Systematic expression of the complete coding sequence of apoB-100 does not reveal transmembrane determinants

James M. Leiper,[1] Georgina B. Harrison, Jayne D. Bayliss, James Scott, and Richard J. Pease[2]

Medical Research Council Molecular Medicine Group, Royal Postgraduate Medical School, Du Cane Road, London W12 0NN, United Kingdom

Abstract We have investigated the hypothesis that apolipoprotein B undergoes a regulated process of translocation into the endoplasmic reticulum (ER) which causes the protein to adopt a transmembrane configuration. Protein segments representing the complete coding sequence of apolipoprotein B were first expressed by in vitro translation of transcripts from seven overlapping transcripts. Two regions were identified (located at residue 2425 and between residues 4149 and 4348) that can undergo incomplete translocation into pancreatic microsomes. Ribosome pausing at these sites uncoupled translation from translocation, leading to the synthesis of large cytoplasmically orientated segments of protein. In contrast, when these two regions were expressed by transfection in cultured cells, transmembrane structures were not detected. Endogenous apolipoprotein B-100 synthesis in HepG2 cells generates a spectrum of nascent chains, indicating that ribosome pausing can also occur in intact cells. However, the cellular pause products were cotranslationally translocated. While endogenous apolipoprotein B-100 in HepG2 cells was fully translocated, discrete proteolytic fragments were generated from the amino terminus of the protein when proteases gained access to the lumen of permeabilized microsomes. These products were similar in size and sequence to apolipoprotein B proteolytic fragments previously ascribed as the luminal domains of transmembrane apoB-100 molecules (Du, E. Z., Kurth, J., Wang, S. L., Humiston, P., and Davis, R. A. 1994. *J. Biol. Chem.* **269:** 24169–24176).—**Leiper, J. M., G. B. Harrison, J. D. Bayliss, J. Scott, and R. J. Pease.** Systematic expression of the complete coding sequence of apoB-100 does not reveal transmembrane determinants. *J. Lipid Res.* 1996. **37:** 2215–2231.

Supplementary key words lipoprotein assembly • cotranslational translocation • ribosome pausing

The primary sequence of apolipoprotein (apo) B-100 (~550 kDa) contains multiple predicted long amphipathic β-sheets and α-helices interspersed with hydrophobic regions (1–3). These may enable apoB to form a 'belt' (4) that stabilizes the surface of lipoprotein particles. Nascent apoB-100 in the ER undergoes two distinct intracellular fates. It is either cotranslationally assembled into very low density lipoprotein particles (5, 6) or, if it fails to assemble, it is retained on the ER membrane until it is degraded (7–9). Studies on cultured hepatoma cells suggest that the commitment of apoB to secretion or degradation is determined to a great extent by the supply of exogenous fatty acids (10–12). Instead of assuming that all apoB molecules are fully translocated into the lumen of the ER, as occurs with other secretory proteins, it has been widely proposed that apoB undergoes incomplete and regulated translocation (7, 12, 13). In this view, while luminal lipidation is occurring, apoB can undergo full translocation into the ER. However, if lipid supply is restricted, apoB translocation is interrupted and the protein becomes fixed in a transmembrane configuration (13). ApoB translocation has also been reported to be interrupted in heterologous cells which do not express microsomal triglyceride transfer protein, an activity that is required to transfer a neutral lipid core to nascent apoB in the ER lumen (14, 15). Incomplete translocation has also been suggested in the case of apoA-I (16).

A commonly accepted criterion that a protein is transmembrane is that proteolysis of isolated microsomes causes selective cleavage of cytoplasmic domains but

Abbreviations: apo, apolipoprotein; ER, endoplasmic reticulum; LDL, low density lipoprotein; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SLO, streptolysin O; PCR, polymerase chain reaction.

[1]Present address: MRC Laboratory for Molecular Cell Biology and Department of Biology, University College London, Gower Street, London WC1E 6BT, U.K.

[2]Present address: Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K.

TABLE 1. cDNA constructs for apoB expression

Fragment			Cloning Site	Amino Acids	M	Asn-X-(Ser/Thr)
					kDa	
ApoB.A	f	GAC ATG ACT **CCG CGG** CAC GTG	SacII	1196–2152	105	1341, 1350, and 1496
	r	AT **ACT AGT** CTA GAT GTA TCA TAT A	SpeI			
ApoB.B	f	ATC GCT **CCG CGG** GCT TCA	Sac II	1847–2485	70	none
	r	GTA TCT **TCT AGA** GTC TCA CGG AA	Xba I			
ApoB.C	f	AGT ACT **CCG CGG** TGG ATT CAA A	Sac II	2219–2710	54	none
	r	CTG **GGA TCC** TGG TTA GTG AAG	Bam HI			
ApoB.D	f	GAA CTT **CCG CGG** TAC CTG TCT	Sac II	2507–3286	85	2752, 2955, 3074, and 3197
	r	G AGA **AAG CTT** GAG ATT TCA AGG	Hind III			
ApoB.E	f	GAC GAG CTC **CCG CGG** ACC TTT CAA	Sac II	3216–3689	52	3309, 3331, and 3384
	r	TAC AGG GAT **CTA GAA** TTA ATA GCC	Xba I			
ApoB.F	If	GAA GGA CAC **CCG CGG** TTC CTC AA	Sac II	3642–4536	98	3868, 4210, and 4404
	Ir	TCT AG*G TCG ACG* GAT CCC CTG C	Sal			
ApoB.F1	If	GAA GGA CAC **CCG CGG** TTC CTC AA	Sac II	3642–4149	55	3608
	IIr	AGT TCC CTG **TCG ACT** GGC TTA GCC	Sal I			
ApoB.F2	IIf	CTG TTG **CCG CGG** GAA GGC CAA GCC	Sac II	4048–4536	53	4210 and 4404
	Ir	TCT AG*G TCG ACG* GAT CCC CTG C	Sal			
ApoB.F3	IIf	CTG TTG **CCG CGG** GAA GGC CAA GCC	Sac II	4048–4347	33	4210
	r	TTG **GTC GAC** CTA CTG TAA CTC TTG	Sal I			

ApoB cDNA clones (3) were PCR-amplified using mismatched oligos, forward oligos (f) introduced an SacII site and reverse oligos (r) introduced a termination codon (underlined) and a suitable cloning site. In the case of proteins F, two separate forward oligos are used in combination with two separate reverse oligos. Oligo Ir is located in the polylinker region of a cDNA source clone, downstream of the apoB termination codon, and products were subcloned with the polylinker *Sal I* site. The residues of apoB encoded by the cDNA products are shown (amino acids) with the predicted molecular masses (M) exclusive of glycosylation and the residue numbers of asparagine residues in potential N-linked glycosylation sites (Asn-X-Ser/Thr).

allows recovery of protected luminal domains (17). Initial studies using proteolysis of microsomes isolated from hepatocytes or hepatoma cells resulted in the complete degradation of the majority of apoB-100 molecules (7), although it was also shown that this pool of apoB is at least partially glycosylated (7). More recently, Du et al. (18) have detected 69, 55, and 30 kDa apoB fragments after proteolysis of HepG2 cell microsomes. They have suggested that the amino terminus of apoB becomes stranded across the translocation pore with its cytoplasmic protein tail anchored against the outer face of the ER.

We have screened for the presence of topogenic sequences throughout the length of apoB by expressing of a series of overlapping cDNA fragments. If there are biologically functional sequences gating apoB translocation it is a reasonable prediction that they would be functional both by in vitro translation and after expression in heterologous cells (two conditions under which lumenal lipidation will be minimal or absent). Additionally, as generation of the proteolytic fragments was variable between previous studies, we investigated the conditions under which similar apoB fragments arise from proteolysis of microsomes. The results presented below are consistent with a standard cotranslational mechanism of apoB-100 translocation into the ER. However, fully translocated apoB-100 adopts a conformation in the ER that can be partially proteolyzed to discrete fragments when microsomes are deliberately permeabilized with saponin.

MATERIALS AND METHODS

Production of cDNA constructs

Overlapping apoB cDNA fragments were generated by PCR amplification with the oligonucleotides listed in **Table 1.** In each case three independent clones were processed simultaneously. To enable transcription and translation, the products were subcloned into vector pUBSS (19) at an SacII site which enables expression of the proteins fused to an apoB signal sequence. For expression in cultured cells, the products were fused to apoB-15 by subcloning into vector pB15.HS (19).

ApoA-I cDNA (20) (a gift from Dr. Carol Shoulders) was amplified with a mismatched 5′ oligonucleotide AT.CGA.TAA.GCT.TCA.CCG.CGG.TCC.ATG.GAC.GAG.CCA to introduce a 5′ SacII site (underlined) and either of two 3′ oligonucleotides G.GGG.GCG.GTC.GAC.TCA.CTG.GGT.GGT.GAG or GG.GCG.GTC.GAC.TCA.CTG.GGT.**GTA**.GGT.GAG.CTT.CTT.AGT.CTC.CTC.GAG. Both of the 3′ oligonucleotides introduce a Sal 1 site (underlined) and the second also creates

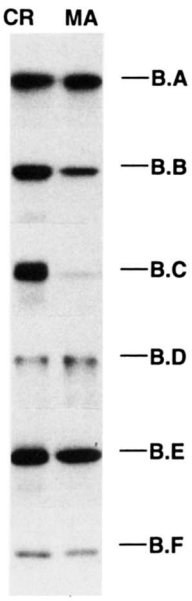


Fig. 1. Membrane association of internal apoB polypeptides. Transcripts encoding apoB.A to apoB.F (see Table 1 for residues) were translated for 60 min in a rabbit reticulocyte lysate containing canine pancreatic microsomal membranes and an inhibitor of glycosylation (N-acetyl-Asn-Tyr-Thr-carboximide (50)). After translation, microsomes were collected by ultracentrifugation and the membrane pellet was extracted with alkaline carbonate. The distribution of [^{35}S]methionine-labeled full-length proteins between carbonate-released (CR) and membrane-associated (MA) fractions is shown.

a carboxyl-terminal glycosylation site by introducing an exogenous Tyr codon (bold). The SacII and Sal I cleaved products were ligated into vector pUBSS (described in ref. 19) to allow expression by in vitro translation. For eukaryotic expression, the constructs in pUBSS were cleaved with EcoRI and SalI to release a fragment containing the 5′ untranslated region from mouse encephalomyocarditis virus fused to the apoB signal sequence followed by apoA-I cDNA and this was transferred to vector pSV7d (21).

In vitro transcription and translation

Transcripts encoding *i)* a type II transmembrane protein from mouse mammary tumor virus (22), *ii)* the EI glycoprotein of coronavirus (23), *iii)* apoB-9 lacking a termination codon (19), and *iv)* yeast α mating factor (19) have been previously described. RNA transcripts of constructs apoB.A–B.F were produced by transcription with T7 RNA polymerase (Promega) after linearization at the 3′ sites listed in Table 1.

In vitro translation in reticulocyte lysates (Promega), translocation into canine pancreatic microsomes (Promega), and protease protection assays were as previously described (19). Where indicated, microsomes from in vitro translation reactions were either *i)* extracted with carbonate or *ii)* subjected to sucrose density gradient centrifugation. *i)* To a 30-µl translation reaction, 1 µg rat liver carrier microsomes was added and the mixture was centrifuged at 30,000 rpm in the TLA100 rotor of a Beckman benchtop ultracentrifuge. The pellet was then resuspended in 100 µl of 100 mM sodium carbonate, pH 11.5, incubated for 1 h at 4°C, and then recentrifuged. Aliquots of the supernatant and pellet were analyzed by SDS-PAGE. *ii)* Translation reactions (30 µl) were layered onto discontinuous sucrose density gradients made up of eight equal steps ranging from 1.4 to 0.25 M sucrose and were centrifuged at 30,000 rpm for 1 h at 4°C in a SW50Ti rotor (Beckman) with adaptors. Gradients were unloaded from the top into twelve 50-µl fractions.

In vitro translated apoA-I comigrated with an abundant lysate protein. To resolve apoA-I it was necessary to either *i)* collect microsomal membranes by ultracentrifugation or *ii)* when apoA-I was translated in the absence of membranes, to immunoprecipitate the protein. Translation reactions (30 µl) were dialyzed for 2 h against 2 liters of complete PBS. The dialysate was mixed with 1 ml immunoprecipitation buffer (19) and immunoprecipitated with 20 µl of sheep anti-human apoA-I polyclonal antiserum (Boehringer) followed by protein A Sepharose (Sigma).

