PROTEASOMAL DEGRADATION OF HEPATIC APOLIPOPROTEIN B

by

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ABSTRACT

Apolipoprotein (apo) B-100 is an atypical secretory protein in that its translocation across the ER membrane can be inefficient. Analysis of truncated apoB variants revealed that sequences in the β1 domain between apoB-39 and apoB-42 and predicted to be composed of amphipathic β-strands, governed the secretion efficiency and intracellular stability of apoB. Unlike smaller truncated apoB proteins, apoB-42 and apoB-100 were secreted poorly and were degraded by an ALLN-sensitive pathway. To test the hypothesis that sequences from the β1 domain are involved in apoB degradation, fusion proteins were created that contained apoB-29 linked to fragments derived from the β1 domain of apoB or to liver FABP, a known β-sheet protein. Fusion proteins containing the segments apoB-34–42 or apoB-37–42 were degraded rapidly, mostly by the proteasome, whereas other fusion proteins were stable and secreted efficiently. The apoB-34–42 segment increased the susceptibility to polyubiquitination and the affinity of apoB to the microsomal membrane by decreasing translocation efficiency. The presence of specific sequences in the β1 domain of human apoB may increase degradation by promoting the cytosolic exposure and polyubiquitination of the protein. Polyubiquitinated apoB proteins can be recognized by cytosolic ER-associated degradation (ERAD) factors involved in the delivery of degradation proteins target to the proteasome. Of these factors, the AAA-ATPase p97 has been shown to have a role in ERAD by recognizing and retro-translocating protein from the ER to the cytosol for delivery to the 26S proteasome. To investigate the potential role of p97 in the retro-translocation and proteasomal degradation of apoB-100, cytosolic proteins were analyzed using a digitonin-permeabilised cell system. Accumulation of cytosolic ubiquitinated apoB proteins was observed when cells were treated with the proteasome inhibitor ALLN or MG132. Using crosslinking agents, we showed that p97 associates with apoB-100. Reducing intracellular levels of p97 by transfecting p97-targeted siRNA increased the intracellular levels of apoB-100 by impairing proteasomal degradation. Reduced levels of p97 also decreased the accumulation of cytosolic apoB-100, supporting the role of p97 in retro-translocation. Taken together, these experiments suggested that p97 is a key player in the recognition and retro-translocation of apoB-100 for proteasomal degradation.

LIST OF ABBREVIATIONS AND SYMBOLS USED

α	Alpha
β	Beta
AAA-ATPase	ATPase associated with various cellular activities
ACAT	Acyl-CoA acyl-transferase
ALLN	Acetyl-leucyl-leucyl-norleucinal
Apo	Apolipoprotein
ATP	Adenosine tri-phosphate
ATPase	Adenosine tri-phosphatase
Bip	Binding protein or glucose-regulated protein 78
CE	Cholesterol ester
COP	Coating protein
COS	Transformed CV1 monkey cells
DGAT	Diacylglycerol acyl-transferase
DMEM	Dulbecco's modified Eagle's medium
EDEM	ER degradation enhancing alpha-mannosidase I like protein
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
FABP	Fatty acid binding protein
FCH	Familial combined hyperlipidemia
FDB	Familial defective apolipoprotein B
FH	Familial hypercholesterolemia
Grp	Glucose-regulated protein
GTP	Guanosine tri-phosphate
HepG2	Human hepatoma cells
Hsp	Heat shock protein
IDL	Intermediate density lipoprotein
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LpB	Apolipoprotein-B containing lipoprotein

McA-RH7777	Rat hepatoma cells
MG132	N-benzoyloxycarbonyl (Z)-leucyl-leucyl-leucinal
MHC	Major histocompatibility complex
MTP	Microsomal triglyceride transfer protein
NMR	Nuclear magnetic resonance
OA	Oleic acid
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PERPP	Post-endoplasmic reticulum presecretory proteolysis
PL	Phospholipid
RIPA	Radioimmunoprecipitation assay
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TAG	Triacylglycerol
TCA	Trichloroacetic acid
TGH	Triacylglycerol hydrolase
TRAM	Translocating chain-associated membrane protein
UC	Unesterified cholesterol
UPR	Unfolded protein response
VCP	Valosin-containing protein
VIMP	Valosin-containing protein-interacting membrane protein
VLDL	Very low density lipoprotein

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CHAPTER 1

INTRODUCTION

1.1 Apolipoprotein B

1.1.1 Lipoprotein Metabolism and Atherosclerosis

Atherosclerosis is a progressive disease characterized by the deposition of cholesterol-enriched macrophages (foam cells) in the intima of the arterial wall [reviewed in (Brown and Goldstein, 1983)]. Atherosclerosis is specific to medium and large arteries and is a subclass of arteriosclerosis, which includes all vessels. Atherosclerosis develops from fatty depositions in the lumen of arteries called plaques. Plaques can grow large enough to reduce blood flow through an artery. Large plaques can become fragile and susceptible to rupture, causing a constriction in blood supply to organs such as the heart myocardium (heart attack) or the brain (stroke), which can be fatal. The development of atherosclerosis leads to cardiovascular events affecting millions of individuals each year (Ford *et al.*, 2002).

Early work in John W. Gofman's laboratory in the 1950's, looking at the plasma lipoprotein profiles of several individuals, revealed a link between circulating lipoproteins and the development of atherosclerosis (Jones *et al.*, 1951). Plasma lipoproteins are spheroidal lipid-protein complexes that contain a surface monolayer of phospholipids (PL) and unesterified cholesterol (UC) and an apolipoprotein surrounding a neutral lipid core of triacylglycerols (TAG) and cholesterol esters (CE) **(figure 1.1)**. Apolipoproteins are amphipathic (both hydrophobic and hydrophilic) proteins able to interact both with lipids, via non-covalent interaction, and with the aqueous environment. Apolipoproteins, such as apolipoprotein (apo) B, solubilize large quantities

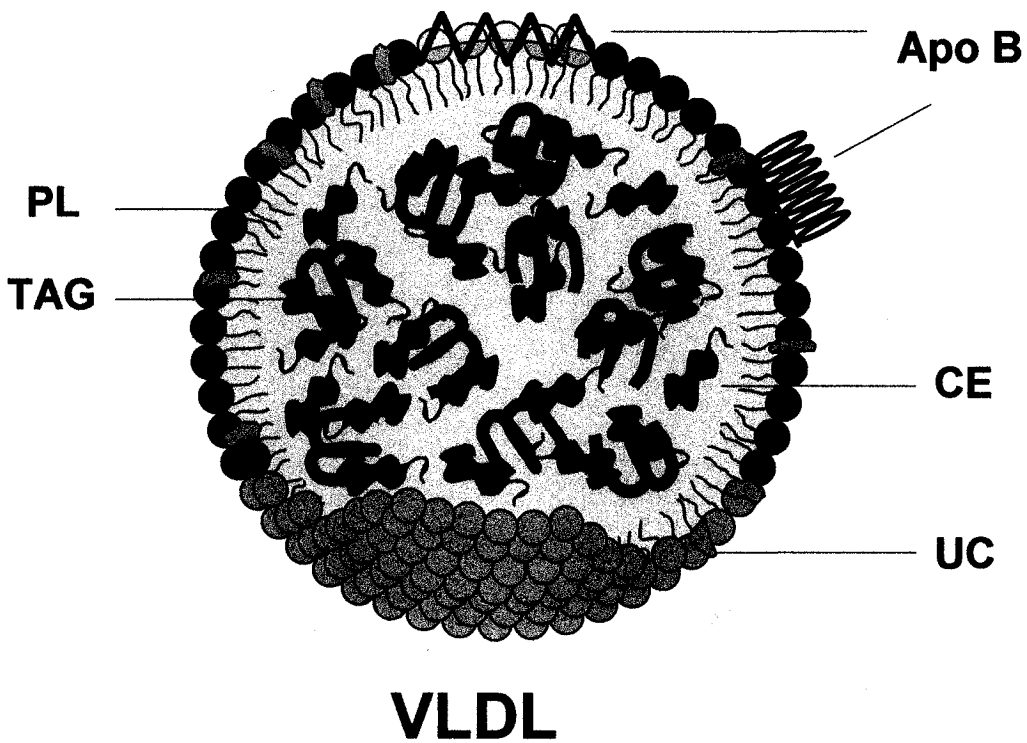

Figure 1.1. Schematic representation of a very low density lipoprotein particle. Phospholipids (PL) are drawn in blue, cholesterol esters (CE) are shown in purple, unesterified cholesterols (UC) are shown in pink and triglycerides (TAG) are drawn in black (ω). Amphipathic β-strand (blue) and α-helix (red) of apolipoprotein (apo) B are also shown. Used with permission, unpublished figure, Martin, D.D.O., 2002.

of lipids for their transport between tissues **(see section 1.1.2)**. To deliver lipids to peripheral tissues, hepatocytes synthesize and secrete apoB in a very low density lipoprotein (VLDL) form. VLDL cargo lipids are hydrolyzed by lipoprotein lipases in the plasma to release fatty acids resulting in an intermediate density lipoprotein (IDL) and further lipolysis leads to a smaller, denser and cholesterol-enriched form called low density lipoprotein (LDL). Analyses in the Gofman laboratory revealed a correlation between high plasma levels of LDL and an elevated risk for the development of atherosclerosis in individuals (Lyon *et al.*, 1956). These observations agreed with earlier work in cholesterol-fed animals showing the development of atherosclerotic plaques (Anitschkow, 1933) and supported the hypothesis that cholesterol may have a central role in atherosclerosis.

Research in the Gofman laboratory initiated strong interest and heated controversy over the role of cholesterol in heart diseases [reviewed in (Steinberg, 2004)]. For several years, some high profile clinicians and researchers remained unconvinced that blood cholesterol was linked to atherogenesis. The following 30 years of research provided converging and solid evidence on the clinical significance of cholesterol in atherosclerosis. With the discovery of the LDL receptor (LDLR) on the surface of cells, a receptor-mediated endocytosis pathway for the hepatic clearance of LDL was defined [reviewed in (Goldstein and Brown, 1977b)]. Analysis of individuals with a genetic defect in the LDLR, impairing LDL clearance by the liver, revealed a hypercholesterolemic phenotype and early atherosclerosis, providing strong evidence that plasma LDL levels are key in the development of atherosclerosis [reviewed in (Brown and Goldstein, 1986)]. In addition, several large-scale clinical studies in the

1980's supported the link between high levels of LDL in the plasma and the development of atherosclerosis leading to coronary artery disease [reviewed in (Musliner and Krauss, 1988; Steinberg, 2006)]. Thus, the scientific community eventually came to the consensus that elevated plasma LDL-cholesterol is one risk factor for atherosclerosis, supporting the importance of the regulation of the production and clearance of LDL in cholesterol homeostasis [reviewed in (Steinberg, 2005a; Steinberg, 2005b).

1.1.2 Apolipoprotein B-100 and B-48

The metabolism of lipoproteins and cargo lipids is directed by their associated apolipoprotein(s). Plasma apolipoproteins (apo) are classified into two groups: the exchangeable (apoA-I, apoA-II, apoA-IV, apoAV, apoC-I, apoC-II, apoC-III and apoE) and non-exchangeable (apoB-100 and apoB-48) apolipoproteins. Exchangeable apolipoproteins are small (10 to 34 kDa) and are soluble in aqueous solution in the absence of lipid ligands. In contrast, apoB-100, one of the largest monomeric polypeptides known (4536 amino acids, ~515 kDa), is highly insoluble in aqueous solution. ApoB-100 cDNA was completely sequenced in 1986 by several groups (Chen *et al.*, 1986; Cladaras *et al.*, 1986; Knott *et al.*, 1986; Yang *et al.*, 1986; Law *et al.*, 1986). The *APOB* gene is 43 kb long and is located on the short arm of chromosome 2 in humans. The *APOB* gene structure consists of 29 exons where exon 26 encodes more than half of the transcript (7572 bp). The nomenclature for apoB polypeptides refers to the percentile of the coding sequence in the *APOB* gene. In this nomenclature, apoB-48 consists of the N-terminal 48% of apoB-100 (2152 amino acids). Both apoB proteins are

important vehicles for the extracellular transport of neutral lipids in the plasma between the intestine, the liver and peripheral tissues. ApoB-100 is expressed in human hepatocytes and assembled into VLDL, precursor of atherogenic LDL. On the other hand, apoB-48, is exclusively expressed in human enterocytes and secreted as an extremely low density lipoprotein called the chylomicron (Innerarity *et al.*, 1987; Powell *et al.*, 1987). ApoB-48 arises from an editing process on the 14.5 kb mRNA, where a cytidine nucleotide at position 6666 of the apoB transcript is converted to uracil by the cytidine deaminase apobec-1 (Navaratnam *et al.*, 1993), resulting in a termination codon [reviewed in (Anant and Davidson, 2001)]. Notably, in rodent hepatocytes, the apoB transcript can undergo RNA editing and both apoB-100 and apoB-48 are secreted (Demmer *et al.*, 1986). Unlike exchangeable apolipoproteins, both naturally occurring forms of apoB remain associated with the lipoprotein particle from their biosynthesis to their degradation and each lipoprotein particle contains only one copy of apoB.

1.1.3 Hepatic VLDL Production and Diseases of Apolipoprotein B Metabolism

The regulation of VLDL production by the liver is important for three reasons. First, the liver secretes VLDL in order to transport TAG to peripheral tissues for energy generation in the unfed state, or to adipocytes for storage. Secondly, TAG synthesis and secretion by the hepatocyte are necessary to prevent the intracellular fatty acid or TAG concentration from reaching cytotoxic levels. Thirdly, hepatic VLDL production serves to distribute UC and CE to peripheral cells, such as steroidogenic tissues, for hormone biosynthesis. Thus, VLDL secretion by the liver must fluctuate in response to different dietary or metabolic conditions to maintain lipid homeostasis in the organism.

Differences in apoB secretion at relatively constant levels of apoB mRNA showed that hepatic production of VLDL is not regulated at the gene level. (Pullinger *et al.*, 1989; Dashti *et al.*, 1989; Moberly *et al.*, 1990; Kushwaha *et al.*, 1991; Sorci-Thomas *et al.*, 1992). Hence, regulation of LpB secretion by hepatic cells involves post-transcriptional mechanisms that result in the presecretory degradation of the apoB polypeptide **(see section 3.1)**.

Hepatic overproduction of VLDL can increase the level of circulating LDL and contribute to the atherosclerotic risk. For example, a genetically determined disorder called familial combined hyperlipidemia (FCH) has been linked to an increased risk to develop atherosclerosis. Kinetic studies of VLDL have demonstrated that individuals with FCH have an approximate doubling of VLDL production (Janus *et al.*, 1980a; Janus *et al.*, 1980b; Cortner *et al.*, 1991). While FCH is the most prevalent lipoprotein disorder, the mutation(s) responsible for FCH has yet to be characterized. Elucidation of the mechanism of hepatic LpB assembly and regulation may provide clues about the factor(s) causing FCH. Other disorders with altered production or/and processing of hepatic VLDL have been linked to genetic defects in the *LDLR* or *APOB* gene [reviewed in (Whitfield *et al.*, 2004)]. Familial hypercholesterolemia (FH), characterized by elevated plasma LDL and high risk to develop atherosclerosis, is most often caused by mutations in the *LDLR* gene that lead to impaired clearance of LDL by the LDLR [reviewed in (Goldstein and Brown, 1977a)]. Also, hepatic VLDL secretion is increased when the LDL receptor activity is decreased [reviewed in (Barrett and Watts, 2002)]. Isotope studies in patients and *in vitro* kinetic analyses suggested that this oversecretion of hepatic VLDL plays a significant role in elevating plasma LDL levels in FH patients

and is perhaps more important than impaired clearance (Thompson *et al.*, 1996; Sniderman *et al.*, 2000). In familial defective apoB disease (FDB), characterized by high levels of plasma LDL and an elevated risk to develop atherosclerosis, a single point mutation in the *APOB* gene impairs the binding of apoB to the LDLR [reviewed in (Humphries and Talmud, 1995)]. Unlike FH, elevated plasma LDL in FDB is caused by a decrease in the catabolism of LDL, not from an increase in hepatic VLDL secretion.

While elevated levels of LDL increase the risk to develop atherosclerosis, low levels of circulating LpB are also detrimental. Hypobetalipoproteinemia, characterized by low concentrations of plasma LpB, is caused by a nonsense mutation in the *APOB* gene resulting in carboxyl-terminal truncated forms of apoB [reviewed in (Schonfeld, 1995)]. The marked reduction of plasma LpB and altered individual plasma lipid levels can severely affect development and survival. The mechanism of these metabolic diseases suggests an intimate link between hepatic VLDL production, LDLR metabolism, plasma lipids and plasma LDL concentrations.

A response-to-retention mechanism was hypothesized for atherosclerosis according to which the deposition of cholesterol in arteries is directly proportional to the level of circulating LpB [reviewed in (Proctor *et al.*, 2002)]. Accumulating evidence supports the link between the number of LpB in the plasma and the risk to develop atherosclerosis. A careful analysis of various clinical studies led to the proposition that plasma levels of apoB may be an appropriate marker for atherosclerotic risk [reviewed in (Sniderman and Rosenbloom, 2005)]. The authors suggest that the broadly practiced measure of plasma cholesterol associated with LDL fails to consider the variation of the cholesterol content of each LDL particle and may result in an incomplete diagnosis. In

contrast, apoB levels more accurately represent the amount of circulating and potential atherogenic particles and may be a better diagnostic tool in therapeutic approaches to prevent cardiovascular diseases. Since the regulation of the production of LpB by the liver may be an important determinant of plasma lipid homeostasis, considerable effort has been expended in the study of apoB structure and hepatic LpB assembly.

1.1.4 Apolipoprotein B Structure and Function

The structure of exchangeable apolipoproteins has been well-characterized and mainly consists of amphipathic alpha (α)-helices, first described by Jere P. Segrest in the 1970's (Segrest *et al.*, 1974). In contrast, little is known about apoB structure. Tryptic digestion of apoB on a lipoprotein particle demonstrated that, in comparison to exchangeable apolipoproteins, large regions of the apoB polypeptide are not accessible to trypsin (Yang *et al.*, 1989b). Protection of these regions from proteolysis suggested that apoB integrates in the lipoprotein surface monolayer and interacts with the lipid core. Lipid-interacting regions of apoB were proposed to consist of amphipathic beta (β)-sheets, a unique feature among the apolipoproteins **(figure 1.1)**. Structural details of these regions have been proposed, but concrete evidence of these functional motifs are lacking. The study of apoB structure is particularly challenging because of its extraordinary size and insolubility in aqueous solutions. Structural and analytical studies indicated that diameter of the LDL particle can range from 18-25 nm, a substantially smaller size than the precursor VLDL that ranges from 60-80 nm in diameter (Tardieu *et al.*, 1976; Atkinson *et al.*, 1977; Krauss and Burke, 1982). The development of monoclonal antibodies against human LDL allowed immunological studies of apoB

structure (Marcel *et al.*, 1982) and revealed that apoB may adopt a quaternary structure on the LDL particle (Marcel et al., 1984). Electron microscopy of LDL showed a ring-shaped elongated structure for the surrounding apoB polypeptide (Phillips and Schumaker, 1989). Studies performed in transfected rat hepatoma (McA-RH7777) cells and human hepatoma (HepG2) cells suggested that the extraordinary length of the apoB polypeptide is necessary for its ability to assemble lipoproteins (Graham *et al.*, 1991; Yao *et al.*, 1991; Boren *et al.*, 1992; Spring *et al.*, 1992; White *et al.*, 1992; McLeod *et al.*, 1994). Using several C-terminally truncated forms of apoB, the lipoprotein core volume and diameter of secreted LpB was suggested to be a function of the apoB polypeptide length (McLeod *et al.*, 1994). The relationship between apoB length and core radius led to the suggestion that apoB must be part of the surface of the LDL particle and may adopt a belt-like conformation (Spring *et al.*, 1992).

Multiple, perhaps repetitive, lipid-binding sequences in the apoB polypeptide appeared to determine the lipid content of the LpB (Schumaker *et al.*, 1994). However, after more than 20 years, the nature of these lipid-binding sequences is still unclear and no evidence of a repeating unit is apparent from the primary amino acid sequence of apoB (Knott *et al.*, 1986; Yang *et al.*, 1986). The distribution of lipid-binding motifs in apoB-100 was first proposed based on the strength of the interaction of particular regions of the polypeptide with the lipid core and five distinct regions, called domains, were defined **(figure 1.2A)** (Yang *et al.*, 1989a; Yang *et al.*, 1989b). Infrared spectroscopy of LDL estimated the β-sheet content in apoB to be between 41 and 50% (Goormaghtigh *et al.*, 1989), whereas the α-helical content of apoB was estimated to be between 25% and 33% from the circular dichroic spectroscopy of LDL (Chen *et al.*,

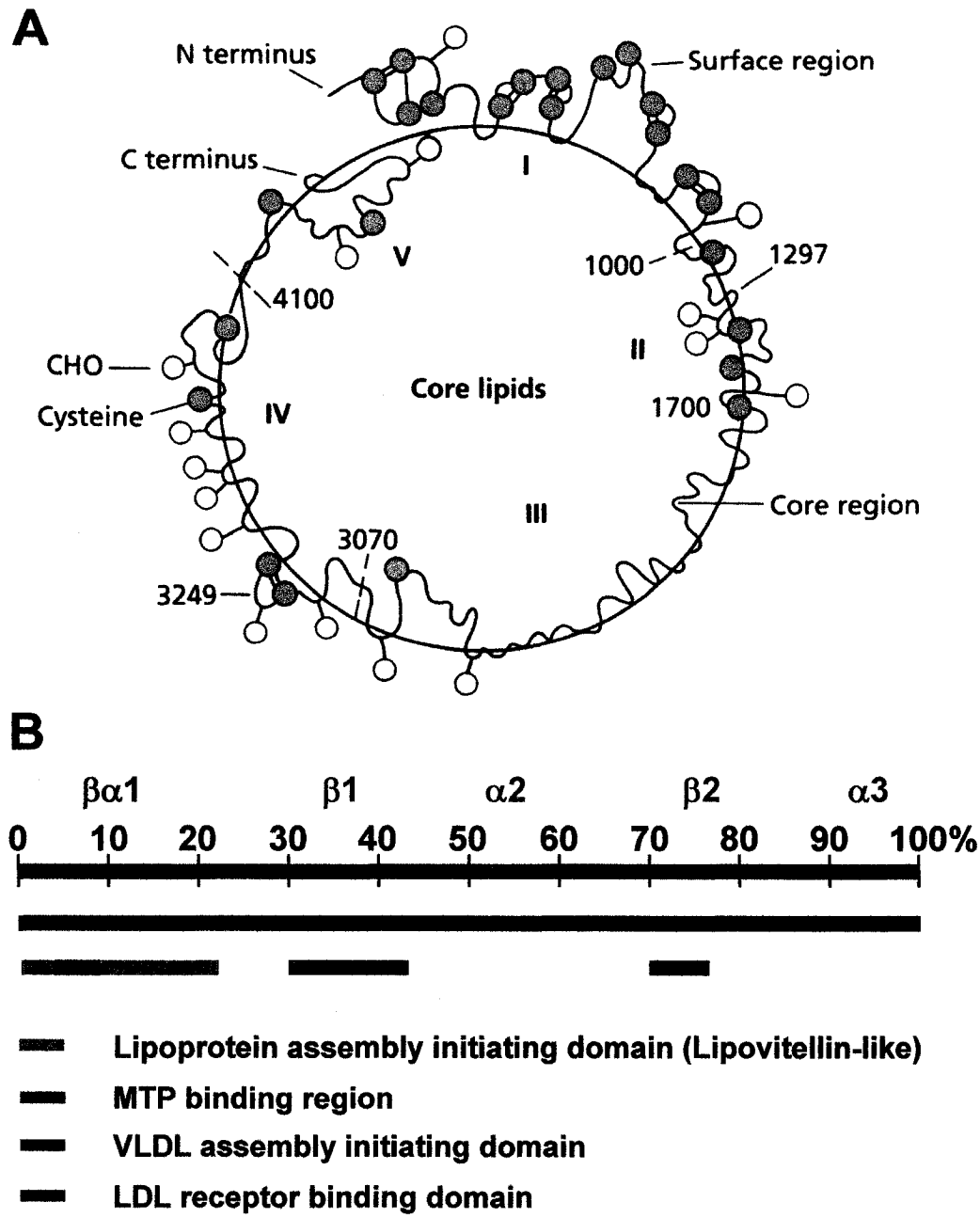

Figure 1.2. ApoB predicted structure. A. Schematic representation of the 5 domains of apoB on the LDL particle. Used with permission (Segrest *et al.* 2001). B. Pentapartite model of apoB structure and speculated functional map.

1983; Singh and Lee, 1986). These observations were later used to develop a predicted structure based on computer algorithms, suggesting the presence of alternating regions enriched in amphipathic α helices or β structures (Segrest *et al.*, 1994) and a pentapartite model (NH_2-βα1-β1-α2-β2-α3-COOH) was proposed **(figure 1.2B)** [reviewed in (Segrest *et al.*, 2001)]. Recent modeling of apoB on the LDL supports a partially flexible configuration for apoB delimited by 5 large modular domains forming a ring around the neutral lipid core (Johs *et al.*, 2006).

ApoB contains 25 cysteines, 16 of which are involved in disulfide linkages. Most (7 of the 8) of the intramolecular disulfide bonds are found in the βα1 domain indicating a compact structure for the N-terminal section of apoB (Shelness and Thornburg, 1996). Sequence homology studies have suggested a structural relationship between the βα1 domain (N-terminal 22%) of apoB and lipovitellin, an egg yolk storage lipoprotein (Mann *et al.*, 1999). In addition, comparative studies of the *APOB* gene with insect apolipophorin, invertebrate and vertebrate vitellogenin (the precursor of lipovitellin) and the microsomal triglyceride transfer protein (MTP) genes have suggested an evolutionary relationship stemming from a common ancestral large lipid transfer module (Babin et al., 1999). The structure of lipovitellin was resolved by X-ray crystallography and consists of a bundle of α helices and β sheets complemented by a large lipid-binding pocket formed by anti-parallel amphipathic β sheets **(figure 1.3)** (Anderson *et al.*, 1998). The structural homology between lipovitellin and the N-terminal region of apoB has been recently supported using circular dichroism and limited proteolysis (Jiang *et al.*, 2005b) and the hydrophobic pocket now serves as a working model for the initiation of LpB assembly (Richardson *et al.*, 2005).

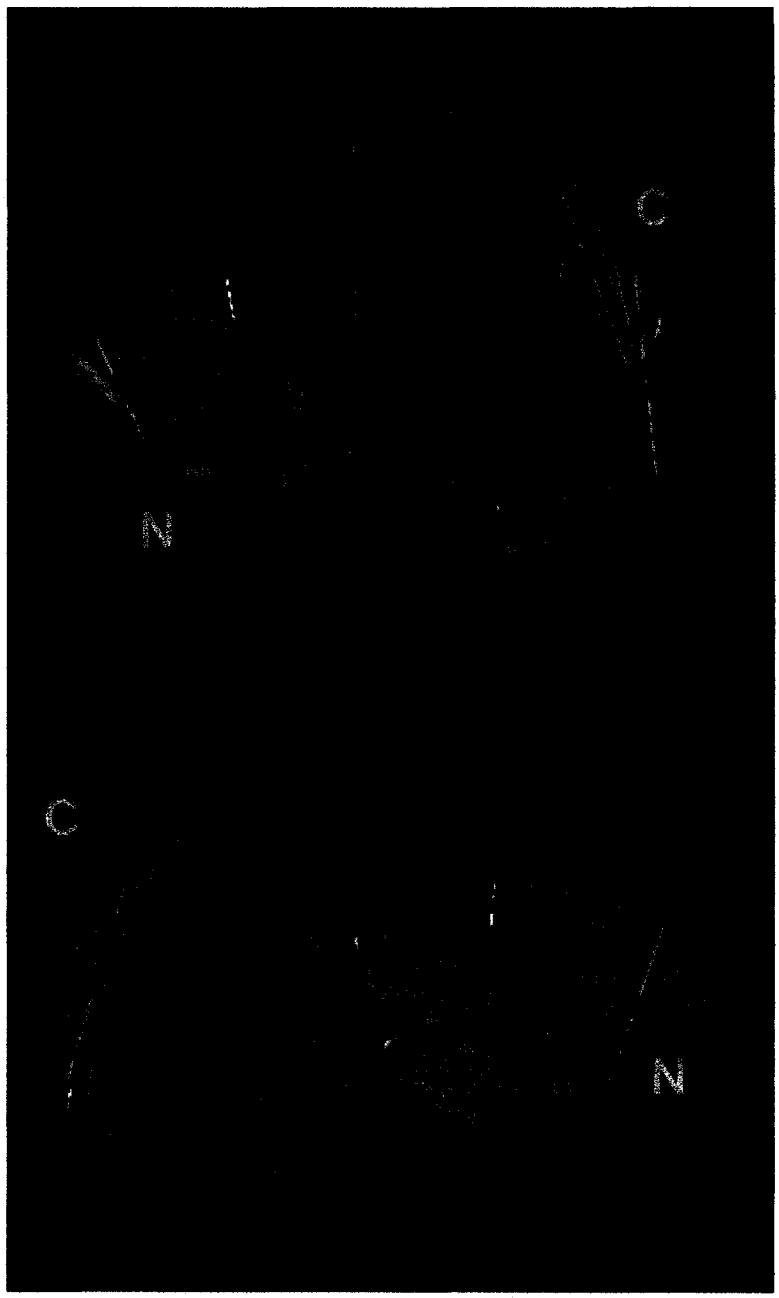

Figure 1.3. Crystal structure of lipovitellin The structure of lipovitellin was visualized with the molecular visualization software Cn3D 4.1.

The structural features of the domains downstream of the βα1 domain (β1-α2-β2-α3) are incompletely understood but cryoelectron microscopy of sodium deoxycholate-solubilized apoB revealed the presence of a globular domain (bead) for the βα1 domain of apoB on the LpB, complemented by a ribbon-like structure (thread) encompassing the rest of the protein (Gantz *et al.*, 2000). This study extended previous observations by immunoelectron microscopy of different monoclonal antibodies for apoB on a LDL suggesting that apoB forms a ribbon (N-terminal 89%) surrounding the spherical core completed by a bow (C-terminal 11%) (Chatterton *et al.*, 1995). One could speculate that coding sequences downstream of the βα1 in the unusually large exon 26 of the APOB gene may represent an innovation from ancestral large lipid transfer modules to allow apoB to transport larger quantities of lipids. Indeed, the β1 (apoB 22-48%) and β2 (apoB 57-88%) domains, representing the two large lipid-associating domains and predicted to consist of several amphipathic β sheets, interact and irreversibly associate with neutral lipids. In contrast, the α2 (apoB 48-57%) and α3 (apoB 88-100%) domains have been predicted by helical wheel analysis to encompass amphipathic α-helix repeats (Wei *et al.*, 1985; Cladaras *et al.*, 1986). These amphipathic helices termed class A and Y are also found in lipid-associating regions of exchangeable apolipoproteins and exhibit reversible lipid binding [reviewed in (Segrest *et al.*, 1992)]. For apoB, these helical regions have been speculated to function as a flexible spring joining the β domains and to regulate the size of LDL particles (Hevonoja *et al.*, 2000). Physicochemical studies of the properties of apoB on lipid/water interfaces have supported the role of the β domains in the irreversible interaction of apoB with lipids and the role of α domains in the reversible interaction of apoB with lipids conferring apoB

its structural flexibility between different lipoprotein classes (VLDL, IDL and LDL) (Wang *et al.*, 2006).

The LDLR binding domain is localized in the C-terminal region of apoB-100 [reviewed in (Segrest *et al.*, 2001)]. Analysis of the affinity of different C-terminally truncated apoB proteins with LDLR demonstrated that the epitope for LDLR recognition is present between apoB70 and apoB75 (included in the β2 domain) (Welty *et al.*, 1995; Parhofer *et al.*, 1996; Chen *et al.*, 1999). Different familial defective mutations in the *APOB* gene, showing impaired binding to LDLR, have supported the localisation of the putative LDLR binding domain suggested in truncated apoB studies (Pullinger *et al.*, 1995; Wenham *et al.*, 1997; Gaffney *et al.*, 2002). Three highly conserved sites were reported in this LDLR binding region (called A, B and C) (Ebert *et al.*, 1988; Law and Scott, 1990; Babin *et al.*, 1995). In particular, site B was shown to bind heparin, which is also a feature of apoE, another ligand for LDLR (Weisgraber and Rall, Jr., 1987; Chatterton *et al.*, 1995). Moreover, apoE was able to functionally replace the site B in truncated apoB studies and restore binding to the LDLR and heparin (Boren *et al.*, 1998).

In the plasma, recognition of apoB by the LDLR requires a change of conformation of the polypeptide to form a ligand for the LDLR. One proposition is that the C-terminal 11% of apoB, suggested to form a bow over the LDLR-binding domain (Chatterton *et al.*, 1995), may regulate the accessibility of the LDLR. Indeed, LDLR binding affinity was increased when the C-terminal 20% of apoB was truncated (Boren *et al.*, 1998). Recognition of apoB by the LDLR has been proposed to depend on the neutral lipid core content of the LpB particle. The hydrolysis of cargo lipids converting

VLDL into IDL and LDL may result in the exposure of the LDLR-binding domain of apoB increasing affinity to the LDLR (Chatterton *et al.*, 1995). Physicochemical analysis of different subspecies of human LDL supported the hypothesis that apoB conformation changes when the lipid core content is modified (McNamara *et al.*, 1996). In summary, the apoB polypeptide consists of functional domains for the initiation of LpB assembly and the recruitment of neutral lipid for VLDL assembly, complemented by dynamic regions necessary for the recognition and clearance of LpB remnants from the plasma. The pentapartite model remains the working guide for apoB structure. Improvements in X-ray crystallography and nuclear magnetic resonance spectroscopy may allow for the resolution of different domains in apoB and eventually lead to a relevant, complete resolution of apoB-100 structure.

1.2 Hepatic VLDL assembly

The assembly of hepatic VLDL has been the subject of intense study for more than 15 years and although the process is not yet completely understood, experiments in hepatoma cells in culture have greatly increased our knowledge of the components and processes involved [reviewed in (Davis, 1999; Davidson and Shelness, 2000; Gibbons *et al.*, 2004)]. LpB assembly is complex and requires the coordination of several factors in the secretory pathway. Coordinated synthesis and lipidation of apoB-100 may be necessary for the proper assembly of hepatic VLDL. A two-step model for LpB assembly was proposed from the observation that apoB-48 has the ability to be secreted as both a lipid-poor or lipid-rich lipoprotein particle from rat hepatocytes and McA-RH7777 cells (Boren *et al.*, 1994; Swift, 1995). Thus, the maturation of apoB into LpB

may follow two sequential steps of lipidation: the formation of a primordial lipoprotein and the lipoprotein core expansion **(figure 1.4)** [reviewed in (Rustaeus *et al.*, 1999; Olofsson *et al.*, 2000)].

