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Apolipoprotein B-Containing Lipoprotein Particle Assembly:

Lipid Capacity of the Nascent Lipoprotein Particle

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Running title: Lipid capacity of nascent apoB lipid pocket

Abbreviations used:

Apo, apolipoprotein

BSA, bovine serum albumin

CE, cholesteryl ester

DAG, diacylglycerol

DMEM, Dulbecco's modified Eagle's medium

ER, endoplasmic reticulum

FBS, fetal bovine serum

HDL, high density lipoprotein

IP, immunoprecipitation

LDL, low density lipoprotein

LV, lipovitellin

MTP, microsomal triglyceride transfer protein

NDGGE, nondenaturing gradient gel electrophoresis

PL, phospholipids

PAGE, polyacrylamide gel electrophoresis

PBS, phosphate-buffered saline

SDS, sodium dodecyl sulfate

S_d , Stokes diameter

TAG, triacylglycerol

VLDL, very low density lipoprotein

SUMMARY

We previously proposed that the N-terminal 1000 residue $\beta\alpha_1$ domain of apolipoprotein B (apoB) forms a bulk lipid pocket homologous to that of lamprey lipovitellin (LV). In support of this “lipid pocket” hypothesis, apoB:1000 (residues 1-1000) was shown to be secreted by a stable transformant of McA-RH7777 cells as a monodisperse particle with HDL₃ density and Stokes diameter of 112 Å. In contrast, apoB:931 (residues 1-931), missing only 69 residues of the sequence homologous to LV, was secreted as a particle considerably more dense than HDL with Stokes diameter of 110 Å. The purpose of the present study was to determine the stoichiometry of the lipid component of the apoB:931 and apoB:1000 particles. This was accomplished by metabolic labeling of cells with either [¹⁴C]oleic acid or [³H]glycerol followed by immunoprecipitation (IP) or nondenaturing gradient gel electrophoresis (NDGGE) of secreted lipoproteins and by immunoaffinity chromatography of secreted unlabeled lipoproteins. The [³H]-labeled apoB:1000-containing particles, isolated by NDGGE, contained 50 phospholipids (PL) and 11 triacylglycerols (TAG) molecules per particle. In contrast, apoB:931-containing particles contained only a few molecules of PL and were devoid of TAG. The unlabeled apoB:1000-containing particles isolated by immunoaffinity chromatography and analyzed for lipid mass, contained 56 PL, 8 TAG, and 7 cholesteryl ester molecules per particle. The surface:core lipid ratio of apoB:1000-containing particles was approximately 4:1 and was not affected by incubation of cells with oleate. Although small amounts of microsomal triglyceride transfer protein (MTP) were associated with apoB:1000-containing particles, it never approached a 1:1 molar ratio of MTP to apoB. These results support a model in which: *i*) the first 1000 amino acid residues of apoB are competent to complete the “lipid pocket” without a structural requirement for MTP; *ii*) amino acids between 931 to 1000 of apoB-100 are critical for the

formation of a nascent lipoprotein particle, and iii) the “lipid pocket” created by the first 1000 amino acid residues of apoB-100 is PL-rich, suggesting a small bilayer type organization and has a maximum capacity on the order of 70 molecules of lipid. This model is supported by the all-atom molecular model of the $\beta\alpha_1$ lipid pocket presented in the accompanying paper.

Keywords: Apolipoprotein B, lipoprotein assembly, lipovitellin, microsomal triglyceride transfer protein, apoB structure.

Introduction

Plasma lipoproteins are submicroscopic particles composed of lipid and protein held together by noncovalent forces. Their general structure is that of a spheroidal microemulsion formed from an outer layer of phospholipids, unesterified cholesterol, and proteins, with a core of neutral lipids, predominantly cholesteryl ester and triacylglycerols (TAG). Although the microemulsion is the basic structural motif of lipoproteins, several different lipoprotein classes exist that differ in relative amount of lipids, in the protein/lipid ratio, and in the protein species present, resulting in differences in size, density, and electrophoretic mobility. Lipoproteins are generally classified by density, size, and/or protein composition.

Apolipoproteins (apo) are amphipathic in nature, in that they have both hydrophobic and hydrophilic regions, and can, therefore, interact with both the lipids of the lipoproteins and with the aqueous environment.¹ Because of the nature of these amphipathic regions, apolipoproteins act as detergents, and have a major role in determining and stabilizing the size and structure of lipoprotein particles. Plasma apolipoproteins can be grouped into two classes, the non-exchangeable apolipoproteins (apoB), and the exchangeable apolipoproteins (apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE).² The exchangeable apolipoproteins are soluble in aqueous solution, and the secondary structural motif responsible for their lipid association, the amphipathic α helix, has been extensively studied.¹⁻³ In contrast, the B apolipoproteins are highly insoluble in aqueous solutions, and thus remain with the lipoprotein particle throughout its metabolism.⁴ Because of the size and insoluble nature of this protein, it has been difficult to confirm the structural motifs responsible for lipid-associating properties of this non-exchangeable protein.^{5,6}

ApoB has a fundamental role in the transport and metabolism of plasma TAG and cholesterol and is synthesized primarily in hepatocytes and enterocytes.⁷⁻⁹ ApoB is present as a single molecule per lipoprotein particle and exists in two forms in humans, apoB-100 and apoB-48. ApoB-100 is the full length protein consisting of 4536 amino acid residues.⁸ ApoB-100 is an essential structural component for the formation and secretion of very low density lipoproteins (VLDL), the precursor of low density lipoproteins (LDL), and is expressed primarily in mammalian liver. ApoB-48 (the N-terminal 48% of apoB-100), is produced by a post-transcriptional modification of the apoB mRNA at codon 2153 that converts a glutamine codon to a stop codon.⁸ ApoB-48 is essential for the formation and secretion of chylomicrons and is expressed in mammalian intestine and in the liver of some non-human mammals.⁸ The assembly of apoB-containing lipoproteins occurs co-translationally, i.e., while the C-terminal portion is still being synthesized on the ribosome of the endoplasmic reticulum (ER), the N-terminal portion is translocated across the ER and is assembled as a small lipoprotein particle. Disulfide-dependent folding of portions of the N-terminal domain of apoB is required for its assembly into lipoprotein.¹⁰⁻¹²

In hepatocytes, the first step in apoB assembly involves the formation of a small particle in the high density lipoprotein (HDL) density range. It has been shown that the translation of the N-terminal 22-29% of apoB-100 is essential for the assembly of apoB-containing particles¹¹⁻¹⁵ and that microsomal triglyceride transfer protein (MTP) has a critical role in the assembly and secretion of apoB-containing lipoproteins.^{9,16} However, the precise domain in the N-terminal region of apoB that is required for the initiation of particle assembly, the structural elements of the assembly-competent domain, the lipid composition of this nascent particle, the mechanisms

by which MTP transfers lipid to the nascent apoB, and the site(s) in the secretory pathway where this transfer occurs are not completely understood and remain controversial.

The full-length apoB, essentially the only protein component of the atherogenic LDL, has a pentapartite structure, NH₂-β_{α1}-β₁-α₂-β₂-α₃-COOH, the β domains containing multiple amphipathic β strands and the α domains containing multiple amphipathic α helices.^{17,18} Because the amphipathic α helices of the α₂ and α₃ domains are mostly class A, the type found in exchangeable apolipoproteins,¹⁻³ we have proposed that these two regions of apoB-100 represented flexible domains with reversible lipid affinity.¹⁹ The two amphipathic β strand domains in apoB-100, β₁ and β₂, we have proposed form β sheets that represent the irreversibly lipid-associated regions of this irreversibly associated apolipoprotein.¹⁻³

The β_{α1} domain of apoB-100, i.e., the first 1000 amino acid residues of the mature protein, is a mixture of amphipathic β strands and amphipathic α helices,¹⁸ has been proposed to be globular in structure,^{17,19} and has sequence and amphipathic motif homologies to lamprey lipovitellin (LV).²⁰⁻²³ Based on sequence homology between the N-terminal domain of apoB and LV, we proposed^{19,20} that formation of a LV-like lipid pocket is necessary for lipid transfer to apoB-containing lipoprotein particles. We suggested^{19,20} that initiation of particle assembly occurs when the β_{α1} domain folds into a three-sided LV-like lipid binding cavity, or alternatively, the lipid pocket is formed by association of the region of the β_{α1} domain homologous to the βA and βB sheets of LV with βD-like amphipathic β sheet from MTP.

