The Microsomal Triglyceride Transfer Protein Catalyzes the Post-translational Assembly of Apolipoprotein B-100 Very Low Density Lipoprotein in McA-RH7777 Cells*

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In cells in which the lipoprotein assembly process had been inactivated by brefeldin A (BFA), membrane-associated apoB-100 disappeared without forming lipoproteins or being secreted, indicating that it was degraded. Reactivation of the assembly process by chasing the cells in the absence of BFA, gave rise to a quantitative recovery of the membrane-associated apoB-100 in the very low density lipoprotein (VLDL) fraction in the medium. These results indicate that the membrane-associated apoB-100 can be converted to VLDL.

A new method was developed by which the major amount (88%) of microsomal apoB-100 but not integral membrane proteins could be extracted. The major effect of this method was to increase the recovery of apoB-100 that banded in the LDL and HDL density regions, suggesting that the membrane-associated form of apoB-100 is partially lipidated. We also investigated the role of the microsomal triglyceride transfer protein (MTP) in the assembly of apoB-100 VLDL using a photoactivatable MTP inhibitor (BMS-192951). This compound strongly inhibited the assembly and secretion of apoB-100 VLDL when present during the translation of the protein. To investigate the importance of MTP during the later stages in the assembly process, the cells were preincubated with BFA (to reversibly inhibit the assembly of apoB-100 VLDL) and pulse-labeled (+BFA) and chased (+BFA) for 30 min to obtain full-length apoB-100 associated with the microsomal membrane. Inhibition of MTP after the 30-min chase blocked assembly of VLDL. This indicates that MTP is important for the conversion of full-length apoB-100 into VLDL. Results from experiments in which a second chase (−BFA) was introduced before the inactivation of MTP indicated that only early events in this conversion of full-length apoB-100 into VLDL were blocked by the MTP inhibitor. Together these results indicate that there is a MTP-dependent “window” in the VLDL assembly process that occurs after the completion of apoB-100 but before the major amount of lipids is added to the VLDL particle. Thus the assembly of apoB-100 VLDL from membrane-associated apoB-100 involves an early MTP-dependent phase and a late MTP-independent phase, during which the major amount of lipid is added.

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There exist two forms of apolipoprotein B (apoB),1 apoB-100 and apoB-48 (1, 2). In humans apoB-100 is expressed in the liver, while apoB-48 is synthesized in the intestine (3). In some animals, such as the rat, both proteins are expressed in the liver. When expressed in liver, both apoB-100 and apoB-48 assemble into VLDL particles (4, 5). When expressed in the intestine, they both have the capacity to assemble into chylomicrons (6).

Lipoprotein assembly is a complex process that is not yet understood in all detail. The availability of the McA-RH7777 rat hepatoma cell line, which assembles bona fide VLDL (4), has facilitated studies of this process. In addition, we have recently demonstrated that brefeldin A (BFA) reversibly inhibits the assembly of all apoB-100 lipoproteins, in particular VLDL (7). During BFA treatment, apoB-100 appeared in a non-VLDL form that pelleted with the microsomal membrane after sodium carbonate extraction of the lumenal content of the microsomes (7). Since it was possible to remove the BFA and reactivate the assembly process, we were able to demonstrate that this membrane-associated form of apoB-100 can be a precursor to apoB-100 VLDL (7). Thus it seems that, under certain conditions, the assembly of apoB-100 VLDL involves the following steps: 1) translation and association with the endoplasmic reticulum membrane and 2) conversion of the membrane-associated VLDL precursor into VLDL.

The microsomal triglyceride transfer protein (MTP) catalyzes the transfer of triglycerides, cholesteryl esters, and phospholipids between phospholipid surfaces (8, 9). Mutations that inactivate or abolish MTP give rise to the phenotype of abetalipoproteinemia, an almost total absence of apoB-containing lipoproteins in the plasma (10, 11). Thus MTP is essential for the assembly and secretion of apoB-containing lipoproteins (12, 13). Very recent results indicate that MTP is important for the early events in the assembly of endogenous rat apoB-48 VLDL. However, MTP was not needed for the addition of the major amount of lipid during the later stages of the apoB-48 VLDL assembly (14). Currently, no direct data describing the role of MTP in the early or late phases of apoB-100 assembly have been reported.

