Translocation Efficiency of Apolipoprotein B Is Determined by the Presence of β-Sheet Domains, Not Pause Transfer Sequences*

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Cotranslational translocation of apoB100 across the endoplasmic reticulum (ER) membrane is inefficient, resulting in exposure of nascent apoB on the cytosolic surface of the ER. This predisposes apoB100 to ubiquitylation and targeting for proteasomal degradation. It has been suggested that pause transfer sequences (PTS) present throughout apoB cause inefficient translocation. On the other hand, our previous study demonstrated that the translocation efficiency of apoB100 is dependent on the presence of a β-sheet domain between 29 and 34% of full-length apoB100 (Liang, J.-S., Wu, X., Jiang, H., Zhou, M., Yang, H., Angkeow, P., Huang, L.-S., Sturley, S. L., and Ginsberg, H. N. [1998] J. Biol. Chem. 273, 35216–35221); this region of apoB has no PTS. However, the effects of the β-sheet domain on the presence of PTS elsewhere in the N-terminal region of apoB100. To further investigate the roles of PTS and β-sheet domains in the translocation of apoB100 across the ER, we transfected McArdle RH7777, HepG2, or Chinese hamster ovary cells with human albumin (ALB)/human apoB chimeric cDNA constructs; ALB/B12–17 (two PTS but no β-sheet), ALB/B29–34 (β-sheet but no PTS), ALB/B36–41 (two PTS and a β-sheet), and ALB/B49–54 (neither PTS nor a β-sheet). ALB/ALB1–40 served as a control. Compared with ALB/ALB1–40, secretion rates of ALB/B12–17, ALB/B29–34, and ALB/B36–41 were reduced. Secretion of ALB/B49–54 was similar to that of ALB/ALB1–40. However, only ALB/B29–34 and ALB/B36–41 had increased proteinase K sensitivity, ubiquitylation, and increased physical interaction with Sec61α. These results indicate that the translocation efficiency of apoB100 is determined mainly by the presence of β-sheet domains. PTS do not appear to affect translocation, but may affect secretion by other mechanisms.

ApoB100 is the major structural protein of very low density lipoproteins and low density lipoproteins. Elevated plasma levels of these lipoproteins are associated with an increased risk factor of atherosclerosis (1). ApoB100 is a large hydrophobic protein of 4536 amino acids with a molecular mass of ~520 kDa and is synthesized in the liver (2, 3). Models of the secondary structure of apoB100 contain three α-helical domains (1–22, 48–56, and 89–100% of apoB100) and two long β-sheet domains (22–48 and 56–89%) (3, 4). Other studies have indicated that the first 17% of the N-terminal end of apoB100 has a lipoprotein lipase-binding domain (5), that the domain between 66 and 83% of full-length apoB100 is the low density lipoprotein receptor-binding domain (6), and that sequences between 1 and 5.8% and between 9 and 16% of apoB100 contain binding sites for microsomal triglyceride transfer protein (7, 8).

Studies by numerous laboratories have demonstrated several points along the intracellular itinerary of apoB100 where it can be targeted for either lipoprotein assembly and secretion or proteasomal degradation (10). Unlike typical secretory proteins, which are efficiently and completely translocated into the lumen of the endoplasmic reticulum (ER) and then efficiently secreted (9). The initial site of regulation of apoB100 occurs during its cotranslational translocation across the ER membrane. Thus, if microsomal triglyceride transfer protein activity and core lipid availability are adequate, nascent apoB100, like typical secretory proteins, is translocated efficiently across the ER membrane. Then it can be assembled, with lipids, into an apoB lipoprotein in the ER lumen and secreted into the medium (10, 11). If instead there is inadequate core lipid or microsomal triglyceride transfer protein is defective, cotranslational translocation of apoB100 across the ER is inefficient, and significant amounts of newly synthesized apoB100 can be degraded cotranslationally by the ubiquitin-proteasome pathway (10, 12–17).

