Cotranslational insertion of apolipoprotein B into the inner leaflet of the endoplasmic reticulum

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APOLIPOPROTEIN (apo) B100 is required for the distribution of hepatic triglyceride to peripheral tissues as very-low-density lipoproteins. The translocation of apo B100 into the endoplasmic reticulum (ER) and its subsequent assembly into lipoprotein particles is of particular interest as the protein is both very large (relative molecular mass 512,000) and insoluble in water. It has been proposed that apo B translocation occurs in discrete stages and is completed post-translationally. Several sites of arrest of translocation were reported to be present in apo B15 (the N-terminal 15% of the protein). We have re-examined this question by in vitro translation coupled with translocation into microsomes, and find no evidence for transmembrane segments in truncated apo B proteins. Translocated apo B17 is strongly associated with the membrane of the ER, being only partially releasable with alkaline carbonate, and remaining bound to the microsomes following disruption with saponin. The efficient binding of short segments of apo B, despite the absence of transmembrane domains, suggests that apo B is cotranslationally inserted into the inner leaflet of the ER. This will obliterate problems caused by the size and insolubility of apo B100, because the growing hydrophobic protein chains will never exist in a lipid-free form during translocation. From the inner leaflet, apo B in association with membrane-derived lipid can bud into the lumen of the ER to form nascent lipoprotein particles.

After biosynthesis apo B100 is either assembled into lipoprotein particles and secreted, or degraded intracellularly.5,6 Studies of cells in culture indicate that a proportion of the molecules are untranslocated and destined for degradation and that the remainder are translocated and quantitatively secreted.7 Studies of apo B15 translated in wheat-germ lysates have suggested that translocation pauses while translation continues, imposing transient transmembrane configurations; we cannot confirm these observations.

To obtain efficient translation of apo B in reticulocyte lysates, a partial S′ untranslated region (UTR) of mouse encephalomyocarditis virus (EMCV) (ref. 4) was fused to apo B complementory DNA. The EMCV–apo B transcripts encode MetAia before the apo B prepeptide sequence, but the extended signal still functions efficiently. Thus, apo B7 and B9 (Fig. 1a, b) increase in size after translation in the presence of microsomes owing to glycosylation,8 and apo B9 is protected from trypsin digestion when translated with microsomes (Fig. 1a). When apo B7 is translated in the presence of the competitive glycosylation inhibitor N-acetyl-AsnTyrThr-carboxamidem,9 the principal product (unglycosylated apo B7) migrates faster than pre-apo B7, consistent with cleavage of the signal sequence (Fig. 1b). Cleavage (−90%) was confirmed by radiosequencing of processed apo B15 (results not shown).

In cultured cells apo B100 is carbonate-resistant1,2 immediately after biosynthesis, which indicates that integration into the ER membrane has taken place.2,6 We therefore investigated whether the truncated apo B proteins bind to microsomes. Under conditions in which soluble secretory proteins are quantitatively released but a transmembrane protein will remain associated with the membrane, membrane binding was significant with proteins in the range apo B9 to B17, but less with apo B7 (Fig. 2a). Similarly, intermediate carbonate releasability has been found with apo E in Golgi membranes: we have confirmed the membrane association of apo B17 following disruption with saponin (Fig. 2b).

Association of apo B with the ER membrane could either involve amphipathic binding to the inner leaflet, or the presence of transmembrane regions. We observed that after translation in reticulocyte lysates for either 40 min (when protein synthesis is ongoing) or for 90 min at 30°C, both apo B15 and apo B17 are protected from trypsin or proteinase K (Fig. 3a, b; and data not shown), thereby excluding the presence of transmembrane domains. To ensure reproducible inactivation of proteinase K, it was necessary to centrifuge the membranes out of the digestion mixture, but this protocol precludes the controls of post-translational addition of microsomes or total disruption with detergent to verify proteinase K susceptibility of apo B17. Instead it was shown that apo B17 becomes susceptible to proteinase K after the membranes are permeabilized with saponin (Fig. 3b). Although we did not find transmembrane domains in apo B, we confirmed that E1 glycoprotein is a transmembrane protein (Fig. 3c).10

