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Invited Review

Cell and molecular biology of the assembly and secretion of apolipoprotein B-containing lipoproteins by the liver

Roger A. Davis *

Mammalian Cell and Molecular Biology Laboratory, Department of Biology, The Molecular Biology Institute, San Diego State University, San Diego, CA 92182-0057, USA

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Abstract

Triglycerides are one of the most efficient storage forms of free energy. Because of their insolubility in biological fluids, their transport between cells and tissues requires that they be assembled into lipoprotein particles. Genetic disruption of the lipoprotein assembly/secretion pathway leads to several human disorders associated with malnutrition and developmental abnormalities. In contrast, patients displaying inappropriately high rates of lipoprotein production display increased risk for the development of atherosclerotic cardiovascular disease. Insights provided by diverse experimental approaches describe an elegant biological adaptation of basic chemical interactions required to overcome the thermodynamic dilemma of producing a stable emulsion vehicle for the transport and tissue targeting of triglycerides. The mammalian lipoprotein assembly/secretion pathway shows an absolute requirement for: (1) the unique amphipathic protein: apolipoprotein B, in a form that is sufficiently large to assemble a lipoprotein particle containing a neutral lipid core; and, (2) a lipid transfer protein (microsomal triglyceride transfer protein-MTP). In the endoplasmic reticulum apolipoprotein B has two distinct metabolic fates: (1) entrance into the lipoprotein assembly pathway within the lumen of the endoplasmic reticulum; or, (2) degradation in the cytoplasm by the ubiquitin-dependent proteasome. The destiny of apolipoprotein B is determined by the relative availability of individual lipids and level of expression of MTP. The dynamically varied expression of cholesterol-7α-hydroxylase indirectly influences the rate of lipid biosynthesis and the assembly and secretion lipoprotein particles by the liver.

Keywords: Apolipoprotein B; Lipoprotein; Lipid; Protein translocation; Protein degradation

Contents

Abbreviations: apo B, apolipoprotein B (note that the number following apo B designates the % of the maximal coding region expressed, apo B100 being the entire coding region); MTP, microsomal triglyceride transfer protein; 7α-hydroxylase, cholesterol-7α-hydroxylase NADPH:oxygen oxidoreductase (EC 1.14.13.17); PDI, protein–disulfide isomerase; VLDL, very low density lipoprotein; DTT, dithiothreitol; ALLN, *N*-acetyl-leucyl-leucyl-norleucinal; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SRP, signal recognition particle

* Corresponding author. Fax: +1-619-594-7937; E-mail: rdavis@sunstroke.sdsu.edu

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1. Introduction

The ability to store energy as fat (about 9 kcal/g) is significantly greater than the amount that can be stored as carbohydrate (about 4 kcal/g). The evolutionary development of processes to transport and store energy in the form of fat has provided organisms with a thermodynamic advantage in surviving catastrophic loss of food and essential fat nutrients. Moreover, nutritional and energy requirements for reproduction further demanded that fat readily be mobilized for transport to reproductive organs in an efficient and specific manner. The evolution of the lipoprotein assembly/secretion pathway provides these functions. Because of the essential role that lipid transport and storage has played in the evolutionary development of complex multi-cellular organisms, the lipoprotein transport system is intimately linked to the most basic biological functions: membrane biogenesis, cell division and gene expression. The movement of a diverse group of lipid substrates through the lipoprotein assembly/secretion/metabolism pathway is dynamically varied in response to nutritional, developmental and reproductive needs. In today's human world where the availability of 'fast-food' is commonplace, this efficient dynamic response, which was essential for survival of ancestral organisms, contributes to three major health threats: atherosclerotic cardiovascular disease, obesity and diabetes. Much effort has been invested into research whose major goal has been to gain an understanding of the molecular mechanisms that control the hepatic assembly and secretion of apo B-containing lipoproteins. This investment has yielded significant rewards in terms of new knowledge (reviewed by [1–5]). Moreover, these insights have been successfully applied in the development of a new class of therapeutics that act specifically on the intracellular process responsible for lipoprotein assembly [6]. We will review the progress made toward understanding the cell and molecular biology of the assembly and secretion of apo B-containing lipoproteins by the liver.

2. Early studies of inherited human hypolipidemic disorders indicated that the secretion of triglyceride-rich lipoproteins required the 'B' apoprotein and at least one additional protein

Analysis of the data obtained in one of the most comprehensive epidemiologic studies provided compelling evidence that plasma LDL is a major risk factor in the development of atherosclerosis cardiovascular disease [7,8]. The recognition of LDL as a potentiator of cardiovascular disease stimulated interest in determining its structure, biogenesis and metabolism. These efforts would ultimately pay off in the development of effective therapeutic interventions to lower LDL levels and significantly reduce both the morbidity and mortality associated with cardiovascular disease [9,10]. One of the major intellectual hurdles to overcome was to delineate the structure of the protein in LDL: apo B. As discussed below, many of the structural aspects of apo B as well as many of the processes required for the biogenesis of apo B-containing lipoproteins were without precedence.

Technical advances in chemistry, genetics and physiology preceded those required for characterization of proteins, DNA, RNA and molecular genetics. Thus, phenotypic characterization of inherited human disorders were established years before the responsible proteins, genes and mRNAs were identified. While the genetic defect responsible for the abetalipoproteinemic phenotype would take almost 30 years to unravel, it was recognized as being a familial trait associated with an almost complete inability by the liver and intestine to secrete apo B-containing lipoproteins (reviewed in [11–14]). This recessive phenotypic disorder provided a clear link between the presence in plasma of the β-migrating lipoproteins and the ability of the intestine and liver

to package and transport exogenous ingested and endogenous de novo synthesized fat [15,16]. Additional studies showing that the protein component in LDL was absent in the plasma of abetalipoproteinemics, suggested it played an important role in the assembly of lipoproteins [17]. The autosomal inherited disorder hypobetalipoproteinemia displayed a phenotype somewhat similar to that of abetalipoproteinemia (i.e., inability to absorb and transport fat [18–20]). The insightful recognition that the co-dominant inheritance of hypobetalipoproteinemia was distinct from the recessive inheritance of abetalipoproteinemia, led to the prescient prediction that the two disorders involved at least two different genes [20]. Thus, these findings suggested that apolipoprotein B and at least one additional gene product were essential for the assembly and secretion of triglyceride-rich lipoproteins.

2.1. *The structure of apolipoprotein B is as unprecedented as the processes that are responsible for its biogenesis and assembly into triglyceride-rich lipoproteins; LDL is derived from hepatic VLDL*

It was clear from early studies of the properties of apolipoprotein B that it behaved differently from the other apolipoproteins. Unlike most other apolipoproteins, apo B can not exchange freely among lipoprotein particles. While the functional significance of this unique property in regard to lipoprotein assembly would not become apparent for several years, it served useful in turn-over studies which established that VLDL was the precursor to the major protein in LDL [21]. The development of systems to culture primary hepatocytes provided the additional finding that essentially all the apo B that was secreted by rat hepatocytes was in the VLDL fraction [22]. These combined data suggested that LDL was not a secretory product of the liver, but was derived in plasma via the metabolism of VLDL. Without knowing the molecular mass of apo B, investigation of the molecular aspects of apo B biogenesis was greatly hampered. While one could produce antibodies using LDL or extracts of LDL as the antigen, one could not unambiguously identify the epitopes recognized by the antibodies.

2.2. *There are two distinct molecular mass forms of apo B*

Until around 1980, the molecular mass of apo B remained unknown. Obtaining this information was an exceedingly intractable problem. Those who attempted to elucidate the structure of apo B were confounded with its unusual physical/chemical properties. For example, when delipidated, the major protein in LDL was observed to be water insoluble and formed large molecular mass aggregates. Subsequent studies showed that delipidated apo B formed intermolecular disulfide-linked 'polymers', suggesting that free sulfhydryl groups were buried in the lipid core [23]. Application of common SDS–PAGE and chromatography systems suggested that the molecular mass of apo B was 4–10% of what was eventually delineated using molecular genetics [24–26]. In later studies of the cellular processing of apo B, the physiologic significance of these small apo B peptides would become apparent [27,28]. The insightful use of low percentage polyacrylamide gels provided a major technical advance that allowed the molecular mass of apo B to be determined [29,30] as well as providing the means to prepare pure preparations of apo B. The discovery that two distinct large molecular mass forms of apo B existed in rat [29] and human [30] plasma raised as many questions about the biogenesis of apo B as it answered questions about its structure and metabolic relationships. The molecular mass of the larger form of apo B ($>550\,000$ kDa), found mostly on LDL, was $\sim$twice that of the smaller molecular mass form. Subsequent studies in rats showed that the smaller form (hereafter referred to as apo B48) was removed from plasma by the liver much more rapidly than the larger form (hereafter to be referred to as apo B100) [31,32]. Additional studies showed that apo E enhanced the hepatic uptake of apo B48-containing remnants [33,34] via a process blocked by the C apoproteins [35]. The more rapid uptake of apo B48-containing lipoproteins by the liver may help to explain why apo B100 is retained as a component of human LDL [36].

2.3. Two distinct molecular mass forms of apo B, derived from the same gene, led to the discovery of apo B mRNA editing

The finding of two distinct molecular mass forms of apo B was consistent with two possibilities: (1) that the smaller apo B48 may have been derived from the larger apo B100; or, (2) that apo B48 and apo B100 were each produced from separate genes. (The unlikely possibility that the larger apo B100 was derived by linking two apo B48 molecules was ruled out by analysis of [^{125}I]apo B48 turnover studies [36].) The additional observation that one abetalipoproteinemic patient produced intestinal apo B48 without an impairment of intestinal fat absorption, whereas the liver produced no apo B100 was interpreted to suggest that there were two independently regulated apo B genes [37]. Considering that in the human, the liver selectively synthesizes apo B100, whereas the intestine selectively synthesizes apo B48, the proposal that one gene encodes liver apo B100 and the other gene encodes intestinal apo B48 seemed to be a logical explanation at the time. As described below, the molecular events responsible for apo B biogenesis would deviate considerably from what one might have predicted.

During the 1980s, molecular cloning and nucleic acid chemistry techniques became available for general laboratory use. For many in the field who had experienced the intrinsic technical challenges of working with apo B, it was soon realized that the structure of apo B would be more likely delineated from its mRNA sequence than its amino acid sequence. These predictions were soon to become reality. The first report of the successful cloning of a apo B cDNA immediately revealed some surprising new insights [38]. As expected the size of the apo B mRNA in the liver of rats was large ($\sim$20 kb) [38]. More striking was the unexpected finding that the intestine, which produces only apo B48, showed a single mRNA having the same size as the one in the liver [38]. Similar findings were obtained for the human apo B mRNA [39–43] and the monkey apo B mRNA [44]. Furthermore, using the human apo B cDNAs, which obviously contained nucleotide sequences common to both apo B100 and apo B48, a single gene on the short arm of chromosome 2 (2p23–2p24) was identified [40,42,44–46]. The combined findings suggested that a single apo B gene, produces a single sized mRNA which is translated into the two different molecular mass forms of apo B! Using an immunologic experimental approach, both apo B100 and apo B48 were found to contain a monoclonal antibody epitope attributed to a specific genetic polymorphism [47]. These data provided additional support indicating that apo B100 and apo B48 are derived from the same gene. The mechanism accounting for the production of the two molecular mass forms of apo B would provide an additional example of the unprecedented characteristics of apo B biology.

