The Amino Terminus of Apolipoprotein B Is Necessary but Not Sufficient for Microsomal Triglyceride Transfer Protein Responsiveness*

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Human apolipoprotein (apo) B mediates the formation of neutral lipid-containing lipoproteins in the liver and intestine. The association of apoB with lipid is thought to be promoted by the microsomal triglyceride transfer protein complex. We have reconstituted lipoprotein assembly in an insect cell line that normally does not support this process. Expression of human microsomal triglyceride transfer protein (MTP) and apolipoprotein B48 (apoB48) together enabled Sf-21 insect cells to secrete 10-fold more lipoprotein-associated triacylglycerol than control cells. This dramatic effect demonstrates that effective partitioning of triacylglycerol into the secretory pathway requires an endoplasmic reticulum-associated neutral lipid transporter (provided by MTP) and an apolipoprotein to shuttle the lipid through the pathway. Expression of the human apoB48 gene in insect cells resulted in secretion of the protein product. Including both MTP subunits with apoB48 and oleic acid specifically increased apoB48 secretion 8-fold over individual subunits alone. To assess whether specific regions of apoB are necessary for MTP responsiveness, nine apoB segments were expressed. These included NH2-terminal segments as well as internal and COOH-terminal regions of apoB fused with a heterologous signal sequence. ApoB segments containing the NH2-terminal 17% of the protein were secreted and responded to MTP activity; however, a segment containing only the NH2-terminal 17% of the protein was not significantly responsive to MTP. Segments lacking the NH2 terminus were not MTP-responsive, and five of six of these proteins were trapped intracellularly but, in certain cases, could be rescued by fusion to apoB17. These results suggest that the NH2 terminus of apoB is necessary but not sufficient for MTP responsiveness.

The transport of water-insoluble lipids through the circulation of all mammals is mediated by lipoprotein particles. Apolipoprotein B (apoB) is an unusually large secretory protein (514 kDa) that is required for the assembly and secretion of triacylglycerol-rich lipoproteins from the liver and the intestine (1). ApoB production rates are highly variable in human populations. Clinically important lipoprotein disorders such as familial combined hyperlipidemia are often associated with apoB overproduction (2).

The microsomal triglyceride transfer protein (MTP) plays an essential role in the assembly of apoB-containing lipoproteins. MTP is a protein complex found in the endoplasmic reticulum (ER) lumen of liver and intestinal cells (3). This complex primarily transfers neutral lipids between membranes in vitro (4). MTP has two subunits: protein disulfide isomerase (PDI) and a 97-kDa subunit that possesses lipid transfer activity in vitro when coupled with PDI (5). Mutations in the 97-kDa subunit of MTP have been found in patients with abetalipoproteinemia (6–8). This condition leads to only trace amounts of apoB-containing lipoproteins in the plasma and a substantial decrease in plasma neutral lipid content.

Recent studies have shown that introduction of the 97-kDa subunit of MTP into cells that are nonhepatic and nonenteric in origin, enables these cells to secrete segments of apoB (9, 10). ApoB secretion in these cases is increased when the cells are supplemented with oleic acid, a substrate for neutral lipid biosynthesis. The tissue-specific production of apoB-containing lipoproteins correlates with the tissue distribution of the 97-kDa subunit of MTP. These studies, coupled with our understanding of abetalipoproteinemia, reinforce the hypothesis that MTP promotes lipoprotein assembly and secretion by facilitating the coupling of apoB with lipid. The secretion of apoB and lipid appear to be interdependent in that apoB requires adequate neutral lipid for secretion and neutral lipid secretion requires apoB.

Studies of invertebrates (primarily insects) indicate that they have evolved an alternative mechanism for the release of neutral lipids from cells (11). While adult insects have large triacylglycerol stores in their lipoprotein-producing fat body tissue, insects cannot target triacylglycerol into the secretory pathway. Insects fail to assemble lipoproteins intracellularly and instead secrete the protein component of their lipoprotein in a lipid poor form (12). Mobilization of stored triacylglycerol occurs only after it is hydrolyzed to diacylglycerol. The diacylglycerol is released from the cell to the extracellular fluid, the
hemolymph, where lipoprotein assembly takes place (13). This assembly process requires an extracellular lipid transfer particle (14) which, like MTP, has been shown to possess a lipid transfer activity in vitro (15).

No molecular explanation exists for the contrasting mechanisms of triacylglycerol mobilization that have evolved within the animal kingdom. We hypothesized that the ability of vertebrates to partition triacylglycerol into the secretory pathway is dependent upon E.R. retention of their neutral lipid transfer activity. If this hypothesis is correct, then providing invertebrate cells with an intracellular neutral lipid transfer activity should enable them to directly secrete triacylglycerol from their intracellular stores.

Sf-21 cells (from the fall armyworm Spodoptera frugiperda) are invertebrate cells that do not produce lipoprotein particles. We have utilized these cells to assess the requirements for the secretion of triacylglycerol and apoB. Although they accumulate high levels of intracellular triacylglycerol, Sf-21 cells release little triacylglycerol into the media. Here we show that expression of human MTP and the intestinal form of human secreted triacylglycerol and apoB. Two oligonucleotides, 5'-gAT CCG CCG CCG CAT AGG CCA CTG GGT-3' and 5'-AAT TCA CTA GGT GCG TAT GCG GGC GGC GGC-3', were synthesized and annealed to generate the "Bam-Eco" polylinker containing (from the 5' end) BamHI, SacI, NotI, SfiI, SspI, and EcoRI sites. The polylinker was phosphorylated with polynucleotide kinase (U.S. Biochemical Corp.) prior to use. The Bam-Eco linker was ligated with a 2.3-kb EcoRI-BamHI fragment of apoB cDNA from pBl18 (24) and pCB17, not digested with PstI, to generate plasmid pAcB100. pBl18L11 was digested with BstEII (+1359 of the apoB cDNA) and KpnI (in the linker region of pCMV5). This product was ligated to a BstEII-KpnI fragment from pBl100 (24) that was generated by a complete digestion with KpnI and a partial digestion (at +1359 of apoB cDNA) with BstEII. The resulting plasmid is pBl100L11.