In this study, we report that, consistent with our previous study,²⁴ a domain between amino acids 931 and 1000 of apoB-100 is critical for the initiation of particle assembly and formation of a lipid-containing particle. Lipid composition and the number of lipid molecules associated with the secreted apoB-containing particles demonstrated that within the 69 amino acid residues

between apoB:931 to apoB:1000, the nature of particles is changed from a lipid-poor to a lipidated particle in the HDL₃-like density range. The lack of a 1:1 molar ratio of apoB to MTP observed in this study supports a model in which the first 1000 amino acid residues of apoB are competent to complete the “lipid pocket” without a structural requirement for MTP. This nascent lipoprotein intermediate has a relatively constant Stokes diameter of 112 Å, a mean density of 1.21 g/ml, and has a maximum capacity on the order of 70 molecules of lipid per particle, primarily phospholipids. The surface:core lipid ratio of approximately 4:1 supports a bilayer type arrangement that is not responsive to the presence of oleate in the culture medium.

Results

Amino Acids 931-1000 of ApoB-100 are Critical for the Formation of a Lipoprotein Particle.

To determine the effect of the 69 amino acid residues between apoB:931 and apoB:1000 on the relative flotation density of apoB:931- and apoB:1000-containing particles, cells were incubated overnight in serum-free Dulbecco's modified Eagle's medium (DMEM). Lipoproteins ($d < 1.23$ g/ml) and infranatant ($d > 1.23$ g/ml), isolated from the conditioned media, were analyzed by nondenaturing gradient gel electrophoresis (NDGGE) and immunoblotted with monospecific polyclonal antibody to apoB-100. As shown in **Fig. 1 A, lane 1**, apoB:931 expressing cells secreted a major particle with Stokes diameter (S_d) of 110 Å and a minor particle with S_d of 96 Å. Only a small fraction of the larger apoB:931-containing particle was recovered in the $d < 1.23$ g/ml fraction (compare **lane 2** to **lane 3**), indicating that these particles are predominantly lipid-poor. The apoB:1000 expressing cells secreted a major particle with S_d of 112 Å and trace amount of smaller particle with S_d of 95 Å (**Fig. 1 B, lane 1**). In contrast to apoB:931-containing particles, a larger fraction of apoB:1000 was recovered in $d < 1.23$ g/ml (**Fig. 1 B, lane 2**) and a smaller fraction was recovered in $d > 1.23$ g/ml (**Fig. 1 B, lane 3**), suggesting that the apoB:1000-containing particles are relatively lipid-rich. As shown in **Fig. 2 B, lane 2**, only the larger, apparently monodisperse, apoB:1000-containing particle floated.

Cells were incubated with 0.4 mM [14 C]-labeled oleic acid bound to 0.75% bovine serum albumin (BSA) and conditioned medium was subjected to NDGGE followed by autoradiography. As shown in **Fig. 2 A**, we did not detect any human apoB-containing particles in the conditioned medium of parental McA-RH777 cells (**lane 1**), LNCX (neo)-transfected cells (**lane 2**), or apoB:800-expressing cells (**lane 3**) by immunoblotting with monospecific polyclonal antibody to human apoB-100. In contrast, and consistent with results shown in **Fig. 1 A**,

apoB:931-expressing cells secreted two forms of apoB-containing particles with S_d of 110 Å and 96 Å (**Fig. 2 A, lane 4**) and apoB:1000-expressing cells secreted a major monodisperse particle with S_d of 112 Å (**Fig. 2 A, lane 5**). Autoradiography of a duplicate gel demonstrated that the apoB:931-containing particles contained little, if any, radioactivity (**Fig. 2 B, lane 4**). In contrast, the band corresponding to apoB:1000-containing particles demonstrated approximately a 10-fold greater level of radioactivity (**Fig. 2 B, lane 5**) than that associated with the apoB:931 band (**Fig. 2 B, lane 4**). The bands on top of the gel might be aggregated proteins that remained in the large pore region of the gel or endogenous rat lipoproteins labeled in the lipid moiety. Similar results were obtained when studies were carried out using [^3H]-labeled glycerol (data not shown).

Cells were then labeled with [^3H]glycerol or [^{14}C]oleic acid and secreted apoB-containing lipoproteins were isolated by immunoprecipitation or NDGGE and analyzed for lipids as described in Materials and Methods. The results shown in **Table I** demonstrate that the apoB:1000-containing particles contain at least 4 times as much labeled lipid as the apoB:931-containing particles. The above results clearly show that the 69 amino acid residues between apoB:931 and apoB:1000 are necessary for the formation of a lipidated particle.

Analysis of [^3H]-Labeled Lipids Associated With Secreted ApoB:1000-Containing Lipoproteins Isolated by Immunoprecipitation. Although we observed similar results with [^{14}C]-labeled oleic acid and [^3H]-labeled glycerol as precursors (**Table I**), we decided to use [^3H]-labeled glycerol as the lipid precursor in all subsequent experiments. This is based on our observation (data not shown) that in the conditioned medium, TAG-53 (containing two fatty acids of 16 carbons and one fatty acid of 18 carbons), TAG-55 (containing two fatty acids of 18 carbons and one fatty acid of 16 carbons), TAG-57 (containing three fatty acids of 18 carbons), and TAG-59 (containing two fatty acids of 18 carbons and one fatty acid of 20 carbons) account

for 29%, 34%, 35%, and 2%, respectively, of the total TAG subspecies in the control medium (without oleic acid) and 8%, 18%, 71%, and 3%, respectively, in the oleate-supplemented medium. This variation in the two experimental conditions, together with uncertainty of the exact number of 18-carbon chain fatty acids that might be labeled, could potentially introduce error in calculations of the number of lipid molecules, especially TAG, per apoB.

We used immunoprecipitation as the first step in determining the lipid composition of the secreted apoB-containing particles. Since apoB:931 was secreted mostly as a lipid-poor particle with peak density of 1.25 g/ml or greater²⁴ and contained very low levels of radiolabeled lipids (**Table 1** and **Fig. 1 B**), the number of lipid molecules per apoB:931 particle could not be accurately calculated by equations used in this study. Therefore, we have not shown any results on the lipid composition of apoB:931 that consisted of only a few PL molecules per particle.

Results of the calculation of the lipid composition of metabolically labeled apoB:1000-containing particles isolated by immunoprecipitation with monospecific polyclonal anti-human apoB-100 are shown in **Table II**. ApoB:1000-containing particles secreted by control cells (incubated without oleic acid) contained 57% PL and 33% TAG and those secreted by the oleate-supplemented cells contained a higher content of TAG (58%) and a lower content of PL (39%). Based on these results, the calculated stoichiometries of PL and TAG molecules per apoB:1000 were 36 and 19, respectively, in the absence of oleate and 22 and 31, respectively, in the presence of oleate.

Analysis of ApoB:1000-Containing Particles by NDGGE Demonstrates the Formation of Stable, Monodisperse Lipidated Particle. To circumvent the potential nonspecific precipitation and/or adsorption of rat TAG-rich particles by immunoprecipitation, we isolated apoB:1000-containing particles by NDGGE. Cells were metabolically labeled with [³H]glycerol in the

presence and absence of 0.4 mM oleic acid bound to 0.75% BSA and the labeled conditioned medium was concentrated and applied to NDGGE. To determine the lipid composition of apoB:1000-containing particles that floated, $d < 1.23$ g/ml fraction was isolated from the conditioned medium and was also subjected to NDGGE. Bands corresponding to apoB:1000-containing particles with S_d of 112 Å in total medium (**Fig. 3 B, lanes 1-3**), identified by immunoblotting of a duplicate gel (**Fig. 3 A, lane 1**), and $d < 1.23$ g/ml lipoproteins (**Fig. 3 B, lanes 4-6**), identified by immunoblotting of a duplicate gel (**Fig. 3 A, lane 2**), were excised, digested and extracted for lipids as described in Material and Methods. In the absence of oleic acid, the isolated particles, from both the total medium and $d < 1.23$ g/ml fraction, contained 71-74% PL and 18-21% TAG and this composition was not altered by incubation of cells with oleate (**Table III**). The calculated stoichiometries of PL and TAG per apoB:1000 in both the total conditioned medium and $d < 1.23$ g/ml fraction were 50 and 12, respectively, and were not responsive to the presence of oleate in the culture medium (**Table III**). Thus, the surface to core lipid ratio of apoB:1000-containing particles measured in this way was approximately 4:1 and was not affected by the addition of oleic acid to the culture medium.

