A Two-site Model for ApoB Degradation in HepG2 Cells*

(Received for publication, June 26, 1996, and in revised form, February 19, 1997)

Xujun Wu‡, Nobuhiro Sakata, Karen M. Lele, Mingyue Zhou, Hongshi Jiang, and Henry N. Ginsberg
From the Department of Medicine, College of Physicians and Surgeons of Columbia University, New York, New York 10032

Newly synthesized apolipoprotein B (apoB) undergoes rapid degradation in a pre-Golgi compartment in HepG2 cells. A major site of this early degradation seems to be on the cytosolic side of the endoplasmic reticulum (ER) membrane and is sensitive to N-acetyl-leucinyl-leucinyl-norleucinal (ALLN), which can inhibit neutral cysteine proteases and/or proteasome activity. Oleate (OA) treatment, which facilitates translocation of nascent apoB across the ER membrane, also reduces early degradation. In the present studies, we have used brefeldin A (BFA), which inhibits vesicular transport from the ER to the Golgi, to demonstrate that apoB can also be degraded by an ER luminal proteolytic activity that is distinct from the ALLN-sensitive proteases. Thus, when BFA-treated HepG2 cells were co-treated with ALLN, which protects apoB but does not facilitate its translocation into the ER lumen, degradation of newly synthesized apoB was significantly reduced compared with cells incubated with BFA alone. However, apoB degradation was rapid and complete when OA was added to media containing either BFA or ALLN/BFA. These results suggested that OA, by increasing translocation of nascent apoB into the ER lumen, exposed apoB to an ALLN-resistant proteolytic pathway. When we incubated HepG2 cells with dithiothreitol (DTT)/OA/BFA or DTT/OA/ALLN/BFA, degradation of apoB was inhibited. Furthermore, addition of DTT resulted in the accumulation of a 70-kDa amino-terminal fragment of apoB. Both full-length and amino-terminal apoB were degraded if DTT was removed from the incubation media; both were secreted if only BFA was removed. Thus, even after apoB is translocated into the ER lumen (thereby avoiding the initial proteolytic pathway), it can potentially be degraded by a luminal proteolytic process that is ALLN-resistant but DTT-sensitive. The present results, together with previous studies, suggest that at least two distinct steps may be involved in the posttranslational degradation of apoB: 1) the first occurs while apoB is partially translocated and is ALLN-sensitive; and 2) the second occurs in the ER lumen and is DTT-sensitive. Finally, our results support the hypothesis that degradation of partially translocated apoB generates a 70-kDa amino-terminal fragment that is mainly degraded in the ER lumen by a DTT-sensitive pathway.

ApoB secretion from cultured liver cells is regulated mainly at the posttranslational level. Thus, apoB mRNA levels are relatively stable under many conditions, whereas secretion of apoB-containing lipoproteins is altered (1–5). The impact of this posttranslational regulation is demonstrated by the observations that only a small to moderate proportion of the newly synthesized apoB is eventually secreted from primary rat hepatocytes (6, 7), McArdle cells (8), and HepG2 cells (9). A major portion of newly synthesized apoB undergoes rapid intracellular degradation in a pre-Golgi or ER compartment (10–12) in HepG2 cells as well as in apoB cDNA-transfected Chinese hamster ovary cells (13). ApoB can be protected from early intracellular degradation by incubation of cells with OA (9) or with the aldehydic tripeptide ALLN (13–15). OA treatment seems to stimulate apoB secretion by providing triglyceride to newly synthesized apoB and facilitating apoB translocation across the ER membrane (14). OA-induced translocation is probably the result of enhanced interaction between apoB and the microsomal triglyceride transfer protein (16). ALLN does not seem to facilitate the translocation of apoB across the ER membrane (14, 15). Direct protection of apoB, either by inhibition of a neutral cysteine protease or proteasome activity (17), is the likely mechanism underlying the effect of ALLN.

The ALLN-sensitive degradation of nascent apoB is thought to occur on the cytosolic side of the ER membrane or in the cytoplasm. This is based on the finding of 70–85-kDa amino-terminal fragments of apoB in the ER lumen and in the media of HepG2 cells (12, 18, 19). A cytosolic site for the protease(s) involved in early degradation of apoB is also compatible with the ability of OA to prevent degradation by facilitating translocation of nascent apoB across the ER membrane (14). On the other hand, later, alternative sites of degradation have been postulated from studies in rat hepatocytes and McArdle cells (20, 21). Our previous observation (11) that OA treatment did not prevent degradation of apoB in cells that were co-treated with BFA, a drug that prevents vesicular transport from the ER to the Golgi, also suggested a posttranslocation ER-luminal site for apoB degradation. The aim of the present study was to further investigate this potential second site of apoB degradation pathway in HepG2 cells. Two specific questions were addressed: (a) Can apoB undergo degradation after translocation into the ER lumen? and (b) Is this second degradative process sensitive to ALLN?

