Overexpression of the Tumor Autocrine Motility Factor Receptor Gp78, a Ubiquitin Protein Ligase, Results in Increased Ubiquitinylation and Decreased Secretion of Apolipoprotein B100 in HepG2 Cells*

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Jun-shan Liang‡§, Tonia Kim¶§, Shengyun Fang†, Junji Yamaguchi‡, Allan M. Weissman‡, Edward A. Fisher¶, and Henry N. Ginsberg‡**

From the ‡Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032, ¶Cardiovascular Institute and Departments of Medicine and Biochemistry, Mount Sinai School of Medicine, New York, New York 10025, and §§Regulation of Cell Growth Laboratory, National Cancer Institute, Frederick, Maryland 21702

Apolipoprotein B100 (apoB) is a large (520-kDa) complex secretory protein; its secretion is regulated post-transcriptionally by several degradation pathways. The best described of these degradative processes is cotranslational ubiquitinylation and proteasomal degradation of nascent apoB, involving the 70- and 90-kDa heat shock proteins and the multiple components of the proteasomal pathway. Ubiquitinylation involves several proteins, including ligases called E3s, that coordinate the covalent binding of ubiquitin to target proteins. The recent discovery that tumor autocrine motility factor receptor, also known as gp78, is an endoplasmic reticulum (ER)-associated E3, raised the possibility that this E3 might be involved in the ER-associated degradation of nascent apoB. In a series of experiments in HepG2 cells, we demonstrated that overexpression of gp78 was sufficient for increased ubiquitinylation and proteasomal degradation of apoB, with reduced secretion of apoB-lipoproteins. This action of gp78 was specific: overexpression of the protein did not affect the secretion of either albumin or apolipoprotein A1. Furthermore, overexpression of a cytosolic E3, Itch, had no effect on apoB secretion. Finally, using an in vitro translation system, we demonstrated that gp78 led to increased ubiquitinylation and proteasomal degradation of apoB48. Together, these results indicate that an ER-associated protein, gp78, is a bona fide E3 ligase in the apoB ER-associated degradation pathway.

Apolipoprotein B100 (apoB) is the essential protein component of atherogenic very low density and low density lipoproteins (1), and overproduction of these lipoproteins is a common feature of human dyslipidemia (2). Extensive studies of cultured primary hepatocytes and hepatoma cells have established that significant control over apoB secretion can be achieved at the post-transcriptional level by degradation. One form of presecretory degradation, namely the ER-associated degradation (ERAD) of apoB, is regulated by the availability of newly synthesized core lipids (triglyceride, cholesterol ester) and microsomal triglyceride transfer protein activity (1, 3, 4). When lipid availability or microsomal triglyceride transfer protein activity is limited, translocation of apoB is incomplete, and apoB is ubiquitinated co-translationally and degraded by the cytosolic ubiquitin-proteasome pathway (5–9).

Cytosolic and nuclear proteins are targeted for proteasomal degradation by the addition of multi-ubiquitin chains. The specificity of this process is largely conferred by ubiquitin (Ub) protein ligases (E3s) (10–12). E3s interact directly or indirectly with substrate and mediate the transfer of Ub from Ub-conjugating enzymes to target proteins. Two major E3 classes have been identified: HECT domain E3s and RING finger E3s (10–12). E3s interact directly or indirectly with substrate and mediate the transfer of Ub from Ub-conjugating enzymes, thereby forming thiol-ester intermediates with Ub. The Ub is then transferred to the target protein. In contrast, RING finger E3s bind Ub-conjugating enzymes and apparently mediate the direct transfer of Ub from an Ub-conjugating enzyme to the target protein. Gp78 was originally isolated as a membrane glycoprotein from murine melanoma cells and was implicated in cell migration (13). Subsequently, gp78 was identified as the tumor autocrine motility factor receptor mediating tumor invasion and metastasis (14). Recently, we identified the tumor autocrine motility factor receptor gp78 as a RING finger-dependent Ub protein ligase, the first mammalian ER-resident E3 (15). This protein localizes primarily to the ER and targets itself, as well as a well-characterized ERAD substrate, the T cell antigen receptor CD3 δ-subunit, for proteasomal degradation.

