Intracellular Translocation and Stability of Apolipoprotein B Are Inversely Proportional to the Length of the Nascent Polypeptide

(Received for publication, June 12, 1998, and in revised form, August 31, 1998)

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We have studied the relationship between the length of apolipoprotein B (apoB) and its intracellular translocation and stability using McArdle RH7777 (McA-RH7777) cells expressing recombinant human apoB variants, ranging in size from B15 to B100. The translocation status of apoB was assessed based on trypsin sensitivity of apoB using isolated microsomes as well as permeabilized cells. In isolated microsomes, shorter apoB variants (≤B48) were 75–100% resistant to exogenous trypsin digestion, whereas apoB variants larger than B48 were less than 40% trypsin-resistant. Experiments with hepatic microsomes isolated from rat or transgenic mice expressing human B48 and B100 also confirmed the high trypsin accessibility of B100 compared with B48. In permeabilized cells, apoB variants shorter than B48 were relatively resistant to exogenous trypsin (percentage of trypsin-resistant apoB greater than 70%) in contrast to recombinant human B72 and B100, which were only 55 and 42% trypsin-resistant, respectively. The trypsin sensitivity of human B100 was comparable with that of endogenous rat B100 in McA-RH7777 cells as well as endogenous B100 in HepG2 cells (percentages of trypsin-resistant cells were as follows: for human B100 construct, 42 ± 7.5%; for endogenous McA-RH7777 B100, 52 ± 2.9%; and for endogenous HepG2 B100, 46 ± 6.3%). Overall, an inverse correlation between the length of apoB and its resistance to exogenous trypsin was evident irrespective of the model system examined.

An inverse relationship was also observed between the size of apoB and its co-translational resistance to proteasomal degradation. Truncated apoB constructs were relatively insensitive to proteasome inhibition by MG132 co-translationally (during the pulse) compared with the full-length B100, which was highly sensitive (apoB recovered in the presence of MG132 as a percentage of control was as follows: B15, 127%; B29, 94%; B48, 110%; B72, 140%; B100, 282%). Post-translational (over a 2-h chase), a similar inverse relationship was found, with B100 being the least stable in comparison with truncated apoB variants. In summary, as the size of the nascent apoB chain increases, there appears to be a greater cytosolic exposure of the polypeptide, leading to a higher sensitivity to proteasomal degradation.

Apolipoprotein B100 (apoB100 or apoB) is a large hydrophobic protein (~550 kDa) that is predicted to be composed of three amphipathic α-helix domains and two amphipathic β-strand domains (1). The unusual length of apoB100, as well as its composition, is believed to be responsible for its ability to recruit lipids in the assembly of lipoprotein particles (2, 3). In addition, the length of apoB appears to play a role in the metabolic fate of apoB-containing lipoproteins and may influence the intracellular biosynthesis and extracellular secretion (4, 5).

Hepatic secretion of apoB-containing lipoproteins appears to be subject to post-transcriptional control (6–9). ApoB translocation across the membrane of the endoplasmic reticulum (ER) is considered to be an important step in the secretion of lipoprotein particles (10–12). Translocation of apoB appears to be dependent upon both lipid availability and the conformation of the nascent polypeptide (13). Two functionally distinct pools may form during translocation of apoB (10). One pool is composed of intraluminal, trypsin-insensitive apoB that is assembled into lipoprotein particles, whereas the second pool is trypsin-accessible and appears to be associated with the ER membrane (10). Davis et al. (10) have suggested that the β-strand domains within apoB may be responsible for the interaction between apoB and the ER membrane and that this interaction may be a determinant of the translational efficiency of the protein. It is important to note, however, that apoB translocation and its role in lipoprotein assembly and secretion remain controversial. While some laboratories have shown that apoB translocation is relatively efficient (14–16), evidence from most other laboratories suggests that apoB is inefficiently translocated across the ER membrane (10, 12, 13, 17–27), resulting in the formation of a large membrane-associated apoB pool. Ingram and Shelnitz (15) have reported that as much as 80% of apoB100 in HepG2 cells is resistant to trypsin digestion, while other groups (28, 29) have observed a significantly lower resistance of apoB100 to trypsin digestion (0–25%). In McA-RH7777 cells, McLeod et al. (18) have found that endogenous rat apoB100 was 10–26% trypsin-resistant, whereas the endogenous rat apoB48 was 100% trypsin-resistant. In rat hepatocytes, endogenous apoB48 was reported to show 78% resistance to exogenous trypsin by Rusinol et al. (30) and 30% resistance by Davis et al. (10) and Verkade et al. (24). It is thus clear that considerable controversy exists in the...
literature on the translocation behavior of apoB variants of different size.

