Translocational pausing of apolipoprotein B can be regulated by membrane lipid composition

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Abstract One potential mechanism by which apolipoprotein (apo) B secretion is regulated is via transient pausing during translocation across the endoplasmic reticulum membrane. We have previously shown that translocation and secretion of full-length and truncated variants of apoB 100 are impaired in hepatocytes in which microsomal membranes are enriched in the phospholipid phosphatidylmonomethylethanolamine (PMME). We have now investigated whether or not the decreased translocation of apoB is the result of altered membrane lipid composition having an impact on translocational pausing. Our experiments showed that less in vitro translated apoB-15 (the N-terminal 15% of human apoB-100) was translocated into the lumen of PMME-enriched microsomes than of control microsomes. Proteinase K treatment of the translocation products yielded discrete N-terminal fragments of apoB indicating that both types of microsomal membranes contained translocationally paused nascent chains. Similarly, apoB generated from a truncated mRNA lacking a stop codon was also found to be translocationally paused. However, restarting of translocation after translocational pausing was impaired in PMME-enriched, but not in control, microsomes. These data suggest that secretion of apoB-containing lipoproteins can be regulated by membrane lipid composition at the level of translocational pausing.—Rusiñol, A. E., R. S. Hegde, S. L. Chuck, V. R. Lingappa, and J. E. Vance. **Translocation pausing of apolipoprotein B can be regulated by membrane lipid composition. *J. Lipid Res.* 1998.** 39: **1287–1294.**

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Apolipoprotein (apo) B-100 is a large (4536 amino acids), hydrophobic protein that is essential for secretion of very low density lipoproteins (VLDL) from the liver. High levels of plasma apoB have been implicated as a major risk factor for the development of atherosclerosis (1). Hepatic apoB secretion is thought to be regulated primarily post-transcriptionally by lipid supply (2, 3) and excess apoB that is not assembled into lipoproteins is degraded intracellularly (reviewed in ref. 4). Transport of apoB out of the endoplasmic reticulum (ER) is most likely the rate-limiting step for secretion of VLDL (5). Moreover, the translocation of apoB across the ER membrane has been suggested to be a key regulated event (4, 6).

ApoB is predicted to lack hydrophobic sequences that are characteristic of the stop-transfer sequences found in integral membrane proteins (7, 8). However, a new class of topogenic sequences called "pause-transfer sequences" has been identified in apoB (9–11). In contrast to the well-established model for translocation of typical secretory proteins (12), apoB has been shown to pause transiently at distinct sites along the nascent chain during translocation through the aqueous, protein-conducting channel of the ER (9–11). Translation of apoB continues during translocational pausing and the normally tight junction between the ribosome and the ER membrane opens, exposing domains of apoB to the cytosol (13). Moreover, translocational pausing is accompanied by structural alterations of proteins within the translocational machinery (13). However, although translocational pausing has been demonstrated in cell-free systems, so far no evidence has been provided that translocational pausing regulates apoB secretion in vivo and the nature of the in vivo signal for restarting translocation of apoB after pausing has not yet been identified. ApoB-100 can be secreted only when associated with lipids. Therefore, one attractive possibility is that during lipoprotein formation partial lipidation and/or the correct folding of apoB might be prerequisites for restarting translocation after pausing. Nascent paused chains that fail to restart translocation might remain spanning the membrane, exposing domains of apoB in a regulated manner to the cytosol and to proteases (14–18) including the proteasome (19–21).

Abbreviations: AcNYT, acetylated-asparagine–tyrosine–threonine; apo, apolipoprotein; ER, endoplasmic reticulum; PMME, phosphatidylmonomethylethanolamine; VLDL, very low density lipoproteins.

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Translocation of apoB across the ER membrane is thought to be primarily regulated by lipid supply. For example, when HepG2 human hepatoma cells are supplemented with oleic acid, both translocation of apoB into the microsomal lumen and secretion of apo B-containing lipoproteins from cells are increased (14, 15). In addition, when the assembly of apoB-48-containing lipoproteins is reconstituted in vitro, translocation of apoB into the microsomal lumen and formation of buoyant lipoprotein particles are stimulated when glycerolipid synthesis is reconstituted co-translationally (22). The lumenal microsomal triacylglycerol transfer protein, which catalyzes the transfer of neutral lipids between membranes in vitro, is thought to be required for efficient translocation, lipidation and/or secretion of apoB-containing lipoproteins (23–25).

Lipids also regulate apoB secretion at the level of membrane lipid composition. When the phosphatidylmonomethylethanolamine (PMME) content of microsomal membranes of hepatocytes is increased from 0.1% to ~10% of total phospholipids, apoB secretion is decreased by 50–70% whereas secretion of other proteins is unaffected (26). Moreover, enrichment of rat liver membranes with PMME reduces the plasma content of triacylglycerols and apoB by ~50% (27). Our studies have also shown that inhibition of the secretion of apoB-100 and B-48 by PMME is not due to a defect in the assembly of apoB with a neutral lipid core, as PMME also inhibits the secretion of apoB-15 (the N-terminal 15% of human apoB-100) and other small apoB variants that do not form buoyant lipoprotein particles (28). In addition, data from experiments using PMME-enriched hepatocytes indicate that the defect in apoB secretion in these cells is the result of an impaired translocation of the protein into the microsomal lumen (17). We have used this model, in which PMME perturbs apoB secretion, to investigate the events occurring during the translocation and secretion of apoB.

In the present study we have investigated whether or not PMME enrichment of microsomes inhibits apoB translocation via an effect on translocational pausing. In cell-free translation/translocation experiments we show that translocation of apoB-15 is impaired in rat liver microsomes enriched in PMME. We also demonstrate that newly synthesized apoB pauses during translocation in both control and PMME-enriched membranes. However, resumption of translocation after pausing is defective in membranes containing the altered phospholipid composition.

MATERIALS AND METHODS

Chemicals

[^{35}S]methionine and Amplify were purchased from Amersham Canada, and Tran[^{35}S]label was from ICN. Phospholipid standards were from Avanti Polar Lipids, Birmingham, AL. Reagents used for transcription of cDNAs were from Promega, as was factor Xa and the reticulocyte lysate. Triton X-100, TPCK-treated trypsin, soybean trypsin inhibitor, proteinase K, and micrococcal nuclease were from Sigma. Other chemicals and reagents were from either Sigma or Fisher Scientific.

