In HepG2 Cells, Translocation, Not Degradation, Determines the Fate of the de Novo Synthesized Apolipoprotein B*

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Previous studies show that translocation and degradation of apolipoprotein B (apoB), two processes occurring on or within the endoplasmic reticulum, determine how much de novo synthesized apoB is secreted. We determined which of these processes regulates the intracellular fate of apoB by examining whether degradation determines how much apoB is translocated or if translocation determines how much apoB is degraded. HepG2 cells, treated with the cysteine active site protease inhibitor ALLN, previously shown to block the degradation of translocation-arrested apoB in Chinese hamster ovary cells (Du, E., Kurth, J., Wang, S.-L., Humiston, P., and Davis, R. A. (1994) J. Biol. Chem. 269, 24169–24176), showed a 10-fold increase in the accumulation of de novo synthesized [35S]methionine-labeled apoB. The majority (80%) of the apoB accumulated in response to ALLN was in the microsomal fraction. In contrast, ALLN did not effect apoB secretion. Since ALLN did not effect the intracellular accumulation of [35S]methionine-labeled albumin and other proteins (trichloroacetic acid-precipitable [35S]methionine-labeled proteins), its effect on apoB was specific. Pulse-chase studies showed that ALLN dramatically reduced the first-order rate of removal of [35S]methionine-labeled apoB from the cell but did not effect its rate of secretion. The finding that ALLN caused the intracellular accumulation of incompletely translated chains of apoB suggests that at least some of the degradation occurs at the ribosomal level. Moreover, 85% of the apoB that accumulated in isolated microsomes in response to ALLN was accessible to exogenous trypsin, indicating this pool of apoB was incompletely translocated. The combined data suggest that translocation, not degradation, determines the intracellular fate of de novo synthesized apoB.

apoB is the major structural protein responsible for the assembly of triglyceride-rich lipoproteins by the intestine and liver (1–3). After secretion into blood plasma by the liver, VLDL triglycerides are rapidly hydrolyzed into free fatty acids, which are taken up by peripheral tissues where they are utilized for energy and anabolic purposes. The remaining VLDL remnants, containing apoB100, are then either removed by the liver or converted into LDL, a major risk factor for atherosclerosis (reviewed in Ref. 4). Because of the importance of hepatic secretion of VLDL apoB in determining blood levels of LDL, a major goal of our research has been directed toward identifying the regulatory factors and processes.

We have made two observations, both of which have shown unusual characteristics governing the hepatic secretion of apoB: 1) a large amount of de novo synthesized apoB is not secreted but is degraded intracellularly (5) and 2) the translocation of apoB across the endoplasmic reticulum is unusually inefficient (6). These results led us to hypothesize that as a result of incomplete translocation, apoB is diverted from the secretory pathway into a degradative pathway that occurs in the endoplasmic reticulum (3, 6). Regulated translocation of apoB across the endoplasmic reticulum could explain the posttranslational control of apoB secretion. In several studies, the amount of apoB that is secreted is less than the amount that is synthesized (5, 7–14), leading to the conclusion that the unaccounted for apoB is degraded intracellularly. The consistent observation that in different types of cultured cells and perfused organs obtained from several species, a diverse group of hormones, nutritional states, stimulatory and inhibitory lipids, and mutations in the coding region of apoB alter the rate of apoB secretion by reciprocal changes in its rate of intracellular degradation suggests that this unusual regulatory mechanism of secretion is of general importance (reviewed in Refs. 3, 15–17).

An important question toward which this research was directed was to determine whether translocation governs degradation or if degradation governs translocation. To this end we employed the proteolytic inhibitor ALLN, which we have shown blocks the degradation of apoB in CHO cells, which normally degrade all of the apoB expressed by a plasmid construct (18, 19). Based on the results showing that ALLN also blocks the degradation of apoB100 in HepG2 cells (20), we examined if blocking the intracellular degradation of apoB would either increase the amount secreted or would cause more to accumulate as an incompletely translocated form. If blocking degradation leads to increased secretion, the data would indicate that degradation determines the fate of apoB. Conversely, if blocking degradation leads to an accumulation of incompletely translocated apoB, the data would indicate that translocation determines the fate of apoB.

EXPERIMENTAL PROCEDURES

Cell Culture—HepG2 cells were obtained from American Type Culture and were cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 4% fetal bovine serum, 4% enriched calf serum, and antibiotics as described (21). [35S]Methionine Labeling—HepG2 cells were grown to 90% confluence. On the day of the experiments, the culture medium was changed to methionine-free DMEM containing 8% serum. Cultures were incubated for 30 min at 37 °C at which time some plates (see figure legends)
received the indicated amount of cysteine active site protease inhibitor ALLN (Boehringer Mannheim). 20 min later, 100 μCi/ml [35S]methionine (DuPont NEN) was added to each plate, and the cultures were incubated an additional 20 min. Cells and medium were harvested as described (19).