1.2.1 Initiation of VLDL Assembly

A polymorphic signal peptide of 24 or 27 amino acids targets the translocation of apoB to the lumen of the rough endoplasmic reticulum (ER) (Boerwinkle and Chan, 1989) where coordinate folding and lipidation of the nascent polypeptide is initiated. The assembly of LpB in the lumen of the ER involves several post-translational modifications. LpB assembly requires the formation of appropriate disulfide bonds facilitated by the protein disulfide isomerase (PDI). (Huang and Shelness, 1997; Tran *et al.*, 1998b; Burch and Herscovitz, 2000). PDI catalyzes the shuffling or rearrangement of pre-existing disulfide bonds and the breakage and formation of disulfide bonds, depending on the reduction potential of the ER. In addition to disulfide linkages, apoB is both N- and O-glycosylated (Taniguchi *et al.*, 1989; Sasak *et al.*, 1991). Glycosylation occurs early in apoB biogenesis and the appropriate modification of apoB glycans, likely accompanied by polypeptide folding, is monitored by the transient interaction of apoB with membrane-bound calnexin and soluble calreticulin (Chen *et al.*, 1998; Linnik and Herscovitz, 1998; Tatu and Helenius, 1999; Stillemark *et al.*, 2000). ER-resident proteins calreticulin and calnexin are key chaperones in glycoprotein folding [reviewed in (Helenius and Aebi, 2004)]. The apoB polypeptide is also palmitoylated, a modification that may facilitate membrane association and targeting of apoB proteins in the secretory pathway (Zhao *et al.*, 2000; Vukmirica *et al.*, 2003; Vilas and Berthiaume, 2004). Other ER resident chaperones [including glucose-regulated protein 78 (Grp78 or

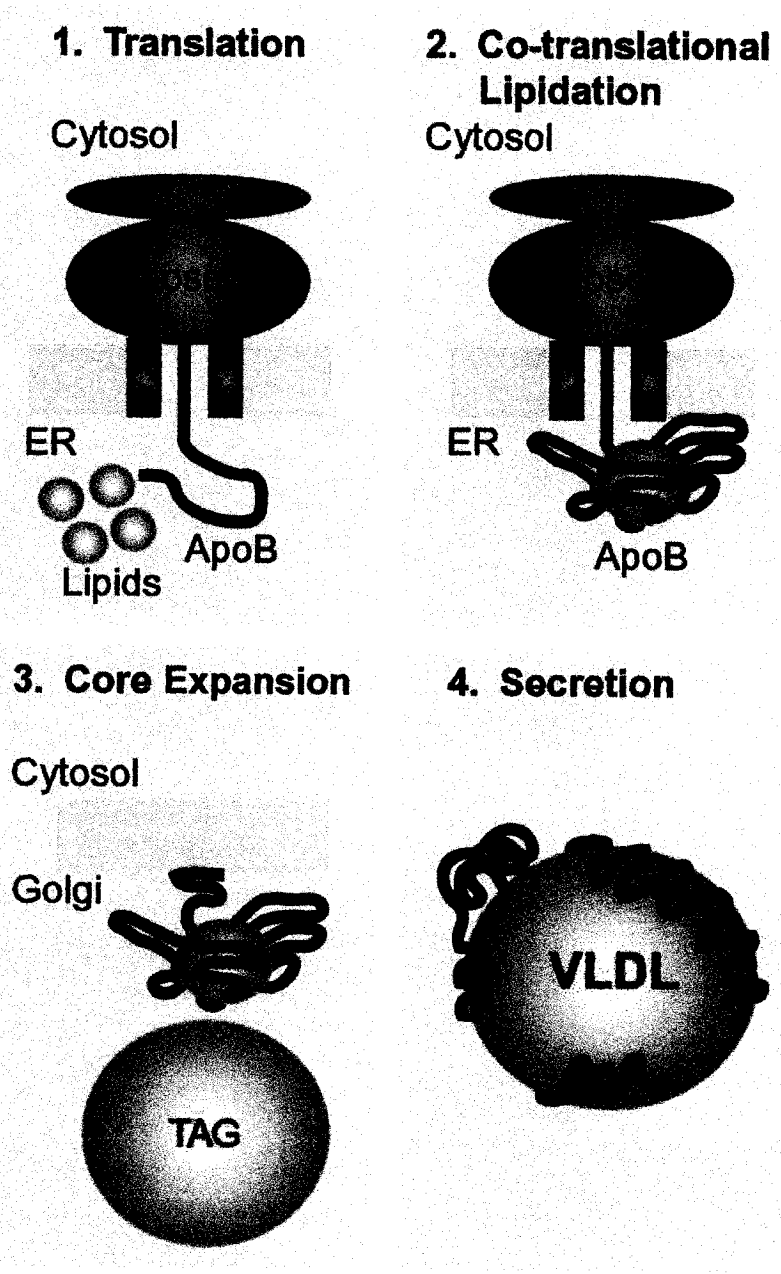

Figure 1.4. Hepatic VLDL assembly. Schematic representation of the assembly of a VLDL particle. 1. Translation and translocation across the ER membrane. 2. Co-translational lipidation of the apoB polypeptide in the ER. 3. Fusion of a lipid droplet to apoB and core expansion in the Golgi apparatus. 4. Secretion of the VLDL particle.

Bip), Grp94, ERp72, and cyclophilin B] shown to interact with apoB may also facilitate the folding of the globular N-terminal domain (Linnik and Herscovitz, 1998; Zhang and Herscovitz, 2003). Therefore, early in assembly, apoB is subjected to several post-translational modifications that require the coordinate action of different ER lumenal proteins.

The βα1 domain of apoB, suggested to form a lipid pocket, may associate with PL of the inner leaflet of the ER membrane bilayer via solvent-exposed hydrophobic sequences (Herscovitz *et al.*, 1991; Pease *et al.*, 1991; Herscovitz *et al.*, 2001; Ledford *et al.*, 2006). Conveniently, apoB may remain anchored on the membrane until the assembly and lipidation processes, localized on the inner leaflet of the ER membrane, are completed and the LpB is formed. The βα1 domain itself is unable to recruit sufficient neutral lipids (Shelness *et al.*, 2003), but the downstream synthesis of the β1 domain serves as the framework for core lipid addition. Sequences in the β1 domain (between apoB-29 and apoB-42) have been shown to endow the apoB polypeptide with the ability to recruit neutral lipids for the assembly of VLDL (McLeod *et al.*, 1996; Carraway *et al.*, 2000). Since apoB-48 is able to assemble into a large neutral lipid-rich chylomicron, the role of sequences downstream of the β1 domain of apoB in LpB assembly is unclear. C-terminally truncated apoB studies have suggested that these sequences may increase the ability of apoB to form buoyant, TAG-rich lipoprotein particles (Boren *et al.*, 1992; Spring *et al.*, 1992; McLeod *et al.*, 1994).

Radiolabeling analyses in HepG2 cells determined that apoB-100 is translated in 14 minutes and is secreted within 30 minutes of the initiation of polypeptide synthesis (Bostrom *et al.*, 1986; Olofsson *et al.*, 1987). Thus, apoB is synthesized at 5 amino acids

per second which is relatively average compared to the range of approximately 3 to 8 amino acids per second for proteins translated in mammalian cells [reviewed in (Hershey, 1991)]. This synthetic rate may be dictated by apoB folding efficiency during co-translational lipidation in the ER.

1.2.2 Lipidation of Apolipoprotein B

The initiation of lipidation of apoB in the lumen of the ER is facilitated by the microsomal triglyceride transfer protein (MTP), which catalyzes the transfer of PL, CE and TAG to the ER lumen for VLDL assembly [reviewed in (Gordon and Jamil, 2000; Hussain *et al.*, 2003)]. Several lines of evidence support the necessity of MTP in LpB assembly. First, genetic defects that prevent the synthesis of functional MTP result in abetalipoproteinemia, a disease characterized by the absence of LpB in the plasma (Wetterau *et al.*, 1992). Second, in cells that do not normally assemble LpB, transfection of MTP enables the cell to assemble and secrete LpB (Gordon *et al.*, 1994; Leiper *et al.*, 1994; Wang *et al.*, 1996). Third, studies using inhibitors of the lipid transfer activity of MTP (Gordon *et al.*, 1996; Jamil *et al.*, 1996; Benoist and Grand-Perret, 1997; Wang *et al.*, 1997b; Wetterau *et al.*, 1998) and conditional knockout mouse experiments (Raabe *et al.*, 1999; Leung *et al.*, 2000) demonstrated the role of MTP in the assembly of LpB. Finally, analysis of the kinetics of LpB assembly in McA-RH7777 cells and rat hepatocytes with a photoactivatable MTP inhibitor have shown that MTP activity is rate-limiting for the assembly of LpB (Jamil *et al.*, 1998). Unlike abetalipoproteinemia in humans, complete knockout of MTP or apoB in mice revealed an embryonic lethal phenotype (Nielsen *et al.*, 1998). In mouse embryonic development, LpB secretion was

proposed to play a role in transporting lipid nutrients from the yolk sac to the embryos (Terasawa *et al.*, 1999). In addition, the presence of apoB and MTP mRNA in cardiac myocytes (Nielsen *et al.*, 1998) and endothelial cells (Sivaram et al., 1996) suggested a role for LpB secretion in fatty acid clearance. However, the contribution of these cells to plasma lipoproteins is unknown. Thus, MTP and apoB are co-expressed in organs that assemble and secrete LpB, primarily the liver and the intestine.

The mechanism of action of MTP during apoB biosynthesis is unclear and the lipid transfer activity may be one among other functions of MTP in LpB assembly. To prevent aggregation and to remain catalytically active, MTP (97 kDa) is associated with PDI (58 kDa) (Wetterau *et al.*, 1991a; Wetterau *et al.*, 1991b). Early in LpB assembly, this heterodimer has been suggested to physically interact with the N-terminus of apoB as it translocates in the ER (Patel and Grundy, 1996; Wu *et al.*, 1996b; Hussain *et al.*, 1997; Bakillah *et al.*, 1998; Hussain *et al.*, 1998) and may act as a chaperone prior to the co-translational lipidation of apoB (Ingram and Shelness, 1997; Richardson *et al.*, 2005). In HepG2 cells, the interaction of apoB with MTP was shown to be required in the secretion of truncated and chimeric apoB proteins containing sequences of the β1 domain involved in VLDL assembly (Liang and Ginsberg, 2001). When apoB-48, a protein able to assemble TAG-rich lipoproteins, was overexpressed in McA-RH7777 cells, apoB-100 secretion was decreased, suggesting that apoB proteins with higher requirement for MTP lipid transfer activity, in particular, can compete with apoB-100 for co-translational lipid loading (Hussain *et al.*, 1995). In cultured cells or in transgenic mice, the overexpression of apoB17, which may bind to MTP but does not require MTP

lipid transfer activity for secretion (Thrift *et al.*, 1992; Wang *et al.*, 1996), did not affect endogenous apoB-100 secretion (Li *et al.*, 2000).

MTP was found to preferentially transfer TAG and CE *in vitro* (Jamil *et al.*, 1995) suggesting that neutral lipid delivery may be important in LpB assembly. However, transformed CV1 monkey cells (COS) were able to assemble LpB when transfected with apoB and an MTP ortholog from drosophila that can transfer PL but is unable to transfer neutral lipids (Rava *et al.*, 2006). This study demonstrated that the PL transfer activity of MTP is necessary and sufficient in the assembly of a primordial LpB. Perhaps, the capacity of human MTP to transfer neutral lipids represents an evolution to enable cells to assemble larger neutral lipid-rich LpB particles. Therefore, MTP appears to be essential in the transfer of lipids onto the apoB polypeptide in the lumen of the secretory pathway for LpB assembly.

Several studies have demonstrated that hepatic intracellular levels and availability of PL, CE and TAG can influence VLDL secretion and may be rate-limiting in the assembly of VLDL. During assembly and expansion of LpB, PL provides surface polar lipids to surround the neutral lipid core (Vermeulen *et al.*, 1997). PL in LpB consist mainly of phosphatidylcholine (PC) and work in rat hepatocytes suggested that VLDL secretion required active synthesis of PC (Yao and Vance, 1988). Subsequent work in McA-RH7777 cells and in mice fed a choline-deficient diet have indicated that active PC synthesis, particularly via the phosphatidylethanolamine methylation pathway, may be important in bulk lipid incorporation in VLDL assembly (Nishimaki-Mogami *et al.*, 1996; Nishimaki-Mogami *et al.*, 2002; Noga *et al.*, 2002; Kulinski *et al.*, 2004). In addition, it was proposed that intracellular PL, also a precursor for TAG, can contribute

substantially to TAG synthesis (Wiggins and Gibbons, 1996). Therefore, the availability of PL and especially PC may be important throughout LpB assembly.

Several studies have suggested a role and necessity for CE in LpB assembly, but this remains a subject of controversy. In hamster hepatocytes and HepG2 cells treated with inhibitors of the CE biosynthetic enzyme acyl-CoA acyl-transferases (ACAT1 and ACAT2), LpB secretion is decreased (Cianflone *et al.*, 1990; Avramoglu *et al.*, 1995; Wilcox *et al.*, 1999; Zhang *et al.*, 1999; Taghibiglou *et al.*, 2002). In addition, ACAT1 or ACAT2 overexpression can stimulate the secretion of VLDL in McA-RH7777 cells (Liang *et al.*, 2004). In particular, the cellular mass of CE was correlated with apoB secretion efficiency in HepG2 cells (Sniderman and Cianflone, 1993). Other studies in HepG2 cells, however, have shown no relationship between cellular CE mass or biosynthesis and LpB secretion (Wu *et al.*, 1994a; Wu *et al.*, 1994b) and ACAT1 and ACAT2 knockout mice were still able to secrete LpB (Buhman *et al.*, 2000; Accad *et al.*, 2000). Differences in models and methods may explain the different conclusions, but it appears that CE availability may be particularly important in the assembly of larger neutral lipid-rich VLDL particles.

The regulatory role of active synthesis of TAG in VLDL assembly is incompletely understood, but intracellular levels of TAG have been proposed to modulate the production and secretion of large VLDL particles (Wang *et al.*, 1999). The activity of TAG biosynthetic enzymes diacylglycerol acyl-transferases (DGAT1 and DGAT2) [reviewed in (Yu and Ginsberg, 2004)] has been suggested to influence VLDL secretion (Zhu *et al.*, 2002; Ganji *et al.*, 2004; Liang *et al.*, 2004; Yamazaki *et al.*, 2005). However, the role of each DGAT differs as DGAT1 knockout mice could still secrete

LpB (Buhman *et al.*, 2002) while DGAT2 knockout mice died shortly after birth (Stone *et al.*, 2004). The necessity of *de novo* TAG synthesis on LpB assembly is not clear but stimulating synthesis and secretion of TAG by treating HepG2 or McA-RH7777 cells with oleic acid (OA) increased LpB secretion (Dixon *et al.*, 1991; White *et al.*, 1992; Boren *et al.*, 1993). Moreover, OA-treated McA-RH7777 cells produced larger VLDL particles, via the lipid transfer activity of MTP (Wang *et al.*, 1997b; Wang *et al.*, 1999). Interestingly, exogenous OA does not provide a direct source of lipids for LpB assembly (Gibbons *et al.*, 1992; Wu *et al.*, 1996a) but instead it may enhance the incorporation and processing of the secretion-coupled pool of TAG already existing in the secretory pathway into VLDL, thereby favoring VLDL secretion (Zammit and Lankester, 2001). In addition, stable isotope studies in mice have shown that the majority of TAG in VLDL comes from hydrolysis and reesterification of cytosolic TAG stores rather than from *de novo* synthesis (Yang *et al.*, 1995; Yang *et al.*, 1996). Recently, a TAG hydrolase (TGH) was characterized in the lumen of the ER (Gilham *et al.*, 2003). A study with a TGH inhibitor suggested that, while TGH is not hydrolyzing lipids associated with apoB, it may still play a role in VLDL assembly in the ER by remodeling lumenal TAG (Gilham *et al.*, 2005).

It is important to note that there are differences between McA-RH7777 and HepG2 cells concerning the effect of the active synthesis of lipids, the intracellular lipid mass and the presence of exogenous lipids on LpB metabolism. While McA-RH7777 cells can secrete VLDL particles, HepG2 cells are unable to assemble and secrete authentic VLDL. Deficiency in TGH (Gilham *et al.*, 2003) or/and the presence of a small secretion-coupled pool of TAG (Wu *et al.*, 1996a) have been speculated to

decrease the ability of HepG2 cells to assemble VLDL. These issues may render HepG2 cells more responsive to exogenous lipids than McA-RH7777 cells and particularly sensitive to lipid biosynthetic enzyme inhibitors. These disparities may explain the different conclusions from different cultured cells. In summary, LpB lipidation relies particularly on the availability of PL, while the presence of neutral lipids appears to influence the VLDL particle size in the core lipidation step.

1.2.3 Translocation of Apolipoprotein B

Apolipoprotein B translocation into the ER lumen is mediated by the translocation channel spanning the bilayer of the ER membrane. The translocation channel (translocon) is composed of 3 subunits of sec61 (α, β, γ) forming the pore [reviewed in (Osborne *et al.*, 2005)] and an accessory protein called translocating chain-associated membrane protein (TRAM) controlling the size of the pore (Hegde *et al.*, 1998). An interesting feature of apoB is that, unlike typical secretory proteins, translocation into the ER lumen can be inefficient. Early studies using microsomes isolated from rat hepatocytes revealed that apoB in the secretory pathway is in two different pools, a trypsin-accessible (cytosolically exposed) pool and a trypsin-resistant (translocated) pool (Davis *et al.*, 1990). The observation of different pools of apoB in microsomes was reproduced by protease protection assays in several different hepatic cell systems (Dixon *et al.*, 1992; Furukawa *et al.*, 1992; Rusinol *et al.*, 1993; Verkade *et al.*, 1993; Wilkinson *et al.*, 1993; Du *et al.*, 1994; Bonnardel and Davis, 1995; McLeod *et al.*, 1996) and with antibodies against apoB (Davis *et al.*, 1990; Wilkinson *et al.*, 1993). Apparently, a significant proportion of apoB that is synthesized by hepatocytes

fails to translocate completely and becomes exposed to the cytosol, a topology similar to a single spanning transmembrane protein and termed bitopic configuration.

The presence of cytosolically exposed apoB was suggested to arise from transient pausing of the translocation of the polypeptide across the ER membrane, a process characterized by the arrest and reinitiation of translocation (Chuck and Lingappa, 1992). This study used an *in vitro* translation system coupled with microsomes and observed by protease protection assays that different levels of translocation of the N-terminal apoB-15 gave rise to either transmembrane or bitopic intermediates. Similar intermediates were also observed *in vitro* with other secretory and transmembrane proteins (Nakahara *et al.*, 1994). Pause transfer sequences in the amino acid sequence of apoB were characterized and suggested to transiently impair translocation without interfering with translation (Chuck and Lingappa, 1992; Chuck and Lingappa, 1993). Notably, the bitopic configuration of apoB appears not to be a consequence of the presence of a transmembrane domain since the primary sequence of apoB is devoid of any classical transmembrane regions (Knott *et al.*, 1986; Yang *et al.*, 1986; Yang *et al.*, 1989a). Moreover, unlike a transmembrane protein, apoB is not integrated into the membrane, instead translocation pausing may result in the prolonged association of apoB with proteins that form the translocon (Chen *et al.*, 1998; Mitchell *et al.*, 1998; Pariyarath *et al.*, 2001). Following translocation pause, the junction between the ribosome and the translocation channel may be dynamic and flexible allowing the release (loop) of a portion of a polypeptide out into the cytosol (Hegde and Lingappa, 1996; Beckmann *et al.*, 1997; Menetret *et al.*, 2000). The exposure of a particular region of a translating polypeptide to the cytosol was suggested to be regulated by TRAM

(Hegde *et al.*, 1998). Translocation pausing was recently proposed to be a common feature among secretory and membrane proteins [reviewed in (Lingappa *et al.*, 2002)].

Another group proposed that the secondary structure of apoB mRNA decreased translation and translocation efficiency (Pease *et al.*, 1991). This group suggested that translocation pause is actually a consequence of the transient arrest of ribosomal translation (Pease *et al.*, 1992; Pease *et al.*, 1995). Subsequently, paused translocation combined with the reinitiation of translation may result in the gradual exposure of the apoB polypeptide to the cytosol. In addition, using the same *in vitro* system as described above, this group did not observe translocation pausing for the N-terminal apoB-17 and suggested that translation was coupled to the insertion of apoB into the ER, at least for the N-terminus of apoB (Pease *et al.*, 1991). The reconstitution of apoB translation and translocation *in vitro* presents issues since the translation rate may be slower than in intact cells.

The distribution of translocation pausing across the apoB polypeptide was suggested (Kivlen *et al.*, 1997). In HepG2 cells, however, apoB translocation pausing was shown to occur only after the βα1 domain indicating that the presence pauses in the N-terminal apoB-17 is unlikely (Du *et al.*, 1994). Translocation pauses have been proposed to occur in the β1 (Liang *et al.*, 1998), the α2 (Nicodeme *et al.*, 1999) and the β2 domain of apoB (Cavallo *et al.*, 1998). These regions of apoB susceptible to pause during translocation are hydrophobic and have a particularly high dependence on MTP. The high requirement of these regions for MTP may decrease the translocation efficiency of apoB (Wang *et al.*, 1997b), perhaps because translocation efficiency is related to the rate of lipidation by MTP. Several different approaches have suggested a

direct role for MTP in facilitating translocation. The accumulation of bitopic apoB was found in cells lacking MTP (Thrift *et al.*, 1992; Du *et al.*, 1996) or in HepG2 cells treated with an inhibitor of MTP lipid transfer activity (Pan *et al.*, 2000). Translocation of apoB was also suggested to be inefficient in hepatocytes of individuals afflicted with abetalipoproteinemia (dysfunctional MTP) (Du *et al.*, 1996). Conversely, co-expression of MTP enhanced apoB translocation in COS-7 cells (Wang *et al.*, 1996). The lack of lipids for MTP lipid transfer interrupted apoB translocation in HepG2 cells (Wu *et al.*, 1996b). Thus, treating HepG2 cells with exogenous OA was found to promote apoB translocation (Macri and Adeli, 1997b) and enhance the movement of apoB through the ER (Du *et al.*, 1998).

The role of MTP and neutral lipids in apoB translocation has been challenged since protease protection assays in permeabilised cells and *in vitro* translocation studies have shown that the lack of MTP activity does not influence translocation of apoB proteins (Rusinol *et al.*, 1997; Macri *et al.*, 2000). In addition, treatment of McA-RH7777 cells with OA failed to enhance the translocation of various C-terminally truncated proteins (Rusinol and Vance, 1995). Instead of the neutral lipid availability, the ER membrane PL composition was proposed to modulate the translocation efficiency of apoB (Rusinol *et al.*, 1998). Recent work highlighting the necessity of phospholipid transfer activity from MTP in primordial LpB assembly (Rava *et al.*, 2006) may explain the influence of ER membrane phospholipids and MTP in apoB translocation. Despite the bulk of the literature on translocation paused apoB, its relevance has been challenged by one group that has shown that translocation of apoB across the ER is not compromised during translation of truncated or full length apoB

proteins (Shelness *et al.*, 1994; Ingram and Shelness, 1996). Thus, the effect of neutral lipids, membrane phospholipids and MTP activity on apoB translocation and the importance of translocation pause in LpB assembly remain to be clarified.

Early microsome studies in HepG2 cells have suggested that apoB becomes fully translocated and is released in the water-soluble lumen of the ER during LpB assembly (Boren *et al.*, 1990; Boren *et al.*, 1992; Boren *et al.*, 1993; Adeli *et al.*, 1997b). However, translocation of apoB across the ER membrane may not be completed as some apoB proteins were found to be exposed to the cytosol in vesicles derived from the ER of McA-RH7777 cells (Gusarova *et al.*, 2003). Perhaps, exposure of the polypeptide to the cytosol may allow a window of time for sufficient lipidation and folding in the ER to occur, with subsequent reinitiation of translocation to yield a secretion-competent LpB in HepG2 cells (Mitchell *et al.*, 1998). It is likely, however, that translocation pausing and cytosolic exposure promote the association of hydrophobic sequences of apoB with the outer leaflet of the ER membrane preventing the reinitiation of translocation.

Subcellular fractionation analyses of rat hepatocytes demonstrated that the majority (90%) of apoB proteins in the ER remained associated with the membrane (Cartwright and Higgins, 1992). Finally, recent studies in rat hepatocytes and McA-RH7777 cells have determined that membrane-associated apoB is the precursor of VLDL (Hebbachi and Gibbons, 2001; Tran *et al.*, 2002). Thus, the two-step model for LpB assembly suggests that the completion of the translation of the apoB polypeptide in the ER yields a modestly lipidated, membrane-associated, dense primordial LpB, which is then fully lipidated in the Golgi. In summary, the initial stages of LpB assembly in the

ER and the translocation of apoB may be influenced by lipid availability, the presence of hydrophobic regions in apoB and the lipid transfer activity of MTP.

1.2.4 Post-ER Processing of LpB

The maturation of the apoB polypeptide may not be completed in the ER, as there is evidence that an incompletely folded, chaperone-bound intermediate is transported to the Golgi apparatus (Zhang and Herscovitz, 2003). Fully translocated apoB may only be released in the lumen when it reaches the Golgi as highly glycosylated apoB proteins were found associated with the microsomal membrane of rat hepatocytes (Wong and Torbati, 1994). Extraction of the lumenal content of cis and trans-Golgi enriched fractions indicated that apoB gradually became predominantly lumenal (33% and 15% membrane-associated apoB, respectively), before it was secreted (Cartwright and Higgins, 1992). The transport of LpB from the ER to the Golgi apparatus represents a challenge for the cell because normal vesicle of ~50 nm are not suitable to for carrying large and lipidated apoB. Vesicle formation is mediated by coating protein (COP) II specialized for trafficking apoB toward the Golgi (Brodsky *et al.*, 2004). In these vesicles, apoB appeared to be the sole cargo while other secretory proteins were transiting together. The mechanism of LpB cargo recognition and vesicle formation is poorly understood but may require the activity of the Sar1 GTPase proteins (Sar1a and Sar1b) (Gusarova *et al.*, 2003), functional components of the COPII complex (Barlowe *et al.*, 1994; Kuge *et al.*, 1994). Indeed, it has recently been shown that Anderson's disease (also called chylomicron-retention disease or CMRD), characterized by the retention of ER-membrane bound LpB in intestinal enterocytes and the inability

to absorb fat (Anderson *et al.*, 1961; Bouma *et al.*, 1986; Roy *et al.*, 1987), is a consequence of a defect in the Sar1b gene (Jones *et al.*, 2003). Hepatic LpB production, however, is not affected in Anderson's disease, suggesting that different factors may be involved in the vesicular transport of LpB in the liver and the intestine. In addition, during pre-Golgi sorting of vesicles, a CopI/Arf-dependent pathway is also implicated in LpB transit as dominant-negative Arf1p impairs VLDL secretion (Asp *et al.*, 2000). Vesicular transport of apoB proteins is currently incompletely understood and unique features in hepatic (VLDL) and intestinal cells (chylomicrons) may present different requirements for the transit of LpB between the ER and the Golgi.

While the initial stages of LpB assembly occur in the ER, bulk lipidation appears to occur after the LpB has exited the ER. This second step of lipidation, termed "core expansion" [reviewed in (Hussain, 2000)] involves the addition of a large quantity of neutral lipids onto the primordial LpB resulting in a triglyceride-rich VLDL particle. ApoB-free lipid droplets formed in the ER, seemingly through MTP (Raabe *et al.*, 1999), are transported to the Golgi where they serve as the major neutral lipid source for the core lipidation of VLDL. Although there is no evidence yet to clarify the mechanism of core expansion, the accepted model suggests that the primordial LpB fuses with a neutral lipid droplet in the lumen of the Golgi. Early analyses of the formation of VLDL in rat hepatocytes using radiolabeled lipids (Higgins, 1988) and with radiolabeled glycerol in chicken hepatocytes (Bamberger and Lane, 1990) demonstrated that the Golgi apparatus was an important site for the extensive lipidation of the apoB polypeptide. Studies of the distribution of apoB in the secretory pathway of McA-RH7777 cells showed that VLDL formation is followed rapidly by secretion suggesting

that VLDL core lipidation occurs in the distal Golgi (Tran *et al.*, 2002). Another group has suggested otherwise as they observed the presence of VLDL in the ER of McA-RH7777 cells (Yamaguchi *et al.*, 2003). However, the majority of studies in McA-RH7777 cells (Stillemark *et al.*, 2000; Tran *et al.*, 2002; Gusarova *et al.*, 2003) and one study in mouse liver (Swift *et al.*, 2001) have demonstrated that the first and second step of lipidation occur in distinct compartments. While differences in cellular fractionation methodology may explain the disparate observations, the site of the second lipidation is yet to be confirmed.

The accumulation of apoB-free neutral lipid droplets in the lumen of the secretory pathway used in the assembly of VLDL was proposed to require MTP (Raabe *et al.*, 1999; Kulinski *et al.*, 2002). Two studies have suggested that MTP directly participates in the lipidation of LpB in the Golgi (Levy *et al.*, 2002; Valyi-Nagy *et al.*, 2002). In contrast, several hepatic cell culture studies have demonstrated that there is a temporal window during which MTP is necessary for the early stages of hepatic VLDL assembly in the ER, after which MTP may no longer be required (Gordon *et al.*, 1996; Wang *et al.*, 1997b; Mitchell *et al.*, 1998; Rustaeus *et al.*, 1998; Pan *et al.*, 2002; Tran *et al.*, 2002). These studies also suggested that coordinated *de novo* synthesis of TAG may not be required for the second step of lipidation and the secretion of VLDL. In summary, co- and post-translational hepatic assembly of LpB requires adequate lipidation, which is influenced by lipid availability and the lipid transfer activity of MTP. ApoB folding is facilitated by chaperones that coordinate the production of VLDL particle.

1.2.5 Pathways for Apolipoprotein B Degradation

Throughout maturation, spatially regulated protein quality control systems oversee LpB assembly and may regulate the secretion of VLDL. Presecretory degradation of apoB was first described in 1987 in rat hepatocytes (Borchardt and Davis, 1987). This metabolic labeling study demonstrated that a significant portion of radiolabeled apolipoprotein B was not recovered from the medium or the cells during a pulse-chase analysis. In the following 10 years, many laboratories have corroborated this observation in hepatocytes and transformed hepatic cell models (HepG2, McA-RH7777) [reviewed in (Yao *et al.*, 1997)]. Early studies suggested that degradation occurred before apoB left the ER (Davis *et al.*, 1990; Sato *et al.*, 1990; Furukawa *et al.*, 1992). Since then, three pathways for apoB degradation, elegantly termed the "triple threat" (Fisher *et al.*, 2001), have been described in hepatic cell models and hepatocytes: ER-associated degradation (ERAD), post-ER presecretory proteolysis (PERPP) and cell surface re-uptake. ERAD of apoB involves the cytosolic proteasome system (Yeung *et al.*, 1996; Fisher *et al.*, 1997) as well as ER lumenal proteases (Adeli *et al.*, 1997a; Cardozo *et al.*, 2002). A non-proteasomal, Golgi-localized degradation pathway has also been observed for apoB (PERPP) (Wang *et al.*, 1993; Wang *et al.*, 1995; Fisher *et al.*, 2001; Pan *et al.*, 2004; Jiang *et al.*, 2005a). In addition, shortly after secretion, VLDL particles may be rerouted back into the hepatocyte through endocytosis via the LDLR on the plasma membrane and degraded in the lysosome compartment (Williams *et al.*, 1990; Twisk *et al.*, 2000).

There is evidence in cultured rodent primary hepatocytes suggesting *in vivo* significance for ERAD (Taghibiglou *et al.*, 2000), PERPP (Pan *et al.*, 2004) and cell

surface reuptake (Twisk *et al.*, 2000) in modulating LpB levels in the plasma. Corroborating observations from cell cultures to human subjects is important in clarifying the role of degradation in the regulation of VLDL production. Recently, clinical studies on ritonavir, a protease inhibitor included in the anti-retroviral treatment against HIV, have suggested that inhibition of ERAD, in particular, can increase hepatic LpB secretion. The inhibition of the chymotrypsin-like activity of the proteasome by ritonavir (Schmidtke *et al.*, 1999), a proteolytic activity particularly important in apoB degradation (Cardozo *et al.*, 2002), was proposed to be a causative factor in the increased level of plasma VLDL, hyperlipidemia and lipodystrophy observed in patients subjected to the anti-retroviral therapy (Carr *et al.*, 1998; Tsiodras *et al.*, 2000; Liang *et al.*, 2001). Metabolic labeling data showed that, by inhibiting proteasomal function, ritonavir protected apoB from degradation and could increase the opportunity for apoB to be secreted as VLDL (Liang *et al.*, 2001). In cultured cells, proteasome inhibition, while increasing the accumulation of intracellular apoB, failed to increase apoB secretion significantly unless a source of OA was present in the media (Mitchell *et al.*, 1998). Perhaps, the development of hyperlipidemia in treated individuals may be a consequence of both the action of ritonavir on the proteasome and dietary lipids. Therefore, ERAD appears to be an important determinant in the rate of secretion of hepatic VLDL.

1.3 ER-associated Degradation

ERAD is a key quality control system managing the removal of unsalvageable misfolded proteins. Misfolded proteins can form aggregates, posing a threat to normal

cellular functions. ERAD utilizes the ubiquitin-proteasome system, an essential degradation pathway involved in the regulatory proteolysis and turnover of several intracellular proteins in various cellular pathways [reviewed in (Voges *et al.*, 1999; Goldberg, 2003)]. The mechanistic features of ERAD have been studied extensively in yeast and mammalian cell systems [reviewed in (Hampton, 2002; McCracken and Brodsky, 2003; Meusser *et al.*, 2005)]. Three sequential steps occur: selection of proteins destined for ERAD, export of ERAD substrates out of the ER through the translocation channel (retro-translocation) to the cytosol and delivery to the proteasome for degradation.

The accumulation of misfolded protein in the ER increases chaperone binding and decreases the level of free chaperones in the ER eliciting an unfolded protein response (UPR) [reviewed in (Zhang and Kaufman, 2004)]. To adapt to ER stress, the UPR triggers the expression of factors to accelerate protein folding and maturation and to attenuate protein translation reducing the import of newly synthesized proteins in the ER. In addition, UPR induces the expression of ERAD factors to facilitate the disposal of misfolded proteins (Patil and Walter, 2001). Mutant variants of yeast and mammalian proteins have been used to study ERAD and study its components. In yeast, in particular, inhibition of the proteolytic activity of the proteasome induces UPR (Travers *et al.*, 2000). UPR is also elicited in yeast defective in ERAD because of a deleted ERAD factor (Ye *et al.*, 2001; Jarosch *et al.*, 2002). In the regulatory turnover of normal proteins such as mammalian apoB (Liao *et al.*, 2003b) and yeast HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis (Ravid *et al.*, 2000), UPR was not triggered. ERAD is also utilized by viral proteins and toxins to evade the immune

response or to compromise cellular defense. For example, US11, a protein of the human cytomegalovirus, was found to co-opt the retro-translocation pathway to attenuate the immune response by directing nascent major histocompatibility complex (MHC) class I heavy chain toward retro-translocation for proteasomal degradation (Wiertz *et al.*, 1996a). The cholera toxin was also shown to utilize the retro-translocation machinery to reach the cytosol and escape degradation to initiate its cytotoxicity (Schmitz *et al.*, 2000; Abujarour *et al.*, 2005). Such examples and the role of ER stress in the pathophysiology of several diseases have considerably increased the interest in understanding the mechanism of ERAD and its impact on human diseases [reviewed in (Plemper and Wolf, 1999; Aridor and Balch, 1999)].

1.3.1 The Ubiquitin-Proteasome System

Proteins bound for proteasomal degradation are post-translationally tagged on a lysine residue by the formation of a covalent isopeptide bond with ubiquitin, an abundant cytosolic protein of 76 amino acids (8 kDa). The conjugation of ubiquitin is initiated by the E1 ubiquitin activating enzyme that catalyzes the ATP-dependent formation of thiol ester-activated ubiquitin. Activated ubiquitin molecules are transferred to an E2 ubiquitin conjugating enzyme. Ubiquitin is then transferred into an E3 ligating enzyme that covalently links a ubiquitin molecule onto a selected target protein [reviewed in (Hershko and Ciechanover, 1998; Weissman, 2001)]. Polyubiquitination may be facilitated by the E4 multi-ubiquitin chain assembling enzyme (Hoppe, 2005). Two types of ubiquitin modification exist: mono and polyubiquitination. Mono-ubiquitination is involved in non-proteolytic pathways such as

histone regulation, virus particle budding and endocytosis (Hicke, 2001). In contrast, polyubiquitination predominantly directs a protein for proteolysis. Different linkages in the multi-ubiquitin chain signal different cellular processes (Gly76-Lys29, Gly76-Lys48 and Gly76-Lys63) [reviewed in (Weissman, 2001)]. Lys29 and the well-characterized Lys48 linkages trigger proteasomal degradation by binding to a substrate-specific multi-ubiquitin chain binding protein such as S5a/Mcb1/Rpn10 encompassed in the 19S subunit (van Nocker *et al.*, 1996). In contrast, Lys63 linkages are involved in non-proteolytic pathways such as endocytosis, DNA repair and IκB kinase activation [reviewed in (Haglund and Dikic, 2005)]. Lys48 multi-ubiquitinated proteins have been extensively studied and their interaction with the 26S proteasome can also be extended to chaperones and multimeric ATPases **(see sections 1.3.2 and 1.3.4).**

The 26S proteasome is a multi-protein complex involved in the selective proteolysis of proteins tagged with four or more ubiquitin molecules (Thrower *et al.*, 2000). Approximately 30 different subunits (~2.5 MDa) constitute this cytosolic proteolytic machinery. The 26S proteasome consists of two copies of the regulatory subunit 19S (890 kDa) which caps a catalytic core, the 20S subunit (720 kDa). Crystal structures of the 20S subunit have revealed a barrel shaped cylindrical structure with 14 different subunits (α1-7 and β1-7) configured to 4 heptameric stacks (α, β, β and α) (Groll *et al.*, 1997; Unno *et al.*, 2002). The proteolytic activity localized inside the core involves three alternative subunits: subunit β1 (peptidylglutamyl-peptide hydrolyzing activity), β2 (trypsin-like activity) and β5 (chymotrypsin-like activity) (Oberdorf *et al.*, 2001). The central pore of the 20S core is gated at either end by 19S subunits.