This report describes the results of studies designed to follow the fate of apoB-100 after it associates with the microsomal membrane. The formation of VLDL from this pool of apoB-100 was elucidated by pulse-chase analysis. The role of MTP in this assembly process was determined using the photoactivatable inhibitor BMS-192951.

1 The abbreviations used are: apoB, apolipoprotein B; BFA, brefeldin A; CHX, cycloheximide; HDL, high density lipoproteins; LDL, low density lipoproteins; MTP, the microsomal triglyceride transfer protein; VLDL, very low density lipoproteins.
EXPERIMENTAL PROCEDURES

Materials—Eagle’s minimum essential medium, nonessential amino acids, glutamine, penicillin, and streptomycin were from ICN Biomedicals (Costa Mesa, CA). Fetal calf serum was from Biochrom KG (Berlin, Germany), and brefeldin A from Epicentre Technologies (Madison, WI). Methionine, fatty acid-free bovine serum albumin, sodium pyruvate, sodium carbonate, sodium bicarbonate, phenylmethylosulfonyl fluoride, pepstatin A, and leupeptin were from Sigma. Polyclonal antibodies to canine Calnexin was from StressGen Biotechnologies Corp (Victoria, Canada). Rabbit immunoglobulin was from DAKO (Glostrup, Denmark). Trasylol® (aprotinin) was from Bayer Leverkusen (Leverkusen, Germany). Immunoprecipitin and Eagle’s minimum essential medium without methionine were from Life Technologies, Inc. (Paisley, Scotland). N-Acetyl-Leu-Leu-norleucinal was from Boehringer Mannheim (Mannheim, Germany). Ampli®, [35S]methionine-cysteine mix, and Rainbow® protein molecular weight markers were from Amersham (Mannheim, Germany). Amplify®, [35S]methionine-cysteine mix, and Rainbow® protein molecular weight markers were from Amersham Corp. (Amersham, UK). Ready Safe® was from Beckman (Fullerton, CA). All chemicals used for SDS-polyacrylamide gel electrophoresis, the Silver Stain Plus® kit and alkaline-conjugated goat anti-rabbit Ig were from Bio-Rad. Western Blue®-stabilized substrate for alkaline phosphatase was from Promega (Madison, WI).

Cell Culture—McA-RH7777 cells were cultured as described earlier (4) in Eagle’s minimum essential medium, containing 20% fetal calf serum, 1.6 mM glutamine, 8.0 mM sodium bicarbonate, 1.6 mM sodium pyruvate, 140 mg/ml streptomycin, 140 IU/ml penicillin, and 60 mg/ml nonessential amino acids, in 5% CO2 at 37 °C. The cells were split twice a week and fed every day.

Metabolic Labeling—The cells were pulse-labeled and chased as described earlier (4). Brefeldin A was dissolved in ethanol and added to the culture medium to a final concentration of 10 μg/ml, unless stated otherwise. The isolation of cells and the microsomal fraction was carried out as described previously (15). The luminal content of the vesicles was separated from the vesicle membranes by the sodium carbonate method (16), with some modifications as described elsewhere (4, 17). The microsomes were also extracted with a combination of sodium carbonate, 0.025% deoxycholate, and 1.2 M potassium chloride (18). The deoxycholate was dissolved in the high salt solution containing 3 M potassium chloride was 1.2 M and for Tris-HCl 0.008 M. The Tris buffer did not influence the final pH during the extraction. The procedure then followed the procedure for the extraction with sodium carbonate alone. The following protease inhibitors were used: 0.1 mM leupeptin, 1 mM phenylmethysulfonfyl fluoride, 1 mM pepstatin A, 5 mM N-acetyl-Leu-Leu-norleucinal, and 100 kallekrein-inhibitory units aprotinin/ml.

Treatment with the MTP Inhibitor—A photoactivatable MTP inhibitor (20) (9-229 m), was used in some experiments. The inhibitor was concentrated to 10 μM (if not stated otherwise) was dissolved in Me2SO (final concentration, 0.5%) and added directly to the cell cultures. Before photoactivation, the culture dishes were kept in the dark. To activate the inhibitor, the cells were irradiated for 15 min with UV light (365 nm) using a Philips UVA lamp at a distance of 8 cm. The temperature in the cell cultures during this process was measured and found to be 37 °C.