Immunoprecipitation and SDS-PAGE—Cells, medium, and microsomal membrane fractions were solubilized in 1 ml of immunoprecipitation buffer (25 mM Tris-HCl, 5 mM EDTA, 250 mM NaCl, 1% Triton X-100) and incubated overnight with 50 μl of a 50% v/v solution of Sepharose CL-6B beads (Pharmacia Biotech Inc.). The samples were centrifuged for 5 min at 12,000 rpm, and the supernatant was incubated for 2 h on a shaker with an amount of appropriate antisera previously determined to maximally immunoprecipitate apoB or albumin. Protein A-Sepharose beads (Sigma) were added to each sample in an amount shown to bind all the antibody, and the samples were incubated on a shaker for an additional hour. The samples were centrifuged for 5 min at 12,000 rpm, and the antigen/antibody protein A-Sepharose pellet was washed four times with immunoprecipitation buffer and once with phosphate-buffered saline. The pellets were solubilized in sample buffer (0.13 M Tris, 4% SDS, 20% glycerol, 0.4 mM EDTA, 5% β-mercaptoethanol), boiled for 5 min, and separated on a linear 1–20% SDS-polyacrylamide gel as described (5).

Additional aliquots of cells and medium were precipitated with 10% trichloroacetic acid (5).

Pulse-Chase Studies—The amount of de novo synthesized apoB and albumin synthesized and secreted was determined by pulse-chase analysis as described (5). The only change from the previously reported method was that in some samples the preincubation medium contained ALLN. After 30 min, cells were pulsed with 100 μCi/ml [35S]methionine for 10 min. The medium was removed and replaced with 5 ml of serum-free DMEM containing 1000-fold excess unlabeled methionine (chase medium) and 40 μg/ml ALLN. At the times indicated in legends, cells and medium were harvested. Samples were immunoprecipitated, separated on SDS-PAGE, and analyzed as described above.

First-order rate constants and half-lives were calculated using Sigma Plot program having least-squares analysis.

Microsome Isolation and Digestion with Trypsin—HepG2 cells were pulsed for 40 min with [35S]methionine as described above. Microsomes were isolated from HepG2 cells by nitrogen cavitation followed by ultracentrifugation as described (5). The microsomal pellet was immediately resuspended in 0.25 M sucrose, 10 mM Tris-HCl buffer. Protein content was assayed by the method of Bradford using bovine serum albumin as the protein standard. Trypsin digestion was carried out as described (6). Each microsomal sample contained 150 μg of protein and was incubated for 30 min at room temperature with one of the following: 1) no trypsin, 2) 100 μg/ml trypsin (Sigma), 3) 1 mg/ml type I soybean trypsin inhibitor (Sigma) plus 100 μg/ml trypsin. The digestion was stopped by adding inhibitors (6). The microsomes were re-isolated by centrifugation, resuspended, and analyzed by SDS-PAGE (6).

RESULTS

ALLN Increases the Accumulation of ApoB100 in Cells and Microsomes but Does Not Affect Its Secretion—HepG2 cells were incubated with and without the cysteine active site protease inhibitor ALLN, and the amount of de novo synthesized [35S]-labeled apoB100, albumin, and total protein (i.e. trichloroacetic acid precipitable) were quantitated. Preliminary studies show that at the concentration used (40–50 μg/ml), ALLN did not affect the amount of trichloroacetic acid-precipitated [35S]-labeled protein in either cells or medium for all experiments performed (data not shown). In contrast, after 4 h of constant labeling with [35S]methionine, ALLN treatment caused [35S]-labeled apoB100 to accumulate in cells by 10-fold, compared to control cells (Fig. 1). In this and all other experiments described below, there was no detectable change in the amount of [35S]-labeled apoB100 secreted into medium. In contrast to its effect on apoB accumulation in cells, ALLN did not affect the amount of [35S]-labeled albumin in either cells or medium (Fig. 1).

We examined if ALLN caused [35S]-labeled apoB100 to accumulate in the endoplasmic reticulum, where the majority of apoB in liver cells resides (5, 22). Cells were treated with ALLN for 20 min and then labeled with [35S]methionine for 40 min. Cells were isolated and disrupted by nitrogen decavitation, and microsomes were isolated by ultracentrifugation. Total cell homogenates and microsomes were subjected to immunoprecipitation. In three separate experiments, during the 40-min labeling period, ALLN increased the accumulation of [35S]-labeled apoB100 in cells by 3.0 ± 0.4-fold in whole cells and 2.3 ± 0.9-fold in microsomes (Table I). These data show that the majority (77%) of the apoB that accumulated in cells was isolated in the microsomal fraction.

