Interactions between Microsomal Triglyceride Transfer Protein and Apolipoprotein B within the Endoplasmic Reticulum in a Heterologous Expression System

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When apolipoprotein B (apoB) is expressed in heterogeneous cells, it is not secreted but retained and degraded within the endoplasmic reticulum (ER). We have previously characterized carboxyl-terminal truncated forms of apoB expressed in COS cells and have shown that these proteins were readily synthesized but retained within the ER and degraded, if the size of the truncated protein was larger than apoB 29. Below this size, the smaller the size of the apoB truncates, the larger the extent of secretion, although >50% of these smaller proteins were also degraded within the ER. In the present study, we demonstrate that this secretory defect can be overcome by coexpression with microsomal triglyceride transfer protein (MTP); moreover, this complementation is inversely related to the size of apoB. Secretion of apoB 41s larger than B29 required the coexpression of MTP and, in the presence of MTP, was oleate-responsive. MTP, in the presence or absence of oleate supplementation, had little or no effect on the secretion of the shorter truncates. We discovered, however, that MTP was physically associated with all forms of apoB intracellularly (B13–B41). The association of MTP with apoB 41 was stable to high salt washing, as well as to low pH, suggesting that these interactions may be hydrophobic in nature. In addition to the interaction with MTP, apoB was also found to be associated with calnexin, confirming previous studies, and with proteins bearing the KDEL retention signal. However, studies on overexpression of human calnexin and tunicamycin inhibition of glycosylation showed that interaction with calnexin was not necessary for the formation or secretion of apoB 41-containing lipoproteins; moreover, in the presence of MTP, the association of calnexin with apoB 41 was transient or absent. These data suggest that for apoB to attain a folded state sufficient to escape the quality control of the ER, it needs to obtain neutral lipid (supplied by MTP), as well as its ability to keep it packaged as a rudimentary lipoprotein, dependent on its size being larger than B29.

The ER plays a pivotal role in the fate of a nascent protein. All secretory and transmembrane proteins must, in general, attain some structural maturity to escape the "quality control" of the ER. Inability to attain "maturity" within the ER results in retention and degradation (1–4). ApoB must undergo a series of conformational changes that would allow it to proceed through the ER. This step appears to be rate-limiting, since up to 80% of newly synthesized apoB 100 is normally degraded (5–8), a process that seems inefficient, given it takes approximately 10 min to fully synthesize this 550-kDa apoB 100 protein (9, 10).

For a complex secretory protein, such as apoB, maturation involves not only the constitutional machinery of the ER to impart a structure allowing it to proceed onto the Golgi but also specialized chaperones that are necessary for this progression. This requirement is evidenced by the autosomal recessive disorders of abetalipoproteinemia and chylomicron retention disease (11); in both of these cases, apoB synthesis appears to be normal, but there is no secretion of apoB from the appropriate tissues. For abetalipoproteinemia, the genetic defect has been identified, cloned, and shown to involve a protein complex, consisting of the microsomal triglyceride transfer protein (MTP) and protein disulfide isomerase (PDI), that transfers neutral lipid into the core of the nascent lipoprotein particle (12, 13). For chylomicron retention disease, no chromosomal localization or candidate genes have so far been identified, although it is known that both the MTP and apoB genes are not the sites of defects (13–15).

A considerable amount has already been learned about the early biogenesis of apoB-containing lipoproteins, based on in vitro studies. A model of biogenesis of has emerged whereby apoB is cotranslationally inserted into the ER membrane, where it follows one of two fates; it is either inserted into the inner leaflet of the ER membrane, or it may begin to form a rudimentary lipoprotein particle cotranslationally and is translated directly into the ER lumen (6, 16–18). This latter particle is destined for secretion and may represent the "constitutive" pathway. apoB inserted into the inner ER membrane, in contrast, may be destined for ER degradation. It is unclear whether this pool of apoB is diverted to the secretory pathway, at the expense of degradation, when the cells are supplemented with oleate to increase secretion (19). Very few of the specific ER proteins involved in controlling the fate of apoB in the ER have been characterized, although it is known that apoB is one of many denovo synthesized glycoprotein proteins that interact.

