Co-translational Interactions of Apoprotein B with the Ribosome and Translocon during Lipoprotein Assembly or Targeting to the Proteasome*

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Hepatic lipoprotein assembly and secretion can be regulated by proteasomal degradation of newly synthesized apoB, especially if lipid synthesis or lipid transfer is low. Our previous studies in HepG2 cells showed that, under these conditions, newly synthesized apoB remains stably associated with the endoplasmic reticulum (ER) membrane (Mitchell, D. M., Zhou, M., Pariyarath, R., Wang, H., Aitchison, J. D., Ginsberg, H. N., and Fisher, E. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 14733–14738). We now show that independent of lipid synthesis, apoB chains that appear full-length are, in fact, incompletely translated polypeptides still engaged by the ribosome and associated with the ER translocon. In the presence of active lipid synthesis and transfer, translation and lipoprotein assembly are completed, and the complexes exit the ER. Upon omitting fatty acids from, or adding a microsomal triglyceride transfer protein inhibitor to, culture media to reduce lipid synthesis or transfer, respectively, apoB was degraded while it remained associated with the ER and complexed with cytosolic hsp70 and proteasomes. Thus, unlike other ER substrates of the proteasome, such as major histocompatibility complex class I molecules, apoB does not fully retrotranslocate to the cytosol before entering the ubiquitin-proteasome pathway. Although, upon immunofluorescence, apoB in proteasome-inhibited cells accumulated in punctate structures similar in appearance to aggresomes (cytosolic structures containing molecules irreversibly lost from the secretory pathway), these apoB molecules could be secreted when lipid synthesis was stimulated. The results suggest a model in which 1) apoB translation does not complete until lipoprotein assembly terminates, and 2) assembly with lipids or entry into the ubiquitin-proteasome pathway occurs while apoB polypeptides remain associated with the translocon and attached to the ribosome.

Apoprotein B100, the major structural protein of atherogenic very low density and low density lipoprotein particles, is an unusually large secretory hepatic protein with a molecular mass ~550 kDa. The regulation of the assembly and secretion of the apoB100-containing lipoproteins has been studied in various hepatic cell models, including the human hepatocarcinoma cell line HepG2. In human intestine, enterocytes edit the transcript of the apob gene so that a shorter protein, apoB48, is formed. Because HepG2 cells do not have this editing activity (2), throughout this report, the abbreviation apoB will refer exclusively to apoB100 and its incompletely translated polypeptides.

One of the early steps of apoB-lipoprotein biogenesis in the endoplasmic reticulum (ER) is the association of translocated domains of apoB with its “lipid ligands” in a process mediated by the lipid transfer activity of microsomal triglyceride transfer protein (MTP) (1). After the initial co-translational lipidation of apoB, the remainder of the assembly process is thought to occur post-transcriptionally (3). If either the synthesis or transfer of lipid ligands is limited, apoB is rapidly degraded, as illustrated not only by numerous studies in vitro (e.g. Refs. 4 and 5), but also by the human genetic disease abetalipoproteinemia, which results from mutations of MTP (6). We and others have shown in HepG2 cells that most of this metabolically regulated intracellular degradation of apoB occurs through the ubiquitin-proteasome pathway (7–9) in a process that appears to involve the cytosolic chaperone hsp70 (8, 10).

Studies in yeast and mammalian systems have identified the proteasome as the principal means of disposal of a growing list of ER-associated proteins that presumably become misfolded because of either a structural mutation or a failure to assemble properly into a oligomeric complex (11, 12). This disposal process has been called ER-associated degradation (ERAD). Because the components of the ubiquitin-proteasome machinery are located in the cytosol, degradation of secretory and integral membrane proteins by this pathway requires that domains are, or become, accessible to the cytosol. Studies on the degradation of major histocompatibility complex class I heavy chains (13) and the cystic fibrosis transmembrane conductance regulator

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† The abbreviations used are: ER, endoplasmic reticulum; MTP, microsomal triglyceride transfer protein; ERAD, endoplasmic reticulum-associated degradation; OA, oleic acid; BSA, bovine serum albumin; α,2-M, α,2-macroglobulin; PBS, phosphate-buffered saline; DSP, dithiobis(succinimidyl propionate); PAGE, polyacrylamide gel electrophoresis.
Biotech Corp. (Victoria, Canada). Antiserum to human biochem. Monoclonal antibody to hsp70 was purchased from Stressgen and antisera to human apoA-I and calnexin were purchased from Cal- tibody for immunoprecipitation and immunofluorescence, respectively, prepared by Research Genetics (Huntsville, AL) and used as described.

The experiment was moved, and the cells were incubated in chase medium (minimal essential medium containing 3% fetal bovine serum, 2% penicillin/streptomycin and were studied after achieving 80% confluency. In all but the immunofluorescence experiments, cells were pretreated for 60 min with 10 mM proteasomal inhibitor (lactacyc- tin), which was then included in all subsequent labeling and chase media. In addition, unless otherwise noted under “Results,” all pulse-chase experiments were performed either with oleic acid (OA; to stim- ulate lipid synthesis and lipoprotein assembly) complexed to BSA (0.8 mM final concentration of OA in a 5:1 molar ratio with BSA) or with 0.16 mM BSA added to the chase medium.