Sucrose Gradient Ultracentrifugation of Lipoproteins—The lipoproteins present in the microsomal lumen or in the media were separated by sucrose gradient ultracentrifugation as described previously (4).

Immunoprecipitation of ApoB and Electrophoresis—ApoB was immunoprecipitated from cells, medium, and gradient fractions as described elsewhere (4). Electrophoresis in SDS-polyacrylamide gels, autoradiography, and determination of the radioactivity in the proteins separated in the SDS-polyacrylamide gels were carried out as described previously (19). To stain silver stain polyacrylamide gels, we used the Silver Stain Plus® kit (Bio-Rad) following the procedure recommended by the manufacturer.

Immunoblotting were carried out on a Trans-Blot®SD (Bio-Rad) as recommended by the manufacturer. The blots were blocked with 5% non-fat dry milk in 20 mM Tris-Cl (pH 7.4) containing 137 mM sodium chloride (TBS) for 1 h. This was followed by a 1-h incubation with the antibody (a rabbit polyclonal antibody against the carboxyl terminus of canine Calnexin (dilution, 1:200) in TBS with 0.1% Tween 20 (TBS-T) and 5% non-fat dry milk. The blots were washed with TBS-T (twice for 5 min each). To detect bound antibodies, the blots were incubated with an alkaline phosphatase-conjugated goat anti-rabbit Ig (Bio-Rad; dilution, 1:2000) in TBS-T with 5% non-fat dry milk. After a washing twice for 5 min each with TBS-T followed by once for 5 min with TBS, the blots were reacted with Western Blue®-stabilized substrate for alkaline phosphatase as recommended by the manufacturer.

RESULTS

Turnover of Membrane-associated ApoB-100—McA-RH7777 cells were pretreated with BFA for 15 min, labeled for 30 min in the presence of BFA, and chased in the presence of BFA for an additional 30 min. After this chase, the major amount of the labeled intracellular apoB-100 was associated with the membrane of the microsomes (i.e. was resistant to carbonate extraction), while no significant amount of radioactive apoB-100 VLDL could be detected in the sodium carbonate extract (i.e. in the luminal content) of these microsomes (Fig. 1, chase 1). The lack of radiolabeled apoB-100 VLDL in the microsomal lumen was verified by gradient ultracentrifugation of extracts, recovered from the microsomes by the combination of sodium carbonate, 0.025% deoxycholic acid, and 1.2 M potassium chloride (data not shown). This extraction procedure removed the major amount of apoB-100 from the microsomes (see below). Together these results confirm our previous observations (7) that the VLDL assembly is blocked by BFA and that the major amount of intracellular apoB-100 is instead associated with the membrane of the microsomes.

The apoB-100 radioactivity that was present in the carbonate extract of the microsomes banded during gradient ultracentrifugation in fractions with a higher density than VLDL, with the dominating amount present in the density region of HDL (20) (Fig. 1, chase 1). We will refer to these dense apoB-100-containing lipoproteins as apoB-100 “HDL” in the following.

No apoB-100 lipoproteins were secreted into the medium under the conditions used in the experiment shown in Fig. 1, chase 1. This was expected since the cells were treated with BFA.

Continuing the chase for 90 min (Fig. 1, chase 2) in the presence of BFA resulted in a significant decrease in the amount of apoB-100 that was associated with the microsomal membrane, while no apoB-100 VLDL was formed or secreted (based on results from the same type of analyzes as were carried out after chase 1). The amount of radioactive apoB-100 HDL in the microsomal lumen remained virtually the same during this chase. These results indicate that membrane-associated apoB-100 is targeted for intracellular degradation.

After the 90-min chase, BFA was removed and the chase continued for 180 min in the presence of oleic acid (Fig. 1, chase 3). After this chase, almost all radioactive apoB-100 had disappeared from the microsomal membrane and could be recovered as VLDL in the medium. This demonstrates that the membrane-associated pool can assemble into VLDL, indicating a precursor-product relationship. We also observed a significant reduction in the amount of apoB-100 radioactivity associated with apoB-100 HDL in the luminal fraction.

