IDENTIFICATION OF THE LIPOPROTEIN INITIATING DOMAIN OF APOLIPOPROTEIN B

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Running Title: Initiating Domain of ApoB

Abbreviations Used:
AP, alkaline phosphatase; apoB, apolipoprotein B; CE, cholesteryl ester; DG, diglyceride; DMEM, Delbecco’s Modified Eagle Media; ER, endoplasmic reticulum; LV, lipovitellin; MTP, microsomal triglyceride transfer protein; PBS, phosphate buffered saline; PL, phospholipid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TG, Triglyceride; TLC, thin layer chromatography; VLDL, very low density lipoprotein; vtg, vitellogenin
SUMMARY

We have explored the minimum sequence requirement for the initiation of apolipoprotein B (apoB)-mediated triglyceride-rich lipoprotein assembly. A series of apoB C-terminal truncation mutants, spanning a range from apoB34 (amino acid residues 1-1544 of apoB100) to apoB19 (residues 1-862) were transfected into COS cells with and without coexpression of the microsomal triglyceride transfer protein (MTP). ApoB34, 28, 25, 20.5, 20.1 underwent efficient conversion to buoyant lipoproteins when coexpressed with MTP. ApoB19.5 (amino acids 1-884) also directed MTP-dependent particle assembly, although at reduced efficiency. When apoB19.5 was truncated by another 22 amino acids to form apoB19, MTP-dependent lipoprotein assembly was abolished. Analysis of the lipid stoichiometry of secreted lipoproteins revealed that all apoB truncation mutants formed spherical particles containing a hydrophobic core. Even highly truncated assembly competent forms of apoB, such as apoB19.5 and 20.1, formed lipoproteins with surface:core lipid ratios of <1. We conclude that the translation of the first ~884 amino acids of apoB completes a domain capable of initiating nascent lipoprotein assembly. The composition of lipids recruited into lipoproteins by this initiating domain is consistent with formation of small emulsion particles, perhaps by simultaneous desorption of both polar and neutral lipids from a saturated bilayer.
INTRODUCTION

The hepatic assembly of VLDL begins with the cotranslational conversion of a nascent apolipoprotein B (apoB) polypeptide chain to a precursor lipoprotein particle (1-3). The process by which apoB and its dedicated cofactor, microsomal triglyceride transfer protein (MTP), initiates lipoprotein formation is unknown (4,5). One of the most commonly cited mechanisms of assembly involves intercalation of apoB into the inner leaflet of the endoplasmic reticulum, where it nucleates triglyceride (TG) droplet formation within the bilayer; the apoB-associated droplet ultimately buds from the inner leaflet of the bilayer into the ER lumen as a lipoprotein emulsion particle (6,7). More recently, Segrest and colleagues used the sequence similarity between the N-terminal domain of apoB (βα1 domain; Fig. 1) and vertebrate vitellogenin (vtg) to argue that initiation of lipoprotein formation progresses through a proteolipid intermediate that resembles the structure and lipid composition of lipovitellin (LV), the processed form of vtg (8,9). The LV monomer contains a funnel-shaped hydrophobic lipid-binding cavity, formed by a series of amphipathic antiparallel beta sheets that can accommodate the acyl chains of a phospholipid bilayer, which in turn can solubilize a small lens of TG (10). The LV component of the Xenopus laevis yolk system contains ~37 molecules of phospholipid (PL) and ~13 molecules of TG (11).
Identification of the minimum size apoB translation product competent to direct particle assembly, as well as analysis of the number and types of lipids that are acquired during the initiation phase of assembly, could help elucidate the mechanism by which apoB directs this process. Previous studies with truncated forms of apoB suggest that the first ~22-28% of the protein must be translated to achieve nascent lipoprotein assembly (8,12,13). However, no systematic attempt has been made to exactly map the structural boundary in apoB required to initiate lipoprotein assembly, nor has the lipid composition of particles assembled by such a truncation mutant been examined. In the present report we used fine truncation mapping to identify the initiating domain of apoB. We found that a narrow, ~50 amino acid interval (amino acids 862-912), specifies the transition of apoB from a form that is only secreted as a polypeptide to a form competent to initiate lipoprotein assembly. Analysis of the lipid composition of particles formed by apoB mutants containing this domain suggests that simultaneous acquisition of both surface and core lipid components defines the mechanism by which apo B directs the early stage of VLDL and chylomicron assembly.
EXPERIMENTAL PROCEDURES