Rabbit antiserum against the 26 S proteasome and the fluorescent substrate for in vitro proteasome activity assay (benzoyloxycarbonyl- Gly-Gly-Phe-p-amino benzamid) were generous gifts from Drs. M. Or- lowski and C. Cardozo (Department of Pharmacology, Mount Sinai School of Medicine, New York). Rabbit antiserum to Sec61 alpha was pre- pared by Research Genetics (Huntsville, AL) and used as described previously (21). Goat anti-human apoB antiserum and monoclonal anti- body for immunoprecipitation and immunofluorescence, respectively, and antisera to human apoA-I and calnexin were purchased from Cal- biotech. Monoclonal antibody to hsp70 was purchased from Stressgen Biotech Corp. (Victoria, Canada). Antiserum to human alpha M-macroglobu- lin (alpha M) was purchased from BIODIES International (Sanco, ME).

In some apoB immunoprecipitation experiments, we used epitope-spe- cific monoclonal antibodies, Bsol 14 (specific for the N terminus) and Bsol 22 (specific for the C terminus), which were purchased from the Lipoprotein Research Facility of the Ottawa Heart Institute (Ottawa, Canada). Antiserum to alpha M-macroglobulin was provided by one of us (W. J. W.) and is described in Ref. 22.

Interaction between Sec61a and ApoB—Proteasome-inhibited HepG2 cells were incubated for 15 min in the presence of 35S-protein labeling mixture (labeled methionine and cysteine; PerkinElmer Life Sciences). Isotope-containing medium was then re- moved, and the cells were incubated in chase medium (minimal essen- tial medium containing 5 mM methionine and 2 mM cysteine) for 20 or 60 min. At the end of the chase period, cells were harvested in cross-linking buffer (1× PBS, 5 mM EDTA, and 0.4% (v/v) digitonin). The homobifunctional cross-linking agent DSP (Pierce) was added to a final concentration of 0.25 mM, and the cells were incubated on ice for 15 min.

Cells were then lysed in denaturing lysis buffer (150 mM NaCl, 50 mM Tris (pH 8), 5 mM EDTA, 0.5% (v/v) deoxycholate, 1% (v/v) Triton X-100, and 0.1% (w/v) SDS containing 1× protease inhibitor mixture (Roche Mo- lecular Biochemicals)) with shaking overnight at 4 °C. The cell lysates were then removed by centrifugation at 15000 × g for 3 min, and the superna- ntants containing equivalent trichloroacetic acid-precipitable counts were then used for sequential immunoprecipitations. The sample was first fixed to antiserum against Sec61 alpha as described (21), and the resulting immunocomplexes were captured by protein A-Sepharose and released from the beads by boiling in elution buffer (10% (v/v) β-mer- captoethanol, 4% (v/v) SDS, and 20% (v/v) glycerol). The eluate was diluted in 1× NET, and the second immunoprecipitation was performed with anti-apoB antiserum as described previously (8). The elute from the second immunoprecipitation was analyzed by 3–17% gradient SDS-PAGE and fluorography. The results were quantified using a densitometer (Bio-Rad GS 700) or a PhosphorImager ( Molecular Dynamics, Inc.).

Immunoprecipitation of ApoB by Epitope-specific Antibodies—Pro- teasome-inhibited HepG2 cells were incubated for 15 min in the absence of 35S-protein labeling mixture and chased in isotope-free chase medium. At 0, 20, 40, and 60 min, microsomes were harvested and processed for denaturing immunoprecipitation with anti-apoB antiserum as described above. Under these conditions, there was quantitative cleavage of the cytosolic do- main of the transmembrane ER protein calnexin and full protection of the cystic fibrosis transmembrane conductance regulator, as undegraded protein accumu- lated in the cytosol, large aggregates (“aggresomes”), irrevers- ibly lost from the secretory pathway, formed and were visible by fluorescence microscopy (14, 15).

In contrast to these other ERAD substrates, we and others have recently shown that, in HepG2 cells, apoB destined for degradation does not accumulate in the cytosol when the protea- some is inhibited (16), but remains associated with the ER in close proximity to the translocon protein Sec61 alpha (1, 17). Thus, complete retrotranslocation may not be a universal fea- sure of ERAD. Rather, only a subset of domains of the substrate may become exposed to and engaged by cytosolic components. This would be consistent with the localization in yeast of the ubiquitin-conjugating enzymes Ubc6p and Ubc7p to the cyto- solic face of the ER membrane (11, 18) and the demonstration of the 26 S proteasome itself on the cytosolic surfaces of the nuclear envelope and the ER membrane in fission yeast (19) and mammalian cells (20).

The focus of this study is the relationship between apoB and the translational and translocation machinery under meta- bolic conditions favoring either lipoprotein assembly or protea- somal degradation. From a variety of approaches, we have obtained data to support a model in which apoB translation in HepG2 cells is not completed until lipoprotein assembly has concluded. If conditions are not favorable to assemble a lipopro- tein, apoB polypeptides destined for degradation form a com- plex with the proteasome and the cytosolic chaperone hsp70, leading to degradation. Overall, then, for apoB in HepG2 cells, both lipoprotein assembly and ERAD appear to be co-transla- tional rather than post-translational processes.

EXPERIMENTAL PROCEDURES

General Cell Culture Methods and Immunological Reagents—HepG2 cells were maintained in minimal essential medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 200 μM L-glutamine, and 200 units/ml penicillin/streptomycin and were studied after achieving ~80% confluency. In all but the immunofluorescence experiments, cells were pretreated for 60 min with 10 μM proteasomal inhibitor (lactacyc- tin), which was then included in all subsequent labeling and chase media. In addition, unless otherwise noted under “Results,” all pulse-chase experiments were performed either with oleic acid (OA; to stim- ulate lipid synthesis and lipoprotein assembly) complexed to BSA (0.8 mM final concentration of OA in a 5:1 molar ratio with BSA) or with 0.16 mM BSA added to the chase medium.