ALLN Blocks ApoB100 Degradation but Does Not Affect Its Secretion—To determine if ALLN blocks the degradation of apoB100, causing it to accumulate in microsomes, we performed pulse-chase studies. HepG2 cells were incubated with and without ALLN (50 μg/ml) for 0.5 h and then pulsed with [35S]methionine for 10 min. The cells were then chased with methionine replete medium containing the same amount of ALLN as was present in the original pulse medium. At the indicated time of the chase period, cells and medium were harvested and immunoprecipitated using the appropriate antisera.

Analysis of the [35S]-labeled apoB100 during the pulse-chase experiment shows that immediately after the pulse period, there are several small molecular weight forms of apoB that disappeared during the chase period (Fig. 2A). These smaller molecular weight forms of apoB are likely to be incompletely translated nascent chains of apoB residing on the ribosome, as demonstrated by the increased amount of [35S]-labeled apoB100 that appears in cells after 20 min of chase (Fig. 3). After this time, in both groups of cells (with and without ALLN) [35S]-labeled apoB100 was rapidly lost from the cell (Fig. 2A). After
a 30-min period, $^{35}$S-labeled apoB100 appeared in the medium obtained from cells treated both with and without ALLN (Fig. 2). Since even in the presence of ALLN, the amount of $^{35}$S-labeled apoB100 that appeared in the medium was less than the amount that was lost from the cells, under the conditions of this experiment ALLN does not appear to block all of the intracellular degradation of apoB100. It should be pointed out that the time course and amount of ALLN used for our experiments was determined empirically to not cause cytotoxicity as determined by no effect on the amount of $^{35}$S-labeled albumin that accumulated in either cells or medium (Fig. 2B) or the amount of trichloroacetic acid-precipitable $^{35}$S-labeled proteins (data not shown). We have found that higher concentrations of ALLN will inhibit protein synthesis. Because of the cytotoxicity of ALLN, it is not possible to conclude that the dose of ALLN used in our studies was sufficient to block all ALLN-inhibitable proteolysis.

Estimation of the first-order rate of loss of $^{35}$S-labeled apoB peptides (all molecular weight forms) showed that it was 6-fold greater in cells not treated with ALLN compared to cells treated with ALLN (0.05 min$^{-1}$, $t_{1/2} = 13.4$ min without ALLN; 0.008 min$^{-1}$, $t_{1/2} = 90$ min with ALLN). In three separate plates of cells, we examined the quantitative effects of ALLN on apoB accumulation in cells and secretion into the medium during the pulse-chase experimental protocol. ALLN did not significantly affect the rate nor the relative amount of $^{35}$S-labeled apoB100 that appeared in the cultured medium (Fig. 3). These pulse-chase study results confirm the previous data (Fig. 1) showing that while ALLN increases the cellular accumulation of $^{35}$S-labeled apoB100, there is no effect on secretion. Moreover, the data extend these observations by showing that the accumulation of cellular $^{35}$S-labeled apoB100 is caused by a decreased rate of removal (i.e. degradation).

It is interesting to note that during the chase period, ALLN blocked the rate of disappearance of the incompletely translated forms of $^{35}$S-labeled apoB that appear as several bands of lower molecular weight than apoB100 (Fig. 2A). With the proviso that ALLN did not affect the rate of apoB translation, these data suggest that a portion of the intracellular degradation of apoB occurs before it is completely translated.

The ApoB100 That Accumulates in Microsomes from ALLN-treated HepG2 Cells Is Incompletely Translocated. Evidence That Translocation Is Saturated in HepG2 Cells—Microsomes, isolated from HepG2 cells labeled with $[^{35}]$S methionine in the presence and absence of ALLN, were subjected to trypsin digestion to determine how much of the apoB100 that accumulates in response to ALLN remains incompletely translocated. In microsomes from HepG2 cells not treated with ALLN, approximately 82% of the apoB was degraded by trypsin, indicating it was incompletely translocated across the endoplasmic reticulum (Fig. 4). In microsomes from HepG2 cells treated with ALLN, approximately 85% of the total apoB100 present was accessible to trypsin (Fig. 4). Since in both groups of microsomes essentially all (90%) of the albumin was resistant to trypsin digestion (Fig. 4), the microsomes remained intact and oriented in the correct manner. Furthermore, addition of soybean trypsin inhibitor prior to adding the trypsin completely prevented the digestion of apoB100, indicating that proteolysis did not occur after the addition of detergent required to disrupt microsomes prior to immunoprecipitation. Additional studies showed that adding Triton X-100 without inhibitors prior to trypsin digestion caused all of the apoB100 and albumin to be degraded, indicating that sufficient trypsin was present to have digested all exposed protein. The combined data indicate that nearly all of the apoB100 that accumulates in HepG2 cells as a result of ALLN treatment resides in mi-
crosomes as an incompletely translocated form. It should be pointed out that ALLN did increase (2-fold) the amount of apoB that was resistant to trypsin digestion (Fig. 4). Within the context of our experiments, it is not possible to know whether this apoB is fully luminal or is trypsin resistant for other reasons (e.g. folded or complexed with other proteins or lipids). Since ALLN clearly did not augment the secretion of apoB, this trypsin-resistant apoB apparently is not secreted.

