Apolipoprotein B-100 destined for lipoprotein assembly and intracellular degradation undergoes efficient translocation across the endoplasmic reticulum membrane

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Abstract It has been proposed that inefficient translocation across the endoplasmic reticulum (ER) membrane gives rise to transmembrane forms of apolipoprotein B-100 (apoB). However, we previously demonstrated that the amino-terminal 50% of apoB (apoB-50) was efficiently translocated across the ER membrane in the nonhepatic cell line COS-1. To determine whether liver-specific factors modulate apoB membrane translocation or topology, hybrid proteins containing 300 amino acid overlapping segments of apoB-48 were transiently expressed in HepG2 cells and their protease sensitivities were examined in membrane vesicles. The hybrid proteins demonstrated the same range of protection from exogenously added protease (75–100%) as a transfected secretory control protein. When endogenous apoB was examined, its protection from trypsin in intact membranes was ~80%, a value similar to that of two endogenous secretory control proteins, transferrin and α_2-macroglobulin. No discretely sized fragments of apoB were generated by trypsin digestion of membranes unless they were first permeabilized with detergent. In contrast to the behavior of apoB and other control proteins, albumin predominantly resisted degradation by trypsin in both intact and detergent permeabilized membranes. HepG2 cells were treated with ALLN, a protease inhibitor that has been proposed to inhibit the turnover of partially translocated forms of apoB. Although an ~6-fold increase in intracellular apoB was observed in ALLN-treated cells, no corresponding increase in protease sensitivity was observed. These results indicate that the efficient translocation of apoB across the ER membrane occurs independently of its ability to undergo assembly into a secretion competent lipoprotein.—**Ingram, M. F., and G. S. Shelness.** Apolipoprotein B-100 destined for lipoprotein assembly and intracellular degradation undergoes efficient translocation across the endoplasmic reticulum membrane. *J. Lipid Res.* 1996. **37:** 2202–2214.

Supplementary key words very low density lipoprotein • lipoprotein assembly • HepG2 cells • ALLN • protein turnover

The production of secretion competent apolipoprotein B-100 (apoB) requires its assembly with lipids in the form of a spherical emulsion particle, an event that is thought to occur predominantly in the endoplasmic reticulum (ER) (1, 2). The initial assembly of apoB with lipids is cotranslational (3), although production of mature, very low density lipoprotein (VLDL)-sized particles may also require post-translational lipid addition (1, 4). Because apoB devoid of lipid is prone to aggregation (5), it has been suggested that the translocation of apoB across the ER membrane is coupled to its assembly with lipids. In this model, apoB that fails to engage in lipoprotein assembly is inefficiently translocated and undergoes transmembrane integration (6, 7). Transmembrane forms of apoB are subsequently targeted for intracellular degradation by an ALLN-inhibitable protease (7–9). It has also been proposed that transmembrane apoB has the capacity to complete its translocation posttranslationally and undergo lipoprotein assembly (8).

The existence of translocational regulation of apoB assembly and secretion is based largely on the analysis of apoB topology in isolated membranes. These studies indicate that in a variety of hepatic cells, about 50 to 100% of the apoB polypeptide chains contained within membrane-derived vesicles are accessible to exogenously added proteases (6, 9–16) and antibodies (6, 17). While the existence of transmembrane apoB in cells has been widely reported, few efforts have been made to identify the domains responsible for this behavior.

Abbreviations: ALLN, N-acetyl-leucyl-leucyl-norleucinal; apoB-100, apolipoprotein B-100; ER, endoplasmic reticulum; FBS, fetal bovine serum; MEM, minimal essential medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VLDL, very low density lipoprotein.

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The amino terminal ~17% of apoB has been reported to undergo efficient translocation across the ER membrane in both rat hepatoma cells (18) and CHO cells (7, 19) despite the presence of several domains that are reported to transiently uncouple translation from translocation in cell free systems (20–23). The formation of transmembrane apoB apparently requires expression of larger forms of apoB (apoB-48 or larger) and has been observed in both hepatic cells and stably transfected CHO cells (7, 9). In contrast, transiently transfected COS cells were incapable of forming detectable amounts of transmembrane apoB-50 (24) or apoB-36 (25). These results are consistent with the idea that only some cells (hepatic cells and perhaps some stably transfected nonhepatic cell lines) express factors that can interact with apoB and cause it to undergo transmembrane assembly.

This possibility was explored in the current report by expressing a series of apoB-48-containing hybrid proteins in the human hepatoma-derived cell line, HepG2. However, based on the criterion of protease protection, these hybrid proteins were judged to undergo efficient ER membrane translocation, arguing against the existence of a simple primary structural domain capable of directing transmembrane topology.

To further explore the basis for its transmembrane orientation, the topology of endogenous HepG2 cell apoB was examined. Surprisingly, the mean percent protease protection of apoB in membrane vesicles was ~80%, a value similar to that of both transfected and endogenous soluble secretory control proteins. Furthermore, the percentage of apoB that was susceptible to exogenous protease was unaffected by incubation of cells with ALLN, a treatment that has been proposed to increase the relative abundance of transmembrane apoB by preventing its intracellular degradation (8, 16, 19). These data indicate that, as with most other secretory precursor proteins, the translocation of apoB is an efficient, essentially cotranslational process. In contrast to previous models of apoB biogenesis and regulation, we propose that the intracellular retention and degradation of unlipidated or underlipidated apoB is a consequence of its aberrant structure and not its inability to undergo translocation across the ER membrane.

MATERIALS AND METHODS

Reagents

Tissue culture medium and supplements were obtained from JRH Biosciences (Lenexa, KS) and Sigma Chemical Co. (St. Louis, MO). Bovine trypsin (catalog no. 109 819, lot no. 12296621-78), soybean trypsin inhibitor, N-acetyl-leucyl-leucyl-norleucinal (ALLN), and polyclonal antibodies to human apoB, transferrin, α_2-macroglobulin, and albumin were obtained from Boehringer Mannheim (Indianapolis, IN). An antibody directed against the amino-terminus of canine calnexin was obtained from StressGen Biotechnologies (Victoria, BC). Anti-FLAG M2 monoclonal antibody was obtained from Eastman Kodak Co., Scientific Imaging Systems (New Haven, CT). Trans^{35}S-label (an ~5:1 mixture of [^{35}S]methionine and cysteine) was from ICN Biomedicals (Costa Mesa, CA). Protein concentrations were determined using BCA protein assay reagent from Pierce (Rockford, IL).