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The abbreviations used are: ER, endoplasmic reticulum; apoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; PDI, protein disulfide isomerase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; BiP, binding protein; gr, glucose-regulated protein.
with calnexin (20). The identification of MTP has been a major advance in this early process; Gordon et al. (22) have shown that MTP expression can overcome the secretory block on apoB 53 (using the centile numbering (21), full-length is apoB 100, 550 kDa) secretion from HeLa cells, and preliminary findings by Leiper et al. (23) have shown that coexpression of apoB 41 and MTP in COS cells can also lead to apoB 41 secretion (23).

To investigate the early steps involved in apoB secretion, we have characterized heterologous expression of carboxyl-terminal truncated apoB5 in COS cells. Previously, we showed that when apoB 41 is expressed in COS cells, it was fully synthesized, N-glycosylated, but was then degraded in the ER or an ER-related compartment (24). Expression of apoB75 that were progressively truncated led to some secretion of the smaller truncates. Others have reported similar findings in various cell systems; for example, expression of apoB truncates in COS cells led to their intracellular degradation (7), apoB 17 was secreted from mouse mammary tumor line C-127 (25), and expression of apoB 53 in CHO cells resulted in a rapid degradation in the ER, a process that could be inhibited by a protease inhibitor, calpain I (26). Furthermore, coexpression of apoB and MTP in HeLa (22) or COS cells (23) abrogates this ER retention and degradation, resulting in secretion of a lipoprotein particle in the high density lipoprotein range. These studies suggest that the ability of non-hepatic/non-intestinal cells to secrete apoB can be conferred solely by the coexpression of MTP. We have utilized this approach to characterize this system further and suggest that heterologous expression may be useful in defining some of the early events in apoB-containing lipoproteins.

In the present study, we have investigated the interaction of MTP with the various sizes of apoB carboxyl-truncates to determine whether expression of MTP can affect secretion of small apoB proteins that are incapable of forming lipoproteins (27, 28). We have characterized the nature of interaction of MTP with these truncates. Finally, we have studied what role that resident ER proteins, such as calnexin, may play in the biogenesis of apoB-containing lipoproteins.

**EXPERIMENTAL PROCEDURES**

Reagents—Tissue culture medium, sheep antibodies to apoB, and reagents were obtained as described previously (24). Rabbit antiserum to MTP (13) was a gift from Dr. John Watterson (Bristol-Myers Squibb) and mouse antibody, A139, to human calnexin was a gift from Dr. Michael Brenner (29). Although the rabbit antiserum to MTP has been well characterized for Western blotting to detect MTP, we have found that it can also immunoprecipitate MTP, although this may not be quantitative. Rabbit antiserum to canine calnexin and mouse monoclonal antibody to peptide KSEKDEL (clone 10C3, recognizes BiP/grp 78, grp 94, and HSP 40) (30) were obtained from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). Mouse monoclonal antibody against human apoB, C1.4 (31), was a gift from Dr. Gustav Schonfeld. All other chemicals were of reagent grade or better.

DNA Constructs—The pSV7-D-based expression plasmids containing carboxyl-truncated apoB fragments are as described previously (24, 32). The expression clones for MTP (under agreement from Bristol-Myers Squibb, N.J.) and calnexin (gift from Dr. Brenner) are as described previously (29), except the MTP was driven by the Rous sarcoma virus promoter in the vector pRSV.Neo (Invitrogen Corp.).

Cell Culture, Transfection, Metabolic Labeling, and Immunoprecipitation—COS-1 cells, maintained in DMEM supplemented with 10% FCS, were transfected, and quantitative pulse-chase analyses were carried out as described previously (24), except 10 μg of each DNA for cotransfection was used unless otherwise stated. In all experiments where treatment with inhibitors or oleate was performed, the control pool of cells was always derived from the same pool of cells as the treated cells, maintaining identical transfection efficiencies. Briefly, cell lysis was performed in 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, and 1% Triton X-100. The cell lysates were cleared of cell debris by centrifugation at 13,000 × g for 15 min at 4°C; total protein concentrations were determined using a dye-binding assay (Bio-Rad) and immunoprecipitated with the appropriate antibodies, collected by protein A-Sepharose beads and analyzed as described previously (24). Sodium phosphate buffers were used for the investigation of the effects of pH on MTP-apoB complexes at a final concentration of 100 mM. Immunoprecipitates were incubated at room temperature for 5 min between each wash. For calnexin immunoprecipitations, 1% CHAPS was substituted for Triton X-100. In the presence of Triton X-100, calnexin-associated proteins appeared to be less stably bound (data not shown). 2

