Apolipoprotein B in the Rough Endoplasmic Reticulum: Translation, Translocation and the Initiation of Lipoprotein Assembly

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ABSTRACT

Apolipoprotein (apo) B and the microsomal triglyceride transfer protein are essential for the hepatic assembly and secretion of triglyceride-rich VLDL. To understand how apoB initiates the process of lipoprotein formation, interest has focused on the biogenesis of its amino terminal globular domain (α1 domain). When only this domain is expressed in hepatoma cells, no lipoprotein particle will form. However, proper folding of the α1 domain is essential for the internal lipophilic regions of apoB to engage in cotranslational lipid recruitment. The essential function of this domain may be related to its capacity to promote a specific physical interaction with the microsomal triglyceride transfer protein, necessary for apoB's proper folding and lipidation. Alternatively, this domain may promote an autonomous lipid recruitment step that nucleates microsomal triglyceride transfer protein-dependent lipid sequestration by apoB. Forms of apoB that fail to initiate particle assembly or forms associated with aberrant underlipidated particles are targeted for intracellular degradation. Two sites of apoB degradation have been identified. In hepatocarcinoma-derived cells, misassembled apoB may undergo progressive reverse translocation from the endoplasmic reticulum lumen to the cytosol, a process that is mechanistically coupled to polyubiquitination and proteasome-mediated degradation on the cytosolic side of the membrane. Alternatively, studies in primary hepatocytes reveal that apoB may undergo sorting to a post-endoplasmic reticulum compartment for presecretory degradation. In either case, the balance between assembly and presecretory degradation of apoB may represent a control point for the production of hepatic VLDL. J. Nutr. 129: 456S–462S, 1999.

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APOLIPOPROTEIN B IS ESSENTIAL FOR THE BIOGENESIS OF TRIGLYCERIDE-RICHE VLDL

Hepatic VLDL are lipid emulsion particles composed of a triglyceride-rich core and a surface monolayer of phospholipid. Although VLDL emerge from the liver with multiple apolipoproteins, genetic analyses in humans and gene disruption experiments in mice reveal that apolipoprotein (apo) B is the sole apolipoprotein required for VLDL assembly (Homanics et al. 1993, Kane and Havel 1995). Apolipoprotein B is a 4536-amino acid secretory glycoprotein; in contrast to the class of soluble apoproteins epitomized by apoA-I and apoE, it is insoluble in aqueous buffers. Hence, apoB is incapable of exchanging among lipoprotein classes, and its initial biosynthetic association with nascent VLDL particles that occurs in the endoplasmic reticulum (ER) is considered irreversible.

Both experimental and theoretical considerations have provided a broad framework for understanding the interactions of apoB with lipoprotein lipids. Apolipoprotein B possesses a pentapartite structure in which three domains dominated by amphipathic α-helical structures (α1, α2 and α3) alternate with two regions proposed to have a strong tendency to form antiparallel amphipathic β-strands (β1 and β2) (Knott et al. 1986, Schumaker et al. 1994, Segrest et al. 1994) (Fig. 1). The amphipathic α-helix is the basic lipid-binding unit of the class of soluble apolipoproteins and is thought to confer strong but reversible binding of apoB to the lipoprotein surface (Segrest et al. 1994). The β1 and β2 domains may form a series of amphipathic belt-like structures; the hydrophobic face of these structures may interact directly with the triglyceride core (Segrest et al. 1998). The β domains may be responsible for the
irreversible integration of apoB with lipoprotein lipids (Yang et al. 1989).

**MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN IS A DEDICATED ENDOPLASMIC RETICULUM–LOCALIZED ASSEMBLY FACTOR REQUIRED FOR VLDL ASSEMBLY**

Two types of genetic disorders are characterized by the absence of plasma apoB-containing lipoproteins. Severe homozygous hypobetalipoproteinemia is caused by frameshift mutations within the apoB gene that result in premature translation termination. Mutations creating forms of apoB smaller than the amino terminal 28% of apoB (apoB-28) are undetectable in plasma and therefore considered null (Linton et al. 1993). Abetalipoproteinemia is a syndrome that is phenotypically similar to severe homozygous hypobetalipoproteinemia, but is not caused by defects in the apoB gene (Tamud et al. 1993). Using a candidate gene approach, Gregg, Wetterau and colleagues (Sharp et al. 1993) discovered that the absence of apoB-containing lipoproteins in abetalipoproteinemia is caused by mutations in the gene for the large subunit of microsomal triglyceride transfer protein (MTP).