2.4. A single apo B gene produces two distinct proteins; elucidation of apo B mRNA editing

Since a single apo B gene produced a single size mRNA, which produced two distinct proteins (i.e., apo B100 and apo B48), differential splicing of mRNA seemed an unlikely explanation. This conclusion was further supported by the findings that the cDNA prepared from human liver apo B mRNA, which produces only a single protein (apo B100), had almost the same sequence as the cDNA prepared from human intestinal apo B mRNA, which produces only apo B48. The only difference was a single C$\rightarrow$U base substitution at position 6666 (codon 2153) [48]. As a result, the CAA (Gln) codon was converted to a stop codon, suggesting that apo B48 is produced by a premature termination of translation (i.e., editing of the apo B100 mRNA) [48]. These findings were soon corroborated and extended by sequence analysis which indicated human apo B48 obtained from human chylous ascites fluid contained a C-terminal methionine (produced from codon 2151) [49]. It was proposed that the expected C-terminal Ile, produced by codon 2152, missing in human apo B48, was cleaved post-translationally [49]. Additionally, two separate groups reported that following the editing reaction, intestinal apo B48 mRNA was cleaved and polyadenylated 5′ to the polyadenylation site on apo B100 mRNA, making it smaller [49,50]. The significance of the variable finding of a smaller apo B mRNA in the intestine compared to the liver remains unknown.

The unexpected discovery of apo B mRNA editing in 1987 provided an explanation for why the human

liver produced only apo B100 (no apo B mRNA editing), whereas the liver of most rodents produce both apo B100 and apo B48 (high activity of apo B mRNA editing) (reviewed in [51,52]). Moreover, since it had been well-established that apo B48 was more rapidly removed from plasma compared to apo B100, the discovery of apo B mRNA editing provided an opportunity to gain an understanding of why rodents display resistance to dietary-induced hypercholesterolemia and atherosclerosis, whereas humans are susceptible [53]. It was reasoned that if one could understand the molecular events controlling apo B mRNA editing, development of methods to induce this process in the human liver might be therapeutically effective in decreasing the susceptibility to hypercholesterolemia and atherosclerosis. It would take 5 years of intensive research before the gene product responsible for apo B mRNA editing was identified. Once again, a creative experimental approach using molecular biology succeeded to solve a problem which proved to be intractable to a protein chemistry approach. However, insights gained from protein chemistry and enzymology were necessary to implement the successful complementary cloning of apo B mRNA editing enzyme.

In contrast to mammalian intestine, chicken intestine neither edits apo B mRNA nor does it secrete apo B48 [54]. However, when the cytosol of chicken enterocytes was added to extracts from tissues and cells that do edit apo B mRNA, editing activity was enhanced [54]. These findings were interpreted to indicate that apo B mRNA editing evolved by adapting tissue factors that are present in chicken intestine and are not used for apo B mRNA editing [54]. These tissue factors are presumed to be sub-units of the apo B mRNA editing enzyme complex [55–57]. One of these factors is an AU-binding protein that specifically recognizes a stem–loop structure in apo B mRNA [58]. While the function of the factors in chicken intestine remains unknown, their ability to activate apo B mRNA editing was shown to be essential to clone the editing enzyme from rat [59]. Using oocytes to express mRNA from rat intestine, a factor was produced that in the presence of chicken intestinal cytosol allowed functional apo B mRNA editing to occur [59]. The cDNA subsequently isolated was shown to encode a 27 kDa protein that acts as a cytidine deaminase [60]. The zinc-containing deaminase, which has been named APOBEC-1 [61] operates catalytically like the *Escherichia coli* cytidine deaminase that uses monomeric substrates [62]. Consistent with what was predicted based on the metabolic differences between apo B100 and apo B48, over-expression of APOBEC-1 in animals ameliorated dietary induced hypercholesterolemia due to a reduction in hepatic apo B100 secretion [63–65].

2.5. *The structure of apo B shows the same degree of complexity as the processes responsible for its production*

The isolation of cDNAs containing the portions of the coding region of apo B100 provided a technically difficult, but not insurmountable method to at last determine the entire coding sequence [66–69]. It is interesting to note that four different groups of collaborators succeeded in this difficult technical achievement and published their results within a few months of each other. Moreover, there was a remarkable consensus concerning the sequence, secondary structure and functional domains of apo B100. Apo B contains a relatively large 27 amino acid N-terminal signal sequence, which is cleaved prior to being secreted into plasma as a 4536 amino acid protein having a molecular mass of 513 kDa [66–69]. A polymorphism in the human apo B N-terminal signal sequence has been shown to correlate with plasma lipid levels, suggesting that it may play a role beyond its binding to SRP [70]. The additional finding that allelic differences in the N-terminal signal sequence altered the secretion of invertase chimeras in yeast led to the proposal that polymorphisms in the signal sequence may affect the production of lipoproteins by the liver [71].

Apo B100 was predicted to contain a globular N-terminal domain followed by several functionally important structural motifs that may facilitate the association with lipids. Amphipathic β-sheets, having hydrophobic and hydrophilic surfaces are located throughout the apo B-100 molecule. Short stretches of amphipathic α-helices (predicted to be too short to act as typical membrane spanning domains) are mainly distributed in two clusters: one located at amino acids 2100–2700 and the other located at amino acids 4100–4500. These amphipathic α-helices may form the reversible lipid-association domains.

Alternating amphipathic β-sheets allows the formation of a pentapartite structure (NH_2–α_1–β_1–α_2–β_2–α_3–COOH) which has been proposed to explain how apo B100 is situated in the LDL particle [72]. The amphipathic β-sheets may play important roles in directing the cellular processing of nascent VLDL apo B (see below).

Several lines of evidence suggest that residues 3359–3367 in apo B100 are responsible for high affinity recognition by the LDL receptor. First, residues 3359–3367 show a remarkable identify to the sequences in apo E shown to contain the high affinity LDL receptor binding domain [66]. Secondly, monoclonal antibodies having an epitope at residue 3249 specifically impair the binding of human LDL to the LDL receptor [73]. Furthermore, mutations causing a loss of arginine at 3500 [74,75] or at 3531 [76] block binding to the LDL receptor.

Several co- and post-translational modifications of the apo B structure may have significant impact on its structure and function. Within human apo B100 there are 25 cysteine residues of which 16 form intramolecular disulfide bonds. Of these 16 disulfide-linked cysteines, 14 are located on the amino-terminal portion, resulting in a globular intramolecular linked N-terminus. The disulfide cross-linked globular N-terminus may play a functional role in the assembly of apo B-containing lipoproteins via its interaction with MTP (see below). The C-terminal cysteine at residue 3426 forms the disulfide link of LDL apo B100 to glycoprotein a in the Lp(a) complex [77]. The Lp(a) complex has been proposed to be an important factor in the atherogenic process [78]. It is interesting to note that apo B shares homology with vitellogenin [79], whereas glycoprotein a shares homology with plasminogen [80]. There seems to be little functional basis for the structural similarities between glycoprotein (a) and plasminogen, a proteolytic enzyme involved in blood clotting. Recent studies show that transglutaminase, a blood clotting enzyme from crayfish, also shares homology with vitellogenin [81]. These data raise the possibility that lipoprotein transport and blood clotting systems may have evolved from each other or a common ancestor. While plasma LDL levels appear to correlate with blood coagulation activity [82], it is unknown if this is related to the ability of apo B to express transglutaminase enzyme activity.

Apo B100 contains at least 20 potential N-linked glycosylation sites of which four are located in β-turn structures. Since VLDL apo B is secreted containing N-linked high mannose and mature (resistant to endoglycosidase H) carbohydrate chains [83] and tunicamycin-blocked glycosylation has no effect on VLDL apo B secretion [84,85], is seems likely that glycosylation is not essential for VLDL assembly and secretion.

Cultured rat hepatocytes secrete apo B containing phosphoserine residues [86]. Subsequent studies showing that phosphorylated apo B was present in rough microsomal fractions suggest that phosphorylation occurs early in the secretory pathway [87]. Hepatocytes from diabetic rats secrete apo B which contains both phosphoserine and phosphotyrosine residues [88]. Since plasma apo B contains markedly less phosphoserine compared to that secreted by cultured hepatocytes this suggests that dephosphorylation occurs in plasma [86,87]. It is interesting to note that vitellogenin, thought to be the ancestor of apo B [79], also contains several phosphoserine residues [89]. While there appears to be a precedence for lipoprotein transport proteins to contain phosphoserine residues, which may play a role in calcium binding, the functional significance in regard to lipoprotein assembly, secretion and transport are not known. Apo B also contains covalently linked fatty acid groups, whose function remain undefined [90].

2.6. The unusually large size of apo B48 may reflect requirements for lipoprotein assembly

The inability to absorb and transport triglycerides displayed by hypobetalipoproteinemic patients predicted that a large apo B molecule is essential in order to assemble large triglyceride-rich lipoproteins [18–20,91,92]. Sequence analysis of the mutation responsible for the production of truncated apo B in one proband showed that a four base deletion resulted in a frame-shift and a premature stop codon [93]. These data provided compelling evidence that mutations in the apo B gene that result in the production of truncated forms of apo B are responsible for the hypobetalipoproteinemic phenotype [93]. Subsequent studies have shown that while apo B is one of the most polymorphic genes known, most mutations do not impair the ability to assemble lip-

oproteins [94,95], or to bind to the LDL receptor [96]. The most prevalent mutations in apo B that both alter function and metabolism, as defined by plasma lipoprotein levels, are those that result in either truncated translation products (hypobetalipoproteinemia) or impaired binding to the LDL receptor (substitution of either Arg 3500 with Gln or Arg 3531 with Cys) [96,97].

The development of methods to determine the enrichment of stable isotopes has provided the opportunity to perform safe turnover studies in humans. Recent studies show that in hypobetalipoproteinemic patients, the secretion rate of apo B was linearly linked to its length ($r^2 = 0.86$, $P < 0.0001$), with secretion being reduced by 1.4% for each 1% of apo B truncated [98]. The linear regression line of size versus secretion rate displayed a zero intercept for apo B secretion at apo B28, which is consonant with the apparent absence in plasma of truncations smaller than apo B25. Additional stable isotope turnover studies showed that limited C-terminal truncations which produce ~apo B50 or greater are associated with greater rates of removal from plasma [99,100]. These results suggest that removing portions of the extreme C-terminus, which do not include the LDL receptor binding domain, increases the affinity for the LDL receptor [101]. Thus, apo B size both determines its ability to produce secretion-competent lipoproteins and to be removed from plasma via its high affinity binding to the LDL receptor.

The development of methods to obtain monoclonal epitope-specific antibodies and to construct artificial genes for expression in mammalian cells and animals provided a means to delineate how apo B structure affects its function. Studies using rat [102] and human [103–105] hepatoma cells provided clues to why the apo B molecule is so large. Apo B forms that are shorter than ~apo B31 were secreted by McArdle rat hepatoma cells without a triglyceride-rich core (i.e., they were secreted in a form having a density > 1.21 g/ml) [102]. In HepG2 cells, apo B41 was secreted solely as a lipoprotein particle, whereas small apo B (e.g., apo B17) was secreted mainly without a lipoprotein core (i.e., $d > 1.21$ g/ml) [103–105].