Mass Analysis of Lipids Associated with ApoB:1000-Containing Particles Isolated by Immunoaffinity Chromatography Supports the Stoichiometry of the “Lipid Pocket” Determined by Metabolic Labeling. To obtain independent confirmation of the results obtained from metabolic labeling and NDGGE studies, apoB:1000-containing particles were isolated by immunoaffinity chromatography of unlabeled conditioned medium on an immunosorbent with affinity purified monospecific polyclonal antibody to human apoB-100. Aliquots of the retained fraction, before and after 10-fold concentration, were analyzed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and NDGGE in conjunction with

immunoblotting. A single band with the expected apoB:1000 molecular weight of 112 kDa was detected on SDS-PAGE by both Colloidal Blue staining (**Fig. 4 A, lanes 1 and 2**) and immunoblotting with anti-human apoB-100 (**Fig. 4 B, lanes 1 and 2**). Analysis of the retained fraction on NDGGE showed a single band with the predicted S_d of 112 Å by both Colloidal Blue staining (**Fig. 4 C, lane 2**) and immunoblotting (**Fig. 4 D, lane 2**). The isolated apoB:1000-containing particles secreted by control cells contained 87% PL, 7% TAG and 7% CE and particles secreted by oleate-supplemented cells contained 80% PL, 13% TAG and 7% CE (**Table IV**). The calculated numbers of PL, TAG, and CE molecules per particle were 64, 5, and 5, respectively, in the control cells and 56, 8, and 7, respectively, in oleate-supplemented cells (**Table IV**). The surface to core lipid ratio in the presence of oleic acid was approximately 4:1 and thus supports the results obtained with metabolic labeling and NDGGE (**Table III**). These results indicate that the “lipid pocket” created by the first 1000 amino acid residues of apoB-100 has a maximum capacity of approximately 70 molecules of lipid.

As shown in **Tables III** and **IV**, the lipid composition and the lipid capacity of the apoB:1000-containing particles isolated by NDGGE and immunoaffinity chromatography were not responsive to oleate supplementation of the cells. In contrast, the lipid composition and hence the surface to core lipid ratio of apoB:1000-containing particles isolated by immunoprecipitation were altered by oleate supplementation of the cells (**Table II**) and were also different from those isolated by either NDGGE or immunoaffinity chromatography. Particles isolated by immunoprecipitation contained considerably more TAG molecules per particle and this was further increased by the addition of oleate to the culture medium, i.e., the surface:core lipid ratio of particles secreted by control and oleate-treated cells were 2.3 and 0.8, respectively. We suggest that isolation of truncated apoB-containing particles by immunoprecipitation might

introduce an artifact in the lipid composition of secreted particles, especially in hepatic cell lines, perhaps by nonspecific immunoprecipitation and/or adsorption of endogenous rat TAG-rich particles to Protein G.

ApoB:1000-Containing Lipoproteins are Shown by Electron Microscopy to Represent Relatively Monodisperse Particles with Irregular Shapes. ApoB:1000-containing particles secreted by stable transformant of MCA-RH7777 cells were isolated by immunoaffinity chromatography on an immunosorber with affinity purified, monospecific polyclonal antibody to human apoB-100 as described above. The retained fraction from immunosorber was analyzed by negative stain electron microscopy (EM) (**Fig. 5**). **Panel A** shows that this preparation consists of HDL-size particles. The particles differ from typical HDL in two ways: 1) they are asymmetric in shape and 2) their edges are somewhat fuzzy in appearance. **Panel B** represents an enlargement of the boxed region in **Panel A**. It is clear from this image that a majority of the particles display an elongated shape, with dimensions of approximately 100 x 150 Å. In a few views, e.g. view 6, the particles have a spheroidal appearance. **Panel C** shows enlargements of six of the elongated images circled in **Panel B** suggesting that the particles tend to have a pear-like taper along their long axis. A molecular graphics image of the model for apoB:1000, described in the accompanying paper (Richardson et al. submitted), is displayed for comparison at the same magnification as the EM images in **panel C**. This model, whose dimensions are approximately 80 x 100 x 130 Å, is oriented so that its long axis is parallel (upper model) and perpendicular (lower model) to the plane of the figure.

MTP is Not Associated with ApoB:1000-Containing Particles in a 1:1 Molar Ratio. Our previous studies²⁴ showed that when concentrated conditioned medium and cell lysate were immunoprecipitated with anti-MTP 97 kDa large subunit or anti-human apoB-100 under

nondenaturing conditions, analyzed by SDS-PAGE, and immunoblotted with anti-human apoB-100 and anti-MTP 97 kDa large subunit, respectively, MTP and apoB:1000 were co-immunoprecipitated. Additionally, analysis of the concentrated conditioned medium by NDGGE and immunoblotting with anti-human apoB-100 and anti-MTP 97 kDa large subunit indicated the presence of MTP, albeit small in amounts, in the secreted apoB:1000-containing particles.²⁴ Based on these results we suggested that MTP might be an integral structural component of the lipid pocket. This would imply that one molecule of MTP should bind to one molecule of apoB:1000. To test this potential association between MTP and apoB:1000, cells were incubated in serum-free DMEM for 24 h and concentrated conditioned medium was subjected to NDGGE. The gel was stained with Colloidal Blue and the band corresponding to apoB:1000 (**Fig. 3 B**) was excised and analyzed by mass spectroscopy. In separate experiments, the concentrated conditioned medium was subjected to NDGGE and proteins were transferred onto PVDF membranes and stained with Coomassie Blue. The band corresponding to apoB:1000, identified by immunoblotting of a duplicate gel, was analyzed by amino acid sequencing. We were unsuccessful in detecting any measurable MTP in apoB:1000-containing particles by either mass spectroscopy or amino acid sequencing (data not shown). These results suggest strongly that MTP is not a structural component of the “lipid pocket” but do not rule out its role in the lipidation of the apoB:1000-containing particles.

Discussion

In our previous studies^{19,20} based on sequence homology between the $\beta\alpha_1$ domain of apoB-100, i.e., the first 1000 amino acid residues of the mature protein, and LV, we proposed that formation of a LV-like lipid pocket is necessary for lipid transfer to apoB-containing lipoprotein particles. We suggested that initiation of particle assembly occurs when $\beta\alpha_1$ domain folds into a three-sided LV-like lipid binding cavity, or alternatively, the lipid pocket is formed by association of the region of the $\beta\alpha_1$ domain homologous to the β A and β B sheets of LV with β D-like amphipathic β sheet from MTP.^{19,20}

In the present paper, we have shown that apoB:1000 forms stable particles without the 1:1 molar ratio of MTP to apoB required if MTP was necessary as an integral structural component of the lipid pocket. In the accompanying paper (Richardson et al., submitted), we propose an all-atom model for the formation of the “lipid pocket” by the first 1000 amino acid residues of apoB-100 that is completely consistent with the results of the present study. In this model, we describe a hairpin-bridge mechanism for lipid pocket completion in which a portion of a non-homologous loop from one of the two amphipathic β sheets of the lipid pocket folds as an amphipathic helical hairpin to bridge the distance between the two sheets. This creates a third side to the lipid pocket without a structural requirement for MTP.

Based upon the depth of the pocket (approximately 40 Å), similar to the thickness of the hydrophobic core of a phospholipid bilayer, we have suggested it probable that lipids form an asymmetric bilayer assembly, containing a neutral lipid lens as described previously,²⁰ in the nascent lipid pocket. A minimum of 44 POPC molecules were manually docked into the lipid pocket of the model for the $\beta\alpha_1$ domain of apoB, a number quite close to the experimental number of approximately 50 phospholipids per particle found in this study.

We previously demonstrated²⁴ that apoB:931 formed a particle with a constant diameter of 110 Å across a wide range of densities and a mean density of 1.25 g/ml or greater, denser than the classical HDL density range of 1.063-1.21 g/ml. The major particle formed by apoB:1000 had a diameter that remained constant at approximately 112 Å across a wide range of densities and a mean density of 1.208 g/ml, within the HDL₃ density range of 1.125-1.21 g/ml. In that same study, the larger construct, apoB:1200, containing a significant number of the amphipathic β strands located in the β₁ domain, formed a large particle with a diameter that increased with decreasing density, ranging from 118 Å to 127 Å, and a mean density of 1.197 g/ml, within the HDL₃ density range. This suggests that apoB:1200-containing particles possess additional, but varying, numbers of lipids compared to apoB:1000-containing particles.

In the present study we have experimentally confirmed that over a short stretch of 69 amino acids from apoB:931 to apoB:1000, the nature of the secreted particle is changed from an essentially lipid-free particle well outside the HDL density range to a lipidated particle within the HDL₃ density range. As shown in **Table I** and **Fig. 2**, the incorporation of [³H]glycerol and [¹⁴C]oleate into the lipid moieties of apoB:1000-containing particles was 4-10-fold higher than that in apoB:931-containing particles.

The apoB:1000-containing particles contained, on average, 50 molecules of PL, 12 molecules of TAG, and 6 molecules of cholesteryl ester per particle, for a surface:core lipid ratio of approximately 3:1. The X-ray crystal structure of lamprey LV²⁵ suggests a “lipid-pocket” containing approximately 27 molecules of PL and 11 molecules of TAG per LV monomer, a surface:core lipid ratio of approximately 2:1. To put these ratios in perspective, the surface:core lipid ratio of spheroidal HDL₃ particles is 1.5:1,²⁶ supporting the concept that the lipid in the

apoB:1000-containing particles is in the form of a bilayer assembly, rather than in the form of a mixed micelle assembly like HDL₃.