Construction of 6x His tagged apoB truncation mutants. ApoB truncation mutants containing C-terminal 6x His tags were generated by polymerase chain reaction as described previously (14). The amino acid coordinates of each truncation mutant are summarized in Fig. 1. All constructs were cloned into the expression vector, pCMV5 (15). The integrity of each construct was verified by DNA sequencing.

Transfection of COS-1 cells and analysis of apoB secretion. COS-1 cells were cultured as described (16), in 100 mm dishes and transiently transfected with the indicated apoB construct and either MTP large subunit (-MTP) or truncated human placental alkaline phosphatase (AP) (-MTP) at a mass ratio of 2:1 (6 µg total DNA) using the Fugene 6 transfection reagent (Roche Applied Science) (14). Twenty-four hours post-transfection, cells were metabolically radiolabeled with 100 µCi/ml 35[S]Met/Cys (EasyTag Express Protein Labeling Mix, Perkin Elmer Life Sciences) in Met/Cys-deficient DMEM (ICN) for 3 hours. To examine secretion efficiency of selected constructs, COS-1 cells in 150 mm dishes were transfected with 15 µg of apoB and MTP plasmids at the mass ratio described above. Twenty-four h after transfection, cells were trypsinized and plated in 35 mm dishes. Twenty-four h after plating, cells were metabolically radiolabeled for 10 min with 100 µCi/ml 35[S]Met/Cys and chased with
media containing an excess of cold amino acids for either 0 or 3 h (17). ApoB protein from media and cell lysates was immunoprecipitated with anti-apoB antibody (Roche Applied Science or Academy Biomedical, Houston TX) and fractionated by SDS-PAGE (16-18). Dried gels were exposed to Biomax MS film in combination with a Biomax TranScreen-LE intensifying screen (Kodak) at -70°C. Radioactive band intensities were quantified using a Molecular Dynamics 445 SI PhosphorImager.

*Flotation and density gradient analysis of lipoproteins.* COS-1 cells were transfected with the specified apoB truncation mutants and radiolabeled with [35S] Met/Cys for 4 h. After addition of protease inhibitors, media was adjusted to a density of 1.25 g/ml KBr in a final volume of 3 ml and transferred to TLA100.3 polyallomer Quick-Seal tubes (Beckman). The samples were centrifuged at 100,000 rpm in a TL-100 centrifuge for 18 hours at 15°C. The top 1 ml (d<1.25 g/ml) and bottom 2 ml (d>1.25 g/ml) fractions were recovered by tube slicing. ApoB was immunoprecipitated and analyzed by SDS-PAGE and fluorography. Density values for the truncated apoB mutants were obtained by transfecting two, 150 mm dishes of COS-1 cells with the indicated apoB construct and MTP, as described above. Media were pooled, concentrated, and the d<1.25 g/ml lipoprotein fraction was obtained. The density of lipoproteins formed by the truncated apoB proteins was determined by isopycnic gradient centrifugation (18).
Metabolic radiolabeling of apoB and associated lipids and purification of lipoprotein particles. COS-1 cells in 150 mm dishes were transfected, as described above, with MTP or AP. Twenty-four hours post-transfection, cells were washed twice with PBS, and then incubated for 20 minutes at 37°C in Met/Cys deficient DMEM. Labeling was performed in media composed of four parts serum free Met/Cys deficient DMEM, one part serum free complete DMEM, 0.5% fatty acid free BSA (Sigma), 10 µCi/ml [3H] oleate, and 20 µCi/ml [35S] Met/Cys. The media from two dishes was combined and protease inhibitors added (0.5 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin). After centrifugation at 600 rpm for 5 min, supernatant was removed and 0.75 ml bed volume of nickel-nitrilotriacetic (NTA)-agarose resin (Qiagen) was added followed by incubation with slow inversion for 1.5 h at 4°C. The slurry was applied to a 15 ml disposable polypropylene column (BioRad Laboratories). The column was washed 4 times with 10 ml of column buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0) containing 5 mM imidazole, followed by 4, 10 ml washes with column buffer lacking imidazole. The apoB was eluted from the column with 0.75 ml of column buffer containing 250 mM imidazole. The elution step was repeated three times and the eluates were pooled and buffer exchanged into PBS by centrifugal dialysis (Centricon 30, Millipore). Affinity purified material was adjusted to 1.25 g/ml of KBr and centrifuged in a TL100.3 rotor, as described above, to obtain a d<1.25 g/ml lipoprotein fraction. Lipoprotein fractions were
exchanged into PBS by centrifugal dialysis.