An 18-kb NotI-SmaI DNA fragment encoding the human apoB100 minigene was excised from pBl100L11 and inserted into a NotI-SmaI-digested baculovirus transfer vector pVL1392 (Invitrogen) to yield pAcB100.

\[ \text{NH}_2 \text{Terminus of ApoB and MTP Responsiveness} \]

The baculovirus transfer vector pVTBac (26) (a gift from T. Vernet) containing the honeybee melittin signal peptide was used for the production of the internal and C-terminal terminal apoB containing plasmids. These plasmids were used to transfect Spodoptera frugiperda Sf-21 cells which were then infected with recombinant baculovirus. All fusion junctions were in frame with the honeybee melittin signal sequence as confirmed by sequencing at the University of California, San Francisco (CUCF) Sequencing Facility. Initially, an XbaI oligonucleotide linker with translational stop sequences in all three reading frames (CTAGTCTAGACTAG) was inserted into the SmaI site of pVTBac, yielding pVTBac. A 6.5-kb BamHI fragment spanning sequences from apoB33 to apoB80 was removed from pBl100L11 and inserted into the BamHI site of pVTBac* yielding pAcB33-80. A 1.8-kb BamHI-BclI fragment encoding apoB33-46 was inserted into the BamHI site of pVTBac* to produce pAcB33-46. A 4.4-kb BclI-BamHI fragment encoding apoB48-80 was inserted into the BamHI site of pVTBac* to produce pAcB48-80.

To produce apoB69-79, pVTBac was first modified by cutting with KpnI and filling in with the large fragment of DNA polymerase (Klenow) to generate blunt ends. A 63-bp synthetic DNA fragment (CGA ATC GAA GGT CGT AAA GAA ACC GAA ACC GCT GCT GCT AAT TCC GAA CGC CAC ATG AAC AGC TAA) encoding the S peptide of RNase A (as in Ref. 27), a factor Xa cleavage site, and a translational stop codon was ligated to the blunt-ended pVTBac to generate pVTBac-S. pVTBac-S was digested with PstI and filled in with the Klenow to generate blunt ends. pAcB33-80 was digested with AscI and MsdI to generate a 1.4-kb fragment encoding apoB69-79. This fragment was treated with Klenow to generate blunt ends and was subsequently ligated to the linearized blunt-ended pVTBac-S to generate pAcB69-79. This construct was used to produce recombinant pAcB69-79 fused in frame with the S peptide sequence as confirmed by sequencing at the CUCF Sequencing Facility.

To generate apoB78-100, pBl100L11 was digested with BsiHKAI and treated with T4 DNA polymerase to remove 3'-protruding sequences. Following digestion with MunI, a 4.5-kb fragment encoding apoB78-100 was isolated. This fragment was digested with EcoRI and SmaI and the 4.5-kb apoB fragment was inserted to generate pAcB78-100.

To produce apoB88-100, two oligonucleotides were used to amplify the apoB exon 29 coding sequence from human genomic DNA via the polymerase chain reaction. The 5'- primer contained 42 bases (5'-ggat cag gat cgc aag gcc tcc tcc tca gat aat aac ccc acc ccc ctt ct3-9) with the uppercase letters representing apoB sequence. The non-apoB sequence begins with the final two amino acids of exon 28. The 3'- primer contained 27 bases (5'-toc cgg cgg tgt tct act gtt ttt ttt-9) with the uppercase letters representing 3' apoB untranslated sequence. The lower case (non-apoB) sequence contains a Xmal site for cloning purposes. Following amplification, the resulting 1.7-kb fragment was inserted to the pAcB17 site for cloning purposes. Following amplification, the resulting 1.7-kb fragment was inserted to the pAcB17 site for cloning purposes. Following amplification, the resulting 1.7-kb fragment was inserted to the pAcB17 site for cloning purposes. The apoB17 fusion proteins were generated using pAcB17. Initially, pAcB17 was partially digested with BamHI, and the upstream BamHI site was destroyed by filling it in with Klenow to generate blunt ends. This yielded pAcB17-Bam. A 1.8-kb BamHI-BclI fragment (from pBl100L11) encoding apoB33-46 was inserted into the remaining BamHI site of pAcB17-Bam. The BamHI junction was then opened, filled in with Klenow, and religated. This placed the apoB33-46 sequence in frame with the apoB17 sequence and formed pAcB17-(33-46). A 4.4-kb BclI-BamHI fragment encoding apoB48-80 was removed from pBl100L11 and filled in to generate blunt ends using Klenow. This fragment was inserted into the BamHI site of pAcB17-Bam after this site had also been filled in. This placed the apoB48-80 sequence in frame with the apoB17 sequences. This resulted in the 3-kb fragment.

To generate the apoB17-(69-79) fusion, pAcB17-Bam was digested with BamHI and SmaI. A 1.5-kb BamHI-SmaI fragment encoding apoB69-79 was removed from pAcB69-79 and inserted into pAcB17-Bam. This construct was linearized with BamHI and was filled in with the Klenow fragment to place apoB69-79 in frame with apoB17. The resulting product was pAcB69-79, which was confirmed by sequencing at the CUCF Sequencing Facility.

