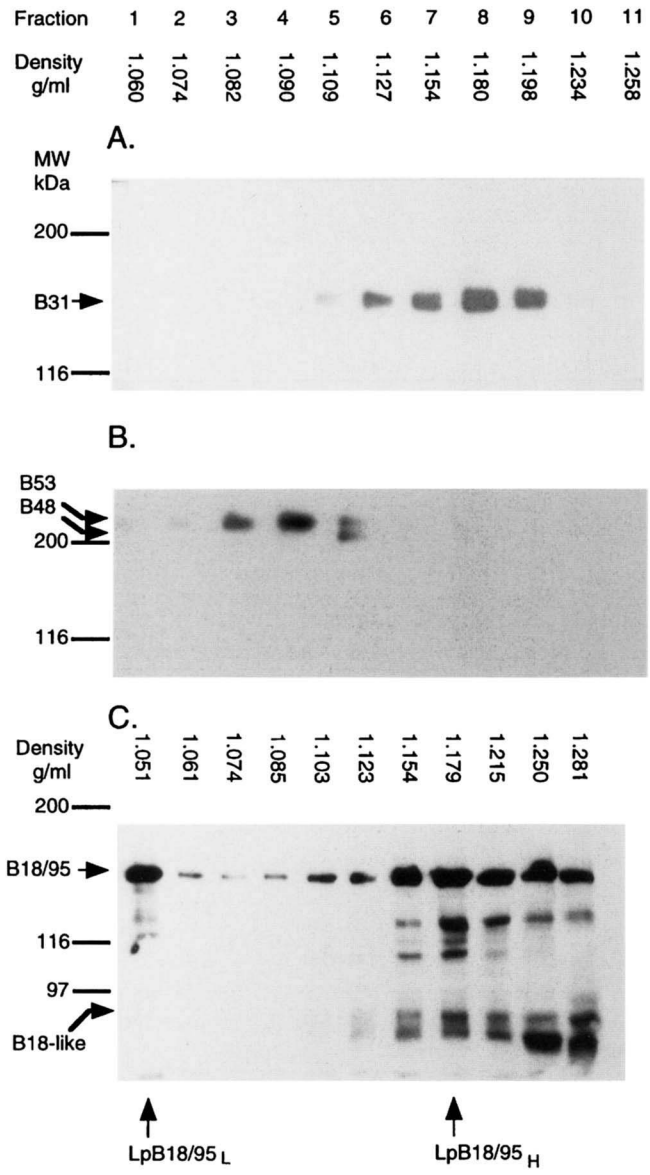

Fig. 2. Density distribution of truncated human apoB-containing lipoproteins isolated from the culture medium of stably transformed McA-RH7777 cells. Stable transformants were cultured in serum-free medium. Isopycnic gradient ultracentrifugation between densities of 1.05 to 1.25 g/ml was performed directly on the cell culture medium at 168,000 *g* for 48 h. Eleven fractions were collected and apoB proteins in each fraction were absorbed on Cab-O-Sil, eluted with SDS-containing sample buffer, and subjected to electrophoresis on SDS–5% polyacrylamide gels. All of the recovered protein in 1 ml of gradient media was placed on the gel. The blots were probed with anti-apoB monoclonal antibody 1D1 to detect the recombinant human apoB proteins. Average buoyant densities were determined from the midpoint of the cumulative intensities across the fractions. A: Immunoblot for the culture media from the apoB-31-secreting cells. B: Immunoblot of culture media from the apoB-53-secreting cells. C: Immunoblot of the culture media from the cells secreting apoB-18/95. All experiments were performed a minimum of three times.

codon for amino acid 2152 of apoB to a stop codon (18, 19). Human apoB-53-containing lipoproteins exhibited a midpoint density of 1.088 g/ml while the apoB-48-containing lipoproteins had a midpoint density of 1.109 g/ml.

The B18/95 cells showed a distinctly different distribution from the C-terminally truncated human apoBs described above. The isopycnic gradient shown in Fig. 2C demonstrated two distinct populations of human apoB-18/95-containing lipoproteins. At the top of the gradient (d 1.06 g/ml) a VLDL and LDL population is denoted by the subscript L (that is, $LpB18/95_L$), while near the bottom of the gradient at a midpoint density of 1.179 g/ml, a high density population is denoted by the subscript H (that is, $LpB18/95_H$).

