We studied the structural requirements of apolipoprotein (apo) B for assembly of very low density lipoproteins (VLDL) using rat hepatoma McA-RH7777 cells expressing human apoB (h-apoB). Recombinant h-apoB48, like endogenous rat apoB48 (r-apoB48), was secreted as VLDL in addition to high density lipoproteins (HDL) by transfected cells, indicating that the N-terminal 48% of apoB contains sequences sufficient for VLDL assembly. Truncation of the C terminus of h-apoB48 to -B42 or -B37 had little effect on the ability of apoB to assemble VLDL, whereas truncation to -B34 or -B29 markedly diminished or abolished VLDL formation. None of the truncations affected the integration of apoB into HDL. To determine whether the ability to assemble VLDL is governed by apoB length or by sequences beyond apoB29, we created chimeric proteins that contained human apoA-I and a segment derived from between the C-terminal 29 and 34%, 34 and 37% or 37 and 42% of apoB100. The resulting chimeras, namely AI/B29–34, AI/B34–37, and AI/B37–42, were secreted by the transfected cells as lipoproteins with buoyant density (d < 1.006 g/ml), electrophoretic mobility (pre–apoB), and size characteristics of human plasma VLDL. The chimeras could assemble discrete VLDL particles devoid of endogenous r-apoB100, and could actively recruit triglycerides and phospholipids into the lipoproteins. However, these chimeras were secreted inefficiently. Pulse-chase analysis showed that less than 5% of the newly synthesized AI/B proteins were secreted, and more than 70% was degraded intracellularly. Degradation of the chimeras could be blocked by the cysteine protease inhibitor N-acetyl-leucyl-leucyl-norleucinal; h-apoA-I, human apoA-I; r-apoB, rat apoB; DMEM, Dulbecco’s modified Eagle’s medium; OS, oleate supplemented.

Human apolipoprotein (apo) B is a large and amphipathic protein which serves as the structural backbone for the assembly of triglyceride-rich lipoproteins. Two forms of apoB are found in human plasma: the full-length apoB100 consisting of 4536 amino acids, and apoB48 representing the N-terminal 2152 amino acids (~48%) of the protein. The enormous size and lipophilic properties of these two polypeptides have been suggested to underlie their ability to recruit triglycerides into hepatic very low density lipoproteins (VLDL) and intestinal chylomicrons (2). Early studies of C-terminally truncated apoB variants found in human familial hypobetalipoproteinemia (3) have provided in vivo evidence that the ability of apoB to recruit lipids is compromised by the truncation. Subsequently, the impact of apoB length on the extent of lipid recruitment during apoB-containing lipoprotein (LpB) formation has been demonstrated by several laboratories using transfected cell lines expressing the truncated forms of human apoB (h-apoB) (4–9). The in vivo observations and cell culture studies combined have unambiguously shown that the length of apoB polypeptide plays an important role in the formation of LpB. Despite this evidence, several other in vivo observations have indicated that the ability of apoB to form VLDL may not be solely determined by the length of the polypeptide. The most compelling evidence is that apoB48, a protein only half the size of apoB100, can mediate the assembly and secretion of both chylomicrons (in the intestine) and VLDL (in the liver of rats and mice). Moreover, some of the C-terminally truncated forms of apoB (e.g. apoB37) have been detected in plasma VLDL of subjects with hypobetalipoproteinemia (3). Therefore, the ability to assemble triglyceride-rich lipoproteins can not be simply a function of apoB length as suggested by the cell culture studies. Rather, amino acid sequences that reside within the N-terminal 48%, or even within 37%, of apoB100 may play...
important roles in the assembly of triglyceride-rich LpB.

Assembly of VLDL in the liver is a complex process perhaps occurring in multiple steps. Experimental evidence accumulated so far suggests that assembly of VLDL, at least the apoB48-containing VLDL in rat liver, may proceed through two steps. The initial step occurs as the apoB polypeptide translocates from the cytosolic to the luminal side of the endoplasmic reticulum (ER) membrane (8), a process presumably facilitated by the microsomal triglyceride transfer protein (MTP) (10) in the presence of sufficient lipid supply. Considerable progress has been made in understanding the apoB sequences involved in the translocation process, showing that the rate of apoB translocation across the ER membrane could regulate the level of apoB secretion. It has been observed that translocation of apoB may pause (11, 12) or even stop (13), producing several transmembrane intermediates of apoB. Indeed, immunoreactive apoB has been detected on the cytosolic side of hepatic microsomal membranes (14–17). It has been suggested by some (18), but disputed by others (19), that translocation pausing is essential for lipidation of the apoB polypeptides. Translocation arrested apoB polypeptides, resulting from insufficient lipid supply, probably do not participate in LpB assembly and are ultimately degraded. Little is known about the mechanism that is responsible for apoB degradation, nor is it clear which specific apoB sequences are involved in lipid recruitment during the initial step of VLDL assembly. Biochemical evidence for the second step of hepatic apoB48-VLDL assembly has recently been presented (20). It has been suggested that following an initial co-translational assembly step which generates an apoB48-containing species resembling high density lipoprotein (HDL), a so-called “second step” takes place in which a large amount of neutral lipid is recruited to convert this primordial apoB48-HDL to apoB48-VLDL. In rat hepatoma McA-RH7777 cells, the second step was dependent upon oleate supplementation of the culture medium (20). This two-step model is in accord with previous observations (21) and is supported by recent studies (22) using primary rat hepatocytes. The current study was intended to search for sequences within h-apoB48 that mediate the second step lipid recruitment during VLDL synthesis.