Effect of Inhibition of MTP during the Early Part of the Assembly of ApoB-100-containing VLDL—In agreement with previous results (14), we found that inhibition of MTP before labeling of the cells profoundly inhibited the assembly and secretion of radioactive apoB-containing lipoproteins. Compared with the control cells, MTP inhibition (10 μM) resulted in only 7.5% (mean of two experiments) of the apoB-100 VLDL secretion.

Effects of MTP Inhibition on the Conversion of the Membrane-associated Form of ApoB into VLDL—In the next experiment, we investigated the effect of inhibiting MTP on the latter part of the VLDL assembly process. This experiment was also designed to delineate a possible time interval during which MTP was important for this part of the assembly process. In these experiments, we pretreated the cells with BFA for 15 min, labeled them for 30 min (+ BFA), and chased them for 30 min (+ BFA). Under these conditions the VLDL assembly was inhibited, and full-length apoB-100 was associated with the
FIG. 1. The turnover of membrane-associated apoB-100 and the assembly of apoB-100 VLDL in cells treated with BFA. McA-RH7777 cells were preincubated for 15 min with BFA, labeled for 30 min with \(^{35}\)S-methionine and \(^{35}\)S-cysteine (+BFA), and chased for 30 min (+BFA, chase 1). This was followed by a second chase for 90 min (chase 2) in the presence of BFA and finally by a third chase for 180 min (chase 3) in the absence of BFA, but in the presence of oleic acid (360 \(\mu\)M). After each chase period, the microsomal fraction as well as the culture media were recovered. The microsomes were extracted with sodium carbonate, and the membrane pellet was separated from the lumenal content. The luminal content and the culture media were subjected to sucrose gradient ultracentrifugation. The sucrose gradient was unloaded into 12 fractions, and apoB-100 was isolated from each fraction by a combination of immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The radioactivity in apoB-100 present in the VLDL fraction as well as in the HDL density region (apoB-100 HDL) was calculated. The filled bar shows the apoB-100 radioactivity present in VLDL of the culture medium (no VLDLs were found in the lumenal content of the microsomes). Open bars show the apoB-100 radioactivity in apoB-100 HDL present in the lumenal content of the microsomes (apoB-100 HDL was not present in the culture medium). ApoB-100 was also recovered from the membrane pellets (hatched bars) by a combination of immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Data shown are the mean \(\pm\) SD of four determinations.

The turnover of membrane-associated apoB-100 and the assembly of apoB-100 VLDL in cells treated with BFA. McA-RH7777 cells were preincubated for 15 min with BFA, labeled for 30 min with \(^{35}\)S-methionine and \(^{35}\)S-cysteine (+BFA), and chased for 30 min (+BFA, chase 1). This was followed by a second chase for 90 min (chase 2) in the presence of BFA and finally by a third chase for 180 min (chase 3) in the absence of BFA, but in the presence of oleic acid (360 \(\mu\)M). After each chase period, the microsomal fraction as well as the culture media were recovered. The microsomes were extracted with sodium carbonate, and the membrane pellet was separated from the lumenal content. The luminal content and the culture media were subjected to sucrose gradient ultracentrifugation. The sucrose gradient was unloaded into 12 fractions, and apoB-100 was isolated from each fraction by a combination of immunoprecipitation and SDS-polyacrylamide gel electrophoresis. The radioactivity in apoB-100 present in the VLDL fraction as well as in the HDL density region (apoB-100 HDL) was calculated. The filled bar shows the apoB-100 radioactivity present in VLDL of the culture medium (no VLDLs were found in the lumenal content of the microsomes). Open bars show the apoB-100 radioactivity in apoB-100 HDL present in the lumenal content of the microsomes (apoB-100 HDL was not present in the culture medium). ApoB-100 was also recovered from the membrane pellets (hatched bars) by a combination of immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Data shown are the mean \(\pm\) SD of four determinations.
Catalysis of Post-translational Assembly of ApoB-100 VLDL