Several prominent smaller bands recognized by human apoB-specific monoclonal antibody 1D1 are also present in Fig. 2C. Either they were proteolytic degradation products of the apoB-18/95 protein or else they were lipoprotein products from an alternative transcript. Because the smaller bands appeared at the same density as the $LpB18/95_H$ particles, we believe that a proteolytic reaction is a more likely explanation. It is interesting to note that the lipoproteins that float to the top of this gradient, the $LpB18/95_L$, are deficient in these prominent smaller bands, although they are not absent. It seems possible they have been selected against in a second step in which lipid is transferred to the intact apoB-18/95 particles.

Lipoprotein radii calculated from lipoprotein density and apoB size

Table 1 lists the sizes of the apoB fragments, the midpoint densities of the gradient fractions containing the

TABLE 1. Buoyant densities, sedimentation coefficients, and radii of lipoprotein particles secreted by McA-RH7777 cells

ApoB Size	Buoyant Density[a]	$s_{20,1.20}$[b]	Radii[c]
	g/ml	*Svedberg*	*Angstrom*
B31	1.176	−1	45.1
$B18/95_H$	1.167	−2	46.7
B48	1.109	−6.4	59.5
B53	1.088	−8.3	65.0
$B18/95_L$	<1.007	S_f = 14–147	

[a]These experiments were repeated three times with very similar results. The buoyant densities were calculated from the experiment shown in Fig. 2, in which the immunoblots were relatively free of background noise.

[b]Sedimentation values are averaged from three different measurements; $s_{20,1.20}$ is the sedimentation coefficient in a solvent with a density of 1.20 g/ml and the viscosity of water. S_f is the flotation coefficient in a solvent with a density of 1.063 g/ml and a viscosity of 1.0260 cp.

[c]Radii were calculated from apoB molecular weights and lipoprotein buoyant densities, as described in Methods.

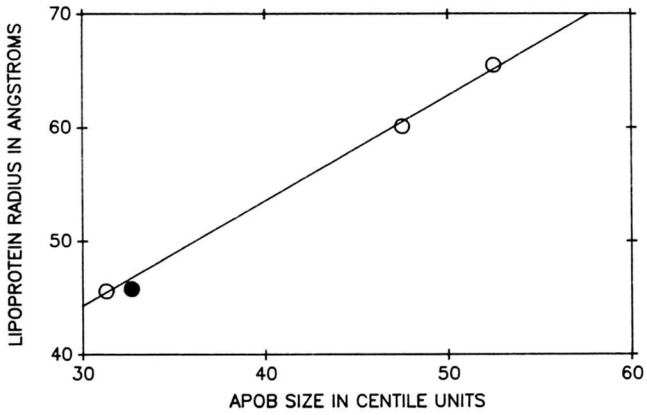

Fig. 3. Radii of the truncated human apolipoproteins as a function of the sizes of the apoB fragments. Lipoprotein radii were calculated from the buoyant densities of lipoproteins and the molecular weights of the apoB fragments and were tabulated in column 4 of Table 1. The value for $LpB18/95_H$ is indicated by the filled circle. Linear regression by least squares analysis yields the expression for lipoprotein radius, $R = 16.7 + 0.924 \times$ centile (apoB size in centile units). This experiment was performed twice.

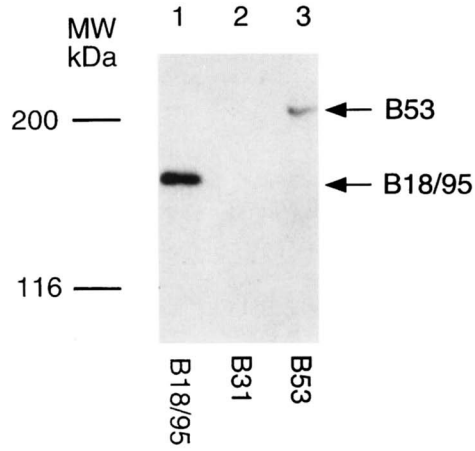

Fig. 4. Immunoblot of VLDL isolated from stable transformants of McA-RH7777 cells. ApoB-18/95-, apoB-31-, and apoB-53-secreting cells were cultured in serum-free media for 48 h. The collected media (d 1.006 g/ml) were centrifuged at 100,000 *g* for 30 h. The top 1-ml fractions were collected, concentrated, resolved in 5% SDS-PAGE, and detected by immunoblotting with monoclonal antibody 1D1. One-tenth (100 μl) of the recovered protein was placed on the gel. Lane 1 contains VLDL from apoB-18/95-secreting cells, lane 2 contains the VLDL fraction (but lacking VLDL) from cells secreting apoB-31-containing HDL, and lane 3 contains VLDL from apoB-53-secreting cells. These experiments were performed twice.