For tunicamycin inhibition, 24 h after transfection, the cells were treated with 0.02% DMSO or 0.02% DMSO and 4 μg/ml tunicamycin (final concentrations). This treatment resulted in greater than 90% inhibition of labeled glucosamine incorporation into total cell acid-precipitable material, as well as into immunoprecipitated apoB 41 (data reviewed but not shown). Immunoprecipitation and SDS-PAGE was performed essentially as described previously. Quantitation was performed by exposure of the dried gels to PhosphorImager screens and analysis using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

For pulse labeling with [3H]oleate (specific activity, 60 Ci/mmol; Moravek Biochemicals, Brea, CA), transfected cells were incubated for 18 h in labeled oleate at 83.3 μCi/ml, the medium was cleared of cellular debris, and subjected to cesium chloride density centrifugation and fractionated as described below. The density of each fraction was determined as described previously, and total lipids were extracted by the Folch method (33), concentrated under a nitrogen stream, loaded onto thin layer silica gel plates, and chromatographed in hexane-ethanol-acetic acid mixture (80:20:1); then the radiolabeled lipid species was detected by autoradiography, after spraying the plates with En- hance spray (DuPont NEN). Specific bands, identified by comparison to standards, were scraped off the plates and quantitated by liquid scintillation counting.

Cesium Chloride Density Gradient Centrifugation—Density gradient ultracentrifugation was performed as described previously (24). ApoB was either immunoprecipitated when radiolabeled protein was present, as above, or the samples were desalted and concentrated in Centricon 30 filters (Amicon, Beverly, MA) separated by SDS-PAGE, and analyzed by Western blotting, using C1.4 primary antibody.

Western Blotting—Immunoprecipitated proteins from equal amounts of cell lysates were separated by SDS-PAGE and electroblotted in 25 mA Tris, pH 8.3, and 192 mA glycine with 20% methanol onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). The membranes were blocked in 5% non-fat milk, incubated with primary antibodies at a dilution of 1:1000, washed extensively, incubated with secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) at a dilution of 1:10,000, and washed; then the antibody conjugates were detected by chemiluminescence using the Renaissance kit from DuPont NEN as per kit instructions.

**RESULTS**

Effects of Coexpression of MTP and apoB on Secretion and Oleate Responsiveness—This study was carried out to determine whether the "secretory block" and lack of oleate responsiveness reported on carboxyl-terminal truncated apoB5 larger than B29 (24) could be overcome by coexpression with MTP. Coexpression of apoB with MTP in COS cells resulted in complementation of the secretory block for apoB truncates that are larger than B29, and the rates of secretion were then responsive to oleate supplementation (Fig. 1). Coexpression of MTP with apoB truncates smaller than B29 did not result in increased secretion of these proteins (data not shown); furthermore, oleate supplementation also had a small or no effect on the secretory rates (Fig. 1). In contrast, supplementation of oleate, in the presence of MTP, had the greatest effect on apoB 41 (Fig. 1). Although there is variability due to transfection efficiencies on expression of both cDNAs in individual cells, we have found that oleate supplementation consistently increased apoB 41 secretion from 10 ± 2% (S.E.) to 30 ± 6.1% (n = 4), for apoB 29 from 14.7 ± 2.4% to 22.6 ± 3.5% (n = 6), and for apoB 23 from 31.9 ± 4.0% to 39.8 ± 4.5% (n = 7) during the 5-h chase period. Oleate and MTP had little or no effect on the

2 S. Rajagopalan, personal communication.
shorter truncates, apoB 13 (32.6 \pm 7.4\% \text{ versus } 33.1 \pm 3.1\%, n = 3) and apoB 17 (30.8 \pm 4.4\% \text{ versus } 28.7 \pm 3.8\%, n = 5).