Cell culture and immunoprecipitation

HepG2 cells or Cos I cells were maintained in RPMI or DMEM (Life Technologies), respectively. Cells were used at 70% confluency. In some experiments, cells were transfected with expression plasmids encoding apoB-36 or apoB-17 (11), apoB-15.cyclin (19) apoB.A–B.F, or apoA-I. Transfection, metabolic labeling of cells, and preparation of membranes (0.5–1.8 M sucrose density gradient interface) were as previously described (19). For some experiments cells were preincubated for 1 h with 10 µg/ml of tunicamycin (Boehringer). Commercially produced rabbit polyclonal antiserum raised against apoB (Boehringer), apoA-I (Boehringer), and alpha 1 antitrypsin (Dako) were used for immunoprecipitation. In some cases membranes were extracted with alkaline carbonate. Membranes (0.3 ml) were diluted with >20 vol. ice-cold 100 mM carbonate (pH 11.5) for 1 h with occasional mixing. The carbonate-treated membranes were ultracentrifuged and the pellets were redissolved in immunoprecipitation buffer. The supernatants were concentrated to 0.5 ml and dialyzed against 2 × 2 ml of PBS in a Centricon 30 concentrator (Amicon) before reconcentration to 0.5 ml and addition of 1 ml immunoprecipitation buffer.

Streptolysin O permeabilization of cells

HepG2 cells were permeabilized essentially as described (24). Approximately 3×10^6 cells were resuspended in SLO buffer (125 mM potassium acetate, 2.5

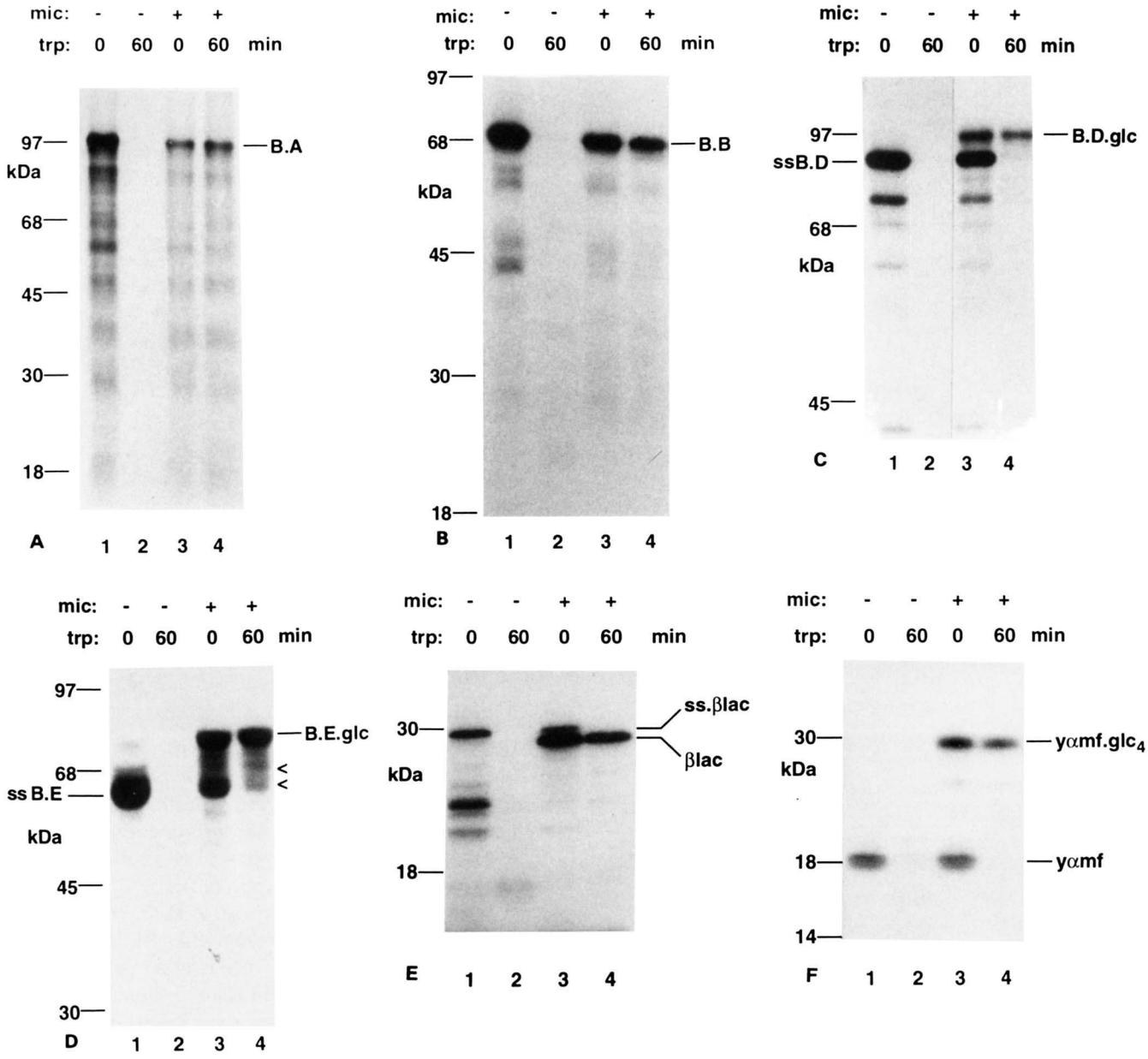


Fig. 2. Membrane topology of internal apoB polypeptides. A, B, C, and D: Transcripts encoding apoB.A, B.B, B.D, and B.E were translated for 60 min in the presence (lanes 3, 4) or absence (lanes 1, 2) of microsomes (mic). Aliquots of translation reactions were digested for either 0 min (lanes 1, 3) or 60 min (lanes 2, 4) with trypsin (trp) and products were resolved by SDS-PAGE on 8% gels and visualized for [^{35}S]methionine by fluorography. Unprocessed proteins with signal sequences (ssB.A, ssB.B, ssB.D, and ssB.E) were fully susceptible to proteolysis. In the presence of membranes, proteins B.D and B.E migrated more slowly owing to glycosylation (glc) and the respective full-length products were each protected from proteolysis. Two full-length partially glycosylated forms of apoB.E are also apparent (open arrows) and these were also protected. Protein apoB.B does not contain glycosylation sites but processed B.B is fully protected from proteolysis. While B.A does contain glycosylation sites, the expected change in mobility of B.A was not apparent although, more importantly, full-length B.A was protected from proteolysis. E and F: Transcripts encoding β-lactamase (βlac) and yeast α mating factor (yαmf) were translated in the presence (lanes 3, 4) or absence (lanes 1, 2) of microsomes (mic) and processed as above. Products were resolved by SDS-PAGE on 12% gels. Both mature proteins showed loss with trypsin when translated in the presence of microsomes. In both cases there is evidence of unprocessed precursors even in the presence of microsomes which are fully trypsin sensitive.

mM magnesium acetate, 1 mM dithiothreitol, 25 mM HEPES, pH 7) plus SLO (1.5 IU/ml Murex Ltd.) and incubated for 20 min at 0°C to allow prebinding. The cells were washed to remove excess SLO and then were collected by centrifugation at 13000 rpm. After resuspension, cells were warmed to 30°C for 5 min to pro-

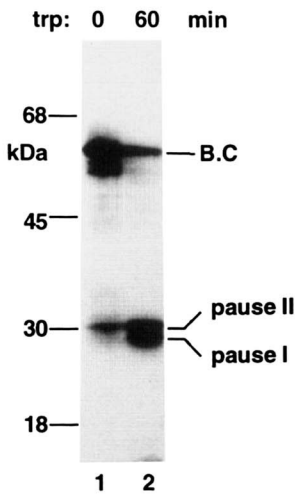


Fig. 3. Protein apoB.C becomes transmembrane at ribosome pause sites. After translation for 60 min in the presence of microsomes, protein apoB.C was trypsinized for 0 (lane 1) or 60 (lane 2) min and products were resolved on 10% SDS-PAGE gels. Only a small proportion of full-length apoB.C chains are fully protease-resistant. The majority of chains are cleaved to products that comigrate with two ribosome pause products (pause I and II) that are present in the undigested sample. Pause product I, although faint, was observed consistently in translations of protein B.C.

mote permeabilization. Trypsin was added to a final ratio of 1:50 per mass of cell protein and digestion was carried out at 0°C or 25°C.

RESULTS

Expression of overlapping apoB fragments by in vitro translation

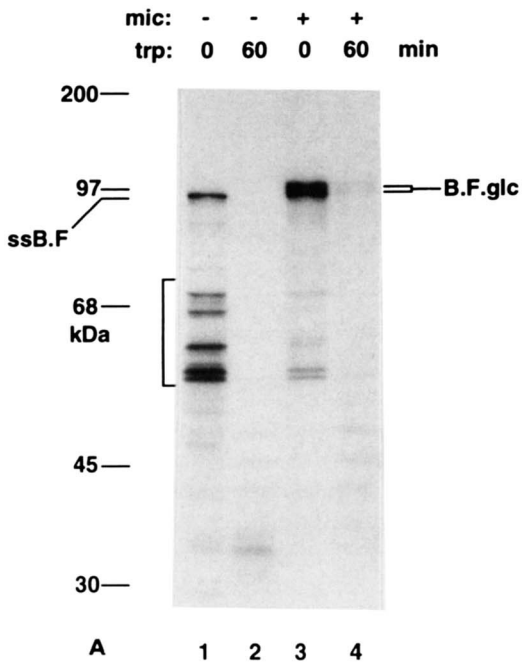
To identify possible sequences that regulate the translocation of apoB into the ER, a series of internal cDNA fragments (Table 1) was expressed by in vitro translation.

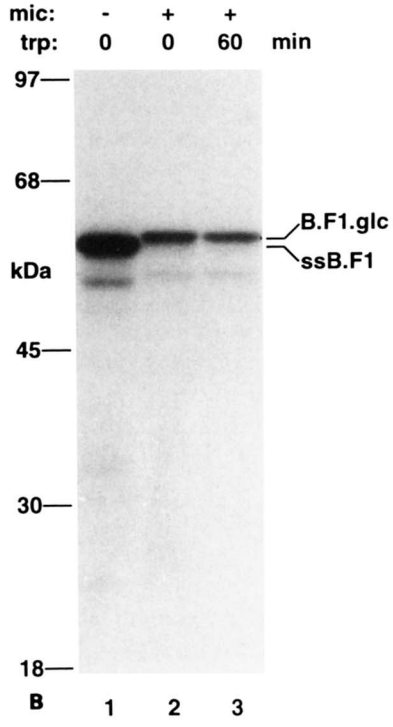
We examined the ability of each of the proteins to cotranslationally bind to canine pancreatic microsomal membranes by coupled in vitro translation/translocation. After alkaline carbonate extraction, the distribution of the translation products between the membrane and soluble fractions was determined (**Fig. 1**). Intermediate carbonate releasibility (similar to that previously observed with apoB-15 (25)) was observed for proteins B.A, B.B, B.D, B.E, and B.F, but not for protein B.C which was completely released. We interpret partial carbonate releasibility as indicating some affinity of the expressed proteins for the ER membrane consistent with the predicted distribution of lipid binding motifs throughout apoB-100 (1–3). A probable explanation for the complete carbonate releasibility of protein B.C becomes apparent in later experiments (Fig. 3 below). Carbonate extraction conditions were verified with a control luminal protein (yeast α mating factor) and a transmembrane protein (E1 glycoprotein of coronavirus) (data not shown).

To determine membrane topology, each of the proteins was translated in the presence of microsomes and digested with trypsin. In the case of proteins B.A, B.B, B.D, and B.E there was no evidence that they adopted a transmembrane configuration as the full-length products were not cleaved to smaller fragments (**Fig. 2**). Small losses of full-length proteins were apparent in these experiments, but this is probably due to overdigestion as similar losses were also observed with the control secretory proteins (Fig. 2 E and F). In the case of proteins B.D and B.E it appears that even in the presence of microsomes some material is completely untranslocated. Evidence that this is indeed untranslocated is that *i)* it comigrates with products synthesized in the absence of microsomes; *ii)* it is protease sensitive; and *iii)* it remained in the soluble fraction rather than cosedimenting with the microsomal membranes when reactions were subjected to ultracentrifugation (not shown). Again, some untranslocated material was also apparent with the control proteins (Fig. 2 E and F).