lipoproteins bearing these fragments, and the radii of the lipoprotein, calculated from Eq. 1.

Figure 3 shows a plot of particle radii as a function of apoB size. The points define a straight line with an intercept of 16 Å. The slope of the line corresponds to 1 Å of core circumference per kilodalton of apoB, as previously reported (9). Figure 3 suggests that all four lipoproteins have homologous structures, consistent with a previously proposed model in which the size of the apoB determines the lipoprotein core circumference.

Isolation of VLDL

In order to extend these observations still further, the VLDL fraction was isolated by centrifugation, without added salt, from the serum-free culture media collected after 48 h of growth. Using the human apoB-specific monoclonal antibody 1D1 to detect apoB, an immunoblot of human VLDL (d < 1.006 g/ml) isolated from the culture media of the three stably transfected McA-RH7777 cell lines, apoB-31, apoB-18/95, and apoB-53 cells, is shown in **Fig. 4.** The apoB-18/95 cells secreted a substantial amount of apoB-18/95-containing VLDL. Flotation experiments were also performed in the presence of 10 mM dithiothreitol to check for the presence of disulfide-bonded complexes formed between rat VLDL and apoB-18/95-containing particles; however, DTT did not alter the distribution of complexes. Although it has been previously reported (13) that the apoB-53 cells secreted a substantial amount of larger, lipid-rich particles in a top fraction of d 1.03 g/ml, only a small amount of human apoB-53-containing VLDL and no human apoB-48-containing VLDL floated at the density of the medium in the absence of serum. The B31 cells secreted no detectable apoB-31 in the VLDL fraction (Fig. 4).

Analysis of the lipoproteins by flotation velocity

The lipoproteins secreted by the three stably transformed McA-RH7777 cell lines were floated at 168,000 *g* (36900 rpm) and 20°C for 380 min. Average sedimentation coefficients ($S_{20,1.20}$), corrected to a density of 1.20 g/ml and viscosity of water at 20°C, were determined by a procedure involving integration over the density and viscosity gradient. The $s_{20,1.20}$ of the apoB-31-containing HDL-sized particles exhibited a peak at −1 S. For the apoB-48- and apoB-53-containing, HDL-sized particles, rather broad distributions were obtained, and sedimentation rates of about −6.4 and −8.3 S were estimated, respectively (Table 1).

The apoB-18/95-containing lipoproteins, in contrast to the others, exhibited two separate populations of particles. The $s_{20,1.20}$ of $LpB18/95_H$ was estimated to be −2 S, while the $s_{20,1.20}$ of $LpB18/95_L$ was calculated to be more negative than −20 S (assuming a density of 1.007 g/ml), because the lipoproteins floated to the top of the gradient. In order to more accurately estimate the sedimentation coefficients of the largest particles, shorter flotation runs at lower speed, 40,000 *g*