Each 19S subunit consists of approximately 19 polypeptides distributed in two sub-complexes: the lid and the base. The base is thought to consist of several ATPases forming an hexameric ring associated with the 20S core. These ATPases are involved in gating the pore and the unfolding of target polypeptides. The lid is composed of 8 proteins (non ATPase) and their role remains mostly unknown. One of the subunits (POH1) is a protease involved in removal of ubiquitin from the target protein (Yao and Cohen, 2002). Removal of the multi-ubiquitin chain is necessary for the entry of unfolded protein into the pore of the 20S core and POH1 has been suggested to also play a role in ubiquitin recycling. The 19S subunit may function as a gate where polyubiquitinated proteins are unfolded, deubiquitinated and translocated to the 20S pore for proteolysis (Horwich *et al.*, 1999; Lee *et al.*, 2001).

1.3.2 Molecular Chaperones

To reach the cytosolic 26S proteasome for ERAD, target proteins are first recognized by chaperones. The fidelity of protein folding is promoted by molecular chaperones that prevent incorrect folds and protein:protein interactions that may lead to aggregation. Primarily, chaperones facilitate the formation of native proteins but they are also responsible for the recognition of aberrant protein and the initiation of ERAD by specifically differentiating normal folding intermediates from proteins that have failed to attain proper tertiary and quarternary structure [reviewed in (Fewell *et al.*, 2001). Following recognition of incorrently folded proteins, chaperones prevent protein aggregation and direct the protein for retro-translocation (Nishikawa *et al.*, 2001). ERAD may be triggered by the prolonged interaction of nascent proteins with

chaperones via hydrophobic patches or particular structural motifs. Degradation of soluble (lumenal) and transmembrane ER proteins involves various chaperones such as ER molecular chaperones Bip and PDI, lectin-like chaperones calnexin and ER degradation enhancing alpha-mannosidase I like protein (EDEM) as well as cytosolic chaperones heat shock protein 40 kDa (Hsp40), Hsp70 and Hsp90 [reviewed in (Nishikawa *et al.*, 2005)].

Degradation of ER proteins requires one or more of these chaperones. ERAD has been proposed to have two systems of recognition involving distinct chaperones for ER lumenal and transmembrane proteins. For example, in the retro-translocation of transmembrane ER proteins, Bip has been found to be dispensable (Hill and Cooper, 2000; Nishikawa *et al.*, 2001; Zhang *et al.*, 2001; Huyer *et al.*, 2004). Conversely, Bip is necessary for the degradation of all known soluble ERAD substrates [reviewed in (Fewell *et al.*, 2001)]. For example, two of the best-characterized yeast ERAD substrates, the misfolding mutant of ER soluble carboxypeptidase yscY (CPY*) and the pro α factor are ER soluble proteins that require Bip for their degradation (Plemper *et al.*, 1997; Brodsky *et al.*, 1999; Taxis *et al.*, 2003). Bip was proposed to be a master regulator of UPR since Bip interacts with transcription factors (IRE1, PERK and ATF6) in the ER thereby preventing the activation of ERAD factors (Dorner *et al.*, 1992; Bertolotti *et al.*, 2000; Shen *et al.*, 2002). When the level of misfolded proteins exceeds the capacity of Bip, these transcription factors are freed and translocate to the nucleus to increase the expression of ERAD factors, including Bip, to attenuate the ER stress. Bip recognizes hydrophobic regions of the polypeptide that are usually inaccessible because they are buried inside native proteins. Binding and release of the partially unfolded

polypeptide is ATP-dependent (Bukau and Horwich, 1998). While Bip can facilitate the folding of the nascent polypeptide, Bip retains terminally misfolded proteins so that high affinity association with Bip may identify ERAD substrates. PDI has been implicated in ERAD downstream of Bip by targeting lumenal ER proteins back to the translocation pore for retro-translocation (Gillece *et al.*, 1999). PDI acts as a redox-dependent chaperone and unfolds proteins for their passage across the ER membrane (Tsai *et al.*, 2001).

In contrast to ER lumenal chaperones Bip and PDI, lectin-like chaperones recognize misfolded glycoproteins by their carbohydrate moieties. Glycan processing during protein folding may function as a timer in glycoproteins [reviewed in (Parodi, 2000)]. Proper oligosaccharide trimming results in the dissociation of nascent glycoproteins from calnexin, allowing newly synthesized proteins to exit the ER (Hebert *et al.*, 1995). Glycoproteins that fail to acquire their native conformation within an appropriate time are removed from the calnexin cycle of glycosylation and directed for degradation. Indeed, calnexin was required in the *in vitro* reconstitution of the ERAD of the pro α factor in yeast (McCracken and Brodsky, 1996) and defects in calnexin impaired the degradation of certain lumenal ERAD substrate (Brodsky *et al.*, 1999). Entry of glycoproteins into ERAD has also been proposed to require a particular mannose trimming and subsequent recognition of the remaining sugar moieties by the glycoprotein chaperone EDEM [reviewed in (Helenius and Aebi, 2004)]. Evidence in yeast ERAD suggests that EDEM, while protecting properly folded nascent glycoproteins from degradation, can also direct misfolded glycoproteins released by calnexin for retro-translocation (Hosokawa *et al.*, 2001; Molinari *et al.*, 2003; Oda *et al.*,

2003). Human EDEM has been recently described and is also involved in ERAD (Karaveg *et al.*, 2005). Thus, lectin-like chaperones have dual functions in glycoprotein processing namely protecting glycosylated folding intermediates from degradation and directing terminally misfolded ER glycoproteins for ERAD.

ERAD substrates that reach the cytosol by retro-translocation avoid aggregation by association with cytosolic chaperones Hsp40, Hsp70 and Hsp90. These chaperones are involved in protein folding but they are also able to deliver an unfolded polyubiquitinated polypeptide to the 19S subunit of the proteasome for degradation (Verma *et al.*, 2000; Luders *et al.*, 2000). Hsp70 is necessary in the ERAD of several ER transmembrane proteins such as Ste6p, the cystic fibrosis transmembrane regulator (CFTR) and Vph1p (Hill and Cooper, 2000; Zhang *et al.*, 2001; Huyer *et al.*, 2004). In addition, Hsp40 has been proposed to be a co-factor of Hsp70 in the degradation of Ste6p and CFTR (Zhang *et al.*, 2001; Huyer *et al.*, 2004). While Hsp70 appears to perform a similar task to Bip, it is dispensable in the degradation of lumenal ERAD substrates (Lee *et al.*, 2004). Similar to Hsp70, Hsp90 has been proposed to facilitate the delivery of transmembrane ERAD substrate to the proteasome by preventing aggregation of the target protein (Imamura *et al.*, 1998; Fuller and Cuthbert, 2000; Mimnaugh *et al.*, 2004). Therefore, various chaperones are involved in initiating ERAD in the ER lumen and targeting proteins for retro-translocation and delivery to the proteasome for degradation.

1.3.3 The Export Channel

The retro-translocation of proteins out of the ER requires a channel to allow the transit of the polypeptide across the lipid bilayer. The translocation channel Sec61 (yeast Sec61p), mediating the anterograde transit of newly synthesized protein into the ER, has been suggested to take part in the retrograde process as well (Wiertz *et al.*, 1996b; Plemper *et al.*, 1997; Pilon *et al.*, 1997). Polypeptides have been proposed to enter the translocation channel for retrograde transport in an unfolded conformation facilitated by the reduction of disulfide bonds by PDI (Tsai *et al.*, 2001). However, native green fluorescent protein and dihydrofolate reductase have been shown to retrotranslocate in a folded state when fused to a degradation target such as the class I MHC molecules (Fiebiger *et al.*, 2002; Tirosh *et al.*, 2003). This suggested that the putative retro-translocation pore may be sufficiently flexible to accomodate these folded polypeptides (24-40 Å). Structural information from the SegYEG complex, the archeal homolog of Sec61 translocon, demonstrated a pore of 10-12 Å (van den Berg *et al.*, 2004; Clemons, Jr. *et al.*, 2004). Other studies have suggested that the pore may flex up to 40-60 Å (Hamman *et al.*, 1997; Wirth *et al.*, 2003). Sec61 may oligomerize to provide a larger pore size, allowing larger polypeptides to cross the ER membrane. Recently, studies have reported that the ER multi-spanning membrane protein Derlin-1 (yeast Der1p) is required for the ERAD of some proteins (Knop *et al.*, 1996; Lilley and Ploegh, 2004; Ye *et al.*, 2004). The interaction of Derlin-1 with class I MHC molecules suggested that Derlin-1 may be involved in forming a retro-translocation pore (Ye *et al.*, 2004). Transmembrane E3 ligases such as Doa10p or Hrd1p may also have an extended role in the formation of a retro-translocation pore for the ERAD of other proteins, but the role

of these factors in the channel for retro-translocation remains speculative. Investigation of the role of these ligases and Derlin-1 on a variety of ERAD substrate may clarify their necessity and function as a channel for retro-translocation.

1.3.4 Retro-translocation and the AAA-ATPase p97

Retro-translocation is an energetically unfavorable process and requires an ATPase to facilitate the retrograde translocation of a protein from the ER to the cytosol. Indeed, polyubiquitination is not sufficient for the release of ERAD substrate to the cytosol (Flierman *et al.*, 2003). The proteasome has been suggested to pull polyubiquitinated proteins out of the ER membrane through the ATPase activity of the 19S subunit (Mayer *et al.*, 1998; Walter *et al.*, 2001). However, only a small portion of proteasomes were bound to the ER membrane (Hirsch and Ploegh, 2000) and a mutation in the 19S subunit of the proteasome failed to prevent retro-translocation (Jarosch *et al.*, 2002).

A highly conserved member of the AAA-ATPase (ATPase associated with various cellular activities) family, the yeast Cdc48p, also named p97 or vasolin-containing protein (VCP) was recently found to be a central component involved in the retro-translocation of some ERAD substrates [reviewed in (Bays and Hampton, 2002; Tsai *et al.*, 2002)]. Defective mutant of cdc48p was found to be lethal in yeast, but conditional mutant showed that cdc48p is involved in the cell division cycle and post-mitotic reassembly of Golgi stacks (Latterich *et al.*, 1995) as well as in proteasomal degradation (Dai *et al.*, 1998). The crystal structure of p97 has revealed an hexameric ring with a central pore (Zhang *et al.*, 2000; Huyton *et al.*, 2003). p97 contains two

ATPase domains and has homology to N-ethylmaleimide-sensitive fusion protein, an ATPase involved in SNARE complex disassembly following vesicle fusion. In association with cofactor p47 (Kondo *et al.*, 1997), p97 is involved in the fusion of the membrane of Golgi vesicles in the reassembly of the Golgi apparutus at the end of mitosis [reviewed in (Patel and Latterich, 1998)]. Bound to ubiquitin-binding proteins Ufd1 and Npl4 (Richly *et al.*, 2005), p97 mediates the retro-translocation of various ERAD substrates (Meyer *et al.*, 2000). Since retro-translocation is an ATP-dependent process, p97 deficient in ATPase activity is unable to mediate retro-translocation (Ye *et al.*, 2001). In particular, ATP binding to p97 has been suggested to induce a conformational change that powers the retro-translocation of a target polypeptide (Rouiller *et al.*, 2000; Rouiller *et al.*, 2002; Albring *et al.*, 2004). The retro-translocation complex of p97/Cdc48p, Ufd1 and Npl4 has been observed in both yeast and mammalian cells (Rape *et al.*, 2001; Hitchcock *et al.*, 2001) and may be anchored in a complex at the ER membrane including Derlin-1 and VIMP (VCP-interacting membrane protein) (Ye *et al.*, 2004). Notably, Cdc48p is also involved in the cell cycle and defective mutant caused lethality in yeast (Latterich *et al.*, 1995). Mutation of Cdc48p (temperature sensitive), Ufd1 or Npl4 impaired the degradation of several ERAD substrates. The ensuing accumulation of misfolded proteins in the ER elicited the UPR (Ye *et al.*, 2001; Bays *et al.*, 2001b; Rabinovich *et al.*, 2002; Jarosch *et al.*, 2002; Braun *et al.*, 2002).

Polyubiquitination has been shown to be a pre-requisite for the retro-translocation of several ERAD substrates (Biederer *et al.*, 1996; Biederer *et al.*, 1997; Bordallo *et al.*, 1998; de Virgilio *et al.*, 1998; Kikkert *et al.*, 2001; Jarosch *et al.*, 2002).

Indeed, polyubiquitinated proteins accumulated on the ER membrane when Npl4 or Ufd1 were mutated in yeast suggesting that ubiquitin tagging preceded retro-translocation (Bays *et al.*, 2001b; Jarosch *et al.*, 2002). The expression of ubiquitin mutated at lysine 48 in yeast or mammalian cells resulted in the accumulation of ERAD substrates in the ER (Ward *et al.*, 1995; Biederer *et al.*, 1996; Hiller *et al.*, 1996; Yu and Kopito, 1999). Accumulation of proteins in the ER was also observed in cells expressing a temperature-sensitive mutant of the E1 ubiquitin activating enzyme (Ward *et al.*, 1995; de Virgilio *et al.*, 1998; Yu and Kopito, 1999; Kikkert *et al.*, 2001). ERAD substrates were polyubiquitinated at the cytosolic face of the ER membrane suggesting that the ubiquitin machinery is associated with the ER (Hiller *et al.*, 1996; Shamu *et al.*, 1999; Shamu *et al.*, 2001). In yeast, two E2 ubiquitin-conjugating enzymes, Ubc6p and Ubc7p (Sommer and Jentsch, 1993; Biederer *et al.*, 1996; Hiller *et al.*, 1996; Biederer *et al.*, 1997) and the E3 ubiquitin ligating enzymes Hrd1/Der3p (Gardner *et al.*, 2000; Deak and Wolf, 2001; Bays *et al.*, 2001a) and Doa10p (Huyer *et al.*, 2004) are associated with the ER membrane and are necessary for the degradation of various ERAD substrates.

p97 can recognize both nonubiquitinated and polyubiquitinated proteins (Ye *et al.*, 2003). As soon as an ERAD substrate emerges from the ER membrane, p97 may interact with the polypeptide, perhaps to prevent its escape back into the ER. p97 may also facilitate polyubiquitination by E3 ubiquitin ligases as it has been shown to interact with E3 ligases such as gp78 (Zhong *et al.*, 2004). The polyubiquitin chain, rather than being a ratcheting molecule, has been suggested to induce the interaction of ERAD substrate with ubiquitin-binding proteins Ufd1 and Npl4, co-factors of p97, and to signal the retro-translocation process via p97 (Flierman *et al.*, 2003). Degradation

intermediates accumulated in cells treated with inhibitors of the proteolytic activity of the proteasome (Ward *et al.*, 1995; Wiertz *et al.*, 1996b; Huppa and Ploegh, 1997; Yang *et al.*, 1998a). Inhibition of the proteolysis by the 20S core was found to uncouple the retro-translocation process from delivery of the target protein via ATPase of the 19S subunit to the 20S core (Oberdorf *et al.*, 2006).

A few exceptions have been described where polyubiquitination may is not required in the retro-translocation process. For example, the cholera toxin has been shown to retro-translocate to the cytosol and induce cellular toxicity by avoiding proteasomal degradation. This toxin contains few lysines allowing it to escape from polyubiquitination upon emergence in the cytosol and preventing its recognition by the proteasome (Rodighiero *et al.*, 2002). Interestingly, since the cholera toxin does not fit the polyubiquitination paradigm of retro-translocation, the role of p97 in the retro-translocation of the cholera toxin remains unclear and somewhat controversial (Abujarour *et al.*, 2005; Kothe *et al.*, 2005). In addition, proteins that are not bound for degradation such as calreticulin were found to retro-translocate independently of p97 (Afshar *et al.*, 2005). For ERAD substrates, p97 powers the retro-translocation process and may also be involved in recognizing target proteins as they become accessible at the outer leaflet of the ER membrane.

In summary, ERAD involves several ER lumenal chaperones to recognize misfolded ER proteins and to direct the polypeptides for retro-translocation. As the protein becomes cytosolically exposed, p97 appears to be involved in recognizing and then retro-translocating the protein following polyubiquitination. Release of protein into the cytosol and delivery to the proteasome is also facilitated by cytosolic chaperones that

direct the polypeptide to the 19S subunit for its subsequent delivery to the 20S proteolytic chamber.

1.4 Regulation of Hepatic VLDL Production by ERAD

The role of the proteasome in apoB degradation was first appreciated in cultured HepG2 cells (Yeung *et al.*, 1996) and then in McA-RH7777 cells (Cavallo *et al.*, 1998), using proteasome inhibitors lactacystin, MG132 and ALLN. Proteasomal degradation was suggested to occur early in apoB biogenesis, even during translation (Zhou *et al.*, 1998; Pariyarath *et al.*, 2001). Impaired primordial LpB assembly steps due to impaired disulfide linkage, MTP lipid transfer activity or glycosylation leads to the proteasomal degradation of apoB. Proteasomal degradation of apoB was enhanced when cysteines in the N-terminus of apoB were substituted to alanine or when the oxidative state of the ER was altered by the reducing agent dithiothreitol, hindering the formation of disulfide bonds (Shelness and Thornburg, 1996; Macri and Adeli, 1997a; Tran *et al.*, 1998a). ApoB susceptibility to proteasomal degradation was increased when lipidation was prevented by inhibitors of MTP lipid transfer activity (Jamil *et al.*, 1996; Benoist and Grand-Perret, 1997). Mutagenesis of the N-terminal glycosylation sites of apoB also increased apoB degradation in McA-RH7777 cells (Vukmirica *et al.*, 2002). These treatments and modifications resulted in the generation of apoB proteins that may be prone to interact with ERAD factors. Translocation-paused bitopic apoB proteins that fail to resume translocation in time and misfolded fully translocated apoB may be potential targets for proteolysis via the ubiquitin-proteasome system **(figure 1.5)**.

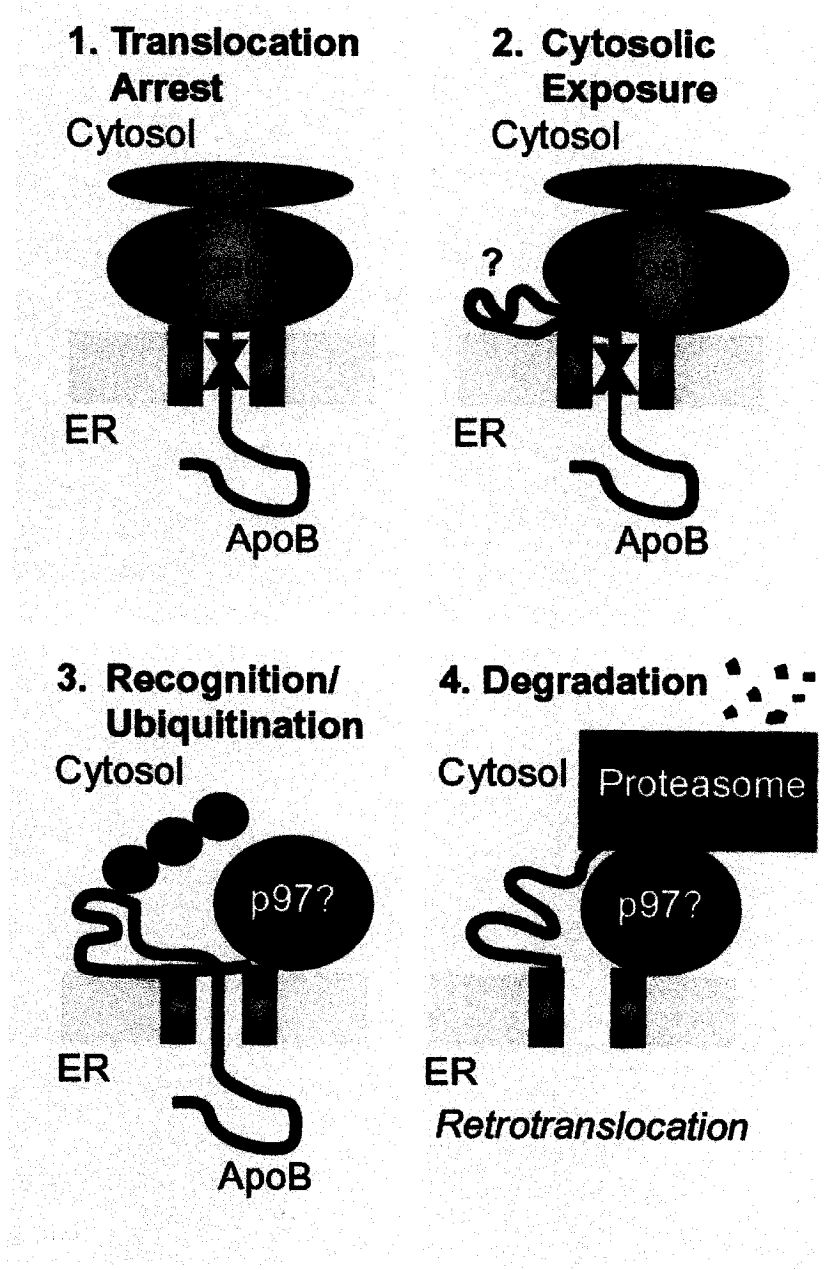

Figure 1.5. ER-associated degradation of apoB. Schematic representation of apoB proteasomal degradation. 1. Arrest of the translocation of the translating apoB polypeptide. 2. Ribosome-Translocon junction opening and looping of the apoB polypeptide in the cytosol. 3. Recognition and polyubiquitination of apoB and speculated interaction of p97 with apoB. 4. Retro-translocation of apoB to the cytosol and delivery to the proteasome for degradation.

Bitopic apoB remains associated with the Sec61 translocation channel (Mitchell *et al.*, 1998) and retrograde movement of the polypeptide to the cytosol may therefore occur in the same channel where translocation was initiated, a feature of ER transmembrane ERAD substrates. That way, following translocation arrest, nascent apoB can be targeted to the proteasome even before translation is completed (Benoist and Grand-Perret, 1997; Chen *et al.*, 1998; Liao *et al.*, 1998; Zhou *et al.*, 1998). This mechanism was supported by a study in HepG2 cells showing that partially synthesized apoB can be associated with the Sec61 translocon and the proteasome (Pariyarath *et al.*, 2001). In accordance with other ERAD studies, immunofluoresence studies have shown proteasomes in close proximity to the ER membrane suggesting a coupling between ubiquitination and degradation (Pariyarath *et al.*, 2001).

The retro-translocation of completely translated apoB has also been suggested as glycosylated apoB proteins were found to be degraded by the proteasome (Ingram and Shelness, 1996; Liao *et al.*, 1998; Huang and Shelness, 1999). Similar to lumenal ERAD substrates, translocated apoB would require targeting to a retro-translocation channel (Huyer *et al.*, 2004). However, the possibility of the complete retro-translocation of apoB has been received with skepticism by other researchers [reviewed by (Fisher and Ginsberg, 2002)]. Immunological analysis of apoB in HepG2 cells suggested that the N-terminal domain of apoB may not retrotranslocate during ERAD (Liang *et al.*, 2000), but is rather cleaved and may be degraded or secreted (Du *et al.*, 1994). Also, analyses of the plasma of abetalipoprotenemia patients (Du *et al.*, 1996) and of the media of non-hepatic cells transfected with apoB (Thrift *et al.*, 1992) have shown the presence of secreted N-terminal fragment of apoB suggesting that apoB is only partially retro-

translocated. Thus, the mechanism surrounding the recognition, retro-translocation and proteasomal degradation of apoB remains to be clarified.

1.4.1 ApoB ERAD and Molecular Chaperones

During LpB assembly, apoB has been shown to interact with various chaperones involved in promoting folding, but that also have a role in ERAD (Bip, PDI, Hsp70, Hsp90 and calnexin) (Fisher *et al.*, 1997; Chen *et al.*, 1998; Bradbury *et al.*, 1999; Gusarova *et al.*, 2001; Zhang and Herscovitz, 2003). For example, the ER lumenal chaperone Bip has been suggested to play a role in the ERAD of apoB. ER stress induced by glucosamine increased Bip expression and stimulated the ERAD of apoB in HepG2 cells (Qiu *et al.*, 2005). Increased levels of Bip may increase its binding to apoB delaying further transport of apoB out of the ER to the Golgi, a possibility suggested for other proteins subjected to higher levels of Bip (Morris *et al.*, 1997; Muresan and Arvan, 1998; Yang *et al.*, 1998b). Retention of apoB in the ER has been proposed to induce the retro-translocation of apoB to the cytosol for proteasomal degradation. In contrast to Bip, there is as yet no evidence to suggest a role for PDI in apoB ERAD. Moreover, a defective PDI in the shufflase activity resulted in a decrease in apoB secretion by increasing the ERAD of apoB (Wang *et al.*, 1997a). While further investigation is required, Bip may potentially be involved in both facilitating apoB folding and specifically recognizing misfolded apoB to initiate the process leading to its degradation.

ApoB interacts transiently with calnexin and calreticulin (Tatu and Helenius, 1999) and similar to other ERAD substrates, calnexin may play a dual role in protecting apoB from ERAD and directing the protein for degradation (Chen *et al.*, 1998).

Glycosylation of apoB in an appropriate time frame may be crucial for apoB translocation and folding and likely allows the polypeptide to escape proteasomal degradation by properly completing the calnexin cycle. For example, trapping apoB N-linked glycans in their monoglucosylated form, by posttranslational inhibition of ER glucosidase activity with castanospermine, increased apoB susceptibility to proteasomal degradation (Chen *et al.*, 1998).

ER quality control systems, other than the proteasome, have been shown to degrade apoB beyond the early stages of its biosynthesis. The increase in apoB degradation caused by tunicamycin treatment in HepG2 cells, which inhibits N-linked glycosylation, was not completely blocked by proteasome inhibitors (Macri and Adeli, 1997a; Liao and Chan, 2001). A combination of proteasome-mediated ERAD and non-proteasomal ER-associated proteolytic pathways has also been suggested for the yeast protein CPY* expressed in mammalian cells (Mancini *et al.*, 2003). Proteases localized in the lumen of the ER have been suggested to degrade apoB (Adeli, 1994; Sallach and Adeli, 1995). ER-60, an ER-resident chaperone (Lindquist *et al.*, 1998) with cysteine protease activity (Urade *et al.*, 1992) was proposed to have a role in the ERAD of apoB. ER-60-mediated proteolysis (Adeli *et al.*, 1997a) may provide an alternative non-proteasomal route for apoB degradation in the ER (Otsu *et al.*, 1995). Moreover, protease-mediated fragmentation of apoB was observed in the ER (Sallach and Adeli, 1995; Qiu *et al.*, 2004). A protease sensitive to (4-amidino-phenyl)-methane-sulfonyl fluoride, a serine protease inhibitor, was also suggested to be involved in degrading apoB (Cardozo *et al.*, 2002). These non-proteasomal pathways of degradation may represent another level of quality control regulating the assembly and secretion of LpB.

Cytosolic chaperones Hsp70 and Hsp90 have been shown to interact with apoB (Zhou *et al.*, 1995; Gusarova *et al.*, 2001). An attractive possibility is that the translocation arrested apoB, similar to a transmembrane protein [reviewed in (Rapoport *et al.*, 2004)], requires the coordinated action of molecular chaperones in both the ER lumen and the cytosol. Thus, Hsp70 and Hsp90 may prevent the polypeptide from aggregating while lipidation of domains downstream of the N-terminal βα1 domain occurs (Wang *et al.*, 1996). However, the induction of Hsp70 expression with herbimycin A increased apoB proteasomal degradation in HepG2 cells (Fisher *et al.*, 1997). Hsp90 was also found to play a role in apoB proteasomal degradation (Gusarova *et al.*, 2001). These studies suggested that both Hsp70 and Hsp90 act as partners in apoB ERAD by maintaining apoB in an aggregation-free unfolded state and by facilitating its delivery to the 19S subunit of the proteasome.

LDLR on the surface of hepatocytes have been extensively studied and have a central role in the clearance of lipoprotein remnants from the plasma [reviewed in (Hussain *et al.*, 1999)]. However, several studies have suggested a direct role for LDLR intracellularly in apoB ERAD. First, individuals with FH with impaired LDL clearance have been shown to have increased hepatic production of VLDL. (Millar *et al.*, 2005). Secondly, studies of LDLR knockout mice suggested that the absence of the LDL receptor may reduce intracellular degradation of apoB (Twisk *et al.*, 2000; Nassir *et al.*, 2004). Finally, modified LDLR (ER-resident) transfected in murine hepatocytes impaired LpB secretion (Gillian-Daniel *et al.*, 2002). LDLR were suggested to bind to the LDLR-binding domain of apoB and to retain apoB in the ER, similar to Bip, and to direct apoB for ERAD. On the other hand, *in vivo* metabolic radiolabeling showed that

the production rate of apoB was similar between wild-type and LDLR knockout mice (Millar *et al.*, 2002). Moreover, decreased affinity of LDLR for apoB failed to increase VLDL secretion in a ligand-defective mutation of apoB (Benn *et al.*, 2005). The controversy over the role of LDLR on apoB stability may be addressed by analyzing the kinetics of nascent apoB in cells where the LDLR has been knocked down.

The regulation of ERAD-related molecular chaperones in apoB degradation is not clear, but MTP inhibitor treatments (which impair LpB assembly) did not cause ER stress in mice and did not trigger UPR, suggesting that the cell has the capacity to efficiently cope with an increase in degradation-prone apoB proteins without initiating UPR (Liao *et al.*, 2003b). In summary, the role(s) of chaperones in the ERAD of apoB is poorly understood, but various chaperones important in the degradation of different ERAD substrates may also be important in apoB degradation.

1.4.2 Ubiquitination and Retro-translocation of ApoB

Polyubiquitinated apoB proteins have been shown to accumulate in cultured cells treated with proteasome inhibitors (Yeung *et al.*, 1996; Fisher *et al.*, 1997). In HepG2 cells, the E3 ubiquitin ligating enzyme gp78 has been shown to play a role in apoB polyubiquitination (Liang *et al.*, 2003). Gp78, also known as the tumor autocrine motility factor receptor (Fang *et al.*, 2001), was previously described as an E3 ligase in the ERAD of CD3δ. In addition, polyubiquitination of the apoB polypeptide during translation (Zhou *et al.*, 1998) suggests that apoB may require ubiquitin tagging for retro-translocation, similar to other ERAD substrates. While polyubiquitination of apoB apparently targets the polypeptide for proteasomal degradation, polyubiquitinated apoB

proteins have been proposed to be salvageable (Mitchell *et al.*, 1998). In proteasome-inhibited HepG2 cells, polyubiquitinated apoB may be de-ubiquitinated, allowing translocation to be reinitiated and apoB to be subsequently secreted when LpB assembly is stimulated by OA. A controversial study in HepG2 cells has also suggested that proteasomal degradation may occur in the Golgi in a ubiquitin-independent manner (Liao *et al.*, 2003a). Nevertheless, the current view suggests that apoB proteasomal degradation is primarily an ER-associated, ubiquitin-dependent process. ApoB ERAD appears to involve similar ERAD factors on each side of the ER membrane to coordinate proteasomal degradation. While the degradation of apoB can occur throughout the secretory pathway, the ubiquitin-proteasome system may be a major determinant in the production of VLDL by hepatocytes.

The role of the AAA-ATPase p97 has not been characterized for apoB ERAD. ApoB possesses all the features of a substrate for p97 retro-translocation. ApoB is polyubiquitinated, cytosolically exposed, associated with the translocation channel and is susceptible to proteasomal degradation. Moreover, p97 has been shown to interact with gp78, an E3 ligase involved in apoB polyubiquitination (Zhong *et al.*, 2004). To determine the retro-translocation pathway of apoB and to clarify the mechanism of proteasomal degradation, the role of p97 in the ERAD of apoB needs to be elucidated.

1.5 Summary

The structural elements within the apoB polypeptide that are responsible for lipid recruitment into a lipoprotein particle and translocation arrest are poorly characterized. Amphipathic α-helix and β-sheet (Segrest *et al.*, 1994) structures are thought to underlie

the unique ability of apoB to assemble lipoproteins with a neutral lipid core. Although the pentapartite model is used widely for the analysis of apoB structure-function relationships, such a structure has not yet been proven. Work with chimeric proteins demonstrated that multiple short segments within the β1 domain, as few as 150 amino acids, mediate neutral lipid recruitment into VLDL-like lipoproteins (McLeod *et al.*, 1996). Other studies have also suggested a specific role for the β1 domain in TAG accretion, particularly sequences between the carboxyl termini of apoB-29 and apoB-41 (Carraway *et al.*, 2000). Characterization of transgenic mice that express truncated human apoB also indicates that the ability of apoB to transport TAG is a function of the β1 domain (Chen *et al.*, 2002). The coordinated addition of lipids to the apoB polypeptide during VLDL assembly may compete with the degradation processes and thereby determine the level of VLDL secretion (Sakata *et al.*, 1993). Translocation arrest has been suggested to be a key process in the regulation of the rate of the production of VLDL. Thus, it was hypothesized that regions of the β1 domain may decrease apoB stability, causing translocation arrest thereby increasing apoB susceptibility to presecretory degradation. Hydrophobic regions in the β1 domain may arrest apoB in a bitopic configuration and associate with the outer leaflet of the ER membrane preventing further reinitiation of translocation and causing prolonged cytosolic exposure of the polypeptide. In addition, regions of the β1 domain may serve as sensors (or flags) to signal proper LpB assembly as they necessitate coordinated lipidation and have high requirements for MTP lipid transfer activity. Inefficient translocation and cytosolic exposure of the β1 domain may ultimately increase apoB susceptibility to proteasomal degradation.

ERAD of apoB may play a regulatory role in the early stages of apoB biosynthesis, but because the proteasome is on the cytosolic side of the ER, exposure of the nascent chains and retro-translocation of the newly synthesized apoB polypeptides is required for this degradation mechanism. The ERAD of misfolded ER transmembrane proteins is accomplished by a series of steps; ubiquitination, retro-translocation and delivery. Steps in the ERAD of apoB may be similar to misfolded ER transmembrane proteins, since bitopic apoB is already accessible to cytosolic ubiquitin-proteasome factors. While several studies have investigated the proteasomal degradation of apoB, the details surrounding the recognition of the apoB polypeptide and the delivery to the proteasome are incompletely understood. The recognition and retro-translocation of ERAD substrate has been proposed to require the AAA-ATPase p97. It was hypothesized that translocation arrest and ubiquitination of apoB renders the polypeptide susceptible for proteasomal degradation by first being recognized by p97 which initiates retro-translocation. Retro-translocation of apoB to the cytosol would allow the polypeptide to be entirely degraded by the cytosolic proteasome.

The focus of this work was to clarify the translocation arrest process by investigating structure-function relationships in apoB and to determine the role of ERAD factors contributing to the retro-translocation process. Chapter 3 describes the role of sequences in the β1 domain of apoB in the assembly of TAG-rich LpB in McA-RH7777 cells and their impact on translocation efficiency, ubiquitination and proteasomal degradation. From this work, it becomes clear that cytosolic exposure is a pivotal step in the recognition of apoB proteins for proteasomal degradation. Chapter 4 encompasses the investigation of the retro-translocation process of apoB-100 in HepG2 cells. Using

digitonin-permeabilised cells, evidence for a complete retro-translocation process is provided. This study also evaluates the role of the ER-associated AAA-ATPase p97 in the recognition of apoB for proteasomal degradation and concludes that p97 is a key player in the retro-translocation process. These studies clarify the mechanism by which the apoB polypeptide in the ER is subject to proteasomal degradation in the cytosol. The data extends previous studies in yeast and mammalian cells indicating that p97 is involved in the retro-translocation and delivery of degradation target to the proteasome.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Cell culture reagents were purchased from Invitrogen Canada Inc. (Guelph, ON, Canada). Electrophoresis-grade chemicals for polyacrylamide gel elecrophoresis (PAGE) were supplied by Bio-Rad laboratories (Hercules, CA, USA). Protein A-Sepharose CL6B and a mixture of [^{35}S]methionine and [^{35}S]cysteine (Promix®) were obtained from Amersham Biosciences (Montreal, QC, Canada). Brefeldin A was bought from Epicentre Technologies (Madison, WI, USA). Protease inhibitors ALLN (acetyl-leucyl-leucyl-norlecuinal) and leupeptin (acetyl-leucyl-leucyl-argininal) were purchased from Roche Diagnostics (Montreal, QC, Canada). Proteasome inhibitor N-benzoyloxycarbonyl (Z)-leucyl-leucyl-leucinal (MG132) and monoclonal antibody for the β1 subunit of the 20S proteasome (PW8140) were supplied by BIOMOL International (Plymouth Meeting, PA, USA). The soybean trypsin inhibitor was bought from Sigma Aldrich (St-Louis, MO, USA). Polyclonal antibodies to apoB (immunoreactive to human and rat apoB) were also purchased from Roche Diagnostics or from Chemicon Inc. (AB742) (Temecula, CA, USA). The monoclonal antibody to rat apoB (LRB 220) was a gift of L. Wong (Louisiana State University, New Orleans, LA, USA). Monoclonal antibodies to human apoB (1D1 and 1C4) were provided by R. Milne and Y. Marcel (Ottawa Heart Institute Research Corp., Ottawa, ON, Canada). Polyclonal antibody to the C-terminal cytosolic domain of canine calnexin (SPA-860), monoclonal antibody to rat protein disulfide isomerase (SPA-891), monoclonal antibody for heat shock protein 70 (SPA-820) were obtained from Stressgen Biotechnologies

(Victoria, BC, Canada). Monoclonal antibody for p97 (PRO65278) was purchased from Research Diagnostic Inc. (Concord, MA, USA).