The intracellular stability of apoB both at the co-translational and post-translational levels has also been under intense investigation. Evidence to date supports the notion of a two-step degradation process involving co-translational degradation of nascent apoB chains and post-translational degradation of fully translated apoB. A number of recent studies have confirmed that nascent apoB molecules undergo rapid co-translational degradation mediated by the cytosolic proteasome (31–34). Nascent apoB chains appear to become ubiquitinated and degraded during the translocation process (31–34). A recent study revealed that ubiquitinated apoB is also associated with the Sec61 complex of the translocon and that alteration in the translocation of apoB appears to induce this association (34). Furthermore, this pool of ubiquitinated apoB has also been shown to be destined for proteasomal degradation (34). In addition to co-translational degradation, there is also evidence for post-translational degradation of apoB, which may occur in the lumen of the ER (27, 35–37) or other subcellular compartments (21, 24, 38, 39). Wu and co-workers (35) have hypothesized that in HepG2 cells, the post-translational degradation of apoB100 may occur in at least two distinct steps (35). The first step is an ALLN-sensitive process that degrades translocation-arrested apoB molecules in the ER membrane, while the second step is a dithiothreitol-sensitive pathway that occurs in the ER lumen (35). Our laboratory has recently identified an ER-60 protease homologue in HepG2 cells that is associated with apoB (40) and may be involved in post-translational degradation of apoB (40). In addition, Chen et al. (34) have speculated that retrograde translocation of apoB from the lumen of the ER to the cytosol may also be a mechanism of apoB degradation. There is thus evidence for both co-translational and post-translational mechanisms that may be involved in intracellular degradation of newly synthesized apoB molecules. The cytosolic proteasome appears to be involved in co-translational degradation of nascent apoB as well as post-translational degradation via a retrograde translocation mechanism.

In the present study, we attempted to further elucidate the translocational behavior of apoB and its intracellular stability by directly analyzing the expression of apoB in a number of different model systems. We analyzed endogenously expressed B100 and B48 in rat hepatocytes, transgenic mice, and HepG2 cells, as well as Mca-RH7777 cells transfected with human apoB cDNA of various lengths ranging from 15 to 100% of full-length apoB100 (hB15–hB100). Overall, studies in a number of model systems using both isolated microsomes and permeabilized cells indicate a clear correlation between the apoB size and its sensitivity to exogenous trypsin. The length of nascent apoB was also found to strongly influence its intracellular stability and its sensitivity to proteasomal degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—**HepG2 cells were maintained in minimal essential medium as described previously (41). Mca-RH7777 cells stably transfected with human apoB15, apoB29, apoB37, apoB48, apoB64, apoB72, and apoB100 cDNA were generated as described previously (2, 18, 29). Cultures were maintained in Dulbecco’s modified Eagle’s medium in the presence of 200 µg/ml Geneticin and serum supplements as described previously (18, 29, 41).

**Animal Studies—**All animal experiments were performed according to animal care guidelines of the University of Ottawa. Male Wistar rat livers were harvested from animals fed either chow or high-fat diet. Human apoB transgenic mice (42) were obtained from Jackson Laboratories.

**Translocational Studies Using Isolated Microsomes from Stable Cell Lines—**Microsomes were prepared from cultured Mca-RH7777 cell lines by ball bearing homogenization. All procedures were performed on ice, unless otherwise indicated. Briefly, cells from two 100-mm dishes were suspended in Tris-sucrose buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose) containing protease inhibitors (0.1 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, aprotinin (100 kallikrein inhibitor units/ml), ALLN (40 µg/ml)) and disrupted by 20 passes through a ball bearing homogenizer (H & Y Enterprise, Redwood City, CA). After removal of intact cells and nuclei by centrifugation (10,000 x g, 10 min), the resulting pellet was resuspended in 5 ml of Tris-sucrose buffer without inhibitors and recovered by 400,000 x g centrifugation. The microsome pellet was resuspended in 100 µl of Tris-sucrose buffer and used immediately for protease protection assays (45). Following digestion, soybean trypsin inhibitor (150 µg/ml) was added, microsomes were separated from trypsin by centrifugation, and the microsomal proteins were solubilized for SDS-PAGE analysis.