In one study, least squares analysis of the relationship between the size of apo B and the estimated core circumference defined a straight line of near-zero intercept [105]. The slope of this line was approximately 1 Å of core circumference/1 kDa of apo B molecular mass. These combined findings suggest that one function of the large size of apo B is to provide a lattice in order to form the neutral lipid core of apo B-containing lipoproteins. Support for this proposal was obtained using an elegant experimental approach in which electron microscopy was used to map the localization of specific epitopes of apo B as determined by the binding of monoclonal antibodies to LDL [106,107]. Since the apo B sequences of the epitopes had been defined and it had been established that there was one apo B molecule/LDL particle [108], the relative 'polar' coordinates of epitopes were assigned in a manner similar to that used for global navigation [106]. This approach provided a three dimensional array of apo B as it surrounds the LDL neutral lipid core. The results led the authors to propose a model in which the first 89% of apo B100 forms a thick ribbon that completes a circle around the particle at residue 4050 and is in contact with the neutral lipid core. There is a kink residing close to the middle of apo B100 ribbon. The C-terminal 11% of apo B constitutes a 'bow,' defined as an elongated structure of about 480 residues, beginning at 4050 and stretching back into one hemisphere and then crossing the ribbon into the other hemisphere between residues 3000 to 3500. As a result, the C-terminal portion of apo B100 may interact with sequences that reside near to the suggested binding site for the LDL receptor [109]. Nascent triglyceride-rich lipoprotein particles made by the liver and intestine contain one apo B100/VLDL particle [110] and one apo B48/chylomicron remnant particle [111], respectively. These data suggest that apo B also circles the core of nascent triglyceride-rich lipoprotein particles in a manner similar to that described for LDL. The combined data suggest that the circumference of the circle made by apo B is likely to be one criterion that determines the size of the neutral lipid core and thus the capacity to transport triglycerides and cholesterol esters. However, apo B size alone can not explain: (1) why apo B48/triglyceride-rich lipoproteins secreted by the intestine are significantly larger than apo B100/triglyceride-rich lipoproteins secreted by the liver [112]; or, (2) why the size of apo B100-containing VLDL is indistinguishable from that of apo B48-containing VLDL secreted by rat hepatocytes [113].

Clearly, there must be criteria in addition to apo B size that influence the size of the lipoprotein particle.

The development of methods to delete specific sequences in the mammalian genome via homologous recombination has provided the opportunity to create mouse models of many human deficiency diseases. Heterozygous mice having one allele of the apo B gene deleted [114] as well as single allelic mutations causing the production of a truncated forms of apo B [115] display a phenotype that is similar to that of hypobetalipoproteinemic human patients. However, the findings that many of the heterozygous mice displayed abnormal neurological development [114,115], whereas homozygosity was embryonic lethal [114,116] suggest that in mice the apo B-containing lipoprotein assembly/secretion pathway is essential for fetal development. This is in contrast to humans, who do not appear to have an absolute requirement for apo B-containing lipoproteins during fetal development. It has been proposed that in mice, the apo B-containing lipoprotein assembly/secretion pathway is essential for transporting essential lipids from the yolk sac to the fetus [114]. Apparently in humans, maternal lipoproteins provide essential lipids to the fetus, thus abrogating the fetal requirement for apo B-containing lipoproteins.

Genetically altered mice have also provided new insights into the functional importance of the C-terminal sequences in apo B100, which affect the removal of LDL from plasma via the LDL receptor [96,97,117]. Human apo B variants expressed in transgenic mice show intramolecular peptide-peptide interactions, predicted by the ribbon-bow model of apo B circling the LDL particle [106,107] which can account for altered binding to the LDL receptor [118]. The arginine at 3500 may facilitate full exposure of the LDL receptor binding domain, thus facilitating recognition and binding. In the absence of this intramolecular association, which presumably occurs in mutant apo Bs lacking the 3500 arginine or in large lipoprotein particles such as VLDL, the LDL receptor binding domain on apo B100 becomes obscured by the C-terminus. This proposal may explain why nascent VLDL is poorly recognized by the LDL receptor and is efficiently secreted. In contrast, following lipolysis in plasma, the resulting smaller remnant particles gain affinity for the LDL receptor [119]. These intramolecular interactions [118] further support a functional basis for the evolutionary conservation of the large size of apo B100.

2.7. The high-affinity proteoglycan-binding site in apo B100

The homologous high affinity binding domains for the LDL receptor present on apo B and apo E bind to negatively charged glycosaminoglycan proteoglycans [120]. While the physiologic function for the binding of LDL to proteoglycans remains unknown, this interaction within the arterial wall may play an important role in the development of atherosclerosis [121–124]. Transgenic mice have been used to examine how proteoglycan binding and LDL receptor binding may be related [125]. Mutations in residues 3359–3369 of human apo B100, which contain sequences that recognize the LDL receptor and a high affinity proteoglycan-binding site, resulted in the production of LDL particles that still bound heparin in a high affinity manner, but did not bind to the LDL receptor [125]. Similarly, other mutations disrupted heparin binding, but did not affect LDL receptor recognition [125]. These findings show that the structural requirements for proteoglycan-binding and LDL receptor binding on apo B are distinct and are not required for both functions. It is interesting to note that the LDL produced in mice expressing the mutant forms of human apo B was similar in size and lipid composition [125]. Based on these observations, it is reasonable to propose that the proteoglycan-binding domain in apo B100 is not essential for metabolism and removal from plasma or for the assembly and secretion of lipoprotein particles.

3. Apo B assembles lipoproteins in the endoplasmic reticulum

The development of electron microscopy allowed intracellular organelles, membranes and their contents to be visualized. The findings obtained from electron microscopy studies and sub-cellular membrane isolation using ultracentrifuges were instrumental in dissecting the functions of individual organelles and how protein synthesis, transport and secretion occurs [126]. The results led to the proposal that proteins transported through the secretory path-

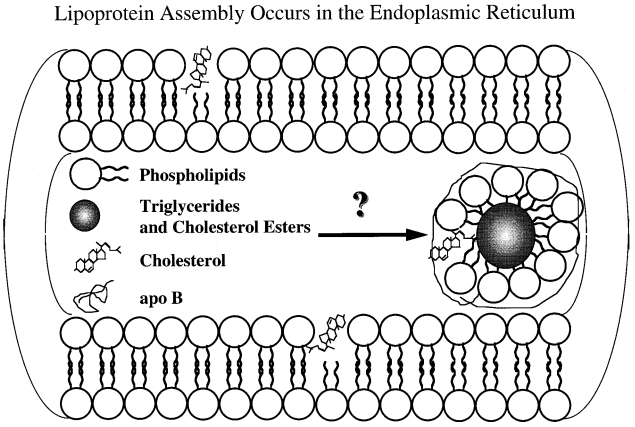

Fig. 1. The assembly of apo B-containing lipoproteins occurs in the endoplasmic reticulum. Early studies using electron microscopy clearly showed that the lumen of the endoplasmic reticulum contains mature sized VLDL particles [127–131]. These data provide compelling evidence that apo B-containing lipoproteins are assembled within the endoplasmic reticulum. The endoplasmic reticulum is also the intracellular site where all the protein (apo B) and lipid (cholesterol, phospholipids, triglycerides and cholesterol esters) components of VLDL are synthesized [1]. The mechanism through which the individual components of VLDL are assembled into a VLDL particle within the lumen of the endoplasmic reticulum remains to be fully elucidated.

way are synthesized on the rough endoplasmic reticulum, translocated into the lumen, transported through the Golgi apparatus and secreted into the sinusoidal space. Some of the earliest studies using electron microscopy concentrated on exploring the absorption of triglycerides [127] and the synthesis of lipoproteins [128]. The observation that spherical particles similar to plasma lipoproteins were commonly present within the lumenal domain of the endoplasmic reticulum of liver [128] supported the proposal that lipoprotein particles were assembled at the proximal beginning of the secretory pathway (Fig. 1). This conclusion was further supported by studies using sub-cellular membrane isolation in combination with pulse-chase analysis of radiolabeled glycerol incorporation into triglycerides and phospholipids [129]. The findings of a sequential movement of the peak of radioactive triglycerides from rough to smooth endoplasmic reticulum suggested that VLDL assembly was nearly complete before entering the Golgi apparatus, where further modifications may also occur [129]. Additional studies suggest that the Golgi apparatus may alter the phospholipid content of immature lipoprotein particles [130].

The clever use of immunology with electron microscopy has provided novel insights into the VLDL assembly process [131]. Immunoreactivity to LDL (presumably apo B100) was present throughout the rough endoplasmic reticulum membrane unassociated with lipid particles [131]. Surprisingly, immunoreactivity to a LDL antibody was not detected associated with the lipid particles present in the lumen of the smooth endoplasmic reticulum, whereas it was observed associated with the lipoprotein particles in the terminal junctions between the rough and smooth endoplasmic reticulum, [131]. With the unproven proviso that the immunoreactivity was reflecting the presence of apo B, these data were interpreted to indicate that: (1) apo B is synthesized on the rough endoplasmic reticulum; (2) the lipid core is produced independent of apo B; and (3) apo B becomes associated with apo B in the terminal junctions of the smooth endoplasmic reticulum [131].

While all of these early studies were consistent with the proposal that lipoprotein particles were assembled within the lumen of the endoplasmic reticulum, many of the responsible molecular details remain to be elucidated (Fig. 1).

3.1. Lipogenic enzymes are asymmetrically localized to the cytoplasmic surface of the endoplasmic reticulum

A clever use of protease digestion of correctly oriented microsomes showed for the first time that the active site of many phospholipid biosynthetic enzymes was facing the cytosolic face [132]. Subsequent studies using similar experimental approaches showed that the topographical distribution of the enzymes responsible for the synthesis of most, if not all, VLDL lipids has a similar orientation [133,134], cholesterol (i.e., HMG-CoA reductase) [135,136] and cholesterol esters (i.e., ACAT) [137]. The cytoplasmic orientation of the active site allows free access to charged CoA- and phosphorylated intermediates, which do not readily enter the lumen. In order to enter the lumen, the site of VLDL assembly, lipids synthesized on the cytoplasmic surface of the endoplasmic reticulum membrane must be translocated across the membrane bilayer. Translocation of phospholipids from one side of the membrane to the other has been shown to be facilitated by a 'flip-

pase' [138–140]. Translocation of triglycerides, cholesterol, cholesterol esters and phospholipids from the cytoplasmic face of the endoplasmic reticulum into the lumen is facilitated by an intraliminal multifunctional protein: microsomal triglyceride transfer protein (MTP; discussed in detail below).