Finally we determined whether generation of transmembrane domains resulted specifically from wheat-germ ribosomes. Reduced expression of some transmembrane domains in reticulocyte lysates relative to wheat-germ lysates has been reported,11 although the topology of protein expression in mammalian cells is better predicted by translation in reticulocyte lysates.12 But we find no evidence for transmembrane domains when apo B15 is translated in wheat-germ lysates and the microsomes are digested with trypsin (results not shown) or proteinase K (Fig. 3d). Using densitometry to quantitate full-length chains after digestion we find (1) 71%±13 s.d. (n=9) recovery of apo B17 in reticulocyte lysates with trypsin and (2) 86%±31 s.d. recovery (n=7) of apo B15 in wheat-germ lysates with proteinase K. In contrast, we find 0% (n=4) recovery of apo B15 or B17 in saponin-disrupted microsomes after proteinase K digestion. Although we routinely included the membrane stabilizer tetracaine13 in digestion mixtures, we cannot detect transmembrane domains when tetracaine is absent but Ca2+14 is present (results not shown); in contrast, substantially reduced levels of putative intermediates have been observed with tetracaine present (S. L. Chuck and V. R. Lingappa, unpublished), which suggests that the data in ref. 1 may be a result of overdigestion.

We conclude that apo B is cotranslationally translocated into microsomes and binds to the inner leaflet of membrane. The binding of the amino terminus of apo B forms a focus for association of the remainder of the elongating protein with the membrane, circumventing the problems of size and hydrophobicity. It is probable that this amphipathic form of apo B
FIG. 1 SDS-PAGE showing expression of apo B7 and apo B9. a. Apo B9 was translated in the presence (tracks 1, 2, 3, 6) or absence (tracks 2, 3, 4, 5) of detergent (Tx) or were undigested (track 1). Apo B9 is protected if cotranslationally imported into microsomes (tracks 4, 5) peptidyl tRNA is apparent at absence (tracks 2, 3, 4, 5) of detergent (Tx) or were undigested (track 1). Glycosylated apo B9. b. Apo B7 was translated in the presence (tracks 2, 3) or absence (1, 4) of microsomes, with (track 3) or without (tracks 1, 2) 100 µM N-acetyl-AsnTyrThr(40)-carboxyamide (NYT). The peptide inhibited glycosylation of apo B7 to generate a protein species (B7) of faster migration than pre-apo B7, indicating cleavage of the MetAla signal sequence. B7.CHO, glycosylated apo B7.

METHODS. To generate the apo B9 transcript, construct EB9 was linearized with SafI and transcribed with T7 RNA polymerase (Boehringer). To generate the apo B7 transcript, construct EB9 was linearized at the Stül (1.199) site of the cDNA and transcribed. RNA was translated in unfraccionated reticulocyte lysates (Amersham), with [35S]methionine (Amersham) and, where indicated, in the presence of canine pancreatic microsomes (Promega). For protease digestion, translation mixtures were treated with 0.5 mg ml⁻¹ tosyl amino phenyl ethyl chloromethyl ketone (TPCK) trypsin (Cooper Bio-medical) for 90 min at 0°C in 20 mM Tris-HCl, pH 7.5, and 2 mM l-triacetyl-OHCl (Sigma)³. Where indicated, membranes were disrupted with 0.1% Triton X-100 during digestion. Digestions were terminated with 6 mg ml⁻¹ final concentration soybean trypsin inhibitor (Sigma). Glycosylated pre-apo B7 is mainly releasable with carbonate, (-80%).

FIG. 2 Membrane of nascent apo B. a. Transcripts of apo B7 to B17 and of control proteins were translated in the presence of microsomes. Carbonate releasable (CR) and membrane associated (MA) proteins were resolved by SDS-PAGE. The tracks show: 1 and 2, mixed apo B9, B13, and B17; 3 and 4, mixed apo B7, B11, and B15; 5 and 6, yeast α-mating factor (YMF); 7 and 8, E₁ glycoprotein (E1gp); 9 and 10, β-lactamase (βL). There is significant association of apo B9, 11, 13, 15, and 17 with the membranes, but apo B7 is mainly releasable with carbonate, (-80%). b. Apo B17 transcripts were translated in the presence of microsomes and were then either untreated (tracks 1, 2) or treated with saponin for 10 min (tracks 3, 4) or 90 min (tracks 5, 6). Transcripts of E₁ glycoprotein (tracks 7, 8), yeast α-mating factor (tracks 9, 10) and β-lactamase (βL) were translated in the presence of microsomes and treated with saponin for 90 min. Solutions and membrane (MM) fraction were resolved and digested by SDS-PAGE. Apo B17 is associated with the membrane fraction under conditions in which soluble products are released. Some apo B17 is present in the saponin-releasable fraction but migrates faster, and represents unprocessed pre-apo B17.