The in vivo data obtained from studies of human hypobetalipoproteinemia also suggest that C-terminal truncation of apoB100 may impair apoB’s ability to recruit lipid during VLDL assembly. While the relatively large truncated apoB forms (e.g., >apoB37) were invariably found in VLDL and low density lipoproteins (LDL) in addition to HDL, the short forms (e.g. apoB31 and -B32.5) were found only in LDL and the d > 1.21 g/ml fractions (3). These data imply that apoB31 and -B32.5 may not contain the sequences necessary for VLDL formation, whereas apoB37 does. A recently proposed pentapartite structural model of human apoB100 (h-apoB100) suggests that the protein is composed of three amphipathic α helix domains and two amphipathic β strand domains (23). One of the β strand domains is located within apoB48 (between apoB18 and -B43) which may represent the functional lipid-binding domain engaged in the second step lipidation to form VLDL. Alternatively, the in vivo data from hypobetalipoproteinemia may suggest that the requirement for the second step lipidation of apoB could be determined by the length of lipid-binding sequences, since hydrophobic structures that potentially mediate apoB binding to lipids are distributed quite evenly throughout the h-apoB100 molecule (2). In this study, we used five C-terminally truncated h-apoB forms and three chimeras containing h-apoB segments that were expressed in stably transfected McA-RH7777 cells to determine whether the sequence requirement for hepatic VLDL assembly is determined by apoB length or by domains within the protein. Our results suggest that the amphipathic β strands between the C termini of apoB29 and -B42 (B29–42) play important roles in VLDL assembly, and segments (as short as 152 amino acids) derived from B29–42 are sufficient to mediate VLDL formation.

**EXPERIMENTAL PROCEDURES**

Materials—DNA restriction and modification enzymes, and oligonucleotide linkers were purchased from New England Biolabs or from Life Technologies, Inc. Sequenase (Version 2.0) was obtained from U. S. Biochemical Corp. All reagents for cell culture were purchased from Life Technologies, Inc. and Sigma. Antisera for polyclonal anti-human apoB antibody were obtained from the Cincinnati Children’s Hospital Research Foundation, and anti-human apoB antibody was obtained from Boehringer Mannheim. Protein A-Sepharose CL4B and cytochrome c from horse heart were purchased from Pharmacia Biotech Inc. (Montreal, Canada). Monoclonal antibodies to human apoB (apoB31) and anti-human apoA-I (apoB37) were provided by Drs. M. Michalak and S. Galloway. Sheep polyclonal antibodies, which recognized human or rat apolipoproteins, and the heparin binding protein inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN) were purchased from Pharmacia Biotech Inc. (Montreal, Canada). Monoclonal antibody to apoB29–34 was obtained from Drs. Milne and Y. Marcel (Ottawa Heart Institute). Polyclonal (26) and monoclonal (27) antibodies to rat apoB (r-apoB) were gifts from R. Davis (San Diego State University) and L. Wong (Louisiana State University), respectively. Rabbit antibody to bovine protein disulfide isomerase was a gift from M. Michalak (University of Alberta).

Construction of Truncated ApoB Expression Plasmids—All expression plasmids were constructed using the vector pCMV5 (28) from which polylinker sequence between the HindIII and XbaI restriction sites had been removed. Truncated apoB cDNAs were prepared by excision of the EcoRI (for pAI/B34–37), or XbaI (for pAI/B37–42) restriction sites from the corresponding apoB cDNA fragments and religation with EcoRI (for pAI/B34–37), or XbaI (for pAI/B37–42). Compatible linkered at the 3′ end of the EcoRI/StuI fragment to release the B29–34 segment (nucleotides 5290–5848 of the apoB cDNA) was generated by excision of this linear fragment from pB35L-L. and ligation into pCMV5 that had been digested with HindIII and XbaI and recircularization of the fragment with T4 DNA ligase. To create the plasmid pB37 (nucleotides 20–5290 of the apoB cDNA) was created by removal of the SalI-Sall fragment from pB35L-L and religation into pCMV5. The plasmid pB29 (nucleotides 20–4124 of the apoB cDNA) was generated by Xhol digestion of pB37, end-filling with Klenow polymerase, ligation with SalI and XhoI, and release of EcoRI and SalI to release the B29 fragment of pCMV5 that had been digested with the SalI and XhoI enzymes. The non-apoB-containing end was excised by donning at the C-termini of the apoB proteins were KL in apoB42, KL in apoB37, FDAYR in apoB34, and DAYR in apoB29.

Construction of Expression Plasmids for ApoA-I/B Fusion Proteins—An EcoRI-Stul fragment encoding the N-terminal 217 amino acids of apoA-I was excised from the pC31 DNA (30). The blunt StuI end was ligated with a Clal linker, and the resulting 500-base pair fragment was ligated into pCMV5 that had been digested with EcoRI and Clal to create pAI/Stul. The expression plasmids, pAl/B29–34, pAI/B34–37 and pAl/B37–42 were assembled using the 800-base pair EcoRI/Stul fragment and corresponding apoB sequences. Appropriate linker oligonucleotides (NcoI linker for pAI/B29–34 and pAl/B37–42, for pAl/B34–37), or NcoI linker for the 3′ end of the EcoRI/Stul fragment to accept the apoB sequences. To obtain the B29–34 segment (nucleotides 4124–4838 of the apoB cDNA), pB34 was digested with XhoI digestion of pB37, end-filling with Klenow polymerase, ligation with SalI and XhoI, and release of EcoRI and SalI to release the B29 fragment and ligation of the fragment into pCMV5 that had been digested with the SalI and XhoI enzymes. The non-apoB-containing end was excised by donning at the C-termini of the apoB proteins were KL in apoB42, KL in apoB37, FDAYR in apoB34, and DAYR in apoB29.