Cell culture

HepG2 cells were cultured in 100-mm dishes containing minimal essential medium (MEM) and 10% fetal bovine serum as described previously (26). For experiments involving ALLN, cells were preincubated for 1 h with 4 ml MEM containing 1.5% fatty acid-free bovine serum albumin (MEM-BSA) (8). MEM-BSA was then removed and replaced with 3 ml of labeling medium (MEM-BSA lacking methionine and cystine). After a 20-min incubation, medium was replaced with 1 ml of labeling medium containing 200 μCi/ml Trans ^{35}S-label. After a 10-min pulse, the cells were washed with 9 ml of chase medium (MEM-BSA with 1 mM additional methionine and cysteine). Four ml of fresh chase medium was then added and cells were incubated for an additional 10 or 60 min. Where indicated (Fig. 4), freshly prepared ALLN (10 mg/ml in ethanol) was added to cells at a final concentration of 40 μg/ml. An equivalent volume of ethanol was added to control media. For transient expression, HepG2 cells in 100-mm dishes were transfected at ~30% confluence by the calcium phosphate method essentially as described by Dawson et al. (27). The cells were labeled ~48 h after transfection.

Membrane preparation, trypsinization, and immunoprecipitation

For protease protection assays utilizing labeled cells, membranes were prepared by Dounce homogenization and treated with 100 μg/ml of trypsin as described (24). Protein concentration in labeled membrane preparations was between 600–700 μg/ml. Proteins of interest were immunoprecipitated from solubilized membranes (24) by addition of the appropriate antiserum (10 μl) or 6 μg of anti-FLAG M2 monoclonal antibody. After a 12–16 h incubation with gentle inversion at 4°C, 20 μl bed volume of protein G-Sepharose was added and incubation was continued for an additional 2 h. Immune pellets were washed and prepared for SDS-PAGE as described (24). For each antibody used, the amount of labeled protein immunoprecipitated was an approximately linear function of the volume of input cell ex-

tract. All immunoprecipitations were performed within the linear range. Control experiments using hybrid proteins secreted from transfected cells (Fig. 1) demonstrated that the anti-apoB polyclonal antibody used in these studies efficiently immunoprecipitated fragments of apoB derived from the amino-terminal 13% of the protein (data not shown). Unless otherwise indicated, immunoprecipitated proteins were resolved by 4–20% SDS-polyacrylamide gradient gels. For fluorography, gels were soaked in 1 M sodium salicylate for 30 min prior to drying (28) and exposed to Kodak XAR or Bio-Max MR film at -70°C. For quantitation, films were scanned using a pdi 325oe scanning densitometer (Protein and DNA Imageware Systems, Inc., Huntington Station, NY) using ImageMaster software (Pharmacia Biotech, Inc.). In cases where a considerable signal was contributed by apoB nascent chains (e.g., Fig. 2), both the full-length band and the predominant grouping of nascent chains were included in the densitometric scans. However, similar levels of protease protection were obtained when only the full-length apoB-100 bands were quantitated.

Immunoblot analyses

Membranes used for immunoblot analyses were prepared as described (24), with the exception that the post-nuclear membrane pellets were resuspended at a protein concentration of 2.25 mg/ml. One hundred µl of membranes was treated for 30 min with 250 µg/ml of trypsin at either 0° or 20°C in the presence or absence of 0.2% Triton X-100. Soybean trypsin inhibitor and phenylmethanesulfonyl fluoride were added to final concentrations of 750 µg/ml and 5 mM, respectively, and membranes were incubated for an additional 30 min at 0°C. After addition of an equal volume of concentrated SDS-PAGE sample buffer (30% glycerol, 6% SDS, 250 mM Tris-HCl, pH 8.8, 2.5 mM EDTA, 0.05% bromophenol blue), samples were immediately transferred to a boiling water bath and incubated for 10 min. For gel electrophoresis, a 140-µl aliquot of each sample was adjusted to 50 mM dithiothreitol and boiled for 5 min. Forty-µl aliquots of each sample were then loaded in triplicate on 4–20% SDS-polyacrylamide gradient gels. After electrophoretic transfer to nitrocellulose membranes (29, 30), blots were cut into sections and blocked overnight with phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) and 5% nonfat dry milk. Blots were then probed for 2 h with 1:500 dilutions of the indicated antibodies in PBS-T containing 2.5% nonfat dry milk. After washing with PBS-T, blots were probed with horseradish peroxidase-conjugated anti-goat or anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO) as required. After a final series of washes, blots were soaked in chemiluminescence reagent (DuPont NEN, Boston, MA) and exposed to X-ray film. After exposure to film, the α_2-macroglobulin blot was stripped by heating at 55°C for 45 min in 50 mM Tris HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol. After extensive washing in PBS-T, the membrane was reprobed with a 1:1000 dilution of antiserum directed against the amino-terminus of canine calnexin.

RESULTS

ApoB-48-containing hybrid proteins undergo efficient ER membrane translocation in HepG2 cells

The construction of a series of epitope-tagged, apoB-containing hybrid proteins was described previously (24). These proteins contain 300 amino acid overlapping segments of apoB-48 appended to the amino-terminal 77 amino acids of a modified form of bovine preprolactin (**Fig. 1A**). We previously demonstrated that, in membrane vesicles from transiently transfected COS-1 cells, each hybrid protein was resistant to exogenously added protease. On this basis we concluded that apoB lacked sequence motifs with the capacity to detectably block membrane translocation in nonhepatic cells (24). To determine whether apoB sequences cause transmembrane localization in cells of hepatic origin, the same set of hybrid proteins were expressed in the hepatoma-derived cell line HepG2.

HepG2 cells were transiently transfected with hybrid protein constructs and labeled with Trans^{35}S-label for 2.5 h. A post-nuclear membrane fraction was prepared as described previously (24) and subjected to digestion with trypsin (100 µg/ml) for 30 min at 0°C. After addition of protease inhibitors and detergent solubilization, hybrid proteins were immunoprecipitated with an antibody directed against the amino-terminal epitope tag (anti-FLAG M2 monoclonal antibody (24, 31, 32)). Results using three of the amino-terminal hybrid proteins, which span the amino-terminal 840 amino acids of apoB, are shown in Fig. 1B. None of the proteins were judged to be transmembrane based on their high degree of resistance to digestion with trypsin (75 to 100% protection based on densitometry). These levels of protection were similar to values obtained for a hybrid protein containing 300 amino acids of human complement component C3, a protein containing no known transmembrane anchor domain (33). The same results were obtained when the transfected cells were subjected to a short (10 min) pulse with radioactive precursor (data not shown). Furthermore, prolonged film exposures failed to identify peptides that arose from trypsin digestion of intact membranes (data not shown). To ensure that trypsin, under our experimental conditions, was able to cleave within a cytosolic domain of a known