Microsomal triglyceride transfer protein was first identified biochemically as a neutral lipid transfer activity localized to the lumen of the endoplasmic reticulum (ER). Its expression is limited to the liver and intestine, the only two tissues that express large amounts of apoB (Wetterau and Zilversmit 1986). Purification of MTP revealed a heterodimer composed of a unique 97-kDa subunit complexed with the ubiquitous ER-localized chaperone/folding enzyme, protein disulfide isomerase (PDI) (Wetterau et al. 1990). The discovery of MTP gene defects associated with abetalipoproteinemia provided the first direct evidence that MTP is essential for the assembly and secretion of apoB-containing lipoproteins. This requirement can also be demonstrated experimentally, i.e., when forms of apoB longer than approximately the length of apoB-28 are transfected into a cell line lacking MTP, the protein is synthesized but does not undergo appreciable secretion. However, if the large subunit of MTP is cotransfected along with apoB, the process of lipoprotein assembly is reconstituted, resulting in a several-fold increase in buoyant apoB and triglyceride secretion (Gordon et al. 1994, Leiper et al. 1994). The mechanism by which MTP functions to promote apoB-containing lipoprotein assembly is not known in detail. However, it is assumed, on the basis of its lipid transfer activity, that MTP transfers lipid from the internal leaflet of the ER membrane to apoB during its initial translation and translocation into the ER (Gordon et al. 1995). Other roles have been attributed to MTP, including that of a factor essential for apoB translocation across the ER membrane (Du et al. 1996, see below) and possibly as a factor required for the post-translational addition of triglyceride to apoB (Alexander et al. 1976, Rustaeus et al. 1998, Wang et al. 1997).

**POST-TRANSCRIPTIONAL REGULATION OF APO B SECRETION**

Many studies of apoB assembly and secretion have been performed in hepatocytes and hepatocarcinoma-derived cell lines, such as HepG2 and MA-RA7777. In these cells, a relatively small percentage of apoB undergoes assembly and secretion, with the remaining population subjected to ER retention and intracellular degradation (Borchardt and Davis 1987, Dixon et al. 1991, Sato et al. 1990, White et al. 1992). In hepatoma cells, the proportion of apoB that is assembled into secretion-competent lipoproteins can be regulated in several ways, but appears ultimately to be determined by an intracellular pool of secretion-coupled lipid (Wu et al. 1996a). Oleic acid promotes apoB secretion by stimulating triglyceride synthesis and availability (Dixon et al. 1991, Wu et al. 1996a), whereas (n-3) fatty acids inhibit apoB secretion presumably by reducing the ability of hepatocytes to synthesize or mobilize triglyceride for secretion (Wang et al. 1994). The relationship between apoB assembly and intracellular cholesterol and cholesterol ester mass and synthesis has been controversial, with some investigators reporting a major regulatory role for the intracellular cholesterol esterifying enzyme, acyl-CoA: cholesterol acyltransferase (Cianflone et al. 1990, Musanti et al. 1996, Thompson et al. 1996) and others observing that it is triglyceride synthesis alone that drives lipoprotein secretion (Benoist and Grand-Perret 1996, Wu et al. 1996a). Irrespective of the effects of lipids and other factors on apoB secretion, a prevailing view has emerged in which the apoB protein is made in excess and directed toward a pool involved in both particle assembly and secretion or intracellular degradation. The question of how the cell is able to distinguish between these two pools of apoB has important implications with respect to the regulation of VLDL triglyceride secretion and the prevention and management of genetically and environmentally induced hyperlipidemias.