EXPERIMENTAL PROCEDURES

Materials—L-[4,5-3H]Leucine (135 Ci/mmol; catalog number EX-005) was purchased from Amersham Corp. Monospecific antihuman apoB antiserum was raised in a rabbit. Monoclonal apoB antibodies (M19 and M47) were kindly provided by Dr. Linda Curtiss

1 The abbreviations used are: apoB, apolipoprotein B; DTT, dithiothreitol; OA, oleic acid; BSA, bovine serum albumin; ER, endoplasmic reticulum; ALLN, N-acetyl-leucinyl-leucinyl-norleucinal; BFA, brefeldin A; MEM, minimum essential medium.
Protein A-Sepharose CL 4B was obtained from Pharmacia Biotech Inc. Minimum essential medium (MEM), nonessential amino acids, sodium pyruvate, and penicillin/streptomycin were from Life Technologies, Inc. laboratories. Fetal bovine serum was from Intergen (Purchase, NY). Leucine-free medium was generated from a minimum essential selection kit (Life Technologies, Inc., catalog number 300 9050AV). Leupeptin and pepstatin A were from Peninsula Laboratories, Inc. (Belmont, CA). Bovine serum albumin (BSA) (essentially fatty acid-free), DTT, and OA (sodium salt) (catalog number 07501) were from Sigma. ALLN was from Boehringer Mannheim. All other chemicals were of the highest purity available.

**RESULTS AND DISCUSSION**

In BFA-treated Cells, OA Treatment Abolishes the Protective Effect of ALLN on Newly Synthesized apoB—We previously reported that OA protects apoB from intracellular degradation and stimulates apoB secretion from HepG2 cells (9). However, when exit from the ER compartment was blocked by BFA, OA lost its ability to protect newly synthesized apoB (11). Because BFA treatment also causes retrograde flow of Golgi contents into the ER, the proteolytic activity observed with ALLN/OA/BFA could have originated in the Golgi compartment. We have no data directly addressing this mechanism.

In BFA-treated Cells, OA Treatment Is Associated with Accumulation of a 70-kDa Amino-terminal Fragment of apoB—Recent experiments have demonstrated that an isolated ER compartment contains apoB-specific proteolytic activity, which is not present in an isolated Golgi compartment (11); 2) when retrograde flow from Golgi to ER was blocked with nocodazole, BFA treatment still resulted in a nearly complete degradation of apoB (11); and 3) in the present study, when HepG2 cells were chased in the presence of OALLN and Monensin (which blocks vesicular transport from the Golgi to the plasma membrane), newly synthesized apoB, which was trapped intracellularly under this condition, was not significantly degraded (data not shown). In contrast to HepG2 cells, apoB seems to be stable in BFA-treated rat hepatocytes (21), suggesting that significant degradation of newly synthesized apoB in rat hepatocytes is not associated with the ER compartment. Indeed, Wang et al. (21) concluded that apoB degradation in rat hepatocytes might occur in a post-ER compartment. We have no data directly addressing these apparent differences between HepG2 cells and primary rat hepatocytes.

**DTT Treatment Inhibits Degradation of Full-Length apoB and Is Associated with Accumulation of a 70-kDa Amino-terminal Fragment of apoB—**In screening agents that might inhibit apoB degradation in BFA-treated HepG2 cells, we first...
found that DTT protected apoB from intracellular degradation in untreated cells. Although DTT seems to affect the secretion of proteins containing disulfide bonds (22), Shelness and Thornburg (23) demonstrated recently that once the amino-terminal disulfide bonds are formed, apoB is no longer sensitive to DTT treatment. Therefore, in our experiments, DTT was always added after the labeling period.

HepG2 cells were first labeled with \[^{3}H\]leucine for 10 min and chased in the presence of either BSA or DTT for 60 min. Fig. 2 shows that DTT did not affect the secretion of \(\alpha\)-antitrypsin (Fig. 2A), which does not contain any disulfide bonds, but did inhibit the secretion of albumin (Fig. 2B), which contains disulfide bonds. DTT, added after labeling was completed, increased the secretion of apoB 1.5 ± 0.3-fold (mean of four separate experiments) (Fig. 2C). Increased apoB in the media was associated with protection of apoB from intracellular degradation in the DTT-treated cells (Fig. 2C); significantly more apoB100 was detected in cells chased in the presence of DTT (2.2 ± 0.5-fold; \(n = 4\)) compared with BSA-treated cells. In both the cell and the medium, DTT also increased a band at the top of the gel; this is probably aggre-
gated apoB. Additionally, three other bands with \(M_o\) of approximately 330,000, 170,000, and 70,000 were co-immunoprecipitated with full-length apoB by anti-apoB antibody from DTT-treated cells but not from BSA-treated cells. The nature of the two larger proteins with \(M_o\) of 330,000 and 170,000 is not known, and they were not reproducibly de-
tected. These two proteins were co-immunoprecipitated with albumin by anti-human albumin antibody in DTT-treated cells (Fig. 2B). Furthermore, the 330-kDa band has been observed in media immunoprecipitation in all of our previous studies and is precipitated by Sepharose beads alone; it is not apoB. The 70-kDa protein, as reported previously (12, 18, 19), was only precipitated by antibody to apoB. It was not precip-
itated by anti-albumin nor was it seen on Western blotting of anti-apoB immunoprecipitates with anti-albumin antibody (data not shown). The 70-kDa band was found to be an amino-
terminal fragment of apoB in the following experiment.