Construction of plasmids and cell-free transcription

The plasmids apoB–FXa and apoB-15 were constructed using a modified pSP64 vector (Promega) (9, 13). The apoB-containing constructs lacked an in-frame stop codon. ApoB–FXa encodes epitope-tagged apoB-15 in which a factor Xa recognition site and a Myc epitope tag (amino acid sequence GTIEGRMGTE QKLISEED) were inserted at amino acid 357 of apoB cDNA, which includes the signal sequence (13). The apoB–FXa plasmid was truncated at the Bgl2 site within the coding region and therefore lacked an in-frame stop codon. ApoB–FXa was transcribed in vitro for 60 min at 40°C using SP6 RNA polymerase (Riboprobe Core System II, Promega) (10).

Cell-free translation and translocation

Purified mRNA transcripts were translated in a rabbit reticulocyte lysate for 2 h at 30°C in the presence of 4.0 O.D.$_{280}$ units of rat liver microsomes per 25 μl (9, 13). Aliquots of translation products were digested either with 0.4 mg/ml of proteinase K for 30 min at 0°C or with trypsin (50 μg/ml) for 30 min at 0°C, in the presence or absence of 1.0% Triton X-100 (29). Proteolysis was terminated as previously described (10). For factor Xa digestions, microsomes were sedimented then resuspended in buffer containing 100 mm NaCl, 50 mm Tris (pH 8.0), 5 mm magnesium acetate, 2 mm $CaCl_2$ and 0.25 m sucrose prior to addition of factor Xa to a final concentration of 0.05 mg/ml for 75 min at 22°C. Digestions were terminated by boiling for 5 min in the presence of 1% SDS, 0.1 m Tris (pH 8.9). In some translations, a competitive inhibitor of *N*-linked glycosylation, acetylated-Asn–Tyr–Thr (AcNYT) (0.5 mm), was added.

Preparation of rat liver microsomes

Male Sprague-Dawley rats (~220 g) were fed a diet of either normal rat chow (control) or chow containing 1.5% (v:w) monomethylethanolamine for 4 days (27). Rat liver microsomes were prepared as described previously for those from dog pancreas (29). The majority of ribosomes was stripped from microsomes by treatment with 100 mm EDTA (pH 7.4) for 30 min at 0°C, then with micrococcal nuclease to reduce background translation products due to endogenous mRNA (29). Microsomes were either stored at −70°C or used fresh. The protein content of microsomes was determined by the BCA protein assay (Pierce Chemicals, Rockford, IL).

Isolation of lumenal proteins

In some experiments, microsomal vesicles were sedimented after translation by ultracentrifugation through a 100-μl cushion of 0.5 m sucrose for 10 min at 55,000 rpm in a Beckman TLA100.1 rotor. The vesicles were resuspended in 100 μl 0.25 m sucrose containing either Tris-HCl (pH 7.5) or 100 mm sodium carbonate (pH 11.5) (30), followed by incubation at 0°C for 30 min. Lumenal contents were separated from membranes by centrifugation for 15 min at 55,000 rpm in a Beckman TLA100.1 rotor.

Electrophoresis and autoradiography

For separation of proteins produced from in vitro translations, microsomal membranes were re-suspended in buffer containing 10 mm Tris (pH 8.3), 2% SDS, and 8 m urea and analyzed by electrophoresis on 10% or 15% polyacrylamide mini-gels containing 0.1% SDS, as indicated. In some experiments, gels were impregnated with Amplify, dried, and autoradiographed for 1 to 6 h at −80°C.

Phospholipid analysis

Lipids were extracted from microsomes prepared from control and PMME-enriched rat livers (31). Phospholipids were separated by thin-layer chromatography in the solvent system chlo-

roform–propionic acid–*n*-propanol–water 30:20:60:10 (v/v) and visualized by either exposure to iodine vapor or by heating at 180°C for 5 min after immersion of the plate in cupric acetate/phosphoric acid solution (32). Bands corresponding to phosphatidylcholine, phosphatidylethanolamine, PMME, and phosphatidylserine/phosphatidylinositol were identified by comparison to authentic standards and scraped from the plates for determination of phospholipid mass (nmol/mg membrane protein) by phosphorus analysis (33).

RESULTS

The secretion of apolipoprotein B variants from PMME-enriched rat hepatocytes (26) and PMME-enriched McArdle 7777 rat hepatoma cells (28), and translocation of these proteins into the lumen of rat hepatocyte microsomes enriched in PMME (17) are known to be impaired. We have now investigated the mechanism by which PMME inhibits apoB translocation as a model for understanding the events that occur during translocation of apoB across the ER membrane and into the secretory pathway.

Disrupted translocation of apoB-15 across rat liver microsomal membranes enriched in PMME

Monomethylethanolamine is a structural analogue of choline and ethanolamine that is readily incorporated into the membrane phospholipid PMME. Normally, PMME is a quantitatively minor component of cellular membranes as it is an intermediate in the metabolic conversion of phosphatidylethanolamine to phosphatidylcholine (34). Rat liver microsomal membranes were enriched in PMME by feeding rats a diet supplemented with 1.5% (v/w) monomethylethanolamine for 4 days (27). The PMME content of liver microsomes from rats fed the control diet without monomethylethanolamine was <0.1% of total phospholipids whereas PMME comprised 6.4 ± 0.3% of microsomal phospholipids from rats fed monomethylethanolamine (**Fig. 1**). The content of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine/phosphatidylinositol in microsomes was not significantly altered by PMME enrichment of the membranes (Fig. 1).