Proteins B.C and B.F each adopted incompletely translocated conformations by in vitro translation. Protein B.C became transmembrane at one or two sites (I and II) each within 30 kDa of the amino terminus of the protein (**Fig. 3**). The resulting proteolytic fragments comigrated with incomplete translation products present in the undigested sample, which result from ribosome pausing and quitting. Using oligonucleotide-directed RNAse H cleavage of transcripts, these sites were mapped close to residue 2425 (not shown). Interruption of translocation at these sites, stranding protein B.C in the translocation pore, could explain why this protein is readily extracted by carbonate (Fig. 1). We assume that transmembrane topology at sites I and II in protein B.C results from artefactual uncoupling of translation and translocation as a result of ribosome pausing, and that this topology is stabilized by folding of a long cytoplasmic domain (285 residues). This could explain why protein B.B is fully translocated despite containing the sequence at which B.C becomes transmembrane (Fig. 2B). In protein B.B the cytoplasmic domain would only be 60 residues long and possibly insufficient to anchor a transmembrane structure.

Protein B.F adopts a complex mixture of topologies (**Fig. 4**). A large proportion is glycosylated (B.Fglc in Fig. 4A) at one, two, or all of the three consensus sites demonstrating that translocation had been initiated. However, as confirmed in nine independent experiments, each of the resultant peptides was sensitive to proteolysis, and yet discrete protected fragments were





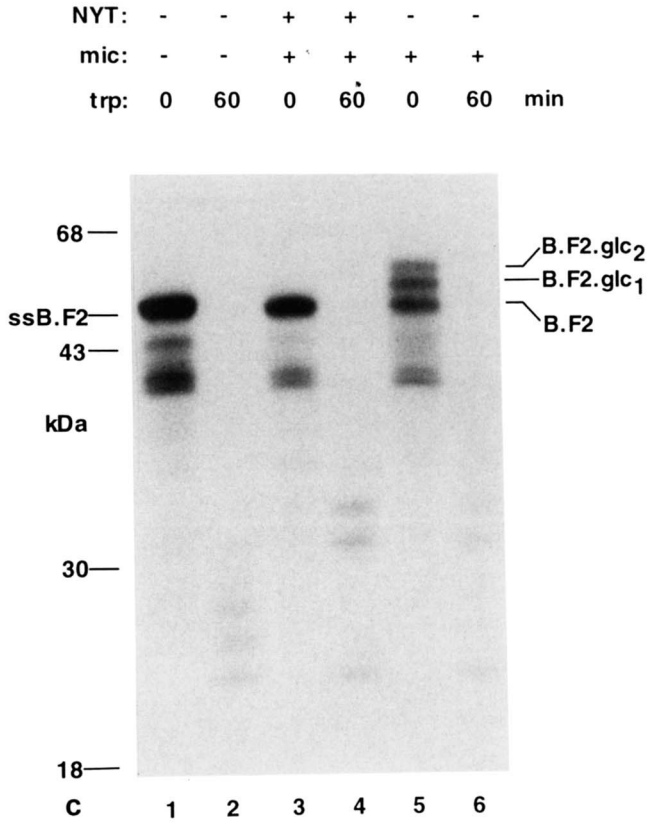


Fig. 4. Proteins containing apoB residues 4048–4348 adopt an anomalous topology when expressed by in vitro translation. A: Protein apoB.F was translated in the presence (lanes 3, 4) or absence (lanes 1, 2) of microsomes (mic) and trypsinized (trp) for either 0 or 60 min. Products were resolved by SDS-PAGE on 8% gels. When translated in the absence of microsomes, unprocessed apoB.F (ssB.F) was wholly susceptible to proteolysis. In the presence of microsomes (lanes 3, 4) glycosylated products (B.F.glc) were apparent but processed B.F.glc was susceptible to proteolysis suggesting that it had not completed translocation into the microsomal lumen. When translated in the absence of microsomes, a series of pause products (bracketed) of apoB.F predominates over full-length apoB.F. From their size it is calculated that these pause products span residues 4149–4318. B: When translated in the absence of microsomes (lane 1), apoB.F1 is unprocessed (ssB.F1) and migrates more quickly than glycosylated apoB.F1 (B.F1.glc) produced in the presence of microsomes (lanes 2, 3). Processed, glycosylated apoB.F1 was completely protected from proteolysis consistent with complete translocation into the microsomal lumen. C: When translated in the absence of microsomes (lanes 1, 2) unprocessed apoB.F2 (ssB.F2) was wholly susceptible to proteolysis. In the presence of microsomes (lanes 3–6), a series of signal cleaved (B.F2) and glycosylated ($B.F2glc_{1+2}$) products were produced but each was wholly sensitive to proteolysis indicating that translocation had not been completed. When translated in the presence of microsomes and an inhibitor of glycosylation (NYT), a single full-length, signal cleaved product was apparent (lane 3) and this was also fully sensitive to proteolysis (lane 4). Proteolysis of apoB.F2 produces traces of 20–35 kDa tryptic fragments; however, as these arise both in the presence and absence of microsomes (lane 2), they cannot be assigned as luminal protected domains of transmembrane protein.

not detected. To map the determinant in protein B.F that generates these complex topologies, we made a series of shorter proteins apoB.F1, F2, and F3 (Table 1). Protein B.F1 was shown not to contain the topogenic determinant as it was quantitatively glycosylated at Asn (3868) and was largely resistant to trypsin (Fig. 4B).

Protein F2 did contain the topogenic determinant and yielded three species: *i)* signal cleaved and unglycosylated, *ii)* signal cleaved and singly glycosylated, and *iii)* signal cleaved and doubly glycosylated. All three species were sensitive to exogenous trypsin (Fig. 4C). In conjunction with the data from B.F1 this localizes the topogenic determinant between residues 4149 and 4536. Additionally we found that protein B.F3 was also incompletely translocated further localizing the topogenic determinant between residues 4149 and 4348 (not shown).

Owing to the atypical protease sensitivity of proteins containing residues 4149–4318, protein B.F2 was translated in the presence of microsomes and then subjected to sucrose density gradient centrifugation (**Fig. 5**). To indicate the sedimentation positions of membrane-associated and soluble proteins, the B.F2 translation reaction was mixed with microsome processed and unprocessed translations of yeast α mating factor. The glycosylated forms of protein B.F2 remained vesicle-associated while the majority of signal cleaved unglycosylated protein was released and cosedimented with unglycosylated yeast α mating factor. The ability of unglycosylated B.F2 to be released from microsomes indicates that although translocation has been interrupted the protein has not become integrated into the bilayer.

How does the destabilizing region of protein B.F exert its effect? Protein B.F generates numerous closely packed ribosome pause products between residues ~55 kDa and ~75 kDa (brackets in Fig. 4A) and this spans the destabilizing region (residues 4149–4347) identified in proteins B.F2 and B.F3. In **Fig. 6** a possible mechanism is suggested in which multiple pause and restart events in this region produce glycosylated, transmembrane polypeptides that are not cleaved to predominant discrete fragments upon proteolysis. However, no background smear of fragments was apparent in Fig. 4A lane 4 which might confirm such a mechanism.

Expression of overlapping apoB fragments in cultured cells

We determined whether the sequences present in proteins B.C and B.F also undergo interrupted translocation in intact cells. When internal apoB cDNA fragments were fused directly to the apoB signal sequence, little or no expressed protein could be detected in the transfected cells. However, we observed that when the internal apoB sequences were fused to the N-terminal 15% of apoB stable protein was produced. Proteins B-15.C and B-15.F appeared fully translocated in transfected Cos cells as judged by proteolysis of isolated microsomes (**Fig. 7A and D**). Comparable protease protection was also seen for proteins B-15.B, B-15.C, and B-15.E (Fig. 7) and for apoB-15.A (data not shown). Minor variability in the degree of protection is apparent among experiments probably reflecting small differences in vesicle stability. To confirm this interpretation, in some experiments apoB fusion proteins were cotrans-

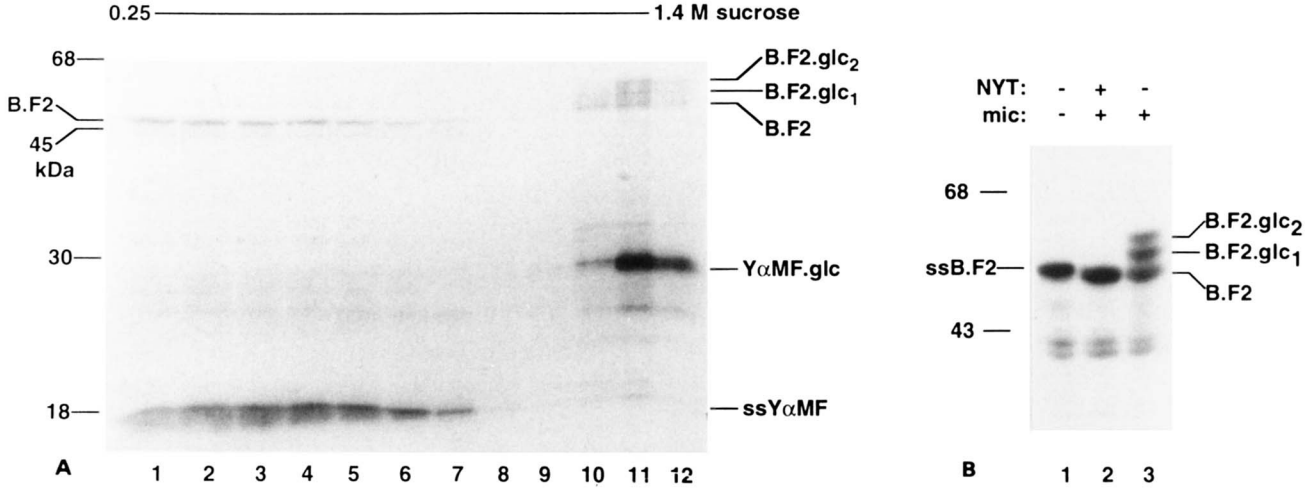


Fig. 5. Membrane association of apoB.F2. A: Transcripts encoding apoB.F2 were translated in the presence of microsomes for 60 min. Subsequently this reaction was mixed with both a translation reaction of yeast α mating factor processed by microsomes (YαMF.glc) and a translation reaction of unprocessed yeast α mating factor (ssYαMF). The mixture was fractionated by sucrose density centrifugation, as described in Materials and Methods, and the fractions were analyzed by SDS-PAGE 10% gels. Singly and doubly glycosylated apoB.F2 sedimented with the microsomal fraction (marked by processed YαMF.glc). Signal-cleaved but unglycosylated apoB.F2 was present both in the soluble and membrane fractions. B: To confirm that complete processing of apoB.F2 had occurred, aliquots of the B.F2 translation reactions were electrophoresed (lane 3) alongside unprocessed (ssB.F2, lane 1) and processed but unglycosylated (B.F2, lane 2) products on 12% SDS-PAGE gels. The three bands in the microsome processed sample (lane 3) are thus identified as singly and doubly glycosylated B.F2 and signal-cleaved, unglycosylated B.F2. Unprocessed B.F2 is not apparent in lane 3 hence the interpretation in A (above) is confirmed.

