Regulated Co-translational Ubiquitination of Apolipoprotein B100

A NEW PARADIGM FOR PROTEASOMAL DEGRADATION OF A SECRETORY PROTEIN

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Presentation of a wild-type secretory protein, apolipoprotein B100 (apoB), to the cytosol for ubiquitin-proteasome proteolysis has been observed in HepG2 cells. A currently accepted model for proteasomal degradation of secretory proteins is retrograde translocation of the substrate polypeptides from the lumen of endoplasmic reticulum (ER) back to the cytosol. In this report, we present evidence that newly synthesized apoB becomes exposed to the cytosol and targeted to the proteasomes in a co-translational manner. Thus, after protein translocation was synchronized with puromycin, partially synthesized apoB polypeptides were found to be conjugated to ubiquitin. The magnitude of co-translational ubiquitination and subsequent degradation of apoB was increased when cells were pretreated with either herbimycin A to induce cytosolic Hsp70 or with an inhibitor of microsomal triglyceride transfer protein; both treatments impede translocation of nascent apoB across the ER membrane. These treatments also decreased secretion of apoB and increased its degradation via the ubiquitin-proteasome pathway. We suggest that translocation arrest with subsequent co-translational exposure to the cytosol provides an alternative model to explain how mammalian secretory proteins can overcome topological segregation by the ER membrane and undergo degradation by the ubiquitin-proteasome pathway.

The endoplasmic reticulum (ER) is the site of synthesis of secretory and membrane proteins. Proteins targeted for secretion ordinarily undergo efficient cotranslational translocation across the ER membrane (1, 2). ER quality control mechanisms ensure that only correctly folded, fully assembled or oligomerized proteins are deployed to distal cellular compartments; proteins failing to acquire their natural conformation are retained in the ER and subsequently degraded (3). This “ER degradation” has long been assumed to occur inside the ER lumen (4). Emerging evidence, however, indicates that degradation of aberrant ER luminal proteins is mediated by the cytosolic proteasome (5–10). Accordingly, an “ER dislocation model has been proposed, which suggests that the translocation of nascent proteins into the ER is a reversible process (5–10). ER luminal proteins that are degraded by proteasomes include the unassembled immunoglobulin k light chain (11); the α-antitrypsin variant piZ (12); a mutant form of carboxypeptidase ysc Y (CPY*), which is a yeast vacuolar protein (13, 14); and a misfolded form of yeast mating pheromone precursor, pro-α-factor (15, 16). Additionally, certain ER proteins with a transmembrane topology, such as the plasma membrane T-cell receptor (4) and the cystic fibrosis transmembrane conductance regulator (16), have also been reported to undergo proteasomal degradation.

Cytosolic degradation has been observed with a wild-type hepatic secretory protein, apolipoprotein B100 (apoB) (17, 18). ApoB is the major protein component of atherogenic plasma very low density lipoproteins and low density lipoproteins (19, 20). Regulation of the assembly and secretion of apoB-lipoproteins is predominately a post-translational event involving both recruitment of lipid ligands for lipoprotein formation/secretion and controlled degradation in a pre-Golgi compartment (21–23). Recently, newly synthesized apoB was demonstrated to undergo degradation mediated by the cytosolic ubiquitin-proteasome pathway: apoB was found in a ubiquitin-conjugated state, and its intracellular degradation could be prevented by proteasome-specific inhibitors, such as MG115 (17). We extended that initial finding and demonstrated that the degree of proteasomal degradation appeared to be regulated by the availability of the lipid ligands for apoB (18). For proteins to be degraded by the 26 S proteasome pathway, conjugation with ubiquitin appears to be both an obligatory and rate-limiting process (24). We felt it was crucial, therefore, to determine how ubiquitination of apoB was regulated. Our hypothesis was that inefficient translocation of apoB across the ER membrane results in exposure of domains of apoB to the cytosol and subsequent ubiquitination.

EXPERIMENTAL PROCEDURES

Reagents—Lactacystin was kindly provided by S. Omura (the Kitasato Institute, Japan (25)), dissolved in water, and used at a final concentration of 10 µM (18). The microsomal triglyceride transfer protein (MTP) inhibitor, CP-10447, was provided by Pfizer Inc. (26) and used at a final concentration of 100 µM. [1-45,5-3H]Heucine was used at a concentration of 150 µCi/ml and was supplied as [1-45,5-3H]Heucine from Amersham Pharmacia Biotech with a specific activity of 150 Ci/mmol. [35S]Methionine/cysteine was used at a concentration of 100 µCi/ml and was purchased from NEN Life Science Products as EXPRESS™ Protein Labeling Mix (specific activity >1000 Ci/mmol). Herbimycin A (HA) and puromycin were from Sigma and used at a final concentration of 1.0 µg/ml and 10 µM, respectively (18). Anti-ubiquitin polyclonal antibody was from StressGen. All tissue culture supplies and other chemicals were obtained from suppliers as described previously (21).