Negative stain electron microscopy of isolated apoB:1000-containing particles showed that these particles differ from typical HDL in two ways: 1) they are asymmetric in shape and 2) their edges are fuzzy in appearance. The majority of the particles display an elongated shape, with dimensions of approximately 100 x 150 Å, and tend to have a pear-like taper along their long axis. A molecular graphics image of the model for apoB:1000 described in the accompanying paper (Richardson et al. submitted), with dimensions of approximately 80 x 100 x 130 Å, displays close similarity to the EM images of apoB:1000-containing particles.

Biochemical studies have suggested that assembly of apoB-100 into a lipoprotein particle occurs co-translationally^{8,9} and requires the activity of MTP.^{9,16} However, little was known about the exact mechanisms by which apoB is assembled into a TAG-rich lipoprotein, i.e., the minimum structural requirement for initiation of lipoprotein assembly, the kinetics of lipid recruitment, specifically bulk lipid addition, the composition and number of lipids in the primordial lipidated particle, and the role of MTP in these processes. One often quoted mechanism for the physical assembly of lipid particles containing apoB is the budding oil droplet.⁷ In this model, the N-terminal portion of apoB is embedded in the inner monolayer of the endoplasmic reticulum (ER) membrane, where it nucleates an oil droplet from the supersaturated rough ER membranes. Upon completion of apoB synthesis this oil-droplet is detached from the bilayer to form the nascent lipoprotein. However, thermodynamic considerations make it unlikely that lipoproteins assemble through the wholesale remodeling or dismantling of membrane bilayers.

An alternate model for the initiation of apoB assembly has been suggested by Small and colleagues.²⁷ In this model, the formation of the “primordial” lipoprotein particle by a multistep process involves the initial recruitment of PL by the N-terminal region followed by incorporation of core lipids directed by the presence of β -sheets at and beyond apoB-29.²⁷ They proposed that the N-terminal 20% of apoB interacts with the internal PL leaflet of the ER by amphipathic structures, mainly β -sheets, present between apoB-13 and apoB-20. They suggested that the recruitment of TAG is due to its association with the region of the 28 predicted amphipathic β -strands extending from apoB-29 to apoB-41.²⁷

Our results demonstrating that apoB:931 does not contain the structural elements to form a lipidated particle are at odds with recent studies by Shelness et al.²⁸ showing that apoB-20.5 (amino acids 1-931) secreted by transfected COS cells contained sufficient structural and functional domains to form a secretion-competent lipoprotein particle. Contrary to our results demonstrating that apoB:931 particles have a mean density of 1.25 g/ml and are lipid-poor, the apoB20.5 secreted by COS cells had a peak density of 1.20 g/ml and contained 34 molecules of surface lipids and 49 molecules of core lipids, predominately TAG.²⁸ These investigators²⁸ showed that truncated apoB as small as apoB-19.5 (amino acids 1-884) and apoB-20.1 (amino acids 912) formed small HDL₃-like particles that contained 23-25 molecules of surface lipids and 28-36 molecules of core lipids. They suggested²⁸ that apoB-containing lipoproteins are initially formed as small, dense emulsion particles, with a surface:core lipid ratio of $\leq 1:1$, where apoB inserts itself into a saturated membrane surface and desorbs lipid as a preformed core-containing lipoprotein, a model consistent with the budding oil droplet mechanism.⁷

We are not sure of the reasons for the observed differences in densities and lipid composition of apoB:931 in our study and that reported by Shelness et al.²⁸ We speculate that this discrepancy

may, in part, be due to the fact that we used hepatic-derived cells and Shelness et al.²⁸ used non-hepatic cells, perhaps reflecting differences in the profile of numerous chaperones known to be involved in apoB maturation⁸ between hepatic McA-RH7777 and non-hepatic COS cells. The uncleaved His-tag used by Shelness, et al.²⁸ may also have contributed to the differences. Consistent with our results, McLeod et al.²⁹ showed that only carboxyl-terminal truncations with a size greater than apoB-23 are able to assemble a neutral lipid core. Our present results, supported by several other studies in hepatic and nonhepatic cells,^{12-14,27,30} suggest rather unambiguously that the N-terminal 931 amino acid domain of apoB-100 does not have the structural and functional elements necessary for the assembly of apoB into a nascent lipoprotein particle.

Although we do not know the exact mechanism by which apoB:1000 acquires PL, the data presented here indicates that gradual lipid transfer into an apoB-containing particle during biosynthesis requires translocation of a critical length of apoB sequence, i.e., the $\beta\alpha_1$ domain (residues 1-1000), necessary for creation of a competent lipid pocket. In support of this hypothesis, several studies³¹⁻³³ have suggested that acquisition of lipid occurs stepwise along the secretory pathway. Our model is also consistent with the hypothesis that the initial step in the assembly of apoB-containing lipoproteins involves the recruitment by the N-terminal domain of apoB of surface lipids, primarily PL, and a small quantity of core lipids during and immediately after translation.³⁴ It has been suggested³⁴ that in the next step, cytosolic TAG droplets are hydrolyzed and TAG is re-synthesized on the ER for assembly with apoB and translation/translocation continues. As more TAG, PL and cholesterol are added to the particle, its size increases and a fully lipidated VLDL particle is eventually formed.³⁴ MTP might be

involved in translocation of apoB, assembly of PL with apoB, and/or transfer of TAG to the core of the nascent VLDL particles.³⁴

Based on the results presented in this paper and the hairpin-bridge mechanism for the formation of the lipid pocket described in the accompanying paper (Richardson et al. submitted), we suggest the following model for the initiation of lipoprotein particle assembly. The lipid pocket formed by apoB:1000 begins to recruit PL and some TAG co-translationally to form a primordial lipoprotein particle. MTP serves as a shuttle to deliver lipids into the lipid pocket upon translation of apoB to and beyond residue 1000. Translation of the amphipathic β strands in the β_1 domain provides a mechanism for stabilization of the particle beyond the nascent apoB:1000 particle. When the particle reaches a critical size, the salt bridges holding the hairpin-bridge in place break and apoB undergoes conformational change. As the hairpin-bridge is unlocked, βA and βB sheets separate; lipids are added to the flexible basal opening of the lipid pocket and the hydrophobic helices of helix-turn-helix associate with the growing particle. Further addition of lipids cause the formation of a V-shaped pocket between βA and βB sheets. This open conformation of apoB would allow its association with a much larger surface than the protein itself. We suggest that this is the conformation that apoB assumes in LDL, IDL, and VLDL.

In summary, we have demonstrated that amino acids 931-1000 of apoB-100 are critical for the initiation of apoB lipoprotein assembly. Based on experimentally derived results and molecular modeling, we propose that initiation of particle assembly occurs when $\beta\alpha_1$ domain (amino acid residues 1-1000) folds into a three-sided LV-like lipid binding cavity to form the “lipid pocket” without the structural requirement of MTP. This hydrophobic cavity is subsequently loaded with PL and forms a particle with lipid composition and surface:core lipid

ratio consistent with LV “lipid pocket”. Lipid composition, total number of lipid molecules per particle, and the particle peak density of apoB:1000-containing particles are not responsive to oleic acid supplementation indicating that the “lipid pocket” formed by the N-terminal 1000 residues of apoB-100 has a fixed lipid capacity on the order of 50 PL for a total stoichiometry of 70 lipid molecules. Negative stain EM of isolated apoB:1000-containing particles revealed a structure consistent with that predicted for the apoB:1000 “lipid pocket” by molecular modeling.

Materials and Methods

Materials. Fetal bovine serum (FBS), horse serum (HS), and antibiotic-antimycotic were obtained from GIBCO BRL Biological Company (Grand Island, NY). Tris-Glycine gels were obtained from Invitrogen-Novex (Carlsbad, CA). Dulbecco's modified Eagle's medium (DMEM), trypsin, and G418 were purchased from Mediatech, Inc. (Herndon, VA). Fatty acid free bovine serum albumin (BSA) was from Miles Inc. (Kankakee, IL). Oleic acid (purity greater than 99% by capillary gas chromatography), sodium deoxycholate, Triton X-100, benzamidine, phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and pepstatin A were from Sigma Chemical Co. (St. Louis, MO). Protein G-Sepharose CL-4B, [³H]glycine, [¹⁴C]oleic acid, and Amplify were from Amersham Pharmacia Biotech. (Piscataway, NJ). Immobilon PVDF transfer membrane and Centriprep Centrifugal Filter Devices YM-30 were purchased from Millipore Corp. (Bedford, MA). Affi-Gel 10 (N-hydroxysuccinimide ester derivative of cross-linked agarose) and all reagents used for gel electrophoresis were from Bio-Rad Laboratories (Richmond, CA). Affinity purified polyclonal antibody to human apoB-100 was prepared in our laboratory and affinity purified polyclonal antibody to bovine MTP 97 kDa large subunit¹⁶ was a generous gift from Dr. J. R. Wetterau (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ). The antibodies to apoB and MTP 97 kDa subunit were biotinylated at Brookwood Biomedical (Birmingham, AL). ApoB-100 cDNA was a gift from Gladstone Institute of Cardiovascular Disease, San Francisco, CA.