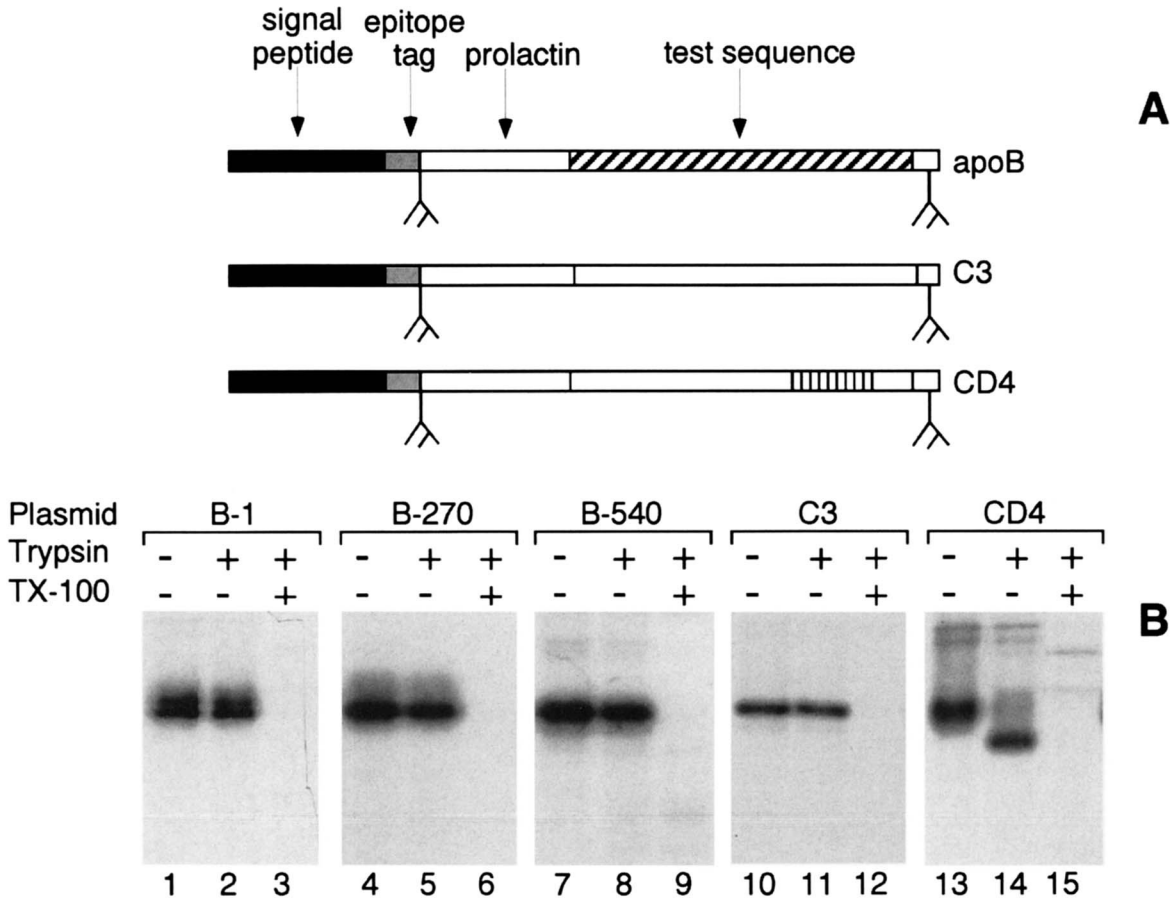

Fig. 1. Trypsin accessibility of apoB-containing hybrid proteins in HepG2 cell membranes. Construction of chimeric proteins diagrammed in A was described previously (24). Each "test sequence" is 300 amino acids in length and is appended to the amino-terminal 77 amino acids of a modified form of bovine preprolactin. Forked symbols indicate the location of engineered glycosylation sites. ApoB-containing plasmids are named after the first amino acid in each test sequence. For example, B-1 contains apoB amino acids 1–300, B-270 contains amino acids 270–570, etc. The C3 construct contains a 300 amino acid segment of human complement component C3, a protein with no known transmembrane anchor domain. CD4 contains a 300 amino acid segment of mouse CD4, which includes a single transmembrane anchor domain (vertically hatched box) positioned ~40 amino acids from its C-terminus. In B, HepG2 cells transiently transfected with the indicated plasmids were labeled for 2.5 h with Trans^{35}S-label. A post-nuclear membrane fraction was prepared and incubated with (+) or without (-) 100 μg/ml trypsin and 1% Triton X-100 (TX-100), as indicated. After inhibition of trypsin with a mixture of protease inhibitors, membranes were solubilized and subjected to immunoprecipitation by addition of an antibody directed against the epitope tag (anti-FLAG M2 monoclonal antibody) and protein G-Sepharose. Immunoprecipitated proteins were resolved by 12.5% SDS-PAGE and visualized by fluorography.

integral membrane protein, the behavior of the CD4 chimera was examined (Fig. 1A). This protein contains a single transmembrane domain positioned about 40 amino acids from its C-terminus. Under the same conditions that gave rise to protected forms of the C3 and apoB-containing proteins, the CD4 chimera was almost quantitatively converted to a more rapidly migrating species, consistent with degradation of its cytosolic domain. The behavior of the other apoB-48-containing hybrid proteins corresponding to amino acids 810–2190 was also examined and found to give results similar to those shown for B-1, B-270, and B-540 in Fig. 1B (data not shown). These results are consistent with those obtained in COS cells (24) and indicate that, irrespective of the cell types we investigated, no single sequence element within apoB-48 is sufficient to direct transmembrane assembly.

Endogenous apoB in isolated HepG2 cell membrane vesicles is predominantly resistant to trypsin digestion

As none of the apoB-containing hybrid proteins underwent transmembrane assembly, we investigated the behavior of endogenous apoB in HepG2 cells. For these experiments HepG2 cells were labeled for 10 min with Trans^{35}S-label and then chased for 10 min with media containing an excess of unlabeled met and cys. Under these conditions, the labeled apoB should be predominantly localized to the ER (34). A post-nuclear membrane fraction was prepared and incubated in the pres-

ence and absence of trypsin. Aliquots of the digests were then immunoprecipitated with antibodies against apoB and control secretory proteins.

Figure 2A shows the results of protease protection assays in which equivalent volumes of control and protease-treated membranes were immunoprecipitated with antibodies to apoB and transferrin. In Fig. 2B, the same experiment was repeated using antibodies to apoB and α_2-macroglobulin. Both apoB and the two control secretory proteins displayed very similar patterns of protease protection. To calculate the percent of each protein protected from trypsin, films were scanned by densitometry. The values obtained are shown in **Table 1** and range from ~50% to 100% protection. Although variable, the mean percentage of protease resistant apoB in HepG2 cell membranes was ~80%, a value similar to that of transferrin and α_2-macroglobulin (68 and 83% respectively). Thus, the protection of apoB from exogenously added protease in HepG2 cell membranes, while not 100%, falls within the experimental limitations for secretory proteins with known lumenal localizations (35–37).