Cell Culture and Treatments—The HepG2 cell culture conditions were as described previously (21). Briefly, cells were seeded into collagen-precocated dishes or six-well tissue culture plates and grown in complete medium containing minimum essential medium (MEM) with...
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0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, penicillin/streptomycin, and 10% fetal bovine serum. Cells were fed fresh complete medium every 3 days and maintained in a 5% CO₂ incubator. The Chinese hamster ovary (CHO) cell lines stably expressing 16% of the NH₂-terminus of human apoB cDNA (CHO-B16) and 50% of the NH₂-terminus of human apoB cDNA (CHO-B50) (27) were grown in MEM containing 5% fetal bovine serum and 300 μg/ml of G418 until 90% confluent before experiments were performed. All experiments were performed using exponentially growing cells at 90–95% confluence. Transient transfection of HepG2 cells with Hsp72 cDNA was carried out with Transfectam (Promega) as described previously (18, 28). For synchronization of protein translation, cells were pretreated for 5 min with serum-free MEM containing 10 μM of puromycin at 37 °C. The plates were then put on ice, and cells were washed three times with ice-cold MEM to remove the puromycin. The cells were then transferred to a 37 °C for radiolabeling as described in the legend to Fig. 6.

Pulse-Chase Experiments and Immunoprecipitation—HepG2 cells were preincubated in serum-free MEM and then radiolabeled with either [³H]leucine in a serum-free, leucine-free medium or with Tran³S-label in a methionine-free, cysteine-free medium. Preincubation and labeling media each contained either 1.5% bovine serum albumin (BSA) alone, or BSA plus other reagents as indicated in the figure legends (18). At the indicated time points, cells were removed from the 37 °C incubator and placed on ice for immediate cell lysis or changed into conditioned medium for chase as indicated by the individual figure legends. For cell lysis, the cells were washed with cold phosphate-buffered saline twice and lysed with buffer containing 1% Triton X-100 and proteinase inhibitors (2 mM phenylmethylsulfonyl fluoride, 2 mM ALLN, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). After a 30-min incubation with lysis buffer at 4 °C, the lysates were centrifuged at 14,000 × g in a microcentrifuge for 5 min, and the supernatants containing equal amounts of trichloroacetic acid-insoluble radioactivity were used for immunoprecipitations with the standard protocols in this laboratory (21). The immunocomplexes were mixed with sample buffer and boiled for 5 min. Following centrifugation, the supernatants were aliquoted for SDS-polyacrylamide gel electrophoresis. The gels were subjected to fluorography. Sequential immunoprecipitation was carried out as described previously (18).

RESULTS

We had previously demonstrated that nascent apoB interacts with a cytosolic molecular chaperone, heat shock protein 70 (Hsp70) (27), a protein that facilitates ubiquitination of certain proteins (29, 30). Further studies had shown that when cell Hsp70 levels were increased either by HA treatment or Hsp70 cDNA transfection, apoB degradation by the proteasome was increased (18). Our first experiment, therefore, was to determine if increased levels of Hsp70 could increase ubiquitination of apoB. Fig. 1 shows that when HepG2 cells were treated with HA for 4 h, Hsp70 levels increased markedly (Fig. 1A). This was associated with decreased apoB secretion into the medium (Fig. 1B, lane 3), which was reversed by co-treatment with lactacystin (Fig. 1B, lane 4). This result suggested that the decreased apoB secretion resulted from increased proteasomal degradation. ApoB levels in the cell lysates were also decreased in the HA-treated cells (Fig. 1C, lane 3), and this was reversed by lactacystin co-treatment as well (Fig. 1C, lane 4). Assessment of apoB ubiquitination was carried out with sequential immunoprecipitation (IP) (anti-apoB immunoprecipitation followed by anti-ubiquitin immunoprecipitation) (18). The results represented in Fig. 1D indicate that HA treatment increased the proportion of ubiquitinated apoB relative to total cell apoB in comparison with that of control cells (Fig. 1D, lane 3 versus lane 1, using panel C, lanes 3 and 1, to normalize for total cell apoB). In particular, the amount of very high molecular weight ubiquitinated apoB conjugates was markedly increased by HA treatment. These effects of HA are even more striking when one considers that total cell apoB was reduced by HA treatment. The ability of HA treatment to increase apoB ubiquitination was even more pronounced when cells were treated with HA in the presence of lactacystin (Fig. 1D, lane 4 versus lane 2). Since proteasomal inhibition by lactacystin blocks the degradation of the ubiquitin-tagged apoB by the 26 S proteasome by 26, maximum accumulation of ubiquitinated apoB by HA treatment was further increased in the Hsp70-transfected cells. Lactacystin treatment of Hsp70-transfected cells was also associated with the appearance of high molecular weight forms of ubiquitinated apoB (Fig. 2A, lane 4), which were barely detectable under control and lactacystin-alone conditions (Fig. 2A, lanes 2 and 3).