Fig. 2. Temporal resolution of the MTP-dependent step in apoB-100 VLDL assembly. A, the effect of an intermediate chase on the ability of the MTP inhibitor to block VLDL assembly. The McA-RH7777 cells were preincubated for 15 min with BFA, labeled for 30 min with [35S]methionine and [35S]cysteine (+BFA), and chased for 30 min (+BFA). A new chase for 0 min (n = 4), 15 min (n = 4), or 30 min (n = 3) in the absence of BFA was introduced (intermediate chase). The MTP inhibitor was added and photoactivated after the intermediate chase. The media were changed, and the cells chased for 180 min in the absence of BFA but presence of 360 µM oleic acid. The culture media were subjected to sucrose gradient ultracentrifugation, and the gradient was unloaded into 12 fractions. ApoB-100 was isolated from each fraction by a combination of immunoprecipitation and SDS-polyacrylamide gel-electrophoresis, and the radioactivity of apoB-100 present in the VLDL fraction by a combination of immunoprecipitation and SDS-polyacrylamide gels, and blotted against antiserum to Calnexin. In these experiments, the membrane pellet was recovered after extraction by the two techniques, electrophoresed in SDS-polyacrylamide gels, and blotted against antiserum to Calnexin. Based on the intensity of the Calnexin band, we concluded that extraction with deoxycholic acid-carbonate (Fig. 3C, III) gave rise to a similar recovery of Calnexin in the membrane pellet as did the original carbonate extraction procedure (Fig. 3C, IV). Together the results in Fig. 3 show that extraction of microsomes with a combination of carbonate, low concentrations of deoxycholate, and 1.2 M potassium chloride led to a very significant improvement in the removal of apoB-100 from the membrane, but did not influence integral membrane proteins more than extraction with carbonate alone.

Analyses of the deoxycholic acid-carbonate extract by gradient ultracentrifugation revealed a general increase of apoB-100 in all density ranges in comparison to extraction with carbonate alone (Fig. 3A). However, there was an enrichment of particles banding in the LDL and HDL density ranges. To confirm this, the microsomes were first extracted with carbonate and then re-extracted with deoxycholic acid-carbonate. The results (Fig. 3D) clearly show that the combination of deoxycholate, carbonate, and 1.2 M potassium chloride increases the efficiency of extraction of apoB-100 present in the HDL and LDL density ranges. Thus, extraction of deoxycholic acid-carbonate yields a more representative recovery of the spectrum of apoB-100 forms associated with the microsomal membranes.

One particular concern with deoxycholate is that it might affect the structure of the lipoprotein particles. In particular it introduced, and this was followed by a third chase for 180 min in the absence of BFA, but in the presence of oleic acid (360 µM). The luminal content of the microsomes was recovered by sodium carbonate extraction after the intermediate chase (cell 30 min and cell 60 min), as well as after the 180 min chase (cell 30 + 180 min and cell 60 + 180 min). New medium was added after the 30- or 60-min intermediate chase and collected after the 180-min chase (medium 180 min). Both the media and the sodium carbonate extract (the luminal contents) of the microsomes were analyzed by gradient ultracentrifugation. ApoB-100 was immunoprecipitated from each fraction of the gradient and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. C, membrane-associated apoB-100. The cells were treated as in B, and the apoB-100 present in the membrane pellet was recovered by immunoprecipitation before and after the 180-min chase that was carried out in the absence of BFA but in the presence of oleic acid (360 µM). The immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. 30 min/160 min, apoB-100 associated with the membrane after the intermediate chase. 30 + 180 min/160 + 180 min, apoB-100 associated with the membrane after the 180-min chase.
may reduce the size of VLDL. To address this issue, we subjected the culture medium obtained after a 2-h labeling of the cells (containing virtually only VLDL particles) to treatment with carbonate or deoxycholic acid-carbonate (Fig. 3E). The media were then subjected to gradient ultracentrifugation, and apoB-100 was recovered from each fraction by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The results demonstrated that the VLDL stayed intact during both extraction procedures. No significant production of smaller particles was seen. Calculation of the distribution of apoB-100 radioactivity between the different density ranges is as follows: for deoxycholic acid-carbonate, 88 ± 2% in VLDL, 5 ± 2% in LDL, and 6 ± 1% in the HDL density range; for carbonate, 85 ± 5% in VLDL, 7 ± 2% in LDL, and 3 ± 1% in the HDL density range (mean ± S.D., n = 3). Thus the total recovery of apoB-100 in VLDL after deoxycholic acid-carbonate versus carbonate alone was not significantly different (92 ± 19%, mean ± SD, n = 3). Carbonate alone did not influence the recovery of apoB-100 VLDL compared with untreated controls.