**APO B HAS BEEN PROPOSED TO UNDERGO A REGULATED FORM OF TRANSLLOCATION ACROSS THE ER MEMBRANE**

One highly cited hypothesis to account for how the cell distinguishes between apoB destined for assembly and degradation involves a phenomenon of regulated translocation across the ER membrane. Under this scenario, the translation and translocation of apoB become uncoupled after synthesis of the first 85 kDa of the protein (Du et al. 1994). This generates a transmembrane form in which the bulk of the protein mass restricts the ability of the cell to synthesize or mobilize triglyceride for secretion (Wang et al. 1994). The relationship between apoB assembly and intracellular cholesterol and cholesterol ester mass and synthesis has been controversial, with some investigators reporting a major regulatory role for the intracellular cholesterol esterifying enzyme, acyl-CoA: cholesterol acyltransferase (Cianflone et al. 1990, Musanti et al. 1996, Thompson et al. 1996) and others observing that it is triglyceride synthesis alone that drives lipoprotein secretion (Benoist and Grand-Perret 1996, Wu et al. 1996a). Irrespective of the effects of lipids and other factors on apoB secretion, a prevailing view has emerged in which the apoB protein is made in excess and directed toward a pool involved in both particle assembly and secretion or intracellular degradation. The question of how the cell is able to distinguish between these two pools of apoB has important implications with respect to the regulation of VLDL triglyceride secretion and the prevention and management of genetically and environmentally induced hyperlipidemias.
is situated on the cytosolic side of the ER membrane. It has been proposed that if lipid becomes available, transmembrane apoB can undergo a process of post-translational “lipid-facilitated translocation” into the lumen of the ER (Sakata et al. 1993). However, if the translocation-arrested form of apoB persists, it is targeted for degradation by the ubiquitin/proteasome–mediated pathway in the cytosol (Fishier et al. 1997, Yeung et al. 1996). In large part, this model of regulated translocation is based on the detection of transmembrane apoB by the use of exogenous proteases that degrade putatively cytosolic domains of apoB present in membrane-derived vesicles and permeabilized cells (Davis et al. 1990, Dixon et al. 1992, Du et al. 1994 and 1998, Macri and Adeli 1997, Rusinol et al. 1993, Thrift et al. 1992). However, others have failed to confirm the existence of cytosolic apoB (Ingram and Shelness 1996, Leiper et al. 1996, Pease et al. 1995, Shelness et al. 1994, see below).

SYSTEMATIC SCREENING OF APO B FOR DOMAINS CAPABLE OF REGULATING TRANSLLOCATION

The proper targeting and compartmentalization of proteins within cells requires topogenic signals, usually in the form of primary structural motifs. As with most proteins destined for secretion, apoB is synthesized with an amino terminal hydrophobic signal peptide that specifies targeting of the ribosome/nascent polypeptide chain complex to the ER membrane. Once the ribosome has been targeted and steps required for the initiation of translocation completed, translation and translocation across the ER membrane are believed to occur in coordinated fashion. The coupling of translation and translocation is due to the existence of a tight junction between the ribosome and the Sec61p translocation pore. Hence, in most cases, the nascent polypeptide chain is transferred from the ribosome directly to the ER lumen without encountering the cytosolic compartment (Johnson et al. 1995).

The interaction between the translating ribosome and the membrane can be dynamic. Studies by Lingappa and colleagues (Chuck et al. 1990, Chuck and Lingappa 1992) utilizing cell-free translation of apoB in the presence of canine pancreas micosomes showed that the translocation of apoB into membrane vesicles becomes transiently uncoupled from its translation, thereby giving rise to cytoplasmic loops (Chuck et al. 1990). The sequence domains responsible for this behavior were termed “pause transfer” (Chuck and Lingappa 1992) and were mapped to multiple sites within apoB. Although the translocational pausing observed in this system is transient, conditions were developed under which apoB could be maintained in the paused condition for prolonged periods of time. Under these conditions, the pausing of translocation was accompanied by a disruption of the normally tight junction between the ribosome and the ER membrane (Hegde and Lingappa 1996). However, when translocation of paused intermediates was restarted, complete translocation of the nascent chain occurred. Hence, the translocation of apoB in a cell-free system occurs efficiently, albeit in a fashion that is not strictly coupled to translation.

The behavior of apoB in cell-free systems is distinct from the behavior in cells in which, at steady state, the majority of apoB is putatively found in a translocation-arrested form. Furthermore, the transmembrane apoB in cells has never convincingly been shown to be a metabolic precursor of fully translocated apoB (Du et al. 1998, Macri and Adeli 1997). In an attempt to identify structural features of apoB required to achieve translocation arrest in cultured cells, a series of fusion proteins were developed in which 300 amino acid overlapping segments of apoB48 were appended to the amino-terminal 77 amino acids of a modified form of bovine preprolactin (Shelness et al. 1994). This strategy enabled the preprolactin region of each construct (considered to be a generic model secretory protein) to initiate ribosome targeting and translocation before the emergence of the apoB test sequence from the ribosome. The extent of translocation arrest or delay associated with each segment of apoB could then be measured by treating ER-derived membrane vesicles with exogenous protease and by examining the extent to which glycosylation sites engineered into the N- and C-terminal sides of the test sequences were utilized. On the basis of these criteria (virtually complete resistance to protease and little glycosylation heterogeneity), none of the apoB sequences was capable of detectably delaying or arresting translocation in transfected COS-1 cells (an MTT-negative cell line) or HepG2 cells (Ingram and Shelness 1996, Shelness et al. 1994). These data demonstrated that neither pause transfer nor any other isolated domain present in apoB48 was capable singly of detectably delaying or arresting translocation in vivo.