2.2 Methods

2.2.1 Construction of Plasmids Encoding Fusion and Truncated Proteins

For the construction of apoB29 fusion protein plasmids, pB29 (McLeod *et al.*, 1994) was digested with *Mlu*I, end-filled with the Klenow fragment of DNA polymerase, and ligated with a *Not*I linker (#1045, New England Biolabs, Ipswich, MA, USA) to generate a modified pB29 vector to assemble fusion constructs. The fusion proteins contained a linker encoded pentapeptide (Asp-Ala-Ala-Ala-Ala) between apoB29 and the remaining protein sequence. ApoB cDNA sections encoding apoB34-37, apoB37-42 and apoB34-42 were amplified from the human apoB-48 cDNA (Hussain *et al.*, 1995) using Vent® polymerase (New England Biolabs). Rat liver fatty acid binding protein (FABP) cDNA was amplified from McA-RH7777 cell total RNA by RT-PCR using primers designed based on the published cDNA sequence (Genbank: gi: 28779, X04714). The 5' end and 3' end primers comprised a *Not*I site and a *Cla*I site, respectively. The pB29 plasmid and the polymerase chain reaction (PCR) product were digested with *Not*I and *Cla*I and ligated mixtures were used to transform *Escherichia coli* strain DH5α; plasmid DNA for McA-RH7777 transfection was purified by cesium chloride ultracentrifugation. For the construction of apoB-39 truncated protein, pGEX B37-42 plasmid was subjected to site directed mutagenesis (Stratagene) at nucleotide 5518 (from complete apoB cDNA) to modify codon TTA to a stop codon TAA, disrupting a *Dra*I site (B39 mut.). *Escherichia coli* strain DH5α was transformed with

the mutated plasmids and enzyme digestion with *Dra*I served as diagnosis for the mutation. The section B37-42 (B39 mut.) of the pGEX plasmid was amplified by PCR and each end was digested with *Cla*I and *Sal*I for ligation. Digested fragments were separated on a 1% agarose gel and isolated using the gel extraction kit Qiaex-II from QIAGEN (Mississauga, ON, Canada). The pCMV-B42 and the pRC-CMV-B48 expression vectors were digested with *Cla*I and *Sal*I and digested fragments of B37-42 (B39 mut.) were ligated with digested expression vectors. Ligation mixtures were used to transform *Escherichia coli* strain DH5α and plasmid DNA was purified by cesium chloride ultracentrifugation.

2.2.2 Cell Culture and Generation of Stable Cell Lines

Parental and transfected McA-RH7777 cells were maintained in 10 cm culture dishes (Falcon) with DMEM containing 10% (w/v) FBS and 10% (w/v) horse serum. For stable cell lines, 200 μg/ml Geneticin was added for selection and maintenance. Stable cell lines were generated using the calcium precipitation technique (McLeod *et al.*, 1994). Cell lines expressing apoB-29, apoB-34, apoB-37, apoB-42, or human apoA-I have been characterized previously (McLeod *et al.*, 1996). Expression of apoB-39 in stably transfected cell lines was confirmed by immunoblotting.

HepG2 cells were obtained from the American Type Culture Collection (ATCC, Manassis, VA; HB-8065). Cells were maintained in 10 cm culture (Falcon) dishes in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Burlington, ON, Canada) containing 2 mM glutamine supplement. Cells were split at approximately 70-80% confluence, every two days, by trypsinization and replating at a ratio of 1:3. More

dilute replating resulted in cells which would no longer grow in monolayer and could therefore not be used for experiments. For most of the experiments, the cells were plated onto 35 mm Primaria dishes, whereas for siRNA transfections the cells were plated onto 12 well plates. Except where indicated, cells were maintained in a 37°C humidified incubator with 5% CO_2 atmosphere.

2.2.3 Immunoblot Analysis of Transfected Cells

The apolipoproteins in total or fractionated medium were concentrated on fumed silica (Sigma-Aldrich), eluted into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (10 mM Tris, pH 8.3, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% Bromophenol Blue), and resolved by SDS-PAGE (3–15% gradient gel) as previously described (Laemmli, 1970). Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting (Yao *et al.*, 1991), and human apolipoproteins were detected using monoclonal antibodies. Anti-mouse immunoglobulin antibodies, labeled with horseradish peroxidase (#170-5047, Bio-Rad Laboratories), and enhanced chemiluminescence (#1500694, Roche Biochemicals) were used to detect the presence of immune complexes on the membrane, according to the manufacturer's recommendations. The liver FABP moiety in the apoB-29/FABP fusion protein was detected using a rabbit polyclonal antibody (a gift of J. Storch, Rutgers University, New Brunswick, NJ, USA).

2.2.4 Density Gradient Ultracentrifugation

Confluent transfected McA-RH7777 cells were incubated in DMEM in the absence or presence of 0.4 mM oleic acid for 24 hours. Media were collected and layered at 12.5% sucrose in 12 ml gradient of 0, 12.5, 25 and 47% sucrose. Gradients were centrifuged at 35000 rpm for 65 hours and fractions of 1 ml were collected. Lipoproteins from each fraction were precipitated with 150 µl of 50 mg/ml of fumed silica for 1 hour. The silica-bound lipoproteins were washed with PBS and released with loading buffer. Proteins were resolved on SDS-PAGE (5% gel) and transferred to PVDF membranes. Human and rat apoB proteins were visualized by immunoblotting with the monoclonal antibody LRB220.

2.2.5 Metabolic Labeling Studies

McA-RH7777 cells were plated on 35 or 60 mm cell culture dishes (Falcon Primaria®) and grown to 50–70% confluence. The medium was removed and replaced with 750 µl or 1 ml, respectively, of serum-free and methionine/cysteine-free DMEM. After 1 hour, medium was removed and cells were labeled for 1 hour in serum-free and methionine/cysteine-free DMEM containing [^{35}S]ProMix® (100 µCi/ml). After a 60 min pulse, the labeling medium was removed and replaced with chase medium (DMEM). Where indicated, the pulse and chase media were supplemented with FBS (20%, v/v), sodium oleate (0.4 mM), MG132 (25 µM), or ALLN (100 µM). At each time point of the chase, medium was collected and cells were solubilized with a radioimmunoprecipiation assay (RIPA) buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate) containing 1% SDS and heated 15 minutes at 85°C.

Lysates were diluted to 0.1% SDS with RIPA buffer and apoB proteins were immunoprecipitated with goat polyclonal antibody (AB742) and recovered on Protein-A Sepharose beads (Amersham Biosciences). Immunoprecipitates were washed 5 times with RIPA buffer containing 0.1% SDS. ApoB proteins were released to SDS-PAGE loading buffer, resolved by 5% SDS-PAGE and visualized by fluorography (Yao *et al.*, 1991). Radioactivity associated with apolipoproteins was quantified by scanning densitometry or by liquid scintillation counting after excision and digestion of the corresponding bands from the gels.

HepG2 cells in 12-well plates or in 35 mm Primaria dishes at approximately 70-80% confluence were incubated in cysteine/methionine-free DMEM for 1 hour and then labeled for up to 1 hour in the same medium containing 100 μCi of [^{35}S] cysteine/methionine (Perkin Elmer, Boston, MA, USA), in the absence or presence of 25 μM proteasome inhibitor MG132. For measurement of initial rates of synthesis, the medium was removed and the cells were recovered by lysis as described above. Pulse-chase analysis of HepG2 cells and apoB immunoprecipitation were conducted as described above. ApoB proteins were resolved by SDS-PAGE (5%) and visualized by autoradiography. Bands were excised and digested and radioactivity was measured by liquid scintillation counting. A polyclonal antibody to human apoAI (Roche Diagnostics, Montreal, QC) was used to immunoprecipitate HepG2 apoAI and immune complexes were resolved by SDS-PAGE on 10% (w/v) acrylamide gels.

2.2.6 Digitonin Permeabilisation and Protease Protection Assay

Confluent monolayers of McA-RH7777 cells in 35 mm dishes were washed with 1 ml cold PBS (41 mM Na_2HPO_4, 4 mM KH_2PO_4, 138 mM NaCl, 2.5 mM KCl) and cells were permeabilised in a cold CSK buffer (10 mM PIPES pH 6.8, 0.3 M sucrose, 0.1 M KCl, 2.5 mM $MgCl_2$, 1 mM sodium-free EDTA) containing digitonin (75 μg/ml). Permeabilised cells were treated with trypsin (100 μg/ml) on ice for 10 minutes after pulse labeling as described previously (Cavallo *et al.*, 1998). Soybean trypsin inhibitor (500 μg/ml) was added, incubated for 10 minutes on ice and cells were lysed in RIPA buffer containing 1% SDS. The apolipoproteins were purified by immunoprecipitation and analyzed by SDS-PAGE (5% gel) and fluorography.

Confluent monolayers of HepG2 cells in 60 mm dishes were permeabilised with digitonin as described above. Cellular and cytosolic fractions were collected and solubilized in 1% SDS as described above. Proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and visualized by immunoblotting with antibodies for apoB (1D1), calnexin (SPA-860), protein disulfide isomerase (SPA-891), p97 (PRO65278), heat shock protein 70 (SPA-820) and the β1 subunit of the 20S proteasome (PW8140). High-salt washing was performed on permeabilised cells by adding 0.5 M KCl to the monolayer for 10 seconds.

2.2.7 Ubiquitination and Retrotranslocation Assays

HepG2 cells were incubated in the absence or presence of MG132 (25 μM) or ALLN (100 μM) for 1 h. Cells were then permeabilised with 75 μg/ml digitonin for 10 min on ice and cytosol and cell fractions were collected. Cells were lysed in RIPA buffer

containing 1% SDS. After addition of 0.1% SDS to cytosol samples and diluting cell samples to 0.1% SDS with RIPA buffer, cellular and cytosolic apoB proteins were collected by immunoprecipitation with goat polyclonal antibody for human apoB or, where indicated, samples were incubated with non-immune goat serum (Chemicon). Immunocomplexes were recovered on protein A sepharose. After washing 5 times in RIPA buffer containing 0.1% SDS, the immunoprecipitant apoB was resolved on SDS-PAGE (5% gel), transferred to nitrocellulose and immunoblotted with monoclonal antibodies for apoB (1D1) or ubiquitin (SPA-203).

For retrotranslocation assays, digitonin-permeabilised HepG2 cells in 60 mm dishes were incubated in 1 ml of DMEM with or without an ATP generating system (5 mM MgCl, 5 mM creatine phosphate, 1 mM ATP, 100 μg/ml creatine kinase). After 2 hours, media were collected and apoB proteins were precipitated with 10% trichloroacetic acid (TCA) and were visualized by immunoblotting as explained above.

2.2.8 Preparation and Analysis of Microsomes

McA-RH7777 cells were grown to confluence in 10 cm culture dishes and treated for 1 h with ALLN (100 μM), brefeldin A (5 μg/ml), ALLN plus brefeldin A, or vehicle control in DMEM/20% FBS. The monolayers were washed and collected in ice-cold PBS using a cell scraper. After low-speed centrifugation (4 min, 500 *g*), the cells (two dishes per treatment) were suspended in 2 ml of microsome buffer (MSB; 10 mM Tris-HCl, pH 7.4, and 250 mM sucrose) with protease inhibitors [leupeptin (100 μM), PMSF (100 μM), aprotinin (10 kallikrein inhibitor units/ml), ALLN (100 μM)] and

disrupted by 20 passes through a ball-bearing homogenizer (H and Y Enterprise, Redwood City, CA, USA).

Postnuclear supernatants (PNS) were prepared by centrifugation of the homogenate at 10,000 *g* for 10 min in an SS-34 rotor at 4°C. Intact microsomes were recovered in the pellet after a 16 min, 100,000 rpm centrifugation of PNS in a TLA-100.4 rotor. Protease protection analysis of microsomes was performed as previously described (Cavallo *et al.*, 1998), and the proteins were resolved by SDS-PAGE (3–15% gradient gel) and revealed by immunoblotting. To separate the lumenal content from the microsomal membranes, PNS was brought to 100 mM sodium carbonate (pH 11) and rotated end-over-end for 30 min at room temperature and then placed on ice. The membranes were collected by centrifugation (100,000 rpm, 16 minutes, 4°C) in a TLA-100.4 rotor. The supernatant (containing cytosol and lumenal contents) was removed and neutralized by the addition of 25 µl of 2.5 N HCl. The membrane pellet was resuspended in MSB and brought to 1% SDS, and proteins were solubilized by heating to 75°C for 15 min. An aliquot of the membrane and lumen samples was used to detect PDI and calnexin by Western blot analysis. The remainder of each sample was diluted 10-fold to reduce the SDS content to 0.1%, and apoB was recovered by immunoprecipitation as described above. ApoB immunoprecipitates were resolved by SDS-PAGE (5% gel), transferred to PVDF membranes, and probed for human apoB using antibody 1D1. Samples of lumenal content and membrane proteins (before immunoprecipitation) were resolved on SDS-PAGE (10% gel), transferred to PVDF membranes, and probed sequentially for PDI and calnexin using the appropriate antibody.

2.2.9 Chemical Crosslinking

Confluent HepG2 cells, grown in 100 mm dishes, were gently scraped into PBS. Dithiobis (succinimidylpropionate) (DSP; Pierce Biotechnology, Inc., Rockford, IL, USA) was added from a 500 mM stock solution (in dimethyl sulfoxide) to a final concentration of 2.5 mM and incubated for 30 minutes at 37°C. Excess DSP was inactivated by incubation for 15 minutes in the presence of 50 mM Tris-HCl. Cells were lysed in RIPA buffer containing 1% SDS and crosslinked apoB complexes were recovered by immunoprecipitation with polyclonal antibody to apoB or with non-immune goat serum. Immunoprecipitates were collected on protein A-Sepharose, washed 5 times in RIPA buffer containing 0.1% SDS and eluted into SDS-PAGE loading buffer with or without the reducing agent dithiothreitol (100 mM). Proteins were boiled for 10 minutes and resolved by SDS-PAGE (5% gel), transferred to nitrocellulose membranes and visualized by blotting with antibodies for apoB or p97.

Protein crosslinking in HepG2 cytosol was performed similarly, except that the water-soluble crosslinking agent dimethyl 3,3'-dithiobispropionimidate·2HCl (DTBP; Pierce Biotechnologies) was used instead of DSP. Cytosol was collected by digitonin permeabilization after treatment of the cells with 25 μM MG132 for 1 h. DTBP was added from a 500 mM aqueous stock solution to a final concentration of 20 mM and incubated for 30 minutes at 37°C. Uncrosslinked DTBP was quenched and apoB proteins were immunoprecipitated and visualized as described above for DSP.

2.2.10 Reduction of HepG2 p97 with siRNA

HepG2 cells were plated in 12 well plates at low density (10% confluence) and grown overnight. The culture medium was removed and replaced with 1 ml of transfection medium containing a recommended volume of 4 μl of Dharmafect-1 transfection reagent (Dharmacon Inc., Chicago, IL, USA) and 1 μM of non-targeting [non-targeting siRNA #1 (Dharmacon, Inc.)], 0.1 μM of validated siRNA for Lamin A/C (Dharmacon, Inc.) or p97 targeted siRNA [pre-designed p97 siRNA sip97#1 ID:119275, or sip97#2 ID:119276, or sip97#3 ID:119277 (Ambion, Inc., Austin, TX, USA)]. Following the addition of the transfection medium, cells were incubated for up to 48 h. The effectiveness of the siRNA was assessed by immunoblotting of cell lysates for p97 and PDI was used as control. Metabolic radiolabeling of transfected cells was conducted 48 h following transfection as described above.

CHAPTER 3

STRUCTURAL REQUIREMENTS IN APOLIPOPROTEIN B ASSEMBLY AND DEGRADATION

3.1 Introduction

Regions within apoB involved in the assembly of VLDL have been suggested to reside in the β1 domain (McLeod *et al.*, 1996; Carraway *et al.*, 2000). However, sequences within apoB that make the polypeptide susceptible to proteasomal degradation are unclear. Some studies have suggested that the β1 domain of apoB reduces the efficiency of translocation across the ER membrane (Liang *et al.*, 1998), which arrests apoB in a bitopic orientation with respect to the membrane (Pariyarath *et al.*, 2001). It has been reported previously that short amino acid sequences from apoB, which do not cause significant translocation arrest but are sensitive to proteolytic degradation, appear to colocalize with the lipid binding regions (McLeod *et al.*, 1996; Cavallo *et al.*, 1998). This chapter encompasses the characterization of the role of sequences within the β1 domain in VLDL assembly and apoB degradation using truncated and fusion apoB proteins. In addition, the role of certain of sequences within the β1 domain in translocation arrest and proteasomal degradation is addressed.

3.2 Results

3.2.1 Secretion and Stability of Truncated apoB Proteins

To locate sequence elements within the β1 domain that are important for degradation, a series of carboxyl-terminally truncated human apoB proteins, namely apoB-42, apoB-39, apoB-37, apoB-34, and apoB-29, were compared. McA-RH7777

cells stably expressing human apoA-I were used as a control, in which the secretion of apoA-I was linear and reached 70% efficiency during the 2 h chase **(figure 3.1A, open triangles)**. The secretion efficiency of truncated apoB-29 and apoB-42 **(figure 3.1A, closed symbols)** varied according to the apoB polypeptide length. Although secretion of apoB-29 was nearly as efficient as that of apoA-I (>50% of initial radiolabel was secreted), the secretion efficiency of apoB-42 was only 20% at the end of the 2 h chase, comparable to that of endogenous apoB-100 **(figure 3.1A, open squares)**. Cell-associated apoB and apoA-I radioactivity were also quantified during the chase; the total radioactivity (i.e., the sum of that in cells plus medium) reflected the posttranslational stability of the proteins. As shown in **figure 3.1B**, the recovery of apoA-I was complete during the chase, whereas only ~50% of apoB-100 and apoB-42 were recovered at the end of 2 h of chase, indicative of extensive degradation. In contrast to the instability of apoB-100 and apoB42, the recovery of apoB29 was nearly complete, with only ~10% degradation at the end of the 2 h chase. In addition, apoB-34, apoB-37 and apoB-39 were also secreted efficiently and, like apoB29, were less susceptible to rapid intracellular degradation **(figure 3.2)**. These observations indicated that secretion efficiency and intracellular stability of the truncated apoB proteins were functions of apoB length between apoB-29 and apoB-42. In addition, a particular region, apoB-39 to apoB-42, was important in determining the fate of apoB (i.e., secretion versus degradation).

3.2.2 Sequence homology between apoB-37-42 and FABP

To gain additional insight into the structural properties of the β1 domain, the protein sequence database (www.ncbi.nlm.nih.gov) was compared with the human

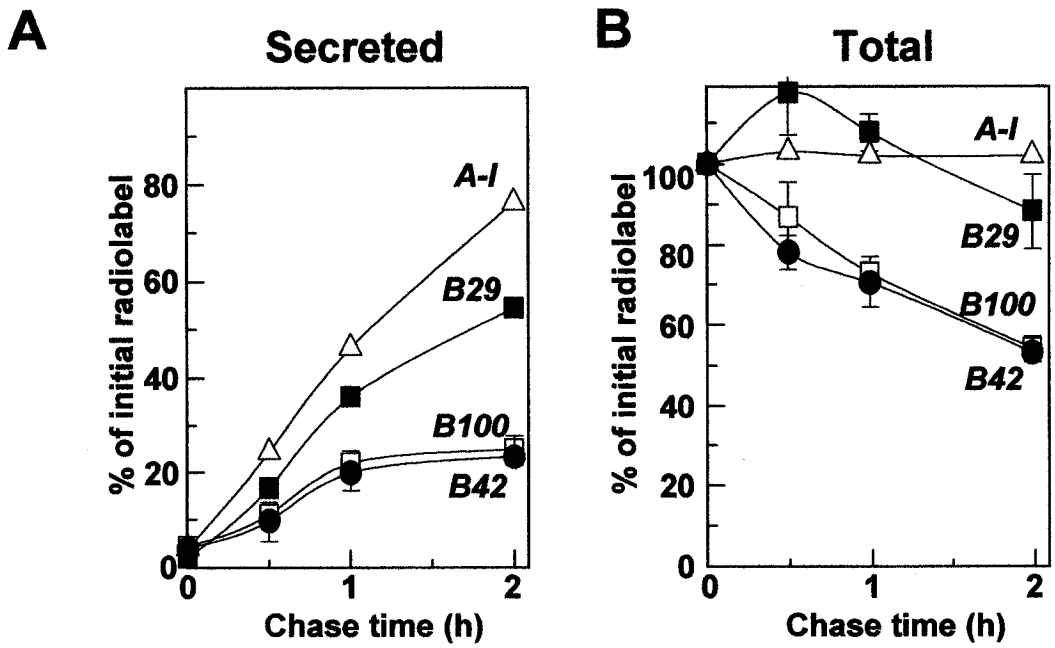

Figure 3.1. Secretion and stability of truncated human apolipoprotein B proteins in transfected McA-RH7777 cell lines. A. Secretion of apoB. Transfected cells were pulse-labeled for 30 min with [^{35}S]methionine/cysteine. At the indicated chase time, the apolipoproteins were recovered from the medium by immunoprecipitation, resolved by SDS-PAGE, and visualized by fluorography. Radioactivity associated with each apolipoprotein species was quantified by liquid scintillation counting. Results are expressed as percentages of the initial radiolabel (in the cells at time 0) secreted into the medium. B. Stability of apoB. Cells were pulse-labeled as in A, and the radioactivity associated with both cell and medium apos was combined. Open triangles, human apoA-I (n = 2); closed squares, apoB-29 (n = 3); open squares, apoB-100 (n = 8); closed circles, apoB-42 (n = 3).

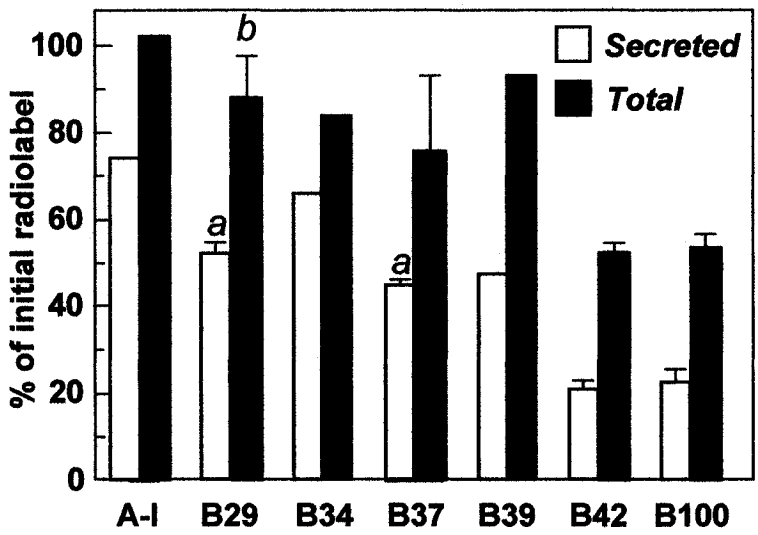

Figure 3.2. Secretion and stability of truncated human apoB proteins in transfected McA-RH7777 cell lines. Comparison of truncated apoB secretion and stability. Experiments were performed as described in figure 3.1. The secretion and stability of apos were determined at the end of a 2 h chase. In all panels, data points represent means ± SEM, except for apoA-I, apoB34 and apoB39 (means of duplicates); [a]: significantly different from apoB-100 ($P < 0.001$); [b]: significantly different from apoB-100 ($P < 0.05$).

apoB-100 sequence as the template. This was performed using the SEQSEE software (Wishart *et al.*, 1994b), and the search revealed that, in addition to the known homology between apoB, MTP, and lipovitellin (Mann *et al.*, 1999), there was significant homology between residues 1724 to 1921 of apoB (corresponding to apoB-38.0 to apoB-42.4) and those of FABP family members. A sequence alignment using the XALIGN software (Wishart *et al.*, 1994a) indicated that apoB residues 1,724 to 1,921 shared 34% sequence identity and 63% sequence similarity with several FABPs of known structure **(figure 3.3)**. Interestingly, the alignment of FABP sequences of four different mammalian species indicated a level of sequence identity and similiraty among FABPs similar to that between the apoB fragment and FABP (human, mouse, pig, and rat FABPs have 36.4% identity and 51% homology). Despite sequence variations, all FABP members share a common structure **(figure 3.4)**, with 10 antiparallel β-strands forming two β-sheets around a central hydrophobic fatty acid binding cavity (Scapin *et al.*, 1992; Sacchettini and Gordon, 1993). The X-ray crystal structure of human muscle FABP (Protein Data Bank code: 1hmr) **(figure 3.4A)** was used as the template to model the apoB fragment using the homology module of the InsightII software package (Accelrys, San Diego, CA). The model predicted that the secondary structure of residues 1,724 to 1,921 of apoB is similar to those of the FABPs, whereas the loop regions in apoB display different lengths.

The presence of potential repeated motifs in the β1 domain was also investigated. Sequences between B29 and B38 were evaluated for sequence similarities with multiple sequence alignments by using the web-based software Clustal W (http://cbr-rbc.nrc-cnrc.gc.ca/services/clustalw_form.html). In addition, structure prediction and tryspin

```
  1hmr_:                                VDAF         LGTWKLVDSKNFD     17
  1adl_:                                CDAF         VGTWKLVSSENFD     17
  1ael_:                                  AF         DGTWKVDRNENYE     15
  1eal_:                                  AF         TGKYEIESEKNYD     15
  LPHUB: FDHTNSLNIAGLSLDFSSKLDNIYSSDKFYKQTVNLQLQPYSLVTTLNSD   1774
Summary: -------------------------D-F--------*-*Y-LV**-N-D
                                   CCCCCCCCCCCCCCBBBBBBBBCHH

  1hmr_: DYMKSL     G VGFATRQVASMTK PTTIIEKNG  DILTLKTHS TFK    58
  1adl_: DYMKEV     G VGFATRKVAGMAK PNMIISVNG  DLVTIRSES TFK    58
  1ael_: KFMEKM     G INVVKRKLGAHDN LKLTITQEG  NKFTVKESS NFR    56
  1eal_: EFMKRL     A LPSDAIDKARNLK IISEVKQDG  QNFTWSQQY PGG    56
  LPHUB: LKYNALDLTNNGKLRLEPLKLH VAGNLKGAYQNNEIKHIYAISSAALSAS  1824
Summary: --*--L-----G-L-*-*-KL*-*A--L--**--N----I**I-S-*-**-
         HHHHHHCCCCCCCCCHHHHHHHHHCCCBBBBBBBCCCCCBBBBBBBBCCCC

  1hmr_:  NTEISFKL GVEFDET T ADDRKVKSIVTL      DGGKLVHLQKW      97
  1adl_:  NTEISFKL GVEFDEI T ADDRKVKSIITL      DGGALVQVQKW      97
  1ael_:  NIDVVFEL GVDFAYS L ADGTELTGTWTM      EGNKLVGKFKRVD    97
  1eal_: HSITNTFTI GKECDIE T IGGKKFKATVQM      EGGKVVVNSP       95
  LPHUB: YKADTVAKVQGVEFS HRLNTDIAGLASAIDMSTNYNSDSLHFSNVFRSVM  1874
Summary: *-*D***K*-GVEF--*-L-*D---L-S*I-M------D*-**--V-*-V-
         CBBBBBBBCCCCBBBBBCCCCCCCBBBBBBBBCCCCCCCCCBBBBBBBBCC

  1hmr_: DGQETTLVRELIDG KLILTLTHGTAV CTRTYEKEA                 132
  1adl_: DGKSTTIKRKRDGD KLVVECVMKGVT STRVYERA                  131
  1ael_: NGKELIAVREISGN ELIQTYTYEGVE AKRIFKKE                  131
  1eal_:   NYHHTAEIVDG  KLVEVSTVGGVS YERVSKKLA                 127
  LPHUB: APFTMTIDAHTNGNGKLALWGEHTGQLYSKFLL KAEPLAFTFSHDYKG    1921
Summary: ----*TI--***GN-KL*L*--H-G-*-S--**-KA-------------
         CCCBBBBBBBBCCCCBBBBBBBBCCCCCBBBBBBBBCCCCCCCCCCCCC
```

Figure 3.3. Sequence alignment of human apoB and FABPs. The human apoB (*LPHUB*) amino acid sequence between amino acid residues 1,724 and 1,921 (positions are indicated to the right of each line) was aligned with various FABP sequences. Amino acid identity between apoB and one or more of the FABP sequences (34.1%) is indicated by the single-letter amino acid designation in the summary line. Amino acid similarity (63.1%) is indicated by the asterisks in the summary line. The consensus structure is indicated beneath as follows: coil (C), β-sheet (B), or α-helix (H), and β-sheet (dark shading) and α-helix (light shading) regions are indicated as well. FABP family members used for the alignment are as follows: 1hmr_, human muscle FABP; 1adl_, mouse adipocyte lipid binding protein; 1ael_, rat intestine FABP; and 1eal_, pig ileal FABP.

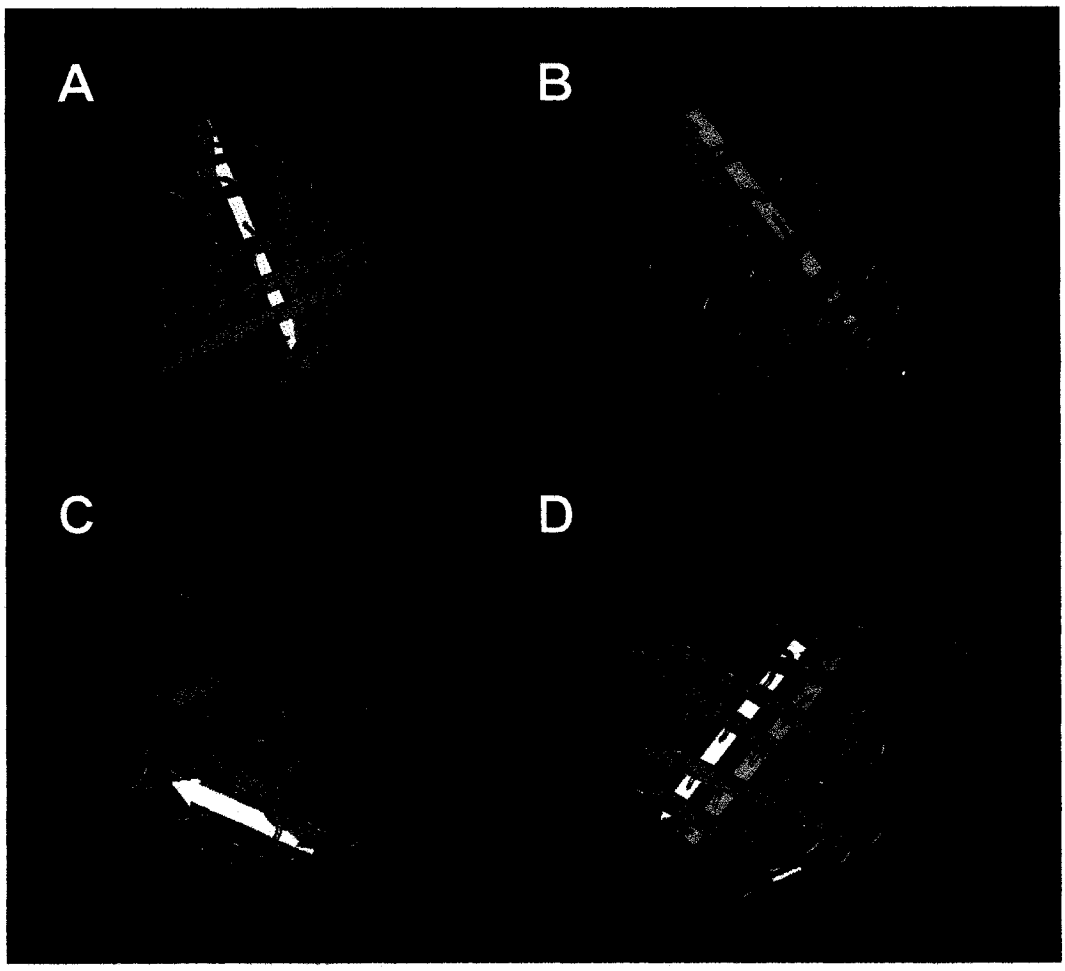

Fig. 3.4. Crystal structure of four FABPs from different species A. Human muscle FABP (1hmr). B. Mouse adipocyte lipid binding protein (1adl). C. Rat intestinal FABP (1ael). D. Pig ileal FABP (1eal). Anti-parallel β strands are represented by gold arrows and α helices are represented by green cylinders.

accessibility of aligned sequences on the LDL were compared (Yang *et al.*, 1989a). Two sets of repeats were found in the sequences between B29.5 and B38.2. Sequences between B29.5 and B31.4 and between B31.4 and B33.3 **(figure 3.5A)** were found to align with 19% identity and 58% similarities. Sequences between B33.3 and B35.8 and between B35.8 and B38.3 **(figure 3.5B)** were found to align with 21% identity and 64% homology. These similarities percentages are in the range of those found between FABPs. Protein structures were predicted with the web-based software 3D-PSSM (http://www.sbg.bio.ic.ac.uk/~3dpssm/index2.html). Each pair of alignments showed similar predicted structure. The alignment pair shown in **figure 3.5A** revealed 53% identity in predicted structure and 28% identity and 48% similarity for trypsin accessibility. In addition, the content of α, β and coiled structures was also similar. The alignment pair shown in **figure 3.5B** revealed 32% identity in predicted structure and 46% identity and 20% similarity for trypsin accessibility. Unlike sequences between B38.4 and B42.4, which are mostly inaccessible to trypsin (Yang *et al.*, 1989a), sequences between B29.5-B38 have mixed accessibility and may not be completely imbedded in the phospholipid monolayer to interact with the neutral lipid core of the LpB particle. Sequence alignments, structure predictions and trypsin accessibility assays suggested that sections of the β1 domain between B29.5-B38.3 may consist of repeats of 85-110 amino acids rich in β-sheets but also containing some α-helical structures. The repeats in the β1 domain may correspond to lipid-binding units involved in initiating the recruitment of TAG for VLDL assembly. In addition, the FABP homology may represent a model for the ability of apoB to bind lipids.

A

```
B29.5-B33.3 (a.a. 1338-1510)
1NLYNWSASYSGGNTSTDHFSLRARYHMKADSVVDLLSYNVQGSGETTYDHKNTFTLSCD--GSLRHKFLDSNIKFSHVEKLGNNPVSKGL
2LIFDASSSWGPQMSASVHLDSKKKQHLFVKEVKIDGQFRVS-----SFYAKGTYGLSCQRDPNTGRLNGESNLRFNSSYLQGTNQITGRY
  ::: *:*:.   ::: *:. : : *: ...*    .:.*.     ::  *.*: ***    .  :   :**::*.     *.* ::
1EEECCEEEECCCCCCCCCEEEEEEEEECCCEEEEEEEEECCCCCEEECCCEEEEEEC--CCHHHHHCCCCCCCHHHHHHCCCCCCCCE
2EEEECCCCCCCCCEEEEEECCCCEEEEEEEEEEEEEEEE-----EEEECCEEEEEEEECCCCEEECCCCCCEECHHHHEEEEEEEEE
 *** *    ****     *    *****   **********     *** ********   **     *****   ****         *
1MMMMMMMMMMMMMMMMMMMMXXOOOOMMMMMMMMMMMMMMMMMMMMMMOOOOOOXO--OOOOXXIIIIIIIIIIIIIIIIIIIIOO
2OOOOOOOOOOOOOOOOOOOOOOXXOOOOOOXXXIIIIIIII-----IIIIIMMMMMXMMXXXXXXIIIIIIIIIIIIIIIIIIIIIM
 :::::::::::::::::::::  ****::   ::::::::     :::::::::::: :        ********************* :
1; 42% Beta, 11% Alpha, 46% Coil
2; 64% Beta, 5% Alpha, 31% Coil
```

B

```
B33.3-B38.2 (a.a 1511-1733)
3EDGTLSLTSTSDLQSG--IIKNTASLKYENYELTLKSDTNGKYKNFATSNKMDMTFSKQNALLRSEYQADYESLRFFSLLSGSLNSHGLE
4QDG-ISTSATTNLKCSLLVLENELNAELGLSGASMKLTTNGRFREHNAKFSLDGKAALTELSLGSAYQAMILGVDSKNIFNFKVSQEGLK
 :** :* ::*::*:..  :::   . :      ::*  ***::::. :. .:* . :  :  * * ***   .:   .::. .:...**:
3ECCEEEECCHHCCCCC--CEECCEEEEEEEEEEEECCCCCCCCCCCCCCCEEEEEEECCCHHHHHHHHHCHHHHHHHHHCCCCCCCCEE
4CCC-CCCCCCCCEEEEEEEECCCCCHHCCCCCEEEEEECCCEEECCCEEEECCCHHHHHHHHHHHHHHHHHCCCCEEEEEEECCCCCC
  **    **          ****          ***   ***   ***  **        ********* ***           ****
3MMMMMMMMMMMMMMM--MMMOOOOOMMMMMMMMMXXXXXXXXMMMMMMMIIIIIIIOOOOOOOOOOOOOOOOOIIIIIIIIIIIIII
4MMMXMMMMMMMMMXOOOOOOOOOOOOOOOOOOOOOOOOOOOXXOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOOXXXXXXOOOOOOO
 *** ********** :  :::******:::::::::::        :::::::       *****************

3LNADILGTDKINSGAHKATLRIG
4LSNDMMGSYAEMKFDHTNSLNIA
 *. *::*:    .  *. :*.*.

3EECCEEEECCCCCHHHHHHHHHC
4CHHHHHCHHHHEEEECCCCEEEE

3IIIIIIIIIIIIIIIIXXXXMM
4IIIIIIIIIIIIIIIIIIIIII
 *****************    ::
3; 32% Beta, 28% Alpha, 39% Coil
4; 34% Beta, 26% Alpha, 40% Coil
```

Figure 3.5. Multiple sequence alignments of sequences in the β1 domain (B29.5-B38.2) A: Sequences between amino acids 1338-1425 and 1426-1510 were aligned with Clustal W (* = identity, : = closely similar, . = distantly similar) and structures were predicted with 3D-PSSM (C = coil, E = beta strand, H = alpha helix). Trypsin accessibility on LDL was noted under each alignments (O = trypsin accessible, M = mixed accessibility, I = trypsin non-accessible, X = not available, - = gap). B: Sequences between amino acids 1511-1620 and 1621-1733 were aligned as explained in A.