**DISCUSSION**

The results presented in this report indicate that the translocation step is responsible for directing apoB into either the secretory or degradation pathways. Furthermore, the data show that under the conditions used to culture HepG2 cells, the amount of apoB that is synthesized is in excess of the amount that is fully translocated, and the "excess" incompletely translocated pool of apoB is shunted into a degradative pathway that can be blocked by ALLN. Taken together, these results indicate that the capacity of HepG2 cells to translocate apoB determines its two major intracellular fates: secretion and degradation. These conclusions are based on the following observations. First, blocking intracellular degradation of apoB by treating HepG2 cells with ALLN caused apoB100 to accumulate intracellularly in microsomes (Fig. 1 and Table I). Most (77%) of the apoB100 that accumulated in the microsomes following ALLN treatment was susceptible to degradation by exogenous trypsin (Fig. 4), indicating that portions of the protein were exposed on the cytoplasmic surface (i.e. it remained incompletely translocated). A previous study indicates that the apoB100 that accumulates in microsomes from both ALLN-treated and untreated HepG2 cells assumes an orientation in which 69 kDa of the N terminus is intralumenal, with the remaining C-terminal portions residing on the cytoplasmic surface (19).

The finding that secretion of apoB and albumin is not affected by ALLN supports the view that under the conditions of these experiments this proteolytic inhibitor did not cause a general impairment of protein synthesis, translocation, or secretion. With the proviso that ALLN does not specifically inhibit apoB translocation, we are compelled to conclude that the inability of ALLN treatment to augment apoB100 secretion is because one or more factors that determine the ability of HepG2 cells to translocate apoB is operating at its capacity (both with and without ALLN treatment).

Our results are consistent with those of a previous study showing that ALLN did not affect the secretion of apoB100 but did increase its intracellular accumulation (20). This study did not examine the translocation status of the accumulated apoB100. Our results showing that essentially all of the apoB100 that accumulates in ALLN-treated HepG2 cells is incompletely translocated extends this earlier work by demonstrating for the first time that translocation, not degradation,
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determines how much apoB is secreted. The additional finding that ALLN caused several small apoB peptides to accumulate in pulse-labeled cells suggests that at least some of the degradation of apoB occurs before it is completely translated. These data raise the interesting possibility that translocation arrest and degradation may be concerted processes.

For most constitutive secretory proteins, rates of secretion are linked to rates of synthesis (23–26). In contrast, under most circumstances the rate of apoB secretion is not linked to its rate of synthesis (reviewed in Ref. 3). As our studies presented here suggest, increasing the availability of apoB by blocking its rapid degradation with ALLN does not lead to increased secretion. Under the conditions used in our experiments, translocation of apoB cannot adapt to accommodate more apoB for entrance into the secretory pathway. However, there are several conditions in which the secretion (hence translocation) of apoB can be augmented (e.g., nutritional status, reviewed in Ref. 15). In a previous study, adding oleic acid to the medium of ALLN-treated HepG2 cells increased the secretion of apoB100 to a greater extent than in HepG2 cells not treated with ALLN (20). These results led to the proposal that oleic acid increased the translocation of apoB100 (20). Moreover, the finding that oleic acid but not ALLN increases the translocation of apoB suggests that lipid, not the amount of apoB, signals an increase in this apparently rate-limiting process.

Two potential recipients for this signal are pulse-transfer sequences in apoB (27, 28) and micosomal triglyceride transfer protein (MTP) (29–31). While our studies clearly show that the capacity of the translocation apparatus is not rate-limiting or paused translocation step (27, 28), it may be situations where other steps in the apoB secretory pathway become limiting. The nutritional and phenotypic state of the cell may have profound influences on one or more steps of the apoB secretory pathway. For example, while in HepG2 cells oleic acid stimulation of lipogenesis augments apoB secretion (8, 10, 20, 36, 37), there is no effect in primary rat hepatocytes (38, 39). Additional studies show that oleic acid increases apoB secretion when infused into livers from fasted rats but not when infused into livers from fed rats (40). A rate-limiting or paused translocation step (27, 28) may act in concert with MTP and the availability of specific lipids to provide the opportunity to efficiently package lipid into lipoproteins for export from the liver and intestine.

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