*Lipid Composition of Affinity Purified Lipoproteins.* Lipids were extracted from affinity-purified lipoproteins by the method of Bligh and Dyer (19). Samples were adjusted to 0.5 ml with PBS and 1.88 ml chloroform:methanol (1:2), 10 µl of a mixture of 5 mg/ml egg yolk lecithin, triolein, dioleoyl glycerol and cholesteryl oleate (in chloroform) and 0.626 ml chloroform was added, followed by vortexing. Samples were acidified with 0.626 ml 0.05% sulfuric acid and vortexed again. After centrifugation at 1500 rpm for 10 minutes at room temperature, the upper phases were removed by aspiration. The lower phases were dried at 45°C under a stream of nitrogen, redissolved in 50 µl chloroform and applied to a polyester-backed silica gel TLC plate (PE SIL G, Whatman). Plates were first developed with chloroform:methanol:acetic acid:water (65:45:12:6) by running the solvent front ~1/3 up the length of the plate, then air-dried and finally developed with hexane:ether:acetic acid (80:20:1). Lipid standards were visualized by incubation in iodine vapor and areas containing phosphatidylcholine, diacylglycerol, triacylglycerol and cholesteryl ester were cut and quantified by liquid scintillation counting. Control experiments, in which the entire length of the TLC plate was cut and counted, revealed that these were the only radiolabeled lipid species present above background. Radiolabeled lipids composition values were converted to particle composition, using the constants and computational techniques described Carraway et al. (20)
RESULTS

*Identification of the initiating domain of apoB.* A series of C-terminal 6x His tagged truncation mutants were generated (Fig. 1) and expressed in COS cells with and without cotransfection of the 97 kDa MTP subunit. With the exception of apoB34, all tested apoB truncation mutants were secreted into media similarly with and without MTP coexpression (Fig. 2). However, density gradient analyses revealed that, in the absence of MTP coexpression, none of the apoB constructs were lipidated, as they were either absent from media (apoB34) or recovered exclusively in the d>1.25 g/ml lipid poor bottom (B) fraction (Fig. 3). Cotransfection with MTP converted apoB34 to a secreted form that floated in the d<1.25 g/ml top (T) fraction (lane 31). About 30-50% of apoB20.1, 20.5, 21, 23, and 25 were recovered from media as buoyant lipoproteins when coexpressed with MTP (e.g., for apoB23 compare lanes 21 and 22 to 23 and 24). Based on these data, it appears that the MTP responsiveness of apoB secretion is lost upon truncation to apoB25 (Fig. 2). However, when lipoprotein formation was considered independently of apoB secretion (Fig. 3), a robust response to MTP was observed for forms of apoB as small as apoB20.1. ApoB19.5 also underwent MTP-dependent conversion to a lipoprotein form, although with reduced efficiency. Further truncation of apoB19.5 by 22 amino acids to form apoB19 completely abolished lipoprotein particle assembly (Fig. 3, lane 3).
The secretion efficiency of selected assembly-competent apoB truncation mutants was examined. Metabolic radiolabeling analysis in transfected COS cells revealed that ~20-25% of newly synthesized apoB20.1, -20.5, and -25 (10 min pulse) was secreted during a 3 h chase (Fig. 4). Although the secretion efficiencies of the exact sized constructs used in the present study have not been examined elsewhere, Yao et al. (12) showed that ~30% of apoB18 and ~25% of apoB23 produced during a 2 h radiolabeling period were secreted into media during a 3 h chase in stably transfected McA-RH7777 cells. Hence, the secretion efficiency in COS cells of constructs terminating near apoB’s initiating boundary are similar to those observed previously in a hepatoma-derived cell line.