3.2.3 Expression of ApoB Fusion Proteins

ApoB fusion proteins were created and contained segments from either the apoB β1 domain (apoB-29/B-34–37, apoB-29/B-37–42, and apoB-29/B-34–42) or the liver FABP (apoB-29/FABP), in which the amino-terminal 29% of apoB was used as a reporter **(figure 3.6A)**. These sequences were chosen to compare different section of the region B34-42 and truncation in the cDNA were done according to restriction site All of the fusion proteins were secreted in a transient transfection assay **(figure 3.6B)**, and using stable cell lines, VLDL assembly, secretion efficiency, and posttranslational stability of the fusion proteins were compared. To determine if the fusion proteins were functional apolipoproteins, sucrose density gradient ultracentrifugation of the culture medium was performed after conditioning with or without exogenous sodium oleate. The apoB-29 was secreted mainly as HDL-like particles (ρ = 1.12–1.21 g/ml, fractions 9–12), regardless of the presence or absence of exogenous oleate **(figure 3.7A, panel B29)**. Fusion protein apoB-29/B-34–42 was secreted as VLDL ($\rho \geq 1.02$ g/ml, fractions 1 and 2) in addition to HDL-like particles in the presence of exogenous oleate **(figure 3.7B, panel B29/B34-42)**, suggesting that this protein had the ability to assemble VLDL. However, other apoB fusion proteins (e.g., apoB-29/B-34–37 and apoB-29/B-37–42) and apoB-29/FABP were unable to form VLDL and were secreted primarily as HDL-like particles. Fusion proteins apoB-29/B37-42 and apoB-29/B34-42 were poorly secreted and smaller, apparently cleaved apoB proteins were found in the medium **(figure 3.7A and 3.7B, panel B29/B37-42)**. In all cell lines, endogenous apoB-48 was secreted as HDL-like particles in the absence of oleate and was secreted as both HDL and VLDL in the presence of oleate. As expected, endogenous apoB-100 was secreted as

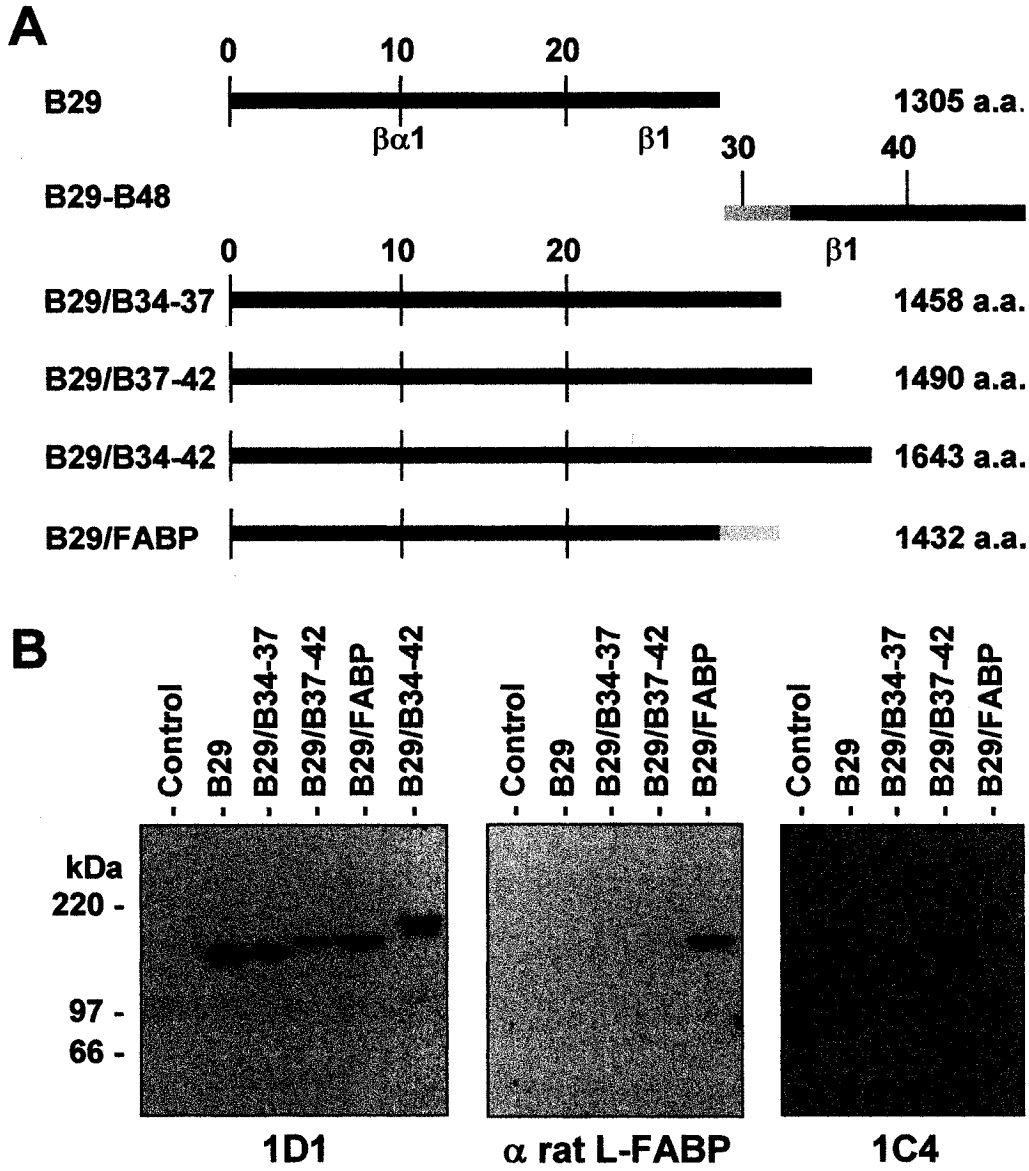

Figure 3.6. Construction of apoB fusion proteins. A. Schematic diagrams of apoB-29 fusion proteins. Purple bars represent the βα1 region of apoB, corresponding to the amino-terminal 22% of apoB-100. Cyan bars represent the β1 region between the carboxyl termini of apoB-22 and apoB-29. Blue bars represent various sequences from the β1 domain (29-34, 34–37, 37–42, 42-48), and the gold bar represents rat liver FABP (βFABP). The length of each fusion protein [in amino acids (a.a.)] is shown. B. Immunoblots of apoB fusion proteins transiently expressed and secreted in McA-RH7777 cells. The medium was collected at 48 h after transfection, and apoB fusion proteins in the conditioned medium were resolved by SDS-PAGE followed by immunoblot analysis using the indicated antibodies. 1C4 epitope is between the carboxyl termini of apoB-37 and apoB-42.

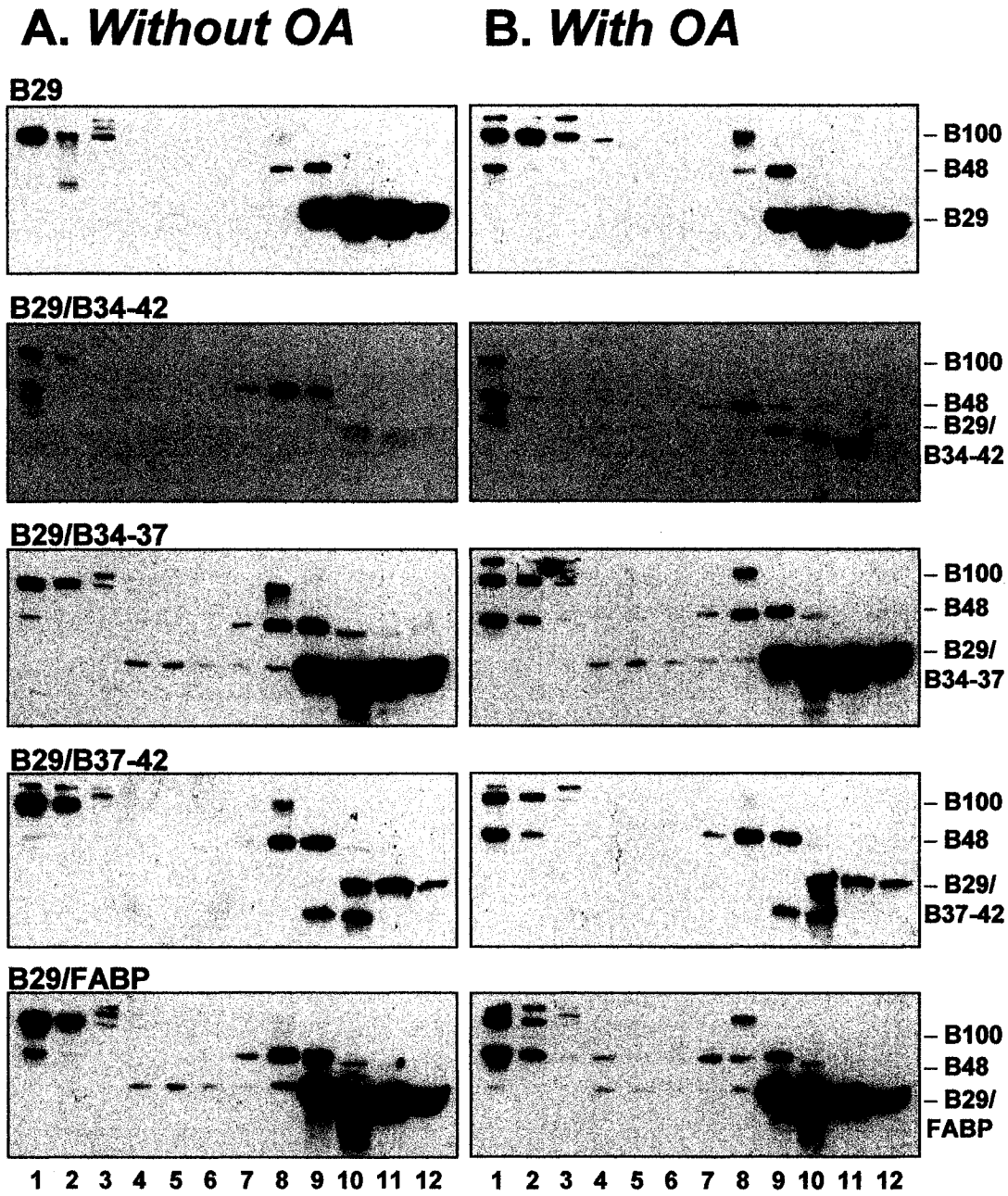

Figure 3.7. Density gradient ultracentrifugation of lipoproteins containing apoB fusion proteins. Stably transfected cells (identified to the left of each panel) were incubated for 16–18 h in medium containing 20% FBS without (A) or with (B) oleic acid (OA; 0.4 mM). The conditioned medium was fractionated by ultracentrifugation in a sucrose density gradient. Twelve fractions were collected, and apoB proteins in each fraction were resolved by SDS-PAGE (5% gel) and visualized by Western blot analysis using mixed monoclonal antibodies (1D1 and LRB220). The location of each apoB protein is indicated to the right of each panel. VLDL density corresponds to fractions 1 and 2, and HDL density corresponds to fractions 9–12.

VLDL whether or not exogenous oleate was added. Thus, short segments from the β1 domain (apoB-34–37 and apoB-37–42) or L-FABP were not sufficient to initiate VLDL assembly, whereas the addition of 8% of apoB sequence from the β1 domain (apoB-34–42) to apoB-29 resulted in a protein that was capable of VLDL assembly.

3.2.4 Pulse-chase Analysis of ApoB Fusion Proteins

The low abundance of apoB-29/B-34–42 and apoB-29/B-37–42 in the conditioned medium **(figure 3.7)** suggested that these fusion proteins may be more susceptible to intracellular degradation than the other fusion proteins. To determine the relationship between posttranslational stability and the β1 domain sequences, pulse-chase analyses were performed on each fusion protein and on the apoB-29 reporter **(figure 3.8).** As shown previously, apoB-29 was efficiently secreted, with >50% recovered in the medium and <30% degraded at the end of a 4 h chase. Thus, apoB-29 may not contain structural elements that mediate rapid posttranslational degradation, even though the protein does contain some β1 domain sequences (between the carboxyl termini of apoB-22 and apoB-29; **(figure 3.6A)**. The fusion proteins apoB-29/B-34–37 and apoB-29/FABP were also quite efficiently secreted (40–50% of the initial radiolabel), although these two proteins were less stable than apoB-29 (up to 50% degraded) at the end of a 4 h chase. In sharp contrast, almost none of the radiolabeled apoB-29/B-37–42 fusion protein was secreted, and essentially all of the nascent chains were degraded. Similar results were obtained for apoB-29/B-34–42, indicating that apoB-29/B-34–42 was also highly susceptible to degradation. These results suggested that some segments from the β1 domain could alter the secretion and stability characteristics of the apoB-29 reporter

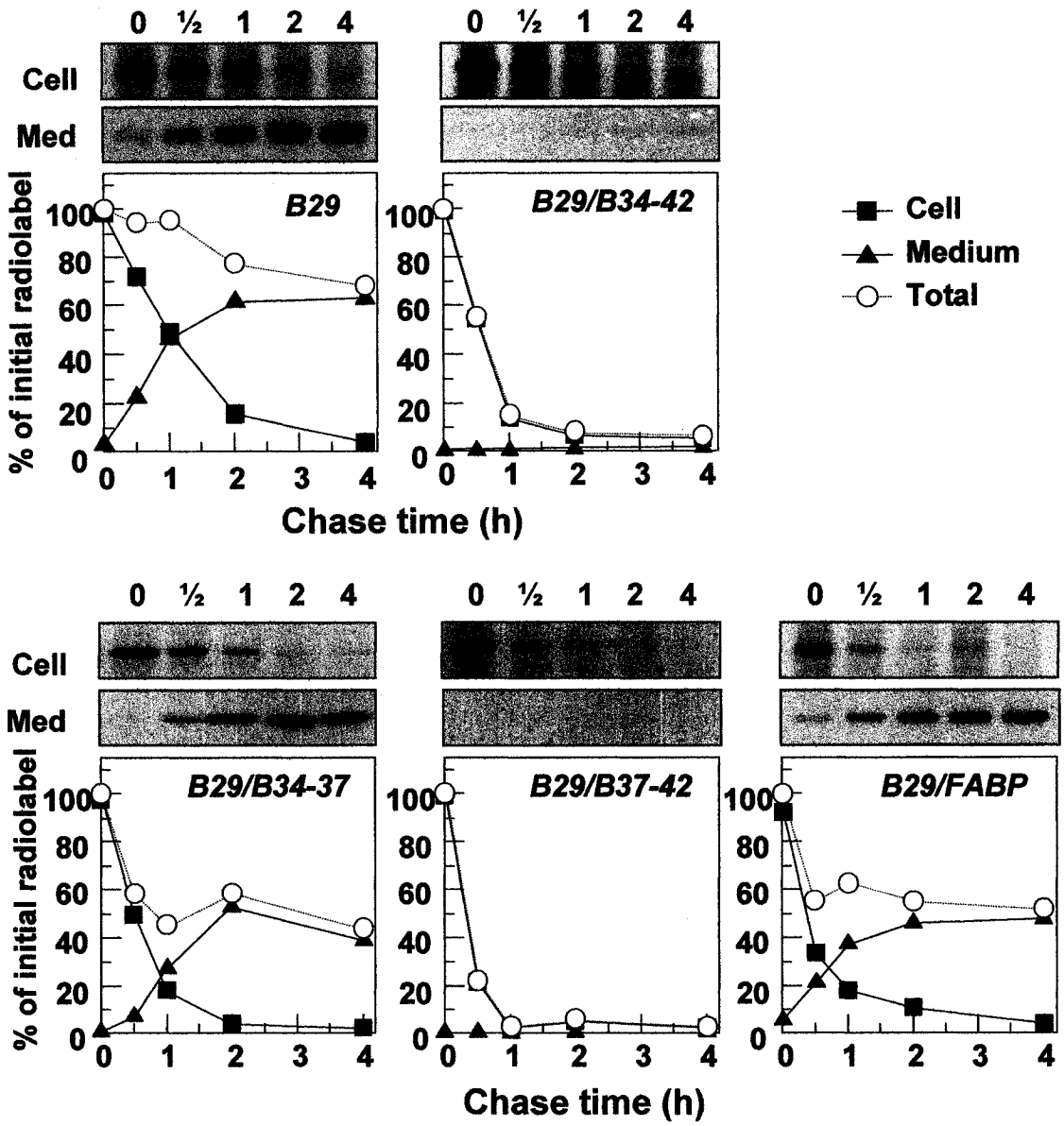

Figure 3.8. Pulse-chase analysis of apoB fusion proteins. Stable cell lines were labeled for 1 h with [^{35}S]methionine/cysteine and chased for up to 4 h in DMEM containing 20% FBS, 2 mM methionine, and 0.6 mM cysteine. The apoB fusion proteins in the medium (Med) and cells were analyzed by SDS-PAGE and autoradiography, and the radioactivity was semiquantified by scanning densitometry. Data are expressed as percentages of the initial radiolabel that was recovered from the cell (closed squares), medium (closed triangles), or cell plus medium (open circles; total). Autoradiographs of cell (top panels) and medium (bottom panels) apoB fusion proteins are shown above each graph.

and that these changes are not associated with the liver FABP or the apoB-34–37 segment. Furthermore, the most remarkable decreases in stability and secretion were observed in proteins containing the apoB-37–42 segment.

3.2.5 Proteasome Inhibition Affects the Degradation of ApoB Proteins

The involvement of the proteasome in apoB protein degradation was analyzed using the inhibitors Acetyl-Leucyl-Leucyl-Norleucinal (ALLN) at 100 μM or MG132 (25 μM). Treatment with ALLN enhanced the stability of endogenous apoB-100 in transfected cell lines by protecting the newly synthesized apoB-100 from intracellular degradation **(figure 3.9A)**. The total amount of radiolabeled apoB-100 recovered from culture at the end of a 1 h chase increased from ~50% to 70–80% of initial radioactivity. Secretion of apoB-100 was only modestly increased by the proteasome inhibition (from 11% secreted in the control to 16% secreted with ALLN) **(figure 3.9A)**. The stability of apoB-42 **(figure 3.9B)** was enhanced by proteasome inhibition in a similar manner to apoB-100. The total apoB-42 radiolabel in culture at 1 h of chase increased from ~65% in the absence of inhibitors to 90% in the presence of ALLN **(figure 3.9B)**. Secretion of apoB-42 was increased by ALLN (from ~15% to ~25–30%), while the stability of apoB-29 was only marginally affected by ALLN or MG132 (from ~85% to 95–100%) **(figure 3.9C and figure 3.11C)**. These observations indicate that apoB-42 and apoB-100 show similar diminished stability and limited secretion resulting from proteasomal degradation, whereas apoB-29 is minimally degraded by the proteasome.

The characteristics of degradation of the apoB-29 fusion proteins were then examined using the proteasome inhibitor ALLN. In the case of apoB-29/B-34–42,

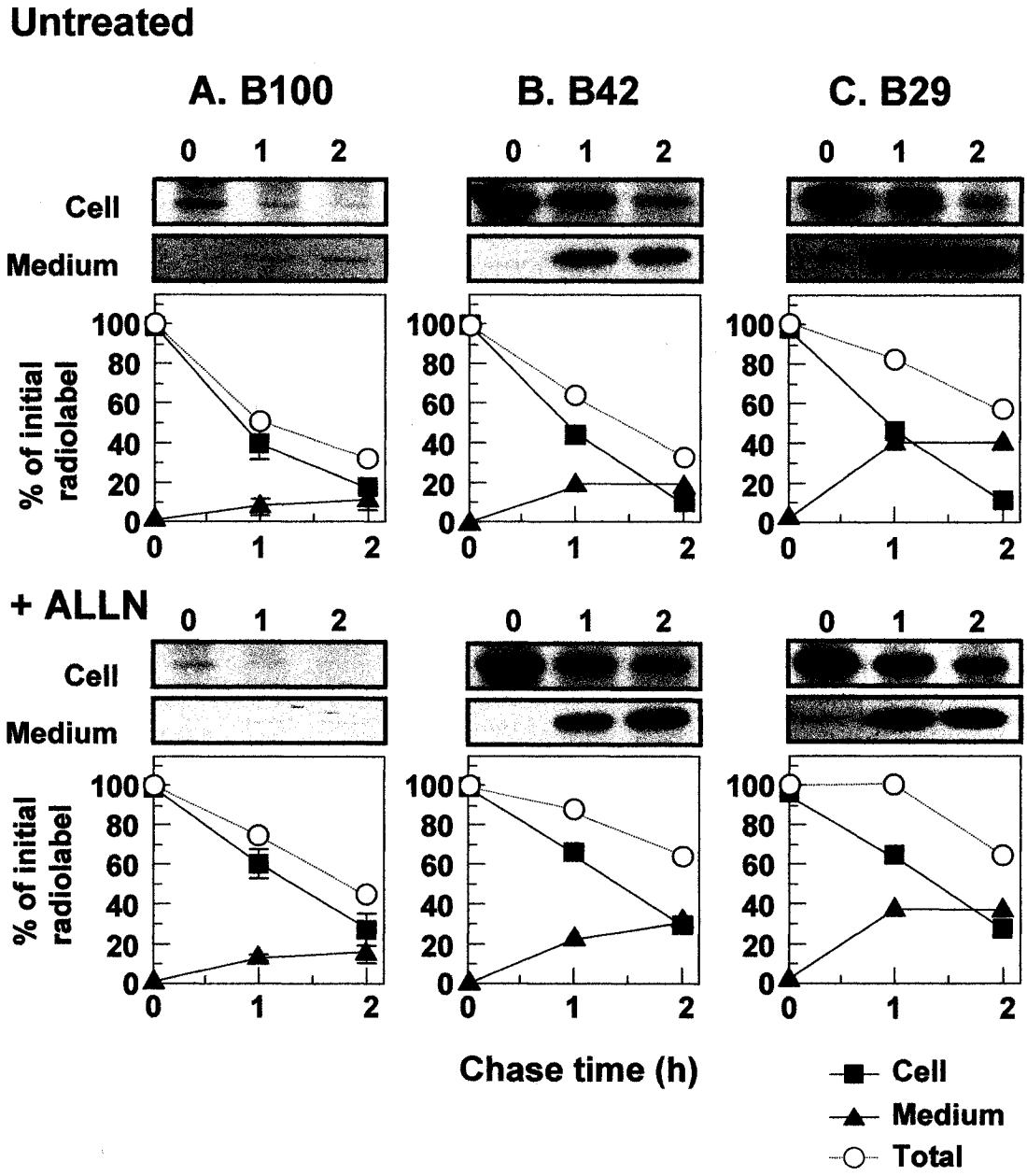

Figure 3.9. Effect of proteasome inhibition on the secretion and stability of truncated apoB proteins. Cells expressing apoB-29 (C) or apoB-42 (B) were incubated for 1 h with 100 μM ALLN or vehicle control, pulse-labeled for 1 h with [^{35}S]methionine/cysteine, and chased for 0, 1, or 2 h under the same conditions. Analysis of apoB proteins was performed as described in the legend to Figure 3.8. Error bars in A represent the range of endogenous apoB-100 values in two cell lines.

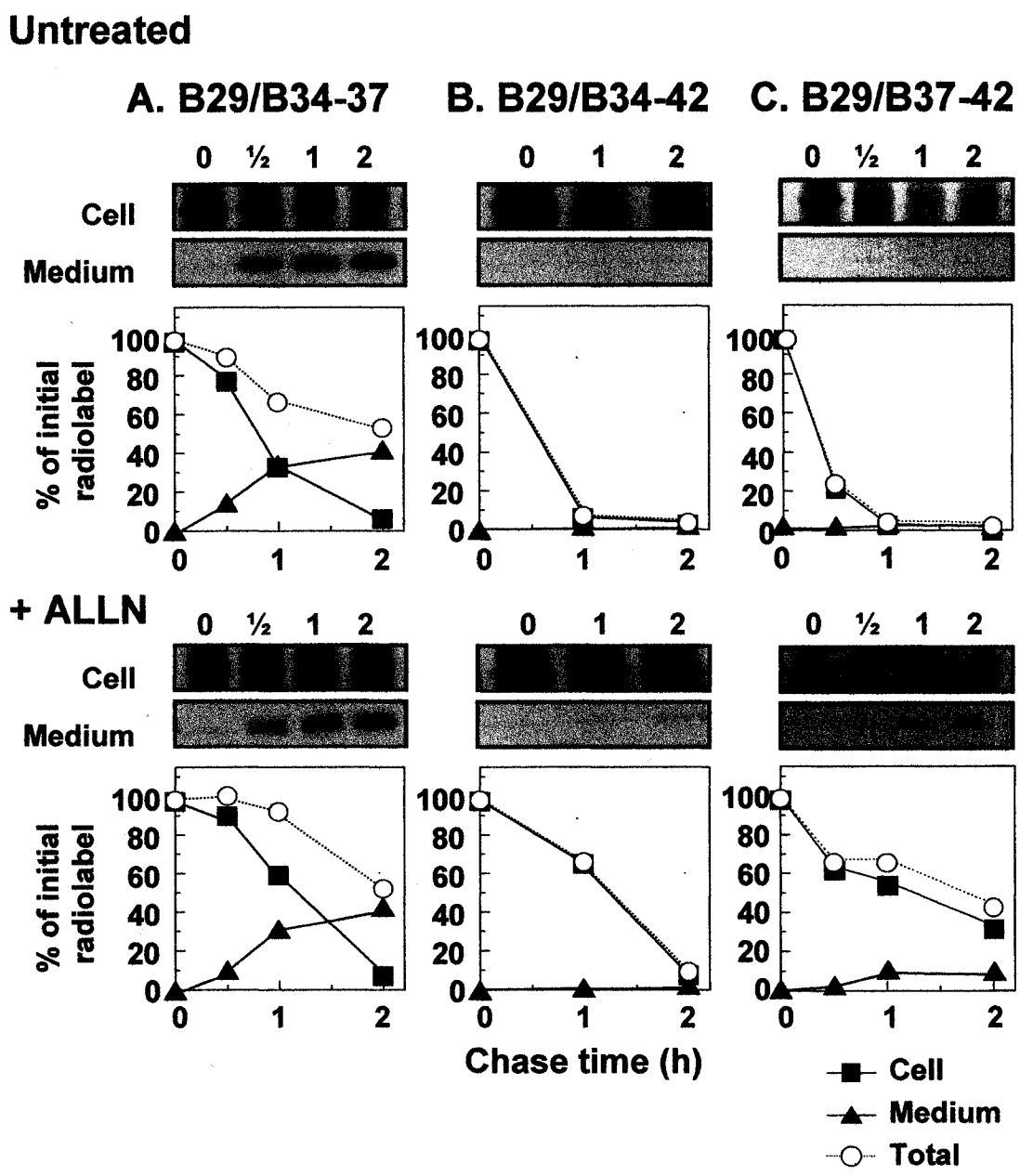

Figure 3.10. Effect of proteasome inhibition on the secretion and stability of apoB-29 fusion proteins. Cells expressing apoB-29/34-37 (A), apoB-29/34–42 (B) or apoB-29/37–42 (C) were incubated for 1 h with 100 μM ALLN or vehicle control, pulse-labeled for 1 h with [^{35}S]methionine/cysteine, and chased for 0, 0.5, 1, or 2 h under the same conditions. Analysis of apoB proteins was performed as described in the legend to Figure 3.8.

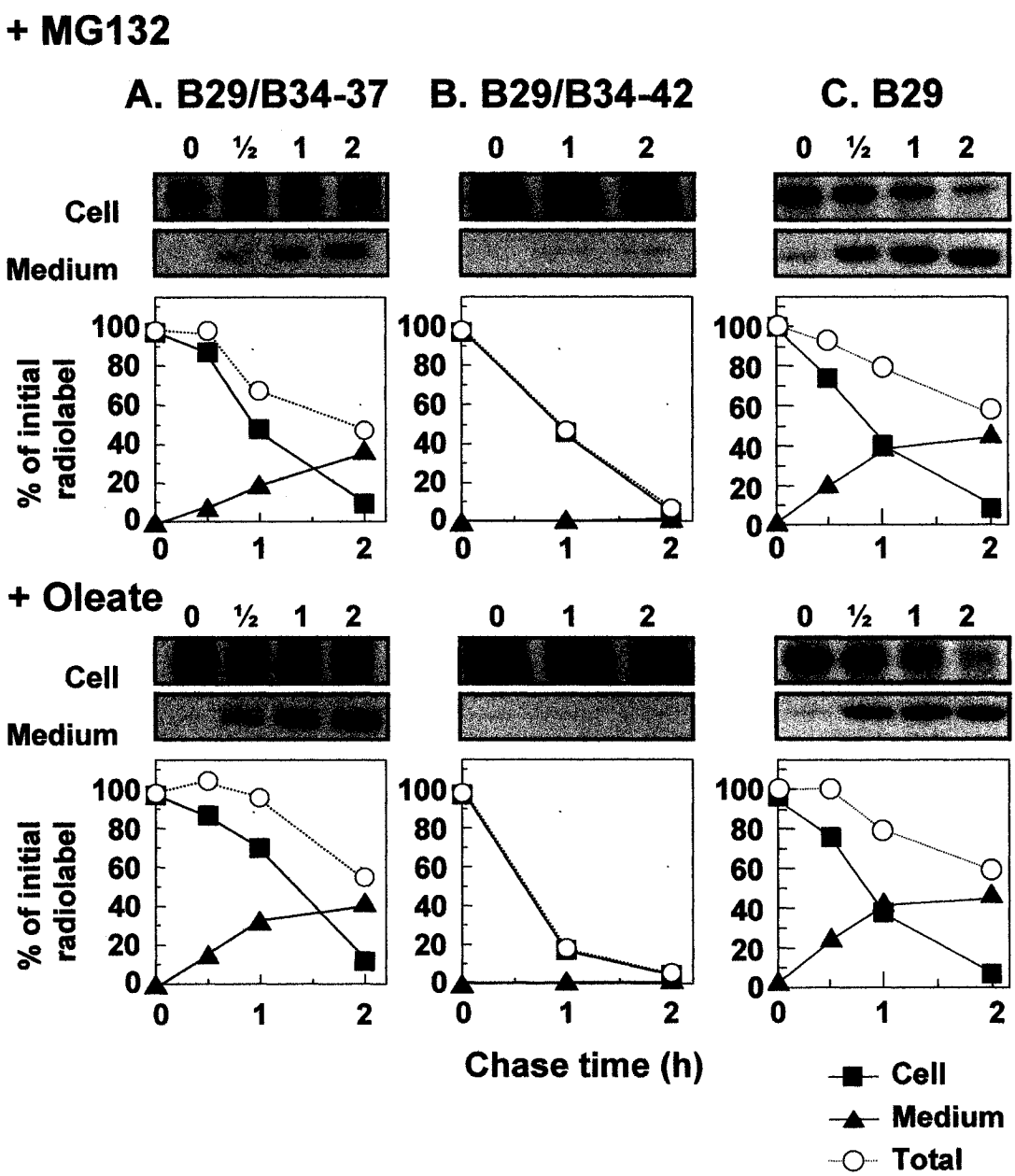

Figure 3.11. Effect of oleate and MG132 on the secretion and stability of apoB29 and apoB-29 fusion proteins. Cells expressing apoB29/B34-37 (A), apoB29/B34-42 (B) or apoB29 (C) were incubated for 1 h with 25 μM MG132 or 0.4 mM oleic acid, pulse-labeled for 1 h with [^{35}S]methionine/cysteine, and chased for 0, 0.5, 1, or 2 h under the same conditions. Analysis of apoB proteins was performed as described in the legend to Figure 3.8.

treatment with ALLN markedly increased apoB-29/B-34–42 stability. Treatment with ALLN markedly increased the recovery of apoB-29/B-34–42 from <10% in untreated cells to 65% in ALLN- treated cells at 1 h of chase **(figure 3.10B).** However, the enhanced stability was not accompanied by increased secretion. Similarly, apoB-29/B-37–42 stability at 1 h of chase increased from <10% to 55% in the presence of ALLN **(figure 3.10C)** accompanied by a noticeable increase in secretion (~10%). In contrast, ALLN did not appreciably affect the stability of apoB-29/B-34–37 at 1 h of chase (from ~70% to ~80%) **(figure 3.10A).** MG132 treatment of cells gave results that were similar to those obtained using ALLN **(figure 3.11A).** Interestingly, the incubation of transfected cells with an exogenous source of oleic acid did not significantly affect the stability and secretion of apoB29 fusion proteins **(figures 3.11A, 3.11B and 3.12).** Notably, the addition of FABP to B29, which does not affect protein stability, was not substantially affected by the addition of proteasome inhibitors or oleic acid at 1 h of chase (75-90%) **(figure 3.12).** Thus, the different regions of the apoB β1 domain have different effects on apoB stability, and instability is not induced by fusion of the β-sheet protein FABP to apoB-29.