It is believed that the complex structure of apoB100 plays an important role in its post-translational regulation, which, under a wide range of conditions, is the predominant way that the assembly and secretion of apoB lipoproteins are controlled (10, 11, 15, 18). Despite demonstration of inefficient translocation of apoB100 by several groups, the basis for this remains unclear and controversial. Lingappa and co-workers suggested that “pause transfer” sequences (PTS) present throughout apoB100 are the cause of inefficient translocation (19–21) and can result in exposure of secretory proteins on the cytosolic surface of the ER (22, 23). However, the importance of PTS in regulating the

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2 The abbreviations used are: ER, endoplasmic reticulum; PTS, pause transfer sequence(s); OA, oleic acid; McA, McArdle RH7777; CHO, Chinese hamster ovary; ALB, albumin; ALLN, N-acetyl-Leu-Leu-norleucinal; BSA, bovine serum albumin; MEM, minimal essential medium; PK, proteinase K.

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translocation of apoB100 was questioned by Pease et al. (24, 25). In addition, our previous data do not support a critical role for PTS; we showed that the simple addition of a β-sheet domain (from 29 to 34% of the full-length apoB100 sequence) to a truncated form of apoB100 consisting of repeats of the globular N-terminal region (apoB13,16), which contains several PTS (26), reduces translocation efficiency and is associated with proteasomal degradation of the protein (27). The addition of that β-sheet domain also makes apoB13,16 responsive to oleic acid (OA). Although the domain between 29 and 34% of full-length apoB100 has no PTS (26), our studies could not rule out the possibility that the effects of the β-sheet domain require the presence of PTS in the N terminus of apoB100. Furthermore, the importance of other β-sheet domains was not addressed.

In this study, to further investigate the relative importance of PTS and β-sheet domains in regulating the translocation of apoB100 across the ER, we transfected McArdle RH 7777 (McA), HepG2, and Chinese hamster ovary (CHO) cells with four different chimeric cDNA constructs derived from human albumin (ALB) and sequences of apoB100. The apoB100 sequences contained either two PTS without a β-sheet domain, a β-sheet domain without any PTS, two PTS and a β-sheet domain, and neither PTS nor a β-sheet domain. We determined how the insertion of PTS or β-sheet domains into ALB, a typical secretory protein, affects its secretion, exposure on the cytosolic side of the ER, proteasomal degradation, and interaction with the translocon. Our results indicate that, although the presence of PTS may affect the secretion of apoB100 and possibly its response to OA, it does not affect the translocation process. By contrast, our results strongly support the view that the efficiency with which apoB100 is translocated across the ER and its predisposition to proteasomal degradation are determined by the presence of β-sheet domains.

EXPERIMENTAL PROCEDURES

Materials—N-Acetyl-Leu-Leu-norleucinal (ALLN), dimethyl sulfoxide, leupeptin, pepstatin A, sodium oleate, and rabbit anti-ALB antibody were purchased from Sigma. Bovine serum albumin (BSA; fatty acid-free) was purchased from VWR International. [35S]Methionine/cysteine was obtained from PerkinElmer Life Sciences. Lipofectamine, protein G-agarose, and all cell culture reagents were purchased from Invitrogen. Sheep anti-apoB100 polyclonal antibodies were from Calbiochem. Rabbit anti-Sec61 antibody was purchased from Santa Cruz Biotechnology. Sheep anti-apoB100 polyclonal antibodies were from Calbiochem. Anti-proteasomal degradation of the protein (27). The addition of that β-sheet domain also makes apoB13,16 responsive to oleic acid (OA). Although the domain between 29 and 34% of full-length apoB100 has no PTS (26), our studies could not rule out the possibility that the effects of the β-sheet domain require the presence of PTS in the N terminus of apoB100. Furthermore, the importance of other β-sheet domains was not addressed.