The deoxycholic acid-carbonate method was used to extract apoB-100 from the microsomes of cells that had been preincubated with BFA for 15 min, pulse-labeled for 30 min (+BFA), chased for 0, 15, or 30 min without BFA. Fig. 4A shows that the membrane was almost completely depleted of apoB-100. Analysis of the extract by gradient ultracentrifugation (Fig. 4B) demonstrated that the major amount of apoB-100 radioactivity was present in the HDL density region. A relatively small amount of apoB-100 was present in the VLDL or intermediate density ranges. Furthermore, the amount of VLDL or intermediate density particles did not differ between the conditions when the assembly was dependent upon MTP (after 0- and 15-min chases) and when the assembly was no longer dependent on MTP (after chases of 30 min or more). In contrast, we did observe a higher amount of apoB-100 radioactivity within the HDL density range after the 0-min chase than after the 30-min chase.

Together this body of data strongly argues against the possibility that the effect of the 30-min intermediate chase was to convert all apoB-100 into luminal VLDL or that VLDL particles were associated with the membrane. Therefore, the accumulation of apoB-100 VLDL in the medium during the final 180-min chase following the 30-min intermediate chase could be explained only by assembly directly from a non-VLDL form of apoB-100 associated with the microsomal membrane (Fig. 2, A and B). Furthermore, the results shown in Fig. 2 indicate that MTP plays a critical role in the process of converting membrane-associated apoB-100 to VLDL. Our results indicate that this influence of MTP on the assembly process occurs before the major amount of lipid is added and the mature VLDL particle is formed. The observation that it takes a 30–60-min chase in the absence of BFA to reactivate the process that adds the major amount of lipids and releases VLDL particles into the lumen of the secretory pathway agrees well with our previous results (7).

**The Effect of MTP Inhibition on the Assembly of VLDL in Cycloheximide-treated Cells—**Data from the BFA-treated cells indicated that MTP is of importance for VLDL assembly after the completion of apoB-100 and that this MTP dependence could be abolished by a short chase. These experiments relied on the ability to reversibly inhibit VLDL assembly by BFA. To show that the observed MTP effect was also applicable to cells

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APOB-100 was isolated from each fraction by immunoprecipitation and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.
polyacrylamide gel electrophoresis followed by autoradiography. Precipitation from each fraction of the gradient and analyzed by SDS-gradient ultracentrifugation, and apoB-100 was recovered by immunoprecipitation from each fraction of the gradient and analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

not treated with BFA, we carried out the following experiment (Fig. 5). The cells were pulse-labeled for 30 min and treated with cycloheximide to block translation. We then introduced a chase for 0, 15, or 30 min (+CHX). These chases were followed by MTP inhibition for 15 min. As a control, the cells were treated with only CHX and UV-irradiated without the inhibitor. Finally, the cells were chased for 180 min (+CHX) in the presence of oleic acid, and the secretion of radioactive apoB-100 VLDL was determined. The results indicate that there was a significant inhibition of VLDL assembly when MTP inhibition was introduced after the intermediate chase of 0 min (Fig. 5, 0 min). Recovery of VLDL in the medium was 35% of that observed for the control. This verifies the observation that MTP influences the assembly of apoB-100 VLDL after translation is complete. After an intermediate chase of 15 min (Fig. 5, 15 min) in the presence of CHX, the recovery increased significantly (to 60% of the control). Finally, after an intermediate chase of 30 min (Fig. 5, 30 min), the recovery was 72%, which is in the range of that observed after the corresponding intermediate chase in cells treated with BFA (see Fig. 2). The same amount of apoB-100 radioactivity was present in the cells after the treatment with the MTP inhibitor (i.e. before the 180-min chase) under the three conditions. These results support the observation that MTP influences the apoB-100 VLDL assembly post-translationally and that there is a short time window during which the process is MTP-dependent.