3.2.6 ApoB-34–42 increases the microsomal membrane association of apoB-29

The presence of sequences in the β1 domain from beyond apoB-29 (particularly apoB-39–42) was hypothesized to lead to an increase in the association of the protein with the membranes of the secretory pathway and thereby increase exposure to the cytosol. Therefore, the distribution of apoB-29, apoB-29/B-34–42, and apoB-42 was analyzed by using sodium carbonate to separate the membrane-associated from soluble

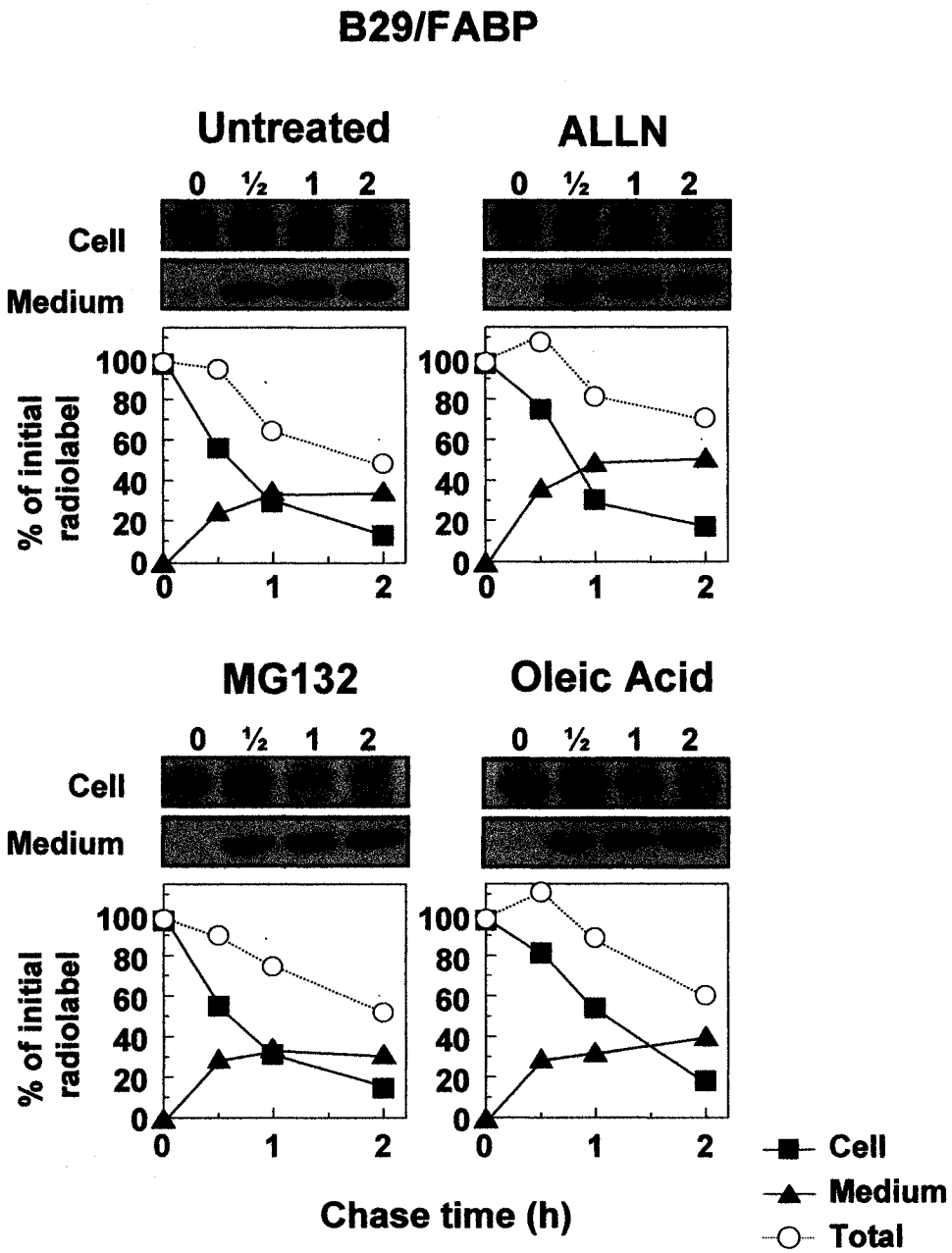

Figure 3.12. Effect of ALLN, MG132 and oleic acid on the secretion and stability of apoB-29/FABP. Cells expressing apoB-29/FABP were incubated for 1 h with 100 μM ALLN, 25 μM MG132, 0.4mM oleic acid or vehicle control, pulse-labeled for 1 h with [^{35}S]methionine/cysteine, and chased for 0, 0.5, 1, or 2 h under the same conditions. Analysis of apoB proteins was performed as described in the legend to Figure 3.8.

content of the post-nuclear supernatant from cells treated with ALLN (to inhibit proteolytic degradation) and brefeldin A (to block ER exit). Cells were pulse-labeled for 1 hour and microsomal membrane and lumenal content fractions were prepared from a mixture of postnuclear supernatants from all three cell lines. The apoB proteins were then purified by immunoprecipitation and quantified by liquid scintillation counting of the gel bands. The apoB-100 was primarily (88%) associated with the membrane fraction **(figure 3.13)**. In contrast, apoB-29 was found primarily in the lumenal content fraction (35% membrane-associated), and apoB-42 and apoB-29/B-34–42 were equally distributed between the lumen and the membrane (54% and 49% membrane-associated, respectively). This suggested that the presence of the segment of apoB beyond apoB-29 affects the distribution of the apoB proteins and that the apoB-29/B-34–42 fusion protein behaves in a similar manner to the truncated apoB-42. The increased association of the apoB proteins with the membrane may indicate that the proteins were arrested at translocation and exposed to the cytosol.

3.2.7 ApoB-34–42 Increases the Cytosolic Exposure of ApoB proteins

To examine the topology of the membrane-associated apoB proteins, the trypsin sensitivity of apoB-29, apoB-42, and apoB-29/B-34–42 in digitonin-permeabilized McA-RH7777 cell lines was analyzed. Digitonin selectively perforates cholesterol-rich regions of the membrane bilayer allowing the escape of proteins contained within the membrane barrier. The plasma membrane is cholesterol-rich and therefore treatment with digitonin causes the components of the cytosol to be released. However, since the ER membrane is cholesterol-poor, the lumenal content of this organelle is not released

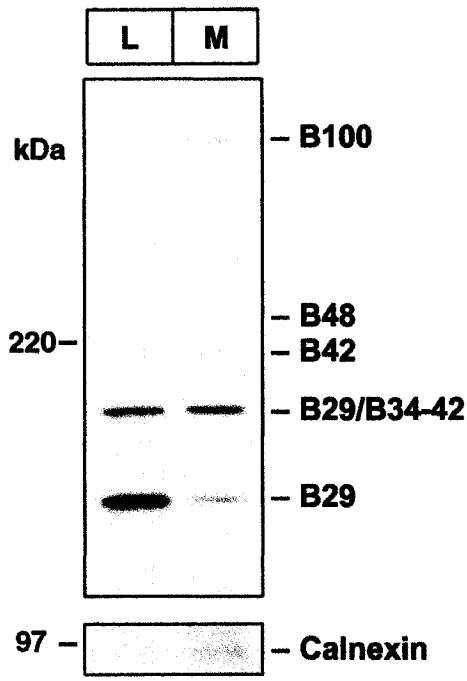

Figure 3.13. Analysis of the distribution of nascent apoB proteins in membrane and lumenal fractions of microsomes. Cells expressing apoB-29, apoB-29/B-34–42, or apoB-42 were pretreated for 1 h with ALLN (100 μM) and brefeldin A (5 μg/ml) and pulse-labeled for 1 h with [^{35}S]methionine/cysteine in the presence of ALLN and brefeldin A. Pooled postnuclear supernatants from cell homogenates were treated with 100 mM sodium carbonate, and the resulting lumenal content (L) and membrane (M) fractions were separated by ultracentrifugation. Lumen and membrane fractions were solubilized separately in 1% SDS, and apoB proteins were precipitated with a polyclonal antibody and visualized by SDS-PAGE with autoradiography. Immunoprecipitation supernatant were reprecipitated with a polyclonal antibody for calnexin and proteins were visualized by SDS-PAGE.

with digitonin. As previously reported (Cavallo *et al.*, 1998), apoB-29 was largely protected from exogenous protease (89 ± 3% protected; mean ± SD, n = 3) **(figure 3.14, bottom panels)**, suggesting that apoB-29 is efficiently translocated (Macri and Adeli, 1997b). In contrast, both apoB-42 and apoB-29/B-34–42 **(figure 3.14, middle panels)** were more sensitive to the added trypsin, as only 41 ± 11% and 53 ± 10%, respectively, of these proteins was protected. ApoB-100 was the least efficiently translocated, as only 15 ± 13% was protected from protease digestion **(figure 3.14, top panels)**. In a separate experiment, the translocation status of each of the model proteins was also examined in isolated microsomes **(figure 3.15)**. Under the conditions of this analysis, the lumenal PDI was protected from protease degradation but the cytosolic epitope of calnexin was completely degraded (lower panels). The protection of apoB proteins from added protease was essentially identical to that in the permeabilized cell system, as 88 ± 11% of apoB-29 (mean ± SD, n = 5), 68 ± 9% of apoB-42, and 65 ± 16% of apoB-29/B-34–42 were protected from trypsin, as assessed by immunoblot analysis. Consistent with previous observations of the translocation arrest of larger apoB proteins, apoB-100 was no longer detectable in microsomes after trypsin treatment. The exposure of apoB to the cytosol suggested that the polypeptide may be susceptible to polyubiquitination.

3.2.8 ApoB-34–42 Increases the Polyubiquitination of ApoB proteins

To determine the effect of the segment of B34-42 on apoB ubiquitination, McA-RH7777 cells transfected with apoB-29 or apoB29/B34-42 were incubated for 1 hour with ALLN and/or Brefeldin A and then homogenized. The post-nuclear supernatant of cell homogenates was treated with sodium carbonate (100 μM) and subjected to

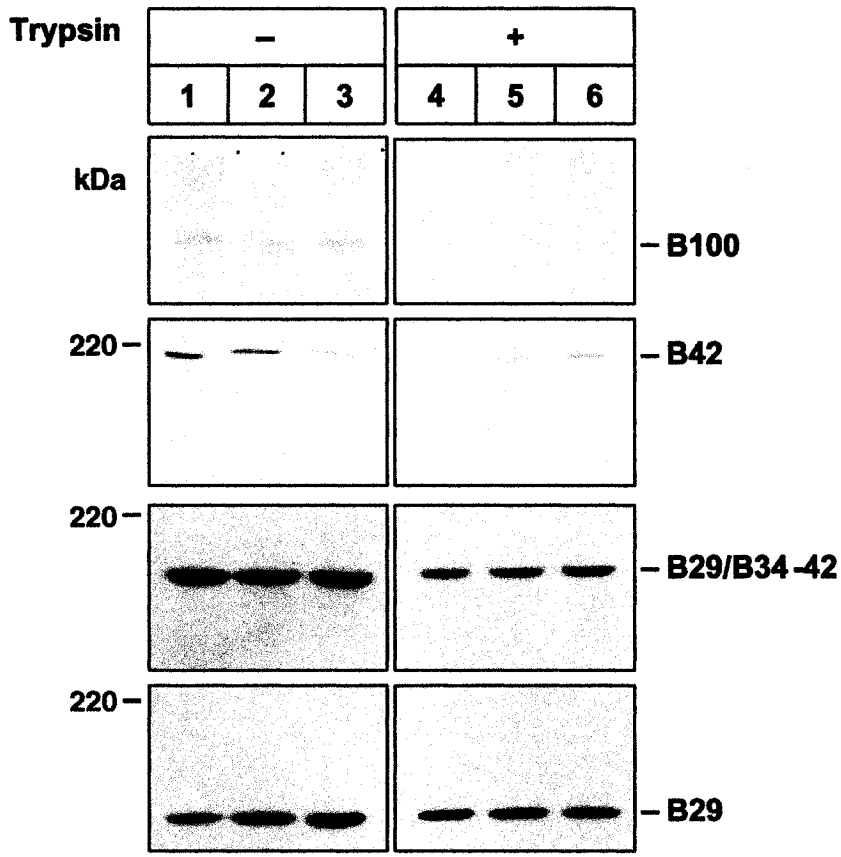

Figure 3.14. Analysis of the topology of nascent apoB proteins Transfected McA-RH7777 cells were labeled for 30 min with [^{35}S]methionine/cysteine and chased with unlabeled amino acids for 15 min in the presence of ALLN and brefeldin A. The cells were then permeabilized with digitonin (75 μg/ml) and incubated in the presence (lanes 4–6) or absence (lanes 1–3) of trypsin (100 μg/ml). After 30 min on ice, the trypsin was inactivated with soybean trypsin inhibitor (500 μg/ml) and the cells were lysed with 1% SDS. The apoB proteins were isolated by immunoprecipitation and visualized by SDS-PAGE with fluorography. Each analysis was performed in triplicate dishes for each cell line.

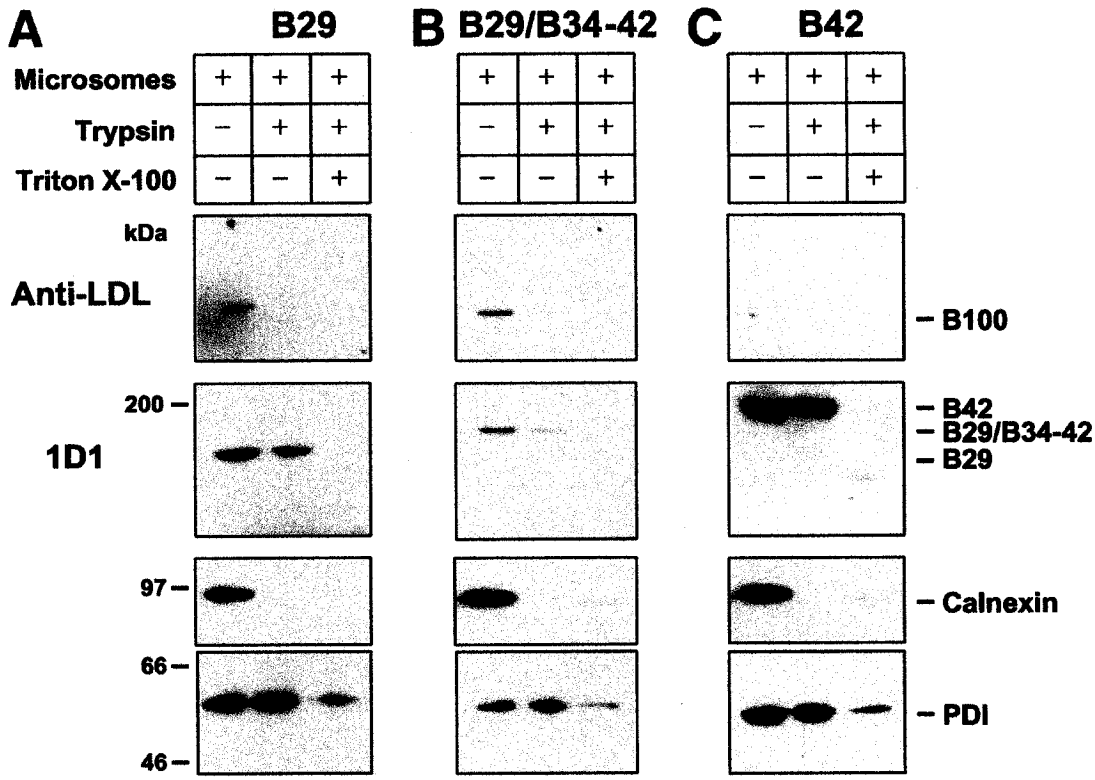

Figure 3.15. Immunoblot analysis of the topology of apoB proteins in isolated microsomes. Microsomes were prepared from ALLN- and brefeldin A-treated B29 (A), B29/B34–42 (B), or B42 (C) transfected cells, as described in Experimental Procedures. Aliquots of each microsome preparation were incubated for 30 min on ice in the presence or absence of trypsin (100 μg/ml) and Triton X-100 (1%, w/v) as indicated. Trypsin was inactivated by the addition of soybean trypsin inhibitor (500 μg/ml) and 2% SDS. Proteins were resolved by 3–15% gradient SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. The protection of individual proteins was revealed by immunoblotting for protein disulfide isomerase (PDI), calnexin, human apoB (1D1), or rat apoB (anti-LDL). The migration of molecular mass markers is shown to the left of each panel (kDa), and the protein decorated by each antibody is indicated to the right. Protease protection was quantified by scanning densitometry of representative fluorographs.

ultracentrifugation and microsomal membranes were separated from cytosolic and lumenal content (soluble fraction). ApoB proteins were then immunoprecipitated and immunoblotted. ALLN treatment did not increase the level of apoB-29 in transfected cells and these cells did not accumulate ubiquitinated apoB proteins **(figure 3.16A, lane 1, 2, 5, 6)**. Intracellular levels of apoB-29 could be increased by blocking secretion with Brefeldin A **(figure 3.16A, lane 3, 4, 7, 8)**. On the other hand, treatment of cells transfected with apoB29/B34-42 with ALLN markedly increased the levels of both apoB-29/B34-42 and ubiquitinated apoB proteins. The increase of apoB29/B34-42 in the soluble fraction suggested that ALLN may cause an accumulation of ubiquitinated apoB-29/B34-42 in the cytosol (soluble fraction) **(figure 3.16B, lane 2 and 4)**. The presence of cytosolic ubiquitinated apoB-29/B34-42 suggests that a mechanism of retro-translocation may extract apoB-29/B34-42 from the ER. Notably, Brefeldin A also increased specifically the level of membrane associated apoB29/B34-42, indicating that apoB29/B34-42 preferably remains associated with the membrane of the ER **(figure 3.16B, lane 7)**. Brefeldin A appeared to decrease the polyubiquitination of apoB29/B34-42 associated with the membrane, but this observation was not consistently reproducible. Also, a very small amount of PDI could be detectable in some membrane fractions suggesting that sodium carbonate extraction of lumenal protein was almost complete. Overall, these observations suggest that the enhancement in membrane association and cytosolic exposure by the fusion of apoB-34–42 to apoB-29 correlates with an increase in the susceptibility of the apoB protein to proteasomal degradation.

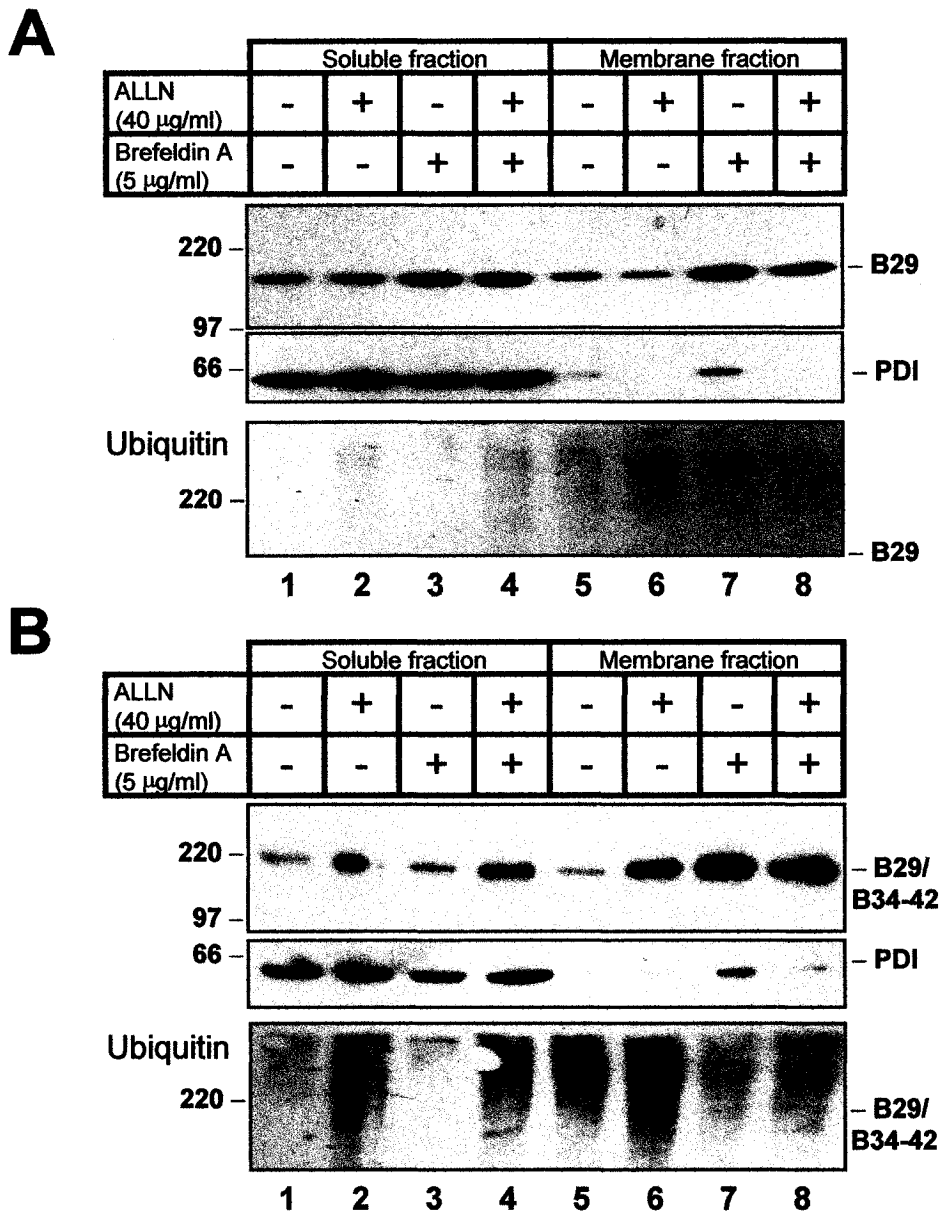

Figure 3.16. Ubiquitination analysis of apoB-29 apoB-29/B34-42. McA-RH7777 cells transfected with apoB-29 (A) or apoB29/B34-42 (B) were incubated with 100 µM ALLN or/and 5 µg/ml of Brefeldin A for 1 hour. Cells were homogenized and post-nuclear supernatants were prepared, incubated with 100 mM sodium carbonate and ultracentrifuged to result in a microsomal membrane and a soluble (cytosol + microsome lumen) fraction. ApoB proteins from each fraction were immunoprecipitated with polyclonal antibodies for apoB. Proteins were resolved by SDS-PAGE, transferred on polyvinylidene difluoride membranes and visualized by immunoblotting with antibodies for human apoB (1D1) and ubiquitin. PDI was used as control for the microsome preparation.

CHAPTER 4

THE ROLE OF THE AAA-ATPASE p97 IN THE RETRO-TRANSLOCATION AND PROTEASOMAL DEGRADATION OF APOLIPOPROTEIN B

4.1 Introduction

ERAD chaperones and factors such as Hsp70, Hsp90, Grp78, Sec61 and E3 ubiquitin ligase gp78 have been suggested to play a role in apoB-100 degradation [reviewed in (Fisher and Ginsberg, 2002)]. These proteins are involved in the processes of recognition and targeting the protein for retro-translocation and degradation. The direct role of the AAA-ATPase p97 in apoB-100 retro-translocation to the cytosol is unknown but intracellular apoB-100 has been shown to be polyubiquitinated suggesting that apoB may be recognized by p97 (Yeung *et al.*, 1996; Fisher *et al.*, 1997). As shown with model truncated and fusion proteins in Chapter 3, polyubiquitination of apoB arises from translocation arrest and cytosolic exposure of the polypeptide. To corroborate these findings, full length apoB-100 was analyzed and the potential role of p97 in retro-translocation was determined. This chapter encompasses different approaches to characterize the retro-translocation process in apoB-100 degradation and to evaluate the role of the AAA-ATPase p97 in apoB-100 proteasomal degradation.

4.2 Results

4.2.1 Characterization of ApoB Metabolism and ERAD Factors in HepG2 cells

HepG2 cells inefficiently assemble LpB causing a substantial amount of apoB to be degraded early in its biosynthesis. Early apoB degradation via the proteasome predominates in these cells making HepG2 a useful model to study the mechanism of the

ERAD of apoB. Initial experiments with HepG2 cells indicated that this cell line was much more sensitive than McA-RH7777 cells to plating density. When HepG2 were plated at low cell density, they tended to form isolated colonies on the culture dish and never reached a confluent monolayer. In contrast, if HepG2 were plated at high cell density, nearly confluent monolayers were obtained within 1 to 2 days in culture. In order to ensure that differences in plating density did not have an influence on apoB metabolism, a pulse-chase analysis was performed and is shown in **figure 4.1**. Cells were radiolabeled at 20, 50 and 80% confluence and stability and secretion of apoB-100 was measured as described in Chapter 3. Despite large differences in the total number of cells per dish, there were no differences in the secretion efficiency or stability of apoB100 at the three different plating densities, expressed as % of initial radiolabel. As expected, the amount of radiolabel incorporated into apoB100 at time 0 of the chase increased with increasing initial cell confluence. Therefore, this analysis indicated that within the range of 20-80% confluence, there was no significant difference in the metabolism of apoB100. In all subsequent experiments, HepG2 cells were utilized at approximately 80% confluence, which gave substantial apoB100 radiolabeling and good reproducibility of monolayer culture. The latter became particularly important when performing digitonin permeabilization studies (**see section 4.2.2**).

Two important cytosolic protein factors in the ERAD system are heat shock protein 70 kDa (Hsp70) and p97. In order to analyse these proteins in HepG2 cells commercial antibody preparations were acquired and the antigens were characterized in HepG2 cell lysates by western blot analysis (**figure 4.2**). This allowed for the assessment of antibody specificity and for the determination of the amount of HepG2

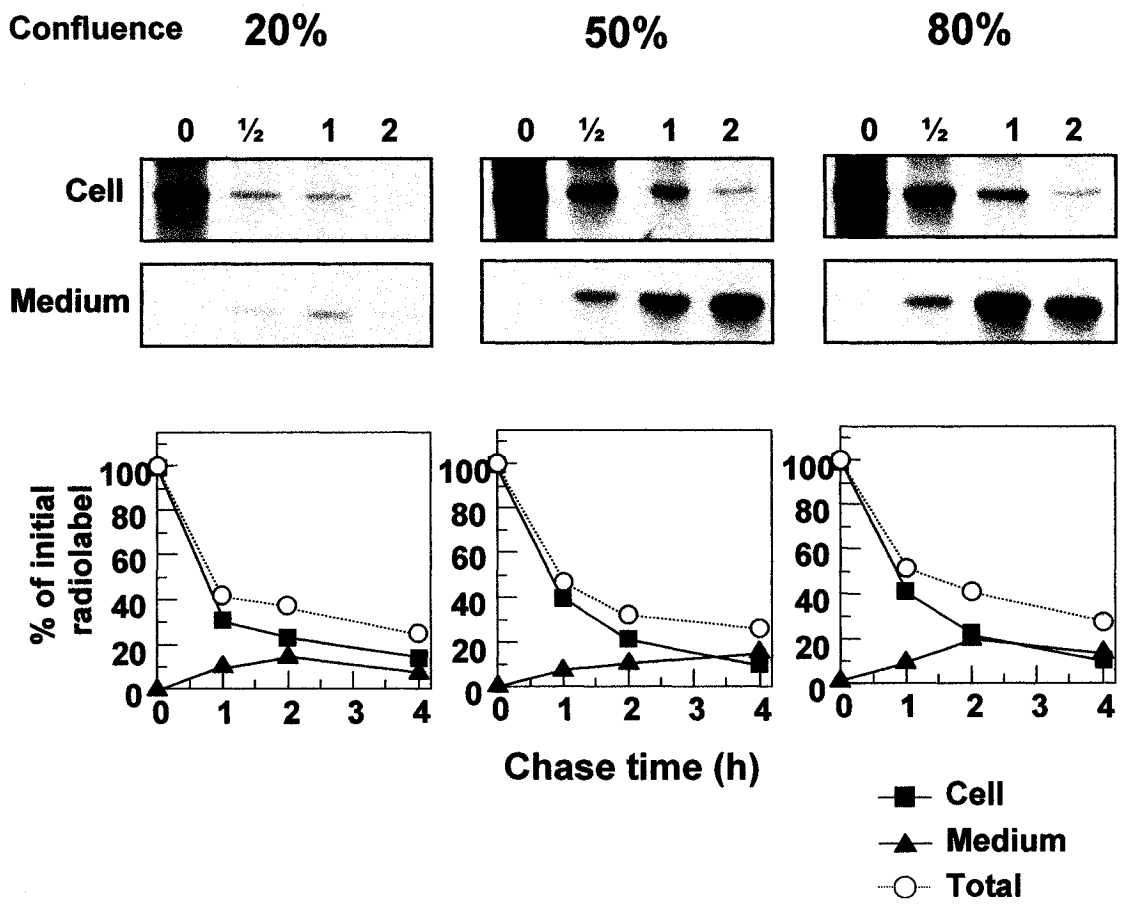

Figure 4.1. Effect of confluency on the stability and secretion of apoB-100 in HepG2 cells. HepG2 cells were plated at different densities and allowed to grow overnight. The following day, when the cells were 20, 50 or 80% confluent, the monolayers were labeled for 1 h with [^{35}S]methionine/cysteine and chased for 0, 1, 2 and 4 h. The apoB proteins were isolated by immunoprecipitation and visualized by SDS-PAGE with autoradiography. Secreted (open squares) and cellular (closed circles) were represented as percentage of initial radiolabel and total apoB (open circles) was calculated.

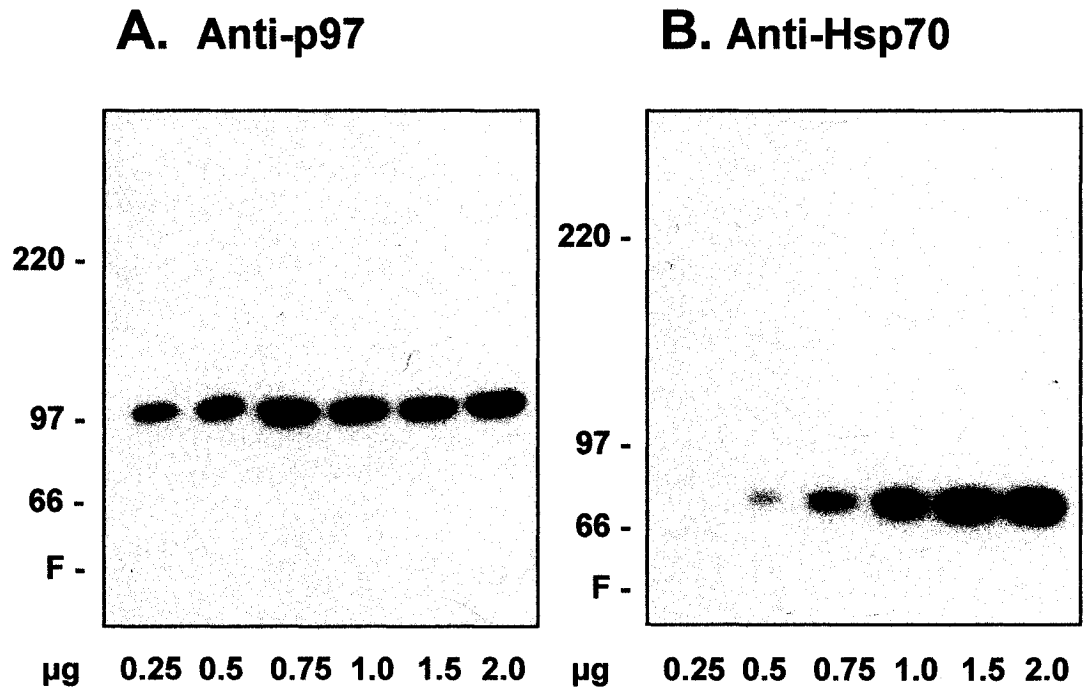

Figure 4.2. Characterization of antibodies against p97 and Hsp70 for immunoblotting in HepG2 cells. HepG2 cells were lysed with RIPA buffer and increments of cell protein (0.25-2.0 µg) were resolved on SDS-PAGE. Proteins were transferred on nitrocellulose membranes and blotted with monoclonal antibodies for p97 (A) and heat shock protein 70 (Hsp70) (B).

cell protein required for reliable detection of the individual proteins. Using 0.5 to 1.0 μg of total cell protein was sufficient for the detection of either Hsp70 or p97 from HepG2 cell lysate. This corresponded to approximately 0.25-0.5% of cell lysate from an 80% confluent 35 mm dish **(figure 4.2)**. Thus, Hsp70 and p97, both abundant cellular proteins, were detectable at low levels of total cell protein. No other bands were detectable, and in particular, no bands were detected in the region of apoB100. Thus, the efficiency of detection of these antibodies and the abundance of the two proteins allowed for the use of only a small portion of cell lysates for analysis.

4.2.2 Digitonin Permeabilisation of HepG2 cells

To study ERAD and examine the retro-translocation of apoB, a method to separate cytosolic from organelle-associated components was required. Digitonin, a non-ionic detergent was utilized to permeabilise HepG2 cells **(see section 3.2.7)**. Digitonin permeabilisation can be used to separate proteins that are in the cytosol from those that are in the ER membrane or lumen. To ensure the integrity of the ER during HepG2 permeabilisation, immunoblotting of the ER-resident transmembrane protein calnexin and ER lumenal protein disulfide isomerase (PDI) was performed **(figure 4.3)**. Calnexin and PDI were found exclusively in the permeabilised cell fraction for concentrations of digitonin up to 75 μg/ml **(figure 4.3, lane 3, 5 and 7)**, indicating that the ER remains intact at this level of digitonin. Concentrations of digitonin higher than 75 μg/ml, however, resulted in the escape of ER lumenal PDI into the cytosolic fraction **(figure 4.3, lane 10 and 12)**. Therefore, subsequent permeabilisations of HepG2 cells were

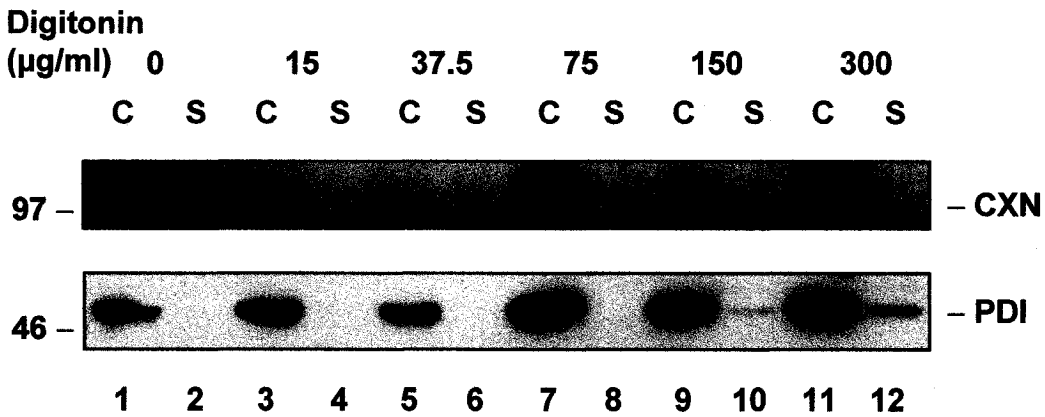

Figure 4.3. Effect of digitonin concentration on the integrity of the ER membrane. Confluent HepG2 cells were incubated in CSK containing different increments of digitonin (0-300 µg/ml) for 10 minutes on ice. Permeabilised cells (C) and cytosols (S) were collected, proteins were resolved by SDS-PAGE, transferred on nitrocellulose and visualized by immunoblotting with antibodies for protein disulfide isomerase (PDI) and calnexin (CXN).

performed at concentration of 75 μg/ml to prevent leakage of ER lumenal material into the cytosolic fraction.

4.2.3 Ubiquitinated ApoB Accumulates in the Cytosol of MG132-treated HepG2 cells

As a first step towards the identification of components involved in retro-translocation of apoB-100, the possibility of dissociating proteasomal degradation from retro-translocation was investigated. To induce the accumulation of apoB in HepG2 cells to allow the characterization of retro-translocation, the proteolytic activity of the proteasome was inhibited with the proteasome inhibitor MG132. MG132 inhibits the chymotrypsin-like, trypsin-like and peptidylglutamyl-peptide hydrolyzing activities of the three β-subunits in the 20S proteolytic core of the proteasome. Thus, HepG2 cells were treated with or without MG132 and permeabilised with 75 μg/ml of digitonin. Ubiquitinated cytosolic and organelle-associated apoB-100 (*i.e.* that remaining with the monolayer following permeabilisation) were visualized by immunoblotting. A small amount of cytosolic apoB-100 could be detected in cells without MG132 treatment **(figure 4.4A, lane 7)** and treatment with MG132 caused the accumulation of cytosolic apoB-100 **(figure 4.4A, lane 8)**. Since only 10% of the total cell lysate and all of the cytosolic fraction was loaded on the gel, the amount of apoB-100 in the cytosolic fraction was estimated to range between 1-5% of the total cellular apoB **(figure 4.4A, lane 5-8)**. Both cytosolic and organelle-associated apoB were polyubiquitinated in MG132-treated cells **(figure 4.4B, lanes 8 and 6, respectively)** but the majority of the polyubiquitinated species were found in the cytosol. Control immunoprecipitations were

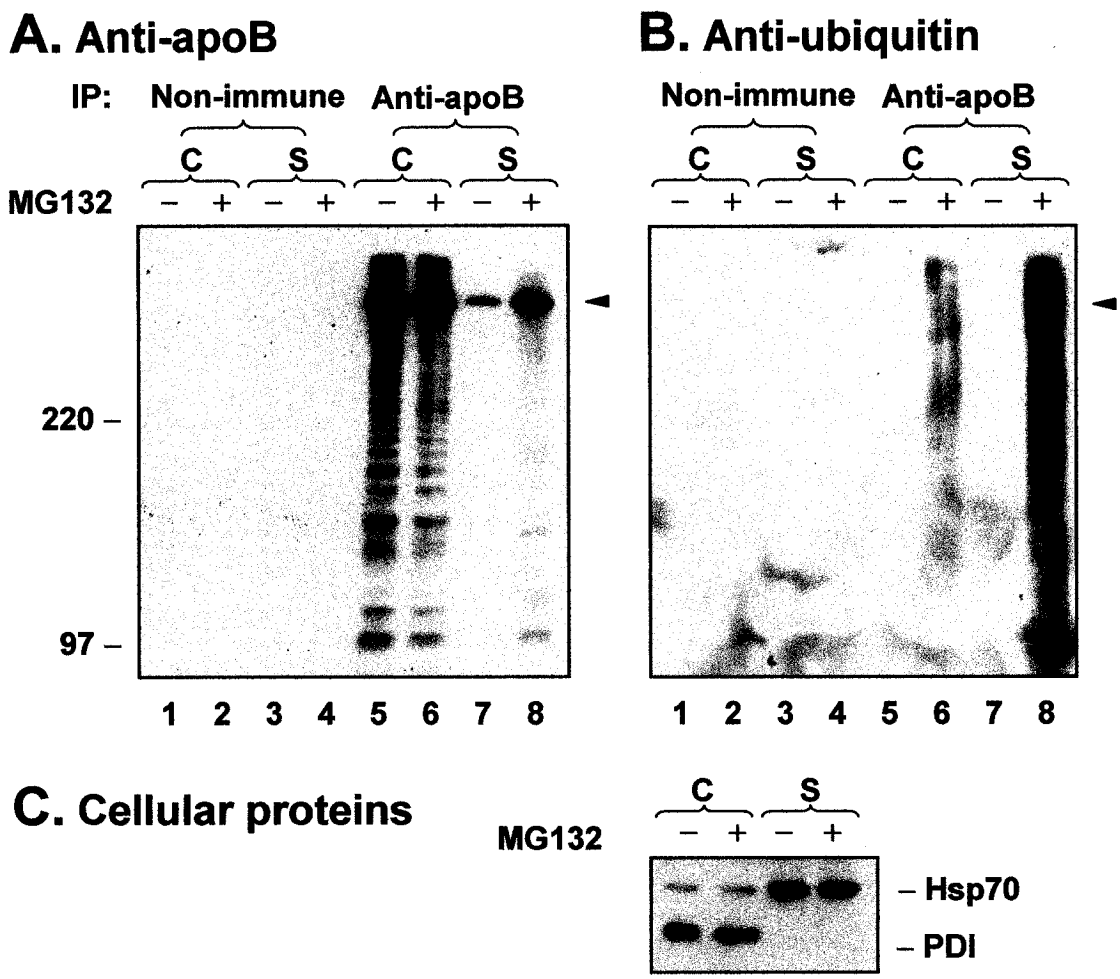

Figure 4.4. Ubiquitinated apoB-100 accumulates in the cytosol of cells treated with MG132. A, HepG2 cells were incubated with or without MG132 (25 μM) for 1 h and the monolayers were then permeabilized with 75 μg/ml of digitonin as described in figure 4.3. The resulting permeabilised cell (C) and supernatant (S, cytosol) fractions were collected and immunoprecipitates were prepared with either polyclonal anti-apoB or non-immune goat serum. Aliquots of each immunocomplex were resolved by 5% SDS-PAGE and, following transfer of the proteins to nitrocellulose, human apoB was revealed by Western blot analysis. Arrowhead indicates the mobility of full-length apoB-100. B, Aliquots of immunoprecipitates prepared as in A were visualized with monoclonal anti-ubiquitin antibody. C, Distribution of cytosolic heat shock protein 70 (Hsp70) and ER-resident PDI were visualized by immunoblotting.

used to rule out the possibility that the ubiquitin-reactive material could be due to non-specific precipitation of unrelated ubiquitinated proteins **(figure 4.4A and 4.4B, lanes 1 to 4)**. No ubiquitinated species were detected in the control precipitation. To ensure permeabilisation efficiency and ER membrane integrity, the distribution of cytosolic heat shock protein 70 kDa (Hsp70) and ER-resident PDI from cytosol and permeabilised cell fractions were analyzed by immunoblotting and showed no leakage and efficient permeabilisation **(figure 4.4C)**. The release of apoB proteins into the cytosol of MG132-treated cells suggests that polyubiquitinated apoB species can be found in the cytosol of MG132-treated cells and that proteasome proteolytic activity is not a requirement in apoB retro-translocation. A similar increase in cytosolic ubiquitinated apoB was also observed when cells were treated with ALLN **(figure 4.5A, lane 5 and figure 4.5B, lane 5)**. Smaller ubiquitinated fragments of apoB were observed in the cytosol **(figure 4.4A, lane 8, Figure 4.5A)** suggesting the presence of non-proteasomal proteolysis or perhaps the disengagement of partially degraded apoB proteins from inhibited proteasomes.