3.2. Pulse-chase analysis suggests that movement of apo B out of the endoplasmic reticulum determines the rate of secretion of apo B by cultured rat hepatocytes

The availability of relatively inexpensive high specific activity [^{35}S]methionine provided the opportunity to analyze the kinetics of apo B biogenesis and transport using pulse-chase analysis. In order to determine the rate of movement of apo B from it site of synthesis on membrane bound ribosomes through the secretory pathway, the time of [^{35}S]methionine pulse labeling of cultured rat hepatocytes (i.e., 5 min) [141] had to be less than the time it takes for $\sim$50% of the apo B molecule to be translated ($\sim$7.5 min) [142]. Following a chase with unlabeled methionine, the peak of [^{35}S]methionine-labeled apo B100, apo B48 and albumin occurred in the following sequential order: rough microsomes, smooth microsomes and Golgi membrane fractions [141]. These data were interpreted to indicate a sequential movement of each secretory protein from rough to smooth endoplasmic reticulum and to the Golgi. For each protein studied, the first-order rate constants for the rate of loss of [^{35}S]methionine-labeled protein from each membrane fraction (i.e., rough microsomes, smooth microsomes and Golgi) were equal to the first-order rate constant for secretion into the culture medium. These data suggest that the rate of movement of each secretory protein out of the first definable membrane fraction (i.e., rough endoplasmic reticulum) governed the subsequent movement through the secretory pathway [141]. Furthermore, the relative first-order rate constants for each secretory protein showed the following: apo B48 $<$ apo B100 $<$ albumin. Thus, while albumin moves through the secretory pathway much faster than apo B, the intracellular transport of apo B100 is faster than that for apo B48. In vivo studies using rats also showed that the rate of movement of apo B48 out of the liver is slower than that for apo B100 [143]. Essentially all of the [^{35}S]methionine labeled albumin that was synthesized was found secreted into the culture medium. In marked contrast, only a fraction of the apo B100 (36%) and apo B48 (50%) that was synthesized was secreted into the culture medium; the remaining fraction was not retained in the cell [141]. These data showed for the first time that a significant portion of de novo synthesized apo B100 and apo B48 is degraded prior to secretion (i.e., within the hepatocyte). Based on these combined findings, it was proposed that the addition of lipid to form a lipoprotein particle was the rate-limiting process responsible for regulating apo B secretion [141]. While similar findings and conclusions were obtained using human hepatoma cells (HepG2) [144], in other studies using estrogen-stimulated chick hepatocytes, movement of apo B out of the Golgi was reported to be slower than movement out of the endoplasmic reticulum [145]. Additional studies using estrogen-stimulated chick hepatocytes showed that apo B100 was quantitatively secreted (i.e., the absence of intracellular degradation) [146] and that VLDL was assembled in the Golgi apparatus [147]. The basis for the differences in the apo B-containing lipoprotein assembly/secretion pathways displayed by mammalian hepatocytes compared to estrogen-stimulated chick hepatocytes remain unresolved.

3.3. The endoplasmic reticulum is the major intracellular site where translocation-arrested apo B is degraded

One of the first studies to suggest that apo B could be degraded in a pre-Golgi compartment of the secretory pathway was based on the finding that in HepG2 cells, brefeldin A treatment did not block the degradation of apo B100 [148]. The additional finding that N-terminal apo B peptides were present in the rough microsomes obtained from primary rat hepatocytes suggested that apo B could be degraded in the same compartment it is synthesized (i.e., the rough endoplasmic reticulum) [149]. Additional processes that actively degrade apo B include a DTT inhibitable proteolytic process degrades apo B which is completely translocated into the lumen of the endoplasmic reticulum [150] and a chloroquine-inhibitable protease has been proposed to degrade apo B

which resides in the lysosomal/endosomal compartment [151].

It is now well established that intracellular degradation of apo B occurs in many different tissues and cells types and involves several different proteolytic processes (recently reviewed in [4]). The most well-characterized apo B degradative process is the process which occurs in the rough endoplasmic reticulum and acts in a concerted manner with translocation arrest (described below) [152]. This degradative process is blocked by the tri-peptide aldehyde (acetylated leucine, leucine, nor-leucal: ALLN) which inhibits cysteine active site proteases [153–155]. Subsequent characterization of the ALLN-inhibitable process showed that it involved ubiquitin conjugation and proteasome degradation [156]. The ubiquitin-dependent degradation of apo B appears to be a consequence invoked in response to impaired translocation across the endoplasmic reticulum [157–161]. Fully translocated apo B can also be degraded in the ER lumen by an ALLN-inhibitable process involving ER-60 protease [162]. In permeabilized HepG2 cells, an ATP generating system together with cytosolic factors also blocks the degradation of a fully lumenal apo B100-containing lipoprotein precursor [163].

3.4. *Unlike most other 'secretory' proteins, apo B is transiently associated with the endoplasmic reticulum membrane*

In an effort to determine the mechanism for the unusually slow rate of movement of apo B out of the endoplasmic reticulum, albumin, apo B48 and apo B100 were characterized in isolated sub-cellular membrane fractions obtained from rat liver and cultured rat hepatocytes. Essentially all of the albumin isolated in microsomes was released by sodium carbonate disruption of the membrane vesicles, indicating a lumenal distribution [164]. In marked contrast, a significant portion of both apo B48 and apo B100 remained with the membrane fraction following carbonate disruption, suggesting it was associated with the membrane [164]. Additional pulse-chase studies using human hepatoma cells showed that membrane associated apo B100 was the precursor to progressively less dense lipoprotein particles which could be isolated in the carbonate extractable lumenal contents [144]. These studies showed for the first time, that VLDL was assembled in the lumen of the endoplasmic reticulum via a process in which as the lipid core of the nascent lipoprotein particle was assembled, apo B was released from the membrane [144].

3.5. *The translocation of apo B across the endoplasmic reticulum is inefficient, causing apo B to accumulate as a trans-membrane protein*

The finding that a majority of the apo B in hepatic microsomes remained with the carbonate-extractable pellet [164] was unexpected and suggested two possibilities: (1) apo B may have been indirectly associated with the membrane through an interaction with a membrane protein that is not affected by carbonate extraction; or, (2) newly synthesized (immature) apo B may exist as an integral, transmembrane protein of the endoplasmic reticulum. Preliminary experiments showed that, in rat liver rough microsomes, a majority of apo B100 and apo B48 was isolated as a Triton X-100 insoluble protein complex (i.e., aggregated cytoskeletal proteins) [164]) providing support for the first possibility. Furthermore, a priori, the second possibility seemed unlikely, since sequence analysis predicted that there were no amphipathic α-helices that were sufficiently long to span the membrane bilayer [66–69], a requirement for the formation of most integral transmembrane proteins [165–167]. Attempts to experimentally support the specific association of apo B with cytoskeleton proteins resulted in data that was ambiguous, making it difficult to prove or deny the first possibility (R.A. Davis, unpublished data). The ability to analyze the topography of membrane proteins using the relative susceptibility to exogenous proteases provided an opportunity to determine if apo B exists in microsomes as a transmembrane protein. Proteins or specific portions of proteins that reside on the cytoplasmic surface of microsomal membranes should be susceptible to cleavage by exogenous proteases, whereas proteins or portions of proteins that reside within the lumen should be resistant. In order to accurately define susceptibility and resistance as reflecting actual topographical orientation, the following criteria must be met: (1) sealed microsomal vesicles must be formed in the correct orientation, (2) lumenal proteins must remain within the lumen during formation of sealed

microsomal vesicles, and (3) proteolysis should cleave the cytoplasmic exposed residues of the putative transmembrane protein while leaving intact proteins that reside completely in the lumen of the endoplasmic reticulum. With the proviso that the aforementioned criteria are experimentally demonstrated, specific proteolysis susceptibility should accurately reflect a cytoplasmic orientation. The converse result (i.e., resistance to proteolytic cleavage) of a putative transmembrane protein may not necessarily indicate a lumenal (protected) localization for the following reasons: (1) the protein may be exposed, but it may not be a useful substrate for the proteolytic enzyme used; (2) the protein may be exposed but may be in an orientation that blocks proteolysis; (3) the protein may be exposed but may be associated with chaperones (e.g., Hsp70) that prevent accessibility to the protease.

Analysis of the susceptibility of apo B48 and apo B100 to digestion with trypsin and proteinase K was determined using isolated microsomal fractions obtained from rat liver (reported first in [168] and later in detail in [169]). The following results were obtained: approximately 50% of the apo B100 and apo B48 present in rat liver rough microsomes was degraded by exogenous trypsin under conditions in which microsomes remained intact (no loss of activity of the intralumenal enzyme mannose-6-phosphatase) and there was no detectable degradation of albumin [169]. Additional data showed that the microsomes remained intact during the proteolysis assay (i.e., following incubation with trypsin, the enzymatic activity of lumenal mannose-6-phosphatase was quantitatively retained within the microsomes) and that apo B100 and apo B48 in the other membrane fractions (i.e., smooth microsomes and Golgi fraction) were resistant to trypsin digestion [169]. Additional studies using pulse-chase analysis of microsomal membranes obtained from primary cultured rat hepatocytes also showed the presence of a pool of both apo B100 and apo B48 which was susceptible to cleavage by exogenous trypsin [169]. To further corroborate these findings, an alternative method to assay apo B topography was developed. This immunological method, not requiring proteolytic susceptibility, measured the amount of cytoplasmic membrane-bound apo B that could bind to magnetic beads that were covalently linked to antibodies that specifically recognize apo B epitopes [169]. The results showed that both polyclonal and monoclonal antibodies specific for rat apo B quantitatively bound rough microsomes from rat liver as demonstrated by recovery of glucose-6-phosphatase enzyme activity. In contrast, magnetic beads that bound antibodies to albumin did not associate with rough microsomes (i.e., no significant recovery of glucose-6-phosphatase) [169]. These combined data were interpreted to indicate that apo B domains are exposed on the cytoplasmic surface of rat liver rough microsomes. Thus, while the data from these studies indicated that apo B exists as a transmembrane protein in the rough endoplasmic reticulum, secondary structure analysis of apo B predicted the absence of canonical membrane spanning domains (i.e., amphipathic α-helices >12 amino acids) [66–69]. The combined findings could be reconciled by proposing that an alternative secondary structure might be responsible for the apparent transmembrane orientation of apo B. Secondary analysis of apo B also predicted the presence of amphipathic β-sheets, which reside throughout the apo B100 molecule and may act to anchor apo B to the lipoprotein particle [72]. Since similar amphipathic β-sheets form oligomeric structures which allow porins to integrate across membrane bilayers [170,171], these structures were proposed to act as the membrane spanning domains in apo B [152,169,172]. Recent structural analysis of the amphipathic β-sheets residing in sequences between apo B21 and apo B41 indicate that they share 43% sequence identity with those of porins [173]. Moreover, recent mutational analysis of apo B demonstrates that β-sheets are responsible for translocation-arrest in HepG2 cells [174]. The finding that as the size of apo B increases, translocation efficiency decreases [175] is consistent with the proposal that the increased content of β-sheets, which reside through-out the entire apo B molecule, provides a barrier to translocation.