**DISCUSSION**

Previous results from our group (21), as well as from other investigators (22), have indicated that membrane-associated apoB-100 is sorted to degradation. Recently however, we obtained results which indicated that this form of apoB-100 can also be a precursor to VLDL (7). In these previous experiments we used BFA to reversibly block the assembly of apoB-100-containing lipoproteins, in particular VLDL. It was demonstrated that BFA caused the association of apoB with the endoplasmic reticulum membrane. When the BFA was removed, the cells resumed assembly of apoB-100 VLDL. In this study, we used the same system, but continued to follow the fate of the membrane-associated apoB-100 during a chase in the presence of BFA, under conditions that inhibited the VLDL assembly process. The results showed a clear decrease in the amount of membrane-associated apoB-100. Since no apoB-100 lipoproteins were formed or secreted, these results indicate that the membrane-associated apoB-100 was removed by degradation. Releasing the BFA block, before the membrane-bound apoB-100 was degraded, resulted in formation of apoB-100 VLDL, which was quantitatively recovered in the medium. These results demonstrate that the membrane-associated apoB-100 is a precursor to VLDL. However, when the assembly process is inhibited, this VLDL-precursor will be degraded in the cell. In agreement with our previous results (20), the present observations indicate that the assembly process plays a critical role in the sorting of apoB-100; apoB-100 that is not recruited to the assembly process will gradually be degraded intracellularly.

The physical nature of the membrane-associated apoB-100 is unknown. In this report we demonstrate that the microsomal membranes could be almost completely depleted of apoB-100 by extraction with sodium carbonate together with small amounts of deoxycholate and 1.2 M potassium chloride. The concentrations of deoxycholate are well below the critical micellar concentration and have been shown to remove luminal proteins and peripheral membrane proteins but not integral membrane proteins (18). In an analogous fashion, we observed that the extraction procedure did not significantly influence recovery of membrane proteins from the membrane pellet during the extraction with ordinary sodium carbonate extraction. Of interest was that, although a general increase in the recovery of apoB-100 in all density classes was seen, we observed that the major effect of deoxycholate and high salt was to liberate apoB-100 that banded in the HDL and LDL density ranges. We have previously demonstrated the presence of such forms of apoB-100 lipoproteins in sodium carbonate extracts of the microsomes, and we have suggested that the form that bands in the HDL density range (apoB-100 HDL) may be a precursor to less dense apoB-100-containing lipoproteins (15). Such a precursor role for apoB-100 HDL is supported by the results of the present study (compare Fig. 1). One possibility is
that apoB-100 HDL represents a lower percentage of the membrane-associated pool that is released during extraction with carbonate alone, implying that the membrane-associated form of apoB-100 and the carbonate-released dense particles belong to the same pool of apoB-100. This in turn may explain why both the membrane-associated form of apoB-100 (i.e. the carbonate-resistant form) and apoB-100 HDL (i.e. the dense particles extracted by sodium carbonate) appear to be precursors for apoB-100 VLDL (compare Fig. 1).

Previous studies have demonstrated that MTP is essential for the assembly of apoB-containing lipoproteins (12, 13). Moreover, it has been shown that mutations in the MTP gene give rise to the phenotype of abetalipoproteinemia (10, 11). Despite the fact that MTP is characterized in great detail, relatively little is known about the molecular details of the role of this protein in the assembly of the lipoprotein. An interaction with apoB has been demonstrated (23, 24) and suggested to involve the N-terminal globular domain of apoB, which is needed for the action of MTP (25, 26). In a recent study, we demonstrated that MTP is necessary for the early events in the assembly of endogenous apoB-48 VLDL in the McA-RH7777 cells, but does not appear to be essential for the step in which the major amount of lipids was added to the particle (14). In addition, it has recently been shown that inhibition of MTP up to 10 min after a pulse of [35S]methionine blocked the secretion of apoB-100 from HepG2 cells (27). After this time, the MTP inhibitor lost its effect, and apoB secretion was observed. This suggests that, as in the case of apoB-48, MTP also plays a role in the early stages of apoB-100 lipoprotein assembly, but not in the later stages. One drawback with the HepG2 cells is that they do not assemble any substantial amount of bona fide VLDL (20), but rather an LDL-size particle rich in triacylglycerol. The assembly of this particle occurs to a significant degree cotranslationally (28).