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Construction of Human ALB/ApoB Expression Plasmids—pCR3.1-ALB was constructed by inserting the cDNA encoding full-length human ALB into pCR3.1 (Invitrogen). 710-bp fragments of partial human apoB or human ALB cDNA were synthesized in PCRs with oligonucleotides containing an additional BglII site (underlined sequences below). The size of the oligonucleotides synthesized was based on the “map” of apoB PTS and β-sheet domains (26); by choosing sequences of apoB that were 5% of the full-length protein, we were able to produce apoB sequences with only PTS, only β-sheet domains, both PTS and β-sheet domains, or neither PTS nor β-sheet domains. ApoB12–17 cDNA (referred to as B12–17), which has two PTS but no β-sheet domain, was synthesized with sense primer 5’-GAGATCTTAAGTTAGTGAAGAAGTTTCTG-3’ and antisense primer 5’-GAGATCT- GTAATCCAGCTCCAGTGGGGAG-3’. ApoB29–34 cDNA (referred to as B29–34), which has a β-sheet domain but no PTS, was synthesized with sense primer 5’-GAGATCTTTTGTATCAACTGCAAGTGCC-3’ and antisense primer 5’-CAGATCTGAGTGTCAAGTTTAAAGGTCAG-3’. ApoB36–41 cDNA (referred to as B36–41) was synthesized with sense primer 5’-GAGATCTTTTGAAGTGATCTCAGTGTT-3’ and antisense primer 5’-CAGATCTGAGTGTCAAGTTTAAAGGTCAG-3’. ApoB49–54 cDNA (referred to as B49–54) was synthesized with sense primer 5’-GAGATCTTTTGAAGTGATCTCAGTGTT-3’ and antisense primer 5’-CAGATCTGAGTGTCAAGTTTAAAGGTCAG-3’. ApoB51–40 cDNA was synthesized with sense primer 5’-GAGATCTTTTGAAGTGATCTCAGTGTT-3’ and antisense primer 5’-CAGATCTGAGTGTCAAGTTTAAAGGTCAG-3’. ApoB51–40, with the first 40% of the amino acid sequence of full-length ALB added to ALB, was chosen because it was approximately the same length as the apoB inserts and would therefore serve as a control for inserting a protein into the middle of full-length ALB (at the same site in which the apoB sequences were inserted). These PCR fragments were digested with BglII and inserted into the linear form of pCR3.1-ALB, which was digested with BglII (corresponding to nucleotide 708 or residue 236 of human ALB, which is ~40% from the N terminus). Each construct was confirmed at the nucleotide level by sequencing. A comparison of these constructs with a schematic representation of the sites of PTS in apoB100 is shown in Fig. 1.

Transfections—McA, HepG2, or CHO cells were transfected with Lipofectamine (Invitrogen) according to the manufacturer’s specifications with each ALB/apoB plasmid or ALB/ALB1–40. Experiments were performed 36–40 h after transfection.

Pulse-Chase Experiments—Transiently transfected McA, HepG2, and CHO cells were preincubated in serum-free MEM containing 1.5% BSA for 2 h and then labeled with Eagle’s MEM containing 1.5% BSA and 200 μCi/ml [35S]methionine/cysteine for 10 min. After being washed, cells were incubated in serum-free Dulbecco’s modified Eagle’s medium containing 10 mM methionine and 3 mM cysteine for 10 or 120 min. The medium was collected at 120 min, and cells were lysed at 10 or 120 min. In the experiment in which OA stimulation was assessed, 0.4 mM OA was added to the incubation medium for 2 h prior to labeling and was also present during the 2-h labeling period. The lysis buffer contained 62.5 mM sucrose, 0.5% sodium deoxycholate, 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.4), 150
mm NaCl, 50 μg/ml leupeptin, 50 μg/ml pepstatin A, and 30 μl/ml protease inhibitor mixture (1 mM benzamidine, 5 mM EDTA, 100 units/ml aprotinin, and 10 mM HEPES (pH 8.0)). Conditioned medium was mixed with protease inhibitor mixture and 0.86 mM freshly made phenylmethylsulfonyl fluoride. Cell lysates and conditioned medium were used for immunoprecipitations.

Immunoprecipitation Experiments—Immunoprecipitation of proteins was carried out according to the method of Dixon et al. (28). Cell lysates or conditioned medium was mixed with NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris (pH 7.4), 0.5% Triton X-100, and 0.1% SDS) and an excess amount of various antiserum. The mixture was incubated at 4 °C for 1 h. Protein G-agarose was added to the reaction solution, and the incubation was continued for an additional 16 h. The beads were washed with NET buffer, and proteins were released with sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 10% β-mercaptoethanol) by boiling for 5 min. Samples were resolved by SDS-PAGE followed by autorgraphy or immunoblotting.