Lipoproteins formed by truncated forms of apoB display discrete density profiles. The lipoproteins generated by the apoB truncation mutants were analyzed by isopycnic gradient centrifugation (18). As shown in Fig. 5, each form of apoB gave rise to a relatively discreet particle population that was recovered in 2-3 density fractions. As expected, the peak densities were inversely proportional to the length of the translation product. Further, the density of apoB25 and 34 was similar to that observed in transfected hepatoma cells (12,21), confirming that under the conditions used, neither MTP nor other factors are limiting in cotransfected COS cells. Even as the truncations approached the region where lipoprotein assembly is abolished, a homogenous set of
particles was produced, suggesting that each construct underwent specific biosynthetic processing to yield a captured static intermediate in the lipoprotein assembly pathway. Although we observed a small amount of apoB19.5 that floated at d<1.25 g/ml when coexpressed with MTP (Fig. 2), we were unable to recover a sufficient amount to perform density gradient analysis. However, based on the trend observed for the other constructs, we assume that its density is at or just below 1.25 g/ml.

Analysis of lipids associated with truncated forms of apoB. The TLC profiles of [3H]oleate-radiolabeled lipids associated with apoB34, 28.8, 25, 20.5, 20.1 and 19.5 are shown in Fig. 6. Of particular note is that use of COS cells with and without MTP cotransfection allows each construct to serve as its own control. In the absence of MTP coexpression, apoB constructs were associated with only trace amounts of lipid (open circles); however, cotransfection with MTP gave rise to reproducible compositional profiles that reflect the relative amounts and types of lipids associated with each protein. Initial inspection of the lipid profiles for each construct indicates that TG is the predominant lipid, and that even apoB19.5 fails to form a phospholipid-rich lipoprotein. For apoB20.1, -20.5, -25, -28.8, and -34, peak TG dpm ranged from ~50,000 to ~190,000, whereas PC dpm ranged only from ~30,000 to 70,000. Although the apoB19.5 values were much lower (PC peak of ~800 dpm and TG peak of ~1,200 dpm),
the values were clearly above the background control and the duplicate determinations were virtually identical. As the total purified apoB-containing lipoprotein fraction was analyzed for lipid content, the absolute value of lipid recovered from the TLC plates reflects the total number of lipid molecules recruited by each form of apoB, the relative abundance of lipids within the particle, and the different levels of apoB expression. As only the relative composition of lipids associated with each particle is relevant for the current analyses (20), the lipid values were not adjusted for apoB content.

Several criteria suggest that the lipids associated with each form of apoB reflect the composition of their respective lipoprotein particles as they emerge from the cell. First, none of the truncated apoB constructs expressed without MTP were capable of associating with sufficient lipid to form a buoyant particle (Fig. 3). Furthermore, when apoB20.5-containing lipoprotein particles generated from unlabeled cells were added to conditioned media containing \[^{3}\text{H}]\text{oleate}-\text{radiolabeled apoB20.5, no radioactive lipid exchange was observed (data not shown). Finally, apoB20.5 particles added to COS cells undergoing radiolabeling with \[^{3}\text{H}]\text{oleate for 24 h did not acquire more than background radiolabeled lipid (data not shown. Hence, we conclude that the lipids associated with each form of apoB analyzed in Fig. 6 are a reflection of intracellular processing events.}
Utilizing the density of the secreted lipoproteins and their relative lipid mass ratios, the number of lipid molecules per particle was calculated using the computational methods described by Carraway et al. (20). For all particles analyzed, PC and TG were the predominant lipid species (Table 1). Furthermore, in all of the particles, the number of surface lipids never exceeded core lipids, even for apoB19.5. Particle size and total volume increased proportionally with apoB length (Table 2); however, the fractional volume occupied by the hydrophobic core lipids rose sharply between apoB19.5 and apoB20.5 (Fig. 7)
DISCUSSION