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Interaction between Sec61a and ApoB—Proteasome-inhibited HepG2 cells were incubated for 15 min in the presence of 35S-protein labeling mixture and then incubated in chase medium. Puromycin (10 μM) was added to the medium either at the start of the chase period (0-min time point) or after 70 min. In either case, incubation was continued for an additional 5 min. ApoB and alpha M (control protein) polypeptides that had been labeled with puromycin were isolated by sequential immuno- precipitation using anti-apoB or anti-alpha M antibodies, respectively, in the first step, followed by anti-puromycin antisera (22) in the second step. The proteins in the final immunoprecipitate were resolved by SDS-PAGE and detected by fluorography as described above. To verify the specificity of anti-puromycin antisera, 100 μM puromycin was added to an aliquot of a control sample before incubation with anti-puromycin an- tiserum (see Fig. 3).

Removal of Ribosomes from the Endoplasmic Reticulum—Protea- some-inhibited HepG2 cells were incubated for 15 min in the presence of 35S-protein labeling mixture and chased for 0, 20, or 60 min. Cells were harvested in 1× PBS containing 0.4% (v/v) digitonin to semi- permeabilize the cells. Equal volume aliquots were treated with or without 1 mM puromycin and 0.5 mM potassium acetate and then incu- bated on ice for 15 min and at 37 °C for 15 min to remove ribosomes from the ER membrane (23). Cell pellets were collected by centrifuga- tion at 1500 × g for 10 min and resuspended in DSP-containing buffer, and apoB cross-linked to Sec61 alpha was isolated by sequential immunoprecipitation.

Interaction between Micosomal ApoB and the Proteasome—Protea- some-inhibited HepG2 cells were incubated for 15 min in the presence of 35S-protein labeling mixture and chased for 20 or 60 min. At each time point, plates of cells were placed on ice and sequentially washed with ice-cold PBS, buffer containing 0.25 mM sucrose and 5 mM EDTA and SH buffer (0.25 mM sucrose and 5 mM HEPES (pH 8.0)). Cells were scraped in SH buffer containing 1× protease inhibitor mixture and sonicated twice on ice at 50% duty cycle for 30 pulses using a Vibra
The pellets were resuspended in 1 ml of 0.5M sucrose and 5 mM HEPES (pH 8.0) and centrifuged again for 1 h at 100,000 × g at 4 °C.

Microsomal pellets were resuspended in nondenaturing lysis buffer (0.5% (v/v) deoxycholate, 150 mM NaCl, and 50 mM Tris-HCl (pH 8)) overnight with rocking at 4 °C. The suspension was clarified by centrifugation for 5 min at 1500 × g. The supernatant containing the microsomal membranes was diluted in nondenaturing immunoprecipitation buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8), and 5 mM EDTA) and incubated with anti-proteasome antisera for 3 h with rocking at 4 °C. Immunocomplexes captured by protein A-Sepharose were eluted by boiling in buffer containing 2% (v/v) β-mercaptoethanol. To isolate proteasome-associated apoB, the eluates were diluted, and a second immunoprecipitation was performed using antiserum to apoB. The final immunoprecipitate was analyzed by SDS-PAGE and fluorography as described above.

**Results**

**Kinetics of the Close Association of ApoB with the Translocon Protein Sec61α**—We have previously reported that pulse-labeled apoB that is protected from degradation by proteasomal inhibitors stays associated with the ER translocon protein Sec61α for at least 1 h after the start of the chase period (1). This prolonged association with Sec61α was disrupted after 40 min when triglyceride synthesis, and therefore lipoprotein assembly, was stimulated by including OA in the chase medium. Based on the time estimated for hepatic lipoprotein assembly and ER exit (37 min) (24), this result suggested that throughout the biogenesis of an apoB-lipoprotein complex in the HepG2 cell, apoB remained in close proximity to the translocon.

The interaction of translocating chains with Sec61α and Sec61β can vary depending on the length of the polypeptide or its transmembrane topology (e.g. Ref. 25). Thus, to test whether the previous results for Sec61β represented a special relationship of that protein with apoB or a more general association of apoB with the translocon, the pattern of interaction of pulse-labeled apoB with Sec61α was determined.

HepG2 cells pretreated with lactacystin were incubated in the presence of [35S]methionine/cysteine for 15 min and chased for up to 60 min in isotope-free medium containing excess methionine/cysteine (chase medium). Cells were harvested at 0-, 20-, or 60-min time points, and apoB adjacent to Sec61α was detected by DSP-based cross-linking and sequential immunoprecipitation (see “Experimental Procedures”). As shown in Fig. 1, similar to the published results for Sec61α-associated apoB (~23% of the total labeled apoB pool) (Fig. 1B), could be detected as late as the 60-min time point when OA was omitted from the chase medium. In the presence of OA, the fraction of apoB adjacent to Sec61α was significantly reduced at 60 min (Fig. 1A, lane 5 versus lane 4). Note that the efficiency of chemical cross-linking of proteins with disuccinimidyl-based reagents typically averages no more than 10% (26), strongly suggesting that the relatively robust results for the apoB-Sec61α interactions summarized in Fig. 1 are representative of the behavior of the bulk of apoB polypeptides studied under these conditions.

To exclude the possibility that OA was affecting apoB-translocon interactions independent of its promotion of lipoprotein assembly, such as by disrupting the ER membrane, the above experiment was repeated in the presence of 0.1 mM BMS-200150, an inhibitor of MTP lipid transfer activity (kindly provided by Drs. David Gordon and John Wetterau, Bristol-Myers Squibb Co.) (27). If the effect of OA were nonspecific, blocking MTP activity and lipoprotein assembly would not alter the interaction of apoB with the translocon. As shown in Fig. 1 (lane 6), however, the decrease in apoB-Sec61α cross-linking at 60 min could be reversed by adding BMS-200150 to the medium in the presence of OA. Thus, the decreased proximity of apoB to Sec61α in the presence of OA most likely reflects successful lipoprotein assembly and exit from the ER, and not a nonspecific effect of the fatty acid on apoB-Sec61α association.