**Translocational Studies Using Isolated Microsomes from Liver Fractions—**Liver fractions were isolated by the method of Croze and Morre (44) with the addition of the protease inhibitors to all buffers as described above. Following the sucrose gradient centrifugation, ER and ERI fractions were combined into a single ER fraction. The crude Golgi fraction was diluted 10-fold with water and recovered after centrifugation to reduce endosomal contamination (45) and to remove protease inhibitors. The Golgi prepared in this manner contained some (~10%) ER contamination as assessed by immunoblotting for the ER marker protein-disulfide isomerase (PDI). ER fraction was similarly diluted 10-fold with water and resolated by centrifugation. Protease protection analysis (43) was performed immediately following removal of the protease inhibitors.

**Determination of Trypsin Sensitivity of Newly Synthesized ApoB Using a Permeabilized Cell System—**In the translocation studies all cells were incubated in methionine-free minimal essential medium for 60 min. MG132 was added 15 min prior to the pulse. The cells were pulsed for 15 min with 35S-methionine, and the radioactivity was chased for 20 min in complete medium containing 10 mM methionine. The cells were then washed and solubilized by incubating in CSK buffer (0.3 M sucrose, 0.1 M KCl, 2.5 mM MgCl2, 1 mM sodium-free EDTA, 10 mM PIPES, pH 6.8) containing 75 µg/ml digitonin for 5 min at room temperature. Digitonized cells were washed once in CSK buffer and were then incubated in CSK buffer for 10 min at room temperature, in the presence and absence of trypsin (100 µg/ml) (13). An equal volume of CSK buffer containing 1 mg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 1.25 µM ALLN, and 100 kallikrein inhibitor units/ml Trasylol was added to all dishes for 10 min at room temperature. The cells were then incubated on ice for 10 min and collected. The collected cells were solubilized in a solubilization buffer (giving a final concentration containing 5% Nonidet P-40, 20% deoxycholate, 5 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 kallikrein inhibitor units/ml Trasylol, 0.1 mM leupeptin, 0.5 µM ALLN) and centrifuged as described above, and supernatants were subjected to immunoprecipitation, SDS-PAGE, and fluorography.

**Analysis of the Translocation Status of the Total Mass of ApoB—**Cells were incubated in the presence of MG132 (25 µM) for 60 min. For translocation studies, the cells were permeabilized with digitonin for 5 min at room temperature following the 60-min incubation period with MG132. Permeabilized cells were then subjected to trypsin treatment as described above. Cells were collected and solubilized, and cell lysates were subjected to chemiluminescent immunoblotting.

**Chemiluminescent Immunoblotting—**Immunoblotting was performed by SDS-PAGE analysis of nonlabeled cell lysates on minigels (8 × 5 cm) with 4.5, 7, or 8–15% polyacrylamide gradient gels. Following SDS-PAGE, the proteins were transferred electrophoretically overnight at 4 °C onto nitrocellulose membranes using a Bio-Rad wet transfer system. The membranes were blocked with a 5% solution of fat-free dry milk powder and then incubated in a 1:1000 dilution of the primary antibody for 1–24 h. After several washes, the membranes were then incubated in 1:3000 or 1:8000 secondary antibody conjugated to peroxidase solution for 1–2 h. Membranes were then incubated in ECL detection reagents for 60 s and exposed to Hyperfilm. Films were then developed, and quantitative analysis was performed using an Imaging Densitometer.

**Immunoprecipitation, SDS-PAGE, and Fluorography—**Immunoprecipitation was performed as described previously (46) with either goat polyclonal anti-human apoB or rabbit polyclonal anti-rat apoB. Immunoprecipitates were washed with wash buffer and were prepared for SDS-PAGE (46). SDS-PAGE was performed as described (47) using gels (16 × 12 cm) containing either 6 or 7% polyacrylamide. Gels were fixed and fluorographed by incubating in Amplify scintillator. The gels were dried and exposed to DuPont autoradiographic film at −80 °C for 1–4 days. Quantitative analysis was performed using a liquid scintillation counter.
RESULTS