The mechanism by which apoB initiates lipoprotein particle assembly is unknown. To address this question, we mapped the minimum sized apoB translation product able to assemble buoyant lipoproteins. We found that expression of the amino-terminal 884 amino acids of apoB (apoB19.5) resulted in MTP-dependent lipoprotein formation. Removal of 22 amino acids from this construct completely abolished assembly, whereas translation of an additional 28 amino acids to form apoB20.1 (amino acids 1–912) resulted in an assembly efficiency of ~30-50%, a value approaching that observed for longer constructs, including apoB20.5, 21, 23 and 25 (Fig. 3). Hence, a narrow interval in the apoB polypeptide chain completes a structural and functional unit competent to achieve initial lipid acquisition during the cotranslational phase (first-step) of apoB-containing lipoprotein assembly (2,22).

The present data demonstrate that MTP is required for the lipidation of all forms of C-terminally truncated apoB equal to or longer than apoB19.5. However, between apoB19.5 and apoB25, MTP-dependent assembly had little impact on apoB secretion (Figs. 2 and 3). The basis for the lipoprotein assembly-independent secretion of these truncated forms of apoB may lie in the nature of the amino-terminal βα₁ domain (Fig. 1) (9). This domain is enriched in intramolecular disulfide bonds (23), can fold...
independently of lipoprotein assembly (18,24), and appears to display a loose
association with the surface of mature lipoprotein particles (25). As a result, constructs
containing primarily the $\beta\alpha_1$ domain may achieve secretion-competence whether or not
they undergo assembly with lipid. In contrast, the $\beta_1$ domain of apoB (Fig.1) (9), by
virtue of its extended amphipathic $\beta$-sheet structures, is specialized for the expansion of
nascent apoB-containing lipoproteins and is believed to irreversibly associate with lipids
(9,26,27). As the length of this domain increases beyond a critical threshold (i.e.
translation beyond $\sim$apoB25), the secretion of apoB becomes increasingly dependent
upon its participation in the lipoprotein assembly process. Hence, in the absence of
MTP, forms of apoB containing a sufficient length of the $\beta_1$ domain may undergo
aggregation and/or nonnative interactions with intracellular membranes, ultimately
resulting in intracellular retention and turnover (28,29).

Lipid analysis of the apoB19.5-containing lipoproteins was consistent with small HDL3-
like particles containing approximately 25 molecules each of surface and core lipid (Fig.
6; Table 1). This suggests that apoB-containing lipoproteins are initially formed as
small, dense emulsion particles, with a surface:core lipid ratio of $\leq$1, rather than as a
phospholipid-rich LV-like intermediate, which would be expected to have a
surface:core lipid ratio of $\sim$2-3 (11). The finding that even the smallest assembly-
competent apoB forms small dense emulsion particles is partially consistent with a prevailing theory of lipoprotein assembly, in which apoB inserts itself into a saturated membrane surface and desorbs lipid as a preformed core-containing lipoprotein (6,7). However, as generally advanced, this model of assembly predicts that particle desorption from the membrane occurs upon completion of translation (6,30). For native forms of apoB, such as apoB100, the implication is that the ~3000 lipid molecules required to form a first step LDL-sized precursor particle are membrane-derived.

Our present data, as well as previous studies on the interfacial properties of apoB’s amino-terminal αβ₁ domain, support a variation of the membrane intercalation model, which posits that the amino terminal ~20% of apoB recruits sufficient membrane lipid to form small primordial particles that thereupon desorb from the ER inner leaflet. Indeed, one of the hallmarks of the amino terminal βα₁ domain of apoB (e.g., apoB17-20) is its capacity to interact spontaneously with lipid surfaces, including phospholipid vesicles (31,32), emulsion particles (33), oil droplets (34), and the inner leaflet of ER-derived microsomal vesicles (35). Thus, the observation that the extension of apoB19.5 to apo B20.5 is associated with a simultaneous, discontinuous rise in mean apoB residue area and fractional particle lipid volume (Fig. 7), argues that with translation of only 931 residues, apoB can assume a conformation that can mediate membrane binding, robust
lipid desorption, and assembly of a hydrophobic core-containing lipoprotein.