Proteinase K Digestion Experiments—Microsomes were prepared according to methods described previously (27). Briefly, 60-min labeled McA cells were scraped in buffer A (250 mM sucrose, 10 mM HEPES (pH 7.4), and 0.5 mM dithiothreitol) with protease inhibitor mixture and disrupted by 10 passes through a ball-bearing homogenizer. Intact cells and nuclei were removed by centrifugation for 10 min at 9000 × g, and the microsomes were isolated after centrifugation for 1 h at 105,000 × g. The microsome pellets were resuspended in buffer A without protease inhibitor mixture. The microsomes were incubated with or without proteinase K (PK; 50 μg/ml) or Triton X-100 (0.5%) for 30 min on ice. After digestion, phenylmethylsulfonyl fluoride (3 mM) was added, and the samples were incubated for another 5 min on ice. The samples were then recentrifuged, and the microsome pellets were dissolved in lysis buffer, immunoprecipitated, and analyzed by SDS-PAGE.

Ubiquitylation Experiments—Transfected CHO cells were pretreated with serum-free Eagle’s MEM containing 1.5% BSA and 40 μg/ml ALLN for 2 h. The medium was removed, and the cells were washed with ice-cold phosphate-buffered saline and lysed in lysis buffer. Ubiquitylated proteins were captured by immunoprecipitation with anti-ubiquitin antibody. Total ALB protein was isolated by immunoprecipitation with anti-A LB antibody. The eluates from the immunoprecipitation were resolved by SDS-PAGE followed by Western blotting using antibody against apoB or ALB.

Physical Interaction of ALB/ApoB and Sec61α—Transfected CHO cells were pretreated with serum-free Eagle’s MEM containing BSA (1.5%) and ALLN (final concentration of 40 μg/ml) for 2 h and then labeled with Eagle’s MEM containing 1.5% BSA and 200 μCi/ml [35S]methionine/cysteine for 20 min. After being washed, cells were incubated with serum-free Dulbeco’s modified Eagle’s medium containing 10 mM methionine and 3 mM cysteine for 15, 30, or 60 min. Cells were collected as described above and either 1) immunoprecipitated with anti-Sec61α antibody as described above, boiled in 2% SDS to dissociate the immunoprecipitated molecules, and then immunoprecipitated with anti-A LB antibody or 2) immunoprecipitated with only anti-A LB antibody as described above. Immunoprecipitated proteins were separated by SDS-PAGE; the bands were cut from the gels; and radioactivity was determined in a Beckman Coulter LS6500 liquid scintillation counter. The percent of each construct that was associated with Sec61α was then calculated, and the data for each ALB/apoB construct was normalized to that for ALB/ALB1–40. Because there were no differences in the results at 15, 30, or 60 min, those data were combined.

RESULTS

The Presence of PTS and/or β-Sheet Domains Significantly Inhibits Secretion of ALB/ApoB from Cultured Hepatocytes—To determine whether the presence of PTS or β-sheet domains affects the secretion efficiency of ALB/apoB chimeric proteins, pulse-chase experiments were performed. 36 h after McA cells (Fig. 2A), HepG2 cells (Fig. 2B), and CHO cells (Fig. 2C) were transiently transfected with full-length ALB, ALB/ALB1–40 (used as a control for the length of the other chimeric proteins), and the ALB/apoB constructs, the secretion of the chimeric
proteins was determined. The quantity of protein in the cell lysates at the 10-min chase time point was taken as 100% of total newly synthesized ALB or ALB/apoB proteins, and the quantities of each construct secreted were normalized based on total construct synthesized. Simply repeating the first 40% of the amino acid sequence of ALB within full-length ALB (Fig. 1) reduced secretion considerably compared with full-length ALB; 34.3–42.2% of newly synthesized ALB/ALB1–40 was
secreted by 120 min versus 71.7–84.4% of ALB. Thus, all other comparisons were against ALB/ALB1–40. After 120 min of chase, 24.3–31.1% of newly synthesized ALB/B49–54, which has neither PTS nor a β-sheet domain, was secreted; this was similar to the secretion of ALB/ALB1–40. In contrast, the secretion of ALB/B12–17 (two PTS), ALB/B29–34 (a β-sheet domain), and ALB/B36–41 (two PTS and a β-sheet domain) was dramatically inhibited, ranging from 1.3 to 9.9% of newly synthesized protein (Fig. 2, A–C). The data in Fig. 2 (A–C) indicate that PTS and β-sheet domains, either alone or together, can reduce apoB secretion.