Trypsin Sensitivity of apoB100 and apoB48 in Subcellular Fractions of Hepatic Cells—In earlier work (18), protease protection assays showed that the trypsin sensitivity of rat apoB100 and apoB48 in transfected McA-RH7777 cell lines was different; apoB100 was much more susceptible to proteolysis than apoB48. To verify that this observation was indicative of the topology of the proteins in vivo and was also relevant to human apoB, we compared the trypsin accessibilities of human or rat apoB in transfected McA-RH7777 cells and liver microsomal fractions. In McA-RH7777 cells (Fig. 1A), more than 70% of rat apoB100 (rB100) was degraded by exogenous trypsin, whereas less than 10% of the rat apoB48 (rB48) was degraded. The differences in trypsin sensitivity were not a result of an inability of trypsin to degrade rB48, since in the presence of the detergent (Triton X-100) both rB100 and rB48 were completely degraded. PDI and rat albumin (not shown) were completely resistant (more than 90% protected) to trypsin digestion in the intact McA-RH7777 microsomes.

In accord with the findings in the rat hepatoma microsomes, the trypsin sensitivities of apoB100 and apoB48 were also different in the ER fractions derived from liver of normal rats or human apoB transgenic mice. Seventy percent of rB100 and 35% of rB48 were trypsin-sensitive in rat liver microsomes (Fig. 1B). In transgenic mouse liver ER, 50% of hB100 was trypsin-sensitive, and less than 10% of human apoB48 (hB48, generated by editing of the hB100 transcript in the mouse liver) was degraded by the exogenous protease (Fig. 1C). However, in contrast to the observations in the ER microsomes, both hB100 and hB48 in the Golgi microsomes isolated from the mouse liver (Fig. 1D) and rat liver (not shown) were trypsin-resistant (>90% protected), suggesting that in the Golgi apparatus, both apoB proteins are predominantly within the lumen. Thus, protease protection analysis of rat or human apoB proteins in microsomal fractions from cultured hepatoma cells or from liver suggest that apoB100 is exposed at the ER membrane, whereas apoB48 is not.

Trypsin Sensitivity of Newly Synthesized Human apoB Variants in Permeabilized Cells—We attempted to further validate the data of microsome protease protection assays by analyzing the translocational status of apoB using a different approach involving trypsin treatment of permeabilized cells. This protocol, which was previously characterized and reported (13), involved a pulse-chase protocol to establish a radiolabeled (newly synthesized) apoB pool followed by permeabilization of the cells, trypsin treatment, and immunoprecipitation of apoB. The protease protection assays were conducted in the presence of the proteasomal inhibitor, MG132, to ensure inhibition of endogenous degradation of apoB. The integrity of microsomal
**Experimental Procedures.** The trypsin accessibility of each protein McA-RH7777 cells expressing the indicated human apoB protein were a function of apoB length. Total microsomal fractions of cultured (percentage of trypsin-resistant apoB, 72% indicated by the high percentage of apoB recovered over control showed little susceptibility to trypsin digestion, indicating that effects of trypsin treatment of cells expressing recombinant McA-RH7777 cells (data not shown). Fig. 3 shows the immunoblotting. Cell lysates were immunoblotted with either monoclonal anti-human apoB (1D1) or polyclonal anti-rat apoB antibodies. The monoclonal human apoB antibody (1D1) distinguished the recombinant hB100 from the endogenous rB100 and thus allowed for the assessment of trypsin sensitivity of recombinant hB100. Immunoblotting revealed that the total mass of hB15 and hB29 were minimally sensitive to trypsin treatment (74 ± 5.4% and 70 ± 3.0% trypsin-resistant, respectively) (Fig. 4). Human B48 and B72 exhibited a greater sensitivity to exogenous trypsin (58 ± 5.3 and 55 ± 4.2% trypsin-resistant, respectively) compared with the shorter apoB variants. However, hB100 showed the highest sensitivity to trypsin digestion of all the apoB variants (42 ± 7.5% trypsin-resistant). Overall, the immunoblotting data were in most part consistent with the data from protease protection experiments in radiolabeled permeabilized cells. We should note that there were some differences between the trypsin sensitivities of larger apoB variants (>B48) using the permeabilized cell system in comparison with isolated microsomes. The sensitivities to trypsin appeared greater when using isolated microsomes, probably due to the higher degree of trypsin accessibility. However, we should emphasize that the inverse relationship between length of apoB polypeptide and its resistance to exogenous trypsin was very well defined in both systems.