Although the data shown for apoB 23 (Fig. 1) would suggest that oleate could have subtle effects, such as a reduction in the rate of degradation of intracellular apoB, this finding was not consistent, and in the majority of the experiments, no significant differences were observed for the effects of oleate supplementation on the kinetics of degradation.

Examination of the buoyant densities of apoBs secreted in the presence of MTP showed that apoBs smaller than apoB 29 were relatively lipid poor (Fig. 2), whereas newly secreted apoB 41 was present at a density of around 1.15 g/ml. Secreted apoB 13 was only found at a density of about 1.24 g/ml, suggesting it did not contain any lipid.

Buoyant Density and Lipid Profile of Secreted ApoB 41 Lipoprotein—To examine the buoyant density of secreted apoB 41 further, COS cells cotransfected with MTP and apoB 41, pulse-labeled and chased in medium containing no serum (Fig. 3, A and B), 10\% fetal calf serum (C), or 10\% fetal calf serum and 0.8 mM oleate (D) were studied. Fig. 3B shows the effect of incubating secreted apoB 41, in the absence of serum, in 10\% bovine serum albumin/0.8 mM oleate complex for 2 h at 37 °C prior to density gradient centrifugation, and fractionation. In the absence of oleate supplementation of the cells, apoB 41 was secreted with a buoyant density of around 1.15 g/ml, and secreted apoB 41 did not appear to obtain lipid directly from the medium, as shown by the mixing experiment (Fig. 3B). Oleate supplementation led to the secretion of apoB 41 particles of densities as low as 1.09 g/ml. To examine the lipid content of the secreted apoB 41 particles, cotransfected cells were pulse-labeled with [3H]oleic acid, and the medium was subjected to density gradient centrifugation and fractionation. Lipid from each fraction was extracted by the Folch method (33) and analyzed by thin layer chromatography, as described under "Experimental Procedures." Fig. 4A shows the chromatograms of the labeled lipid fraction at a density of about 1.14 g/ml secreted from apoB 41/MTP or apoB 41 alone transfected cells. The chromatograms shown are for fractions 4 shown in Fig. 4B. In the presence of MTP, a large amount of triglyceride was detected at buoyant densities where secreted apoB 41 is expected, but very little was seen in the control medium. Fig. 4B shows quantitation of the triglyceride profiles...
across the gradients. Fraction 1 is from the bottom of the density gradient centrifugation and contains some cellular debris not removed by the pre-spin of the medium. As can be seen, there is a significant amount of triglyceride associated with fractions 3–6, in the density range 1.18–1.12 g/ml, where secreted apoB41 would be expected to float (see Figs. 2 and 3 for comparison). Quantitation of the radioactivity migrating at the cholesterol ester position across the gradients did not show a significant difference when compared to the control profile (data not shown).

Hydrophobic Interactions between MTP and ApoB—In the course of our pulse-chase studies, it was noted that, when MTP was cotransfected with any of the apo Bs (B13–B41), a protein species coprecipitated with apoB; this was absent when MTP was omitted (Fig. 5A). To exclude the possibility that the antibodies could cross-react with MTP or apoB, COS cells transfected with apoB41 alone (B: tracks 1 and 2) or MTP alone (B: tracks 3 and 4) and immunoprecipitated with anti-apoB and anti-MTP antibodies. Anti-MTP antibody does not immunoprecipitate apoB (B: track 2), and apoB antiserum does not immunoprecipitate MTP (B: track 3).
ApoB was immunoprecipitated and analyzed by SDS-PAGE. The migration of apoB 41 and MTP are as indicated by the arrowheads. A band comigrating with the mobility of MTP coprecipitates when apoB is immunoprecipitated, and a faint band with the mobility of apoB 41 is apparent when MTP is immunoprecipitated. To further confirm this association, cell lysates from unlabeled cotransfected cells were immunoprecipitated with MTP, Western blotted, and probed for the presence of apoB (track 1) or immunoprecipitated for apoB, Western blotted, and probed for the presence of MTP. Immunoreactive bands are seen in both cases. The immunoreactive bands at around 50 kDa are the heavy chains from the immunoprecipitating antibodies (sheep anti-apoB and rabbit anti-MTP) that cross-react with the secondary anti-mouse immunoglobulin antibody.

appropriate antigen and do not show considerable cross-reactivity to other proteins.