OA stimulates the secretion of apoB from cultured hepatocytes, particularly HepG2 cells (28). When HepG2 cells that had been transiently transfected with chimera cDNAs were incubated with 0.4 mM OA (Fig. 2D), the secretion of ALB/B12–17 (two PTS) and ALB/B36–41 (two PTS and a β-sheet domain) was increased significantly, as was the secretion of endogenous apoB100 in the HepG2 cells. Surprisingly, the secretion of ALB/B29–34 (a β-sheet domain without any PTS) was unaffected by OA. However, there was almost no secretion of this construct in any of the cell lines (Fig. 2, A–C). As predicted, the secretion of ALB/B49–54 (no PTS or β-sheet domain) was also unaffected by OA.

It should be noted that, although OA increased the secretion of ALB/B12–17 and ALB/B36–41, the secretion of these chimeras was still very low. Thus, whereas 52.5% of newly synthesized ALB/ALB1–40 was secreted at 120 min in the presence of OA, only 27.6 and 23.9% of newly synthesized ALB/B12–17 and ALB/B36–41, respectively, were secreted under the same conditions.

The Presence of β-Sheet Domains Increases the PK Sensitivity of ALB/ApoB—The results described above indicate that both PTS and β-sheet domains affect ALB/apoB secretion. To determine whether the reduced secretion is associated with inefficient translocation, as assessed by cytosolic exposure of the translocating protein, we isolated microsomes and digested them with exogenous protease. This method allows for the partial digestion of proteins that are exposed on the cytosolic side of the ER (and other microsomal components) and is able to demonstrate such exposure for apoB100 (27). 36 h after McA cells were transiently transfected with ALB/apoB constructs, cells were labeled for 1 h with [35S]methionine/cysteine. Microsomes were then isolated and incubated with or without PK (50 μg/ml) for 30 min. After the reaction was stopped, the remaining proteins were analyzed by SDS-PAGE. ALB/ALB1–40, ALB/B12–17, and ALB/B49–54 were digested similarly (43.7 ± 8.5, 45.4 ± 7.0, and 47.6 ± 19.1%, respectively), whereas ALB/B29–34 and ALB/B36–41 were both digested more extensively (76.3 ± 5.0 and 75.8 ± 5.4%, respectively) (Fig. 3). All of the protein bands disappeared completely when the microsomes were incubated with both PK and Triton X-100, which disrupted microsomes and exposed all proteins to proteinase activity (data not shown). These results indicate that the extent of cytosolic exposure of ALB/apoB, as it translocates across the ER membrane, is determined by the presence of β-sheet domains, but not PTS.

Because OA appeared to increase the secretion of ALB/B12–17 as well as ALB/B36–41, we examined the effect of OA on PK sensitivity in each construct in McA cells. In two separate experiments performed in duplicate, we did not observe any effect of OA pretreatment of the cells on their sensitivity to PK (data not shown).

The Presence of β-Sheet Domains Increases the Ubiquitylation of ALB/ApoB—Because poor translocation efficiency of apoB100 across the ER results in degradation by the ubiquitin-proteasome pathway, we next determined which of the ALB/apoB constructs could be ubiquitylated. 36 h after CHO cells were transiently transfected with ALB/apoB constructs, they were preincubated for 2 h with serum-free medium containing ALLN (40 μg/ml) to block degradation by the ubiquitin-proteasome pathway. The cell lysates were immunoprecipitated with anti-ubiquitin antibody, and eluates from the immunoprecipitations were analyzed by SDS-PAGE. ALB/ALB1–40, 27.6% ALB/B12–17, 0% for ALB/B29–34, 23.9% for ALB/B36–41, and 56.3% for ALB/B49–54. All pulse-chase experiments were done in triplicate: four separate experiments with McA cells, one experiment with HepG2 cells, and one experiment with CHO cells. The stable labeling experiment in HepG2 cells with OA in the medium was performed twice with triplicates each time.
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**A**

![Graph showing total expression and ubiquitinated states for ALB/ALB1–40, ALB/B12–17, ALB/B29–34, ALB/B36–41, and ALB/B49–54 constructs.]

**B**

![Graph showing percentage ubiquitinated expression for ALB1–40, B12–17, B29–34, B36–41, and B49–54 constructs.]

