Demonstration of a Physical Interaction between Microsomal Triglyceride Transfer Protein and Apolipoprotein B during the Assembly of ApoB-containing Lipoproteins*

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Triglyceride synthesis by Triacsin D, on the other hand, significantly decreased the MTP-apoB binding.

Although the large body of evidence demonstrating the importance of hepatic lipids in the assembly and secretion of apoB-containing lipoproteins, TG synthesis appears to be normal, and lipid droplets accumulate, in livers of patients with abetalipoproteinemia (18), a condition characterized by the absence of apoB secretion. Recently, defective assembly and secretion of apoB-containing lipoproteins in affected patients was found to be associated with mutations in the gene encoding a 97-kDa protein, microsomal TG transfer protein (MTP) large subunit (19, 20). In vitro studies (21, 22) showed that MTP efficiently catalyzes the transfer of TG and other lipids from donor membranes to acceptor membranes. MTP large subunit forms a heterodimer with protein disulfide isomerase in the lumen of the endoplasmic reticulum (ER) in hepatocytes and enterocytes. MTP large subunit appears to be expressed normally only in hepatocytes and enterocytes; protein disulfide isomerase is ubiquitously expressed. Two recent studies (23, 24) convincingly demonstrated that coordinate expression of large apoB truncations and MTP large subunit in cells that normally do not express either of the two proteins resulted in the efficient secretion of apoB-containing lipoproteins. ApoB was not secreted from these cells before MTP large subunit was expressed.

Although the above studies have clearly indicated a role for MTP in the assembly and secretion of apoB-containing lipoproteins from hepatocytes, the in vivo mechanism underlying this activity remains unknown. An unanswered question is how MTP transfers TG molecules to apoB during the assembly of lipoprotein particles. More specifically, does a physical interaction between MTP and apoB play a role in this process? The present studies were conducted to answer this question.

EXPERIMENTAL PROCEDURES

Materials—L-[4,5-3H]Leucine (135 Ci/mmol, catalog number TRK 683) and [2-14C]glycerol (1.0 Ci/mmol, catalog number TRA.118) were purchased from Amersham Corp. Monospecific anti-human apoB antiserum was raised in rabbits. Anti-bovine MTP antiserum (raised in a goat) was generated by one of the authors (J.R.W.). These antibodies were characterized and utilized in previous studies (19, 23). Protein A-Sepharose CL 4B was from Pharmacia LKB Biotechnology Inc. (Upsalla, Sweden). Bovine serum albumin (BSA) and oleic acid (sodium salt) were

1 The abbreviations used are: TG, triglyceride; TGI, TG inhibitor (Triacsin D); OA, oleic acid; MTP, microsomal TG transfer protein; ER, endoplasmic reticulum; BSA, bovine serum albumin; PAGE, polycrylamide gel electrophoresis; MTPL, MTP large subunit; ALLN, N-Acetyl-leucyl-leucyl-norleucinal.

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turing lysis buffer (62.5 mM sucrose, 0.5% sodium deoxycholate, 0.5% cold phosphate-buffered saline and subsequently lysed in a nondenaturating buffer, and immunoprecipitated with anti-apoB, anti-MTP, or anti-MTP large subunit antiserum. With anti-apoB antiserum, labeled apoB was precipitated (α-apoB); with anti-MTP large subunit, labeled apoB was also precipitated (α-MTPL); with anti-MTP, both labeled apoB and labeled MTP small subunit (protein disulfide isomerase, 55 kDa) were precipitated (α-MTP).

Immunoprecipitation—After labeling, the cells were washed with cold phosphate-buffered saline and subsequently lysed in a nondenaturating lysis buffer (62.5 mM sucrose, 0.5% sodium deoxycholate, 0.5% Triton X-100, 50 μg/ml pepstatin A, 50 μg/ml leupeptin, 150 μg/ml phenylmethylsulfonyl fluoride, 5 mM EDTA, 50 mM Tris·HCl, pH 7.4, and 150 mM NaCl). Immunoprecipitation of proteins was carried out according to the method of Dixon et al. (14). Briefly, medium or cell lysate samples were mixed with NET buffer (50 mM Tris·HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS) and excess amounts of various antisera. The mixture was incubated at 4 °C on a shaker for 5 h. Protein A-Sepharose CL 4B was added to the mixture, and the incubation was continued for an additional 2 h. The beads were extensively washed with NET. Proteins were extracted from the beads with sample buffer by boiling for 4 min. An aliquot of the sample was run on SDS-PAGE followed by autoradiography or immunoblotting.