METHODS. To generate the apo B13 transcript construct EB13 was first linearized at the EcoRV (1.952) site of the cDNA. RNA encoding β-lactamase and yeast α-mating factor was obtained from Promega. For carbonate extraction, carrier liver microsomes ( ~ 25 µg) were added post-translationally to reticulocyte lysate translations and the membrane fraction was separated from the soluble fraction by centrifugation for 10 min at 30 p.s.i. in a Beckman airfuge. The membrane fraction was washed and resuspended in 200 µl of 100 mM Na₂CO₃, pH 11.5, for 45 min at 0°C. Membrane-associated proteins were separated from the carbonate releasable proteins by recentrifugation. For saponin extraction, carrier liver microsomes were added post-translationally and the mixture was then adjusted to 0.05% (final concentration) saponin (Sigma)). The membrane fraction was separated from the soluble fraction by centrifugation. Proteins were resolved on 7-17% gradient SDS-polyacrylamide gels. Subcloning: cDNA encoding coronavirus E₂ glycoprotein(19) was amplified by PCR with mismachted oligonucleotides to introduce an Ncol site at the ATG encoding first Met of the open reading frame and a Sall site immediately downstream of the termination codon. This was ligated to the Ncol site of the modified EMCV 5' UTR to encode MetAla-E1gp.
FIG. 3 Luminal disposition of apo B in microsomes. a. Apo B15 (tracks 1, 2) or apo B17 (tracks 3, 4, 5, 6) transcripts were translated in reticulocyte lysates in the presence of microsomal membranes for 40 min (tracks 3, 4) or 90 min (tracks 1, 2, 5, 6) and digested with trypsin for 0 min or 90 min. In tracks 2, 4, 5, 6, 0.1% Triton X-100 (Tx) was included during the digestion. b. Apo B17 was translated in the presence of microsomal membranes and then aliquots were left undigested (tracks 1 and 2) or were digested for 40 min with proteinase K (PrK) (tracks 3, 4) in the presence of microsomes or for 90 min (tracks 1, 3) with trypsin. The clearer of the two products (tracks 1, 2, 5, 6) is generated by cleavage of the cytoplasmic domain of E1gp. The smaller of the apparent product is generated by cleavage of both the cytoplasmic and luminal domains of E1gp. c, d. E1gp glycoprotein transcripts were translated in reticulocyte lysates in the presence of microsomal membranes and digested with trypsin (tracks 1, 2) or proteinase K (tracks 3, 4) for 0 min (tracks 1, 3) or 90 min (tracks 2, 4). E1gp glycoprotein (E1gp) was cleaved from a protein of apparent Mr 23.5K to a major product (FA) of 22K and a minor product (FB) of 19.5K (ref. 10), which migrates here at <18K. The 22K product is generated by cleavage of the cytoplasmic domain of E1gp. The smaller product is generated by cleavage of both the cytoplasmic and luminal domains. c, d. Apo B15 transcripts were translated in wheat germ lysate in the presence of microsomes for 60 min, and then the membranes were collected by ultracentrifugation before (track 1) or after digestion with proteinase K for 0 min (track 2) or 90 min (track 3). Some partial synthesis products are present in all tracks but no new bands are generated by protease digestion.

METHODS. Apo B15 capped transcripts were prepared by linearizing construct GB15 with SalI and transcribing with T7 polymerase in the presence of 0.5 mM GTP with 5 mM 7-methyl GpppG (Pharmacia), and fractionated on a Sephadex-G25 spin column (Pharmacia). Fractionated wheat germ lysate (Amersham) was reconstituted to 120 mM K+, with a 1 mM mixture of unlabelled amino acids excluding Met. Proteinase K digests were diluted with 2 volumes of 250 mM NaCl to 0.5 mM NaCl with 5 mM PMSF. Membranes were collected in an airfuge and washed 3 times with NaCl solution before dissolving in SDS loading buffer plus 5 mM PMSF for SDS-PAGE. In some instances membranes were disrupted with 0.05% saponin before protease digestion. Proteins were resolved on 10-17% SDS-PAGE gels. Subcloning: The 50-nucleotide 5' UTR of human /3-globin containing the natural NcoI site at the first A1G of the coding sequence was synthesized as oligonucleotides with a 5' Smal site and a 3' Sfil site to enable it to be cloned into the polynucleotide pKS. Pre-apo B15 was constructed by ligation of NcoI-HindIII (2,279) fragments of cDNA to generate plasmid GB15.

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