The resulting transfer vectors encoding regions of apoB were used to produce recombinant baculoviruses using linearized viral DNA.
Sf-21 Cells—viruses were identified, plaque-purified, amplified, and titered as described (28, 29).

Analysis of Intracellular Lipids following Oleic Acid Treatment of Sf-21 Cells—Sf-21 cells were grown in suspension using TC 100 medium (Life Technologies, Inc.) with 10% fetal bovine serum (HyClone). Cells were plated in 60-mm diameter tissue culture dishes to a density of 4 × 10^5 cells/dish. Cells were infected for 1 h with wild-type baculovirus at a multiplicity of infection of 5. Following infection, cells were washed and fed SF 900 serum-free media (2 ml) (Life Technologies, Inc.). Twenty-seven hours postinfection, the media were replaced with 2 ml of SF 900 media containing 0.5% bovine serum albumin (BSA) or 0.5% bovine serum albumin with 1 mM oleic acid. In experiments where an oleic acid tracer was used, [14C]oleic acid was included at 6.25 μCi/ml. Seventeen hours later, cells were washed and scraped into 1 ml of phosphate-buffered saline (PBS), pH 6.2 (150 mM NaCl, 2.8 mM KCl, 1.5 mM KH2PO4, 6.5 mM NaHPO4, pH 6.2). Protein measurements were determined by a modified Lowry assay (30) using bovine serum albumin as a standard. Lipids were extracted (31) and subjected to TLC using a hexane/ether/acidic acid (80:20:2, v/v/v) solvent system. Mass measurements of various lipids were determined by charring with concentrated sulfuric acid (32) and compared with standard curves generated using tristearin, diolein, cholesterol ester, and phosphatidylcholine. For measurements involving [14C]oleic acid tracer, TLC was performed as above, and resolved lipids were identified using a Berthold automatic TLC scanner (Berthold CHROMA software package, version 4.1). To ensure that analysis was in the linear range of detection, several sample volumes (20–200 μl) were analyzed, and several timed exposures were made. To monitor levels of total protein expression, cell lysates were prepared as described above, and immunoblot analysis was carried out (using 25 μg of total cell protein) with anti-apoB antibodies.

Analysis of the Effect of MTP on ApoB48 Secretion—Cells were infected as described above with a total viral multiplicity of infection of 5 viruses/cell for each condition tested. The viral ratios used were as follows: for cells expressing apoB48 and PDI (apoB48:PDI) 5:3:2; for cells expressing apoB48 and the 97-kDa subunit (apoB48:97-kDa subunit) 5:3:2: for cells expressing apoB48 and both MTP subunits (apoB48:PDI:97-kDa subunit) 5:3:2:3. Twenty-seven hours postinfection, media were changed as in the oleic acid experiments (above) and were harvested 16 h later. Media harvesting, Cabosil precipitation, and quantitative immunoblotting were carried out as described above. To monitor protein expression, cell lysates were prepared as described above, and individual protein expression was monitored via immunoblotting of cell lysates (for MTP) and media (for apoB48) (not shown).

Analysis of Lipoprotein-associated Lipids—To assess the effect of MTP on apoB48 on lipoprotein secretion from Sf-21 cells, the release of oleic acid-containing lipoproteins was assessed using uninfected cells and cells infected with wild-type (control), apoB48, and MTP (23) viruses. Cells were infected as above with a total multiplicity of infection of 5 viruses/cell. In experiments where multiple viruses were used to infect cells, the following virus ratios were used: wild type:PDI, 3:2; wild type:PDI:97-kDa subunit, 3:1:1; apoB48:PDI, 3:2; apoB48:PDI:97-kDa subunit, 3:1:1. Media changes and harvesting were done as described above. After harvest, the media were centrifuged at 1500 × g for 10 min. Media lipids were extracted and quantitated as described above. To assess the specificity of the MTP effect on apoB48 secretion, cells were infected at a total multiplicity of infection of 5 with the following viruses: wild-type virus (multiplicity of infection of 5). The media introduced at 27 h postinfection contained 0.5% BSA with or without 1 mM oleic acid. The media also contained 100 μCi/ml [35S]methionine (DuPont Express Protein Labeling Mix). To assess the specificity of the MTP effect on apoB48 secretion, cells were infected at a total multiplicity of infection of 5 with the following viruses at the indicated viral ratios: wild-type virus (multiplicity of infection of 5): 3:2; wild-type virus (multiplicity of infection of 5): 3:2:3; apoB48:PDI, 3:2; apoB48:PDI:97-kDa subunit, 3:1:1. The media change at 27 h postinfection included 0.5% BSA complexed with 1 mM oleic acid and 100 μCi/ml [35S]methionine. Eight hours after the media changes, media samples were harvested, and 5 μl of total secreted protein was analyzed by SDS-PAGE under reducing conditions in 4–15% gradient acrylamide gel electrophoresis (SDS-PAGE) (33). Immunoblotting (34) was carried out with anti-human apoB monoclonal antibodies (1D1, CC3.4, D7.2, MB47, MB43, Bsol 16, and Bsol 7) (35–37) and alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma). All expected immunoreactive products were detected (data not shown). The apoB100 construct produced two discrete protein products, one the size of apoB100 and the other the size of apoB48. The mechanism by which this heterogeneity occurs is unknown, although mRNA editing (38, 39) and premature polyadenylation of apoB mRNA (40) are known to result in apoB48 production in other systems. Specific proteolysis of apoB prior to secretion is another possible mechanism of formation of the shortened product.

Expression of ApoB48 Secretion by Recombinant Cells—Cells were plated out and infected with wild-type control virus or virus encoding apoB48 with a multiplicity of infection of 5 as described above. Twenty-seven hours postinfection, the 2 ml of SF 900 media were replaced with 2 ml of SF 900 media containing 0.5% BSA or 0.5% BSA complexed with 1 mM oleic acid. The media were harvested 16 h later. Following centrifugation for 10 min at 1500 × g, the media were analyzed for apoB48 and apoB100 secretion.