Immunoblot—Cell lysates or samples from the immunoprecipitation were run on SDS-PAGE, transferred to a nitrocellulose membrane, and blocked with 3% BSA in phosphate-buffered saline. Either anti-human apoB antiserum or anti-bovine MTP large subunit antiserum was incubated with the membrane for 2 h. After washing with phosphate-buffered saline, secondary antibody conjugated with horseradish peroxidase was then incubated with the membrane for 2 h. 4-Chloro-1-naphthol and H₂O₂ were used as substrates to develop the membrane.

**FIG. 1. Anti-MTP antibodies co-precipitate newly synthesized apoB in HepG2 cells.** HepG2 cells were labeled with [³H]leucine for 4 h, lysed with a non-denaturating buffer, and immunoprecipitated with anti-apoB, anti-MTP, or anti-MTP large subunit antiserum. With anti-apoB antiserum, labeled apoB was precipitated (α-apoB); with anti-MTP large subunit, labeled apoB was also precipitated (α-MTPL); with anti-MTP, both labeled apoB and labeled MTP small subunit (protein disulfide isomerase, 55 kDa) were precipitated (α-MTP).

**FIG. 2. MTP is associated with apoB in HepG2 cells.** HepG2 cells were lysed with a non-denaturating buffer and immunoprecipitated with non-immune serum or anti-MTP large subunit antiserum (α-MTPL). The immunoprecipitates and an aliquot of HepG2 cell whole lysate were run on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with either anti-apoB antibody (panel A) or anti-MTP (panel B). In panel A, immunoblotting with anti-apoB antibody demonstrated that apoB was immunoprecipitated by anti-MTP antibody. In panel B, immunoblotting with anti-MTP demonstrated that MTP was co-immunoprecipitated in the same experiment. In a parallel experiment, HepG2 cell lysates were immunoprecipitated with either non-immune serum or anti-human apoB antiserum (α-apoB). The immunoprecipitates were run on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with anti-MTP large subunit (panel C). The result indicated that MTP large subunit co-immunoprecipitated by anti-apoB antibody. The dark band at the bottom is rabbit anti-human IgG.
large subunit antiserum. The immunoprecipitates and an aliquot of HepG2 cell whole lysate were run on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with anti-apoB antibodies. As shown in Fig. 2A, apoB was not detected with nonimmune serum; apoB was detected, however, with anti-MTP large subunit antiserum. In another experiment, MTP large subunit was detected in an anti-MTP large subunit immunoprecipitate by immunoblotting with anti-MTP large subunit antiserum (Fig. 2B).

HepG2 cell lysates were also immunoprecipitated with nonimmune serum or anti-human apoB antiserum. The immunoprecipitate was run on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blotted with anti-MTP large subunit antiserum. MTP large subunit (97 kDa) was detected in the anti-apoB immunoprecipitate but not in the nonimmune precipitate (Fig. 2C).

The MTP-ApoB Interaction Is Coordinated with the Synthesis of TG—We previously reported that proteolysis and lipid-facilitated apoB translocation are two competitive processes that determine the rate of apoB secretion (15). To further confirm the association between MTP-apoB during the assembly of apoB-containing lipoproteins, we examined the MTP-apoB interaction under various conditions that would alter apoB secretion by affecting either proteolytic activity or lipid availability. HepG2 cells were preincubated with one of the following agents for 1 h: 1) 1.5% BSA alone, 2) 1.5% BSA plus ALLN (40 μg/ml), 3) 1.5% BSA plus OA (0.2 mM), 4) 1.5% BSA plus OA (0.2 mM) and TGI (12.5 μM), or 5) 1.5% BSA plus TGI (12.5 μM). Cells were then labeled with [3H]leucine for 2 h, lysed with a nonblocking buffer, and immunoprecipitated with either anti-apoB or anti-MTP large subunit (panels A and B). Panel B is presented as mean ± S.D. of triplicate wells. The results demonstrated that treatment with OA (which stimulates TG synthesis as shown in panel C) increased the association between apoB and MTP. Co-incubation of OA with TGI abolished the effect of OA. In parallel experiments, HepG2 cells were incubated for 2 h with or without [3H]glycerol and one of the agents described above. For TG determination, [3H]glycerol-labeled cells were extracted with hexane/isopropyl alcohol (3/2), and TG synthesis was determined by thin-layer chromatography. Results in panel C are presented as mean ± S.D. of triplicate wells. Changes in TG synthesis paralleled changes in apoB-MTP association. In a third experiment, unlabeled cells were directly lysed and immunoprecipitated with anti-MTP large subunit antiserum. The immunoprecipitates were run on SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-MTP large subunit (panel D). None of the treatments significantly affected MTP levels in HepG2 cells.