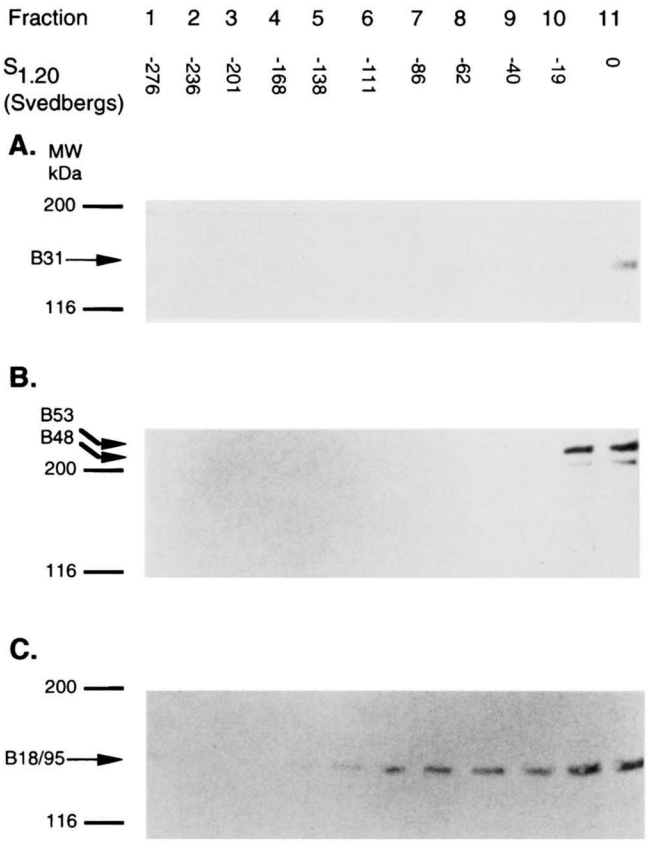

Fig. 5. Determination of sedimentation coefficients for the rapidly sedimenting lipoproteins secreted by the three McA-RH7777 stable transformants. Sample preparation and detection were as described in the legend to Fig. 2. Ultracentrifugation was at 18,000 rpm (40,000 *g*) for 1 h in a linear 1.25–1.30 g/ml gradient, and all of the protein in each 1-ml fraction was placed on the gel. A: Lipoproteins obtained from apoB-31-secreting cells; B: lipoproteins obtained from apoB-53-secreting cells; and C: lipoproteins obtained from apoB-18/95-secreting cells. This experiment was repeated three times.

(18,000 rpm, 1 hour) were also performed. ApoB-31-containing (**Fig. 5A**) and apoB-48-containing (Fig. 5B) lipoproteins remained in the bottom fraction, while a few apoB-53-containing lipoproteins (Fig. 5B) were found in the adjacent (10th) fraction, corresponding to $s_{1.20} = -19$ S (assuming a lipoprotein density of 1.007 g/ml). In contrast, the apoB-18/95-containing VLDL migrated from the bottom fraction into fractions 10 to 3 (Fig. 5C), corresponding to sedimentation values from $s_{1.20} = -19$ to -201 S respectively. Calculations show that in an SW41 rotor centrifuged at 40,000 *g* for 1 h, 20°C, typical LDL (d 1.032 g/ml, 2.5×10^6 Da, dia. 220 Å) would float to the 9th fraction while IDL (d 1.007 g/ml, 5×10^6 Da, dia. 290 Å) would float into the 7th fraction. Small VLDL (d 0.97 g/ml, 9×10^6 Da, dia. 345 Å) would float into the 5th fraction, while VLDL (d 0.95 g/ml, 14×10^6 Da, dia. 400 Å) would float into fraction 3. Thus, the calculated particle diameters for the fractions shown in Fig. 5C ranged from less than 220 Å (fraction 11) to 400 Å (fraction 3).

When the gels shown in Fig. 5 were stripped of 1D1 and reblotted using a polyclonal antibody against rat apoB, a readily detectable amount of rat apoB-100 was found in all fractions, suggesting that the McArdle cells were making at least a small amount of typical VLDL of sizes up to dia. 400 Å, the size of the particle found to be in the third fraction. All three stably transfected cell lines used in this study, B18/95, B31, and B53, secreted rat VLDL in approximately the same quantities and distribution of sizes (data not shown).

ApoB-18/95 protein does not reside together with endogenous rat apoB on the same VLDL particle

In these experiments, serum-free media was used to eliminate the possibility of transfer of apoB-18/95 to calf lipoproteins. In order to test whether the human apoB-18/95 protein was present on VLDL together with rat apoB, VLDL secreted by wild-type McA-RH7777 cells and those secreted by the B18/95 cells were isolated by ultracentrifugation at d < 1.006 g/ml and then further purified by selective immunoprecipitation of the rat or human proteins. Analyses of the secreted proteins are shown in **Fig. 6A**, where the anti-human apoB monoclonal 1D1 was used to detect the human apoB-18/95 protein, and in Fig. 6B where anti-rat antibody AB100-4 was used to detect rat apoB.