To uncover the identity of the protein coprecipitating with apoB, immunoprecipitation from labeled cells as well as Western blotting was used (Fig. 6). Cells were cotransfected with apoB 41 and MTP, pulse-labeled, lysed, and immunoprecipitated for apoB (Fig. 6A, track 1) or MTP (Fig. 6B, track 2). A band comigrating with MTP was present when apoB 41 was immunoprecipitated. A faint band comigrating with apoB 41 was also present when MTP was specifically immunoprecipitated (Fig. 6A, track 2). The protein band above apoB 41 (Fig. 6A, track 2) was also present when nonimmune serum was used. Although a considerable amount of labeled MTP coprecipitated with apoB (Fig. 6A, track 1), very little apoB coprecipitated when MTP was immunoprecipitated (Fig. 6A, track 2). In sequential immunoprecipitation studies, precipitation with apoB antiserum appears to immunoprecipitate more MTP than the rabbit antiserum against MTP (data not shown, but see Fig. 6). The anti-MTP antibody has been used for Western blotting previously but not for immunoprecipitation. Our studies would suggest that this reagent can immunoprecipitate MTP, but this may, therefore, not be used for quantitative purposes. To further prove that MTP coprecipitated with apoB and vice versa, cell lysates from unlabeled cotransfected cells were immunoprecipitated with anti-MTP antiserum (Fig. 6B, track 2) or with anti-apoB antiserum (Fig. 6B, track 2) and Western blotted for the corresponding protein. ApoB 41 could be detected when MTP was immunoprecipitated, and MTP could be detected when apoB 41 was immunoprecipitated. In control experiments where MTP was omitted or when nonimmune sera were used, no such coprecipitation was detected.

To examine the nature of the association of MTP with apoB 41, immunoprecipitation with anti-apoB antiserum was performed, and the immunoprecipitates were washed in increasing concentration of salt. Fig. 7 shows a salt wash profile, when immunoprecipitation was performed in 150 mM NaCl (Fig. 7, track 1) and washed at various salt concentrations as indicated. The association of MTP with apoB 41 appeared to be unaffected by salt; this finding was reproducible, even when initial lysis was performed at the various salt concentrations indicated (data not shown). We have previously reported coprecipitation of an unidentified protein (80 kDa) with apoB 41 from COS cells (24). This protein is indicated by the asterisk (Fig. 7). It also was resistant to high salt washing. The interaction of MTP and apoB 41 was also stable at pH 8.0–pH 5.8 (Fig. 8). Labeled apoB 41 and MTP coprecipitates (using anti-apoB antiserum) were isolated as described under “Experimental Procedures” in 150 mM NaCl at pH 7.5 and washed in buffers at the various pH conditions shown. To account for the dissociation of apoB 41, the apoB 41 and MTP bands were quantitated and expressed as a ratio of apoB 41 to MTP (arbitrary units). Preferential removal of MTP should result in a dramatic rise in this ratio. Lowering the pH of the wash buffers resulted in some loss of apoB 41, presumably via antibody-antigen dissociation (compare apoB 41 intensity, tracks 1 with 5 and 6, Fig. 8). However, the proportion of MTP associated with apoB 41 under these conditions remains comparable (ratio of apoB 41 to MTP is 2.3 at pH 8.0 compared with 1.9 at pH 5.8). Thus, this interaction was also relatively insensitive to pH changes. Note that the unidentified protein that coprecipitates also with apoB 41 appears to dissociate at a pH less than 6.8 (Fig. 8, track 4).