**Differential Recovery of ApoB by Epitope-specific Monoclonal Antibodies**—The above and previous (1) results demonstrate that apoB remains adjacent to the translocon during lipoprotein assembly. We wondered whether the apoB bands in Fig. 1 included some incompletely translated apoB that was indistinguishable upon SDS-PAGE from bona fide full-length apoB100. If translation were incomplete, we would expect that in a pulse-chase study, the appearance of an epitope at the extreme C terminus should be abnormally delayed relative to one at the N terminus. Therefore, we compared the relative recoveries of the N- and C-terminal epitopes from microsomes isolated at different time points during the 60-min chase. As in the experiments above, proteasome-inhibited HepG2 cells were pulse-labeled; and after 0, 20, 40, and 60 min of chase, cells were harvested, and microsomes were collected by ultracentrifugation (see “Experimental Procedures”). Equivalent aliquots of the resuspended microsomal pellets were immunoprecipitated with Bsol 14 or Bsol 22 monoclonal antibodies, which recognize the N-terminal (amino acids 405–539) and C-terminal (amino acids 4521–4536) regions of apoB, respectively. The signals on the resulting fluorograms were quantified by densitometry, and the abundance of labeled apoB recovered by the anti-C terminus antibody was divided by the abundance recovered by the anti-N terminus antibody. This fraction was multiplied by 100 to derive the parameter we have termed the “C/N ratio.”

The results are summarized in Fig. 2A. The C/N ratio was 100 for apoB in the conditioned medium (i.e. medium collected after 2 h of continuous labeling in the presence of OA), demonstrating equal recovery efficiencies of the antibodies when both epitopes were present on fully translated apoB. At the end of the 15-min labeling period (i.e. 0 min of chase), the C/N ratio was only 50, indicating that only 50% of the microsome-associated apoB molecules contained the C-terminal epitope. At 20 min of chase, there was still a relative deficiency of the C-terminal epitope. Based on the average elongation rate of hepatic proteins, apoB translation would be expected to be finished by ~11 min. Therefore, the data indicate a greater than expected lag in the appearance of the C-terminal epitope, re-
Fig. 1. Association of newly synthesized apoB with Sec61α depends on lipid synthesis and transfer. A, proteosome-inhibited HepG2 cells were labeled with [35S]methionine/cysteine for 15 min and chased with excess unlabeled methionine/cysteine for 20 or 60 min in the presence or absence of 0.8 mM OA (OA stimulates lipid synthesis) and the MTP inhibitor BMS-200150 (which decreases lipid transfer). At each time point, cells were harvested and subjected to cross-linking and sequential immunoprecipitation (first step, anti-Sec61α antiserum; second step, anti-apoB antiserum) to detect labeled apoB associated with Sec61α (see “Experimental Procedures”). After SDS-PAGE resolution of the second immunoprecipitate, fluorography was performed to visualize the apoB that was covalently bound to Sec61α. Note the significant decrease in the apoB signal at 60 min when OA was present along with BMS-200150; however, the decrease in the signal for apoB bound to Sec61α was not observed (lane 6 versus lane 5). The results shown are representative of three separate experiments. The increase in apoB signal at 20 min in this and other figures is typical (24) and represents continued incorporation of [35S]methionine/cysteine into apoB polypeptides in the early chase period. B, the fluorograms from the three separate experiments were quantified by densitometry, and the signals for apoB bound to Sec61α were normalized to the recovery of total labeled apoB at the corresponding time points. The results are shown as the mean ± S.E.

Reflecting a delay in completing apoB translation. In the presence of OA, the C/N ratios at the 20- and 40-min time points were similar to those in Fig. 2A, but were lower at 60 min, most likely representing the exit from the ER of full-length apoB molecules assembled into lipoproteins (data not shown).

As shown in Fig. 2A, some apoB polypeptides were gradually elongated to include the C-terminal epitope, as evidenced by the C/N ratio approaching 100 at the 60-min time point. We were interested in the topology of this extreme C-terminal region, so protease protection studies using these epitope-specific antibodies were performed (see “Experimental Procedures”). Representative results are shown in Fig. 2B. Note that at the 60-min time point, relative to the N-terminal epitopes, the C-terminal epitopes were more sensitive to trypsin digestion under conditions of limited lipid synthesis (i.e., without OA). When OA was added to stimulate lipid synthesis and lipoprotein assembly, the relative protection of the C-terminal epitope was significantly increased.

In control experiments, if microsomes were pretreated with Triton X-100 to disrupt membranes, the concentration of trypsin used resulted in 100% disappearance of the signal for apoB. In addition, the concentration of OA (0.8 mM) was not damaging to microsomal integrity, as it degraded only the cytosolic tail of calnexin, an integral ER membrane protein (data not shown). Thus, the results shown in Fig. 2B most likely reflect the topological relationship between apoB and the ER membrane and imply that when lipid synthesis is inadequate, there is incomplete translocation and cytosolic exposure of the extreme C-terminal domain of apoB. In contrast, when lipid synthesis is stimulated, this domain is shielded from the cytosol, most likely because it was fully translocated.

ApoB Remains Functionally Bound to the Ribosome throughout the Time Required for Lipoprotein Assembly—The prolonged proximity of apoB polypeptides to the translocon and the kinetics of the differential recoveries of the C- and N-terminal epitopes suggested that the nascent apoB polypeptides were still functionally bound to the ribosome as peptidyl-tRNAs. This possibility was tested by assessing whether puromycin could be incorporated into labeled apoB chains during the chase period, particularly at the later time points. For comparison purposes, puromycin incorporation into another secretory protein, αM, was also assessed.