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pCMV5 by a three-piece ligation reaction. Exontraneous amino acids, introduced at the junctions between the apoA-I and apoB sequences by the ligation procedures, were: PWL in pAI/B29–34, DAS in pAI/B34–37, and PW in pAI/B37–42. For in vitro transcription and translation studies, the AI/B chimera coding sequences were excised from the pCMV expression constructs by EcoRI and MluI digestion and inserted into the transcription vector SP6. Expression of AI/B chimeras was driven by the SP6 promoter. All expression plasmids, purified twice by CsCl gradient centrifugation, were analyzed by restriction enzyme digestion, and by DNA sequencing to verify the pAI/B junctions and the C-terminal amino acid codons, respectively.

Cell Culture—MCA-RH7777 cells (CRL-1601) were obtained from the American Type Culture Collection (Rockville, MD). Cell cultures were grown in DMEM containing 10% fetal bovine serum. Three cell lines were engineered to secrete apoA-I/B chimeras, and the choice of the culture medium depended on the presence of h-apoA-I, h-apoB, or h-apoB48, as described above.

In Vitro Transcription and Translation of AI/B Chimeras—The AI/B expression plasmids (prepared in pSPT19) were linearized by PvuII digestion and transcribed for 60 min using SP6 polymerase (RiboProbe Core System, Promega). The transcripts were purified and subsequently translated in rabbit reticulocyte lysate (Promega) containing [35S]methionine in the presence of rat liver microsomes (16). Aliquots of the translation mixture were treated with trypsin in the presence or absence of microsomes and digested with proteinase K. The radioactivity associated with the lipid-recruiting moiety was detected using thin layer chromatography as described previously (34). The radioactivity associated with the lipid-recruiting moiety was detected using thin layer chromatography as described previously (34).

RESULTS

C-t�mually Terminated ApoB Proteins Retain the Ability to Assemble VLDL—It was reported that endogenous r-apoB48 of MCA-RH7777 cells could assemble VLDL through two steps in an oleate-dependent manner (20). In this study we first determined the effect of sequential C-terminal truncation of human apoB48 on its ability to assemble VLDL. Four truncated proteins: h-apo-B29, -B34, -B37, and -B42, in addition to h-apoB48 (33) were expressed in MCA-RH7777 cells (Fig. 1A). The h-apoB of the appropriate molecular weight and immunoreactivity was detected in the medium of each stably transfected cell line by immunoblot analysis (Fig. 1B). Under standard cell culture conditions (i.e. DMEM containing 20% serum), all of the secreted h-apoB were associated with HDL (d = 1.08–1.18 g/ml) species (data not shown), consistent with previous results using the same expression system (4, 8). When the culture medium was supplemented with oleate (0.4 mM), a portion of the secreted h-apoB48 was recovered in VLDL (Fig. 1C, fractions 1 and 2) in addition to HDL (fractions 8–10). This result suggests that recombinant h-apoB48, like r-apoB48, can also assemble VLDL in an oleate-dependent manner in the transfected cells. The oleate-dependent VLDL formation was also observed for the C-terminally truncated h-apoBs such as h-apo-B42, -B37, and -B34, indicating that the ability to assemble VLDL was not severely affected by the truncation (although VLDL formation by h-apoB34 was compromised) (Fig. 1C). In contrast, formation of VLDL was entirely abolished by truncation to h-apoB29. None of the truncations affected the secretion of apoB48 as HDL (Fig. 1C). Sequential flocculation of the secreted lipoproteins containing h-apoB48 showed that h-apo-B34 fractionated in HDL, and B37 and -B34 indeed formed VLDL (d < 1.006 g/ml) (data not shown). These data are reminiscent of observations in human hypobetalipoproteinemia indicating that apoB37 forms VLDL but apo-B31 and -B32.5 do not (3). Thus, these results provide in vivo evidence that the lipid-recruiting sequences for VLDL formation most likely reside beyond the N-terminal 30% of apoB100.

A/I- B Chimeras Synthesized by MCA-RH7777 Cells Can Assemble VLDL—The failure of apoB species containing less than 30% of the N-terminal sequences (i.e. ≤ apoB30) to form VLDL might also be explained by the lack of sufficient polypeptide length. To determine whether the ability of apoB to recruit lipid was determined by the protein length or by unique properties of the apoB sequences, we designed chimeras that contained segments of apoB derived from between the C termini of apo-B29 and -B42, a region enriched with amphipathic α helices (23) and is devoid of cysteine residues and N-glycosylation sites. The number of amino acids

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in the h-apoB segments varied from 237 in B29–34 (containing two cysteines and three predicted N-glycosylation sites) (38), 152 in B34–37 (containing one cysteine), to 187 in B37–42 (Fig. 2A). The corresponding chimeras, namely AI/B29–34, AI/B34–37, and AI/B37–42, were stably expressed in McA-RH7777 cells. Fig. 2B shows that proteins with the predicted molecular mass and reactivity with antibodies to either h-apoA-I (left panel) or h-apoB (right panel) were expressed by the transfected cells. No degradation products of the chimeras were detected with the corresponding antibodies to h-apoA-I and h-apoB sequences of the chimeras.