Association of Calnexin and KDEL-bearing ER Proteins with ApoB—ApoB is likely to interact with a variety of other proteins in the ER as it undergoes a complex folding process to
form a nascent lipoprotein particle. We have investigated whether apoB could be coprecipitated from COS cells transfected with either apoB 41, MTP, or both using antibodies directed against calnexin, carboxyl-terminal peptide KDEL (a peptide ER retention signal used by many resident ER proteins) (34) or MTP. In data not shown, when cells transfected with apoB 41 were radiolabeled and immunoprecipitated for calnexin, BiP, or MTP, a band with the migration of apoB 41 was seen to coprecipitate. To show that this protein is indeed apoB 41, Western blotting for apoB was performed on similar immunoprecipitates (Fig. 9). Tracks 1, 4, and 7 represent COS cells transfected with apoB 41 alone; tracks 2, 5, and 8, MTP alone; and tracks 3, 6, and 9, cotransfected with apoB 41 and MTP. Tracks 1–3 were immunoprecipitated with rabbit anti-calnexin antiserum, tracks 4–6 with mouse anti-peptide KSEKDEL monoclonal antibody (10C3), and tracks 7–9 with mouse monoclonal antibody (10C3). As can be seen, only BiP/grp 78 and grp 94 are detected, and no cross-reactivity to apoB 41 or MTP was detected.

Note that in the presence of MTP, apoB could not be readily detected to coprecipitate with calnexin. Calnexin appears to interact with nascent glycoproteins at the early stages of synthesis (20, 35), and this appears to be mediated via the glucosylation state of the de novo synthesized glycoproteins (36). To investigate the interaction of calnexin and apoB 41 further, we carried out kinetic as well as coimmunoprecipitation studies in the presence of tunicamycin, an inhibitor of N-glycosylation (37). Tunicamycin treatment should, therefore, prevent calnexin binding to apoB 41 in the ER. In control experiments, treatment of COS cells transfected with apoB 41 showed that tunicamycin treatment resulted in 90% inhibition of 3H-labeled glucosamine incorporation into acid-precipitable material in total cell lysates as well as immunoprecipitated apoB 41. Fig. 10 shows Western blotting analyses of two experiments of COS cells transfected with apoB 41, cultured for 24 h in tunicamycin prior to cell lysis, and immunoprecipitated with anti-calnexin antibodies. Immunoprecipitates from equal numbers of cells were loaded. In the presence of tunicamycin, very little of apoB 41 was detectable. Tracks 2, 5, and 8 serve as negative controls for the antibodies, because these cell lysates do not contain any apoB 41. As a positive control, when MTP is immunoprecipitated, apoB 41 is also detectable. Tracks 2, 5, and 8 show cross-reactivity to apoB 41 or MTP.

Quantitative analyses showed no significant differences in the kinetics of apoB 41.
and, hence, secretion of apoB. We cotransfected human calnexin cDNA and apoB 41 to investigate whether overexpression of calnexin would lead to secretion of apoB 41. No secretion of apoB 41 was seen under these conditions, although communoprecipitation of apoB and human calnexin was also demonstrable (data not shown). Similarly, transfection of apoB 41, human calnexin, and MTP together did not result in enhancement of apoB 41 secretion when compared to apoB 41 and MTP alone (data not shown).

**DISCUSSION**

MTP Effects on ApoB Secretion and Oleate Responsiveness—When MTP is coexpressed with apoB truncates, apoBs larger than B29 are secreted; moreover, secretion becomes responsive to oleate supplementation and secreted apoB particles contain newly synthesized triglyceride. MTP coexpression does not significantly alter the metabolism of apoBs smaller than B29, and the secretion of these species, in the presence of MTP, is not responsive to oleate supplementation. To place these results in context, studies in human and rat hepatoma cell lines using carboxyl-terminal apoB cDNAs have shown that there is a linear relationship between the size of apoB and its buoyant density and diameter (7, 27, 28, 32). Hence, the larger the apoB, the greater the amount of lipid it is secreted with, and the greater its lipoprotein diameter. However, this relationship appears to breakdown around truncates in the B26–B29 range. Below this size, the apoB proteins do not bind sufficient lipid and have densities of more than 1.2 g/ml (38). In our system, we can now demonstrate that MTP affects the secretion of apoBs larger than B29 and that the oleate responsiveness is also only evident for apoBs larger than B29. Oleate responsiveness has been shown previously to be mediated at the posttranslational level, primarily at the expense of apoB degradation. Our studies demonstrate that MTP mediates oleate responsiveness, since only in the presence of MTP is the oleate effect seen. We cannot distinguish whether this effect results from increased production of cotranslationally formed lipoproteins (and decreased membrane-inserted apoB) or whether MTP attaches to the membrane-inserted apoB and converts it into luminal rudimentary lipoprotein. However, since apoB interaction with calnexin is less pronounced in the presence of MTP, we favor the latter mechanism (see below).

