Assembly and Secretion of Atherogenic Lipoproteins

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A doctoral thesis at a University in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have already been published or are in manuscript at various stages (in press, submitted, or in manuscript).

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Abstract

The classical dyslipidemia seen in patients with type 2 diabetes is characterized by elevated serum triglycerides (TG), low levels of high-density lipoprotein cholesterol and the appearance of small, dense low-density lipoproteins (LDL). It is now recognized that the different components of diabetic dyslipidemia are not isolated abnormalities but are closely linked to each other metabolically, and are initiated by the hepatic overproduction of large triglyceride-rich very low-density lipoproteins ($VLDL_1$). Diabetic dyslipidemia frequently precedes type 2 diabetes by several years, indicating that the disturbance of lipid metabolism is an early event in the development of cardiovascular complications of type 2 diabetes. It is thus of key importance to elucidate the mechanisms involved in the production of $VLDL_1$. The aim of this thesis was to further clarify the molecular mechanisms of the assembly process and secretion of apolipoprotein B (apoB)-containing lipoproteins.

The results indicate that apoB100 assembles into partially lipidated dense pre-VLDL that is retained in the cell unless further converted into $VLDL_2$ by size-dependent lipidation. $VLDL_2$ in turn can proceed through the secretory pathway to be secreted or converted to $VLDL_1$ in the second step of the assembly. Furthermore, an efficient formation of $VLDL_1$ specifically requires a sequence located between apoB46.8 and apoB48. This sequence interacts with the B-cell receptor-associated protein (BAP31), which seems essential for an efficient secretion of $VLDL_1$, but not for the secretion of denser particles.

The formation of lipoproteins depends on the availability of lipids. However, the results show that the accumulation of cytoplasmic lipids is not directly associated with increased secretion of VLDL. The phenol epicallocatehin gallate (EGCG) diverts TG from the secretory pathway for storage in cytosolic lipid droplets. While increasing the cytosolic lipid droplet fusion rate and TG content in the cytsosol, apoB100 secretion from the cells is decreased. As a consequence, apoB becomes degraded.

The results presented advance our understanding of the complex mechanisms underlying the formation of VLDL. Clarification of these molecular mechanisms will hopefully enable development of targeted treatment for diabetic dyslipidemia, which is of key importance given the high risk for coronary vascular disease (CVD) in patients with type 2 diabetes and the metabolic syndrome.

List of publications

The thesis is based upon the following papers, referred to in the text by their roman numerals:

Paper I **Relation of the size and intracellular sorting of apoB to the formation of $VLDL_1$ and $VLDL_2$**

Pia Stillemark-Billton*, Caroline Beck*, Jan Borén, and Sven-Olof Olofsson

J. Lipid Res. 2005. **46**: 104-114

*contributed equally

Paper II **B-cell receptor-associated protein 31 (BAP31) interacts with apolipoprotein B48 and directs $VLDL_1$ secretion**

Caroline Beck, Sven-Olof Olofsson, and Jan Borén

Manuscript

Paper III **Epigallocatechin gallate increases the formation of cytosolic lipid droplets and decreases the secretion of apoB-100 VLDL**

Lu Li*, Pia Stillemark-Billton*, Caroline Beck*, Pontus Boström*, Linda Andersson, Mikael Rutberg, Johanna Ericsson, Björn Magnusson, Denis Marchesan, Anna Ljungberg, Jan Borén, and Sven-Olof Olofsson

J. Lipid Res. 2006. **47**: 67-77

*contributed equally

List of abbreviations

PL	phospholipids
CE	cholesterol esters
VLDL	very low-density lipoproteins
IDL	intermediate-density lipoproteins
LDL	low-density lipoproteins
HDL	high-density lipoproteins
ApoB	apolipoprotein B
CETP	cholesterol ester-binding protein
Apobec-1	apoB mRNA editing enzyme catalytic polypeptide-1
MTP	microsomal triglyceride transfer protein
ER	endoplasmatic reticulum
BiP	binding protein
GRP94	glucose-regulated protein 94
PDI	protein disulfide isomerase
ERGIC	ER–Golgi intermediate compartment
COP	coatomer protein
ARF1	ADP ribosylation factor 1
VTC	vesicular-tubule cluster
BAP31	B-cell receptor-associated protein 31
ERAD	ER-associated degradation
BFA	Brefeldin A
PLD	phospholipase D
MAP	mitogen-activated kinase
PIP2	phosphatidylinositol (4,5)-biphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
GEF	guanine nucleotide exchange factor
PERPP	post-ER presecretory proteolysis
LDLR	low-density lipoprotein receptor
Hsp70	heat shock protein 70
PI3K	phosphoinositol 3-kinase
NEFA	non esterified fatty acids
DGAT	diacylglycerol acyltransferase
ADFP	adipose differentiation-related protein
EGCG	epigallocatechin gallate

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The best way to become acquainted with a subject
is to write a book about it.

Benjamin Disraeli

INTRODUCTION

Lipoproteins

The transport and metabolism of dietary and endogenously synthesized lipids is achieved by the formation of soluble lipid–protein complexes known as lipoproteins. Amphipathic phospholipids (PL) (mainly phosphatidylcholine) and free cholesterol (FC) constitute the outer monolayer of the spherical lipoproteins and shield the insoluble core of triglycerides (TG) and cholesterol esters (CE) from the aqueous blood (Figure 1). Structural proteins known as apolipoproteins are embedded in the monolayer and can interact with the core and surface lipids.

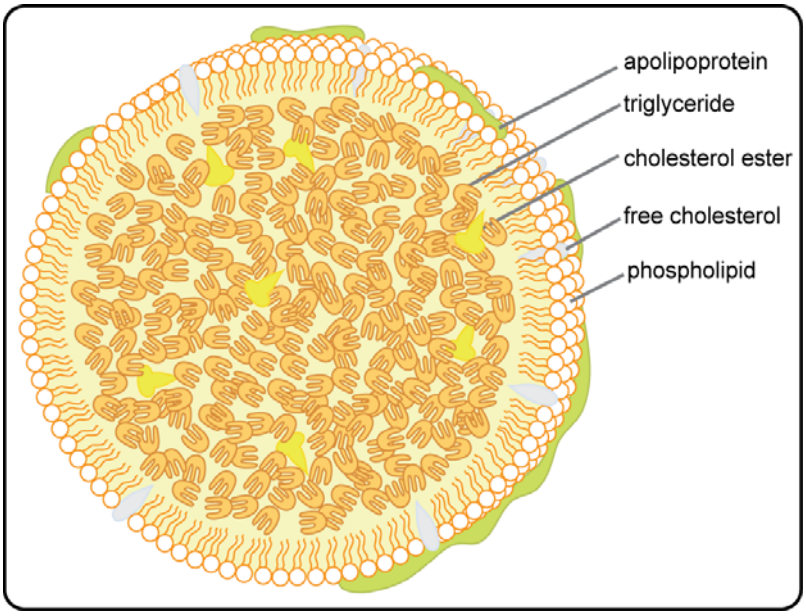


Figure 1. A schematic picture of a lipoprotein showing the surrounding monolayer of phospholipids and free cholesterol that shields the hydrophobic core of triglycerides and cholesterol esters from the aqueous environment. The apolipoprotein is located on the surface of the lipoprotein, interacting with both the surface and core lipids.

There are five different classes of lipoproteins (some of which can be further subdivided into subgroups), see Table 1 (adapted from[1]). The chylomicrons transport dietary lipids while very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL) transport endogenously synthesized lipids. High-density lipoproteins (HDL) are involved in the reversed cholesterol transport but also have antiatherogenic properties such as antioxidative and anti-inflammatory capacities[2, 3].

Apolipoproteins

The apolipoproteins are important for the structure, formation and solubilization of the lipid–protein complex, but also regulate its metabolism through different receptor interactions and enzyme regulations. The main apolipoproteins are divided into three groups based on their solution properties and structural homology[4]. Apolipoprotein A (apoA), apoC and apoE are small, water-soluble proteins that can exchange between different lipoproteins because of their weak association with the lipids. The non-exchangeable apolipoprotein apoB is large and water-insoluble. The third group of apolipoproteins can all bind to and carry monomeric lipids and consists of apoD,

retinol-binding protein, cholesterol ester-binding protein (CETP) and phosphatidylcholine transfer protein[4].

Lipoprotein class	Density (g/ml)	Particle diameter (nm)	TG %	CE %	PL %	FC %	Protein %	Major proteins
Chylomicrons	**<0.95**	**80-100**	**90-95**	**2-4**	**2-6**	**1**	**1-2**	**C, A, E, B48**
VLDL	**0.95-1.006**	**30-80**	**50-65**	**8-14**	**12-16**	**4-7**	**5-10**	**C, B100, E**
IDL	**1.006-1.019**	**25-30**	**25-40**	**20-35**	**16-24**	**7-11**	**12-16**	**B100, C, E**
LDL	**1.019-1.063**	**20-25**	**4-6**	**34-35**	**22-26**	**6-15**	**22-26**	**B100**
HDL	**1.063-1.210**	**8-13**	**7**	**10-20**	**25**	**5**	**45**	**A, C, E**

Table 1. The composition of the different lipoproteins, adapted from[1].