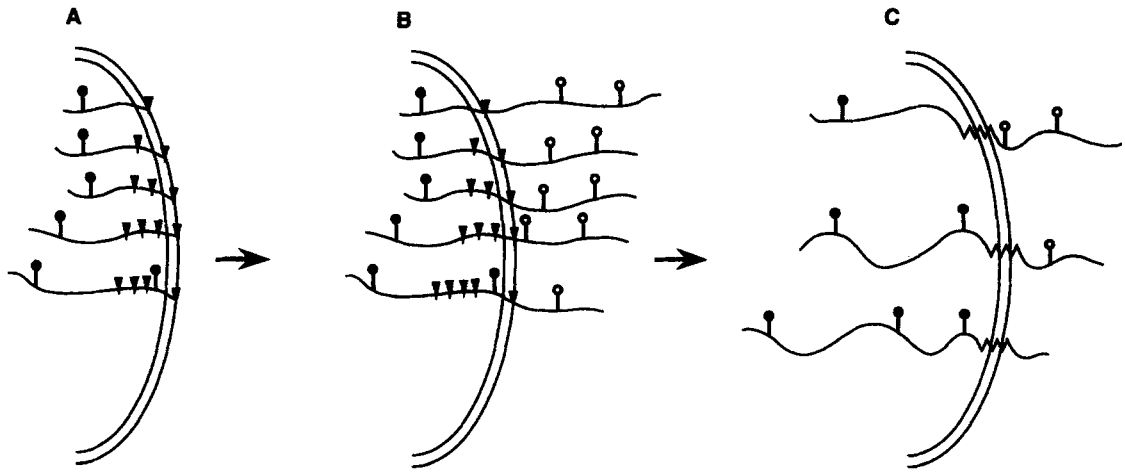


Fig. 6. A model to account for the anomalous topology of protein F. A: At the onset of translation translocation is cotranslational. When the ribosome reaches a series of closely packed pause sites (filled arrow heads) the majority of nascent peptide chains stall at one or other of these sites. Some potential glycosylation sites have been translocated into the lumen and been glycosylated (🍍) while others remain cytoplasmic (🍍). B: Protein synthesis resumes from these pause sites, but with translocation uncoupled from translation. Therefore, newly synthesized protein is located on the cytoplasmic side of the membranes. C: A limited amount of posttranslational translocation may occur but this process is inefficient, resulting in variability at the sites at which the chains become transmembrane (〰). Therefore, a large number of proteolytic products result from digestion, preventing visualization of predominant discrete products.

fected with apoB-15-cyclin. As shown in Fig. 7E, proteolytic losses of B-15.B and B-15.F were matched by parallel losses in B-15.cyclin and in apoB-17, i.e., the loss of B-15.F could not be attributed to specific determinants in the primary sequence of domain B.F. Similar results were obtained for protein B-15.C (not shown).

Identification of partial proteolytic products deriving from fully translocated apoB-36

The transfection experiments above did not identify any topogenic sequences that could account for reports of transmembrane apoB-48 or B-100 in intact cells. However, as shown below, fragments of apoB can arise from the digestion of fully translocated protein in partially disrupted microsomes. In Fig. 8 the topology of apoB-36 expressed in Cos cells was investigated by proteolytic digestion of microsomes. Approximately 60% of the full-length product was protected, consistent with ER translocation. The decrease in intensity of the apoB-36 band was associated with an increase in a 65 kDa band (labeled A in Fig. 8). When the membranes were disrupted with limiting amounts of saponin (0.02%) there was a further absolute increase in band A. Excision and scintillation counting of band A indicated that the proportion of counts that had been lost from apoB-36 but were recovered in band A was the same with or without saponin disruption (see calculation in legend to Fig. 8), i.e., that the origin of band A in the undisrupted sample might be digestion of a proportion of leaky vesicles. Fragment A could also be generated from apoB-15.cyclin after saponin permeabilization of the membrane, indicating that it derives from sequences in the amino terminal 75 kDa of apoB (Fig. 8B). In this and one other preparation of membranes containing apoB-15.cyclin (not shown), band A was not generated in the absence of saponin. This probably represents variability in the integrity of recovered membranes rather than an intrinsic difference between apoB-36 and apoB-15.cyclin as in other experiments proteolysis of undisrupted membranes containing apoB-36 did not invariably produce fragment A (19).

As shown in **Fig. 9A and B,** our data do not suggest that band A represents the luminal domain of transmembrane apoB molecules, but indicate that trypsin acting on leaky or deliberately permeabilized membranes cleaves this fragment from the N-terminus of fully translocated apoB-36 (Fig. 9C).

Translocation of endogenous apoB-100

When HepG2 cells were labeled with [^{35}S]methionine and then chased with unlabeled methionine, the predominant band was apoB-100, and proteolysis indicated that the majority of this protein was translocated (**Fig. 10**). In the absence of a chase period, a series of discrete biosynthetic intermediates of apoB were immunoprecipitated. These nascent chains were not cleaved to smaller products by proteolysis of microsomes (**Fig. 11A**) indicating that they are cotranslationally translocated into the ER. Cotranslational translocation does not apparently support a current model of discontinuous apoB translocation (26, 27) that arose from in vitro translation experiments where membrane spanning in-

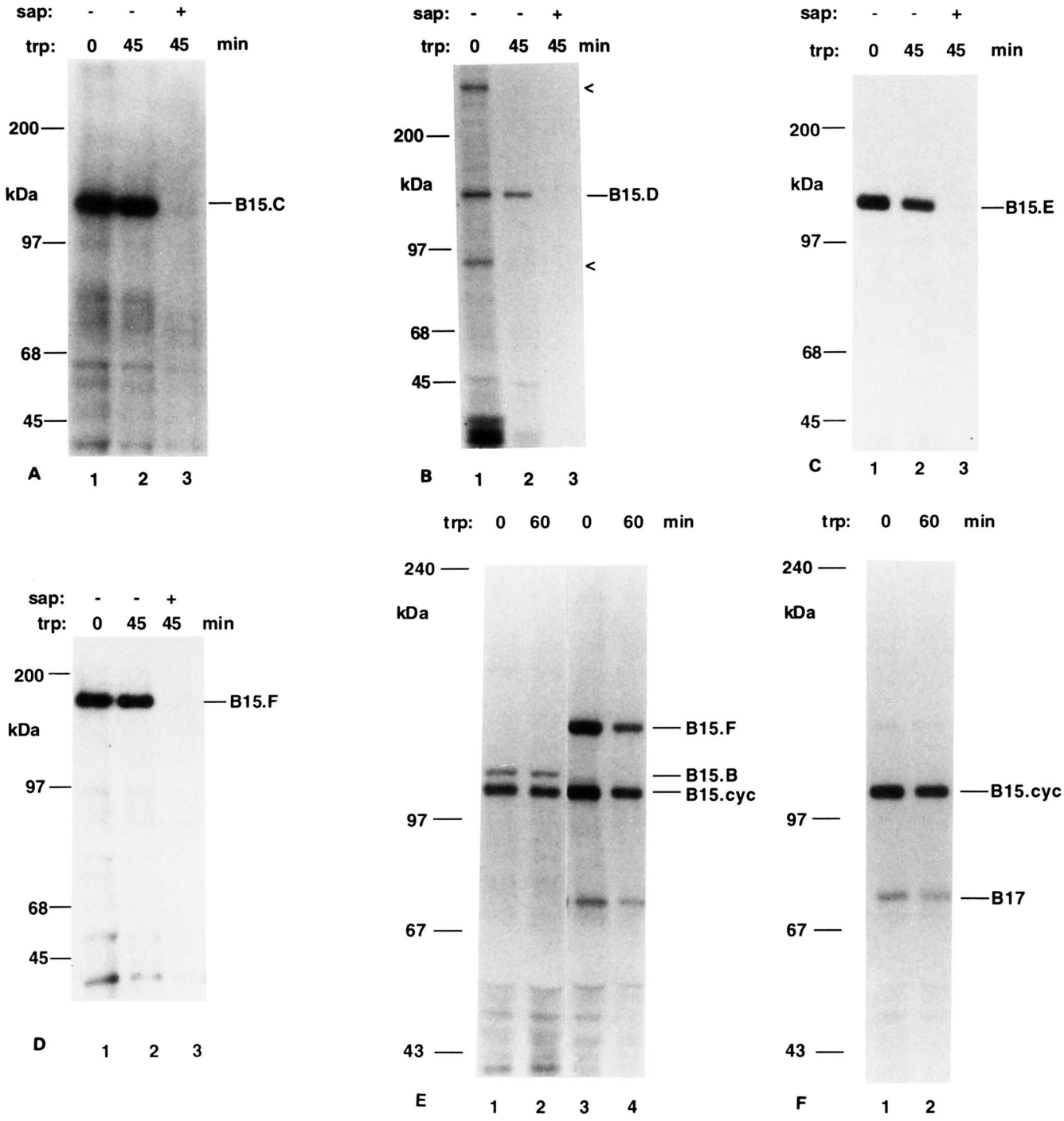


Fig. 7. Internal apoB fragments are fully translocated in Cos cells. In parts A, B, C, and D, Cos cells were transfected with plasmids encoding apoB.C, B.D, B.E, and B.F, respectively, and then labeled for 60 min with [^{35}S]methionine, after which a microsomal fraction was isolated as described. Aliquots were trypsinized (trp) for either 0 (lane 1) or 45 min (lanes 2, 3) in the presence (lane 3) or absence (lanes 1, 2) of 0.2% saponin (sap). After digestion, membranes were collected by centrifugation, immunoprecipitated for apoB, and analyzed by SDS-PAGE on 8% gels. In each case fusion proteins are completely susceptible to trypsin in the presence of saponin but predominantly resistant to digestion in its absence. This is consistent with complete translocation into the ER lumen. In some experiments, e.g., panel B, additional trypsin-susceptible bands are apparent (marked with arrowheads) in undigested samples. However, these were also apparent in some preparations of membranes from untransfected cells and therefore are not related to the transfected apoB proteins. E: Cos cells were co-transfected with a plasmid encoding apoB-15.cyclin and either apoB-15.B (lanes 1, 2) or apoB-15.F (lanes 3, 4). Transfected cells were labeled and fractionated as above. Microsomes were digested for either 0 (lanes 1, 3) or 60 min (lanes 2, 4) with trypsin (trp). F: Cos cells co-transfected with plasmids encoding apoB-15.cyclin and apoB-17 were labeled and fractionated as above. Microsomes were digested with trypsin (trp) for either 0 (lane 1) or 60 min (lane 2). While some losses of apoB-15.B and F are apparent upon proteolysis, these are accompanied by parallel losses of apoB-15.cyclin and apoB-17, indicating that vesicle instability rather than topogenic determinants in apoB segments is responsible for the losses.

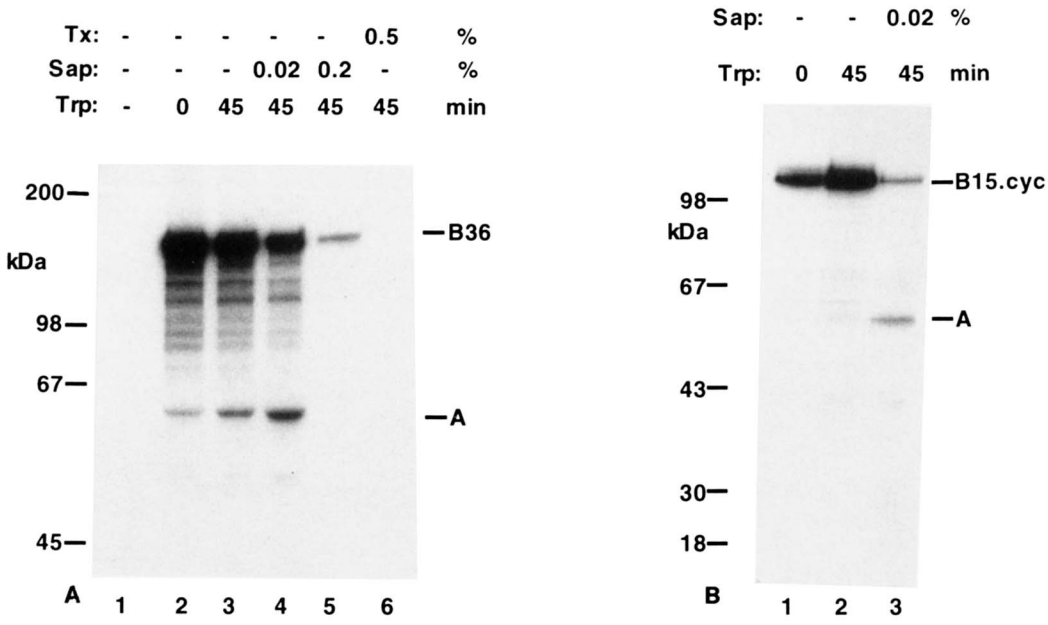


Fig. 8. Partial proteolysis of luminal apoB-36 yields a 65 kDa fragment. A: Cos cells were transfected with a plasmid encoding apoB-36, and then labeled with [^{35}S]methionine, homogenized, and fractionated. Aliquots of intact or saponin (sap) permeabilized membranes were trypsinized (trp) for either 0 (lane 2) or 45 min (lanes 3–6) and immunoprecipitated for apoB. Lane 1 shows an immunoprecipitation from microsomes of untransfected cells. Samples were resolved on 5–15% SDS-PAGE gels. Trypsinization (trp) in the absence of saponin (lane 3) resulted in a decrease in full length apoB-36 (B36) and an increase in a ~65 kDa band (labeled A). The activity in A was further increased when the vesicles were disrupted with 0.02% saponin. ApoB-36 is extensively proteolyzed in the presence of 0.2% saponin or 0.5% Triton X-100 (Tx). Band A was excised from the gel and the activity of ^{35}S was determined by scintillation counting. Proteolysis with 0.02% saponin caused a decrease in activity in apoB-36 from 11165 dpm (undigested) to 4247 dpm (digested) with 805 dpm being recovered in band A. In the absence of saponin [^{35}S]apoB-36 decreased to 6872 dpm with 530 dpm being recovered in band A. In each case 12% of the original activity in apoB-36 was recovered in band A which corresponds approximately to 0.35 mol of A per mol of cleaved apoB-36. B: Cos cells were transfected with a plasmid encoding an apoB-15.cyclin fusion protein and then were labeled for 45 min with [^{35}S]methionine, homogenized, and fractionated. Aliquots of membranes were digested with trypsin (trp) for 0 (lane 1) or 45 min (lanes 2, 3) in the presence (lane 3) or absence (lanes 1, 2) of 0.02% saponin and immunoprecipitated for apoB. Samples were resolved on 5–15% SDS-PAGE gels. Proteolysis of apoB-15.cyclin in the presence of saponin produces a proteolytic fragment of ~65 kDa (A) similar in migration to that produced from apoB-36 (above).