In Fig. 6A, the only proteins detected by 1D1 were apoB-18/95 on VLDL secreted by B18/95 cells and precipitated with fumed silica (lane 1) or precipitated by 1D1 coupled to acrylic beads (lane 3). A control preparation of acrylic beads lacking 1D1 bound only a trace of apoB-18/95. Another control consisting of rat apoB was precipitated with anti-rat AB100-4 (lane 4); this control bound only a trace of apoB-18/95 when tested with 1D1 (lane 4).

Figure 6B shows a second immunoblot prepared simultaneously and identically to the first, and analyzed using the anti-rat AB100-4 to detect rat apoB. In this case, the only proteins detected in substantial amounts were rat apoB-100 and apoB-48 from VLDL secreted by the apoB-18/95 cells precipitated with fumed silica (lane 1) or with anti-rat AB100-4 (lane 4). Importantly, no rat apoB was seen in lane 2, and only a trace in lane 3, although lane 3 had previously shown a strong band with human apoB-18/95.

We conclude that at least most of the apoB-18/95 protein and the endogenous rat apoB are on separate particles. This experiment has been repeated two additional times on different preparations with very similar results. Thus, most of the apoB-18/95 did not reside on preformed rat VLDL, but rather it was capable of initiating new VLDL formation.

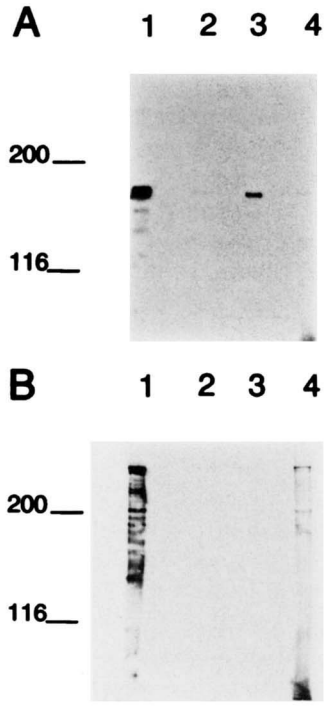

Fig. 6. Immunoblots of VLDL proteins selectively precipitated with antibodies specific for rat and human apoB. VLDL were isolated by flotation at d 1.006 g/ml, and purified either by fumed silica, which adsorbs all apolipoproteins, or else selectively immunoprecipitated. All of the protein in 1 ml from the top of the tube was absorbed or immunoprecipitated, re-extracted, and placed on the SDS-5% polyacrylamide gel. In panel A, proteins were detected using anti-human 1D1 as the primary antibody, while in panel B, proteins were detected using anti-rat AB100-4. Lanes 1–4 show proteins isolated from B18/95 cell VLDL: lane 1, by adsorption to fumed silica; lane 2, by adsorption to acrylic beads; lane 3, by binding to 1D1 coupled to acrylic beads; lane 4, by immunoprecipitation with anti-rat AB100-4. This experiment was repeated three times.

DISCUSSION

Lipoprotein core circumference is proportional to apoB size for apoB-18/95-containing HDL

We have characterized the effects of a large internal deletion in an apoB fusion protein on lipoprotein structure. First, it was found that the average density of high density, apoB-18/95-containing lipoproteins, LpB18/95_H, was close to the density of the C-terminally truncated apoB-31-containing lipoprotein used as a control, as shown in Fig. 2A. When the radius of LpB18/95_H, as calculated from the midpoint of its density values, was plotted as a function of apoB size, it fell upon the line generated by the other three lipoproteins (Fig. 3).

Previous observations have shown that a homologous series of HDL-sized lipoproteins are formed by C-terminally truncated apoBs longer than about apoB-18. Evidently, portions of apoB that lie C-terminal to apoB-18 are not essential for lipoprotein formation. However, those studies showed that each additional length of apoB bound additional lipid and extended the circumference of the non-polar core. Thus, a linear relationship was obtained when lipoprotein radius was plotted as a function of apoB size, showing that all segments of apoB contributed to core circumference, at least over the range of the data which extended from apoB-25 to apoB-80, within the error of the measurements. The least squares straight line through the 13 apoB sizes analyzed in that publication (9) was R = 18.8A + 0.946A × centile, where apoB size was expressed in the centile nomenclature (31). In the present communication, this result has been reproduced, that is, the line shown in Fig. 3 and generated by four points possessed a similar slope and intercept, R = 16.7 + 0.924 × centile. Moreover, the present study extends these observations to an apoB containing a large internal deletion. Evidently, lipid binding and the extension of core circumference by apoB is a local phenomenon, as may be inferred from previous studies (9, 14) and does not depend upon contiguous apoB sequence, as shown here.