Our experiments show that apoB 41, secreted in the presence of MTP, contains a significant amount of newly synthesized triglyceride. Incubation with labeled oleate showed that much of this fatty acid was incorporated as triglyceride into the secreted lipoprotein, and very little was present as cholesterol ester. Although we could not detect any changes in cholesterol ester levels in our experiments, we cannot exclude the possibility that some cholesterol ester is necessary for apoB secretion. Previous studies have shown that newly synthesized triglycerides are preferentially incorporated into the lipoprotein particles, and our studies in a non-hepatic line corroborate this.

**What is not clear is the source of this pool. MTP is not an enzyme but appears to facilitate the transfer of neutral lipid from one compartment to another to equilibrium. MTP would, therefore, need to be in contact with both the triglyceride donor pool (from an active metabolic site) and the nascent apoB particle. Although the specific site of the MTP-PDI complex within the ER has not been localized, it is likely that this complex is luminal, since neither proteins have transmembrane domains. Hence, this complex must be in contact with a metabolically active neutral lipid pool, as well as apoB to facilitate transfer of lipid into the nascent lipoprotein particle.**

**MTP and ApoB Interactions—** MTP associated with apoB intracellularly, and this association was noted with all apoBs, from B13 to B41. Qualitatively, less MTP communoprecipitation with apoB41 was readily detectable, but in its presence, very little of apoB 41 was detectable (tracks 2 and 4).
tated with the smaller truncates (<B23) than with the larger ones. The interaction with apoB 41 was resistant to high salt washing and was also resistant to lowering of pH buffers as low as 5.8. These results suggest that this interaction may be predominantly hydrophobic in nature. Clearly, MTP must dissociate from apoB at some point, because no MTP-PDI complex is secreted under normal circumstances. Since there is evidence that the lipoprotein particle continues to obtain lipid following its exit from the ER, this complex may dissociate in the cis-Golgi, where a more acidic environment is present, in a manner analogous to the disassociation of receptor-associated protein from newly synthesized low density lipoprotein receptor-related protein (39), although our results suggest that changes in pH alone may not suffice for MTP to dissociate from apoB. The KDEL retrieval signal on PDI could then shuttle the MTP complex back to the ER (40). The smaller apoBs, such as B17, are secreted with very little lipid but can obtain further lipid from serum to attain densities in the high density lipoprotein range (24, 25). However, MTP coexpression did not augment secretion of these smaller truncates, in the absence or presence of oleate supplementation, despite being able to bind to them. MTP binding to these smaller apoBs may not be as robust as to the larger apoBs. Cotransfection experiments make it difficult to measure this, but stable heterologous expression of MTP may allow us to study this hypothesis, as well as to better define the domains of apoB that interact with MTP.