**Fig. 1.** Expression of C-terminally truncated forms of h-apoB in McA-RH7777 cells. A, schematic diagram of the C-terminally truncated h-apoBs. The top line shows the centile scale of apoB100. The truncated apoBs are represented by solid bars with the number of amino acids (a.a.) labeled at right. B, immunoblots of h-apoBs. Culture media from individual cell lines (labeled above each lane) were concentrated, resolved by SDS-PAGE, and visualized by immunoblotting using antibody 1D1. The migration of h-apoB100 (550 kDa) and myosin (200 kDa) markers is indicated above the bars. C, density gradient profiles of secreted h-apoBs. The conditioned media (6 h in OS media) were fractionated into 12 fractions, and h-apoBs in each fraction were recovered by immunoprecipitation, separated by SDS-PAGE, and visualized by immunoblot analysis. Shown above the immunoblots are density (g/ml) of each fraction (mean value of eight density gradients) determined gravimetrically.

**Fig. 2.** Expression of A-I/B chimeras in McA-RH7777 cells. A, schematic diagram of the apoA-I/B chimeras. The cDNA encoding h-apoA-I (243 amino acids) was truncated at the StuI restriction site to generate AI/StuI (217 amino acids represented by eight open boxes) or h-apoB100 (291 amino acids represented by eight filled boxes). Filled bars, with the boundary amino acids indicated at both ends, represent h-apoB segments (B29–34, B34–37, and B37–42) that were ligated in-frame to the C terminus of AI/StuI. Positions of cysteines (C) and predicted N-glycosylation sites (N) are indicated above the bars. B, immunoblots of A-I/B chimeras. Samples obtained from medium or cell lysate were resolved by SDS-PAGE and visualized by immunoblotting with antibody 5F6 (left panel, anti-apoAI) or with antisera to h-apoB (right panel, anti-apoB). Monoclonal antibodies 2D8 (24) and 1C4 (R. Milne, unpublished results) were used to detect h-apoB epitopes in AI/B29–34 and AI/B37–42, respectively, and polyclonal antiserum to h-apoB (Boehringer Mannheim) was used to detect AI/B34–37. Molecular mass markers are indicated on the left. C, density gradient profiles of secreted A-I/B chimeras. The experiments were performed as described in Fig. 1, except that 54% (w/v) sucrose was used in the bottom layer to achieve the higher density limit (1.24 g/ml), and antibody 5F6 was used to detect A-I/B chimeras. D, secretion of 35S-labeled r-apoB100 and AI/B29–34 on lipoproteins. The AI/B29–34-producing cells were labeled with [35S]methionine/cysteine in standard medium (f) or OS medium (a). At the indicated labeling time, VLDL (d < 1.006 g/ml) and HDL (d > 1.006 g/ml) were isolated from the medium by ultracentrifugation. 35S-Labeled r-apoB100 (top panel) and AI/B29–34 (bottom panel) were purified from the lipoproteins by immunoprecipitation, resolved by SDS-PAGE, and quantified by liquid scintillation counting.
Density gradient ultracentrifugation of the medium lipoproteins (Fig. 2C) showed that each chimera was secreted as VLDL when cells were cultured in oleate-supplemented media (the top three panels). The ability to associate with VLDL might be correlated with the h-apoB segment length within the chimeras, since the longest chimera (AI/B29–34) was more predominant on VLDL than either AI/B34–37 or AI/B37–42. The control protein AI/Stul, however, was secreted exclusively as HDL (fractions 7–9 of the panel labeled as AI/Stul). Unlike the C-terminally truncated apoB (Fig. 1C), only small amounts of the AI/B chimeras were secreted as HDL (Fig. 2C). Metabolic labeling studies using AI/B29–34 as representative (Fig. 2D) showed that secretion of 35S-AI/B29–34 VLDL (left panels, d < 1.006 g/ml), like endogenous r-apoB100 VLDL, increased 5-fold with oleate supplementation. Secretion of the radiolabeled AI/B29–34 and r-apoB100 in dense lipoproteins (right panels, d > 1.006 g/ml) was less affected by oleate supplementation. These data were the first indication that a small segment of h-apoB in the chimera, either B29–34, B34–37, or B37–42, was sufficient to mediate lipid recruitment to form VLDL.

Characterization of A/I-B Chimera-containing VLDL—It has been shown that formation of apoB48 VLDL in McA-RH7777 cells differs from that of apoB100 VLDL by its oleate dependence (20). We examined the oleate dependence of AI/B chimera VLDL formation and found that it resembled that of apoB48 VLDL. Fig. 3A shows data obtained using AI/B29–34 producing cells as representative. In the absence of oleate, both AI/B29–34 (Fig. 3A, fraction 7–9, without oleate, AI/B29–34) and r-apoB48 (fractions 8–9, without oleate, B48) were secreted exclusively in fractions of d > 1.02 g/ml, whereas r-apoB100 was found primarily in fractions of d < 1.02 g/ml (fractions 1–3, without oleate, B100). Upon oleate supplementation, AI/B29–34, like endogenous r-apoB48, was secreted predominantly as VLDL in addition to small amounts as HDL (Fig. 3A, with oleate, second and third panels). Formation of VLDL in this oleate-dependent fashion was also observed with chimeras AI/B34–37 and AI/B37–42 produced by the respective cell lines (data not shown). Secretion of r-apoB100 as VLDL was stimulated by oleate (Fig. 3A, compare without oleate to with oleate). In experiments where the medium lipoproteins were fractionated by sequential flotation at d = 1.006 g/ml (Fig. 3B), we confirmed that the oleate-induced VLDL indeed had buoyant density less than 1.006 g/ml (compare lanes labeled as −OA and +OA in vivo of the left panels). Secretion of the chimera as VLDL (d < 1.006 g/ml) was not a result of nonspecific association with preexisting VLDL, since mixing of the conditioned medium postsecretionally with 0.4 mM oleate (+OA in vitro) did not result in formation of VLDL containing the chimeras. As was the case for density gradient ultracentrifugation in Fig. 3A, sequential flotation (Fig. 3B, right panels) also demonstrated that secretion of AI/B chimeras in the d > 1.006 g/ml fraction was not affected by oleate. In a separate experiment where triglyceride-rich VLDL secreted by McA-RH7777 cells were mixed with the conditioned medium from the chimera-producing cells, the chimeras also failed to transfer postsecretionally to the lipid-enriched VLDL (data not shown). As shown below, the AI/B chimera VLDL could be separated from r-apoB100 VLDL by immunoaffinity techniques. Thus, these results indicate that the characteristic nonexchangeability of apoB is retained in the AI/B chimeras, and they can, like
endogenous r-apoB100, independently assemble VLDL particles.