Expression of Apolipoprotein B Segments Produced by Recombinant Baculoviruses—Sf-21 cells were plated in 60-mm diameter tissue culture dishes at a density of 4 × 10^5 cells/dish. Cells were infected for 1 h with wild-type virus or wild-type virus expressing electronegatively charged apoB48 (multiplicity of infection of 5). The media introduced at 27 h postinfection contained 0.5% BSA with or without 1 mM oleic acid. Sixteen hours later, the media were removed, and cells were washed in PBS (pH 6.2) and then incubated in SF900 medium free of L-cysteine or methionine for 1 h at 27°C, in order to deplete...
the intracellular pool of the two amino acids. The cells were pulse-labeled with 1 ml of 0.1 mCi/ml [35S]methionine/cysteine mix containing medium and chased in SF900 medium with an excess amount of cold methionine and cysteine. At each time point, the medium was collected after spinning down the cell debris, and the cells were lysed by sonication. Cell extracts and media samples were immunoprecipitated by incubating with polyclonal antibodies against apoB followed by protein G-agarose. After extensive washing, the precipitated proteins were solubilized and subjected to SDS-PAGE fractionation. The bands corresponding to apoB 48 were cut out from the gels and counted for radioactivity. To ensure quantitative recovery of apoB, a second round of immunoprecipitation was performed. As a control for nonspecific interactions with the antibody and/or the protein G beads, cells infected with PDI or MTP viruses alone were also carried through the whole procedure.

Analysis of the Buoyant Density of ApoB48—Cells were infected at a total multiplicity of infection of 5 as described above. The medium change at 27 h postinfection contained 0.5% BSA complexed with 1 mM oleic acid and 50 μCi/ml [3H]methionine. Eight hours later the media were harvested as above, and samples (1.75 ml) were subjected to NaBr density gradient ultracentrifugation (42) in an SW-41 rotor at 175,000 × g for 32 h. The resulting gradient range was 1.016–1.247 g/ml. Twelve 1-ml fractions were collected from the top of each tube. Each fraction was precipitated with Cabosil and resuspended in 30 μl of Cabosil resuspension buffer (see above). Twenty μl from each sample was subsequently analyzed in a 4–15% SDS-PAGE gel under reducing conditions, and fluorography was carried out as described above.

Analysis of ApoB Segments and ApoB17 Fusions for Secretion and MTP Responsiveness—NH2-terminal, internal, and COOH-terminal apoB constructs as well as apoB17 fusion proteins were tested for their secretion and responsiveness to MTP. Cells were infected as described above with individual apoB viruses along with one (PDI) or both subunits of MTP. Media were changed at 27 h postinfection, and new media contained 0.5% BSA complexed with 1 mM oleic acid. Media were harvested 17 h later (as described above), and 1 ml was precipitated with Cabosil. Pellets were resuspended as above in 200 μl of resuspension buffer, and aliquots ranging from 5 to 60 μl were analyzed by SDS-PAGE and quantitative immunoblotting (see above). All immunoblotting was carried out with a rabbit anti-pig apoB polyclonal antibody except for apoB78–100 and apoB88–100 (which required a monoclonal antibody-Bsol16) and apoB69–79 (for which MB47 was used). To monitor protein expression, cell lysates were prepared as described above, and immunoblotting was carried out (using 25 μg of total cell protein) with anti-apoB, anti-PDI, and anti-97-kDa subunit antibodies.

**RESULTS**

**Sf-21 Cells Actively Synthesize Triacylglycerol and Store It Intracellularly—**To stimulate the production of neutral lipids in Sf-21 cells, virally infected cells were exposed to 1 mM oleic acid using BSA as a carrier. The intracellular levels of lipid species known to participate in either vertebrate or invertebrate lipoprotein assembly were quantitated. The 17-h oleic acid treatment resulted in an 11-fold increase in intracellular triacylglycerol content (Fig. 1). No significant change in the levels of phospholipids or diacylglycerols was observed. This increase in triacylglycerol content transformed the phenotype of the Sf-21 cells so that they more closely approximated that of both vertebrate and invertebrate lipoprotein-producing cells.

Introduction of a [3H] oleic acid tracer, along with the 1 mM oleic acid, resulted in effective incorporation of the tracer in all lipid pools analyzed except cholesteryl ester (Fig. 2). The lack of detectable cholesterol ester is consistent with the observation that Sf-21 cells lack the enzyme required for its formation (43). The triacylglycerol pool contained two-thirds of the intracellular tracer, supporting the mass analysis and indicating that synthesis of triacylglycerol was a major element of the lipogenic response. Infections with recombinant viruses did not significantly alter the intracellular lipid mass levels or tracer distributions when compared with wild-type virus infection (not shown).

**Sf-21 Cells Secrete Little Triacylglycerol from Their Intracellular Stores—**In order to characterize the neutral lipids released from oleic acid-stimulated Sf-21 cells, measurements were made of [3H]-media lipids following exposure of the cells to oleic acid for 17 h. Use of a [3H]oleic acid tracer ensured that the species measured were products of cellular lipid biosynthesis and secretion and not lipid component. Despite a large elevation in intracellular triacylglycerol levels in response to oleic acid, very little was released into the culture media (Fig. 3). This inability of Sf-21 cells to effectively secrete triacylglycerol, despite high intracellular levels, is consistent with invertebrate physiology; invertebrates release diacylglycerol rather than triacylglycerol.