HepG2 cells were incubated with either BSA or DTT for 2 h and lysed. Aliquots of the lysate were run on SDS-poly-
acrylamide gel electrophoresis, transferred to a nitrocellulose mem-
brane, and blotted with one of the following antibodies: a polyclonal

antibody against full-length apoB (Mab-N), or a monoclonal antibody against the amino-terminal fragment of apoB (Mab-C). This experi-
ment was performed twice with similar results.

Together, these experiments (Figs. 2C and 3) indicated that DTT treatment could protect both full-length apoB and its amino-terminal fragment from intracellular degradation in HepG2 cells incubated in BSA. These findings raised several questions: 1) Would DTT protect apoB from degradation in BFA-treated cells? 2) Was DTT inhibiting a proteolytic process that was different from the one affected by ALLN? 3) Was the DTT-sensitive degradation occurring in the lumen of the ER? and 4) What was the relationship of the 70-kDa amino-termi
nal fragment to the degradation of full-length apoB? Several experiments were performed to examine these questions.

### DTT Prevents Degradation of apoB in BFA-treated Cells by Inhibiting a Proteolytic Process That Is Distinct from the ALLN-sensitive Pathway

In Fig. 1, the addition of OA to ALLN-treated cells in the presence of BFA resulted in a nearly complete degradation of apoB. This is in sharp contrast to the additive effects of OA and ALLN on the protection of apoB in native cells not treated with BFA (14). To determine if DTT inhibited a proteolytic pathway distinct from ALLN, HepG2 cells were preincubated with ALLN for 1 h, pulse-labeled for 10 min with \[^{3}H\]leucine, and chased in the presence of BFA plus ALLN, ALLN/OA, or ALLN/OA/DTT for 2 h. The results (Fig. 4) indicated that although the addition of OA to BFA/ALLN-
treated cells is associated with complete degradation of nascent apoB, further addition of DTT to the BFA/ALLN/OA incubation at the start of the chase results in protection of both full-length apoB and the 70-kDa fragment. This result supports the hy-

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**Fig. 2. Effects of DTT on the secretion of antitrypsin, albumin, and apoB.** HepG2 cells were pulse-labeled with \[^{3}H\]leucine for 10 min and chased in the presence of either BSA or DTT for 60 min. Immunoprecipitation of the medium and cell lysate was carried out using the following antibodies: (A) antitrypsin antibody, (B) albumin antibody, and (C) apoB antibody. This experiment was performed four times with similar results.

**Fig. 3. Immunoblot demonstrating that DTT treatment results in the accumulation of the 70-kDa amino-terminal fragment of apoB in HepG2 cells.** HepG2 cells were incubated with either BSA or DTT for 2 h and lysed. Aliquots of the lysate were run on SDS-poly-
acrylamide gel electrophoresis, transferred to a nitrocellulose mem-
brane, and blotted with one of the following antibodies: a polyclonal

antibody to full-length apoB (Poly), a monoclonal antibody against the amino-terminal fragment of apoB (Mab-N), or a monoclonal antibody against the carboxy-terminal fragment of apoB (Mab-C). This experi-
ment was performed twice with similar results.
prothesis that DTT inhibits a process that is distinct from the ALLN-sensitive proteolytic pathway. Furthermore, these results, together with those presented in Fig. 1, suggest that the DTT-sensitive proteolytic process is situated in the ER lumen and exerts its effects on apoB that has been translocated across the ER membrane. The latter conclusion is based on two observations: 1) the addition of OA, which facilitates translocation of nascent apoB into the ER lumen, abolished the ability of ALLN to prevent apoB degradation in BFA-treated cells but did not affect DTT-associated protection; and 2) DTT, but not ALLN, prevented degradation of the 70-kDa amino-terminal fragment of apoB, which is believed to efficiently translocate into the ER lumen.