Because of the demonstrated role of gp78 in the ERAD for one target protein, we investigated whether it played a similar role for apoB, a secretory protein. When apoB’s “lipid-ligands” are limited, it undergoes extensive degradation by the Ub-proteasome system (1). We investigated this role by two approaches: transfection studies in HepG2 cells and co-expression of gp78 and apoB in a cell-free system. Our results with both approaches were consistent with each other and together indicate that gp78 is a bona fide E3 ligase in the apoB ERAD pathway.
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FIG. 1. A, specific expression of gp78 in Hep G2 cells. Thirty-six hours after transfection (with mock reagents (c) or with cDNA encoding either gp78 (ER-E3) or myc-tagged Itch (cytosolic E3), the cells were lysed and cell lysates were analyzed by Western blotting analysis with anti-gp78 antibodies (left) or anti-myc antibodies (right). The arrows indicate the expected positions of gp78 and Ith. Both were efficiently expressed after transfection. B, overexpression of gp78 reduces secretion of apoB from HepG2 cells. After being transiently transfected with gp78 or Itch cDNAs, HepG2 cells were labeled for 2 h with $^{35}$S methionine. After labeling, cellular and medium apoB were analyzed by immunoprecipitation with anti-human apoB antibody. The data shown are representative of three experiments. D, ALLN reverses the decreased secretion of apoB associated with overexpression of gp78 in HepG2 cells. After being transiently transfected with mock reagents (c) or with gp78 or Itch for 36 h, HepG2 cells were labeled for 2 h with $^{35}$S methionine in the presence or absence of ALLN (40 μM). After labeling, cellular and medium apoB were analyzed by immunoprecipitation with anti-human apoB antibody. The data are representative of four experiments.

MATERIALS AND METHODS

Reagents—N-Acetyl-leucinyl-leucinyl-norleucinal (ALLN), oleic acid, Triton X-100 and protein A-Sepharose CL-4B were purchased from Sigma. ALLN was used at a concentration of 100 μM (40 μg/ml). Sheep anti-human apoB polyclonal antibody was purchased from Roche Applied Science. $^{35}$S Methylamine was from PerkinElmer Life Sciences. LipofectAMINE was purchased from Invitrogen.

Growth of Cells—HepG2 cells were obtained from the American Type Culture Collection. Briefly, cells were maintained at 37°C in 5% CO₂ in 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. The medium was changed every 3 days, and experiments were started after the cells were 70–90% confluent. During the experiments, cells were maintained at 37°C in 5% CO₂ in serum-free minimum essential medium containing 1.5% bovine serum albumin, with the indicated additions or treatments.

In transfection experiments, HepG2 cells were treated with LipofectAMINE with or without human gp78 cDNA (15) or murine Itch cDNA (15). Itch is a cytosolic E3 that is not involved in ERAD (16, 17). After 36 h, cells were labeled with $^{35}$S methionine. After labeling, cellular and conditioned medium were analyzed by immunoprecipitation as described below.

Immunoprecipitation—Briefly, after samples were incubated on a shaker for 10 h at 4°C with an excess amount of anti-apoB antisera, the immune complexes were precipitated by an additional 3-h incubation with protein A-Sepharose CL-4B. The samples were analyzed on 4% SDS-polyacrylamide gels. For analysis of ubiquitylated apoB, cellular immunoprecipitates were incubated with the addition of [35S]methionine. Antibodies were resolubilized by boiling 4 min in 2% SDS and subjected to a second immunoprecipitation with anti-apoB antibody after adjusting the SDS concentration to 0.1%.

Cell-free System Studies—The cell-free expression of human apoB48 cDNA in the Promega TNT system was described previously (18). Briefly, human apoB48 cDNA (1.5 μg of circular plasmid) was transcribed/translated in the presence of rabbit reticulocyte lysate, canine pancreatic microsomes, and $^{35}$S protein labeling mix (1000 Ci/mmol; PerkinElmer Life Sciences); hereafter, this is referred to as the “reaction.” ApoB48 was studied either as translated alone or co-translated with gp78 cDNA (0.5 μg) and either with or without 50 μM benzoyl-carbonyl-Ile-Glu(3-butyl)-Ala-leucinal (PSI; proteasome inhibitor obtained from S. Wilk, Mount Sinai School of Medicine) (18, 19) dissolved in Me2SO. In the reaction in which PSI was absent, an equivalent volume of Me2SO was substituted. Incubation of the coupled transcription/translation reactions (total volume, 50 μl) was carried out at 30°C for 1.5 h. Aliquots (25% of the transcription/translation reaction mixture) were then taken to isolate microsomes by centrifugation at 90,000 × g for 30 min at 4°C. The microsomes were then solubilized in gel loading buffer and proteins separated by SDS-PAGE (3–17% acrylamide gradient). Fluorography was used to detect the radioactive apoB48 protein bands.

The remaining 75% of the reaction mixture was used to study apoB48 ubiquitylation. A 100× excess of unlabeled Met/Cys was added, and the reaction mixture was composed of 2 mM ATP, 10 mM creatine kinase, and 5 μl of anti-human apoB antibody (Roche Diagnostics). The final immunoprecipitates were then analyzed by SDS-PAGE and fluorography as above.