Control and PMME-enriched microsomes isolated from rat livers were used for in vitro translation of a control secretory protein, β-lactamase, as well as apoB-15 (the N-terminal 15% of human apoB-100), using a rabbit reticulocyte lysate. Translation proceeded for 2 h to ensure that translocation of all nascent proteins was complete (9). The degree of translocation of the proteins into the microsomal lumen was assessed by measurement of the extent of protection of the proteins from exogenously added trypsin. **Figure 2** shows that β-lactamase was almost entirely resistant to digestion by trypsin in both control (lane 4) and PMME-enriched (lane 7) microsomes. The lack of proteolysis of β-lactamase was not due to an inherent resistance to trypsin or to the protease being inactive because *i*) β-lactamase translated in the absence of microsomes was completely degraded by trypsin (lane 2), and *ii*) β-lactamase was completely proteolyzed by trypsin in detergent-treated microsomes (Triton-X100, lanes 5 and 8). Figure 2 also confirms that control and PMME-enriched microsomes are essentially impermeable to exogenously added trypsin. Although the majority (~76%) of apoB-15 was protected from trypsin in control microsomes (lane 4), only ~20% (as determined by densitometric scanning) of apoB-15 was protected from trypsin (i.e., was lumenal) in PMME-enriched microsomes (lane 7), supporting the idea that in these microsomes much of the apoB-15 was not fully translocated into the lumen.

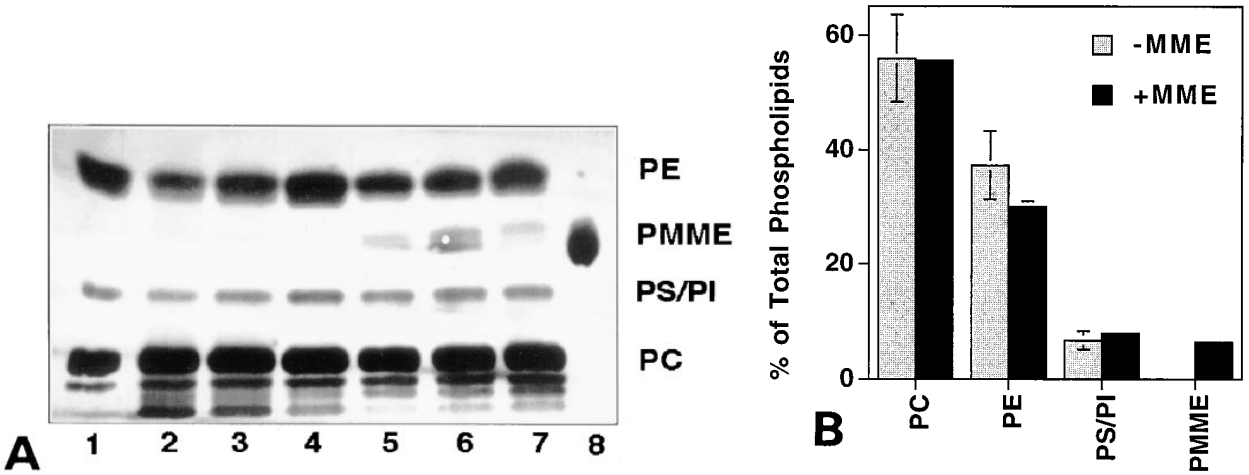

Fig. 1. Phospholipid composition of microsomal membranes from control and PMME-enriched rat livers. Rats were fed either a control chow diet (−MME) or a diet of chow enriched in 1.5% (v/w) monomethylethanolamine (+MME) for 4 days. Liver microsomes were isolated; lipids were extracted then separated by thin-layer chromatography. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS/PI, phosphatidylserine combined with phosphatidylinositol; PMME, phosphatidylmonomethylethanolamine. (A) Thin-layer chromatograph of phospholipids visualized by charring after exposure to cupric acetate/phosphoric acid (32). Lane 1, standard phospholipids; lanes 2–4, microsomal phospholipids from livers of rats fed control diet; lanes 5–7, microsomal phospholipids from livers of rats fed monomethylethanolamine; lane 8, standard PMME. (B) Phospholipid content (nmol/mg membrane protein) of control and PMME-enriched microsomes. Data are averages ± SD from 3 rats fed the control diet (shaded bars) and 3 rats fed the monomethylethanolamine-supplemented diet (solid bars).

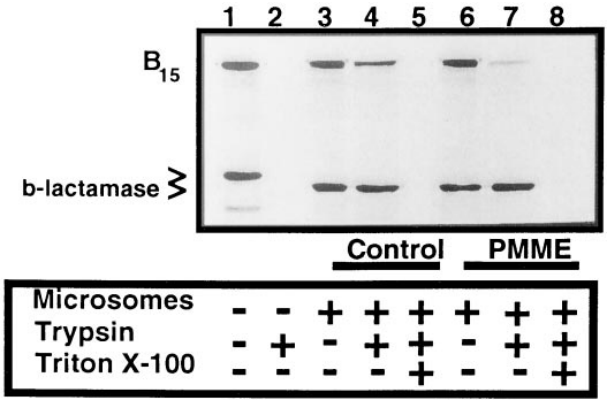

Fig. 2. Translocation of apoB-15 across PMME-enriched and control rat liver microsomes. Purified transcripts encoding apoB-15 and β-lactamase were translated in a rabbit reticulocyte lysate in the presence of liver microsomes isolated from either control or monomethylethanolamine-fed rats. Aliquots of translation products were digested with trypsin in the presence or absence of 1% Triton X-100. Products were analyzed by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, followed by autoradiography. Upper arrowhead, unprocessed β-lactamase; lower arrowhead, signal sequence-cleaved β-lactamase.

In the presence of microsomes, a single species of β-lactamase, of size smaller than that produced in the absence of microsomes, was generated (compare lanes 1 and 3, Fig. 2) indicating that β-lactamase had entered the ER lumen where the N-terminal signal peptide had been cleaved. From Fig. 2, however, we cannot distinguish whether or not the signal sequence of apoB-15 had been cleaved as there was no apparent difference in the size of apoB-15 synthesized in the presence and absence of microsomes (e.g., compare lanes 1 and 3). Upon entry of nascent apoB-15 into the lumen one would anticipate that both signal sequence cleavage and glycosylation would occur, resulting in a mature protein of approximately the same size as the apoB-15 that is synthesized in the absence of microsomes. Therefore, as a means of determining whether or not the signal sequence of apoB-15 had been removed, a competitive inhibitor of glycosylation (AcNYT) was included in some translations. **Figure 3A** shows that in both control and PMME-enriched microsomes the species of apoB-15 synthesized in the presence of AcNYT was smaller than that made in the absence of AcNYT. This experiment supports the idea that the signal sequence was cleaved from most of the apoB-15 molecules synthesized in both control and PMME-enriched microsomes in the presence of AcNYT. These data indicate that translocation was initiated, and signal sequence cleavage occurred normally, for virtually all full-length apoB-15 chains in both types of microsomes. Thus, the trypsin accessibility of apoB-15 observed in Fig. 2 is the result of defective translocation, not a defect in the targeting or initiation of translocation or signal sequence cleavage.