Apolipoprotein B - from gene to protein

The *ApoB* gene spans 43 kb and is located on human chromosome 2[5]. It is expressed mainly in hepatocytes and enterocytes, although lower expression has been recognized in human yolk sac[6], placental cells[7] and cardiomyocytes[8]. The gene contains 29 exons comprising approximately one third of the gene, which makes the *ApoB* gene rather small relative to the size of its gene product[4]. The introns are asymmetrically distributed, shown by the fact that 19 of the 28 introns are found within the first 1000 codons[9].

Constitutive expression of *ApoB* results in a protein of 4536 amino acids, referred to as apoB100[10]. In the mammalian intestine, a shorter form, apoB48, is synthesized due to a C-to-U mRNA editing process[11]. ApoB48 is colinear with the amino-terminal 2152 amino acids of the full-length form (Figure 2 A). The apoB mRNA editing enzyme catalytic polypeptide-1 (apobec-1) deaminates nucleotide 6666 (a cytidine becomes a uridine) thereby converting the glutamine codon into a stop codon[12]. Hence, the chylomicrons secreted from the enterocytes assemble with apoB48, while the lipoproteins secreted from the liver contain apoB100. In several species (e.g., rodents and dogs[11]) apoB100-as well as apoB48-containing lipoproteins are secreted from the liver, due to apobec-1 expression also in this organ[13, 14].

In the primary DNA sequence of apoB100, there are 25 cysteines, 16 of which form intra-molecular disulfide bonds that stabilize and enable correct folding of the mature protein[15]. There are also 20 amino-linked glycosylation sites present that further ensures correct folding, transport and secretion of the protein[16], as well as protection

from proteolytic degradation[17]. The molecular mass of apoB100 is hard to predict due to technical insufficiencies but the theoretical molecular mass of 512 kDa differs from the 550 kDa found by SDS-gel electrophoresis[18].

The secondary structure of apoB100 contains, in contrast to the exchangeable apolipoproteins, not only amphipathic α-helical domains but amphipathic β-strand domains as well. Both these structures contain a polar and a non polar side that faces the surrounding aqueous and lipid phases, respectively. The current knowledge around the structural domain composition of apoB100 relies only on computational approaches. Different approaches show slightly different results, and hence a consensus model has not yet been established[19, 20]. Analysis using the software program LOCATE suggests a model that contains five structures: a globular amino-terminal structure containing both α-helices and β-strands, followed by two alternating α-helix and β-strand motifs (NH2–$\beta\alpha_1$-β_1-α_2-β_2-α_3–COOH)[21]. In a more recent analysis, using further developed prediction methods that were compared with experimental data, the protein was decomposed into eight domains each with a proposed 3D structure[22] (Figure 2 B). The conformation, and hence the function, of apoB100 depends on its lipid environment. Thus, the lipid composition of LDL directly influences the LDL receptor and proteoglycan binding[23].

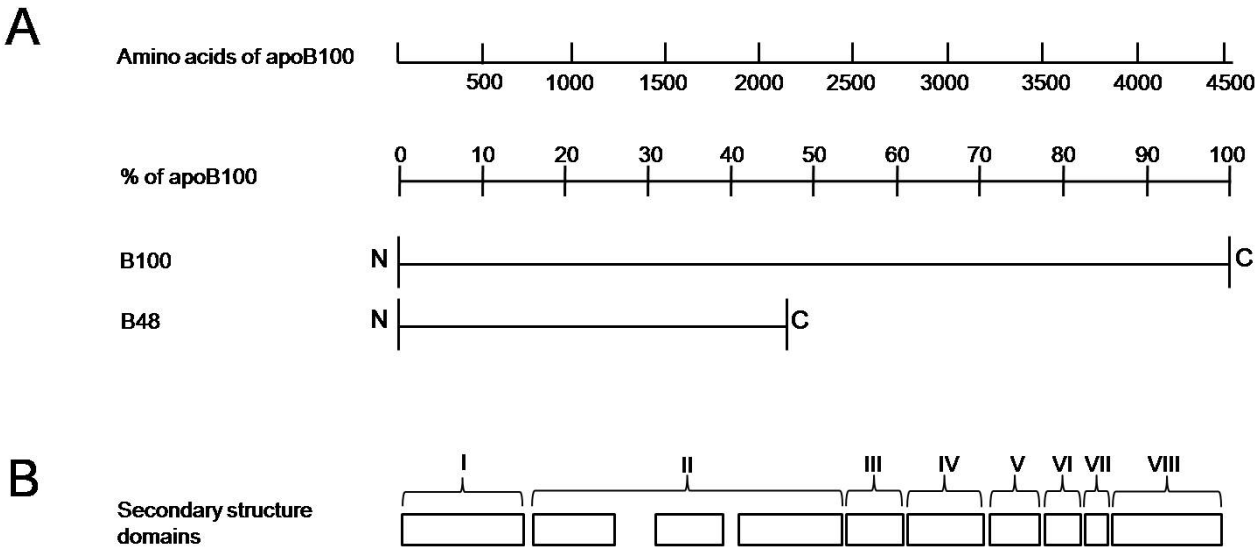


Figure 2. Schematic drawings of apoB100. The 4536 amino acids represents the complete protein of apoB100. ApoB48 corresponds to the amino-terminal 48% of apoB100 as shown in A. In B, the eight domains of secondary structures distributed over apoB100, adapted from[22].

The globular amino-terminal structure of apoB shows conserved sequence homology with several lipid binding proteins from the same multigene super-family referred to as the large lipid transfer protein family. Among these, the insect apolipophorin II/I precursor, invertebrate and the vertebrate vitellogenins, as well as the large subunit of mammalian microsomal triglyceride transfer protein (MTP) are included[24]. They all show amino-terminal homologies, but differ in the amount and type of lipid binding

properties of their carboxyl-terminal ends[25]. This end contains a lipid pocket that enables the initiation of the lipoprotein particle assembly process. The proposed model is based on the homologous regions of vitellogenin but in contrast to vitellogenin, one side of the pyramidal cavity required to create this lipid pocket is missing. In earlier studies, it was proposed that MTP would fill in as the third side[21], but further analysis of the sequence resulted in the identification of salt-bridges. The salt-bridges create interactions between the amino-terminal and carboxyl-terminal part of the globular domain, which creates a partially closed lipid pocket[26]. The structure of this model is supported by proline positioning as well as its preservation of the hydrophobic faces of the amphipatic β-sheets within this region. Moreover, it gives room for the structural changes that take place as the nascent lipoprotein particle expands into the mature particle.

Although the 3D model of apoB100 on VLDL is hard to elucidate, the organization of the protein on LDL has been mapped by monoclonal antibodies and electron microscopy[27], as well as by functional binding studies combined with expression of recombinant forms of apoB[28]. In this model, the first 89% of apoB100 wraps around LDL and encircles the whole particle. The rest of the protein folds back over the preceding amino-terminal part and an interaction between amino acids Arg 3500 and Trp 4369 is established. This interaction prevents the carboxyl-terminal part of apoB from sliding over the binding site for the LDL receptor (LDLR) binding site between amino acids 3359 and 3369.

The secretory pathway

Secretory proteins, including apolipoproteins, are synthesized on the endoplasmic reticulum (ER) by the ribosomes and released into the ER lumen. Once packaged into vesicles, they are transported from one organelle to another by anterograde vesicle transport. Finally they fuse with the plasma membrane and are secreted.

Translation within the ER

Most proteins in higher eukaryotes are simultaneously translated and transported across the ER membrane. A signal peptide on the nascent polypeptide causes a halt in the translation until the complex has been directed to receptors at the membrane surface of the ER. Upon receptor binding, the signal peptide is displaced and the translation is allowed to continue. The protein is transferred into the lumen of the ER through the translocon – a membrane complex that forms an aqueous pore.

Chaperones and folding enzymes

Once in the lumen of the ER, chaperones and folding enzymes associate with the nascent protein. This primary quality control regulates the transport of proteins from the ER to the Golgi and involves posttranslational modifications and folding of the proteins. It is applied to all synthesized proteins and is based on structural and biophysical features, such as exposure of hydrophobic regions, the presence of unpaired cysteine residues, lack of compactness and aggregate formation. In normal

protein assembly, interaction with chaperones occurs transiently as these features are exposed for only a very short time. If correction of aberrant proteins fails, they are recognized, retracted through the translocon back to the cytosol, covalently tagged with ubiquitin and then degraded by the proteasomes (reviewed in[29]).

The ER-abundant chaperones include binding protein (BiP), the lectins calnexin and calreticulin, glucose-regulated protein 94 (GRP94) and thiol oxidoreductases of the protein disulfide isomerase (PDI) family (for review see [30]). BiP assists protein folding by recognizing hydrophobic regions of peptides that are usually buried inside native proteins[31]. GRP94 has peptide-binding properties while calnexin and calreticulin identify monoglucosylated glycoproteins and assist their folding[32]. PDI has several different functions to assist protein folding and to avoid protein aggregation, such as the catalyzed oxidation of disulfide bonds as well as the isomerization and reduction of disulfide bonds[32].