Presumably, during synthesis of native apoB48 and apoB100, after the amino terminal ~19.5-20.1% of apoB is translated, the precursor emulsion particle remains tethered to the ER membrane via the nascent polypeptide chain, or perhaps by an interaction between apoB and membrane lipids, or with other ER proteins.

A novel alternative model of lipoprotein assembly proposed by Segrest, Dashti and colleagues is based on sequence conservation between apoB and vtg (36-38). In this model, initiation of particle assembly occurs when apoBs αβ₁ domain folds into a three-sided LV-like lipid binding cavity, or alternatively, forms two sides of a binding cavity that is completed upon heterodimerization with MTP (8,9,39). The hydrophobic interior of the cavity would stabilize the acyl chains of a discoidal PL-rich complex similar to the known composition of LV, which contains about 37 molecules of PL and 13 molecules of TG per LV monomer (11). Although it is possible that the methodologies used in the present study cannot trap such an LV-like intermediate, it is clear that the lipid composition of apoB19.5 and apoB20.1-containing lipoproteins is more consistent with emulsion particles (surface:core lipid ratio <1), than an LV-like particle, (PL:TG ratio of ~2-3). In this regard, it is important to note that while the structure of vtg may provide clues to the mechanism of lipid acquisition by apoB, the mechanism by which vtg itself
acquires lipid is unknown. One possibility is that vtg forms a hydrophobic binding cavity that is subsequently loaded with lipid; however, it is equally possible that vtg acquires lipid during translation by desorption from a membrane surface, as proposed for apoB. Hence, the lipid binding pocket and membrane desorption models of lipoprotein assembly may not be mutually exclusive.

Although many previous studies have examined the relationship between the length of apoB translated and the size of the particle generated (6,12,21,30), few have determined the lipid composition of the precursor particles formed. In one study, Carraway et al. (20) examined the composition of particles produced by apoB29, 32.5, 37 and 41 in stably transfected C127 cells (a murine mammary tumor-derived cell line), and found that apoB29 and 32.5 were secreted with densities of 1.25 and 1.22 g/ml, and surface:core lipid ratios of 8 and 2.5, respectively. These values are clearly at odds with our observation that particles generated by truncation mutants as small as apoB19.5 and apoB20.1 contain sufficient TG to form neutral lipid cores. In part, this discrepancy may be due to the limiting expression of MTP in C127 cells, which may give rise to underlipidated particles, for although C127 cells were presumed to be devoid of MTP expression (27), we recently demonstrated that they express a low level of endogenous MTP (14). Indeed, in the COS cell system, the density of the particles generated were consistent with values reported previously for similar constructs expressed in rat (McA-
RH7777) and human (HepG2) hepatoma cells (12,21,30).

The location of the functional boundary identified in the present work is in partial agreement with the predictions of Segrest et al. (8,9) who proposed that discontinuous amino acid domains situated between amino acids 1-1000 are necessary to form a lipid pocket necessary for nascent lipoprotein formation. In their recent study, apoB22 (amino acids 1-1000) was secreted from stably transfected McA-RH7777 cells as a buoyant particle, whereas apoB20.5 was secreted in lipid poor form (39). However, our data showing that forms of apoB as small as apoB19.5 (amino acids 1-884), and certainly apoB20.1, respond to MTP by formation of a buoyant lipoprotein, suggests that the initiating domain of apoB is shorter than previously predicted. Our ability to discern a discreet initiating domain may be due to the finer truncation mapping performed across the initiating domain and the use of the COS cell system, which provides the capacity to control the key elements of the assembly mechanism (i.e., apoB and MTP).

In conclusion, we have found that the amino terminal 19.5-20.1% of apoB constitutes a discreet functional unit, which is capable of responding to MTP to direct the assembly of homogenously sized apoB-containing lipoproteins. The lipid composition of these nascent lipoproteins is consistent with small, dense emulsion-like particles, which suggests that the initiation of particle assembly involves simultaneous desorption of both
surface and core lipids from the ER membrane. The identification of a relatively small and discreet structural unit, which is capable of performing all of the steps required to initiate particle assembly, has important implications for the design of structural and functional studies to fully elucidate the nature of the lipoprotein assembly mechanism. Head to head comparisons of apoB truncation mutants terminating on either side of the initiating boundary, including examination of the conformational and interfacial properties that accompany the completion of the initiating domain, may reveal the mechanism by which apoB functions to achieve nascent lipoprotein assembly.
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FIGURE LEGENDS