termediates with large cytoplasmic tails predominated over translocated products. However, the in vitro translated transmembrane products in this system could complete translocation after alteration of the ionic environment (19, 27). It therefore seemed possible that the nascent chains had indeed been stalled across the translocation pore in intact cells but were induced to undergo posttranslational translocation during membrane isolation. This possibility was excluded in Fig. 11B, where we show that apoB-9 translated in vitro from transcripts lacking termination codons, and therefore in a transmembrane configuration (19, 27), retains this conformation during the steps used to isolate membranes from cells. The conclusion that apoB nascent chains are cotranslationally translocated was also supported by proteolysis of HepG2 cells in which the plasma membrane had been deliberately permeabilized with SLO (24) to expose internal membranes but avoiding membrane damage during membrane isolation (Fig. 11C). In this experiment some loss of material is apparent, however, all bands appear to change equally in intensity. In contrast, if a discontinuous process of translocation was occurring, preferential cleavage of certain intermediates would be predicted.

Detection of proteolytic fragments from fully translocated apoB-100

The majority of endogenous apoB-100 appears to be fully translocated in the above experiments. Further, apoB-100 shows equivalent protection to the secretory protein, α-1 macroglobulin in the accompanying article (28). However, translocated apoB-100 can be cleaved to discrete fragments in permeabilized membranes. After digestion of endogenous apoB-100 in saponin-treated HepG2 cell microsomes, fragments of 60–70 kDa are apparent including a major fragment of 65 kDa (A′ in **Fig. 12A**). This is similar in size to fragment A previously observed with transfected apoB-36 (Fig. 8A) and apoB-15.cyclin (Fig. 8B) and also similar to an amino terminal fragment recently reported by Du et al. (18). Fragment A′ (Fig. 12 lanes 2, 3) migrates marginally faster than a band, B, present in the undigested sample (lane 1). To confirm that A′ and B are indeed different (i.e., that A′ is indeed a proteolytic product), we also examined par-

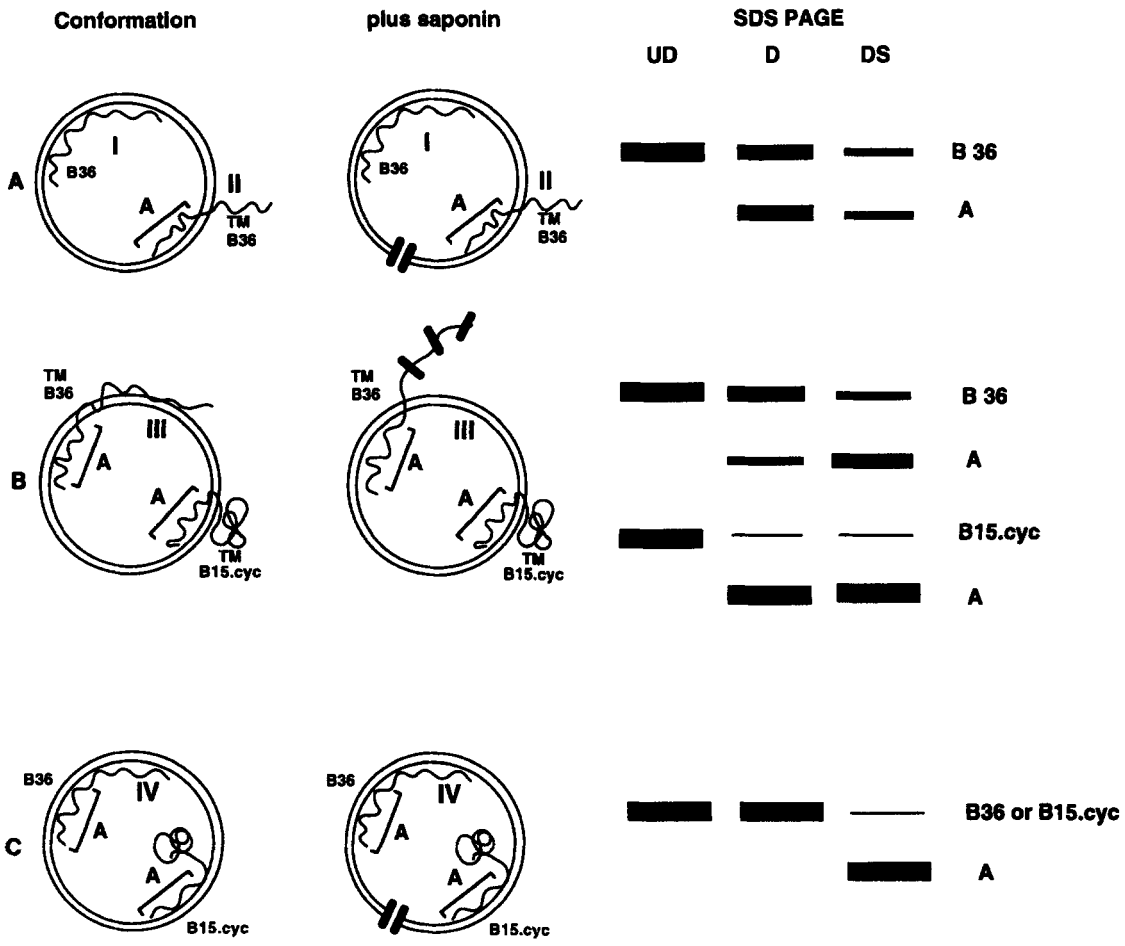


Fig. 9. Possible topologies of apoB-36 and apoB-15.cyclin in secretory membranes. Three possible arrangements of apoB-36 in the ER membrane are shown, with the predicted proteolytic fragments apparent by SDS-PAGE in undigested (UD), digested (D), and digested in the presence of saponin (DS) samples. A: ApoB-36 is present as a mixed population of fully translocated (conformation I) and transmembrane (II) molecules such that A is the protected luminal domain of transmembrane molecules. However, if A represented the luminal domain of transmembrane apoB-36, saponin disruption of the vesicles would cause a parallel loss in full length apoB-36 (conformation I) and fragment A and not the increase in A that is observed (Fig. 7A). B: ApoB-36 molecules are transmembrane at site A with the cytoplasmic protein tail closely anchored to the outer face of the ER (conformation III) thus restricting trypsin cleavage. Saponin might alter the configuration of the cytoplasmic tail making cleavage more probable. However, in this case B-15.cyclin must also be transmembrane at site A with cyclin exposed to the cytoplasm. As cyclin is not membrane-binding, B-15.cyclin should not depend on the presence of saponin to reveal a transmembrane configuration. C: We conclude that apoB-36 is fully luminal (conformation IV) and that segment A is relatively protected against digestion in broken membranes. This conformation is consistent with the saponin-dependent generation of fragment A from apoB-15.cyclin.

tially digested saponin-treated membranes where a double of A′ and B is apparent (Fig. 12B). To ensure that the apoB-100 in these experiments really was fully translocated, we used tunicamycin (29) to examine the glycosylation state of the membrane-associated and carbonate-soluble (luminal) pools of apoB in HepG2 cells (Fig. 12C). In this experiment it is apparent that both membrane-associated and luminal pools of apoB-100 are glycosylated to a similar extent, which is not consistent with a large proportion of membrane-associated apoB being in a conformation where only 65 kDa from the amino terminus is translocated. Therefore, fragments of apoB produced during proteolysis of microsomes isolated from HepG2 cells must be assumed to have originated from fully translocated apoB molecules.

Topology of apoA-I

It was previously reported that apoA-I shows quantatively similar cytoplasmic exposure to apoB in chick hepatocytes (16). We examined the topology of endogenous apoA-I in HepG2 cells. Although some losses in apoA-I are apparent after trypsin digestion (**Fig. 13B**), parallel losses were apparent with both the ER glycosylated and Golgi glycosylated forms of α1 antitrypsin (Fig. 13A), proteins which must be translocated. The

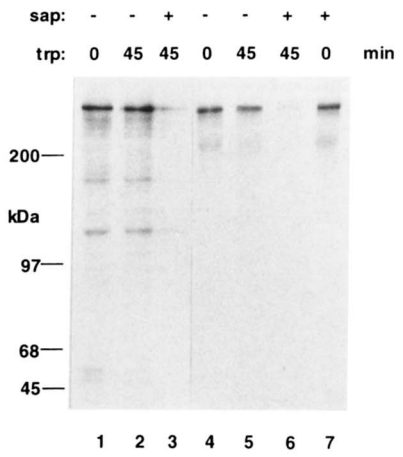


Fig. 10. Topology of apoB-100 in hepatoma cells. HepG2 cells were labeled with [^{35}S]methionine for 60 min and then chased with unlabeled methionine for 15 min (lanes 1, 2, 3) or 60 min (lanes 4, 5, 6, 7). Membranes were isolated and trypsinized for 0 min (lanes 1, 4), 45 min (lanes 2, 5) or for 45 min in the presence of 0.2% saponin (sap) (lanes 3, 6). In lane 7, membranes were treated with saponin but were undigested. Digests were immunoprecipitated for apoB and samples were resolved on 4–13% SDS-PAGE gels. In the absence of saponin, the majority of apoB-100 is resistant to proteolysis; however, when membranes are permeabilized with saponin, apoB-100 is completely digested.

native apoA-I sequence does not contain glycosylation sites. To confirm that apoA-I undergoes a normal process of translocation, we engineered an exogenous tyrosine residue to create a C-terminal glycosylation site (KKLNTQter-KKLNYTQter) (Fig. 13C). Proteolysis of in vitro translocated apoA-I either with or without a glycosylation site failed to detect transmembrane structures (Fig. 13D). Finally, apoA-I with or without the glycosylation site was expressed by transfection in Cos cells. The engineered glycosylation site was efficiently used, indicative of complete translocation in these cells (Fig. 13E).