Fig. 3. The MTP-apoB interaction is coordinated with the synthesis of TG. HepG2 cells were preincubated with one of the following agents for 1 h: 1) 1.5% BSA alone, 2) 1.5% BSA plus ALLN (40 μg/ml), 3) 1.5% BSA plus OA (0.2 mM), 4) 1.5% BSA plus OA (0.2 mM) and TGI (12.5 μM), or 5) 1.5% BSA plus TGI (12.5 μM). Cells were then labeled with [3H]leucine for 2 h, lysed with a nondenaturing buffer, and immunoprecipitated with either anti-apoB or anti-MTP large subunit antiserum (panels A and B). Panel B is presented as mean ± S.D. of triplicate wells. The results demonstrated that treatment with OA (which stimulates TG synthesis as shown in panel C) increased the association between apoB and MTP. Co-incubation of OA with TGI abolished the effect of OA. In parallel experiments, HepG2 cells were incubated for 2 h with or without [3H]glycerol and one of the agents described above. For TG determination, [3H]glycerol-labeled cells were extracted with hexane/isopropyl alcohol (3/2), and TG synthesis was determined by thin-layer chromatography. Results in panel C are presented as mean ± S.D. of triplicate wells. Changes in TG synthesis paralleled changes in apoB-MTP association. In a third experiment, unlabeled cells were directly lysed and immunoprecipitated with anti-MTP large subunit antiserum. The immunoprecipitates were run on SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with anti-MTP large subunit (panel D). None of the treatments significantly affected MTP levels in HepG2 cells.
were pulse-labeled for 10 min with [3H]leucine and chased. In this possibility we carried out studies in which HepG2 cells were investigated. These data suggest that in HepG2 cells, MTP might play an important role in the early stages of lipoprotein assembly. To examine this possibility, we conducted experiments in which HepG2 cells were pulse-labeled with [3H]leucine for 10 min and chased in serum-free medium up to 180 min. At each time point, cells were lysed and immunoprecipitated with either anti-apoB (apoB) or anti-MTP large subunit (anti-MTPL). The highest band on the top of the gel represents aggregated material. Interaction between MTP and apoB was greatest at 10 min of chase and rapidly diminished thereafter.

The results of these two experiments indicated that the MTP-apoB interaction was closely coupled with, or dependent upon, TG synthesis. These results are consistent with a pathway in which MTP is abundant but only binds to apoB in the presence of newly synthesized triglyceride. Additionally, our findings support the idea that a MTP-apoB physical interaction may be important in both the TG transfer process and lipoprotein assembly. On the other hand, the accumulation of apoB, in the absence of increased TG availability (as would occur in the presence of ALLN), does not appear to significantly modulate the MTP-apoB interaction.