Construction of Truncated ApoB Expression Plasmids. Truncated apoB cDNAs spanning nucleotides 1-2481, 1-2874, and 1-3081, respectively, of the full-length apoB-100 cDNA, were prepared from pB100L-L¹³ as a PCR template and appropriate primers as previously described in detail.²⁴ The amplified PCR products were cloned and apoB fragments were excised from the

vector, extracted and purified, and ligated into the mammalian expression vector, the Molony murine leukemia virus based retrovirus LNCX³⁵ containing the neomycin phosphotransferase gene which confers G418 resistance for use as selectable marker. The apoB expression vectors, pLNCB:800, pLNCB:931 and pLNCB:1000 were used to transform cells and clones harboring plasmids containing apoB gene with the correct orientation were identified by restriction enzyme digestion and confirmed by nucleotide sequencing as previously described.²⁴

Cell Culture and Transfection. Clonal stable transformants of rat hepatoma McA-RH7777 cells expressing apoB:800, apoB:931 and apoB:1000, denoting amino acid residues 1-800, 1-931 and 1-1000, respectively, of the mature protein lacking the signal peptide, were generated as previously described in detail.²⁴ Since apoB:800, which lacks the β B domain was shown to be secreted as a lipid-poor aggregate,²⁴ cells expressing this truncated form of apoB, parental non-transfected McA-RH7777, and LNCX (neo)-transfected cells were used as controls in only a few experiments. Cells were grown in DMEM containing 20% horse serum, 5% FBS, and 0.2 mg/ml G418 and medium was changed every 48 h. All experiments were conducted with 4- to 5-day old cells as previously described.²⁴

Metabolic Labeling Studies. Clonal stable transformants of McA-RH7777 cells, expressing apoB:931 and apoB:1000, were grown for 4 days in either 6-well dishes for immunoprecipitation (IP) studies or in 100-mm dishes for nondenaturing gradient gel electrophoresis (NDGGE) and immunoaffinity chromatography studies as described above. At the start of experiments, maintenance medium was removed, monolayers were washed twice with phosphate-buffered saline (PBS) and serum-free DMEM containing [³H]-labeled glycerol or 0.4 mM [¹⁴C]-labeled oleic acid bound to 0.75% BSA was added. After the indicated incubation time, labeled conditioned medium was collected, preservative mixture at a final concentrations of 500 units/ml

penicillin-G, 50 µg/ml streptomycin sulfate, 20 µg/ml chloramphenicol, 50 µg/ml leupeptin, 50 µg/ml pepstatin A, 1.3 mg/ml ε-amino caproic acid, 1 mg/ml EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, and 100 kallikrein-inactivating units of aprotinin/ml was added to prevent oxidative and proteolytic damage. The medium was centrifuged at 2,000 rpm for 30 min at 4°C to remove broken cells and debris. The incorporation of [³H]-labeled glycerol or [¹⁴C]-labeled oleate into various lipid moieties of the secreted apoB-containing lipoproteins was determined by immunoprecipitation with polyclonal antibody to human apoB-100 or by NDGGE of labeled conditioned medium as described below. Cell monolayers were washed with cold PBS, scraped off the plate in PBS and sonicated for determination of protein content by the method of Lowry et al.³⁶

Lipoprotein Isolation. Cells were incubated overnight in 6 ml of serum-free DMEM with or without the labeled precursors. In studies without the labeled precursor, conditioned medium from four 100-mm dishes were combined, preservative mixture was added, and medium was concentrated 4-fold using Centricon YM-30. The density of the concentrated conditioned medium was adjusted to 1.23 g/ml using solid KBr and lipoproteins ($d < 1.23$ g/ml) were isolated by centrifugation for 40 h at 50,000 rpm. The lipoprotein fraction ($d < 1.23$ g/ml) and infranatnat ($d > 1.23$ g/ml) were dialyzed and concentrated 5- to 6-fold. In metabolic labeling studies, medium from two 100-mm dishes were pooled, processed as above and was concentrated 2-fold. The density of the concentrated medium was adjusted to 1.23 g/ml and was subjected to ultracentrifugation as described above.

Immunoprecipitation. After an overnight (17-20 h) incubation with serum-free medium and [³H]-labeled glycerol or 0.4 mM [¹⁴C]-labeled oleic acid bound to 0.75% BSA, the labeled apoB-containing lipoproteins secreted into the conditioned medium were immunoprecipitated under

nondenaturing conditions³⁷⁻³⁹ using monospecific polyclonal antibody to human apoB-100 coupled to Protein G-Sepharose CL-4B as previously described.^{37,38} The beads were washed six to seven times until background count was obtained in the wash and then extracted for lipids as described below.

Nondenaturing Polyacrylamide Gradient Gel Electrophoresis (NDGGE). In unlabeled experiments, aliquots of concentrated total medium, lipoprotein fraction ($d < 1.23$ g/ml), and infranatant fraction ($d > 1.23$ g/ml) were subjected to 4-20% NDGGE for 48 h at 4°C in buffer containing 24 mM Tris-HCl, pH 8.3 and 192 mM glycine. Proteins were transferred onto PVDF membrane and immunoblotted with anti-human apoB-100 as described below. In metabolic labeling studies, aliquots of total medium and $d < 1.23$ g/ml lipoprotein fraction were run on 4-20% NDGGE; gels were stained and the bands corresponding to apoB:931 and apoB:1000, identified by their Stokes diameter and immunoblotting of a duplicate gel, were excised and analyzed for lipids described below. The incorporation of [³H]-labeled glycerol or [¹⁴C]-labeled oleic acid into total lipids of intact apoB-containing lipoproteins was also determined by NDGGE of the labeled conditioned medium and autoradiography of the amplified and dried gels.

Isolation of Truncated ApoB-Containing Particles by Immunoaffinity Chromatography. Affinity purified monospecific polyclonal antibody to human apoB-100 was coupled to the cross-linked agarose activated with N-hydroxysuccinimide (Affi-Gel 10). After exhaustive washing of the Affi-Gel with cold, double-distilled water, the antibody solution was added to the gel slurry (2 mg protein/ml of gel) and the mixture was gently shaken for 2-3 h at room temperature. The supernatant was removed and the remaining active sites were blocked; gel was washed and equilibrated with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1.5 mg/ml EDTA as previously described.⁴⁰ Column was packed with the gel flanked with two

layers of Sephadex G-25, a 25 ml bottom layer to minimize the exposure time of lipoproteins to dissociating agent and a 3 ml protective top layer, as previously described.⁴⁰

Stable transformants of McA-RH7777 expressing apoB:1000 were grown in 100 mm dishes in serum-containing DMEM for 4 days. Monolayers were washed 3 times with PBS to remove residual serum and were incubated for 24 h in serum-free DMEM with or without 0.4 mM oleic acid bound to 0.75% BSA. The conditioned medium from 10 plates were pooled, preservatives were added and medium was centrifuged for 30 min at 2000 rpm to remove broken cells and debris. The conditioned medium was concentrated 10- to 12-fold and applied to the immunoaffinity column. The unretained fraction was eluted with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 1.5 mg/ml EDTA and the retained fraction was eluted with 5 ml of 4 M NaSCN as previously described.⁴⁰ After addition of preservative cocktail, retained fraction was concentrated and analyzed for the mass of PL, TAG, and cholesteryl ester.