Overall, these results indicated that increased levels of Hsp70 stimulated apoB ubiquitination as a result, we believe,
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**Fig. 2.** *hsp70* cDNA transient transfection increases apoB ubiquitination. HepG2 cells of 70% confluency were transfected with rat *hsp70* cDNA for 48 h using Transfectam. Cells were then pretreated with or without lactacystin (10 μm) for 1.5 h at 37 °C, followed by radiolabeling with [3H]leucine (150 μCi/ml) in serum-free, leucine-free MEM for 15 min. After a 20-min chase with or without lactacystin, cells were lysed, and immunoprecipitation was performed as described. A, sequential immunoprecipitation, with anti-apoB first and anti-ubiquitin second, was carried out to measure the extent of ubiquitin conjugation of apoB. B, aliquots of cell lysates were immunoprecipitated with anti-apoB antibody only.

**Fig. 3.** ApoB50, but not apoB16, expressed in CHO cells is conjugated with ubiquitin. Confluent CHO cells stably transfected with apoB50 cDNA or apoB16 cDNA (27) were radiolabeled at 37 °C for 4 h with [3H]leucine (150 μCi/ml) in serum-free, leucine-free MEM containing 1.5% BSA and lactacystin (20 μM). Cells lysis and immunoprecipitation (IP) were performed as described under “Experimental Procedures.” A, cell lysates were immunoprecipitated with anti-apoB antibody; B, conditional medium was collected and immunoprecipitated with anti-apoB antibody. C, aliquots of cell lysates were immunoprecipitated with anti-apoB antibody first, followed with anti-ubiquitin antibody.

**Fig. 4.** Inhibition of MTP blocks apoB secretion and increases apoB ubiquitination. HepG2 cells were pretreated for 2 h at 37 °C with serum-free MEM containing 1.5% BSA (lane 1), MTP inhibitor (100 μM, lane 2), lactacystin (10 μM, lane 3), or lactacystin plus MTP-inhibitor (lane 4). Cells were then radiolabeled with [35S]-trans (200 μCi/ml) for 30 min followed by 30 min of chase under the same conditions as that of pretreatment. Cell lysis and immunoprecipitation were performed as described before. A, aliquots of cultured medium were immunoprecipitated with anti-apoB antibody. B, aliquots of cell lysates were immunoprecipitated with anti-apoB antibody. C, equal amounts of radiolabeled apoB immunoprecipitates from cell lysates were aliquoted for anti-ubiquitin immunoprecipitation.

of inhibiting apoB translocation across the ER membrane. To further investigate the relationship between apoB translocation and ubiquitination, we conducted the following experiments. First, we were guided by the observation that carboxyl-truncated apoB containing approximately 50% of apoB100 does not undergo lipoprotein assembly and secretion after transfection into CHO cells (19, 31, 32). Translocation of nascent apoB50 across the ER membrane appears to be inhibited at the point where the lipid-binding β1-sheet domain of the polypeptide (which extends from approximately apoB18 to apoB46) (33) would be predicted to enter the translocon in the ER membrane (31, 32). In contrast, apoB16, which contains no lipid-binding β1-sheet domains (33), is efficiently translocated across the ER membrane and secreted as a lipid-free polypeptide (31, 32). ApoB16 does not interact with cytosolic Hsp70, whereas apoB50 does (27). In order to determine if inefficient translocation of apoB can predispose it to ubiquitination, we determined the ubiquitination status of apoB16 and apoB50 that were stably expressed in CHO cells (27). Fig. 3 shows that apoB16, depicted as an 85-kDa band in both the cell lysate (Fig. 3A, lane 1) and the medium (Fig. 3B, lane 1), was minimally ubiquitinated in the cells (Fig. 3C, lane 1). In contrast, apoB50, which showed a 250-kDa band plus some aggregates and fragments in the cell lysate (Fig. 3A, lane 2), displayed minimal, if any, secretion into the medium but was heavily ubiquitinated in the cells (Fig. 3, B and C, lane 2). Thus, translocation arrest and subsequent trapping in the ER membrane can render apoB susceptible to ubiquitination.