Apo B15, which contains no amphipathic β-sheets and does not exist as a transmembrane protein [27,153] displays an unusual translocational pause using a reticulocyte translation system with pancreatic microsomal membranes [176]. A similar translocational pause was observed using rat liver microsomes [177], suggesting that this finding is not likely to be an artifact associated with microsomes from

tissue not active in lipoprotein production. Transient translocational pausing has been proposed to play a role in the formation of lipoprotein particles [176]. Additional studies have identified several distinct translocation pause sequences present throughout the apo B100 molecule [178–180]. The existence of apo B translocational pausing has been questioned based on studies using apo B17 and chimeric proteins containing putative apo B pause sequences [181,182]. In one study, translocational pausing was proposed to be due to translational pausing [182]. However, more recent studies provide evidence that translocation pausing may play a role in the formation of several different 'unconventional' secretory and membrane proteins in addition to apo B [183]. The observations showing that apo B can exist as a stable trans-membrane protein in the endoplasmic reticulum was subsequently corroborated by additional studies which used different models of hepatocytes including rat liver [184], HepG2 cells [155,185–187], chicken hepatocytes [188] and rabbit livers [189].

The hypothesis that apo B can exist as a transmembrane protein in the endoplasmic reticulum and can exhibit translocation-arrest has been questioned by one group of co-workers [190,191]. They were unable to detect protease susceptible forms of apo B chimeras that were expressed in COS cells and concluded that the amino-terminal 50% of apo B lacks autonomous signals or properties that can block translocation [190]. They same group reported findings showing that apo B does not display translocation arrest in HepG2 cells [192]. The findings reported by other groups showing that apo B did exhibit translocation arrest in HepG2 cells [155,185–187], were interpreted to be due to leaky microsomes rather than transmembrane topography [190,192]. However, the finding that microsomes displaying apo B forms that were susceptible to trypsin digestion quantitatively retained latent (mannose-6-phosphatase) enzymatic activity of the intralumenal enzyme glucose-6-phosphatase [169] argues against this interpretation. The most likely explanation for the apparent discrepancy in detecting translocation-arrested forms of apo B in microsomes is their transient nature. Since in HepG2 cells, translocation arrest and degradation by the cytoplasmic proteasomes occurs in a concerted manner [155,158,160,193], the failure to find cytoplasmic exposed forms of apo B [190,192] may be because they were degraded before detection. Additional data supporting this proposal were derived in cells that are unable to rapidly degrade translocation-arrested apo B via the ubiquitin-dependent proteasome pathway (see CHO cell studies, below).

4. In hepatoma cells, oleic acid rescues apo B from intracellular degradation

In some of the earliest studies using cultured rat hepatocytes to examine VLDL secretion, it was noted that while oleic acid increased the secretion of triglycerides and the size of the secreted lipoprotein particle, there was no significant effect on the secretion of apo B48 or apo B100 [194,195]. The ability of the fatty acid to stimulate triglyceride secretion was reduced with chain length and degree of saturation ($18{:}1 > 14{:}0 > 16{:}0 > 18{:}0$) [194]. In marked contrast, in human HepG2 hepatoma cells oleic acid stimulated the secretion of apo B100 [196]. Subsequent studies showed that the oleic acid stimulation of apo B100 secretion by HepG2 cells was not associated with any detectable change in apo B100 mRNA suggesting that oleic acid increased the secretion of apo B via a co- or post-translational process [197]. Pulse-chase studies showing that oleic acid decreased the degradation of apo B within 20 min of the chase, provided evidence that it acted on a process that occurred in an early compartment of the secretory pathway (i.e., before the majority of apo B is secreted) [198]. Subsequent studies provided data indicating that a pre-Golgi compartment (likely to be the endoplasmic reticulum) was the major site where oleic acid acts to block apo B degradation in HepG2 cells [185]. The use of ALLN provided compelling data indicating that in HepG2 cells, oleic acid blocked the degradation of apo B indirectly by increasing the efficiency of the translocation process [154,155]. Additional studies showed that in HepG2 cells the cytosolic chaperone Hsp70 binds to apo B100; oleic acid decreases this association, causing more to be translocated and subsequently secreted as a lipoprotein particle [199]. Based on these studies, it was proposed that Hsp70 maintains the translocation competence of apo B [199]. Thus, either

the translocation process or a process that acts in concert with translocation, appears to be rate-limiting for apo B secretion. The rate-limiting translocation processing of apo B, which in HepG2 cells is activated by oleic acid, determines whether apo B is secreted or is degraded in the endoplasmic reticulum.

4.1. The relative availability of all VLDL lipids (phosphatidylcholine, cholesterol, triglycerides and cholesterol esters) influence the assembly and secretion of apo B-containing lipoproteins

Essentially all de novo synthesized apo B-containing lipoprotein particles contain phosphatidylcholine, free cholesterol, triglycerides and cholesterol esters, albeit in varying amounts. Oleic acid stimulation of apo B secretion by hepatoma cells may involve increasing the synthesis of one or more of these lipids. However, the findings that in HepG2 cells Triacsin D blocked oleic acid stimulated apo B secretion by inhibiting triglyceride synthesis without affecting the stimulation of cholesterol ester synthesis suggests that in these cells cholesterol ester synthesis is not rate-limiting for apo B secretion [200]. This conclusion is further supported by studies showing that while cholesterol feeding caused a marked increase in hepatic cholesterol ester levels, there is no effect on apo B secretion [201]. While it appears that under most circumstances, cholesterol ester levels are not rate-limiting for apo B secretion, the relative availability of cholesterol esters to triglycerides determines the relative composition of these hydrophobic lipids in the core of newly secreted VLDL [202]. These findings may explain the accumulation of cholesterol ester-enriched apo B-containing lipoproteins in the plasma of animals susceptible to diet-induced atherosclerosis [203]. While there are no data indicating that free cholesterol acts as a specific regulator of the assembly and secretion of apo B-containing lipoproteins, there are several reports indicating that its availability can become rate-limiting [204–206].

The availability of phosphatidylcholine, the major lipid component of the surface monolayer of lipoprotein particles, when reduced via a choline-deficient diet, leads to impaired secretion of apo B-containing lipoproteins [207]. The mechanism responsible for decreased apo B secretion by choline-deficient rat hepatocytes appears to involve degradation in the Golgi apparatus [184]. Further studies show that choline deficiency only affects the secretion of apo B forms that are sufficiently large to form a core-containing lipoprotein particle [208]. Based on the essential role that phosphatidylcholine plays in lipoprotein particle assembly, the liver specific expression of the phosphatidylethanolamine methylation pathway has been proposed to have evolved in order to provide phosphatidylcholine for essential functions (e.g., membrane biogenesis and lipoprotein assembly) in the absence of dietary choline [209]. Thus, without methylation, phosphatidylethanolamine can not replace the requirement for phosphatidylcholine in lipoprotein particle formation. The head-group specificity of phospholipids for lipoprotein particle formation is further exemplified by experiments showing that monoethanolamine cannot replace choline in order to assemble and secrete apo B-containing lipoproteins [210]. Subsequent studies indicate that phosphatidylmonoethanolamine blocks the translocation of apo B across the endoplasmic reticulum membrane [211].

The type of fatty acid supplied to hepatocytes has a marked influence on the assembly and secretion of apo B-containing lipoproteins. For example, while oleic acid stimulates apo B secretion in hepatoma cells, $n-3$ fatty acids (eicosapentaenoic and docosahexaenoic acids) may decrease secretion of both apo B and triglycerides [212–214].

5. Studies of apo B processing in CHO cells indicate that tissue-specific factors are required for translocation, lipoprotein assembly and secretion of apo B-containing lipoprotein particles

While in McArdle rat hepatoma cells, expression of truncated forms of human apo B $>33\%$ of apo B100 led to the assembly and secretion of core-containing lipoproteins, when the same CMV-driven apo B plasmids were transfected into COS-7 cells, no lipoproteins were secreted [102]. These findings raised the possibility that tissue-specific factors present in McArdle rat hepatoma cells may be required for

processing apo B into lipoprotein particles. To examine this possibility, two apo B expression plasmids, shown by the McArdle rat hepatoma cell studies to produce apo B forms that were either too small (apo B15) or sufficiently large (apo B53) to form a lipoprotein particles were stably expressed in CHO cells [153]. Expression of apo B15 in CHO cells resulted in the accumulation of apo B15 protein in both medium and cells. In contrast, intact apo B53 was not detected in either the culture medium or within CHO cells transfected with the plasmid encoding apo B53, despite the expression of apo B53 mRNA. However, treating these cells with ALLN caused intact apo B53 to accumulate in cells as a transmembrane protein of the endoplasmic reticulum suggesting that it is synthesized but completely degraded in the absence of ALLN due to a block in its translocation [153]. The topography of the translocation-arrested apo B53 that accumulates in microsomes from ALLN-treated CHO cells was determined using epitope specific antibodies to map trypsin susceptible and resistant portions of the apo B53 molecule [27]. The results showed that apo B assumes a transmembrane orientation having almost 69 kDa of its N-terminus in the lumen of the endoplasmic reticulum and the remaining portion of the C-terminus residing on the cytoplasmic surface [27]. Endogenous proteolytic cleavage of the translocation arrested apo B53 by a process that was blocked by ALLN produced an 85 kDa N-terminal fragment that apparently resumes translocation and is secreted [27]. Interestingly, the same topography was observed for the apo B100 that accumulates as a transmembrane protein in microsomes obtained from ALLN-treated HepG2 cells [27]. These combined findings suggest that CHO cells lack a factor present in cells that actively secrete apo B-containing lipoproteins and is necessary to completely translocate apo B forms that are sufficiently large to assemble core-containing lipoproteins (e.g., apo B53). Furthermore, these data indicate that in the absence of this cell-type specific factor, translocation-arrested apo B53 is rapidly degraded by an ALLN-inhibitable process. Based on the combined data it was proposed that in the liver, the apo B translocation process is the branch point in the secretory pathway which determines whether apo B will enter the assembly/secretion pathway or if it will be diverted to degradation [152].

5.1. MTP, whose functional deletion is responsible for abetalipoproteinemia, facilitates apo B translocation and lipoprotein assembly, while blocking ALLN-inhibitable degradation

The discovery of MTP provided new insights linking apo B translocation, lipoprotein particle assembly and intracellular degradation. MTP was first described as a neutral lipid transfer protein found in the lumen of the endoplasmic reticulum of liver [215,216]. MTP exists as a heterodimer non-covalently associated with the ubiquitous endoplasmic reticulum lumenal chaperone: protein disulfide isomerase (PDI) [217]. The finding that MTP facilitates the transfer of lipids between membranes and liposomes, led to the proposal that it functions in the assembly of lipoproteins in the lumen of the endoplasmic reticulum [218]. This prediction was validated by the discovery that MTP is the defective 'abetalipoproteinemia' gene responsible for the inability to secrete apo B-containing lipoproteins [219,220]. The recent finding that heterozygous mice expressing a mutated (inactive) MTP gene have decreased plasma levels of apo B-containing lipoproteins further indicates that MTP plays an essential and rate-limiting role in the secretion of apo B in mice [221]. Unlike human homozygous abetalipoproteinemics, inactivating both MTP genetic loci in mice causes embryonic lethality [221]. These findings raise the possibility that MTP may not be rate-limiting for the secretion of lipoproteins in humans.