All these proteins contain a carboxyl-terminal retrieval signal known as the KDEL sequence[33] which interacts with the integral membrane KDEL receptor localized in the Golgi complex and ER–Golgi intermediate compartment [ERGIC, also known as the vesicular-tubule cluster (VTC)][34]. This interaction prevents KDEL-containing proteins from entering the later parts of the secretory pathway[35]. The formation of coatomer protein (COP) I vesicles ensures the retrograde transport of KDEL-containing proteins back to the ER. The budding and transport of COPI vesicles are dependent on the GTPase ADP ribosylation factor 1 (ARF1)[36]. Ligand interaction with the KDEL receptor on the luminal side has been shown to result in activation of ARF1, thereby indicating that the KDEL receptor itself can actually modulate the retrograde transport pathway[37].

ER exit and anterograde transportation

Once the proteins are folded correctly, they enter ER exit sites from where vesicles assemble and bud off. This is accomplished by the COPII complex (Sar1p, Sec23/24p, Sec13/31p) and its regulator Sec12p[38]. The vesicles are typically 60–70 nm in diameter[39], but, as will be discussed later, are suggested to vary in size (see[40] for a review).

The vesicles shed their coat proteins and fuse to form the next organelle in the transport pathway, the ERGIC. The ERGIC fuses with the cis-Golgi in preparation for the next step, which is known as cisternal maturation (reviewed in[41]). This maturation is dependent on the retrograde delivery of the organelle-specific proteins (Figure 3).

The final event in which the secretory proteins are delivered to the plasma membrane and released into the circulation is not well defined[40].

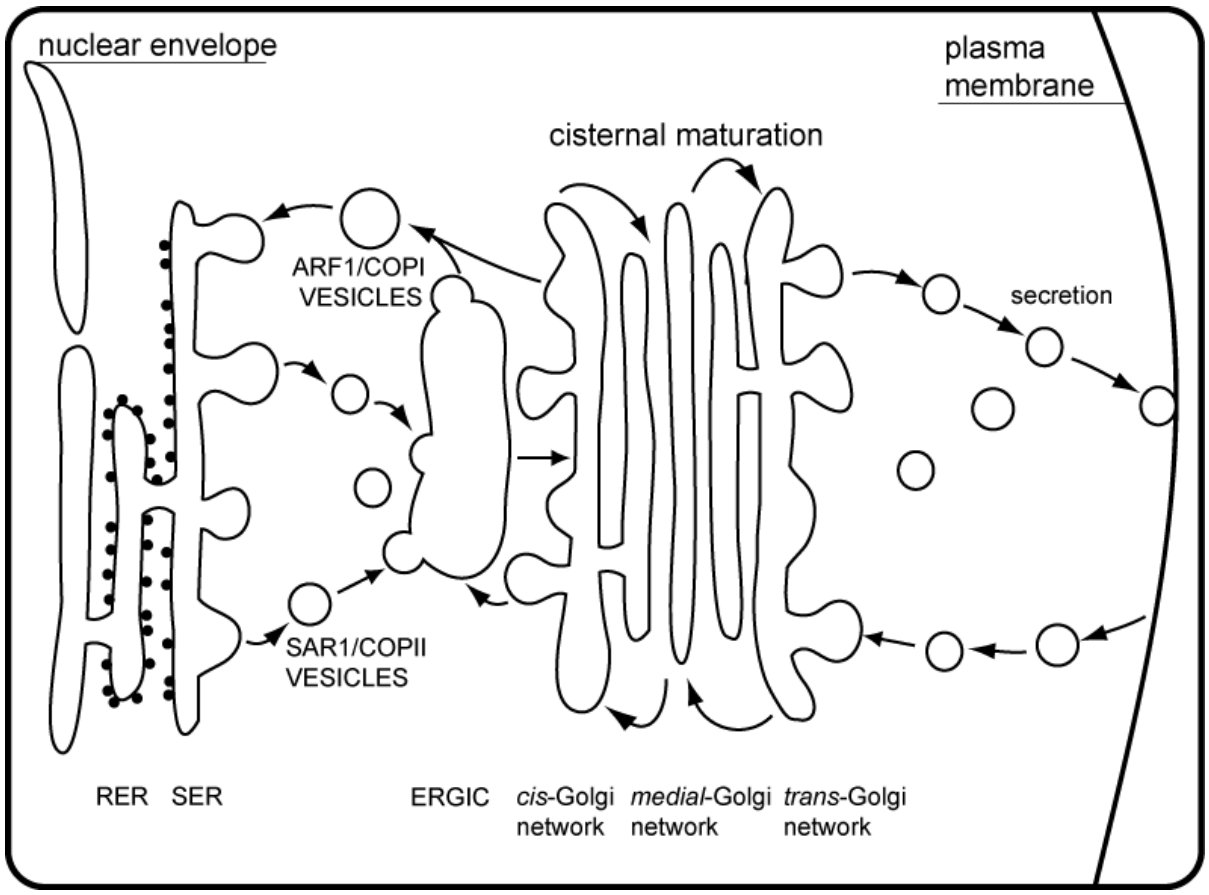


Figure 3. The secretory pathway of the cell. The ribosomes on the rough ER (RER) synthesize the proteins that are internalized into the lumen of the ER. Translated proteins are folded correctly by the help of chaperones and sorted to exit sites in the smooth ER (SER). SAR1 and COPII vesicles transport the proteins and merge to form the ERGIC. ERGIC fuse with the cis-Golgi network and by mechanisms incompletely understood, cis-Golgi will mature into medial Golgi that in turn will mature into trans-Golgi by cisternal maturation. Finally, when the proteins reach the trans-Golgi they will be further modified and sorted for secretion.

BAP31

The ubiquitously expressed B-cell receptor-associated protein 31 (BAP31) is a membrane protein important for the control of ER release of certain secretory proteins[42, 43]. It has been found both in the ER and associated to vesicles and cisternae of the Golgi and is thought to shuttle between these[44]. The protein contains an ER retention signal - different from the KDEL sequence - the KKXX signal[45] and besides having a role in cellular traffic, BAP31 is also apoptosis-related. Its carboxyl-terminal part contains two caspase-sensitive motifs that can be cleaved off by caspase-8[46]. In its full length, BAP31 is anti-apoptotic, but when cleaved, it promotes Ca^{2+}-release from the ER followed by mitochondria-induced apoptosis.

The assembly mechanism of apoB-containing lipoproteins

Much of the knowledge concerning the assembly mechanism of lipoproteins is based on studies using the human and rat hepatoma cell lines, HepG2 and McArdle 7777 respectively, and primary hepatocytes. Despite many reports, the mechanism is still not fully understood. It is well established though that the VLDL[47-50] as well as the chylomicrons[51] assemble with a consecutively increased lipidation, involving at least two more defined steps.

The first step that occurs during the apoB translation gives rise to a lipid-poor primordial particle, pre-VLDL, with a density as HDL[50, 52]. This has been shown (in HepG2 cells) to be initiated when apoB100 has reached the size of approximately 80 kDa[52]. The lipid load and size of the pre-VLDL particle increases as the translation continues and displays a direct relationship between the nascent polypeptide size and the size of the assembled particle[52, 53]. With a translation rate of 6 amino acids/s, the apoB100 is fully translated after approximately 14 min[54] and the formed dense particle mainly remains loosely attached to the ER membrane awaiting its fate[55, 56]. If apoB48 is the assembling lipoprotein, the dense particle is secreted but might, if enough TG present, combine with them in the second assembly step[56].

In the second assembly step, the lipid-poor particle is thought to be transported into a compartment separate from the ER where the bulk addition of lipids occurs[47, 56]. Unfortunately, the details of these processes are still unclear, particularly how and exactly where this bulk addition takes place. Results from classical immuno-electron microscopy experiments showed the presence of apoB-free VLDL-sized particles in the SER[47]. These were thought to constitute the lipid core of VLDL that in the second step of VLDL assembly associates with the pre-VLDL particle to form the mature VLDL. The mechanistic events though are still not determined, but results from other *in vitro* experiments[50] and human *in vivo* data[57] support this hypothesis. The latter report showed that mature VLDL particles are secreted 33 min after their initiated synthesis and there is a time delay of approximately 15 min seen between the newly synthesized apoB100 and its major lipidation step. These results further strengthen the concept that TG can be added to a pre-existing pre-VLDL particle in the human liver.

ApoB100 can be secreted in two forms: large TG-rich $VLDL_1$ and smaller, TG-poor $VLDL_2$, where an overproduction of $VLDL_1$ is linked to insulin resistance and type II diabetes[58]. There are however indications that the protein can be secreted on denser lipoproteins such as IDL and LDL[59, 60]. The size of the mature circulating apoB100-containing VLDL is typically 80-100 nm in diameter[1, 61]. This suggests that it would not fit into the COPII-assembled vesicles if formed in the ER. On the other hand, both pro-collagen (>300 nm long) and chylomicrons (up to 1200 nm in diameter) are exported, which favours the belief that COPII vesicles might vary in size. However, as will be discussed below, the lipoproteins thought to be assembled in the ER might not be the mature lipoprotein and therefore smaller, fitting the vesicles. Due to the small size, these particles cannot be seen by routine electron microscopy and have therefore not been documented.

There is another retrograde transport model proposed where the exported material move in large pleiomorphic carriers instead of in vesicles. These large (100-1000 nm) carriers form via extrusion from the donor membranes and could account for the transportation of large secretable proteins from the ER to the Golgi, and could also explain the transportation from Golgi to the plasma membrane (reviewed in[62]).