EVIDENCE THAT APO B DESTINED FOR LIPOPROTEIN ASSEMBLY OR INTRACELLULAR DEGRADATION UNDERGOES EFFICIENT TRANSLLOCATION ACROSS THE ER MEMBRANE

In unstimulated HepG2 cells, ~20% of the endogenously expressed apoB100 has been reported to undergo complete translocation into the lumen of the ER (Bonnardel and Davis 1995, Du et al. 1994). This is similar to the amount of apoB that undergoes assembly into a secretable lipoprotein (Dixon et al. 1991) and is consistent with the idea that successful translocation is observed only when apoB assembles with lipids. However, when the percentage of transmembrane apoB in unstimulated HepG2 cells was systematically studied with the use of a protease protection assay, the mean percentage protection was 84 ± 18% (n = 7), a value similar to that observed for several control secretory proteins (Ingram and Shelness 1996, Leiper et al. 1996). Furthermore, no significant difference in protease protection was observed in control cells or cells treated with N-acetyl-Leu-Leu-norleucinal (ALLN). N-Acetyl-Leu-Leu-norleucinal is a tripeptide derivative that inhibits a variety of cysteine proteases, including the cytoplasmic proteasome (Coux et al. 1994). Because it has been proposed that the substrate for the ALLN-inhibitable protease is the translocation-arrested form of apoB (Bonnardel and Davis 1995, Sakata et al. 1993, Thrift et al. 1992, Yeung et al. 1996), one should see a relative increase in transmembrane apoB in ALLN-treated cells. However, despite a sixfold increase in the amount of intracellular apoB in ALLN-treated vs. control cells, the percentage of protease-resistant apoB remained uniformly high (Ingram and Shelness 1996). These results suggest that apoB destined for intracellular assembly into lipoprotein particles or destined for intracellular degradation by the ALLN-sensitive proteolytic system are both efficiently translocated into the ER lumen.

If apoB is efficiently translocated into the ER, how does it become exposed to the cytosolic proteasome? A general pathway for the disposal of misfolded secretory proteins at the level of the ER has been described recently. Surprisingly, these proteins appear to undergo a retrograde translocation (dislocation) reaction before being transported back to the cytosol (Fishier et al. 1996, Qu et al. 1996). Apolipoprotein B may therefore undergo complete translocation into the ER followed by either a co- or post-translational dislocation of all or part of the apoB
polypeptide chain into the cytosol (Fig. 2). The fact that this process generates little detectable transmembrane apoB indicates that the dislocation reaction may be temporally and mechanistically coupled to proteasome-mediated degradation (Kopito 1997). Although the disposal of apoB in hepatocarcinoma-derived cell lines appears to occur predominantly at the level of the ER, apoB turnover in primary rat hepatocytes may involve vesicular sorting to a post-ER compartment (Wang et al. 1995). In either case, the balance between assembly and precocious degradation may represent a major control point for the production of hepatic VLDL.

**INITIATION OF APO B–CONTAINING LIPOPROTEIN ASSEMBLY: ROLE OF THE α1 DOMAIN**

The initial association of apoB with lipids is believed to occur during its cotranslational translocation into the ER. The basis for this prediction lies in the fact that nascent chains of apoB in HepG2 cells are released from the ribosome with puromycin, they are recovered from the ER in the form of nascent lipoprotein particles whose diameter increases as a function of apoB length (Borén et al. 1992, Spring et al. 1992). The same relationship between apoB length and lipoprotein size was observed for a series of C-terminally truncated forms of apoB expressed in rat hepatoma cells (Graham et al. 1991, Shelness and Thornburg 1996). These studies revealed the following features distinct from those of the rest of the apoB protein: 1) it is globular and highly disulfide bonded [6 of the 8 disulfide bonds in apoB-100 are positioned within the amino-terminal 11% of the protein (Yang et al. 1990)]; 2) it demonstrates a lower affinity for plasma LDL particle than internal and carboxyl-terminal domains (Chan 1992, Segrest et al. 1994); and 3) in transfected cells, forms of apoB containing only the α1 domain (e.g., apoB-18) lack the capacity to recruit a significant amount of lipid and can be secreted independently of MTP (Graham et al. 1991, Yao et al. 1991).