Infection of Sf-21 cells with wild-type baculovirus did little to affect the media neutral lipid composition (Fig. 3). This indicates that any change, upon infection with recombinant viruses, would likely reflect the effect of heterologous proteins expressed by these viruses.

**Human MTP and Apolipoprotein B Stimulate Triacylglycerol Secretion from Sf-21 Cells—**Following introduction of the

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**Figure 1. Changes in intracellular lipid levels in response to oleic acid treatment.** Wild-type virus-infected Sf-21 cells were treated with BSA or BSA complexed with oleic acid. Following treatment, the cellular lipids were extracted and separated via thin layer chromatography. Mass measurements of lipids known to be released from vertebrate and invertebrate cells were made via charring with concentrated sulfuric acid. PL, phospholipid; DG, diacylglycerol; TG, triacylglycerol; CE, cholesteryl ester. The results are means of three determinations ± S.D. ND, none detected.

**Figure 2. Distribution of a [3H]oleic acid tracer within intracellular lipids from cells infected with wild-type virus.** The data show the distribution of labeled oleic acid within lipid classes that are released by vertebrate and invertebrate cells. The values are expressed as percentage of total intracellular lipid counts. Each value is the mean of three determinations ± S.D. ND, none detected; PL, phospholipid, DG, diacylglycerol, TG, triacylglycerol, CE, cholesteryl ester.
apoB48 and MTP genes (either separately or together), triacylglycerol secretion was measured. While expression of both MTP subunits in SF-21 cells resulted in neutral lipid transfer activity (23), this was insufficient to promote triacylglycerol secretion (Fig. 3). Expression of apoB48 in SF-21 cells had a modest effect on triacylglycerol release, consistent with apoB48 having a limited ability to bind neutral lipids and carry them through the secretion pathway without the assistance of MTP.

In contrast, co-expression of apoB48 with MTP resulted in a dramatic increase in triacylglycerol secretion by the invertebrate cells. The total media triacylglycerol level under these conditions was 10-fold higher than in media from uninfected cells. Thus, apoB48 and MTP are sufficient to efficiently partition cellular triacylglycerol into the secretory pathway. No effect on media diacylglycerol levels was observed in the presence of apoB48 and MTP (not shown), demonstrating that the effect is specific for triacylglycerol.

MTP-mobilized Triacylglycerol Is Assembled into Lipoprotein Particles—The previous experiments suggested that MTP redistributes triacylglycerol into the secretory pathway of SF-21 cells and combined it with apoB48. To quantitate the amount of triacylglycerol secreted in association with lipoprotein particles, the media was subjected to ultracentrifugation in sodium bromide at a density of 1.20 g/ml. Sixty times more triacylglycerol was present in the d < 1.20 g/ml fraction from cells expressing both apoB48 and MTP when compared with control cells (Fig. 4). This large difference clearly demonstrated that vertebrate expression of these vertebrate gene products dramatically stimulated triacylglycerol secretion in the form of nascent lipoprotein particles. By contrast, little difference was detected in the diacylglycerol found at this density, demonstrating a triacylglycerol-specific effect.

In addition to neutral lipid and protein, lipoproteins contain a surface layer of phospholipid. We therefore assessed the phospholipid content of the SF-21 cell-produced apoB48 lipoproteins. Analysis of the phospholipids that floated with the apoB48 and triacylglycerol indicated that phosphatidylcholine and phosphatidylethanolamine were both present in the lipoprotein particles (data not shown).

ApoB48 Secretion by SF-21 Cells Is Specifically Stimulated by Oleic Acid and MTP—In mammalian liver, apoB secretion is regulated post-translationally, primarily by the amount of triacylglycerol synthesized in the cells. To determine if insect cells expressing apoB are also capable of responding to the regulatory effects of triglyceride, apoB secretion was measured in the absence and presence of 1 mM oleate. Under these conditions, the triacylglycerol content of the SF-21 cells increases 11-fold (Fig. 2). The level of apoB48 secretion was increased 6-fold in the presence of exogenous oleic acid (Fig. 5). No detectable change in the level of intracellular apoB48 was observed in the presence of oleic acid (data not shown).

To investigate whether the effect of oleic acid treatment was specific for apoB48 secretion or if it was influencing general protein secretion, analysis of total cell protein secretion was carried out. Total 35S-labeled secreted proteins were analyzed in the presence and absence of exogenous oleic acid (Fig. 6). In cells expressing apoB48, oleic acid stimulated the secretion of apoB48, while the levels of other secreted proteins were unaffected. The secretion of apoB17 from control cells was unaffected by the presence of oleic acid, further demonstrating that the longer apoB48 protein was specifically influenced by the presence of oleic acid.

In order to assess the effect of MTP on apoB secretion, cells expressing apoB48 were co-infected with either the individual MTP subunits or with both subunits together. Expression of both MTP subunits with apoB48 in the presence of oleic acid dramatically increased apoB48 secretion (Fig. 7). This increase was 8-fold more than that observed in the presence of either individual subunit alone. This pronounced stimulation of apoB48 secretion by MTP is not seen in the absence of exogenous oleic acid (not shown), demonstrating that MTP’s activity is dependent on triacylglycerol availability. By quantitative Western blot analysis of oleate-treated cells, we estimate that the mass of apoB accumulating in the tissue culture medium in the presence of MTP after 17 h was 2-10% of the intracellular apoB mass (data not shown).

To assess whether or not the effect of MTP on apoB48 secretion was specific, total protein secretion was analyzed in cells expressing apoB48 and one or both subunits of MTP (Fig. 8). Induction of apoB48 secretion was clearly seen in the presence of both MTP subunits. No other secreted proteins were stimulated by MTP, demonstrating its specificity for apoB48. An additional media protein (97 kDa) is detectable as a result of
MTP expression. We have determined immunochemically that this is the large subunit of MTP (some of which is secreted during overexpression) and not an MTP-stimulated protein (not shown).