Lipid Analysis of Isolated Truncated ApoB-Containing Particles. Total lipids associated with the immunoprecipitated labeled apoB-containing particles were extracted from Protein G with chloroform/methanol (2:1). In NDGGE studies, the bands corresponding to labeled apoB:931- or apoB:1000-containing particles were excised from the gel and homogenized using Dounce homogenizer. Sodium dodecyl sulfate (SDS) was added to the final concentration of 0.2% and the volume of the homogenate was adjusted to 5 ml with distilled water. The homogenate was incubated at 37° C for 2 h to dissociate the lipids from the polyacrylamide gel and then lyophilized. Total lipids were extracted from the lyophilized homogenate five times in a total volume of 10 ml of chloroform/methanol (2:1); complete extraction was assessed by counting the final gel homogenate. Total labeled lipids extracted from immunoprecipitated and gel isolated apoB-containing lipoproteins were washed by the Folch method⁴¹ as previously

described.⁴² The final washed extracts were dried under a nitrogen stream, dissolved in small volume of ether, applied to thin layer chromatography (TLC) plate, and chromatographed in hexane:diethylether:acetic acid (80:20:1) solvent system. The bands corresponding to PL, DAG, and TAG, identified by comparison to known standards, were visualized with iodine; each band was scraped off the plate, placed in vials and quantified by liquid scintillation counting. In immunoaffinity chromatography studies, the concentrated retained fraction from the anti-apoB immunosorber containing unlabeled apoB:1000-containing particles was analyzed for the mass of TAG, and cholesteryl esters by gas chromatography⁴³ and for the mass of PL according to the micromethod of Gerlach-Deuticke.⁴⁴

Immunoblot Analysis. The isolated truncated apoB-containing particles were run on 4-12% SDS-PAGE⁴⁵ or on 4-20% NDGGE. After electrophoresis, proteins were detected by Western blot analysis⁴⁶ using biotinylated antibodies to either human apoB-100 or bovine MTP 97 kDa large subunit as previously described.²⁴

Calculation of Number of Lipid Molecules per Particle. Calculations were made essentially as described by Carraway et al.²⁷ The number of lipid molecules per apoB particle was calculated from percent composition of PL, DAG, and TAG determined by metabolic labeling and TLC analysis or by mass determination of PL, TAG, and CE in retained fraction from anti-apoB immunosorber. The equation for calculating lipid:apoB molar ratios of apoB:1000-containing particles was derived by using the calculated molecular weight of 111,375 kDa for nonglycosylated apoB:1000, based on amino acid sequence using DNAMAN program, and particle density of 1.208 g/ml, determined by density gradient ultracentrifugation followed by NDGGE and immunoblotting as previously described.²⁴

Electron Microscopy. For electron microscopy, concentrated retained fraction from anti-apoB immunosorber was dialyzed against ammonium acetate buffer (2.6 mM, pH 7.4) and negatively stained with 2% sodium phosphotungstate as previously described.⁴⁷ Samples were examined in a JEOL 100C electron microscope.

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Figure Legends

Figure 1. Ability of apoB:931 and apoB:1000 to form lipoproteins. Stable transformants of McA-RH7777 cells expressing apoB:931 and apoB:1000 were grown in DMEM containing 20% horse serum and 5% FBS for 4 days. Maintenance medium was removed; cells were washed with PBS and were incubated for 20 h in serum-free DMEM containing 0.4 mM oleic acid bound to 0.75% BSA. The density of the concentrated conditioned medium was adjusted to $d = 1.23$ g/ml and lipoproteins were isolated by centrifugation for 40 h at 50,000 rpm. The lipoprotein ($d < 1.23$ g/ml) and infranatant ($d > 1.23$ g/ml) fractions were dialyzed and concentrated. Aliquots of concentrated conditioned medium (**lane 1**), $d < 1.23$ g/ml lipoproteins (**lane 2**), and $d > 1.23$ g/ml infranatant fraction (**lane 3**) of apoB:931-expressing cells (**Panel A**) and apoB:1000-expressing cells (**Panel B**) were subjected to 4-20% NDGGE at 4 °C for 48 h. Proteins were transferred onto PVDF membrane and detected by immunoblotting with anti-human apoB-100.

Figure 2. Amino acids 931 to 1000 of apoB-100 are critical for the formation of a bulk lipid-containing particle and initiation of lipoprotein assembly. Stable transformants of McA-RH7777 cells expressing truncated forms of apoB were grown for 4 days in DMEM containing 20% horse serum and 5% FBS. Maintenance medium was removed; cells were washed with PBS and incubated in serum-free DMEM containing 0.4 mM [¹⁴C]-labeled oleic acid bound to 0.75% BSA. Aliquots of conditioned medium from parental nontransfected McA-RH7777 cells (**lane 1**), LNCX (neo)-transfected cells (**lane 2**), apoB:800 (**lane 3**)-, apoB:931 (**lane 4**)-, and apoB:1000 (**lane 5**)-expressing cells were concentrated and subjected to 4-20% NDGGE at 4°C for 48 h; gels were run in duplicates. In **Panel A**, proteins were transferred onto PVDF membrane and detected by immunoblotting with anti-human apoB-100 to confirm the identity of apoB:931 and apoB:1000. Stokes diameter of the secreted particles were determined by comparison to known

standards. In **Panel B**, gel was stained with Colloidal Blue, amplified, dried, and labeled apoB-containing particles were visualized by autoradiography.

Figure 3. Isolation of [³H]glycerol-labeled apoB:1000-containing particles by NDGGE for determination of lipid composition. Stable transformants of McA-RH7777 cells expressing apoB:1000 were grown for 4 days in DMEM containing 20% horse serum and 5% FBS. Maintenance medium was removed, cells were washed with PBS and incubated in serum-free DMEM containing [³H]-labeled glycerol (7 μCi/ml). Aliquots of concentrated labeled conditioned medium and $d < 1.23$ g/ml lipoproteins, isolated from conditioned medium, were subjected to 4-20% NDGGE at 4°C for 48 h; gels were run in duplicates. In **Panel A**, proteins in total medium (**lane 1**) and $d < 1.23$ g/ml lipoproteins (**lane 2**) were transferred onto PVDF membrane and detected by immunoblotting with anti-human apoB-100 to confirm the identity of apoB:1000. Stokes diameter of the secreted particles was verified by comparison to known standards. In **Panel B**, gel was stained with Colloidal Blue and bands corresponding to apoB:1000 in total medium (**lanes 1-3**) and $d < 1.23$ g/ml lipoprotein (**lanes 4-5**), indicated by an arrow, were excised and extracted for lipids as described in Materials and Methods.

Figure 4. Characterization of apoB:1000-containing particles isolated from the conditioned medium of McA-RH7777 cells by immunoaffinity chromatography. Stable transformants of McA-RH7777 cells expressing apoB:1000 were grown for 4 days in DMEM containing 20% horse serum and 5% FBS. Maintenance medium was removed; cells were washed three times with PBS and incubated for 24 h in serum-free DMEM with or without 0.4 mM oleic acid bound to 0.75% BSA. Conditioned medium was concentrated and applied to immunosorbent with affinity purified monospecific antibody to apoB-100. The retained fraction containing apoB:1000-containing particles was concentrated and analyzed for purity by SDS-PAGE (**Panels**

A and B) and NDGGE (**Panels C and D**). In **Panel A**, aliquots of the retained fraction before (**lane 1**) and after (**lane 2**) 10-fold concentration were applied to SDS-PAGE and stained with Colloidal Blue. In **Panel B**, proteins separated on SDS-PAGE were transferred onto PVDF membrane and detected by immunoblotting with polyclonal anti-human apoB-100. In **Panel C**, aliquots of concentrated retained fraction (**lane 2**) were applied to 4-20% NDGGE at 4 °C for 48 h and gel was stained with Colloidal Blue. In **Panel D**, apoB:1000-containing particles (**lane 2**) separated on NDGGE were transferred onto PVDF membrane and detected by immunoblotting with anti-human apoB-100. The molecular weight of secreted apoB:1000 and Stokes diameter of the intact particles were verified by comparison to known standards (**Panels A and C, lane 1**).

Figure 5. Electron micrograph of apoB:1000-containing lipoproteins isolated by immunoaffinity chromatography. ApoB:1000-containing particles secreted by stable transformants of McA-RH7777 cells were isolated by immunoaffinity chromatography on an immunosorbent with affinity purified monospecific polyclonal anti-human apoB-100. **Panel A** shows that this preparation consists of HDL-sized particles. The particles differ from HDL in two ways: they are asymmetric in shape and their edges are somewhat fuzzy in appearance. **Panel B** represents an enlargement of the boxed region in **Panel A**. It is clear from this image that a majority of the particles display an elongated shape, with dimensions of approximately 100 x 150 Å. In a few views, e.g. view 6, the particles have a spheroidal appearance. **Panel C** shows enlargements of six of the elongated images circled in **Panel B** suggesting that the particles tend to have a pear-like taper along their long axis. A molecular graphics image of the model for B:1000 described in the following paper is displayed for comparison at the same magnification as the EM images in **Panel C**. This model, whose dimensions are approximately 80 x 100 x 130 Å, is oriented so that its long axis is parallel to the plane of the figure.

Table I

Relative Incorporation of [¹⁴C]Oleic Acid and [³H]Glycerol into the Lipid Moiety of Truncated ApoB-Containing Lipoproteins Secreted by McA-RH7777 Cells

Truncated ApoB	Total Lipids of Immunoprecipitated	Total Lipids of Gel-Isolated
	[¹⁴ C]-Labeled Particles	[³ H]-Labeled Particles
	cpm/mg cell protein	cpm/mg cell protein
ApoB:931	758 ± 71	568 ± 69
ApoB:1000	2810 ± 184	2537 ± 667

McA-RH7777 cells, expressing apoB:931 and apoB:1000 were grown under conditions described in Material and Methods. Cells were incubated in serum-free DMEM containing either 0.4 mM [¹⁴C]oleic acid bound to 0.75% BSA or serum-free DMEM containing 0.4 mM unlabeled oleic acid bound to 0.75% BSA and [³H]glycerol (7 μCi/ml of medium). The [¹⁴C]-labeled and [³H]-labeled apoB-containing lipoproteins were isolated by immunoprecipitation with monospecific polyclonal antibody to human apoB-100 and NDGGE, respectively, and their total lipid content was determined as described in Material and Methods. In immunoprecipitation experiments, radioactivity in total lipids secreted by neo-transfected cells was subtracted from that secreted by apoB-expressing cells. Values are means ± SE of three experiments and are normalized for the total volume of medium and cell protein.