RESULTS AND DISCUSSION

We first investigated the effect of overexpression of gp78 in HepG2 cells on the synthesis and secretion of apoB. Itch (16, 17), a cytosolic E3, was used as a control. Thirty-six hours after transient transfection with cDNAs for gp78 or myc-tagged Itch, we observed a decrease in the secretion of apoB associated with the expression of gp78 in HepG2 cells. After being transiently transfected with mock reagents (c) or with gp78 or Itch for 36 h, HepG2 cells were labeled for 2 h with $^{35}$S methionine (in the presence or absence of ALLN (40 μM)). After labeling, cellular and medium apoB were analyzed by immunoprecipitation with anti-human apoB antibody. The data are representative of four experiments.

A C

B cell medium

D cell medium

FIG. 1.
HepG2 cells were labeled for 2 h with [35S]methionine. After labeling, cellular and medium were analyzed by immunoprecipitation with anti-human apoB, anti-human albumin, or anti-human apolipoprotein AI antibodies. The latter two proteins are known to be ERAD substrates and served as controls. Specific expression of gp78 was confirmed by Western blotting with an anti-gp78 polyclonal antibody (Fig. 1, left). The second approach we took to establish the role of gp78 in apoB ERAD was to co-express gp78 cDNA and apoB48 cDNA in a cell-free system that we had previously demonstrated could recapitulate the major features of apoB100 ERAD (18). As shown in Fig. 4, the co-expression of gp78 led to decreased recovery of microsome-associated apoB48 (Fig. 4A, compare lanes 1 and 3; for each sample, microsomes from 12.5 μl of the transcription/translation reaction mixture were solubilized directly in gel buffer and subjected to SDS-PAGE). Concomitantly, overexpression of gp78 was associated with an increase in Ub-apoB48 (Fig. 4A, compare lanes 2 and 4; for each sample, microsomes from 37.5 μl of the transcription/translation reaction mixture were subjected to a two-step immunoprecipitation to isolate ubiquitinylated apoB48). Nonspecific effects of co-expression could not explain these results, because the co-expression of luciferase and apoB48 had no impact on apoB48 recovery or ubiquitinylation relative to results when only apoB48 was expressed (data not shown).
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To determine apoB synthesis. The result is representative of two experiments.

Fig. 3. Oleic acid treatment partially reverses the effect of gp78 overexpression on secretion of apoB in HepG2 cells. After being transiently transfected with gp78 or Itch cDNAs for 36 h, Hep G2 cells were labeled for 30 min with [35S]methionine in the presence of ALLN (40 μg/ml). After labeling, the original medium was washed out, and cells were first incubated for an additional 15 min in new chase media without ALLN. Then, the chase medium was changed again to fresh chase medium with or without 0.4 mM oleic acid) for 15, 30, and 60 min. Chase medium was collected at each time point and analyzed by immunoprecipitation with anti-apoB antibody. Cell lysates from some wells at the end of labeling were used to determine apoB synthesis. Cell lysates from some wells at the end of labeling were used to determine apoB synthesis. The result is representative of two experiments.

Fig. 4. Gp78 increases ubiquitinylation and degradation of apoB48 in the cell-free system. ApoB48 was labeled with [35S]methionine/cysteine in a coupled transcription/translation reaction (noted Reaction in figure) containing rabbit reticulocyte lysate and canine pancreatic microsomes (total volume, 50 μl). ApoB48 was expressed either alone (lanes 1 and 2) or co-expressed with gp78 (lanes 3 and 4). After 1.5 h at 30 °C, an aliquot (25% of total volume) was removed and microsomes isolated. Hemagglutinin-ubiquitin was added to the remaining mixture (75% of total volume), the incubation was continued (30 min at 37 °C), and microsomes were isolated. Microsome-associated total apoB48 (lanes 1 and 3) was recovered by solubilizing microsomes in gel loading buffer. Ubiquitinylated (Ub)-apoB48 species (lanes 2 and 4) were recovered by a two-step immunoprecipitation of isolated microsomes using anti-hemagglutinin followed by anti-apoB antibodies as described under “Materials and Methods.” Analysis of the solubilized microsomes or the immunoprecipitates was by SDS-PAGE/fluorography; in each case, the total volume available was loaded onto the gels. A and B comprise data from a representative experiment (three total experiments) conducted in the absence (A) or presence (B) of the proteasome inhibitor PSI. ApoB48 denotes the site on the gel where native, non-ubiquitinylated apoB48 (lanes 1 and 3) or minimally ubiquitinylated apoB48 (lanes 2 and 4) would be found; Ub-apoB denotes the site on the gel where polyubiquitinylated apoB48 would be found. Co-expression of gp78 significantly reduced the recovery of apoB48 in microsomes; this was caused by increased ubiquitinylation and proteasomal degradation.