Proteins necessary for the lipoprotein assembly process

MTP: the lipid transporter

MTP is a heterodimeric protein composed of PDI and a unique 97 kDa subunit[63]. It resides in the ER lumen[64], but has also been recognized in the Golgi apparatus[65]. PDI is a ubiquitous multifunctional protein while the larger subunit of MTP, expressed mainly in liver and intestine, has the ability to transfer various lipids between membranes[66]. The protein complex transfers the different lipids according to their hydrophobicity and although TG and CE are preferred, PL are also transferred[67]. A genetic lack of MTP results in the human disorder abetalipoproteinemia where both intestinal as well as liver-derived apoB-containing lipoproteins are almost completely absent from plasma[66, 68, 69].

The amino-terminal end of MTP - which has been modelled using its relative, the vertebrate vitellogenin - contains a lipid binding cavity and two adjacent conserved α-helices, which are thought to mediate the acquisition and binding of lipids. While one helix is suggested to interact with the membrane in a similar manner as a viral fusion peptide, the other is necessary for the transfer of lipids into the cavity of MTP[70].

The thiol oxidoreductase PDI is commonly found as a part of multimeric protein complexes. Its chaperone activities would be plausible during VLDL assembly, but cell experiments using PDI where the two sites for chaperone activity have been mutated, showed that the disulfide isomerase activity was not required for the MTP lipid transfer activity. Reports indicate that PDI when part of the MTP complex assists as a solubilizer of the larger subunit and due to the KDEL sequence localizing MTP to the ER and Golgi compartments[71].

MTP has been stated to have several different roles in VLDL assembly and is widely believed to be essential during the initial co-translational lipidation step of apoB[64, 72-74]. During this co-translational lipidation, the lipid-loaded MTP binds to the amino-terminal part of apoB and forms a complex that allows the lipid cavity of MTP in close proximity to the growing nascent polypeptide chain, possibly to facilitate lipid transport[75].

There are findings that MTP activity seems necessary also during a short time period just after the translation of apoB100 is completed[55, 76]. Inhibition with specific MTP inhibitors during either of these phases will sort the particle for ER-associated degradation (ERAD)[55] . MTP does not seem to be crucial for the major lipidation step[64]. However, conflicting results have been reported[77] (reviewed in[78]).

Likewise, results from MTP knockout mice showed different results. In one of the studies, the total plasma triglyceride level was reduced[82] while in the other no such effect was seen[83]. Furthermore, there were discrepancies regarding the secretion of hepatic apoB48-lipoproteins in the liver-specific knockout mice: a 20% reduction[82] vs. an almost absence of apoB48 in the plasma[83]. Both studies though, showed an essentially complete absence of apoB100-containing lipoproteins in the plasma of these mice. The reason for these differences is not obvious, but needs further investigation.

ARF1 and the PLD activation

The second step of lipoprotein assembly requires ARF1 and its activation of phospholipase D1 (PLD1)[79]. ARF1 is a small ubiquitously expressed GTPase that has been shown to be necessary for the conversion of pre-VLDL to the mature VLDL[79]. Like all GTPases, it switches between an active GTP-bound and an inactive GDP-bound conformation. Their regulation is determined by GTPase-activating proteins that inactivate them by accelerating the hydrolysis of bound GTP and GEFs (guanine nucleotide exchange factors) that facilitate the substitution of GDP with GTP (reviewed in[80]). The GDP–GTP exchange is believed to occur via transient intermediates, of which ARF1 first forms a complex with GDP and the Sec7 domain of the GEF[81]. Brefeldin A (BFA) is a fungal metabolite that stabilizes this intermediate, inhibiting the release of GDP following binding of GTP[82, 83]. As a result, the intracellular transfer and secretion of proteins are blocked. BFA has been used to investigate the function and activity of ARF1[84] and hence has clarified some important features and regulations of the VLDL assembly.

Class I ARF proteins, which includes ARF1, activate lipid-modifying proteins and regulates the formation of budding vesicles along the secretory pathway[85]. It is well established that transport vesicles from the Golgi to the ER and within the Golgi are mediated by ARF-recruited COPI (reviewed in[85]), but ARF1 is also involved in the anterograde transport from the ER to Golgi[86] as well as in the formation of ERGIC (for review see[87]). Hence, the ARF1 dependency in VLDL assembly could be explained by its function in ERGIC formation, and/or the following fusion between ERGIC and *cis*-Golgi.

In addition, the VLDL assembly is dependent on yet another function of ARF1, namely by its activation of the ubiquitously expressed PLD1[88]. The ER-localized PLD1[89] is a generator of second messengers involved in signaling pathways. For instance, experiments in a cell-free system demonstrated that the PLD1-catalyzed formation of phosphatidic acid was necessary for VLDL assembly[79] (see[89] for review). Phosphatidylinositols are considered regulators of PLD1 activity and *in vitro* studies showed that phosphatidylinositol (4,5)-biphosphate (PIP2) and phosphatidylinositol (3,4,5)-triphosphate (PIP3) activated PLD1 with similar potency in the presence of ARF1[90], but PIP3 had lessened efficacy[88]. A positive feedback loop is also plausible in which the kinase that phosphorylates phosphatidylinositol (4)-phosphate is activated by phosphatidic acid, resulting in more active PLD1[91]. Furthermore, PLD1 might also be regulated by members of the large and variable group of protein kinase C enzymes

(reviewed in[91]) as well as specific Rho GTPases[92]. The regulation of PLD1 and its signalling events remains poorly defined but it is clear that the localization of both PLD1 and its activators play a substantial role in the downstream signaling cascade.

ApoB degradation pathways

Of all new polypeptides synthesized in the cell, one third are degraded during or very soon after their synthesis. This is thought to be the result of a highly active protein-quality control system present in the cells. Studies in both HepG2 cells and primary hepatocytes show that a significant amount (between 40-60% in the latter[93] and up to almost 80% in the former[54]) of newly synthesized apoB is degraded. The understanding of the mechanisms behind this intracellular degradation are yet not complete, but the degradation is decreased when the supply of fatty acids is increased which supports the fact that the lipidation of apoB is a determining factor[54, 94]. Even after the translation of apoB, the nascent polypeptide stays in close proximity to the translocon, and so can be targeted either for degradation or assembly into lipoproteins depending on the lipid availability[95].

There are three different pathways of hepatic apoB secretory control: 1) the ERAD; 2) the post-ER presecretory proteolysis (PERPP); and 3) reuptake from the unstirred water layer around the outside of the plasma membrane via the LDLR[96].

ER-associated degradation

ERAD has been extensively investigated and involves the cytosolic heat shock protein 70 (Hsp70)[97]. ApoB is retracted from the lumen of the ER back through the translocon to the cytosol where it interacts with Hsp70[98]. The degradation pathway is stimulated when either MTP activity or co-translational lipidation is limited. Less newly synthesized apoB is needed during these circumstances, and the excess is ubiquitinated and ultimately degraded by proteasomal mechanisms[97], presumably by a chymostrypsin-like activity[99]. Since apoB is prone to form irreversible aggregates when poorly lipidated, Hsp70 might maintain apoB in a conformation that promotes the ubiquitinylation enzymes and/or the proteasome [97].

Post-ER presecretory proteolysis

The mechanism of the post-translational degradation PERPP (that appears to occur in a compartment other than the rough ER, hence the name) is less well known. It is possible that there are several different pathways of non-proteasomal degradation since different studies show effects from agents in one context but not in others[99]. Interestingly, ω-3 fatty acids have been shown to stimulate PERPP and as a result less apoB-containing lipoproteins were secreted (consistent with their dietary effects on plasma lipoproteins)[100]. The ω-3 fatty acids seem to increase the cellular content of lipid peroxidation products, which triggers the post-translational degradation of apoB[101]. This link between cellular stress and apoB degradation is strengthened by experiments in which agents with antioxidant activity are included and, despite the presence of the ω-3 fatty acids, reduce the degradation, resulting in increased secretion of apoB[101].

LDLR-mediated degradation

The LDLR is responsible for the last degradation pathway. The receptor mediates the reuptake of newly secreted lipoproteins by binding to the specific LDLR binding region of apoB, internalizing and returning the lipoproteins into the cell for degradation. The degradation pathway is thought of as a post-secretion degradation pathway, although more recent data also have shown that the LDLR does interact with apoB early in the secretory pathway as well[102, 103]. While they are still within the unstirred waterlayer, very close to the plasma membrane, the lipoproteins can bind to the receptor and be internalized into the cells[96].

The regulation of lipoprotein assembly

As stated above the lipoprotein assembly is mainly regulated by the availability of its lipid ligands. The metabolic status, signalling pathways as well as genetic disorders determine the production rate and catabolic fate of lipoproteins.