The translocation status of a protein can also be independently assessed by extraction of microsomal membranes with sodium carbonate at pH 11.5 (30). This treatment disrupts protein–protein interactions, but not

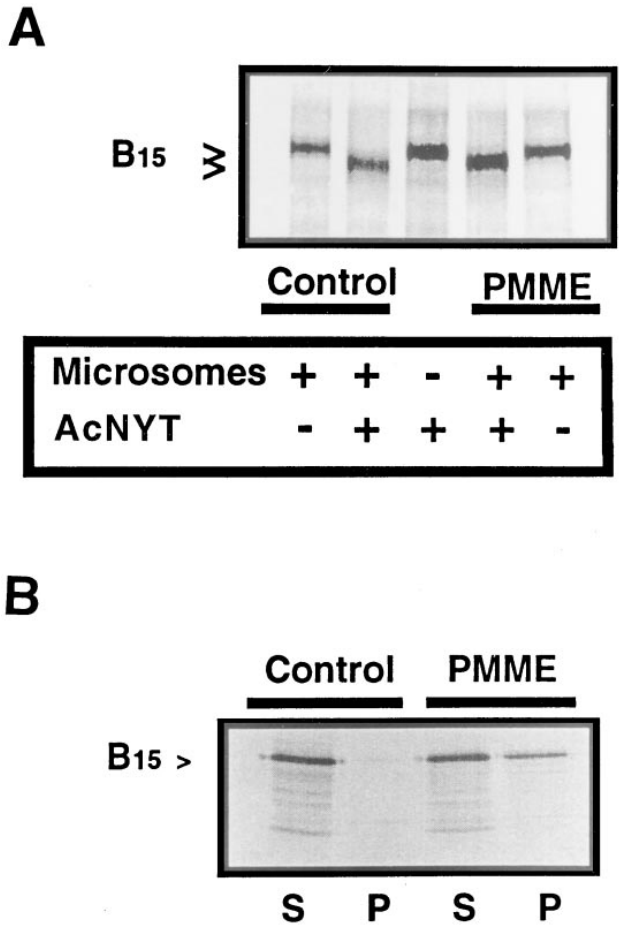

Fig. 3. (A) Signal sequence cleavage from apoB-15. The transcript encoding apoB-15 was translated as described in the legend to Fig. 2. A tripeptide inhibitor of *N*-linked glycosylation (AcNYT) was included in some translations. Proteins were electrophoresed to near the bottom of a 15% polyacrylamide gel in the presence of 0.1% SDS. Upper arrowhead: signal sequence-cleaved, glycosylated apoB-15 from incubations that included microsomes (lanes 1 and 5), and unglycosylated apoB-15 from which the signal sequence has not been cleaved in an incubation without microsomes (lane 3). Lower arrowhead: non-glycosylated apoB from which the signal sequence has been cleaved (lanes 2 and 4). (B) Co-translationally translocated apoB-15 is partially membrane-associated in PMME-enriched microsomes. mRNA encoding apoB-15 was translated in the presence of control or PMME-enriched microsomes. Microsomes were re-isolated then incubated with sodium carbonate (pH 11.5). The supernatant (S), containing apoB that was not tightly associated with microsomal membranes, was separated from membranes (P) by ultracentrifugation. Proteins were subjected to polyacrylamide gel electrophoresis on a 10% gel in the presence of 0.1% SDS, followed by autoradiography. The arrowhead indicates apoB-15.

protein–lipid interactions, and converts membrane vesicles into sheets. Consequently, lumenal and peripheral membrane proteins are released from microsomes. When PMME-enriched microsomes were incubated with sodium carbonate (pH 11.5) after translation, a significant fraction of apoB-15 remained membrane-associated (Fig. 3B). In contrast, sodium carbonate treatment released the majority of apoB-15 from control microsomes. It should be noted that more molecules of apoB-15 were exposed to exogenously added protease in PMME-enriched micro-

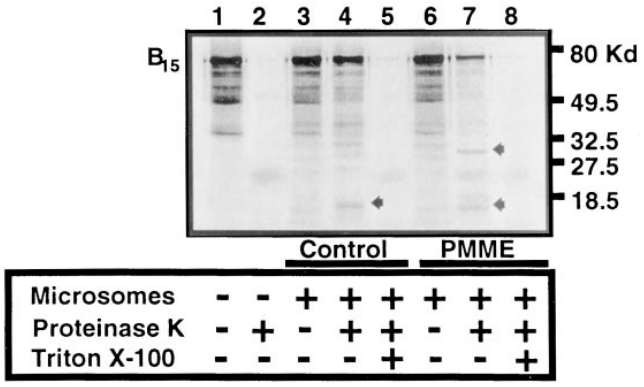

Fig. 4. Proteinase K proteolysis of in vitro translated apoB-15. ApoB-15 mRNA was translated in the presence of control or PMME-enriched microsomes as described in the legend to Fig. 2. Aliquots of translation products were digested with proteinase K in the presence or absence of 1% Triton X-100. Products were analyzed by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, followed by autoradiography. Molecular mass size standards are indicated at the right-hand side in kD. Arrowheads indicate proteolytic fragments of apoB-15.

somes, than in control microsomes (Fig. 2), and more molecules of apoB-15 were membrane-associated in PMME-enriched membranes than in control microsomes. These observations make unlikely the possibility that in PMME-enriched microsomes the extra "membrane-associated" apoB-15 was fully translocated but remained tightly associated with the membranes. Thus, these data indicate that apoB-15 translocates less completely into the lumen of PMME-enriched microsomes than of control microsomes.