**Comparison of Trypsin Sensitivities between ApoB Variants and Endogenous ApoB**—The translocation status of recombinant hB100 expressed in McA-RH7777 cells was compared with those of the endogenous rB100 of the same cell line as well as the endogenous hB100 in HepG2 cells. The different species of human and rat apoB100 had comparable trypsin sensitivities (Fig. 5). The percentage of trypsin-resistant apoB was 42 ± 7.5% for recombinant hB100 expressed in McA-RH7777 cells, 52 ± 2.9% for endogenous rB100 in McA-RH7777 cells, and 46 ± 6.3% for endogenous hB100 in HepG2 cells (Fig. 5). Thus, the high trypsin sensitivity observed with the recombinant hB100 appears to be consistent with the trypsin sensitivity of the endogenous B100 of both rat and human origin.

Finally, based on data obtained from protease protection experiments in permeabilized cells, we attempted to establish a correlation between the apoB size and its trypsin sensitivity for both human apoB variants and endogenous rat apoB species. Fig. 6 shows the percentage of trypsin-resistant apoB plotted against the length of apoB protein. ApoB variants ranging in size from B15 to B48 were relatively insensitive to trypsin digestion. Further increases in apoB size appeared to have a striking effect on the trypsin sensitivity of the apoB protein as seen with hB72 and the endogenous rB100 exhibiting significantly higher sensitivities to exogenous trypsin.

**Co-translational Stability of Human ApoB Variants in McA-RH7777 Cells**—The co-translational stability of apoB variants expressed in McA-RH7777 cells was investigated using a pulse-labeling protocol. Cells were pretreated with or without MG132 for 15 min prior to the pulse. Control cells were pretreated with 0.1% Me2SO (the solvent used to dissolve MG132). Following the pulse, cells were collected, solubilized, and immunoprecipitated for apoB using monospecific anti-human and anti-rat antibodies. Fig. 7A shows the immunoprecipitable apoB recovered after a 15-min pulse in the absence or presence of MG132. Quantitatively, the percentage of apoB recovered in the presence of MG132 compared with control were 130 ± 7.0% for hB15, 94 ± 8.0% for hB29, 110 ± 0.1% for rB48, and 140 ± 6.0% for hB72 (Fig. 7B). Among the truncated apoB constructs, hB15 and hB72 were slightly sensitive to the MG132, since their accumulation was stimulated by the presence of proteasome inhibitor. There was no noticeable change in stability of hB29 and rB48 with MG132 treatment. In contrast, the presence of the proteasome inhibitor caused a significant increase in recovery of the endogenous rB100 (280 ± 17% of the control).
Post-translational Stability of Human ApoB Constructs in McA-RH7777 Cells—The stability of human apoB variants following translation was also assessed using a pulse-chase labeling protocol. Post-translational degradation was estimated by comparing the amount of apoB recovered after a 2-h chase with that recovered initially at time 0 (following the pulse and before the chase), in the presence and absence of MG132. All apoB variants appeared to be degraded post-translationally (Fig. 8). In the absence of MG132, approximately half of all newly synthesized apoB nascent chain increases, its intracellular stability during translation decreases principally due to increased sensitivity to the proteasome.

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Post-translational turnover of all apoB variants as well as the endogenous apoB species was sensitive to the presence of MG132, although to varying degrees. In the presence of MG132, the recovery of hB15 and hB29 increased 1.5 ± 0.10- and 1.7 ± 0.06-fold, respectively, after a 2-h chase compared with untreated control, whereas hB72 showed a 1.3 ± 0.12-fold increase (Fig. 9). In addition, the amount of endogenous rB48 recovered after a 2-h chase in MG132-treated cells was 1.3 ± 0.10-fold greater than controls (Fig. 9), whereas the amount of endogenous rB100 recovered in the presence of MG132 increased by approximately 2.5 ± 0.19-fold (Fig. 9). Thus, the presence of a proteasome inhibitor resulted in significant increases in recovery of both expressed apoB variants as well as endogenous apoB species, suggesting the involvement of the proteasome in their post-translational turnover, regardless of size. Furthermore, there appears to be a correlation between the size of the apoB polypeptide and the degree of susceptibility to post-translational proteasomal degradation (Fig. 9), with full-length apoB100 being the most sensitive to the proteasome.