**FIGURE 4.** The presence of a β-sheet domain increases ubiquitinylation. CHO cells were transiently transfected with ALB/apoB constructs. The cells were pretreated with ALLN (40 μg/ml) for 2 h and then lysed on ice. Anti-ALB or anti-apoB antibody was used to immunoprecipitate total ALB or apoB in the cells, respectively. Anti-ubiquitin antibody was used to immunoprecipitate all ubiquitinylated proteins. All immunoprecipitates were resolved by SDS-PAGE followed by Western blotting using anti-apoB or anti-ALB antibody. The ubiquitinylated proteins were loaded onto the gels at 10 times the level of total ALB or apoB (A). The percent of ubiquitinylated proteins for each construct was normalized to the data on the left side of the gel, which depicts results from immunoprecipitation with either anti-ALB antibody (for ALB/ALB1–40) or anti-apoB antibody (for the rest of the constructs) and Western blotting with either anti-ALB or anti-apoB antibody, respectively. The normalized data for two separate experiments performed in triplicate are depicted (B).

**FIGURE 5.** The presence of a β-sheet domain increases the physical interaction between ALB/apoB constructs and Sec61α. CHO cells were transiently transfected with ALB/apoB constructs. The cells were pretreated with ALLN (40 μg/ml) for 2 h, labeled for 20 min with [35S]methionine/cysteine, chased for 15–60 min, and immunoprecipitated with anti-Sec61α and anti-ALB antibodies as described under “Experimental Procedures.” We performed five separate experiments with ALB/ALB1–40 and three separate experiments for each of the ALB/apoB constructs. Each experiment was done in triplicate at 15, 30, and 60 min, and the data for the three time points were pooled because no differences over time were observed. The percent of each of the ALB/apoB constructs that was associated with Sec61α was normalized to the percent of ALB/ALB1–40 that was associated with Sec61α.* * p < 0.001 compared with ALB/ALB1–40; #, p < 0.005 compared with ALB/B12–17.

This atypical behavior is manifested by an incomplete translocation and a bitopic orientation of apoB100 in which some domains are exposed to the cytosol and some are exposed to the ER lumen (34–37). The availability of the core lipids that apoB100 carries out of the cell is thought to be the most crucial physiological factor determining the translocation efficiency of apoB100. When lipid synthesis is inadequate and/or microsomal triglyceride transfer protein activity is impaired, apoB100 associates with Hsp 70 and undergoes rapid intracellular degradation by the ubiquitin-mediated proteasomal degradation pathway (10, 12–14). Although several laboratories, including ours, have demonstrated that apoB100 is inefficiently translocated across the ER membrane, there have been differing views as to the molecular basis for this atypical behavior of a secretory protein.

Lingappa and co-workers (19–21, 26) suggested that PTS, which are present throughout apoB100, can cause a transient interaction of apoB100 with the translocon within the ER membrane. Pease et al. (24, 25) published data to the contrary and concluded that translation pausing was the basis for the observations by Lingappa and co-workers. We reported that the translocation efficiency of apoB100 is dependent on the presence of a lipid-binding β-sheet domain between 29 and 34% of the full-length protein (27). The simple addition of that β-sheet domain to the globular N terminus of apoB confers PK sensitivity, predisposition to proteasomal degradation, and sensitivity to OA. However, our studies could not rule out the possibility that the effects of the β-sheet domain depend on the presence of PTS or the N terminus of apoB100.

In this study, we attempted to more definitively determine the cause of the atypical interaction of apoB100 with the translocon, an interaction that results in inefficient translocation of apoB100 across the ER membrane and a predisposition to cotranslational degradation by the proteasome. To investigate this question, we decided to use a strategy centered upon chimeric proteins made of ALB, a typical secretory protein that is transported through the secretory pathway efficiently with little intracellular degradation (28), and sequences of apoB100 containing either PTS or β-sheet domains, neither, or both. We
characterized the secretion of these chimeric proteins, including their sensitivity to OA and their interaction with the translocon. The latter was studied using PK sensitivity, ubiquitylation, and their association with Sec61α. There are several potential problems with our approach. First, the insertion of any sequence of amino acids into the mid-portion of ALB could significantly alter its translocation and passage through the secretory pathway. To address this, we made a cDNA construct in which 708 bp starting with the N terminus of ALB cDNA were inserted into full-length ALB cDNA at the same site into which the apoB sequences were inserted. We chose the size of the construct so that the inserted amino acid sequence would be the same length as the apoB sequences inserted into the ALB/apoB chimera. Indeed, there were differences between ALB/ALB1–40 and ALB. ALB was secreted with high efficiency (70–80%), which was similar to what we observed in previous studies (28), whereas only ~40% of ALB/ALB1–40 was secreted (Fig. 2). We demonstrated previously that ALB is not sensitive to PK (35); microsomal ALB/ALB1–40 was reduced by ~40% when exposed to PK (Fig. 2). Finally, ALB/ALB1–40 was minimally ubiquitylated (Fig. 4). Because ALB/ALB1–40 did not behave like ALB, we used the ALB/ALB1–40 construct as the appropriate control for our ALB/apoB constructs. We accept, however, that our findings must be viewed in a relative and not absolute sense. However, within those limits, we believe that direct comparisons of the effects of PTS and β-sheet domains on apoB100 translocation across the ER membrane are valid.