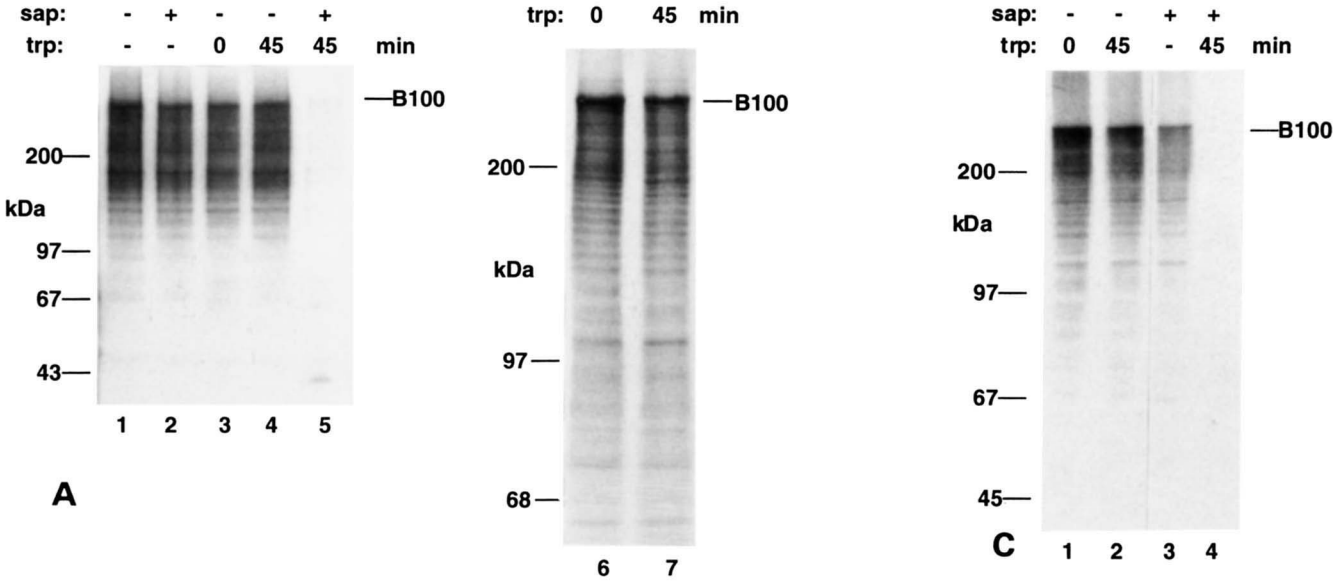
DISCUSSION

Various reports have suggested that apoB undergoes a novel process of translocation into the ER (30–36). In several cases, proteolysis of apoB in ER membranes has led to a complete loss of immunoreactive protein precluding the mapping of its precise membrane topology. However, other studies have proposed that specific transmembrane structures can be identified in apoB. First, proteolysis of microsomes isolated from cell lines expressing apoB-100 has generated amino terminal fragments that were suggested to be the luminal domains of partially translocated apoB-100 molecules (7–9). Second, coupled in vitro translation/translocation experiments have indicated that apoB translocation can stall leaving the protein threaded through the translocation pore (26, 27). It has been proposed that this stalling is controlled by specific membrane receptors that recognize pause transfer determinants in apoB (27). Du et al. (18) have advanced a composite model where pause transfer sequences in apoB, similar to those detected in in vitro translation experiments, uncouple translation from translocation in hepatic cells. Subsequent protein synthesis would generate cytoplasmic apoB, and this could bind to the outer face of microsomes anchoring apoB across the translocation pore.

To further evaluate this model, we have now expressed segments of apoB that, together with apoB-15 (expressed previously (19)), represent the entire apoB-100 coding region. Two regions in apoB.C and apoB.F (residues 2219–2710 and 3642–4536, respectively), undergo incomplete translocation in reticulocyte lysates, and hence they may contain pause-transfer sequences as previously defined in the studies of Chuck et al. (26) and Chuck and Lingappa (27). However, all regions of apoB, including C and F, appeared to be quantitatively translocated when expressed by transfection into Cos cells. We saw no evidence that C, F, or indeed any other determinants in apoB can act to generate stable transmembrane structures in intact cells similar to those proposed by Du et al. (18). This is in agreement with previous studies of apoB translocation by Shelness, Morris-Rogers, and Ingram (37). This does not, however, exclude the possibility that regions C and F might cause apoB to undergo a process of discontinuous translocation similar to that proposed for prion protein (38). In this case it has been proposed that transient 'U'-shaped structures form in the translocation pore which are then rapidly resolved to fully luminal products at the termination of translation (39).

A caveat, however, is that artefactual uncoupling of translation from translocation has been reported by other workers to occur during in vitro translation: notably prolactin in wheat germ lysates (40), and also chimeric proteins with segments of hemaglutinin in wheat germ and in reticulocyte lysates (41). Similarly, we have observed transmembrane structures generated during the translation of cyclin that may not have been directed by physiological signals, but may have resulted from ribosome pausing.

In view of these uncertainties over defining pause sequences and predicting their ultimate effect on protein topology, we have also examined the partial translation products of apoB-100 in HepG2 cells as these may identify any translocation intermediates that occur naturally. We found that the apoB nascent chains are protected from proteolysis. As the lifetime of each of these intermediates must be very short to enable apoB-100 translation to be completed in 15 min (42), we believe



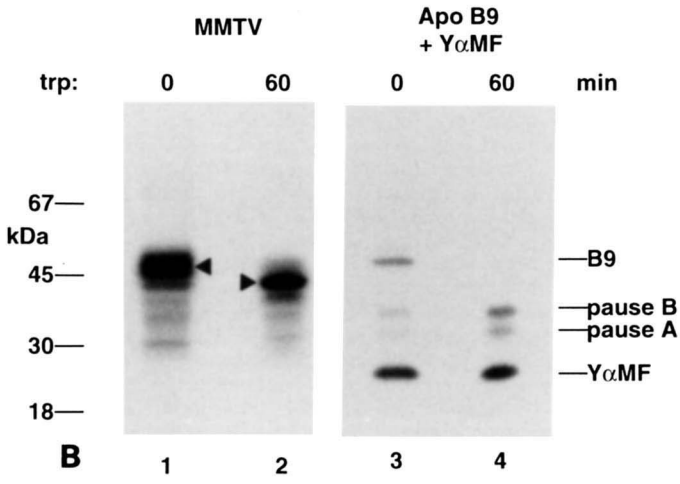


Fig. 11. ApoB nascent chains are cotranslationally translocated in HepG2 cells. A: HepG2 cells were labeled for 1 h (lanes 1–5) or 10 min (lanes 6, 7) with [^{35}S]methionine, homogenized, and fractionated. Aliquots were undigested (-) or trypsinized for 0 (lanes 3, 6) or 45 (lanes 4, 7) min. Lanes 2 and 5 show undigested and digested products after the inclusion of 0.2% saponin. Samples were immunoprecipitated for apoB and resolved by SDS-PAGE on 4–11% gels. Biosynthetic intermediates of synthesis (95–500 kDa) are protected from proteolysis in intact membranes (compare lanes 3 and 4) but not in permeabilized membranes (5). Similar protection from proteolysis is observed after 10 min of labeling (lanes 6 versus 7) where a greater proportion of apoB chains are present as intermediates of synthesis. B: ApoB-9 transcripts lacking termination codons were translated in the presence of canine pancreatic microsomes to generate transmembrane apoB-9. A type II transmembrane protein (MMTV) and a soluble control protein (YαMF) were also translated. In vitro translation reactions were mixed with unlabeled HepG2 cells then homogenized and fractionated. The membranes were trypsinized (trp) for 0 (lanes 1, 3) or 60 min (lanes 2, 4) at 25°C and the products were resolved by SDS-PAGE on 8–15% gels. MMTV protein was cleaved to products 2 kDa smaller (lanes 1, 2) and YαMF fully protected form proteolysis (lanes 3, 4). ApoB-9 was cleaved to products comigrating with pause products A and B present in the undigested sample and had thus maintained its transmembrane configuration during membrane preparation. C: HepG2 cells were labeled with [^{35}S]methionine and then treated with streptolysin O to selectively permeabilize the plasma membrane. After washing the cells were trypsinized (trp) for 0 (lane 1) or 45 min (lane 2) or they were treated with 0.2% saponin and trypsinized for 0 min (lane 3) or 45 min (lane 4). ApoB nascent chains are not cleaved by trypsin but become trypsin-accessible in the presence of saponin (lane 4). In this experiment some release of apoB peptides is seen with saponin alone (lane 3).

that apoB-100 translocation is effectively continuous. Certainly there is no evidence for predominant transmembrane intermediates with half lives approaching 1 h and with up to 50 kDa exposed on the cytoplasmic face as were apparent in the initial wheat germ translations of apoB-15 upon which the hypothesis of discontinuous translation was based (26). More recently, Hegde and Lingappa (43) suggested that the lifetime of paused transmembrane structures may be a function of translation conditions, and that they may resolve more quickly in reticulocyte lysates than in wheat germ lysates. Based upon this observation, these authors have predicted that the halftime of paused transmembrane structures in vivo may be as short as a few seconds. In this case transmembrane products would be at the limit of detection by proteolysis of the nascent chains whose lifetimes must also be seconds.

However, two issues remain to be addressed. First, if the transmembrane structures are so transient they would not lead to the establishment of structures as

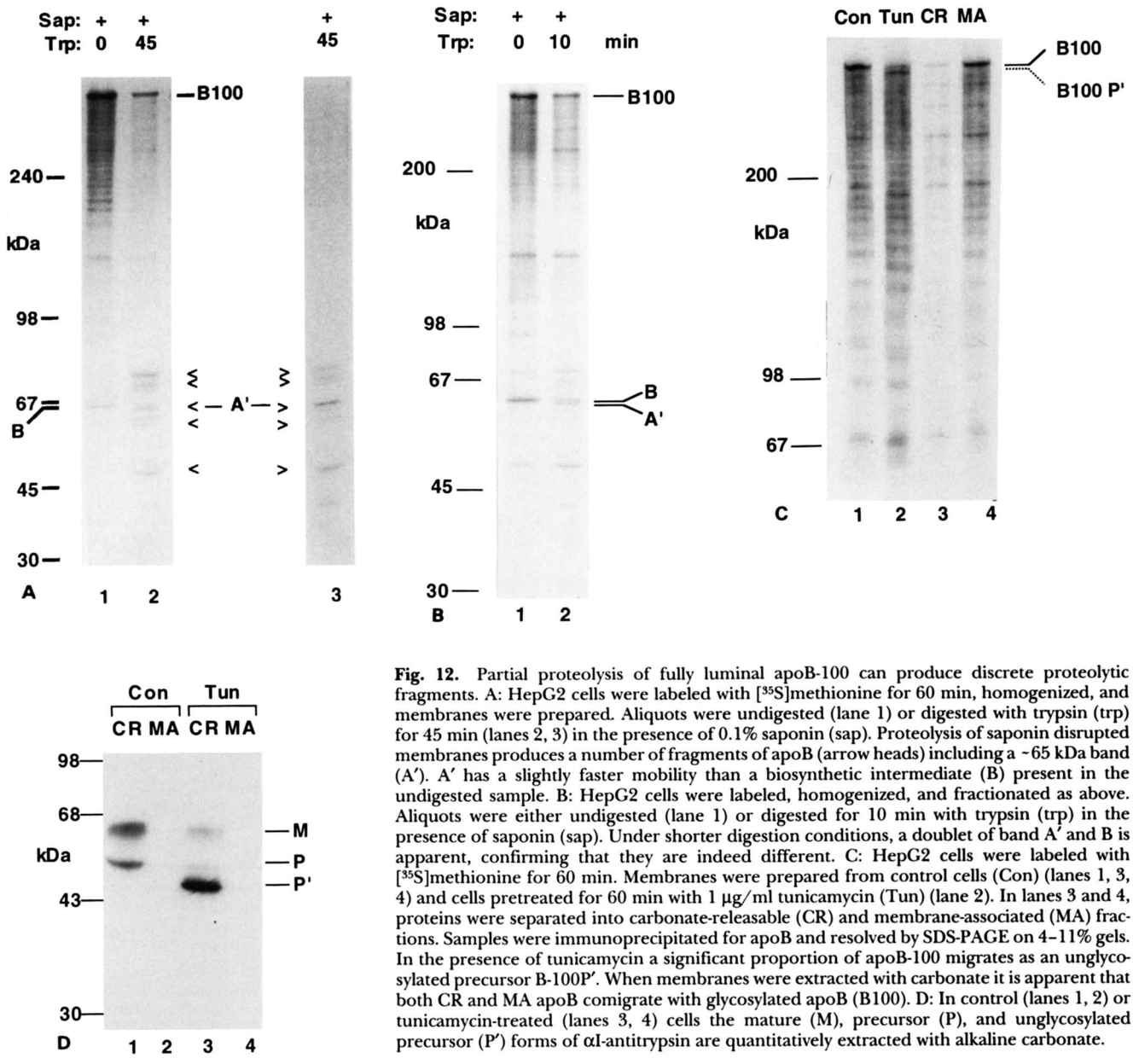


Fig. 12. Partial proteolysis of fully luminal apoB-100 can produce discrete proteolytic fragments. A: HepG2 cells were labeled with [^{35}S]methionine for 60 min, homogenized, and membranes were prepared. Aliquots were undigested (lane 1) or digested with trypsin (trp) for 45 min (lanes 2, 3) in the presence of 0.1% saponin (sap). Proteolysis of saponin disrupted membranes produces a number of fragments of apoB (arrow heads) including a ~65 kDa band (A′). A′ has a slightly faster mobility than a biosynthetic intermediate (B) present in the undigested sample. B: HepG2 cells were labeled, homogenized, and fractionated as above. Aliquots were either undigested (lane 1) or digested for 10 min with trypsin (trp) in the presence of saponin (sap). Under shorter digestion conditions, a doublet of band A′ and B is apparent, confirming that they are indeed different. C: HepG2 cells were labeled with [^{35}S]methionine for 60 min. Membranes were prepared from control cells (Con) (lanes 1, 3, 4) and cells pretreated for 60 min with 1 μg/ml tunicamycin (Tun) (lane 2). In lanes 3 and 4, proteins were separated into carbonate-releasable (CR) and membrane-associated (MA) fractions. Samples were immunoprecipitated for apoB and resolved by SDS-PAGE on 4–11% gels. In the presence of tunicamycin a significant proportion of apoB-100 migrates as an unglycosylated precursor B-100P′. When membranes were extracted with carbonate it is apparent that both CR and MA apoB comigrate with glycosylated apoB (B100). D: In control (lanes 1, 2) or tunicamycin-treated (lanes 3, 4) cells the mature (M), precursor (P), and unglycosylated precursor (P′) forms of αI-antitrypsin are quantitatively extracted with alkaline carbonate.