Calnexin and ApoB Interactions—Only one other ER protein has been shown to interact with apoB, i.e. calnexin (20). One of the proposed roles of calnexin is to recognize incompletely folded newly synthesized glycoproteins, and that it may do by recognition of the glucosylation state. The binding to calnexin is thought to engage a round of folding, perhaps by recruiting other ER proteins to help in this process (36, 41). In the process, glucose residues are cleaved from the N-glycosylated moieties, and the protein is released. If the protein is incompletely folded, it is recognized by UDP-glucosyltransferase and glucosylated (42, 43), making it a substrate for calnexin binding, and a further round of folding ensues. Once correctly folded, the protein can then leave this quality control onto to its final destination. The binding of calnexin to the newly synthesized glycoproteins is transient, but in cases where cell lines fail to express one chain of a multichain complex, such as the T-cell receptor, the binding of calnexin to the residual chains is prolonged (35). These authors suggested that calnexin may, therefore, play a role in ER degradation. However, it has been demonstrated previously that tunicamycin treatment of cells, which inhibits N-glycosylation, does not affect apoB secretion from cultured hepatocytes (44), and some secreted apoB 100 from human hepatoma cell lines may also not be fully glucosylated (45). We have investigated the interaction of calnexin and apoB in COS cells. Overexpression of human calnexin in COS cells with apoB did not lead to attainment of a “folded” state for apoB such that it could leave the ER, nor were the degradation kinetics altered. Indeed, although apoB could be readily demonstrated to coprecipitate with endogenous calnexin, in the presence of MTP this interaction was absent or very transient. Tunicamycin treatment of COS cells inhibited N-glycosylation, and thus calnexin binding, but did not alter the secretion kinetics and lipoprotein formation of apoB in the presence of MTP, confirming previous findings that N-glycosylation was not necessary for apoB secretion. Calnexin has been shown to bind to nonglycosylated proteins (46, 47), but our Western blotting data (Fig. 9) suggest that the binding of calnexin to apoB is likely to be mediated via the glycosylation interactions. In the presence of overexpressed MTP, the interaction with calnexin is diminished in COS cells, but in hepatoma cells, calnexin can be readily seen to associate with apoB (20). One explanation for this may be that MTP may be rate-limiting in hepatoma cells. This would allow for the relative excess of apoB synthesized to interact with calnexin, even though this apoB is ultimately destined for degradation. The possibility that the pool of apoB that interacts with MTP in hepatoma cells is the luminal fraction compared with the membrane-inserted fraction that interacts with calnexin should be amenable to testing. Experiments to address the partition of MTP may allow us to distinguish whether this model is correct.

ApoB Interactions with KDEL Proteins—We have attempted to identify some of the other proteins that may be involved in apoB folding within the ER. Using antibodies directed against the carboxyl-terminal peptide KSEKDEL, apoB coprecipitated with proteins recognized by this antibody. This interaction was present in the absence of MTP (which is associated with the KDEL signal of PDI), suggesting that other ER proteins are involved. One obvious candidate is BiP grp 78, a ubiquitous and relatively abundantly expressed ER chaperone known to bind to misfolded proteins. BiP has a preference for hydrophobic sequences (48), a large number of which are present on apoB. There is a protein of about 80 kDa that coprecipitates with apoB, irrespective of MTP expression, and which appears to disassociate from the immunoprecipitates at a pH of 6.0 but is stable to high salt (see Figs. 7 and 8). In data not shown, this protein band has a half-life of more than 5 h, is not secreted, is present at short and long chase time points, and absent when nonimmune antiserum is used, suggesting that it may be an ER-resident protein. Our antibodies at present do not allow us to specifically identify simian BiP, although we strongly believe that the coprecipitating protein is BiP.

In this study, we show that the “secretory block” imposed on heterologously expressed apoB can be overcome by coexpression with MTP. This coexpression also leads to responsiveness to oleate supplementation, and the secreted lipoprotein particles contain newly synthesized triglyceride. In addition, oleate supplementation is most effective for truncates larger than B29, and the secretion of the smaller truncates is not augmented by either oleate or MTP coexpression. MTP was found to be physically associated with intracellular apoB 41, and this interaction is hydrophobic in nature. Other ER proteins were also found to interact with apoB, i.e. calnexin and an unidentified protein of 80 kDa. Interaction with calnexin did not appear to be necessary for lipoprotein secretion, and interestingly, in the presence of MTP, this interaction may be transient or absent. Although the identification of the 80-kDa protein is not known at this stage, it may interact with apoB via hydrophobic bonding, and it dissociates from apoB at pH conditions present in the Golgi. These studies suggest that MTP plays a role in the folding of apoB by providing it with neutral lipid, and neutral lipid is necessary for the formation of a lipoprotein but also for allowing apoB to attain a conformation sufficient for it to escape the quality control of the ER.

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