The MTP Induction of ApoB Secretion Is Not a Consequence of Rescue from Early Intracellular Degradation—Previous studies in HepG2 cells have shown that oleate can increase apoB secretion by diminishing the proportion of apoB subject to early post-translational degradation (44). ApoB proteolysis is very efficient, resulting in the degradation of 80% of intracellular apoB within 40 min (44). We assessed apoB48 degradation in SF-21 cells over a period of 4 h. The cells were subjected to a 30-min pulse with [35S]methionine and [35S]cysteine followed by a chase in tracer-free medium for times up to 4 h. The experiments showed that the rate of tracer incorporation into apoB is not affected by oleate or by co-expression of MTP, indicating that oleate and MTP do not increase apoB secretion by increasing apoB biosynthesis (data not shown). In contrast to primary hepatocytes or HepG2 cells, SF-21 cells do not rapidly degrade significant proportions of newly synthesized apoB (Fig. 9). ApoB radioactivity was not detectable in the culture media until after 4 h, explaining why there was no decline in intracellular apoB radioactivity during this chase period. At longer times, however, oleate or MTP exerted a major effect on the amount of apoB secreted (Figs. 5 and 7).

ApoB48 Secreted by SF-21 Cells Has the Buoyant Density of a High Density Lipoprotein—While oleic acid and MTP were able to stimulate the secretion of apoB48, density gradient ultracentrifugation was required to assess whether the secreted apoB48 was in the form of a lipoprotein particle. Analysis of
apoB48 buoyant density showed that oleic acid and MTP stimulation of apoB48 secretion correlated with the formation of apoB48 containing high density lipoprotein particles (Fig. 10). The density distribution of these particles is comparable with that of particles from apoB48-producing cells of hepatic origin (16–18). MTP expression resulted in a higher level of apoB48 secretion but did not significantly alter the buoyant density of the secreted lipoprotein particles, indicating an increase in the number but not in the size of the particles. The apparent difference in extent of the MTP effect between Figs. 7 and 10 is due to the fact that additional viruses were included in the experiment shown in Fig. 7 to control for the total multiplicity of infection. This was not done in the experiments depicted in Fig. 10.

Secretion and MTP Sensitivity of ApoB Segments Correlates with the Presence of the NH2 Terminus of ApoB—To assess whether specific regions of apoB are required for secretion of the protein and MTP responsiveness, nine apoB segments (Fig. 11) were expressed in oleic acid-treated Sf-21 cells, in the presence and absence of MTP. All segments containing the NH2-terminal 17% of apoB were secreted from the cells. MTP stimulated the secretion of all the segments containing this NH2-terminal apoB segment (Fig. 12). In contrast, none of the apoB segments that lacked the NH2 terminus were responsive to MTP. In fact, five of these gene products were only detectable inside the Sf-21 cells and were not secreted, even in the presence of MTP. The most COOH-terminal protein product of apoB (B88–100) was secreted by the cells but was unresponsive to MTP.

The NH2 Terminus of ApoB Confers Secretion Competence and MTP Responsiveness to ApoB Internal Segments—To determine if the NH2 terminus of apoB could confer secretion competence and MTP responsiveness to internal apoB segments, the NH2-terminal 17% of apoB (B17) was fused with three internal apoB fragments. Cells expressing these three segments (B33–46, B69–79, and B48–80) were unable to secrete them (Fig. 12). Fusion of these internal segments to apoB17 enabled all three of these apoB segments to be secreted by Sf-21 cells (Fig. 13). Co-expression of the fusion proteins with MTP resulted in a 3.6-fold induction in the secretion of the longest construct (B17-(48–80)) while the shorter fusion proteins were insensitive to MTP. This is consistent with the size correlation observed in Fig. 12 but does not rule out a role for specific sequences in lipid binding.

DISCUSSION

To understand the mechanism of triacylglycerol targeting to the secretory pathway of cells, we introduced mammalian gene products into invertebrate host cells. Invertebrate cells are unable to effectively secrete triacylglycerol, although they actively synthesize triacylglycerol when given media supplemented with free fatty acid.

Individually, expression of the mammalian genes for apoB or MTP did not promote substantial triacylglycerol secretion. However, in combination, apoB and MTP promoted a striking increase in the levels of triacylglycerol secreted from the cells. This observation suggests that the productive partitioning of triacylglycerol into the secretory pathway requires an ER-associated neutral lipid transfer activity (provided by MTP) and a vehicle by which the lipid can be shuttled through the pathway and out of the cell (provided by apoB).

The lipoproteins secreted by Sf-21 cells expressing apoB and MTP contained phospholipid and triacylglycerol. Unlike mammalian cells, cultured Sf-21 insect cells do not synthesize cho-
lesterol esters (43), thus none were detected in the secreted lipoproteins. This suggests that cholesteryl ester is not a required substrate for the formation of apoB-containing lipoproteins.

Invertebrate fat body tissue produces apolipoproteins that are capable of binding and transporting neutral lipids. However, their neutral lipid transfer activity (lipid transfer particle) is localized extracellularly, in the hemolymph. It is therefore likely that the differences in lipoprotein assembly that have arisen within the animal kingdom (substrate usage and site of assembly) are due to differences in the secretion or retention of the respective lipid transfer activities.