Additional physicochemical analysis provided further evidence that the Al/B chimera VLDL had the characteristics of VLDL. Gel filtration chromatography using Superose 6 columns (Fig. 3C) revealed that each chimera VLDL eluted in the void volume (V0), earlier than human LDL (hLDL). Agarose gel electrophoresis (Fig. 3D) showed that the chimeras (e.g. Al/B29–34 and Al/B37–42) formed VLDL with pre-β mobility, and there was no indication of aggregation of these lipoproteins. The same gel filtration characteristics (i.e. elution as VLDL) and electrophoretic mobility (i.e. pre-β) were also observed for VLDL containing the C-terminally truncated h-apoB such as apoB48 and -B34 (data not shown).

Overexpression of Al/B Chimeras Stimulates Lipid Secretion—The following experiments were performed to demonstrate that the Al/B chimeras were able to actively recruit lipids for VLDL synthesis. Using Al/B29–34 as a representative and Al/Stu as control, we metabolically labeled the respective cell lines with both [3H]glycerol and [35S]methionine/cysteine. The VLDL (d < 1.02 g/ml) were isolated from the medium by ultracentrifugation and the Al/B-containing VLDL was purified by immunoadsorption. In a preliminary experiment which is shown in Fig. 4A, we found that the purified VLDL contained only Al/B29–34 and lacked r-apoB100 (right lane, 5F6 bound). Unfortunately, intact Al/B containing VLDL particles could not be eluted from the immunoaffinity adsorbant for further analysis. However, using the immunoaffinity technique, we could purify the radiolabeled Al/B chimera VLDL and analyze their lipid and protein composition. The autoradiograms shown in Fig. 4B demonstrated that the separation of 35S-labeled r-apoB100 VLDL and Al/B29–34 VLDL secreted by Al/B29–34-producing cells was essentially complete, and the immunoaffinity recovery of the Al/B29–34 VLDL was 85% as determined by counting of the radiolabeled VLDL. The 35S-labeled Al/B29–34 specifically bound to the anti-h-apoAI affinity beads (left panel, 5F6 bound), whereas 35S-labeled r-apoB100 bound predominantly to the anti-apoB immunoadsorbant (right panel, αB-VLDL). Less than 5% of the recovered 35S-labeled r-apoB100 bound nonspecifically to the anti-apoAI affinity beads as determined by scanning densitometry. As shown in the immunoblots of Fig. 2C, Al/Stu-producing cells secreted none of the Al/Stu protein in the VLDL fraction. In control dual labeling experiments using the Al/Stu-producing cells, 35S-labeled r-apoB100 was recovered only in VLDL fractions treated with the anti-apoB immunoadsorbant (Fig. 4C, right panel, αB-VLDL). Notably, VLDL that contained apoE and/or apoA-I and essentially no r-apoB100 was secreted by Al/Stu-producing cells (Fig. 4C, 5F6 bound). The level of synthesis (data not shown) and secretion (Fig. 4, compare B and C) of endogenous r-apoB100 was similar between Al/Stu and Al/B29–34-producing cells. The McA-RH7777 cells produced very little endogenous r-apoB48 as compared with r-apoB100 (9, 34) (also see below). Endogenous rat apoE and rat apoB-1 were co-precipitated with VLDL (d < 1.02 g/ml) from Al/B29–34-producing cells with both immunoadsorbants (Fig. 4B). Association of these exchangeable apolipoproteins with VLDL may reflect their physical preference for large particles (39). Lipid composition of the affinity purified VLDL samples was also determined. Fig. 5A shows that the amount of [3H]triglyceride and [3H]phosphatidylcholine (left panels) secreted as Al/B VLDL (5F6 bound) by Al/B29–34-producing cells was 2-fold higher than by the control Al/Stu-producing cells. Notably, the amount of [3H]triglyceride and [3H]phosphatidylcholine associated with Al/B chimera VLDL (5F6 bound) was comparable to that associated with r-apoB100 VLDL (αB bound). The 5F6-bound VLDL species secreted by Al/Stu-producing cells that contained apoE and/or apoA-I but little apoB also contained triglyceride and phospholipid (Fig. 5A, 5F6 bound). The origin of these VLDL-like particles (Figs. 4C and Fig. 5A, 5F6 bound) is unclear and remains to be determined. The amount of [3H]triglyceride and [3H]phosphatidylcholine associated with r-apoB100 VLDL (αB bound) did not differ between the two cell lines. The increase in triglyceride concentration in the Al/B29–34 VLDL was also evident by high performance thin-layer chromatography (data not shown). The initial rate of incorporation of [3H]glycerol into triglyceride and phosphatidylcholine was similar in the two cell lines (Fig. 5B). These data demonstrate that the chimeras can actively recruit lipids (both neutral lipid and phospholipid) to form VLDL.