To examine further the essential requirement of MTP for lipoprotein secretion in mice, a conditional cre/lox liver MTP gene knockout was created in mice [222]. The results showed a marked impairment in the production and secretion of lipoproteins containing both apo B100 and apo B48 mice [222]. These findings were in contrast to other studies showing that in other mice, conditional liver MTP gene knockouts only blocked the secretion of apo B100 lipoproteins, with no little effect on the secretion of hepatic apo B48 lipoproteins (S.G. Young and M. Raabe, personal communication). Whether the differences in the response of apo B48 to MTP deletion are due to unique aspects of the experimental mouse model or represent fundamental differences in the dependency of mouse apo B100 and apo B48 for MTP remain unanswered.

Based on the initial discovery of MTP as a lipid transfer protein [216], it is reasonable to assume that MTP plays a similar function in lipoprotein assembly. The finding that plasma of abetalipoproteinemics display a marked 2000-fold increase in N-terminal apo B peptides, which are similar to those that are formed from the degradation of translocation-arrested apo B [27], led to the conclusion that MTP facilitates the translocation of apo B across the endoplasmic reticulum [223]. These findings and conclusions were further supported by studies showing that forms of apo B that are capable of assembling a core-containing lipoprotein could not be secreted by HeLa and COS cells, which do not express MTP [224–226]. In contrast, plasmid-driven expression of MTP in these cells allowed the assembly and secretion of apo B-containing lipoproteins [224–226].

While the findings discussed above provide strong support for the proposal that MTP plays an essential function in the translocation of apo B forms that are capable of assembling a core containing lipoprotein, there are two examples in which translocation has been detected in the absence of MTP. Mouse mammary-derived cells (C127), which do not express MTP, secrete apo B-containing lipoproteins having a neutral lipid core (albeit the efficiency of secretion may be much lower than the secretion of VLDL by the liver) [227]. Another example of an experimental system that allows apo B translocation in the absence of MTP were from in vitro translation/translocation experiments using dog pancreatic microsomes [228]. Although dog pancreatic microsomes contain no detectable MTP, apo B48 became resistant to exogenous protease suggesting it was fully translocated [228]. Furthermore, in the presence of active glycerolipid synthesis and hepatic microsomes, apo B48 was assembled into a $d < 1.10$ g/ml core containing lipoprotein [228]. These findings raise the interesting possibility that gene products (present in the pancreas and C127 cells) may complement the function that MTP apparently plays in cultured hepatoma cells [158,229], COS-1 cells [225], HeLa cells [224] and CHO cells [27,153,161, 230].

The development of specific inhibitors of MTP lipid transfer activity has provided scientists with a tool to further examine the role of MTP in lipoprotein assembly and secretion. Inhibitors of MTP lipid transfer activity impair the assembly and secretion of apo B-containing lipoproteins by blocking an early event in the secretory pathway [13,158,226, 231,232]. At concentrations of MTP inhibitors that are not toxic to cells, there is a relationship between the size of apo B and its sensitivity to inhibition of secretion [233]. For example, the ability of a MTP inhibitor to block the secretion follows the order apo B100 > apo B53 > apo B48 [233]. An amphipathic α-helical domain, which is followed by a β-sheet lipid binding domain (present on the C-terminus of apo B53) has been proposed to be responsible for the greater sensitivity of apo B53 compared to apo B48 to MTP inhibitors [233]. The relative difference in the sensitivity of different forms of apo B to inhibited secretion by MTP inhibitors may explain the earlier finding of normal secretion of intestinal apo B48-containing lipoproteins in an abetalipoproteinemic patient lacking hepatic apo B100 [37]. These differences may also explain why hepatic MTP gene deletion in mice appeared to decrease the plasma concentration of apo B100 to a greater extent than apo B48 (M. Raabe, S.G. Young, personal communication).

Chemical inhibition of MTP appears to block apo B secretion by two distinct processes: translocation and maturation of the lipid core. Pulse-chase studies in HepG2 cells suggest that chemical inhibition of MTP lipid transfer blocked the translocation of apo B100 causing it be co-translationally degraded by a process attributed to the proteasome [158]. Subsequent studies show that chemical inhibition of MTP interrupts apo B translocation in a manner that is linked to rapid degradation by the ubiquitin-dependent proteasome [229]. If proteasome degradation is also inhibited, the translocation-arrested apo B remains associated with the major protein constituent of the translocon (Sec61) as a ubiquitin conjugated protein [193].

In addition to blocking apo B translocation, chemical inhibition of MTP lipid transfer also impairs a later process in the secretory pathway (i.e., the addition of lipid to form a more fully lipidated lipoprotein particle). In separate studies using McArdle rat hepatoma cells, chemical inhibition of MTP lipid transfer activity was shown to block the oleic acid stimulation of apo B48 secretion [234] and the for-

mation and secretion of VLDL containing apo B100 [235].

For most secretory proteins, the receptor for the signal recognition particle (SRP), Sec61 complex, and the translocating chain-associated membrane protein (TRAM) are the only endoplasmic reticulum proteins required to allow translocation [236,237]. Apo B translocation has an additional requirement for MTP and at least two chaperones PDI and calnexin. PDI contributes two distinct functions to apo B translocation: (1) formation of a heterodimeric complex between MTP and PDI is absolutely required in order to express active lipid transfer activity [238]. In addition, while the PDI disulfide shuffling and redox activities are not required for MTP facilitated lipoprotein particle assembly and secretion by insect cells, these PDI enzyme activities are required for MTP independent processes [239]. The additional finding that in HepG2 cells, apo B100 associates with calnexin [240] and impairing this association blocks translocation and causes rapid ubiquitin-dependent proteasome degradation [160] suggests that intralumenal chaperones allow apo B to attain the proper folding required for its maturation as a component of a lipoprotein particle. In other studies, apo B was shown to associate with several intralumenal chaperones (ERp72, GRP94, calreticulin, and BiP) [241].

Several lines of evidence suggest that the MTP/PDI complex also acts as an intralumenal chaperone through its association with the N-terminus of apo B. Studies using insect cells showed that MTP-facilitated lipid transfer requires the N-terminal ~17% of apo B [242]. The additional findings of a physical interaction between apo B and MTP in the lumen of the endoplasmic reticulum is reminiscent of a chaperone/substrate interaction [243,244]. N-terminal amino acid residues 430–570 [245] and 512–721 [246] have been proposed to contain the site responsible for MTP binding. It is interesting to note that the site on MTP which has been proposed to bind to apo B also appears to be the site that interacts with PDI [246]. Since the association of MTP with PDI is required for lipid transfer activity [238], the possible formation of the MTP-apo B complex, which may release PDI, suggests that MTP may facilitate apo B folding independent of its lipid transfer activity.

6. Cholesterol-7α-hydroxylase influences production of apo B-containing lipoproteins: complementing CHO cells with gene products expressed by the liver

Over-production of triglyceride-rich lipoproteins is responsible for the human disease familial hypertriglyceridemia [247]. In these patients, the secretion of triglyceride-rich lipoproteins varies in parallel with the rate of bile acid synthesis and cholesterol-7α-hydroxylase [248,249]. Only one of three familial combined hyperlipidemic patients showed increased rates of bile acid synthesis [249]. The increased synthesis of bile acids in hypertriglyceridemic patients has been ascribed to decreased bile acid absorption [250]. Interestingly, treatment of hypertriglyceridemic patients with chenodeoxycholic acid, which would be expected to decrease the activity of cholesterol-7α-hydroxylase, reduces fasting plasma triglyceride levels [251].

In order to determine the mechanism linking hepatic lipoprotein production to the bile acid synthetic pathway, CHO cells were chosen to examine how expression of the liver-specific enzyme that regulates bile acid synthesis, cholesterol-7α-hydroxylase, affected the processing apo B [161,230]. CHO cells expressing apo B53, but not MTP, displayed an inability to translocate apo B53, which results in its rapid degradation [27,153]. While CHO cells expressing apo B53 and cholesterol-7α-hydroxylase remained incapable of translocating apo B53, intact apo B53 accumulated as a stable transmembrane protein of the endoplasmic reticulum [161]. Further studies showed that expression of cholesterol-7α-hydroxylase blocked the proteasome degradation of translocation-arrested apo B53 by blocking its conjugation with ubiquitin in a manner that was reversed by oxysterols (7-oxo-cholesterol plus 25-hydroxycholesterol) [161]). These findings provide a link between the sterol-status of cells, altered by the expression of cholesterol-7α-hydroxylase, and the ubiquitin-dependent proteasome degradation of apo B. Furthermore, the accumulation of intact apo B53 as a stable transmembrane protein in the isolated domains of the endoplasmic reticulum which are reminiscent of 'Russell bodies' [252] provided further evidence for the translocation arrest of this unusual 'secretory' protein [161].

Complementing the CHO cells with MTP allowed the two functions of MTP (translocation and lipoprotein particle assembly via lipid transfer) to be experimentally separated [230]. Unlike HeLa and COS cells which require only the expression of MTP to exhibit MTP-lipid transfer activity and the assembly and secretion of apo B-containing lipoproteins [224,225], CHO cells expressing apo B53 were shown to require both MTP and cholesterol-7α-hydroxylase [230]. While expressing MTP by itself allowed CHO cells to translocate apo B53, it was rapidly degraded in an acidic compartment via a process that was inhibited by DTT and chloroquine, but was resistant to inhibitors of the proteasome [230]. The expression of MTP mRNA and protein was unaffected by co-expression of cholesterol-7α-hydroxylase. However, in CHO cells expressing cholesterol-7α-hydroxylase, MTP expression led to expression of lipid transfer activity and the assembly and secretion of apo B53 as a core containing lipoprotein particle. These studies showed that in CHO cells: (1) MTP-facilitated lipid transfer is not required for apo B translocation; and (2) the phenotype of the cell (e.g., expression of cholesterol-7α-hydroxylase by the liver) may profoundly influence the metabolic relationships determining how apo B is processed into lipoproteins and/or degraded. If the findings in CHO cells reflect processes that occur in vivo, the unexpected requirement of cholesterol-7α-hydroxylase expression for MTP-facilitated lipid transfer activity and lipoprotein particle assembly and secretion may provide insights into the basis for chylomicron retention disorder. Chylomicron retention disorder is characterized by a block in the secretion of apo B-containing lipoproteins by the intestine, but not by the liver [253]. Affected patients produce immunoreactive apo B48 in enterocytes, process apo B mRNA to the intestinal (edited) form and express MTP [254–256]. These data suggest that the inability of the intestine of chylomicron retention disease patients to secrete apo B-containing lipoproteins is due to the functional loss of a gene product other than apo B or MTP. The findings showing that the liver specific enzyme cholesterol-7α-hydroxylase is required for CHO cells expressing apo B and MTP to secrete apo B-containing lipoproteins support the possibility that expression of cholesterol-7α-hydroxylase in the liver of chylomicron retention disease patients may complement a defective process that is common to both intestinal and hepatic lipoprotein assembly/secretion pathways.