Fig. 1. **Relationship of truncation mutants to the domain structure of apoB.** The diagram depicts the first two domains of the apoB100 pentapartite domain structure as defined by Segrest et al. (8,9,26). The globular $\beta\alpha_1$ domain displays similarity to the amino-terminal domain of LV. Shown below the domain structure are the apoB truncation mutants used in this study. Each is defined on the left by the apoB centile system (percent of amino terminus of mature apoB) and on the right by the C-terminal amino acid residue. Each construct terminates with a C-terminal 6x His tag.

Fig. 2. **MTP-independent secretion of apoB truncation mutants.** COS cells were transfected with the indicated apoB truncation mutant without (-) or with (+) MTP coexpression. After metabolic radiolabeling with $[^{35}\text{S}]\text{Met/Cys}$ for 3 h, the media was collected and the apoB immunoprecipitated with anti-apoB antibodies. The samples were subjected to SDS-PAGE and visualized by fluorography.

Fig. 3. **Identification of the minimum translation product of apoB required for MTP dependent lipoprotein assembly.** The indicated apoB truncation mutants were transiently transfected into COS cells without (-) and with (+) MTP coexpression. The cells were radiolabeled with $[^{35}\text{S}]\text{Met/Cys}$ for 3 h and the media was adjusted to a
density of 1.25 g/ml KBr. After equilibrium centrifugation the top (T) 1 ml (d<1.25 g/ml), and bottom (B) 2 ml fractions (d>1.25 g/ml) were collected by tube slicing. The samples were immunoprecipitated with anti-apoB antibodies, fractionated by SDS-PAGE, and visualized by fluorography.

Fig. 4. **Secretion efficiency of apoB truncation mutants.** COS cells cotransfected with MTP and the indicated truncation mutants were subjected to metabolic pulse-chase analysis as described under “Experimental Procedures”. Each bar represents the mean percentage of newly synthesized radiolabeled protein (10 min pulse) secreted into media during a 3 h chase ± S.D. (n=3).

Fig. 5. **Truncation mutants form lipoprotein particles with discrete densities.**

The indicated apoB truncation mutants and MTP were cotransfected into COS cells and radiolabeled for 3 h with [35S]Met/Cys. The apoB was isolated by Ni-NTA chromatography and the lipoproteins were floated at d<1.25 g/ml gradient as described in Fig. 2. The top 1 ml was isolated from each tube and subjected to isopycnic gradient centrifugation as described (18). Twelve, 1 ml fractions were collected from the top of each tube and their densities were determined gravimetrically. Samples were then concentrated, subjected to SDS-PAGE and visualized by fluorography.
Fig. 6. **Compositional profiles of apoB truncation mutants.** COS-1 cells were transfected with the designated apoB constructs without MTP (*open circles*) or with MTP (*black squares*), and then radiolabeled with [\(^{35}\)S]Met/Cys and [\(^{3}\)H]oleate for 24 h. ApoB was affinity purified by Ni-NTA binding, and the lipoprotein particles separated by centrifugation at d=1.25 g/ml as described in Fig. 2. The top 1 ml was isolated by tube slicing, concentrated and the lipids extracted by the method of Bligh and Dyer (19). The samples were subjected to TLC to separate the lipid species, and the radioactivity in corresponding regions of the TLC plate was quantified by liquid scintillation counting. PC, DG, TG, and CE are represented in fractions 2, 5, 7, and 8, respectively. Data points represent the average of duplicate experiments. Error bars, where visible, depict the positive data range. The radioactivity recovered in the four lipid classes associated with each purified apoB-containing lipoprotein was used to calculate the percent radiolabeled lipid composition (Table 1).