Immunoblot analysis of trypsinized membrane preparations

Many previous reports of transmembrane apoB have utilized immunoblotting as an analytical method rather than immunoprecipitation (6, 9, 11). We, therefore, repeated protease protection assays using post-nuclear membranes prepared from unlabeled cells. For these experiments membranes were prepared at a higher protein concentration (2.25 mg/ml) so that samples could be directly loaded on SDS gels without having to first concentrate them by centrifugation or acid precipi-

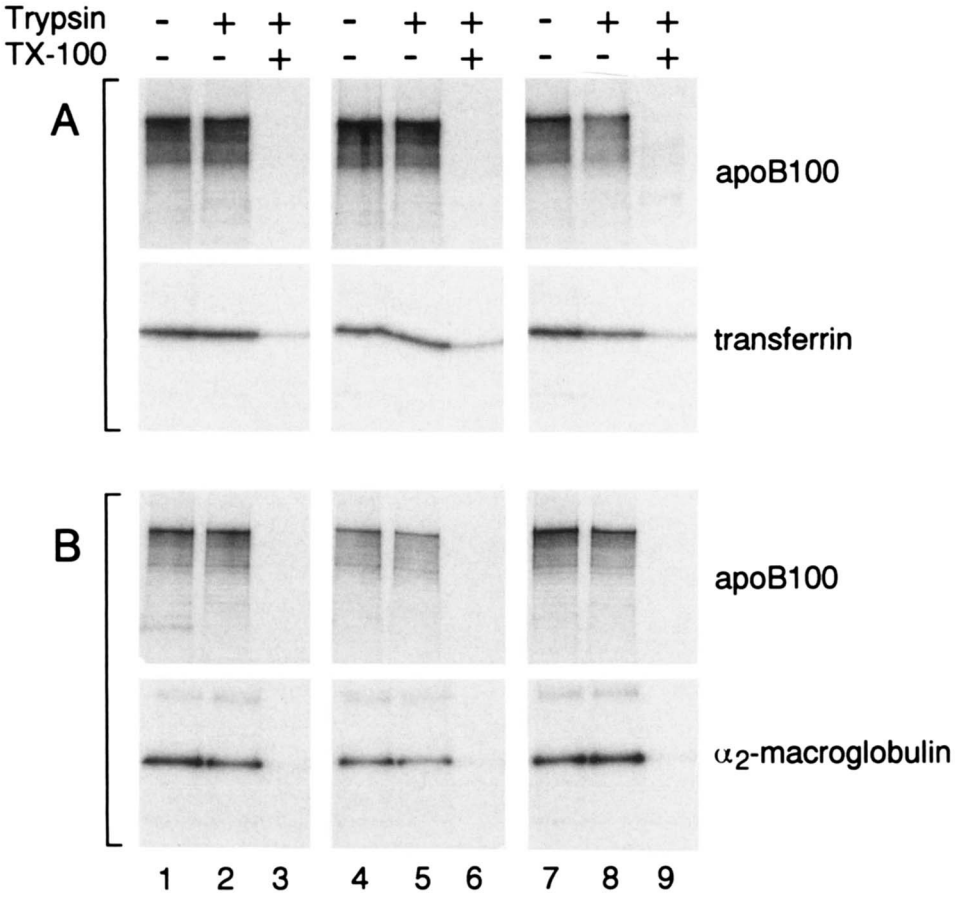

Fig. 2. Trypsin accessibility of endogenous apoB, transferrin, and α_2-macroglobulin in HepG2 cell membranes. HepG2 cells were pulsed for 10 min with Trans^{35}S-label and then chased for 10 min in media containing an excess of unlabeled amino acids. Post-nuclear membranes were isolated and incubated with or without trypsin (100 μg/ml) and 1% Triton X-100 as indicated. Equal aliquots of the membrane lysates were subjected to immunoprecipitation with either anti-apoB (apoB-100) and anti-transferrin antibodies (A), or anti-apoB and anti-α_2-macroglobulin antibodies (B). Immunoprecipitated proteins were fractionated by SDS-PAGE and visualized by fluorography. Each vertically paired set of panels in A and B represents independent membrane preparations and protease protection assays (n = 6).

TABLE 1. Percentage of trypsin-resistant apoB, transferrin and α_2-macroglobulin in HepG2 cell membrane vesicles[a]

Protein	Membrane Preparation 1	2	3	Mean ± SD
A				
ApoB-100	97	92	49	79 ± 26
Transferrin	96	67	42	68 ± 27
B				
ApoB-100	103	74	73	83 ± 17
α_2-Macroglobulin	74	62	114	83 ± 27
ApoB-100 (total)				84 ± 18[b]

[a]Percent protection from trypsin in each membrane preparation was determined by densitometric scanning of fluorograms displayed in Fig. 2A and B, as indicated.

[b]ApoB-100 (total) mean and SD were determined from the six protease protection assays displayed in Fig. 2A and B and the eight additional assays shown in Fig. 4 and described in the text (n = 14).

tation. To compensate for this higher protein concentration, a higher concentration of trypsin (250 μg/ml) was used. We also compared results obtained by trypsinization at both 0° and 20°C. As observed in **Fig. 3,** apoB from membranes subjected to treatment with 250 μg/ml of trypsin at either 0° or 20°C was predominantly intact, unless Triton X-100 (0.2%) was added. In the presence of Triton X-100, apoB was converted to a series of lower molecular weight peptides. In the same membranes, α_2-macroglobulin behaved similarly to apoB whereas albumin resisted proteolysis both in the absence and presence of Triton X-100. Analysis of calnexin, a type I integral membrane protein (38), showed that its cytosolic domain was completely digested by trypsin in intact membranes. Interestingly, calnexin's lumenal domain resisted trypsin digestion even in the presence of Triton X-100, a finding that confirms previous reports (39). Similar resistance to proteolysis has been observed for other resident membrane proteins of the ER (40, 41).

Effect of ALLN on intracellular apoB accumulation and localization in HepG2 cells

It has been suggested that transmembrane forms of apoB may represent polypeptide chains destined for intracellular degradation rather than lipoprotein assembly (6, 8). Incubation of HepG2 cells with the protease inhibitor ALLN selectively protects apoB from intracellular degradation without appreciably affecting its secretion (8, 16). Therefore, the relative amount of apoB containing cytosolic domains should be increased by ALLN treatment (19). This predication, however, has not been confirmed using protease protection assays (16). In an attempt to increase the relative amount of putative transmembrane apoB, HepG2 cells were preincubated in the presence or absence of ALLN. Cells were then labeled for 10 min and chased for either 10 or 60 min, also in the presence or absence of ALLN. Aliquots of cell suspension from each of the four experimental conditions were used to measure the effects of ALLN on both intracellular apoB accumulation and topology.