Secretion of Al/B Chimera-containing VLDL Is Inefficient and the Majority of Newly Synthesized Chimera Is Degraded by...
an ALLN-sensitive Protease—Although oleate supplementation stimulated chimera secretion as VLDL, the secretion efficiency of these proteins was low. Pulse-chase analysis after 4-h pre-treatment with oleate (using AI/B29–34 as representative), revealed that less than 2% of the newly synthesized chimeras were secreted as VLDL ($d_{1.02}$ g/ml) at the end of 3-h chase (Fig. 6, A and B). Under the same conditions, the secretion efficiency of endogenous r-apoB100 was over 40% (Fig. 6, A and B). However, since the number of $^{35}$S-labeled AI/B29–34 molecules synthesized was nearly 20-fold higher than that of r-apoB100, the number of label incorporated was about the same as at time 0 (Fig. 6B). The number of chimera molecules secreted was comparable to that of r-apoB100. This calculation assumes that the cysteine/methionine content of r-apoB100 is the same as h-apoB100. Both AI/B29–34 and endogenous r-apoB100 were secreted as VLDL (Fig. 6A, left panels) but not as HDL (right panels) by cells cultured with oleate-supplemented medium. The AI/B29–34 chimera, like endogenous r-apoB100, displayed rapid post-translational degradation within the cells (Fig. 6B). At the end of the chase, less than 20% of the initial labeled proteins were left in the cells. The McA-RH7777 cells synthesized (Fig. 6B) and secreted (Fig. 6A) little endogenous r-apoB48 as compared with r-apoB100.

The effects of lipid supplementation and protease inhibitor on the secretion of AI/B chimeras were further examined and compared with endogenous r-apoB100 (Table I). In the absence of lipid supplement, virtually no chimeras were secreted, nor did 20% serum alone stimulate their secretion. In contrast, low levels of secretion (~12% of total) were observed for endogenous r-apoB100 under serum-free conditions. Secretion of the chimeras was stimulated (2–4% of total) only when 0.4 mM oleate, together with serum, was added to the medium. Secretion of endogenous r-apoB100, however, was stimulated (~24% of total) even by serum alone. Oleate supplementation of the serum-containing media had no further stimulatory effect on the secretion of r-apoB100. The cysteine protease inhibitor ALLN blocked the intracellular degradation of endogenous r-apoB100 and the chimeras. However, ALLN did not enhance their secretion. Thus, even under conditions most favorable for VLDL synthesis and secretion, the secretion efficiency of the AI/B chimeras was significantly lower than that of endogenous r-apoB100. The protection of AI/B chimeras from intracellular degradation by ALLN suggests that sequences susceptible to the ALLN-sensitive proteolysis may colocalize with the lipid-binding regions of apoB.

The low secretion of AI/B chimeras might result from inefficient translocation of the proteins across the ER membrane. We tested this possibility by assessing the topology of these
proteins with respect to the microsomal membranes using protease protection assay. In the intact microsomes isolated from the transfected cells, a significant portion of AI/B29–34 (67%), AI/B34–37 (68%), and the control AI/Stu (60%) were protected from exogenous trypsin as determined by scanning densitometry of the immunoblots (Fig. 7A, compare lane 1 with lane 3 of the left panels). Prolonged exposure of the immunoblots (lane 4 of the left panels) revealed several smaller protected fragments derived from trypsin digested AI/B29–34 and AI/B34–37, but not the control protein AI/Stu, suggesting that only a small portion of the AI/B chimeras are found in a transmembrane topology. There was a striking difference in the trypsin accessibility of apoB100 and -B48. While only 10–26% of r-apoB100 was trypsin resistant (as determined in two independent experiments), apoB48 was almost totally resistant to trypsin; 91% of recombinant h-apoB48 expressed in transfected cells, and 100% of endogenous r-apoB48 were trypsin-resistant (Fig. 7A, compare lane 1 with lane 3 of the right panels). Protected fragments with Mr ≈ 250,000 and <200,000 (indicated by arrowheads) derived from trypsin digestion of r-apoB100 were also observed, indicating a significant portion of r-apoB100 may exist as transmembrane intermediates. As expected, the E.R. resident protein disulfide isomerase was inaccessible to exogenous trypsin and was fully protected (Fig. 7A). These data suggest that the translocation efficiency is markedly different between apoB100 and -B48. In separate experiments, AI/B chimeras were translated and translocated in vitro using microsomes isolated from rat liver, and the resulting samples were subjected to trypsin digestion to assess the topology of the AI/B chimeras (Fig. 7B). One predominant protein species was observed when chimera RNA was translated in the absence of microsomal membranes (lane 1). In the presence of microsomal membranes (lane 2) an additional processed AI/B29–34 polypeptide of larger apparent molecular weight was detected (downward arrowhead) reflecting N-glycosylation of the primary translation product, as predicted from the primary sequence of the apoB segment (see Fig. 2A). Addition of microsomal membranes to AI/B34–37, AI/Stu (lane 2, middle and lower panels) and AI/B37–42 (not shown) translation mixtures produced polypeptides of smaller apparent molecular weight than the primary translation product (upward arrowheads), reflecting signal peptide cleavage. In all instances the unprocessed species were totally degraded by exogenous trypsin (lane 3). In contrast, the majority of the processed species of AI/B...
chimeras were inaccessible to exogenous trypsin: 76.8 ± 0.8% of AI/B29–34, 87.7 ± 11.6% of AI/B34–37, and 91.8 ± 12.0% of AI/B37–42 (mean ± S.D., n = 3). These data together suggest that, unlike endogenous r-apoB100, AI/B chimeras are efficiently translocated across the ER membranes.