The DTT-sensitive Proteolytic Pathway Is Active in the ER Lumen and Dephosphorylates Both Full-Length apoB and the 70-kDa Amino-Terminal Fragment of apoB—To further support the hypothesis that DTT inhibiting proteolysis of apoB that had already translocated across the ER membrane, the following experiment was carried out. HepG2 cells were labeled with [3H]leucine for 10 min and chased in serum-free medium in the presence of OA, DTT, and BFA for 10 min or 2 h. To determine the topology of newly synthesized apoB under this experimental condition, ER was isolated at both the 10-min and 2-h time points by the ball-bearing homogenization/sucrose gradient ultracentrifuge method described in our previous study (11). Proteinase K sensitivity experiments were performed using the isolated ER. As shown in Fig. 5, at 10-min chase, approximately 70% of newly synthesized apoB was sensitive to proteinase K treatment. After 2-h chase in the presence of OA/DTT/BFA, this apoB had lost its sensitivity to proteinase K treatment, indicating that translocation into the ER lumen had occurred. In addition, the 70-kDa fragment, presumably generated during the degradation of initially labeled apoB, was easily detected at this time and was also insensitive to proteinase K treatment. When the ER preparations were treated with proteinase K plus Triton X-100, apoB was completely sensitive to proteinase K at both times.

In a separate experiment using the same incubation and labeling protocol and a 2-h chase, cells in some dishes were lysed at the end of chase, and intracellular apoB was determined. Cells in the remaining dishes were divided into two groups. For the first group, the chase medium was changed to serum-free medium in the presence of OA, DTT, and BFA for 10 min or 2 h. To determine the topology of newly synthesized apoB, cells were divided into two groups. For the first group, the chase medium was changed to serum-free medium in the presence of OA and DTT; BFA was withdrawn from the incubation (−BFA). The cells were chased for an additional hour. At the end of the chase, the medium was collected, and the cells were lysed for apoB immunoprecipitation. This experiment was performed three times with similar results.

The 70-kDa Amino-terminal Fragment of apoB Is Generated by the ALLN-sensitive Proteolytic Pathway and Is Degraded by the DTT-sensitive Pathway—To better characterize the relationship between the 70-kDa amino-terminal fragment and apoB degradation, the following experiments were performed. HepG2 cells were preincubated with either BSA or ALLN for 1 h, labeled for 10 min, and chased for 10, 30, and 60 min in serum-free medium containing one of three agents (BSA, DTT, or ALLN) or the combination of DTT plus ALLN (Fig. 7A). The results demonstrate that in BSA-treated cells, full-length apoB was rapidly degraded (13.3 ± 2.7% of the initial amount was detected at 60-min chase; n = 4), and no visible 70-kDa fragment accumulated during the chase period. Addition of DTT to the chase medium protected full-length apoB (28.5 ± 5% of the initial amount was detected at 60-min chase; n = 4), and the 70-kDa fragment was now easily detected. When ALLN was present, postlabeling and in the chase medium, full-length apoB was also protected (51.2 ± 13.2% of the initial amount was detected at 60-min chase; n = 3). However, with only ALLN,
very little 70-kDa fragment was detected. In contrast, when both DTT and ALLN were added at the start of the chase to ALLN-preincubated cells, both full-length apoB (58.1 ± 11% of the initial amount was detected at 60-min chase; n = 3) and the 70-kDa fragment were protected. Of note was the observation that significantly less 70-kDa fragment was present when ALLN/DTT was added to the media than when DTT alone was added. Similar results were observed in studies with OA (Fig. 7B). This less 70-kDa fragment was detected during chase with OA/DTT compared with DTT alone, even though the combination of OA/DTT protected full-length apoB to a greater extent (DTT:28.5 ± 5%; n = 4; OA/DTT:37.7 ± 6.4%; n = 3) (Fig. 7B). These results indicated that the 70-kDa fragment of apoB is degraded by an ALLN-resistant proteolytic process and that the ALLN-sensitive pathway (which is also affected by OA) is efficiently translocated into the ER lumen independent of core-lipid availability or the presence of microsomal triglyceride transfer protein (18, 26, 27). The remaining domains of apoB have not been detected in pulse-chase studies and are probably rapidly degraded by a cytosolic pathway. Under conditions in which triglyceride availability is increased (e.g. OA in the medium), a larger fraction of the newly synthesized apoB is targeted for translocation and secretion, and less 70-kDa fragment is generated. However, after translocation of nascent apoB is complete, a second proteolytic pathway present in the lumen of the ER can degrade apoB that is not efficiently transferred to the Golgi. This second-stage degradative process, which is ALLN-resistant but DTT-sensitive, probably controls against the secretion of apoB that has misfolded after translocation. It is also possible that inadequate lipidation of the core of the primordial apoB particle, possibly during a second stage of lipoprotein formation (28–32), might target apoB for degradation by the DTT-sensitive pathway. Characterization of the DTT-sensitive proteolytic pathway could provide new approaches to reducing the secretion of apoB-containing lipoproteins in dyslipidemic patients.

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