Second, we exploited the effects of MTP on apoB translocation and degradation via the ubiquitin-proteasome pathway. MTP expression is restricted to cells of hepatic and intestinal origin, and assembly of apoB into lipoprotein particles in these organs requires functional MTP activity (34). MTP may act as a molecular chaperone on the luminal side of the ER membrane and facilitate the translocation of apoB (35). Inhibition of MTP blocks apoB secretion and increases apoB intracellular degradation (26, 34). We determined, therefore, if inhibition of MTP would affect apoB ubiquitination. HepG2 cells were pretreated with a specific MTP inhibitor (CP-10447) (26) for 2 h with or without the addition of lactacystin, followed by 30 min of radiolabeling and 30 min of chase under the same conditions. As shown in Fig. 4A, cells that were treated with the MTP inhibitor alone had minimal, if any, apoB secretion into the medium (Fig. 4A, lane 2). The cell apoB level was also lower than that of control cells (Fig. 4B, lane 2 versus lane 1), suggesting increased intracellular degradation. Co-treatment with MTP inhibitor and lactacystin blocked apoB degradation and, therefore, increased the apoB level in the cell lysate (Fig. 4B, lane 4). The defect in apoB secretion was not, however, reversed by lactacystin (Fig. 4A, lane 4), since lipid ligands could not be transferred to apoB molecules. Fig. 4C represents the results from sequential immunoprecipitation; after apoB immunoprecipitation, equal amounts of radiolabeled apoB immunoprecipitates from cell lysates were aliquoted for anti-ubiquitin immunoprecipitation.
These data suggest strongly that MTP inhibition leads to increased cytosolic exposure and ubiquitination of nascent apoB.

The results of the preceding experiments implied that when apoB translocation was impeded, there was increased exposure of apoB domains to ubiquitin-conjugating enzymes. These results did not tell us, however, how early in the translation-translocation process exposure to the cytosol occurred. Domains of apoB could have been exposed to the cytosol either during or after translation. Furthermore, if cytosolic exposure occurred after translation, Hsp70 and ubiquitinating enzymes could have been targeted to apoB domains that had not entered the ER lumen (i.e., apoB that was translated, but translocation-arrested) or to apoB domains that had completed translocation but were now being transported back across the ER membrane in a retrograde fashion. Two possible pathways linking nascent apoB to the cytosolic ubiquitin-proteasome pathways are depicted in Fig. 5.

A model of translocation arrest and subsequent looping out of elongating polypeptides to the cytosol (36) predicts that apoB ubiquitination could take place before the polypeptide reached full-length (co-translational ubiquitination; Fig. 5A), whereas a retrograde translocation model implies that only full-length apoB would be ubiquitinated (post-translational ubiquitination; Fig. 5B). Of note, Benoist and Grand-Perret (37) recently reported that apoB degradation by the proteasome could occur in a co-translational manner. To ascertain if nascent apoB is exposed to the cytosol co-translationally, we used puromycin pretreatment to synchronize protein translation before radiolabeling cellular proteins. Puromycin treatment is known to expose the ribosomes to the cytosol co-translationally. To initiate protein synthesis, puromycin binding to the ribosome (37). After removal of puromycin, therefore, all of the ribosomes exist in an unengaged state and would be ready to initiate protein synthesis synchronously. As demonstrated in Fig. 6A, following puromycin synchronization, the length of newly synthesized, radiolabeled apoB polypeptide chains increased in proportion to the duration of labeling period. Thus, after 3 min of labeling, nascent apoB chains reached lengths up to about 20% of full-length apoB (80–100 kDa) (Fig. 6A, lane 2). Labeling for 8 min allowed synthesis of about 50% of full-length apoB (~250 kDa) (Fig. 6A, lane 3), while 15 min of labeling allowed apoB synthesis to reach about 70–80% of full length (~400 kDa). These apoB peptides were, therefore, still undergoing translation. Sequential immunoprecipitation was performed to detect if these premature apoB polypeptides had been ubiquitinated (Fig. 6B). Compared with lane 1, which served as a positive control for ubiquitination of full-length apoB from cells not pretreated with puromycin, the results in lanes 3 and 4 demonstrated that apoB polypeptide chains that reached 50–80% of full length were clearly ubiquitinated. In contrast, shorter stretches of the apoB NH2-terminal region showed minimal ubiquitination (lane 2). It is possible that the sites for ubiquitination of apoB are located in the carboxy-terminal 50% of the molecule. There are no studies of the sites of apoB ubiquitination, but apoB has lysine residues distributed from the amino to the carboxyl terminus. We believe that it is unlikely that a lack of preferred lysine sites was the basis for the absence of ubiquitination of apoB peptides that were about 20% of the full-length protein. By contrast, we believe that the absence of cytosolic exposure of these very short apoB peptides prevented ubiquitination. This conclusion is supported by the results shown in Fig. 3, demonstrating that apoB16 was secreted and only minimally ubiquitinated while apoB50 was not secreted but was heavily ubiquitinated in CHO cells, and by our prior demonstration of the lack of association of apoB16 with Hsp70 (27).
isolation of polysome-associated, partially synthesized apoB polypeptides; we observed that polysome-associated apoB polypeptides were conjugated with ubiquitin (data not shown). Overall, these experiments demonstrate clearly that apoB ubiquitination can occur co-translationally.