Structures in apoB that determine the lipidation

There are several different genetic disorders that may result in an over- or underproduction of apoB-containing lipoproteins[104-108] (reviewed in[109, 110]). Regarding the heterogeneous autosomal trait, familial hypobetalipoproteinemia, the majority of the affected subjects are heterozygotes who are asymptomatic but have very low levels of cholesterol and apoB-containing lipoproteins in the plasma. The best characterized cause is early carboxyl-terminal truncations of the apoB, ranging from as short as apoB2 to apoB89, which mirrors the heterogeneity of the syndrome[111]. Truncated forms shorter than apoB27 have not been reported isolated from plasma[56], but the longer forms of truncated apoB molecules give rise to apolipoproteins of a variety of densities, ranging from HDL to VLDL. The shorter the apoB, the smaller amount of the TG are incorporated. It has been shown *in vitro* that carboxyl-terminal-truncated apoB molecules must reach the size of apoB40 to assemble VLDL, but not until the apoB molecule reaches apoB48 is a substantial amount of VLDL formed[56]. This result argues that the carboxyl-terminal part of apoB48 contains a sequence that is critical for recruiting TG.

The effects of insulin

There are many levels of regulation of lipid availability and the influence of insulin is of great importance. The cascade signalling initiated by insulin when binding to its receptor on the cell surface, is mediated via two major pathways: the mitogen-activated protein (MAP)-kinase pathway and the metabolic, phosphoinositol 3-kinase (PI3K) pathway. Insulin regulates the VLDL assembly at several levels and one direct consequence mediated by PI3K is reduced apoB mRNA levels simultaneously as the degradation of newly synthesized apoB is increased[112-115]. Moreover, the mitogenic MAP-kinase-mediated pathway signals to downregulate the transcription of MTP due to binding of the sterol regulatory element binding protein 1-c to the sterol-response

element in its promoter[116]. This will also result in an increased degradation of newly transcribed apoB, since the pre-VLDL formed will be underlipidated.

Insulin has been shown in rat hepatocytes to inhibit the conversion of pre-VLDL to $VLDL_2$[114, 117] and, furthermore, is also involved in the second lipidation step. Insulin infusion studies in human subjects showed that the $VLDL_1$ production was suppressed with little effect on the $VLDL_2$ production[118]. This indicates that $VLDL_1$ and $VLDL_2$ assembly and/or secretion are separately regulated.

PLD1 – necessary for the second step of lipoprotein assembly – is activated by PIP2, and a lack of PIP2 prevents activation of PLD1 and subsequently the second step of VLDL assembly. Under normal intracellular insulin signalling, PI3K, which converts PIP2 into PIP3, is activated and inhibits the second step of VLDL assembly[92]. During defective insulin signalling, however, as in the insulin-resistant state or in subjects with type II diabetes, the absence of insulin's inhibitory effect results in excess of PIP2 and a subsequent activation of PLD1[114]. This could be a plausible explanation to the increased plasma $VLDL_1$ level seen in type II diabetic patients[115]. Interestingly, the amount of lipids added to $VLDL_1$ is not increased in subjects with type II diabetes compared with controls, but the rate of conversion from $VLDL_2$ to $VLDL_1$ is increased in patients suffering from this disease[57]. Thus, it seems that the assembly process in patients with type II diabetes is not defective and more, not larger, $VLDL_1$ are secreted in these patients. This is a very important finding since patients with diabetes have an increased mortality rate from cardiovascular disease (CVD)[119], the leading cause of morbidity and mortality in the world[120, 121]. The dyslipidemia displayed in these patient (a common CVD risk factor associated with type II diabetes) is indeed characterized by increased plasma triglycerides (mainly $VLDL_1$) accompanied by the formation of small, dense LDL, and decreased HDL[122].

Another level of insulin regulation is its effect on the adipose tissue. The hormone has anti-lipolytic effects on adipocytes, which lead to a reduced export of non esterified fatty acids (NEFA), followed by negative effects on the $VLDL_1$ assembly and secretion (reviewed in[123]). A further regulating step by insulin is to channel the hepatic lipids into cytosolic droplets and storage instead of incorporation into VLDL[114, 124, 125].

The hepatic triglyceride pool

TG stored in hepatic cells can have different origins and the relative amount of contribution from these sources for utilization in the secretion of lipoproteins from the liver is partly variable[126]. The main three sources of NEFA that constitutes the hepatic TG pool are: *de novo* lipogenesis, hepatic uptake of remnant particles and from peripheral tissue-derived NEFA in the plasma[127].

The majority (at least 60%) of the TG in VLDL are derived from cytosolic lipid droplets that follow a lipolysis-re-esterification cycle[127-129] (reviewed in[130]). This means that the majority of NEFA, taken up by the liver from the circulation, are not immediately incorporated into the VLDL, but directed into storage as TG in cytosolic

lipid droplets. When needed, the stored TG are hydrolyzed back to NEFA, used as TG in the lipoprotein core, and excess amounts are then re-esterified into TG and stored until further notice.

The enzymes participating in the lipolysis-re-esterification cycle and TG transport within the cell are obscure and very complex. Extracellular NEFA entering the hepatocytes are esterified by diacylglycerol acyltransferase (DGAT)-1 and give TG for storage in the cytosol. These TG from cytosolic storage pools are mobilized possibly by the lipolytic action of the two lipases: arylacetamide deacetylase and triacylglycerol hydrolase, and re-esterified on the luminal side of the secretory appartus. This event requires DGAT-2, which is a separate gene product from its isoenzyme DGAT-1 and differently regulated[131-133].

In *in vitro* studies where an exogenous lipid (e.g. oleic acid) is supplemented, increased assembly of both apoB48- and apoB100-containing lipoproteins is seen[134]. Also, in an insulin-resistant mouse model, where the NEFA flux from adipose tissue to the liver is increased, the lipoprotein secretion was also elevated[128]. These results suggest that an increased FFA supply leads to an increased secretion of lipoproteins.
This conclusion is supported by a recent report where stable isotopes were used, showing that the major amount of TG for lipoprotein assembly is attained from plasma NEFA both in the fed and fasted state[135].

Although the lipid availability is a determining factor for lipoprotein secretion, there are arbitrary results concerning this topic. For instance, the NEFA flux towards the liver is increased in mice deficient in the receptor CD36, responsible for the NEFA transport in peripheral tissue. None the less, there is no evidence of an increased VLDL production rate in these mice despite increased hepatic TG storage[136]. This suggests that the hepatic TG content *per se* does not necessarily result in an increased lipoprotein secretion[137, 138].

The hepatic cholesterol content as well as the phospholipid content might also influence the lipoprotein assembly. For instance, the cholesterol synthesis correlates with $VLDL_2$ but not with the $VLDL_1$ production[139]. This finding may explain why only the $VLDL_2$ and not the $VLDL_1$ are increased in patients with increased plasma cholesterol[60, 140].

Lipid droplet formation

The ability to store lipids seems to be a fundamental process in all cell types investigated[141]. This is logical since not only are TG the most concentrated form of energy available to the tissue, they are also a very benign form of NEFA storage[142]. The TG are stored in intracellular cytosolic lipid droplets ranging from 0.1-50 μm in diameter depending on the cell type and nutritional status[143]. The droplets constitute a TG and/or CE core surrounded by a monolayer of PL and proteins[144], and there are several enzymes critical for the initiation and growth of the lipid droplets. The most well known are the PAT-domain protein family, a group of proteins reported to associate with the lipid droplets and involved in the metabolic and catabolic fate of

these lipids. The family includes the adipose differentiation-related protein (ADFP), tail interacting protein 47 (TIP47), S3-12 and lipid storage droplet 5 (LSDP5). ADFP is the protein located on lipid droplets during the adipocyte differentiation[145]. Its precise role is unclear but it is a marker for non-adipocytes and becomes replaced by perilipin at a later stage of adipocyte maturation. Perilipin is the most well studied PAT-protein and prevents TG hydrolysis when unphosphorylated and inactive, but is reported to stimulate the same reaction via hormone-sensitive lipase when phosphorylated (see[146] for review). The ubiquitously expressed TIP47 and S3-12 (mainly expressed in white adipose tissue) appear to translocate from the cytosol to the lipid droplets upon fatty acid stimulation[147, 148], while the LSDP5 is thought to prevent lipid droplet TG hydrolysis in muscle, heart, liver and brown adipose tissue[149].

The lipid droplet formation takes place in the microsomal membranes[150], which fits well with the presence of the enzyme machinery needed for TG synthesis (reviewed in[151]). Also, an active PLD1 and extracellular signal regulated kinase (ERK) 2 have been recently shown to play central roles in the lipid droplet formation[125, 150]. The synthesized TG are deposited within the ER bilayer. They then oil out and become a lens-shaped droplet that is the core of the future lipid droplet. This lens will increase in size until it finally buds off into the cytosol. The initial droplets have a diameter of only 0.1-0.4 μm[152] that later in the cytosol will have increased in size to between 0.5-5 μm (in adipocytes much larger). Lipid droplets increase in size by fusion, a process dependent on intact microtubules and the phosphorylated (by ERK2) motor protein dynein[153].

The fusion process of lipid droplets is thought to occur in a simpler but similar way to the fusion of transport vesicles and target membranes[154]. The machinery involves the N-ethylmaleimide-sensitive factor (NSF), α-soluble NSF adaptor protein (α-SNAP) and different SNAREs (SNAP receptors). The SNAREs present on the target membrane and on the transport vesicle interact with each other and form a four-helix bundle that forces the two membranes together, causing them to fuse with each other. After the fusion event the SNARE complex is dissolved by NSF and α-SNAP (reviewed in[155]).

METHODOLOGICAL CONSIDERATIONS

Below follows discussion of some of the methods and different models used in this thesis. A more detailed description of them is given in each of the papers and their references.