Finally, we determined the distribution of AI/B chimeras and endogenous r-apoB100 between lumen and membrane of the microsomes by sodium carbonate treatment. The microsomes were isolated from transfected cells that had been labeled with [35S]methionine/cysteine for 4 h. Only a minor portion (<15%) of the [35S]-labeled AI/B chimeras and endogenous r-apoB (B100 and B48) was recovered in the lumen irrespective of oleate supplementation. Under the same conditions, the majority (>80%) of the luminal marker PDI was released from the membrane fraction by the sodium carbonate treatment (data not shown). These results suggest that the majority of the chimera and apoB are associated with the microsomal membranes.

**DISCUSSION**

Little is known about how lipids are recruited by apoB during VLDL assembly. The current study has attempted to determine if the ability to assemble apoB48-VLDL is governed by length or by unique sequences of the protein, and provided evidence that VLDL assembly is probably mediated by hydrophobic, lipid-binding domains within apoB48. Results obtained from experiments with both the truncated h-apoB (ranging from apo-B29 to -B48) and the AI/B chimeras clearly demonstrate that the full-length of apoB48 is not a prerequisite for VLDL assembly. Rather, short h-apoB sequences (152–237 amino acids), most likely the amphipathic β strands present as multiple copies within B29–42, can mediate the recruitment of a large quantity of lipids in a manner similar to the proposed "two-step" assembly for apoB48-VLDL. Thus, the apoB sequences required for VLDL assembly are much shorter than was previously expected.

Structural Requirement of Human ApoB for Hepatic VLDL Assembly—The finding that AI/B chimeras could form VLDL was surprising and prompted us to analyze extensively the physicochemical characteristics of these VLDL particles. The experimental data obtained from density ultracentrifugation (Figs. 2C and 3A and 3B), gel permeation chromatography (Fig. 3C), and agarose gel electrophoresis (Fig. 3D), combined with the apparent absence of endogenous r-apoB by immunoaffinity purification (Fig. 4), supported the suggestion that the AI/B chimeras (containing 3–5% of apoB sequences) can form discrete, secretion competent VLDL. The VLDL containing AI/B chimeras are not formed by nonspecific exchange of the chimeras onto pre-existing VLDL (Fig. 3B). Rather, they are formed by active recruitment of triglyceride and phosphatidylcholine into the lipoproteins (Fig. 5A). To date, it has not been possible to ascertain the stoichiometry of AI/B chimeras per VLDL particle. Nor could the morphology of the AI/B chimera VLDL be examined because elution of intact particles from the immunoaffinity adsorbants was unsuccessful. However, even considering the possibility that more than one AI/B chimera could be present per VLDL particle, one might still wonder how a protein containing only 3–5% of the apoB sequence can mediate VLDL assembly? We have searched for structural features within the three apoB segments (i.e. B29–34, B34–37, and B37–42) that could potentially be responsible for the ability of AI/B chimeras to recruit lipids. Since N-glycosylation sites are present in some (e.g. B29–34) but not the other segments (e.g. B34–37 and B37–42) (Fig. 2A), we have eliminated N-glycosylation as a requirement for VLDL assembly. Likewise, since cysteines (two in B29–34, one in B34–37, and zero in B37–42), proline-rich clusters (2) (one in B29–34, and zero in B34–37 or B37–42), or the postulated “pause-transfer” motifs (11, 12) (four in B37–42, one in B29–34, and zero in B34–37) are not uniformly distributed among the three segments, we have concluded that none of these structural features is essential for the AI/B VLDL assembly. The only common feature among the three apoB segments is their hydrophobicity. The hydrophathy values, as determined by the Kyte and Doolittle algorithm (PCGene™, Intelligenetics, Inc., 15-residue window) revealed that each apoB segment generated a chimera which was more hydrophobic (grand average hydrophobicity = −6.59 for AI/B29–34, −6.20 for AI/B34–37 and −5.67 for AI/B37–42) than AI/Stu (grand average hydrophobicity = −8.06). Furthermore, according to the algorithm of Segrest et al. (23), each apoB segment was predicted to consist predominantly of amphipathic β strands. It is conceivable that these uniquely enriched β strands within B29–42 are the critical determinants of VLDL assembly. However, although the presumed role of the amphipathic β strands in VLDL has been demonstrated by the current study, the role of other structural elements, such as amphipathic α helices, in VLDL assembly has not been excluded. The present study, nevertheless, suggests that the structural determinants for VLDL could be relatively short (3–5% of apoB100) and present in multiple copies. The presence of multiple segments enriched in hydrophobic sequences within apoB48 would certainly enhance the efficiency of lipid recruitment during VLDL synthesis. It should be noted that according to Segrest et al. (23) amphipathic β strands also occur within apoB29 (between apo-B18 to -B29) but the protein does not assemble VLDL (Fig. 1C). Thus, the lipid recruiting function for VLDL assembly may not be an indiscriminant property of all the amphipathic sequences.