7. MTP and apo B are co-expressed in organs that assemble and secrete apo B-containing lipoproteins

Elucidation of the genetic basis for the abetalipoproteinemic phenotype (inability to assemble and secrete apo B-containing lipoproteins) [11,14] demonstrates that MTP is essential. Based on this conclusion, one would predict that the tissue specific expression of the apo B-containing lipoprotein assembly/secretion pathway requires the co-expression of apo B and MTP. This prediction is fulfilled by the unexpected findings that in mice the yolk sac [257] heart [258] and kidney [12] express both apo B and MTP and display active synthesis and secretion of apo B-containing lipoproteins. These data further support the proposal that efficient assembly of apo B-containing lipoproteins requires MTP.

8. Inbred, transgenic and gene-deleted mouse models provide new insights into the physiological functions of lipoprotein assembly and secretion

With technical advances in being able to manipulate the genome of mammals, the physiological in vivo relevance of hypotheses obtained from in vitro and cell based experiments can be explored. Studies in transgenic and gene deleted mice have provided unexpected insights of the essential role of apo B in the transport of essential lipid nutrients for fetal development and adult energy balance (reviewed in [5]).

Targeted mutagenesis of the mouse apo B gene resulting in the expression of apo B70 and no apo B100 caused developmental neurological abnormalities [115]. The essential role for the apo B-containing lipoprotein assembly/secretion pathway in embryonic development was dramatically demonstrated by the findings that complete disruption of either the apo B gene [116] or the MTP gene [221] resulted in resorption of the fetus. These findings are distinct from those of the human fetus which can survive in the absence of either apo B or MTP [5]. Elegant studies by Farese and collaborators provided an explanation for this apparent species difference in the require-

ment for the apo B-containing lipoprotein assembly/secretion pathway [114]. These studies showed that in the mouse, the yolk sac produces lipoproteins for fetal nutritional requirements. In contrast, the human placenta transports maternal lipoproteins to the fetus. Thus, in humans the apo B-containing lipoprotein assembly/secretion pathway is not essential for fetal survival. However, the finding that neurological developmental abnormalities are common occurrences in homozygous hypobetalipoproteinemia (mutated apo B gene) and abetalipoproteinemia (mutated MTP gene) suggests that the transport of lipid soluble nutrients to the fetus is nevertheless impaired [5].

8.1. Studies of the assembly and secretion of apo B in animal models show that both the production of apo B and the enzyme activity of MTP can become rate-limiting

From the studies discussed above using cultured cells it appears that the availability of lipid, apo B and MTP activity can be rate-limiting for lipoprotein assembly and secretion. Analysis of the plasma lipoprotein levels in genetically altered mice may offer some insight into this issue as it relates to in vivo physiology. Mice exhibiting one inactivated apo B allele (via gene targeting disruption) show reduced plasma apo B levels and a dramatic insensitivity to diet-induced hyperlipidemia [116]. These data suggest that apo B has become rate-limiting for secretion. Furthermore, under these circumstances, the requirement for lipid for driving VLDL assembly has become reduced. In contrast, transgenic expression of human apo B in mice showed increased plasma levels of LDL, human apo B and a markedly increased sensitivity to diet-induced hyperlipidemia [259,260]. Similar results were found in transgenic rabbits expressing human apo B [261]. These data suggest that under these circumstances, the requirement for lipid for driving VLDL assembly is greater, thus dietary lipid has more influence on plasma lipoprotein levels. Conversely, in heterozygous MTP gene deleted mice, hepatic secretion of apo B is decreased and mice display a lower sensitivity to diet-induced hyperlipidemia [221]. These data suggest that under these circumstances, the enzymatic activity of MTP has become rate-limiting for VLDL assembly. As a result, MTP is 'saturated' with lipid substrates and the requirement for lipid for driving VLDL assembly decreased. Thus, one would predict that the effect of dietary and de novo synthesized lipid on plasma lipoprotein levels would be less in situations in which MTP lipid transfer activity was rate-limiting. Data supporting this prediction have been recently obtained using a chemical inhibitor of MTP in Watanabe-heritable hyperlipidemic (WHHL) rabbits, which have a deficiency in functional LDL receptors [6]. In spite of having a catabolic defect in plasma LDL, chemical inhibition of MTP restored plasma LDL levels to normal by blocking the assembly and secretion of apo B-containing lipoproteins [6].

8.2. A gene other than apo B or MTP appears to be responsible for one form of familial combined hyperlipidemia

The familial combined hyperlipidemia (FCH) phenotype, thought to be caused by over-production of hepatic apo B-containing lipoproteins, accounts for a significant portion of the human population having premature atherosclerosis [262]. The HcB-19 inbred mouse displays the FCH phenotype (i.e., high plasma levels of triglyceride and cholesterol as well as increased hepatic secretion of apo B-containing lipoproteins) [263]. Furthermore, the gene loci responsible for the FCH phenotype in HcB-19 mice is syntenic with that of a human loci for FCH (1q21–q23) in a Finnish cohort [264]. These data suggest that a single (as yet unidentified) gene other than apo B or MTP is responsible for the inappropriately induced rate of assembly and secretion of apo B-containing lipoproteins.

9. Coordinate regulation of lipogenesis, VLDL assembly and secretion occurs in vivo

In vivo the assembly and secretion of apo B-containing lipoproteins by the liver show marked changes in response to nutritional and hormonal signals [172]. The most dramatic changes in VLDL secretion were observed in hepatocytes obtained from rats that were fasted (48 h) [113,265,266] or fed carbohydrate enriched diets (sucrose) [267]. Hepatocytes from fasted rats displayed a marked decrease in the

secretion of apo B100 and apo B48 [266]. In contrast, hepatocytes from rats fed carbohydrate enriched diets displayed increased secretion of apo B100 and apo B48 [267]. Since there was no detectable change in the content of apo B mRNA, the changes observed in apo B secretion were due to a post-transcriptional mechanism (i.e., decreased degradation of apo B) [266]. Moreover, the synthesis of all VLDL lipids was coordinately changed in response to nutritional state. Fasting decreased, whereas carbohydrate feeding increased the synthesis of all VLDL lipids [267]. Based on these findings, it was hypothesized that metabolic signals, invoked in response to metabolic state, coordinately regulate seemingly independent steps of the VLDL assembly/secretion pathway (apo B translocation and particle assembly in the ER lumen vs. degradation of translocation-arrested apo B) [268]. The recent finding that carbohydrate induction of hepatic lipogenesis is associated with increased cellular content of the mature form of the transcription factor mature SREBP1a [269] suggests that this might be the mediator through which metabolic signals coordinately regulate the VLDL assembly/secretion pathway.

9.1. *The expression of cholesterol metabolic and lipogenic enzymes is regulated by a common transcription factor family: SREBP*

SREBP was first identified as the sterol responsive transcription factor that mediates regulation of the human LDL receptor gene [270]. Its rat homologue ADD1 was identified as a transcription factor involved in adipocyte differentiation [271], suggesting that it may play a diverse role in regulating the expression of genes involved in the biosynthesis and metabolism of several classes of lipids. Subsequent studies showed that many, if not most, of the genes encoding isoprenoid/cholesterol biosynthetic enzymes are SREBP responsive: HMG-CoA synthase [272], HMG-CoA reductase [273], isopentyfarnesyldiphosphate synthase [274] and squalene synthase [275]. More recently, data have been reported showing that the transcription of some of the genes responsible for regulating the synthesis of fatty acids and phospholipids are induced by SREBP: fatty acid synthase [276], acetyl-CoA carboxylase [277], stearoyl-CoA desaturase-1 [278] and glycerol-3-phosphate acyltransferase [279].

9.2. *Coordinate induction of hepatic lipogenesis and lipoprotein production can be mediated via changes in the expression of cholesterol-7α-hydroxylase*

Studies in which cholesterol-7α-hydroxylase was expressed in McArdle rat hepatoma cells suggest a role in regulating lipoprotein assembly and secretion. Stable expression of cholesterol-7α-hydroxylase in McArdle rat hepatoma resulted in the following [280]: (1) a linear relationship between the cellular content of mature SREBP1 and cholesterol-7α-hydroxylase, (2) a linear relationship between the relative expression of cholesterol-7α-hydroxylase mRNA and the mRNAs encoding the enzymes regulating fatty acid and sterol synthesis: i.e., acetyl-Co A carboxylase and HMG-CoA reductase, (3) a linear relationship between the relative expression of cholesterol-7α-hydroxylase mRNA and MTP mRNA, (4) increased synthesis of all lipoprotein lipids (cholesterol, cholesterol esters, triglycerides and phospholipids), and (5) increased secretion of apo B100 without any change in apo B mRNA. The increased cellular content of mature SREBP1 and increased secretion of apo B100 were concomitantly reversed by 25-hydroxycholesterol, suggesting that the content of mature SREBP1, known to be decreased by 25-hydroxycholesterol [272], mediates the changes in the lipoprotein assembly and secretion pathway that are caused by cholesterol-7α-hydroxylase. It is interesting to note that McArdle cells expressing cholesterol-7α-hydroxylase become resistant to oleic acid stimulated apo B100 secretion [280]. These findings may explain the discrepancy between primary rat hepatocytes (expression of cholesterol-7α-hydroxylase, no oleic acid-stimulated apo B secretion) and hepatoma cells (no expression of cholesterol-7α-hydroxylase, but oleic acid-stimulated apo B secretion). Additional findings showed that oleic acid stimulated apo B secretion by perfused liver from fasted rats, whereas livers from fed rats showed no increased apo B secretion [281]. These findings further support the proposal that cholesterol-7α-hydroxylase expression, which is markedly decreased in response to fasting,

influences the requirement of apo B secretion for oleic acid stimulated lipogenesis.

9.3. Coordinate, diurnal cyclic changes in hepatic cholesterol-7α-hydroxylase expression, lipogenesis and lipoprotein secretion

The findings that cholesterol-7α-hydroxylase expression markedly induced the processes in the VLDL assembly/secretion pathway, suggests the possibility that this liver-specific gene product may play an important role in vivo in the coordinate regulation of this pathway. One of the unique characteristics of cholesterol-7α-hydroxylase is its diurnal variation [282]. Circadian (diurnal) rhythms of several distinct metabolic pathways, a common characteristic of multi-cellular organisms [283], provide an efficient and productive utilization of energy and nutrients. Diurnal changes in hepatic lipid and sterol metabolism in rodents and humans are well established [282]. Several studies provide indirect support suggesting that diurnal changes in hepatic lipogenesis and lipoprotein metabolism significantly affect the apo B-containing lipoprotein assembly/secretion pathway.

Coordinate diurnal changes in hepatic expression of cholesterol biosynthetic enzymes (e.g., HMG-CoA reductase [284,285] and the LDL receptor [286]) are temporally linked to diurnal changes in cholesterol-7α-hydroxylase. The hypothalamic/pituitary endocrine system initiates the hepatic diurnal cycle via a dramatic induction of the expression of two transcription factors: DBP and TEF [287,288]. TEF and DBP have different cognate targets and genetic transcriptional responses [288]. The finding that DBP binds to the cholesterol-7α-hydroxylase promoter and activates gene expression is consistent with the proposal that this may be the initial step leading to subsequent activation of other liver processes [289,290].