As observed in **Fig. 4A,** cells pulsed for 10 min and chased for 10 min in the presence or absence of ALLN gave similar amounts of immunoprecipitable apoB (compare labeling condition 1 and 2). However, after the 60-min chase the presence of ALLN increased the amount of labeled intracellular apoB by ~6-fold (0.365 versus 2.285 OD units/mg cell protein when conditions 3 and 4 were compared). Precipitation of cell extracts with 10% trichloroacetic acid followed by liquid scintillation counting revealed that ALLN produced a 10–20% reduction in the amount of labeled protein in the post-nuclear detergent extracts (**Table 2,** compare labeling conditions 1 and 2; 3 and 4). It was previously reported that ALLN used at concentrations above 50 μg/ml significantly inhibited protein synthesis in HepG2 cells (8). The slightly reduced protein synthesis observed in cells treated with 40 μg/ml ALLN is consistent with this finding and would tend to slightly underestimate the fold increase in apoB accumulation caused by the ALLN-induced inhibition of apoB degradation.

To determine whether the apoB stabilized by ALLN accumulated as a transmembrane protein, aliquots of control and ALLN-treated cells were used to prepare post-nuclear membranes. When membranes were subjected to digestion with trypsin, the percentage of apoB protected from protease was 57, 96, 103, and 83% for labeling conditions 1–4, respectively (Fig. 4B). While these values are variable they are within the experimental limitations of the protease protection assay as defined earlier (Table 1). Of particular significance is the similarity between the percent protease protection observed for labeling conditions 3 and 4. Although there was an ~6-fold difference in the amount of apoB that accumulated in ALLN-treated cells after a 60-min chase, there was clearly no corresponding increase in the percentage of apoB susceptible to degradation by exogenous trypsin. The experiment shown in Fig. 4 was repeated with essentially the same results (data not shown). As no relationship was observed between ALLN treatment and the extent of protease protection, these data indicate that the apoB protected from degradation by ALLN (i.e., apoB that accumulates under labeling condition 4) has the same lumenal localization as apoB that is destined for lipoprotein assembly and secretion.

ApoB and albumin display different sensitivities to trypsin in detergent-permeabilized HepG2 cell membranes

As discussed previously, the relative amount of protease accessible apoB in hepatic cells has been reported between ~50–100% (6, 9–16). In HepG2 cells specifi-

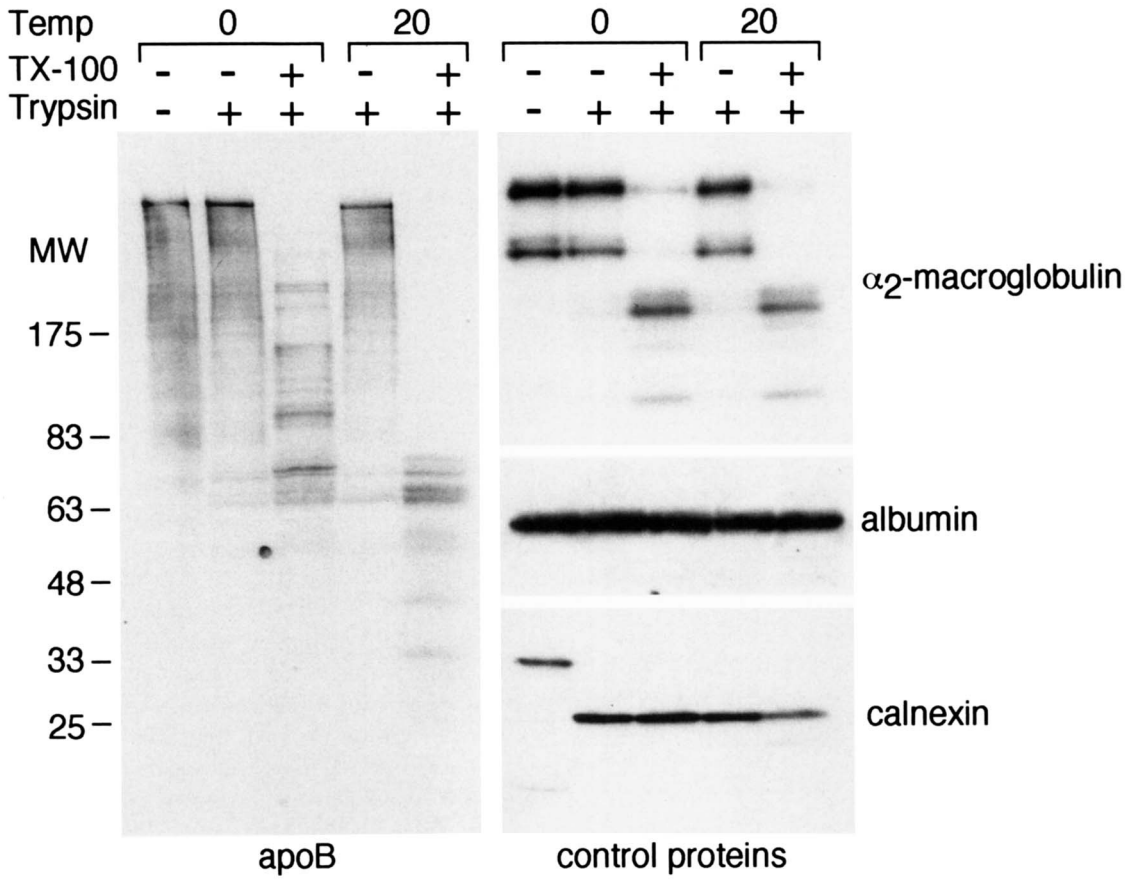

Fig. 3. Immunoblot analysis of trypsin-treated HepG2 cell membranes. A post-nuclear membrane preparation was divided into five, 100-µl aliquots. Each aliquots was incubated for 30 min at either 0° or 20°C in the absence or presence of trypsin (250 µg/ml) and Triton X-100 (0.2%), as indicated. After addition of protease inhibitors, samples were mixed with an equal volume of concentrated SDS-PAGE sample buffer and immediately transferred to a boiling water bath. Equal aliquots of each sample were fractionated by SDS-PAGE. After electrophoretic transfer to nitrocellulose, membranes were probed with antibodies to apoB, α_2-macroglobulin, or albumin as indicated. Immunoreactive bands were visualized by chemiluminescence detection. The membrane that was first probed with the α_2-macroglobulin antibody (top right panel) was stripped and reprobed with an antibody directed against the amino-terminal domain of canine calnexin.