MTP-ApoB Interaction Occurs at an Early Stage in the Assembly of ApoB-Containing Lipoproteins—The assembly of apoB-containing lipoproteins in HepG2 cells begins cotranslationally in the ER compartment (13). Data from rat hepatocytes suggest, however, that the bulk of the core lipid is added after translation and translocation have been completed (27–29). This appears to be particularly true for apoB48-containing particles (28, 29), where a two-step process of lipoprotein assembly seems to predominate. The two-step assembly model was essentially drawn from studies conducted in rat hepatocytes. The assembly of apoB-containing lipoprotein particles in HepG2 cells is probably distinct in that the second step observed in rat hepatocytes may not exist in HepG2 cells. Thus, even in the presence of OA, HepG2 cells secrete predominantly intermediate density lipoproteins and low density lipoproteins (30). These data suggest that in HepG2 cells, MTP might play a role in the early stages of lipoprotein assembly. To examine this possibility, we conducted experiments in which HepG2 cells were pulse-labeled for 10 min with [3H]leucine and chased up to 180 min. At each time point, total apoB and MTP-bound apoB were determined (Fig. 4). As we have demonstrated previously (14), apoB was rapidly degraded during the chase. Thus, over 50% of initially labeled apoB was degraded within the first 20 min of chase. No secretion had occurred by that time (data not shown). After chase for 60 min, less than 20% of initially labeled apoB was found in the cells. At the same time, only about 5% of the labeled apoB was in the medium (data not shown). On the other hand, the maximum MTP-apoB binding was detected at 10 min of chase; the binding was rapidly decreased by 20 min of chase and minimal thereafter (Fig. 4).

This result indicated that in untreated HepG2 cells, the MTP-apoB interaction occurred transiently at an early stage of apoB lipoprotein assembly. As noted earlier, only a very small proportion of newly synthesized apoB interacts with MTP, a finding consistent with the very low level of apoB secretion from HepG2 cells under basal conditions.

MTP-ApoB Interaction Parallels the Extent of ApoB Translocation across the ER Membrane—When the cells were treated with OA, the MTP-apoB interaction was increased significantly at 10 min of chase (Fig. 5). The high level of interaction remained relatively constant during the next 10 min of chase.

**Fig. 4.** MTP-apoB interaction occurs at an early stage of the assembly of apoB-containing lipoproteins. HepG2 cells were labeled with [3H]leucine for 10 min and chased in serum-free medium up to 180 min. At each time point, cells were lysed and immunoprecipitated with either anti-apoB or anti-MTP large subunit (anti-MTPL). The highest band on the top of the gel represents aggregated material. Interaction between MTP and apoB was greatest at 10 min of chase and rapidly diminished thereafter.

**Fig. 5.** MTP-apoB interaction parallels the extent of apoB translocation across ER membranes. HepG2 cells were preincubated with BSA, OA, or ALLN for 1 h, pulse-labeled with [3H]leucine for 10 min, and chased in serum-free medium up to 60 min. At each time point, the chase, cells were lysed and immunoprecipitated with either anti-apoB or anti-MTP large subunit (anti-MTPL). The highest band on the top of the gel represents aggregated material. The addition of OA after a 30-min chase, which would increase translocation of nascent apoB (15), sharply increased MTP-apoB binding (compare ALLN at the 60-min time point with ALLN at 30 min + OA at the 20- or 30-min time point). Overall, the results suggest that increased translocation of apoB into the ER lumen (OA treatment) is associated with greater interaction of apoB with MTP. On the other hand, simply increasing apoB content without effective translocation (ALLN treatment) mainly prolongs the low, basal level of interaction.
after which it decreased rapidly. This is compatible with the ability of OA to protect apoB from intracellular degradation by facilitating its translocation across the ER membranes and targeting it for secretion (15, 31). By contrast, when the cells were treated with ALLN, the MTP-apoB interaction was only minimally increased compared with control cells at 10 min of chase (Fig. 5A). However, ALLN treatment markedly prolonged the association of MTP with apoB, a result compatible with the direct inhibition of apoB degradation by ALLN, which appears to allow more nascent apoB to slowly translocate across the ER membranes (15). Similar results were obtained in a second experiment (Fig. 5B). In addition, when OA was added to some of the ALLN-treated cells after they had been chased for 30 min, the MTP-apoB interaction was significantly increased, reaching the level observed at the 10 min point in the ALLN-treated cells (Fig. 5B). The molecular characteristics of this interaction remained to be determined.

In summary, the present studies demonstrate that the interaction between MTP and nascent apoB participates in the assembly of apoB-containing lipoproteins. This interaction is very closely coordinated with TG synthesis and appears to be linked to completion of apoB translocation and targeting for secretion. The molecular characteristics of this interaction remain to be determined.

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