To further characterize ubiquitinated apoB proteins found in the cytosol, the cytosolic and secreted apoB-100 were analyzed by density gradient ultracentrifugation. While secreted apoB-100 was found primarily in the LDL fraction **(figure 4.6A, fractions 4 and 5)**, cytosolic apoB-100 was found in higher density fractions of the gradient **(figure 4.6A, lane 10, 11 and 12)** suggesting that these apoB proteins contained much less lipid than the secreted forms. Blotting for cytosolic Hsp70 and ER-resident PDI showed that cells were permeabilised with digitonin **(figure 4.6B)**. To

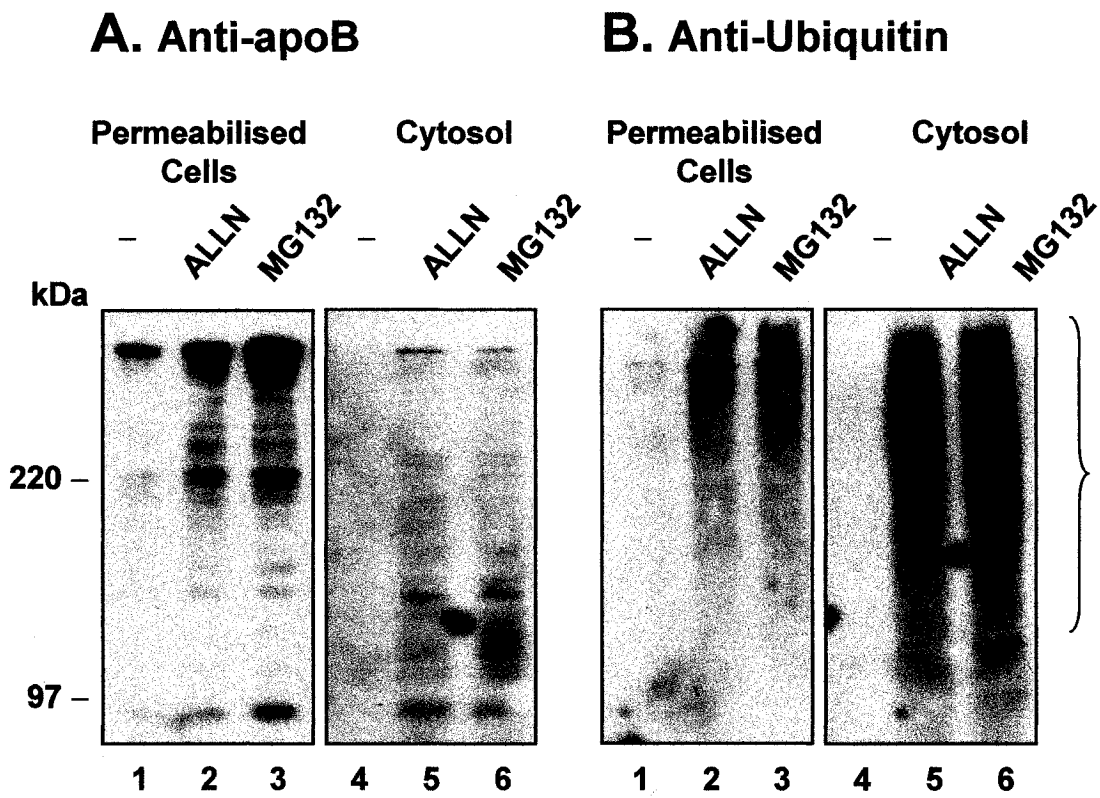

Figure 4.5. ApoB-100 accumulates in the cytosol of cells treated with ALLN or MG132. HepG2 cells were incubated for 1 h in the presence of 100 μM ALLN, 25 μM MG132 or vehicle and cells were permeabilised with 75 μg/ml digitonin. Cellular and cytosolic fractions were collected and analyzed as described in Figure 4.4.

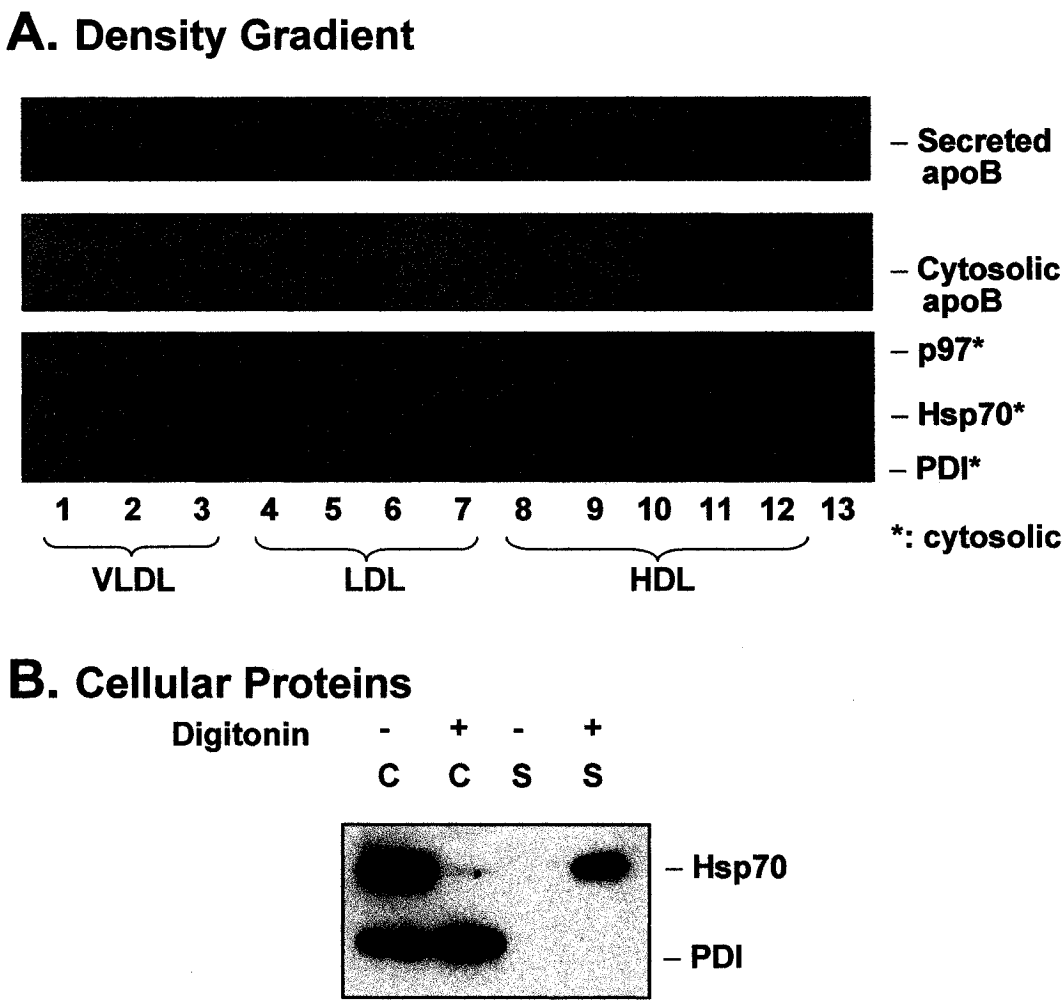

Figure 4.6. Cytosolic apoB proteins are associated with the very high density lipoprotein fraction HepG2 cells were incubated with 25 μM MG132 for 1 h. The medium was collected and cells were permeabilised with digitonin. Collected cytosol and medium were subjected to sucrose density gradient ultracentrifugation as described in Chapter 2. A. Gradients were fractionated and apoB100 in the individual fractions was visualized by immunoblotting. Cytosolic fractions were also analyzed for the presence of p97, heat shock protein 70 (Hsp70) and protein disulfide isomerase PDI. B. Cellular (C), medium or cytosol (S) proteins were resolved by SDS-PAGE, transferred on nitrocellulose and blotted for PDI and Hsp70.

understand the mechanism of the release of apoB-100 into the cytosol, potential ERAD factors involved in apoB retro-translocation were investigated.

4.2.4 Distribution of ERAD factors in HepG2 cells

To identify candidate proteins that may be involved in the delivery of apoB to the cytosol for degradation, the distribution of key ERAD factors (Hsp70, p97 and the proteasome) between the membranes and cytosol of HepG2 cells was examined. When HepG2 cells were permeabilised with digitonin, essentially all of the Hsp70 was recovered in the cytosol fraction **(figure 4.7, Hsp70)**, whereas ER-resident PDI and calnexin remained associated with the cell membranes **(figure 4.7, PDI and calnexin)**. Similarly, apoB-100 was found almost exclusively with the cell membrane fraction although, as described in section 4.2.3., a small amount of apoB-100 could be detected in the cytosol on longer exposures. In contrast, components of the 20S subunit of the proteasome **(figure 4.7, 20S)** and the AAA-ATPase p97 **(figure 4.7, p97)** were equally distributed in the cell and cytosol fractions. To characterize the interaction between p97 and the membrane, permeabilised cells were washed with 0.5 M KCl. This high salt wash was able to remove most of the p97 associated with organelles **(figure 4.8, lane 10)**, suggesting an electrostatic interaction of p97 with the membrane or with protein components of the membrane. Membrane-associated p97 could also be released from the membrane by incubating permeabilised cells in serum-free medium for 30 minutes **(figure 4.8, lane 8)**. This suggested that the association between p97 and the membranes may be reversible. Conversely, apoB was unaffected by high-salt and remained predominantly associated with the organelle fraction **(figure 4.8, lanes 9 and 11)**.

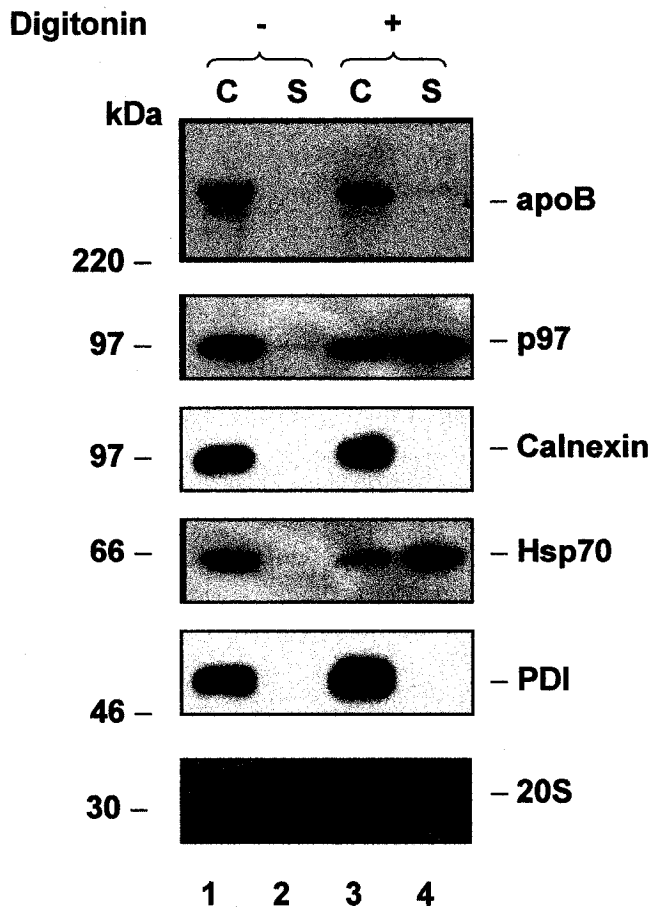

Figure 4.7. Distribution of cellular markers and ERAD factors in digitonin-permeabilised HepG2 cells. Western blot analysis of cell (C) and supernatant (S, cytosol) fractions of HepG2 cells following digitonin permeabilization. Cells were treated with or without digitonin and the cell (C) and supernatant (S) fractions were collected. Aliquots of cell and supernatant protein were resolved by 3-15% gradient SDS-PAGE and transferred to nitrocellulose. Membranes were incubated with the indicated antibody and visualized by enhanced chemiluminescence. AAA-ATPase p97 (p97); 20S proteasome β1 subunit (20S), cytosolic heat shock protein 70 (Hsp70); calnexin; protein disulfide isomerase (PDI).

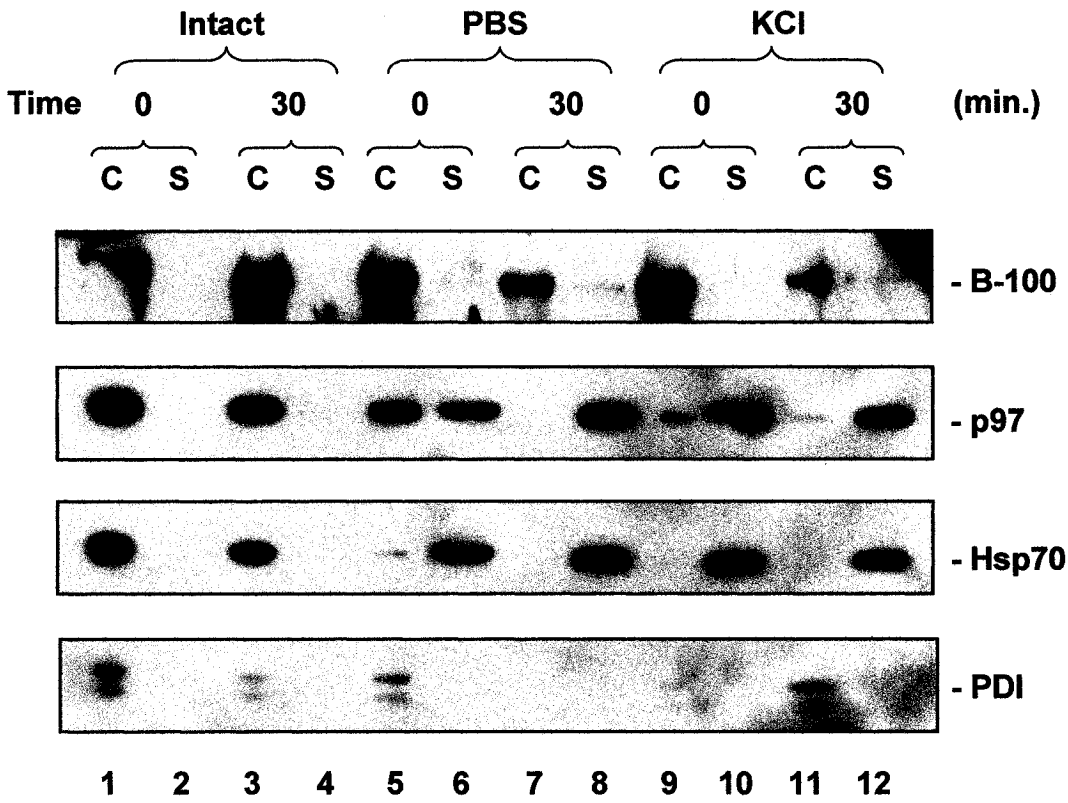

Figure 4.8. p97 is loosely associated with the membrane. HepG2 cells were incubated in CSK with or without digitonin for 10 minutes and cells were washed with PBS or 0.5 M KCl. Cytosol and washing buffer were pooled (S) and cells (C) were collected by lysis. Some cells were incubated for 30 minutes after washing in DMEM and cytosol, washing and media were pooled (S) and cells were collected (C). Proteins from pooled buffers were precipitated with TCA and proteins from cells and from buffers were resolved on SDS-PAGE, transferred onto nitrocellulose and visualized by immunoblotting with antibodies for apoB-100 (B-100), p97, heat shock protein 70 (Hsp70) and protein disulfide isomerase (PDI).

Intracellular apoB decreased significantly in permeabilised cells incubated for 30 minutes **(figure 4.8, lanes 7 and 11)**, suggesting that intracellular apoB may be susceptible to degradation in the absence of cytosol under some conditions. Since both apoB-100 and p97 were found in the membrane fraction, potential interactions were examined using a crosslinking approach.

4.2.5 Intracellular ApoB-100 is in Complex with the AAA-ATPase p97

Potential interacting partners of apoB-100 were captured using thiol-cleavable crosslinking agents. Intact HepG2 cells were incubated with the membrane permeable crosslinker dithiobis[sulfosuccinimidylpropionate] (DSP) and apoB-containing complexes were collected by immunoprecipitation. Under non-reducing electrophoresis conditions **(figure 4.9, lane 1)** apoB-100 was found as a monomer and as a higher molecular weight form whereas p97 was found predominantly as the 97 kDa monomer **(figure 4.9, lane 3)** in the absence of DSP. In the presence of DSP, only the larger form of apoB was detected on non-reducing gels **(figure 4.9, lane 2)** and p97 was found in monomeric form as well as a diffuse band co-migrating near apoB-100 **(figure 4.9, lane 4)**. This suggested that a portion of the cellular p97 was in complex with apoB. This was verified on reducing gels where the cross-linked apoB immunoprecipitates contained both apoB-100 **(figure 4.9, lane 8)** and p97 **(figure 4.9, lane 12)**. Monomeric and multimeric (perhaps trimer) forms of p97 were observed despite the reducing conditions **(figure 4.9, lane 12, *)**. Neither apoB nor p97 were found in immunocomplexes prepared with non-immune serum **(figure 4.9, lane 5, 6 and 9, 10)**. Of note, the signal for apoB-100 was decreased when the crosslinker was added suggesting that

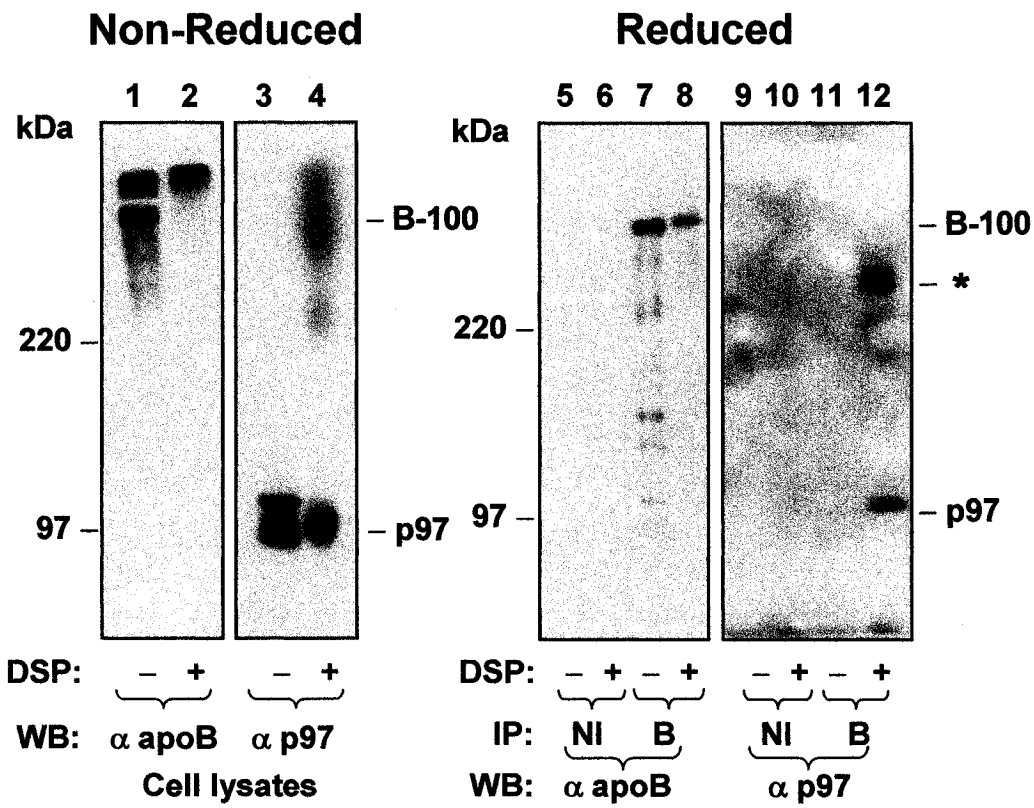

Figure 4.9. AAA-ATPase p97 is associated with apoB-100 in intact HepG2 cells HepG2 cells were incubated with or without the membrane-permeable, thiol-cleavable crosslinking agent DSP (2.5 mM) for 1 h at room temperature. Tris-HCl, pH 7.4 was added to 50 mM and the incubation was continued for an additional 15 min. The cells were then lysed in 1% SDS buffer. and subjected to immunoprecipitation (IP) with polyclonal anti-apoB serum (B) or with non-immune serum (NI). Immunocomplexes were released into reducing buffer (100 mM DTT), separated on 5% SDS-PAGE gels, transferred to nitrocellulose and visualized using monoclonal antibodies to apoB (B-100) or p97 with chemiluminescence detection.

immunoreactivity may be lost as a result of crosslinking. Covalent linkage of crosslinker bound to lysine residues on apoB may disrupt the epitope for the apoB monoclonal antibody used for immunoblotting. In addition, immunoprecipitation efficiency may also be affected by crosslinking. Therefore, a larger portion of cellular p97 than observed may be associated with apoB in HepG2 cells.

To determine if cytosolic apoB-100 was associated with p97, cytosol from MG132-treated HepG2 cells was prepared and proteins were crosslinked with the water-soluble agent dimethyl 3,3'-dithiobisproprionimidate·2HCl (DTBP). The analysis of immunoprecipitated apoB-containing complexes suggests that p97 did not interact with cytosolic apoB **(figure 4.10, lane 8)**. It is important to note that the lack of signal for p97 when the cytosol is crosslinked may also be due to a lost in immunoreactivity. Nevertheless, p97 may not interact with apoB after its release into the cytosol.

4.2.6 Optimization of p97 Silencing in HepG2 cells

The permeabilisation and crosslinking results suggested that p97 might be involved in retro-translocation of apoB-100. If p97 is necessary and sufficient for retro-translocation of apoB, a reduction of the level of p97 in HepG2 would be expected to impair the retro-translocation process and decrease proteasomal degradation of apoB-100. The AAA-ATPase p97 is very abundant comprising about 1% of cellular proteins and to reduce the expression of p97, a silencing approach with pre-designed double stranded siRNA targeting the p97 transcript, was used. First, transfection conditions were established with a validated siRNA for Lamin A/C. The transfection reagent Dharmafect-1 was utilized since it has been shown that, unlike other lipid-based

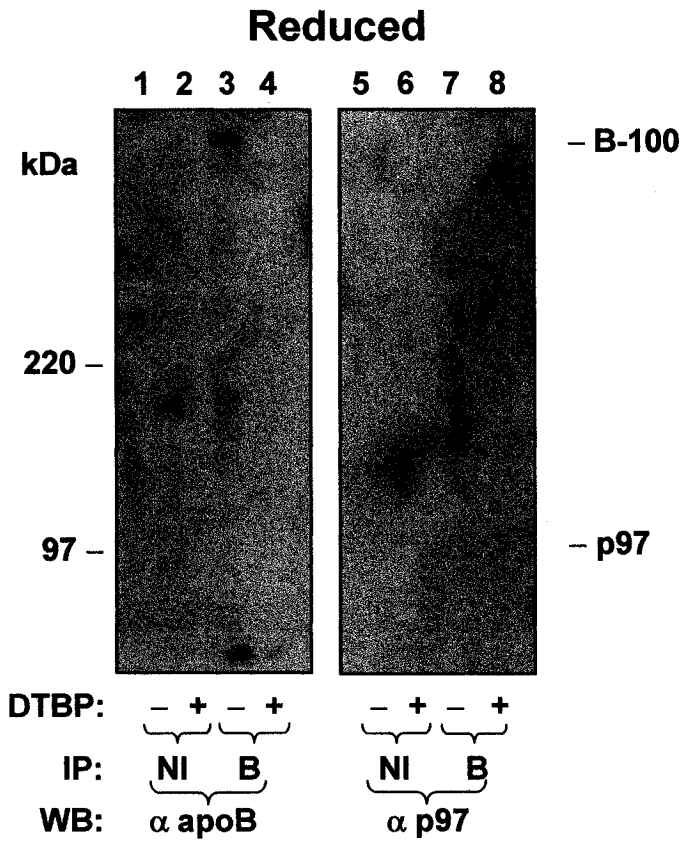

Figure 4.10. AAA-ATPase p97 is not associated with cytosolic apoB. Cytosol prepared by permeabilising HepG2 cells was incubated with or without 20 mM of the membrane-permeable, thiol-cleavable crosslinking agent DTBP for 1 h at room temperature. Tris-HCl, pH 7.4 was added to 50 mM and the incubation was continued for an additional 15 min. The cells were then lysed in 1% SDS buffer and subjected to immunoprecipitation (IP) with polyclonal anti-apoB serum (B) or with non-immune serum (NI). Immunocomplexes were released into reducing (100 mM DTT), separated on 5% SDS-PAGE gels, transferred to nitrocellulose and visualized using monoclonal antibodies to apoB (B-100) or p97 with chemiluminescence detection.

transfection reagents, it does not affect the viability of HepG2 cells. The recommended concentration of 4 µl of Dharmafect-1 per ml of transfection medium was used in the transfection mix. Cells were plated in 12 well plates at 10-15% confluence and grown overnight prior to transfection. HepG2 cells were transfected with non-targeting siRNA (0.1 µM), siRNA validated for Lamin A/C (0.1 µM) or siRNA targeted for sip97 #2 (1 µM) for 48 hours and silencing was assessed by immunoblotting. Lamin A and Lamin C were efficiently silenced (~90% and ~80%, respectively) as measured by scanning densitometry **(figure 4.11, Lamin A/C)**. In this experiment, the siRNA for p97 was used at 1 µM as previously suggested (Wojcik *et al.*, 2004) and p97 was reduced to ~50% after 48 hours **(figure 4.11, p97)**. Control proteins calnexin, Hsp70 and PDI were not affected by any of the siRNAs. Interestingly, apoB100 and p97 were both reduced with lamin siRNA transfection.

In a second experiment, the concentration of p97 siRNA was varied and the levels of p97 at 48 hours were determined. HepG2 cells were transfected with non-targeting siRNA and 3 different concentrations (0.01 µM, 0.1 µM and 1 µM) of 3 different p97 siRNA (sip97#1, #2 and #3). Transfection of 1 µM p97 siRNA resulted in ~85% reduction of cellular p97. Lowering the concentration of siRNA to 0.1 µM and 0.01 µM resulted in a substantial decrease in the silencing efficiency (reduction of ~20% and ~10%, respectively) **(figure 4.12)**.

In a third experiment, the time course of p97 silencing was determined for all 3 siRNAs at 1 µM. As shown in **figure 4.13A,** there was a time-dependent decrease in cellular p97 in the presence of the targeting siRNA. No decrease of p97 was found with the nontargeting siRNA or in mock-transfected cells. In the 48 hours following

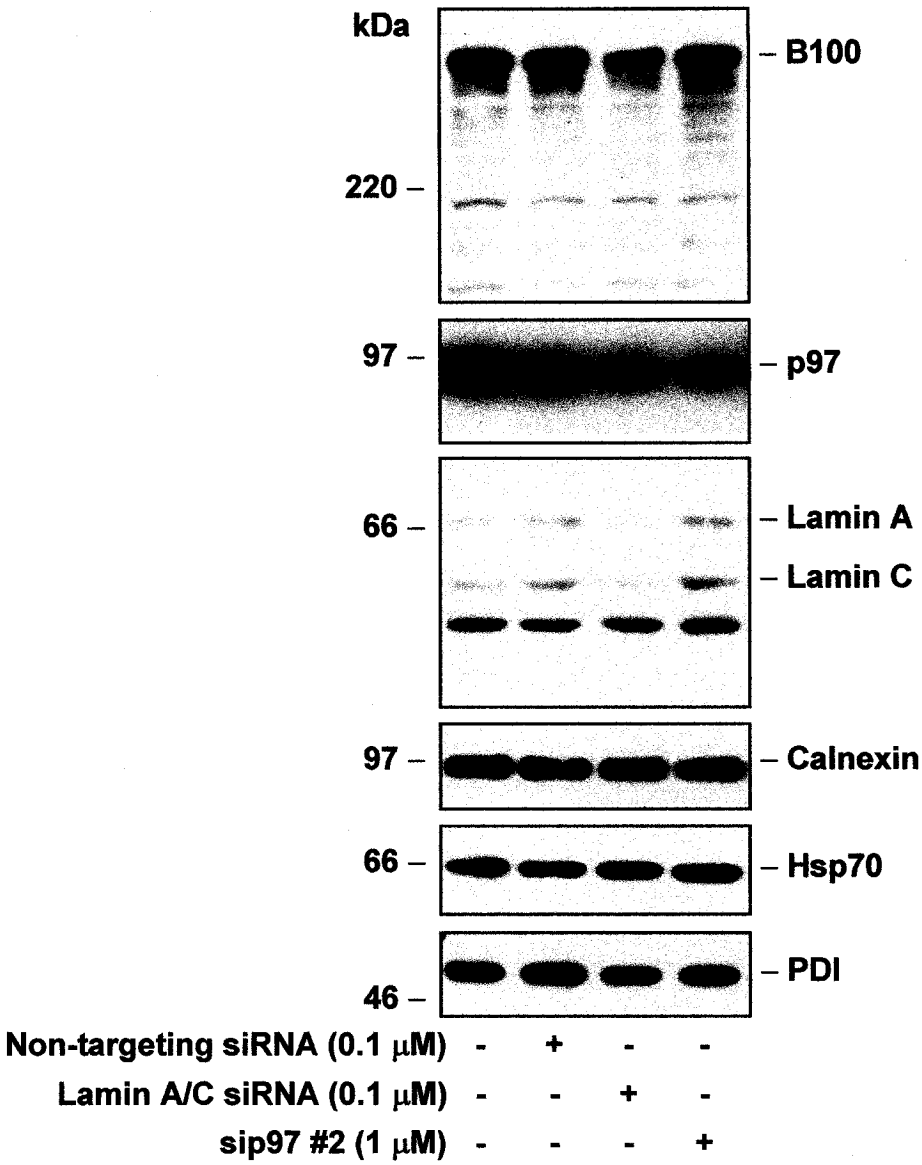

Figure 4.11 Optimization of the transfection of siRNA in HepG2 cells. HepG2 cells were transfected with non-targeting siRNA (0.1 μM), Lamin A/C specific siRNA (0.1 μM) or p97-specific siRNA (1 μM) for 48 hours. Cells were lysed and proteins were resolved by SDS-PAGE, transferred on nitrocellulose and visualized by immunoblotting using antibodies for apoB (B100), p97, lamin A/C, calnexin, heat shock protein 70 (Hsp70) and protein disulfide isomerase (PDI).

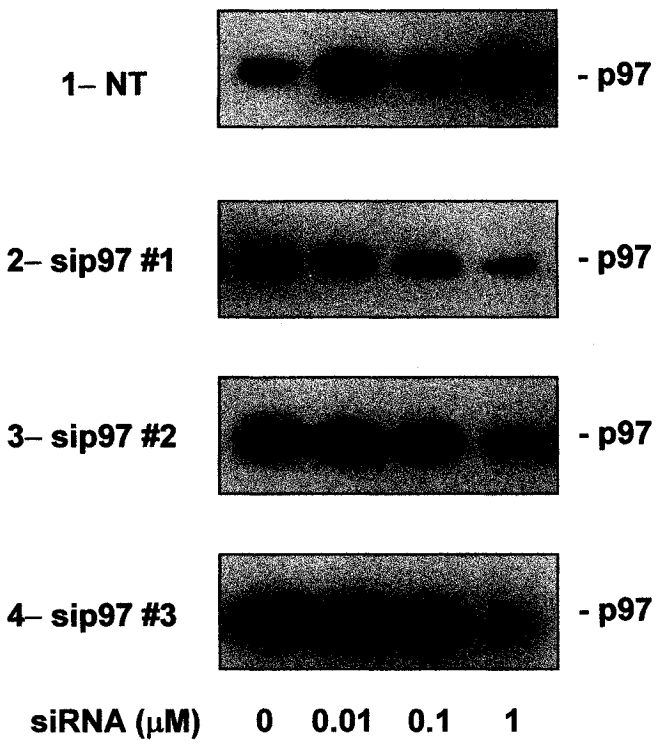

Figure 4.12 Effect of different concentration of siRNA on p97 silencing in HepG2 cells. HepG2 cells were transfected for 48 hours with non-targeting (NT) siRNA or p97-specific siRNA (sip97 #1, #2 and #3) at concentrations ranging from 0 to 1 μM for 48 hours. Cells were lysed and proteins were resolved by SDS-PAGE, transferred on nitrocellulose and visualized by immunoblotting using a monoclonal antibody for p97.