cally, the relative amount of protease accessible apoB is reported to be between 75–100% (9, 12, 16). The cumulative results presented above, in which ~80% of apoB is protected from proteolysis, are clearly inconsistent with the existence of appreciable amounts of transmembrane apoB. In an attempt to explain this discrepancy we investigated the proteolytic susceptibility of apoB and control proteins in membrane vesicles that were progressively permeabilized with increasing concentrations of sodium deoxycholate. We hypothesized that, under conditions in which membranes are permeabilized by deoxycholate but otherwise remain intact (42), apoB may display a greater sensitivity to proteolysis than control proteins. To address this issue, HepG2 cells were labeled for 2.5 h and a post-nuclear membrane fraction was prepared. Membrane suspensions were incubated with deoxycholate at concentrations from 0.01% to 0.1%, and then subjected to trypsin digestion for 30 min. After addition of protease inhibitors, membranes were adjusted to 1% Triton X-100 and aliquots were immunoprecipitated with antibodies to apoB, α_2-macroglobulin, and albumin. As observed in **Fig. 5,** the protection of all three proteins was essentially complete in unpermeabilized microsomes (compare lanes 1 and 2; 8 and 9; 15 and 16). Inclusion of deoxycholate at concentrations of 0.02% and higher clearly reduced the protection of apoB and α_2-macroglobulin and gave rise to specific trypsin digestion products, particularly in the case of apoB. The size of the apoB tryptic peptides generated in the presence of detergent correspond in size to those seen in Fig. 3 and are similar to those reported by others to have arisen from trypsin digestion of apparently intact membranes (9). However, as was predicted from the immunoblot analysis in Fig. 3, the protection of albumin from protease is not appreciably altered by addition of deoxycholate (lanes 16–20). Even

addition of Triton X-100 to 1% failed to cause appreciable degradation of albumin (lane 21). It is clear from this analysis that, under conditions where apoB was partially or fully susceptible to proteolysis, albumin was predominantly resistant. However, the protection of albumin from proteolysis was due to an inherent property of the protein and not the integrity of the membrane vesicles.

DISCUSSION

In the current report we attempted to experimentally define domains within apoB that are responsible for its proposed transmembrane topology in hepatic cells. Hybrid proteins containing 300 amino acid overlapping segments of apoB-48 were transiently expressed in HepG2 cells. Based on the criterion of protease protection, all of the hybrid proteins appeared to undergo efficient ER membrane translocation, as was previously observed in the nonhepatic cell line COS-1 (24). These results confirmed that, unlike a classical topogenic sequence such as the transmembrane domain in the CD4 chimera (Fig. 1), no single sequence element within apoB-48 appeared capable of conferring transmembrane localization in a heterologous protein context, in vivo. To further explore the basis for transmembrane apoB we examined the trypsin sensitivity of endogenous apoB in HepG2 cell membranes. Under standard conditions of protease digestion, in which known transmembrane proteins were degraded, apoB displayed the same degree of protease resistance as two soluble secretory control proteins, transferrin and α_2-macroglobulin.

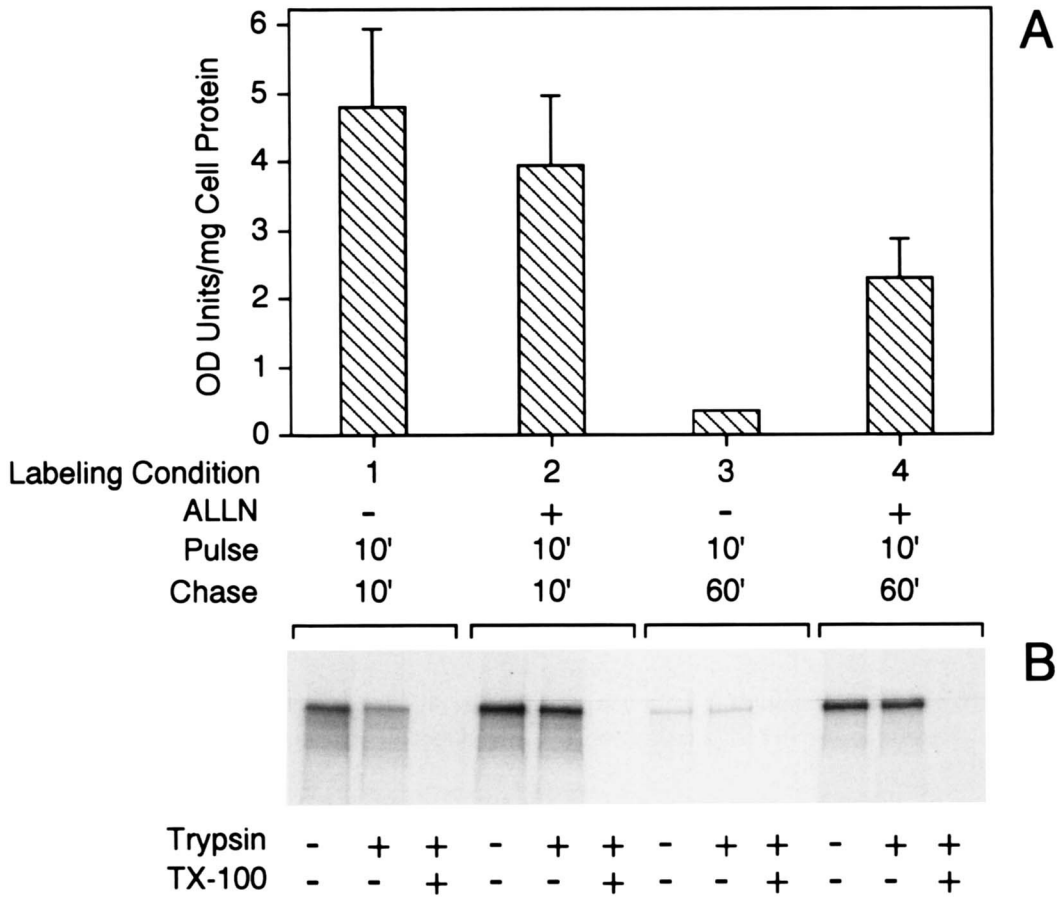

Fig. 4. Effects of ALLN on intracellular apoB accumulation and localization in HepG2 cells. HepG2 cells were pulsed with Trans^{35}S-label for 10 min and chased for either 10 or 60 min in the presence or absence of 40 μg/ml ALLN, as indicated. In A, an aliquot of cells from each experimental group was lysed and immunoprecipitated with anti-apoB antibodies. After electrophoresis, gels were soaked in fluor, dried, and exposed to pre-flashed X-ray film. Signal density was quantitated by densitometry. The amount of labeled apoB is expressed as mean optical density (OD) units/mg cell protein (mean ± SD of triplicate immunoprecipitations). For labeling condition 3, the SD bar was too small to project beyond the symbol. In B, the remainder of the cell samples was used to prepare post-nuclear membranes that were incubated in the absence or presence of trypsin (100 μg/ml) and 1% Triton X-100. After inhibition of trypsin, membranes were solubilized and aliquots of lysate were subjected to immunoprecipitation with anti-apoB antibodies. Immunoprecipitated proteins were fractionated by SDS-PAGE and visualized by fluorography.