The work in this thesis involves both *in vitro* and *in vivo* experiments. Usually, both these approaches are used to obtain detailed information about structure–function relationships in genes and their protein products. *In vitro*, which literally means "in glass", experiments may not necessarily represent the exact scenario seen inside an organism, but there are fewer variables that can confound an experiment. In principle, in an *in vitro* experiment the conditions are artificial and are reconstructions of what might happen *in vivo.* On the contrary, *in vivo* (latin for (with)in the living) experiments have an advantage over *in vitro* research as the experiments are performed in a more complex biological system, and usually therefore more properly portray the "true picture".

In vitro approaches

When looking at specific events in specific cell types, the use of cell lines is often superior. The immortality of cell lines is due to either tumour cell transformation, transformation induced using viral oncogenes or by chemical treatments. The advantage of cell lines is of course, unlimited (almost) availability, but a disadvantage is loss of *in vivo* characteristics. Cells *in vitro* can grow either in suspension or as a monolayer that is attached to the culture flask.

McArdle 7777 cells

In all three papers, we used the rat hepatoma cell line McArdle 7777 (McA-RH7777), which has been extensively used to study the assembly and secretion of lipoproteins. Since it is a cell line derived from a rodent, the monolayer growing cells when cultured in the presence of oleic acid assemble and secrete both apoB48- and apoB100-containing lipoproteins. Despite this discrepancy from human liver cells (which secrete only apoB100), this cell line resembles the assembly and secretion patterns of lipoproteins in man. Unlike for example the human hepatocarcinoma cell line HepG2, which is unable to secrete fully lipidated lipoprotein, the McA-RH7777 cell line completes the assembly steps of lipid-rich lipoproteins. In paper I and II, truncated and chimeric constructs of apoB100 were transfected into McA-RH7777 cells. Stable clones were selected and maintained by adding the aminoglycoside antibiotic G418 (neomycin) in the cell culture medium, since the cells were co-transfected with a vector that encodes the neomycin resistance. As always, when using stable clones, it is very important to avoid selecting subclones. It is therefore important to have internal controls while running the experiments. In our case, we used endogenously expressed apolipoproteins as internal standards of selected stable clones.

Primary mouse hepatocytes

When preparing primary cell cultures, animal tissue is excised and cultured either as an explant or as single cell suspension following enzyme digestion. These cell cultures survive *in vitro* only for a limited period. Both in papers I and III, the method of nonrecirculating collagenase perfusion was used to isolate mouse hepatocytes. The cells were used shortly after isolation since control experiments showed stable lipoprotein secretion 24 h after seeding of the cells.

In vivo approaches

As discussed above, the *in vivo* approaches add great complexity to the experiment and are more accurate in regard to how mechanisms and events are affected by the physiological surroundings. During the past decade, the use of transgenic mice has vastly improved the biomedical research and today is a common tool when investigating molecular mechanisms, different diseases and their treatments. In papers I and III, we took the advantage of using the transgenic apoB100×LDLR$^{-/-}$ mice. The role of the LDLR in the retention of apoB100-particles as well as the mechanism of how the phenol epigallocatechin gallate (EGCG) affect the TG utilization was investigated in primary hepatocytes from these mice.

Lipid droplet quantification

In paper III, the amount of cytosolic lipid droplets was quantified. The stored neutral lipid pool was stained with the red dye Oil Red-O and since the cells were cultured on cover slips, once mounted the cells were photographed in a light microscope. The degree of lipid droplet formation was quantified by the software program Biopix that can not only quantify the amount of pixels within a specific colour range and intensity, but also has the ability to categorize adjacent coloured pixels and identify them as parts of a lipid droplet. The result is a measurement of the total lipid area content per cell as well as the droplet size distribution. The method has been evaluated previously and detailed descriptions of these experiments are presented as supplemental data in[125]. In summary, the quantification made with this software shows an agreement with manually lipid droplet measurements and is a valuable tool when determining droplet size.

AIM

The general aim of this thesis was to further clarify the molecular mechanisms of the assembly process and secretion of apoB-containing lipoproteins.
The specific aims were:

Paper I

- To test the hypothesis that two separate pathways, the two-step process and an apoB size-dependent lipidation process, give rise to different lipoprotein

Paper II

- Identify and elucidate the minimal sequence of apoB needed for $VLDL_1$ secretion
- Investigate a possible reason for this sequence requirement

Paper III

- To study the impact of EGCG on hepatic VLDL secretion
- Investigate the relationship between cytosolic triglycerides and VLDL secretion

SUMMARY OF RESULTS

Paper I

The aim of the study was to test the hypothesis that two separate pathways, the two-step process and an apoB size-dependent lipidation process, give rise to different lipoproteins.

McA-RH7777 cells were transfected with carboxyl-terminal truncated forms of apoB (apoB41, apoB53, apoB72 and apoB80). Analysis of secreted lipoproteins revealed that all recombinant forms except apoB41 assembled substantial amounts of $VLDL_1$. To test if the ability of apoB to form $VLDL_1$ depends on specific sequences located between apoB41 and apoB48, or simply is length-dependent, apoB41 was fused to the carboxyl-terminal 28% of apoB100 and expressed in McA-RH7777 cells. Results showed that the chimeric apolipoprotein failed to assemble $VLDL_1$.

All carboxyl-truncated recombinant apolipoproteins, as well as endogenous apoB48 and apoB100, were also secreted on less lipidated lipoprotein particles whose densities were inversely related to the size of apoB. This lipidation results in a lipoprotein particle with density as LDL-$VLDL_2$ in full-length apoB100. Kinetic studies indicated that these lipoproteins are precursors of intracellular $VLDL_1$. To test if the formation of these lipoproteins was dependent on the length of apoB only, apoB50 was fused to the carboxyl-terminal 18% of apoB100 and expressed in Mc-RH7777 cells. Results showed that the chimeric apolipoprotein assembled a lipoprotein protein with predicted density of apoB68. In contrast, B41 + (72–100) failed to acquire the expected density of apoB69. Thus, the size-dependent lipidation is not simply length-dependent.

ApoB80 and apoB100 were also detected on intracellular, retained dense pre-VLDL particles (d=1.10 g/ml). Their formation was dependent on the sequence between apoB72 and apoB90. Kinetic studies of these lipoprotein particles revealed a precursor–product relationship with secreted $VLDL_1$.

We next explored potential mechanisms for the intracellular retention of pre-VLDL particles. First we tested the importance of the LDLR using hepatocytes isolated from human apoB100 and apoB100×$LDLR^{-/-}$ transgenic mice. The results showed that the LDLR is not involved in the intracellular retention. Next we isolated microsomal dense lipoproteins and analyzed their protein content. Results showed that the dense lipoprotein particles contained the chaperones PDI and BiP, indicating that the lipoproteins might be retained by chaperones.

We propose that apoB100 assembles into partially lipidated dense pre-VLDL that are retained in the cell unless further converted into LDL-$VLDL_2$ by size-dependent

lipidation. LDL-$VLDL_2$ in turn can proceed through the secretory pathway to be secreted or converted to $VLDL_1$ in the second step of the assembly. Furthermore, an efficient formation of $VLDL_1$ requires that apoB has reached the size of apoB48.

Paper II

The aims of the study were to identify and elucidate the minimal sequence needed for $VLDL_1$ secretion, as well as to elucidate the mechanism for this requirement.

McA-RH7777 cells were transfected with the carboxyl-terminally truncated apoB, apoB46.8. Analysis of the secreted lipoproteins showed that although less lipidated forms of apoB were assembled, the cells failed to secrete any significant amount of $VLDL_1$. This result indicates that amino acid residues between apoB46.8 and apoB48 are crucial for the assembly of $VLDL_1$.

The ubiquitously expressed BAP31 was found to interact with a synthetic peptide corresponding to the sequence between apoB46.8 and apoB48. We next explored the impact of this ER-resident protein on lipoprotein secretion. Incubating McA-RH7777 cells with BAP31 siRNA resulted in a decreased secretion of apoB48-$VLDL_1$ while there was a corresponding increase of apoB48-containing lipoproteins with higher densities. The effect was similar on lipoproteins assembled by apoB100 but not as pronounced. These results were not caused by decreased MTP availability or activity, and the siRNA treatment did not result in any increased intracellular lipid accumulation. Hence, BAP31 interacts with the sequence downstream of apoB46.8 and seems important for the secretion of $VLDL_1$.

We next used immunocytochemistry studies to identify *in vivo* colocalization of apoB and BAP31. Both apoB and BAP31 localized to the secretory pathway and indeed did seem to colocalize at places, indicating that an *in vivo* interaction is possible.

These results show that the C-terminal part of apoB48 (i.e. the sequence between apoB46.8 and apoB48) is crucial for the secretion of $VLDL_1$. Furthermore, this sequence interacts with the shuttle protein BAP31, which seems essential for an efficient secretion of $VLDL_1$, but not for the secretion of denser particles.

Paper III

The aims of the study were to elucidate the impact of EGCG on hepatic VLDL secretion and at the same time investigate the relationship between stored and secreted TG.

The phenol EGCG was incubated in the cell culture medium of two different cell lines – NIH 3T3 and McA-RH7777 – that both responded by an increased cytosolic lipid

droplet storage. The TG synthesis, turnover and degradation was unchanged by EGCG treatment. However, the secretion of TG from the McA-RH7777 cells was decreased.