We initially determined the secretory efficiency of each of the chimeras using a pulse-chase protocol. As noted above, only ~40% of initially synthesized ALB/ALB1–40 was secreted at 120 min compared with 70% secretion of native ALB, a value similar to what we observed previously for ALB secretion in cultured liver cells (28). The results from the pulse-chase experiments (Fig. 2, A–C) showed that the presence of either PTS or a β-sheet domain or both together reduced the secretion of ALB/apoB compared with ALB/ALB1–40. By contrast, insertion of apoB49–54, a region of apoB100 lacking both PTS and β-sheet domains, had no effect on the secretion of the chimeric protein compared with ALB/ALB1–40. We were struck by the consistency of these findings in each of three different cell types, including CHO cells, which do not normally synthesize either ALB or apoB100. We also determined the sensitivity of each of the chimeras to OA, a well described stimulus for apoB secretion (28). On the basis of our previous studies (27, 28, 38), we were surprised that OA stimulated the secretion of ALB/B12–17, which contains only PTS (Fig. 2D). In our previous work, truncations containing apoB16, apoB13,16, and apoB13,13,16 were efficiently translocated across the ER and were not sensitive to OA (27). It is possible that the additional 1% of the N terminus of apoB100 that we used (apoB12–17) in this construct has enough lipid binding capacity; although there is some controversy regarding the minimal number of N-terminal amino acids required for apoB either to bind to microsomal triglyceride transfer protein or to form a spherical lipoprotein with a lipid core (39–42), we believe it to be more likely that our chimeric protein ALB/B12–17 was not a good model of the globular N-terminal component of apoB100. It is also important to note that the actual rate of secretion of ALB/B12–17 was only 27.6% in the presence of OA, which was much lower than the 52.5% of ALB/ALB1–40 that was secreted under the same conditions. Lacking other data, however, we must conclude that the two PTS in ALB/B12–17 directly reduced secretion of and conferred OA sensitivity to the chimeric protein and that, in apoB100, the two PTS in the N-terminal region may play a role in the translocation of apoB across the ER.

Even more surprising was the lack of response to OA by ALB/B29–34 (Fig. 2D). In our previous work, the presence of this β-sheet domain conferred OA sensitivity to apoB13,16 and apoB13,13,16 (27). Both of these unexpected findings, that PTS within apoB12–17 reduced secretion and conferred OA sensitivity to ALB, whereas apoB29–34 did not confer OA sensitivity (although its presence markedly reduced secretion of ALB), could have been related to our use of ALB, rather than apoB13,16 or apoB13,13,16, as the protein “surrounding” the β-sheet domain. On the other hand, we did see OA sensitivity when both PTS and a β-sheet domain were present in ALB/B36–41. The present results therefore support the view that the effect of β-sheet domains on apoB translocation requires the presence of PTS. However, this result must be viewed with caution because either the absence of the first 10% of the N terminus of apoB or the presence of sequences in ALB may have altered the function of the β-sheet domain in apoB29–34.