Fig. 7. **Effect of apoB construct length on surface conformation and fractional core lipid volume.** ApoB-containing lipoproteins were isolated from the media of COS cells transfected with truncated apoB constructs (apoB19.5-apoB34), and their density and lipid composition were determined and used to calculate particle radius and core lipid volume (20). Particle surface area/apoB amino acid residue (I) and core lipid (TG and
CE) volume/total particle volume ($\omega$) are plotted as a function of apoB length.
Table 1: Radioactive Lipid Composition of Lipoproteins Secreted Lipoproteins

|       | B19.5 | B20.1 | B20.5 | B25  | B28.8 | B34  |
|-------|-------|-------|-------|------|-------|------|
| **Percent Radiolabeled Lipids** |       |       |       |      |       |      |
| PL    | 37.0  | 32.0  | 34.7  | 24.8 | 29.7  | 18.8 |
| DG    | 7.4   | 4.7   | 4.4   | 5.8  | 5.3   | 5.7  |
| TG    | 52.3  | 62.0  | 57.3  | 67.4 | 62.8  | 66.9 |
| CE    | 3.3   | 1.4   | 3.6   | 1.9  | 2.2   | 8.7  |
| **Lipid Molecules Per Particle** |       |       |       |      |       |      |
| PL    | 20    | 19    | 29    | 30   | 44    | 36   |
| DG    | 5     | 4     | 5     | 9    | 10    | 14   |
| TG    | 26    | 35    | 45    | 74   | 85    | 118  |
| CE    | 2     | 1     | 4     | 3    | 4     | 20   |
| **Surface and Core Lipid Stoichiometry** |       |       |       |      |       |      |
| Surface<sup>a</sup> (S) | 25    | 23    | 34    | 39   | 54    | 50   |
| Core<sup>b</sup> (C)   | 28    | 36    | 49    | 77   | 89    | 138  |
| S:C Ratio | 0.89 | 0.64  | 0.69  | 0.51 | 0.61  | 0.36 |

<sup>a</sup> PL+DG

<sup>b</sup> TG+CE
Table 2: Physical Properties of Secreted Lipoproteins

|                | B19.5 | B20.1 | B20.5 | B25   | B28.8 | B34   |
|----------------|-------|-------|-------|-------|-------|-------|
| No. AA<sup>a</sup> | 884   | 912   | 931   | 1134  | 1306  | 1544  |
| Protein MW<sup>b</sup> | 99,537.6 | 102,474.8 | 104,562.1 | 127,181.9 | 147,024.9 | 173,591.0 |
| Particle Density (g/ml) | 1.24 | 1.23 | 1.20 | 1.18 | 1.18 | 1.16 |
| Particle Volume<sup>c</sup> (Å³) | 195,541 | 209,085 | 244,530 | 322,732 | 384,982 | 481,536 |
| Particle Diameter<sup>c</sup> (nm) | 7.2 | 7.4 | 7.8 | 8.5 | 9.0 | 9.7 |
| Lipid Core Radius<sup>c</sup> (Å) | 16.0 | 16.8 | 18.8 | 22.6 | 25.1 | 28.6 |

<sup>a</sup>Number of residues includes 6x His tag

<sup>b</sup>Calculated molecular weight based on amino acid composition

<sup>c</sup>Calculated according to Carraway et. al (20)
Fig. 1

\[ \beta_{\alpha_1} \text{ (LV)} \quad \beta_1 \]

N \hspace{1cm} C

| 19 | 19.5 | 20.1 | 20.5 | 21 | 23 | 25 | 28.8 | 34 |
|----|------|------|------|----|----|----|-------|----|
| 862| 884  | 912  | 931  | 953| 1043| 1134| 1306  | 1544|
Fig. 2
Fig. 3

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T B T B T B T B T B T B T B T B T B T B T B T B

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ApoB19

T B T B

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ApoB19.5

T B T B

---

ApoB20.1

T B T B

---

ApoB20.5

T B T B

---

ApoB21

T B T B

---

ApoB23

T B T B

---

ApoB25

T B T B

---

ApoB34

T B T B

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Fig. 4

![Graph showing secretion efficiency for different truncation mutants (B20.1, B20.5, B25).](image-url)
Fig. 5

ApoB34

ApoB25

ApoB20.5

ApoB20.1
Fig. 7

![Graph showing surface area/Residue and lipid volume/Particle vol vs % N-Terminus of ApoB](graph.png)