These results strongly indicate that apoB undergoes the same, efficient degree of translocation into the ER as other soluble secretory precursor proteins.

One possible concern associated with these studies is the variable nature of the protease protection assay. The percent protection of transferrin, α_2-macroglobulin, and apoB ranged from ~40% to greater than 100% (Table 1). However, the mean protection of apoB from protease was 84% ± 18 (n = 14), a value similar to both the transfected and endogenous secretory control proteins and well within a range indicative of complete segregation within membrane vesicles (37).

The observation that our protease protection values were variable and consistently less than 100% is not altogether unexpected. In the first gel electrophoretic analysis of a protease protection experiment it was noted that only 60% of processed IgG light chain synthesized by membrane-bound polysomes was protected from proteolysis (35). In subsequent cell-free experiments, protease protection values ranged from 46–71% with SD of 9–12% (36). While this degree of variability was lower than that reported here, the additional steps required to detect apoB and control proteins, involving immunoprecipitation or Western blotting, may further increase experimental variability. In one of the few previous reports in which multiple determinations of apoB-100 protease accessibility were performed (6), the coefficient of variation (SD/mean) ranged from 35–53%. The coefficient of variation for the data reported here was in a similar range (21% for apoB, 33% for α_2-macroglobulin, and 40% for transferrin).

The basis for the inability to consistently achieve 100% protection of proteins during protease protection assays is not fully understood. In the seminal early studies in which this assay was devised it was noted that "the extent of protection of pancreatic bands and of the processed light chain was not complete, probably due to the leakiness of some membrane vesicles" (36). In subsequent discussions of the nonspecific degradation of secretory proteins in such assays it was noted that, while protease protection in the range of 80–100% was attainable, "Too extensive protease digestion leads to degradation of even the segregated forms, presumably owing to breakdown of the membrane barrier. The reasons for this permeabilization are not clear and have not been systematically investigated" (37). Hence, the protection from proteolysis afforded by segregation of proteins within membrane vesicles is a relative property dependent upon both the integrity of the membrane barrier and the inherent proteolytic sensitivity of the proteins of interest. Although well suited to distinguish between a translocated protein (~50–100% protection) and an untranslocated protein or protein domain (<5% protection) (35), problems may arise when the protease protection assay is applied to the unprecedented case of apoB where a distinction is made between "efficient" and "inefficient" translocation.

TABLE 2. Effect of ALLN on incorporation of [^{35}S]met and cys into cellular protein

Labeling Conditions[a]			
1	2	3	4
cpm/mg cell protein (10^7)			
4.22 ± 0.10	3.70 ± 0.07	2.47 ± 0.05	1.90 ± 0.07

Values are given as means ± SD (n = 3).
[a]Labeling conditions are defined in Fig. 4 and text.

In contrast to our data, in which ~80% of apoB was protected from exogenous protease, other investigators have observed only 0–25% protection in HepG2 cell membranes (9, 12, 16). While we cannot fully reconcile the discrepancy between our results and those of others, one explanation may be related to both the qualitative nature of the protease protection assay, discussed above, coupled with the wide range of inherent sensitivities that different proteins display towards proteolysis (43). As observed in Figs. 3 and 5, albumin resisted trypsinization whether or not it was sequestered within a membrane vesicle. While many previous reports have not displayed data in which the protease sensitivities of apoB and albumin were compared in the presence of detergent (6, 9, 13, 15, 16) the results of Furukawa et al. (12) partially support the notion that human serum albumin is more inherently resistant to proteolysis than apoB. When HepG2 cell membranes were treated with 280 μg/ml of proteinase K in the presence of Triton X-100, about 30% of the albumin remained undegraded and another ~30% underwent a limited cleavage giving rise to a form only slightly smaller than the full-length protein (12). Considering that proteinase K possesses a broader cleavage specificity than trypsin (44, 45), this behavior is consistent with our own observations that human serum albumin is relatively resistant to proteolytic degradation. Therefore, under conditions of mild membrane permeability, albumin, but not apoB, may completely resist proteolysis giving rise to the potentially erroneous conclusion that apoB possesses cytosolic domains.

In addition to the discrepancy between our results and those of others, it should be noted that even among reports of transmembrane apoB, little consensus exists as to the percentage of apoB that is transmembrane or the membrane systems that contain the preponderance of transmembrane apoB. For example, in rat hepatocyte membranes, the percentage of apoB-48 susceptible to proteolysis has been reported as "only a small portion" (i.e., completely lumenal) (15), 22% (11), and 70% (6, 13). For apoB-100, protease protection values range from 10–44% in ER fractions (6, 15) and ~40–100% protection in Golgi fractions (6, 15). Again, this large