suggested by Du et al. (18). Second, Hedge and Lingappa (43) based their arguments on the lifetime of transmembrane intermediates on data from studies of the translocation of residues 234 to 263 of apoB which are inferred to pause for ~80 seconds in vitro, but which appeared to be continuously translocated in our in vitro translation experiments (19). In contrast, where we have seen long-lived transmembrane structures in proteins C and F in vitro, we saw no evidence for a disturbance of translocation in intact cells, therefore simple extrapolation between systems may not hold true.

In contrast to other reports (7–9), the results that we observed with apoB have not suggested that a large proportion of endogenous apoB-100 in our preparations of HepG2 cells is untranslocated or transmembrane even though greater than 90% of newly synthesized apoB underwent intracellular degradation (data not shown). This conclusion is also apparent from the data of Ingram and Shelness (28). However, we cannot exclude the possibility that a small proportion of apoB-100 may adopt a transmembrane conformation that might not be detected in our experiments, particularly if several distinct pools of apoB-100 undergo separate trafficking pathways in these cells (44).

During our proteolysis experiments it became apparent to us that proteolytic fragments (~65 and ~40 kDa) can be generated from fully translocated apoB-100, apoB-36, and apoB-15.cyclin if trypsin gains access to the

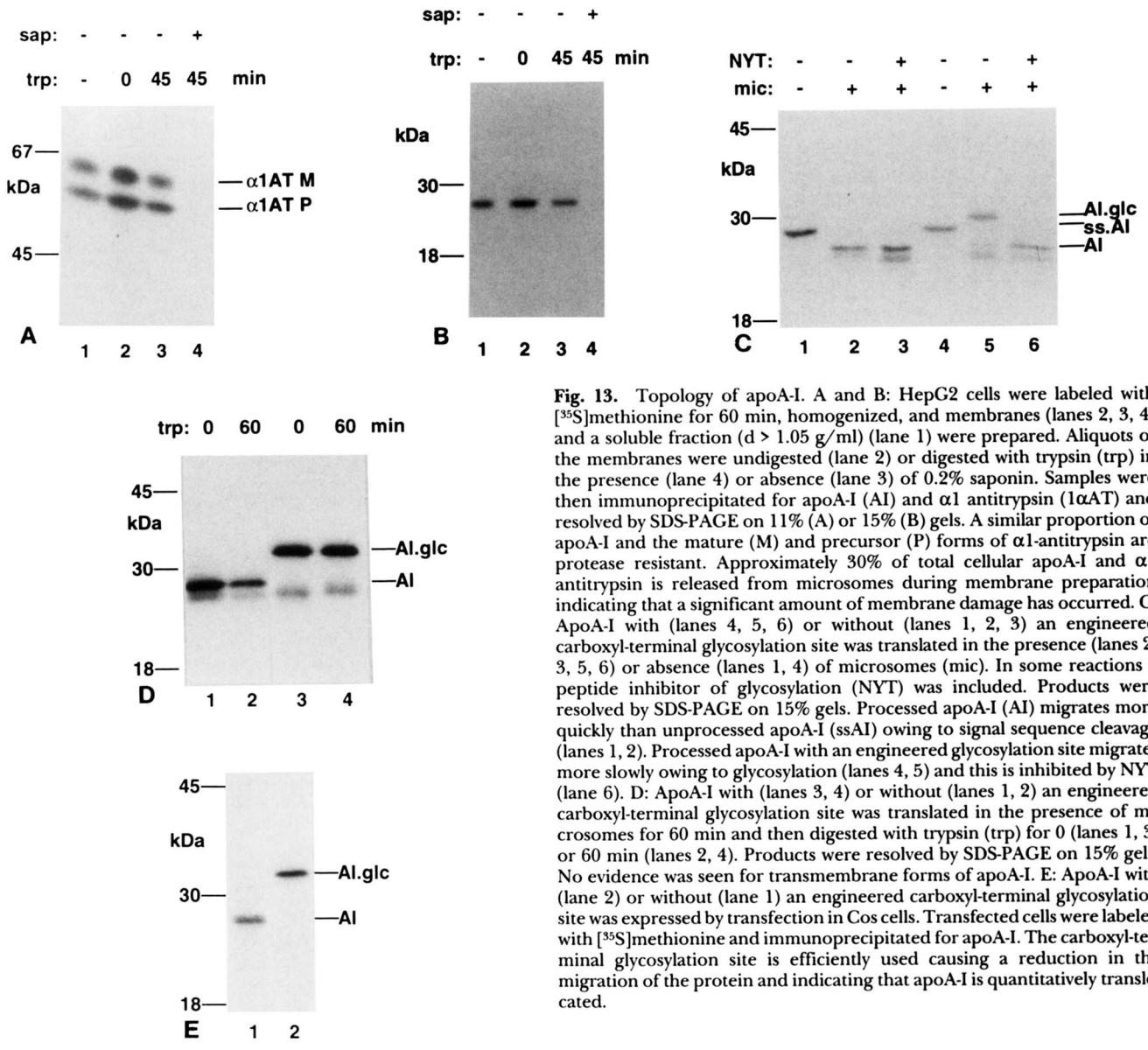


Fig. 13. Topology of apoA-I. A and B: HepG2 cells were labeled with [^{35}S]methionine for 60 min, homogenized, and membranes (lanes 2, 3, 4) and a soluble fraction (d > 1.05 g/ml) (lane 1) were prepared. Aliquots of the membranes were undigested (lane 2) or digested with trypsin (trp) in the presence (lane 4) or absence (lane 3) of 0.2% saponin. Samples were then immunoprecipitated for apoA-I (AI) and α1 antitrypsin (1αAT) and resolved by SDS-PAGE on 11% (A) or 15% (B) gels. A similar proportion of apoA-I and the mature (M) and precursor (P) forms of α1-antitrypsin are protease resistant. Approximately 30% of total cellular apoA-I and α1 antitrypsin is released from microsomes during membrane preparation indicating that a significant amount of membrane damage has occurred. C: ApoA-I with (lanes 4, 5, 6) or without (lanes 1, 2, 3) an engineered carboxyl-terminal glycosylation site was translated in the presence (lanes 2, 3, 5, 6) or absence (lanes 1, 4) of microsomes (mic). In some reactions a peptide inhibitor of glycosylation (NYT) was included. Products were resolved by SDS-PAGE on 15% gels. Processed apoA-I (AI) migrates more quickly than unprocessed apoA-I (ssAI) owing to signal sequence cleavage (lanes 1, 2). Processed apoA-I with an engineered glycosylation site migrates more slowly owing to glycosylation (lanes 4, 5) and this is inhibited by NYT (lane 6). D: ApoA-I with (lanes 3, 4) or without (lanes 1, 2) an engineered carboxyl-terminal glycosylation site was translated in the presence of microsomes for 60 min and then digested with trypsin (trp) for 0 (lanes 1, 3) or 60 min (lanes 2, 4). Products were resolved by SDS-PAGE on 15% gels. No evidence was seen for transmembrane forms of apoA-I. E: ApoA-I with (lane 2) or without (lane 1) an engineered carboxyl-terminal glycosylation site was expressed by transfection in Cos cells. Transfected cells were labeled with [^{35}S]methionine and immunoprecipitated for apoA-I. The carboxyl-terminal glycosylation site is efficiently used causing a reduction in the migration of the protein and indicating that apoA-I is quantitatively translocated.

lumen of permeabilized microsomes. Presumably trypsin has preferential access to certain cleavage sites in apoB whilst the remainder may be relatively protected owing to the close apposition of its peptide backbone to the lipid bilayer. This situation is reminiscent of the apparent conformation of apoB in LDL particles. Epitopes for the amino terminal monoclonal antibodies IDI and Bsol14 are fully expressed on the surface of native LDL particles, showing that this region of the protein is not buried within the lipid of LDL (45). Despite this, proteolysis yields 2–3 discrete fragments of 40–45 kDa from the amino terminal region of apoB that contain IDI and Bsol14 epitopes and some fragments of 60–65 kDa that extended to the extreme amino terminus defined by the epitope for Bsol12 (45–47).

Du et al. (18) reported similar sized (69, 55, and 30 kDa) proteolytic fragments of apoB in microsomes isolated from HepG2 cells and interpreted these as being the luminal amino termini of transmembrane apoB-100 molecules. To control for proteolysis arising from membrane instability, these authors also monitored the recovery of albumin. However, as demonstrated by Ingram and Shelness (28), this may have been misleading as the intrinsic protease sensitivity of albumin can be relatively low. Similarly, the pool size of untranslocated apoB may have been overestimated in previous studies where albumin was used as a control for membrane integrity. In one such study it was also reported that apoA-I shows membrane exposure similar to apoB (16), although our results and the results of previous studies

of apoA-I translocation (48) suggest that the majority of apoA-I is fully translocated.

One criterion that may unequivocally identify untranslocated apoB-100 is the glycosylation state of the protein. Previously, Wong and Torbati (49) performed double-labeling of rat hepatocytes with [^{35}S]methionine and [^{3}H]mannose and concluded that intracellular (membrane-bound) and secreted apoB are both fully glycosylated, showing that the majority of the protein is fully translocated. This appears consistent with our observations of the glycosylation state of membrane-bound apoB in HepG2 cells (Fig. 12C). If, on the other hand, in our HepG2 cell preparations greater than 80% of apoB was in the conformation described by Du et al. (18) with only ~65 kDa translocated, then it could only be glycosylated at two out of a total of the sixteen available glycosylation sites (1). Such a major underglycosylated pool of apoB was not apparent in our experiments and has not been demonstrated to corroborate the findings of Du et al. (18).

In conclusion, we do not believe that there is any unequivocal evidence at present that apoB undergoes a discontinuous process of translocation or that a significant pool of untranslocated apoB is established in hepatic cells.

Manuscript received 2 April 1996 and in revised form 29 July 1996.