ApoB-15 undergoes translocational pausing in control and PMME-enriched microsomes

Because PMME enrichment of microsomal membranes inhibits the translocation of apoB-15 but not other secretory proteins (17, 28), we hypothesized that the target for the action of PMME might be translocational pausing. Transmembrane protein fragments indicative of translocational pausing of apoB-15 can be readily detected when microsomes are incubated with proteinase K (9, 10, 13). Therefore, we performed an experiment parallel to the one depicted in Fig. 2 except that microsomes were incubated with proteinase K instead of trypsin. In support of the findings shown in Fig. 2, fewer full-length chains of apoB-15 were protected from proteinase K digestion in PMME-enriched microsomes than in control microsomes (**Fig. 4**, compare lanes 7 and 4). In both types of microsomes, a discrete fragment of apoB-15 (lower arrowhead, lanes 4 and 7) was generated upon proteinase K treatment. This peptide was protected from further digestion by proteinase K and was therefore lumenal. Digestion of PMME-enriched microsomes with proteinase K revealed an additional protease-resistant peptide fragment (upper arrowhead, lane 7) that was absent from control microsomes. Similar fragments have been detected previously and have been shown by immunoprecipitation to be derived from apoB-15 (9). Generation of these peptides indicates that translocational pausing of apoB-15 occurs in both types of membranes. However, more apoB-15 molecules span the membrane in the paused state in PMME-enriched microsomes than in control microsomes (Fig. 4).

Impaired restarting of translocation after translocational pausing in PMME-enriched microsomes

The above experiments suggested that both control and PMME-enriched membranes support translocational pausing (which is normally a transient event), but that pausing of apoB-15 might be prolonged in PMME-enriched microsomes compared to control microsomes. These observations might, therefore, explain why significantly less apoB-15 was translocated into the lumen of PMME-enriched microsomes than of control microsomes (Figs. 2 and 3). We investigated this possibility by utilizing a nascent-chain intermediate of apoB that has previously been demonstrated to be engaged in translocational pausing (13). Such an intermediate is generated upon translation of a mRNA that is truncated within the coding region and thus lacks an in-frame stop codon. Consequently, when the translating ribosome reaches the end of the mRNA, chain termination does not occur and the nascent chain is not released. Translocation intermediates produced in this manner appear to maintain the correct architecture of the translocon and ribosome around the nascent chain. By selecting a truncation point just beyond the coding segment for a pause transfer sequence, one can "trap" the nascent chain as it is engaged in a translocational pause. Furthermore, the pause can be released by treatment of the translocation intermediate with EDTA which disassembles the ribosome and releases the nascent chain, thereby allowing continued translocation across the membrane and movement of the peptide into the microsomal lumen (10, 13). Thus, translocational pausing can be experimentally dissected into discrete stopping and starting steps.

We examined which, if either, of these steps was affected by PMME-enrichment of microsomal membranes. As a model protein substrate we chose an epitope-tagged apoB-15, apoB–FXa, which contains a recognition site for factor Xa (**Fig. 5a**) (13). This construct allowed us to use the highly specific protease, factor Xa, as a probe for examining the exposure of nascent apoB chains to the cytosolic surface of membranes during a specific translocational pause. Previous studies have demonstrated that translocationally paused apoB–FXa nascent chains are exposed on the cytosolic side of the membrane rendering the factor Xa site accessible to factor Xa protease. Moreover, upon EDTA treatment, apoB–FXa resumes translocation and the factor Xa site becomes no longer exposed to the protease (13).

ApoB–FXa mRNA, generated from transcription of a cDNA truncated at the Bgl2 site, was translated in the presence of either control or PMME-enriched microsomes. Truncation at the Bgl2 site generated a paused translocation intermediate and exposed the factor Xa site on the cytosolic side of the membrane. After the translation reaction, the microsomes were isolated and incubated with factor Xa protease. Figure 5B shows that ~50% of nascent chains were cleaved by factor Xa in both con-

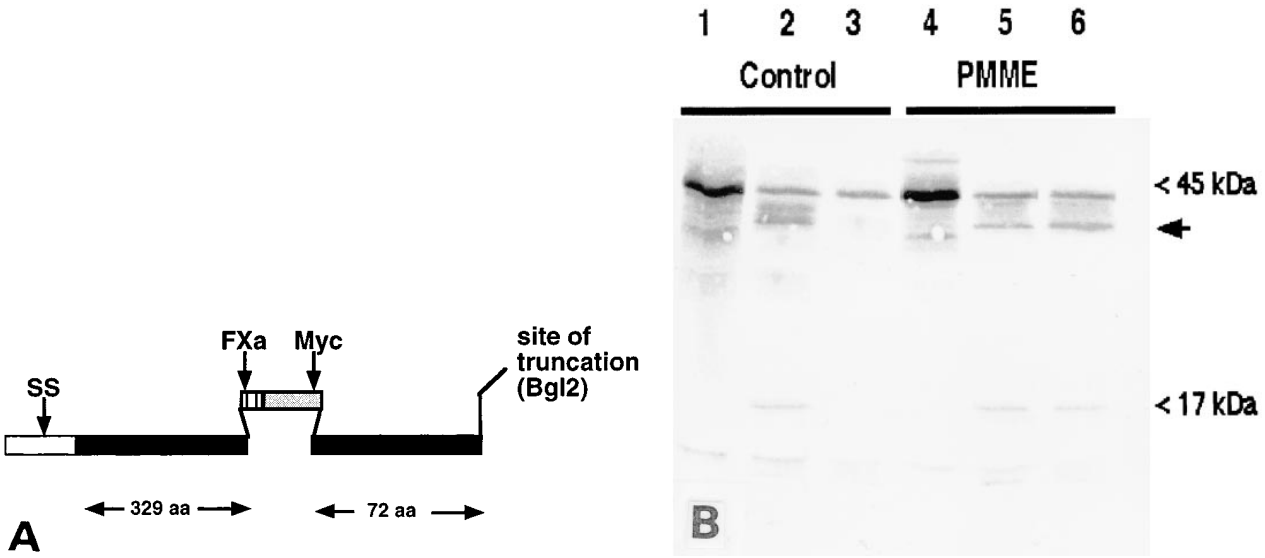

Fig. 5. Restarting of translocation of apoB–FXa after pausing is impaired in PMME-enriched microsomes. (A) The construct apoB–FXa. The signal sequence (SS, open box), the factor Xa cleavage site (FXa, vertical striped box) and sequences from human apoB-100 (solid boxes) are indicated. The *myc* epitope (Myc, stippled box) is also included in this construct. (B) The cDNA encoding apoB-FXa was truncated at the Bgl2 site and contained no in-frame stop codon. The corresponding mRNA was translated in the presence of PMME-enriched or control microsomes. One aliquot of translation products was incubated with EDTA, then microsomes were re-isolated and incubated with or without factor Xa protease. Products were analyzed by electrophoresis on a 10% polyacrylamide gel containing 0.1% SDS, followed by autoradiography. N- and C-terminal fragments of apoB–FXa are indicated by upward- and downward-pointing arrowheads, respectively.