While not widely recognized, there are several other physiologically important diurnal changes that occur within the liver. The size of the liver diurnally varies in parallel with the expression of DBP [291]. Concomitant diurnal variations in the amount of hepatic endoplasmic reticulum [292], may reflect increased membrane biogenesis. The diurnal anabolic cycle displayed by the liver is further expressed by parallel changes in the synthesis and secretion of lipoprotein lipids (i.e., triglycerides and cholesterol [293–295]). Studies using Triton WR-1339 to block the clearance from plasma and metabolism of newly secreted lipoproteins showed that in rats the hepatic secretion of newly synthesized triglycerides was increased in the dark phase of a 12 h light cycle [295]. In hamsters, the secretion of VLDL cholesterol esters was significantly increased in the mid-dark period [296]. Interestingly, the influence of diurnal cycle on VLDL secretion was enhanced when hamsters were fed a high fat diet [296]. The finding that intestinal lymph apo AIV varies in parallel to hepatic biliary lipid output, further exemplifies the pervasive influence of the hepatic diurnal anabolic cycle on lipoprotein production [297].

Several lines of evidence suggest that glucocorticoid induction of cholesterol-7α-hydroxylase potentiates diurnal changes in hepatic cholesterol metabolism, lipogenesis and VLDL secretion. In rodents adapted to a 12:12 h light/dark cycle, plasma levels of glucocorticoids exhibit a diurnal zenith at light/dark boundary [298], which is just prior to the induction of cholesterol-7α-hydroxylase. It is well-established that glucocorticoids induce the genetic expression and activity of cholesterol-7α-hydroxylase [299–303]. The finding that dexamethasone increases the secretion of apo B-containing lipoproteins by cultured rat hepatocytes [304] is consistent with proposal that cholesterol-7α-hydroxylase may indirectly regulate VLDL secretion.

10. Closing remarks: A look toward the future

Technical developments have played an important role in providing scientists with the opportunity to define many of the gene products and processes responsible for regulating the assembly and secretion of apo B-containing lipoproteins. The advantages offered by this mainly reductionist experimental approach have provided the opportunity to gain many new insights into the complex relationships which in toto comprise the lipoprotein anabolic pathway. Based on these new insights, we offer a general scheme describing the post-translational regulation of the apo B-containing lipoprotein assembly/secretion pathway (Fig. 2). This model suggests that

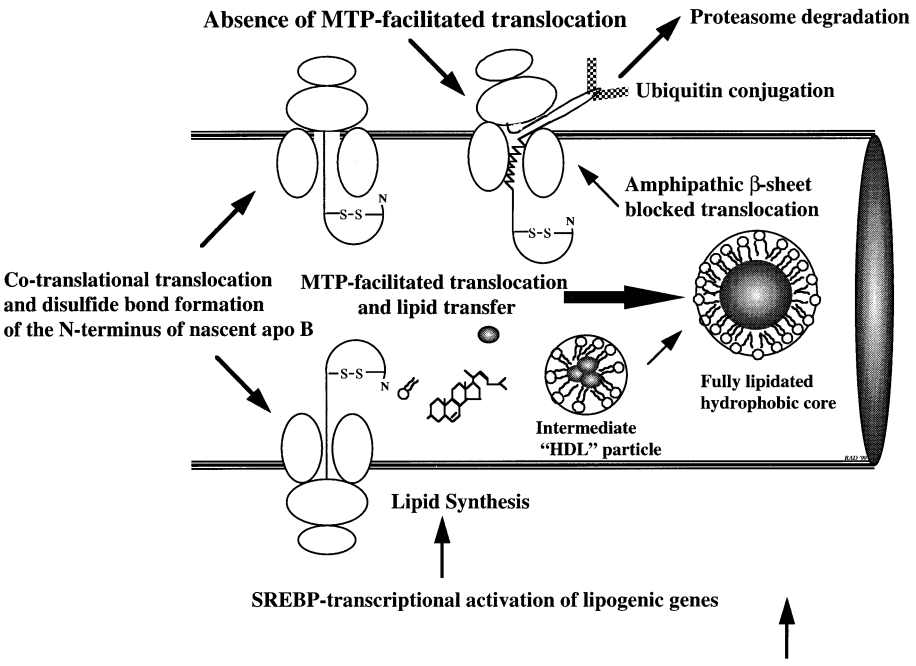

Fig. 2. In the endoplasmic reticulum apo B has two fates: degradation by the ubiquitin-dependent proteasome in the cytoplasm or translocation and assembly into lipoproteins in the lumen. Both in the presence and absence of functional MTP, the ~70 kDa of N-terminus of apo B (~apo B17) enters the lumen of the endoplasmic reticulum. It is at the next step that the metabolic fate of apo B is determined.

Top (ubiquitin-dependent proteasome degradation): In the absence of functional MTP (e.g., abetalipoproteinemia, CHO cells, treatment with an inhibitor of MTP or insufficient lipid), the translocation of apo B becomes blocked by an interaction of amphipathic β-sheets with the translocation apparatus (e.g., Sec61). The translocated arrested apo B is rapidly conjugated with ubiquitin and degraded in the cytoplasm by the proteasome. In CHO cells expressing apo B53, the N-terminus, which is intraliminal, enters the secretory pathway and becomes secreted without hydrophobic lipid (i.e., triglycerides and cholesterol esters) [27]. In HepG2 cells, a portion of apo B100 may move from the lumen to the cytoplasm (retrotranslocation) [159].

Bottom (translocation and lipoprotein particle assembly): In the presence of MTP facilitated translocation and lipid transfer activity, apo B continues translocation in a manner that is dependent upon the addition of lipids and appropriate chaperone-assisted folding. The disulfide-linked N-terminus of apo B interacts with the MTP/PDI complex. This interaction facilitates both translocation and addition of neutral lipid to the nascent lipoprotein particle. Within the lumen of the endoplasmic reticulum, the formation of a lipoprotein particle containing its full complement of neutral lipids in its core may be either assembled as a concerted series of chemical transformations involving protein folding and lipid addition (large bold arrow) and/or it may occur as several steps in which partially completed lipoprotein particles may exist as intermediates (e.g., 'HDL'). Improper folding of apo B and/or addition of lipid to both the surface monolayer and the core can result in the production of a lipoprotein particle that is degraded at a site in the secretory pathway that is distal to the endoplasmic reticulum (not shown).

under most circumstances and physiologic situations, MTP lipid transfer activity determined by the availability of each individual lipoprotein lipid (triglycerides, cholesterol, phosphatidylcholine and cholesterol esters), drives the translocation of apo B into the ER lumen where it forms a core-containing lipoprotein particle. In the absence of sufficient lipid or MTP lipid transfer activity, apo B is diverted into the ubiquitin-dependent proteasome degradation pathway. There appears to be pervasive and compelling data showing that the phenotype of the cell has a profound influence on how the basic members of the lipoprotein assembly/secretion pathway interact and orchestrate the processing of apo B. Thus, there remains ample room within this scheme to accommodate different apo B degradative pathways, different

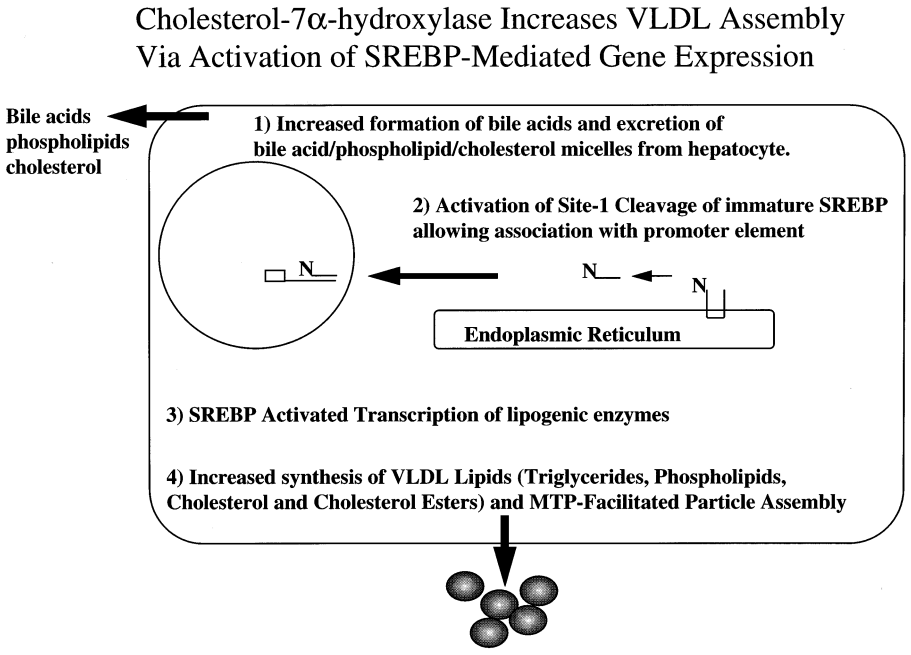

Fig. 3. Influence of cholesterol-7α-hydroxylase on SREBP activation of gene transcription, lipogenesis and VLDL assembly/secretion. The liver-specific expression of cholesterol-7α-hydroxylase, a cytochrome P450 enzyme in the endoplasmic reticulum, regulates the production of bile acids. The excretion of bile acid/phospholipid/cholesterol micelles into the bile by the hepatocyte provides the major quantitative pathway through which cholesterol is eliminated from the mammalian body. As a result of depleting hepatic sterols via biliary elimination, cholesterol-7α-hydroxylase indirectly activates the site-1 (sterol sensitive) cleavage of SREBPs, causing more mature transcriptionally active SREBPs to bind to cognitive promoter elements and activate transcription [272]. Since the transcription of many of the genes controlling the synthesis of the lipid components of VLDL (i.e., cholesterol, fatty acids, triglycerides, phospholipids and cholesterol esters) are activated by SREBPs [268–280], cholesterol-7α-hydroxylase provides more lipid substrates for the production of VLDL. In addition, cholesterol-7α-hydroxylase may also decrease the intracellular degradation of apo B [230] and increase both the expression [280] and lipid transfer activity of MTP [223]. The net result of increased expression of cholesterol-7α-hydroxylase, as indicated schematically in this figure, is increased lipogenesis and VLDL assembly via activation of SREBP-mediated gene expression.

modes of regulation and possible unknown gene products that may complement or antagonize one or more of the requisite processes.

Whether and how this scheme conforms to the complexity expected of the in vivo lipoprotein assembly/secretion pathway remains to a large degree unanswered. Most studies of lipoprotein assembly secretion in animals are performed with tissues and cells isolated during the day (when the hepatic diurnal expression of lipogenic enzymes and cholesterol-7α-hydroxylase are at their nadir). Considering the potential important influence that cholesterol-7α-hydroxylase and SREBP may play in regulating the apo B-containing lipoprotein assembly/secretion pathway (Fig. 3), future studies of this pathway should be directed toward examining diurnal effects.

While it appears that MTP and apo B are two of the gene products that are essential for the apo B-containing lipoprotein assembly/secretion pathway, clearly other genes also are important. Identifying these genes and how that they may affect the relationship between lipogenesis, MTP function and lipoprotein assembly may provide new insights into the molecular basis for familial forms of hyperlipidemias caused by excessive production of apo B-containing lipoproteins.

The advances in genomics and molecular genetics should provide new windows to further our understanding how this pathway occurs and is regulated in vivo.

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