DISCUSSION

We performed a series of protease protection experiments to explore a correlation between the size of the apoB polypeptide and its sensitivity to exogenous trypsin using isolated microsomes and the permeabilized cell system (13). The most beneficial advantage of using a permeabilized cell system is that permeabilization allows the organelles (ER and Golgi) to maintain both their morphology and integrity while permitting exposure to exogenous trypsin (48). We have shown that control proteins such as α1-antitrypsin, transferrin, and albumin, secretory proteins found within the lumen of the ER, are completely resistant to trypsin digestion using this permeabilized cell system (13). Based on protease protection experiments in permeabilized cells, the truncated apoB constructs examined, including hB15 and hB29, were found to be relatively insensitive to exogenous trypsin, suggesting minimal exposure to the cytosol. However, a significant increase in trypsin sensitivity was observed for hB72 and the full-length endogenous apoB100. A similar relationship was observed when investigating the trypsin sensitivity of the total steady state mass of apoB. The intracellular mass of the hB100 construct exhibited the greatest sensitivity to trypsin digestion, compared with the mass of shorter apoB variants, which generally showed a significantly lower trypsin sensitivity. Therefore, results observed in the protease protection assays using a permeabilized cell system appeared to be comparable with the data obtained from experiments in isolated microsomes. As a whole, the data from protease protection assays in different model systems appear to suggest that sequences within the extreme C-terminal domain of the full-length apoB (the region absent in truncated apoB variants) may be responsible for the increase in trypsin sensi-
tivity of apoB100, which may reflect increased membrane as-
sociation and higher cytosolic exposure.

ApoB100 contains two amphipathic \( b \)-strand domains, \( b_1 \) and \( b_2 \), located within the 18–43% and 58–85% regions of apoB, respectively (1). It is believed that these \( b \)-strand do-
mains are responsible for the association of the apoB polypep-
tide with the ER membrane, thus slowing or stopping its trans-
location (10). The C-terminally truncated apoB constructs used
in the present study lacked either part or all of the \( b_1 \) and/or \( b_2 \) domain. The deletion of part or all of the \( b \) domains may be the
underlying factor for the differences observed in the trypsin
sensitivities of various apoB constructs. Based on the data from
protease protection studies in permeabilized cells, the con-
structs that did not contain the \( b_2 \) domain (hB15, hB29, and
hB48) had the highest resistance to exogenous trypsin, whereas those containing part or all of the \( b_2 \) domain (B72 and
B100) were more sensitive to trypsin digestion. Moreover, the
increase in trypsin sensitivity of these larger sized apoB pro-
teins may be a result of inefficient translocation of these
polypeptides across the ER membrane, thus resulting in in-
creased accessibility to exogenous trypsin. Further studies are
needed to elucidate the mechanisms by which specific cis-ele-
ments such as C-terminal \( b \)-strand domains may modulate
apoB translocational status as well as its susceptibility to the
proteasome.

Truncated apoB constructs expressed in various cell lines
have been used in several studies examining the apoB trans-
location process (12, 49). The translocational efficiency of
some of these short apoB constructs has also been assessed
using protease protection assays. Rusinol and Vance (43) re-
vealed that human B28 and B18 expressed in McA-RH7777
cells were 84 and 99% resistant to trypsin digestion, respecti-
vally. Thrift et al. (12) reported that Chinese hamster ovary
fibroblasts expressing recombinant apoB were capable of ef-
ciently translocating and secreting the B15 construct but not
B53. The B53 was found to be associated with the ER mem-
brane and thus sensitive to exogenous trypsin digestion (12).
Wang et al. (49) observed that transfection of COS-7 cells with
MTP catalytic subunit allowed these cells to translocate trans-
sected apoB constructs larger than B53. On the other hand,
Shelness and co-workers (16) have reported that apoB50 ex-
pressed in COS-1 cells (not expressing MTP activity) was effi-
ciently translocated across the ER membrane and was mini-
mally sensitive to exogenous trypsin. It is apparent that
considerable variation exists in the assessment of transloca-
tional efficiencies of both endogenous apoB as well as truncated
apoB variants. At least some of this variability may be due to
differences in experimental conditions used in the isolation of
microsomes and the protease protection assays. In the present
study, we have attempted to circumvent some of these vari-
bles by using a permeabilized cell system, which did show
differences in the percentage of trypsin sensitivity of apoB in
comparison with the percentages obtained from isolated micro-
somes. However, the correlation between the apoB size and its
trypsin sensitivity was observed in both experimental systems.