The location of the animal's lipid transfer activity is likely to influence the neutral lipid species (triacylglycerol or diacylglycerol) that is released from the cell. Vertebrates and invertebrates both appear to mobilize cytosolic triacylglycerol stores by first hydrolyzing them into more soluble diacylglycerol (11, 45). In vertebrates, it appears that the diacylglycerol is re-esterified to triacylglycerol at the cytoplasmic face of the ER (45). The ER localization of MTP places it in close proximity to the re-esterification reaction, making it accessible to triacylglycerol. In addition MTP transports nonpolar lipids more actively than amphipathic lipids (46). MTP's subcellular location and substrate specificity are therefore likely to contribute to the partitioning of triacylglycerol rather than diacylglycerol into the secretory pathway where it is complexed with apoB. In contrast, the invertebrate neutral lipid transfer activity (lipid transfer particle) is extracellular. The lack of triacylglycerol re-esterification activity at the plasma membrane would likely limit the availability of this lipid for extracellular assembly. However, diacylglycerol is more soluble and membrane-permeable than triacylglycerol (47, 48), making it a better candidate for transfer across the plasma membrane for extracellular lipoprotein assembly.

The ability of Sf-21 cells to secrete apoB48 enabled us to study the effect of MTP on apoB secretion and determine whether specific regions of apoB are important for its secretion and MTP-responsiveness. These experiments demonstrate a clear role for both subunits in apoB secretion. In Sf-21 cells, co-expression of apoB48 with the 97-kDa MTP subunit alone had little or no effect on apoB48 secretion (Fig. 7). This suggests that the 97-kDa subunit alone is incapable of stimulating apoB secretion and that this subunit is also incapable of utilizing endogenous insect PDI as a productive subunit. Co-expression of both subunits of MTP in Sf-21 cells has been shown to result in detectable levels of neutral lipid transfer activity (23).

**NH₂ Terminus of ApoB and MTP Responsiveness**

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**Fig. 11. Apolipoprotein B constructs used in this study.** The figure represents nine apoB constructs that were expressed by recombinant baculoviruses during the course of this study. The construct names reflect the region of apoB they encompass on a centile basis. For example, apoB-17 contains the NH₂-terminal 17% of apoB, while apoB-33-46 contains sequences between 33% of full-length apoB and 46% of the protein. The three NH₂-terminal constructs all utilize the apoB signal sequence (black box), while the internal and COOH-terminal segments are fused in frame with the honeybee melittin signal peptide (shaded box).

**Fig. 12. MTP stimulation of different apoB segments.** To determine if specific regions of apoB are required for secretion and MTP responsiveness, nine apoB segments (Fig. 11) were expressed with or without both MTP subunits in oleic acid-treated cells. B-"48" represents the apoB48-like protein secreted by cells infected with the apoB100-encoding virus. B-100H (B-100 heavy) represents the full-length protein secreted by the same cells. Quantitative immunoblotting was used to assess levels of induction. For each construct, secretion levels in the presence of PDI alone were normalized to a value of 1, and levels in the presence of both subunits are expressed relative to that. Five of the segments were retained intracellularly and were not detected in the media (ND), even in the presence of MTP. Error bars represent the standard deviation from three independent determinations. Independent t-test analysis for apoB17 resulted in p = 0.06005 and for apoB88-100 p = 0.32946. Representative immunoblots are shown for secreted and intracellular apoB. Odd-numbered lanes represent samples from cells expressing the apoB segment and PDI. Even-numbered lanes represent samples expressing apoB and both MTP subunits.
In the present study, expression of apoB17 in the presence of both MTP subunits increased apoB17 secretion 8-fold over either individual subunit alone. This demonstrates a requirement for both MTP subunits and active lipid transfer for the stimulation of apoB secretion. Analysis of total protein secretion demonstrated specificity of the MTP effect for apoB48 (Fig. 8).

ApoB produced in hepatoma cells is subject to rapid intracellular proteolysis under conditions that do not favor apoB secretion. Its secretion is enhanced by oleate through rescue from proteolysis (44, 49). In primary rat hepatocytes, apoB is rapidly degraded, but its secretion rate is unaffected by oleate (50). Pulse-chase experiments demonstrated that apoB is not rapidly degraded in SF-21 cells (Fig. 9). Accordingly, the protease inhibitor, N-acetyl-Leu-Leu-norleucinal, which inhibits apoB degradation in mammalian cells (51, 52), seems to have little if any effect on SF-21 apoB levels.\(^2\) The fact that we see a basal level of apoB secretion in the SF-21 cells in the absence of MTP might be attributable to the lack of an intracellular apoB degradation system. Therefore, the ability of oleate and MTP to stimulate apoB secretion in this system might not occur as a result of rescue of apoB from proteolysis. Our data also suggest that apoB synthesis is not changed by oleate or MTP. Our results are consistent with intracellular transport rather than degradation being rate-limiting for apoB secretion, as recently suggested by Bonnardel and Davis (53). Therefore, this system might be useful for identifying the primary impediment to apoB secretion and the mechanism by which MTP exerts its effect.

Many studies have been carried out to define regions of apoB that may participate in lipoprotein formation. Limited trypsin proteolysis has demonstrated that some regions of apoB100, when trypsinized, readily dissociate from LDL, while other regions remain tightly associated with the lipoprotein particle (54). Complementary studies where proteolytic fragments of apoB100 were incubated with lipid microemulsions demonstrated that specific regions of apoB100 are more lipophilic than others (55, 56). Although these studies suggest that certain regions of apoB may readily bind lipid, they provide little information about which regions are essential for lipoprotein assembly.

Sequence based predictions suggest that apoB100 contains several amphipathic motifs throughout its sequence. A recent analysis suggests that apoB has a pentapartite structure of three amphipathic \( \alpha \)-helical stretches alternating with two amphipathic \( \beta \)-sheet stretches (57). The \( \beta \)-sheet regions are predicted to exist as irreversible lipid-associating domains that encompass amino acids 827-1961 and 2611-3867.