trol and PMME-enriched microsomes (lanes 2 and 5, respectively), generating the expected N- and C-terminal fragments (indicated by the solid arrow below the 45 kDa marker and the arrowhead at 17 kDa, respectively) (13). Thus, in both control and PMME-enriched membranes, the apoB–FXa translocation intermediate pauses and the factor Xa site of the nascent chain becomes exposed on the outside of the microsomes. Upon treatment of control microsomes with EDTA after translation, nascent chains were no longer accessible to digestion by factor Xa (Fig. 5B, lane 3), indicating that the polypeptide had resumed translocation after the pause. In marked contrast, EDTA treatment of PMME-enriched microsomes had little or no effect on the accessibility of the nascent chains to factor Xa, as shown by the observation that ~50% of the chains were digested to their N- and C-terminal fragments (lane 6). These experiments demonstrate that in PMME-enriched microsomes, restarting of translocation of the paused apoB–FXa translocation intermediate was reduced compared to that in control microsomes, resulting in decreased translocation of the protein into the lumen.

DISCUSSION

Translocation and secretion of the atypical secretory protein apoB-100 are known to depend upon a supply of lipids that are required for assembly of lipoprotein particles such as VLDL (4). We now show that alteration of the phospholipid composition of microsomal membranes, by enrichment of the membranes in the normally quantitatively insignificant phospholipid PMME, can also regulate apoB translocation. In in vitro translation/translocation experiments we demonstrate that: *i*) translocation of apoB-15 into the microsomal lumen of PMME-enriched membranes is impaired, *ii*) more apoB-15 molecules are translocationally paused in PMME-enriched, than in control, microsomes, *iii*) a truncated, nascent-chain intermediate of apoB, previously demonstrated to be translocationally paused, is similarly paused in PMME-enriched microsomes, and *iv*) restarting of translocation of the paused translocation intermediate is defective in PMME-enriched, but not in control, microsomes. These observations suggest that the mechanism by which PMME inhibits apoB translocation and secretion is via impaired resumption of translocation after pausing.

The reason why pause transfer sequences in apoB induce a transient arrest in translocation, during which translocation is temporarily uncoupled from translation and domains of apoB become exposed to the cytosol, has not yet been established. However, as the secretion of apoB requires assembly with multiple lipid constituents (i.e., phospholipids, triacylglycerols, cholesterol and cholesteryl esters) one might speculate that lipid assembly and translocational pausing of apoB are related events. In our experiments, we have used a truncated variant of apoB (the N-terminal 15% of apoB-100) which is secreted in association with only small quantities of lipids, primarily phospholipids, but nevertheless undergoes translocational pausing.

In one model of apoB translocation, the translocation channel might partially and transiently disassemble during translocational pausing, thereby permitting contact between apoB and bilayer lipids. When a signal (the nature of which is so far unknown) is given for resumption

of translocation, the proteins of the translocon would re-assemble and translocation would resume. Possibly, in PMME-enriched microsomes, the paused apoB-15 chain becomes "derailed", escapes the translocation channel, and becomes anchored in the bilayer despite lacking a typical membrane-spanning sequence. Alternatively, during translocational pausing, domains of apoB that emerge from the lumenal side of the translocon might associate with lipids on the inner leaflet of the ER bilayer.

Analysis of the apoB-100 sequence has predicted that multiple pause–transfer sequences are present and are unevenly distributed throughout the molecule (35). Pause–transfer sequences themselves probably do not bind lipids because at least some of these sequences contain several charged amino acid residues (10, 11). More likely, lipids bind hydrophobic domains that flank pause–transfer sequences. The existence of several lipid binding domains of apoB-100 has been proposed (36). Similarly, a hydrophobic domain downstream of a pause–transfer sequence in the prion protein has been proposed to be responsible for integration of this protein into the membrane (37).

In recent in vitro translation experiments, in which lipoprotein assembly was reconstituted in the presence of active lipid synthesis, we demonstrated that apoB-15 and apoB-48 associate co-translationally with phosphatidylcholine, and that apoB-48 also associates with newly synthesized triacylglycerols during translation/translocation (22). We speculate that co-translational "painting" of apoB with lipids and/or the correct folding of the protein might be signals for resumption of translocation after pausing. Enrichment of microsomal membranes with PMME might therefore interrupt the association of apoB with some lipids during translocational pausing and thereby impair further translocation. Alternatively, modification of the phospholipid composition of the bilayer might alter the affinity of the pause–transfer sequence for a protein of the translocation apparatus [for example, TRAM (38) or the 11 kDa protein of the translocon which has been shown (13) to bind a protein containing a pause–transfer sequence from apoB], and consequently prevent or delay the restarting of translocation. Another possibility is that PMME enrichment of the membranes might interfere with the gating mechanism of the translocon on the lumenal side of the membrane (39).