HepG2 cells pretreated with lactacystin were incubated for 5 min in the presence of [35S]methionine/cysteine and then incubated for up to 70 min in chase medium containing either OA/BSA or BSA alone. Puromycin (10 μM) was added to the medium after 70 min of chase, and incubation was continued for another 5 min. Labeled apoB or αM chains that had incorporated puromycin were recovered by sequential immunoprecipitation with either anti-apoB or anti-αM antibody in the first step and anti-puromycin antibody in the second step.

As shown in Fig. 3 (lanes 6 and 7), in the presence or absence of OA, there were no αM-puromycin conjugates detected. Surprisingly, there were apoB-puromycin conjugates (lanes 3 and 4), with the labeled apoB species appearing full-length (based on the migration of apoB recovered from the conditioned medium). The percent of apoB conjugated to puromycin was ~20% of total labeled apoB, consistent with the 23% recovery of apoB cross-linked to Sec61α late in the chase period when OA was absent (Fig. 1). The comparable quantitative recoveries under
essentially the same experimental conditions strongly imply that the apoB polypeptides conjugated to puromycin or cross-linked to Sec61α belong to the same pool of molecules. If OA was present in the chase medium, puromycin incorporation into apoB was significantly lower (Fig. 3, lane 4 versus lane 3), consistent with the loss of association of apoB with the translocon at the time when lipoprotein assembly should be completed, resulting in exit from the ER (Fig. 1) (1, 24). In contrast to these results, if puromycin was added at the end of the labeling period, both labeled nascent apoB and α1-M chains incorporated puromycin, as expected, and a range of incomplete translation products from 45 to 550 kDa for apoB and from 70 to 180 kDa for α1-M was observed (Fig. 3, lanes 2 and 5). That the sequential immunoprecipitation was specific for apoB-puromycin conjugates was indicated by a large reduction in the recovery of apoB when excess puromycin was added to the reaction mixture just before adding the anti-puromycin antibody (lane 1).

These results imply that apoB translation either slows or pauses very close to its carboxyl terminus. Overall, the data in Figs. 1–3 suggest that when lipid synthesis is stimulated, apoB translation is completed, and assembled lipoproteins are transported out of the ER.

Association of ApoB with the Translocon Is Independent of the Presence of the Ribosome—Since nascent apoB is bound to the ribosome via the tRNA, and the ribosome is bound to the translocon, the prolonged proximity of apoB to the translocon (Fig. 1) may not be due to direct apoB-translocon interactions. To determine whether the release of the ribosome from the ER membrane would result in the loss of apoB-Sec61α cross-linking, HepG2 cells were pretreated, labeled, and chased for the Sec61α cross-linking experiment as described above. At the indicated time points (Fig. 4), cells were scraped into buffer, and the suspension was divided into two aliquots. One was treated with 1 mM puromycin in the presence of 0.4 M potassium acetate prior to cross-linking (to disrupt the ribosome-translocon interaction) (23), and the other was similarly, but without adding puromycin (see “Experimental Procedures”). Both samples were then treated with the DSP cross-linker, lysed, and subjected to sequential immunoprecipitation with anti-Sec61α and anti-apoB antisera (see “Experimental Procedures”). As shown in Fig. 4, the puromycin/high salt treatment, which releases ribosomes from translocons (28), did not affect the extent of cross-linking of apoB to Sec61α in samples taken at the different chase points. This suggests that along with apoB engagement by the ribosome (Fig. 3), there are...
also interactions with ER proteins or lipids that serve to retain apoB in the translocon during lipoprotein assembly.

Interactions of Microsome-associated ApoB with the Proteasome and the Cytosolic Chaperone hsp70—The results above and in our previous reports (1, 8, 16) imply that apoB can be directed to either lipoprotein assembly or degradation while still associated with the translocon and the ribosome. Thus, rather than interacting with cytosolic factors, such as hsp70 and proteasomes, only after being completely retrotranslocated, apoB may be engaged by these factors while still bound to the ER membrane in a “bitopic” state (i.e. with part of apoB accessible to the cytosol and part in the ER lumen). To test this possibility, HepG2 cells were labeled to steady state for 2 h in the presence of 10 μM lactacystin and then semi-permeabilized with digitonin. To test for the ATP dependence of interactions among apoB, hsp70, and the proteasome, some samples were treated with 50 units/ml apyrase to deplete ATP. Microsomes were collected by ultracentrifugation from both apyrase-treated and buffer-treated lysates, and the pellets were resuspended in nondenaturating lysis buffer. hsp70-associated proteins were isolated by nondenaturing immunoprecipitation with anti-hsp70 monoclonal antibody, and one-half of this immunoprecipitate was resolved by SDS-PAGE and immunoblotted with anti-proteasome antisera (see “Experimental Procedures”).

As shown in Fig. 5, 26 S proteasome subunits ranging from 25 to 35 kDa were detected. This interaction with hsp70 was specific as shown by its ATP dependence (Fig. 5, lane 3 versus lane 4). The other half of the hsp70 immunoprecipitate was diluted, and a second immunoprecipitation was performed with anti-apoB antiserum. Labeled apoB was recovered (lanes 1 and 2), and the amount recovered increased after apyrase treatment (lane 1 versus lane 2), indicating an ATP dependence consistent with previous results for hsp70-apoB interactions (10).