Upon EGCG treatment, the NIH 3T3 cells decreased their amount of small lipid droplets (< 2 μm), while the amount of large ones (> 2 μm) increased. This shift in lipid droplet size was further investigated by ADRP-green fluorescent protein microinjection or Nile red staining, followed by time lapse studies. These results showed that the increased lipid droplet size is attributed to increased lipid droplet fusion, independent of TG biosynthesis.

We next investigated the decreased TG secretion. McA-RH7777 cells decreased its apoB100-lipoprotein secretion – both $VLDL_2$ and $VLDL_1$ – when EGCG was included in the cell culture medium. First we tested if this was due to an inhibition of MTP. Results showed that both the MTP amount and activity were unchanged and hence not the cause. Next we investigated the importance of the LDLR for the decreased apoB100 secretion seen upon EGCG treatment. Heparin treatment failed to restore the secretion of apoB100 in EGCG-treated McA-RH7777 cells indicating that the LDLR was not involved. The independence of the LDLR was confirmed using the human apoB100 and apoB100×$LDLR^{-/-}$ transgenic mice that had received EGCG intraperitoneally for seven days. The lipoproteins were isolated and analyzed, and the results again showed that the LDLR did not mediate the effect of EGCG on apoB100 secretion.

The intracellular degradation of apoB in McA-RH7777 cells was increased upon EGCG treatment, which only partly could be blocked by the protease inhibitor lactacystin. Thus, EGCG induces an increased intracellular degradation of apoB100, probably not only by the early proteasomal degradation but also by PERPP.

In conclusion, EGCG promotes fusion of cytosolic lipid droplets, while reducing the assembly of apoB100-lipoproteins. The TG used for the droplets are diverted from the VLDL assembly pathway during EGCG treatment, resulting in underlipidated apoB that is sorted to degradation. Furthermore, this study shows that the accumulation of lipids in the hepatic cytosol is not always associated with increased secretion of VLDL.

DISCUSSION

Three forms of lipidations in the assembly of apoB100-lipoproteins

One of the major observations in this thesis is the identification of three forms of lipidations occurring in the assembly of apoB100-containing lipoproteins: 1) a bulk lipidation step resulting in the formation of $VLDL_1$, 2) an apoB size-dependent lipidation leading to $VLDL_2$ and 3) a partial lipidation forming a retained primordial particle (pre-VLDL), summarized in Figure 5.

The formation of $VLDL_1$

The pulse chase experiments showed that apoB must be close to at least apoB48 size to be able to assembly $VLDL_1$ in McA-RH7777 cells. The sharp increase in the ability to assemble $VLDL_1$ at an apoB length corresponding to apoB48 is interesting since apoB48 is the natural occurring truncated form of apoB known to assemble TG-rich lipoproteins not only in the liver but also in the intestine. Indeed apoB48 is even more associated with the ability to assemble TG-rich lipoproteins than apoB100.

Is it then just the length of apoB that determines if $VLDL_1$ can be assembled or not? We find a step increase in the efficiency of $VLDL_1$ formation when increasing the apoB length from apoB46.8 to apoB48. One caution when interpreting these results: we compare endogenous apoB48 with transfected apoB46.8. Is it possible that transfection *per se* distorts the $VLDL_1$ assembly? An argument against this is that transfected apoB50 avidly assembles $VLDL_1$. This stepwise increase may indicate that the $VLDL_1$ formation is only dependent on the length of apoB, i.e. when apoB48 is reached the apolipoprotein is large enough to enter into $VLDL_1$. However, increasing the length of apoB41 to an apoB48 size protein with a similar carboxyl-terminal sequence does not promote the formation of $VLDL_1$ and argues against this hypothesis. The observation that the sequence between apoB46.8 and apoB48 binds the ER protein BAP31 argues in favour of a specific role of this sequence. When BAP31 is suppressed, apoB48 fails to assemble $VLDL_1$.

Taken together, we propose that there is a sequence between apoB46.8 and apoB48 that is essential for the formation of $VLDL_1$ and that the reason is a specific protein-protein interaction with BAP31 that could be involved in the transfer of apoB to the site of $VLDL_1$ assembly.

$VLDL_1$ is formed from $VLDL_2$

The results from the pulse chase studies clearly showed that $VLDL_2$ is indeed a precursor to $VLDL_1$. This finding agrees with reports that suggest a separate regulation of $VLDL_1$ and $VLDL_2$[118, 156-158], as well as with a similar finding from an *in vivo* insulin clamp study where a similar result was reported[158]. Upon insulin infusion in healthy subjects, an inverse response between $VLDL_2$ and $VLDL_1$ production was seen, i.e. the amount of $VLDL_1$ was decreased while the amount of $VLDL_2$ was increased.

Where does the conversion from $VLDL_2$ to $VLDL_1$ take place? Earlier pulse chase studies have shown that secreted $VLDL_1$ can be secreted in the culture medium approximately 15 min after the completion of apoB100[50]. Again, this is confirmed in experiments *in vivo* where mature VLDL were secreted into the circulation 33 min after their initiated synthesis[57]. Interestingly, newly formed $VLDL_1$ triglycerides entered the circulation approximately 20 min before the newly formed $VLDL_1$ apoB100, indicating that the triglycerides were added to already formed apoB100-containing particles that had transferred for 20 min in the secretory pathway. This suggests a formation of $VLDL_1$ within the Golgi apparatus. *In vitro* experiments showed that when ARF1 was blocked by either the fungus metabolite BFA or the expression of a dominant negative ARF1 mutant, the $VLDL_1$ assembly was decreased. Inhibition of ARF1 usually results in a collapse of the Golgi apparatus; however, by titrating the dominant negative mutant, authors were able to inhibit $VLDL_1$ assembly with intact ERGIC and Golgi apparatus. Under these circumstances, the transport between ER and Golgi was inhibited. This supports the idea that the $VLDL_1$ assembly occurs in the Golgi apparatus. Finally, lowering the temperature to 15°C, which is known to block the transport between ERGIC and *cis*-Golgi, also inhibited the assembly of $VLDL_1$. These results argue that the maturation step of $VLDL_1$ is located in a smooth compartment, such as the Golgi.

The ER-resident protein BAP31 was found to play an important role in the $VLDL_1$ assembly. When suppressed by siRNA, the $VLDL_1$ secretion decreased, while the $VLDL_2$ (and LDL-$VLDL_2$-analog) secretion increased, resembling the response of insulin. Since BAP31 is reported to function as a shuttle protein between ER and Golgi, it is tempting to speculate that this protein is needed to shuttle apoB-containing particles to the final lipidation compartment. How then could the $VLDL_2$ (and the LDL-$VLDL_2$-analogs) be secreted despite inhibition of BAP31? The answer to this question still remains unanswered, and further information on the interaction between apoB100 and BAP31 is needed. In particular, if such an interaction may be influenced by the availability of triglycerides in the cell.

The lipidation pattern for $VLDL_2$ is different than that for $VLDL_1$

The pulse chase experiments also showed that the lipidation pattern seen for $VLDL_2$ differed from the bulk lipidation seen in the $VLDL_1$ assembly. Both apoB48 and apoB100 could form $VLDL_1$ of the same density, but the non-$VLDL_1$ particles differed in density. Indeed, when expressing truncated versions of apoB100, both shorter and longer than apoB48, we found an almost linear inverse relation between the size of apoB and the density of the formed particle, i.e. the longer a portion of apoB that was translated the more lipids were associated with the formed non-$VLDL_1$. It should be pointed out that this relation has been seen by other authors previously. However, our interpretation of the results is different. Thus, we conclude that two forms of VLDL is formed: the $VLDL_1$ and the non-$VLDL_1$. Only the formation of the non-$VLDL_1$ seemed to be dependent on the size of apoB. It would be plausible that apoB100-$VLDL_2$ represents the end stage of such an apoB size-dependent lipidation. What about the apoB48-containing HDL particle, apoB48-"HDL" (the partially lipidated apoB48-containing lipoprotein with density as HDL)? In agreement with

apoB100-$VLDL_2$, the formation of this apoB48-"HDL" follows the prerequisite for an apoB size-dependent particle, i.e. its formation is analogous to that of apo100-$VLDL_2$. Moreover, like apoB100-$VLDL_2$, apoB48-"HDL" is secreted from the cell. Finally, also in agreement with apoB100-$VLDL_2$, the apoB48-"HDL" is a precursor to $VLDL_1$. Taken together, we believe that this indicates that apoB48-"HDL" are analogs to apoB100-$VLDL_2$.

The primordial particle

In the secretory pathway, apoB80 and apoB100 were seen in yet another kind of particle: the primordial pre-VLDL. These particles had densities that did not follow the relation between size of apoB and density of the particle. Instead these particles had a much higher density than could be expected, suggesting that they contained much less lipids or were associated with more proteins (or a combination of both). These particles were retained and degraded unless converted to $VLDL_2$ (or $VLDL_2$-analogs as for apoB80). It should be pointed out that to account for all $VLDL_1$ and $VLDL_2$ secreted, the pre-VLDL apoB had to be taken into account.