We consider it unlikely that the microsomal triacylglycerol transfer protein is the target for the action of PMME on apoB translocation. This ER lumenal protein, which is required for assembly/secretion of apoB-containing lipoproteins, transfers lipids, particularly neutral lipids but also phospholipids, between membranes in vitro (40, 41). Roles for the microsomal triacylglycerol transfer protein in supplying lipids to apoB during and/or after translocation, and in facilitating translocation, have been proposed (23–25, 42). However, secretion of the N-terminal 17% of apoB does not depend on expression of the microsomal triacylglycerol transfer protein, at least in an insect system (43). In addition, PMME inhibits the secretion of truncated apoB variants (such as apoB-15) which do not assemble buoyant lipoprotein particles demonstrating that PMME affects the translocation event per se (28). We also demonstrated that in an in vitro translation/translocation system reconstituted in the presence of active lipid synthesis, the translocation of apoB-48 was not absolutely dependent on the presence of the transfer protein. Our experiments showed that in rat liver microsomes, which contain the transfer protein, apoB-48 was translocated across the microsomal membrane (i.e., was protected from exogenously-added protease) and buoyant apoB-48-containing lipoproteins were generated. However, in dog pancreatic microsomes, which lack the microsomal triacylglycerol transfer protein, apoB-48 was able to translocate across the membrane but did not assemble into buoyant lipoprotein particles (22). Therefore, it appears likely that the event in the secretion of apoB that is affected by PMME precedes the step requiring the microsomal triacylglycerol transfer protein.

In conclusion, our experiments support the concept that apoB undergoes a transient pausing event during translocation into the ER lumen. The studies also provide an explanation for why translocation and secretion of apoB are decreased in PMME-enriched microsomes (17, 26). We show that PMME enrichment of microsomal membranes impairs the restarting of translocation of a paused apoB translocation intermediate. Thus, more apoB molecules remain spanning the membrane and are accessible to the cytosolic environment. In intact cells, the consequence of prolonged exposure of apo B to the cytosol might be that apoB is targeted for destruction by cytosolic proteases such as the proteasome (19–21). Thus, the inability of apoB to restart translocation after a translocational pause would decrease apoB secretion by decreasing translocation of apoB into the ER lumen, and consequently apo B degradation would be increased. That these events can be modulated by membrane lipid composition raises the possibility that lipoprotein secretion can be regulated, physiologically as well as pharmacologically, by translocational pausing. Although we cannot extrapolate these in vitro findings, in which we used apoB peptides that did not assemble lipoproteins, to the in vivo situation in which larger apoBs (apoB-100 and B-48) assemble with lipids, we have no reason to think that physiologically relevant apoBs do not undergo translocational pausing in vivo. Nevertheless, confirmation of this assumption awaits the demonstration that translocational pausing occurs in cultured cells and/or intact animals.

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REFERENCES

1. Brunzell, J. D., A. D. Sniderman, J. J. Albers, and P. O. Kwiterowich. 1984. Apolipoproteins B and A-1 in coronary artery disease in humans. *Arteriosclerosis.* **4:** 79–83.