REFERENCES

1. Yang, C-Y., Z-W. Gu, S-A. Weng, T. W. Kim, S-H. Chen, H. J. Pownall, P. M. Sharp, S-W. Liu, W-H. Li, A. M. Gotto, Jr., and L. Chan. 1989. Structure of apolipoprotein B-100 of human low density lipoproteins. *Arteriosclerosis.* **9:** 96–108.
2. Yang, C-Y., S-H. Chen, S. H. Gianturco, W. A. Bradley, J. T. Sparrow, M. Tanimura, W-H. Li, D. A. Sparrow, H. DeLoof, M. Rosseneu, F-S. Lee, Z-W. Gu, A. M. Gotto, Jr., and L. Chan. 1986. Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. *Nature.* **323:** 738–742.
3. Knott, T. J., R. J. Pease, L. M. Powell, S. C. Wallis, S. C. Rall, Jr., T. L. Innerarity, B. Blackhart, W. H. Taylor, Y. Marcel, R. Milne, D. Johnson, M. Fuller, A. J. Lusis, B. J. McCarthy, R. W. Mahley, B. Levy-Wilson, and J. Scott. 1986. Complete protein sequence and identification of structural domains of human apolipoprotein B. *Nature.* **323:** 734–738.
4. Chatterton, J. E., M. L. Phillips, L. K. Curtiss, R. W. Milne, Y. L. Marcel, and V. N. Schumaker. 1991. Mapping apolipoprotein B on the low density lipoprotein surface by immunoelectron microscopy. *J. Biol. Chem.* **266:** 5955–5962.
5. Boren, J., L. Graham, M. Wettesten, J. Scott, A. White, and S-O. Olofsson. 1992. The assembly and secretion of apoB-100-containing lipoproteins in HepG2 cells. *J. Biol. Chem.* **267:** 9858–9867.
6. Rusinol, A. E., H. Verkade, and J. E. Vance. 1993. Assembly of rat hepatic very low density lipoproteins in the endoplasmic reticulum. *J. Biol. Chem.* **268:** 3555–3562.
7. Davis, R. A., R. N. Thrift, C. C. Wu, and K. E. Howell. 1990. Apolipoprotein B is both integrated into and translocated across the endoplasmic reticulum membrane. *J. Biol. Chem.* **265:** 10005–10011.
8. Dixon, J. L., S. Furukawa, and H. N. Ginsberg. 1991. Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from HepG2 cells by inhibiting early intracellular degradation of apolipoprotein B. *J. Biol. Chem.* **266:** 5080–5086.
9. Furukawa, S. N., N. Sakata, H. N. Ginsberg, and J. L. Dixon. 1992. Studies on the sites of intracellular degradation of apolipoprotein B in HepG2 cells. *J. Biol. Chem.* **267:** 22630–22638.
10. Pullinger, C. R., J. D. North, B-B. Teng, V. A. Rifici, A. E. Ronhild de Brito, and J. Scott. 1989. The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. *J. Lipid Res.* **30:** 1065–1077.
11. White, A. L., D. L. Graham, J. LeGros, R. J. Pease, and J. Scott. 1992. Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. *J. Biol. Chem.* **267:** 15657–15664.
12. Rusinol, A. E., L. Chan, and J. E. Vance. 1993. Movement of apolipoprotein B into the lumen of microsomes from hepatocytes is disrupted in membranes enriched in phosphatidyl monomethyl ethanolamine. *J. Biol. Chem.* **268:** 25168–25175.
13. Sakata, N., X. Wu, J. L. Dixon, and H. N. Ginsberg. 1993. Proteolysis and lipid-facilitated translocation are distinct but competitive processes that regulate secretion of apolipoprotein B in HepG2 cells. *J. Biol. Chem.* **268:** 22967–22970.
14. Gordon, D. A., H. Jamil, D. Sharp, D. Mullaney, Z. Yao, R. E. Gregg, and J. Wetterau. 1994. Secretion of apolipoprotein B-containing lipoproteins from Hela cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. *Proc. Natl. Acad. Sci. USA.* **91:** 7628–7632.
15. Leiper, J. M., J. D. Bayliss, R. J. Pease, D. J. Brett, J. Scott, and C. C. Shoulders. 1994. Microsomal triglyceride transfer protein, the abetalipoproteinemia gene product, mediates the secretion of apolipoprotein B-containing lipoproteins from heterologous cells. *J. Biol. Chem.* **269:** 21951–21954.
16. Dixon, J. L., R. Chattapadhyay, T. Huima, C. M. Redman, and D. Banerjee. 1992. Biosynthesis of lipoprotein: location of nascent apoA-I and apoB in the rough endoplasmic reticulum of chicken hepatocytes. *J. Cell Biol.* **117:** 1161–1169.
17. Morimoto, T., M. Arpin, and S. Gaetani. 1983. Use of proteases for the study of membrane insertion. *Methods Enzymol.* **96:** 121–150.
18. Du, E. Z., J. Kurth, S. L. Wang, P. Humiston, and R. A. Davis. 1994. Proteolysis-coupled secretion of the N-terminus of apolipoprotein B. Characterization of a transient, transmembrane arrested intermediate. *J. Biol. Chem.* **269:** 24169–24176.
19. Pease, R. J., J. M. Leiper, G. B. Harrison, and J. Scott. 1995. Studies on the translocation of the amino-terminus of apolipoprotein B into the endoplasmic reticulum. *J. Biol. Chem.* **270:** 7261–7271.

20. Shoulders, C. C., A. R. Kornblihtt, B. S. Munro, and F. E. Barelle. 1983. Gene structure of human apolipoprotein A-I. *Nucleic Acids Res.* **11:** 2827–2837.
21. Truett, M. A., R. Blacher, R. L. Burke, D. Caput, C. Chu, D. Dina, K. Hartog, C. H. Kuo, F. R. Masiarz, J. P. Merryweather, R. Najarian, C. Pachl, S. J. Potter, J. Puma, M. Quiroga, L. B. Rall, A. Randolph, M. S. Urdea, P. Valenzuela, H. H. Dahl, J. Favalaro, J. Hansen, O. Nordfang, and M. Ezban. 1985. Characterization of the polypeptide composition of human factor VIII:C and the nucleotide sequence and expression of the human kidney cDNA. *DNA.* **4:** 333–349.
22. Knight, A. M., G. B. Harrison, R. J. Pease, P. J. Robinson, and P. J. Dyson. 1992. Biochemical analysis of the mouse mammary tumor virus long terminal repeat product. Evidence for the molecular structure of an endogenous superantigen. *Eur. J. Immunol.* **22:** 879–882.
23. Armstrong, J., H. Niemann, S. Smeekens, P. Rottier, and G. Warren. 1984. Sequence and topology of a model intracellular membrane protein, E1 glycoprotein, from a coronavirus. *Nature.* **308:** 751–752.
24. Stafford, F. J., and J. S. Bonifacino. 1991. A permeabilized cell system identifies the endoplasmic reticulum as a site of protein degradation. *J. Cell Biol.* **115:** 1225–1236.
25. Pease, R. J., G. B. Harrison, and J. Scott. 1991. Cotranslocational insertion of apolipoprotein B into the inner leaflet of the endoplasmic reticulum. *Nature.* **353:** 448–450.
26. Chuck, S. L., Z. Yao, B. D. Blackhart, B. McCarthy, and V. R. Lingappa. 1990. A new variation on protein translocation occurs in the early biogenesis of apoB. *Nature.* **346:** 382–385.
27. Chuck, S. L., and V. R. Lingappa. 1992. Pause transfer: a topogenic sequence in apolipoprotein B mediates stopping and restarting of translocation. *Cell.* **68:** 9–21.
28. Ingram, M. F., and G. S. Shelness. 1996. ApoB-100 destined for lipoprotein assembly and intracellular degradation undergoes efficient translocation across the endoplasmic reticulum membrane. *J. Lipid Res.* **37:** 2202–2214.
29. Elbein, A. D. 1983. Inhibitors of glycoprotein synthesis. *Methods Enzymol.* **98:** 135–154.
30. Zhou, M., X. Wu, L. S. Huang, and H. N. Ginsberg. 1995. Apoprotein B-100, an inefficiently translocated secretory protein, is bound to the cytosolic chaperone, heat shock protein 70. *J. Biol. Chem.* **270:** 25220–25224.
31. Adeli, K., A. Kohammadi, and J. Macri. 1995. Regulation of apolipoprotein B biogenesis in human hepatocytes: posttranscriptional control mechanisms that determine the hepatic production of apolipoprotein B-containing lipoproteins. *Clin. Biochem.* **28:** 123–130.
32. Hussain, M. M., Y. Zhao, R. K. Kancha, B. D. Blackhart, and Z. Yao. 1995. Characterization of recombinant human apoB-48-containing lipoproteins in rat hepatoma McA-RH7777 cells transfected with apoB-48 cDNA. Overexpression of apoB-48 decreases synthesis of endogenous apoB-100. *Arterioscler. Thromb. Vasc. Biol.* **15:** 485–494.
33. Sturley, S. L., P. J. Talmud, R. Brasseur, M. R. Culbertson, S. E. Humphries, and A. D. Attie. 1994. Human apolipoprotein B signal sequence variants confer a secretion-defective phenotype when expressed in yeast. *J. Biol. Chem.* **269:** 21670–21675.
34. Boren, J., M. Wettesten, S. Rustaeus, M. Andersson, and S-O. Olofsson. 1993. The assembly and secretion of apoB-100-containing lipoproteins. *Biochem. Soc. Trans.* **21:** 487–493.
35. Wang, C. N., T. C. Hobman, and D. N. Brindley. 1995. Degradation of apolipoprotein B in cultured rat hepatocytes occurs in a post endoplasmic compartment. *J. Biol. Chem.* **270:** 24924–24931.
36. Wilkinson, J., J. A. Higgins, P. H. E. Groot, E. Gherardi, and D. E. Bowyer. 1992. Membrane bound apolipoprotein B is exposed at the cytosolic surface of liver microsomes. *FEBS Lett.* **304:** 24–26.
37. Shelness, G. S., K. C. Morris-Rogers, and M. F. Ingram. 1994. Apolipoprotein B48-membrane interactions. Absence of transmembrane localization in nonhepatic cells. *J. Biol. Chem.* **269:** 9310–9318.
38. De-Fea, K. A., D. H. Nakahara, M. C. Calayag, C. S. Yost, L. F. Mirels, S. B. Prusiner, and V. R. Lingappa. 1994. Determinants of carboxyl-terminal domain translocation during prion protein biogenesis. *J. Biol. Chem.* **269:** 16810–16820.
39. Nakahara, D. H., V. R. Lingappa, and S. L. Chuck. 1994. Translocational pausing is a common step in the biogenesis of unconventional integral membrane and secretory proteins. *J. Biol. Chem.* **269:** 7617–7622.
40. Connolly, T., P. Collins, and R. Gilmore. 1989. Access of proteinase K to partially translocated nascent polypeptides in intact and detergent solubilized membranes. *J. Cell Biol.* **108:** 299–307.
41. Spiess, M., C. Handschin, and K. P. Baker. 1989. Stop transfer activity of hydrophobic sequences depends on the translation system. *J. Biol. Chem.* **264:** 19117–19124.
42. Wettesten, M., K. Bostrom, G. Bondjers, M. Jarfeldt, P-I. Norfeldt, M. Carrella, O. Wiklund, J. Boren, and S-O. Olofsson. 1985. Pulse-chase studies of the synthesis of apolipoprotein B in a human hepatoma cell line, HepG2. *Eur. J. Biochem.* **149:** 461–466.
43. Hegde, R. S., and V. R. Lingappa. 1996. Sequence-specific alteration of the ribosome-membrane junction exposes nascent secretory proteins to the cytosol. *Cell.* **85:** 217–228.
44. Rustaeus, S., K. Lindberg, J. Boren, and S. O. Olofsson. 1995. Brefeldin A inhibits the assembly of apoB-containing lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **270:** 28879–28886.
45. Pease, R. J., R. W. Milne, W. K. Jessup, A. Law, P. Provost, J-C. Fruchart, R. T. Dean, Y. L. Marcel, and J. Scott. 1990. Use of bacterial expression cloning to localize the epitopes for a series of monoclonal antibodies against apolipoprotein B-100. *J. Biol. Chem.* **265:** 553–568.
46. Milne, R. W., L. Blanchette, R. Theolis, Jr., P. K. Weech, and Y. L. Marcel. 1987. Monoclonal antibodies distinguish between lipid-dependent and reversible conformational states of human apolipoprotein B. *Mol. Immunol.* **24:** 435–447.
47. Marcel, Y. L., T. L. Innerarity, C. Spilman, R. W. Mahley, A. A. Protter, and R. W. Milne. 1987. Mapping of human apolipoprotein B antigenic determinants. *Arteriosclerosis.* **7:** 166–175.
48. Stoffel, W., G. Blobel, and P. Walter. 1981. Synthesis in vitro and translocation of apolipoprotein A-I across microsomal vesicles. *Eur. J. Biochem.* **120:** 519–522.
49. Wong, L., and A. Torbati. 1994. Differentiation of intrahepatic membrane-bound and secretory apolipoprotein B by monoclonal antibodies: membrane-bound apolipoprotein B is more glycosylated. *Biochemistry.* **33:** 1923–1929.
50. Kassenbrock, C. K., P. D. Garcia, P. Walter, and R. B. Kelly. 1988. Heavy-chain binding protein recognizes aberrant polypeptides translocated in vitro. *Nature.* **333:** 90–93.