Table II

Composition of [³H]Glycerol-Labeled Lipids Associated With ApoB:1000-Containing Lipoproteins
Secreted by McA-RH7777 Cells and Isolated by Immunoprecipitation

Addition	PL	DAG	TAG	Total Lipid Molecules
Percent of Total Lipids				
Control	57.0 ± 0.3	10.0 ± 0.6	33.0 ± 2.0	
Oleic Acid	39.0 ± 1.6	3.0 ± 0.4	58.0 ± 1.0	
Calculated Number of Lipid Molecules per Particle				
Control	36.0 ± 2.0	8.0 ± 0.6	19.0 ± 1.0	63.0 ± 1.0
Oleic Acid	22.0 ± 0.7	2.0 ± 0.6	31.0 ± 0.6	56.0 ± 0.3

Cells were incubated in serum-free DMEM with or without 0.4 mM oleic acid bound to 0.75% BSA and [³H]-labeled glycerol (7 μCi/ml of medium). The secreted apoB-containing lipoproteins were immunoprecipitated using monospecific polyclonal antibody to apoB-100 and their lipid composition was determined as described in Materials and methods. Values are means ± SE of triplicate dishes representing three separate experiments.

Table III

Composition of [³H]Glycerol-Labeled Lipids Associated with ApoB:1000-Containing Lipoproteins
 Secreted by McA-RH7777 Cells and Isolated by Nondenaturing Gradient Gel Electrophoresis

Fraction	Addition	PL	DAG	TAG	Total Lipid Molecules
Percent of Total Lipids					
Total Medium	Control	74.0 ± 1.0	8.0 ± 0.4	18.0 ± 0.8	
d < 1.23 g/ml	Control	71.0 ± 3.0	8.0 ± 0.8	21.0 ± 2.6	
Total Medium	Oleic Acid	70.0 ± 3.0	8.0 ± 0.7	22.0 ± 3.0	
d < 1.23 g/ml	Oleic Acid	73.0 ± 3.0	6.0 ± 0.7	21.0 ± 3.0	
Calculated Number of Lipid Molecules per Particle					
Total Medium	Control	50.0 ± 1.0	7.0 ± 0.4	11.0 ± 0.3	69.0 ± 0.3
d < 1.23 g/ml	Control	50.0 ± 2.2	7.0 ± 0.5	13.0 ± 1.0	69.0 ± 1.0
Total Medium	Oleic Acid	48.0 ± 3.0	6.0 ± 0.5	14.0 ± 1.5	68.0 ± 1.3
d < 1.23 g/ml	Oleic Acid	51.0 ± 2.4	5.0 ± 0.5	13.0 ± 1.5	69.0 ± 1.0

Cells were incubated with serum-free DMEM with or without 0.4 mM oleic acid bound to 0.75% BSA. The incorporation of [³H]glycerol (7 μCi/ml of medium) into the lipid moiety of the secreted particles was determined by NDGGE as described in Materials and Methods. Values are means ± SE of seven separate experiments.

Table IV

Lipid Composition of ApoB:1000-Containing Lipoproteins Secreted by McA-RH7777 Cells
and Isolated by Immunoaffinity Chromatography

Addition	PL	TAG	CE	Total Lipid Molecules
Percent of Total Lipids				
Control	87.0 ± 1.2	6.7 ± 0.7	6.4 ± 1.3	
Oleic Acid	79.6 ± 2.6	12.8 ± 2.6	7.6 ± 0.8	
Calculated Number of Lipid Molecules per Particle				
Control	64.0 ± 1.3	5.0 ± 0.5	5.0 ± 1.0	74.0 ± 0.4
Oleic Acid	56.0 ± 2.6	8.0 ± 1.6	7.0 ± 0.7	71.0 ± 1.0

Cells were incubated for 24 h in serum-free DMEM (control) or serum-free DMEM containing 0.4 mM oleic acid bound to 0.75% BSA. Conditioned medium was concentrated 10-fold and applied to immunoaffinity column with monospecific, affinity purified polyclonal antibody to human apoB-100. The retained fraction was concentrated and analyzed for the mass of PL, TAG and CE as described in Material and Methods. Values are means ± SE of six separate experiments.