2. Pullinger, C. R., J. D. North, B. B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* **30:** 1065–1077.
3. Dixon, J. L., and H. N. Ginsberg. 1993. Regulation of hepatic secretion of apolipoprotein B-containing lipoproteins: information obtained from cultured liver cells. *J. Lipid Res.* **34:** 167–179.
4. Davis, R. A., and J. E. Vance. 1996. Structure, assembly, and secretion of lipoproteins. In Biochemistry of Lipids, Lipoproteins and Membranes. D. E. Vance, and J. E. Vance, editors. Elsevier Science, Amsterdam. 473–493.
5. Borchardt, R. A., and R. A. Davis. 1987. Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. *J. Biol. Chem.* **262:** 16394–16402.
6. Bonnardel, J. A., and R. A. Davis. 1995. In HepG2 cells, translocation, not degradation, determines the fate of the de novo synthesized apolipoprotein B. *J. Biol. Chem.* **270:** 28892–28896.
7. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
8. Yang, C. Y., S. H. Chen, S. H. Gianturco, W. A. Bradley, J. T. Sparrow, M. Tanimura, W. H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F. Lee, Z. W. Gu, A. M. Gotto, and L. Chan. 1986. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature.* **323:** 738–742.
9. Chuck, S. L., Z. Yao, B. D. Blackhart, B. J. McCarthy, and V. R. Lingappa. 1990. New variation on the translocation of proteins during early biogenesis of apolipoprotein B. *Nature.* **346:** 382–385.
10. Chuck, S. L., and V. R. Lingappa. 1992. Pause transfer: a topogenic sequence in apolipoprotein B mediates stopping and restarting of translocation. *Cell.* **68:** 9–21.
11. Chuck, S. L., and V. R. Lingappa. 1993. Analysis of a pause transfer sequence from apolipoprotein B. *J. Biol. Chem.* **268:** 22794–22801.
12. Skach, W. R., and V. R. Lingappa. 1992. Intracellular trafficking of pre(pro)protein across RER membranes. *In* Mechanisms of Intracellular Trafficking and Processing of Proteins, Y. P. Loh, editor. CRC Press, Boca Raton, FL. 19–77.
13. Hegde, R. S., and V. R. Lingappa. 1996. Sequence-specific alteration of the ribosome-membrane junction exposes nascent secretory proteins to the cytosol. *Cell.* **85:** 217–228.
14. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266:** 5080–5086.
15. Borén, J., L. Graham, M. Wettesten, J. Scott, A. White, and S-O. Olofsson. 1992. The assembly and secretion of apoB-100-containing lipoproteins in Hep G2 cells. ApoB-100 is cotranslationally integrated into lipoproteins. *J. Biol. Chem.* **267:** 9858–9867.
16. Thrift, R. N., J. Drisko, S. Dueland, J. D. Trawick, and R. A. Davis. 1992. Translocation of apolipoprotein B across the endoplasmic reticulum is blocked in a nonhepatic cell line. *Proc. Natl. Acad. Sci. USA.* **89:** 9161–9165.
17. Rusiñol, A .E., E. Y. W. Chan, and J. E. Vance. 1993. Movement of apolipoprotein B into the lumen of microsomes from hepatocytes is disrupted in membranes enriched in phosphatidylmonomethylethanolamine. *J. Biol. Chem.* **268:** 25168–25175.
18. Verkade, H. J., D. G. Fast, A. E. Rusiñol, D. G. Scraba, and D. E. Vance. 1993. Impaired biosynthesis of phosphatidylcholine causes a decrease in the number of very low density lipoprotein particles in the Golgi but not in the endoplasmic reticulum of rat liver. *J. Biol. Chem.* **268:** 24990–24996.
19. Yeung, S. J., S. H. Chen, and L. Chan. 1996. Ubiquitin–proteasome pathway mediates intracellular degradation of apolipoprotein B. *Biochemistry.* **35:** 13843–13848.
20. Fisher, E. A., M. Zhou, D. M. Mitchell, X. Wu, S. Omura, H. Wang, A. L. Goldberg, and H. N. Ginsberg. 1997. The degradation of apolipoprotein B-100 is mediated by the ubiquitin–proteasome pathway and involves heat shock protein 70. *J. Biol. Chem.* **272:** 20427–20434.
21. Benoist, F. and T. Grand-Perret. 1997. Co-translational degradation of apolipoprotein B-100 by the proteasome is prevented by microsomal triglyceride transfer protein. *J. Biol. Chem.* **272:** 20435–20442.
22. Rusiñol, A. E., H. Jamil, and J. E. Vance. 1997. In vitro reconstitution of assembly of apolipoprotein B-48-containing lipoproteins. *J. Biol. Chem.* **272:** 8019–8025.
23. Sharp, D., L. Blinderman, K. A. Combs, B. Kienzle, B. Ricci, K. Wager-Smith, C. M. Gil, C. W. Turck, M-E. Bouma, D. J. Rader, L. P. Aggerbeck, R. E. Gregg, D. A. Gordon, and J. R. Wetterau. 1993. Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinemia. *Nature.* **365:** 65–68.
24. Gordon, D. A., J. R. Wetterau, and R. E. Gregg. 1995. Microsomal triglyceride transfer protein: a protein complex required for the assembly of lipoprotein particles. *Trends Cell Biol.* **5:** 317–321.
25. Du, E. Z., S.-L. Wang, H. J. Kayden, R. Sokol, L. K. Curtiss, and R. A. Davis. 1996. Translocation of apolipoprotein B across the endoplasmic reticulum is blocked in abetalipoproteinemia. *J. Lipid Res.* **37:** 1309–1315.
26. Vance, J. E. 1991. Secretion of VLDL, but not HDL, by rat hepatocytes is inhibited by the ethanolamine analogue *N*-monomethylethanolamine. *J. Lipid Res.* **32:** 1971–1982.
27. Rusiñol, A. E., P. S. Lysak, G. T. Sigurdson, and J. E. Vance. 1996. Monomethylethanolamine reduces plasma triacylglycerols and apolipoprotein B and increases apolipoprotein A-1 in rats without induction of fatty liver. *J. Lipid Res.* **37:** 2296–2304.
28. Rusiñol, A. E., and J. E. Vance. 1995. Inhibition of secretion of truncated apolipoproteins B by monomethylethanolamine is independent of the length of the apolipoprotein. *J. Biol. Chem.* **270:** 13318–13325.
29. Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for co-translational protein translocation. *Methods Enzymol.* **96:** 84–93.
30. Fujiki, Y., A. L. Hubbard, S. Fowler, and P. B. Lazarow. 1982. Isolation of intracellular membranes by means of sodium carbonate treatment: application to endoplasmic reticulum. *J. Cell Biol.* **93:** 97–102.
31. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
32. Macala, L. J., R. K. Yu, and S. Ando. 1983. Analysis of brain lipids by high performance thin layer chromatography and densitometry. *J. Lipid Res.* **24:** 1243–1250.
33. Rouser, G., A. N. Siakatos, and S. Fleischer. 1966. Quantitative analysis of phospholipids by thin-layer chromatography and phosphorus analysis of spots. *Lipids.* **1:** 85–86.
34. Ridgway, N. D. and D. E. Vance. 1992. Phosphatidylethanolamine *N*-methyltransferase from rat liver. *Methods Enzymol.* **209:** 366–374.
35. Kivlen, M. H., C. A. Dorsey, V. R. Lingappa, and R. S. Hegde. 1997. Asymmetric distribution of pause transfer sequences in apolipoprotein B-100. *J. Lipid Res.* **38:** 1149–1162.
36. Segrest, J. P., M. K. Jones, V. K. Mishra, G. M. Anantharamaiah, and D.W. Garber. 1994. ApoB-100 has a pentapartite structure composed of three amphiphilic α-helical domains alternating with two amphipathic β-strand domains. *Arterioscler. Thromb.* **14:** 1674–1655.
37. Nakahara, D. H., V. R. Lingappa, and S. L. Chuck. 1994. Translocational pausing is a common step in the biogenesis of unconventional integral membrane and secretory proteins. *J. Biol. Chem.* **269:** 7617–7622.
38. Hegde, R. S., S. Voigt, T. A. Rapoport, and V. R. Lingappa. 1998. TRAM regulates the exposure of nascent secretory proteins to the cytosol during translocation into the endoplasmic reticulum. *Cell.* **92:** 621–631.
39. Liao, S., J. Lin, H. Do, and A. E. Johnson. 1997. Both lumenal and cytosolic gating of the aqueous ER translocon pore are regulated from inside the ribosome during membrane protein integration. *Cell.* **90:** 31–41.
40. Wetterau, J. R., and D. B. Zilversmit. 1985. Purification and characterization of microsomal triglyceride and cholesteryl ester transfer protein from bovine liver microsomes. *Chem. Phys. Lipids.* **38:** 205–222.
41. Jamil, H., J. K. Dickson, C-H. Chu, M. W. Lago, J. K. Rinehart, S. A. Biller, R. E. Gregg, and J. R. Wetterau. 1995. Microsomal triglyceride transfer protein: specificity of lipid binding and transport. *J. Biol. Chem.* **270:** 6549–6554.
42. Gordon, D. A., H. Jamil, R. A. Gregg, S-O. Olofsson, and J. Borén. 1996. Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. *J. Biol. Chem.* **271:** 33047–33053.
43. Gretch, D. G., S. L. Sturley, L. Wang, B. A. Lipton, A. Dunning, K. A. A. Grunwald, J. R. Wetterau, Z. Yao, P. Talmud, and A. D. Attie. 1996. The amino terminus of apolipoprotein B is necessary but not sufficient for microsomal triglyceride transfer protein responsiveness. *J. Biol. Chem.* **271:** 8682–8691.