That the decreased recovery of apoB48 observed when gp78 was co-expressed was caused by proteasomal degradation is supported clearly by the data in Fig. 4B, which employed PSI (19), an effective inhibitor of the proteasome in the cell-free system (18). As shown in Fig. 4B, PSI did not alter the recovery of microsome-associated apoB48 when it was expressed alone (Fig. 4, compare lanes A1 and B1), but did significantly increase recovery of translated apoB48 from reaction mixtures where gp78 was co-expressed (Fig. 4, compare lanes A3 and B3). We have previously shown that the most highly ubiquitinylated apoB species are stable and do not undergo de-ubiquitinylation (23). Thus, the appearance of a wider molecular mass range of Ub-apoB48 species when PSI is added to inhibit proteasomal degradation is not surprising (Fig. 4, lanes A4 and B4, compare the regions above the expected migration of unmodified apoB48). The more labile forms of moderately ubiquitinylated apoB48 that run between the top of the gel and where minimally ubiquitinylated apoB48 runs were now able to accumulate.

ApoB is an atypical secretory protein that, after undergoing early translational targeting to the ER and initiation of cotranslational translocation into the ER lumen, assumes a bipartite orientation in the ER membrane. Thus, concomitant with a prolonged interaction between apoB and the Sec61 proteins of the translocon (24), partially translocated apoB has domains in both the ER lumen and in the cytosol (25). Because of this atypical orientation relative to the ER membrane, sequences of apoB are exposed to the cytosol, leading to an interaction with the 70- and 90-kDa heat shock proteins and the 26 S proteasome (18, 26). These associations can lead to cotranslational ubiquitinylation (27) and degradation (7). The proportion of newly synthesized apoB that undergoes degradation by the proteasome is critically dependent on the availability of the lipid-ligands for apoB, especially triglycerides (4, 28, 29), and varies by cell type. However, ubiquitinylation and proteasomal degradation of apoB has been shown to occur in all systems, including primary hepatocytes (1).

Ubiquitinylation is dependent on several proteins that activate and transfer ubiquitin to the targeted protein substrate (10–12). The fact that ubiquitinylation and proteasomal degradation of apoB occurs cotranslationally raised the question as to whether any of the proteins involved in those processes were associated with the ER. It has been demonstrated in yeast, for example, that mutant carboxypeptidase Y (30) or misfolded hydroxymethyl glutaryl-CoA reductase (31) interacts with E3 ligases that are members of the DER3/HRD complex, which contains multiple integral ER membrane proteins (32). The recent identification of a mammalian transmembrane E3 associated with the ER (15) allowed us to address that question. The results of the present studies provide clear evidence that the ER-associated E3, gp78, can participate in the ubiquitinylation and proteasomal degradation of apoB. This finding adds another important piece to a model of post-transcriptional reg-
ulation of apoB secretion in which, after initial insertion of the amino terminus of apoB into the ER lumen, degradation can be initiated in the original translocon (33) after only 50–60% (7, 27) of the protein has been translated. Considering the size of apoB (4536 amino acids) and the complexity of its secondary and tertiary structure (34), the presence of all essential components of a major system for degradation at the site of synthesis of this protein provides the most efficient system for regulation. In this regard, the results of the present study are consistent with our previous data, which demonstrated that other cytosolic components involved in the targeting and degradation of apoB, such as the 70- and 90-kDa heat shock proteins and proteasomes, are all associated with microsomal apoB and degradation remains to be determined.

Because of the large size of apoB, it would not be surprising if associated E3s, or cytosolic E3s other than Itch, can participate significantly in apoB degradation. Consistent with our previous data, which demonstrated that all essential components of a major system for degradation at the site of synthesis of this protein provides the most efficient system for regulation of apoB secretion in which, after initial insertion of the amino terminus of apoB into the ER lumen, degradation can be initiated in the original translocon (33) after only 50–60% (7, 27) of the protein has been translated. Considering the size of apoB (4536 amino acids) and the complexity of its secondary and tertiary structure (34), the presence of all essential components of a major system for degradation at the site of synthesis of this protein provides the most efficient system for regulation. In this regard, the results of the present study are consistent with our previous data, which demonstrated that other cytosolic components involved in the targeting and degradation of apoB, such as the 70- and 90-kDa heat shock proteins and proteasomes, are all associated with microsomal apoB and degradation remains to be determined. Because of the large size of apoB, it would not be surprising if its different domains are processed by distinct E3s, given the selectivity of these ligases.

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