To investigate the kinetics of the association of microsomal apoB with the proteasome, HepG2 cells were pretreated with lactacystin; pulse-labeled for 15 min; and then chased for 0, 20, or 60 min as before. Microsomes were isolated at these time points and subjected to sequential immunoprecipitation under nondenaturing conditions (see “Experimental Procedures”) using anti-proteasome antisera in the first step and anti-apoB antisera in the second step. As shown in Fig. 6, proteasomes and labeled microsomal apoB co-immunoprecipitated at all time points, and the extent of this interaction was lower in the presence of OA. These results are consistent with the decreased degradation of apoB (29) and the decreased cytosolic exposure of the C-terminal domain when HepG2 cells are incubated with OA (Fig. 2B).

The interaction between apoB and the proteasome was specific as judged by the absence of the apoB band if nonimmune serum was used in the first immunoprecipitation step (Fig. 6, lane 1). We also tested whether proteasomes co-immunoprecipitating with microsomal apoB were functional by performing assays for the chymotrypsin-like activity of the proteasome. By incubating the co-immunoprecipitated proteasomes with a syn-

![Image](65x274 to 282x392)

**FIG. 3.** ApoB remains functionally bound to the ribosome throughout the time required for lipoprotein assembly. Proteasome-inhibited HepG2 cells were labeled with [35S]methionine/cysteine for 5 min and chased for either 0 or 70 min in the presence or absence of OA. Puromycin was then added, and incubation was continued for 5 min. Cells were lysed, and labeled apoB and αM polypeptides that incorporated puromycin were isolated by immunoprecipitation with anti-apoB or anti-αM antiserum, respectively, in the first step and anti-puromycin antiserum in the second step. Immunoprecipitates were resolved by SDS-PAGE, and a representative fluorogram is shown. Note that when puromycin was added at 0 min, labeled apoB and αM chains of various lengths that had incorporated puromycin were visible (lanes 2 and 5, respectively). In contrast, when puromycin was added after 70 min of chase, only apoB polyepitides were visible (lanes 3 and 4 versus lanes 6 and 7). Note that the αM lanes were deliberately overexposed to ensure that there was no residual signal detectable at the 70-min time point. In the presence of OA, apoB-puromycin conjugates were decreased (lane 3 versus lane 4). To control for the specificity of the anti-puromycin antibody. The immunoprecipitate was divided in two aliquots; one was subjected to nondenaturing immunoprecipitation (IP) with anti-hsp70 antibody. The immunoprecipitate was divided in two aliquots; one was processed for a second immunoprecipitation with anti-apoB antiserum, and the other was used for immunoblotting (Western blotting (WB)) with anti-proteasome antiserum. Both apoB (lanes 1 and 2) and 26 S proteasome subunits ranging from 25 to 35 kDa (lanes 3 and 4) were recovered from the hsp70 immunoprecipitate. Note that the signals increased in apyrase-treated samples, indicating that the interactions with hsp70 were ATP-dependent.

![Image](333x625 to 530x729)

**FIG. 4.** Effect of the removal of ribosomes on the proximity of apoB to Sec61α. Proteasome-inhibited HepG2 cells were labeled with [35S]methionine/cysteine for 15 min and chased for 0, 20, or 60 min. Cells were harvested in 1× cross-linking buffer. One-half of the cells were treated with 1 mM puromycin (Puro) and 0.5 mM potassium acetate and then incubated (15 min on ice, followed by 15 min at 37 °C) to release the ribosomes from the nascent polypeptide chains. The other half were treated similarly, but without puromycin. After cross-linking with DSP (see “Experimental Procedures”), cell lysates were sequentially immunoprecipitated with anti-Sec61α antiserum in the first step and anti-apoB antiserum in the second step. Immunoprecipitates were resolved by SDS-PAGE, and a representative fluorogram is shown.
thetic substrate (benzyloxycarbonyl-Gly-Gly-Phe-p-aminobenzamide) (30), lactacystin-inhibitable activity was readily detected, suggesting that the microsomal apoB-associated proteasomes were indeed functional (data not shown).

Overall, these results imply that the proteasome and hsp70, both of which we have previously shown to be involved in ERAD of apoB (8), interact with apoB while it is still associated with the ER membrane. In other words, complete retrotranslocation of apoB is not required for its interaction with cytosolic factors and its entry into the ubiquitin-proteasome pathway.

ApoB Accumulates in Non-aggresomal Structures when Proteasome Activity Is Inhibited—We have previously shown by indirect immunofluorescence in HepG2 cells that when proteasomes are inhibited, apoB accumulates and assumes a punctate appearance that co-localizes with ER markers (1, 8). Biochemical studies suggested that apoB accumulating under these conditions can still be recruited to lipoprotein assembly and the secretory pathway when OA is added to stimulate lipid synthesis (1). This implies that the intracellular localization of proteasome-protected apoB, as detected by immunofluorescence, will be changed by OA.

To test this, HepG2 cells were treated with lactacystin for 2 h, and then the cells were chased in either BSA- or OA/BSA-containing medium for 30 or 90 min. The cells were immunostained using either anti-apoB or anti-apoA-I antibody (control protein) as the primary antibody. As shown in Fig. 7 (left panel), a punctate appearance of apoB, similar to that shown previously (1, 8), was apparent at the start of the chase period. Note that in the presence of BSA, there were no obvious changes in the level or distribution of the immunofluorescent signals over time (upper center and right panels). In marked contrast, with OA, the signal became diffuse by 30 min and was significantly lower in intensity by 90 min, consistent with the mobilization and secretion of the accumulated apoB after lipid synthesis was stimulated. Supporting this interpretation is that the apoB signal at 30 min overlapped with a marker (ERGIC53) (31) for the intermediate compartment, which is enriched in secretory vesicles (data not shown). These results were not due to nonspecific effects of OA, based on the lack of changes in the intensity or distribution of the signal for apoA-I (data not shown).