The pre-VLDL were not present in the clones expressing apoB72 or shorter, and hence their formation seemed dependent on the sequence downstream the carboxyl-terminal of apoB72. This was supported by the fact that the chimera apoB50 + (72-90) assembled an intracellular particle with a higher density than expected. In fact, it had the same density as the pre-VLDL assembled by apoB80 and apoB100. Furthermore, this chimera secreted both LDL-$VLDL_2$ analogs and $VLDL_1$, and hence behaved exactly as apoB80 and apoB100.

Why is this dense particle retained in the cell? We found that only apoB80 and apoB100 were relatively stably associated with PDI and BiP in the cell. This suggests that these apoB species are relatively strongly associated with ER-resident chaperones. This could explain the retention. Why are they associated with the chaperones? It is tempting to speculate that the carboxyl-terminal region of apoB100 changes the way in which the protein is lipidated inducing a misfolding of the protein. The reason for this effect of the carboxyl-terminus is unknown and needs more investigation. It should be pointed out that this part of the molecule has a complex folding and contains physiological important binding sites for receptors and other bio-molecules.

From these results, we conclude that apoB100, contrary to apoB48, exists in a partially lipidated, chaperone-associated, pre-VLDL form in the ER and that the formation of this pre-VLDL is dependent on the sequence downstream the carboxyl-terminal of apoB72.

Regulation of $VLDL_1$ formation

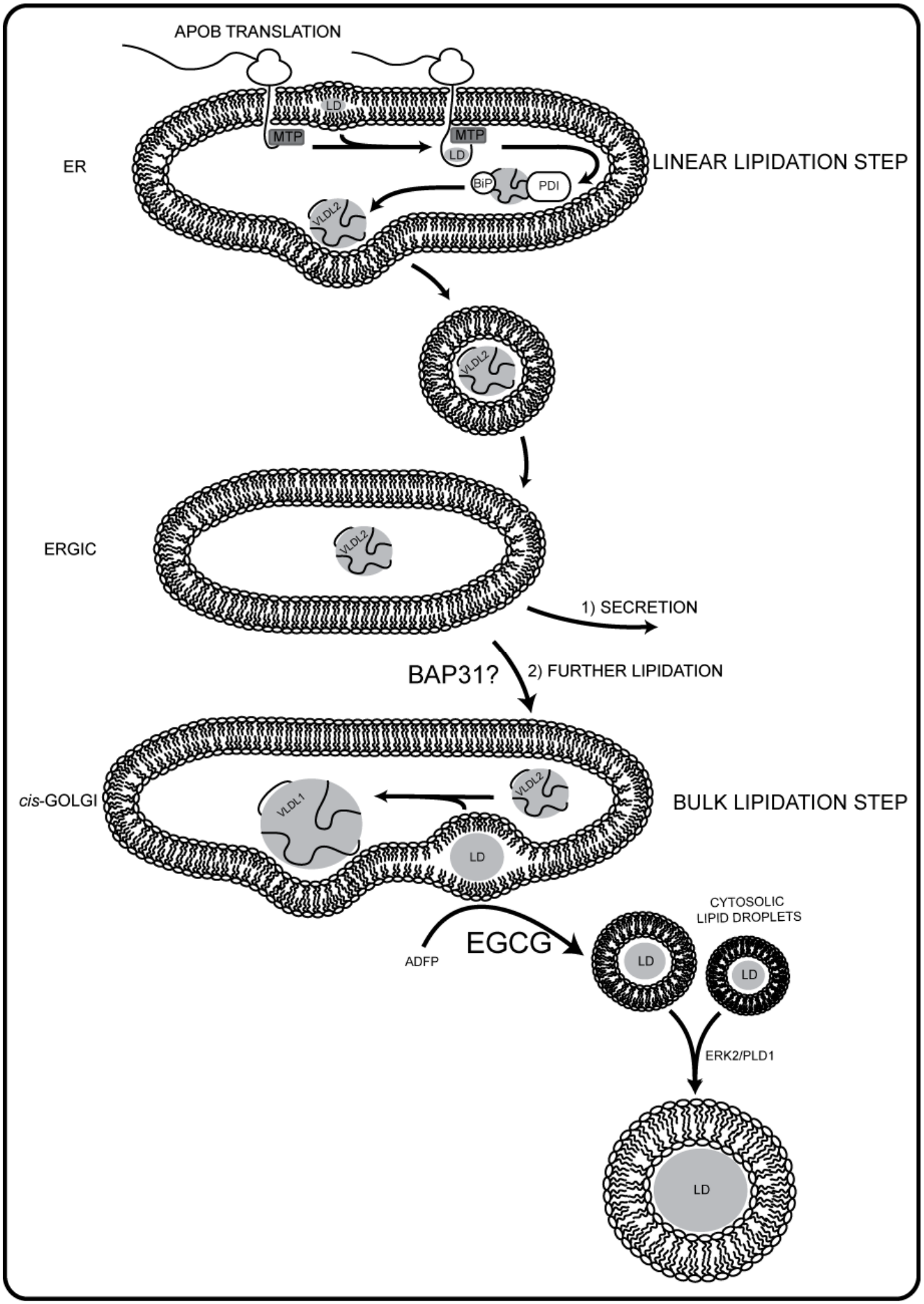
It is well known that the amount of hepatic TG is important for the lipoprotein secretion[128, 142, 159]. However, does increased liver fat deposition always result in an increased lipoprotein secretion?

In paper III we manipulated the lipid droplet formation by EGCG, which influenced the assembly and secretion of VLDL. Since neither the amount of TG synthesized by the cell was affected nor was the oxidation, the increased droplet formation was due to increased lipid droplet fusion and resulted in lipid depletion of the secretory pathway.

This caused a reduced assembly and secretion of apoB100-lipoproteins. Probably, the pre-VLDL formed during these circumstances were not properly lipidated and therefore sorted to degradation.

Overexpression of the PAT-protein ADFP results in a similar situation, where the VLDL secretion was reduced while the cytosolic lipid storage increased[160]. Interestingly, the hydrolyzed fatty acids were not channelled to the lipoprotein assembly pathway, but mainly to oxidation when suppressing the protein. This argues that there are still many questions unanswered that need to be solved before we understand the interplay between TG storage and lipoprotein secretion. Our results show that an accumulation of lipids in the cytosol is not always associated with increased secretion of VLDL.

Figure 5. The assembly of apoB100-containing lipoproteins. ApoB is co-translationally translocated into the ER lumen. The amino-terminal part of apoB interacts with MTP, which transfer TG from microsomal lipid droplets (LD) to the growing chain and forms a pre-VLDL. Pre-VLDL interacts with the chaperones BiP and PDI that presumably protect apoB from degradation (due to misfolding) until enough lipids are added for correct folding. If this lipidation fails (due to MTP inhibition, TG deprivation, EGCG treatment) pre-VLDL will be sorted to proteasomal degradation. The linear lipidation – when apoB100 is the assembled apoprotein – ends with the formation of $VLDL_2$. The $VLDL_2$ can be transported downstream the secretory pathway and may be secreted or converted to $VLDL_1$ in a bulk lipidation step. The transport has in this thesis been postulated to be dependent on the shuttle protein BAP31. If $VLDL_2$ is further lipidated, apoB-deficient lipid droplets from the membrane leaflet associate with $VLDL_2$ - a process also inhibited by EGCG. Instead, the TG in the lipid droplets will be diverted to cytosolic lipid droplets. The EGCG treatment also affects the TG pool by increasing the cytosolic lipid droplet fusion events, thereby increasing the amount stored TG while reducing the lipoprotein secretion. This results in an increased degradation of apoB.


APOB TRANSLATION
LD
MTP
MTP
LD
ER
LINEAR LIPIDATION STEP
BiP
PDI
VLDL2
VLDL2
ERGIC
VLDL2
1) SECRETION
BAP31?
2) FURTHER LIPIDATION
cis-GOLGI
VLDL1
VLDL2
LD
BULK LIPIDATION STEP
CYTOSOLIC
LIPID DROPLETS
ADFP
EGCG
LD
LD
ERK2/PLD1
LD

CONCLUDING REMARKS

The assembly and regulation of lipoproteins is, to a large extent, a matter of adding enough lipids to the growing chain. The lipidation will protect apoB from proteasomal degradation. While apoB48 can be secreted on LDL-$VLDL_2$-analogs (with the density of HDL), apoB100 is more sensitive to such underlipidation. This is probably due to its misfolding on a particle too small, forcing it to interact with chaperones that keep this pre-VLDL in the ER for proper lipidation and $VLDL_2$ assembly.

$VLDL_1$ is formed by further bulk lipidation of $VLDL_2$ in a smooth ER compartment, an event dependent on the sequence downstream the carboxyl-terminal of apoB46.8. This “alternative lipidation” is not necessary for the secretion of apoB, but depends on the availability of TG. The transport protein BAP31 might be important for this transport of $VLDL_2$ to the final lipidation location, an hypothesis that needs further investigation.

The relationship between hepatic TG storage and lipoprotein assembly can be interrupted by the phenol EGCG, which decreases the apoB100-lipoprotein secretion despite TG availability. The TG seems to be depleted from the secretory pathway and instead stored in cytosolic lipid droplets. EGCG affects this process by stimulation of lipid droplet fusion, resulting in an increased amount of larger lipid droplets found in the cells. Since apoB100 is not properly lipidated, it is sorted to degradation.

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