Finally, we tested the hypothesis that perturbations impeding the translocation of apoB would increase co-translational ubiquitination of nascent apoB polypeptides. HepG2 cells were pretreated with either HA or the MTP inhibitor. After puromycin synchronization and 10 min of radiolabeling, apoB peptide chains were synthesized up to about 50% of full length (Fig. 7A, lanes 2–4). The magnitude of co-translational apoB ubiquitination, as shown in Fig. 7B, was increased by both HA and MTP inhibitor treatment (lanes 3 and 4) in comparison with that of control cells (lane 2). Thus, co-translational ubiquitination of apoB was increased when apoB translocation was inhibited. Furthermore, high molecular weight apoB-ubiquitin conjugates (>500 kDa) were easily detected (lanes 3 and 4) in cells treated with either HA or the MTP inhibitor.

DISCUSSION

Our demonstration that apoB ubiquitination can occur even before the nascent apoB polypeptide chain reaches full length makes it very unlikely that retrograde translocation of apoB from the ER lumen back to the cytosol is necessary to explain its proteasomal degradation. Our observations are, by contrast, compatible with a model in which the initial co-translational targeting and translocation of the amino-terminal domain of apoB across the ER membrane is followed by translocation slowing or even translocation arrest, resulting in exposure of carboxyl-terminal domains to the cytosol. This model emphasizes that translocation of apoB across the ER membrane is an early sorting process that can determine the ultimate fate of newly synthesized apoB molecules; efficient translocation allows apoB to enter the secretory pathway, where it is assembled into a lipoprotein, whereas inefficient translocation exposes apoB to cytosolic degradation via the ubiquitin-proteasome pathway. The efficiency of translocation appears to be determined mainly by the availability of the other components of apoB-containing lipoproteins, particularly the core lipids, triglyceride, and cholesterol ester. When lipid availability is limited, apoB co-translationally assumes a transmembrane topology and interacts with cytosolic Hsp70. This interaction facilitates degradation by both impeding further translocation (18, 27) and promoting ubiquitination (Figs. 1, 2, and 7) (29, 30). The finding that both ubiquitin-conjugating enzymes and proteasomes can be associated with the cytosolic face of the ER membrane is compatible with coupling of degradation to the translocation process (38–40). Interestingly, the NH2-terminal 70 kDa of apoB polypeptide, which would be expected to co-translationally enter the ER lumen before translocation arrest occurs (31), has been detected in both the medium of cultured cells and the circulation of patients with abetalipoproteinemia, a disorder in which MTP is absent (31, 32). This suggests that proteasomal degradation might act as a molecular “razer,” shaving off cytosolically exposed domains of apoB while sparing the already translocated amino-terminal domain (8).

In summary, apoB100 is a very large, extremely hydrophobic secretory protein that is constitutively synthesized and functions as a lipid transporter in hepatocytes. Our results indicate that these cells have evolved a very efficient quality control strategy to monitor the biogenesis of apoB-containing lipoproteins such that elimination of “lipid-challenged” nascent apoB can occur well before apoB synthesis is completed. Since degradation of a large secretory protein such as apoB requires significant investment in energy and time, it is reasonable that liver cells would favor a process of co-translational targeting to the ubiquitin-proteasome pathway over a process involving retrograde translocation of full-length apoB. Whether this pathway is unique for apoB or is a model for other nascent secretory proteins that are degraded by the proteasome remains to be determined.

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