Taken together with the published biochemical results (1, 8), these data demonstrate that apoB accumulating in punctate structures after proteasomal inhibition can be recruited to lipoprotein assembly and secretion. As will be described further under “Discussion,” the apoB-associated punctate structures are unlikely to represent aggresomes, which are also punctate in immunofluorescent appearance. Although they also consist of an ERAD substrate (the cystic fibrosis transmembrane conductance regulator) that accumulates when the proteasome is inhibited, they contain protein molecules irreversibly lost from the secretory pathway (14, 15).

**DISCUSSION**

As polypeptides emerge from the translocon, they encounter ER chaperones, such as BiP and calnexin, and undergo a “quality control” process in which conformation-dependent sorting...
results in either a native folded state that exits the ER or a malfolded state that is retained and subject to ERAD (see Ref. 32 for a recent review). We have previously shown that when HepG2 cells are deprived of fatty acids, the association of apoB with its lipid ligands is reduced, and ERAD mediated by the ubiquitin-proteasome pathway ensues (1, 8). Complete assembly of apoB with lipids to form a hepatic lipoprotein particle culminating in ER exit has been estimated to take ~40 min based on the kinetic analysis of subcellular fractionation data (24). It has also been shown that the initiation of lipid association with nascent apoB polypeptides in HepG2 cells is co-translational (33), consistent with the interaction of metabolically labeled apoB with either Sec61β (1) or Sec61α (Fig. 1A) (17). In the common models proposed for hepatic lipoprotein formation (e.g. Refs. 3, 34, and 35), however, it has been proposed that the initial lipidation is co-translational, but subsequent lipidation (referred to as a “second step”) occurs post-translationally to complete the assembly process.

The data in this report argue strongly that not only the initial, but the bulk, if not all, of the assembly process in HepG2 cells occurs co-translationally. A key line of evidence to support this view derives from the finding that apoB is adjacent to translocon proteins throughout the time period required for lipoprotein assembly. Furthermore, the disappearance of apoB-translocon interactions appears to coincide with the expected exit of the completed lipoprotein particle from the ER. A simple explanation for the persistent interaction between apoB and the translocon is that apoB does not leave the translocon until translation has terminated. Yet, in many studies of HepG2 cells, including our own, labeled apoB appears to be full-length (i.e. apoB100, ~550 kDa) at early time points in the chase period. SDS-PAGE, however, does not have the resolution to reveal minor differences in size among apoB polypeptides that would occur if translation slowed or paused near the carboxyl terminus of the protein. Thus, we determined directly whether apoB polypeptides were still bound to functional ribosomes as peptidyl-tRNAs late in the chase period by their ability to react with puromycin, an aminoacyl-tRNA analog, and by the kinetics of C-terminal synthesis.

As shown in Figs. 2 and 3, the results independently support the notion that the assembly of a lipoprotein particle and the termination of apoB translation are coupled events. In proteasome-inhibited cells, puromycin could be incorporated into [35S]-labeled apoB polypeptides as long as 70 min following pulse labeling, indicating the functional engagement of nascent apoB chains with the ribosome well into the chase period. In the presence of OA, the decrease in incorporation of puromycin added after 70 min of chase (Fig. 3), a time sufficient for the complete assembly of a lipoprotein, most likely reflects the completion of translation and exit from the ER. Because OA is not totally effective in sorting apoB to lipoprotein assembly (29), the residual apoB at 70 min in +OA samples most likely represents nascent apoB that is not associated with a full complement of lipid ligands.

Another indication of a delay in completing apoB translation is the significant lag in the appearance of the C-terminal epitope of apoB relative to that of the N-terminal epitope (Fig. 2). This lag can only be explained by a slower rate of, or pause in, the translation of the region of apoB mRNA encoding the extreme carboxyl-terminal portion of the full-length protein. Interestingly, the location of the C-terminal epitope is just distal to one of the strongest pause-transfer sequences detected by Lingappa and co-workers (36) in their survey of the entire length of apoB. In a cell-free assay system, they have shown that sequences dispersed throughout the length of apoB can mediate pauses in translocation (37, 38). In addition, we (39) and others (40) have observed pauses in translation of apoB. Both types of pauses have been hypothesized to allow time for the assembly of nascent apoB chains with lipids (3). Making an analogy to the coordinated efforts of the ribosome and translocon during the insertion of a transmembrane domain into the ER membrane bilayer (41), the results (puromycin incorporation, delayed C-terminal epitope appearance, and prolonged interaction with Sec61p) imply that the translational and translocon apparatuses cooperate to stabilize a topographical state of apoB necessary to achieve the final stage of lipoprotein assembly in HepG2 cells. Relevant to this point is a proposed “stop-translation” sequence (in the bacterial λ-receptor), which is thought to halt translation to allow sufficient time for important interactions between the nascent polypeptide and membrane lipids and proteins that are required for secretion (42). The fact that a considerable length of apoB polypeptide is translated prior to a final assembly stage is consistent with recent studies showing that glycosylation sites in apoB as distal as apoB68 are modified in HepG2 cells (43) in the absence of OA.