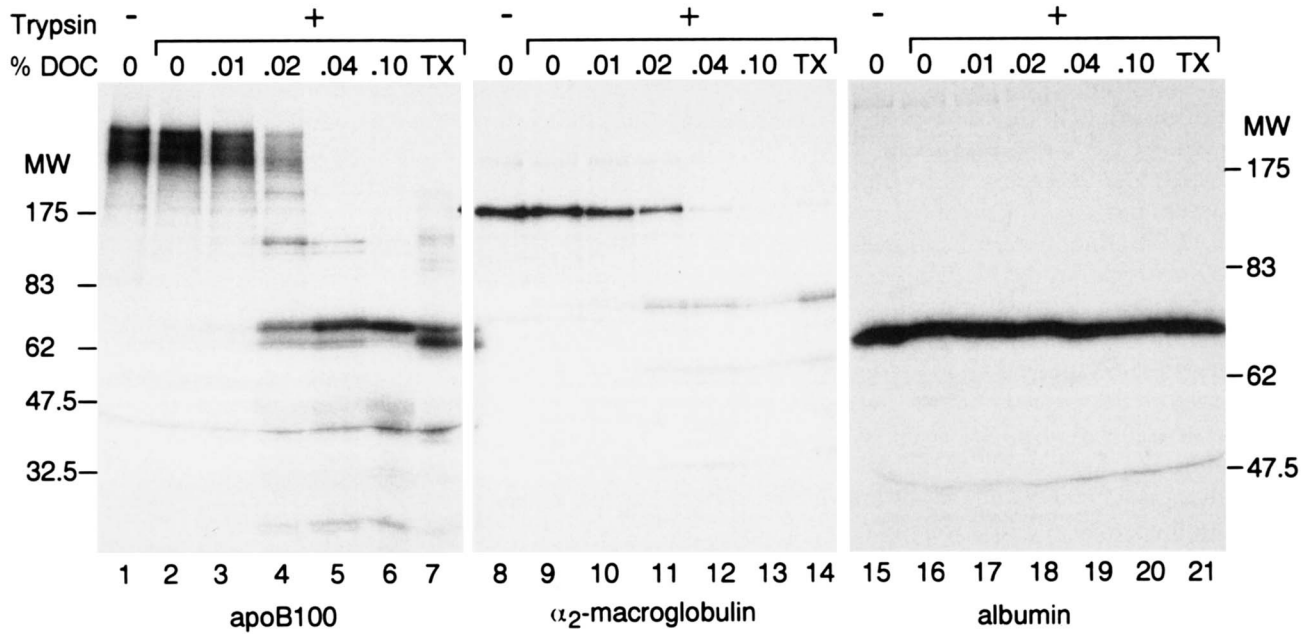

Fig. 5. Detergent permeabilization of HepG2 cell membranes. HepG2 cells were metabolically labeled with Trans^{35}S-label for 2.5 h. A post-nuclear membrane fraction was prepared and incubated in the absence or presence of trypsin (100 μg/ml) and either 1% Triton X-100 (TX) or the indicated concentrations of deoxycholate (DOC). After inhibition of trypsin, membranes were solubilized and aliquots of the lysates were subjected to immunoprecipitation with antibodies to apoB, α_2-macroglobulin, or albumin. Immunoprecipitated proteins were resolved by 4–20% SDS-PAGE (apoB-100 and α_2-macroglobulin) or 8% SDS-PAGE (albumin) and visualized by fluorography.

variability may be related to the qualitative nature of the protease protection assay as opposed to differences in the localization of apoB.

Another finding revealed by the present studies is that an ~60–70 kDa domain of apoB appears to predominately resist proteolysis by trypsin (Figs. 3 and 5). Similarly sized fragments derived from the amino-terminal domain of apoB were observed by Du et al. (9) upon digestion of HepG2 cell membranes with trypsin. The finding that these fragments can be produced even in the presence of detergent indicates that they may represent limit digestion products resulting from their inherent resistance to proteolysis rather than protection by the membrane.

That a globular protein or protein domain can resist proteolysis is not uncommon. As noted by Price and Johnson (43), "In general, native proteins are rather resistant to the action of proteinases, a consequence of the tight, compact or 'domain' structures adopted by most globular proteins". As shown in Fig. 3, the cytosolic domain of calnexin is clearly sensitive to trypsin in intact membranes; however, addition of detergent does not alter its pattern of proteolytic degradation. This behavior of calnexin has been described previously (39) and is similar to a number of other integral membrane proteins such as ribophorin II (41) and subunits of the signal peptidase complex (40). In addition to membrane proteins, soluble proteins and domains of soluble proteins can display a high degree of resistance to proteolysis (43, 44, 46). As shown in Figs. 3 and 5, trypsin digestion of α_2-macroglobulin generates several distinct limit digestion products. The predication that the amino-terminal domain of apoB has globular character (47) may explain its relative resistance to proteolytic degradation both in vitro and during the intracellular degradation of unlipidated or underlipidated apoB in vivo (9, 48–50).

Another issue related to the putative existence of transmembrane apoB is the inability to experimentally address its metabolic significance. In HepG2 cells, where ~75–100% of apoB has been reported to exist in transmembrane topology, it has been proposed that transmembrane apoB represents a pool that is subsequently targeted for intracellular degradation (8, 9, 16). Under this scenario, it has been proposed that treatment of cells with oleate stimulates apoB secretion by facilitating its translocation across the membrane (8). On the other hand, the protease inhibitor ALLN has been proposed to selectively protect transmembrane apoB from intracellular degradation (8, 16). Consistent with this model, it has been reported that oleate decreases the percentage of apoB associated with the cytosolic chaperone protein family, Hsp70, whereas ALLN increases this percentage (19). Assuming that the post-cell lysis coimmunoprecipitation assay used to detect apoB-Hsp70 interactions reflects their association in vivo, similar

alterations in the amount of cytosolic apoB should be detected using a protease accessibility assay. However, no such relationship between the apparent translocation state of apoB and its degree of protease accessibility has been reported (8, 16). In an attempt to establish such a relationship, HepG2 cells were treated with ALLN to enrich them in putative transmembrane forms of apoB. Although the accumulation of intracellular apoB was increased by ~6-fold in ALLN-treated cells, no evidence was obtained for a corresponding increase in its protease accessibility (Fig. 4).

A second method that has been used to establish that apoB resides on the cytosolic side of intracellular membranes has employed antibody reactivity. In one such study, antibodies conjugated to magnetic beads effectively immunoisolated hepatic microsomal membranes, whereas antibodies to albumin were ineffective (6). In a second series of studies, subcellular fractions from rabbit liver were shown to compete for antibody binding to LDL (17). It is difficult to reconcile these results with those presented here as extensive analysis of apoB using protease protection assays, an established method for deducing membrane protein topology (51, 52), reveals that apoB in HepG2 cells resides predominantly on the trans side of intracellular membranes, a localization it shares with other soluble secretory precursor proteins. In addition to the protease protection data, however, the glycosylation status of apoB also indicates that it has undergone complete translocation (53). While the translocation of both secretion competent and nonsecretion competent forms of apoB may occur efficiently, a large proportion of apoB may bind to the inner leaflet of the ER membrane during or after translocation. This behavior is consistent with the resistance to sodium carbonate extraction displayed by some apoB contained in membrane fractions (6, 34, 54). However, it has also been shown that the carbonate resistant membrane-bound pool of apoB in rat hepatocytes is more highly mannosylated than plasma apoB (53). As 4 of the 5 sites for N-linked glycosylation within apoB-48 and 15 of the 16 sites in apoB-100 (55) are positioned well downstream of the putative site (~apoB-15) at which translocation arrest is believed to occur (9), this finding is consistent with the idea that membrane-bound apoB has undergone complete translocation into the lumen of the ER where it gained access to the active site of oligosaccharyltransferase (56).

In conclusion, our data, as well as those presented by Leiper et al. (57) and of Wong and Torbati (53), strongly support the notion that apoB undergoes the same degree of translocation across the ER membrane as other soluble secretory precursor proteins. These data indicate the need to reconsider certain aspects of previous models of apoB biosynthesis and regulation. As ~85–90% of apoB synthesized in HepG2 cells is retained and degraded (58), it is clear that the translocation of apoB across the ER membrane is not dependent upon its ability to undergo assembly into a secretion competent lipoprotein. This is true of other proteins that are misfolded in the ER due either to mutations or the absence of a subunit partner. While many such proteins are retained intracellularly and degraded (59), in no case is the underlying defect in secretion attributed to the inability to translocate the protein. We propose, therefore, that the inability of cells to secrete unlipidated or underlipidated apoB may also be a function of its aberrant structure and not a defect in translocation.

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