Figure 1

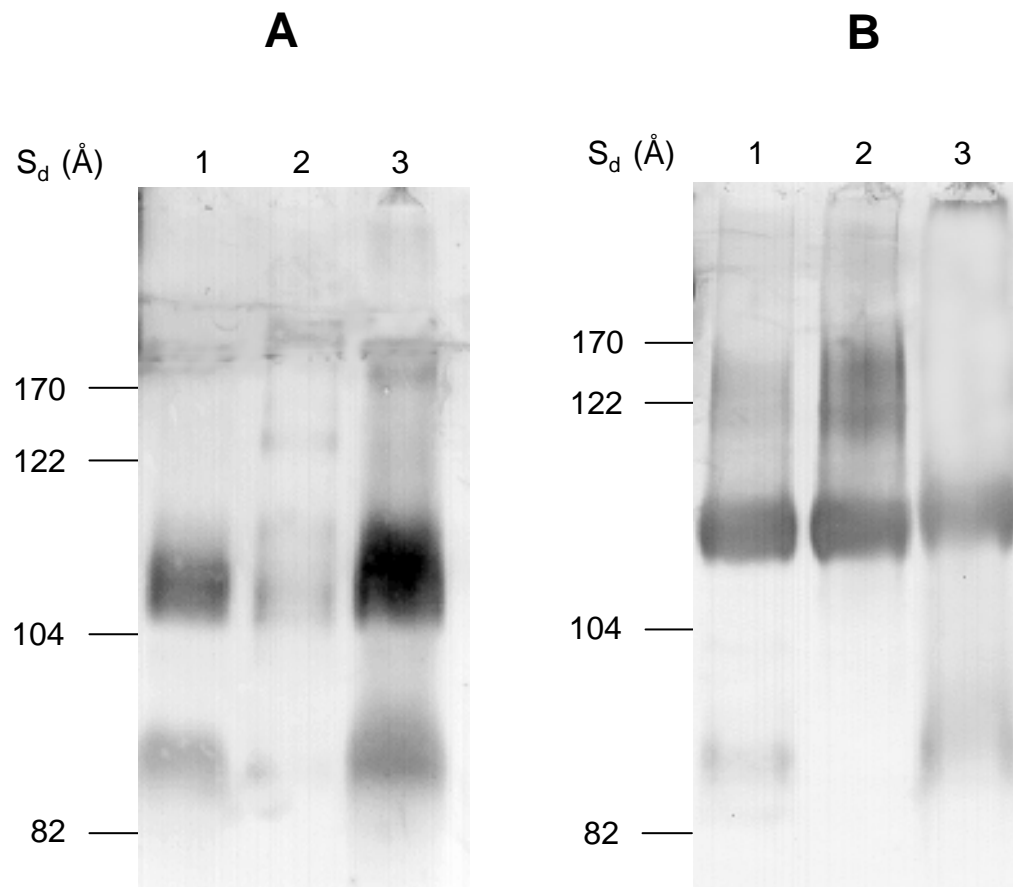


Figure 2

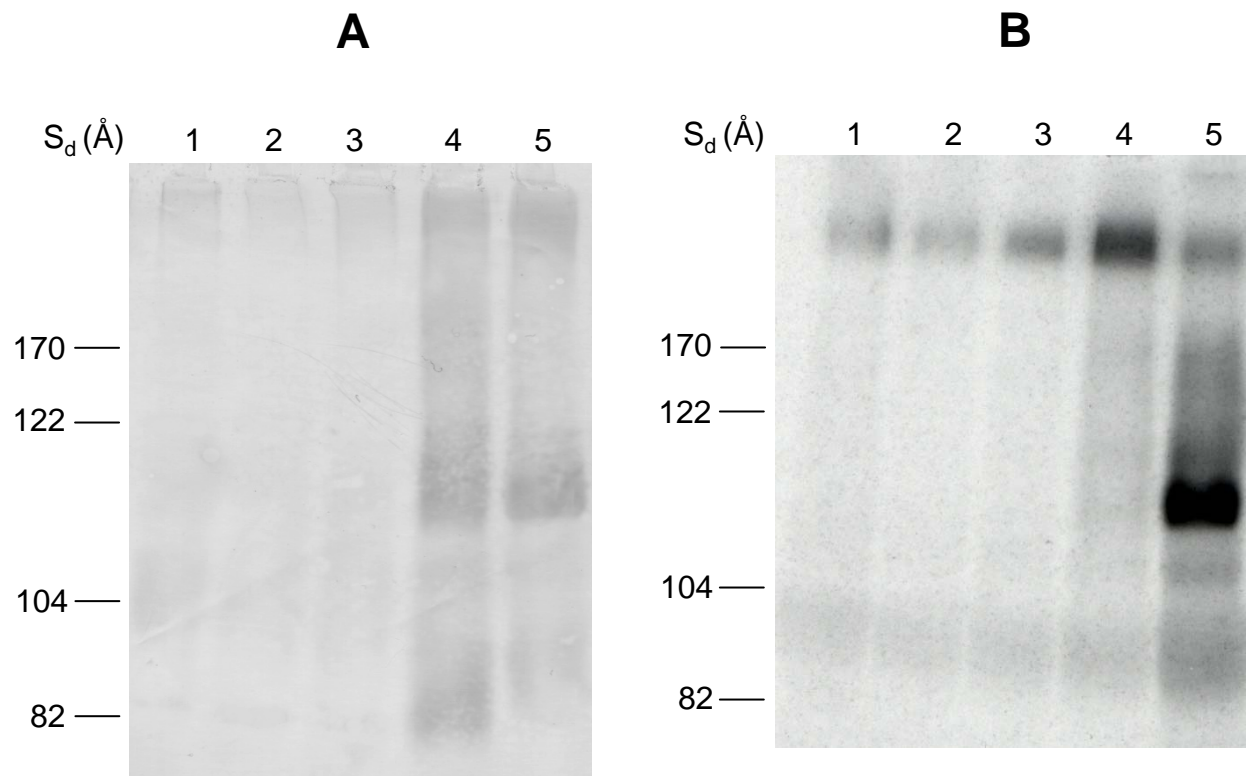


Figure 3

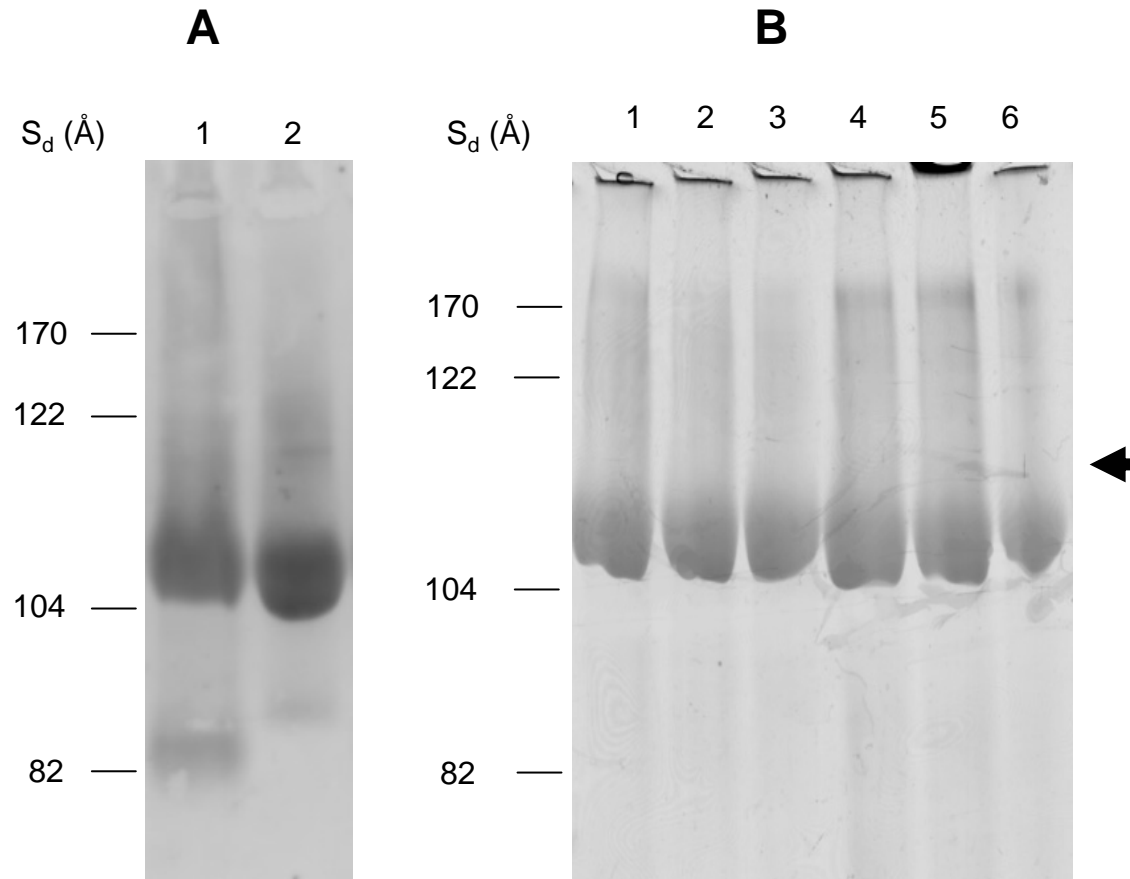


Figure 4

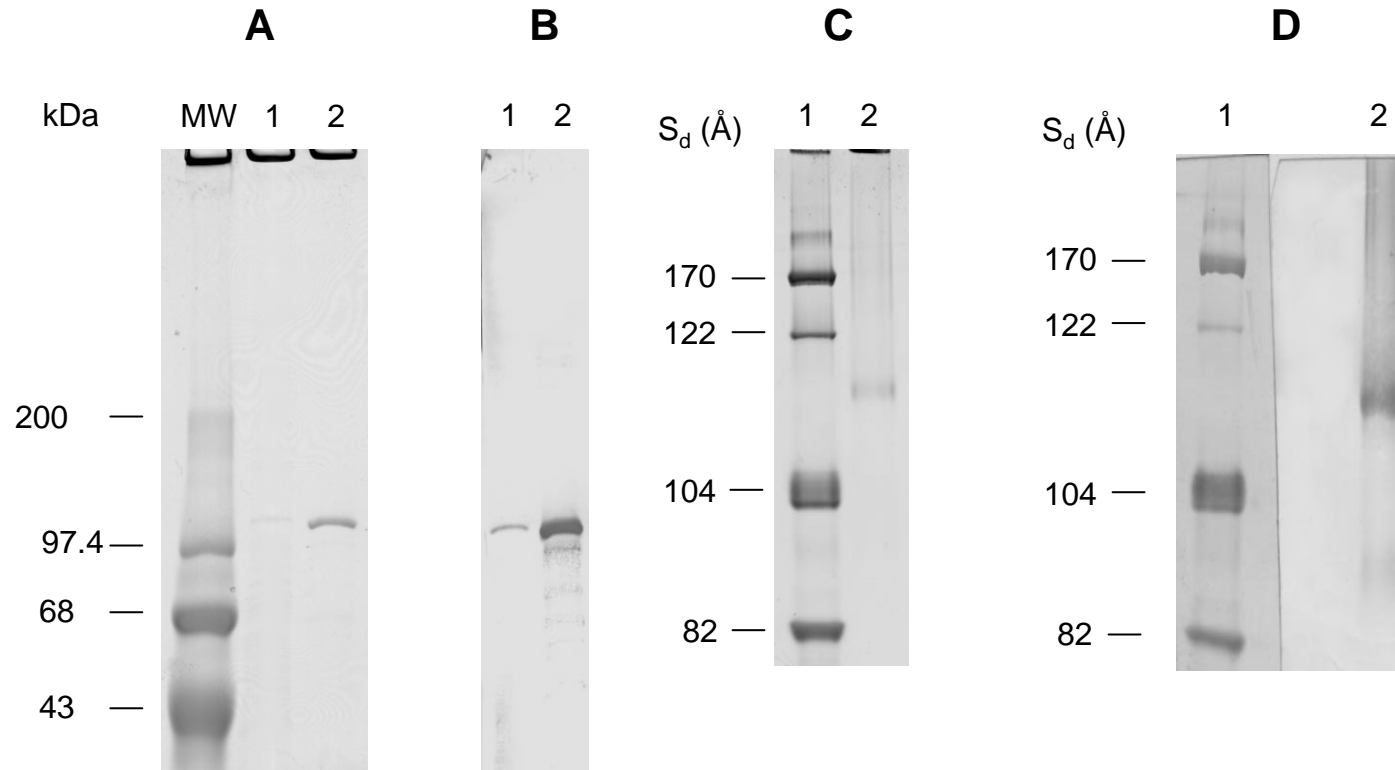


Figure 5