Systematic COOH-terminal truncation of apoB100 suggests that there are no specialized regions within the COOH-terminal 70% of the protein that are essential for lipoprotein assembly (16-18). These studies suggest that the lipid binding capacity of apoB is a function of its total length. While these studies thoroughly examined the effect of apoB COOH-terminal deletions, all constructs retained the NH\(_2\) terminus of apoB.

The present study tested whether apoB contains specific regions that are required for protein secretion and MTP responsiveness. Nine apoB segments spanning the entire length of the protein were expressed with and without MTP. These segments included NH\(_2\)-terminal, COOH-terminal, and internal apoB fragments (Fig. 11) and varied in length from 10% of apoB through the full-length apoB100.

Of the initial constructs tested, only those containing the NH\(_2\) terminus of apoB were secreted by the cells and were stimulated by MTP (Figs. 7 and 12). In contrast, none of the constructs that lacked the NH\(_2\) terminus of apoB were stimulated by MTP. The majority of these apoB segments (5 out of 6) were trapped inside the cells and remained so, even in the presence of MTP. The trapped segments consisted mainly of internal regions of apoB. All of the trapped segments overlapped with apoB sequences predicted to contain irreversible lipid-associating amphipathic \( \beta \)-sheets (57). Secretion-negative apoB segments obtained from tunicamycin-treated cells showed greater electrophoretic mobility than those from untreated cells, indicating that the trapped internal segments were at least targeted to the ER.\(^3\) This suggests that the heterologous signal sequence functioned properly.

The one segment that lacked the NH\(_2\) terminus of apoB but was secreted was apoB888–100. This segment corresponds almost exactly with the final exon of apoB (exon 29). The apoB888–100 region does not overlap with a predicted irreversible lipid-associating region of apoB, but it contains sequences predicted to form reversible lipid-binding \( \alpha \)-helices much like non-apoB apolipoproteins. Although apoB888–100 was capable of being secreted, it was not responsive to MTP. This is also the case for non-apoB apolipoproteins.

Analysis of the nine apoB segments tested suggested that the NH\(_2\) terminus may be important for secretion of internal regions of apoB and for MTP responsiveness. To further explore this idea, three fusion proteins were generated. These contained three internal fragments that were secretion-defective and nonresponsive to MTP. Each was fused in frame with the NH\(_2\)-terminal 17% of apoB (apoB17). Following fusion with apoB17, all of the internal fragments could be detected in the media of expressing cells (Fig. 13), suggesting that apoB17 had conferred secretion competence on them. Only the longest of the three was responsive to MTP. This construct, apoB17-(48–80), represents 49% of the total apoB sequence, and its secretion was stimulated 3.6-fold by MTP.

The reason for the importance of the NH\(_2\) terminus of apoB in directing apoB secretion and enabling MTP responsiveness is currently unclear. This region may interact directly with MTP and mediate MTP sensitivity by providing a nucleation point for lipid acquisition by apoB. Previous work suggests this region must be completely translated before nascent apoB can be lipidated (58). Other studies have suggested that the NH\(_2\) terminus of apoB undergoes novel translocation pausing, resulting in transient transmembrane intermediates (19, 20) and may integrate into the inner leaflet of the mem-

\(^2\) D. G. Gretch and A. D. Attie, unpublished observations.

\(^3\) L. Wang and A. D. Attie, unpublished observations.
brane (59). An alternative model is that apoB pauses during translation rather than translocation (59). Additional studies suggest that the NH₂-terminal events transiently translocate into the ER lumen (21). It has been suggested that translocation then pauses so that apoB exists as a transient transmembrane protein with the NH₂-terminal extending into the lumen of the ER, while the remainder of the protein remains cytosolic. Without the impetus to resume translocation (i.e. binding of lipid presented by MTP within the ER lumen), the arrested apoB is susceptible to cytoplasmic degradation and subsequent secretion of the already luminal NH₂-terminal (21).

The NH₂-terminal of apoB is very rich in cysteine residues. Twelve of the protein's 25 cysteine residues are found in the NH₂-terminal 11% of the protein (60, 61). All 12 of these cysteines are involved in disulfide bond formation, while only 4 of the remaining 13 cysteines are found in disulfide bonds (62). Without ER luminal proteins to hold them in place and promote their forward translocation, secretory proteins are free to retrotranslocate and free themselves from the ER membrane (63). A protein such as apoB that is capable of periods of translocation arrest may require additional mechanisms to prevent retrotranslocation. A compact, luminal NH₂-terminal (held together with disulfide bonds) may form a disulfide knot, thus preventing retrotranslocation and keeping apoB accessible to lipids presented by MTP. Subsequent lipid acquisition is likely to stimulate the forward translocation of the rest of the protein, ultimately leading to lipoprotein maturation and the subsequent secretion of the lipid and apoB complex.

In summary, we have created a system with which to study the process of apoB and MTP-mediated lipoprotein assembly. Our ability to promote lipoprotein formation in insect cells utilizing several gene products encoded by separate viruses has allowed us to probe the importance of each individual protein. A detailed analysis of apoB suggests that the NH₂-terminal protein is essential but not sufficient for MTP-mediated lipoprotein formation. Future studies should allow a better understanding of the mechanism by which the NH₂-terminal has its effect and of which NH₂-terminal elements are essential. Similar studies may help define the functional elements of MTP and should aid in the analysis of other gene products that might participate in lipoprotein formation.

Acknowledgments—We thank Drs. Roger A. Davis and Darren Fast for comments regarding the manuscript. We thank Drs. Linda Curtiss, Yves Marcel, and Tom Innerarity for generosity in supplying essential support and encouragement.

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