Topological Considerations for AI/B Chimera-containing VLDL Assembly—When the microsomal membrane topology of AI/B chimeras was compared with that of endogenous r-apoB100, similarities and differences between the two species were observed. In the microsomal fractionation studies, both r-apoB100 and AI/B chimera were similarly resistant to the sodium carbonate treatment and associated with the microsomal membranes (~85% of total). The avid membrane association of AI/B chimeras is most likely attributable to the presence of the hydrophobic apoB segments within these chimeras. Although such a high proportion of apoB on the microsomal membranes was previously reported using rat liver (40) and was unlikely an artifact of the sodium carbonate treatment, other researchers (22) have found less apoB associated with the membranes (as low as 20–40%). It is possible that the high proportion of membrane associated apoB observed in the present study was attributable to the addition of ALLN during microsome preparation, preventing degradation of a labile pool of membrane-bound apoB. Although the AI/B chimera and r-apoB100 showed similar membrane affinity, their topologies were different, as demonstrated by protease protection analysis. The former was predominantly resistant (~70% protection in microsomes isolated from transfected cells (Fig. 7) and 80–90% protection by in vitro translation assay using rat liver microsomes), whereas the latter was extremely sensitive (~20% protection) to proteolysis. These data could be interpreted as indicating that the AI/B chimeras are predominantly on the luminal side of the membrane while r-apoB100 is to a large extent in a transmembrane configuration. In this regard, the AI/B chimeras were similar to apoB48 which was also fully resistant to exogenous trypsin. At present, the reason for the striking differences in trypsin sensitivity between apoB100 and apoB48 is not clear. If the accessibility to trypsin at steady state indeed reflects apoB translocation efficiency, then the transmembrane topology of apoB100 is most likely determined
by sequences within the C-terminal 50% of the protein. The translocation efficiency of apoB48 and apoB100 needs to be further examined. Furthermore, although it is reasonable to speculate that the membrane associated AI/B chimeras (on the luminal leaflet of the ER membrane) may serve as precursors of the secreted VLDL, whether or not the formation of AI/B chimeras VLDL indeed occurs on the ER membrane also remains to be determined experimentally. Recently, it was reported that the ER membrane associated apoB100, and probably apoB48 as well, was the precursor for the formation of VLDL in McA-RH7777 cells (41). Results of the AI/B chimera studies suggest strongly that sequences beyond the N-terminal 30% of apoB100 probably play important roles in VLDL assembly through their unique interaction with the ER membrane. Further studies using the AI/B chimera approach may allow us to define the structural features of h-apoB involved in VLDL assembly.

AI/B Chimeras Are Less Able than Truncated ApoB37 to Form Secretion Competent HDL—There were two unique features associated with the secretion kinetics of the AI/B chimeras. First, secretion of the chimeras under standard conditions (i.e. DMEM containing 20% serum) was extremely poor, and the vast majority of them was degraded within the cells (Table I). This is in sharp contrast to the truncated h-apoB37 such as apoB37 which could be efficiently secreted as HDL without significant intracellular degradation (4). Second, under the oleate-supplemented conditions, the chimeras were secreted predominantly as VLDL (Fig. 2C), unlike the truncated h-apoB37 (e.g. apoB42 and apoB37) which could be secreted as both HDL and VLDL (Fig. 1C). Although currently there is no satisfactory explanation for the two features, several possible mechanisms have been considered. Since degradation of AI/B apoB48 could be specifically inhibited by ALLN, it is unlikely that their degradation is the result of nonspecific proteolysis. Rather, degradation of the chimeras is mediated by an ALLN-sensitive mechanism characteristic of apoB degradation. Likewise, since no proteolytic cleavage at the junction between apoA-I and apoB sequences was observed in the transfected cells, it is unlikely that degradation of the chimeras results from instability of the protein per se. The inability to recover substantial quantities of AI/B chimera HDL from the medium may suggest that the chimeras are less able than truncated apoB48 to form HDL or that HDL particles can be formed but are unstable. The reason for the impaired ability of chimeras to form stable HDL particles is not immediately clear. Although it is plausible that the impairment is attributable to the lack of sufficient polypeptide length of the chimeras, more likely it is because they do not contain the unique N-terminal apoB domain. Distinct from the rest of the molecule, the N-terminal portion of apoB (~15%) contains several intrachain disulfide linkages and probably assumes a globular conformation (2, 23). Recently, several groups (41–43) working with different systems have reported that the N-terminal cysteine-rich domain of apoB plays an important role in the initial cotranslational assembly of LPB. In the case of AI/B chimeras, the apoA-I portion of the proteins may not be sufficient to initiate lipida- tion to form a primordial HDL particle. Thus, the insufficient lipidation of AI/B chimeras on the ER membrane may hinder their susceptibility to intracellular degradation. However, in the presence of abundant lipid supply, these membrane associated AI/B chimeras are able to recruit lipid through the h-apoB portion of the proteins to form VLDL.

We have thus provided evidence that massive lipid recruitment during VLDL synthesis is governed by discrete hydrophobic sequences within apoB48. In mammals, apoB48 is synthesized from the same apoB gene that encodes apoB100 through the apoB mRNA editing (44). Although both apo-B100 and -B48

are essential for triglyceride transport, it has been shown that in the intestine (45) and rat liver (46) where apoB48 is synthesized, the turnover rate of apoB48 in response to the lipid is higher than that of apoB100, suggesting that the N-terminal portion of apoB is more efficient than the full-length protein in mobilizing lipid. The ability of apoB48 to assemble VLDL makes the C-terminal portion of apoB100 superfluous for triglyceride transport, this might be the rationale for the apoB mRNA editing mechanism to have evolved in the intestine to preclude futile translation. The physiological advantage for the evolution of apoB48 is also evident from the inverse relationship between apoB48 concentration and atherogenic lipoproteins (VLDL and low density lipoproteins) in the plasma (47). An improved understanding of the mechanisms responsible for apoB48 VLDL synthesis may help find means to lower plasma cholesterol and triglyceride levels.
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