An attempt was also made to provide a correlation between
apoB size and its intracellular stability at both the co-transla-
tional and post-translational levels. MG132, a proteasomal in-
hibitor, was used in assessing the intracellular stability of both
endogenous and exogenous apoB variants. Our data indicated

![FIG. 4. Trypsin accessibility of total intracellular mass of C-terminally truncated apoB variants in permeabilized McA-RH7777 cells.](http://www.jbc.org/Downloaded from)

**FIG. 4.** Trypsin accessibility of total intracellular mass of C-terminally truncated apoB variants in permeabilized McA-RH7777 cells. McA-RH7777 cells transfected with various apoB constructs were incubated in the presence of 25 \( \mu \)M MG132 for 1 h. Cells were then permeabilized with digitonin (75 \( \mu \)g/ml) for 5 min, and permeabilized cells were treated with trypsin as described in the legend to Fig. 3. Cells were then solubilized, cell lysates were resolved by SDS-PAGE, and apoB was visualized by immunoblot analysis using antibody 1D1. The autoradiographs show representative experiments performed in the presence and absence of trypsin. Graphs show the mean \pm S.E. of several experiments for each apoB variant. A, hB15 (n = 6); B, hB29 (n = 3); C, hB48 (n = 3); D, hB72 (n = 3); E, hB100 (n = 3).
that co-translationally there was a slight increase in the intracellular stability of most truncated apoB variants (hB15, hB72, and rB48) in the presence of MG132. However, in the presence of the proteasomal inhibitor, there was almost a 3-fold increase in the amount of endogenous rat apoB100 recovered co-translationally. Thus, the data revealed a substantial difference between truncated apoB variants and the full-length apoB100 with respect to their co-translational stability. It also suggests that truncated apoB variants are much less accessible to proteasomal degradation, most likely as a result of more efficient translocation.

It is believed that incomplete translocation may result in the formation of a pool of apoB that becomes associated with the membrane and that a percentage of this pool of protein is exposed to the cytosolic face of the ER (10, 31) and destined for proteasomal degradation (31–35). Based on our current observations, we hypothesize that the b2 domain in the C-terminal region of the polypeptide may be responsible for incomplete translocation and thus co-translational instability of full-length apoB. In addition, the fact that MG132 blocks the degradation of both exogenous and endogenous apoB100 also supports the notion that incomplete translocation leads to cytosolic degradation of apoB by the proteasome at the co-translational level. In addition, Benoist and Grand-Perret (32) have shown that MTP activity prevents the proteasome mediated co-translational degradation of apoB100, particularly at the carboxyl-terminal sequences downstream of apoB65. They hypothesize that as the apoB polypeptide increases in size, there is a greater accumulation of lipophilic β-sheets in the protein (1), and this therefore increases the amount of MTP activity required by apoB during translocation. Furthermore, studies by Zhou et al. (50) have revealed that while apoB100 in HepG2 cells became associated with the cytosolic Hsp70 (a chaperone...
Fig. 8. Post-translational sensitivity to proteasomal degradation of apoB variants expressed in McA-RH7777 cells and endogenous rat apoB. Cells were incubated in the presence and absence of 25 μM MG132 for 15 min and labeled for 15 min with [35S]methionine. Cells were chased for 2 h in the presence or absence of 25 μM MG132, and immunoprecipitated for apoB. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. In addition, the percentage of apoB remaining over a 2-h chase in the presence of MG132 was compared with controls. A–E show representative experiments for each apoB variant, and the graphs show the mean ± S.E. of several experiments. A, hB15 (n = 6); B, hB29 (n = 8); C, endogenous rB48 (n = 2); D, hB72 (n = 7); E, endogenous rB100 (n = 16).