Because of the asymmetric distribution of disulfide bonds in the α1 domain, the thiol-reducing agent dithiothreitol was used to perturb its folding and assess the effects on the capacity of apoB to initiate lipoprotein formation (Ingram and Shelness 1997, Shelness and Thornburg 1996). These studies revealed that the following features of α1 domain that are relevant to understanding how apoB-containing lipoprotein assembly may be initiated: 1) The α1 domain of apoB was observed to fold during the process of translation, consistent with a domain-specific folding reaction that is completed before the translocation and translation of the more internal lipophilic regions of apoB (i.e., the β2 domain). 2) The cotranslational folding of the α1 domain occurred to a similar extent and with similar kinetics in both hepatoma and Chinese hamster ovary (CHO) cells, which lack MTP. Therefore, the folding of this domain is achieved independently of MTP and before events associated with buoyant lipoprotein formation. 3) Forms of apoB in which folding of the α1 domain was blocked were incapable of undergoing detectable assembly with lipid. Hence, in addition
FIGURE 3  Hypothetical functions of the \( \alpha_1 \) domain of apolipoprotein (apo) B in the initiation of apoB-containing lipoprotein assembly. Previous results demonstrated that if folding of the amino-terminal disulfide-bonded domain of apoB is disrupted by dithiothreitol (Ingram and Shelness 1997, Shelness and Thornburg 1996) or mutagenesis (Gretch et al. 1996, Huang and Shelness 1997, Tran et al. 1998), apoB is incapable of engaging in lipoprotein formation. Several hypothetical functions of the amino terminal domain of apoB that may be essential for the initiation of lipoprotein formation are diagrammed and discussed in text. Abbreviations: MTP, microsomal triglyceride transfer protein; PL, phospholipid; TG, triglyceride.

to its unique structural properties and folding kinetics, the amino terminal \( \alpha_1 \) region of apoB was indeed shown to be essential to initiate lipoprotein formation in the ER.

**HYPOTHETICAL FUNCTIONS OF THE \( \alpha_1 \) DOMAIN OF APO B IN THE INITIATION OF LIPOPROTEIN ASSEMBLY**

Although apoB-17 is secreted in lipid-poor form from both hepatic and nonhepatic cells, Herscovitz et al. (1991) demonstrated that apoB-17 can bind to dimyristoylphosphatidylcholine multimamellar vesicles and convert them to discoidal recombinant lipoproteins in vitro. In addition, apoB-15, translated in vitro in the presence of rat liver microsomes, undergoes association with newly synthesized phosphatidylcholine (Rusnàk et al. 1997). Hence, one potential function of the \( \alpha_1 \) domain may be to recruit a phospholipid surface early in translation that would then serve as an acceptor site for MTP-mediated lipid transfer (lower right corner of Fig. 3). The subsequent delivery of neutral lipid to this site, concomitant with the translation and translocation of the internal lipophilic regions of apoB into the ER, would drive the formation of a primordial core-containing lipoprotein, akin to the maturation of discoidal HDL to spherical HDL (Hamilton et al. 1976, Tall and Small 1978). Another potential function of the \( \alpha_1 \) domain of apoB may be to associate with the inner leaflet of the ER membrane. This association may initiate formation of a triglyceride droplet (Fig. 3, upper right corner). At some point during or after translation, this droplet may bud from the membrane into the lumen of the ER (Atkinson and Small 1986, Olofsson et al. 1987, Schumaker et al. 1994). In support of this model, Pease et al. (1991) showed that during cell-free translation in the presence of dog pancreas microsomes, apoB-17 translocates into the inner leaflet of the ER membrane in a form that cannot be extracted with sodium carbonate, pH 11.5. Yet another potential function of the \( \alpha_1 \) domain of apoB is to provide a binding site for MTP (Fig. 3, upper left corner). Wu et al. (1996b) demonstrated that MTP is physically associated with apoB during lipoprotein assembly in the ER on the basis of co-immunoprecipitation of both apoB and MTP with antibodies directed against either of the two proteins. Microsomal triglyceride transfer protein interacts with forms of apoB containing as little as the amino-terminal 17% of the protein (Hussain et al. 1997, Patel and Grundy 1996). These studies indicate that apoB may engage MTP at an early stage of its translation and that this interaction is critical to achieve net lipid transfer to apoB during its translation and/or to serve as a chaperone for critical folding and assembly events necessary for lipoprotein particle assembly (Fig. 3, upper left panel).

Although the various properties of the \( \alpha_1 \) domain of apoB diagrammed in Figure 3 and discussed above are intriguing, their relationship to the mechanism of apoB-containing lipoprotein assembly remains speculative. A molecular description of this process awaits the development of more detailed structural and functional characterization of the apoB protein, a task that is currently underway in several laboratories.

**LITERATURE CITED**