As discussed above, a precursor to apoB-100 VLDL appears to be a membrane-associated form of full-length apoB-100 (7). We observed that a photoactivatable MTP inhibitor could block the assembly and secretion of apoB-100 VLDL not only when the inhibitor was photoinactivated prior to the labeling of the cells, but also when the photoinactivation was carried out after completion of the membrane-associated apoB-100 (a VLDL precursor). This indicates that MTP takes part in the post-translational conversion of membrane-associated apoB-100 to VLDL. Our results also indicate that there is a “window” within which this process is MTP-dependent. This window occurs in BFA-treated cells after translation is complete, but before they have recovered their capacity to assemble and release VLDL particles into the secretory pathway after BFA is withdrawn. This in turn indicates that MTP is not involved in the loading of the major amount of lipid to the particle, but rather in the preparation of apoB-100 for this process. This agrees with our observations that the addition of the major amount of lipid to apoB-48 VLDL occurs in an MTP-independent step (14).

The identification of the window during which the assembly process was MTP-dependent was based on BFA-treated cells. To confirm that this was not due to an artifact introduced by the BFA treatment, an alternate strategy was applied. In this case we used CHX to achieve the necessary manipulations. Qualitatively, the results were nearly identical, showing a distinct post-translational window during which apoB-100 lipoprotein assembly was MTP-dependent. However, since the CHX-treated cells, contrary to BFA-treated cells, continue to assemble and secrete apoB-100 VLDL (4), the exact time window of MTP dependence cannot be compared.

Our results thus indicate that MTP is of importance for the creation of a situation that allows full-length membrane-associated apoB-100 to be assembled into VLDL and secreted. This suggests that an important function of MTP is to prepare apoB-100 for the assembly with the major amount of lipid. The nature of this preparation is a key question that needs to be addressed in future studies; however, possible mechanisms can be discussed.

The fact that inhibition of MTP mediated lipid transfer has such a profound effect on the assembly process during translation of apoB suggests that MTP is essential for stabilizing the protein during or immediately after translocation, perhaps by facilitating the folding of the protein. It has been pointed out that mature apoB contains large regions of amphipathic structures, in particular long regions of amphipathic β-strands that fold into amphipathic β-sheets (29). The amphipathic β-sheets, that are unique for apoB, are generally believed to mediate the irreversible binding between apoB and the lipoprotein particle (29). It could be speculated that these amphipathic structures require MTP-mediated lipid transfer to fold correctly to form a structure capable of receiving the bulk of the core neutral lipid. In the absence of MTP, these structures would misfold leading to the degradation of the apoB polypeptide.

The observation that MTP is also needed after the completion of apoB-100 could suggest that portions of the protein remains unfolded after the translation of the protein. There is a possibility that at least part of apoB-100 is post-translationally translocated to the lumenal side of the membrane (20, 30, 31). MTP may participate in such a translocation indirectly by allowing this part of the protein to properly fold as it enters the interior of the endoplasmic reticulum. In this way the protein may acquire a structure that allows it to interact with the downstream VLDL assembly process. It should, however, be kept in mind that the association between apoB-100 and the microsomal membrane is not completely understood. Contradictory results have appeared in the literature concerning the exposure of apoB-100 on the cytosolic side of the endoplasmic reticulum membrane (17, 20, 30–33). An alternative explanation is that apoB-100 is completely translocated cotranslationally (like ordinary secretory proteins) and that the folding of the C terminus occurs post-translationally, involving chaperone proteins in addition to MTP. The observation that the tentative membrane-associated precursor could be extracted under conditions that have been shown not to extract integral membrane proteins (18) (verified in this study) would favor the possibility that apoB-100 is more loosely associated with the membrane than is an integral protein. This does not rule out a transmembrane orientation mediated by chaperones or membrane channels. It should be remembered that the translocation channel Sec 61 has recently been demonstrated to participate not only in the translocation of proteins to the endoplasmic reticulum lumen but also in the retraction of luminal protein for degradation by proteasomes (see, for example, Cresswell and Hughes (34) for a review and references therein).

Together our data suggest a model for the assembly of apoB-100 VLDL. According to this model, apoB-100 is translated and at least partially translocated into a membrane-associated VLDL precursor form. This process is highly dependent on MTP. The assembly of VLDL from this precursor pool occurs first in an MTP-dependent step during which an immediate VLDL precursor is formed. The final step(s) is MTP-independent and allows the major amount of lipids to be loaded onto the protein.

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