ApoB size determined lipoprotein core circumference only for the small LDL-sized or smaller particles, falling in the HDL-LDL density range. In contrast, for VLDL, no such rule was obeyed, and indeed, on VLDL, apoB sequence and not apoB size determined its presence, as will be discussed next.

ApoB sequence and not apoB size determines VLDL assembly

The second significant finding of this study was that the secreted VLDL contained apoB-18/95 (Fig. 5C). Such VLDL were unexpected, as the length of apoB-18/95 was only equal to the length of apoB-33, reported to be present exclusively on dense lipoproteins in human hypobetalipoproteinemia (15–19). In the absence of oleate supplementation, no apoB shorter than apoB-48 were found to be secreted on VLDL by transfected McA-RH7777 cells (23, 32). With oleate supplementation, shorter apoBs appeared on VLDL, and in this case, apoB-33 fell close to the minimum length of apoB required for VLDL assembly in stably transfected McA-RH7777 cells. This minimum length has been estimated to lie somewhere between apoB-29, which was not found on VLDL, and apoB-34, which was present on VLDL in small amounts when the medium was supplemented with oleate (33). McLeod and coworkers (33) also have studied chimeras formed between apoAI and short sequences of apoB. ApoA-I/B chimeras containing the segment lying between apoB-29 and apoB-34 did appear to assemble a VLDL-like particle, and these authors suggested that apoB sequences with this region, which have greater than average hydropho-

bicity and are predicted to consist predominately of amphipathic β strands (34), are the critical determinants of VLDL assembly. The apoB-18/95 was found over a wide range of VLDL particle sizes, from S_f 6 to 60 (Fig. 5C). Moreover, these VLDL-like particles did not contain rat or bovine apoB. Therefore, we may conclude that the ability to assemble a VLDL was not determined by the length of apoB, because internally deleted apoB-18/95, which appeared on VLDL, was only about the length of C-terminally truncated apoB-33, which does not appear on VLDL in the absence of oleate supplementation.

C-terminal sequence of apoB-18/95

The C-terminal sequence of the apoB-18/95 protein comprises residues 3636–4343 (apoB-80–apoB-96) of apoB-100. When placed on the pentapartite model for apoB-100 proposed by Segrest et al. (34), these residues correspond to the final 18% of the β_2 domain (residues 2611–3867), and all of cluster III of the α_3 domain (residues 4061–4338). Thus, a potentially strong lipid-associating domain of apoB-100, cluster III, preceded by 231 residues of a possible amphipathic β strand, may form the last half of the apoB-18/95. This combination of structural features could be involved in the apparent affinity of this protein for lipid and explain why it is unusually capable of forming VLDL.

Conclusions

How do the results of these experiments advance our knowledge of lipoprotein biosynthesis, and what are the implications for the design of future studies? The results demonstrate that at least in the case of apoB-18/95, a large deletion is compatible with the biosynthesis of a primary particle homologous in structure to the core-containing primary particles described previously (9, 14). Thus, the binding of lipid appears to be a local phenomenon along the length of apoB that is not significantly interrupted by a large deletion. This result, together with extensive studies of C-terminally truncated apoB fragments, indicates that most of apoB larger than apoB-18 has this property. Future studies should explore the initial portion of apoB, and ask how much of the N-terminal domain (amino acid residues 1–782) is critical for the initiation of lipoprotein formation.

The results also demonstrate that large, buoyant VLDL-like particles can be secreted bearing apoB-18/95, which has a length equivalent to a C-terminally truncated apoB-33. This result was unexpected, as apoBs of this size have not been previously associated with VLDL, especially when oleate supplementation was not provided. The results of these current studies only hint at the answers, and all the fundamental questions still remain concerning the detailed molecular mechanism used in VLDL assembly in the intact animal. The answers to these questions await future studies.

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