The prolonged binding of the nascent apoB chain to the ribosome via the RNA and the affinity of the ribosome for the translocon lead, not surprisingly, to the positioning of the apoB within or near the translocon late in the assembly process. Yet, when ribosomes were stripped off the ER membrane, apoB was still adjacent to Sec61α (Fig. 4). Thus, the cross-linking of apoB to Sec61α results not only from their spatial proximity in the ribosome-translocon complex, but also from protein-protein interactions. These interactions with the nascent chain may be mediated by translocon components or by translocon-associated proteins, as suggested by studies on β-sheet and pause-transfer sequences contained in apoB (44, 45). Also consistent with the data, apoB may remain in association with the ER membrane by interacting with ER proteins in the vicinity of the translocon (as suggested by studies of apoB and ER chaperones) (46, 47) or with lipid components (because of the many hydrophobic domains of apoB). This last possibility is compatible with the inability of urea to effectively extract apoB from microsomes.2 Delayed translation (and hence termination of translation) then may not reflect the need of the ribosome to be a structural anchor of apoB polypeptides, but may provide the length of polypeptide and time required to position nascent apoB properly so that it interacts with a variety of protein and lipid factors that help achieve or maintain the conformation needed to complete lipoprotein assembly. This would not be surprising, given that many transmembrane and secretory proteins, albeit more typical than apoB, undergo chaperone-mediated conformational maturation to the native folded state before ER exit is allowed (32). That the maturation process is prolonged for apoB is consistent with its previously reported tardiness (relative to other secretory proteins) in exiting the ER of HepG2 and rat hepatic cells (24, 48).

Our results also speak to the fate of nascent apoB chains not successfully assembled into a lipoprotein particle. In contrast to the paradigm for other substrates of ERAD, such as major histocompatibility complex class I molecules in cytomegalovirus-infected cells and mutant carboxypeptidase Y in yeast (see Ref. 49 for a recent review), we have previously shown in proteasome-inhibited HepG2 cells maintained in OA-deficient medium that apoB does not appear to be completely retrotranslocated or “dislocated” to the cytosol prior to degradation (1). The proteasome and cytosolic factors involved in apoB degradation therefore appear to interact with ER-associated apoB polypeptides, as supported by the finding of ATP-dependent

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2 H. Wang, unpublished data.
interactions among microsome-bound apoB, proteasomes, and cytosolic hsp70 (Figs. 5 and 6). It should be noted that the pretreatment of fatty acid-deficient HepG2 cells with lactacystin allowed the study of apoB intermediates of greater length than would have been ordinarily present in this metabolic state, based on data showing that targeting of apoB polypeptides to the ubiquitin-proteasome pathway can occur as early as midway through translation (16, 50). Nonetheless, many features of the proteasomal targeting of apoB appear to be independent of polypeptide length, namely, stimulation by a deficiency in lipid synthesis or transfer, the increased exposure of the C-terminal region relative to the N terminus when lipoprotein assembly is not successful, and an increased interaction with hsp70 (16, 51). We have previously shown that increased hsp70 expression promotes apoB ubiquitylation (16) and proteasomal degradation (8), and we presume that the hsp70 bound to microsomal apoB (Fig. 5) is fulfilling functional roles, consistent with the decrease in apoB-proteasome interaction when OA is present (Fig. 6).

The lack of cytosolic accumulation of apoB in proteasome-inhibited cells (1) indicates that, unlike other ERAD substrates, such as the cystic fibrosis transmembrane conductance regulator (14, 15) and influenza virus antigen (52), apoB does not form aggresomes, cytosolic structures containing proteins that are irreversibly lost to the secretory pathway. Consistent with this was the finding that the stimulation of lipid synthesis and lipoprotein assembly by OA resulted in the disappearance of the apoB-containing punctate immunofluorescent structures that formed after proteasome inhibition (Fig. 7). That this disappearance represented the rapid recruitment of the accumulated apoB to lipoprotein assembly and secretion is supported by our two previous studies (1, 8) in which there was quantitative recovery of metabolically labeled apoB from the conditioned medium of HepG2 cells treated similarly to the cells in Fig. 7. Because these apoB-containing punctate structures co-localized with ER markers in previous immunofluorescence studies (1, 8), it is plausible that they are part of the recently described “ER-associated bodies” formed at exit sites in which another proteasomal ERAD substrate, a mutant form of a yeast ABC transporter Ste6p, has been shown to accumulate (53). It is therefore intriguing to speculate that the diffuse apoB immunofluorescent signal observed 30 min after OA addition represents a plethora of secretory vesicles containing apoB-lipoprotein complexes that budded off the ER after lipid synthesis and assembly were stimulated.

In summary, we have used proteasome-inhibited HepG2 cells to study the molecular events in the two possible paths of apoB metabolism: lipoprotein assembly or ERAD. Both paths appear to be co-translational. The assembly process, which occurs when there is sufficient transfer of lipid ligands, functions while apoB polypeptides are incompletely translated and are interacting with the ribosome, translocon, ER chaperones, and, perhaps, the lipid bilayer. Lipoprotein assembly, apoB translocation, and apoB mRNA translation appear to be coordinated events that serve to maintain apoB in an appropriate functional and topographical state. If lipid ligands are insufficient, then ER-associated apoB polypeptides interact with cytosolic factors that target it to the ubiquitin-proteasome pathway. Given the potential of the proteasome (54) or chaperones (55, 56) to act as molecular motors by virtue of their intrinsic ATPase activities, apoB is most likely extracted from the ER membrane rather than becoming fully retrotranslocated into the cytosol prior to its degradation.

A schematic summarizing the essential features of the proposed model is given in Fig. 8. Basically, lipoprotein assembly and apoB degradation resemble the quality control mechanisms governing the molecular sorting of other secretory and transmembrane proteins in the ER, in that exit and retention (and ultimate degradation) represent the successful separation of conformational variants. The differences between apoB and other ERAD substrates most likely reflect special requirements of lipoprotein assembly, unusual structural features of the apoB protein, and the regulation of the quality control process by the lipid metabolic state.

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