Fig. 9. The relationship between post-translational susceptibility to proteasomal degradation and the size of the apoB polypeptide. Data from pulse-chase proteasomal inhibitor studies were analyzed to explore a correlation between the size of the apoB variant and its post-translational sensitivity to proteasomal degradation. In addition, the results were compared with the proteasomal sensitivity of endogenous McA-RH7777 apoB. The graph shows the mean ± S.E. of several experiments (n ≥ 3). Closed circles, apoB constructs expressed in McA-RH7777 cells; open circles, endogenous rat apoB.

apparently involved in proteasomal degradation process), the shorter apoB15, which was efficiently translocated, exhibited minimal if any association with this chaperone. They speculated that cytosolic Hsp70 may specifically associate with the cytosolically exposed hydrophobic domains in the C-terminal regions of the apoB molecule such as the β2 domain. A B50 construct expressed in Chinese hamster ovary cells was also found to efficiently associate with Hsp70, indicating its sensitivity to proteasomal degradation (50). The interaction of B50 with Hsp70 may have resulted from the fact that this construct was expressed in a nonhepatic cell line devoid of MTP activity (12). In the present study, expression of a similarly sized construct (hB48) in a hepatic cell line resulted in efficient translocation and thus a lower sensitivity to proteasomal degradation. More recently, Chen et al. (34) have shown that alteration in the translocation of apoB leads to the association of ubiquitinated forms of apoB with the Sec61 complex of the translocon, which is ultimately degraded by the proteasome.

Evidence from the present report and other studies (27, 37, 46) appears to suggest that following translation and translocation, apoB may also be subject to post-translational degradation. Post-translationally, all truncated apoB variants as well as full-length apoB were found to be unstable, with 40–60% newly synthesized apoB degraded over a 2-h chase. Furthermore, our studies revealed that all apoB constructs, independent of size, were susceptible to proteasomal degradation, post-translationally. Studies using the proteasomal inhibitor, MG132, revealed that full-length apoB100 is more susceptible to post-translational proteasomal degradation than the shorter constructs and that this may be due to a higher degree of membrane association of apoB100 and thus a greater accessibility to the cytosolic proteasome. The degradative mechanisms involved in such post-translational instability of truncated apoB constructs are unknown; however, it is interesting to note that we found that such post-translational degradation was partially sensitive to MG132. This is somewhat puzzling, since efficient translocation of short apoB constructs is initially thought to preclude sensitivity to proteasomal degradation. How the proteasome is involved in the degradation of these efficiently translocated proteins is unclear, considering that the proteasome resides predominantly in the cytosol (51, 52). One possibility that may explain the above findings is the retrograde translocation of the luminal proteins from the secretory pathway into the cytosol. Several recent studies have shown that retrograde translocation acts as a form of quality control, where some proteins may be transported back out of the ER and into the cytosol (53–55). Once in the cytosol, ubiquitination of these proteins acts as a signal for their destruction via a proteasome-mediated process (53–55). Thus, it is possible that under certain conditions, such as limited availability of lipids, apoB, although fully translocated into the ER, may be shipped back out of the ER for degradation. Chen et al. (34) have speculated that retrograde translocation may be involved in the degradation of ubiquitinated apoB identified in the translocon of HepG2 cells. Further studies are currently under way in our laboratory to examine the role of retrograde translocation in the process of proteasome-mediated quality control of misfolded apoB. It is important to note that post-translational degradation of apoB constructs was only partially sensitive to the proteasome inhibitor. This would suggest that other degradative mechanisms, independent of the proteasome, may also modulate post-translational stability of apoB. Potential
candidates for such protease systems include the recently identified ER-60 protease. ER-resident proteases such as ER-60 protease have recently been detected in the ER lumen of rat hepatocytes (56) as well as HepG2 cells (40).

Acknowledgments—Monoclonal antibodies to human apoB (1D1) and antibodies to rat apoB (LBB220) were kind gifts from Drs. Ross Milne and Yves Marcel (University of Ottawa Heart Institute) and L. Wong (Louisiana State University), respectively. A polyclonal rabbit antibody to rat apoB (LRB220) was kind gifts from Drs. Ross Milne and Yves Marcel (University of Ottawa Heart Institute) and L. Wong (Louisiana State University), respectively. A polyclonal rabbit antibody to rat PDI was kindly provided by M. Michalak (University of Alberta).

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Intracellular Translocation and Stability of Apolipoprotein B Are Inversely Proportional to the Length of the Nascent Polypeptide

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*J. Biol. Chem.* 1998, 273:33397-33405.
doi: 10.1074/jbc.273.50.33397

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