The atypical interaction of apoB100 with the translocon not only makes the protein responsive to OA, but predisposes it to an association with heat shock proteins (43, 44) and cotranslational degradation by the proteasome (12, 13). The latter requires both cytosolic exposure of the partially translocated apoB100 and ubiquitylation of the protein while it is still in the translocon. Therefore, we next determined the sensitivity of each of the chimeric proteins to PK. The results showed that, although ALB/ALB1–40 was exposed moderately on the cytosolic surface of the microsomes, the chimeric proteins containing a β-sheet domain either within apoB29–34 or apoB36–41 were nearly completely accessible to PK under the same conditions (Fig. 3). The chimeric proteins without a β-sheet domain, whether they did (apoB12–17) or did not (apoB49–54) have PTS, were not any more accessible to PK than ALB/ALB1–40, indicating that they had no cytosolic exposure related to their apoB sequences (Fig. 3). These results indicate that the presence of an apoB β-sheet domain is necessary for the atypical interaction with the translocon that leads to uncoupling of translocation from translation, followed by opening of the cytosol-ribosome junction and exposure of the translocating protein to the cytosol (22). Furthermore, these findings indicate that two separate regions of the first β-sheet domain of apoB100 have the capacity to cause an atypical interaction with the translocon.

By contrast to previous reports with apoB100 and apoB constructs containing β-sheet domains (27, 37), treatment of cells with OA did not alter the PK sensitivity of any of the chimeric constructs. This is consistent with the lack of any effect (or at most, a modest effect) of OA on the rates of secretion of these constructs (Fig. 2D).

Cytosolic exposure of apoB results in its association with chaperone proteins, especially Hsp 70 and Hsp 90 (43, 44).
Although this association may initially afford some protection for apoB from aggregation in the cytosol, both of these chaperones can in fact also facilitate the ubiquitinylation and subsequent proteosomal degradation of apoB (45). Therefore, we determined whether PTS or β-sheet domains affect the ubiquitinylation of the chimeric proteins. The results from this experiment were in complete accord with those observed for PK sensitivity; chimeric proteins containing either of the two β-sheet domains were significantly ubiquitinylated (Fig. 4). In the absence of a β-sheet domain, there was almost no ubiquitinylation whether or not PTS were present. The congruence of the results from the PK sensitivity and ubiquitinylation experiments provide strong support for the view that β-sheet domains in apoB100 are critical for an atypical interaction of this secretory protein with the translocon, an interaction that leads to cytosolic exposure and predisposition to proteosomal degradation.

Proteins destined for translocation across the ER membrane interact with proteins in the cytosol, translocon, and ER lumen that facilitate and regulate the process (9). The Sec family of proteins, which make up the translocon, plays a crucial role in translocation (29, 42, 46), and we (31, 32) and others (33) have demonstrated that apoB has a prolonged interaction with Sec family proteins. To determine whether PTS or β-sheet domains affect the association of apoB100 with the Sec complex, we performed two-step immunoprecipitation experiments with the chimeric proteins and Sec61α. Our finding that only the chimeras containing a β-sheet domain were associated with Sec61α to a greater extent than ALB/ALB1–40 (Fig. 5) were completely consistent with the results of the PK sensitivity and ubiquitinylation experiments. Indeed, all three of these approaches to determine the efficiency with which apoB is translocated and how it interacts with the translocon provided nearly identical results.

In summary, we have used a strategy whereby PTS or β-sheet domains of apoB were inserted into a typical secretory protein (albumin) in an attempt to gain insight into the “controversy” regarding the relative roles of those two sequences/domains in the interaction between apoB100 and the ER translocon. Our results provide support for each of these components of apoB having a role. Based on this study and in contrast with our previous observations (27), PTS appear to play a role in regulating the secretion of apoB100 and possibly the responsiveness of apoB100 secretion to OA. In this regard, our results support the proposed model of Lingappa and co-workers (19–21, 26). On the other hand, our results not only support a crucial role for β-sheet domains in regulating the secretion and OA sensitivity of apoB100 (albeit the latter possibly only in the presence of PTS), but provide consistent and convincing evidence that it is the presence of a β-sheet domain that results in an atypical interaction with the translocon. This interaction, defined by an increased association of apoB100 with Sec family proteins, predisposes apoB100 to cytosolic exposure, ubiquitinylation, and proteosomal degradation. Notably, based on our results with chimeric proteins, PTS do not play a role in the atypical interaction of apoB100 with the translocon, and they are not needed for the β-sheet domain to lead to cytosolic exposure and ubiquitinylation. Within the limitations of our study design, our results showing the importance of β-sheet domains for the interaction of apoB with the translocon are, we believe, stronger than our results regarding secretion of the various constructs and their sensitivity to OA. The mechanism whereby PTS may affect the secretion and OA sensitivity of apoB100 remains to be determined.

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