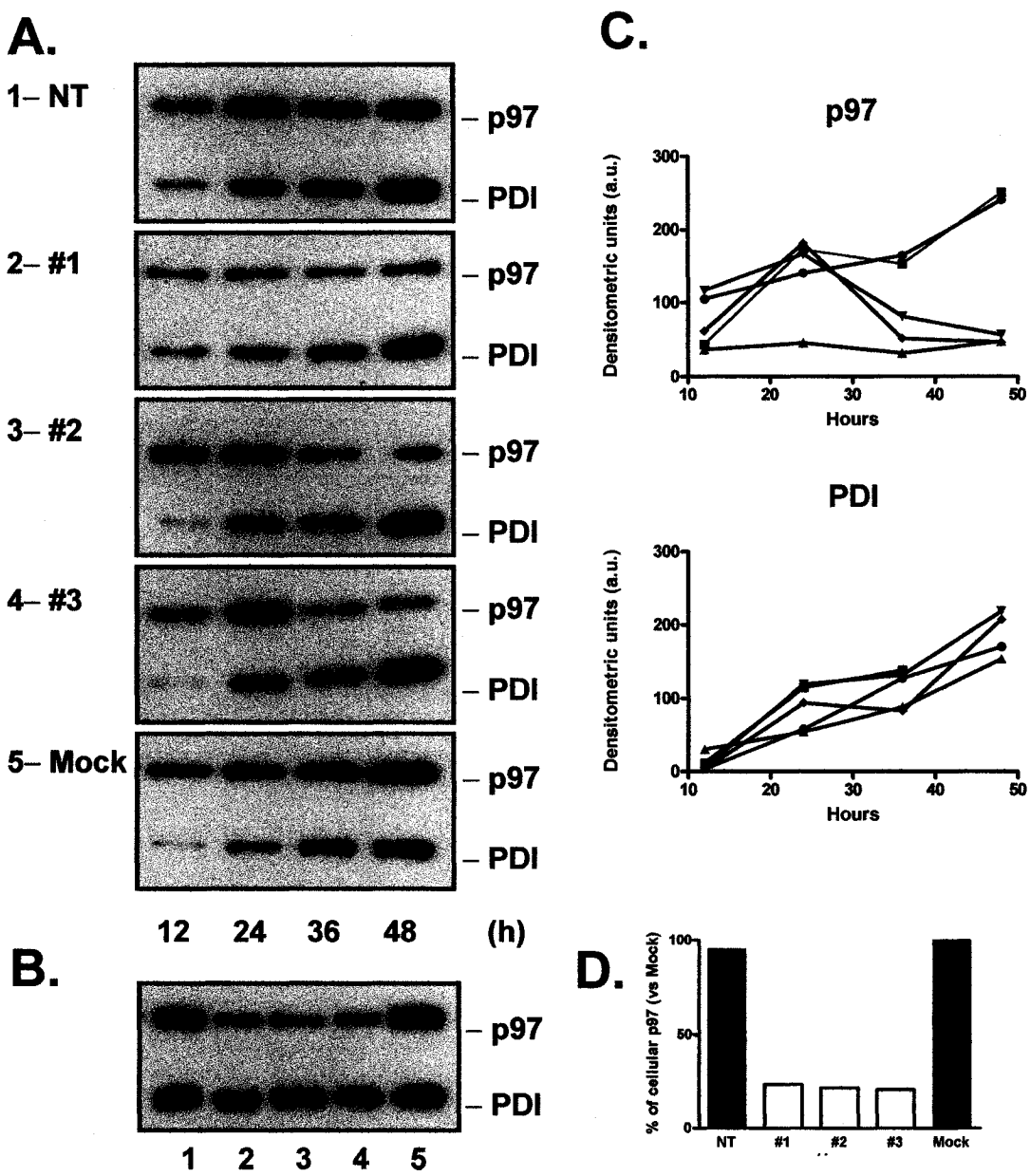

Figure 4.13 Transfection efficiency of three different siRNA for p97. A. HepG2 cells were incubated with transfection reagent (Mock) or transfected with non-targeting siRNA (NT) or p97-specific siRNA (sip97 #1, #2 and #3) for 12, 24, 36 or 48 hours. Cells were lysed and proteins were resolved by SDS-PAGE, transferred on nitrocellulose and visualized by immunoblotting using a monoclonal antibody for p97 and protein disulfide isomerase (PDI). B. Transfected HepG2 cells after 48 hours. C. Graphical representation of results in A. Closed squares (NT), closed triangle up (sip97 #1), closed triangle down (sip97 #2), closed diamonds (sip97 #3) and closed circle (Mock) D. Histogram of data in B.

transfection, the cellular levels of the control protein PDI increased regardless of the siRNA construct. When treated with the non-targeting (NT) siRNA or mock-transfected (Mock), p97 levels also increased. Both increases are consistent with the increase in the number of cells per well during this time frame. When the p97-targeted siRNAs were present **(figure 4.13A, panels 2-4)** there was no increase in p97 with cell growth; and with some oligonucleotides **(figure 4.13A, panels 3 and 4)** there was a decrease. At 48 hours **(figure 4.13B)** the level of p97 was 22% ± 3% (mean ± S.D.) of the level in the control transfections (non-transfected, mock-transfected and NT). Viability was maintained after 48 hours of transfection but beyond 48 hours, viability decreased substantially. Therefore, all subsequent experiments were conducted 48 hours after transfection and with 1 μM siRNA to obtain the maximum reduction of p97.

4.2.7 Reduction of Cellular p97 Increases Cellular apoB-100.

To evaluate the effect of reduced levels of p97 on apoB synthesis and stability, transfected HepG2 cells were subjected to metabolic radiolabeling. In cells transfected with siRNA for p97 **(figure 4.14, sip97#2)**, the extent of radiolabel incorporation into apoB-100 was increased approximately 2-fold, compared to the non-targeting control siRNA, while the incorporation into apoAI was not affected. These studies indicate that more apoB chains reached full-length when p97 levels are reduced.

Pulse-chase experiments were then performed to assess the effect of reduced p97 on the post-translational stability of apoB-100. Compared to non-targeting siRNA, non-transfected and mock-transfected cells, treatment with p97 siRNA only modestly affected the post-translational degradation of apoB-100. As shown in **figure 4.15**, the

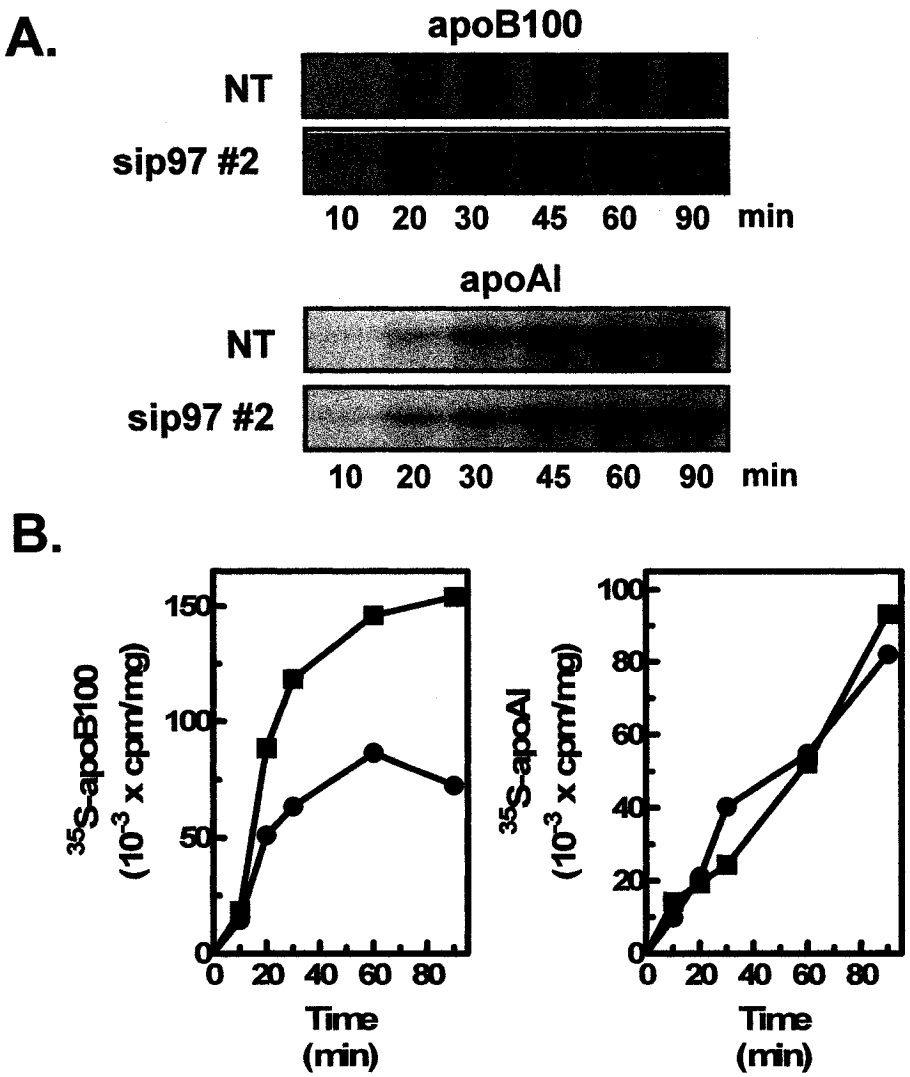

Figure 4.14 Biosynthesis of apoB-100 and apoA-I in siRNA-transfected HepG2 cells. Forty-eight hours following transfection with either non-targeting (NT) or p97-targeting (sip97 #2) siRNA, cells were pulse-labeled with 100 μCi of [^{35}S] methionine/cysteine for up to 90 minutes. A. ApoB and apoAI proteins were immunoprecipitated from cell lysates, resolved by SDS-PAGE and visualized by autoradiography. B. Radioactivity in each protein band was quantified by scintillation counting and normalized for cell protein. NT (closed circles), sip97 #2 (closed square).

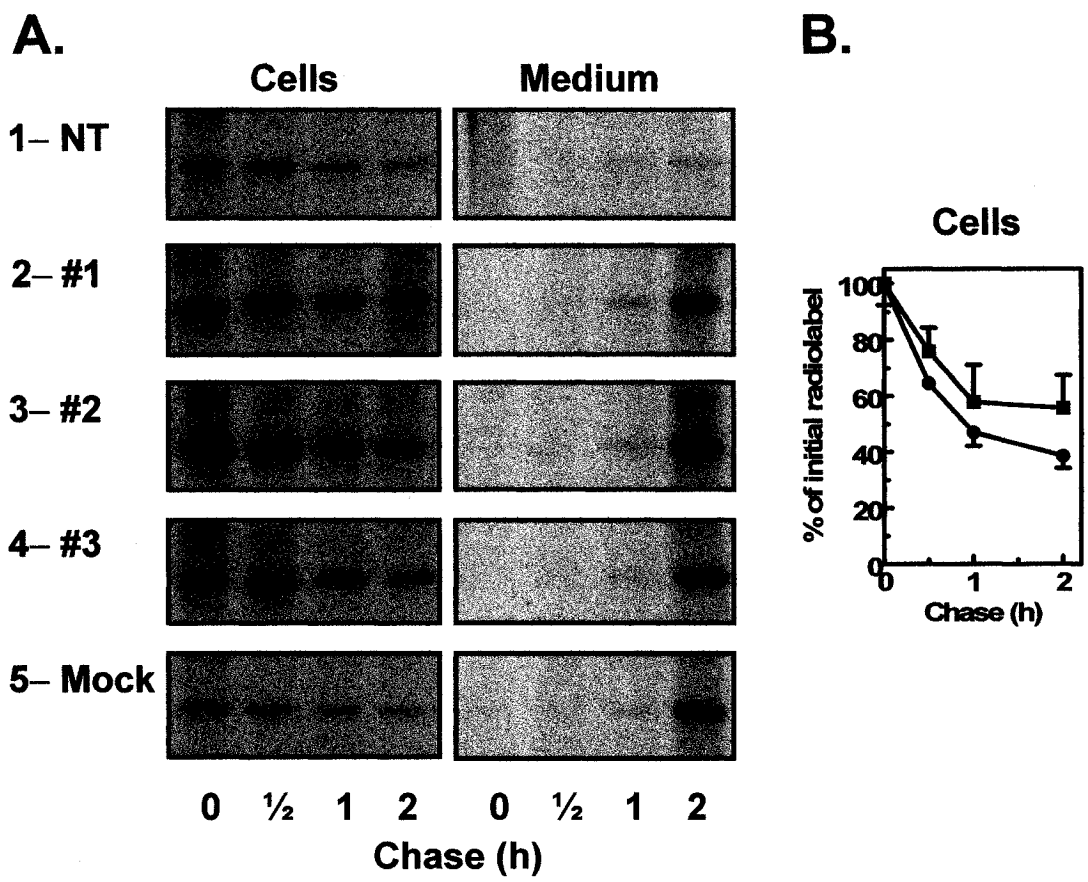

Figure 4.15 siRNA-mediated reduction of cellular p97 does not affect apoB-100 secretion or stability. A. Autoradiographs of apoB100 in cells and medium by pulse-chase analysis. Forty-eight hours following transfection, HepG2 monolayers were labeled with [^{35}S]methionine/cysteine for 1 h and then chased for up to 2 h as described in Materials and Methods. At each time point apoB was recovered from cells and medium and visualized by autoradiography. B. Decrease of radiolabeled apoB100, expressed as percent of initial radiolabel (filled squares: NT and mock controls, filled circles sip97 #1, #2 and #3). Control, mean and range for mock and NT samples; p97 siRNA, mean ± S.D. for the p97-targeting siRNAs.

decrease of radiolabeled apoB-100 was less at each time point in the cells treated with p97 siRNA, but was not significantly different from the control transfections. While the half-time of apoB-100 was prolonged, this was similar to the effect of MG132 (R.S. McLeod, unpublished data): predominant after approximately 30 mins, but minimal thereafter. ApoB-100 secretion in the siRNA-treated cultures was not substantially different, suggesting that the role of p97 may be restricted to the early stages of apoB biogenesis.

4.2.8 Reduction of Cellular p97 Impairs Retro-translocation and Proteasomal Degradation of ApoB

To look directly at the effect of reduced p97 on the retro-translocation of apoB-100, the accumulation of apoB-100 in the cytosol was analyzed. HepG2 cells transfected with non-targeting siRNA or one of 3 different siRNAs targeting p97 were incubated at 48 hours after transfection with MG132 for 1 hour. The mass of apoB100 in the cytosol and residual cell was determined by immunoblotting after digitonin treatment. Reduction of p97 **(figure 4.16, lanes 2-4)** increased cellular apoB-100 125% ± 10% and decreased the accumulation of cytosolic apoB-100 to 35% ± 12% of the non-targeted siRNA control **(figure 4.16, lane 1)**. The amount of cellular apoB-100 found in sip97 transfected cells was increased compared to non-targeting siRNA transfected cells. This observation suggests that not only does p97 knockdown increase nascent apoB-100 biosynthesis, it also suggests that p97 is directly involved in the retro-translocation process.

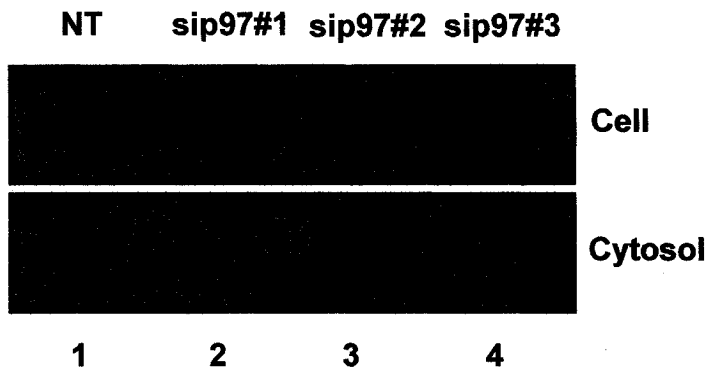

Figure 4.16 Reduction of p97 decreases retrotranslocation of apoB-100. HepG2 cells transfected with non-targeting (lane 1) or siRNA targeted for p97 (lane 2-4) were permeabilised with digitonin 48 hours post-transfection. ApoB proteins from permeabilised cells and cytosol were immunoprecipitated, resolved by SDS-PAGE, transferred on nitrocellulose and immunoblotted for apoB.

To analyse the role of the proteasome in apoB-100 after p97 knockdown, the effect of MG132 on the synthesis of apoB was evaluated. It was hypothesized that MG132 should have less effect on apoB-100 synthesis when cellular p97 levels are reduced. HepG2 cells were transfected with non-targeting siRNA **(figure 4.17A, lane 1 and 5)** or one of 3 different siRNAs targeting p97 **(figure 4.17A, lane 2-4 and 6-8)**. 48 hours post-transfection, cells were radiolabeled for 1 hour in the absence or presence of MG132. The amount of radiolabel incorporated into apoB-100 was used as a direct comparison of the quantity of apoB-100 susceptible to proteasomal degradation between cells with normal levels of p97 and cells where p97 was reduced. Untreated cells showed that reduced p97 increases the levels of apoB by approximately 1.5 to 2-fold, similar to previous experiments shown in **figure 4.14 and 4.15**. In contrast, cells treated with MG132 showed similar levels of apoB-100 regardless of the cellular levels of p97. This suggested that the increase of apoB-100 synthesized when p97 is reduced is due to an increase in the protection of nascent apoB-100 from proteasomal degradation. Conversely, the protective effect of MG132 in cells transfected with p97-targeted siRNA is reduced because a reduction of p97 alters the same pathway. In summary, the use of siRNA to silence p97 resulted in an increase in apoB synthesis by impairing the retro-translocation process thereby protecting nascent apoB proteins from proteasomal degradation. Taken together, these silencing experiments indicate that p97 has a role in apoB-100 retro-translocation leading to proteasomal degradation.

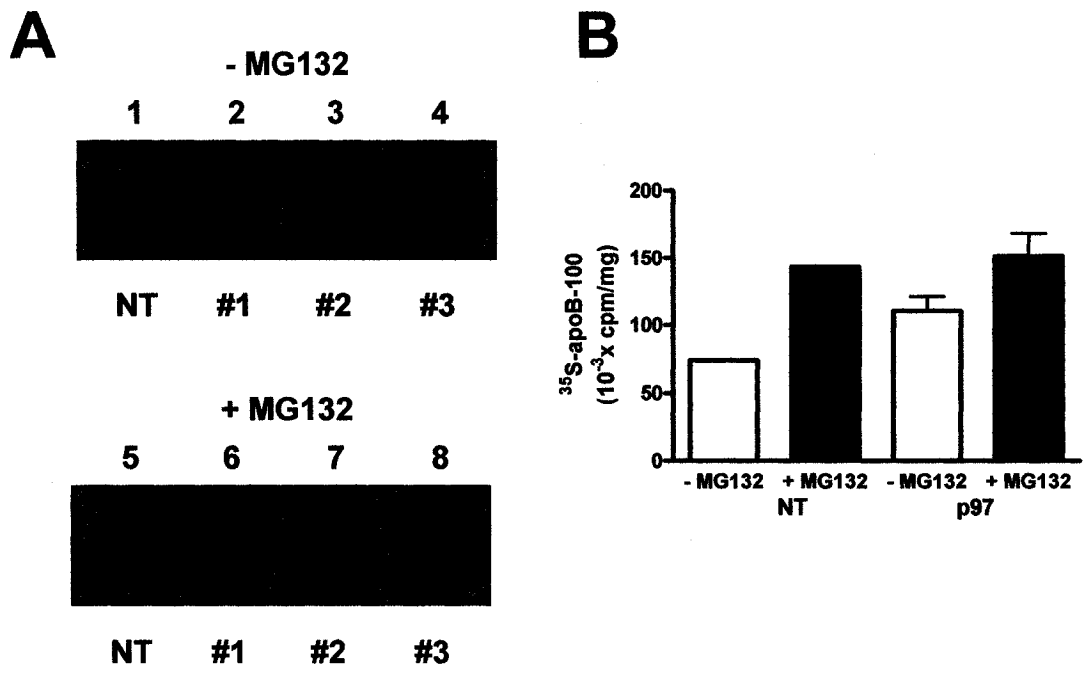

Figure 4.17 Reduction of p97 decreases the protective effect of MG132. A. HepG2 cells transfected with non-targeting (lane 1 and 5) or siRNA targeted for p97 (lane 2-4 and 6-8) were subjected to radiolabeling 48 hours after transfection. Cells were pulse-labeled for 1 hour in DMEM with 100 μCi of [^{35}S] methionine/cysteine with or without 25 μM of MG132. ApoB proteins were immunoprecipitated and visualized by autoradiography. B. ApoB proteins in A were quantified by scintillation counting and normalized for cell protein (n=3, ±SD.).

4.2.9 Attempts to Establish a Retro-translocation Assay

To look at the requirements in the retro-translocation of apoB-100, another approach was developed using HepG2 digitonin-permeabilised cells. It was hypothesized that following permeabilisation, remaining organelle-associated apoB-100 may be retro-translocated under specific conditions. HepG2 cells were permeabilised with digitonin and cytosol was removed and replaced with DMEM with or without an ATP-generating system based on the assumption that the retro-translocation mediated by p97 was an ATP-dependent process. During the 2 hour incubation, Brefeldin A was used to prevent the secretion from cells that might not have been permeabilised by digitonin. Cellular proteins Hsp70, PDI and calnexin were analyzed to establish the integrity of the ER membrane during the experiment **(figure 4.18A, lane 1-4)**. Brefeldin A abolished the secretion from intact cells **(figure 4.18A, lane 6)**. Some apoB-100 could be released to the supernatant after 2 hours **(figure 4.18, lane 7)**, which was stimulated by the addition of an ATP-generating system. These studies suggested that retro-translocation of apoB-100 could be stimulated in the permeabilised cell system and that it may require ATP.

Retro-translocated apoB-100 was further characterized by density gradient ultracentrifugation and the protein was found in the high density fractions of the gradient **(figure 4.18B, fractions 11-13)**, suggesting that apoB-100 released into the medium was distinct from secreted apoB **(figure 4.6A)** and may be associated with little or no lipids. The distribution of cellular proteins p97, Hsp70 and PDI was also analyzed and in contrast to **figure 4.18A**, some PDI was found in the medium fraction suggesting that the apoB-100 release into the supernatant of permeabilised cells might derive from

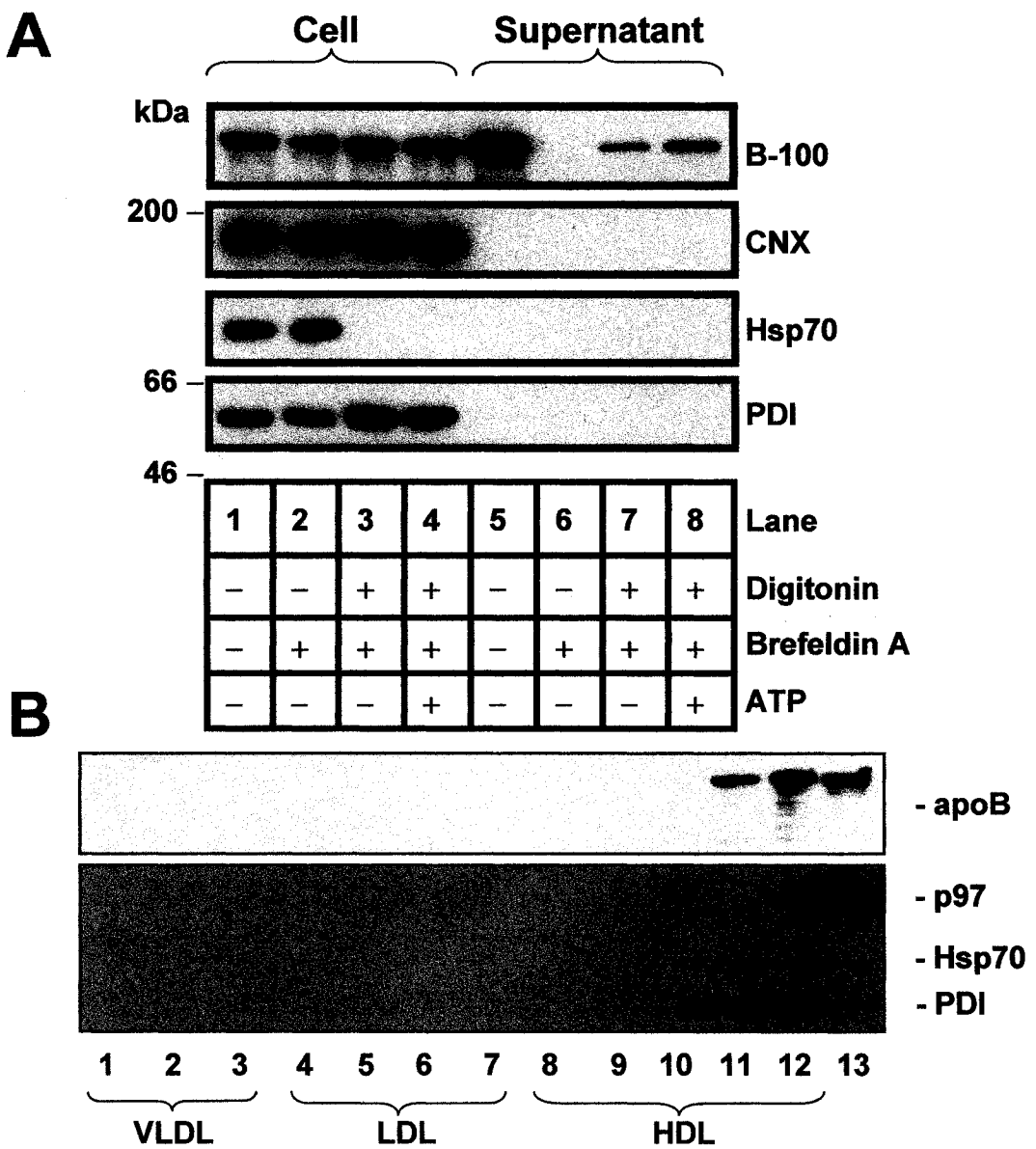

Figure 4.18 Effect of digitonin permeabilisation on HepG2 cells after 2 hours. A. HepG2 cells were incubated in CSK in the absence or presence of 75 μg/ml of digitonin for 10 minutes. Cells were then incubated for 2 hours in DMEM containing or devoid of 5 μg/ml of Brefeldin A and in the absence or presence of an ATP generating system. Proteins from the intact and permeabilised cells as well as the proteins found in the medium were TCA precipitated, resolved by SDS-PAGE, transferred onto nitrocellulose membranes and visualized by immunoblotting. B. HepG2 cells were permeabilised and incubated for 2 hours in DMEM with an ATP-generating system. Medium apoB proteins were analyzed by density gradient ultracentrifugation and 13 fractions were collected. Proteins were analyzed as described in A. Fractions were also analyzed for PDI, Hsp70 and p97.

permeabilised ER membranes. While digitonin was previously shown not to affect ER permeability initially (10 minutes permeabilisation), the integrity of the endomembrane system became compromised after extended incubation at 37°C (2 hours). In addition, the analyses of cellular proteins and apoB-100 in both **figure 4.18A** and **figure 4.18B** were difficult to reproduce, perhaps because of instability of the permeabilised cells. These observations showed the limitations of the digitonin-permeabilised cell system even though digitonin-permeabilisation of HepG2 cells successfully and reproducibly separated organelles from cytosolic components (as shown in **figure 4.7**).

CHAPTER 5

DISCUSSION

5.1 Structural Requirements in Apolipoprotein B Assembly and Degradation

The accepted model for the biosynthesis of secretory proteins is that, during translation, nascent polypeptides are efficiently inserted into the translocation pore and translocated across the ER membrane [reviewed in (Corsi and Schekman, 1996)]. Several studies have shown that apoB-100, however, is not efficiently translocated across the ER membrane, leading to the formation of a bitopic conformation where the polypeptide is exposed simultaneously to both cytosol and ER lumen [reviewed in (Fisher and Ginsberg, 2002)]. This conformation ultimately results in degradation. Since the discovery of this bitopic conformation of apoB-100, translocation of the polypeptide across the ER membrane has been considered an important step in the assembly of VLDL (Davis *et al.*, 1990; Thrift *et al.*, 1992). This thesis has presented evidence demonstrating that sequences within the β1 domain of apoB decrease translocation efficiency and can mediate rapid degradation of apoB proteins. The bitopic topology and increased membrane affinity of apoB proteins containing sequences between apoB39 and apoB42 correlated with an increase in polyubiquitination and proteasomal degradation. Sequences between apoB39 and apoB42 are encompassed by the region (apoB-29-42) involved in mediating core lipid recruitment into VLDL in transfected hepatoma cells (McLeod *et al.*, 1996; Carraway *et al.*, 2000). Furthermore, the region apoB-38–42 showed homology to the FABP family. Comparison of different portions of the apoB β1 domain fused to apoB-29 indicated that the predicted β-sequences in apoB between apoB-29 and apoB-37 are not functionally equivalent to sequences between

apoB-37 and apoB-42. Although truncated and fusion proteins containing apoB-37–42 were rapidly degraded in transfected rat hepatoma cells, fusion proteins containing apoB-29 and FABP, a known β-sheet polypeptide, or apoB-34–37 were not. These findings extend previous observations implicating sequences in the β1 domain in apoB regulated production (Liang *et al.*, 1998) by suggesting that the susceptibility of apoB proteins to degradation may be related to their ability to form buoyant lipoproteins.

Previous studies (McLeod *et al.*, 1996; Cavallo *et al.*, 1998; Liang *et al.*, 1998) and the present work have implicated sequences beyond the carboxyl terminus of apoB-28 in apoB function. Experiments in this thesis indicated that apoB-42 and the apoB-29/B-34–42 fusion protein have a higher affinity for the microsomal bilayer than apoB-29 and are more susceptible to trypsin digestion suggesting that apoB translocation arrest and cytosolic exposure is mediated by sequences between apoB34 and apoB42. Previous work did not show that apoB-48 and apoB-29 were arrested at translocation in rat hepatoma cells leading to the conclusion that the β1 domain was translocated efficiently (Cavallo *et al.*, 1998). The variations in topology between apoB-42 and apoB-48 in rat hepatoma cells may suggest that apoB-48 has unique properties, perhaps related to sequences between the carboxyl termini of apoB-42 and apoB-48.

Fusion proteins have been useful tools in defining apoB functional sequences in this and other studies (McLeod *et al.*, 1996; Liang *et al.*, 1998; Liang and Ginsberg, 2001). One must always be cautious in studies of this type because the model proteins that are used to study structure and function might have properties that are not present in physiologic forms of the protein. Creation of a novel model protein from amino acid sequences that are normally not contiguous in the native protein could introduce

functional artifacts associated with misfolding. This is of particular concern in studies of apoB because there is no way of experimentally determining whether the sequences are folded correctly. Because several of the truncated proteins and fusion proteins that were examined in this thesis retained the ability to assemble lipoproteins and were quite efficiently secreted, this approach to functional analysis is valid for at least some of the model proteins. One could argue that the proteins that contain the apoB-39–42 region are misfolded in this context and therefore are degraded because they do not retain physiological function. Perhaps, C-terminal truncation at apoB42 resulted in the disruption of a functional domain thereby decreasing the protein stability, a question that may be answered by the study of truncated apoB-41 and apoB-43. Although these possibilities cannot be excluded, the artificial junction of the C-terminus of apoB29 and segments of the β1 domain was not responsible for the increase in the degradation of fusion proteins apoB29/B34-42, as apoB29/B34-37 stability and secretion efficiency were similar to apoB29. In addition, at least one of the fusion proteins is able to function in a manner similar to that of native forms of apoB, in that apoB-29/B-34–42 can assemble VLDL, consistent with the role of the β1 domain in recruiting TAG for VLDL assembly (Carraway *et al.*, 2000).

A substantial amount of sequence from the β1 domain between apoB-29 and apoB-42 enables the protein to accrete sufficient TAG for VLDL assembly. The presence of 8% of apoB such as B34-42 on apoB29 (apoB29/B34-42) or B29-B37 (apoB-37) (McLeod *et al.*, 1996) endows apoB proteins with the ability to assemble VLDL in transfected McA-RH7777 cells. Analysis of amino acid sequences between apoB-29 and apoB-38 revealed the presence of repeated units in the β1 domain. Two

different predicted repeats were suggested from similarities in their amino acid sequence, predicted structures and accessibility to trypsin on a lipoprotein. Downstream of apoB29, sequences in apoB have been shown to gradually increase the ability of the protein to incorporate TAG necessary for VLDL assembly (Carraway *et al.*, 2000). The α helical region in repeats between apoB-33.3 and apoB-38.2 coincides with regions of apoB that are accessible to trypsin on LDL (Yang *et al.*, 1989a). In contrast, β-sheets are not all accessible to trypsin suggesting the integration of β-sheets of apoB in the phospholipid monolayer of the LpB. The presence of alternating α and β structures in each repeat may correspond, on a smaller scale, to the model for apoB conformational flexibility in a lipoprotein form (Wang *et al.*, 2006). Even though the β1 domain is predicted to be mostly composed of β-sheets, a potential role of amphipathic α helices in the assembly of VLDL is not excluded. The contribution of similar structures between apoB29 and apoB38 in VLDL assembly suggests that the ability of apoB to assemble a neutral lipid core relies on the presence of sufficient repeated amphipathic structures to attract and package neutral lipids. Further studies of the structure of sections apoB-29-33 and apoB-33-B38 may indicate the presence of flexible repeated hydrophobic lipid-binding domains.

A significant structural homology was identified between apoB-38.0-42.4 and FABP, a well-defined β-sheet protein, providing support for the predicted β-structure of this region of apoB (Segrest *et al.*, 1994; Segrest *et al.*, 1998). The presence of this section in truncated and fusion proteins greatly increased apoB degradation. For example, apoB29/B34-42 and apoB-37 stability differs significantly as apoB-37 is efficiently secreted and apoB29/B34-42 is rapidly degraded and poorly secreted.

Apparently, the stability of apoB is related but may not be entirely dependent on its ability to form a VLDL particle. Similar to fusion proteins, truncated protein studies suggested that unique features of amino acid sequences in the β1 domain between apoB-39 and apoB-42 substantially affect the protein stability. Sequence alignments of apoB-38-42 and FABP revealed several gaps that suggest longer loop regions between each β-strand of apoB. While each FABP has a compact pocket-like structure, the section between apoB-38 and apoB-42 may be more flexible and present a ribbon-like anti-parallel β-sheet structure, a structure predicted for the β1 domain. This flexibility in structure may substantially increase the affinity of the polypeptide to interact with the ER membrane bilayer and perhaps also with molecular chaperones, resulting in the retention of apoB in the ER. If exposed to the cytosol, these sequences may interact strongly with the outer leaflet of the ER membrane, preventing the reinitiation of translocation and predisposing the polypeptide for degradation. Prolonged cytosolic exposure and interaction with the translocon may render the polypeptide susceptible to polyubiquitination and proteasomal degradation (Pariyarath *et al.*, 2001). Thus, unique features in the sequence, in addition to the presence of hydrophobic lipid-binding β-sheets (Liang *et al.*, 1998), may play a role in decreasing apoB stability. Variations between the structural features of sequences between apoB-29 and apoB-38 and the FABP homology in apoB-38 to apoB-42 may explain the different effect that these sequences have on apoB translocation efficiency. Currently, the structure of an apoB fragment from apoB37 to apoB42 is being investigated in the laboratory of Roger S. McLeod and structural information may provide conclusive evidence supporting the unique nature of this region.

Translocation arrest has been proposed as a mechanism for the cytosolic exposure of apoB during translation, leading to ubiquitination and co-translational proteasomal degradation (Pan *et al.*, 2000). Studies in HepG2 cells have suggested that translocation may be influenced by lipid availability (Pullinger *et al.*, 1989; Dixon *et al.*, 1991; Fisher *et al.*, 1997). Moreover, a study in HepG2 cells indicated that the presence of β-sheet domains in the β1 domain of apoB (between apoB-28 and apoB-34) increased apoB susceptibility to proteasomal degradation and increased the protein responsiveness to exogenous lipids such as oleic acid. In transfected McA-RH7777 cells, however, treatments with oleic acid did not increase the translocation efficiency or stability of apoB fusion proteins containing lipid-responsive sequences. In addition, apoB-34, which contains the segment apoB-28-34, was efficiently secreted in McA-RH7777 cells (McLeod *et al.*, 1996). Differences between HepG2 and McA-RH7777 cells in the accessibility of apoB to lipids during assembly in the ER may explain the discrepancies in the effect of oleic acid on apoB translocation. It is important to acknowledge that the effect of oleic acid may be more noticeable on larger apoB proteins because of the increase in TAG requirement in their assembly into VLDL. While small segments of the β1 domain fused to ApoA-I were able to assemble into VLDL in McA-RH7777 cells and were subjected to rapid degradation, oleic acid did not substantially improve their intracellular stability (McLeod *et al.*, 1996). In addition, this study showed that small proteins that contain segments of the β1 domain can translocate efficiently suggesting that the size and type of the reporter protein may also have an effect in translocation efficiency.

Issues concerning the reproducibility of protease protection assays have been addressed by comparing trypsin treatments in microsomes and in digitonin-permeabilised cells. These experiments showed that both methods gave similar results, consistent with previous work in McA-RH7777 cells (Cavallo *et al.*, 1998). In this thesis, protease protection assays in both permeabilised cells and microsomes showed that apoB-29 was completely translocated and primarily in the lumen of microsomes. In contrast, apoB-42, encompassing functional regions for VLDL assembly, resulted in a decrease in the efficiency of translocation. In particular, there was a correlation between the increase in membrane association and the decrease in translocation efficiency suggesting that apoB proteins containing apoB-39 to apoB-42 were in a bitopic configuration.

The process of extracting apoB from the ER for proteasomal degradation is unclear but a mechanism was proposed in HepG2 cells by which apoB is co-translationally degraded in its original translocon (Pariyarath *et al.*, 2001). Other evidence has suggested that completely translocated apoB can be destined for degradation in HepG2 and McA-RH7777 cells (McLeod *et al.*, 1996; Adeli *et al.*, 1997b; Huang and Shelness, 1999). Moreover, Chan and colleagues (Liao *et al.*, 1998) demonstrated that the degradation of apoB-100 in HepG2 cells requires targeting with ubiquitin of the nascent polypeptide before its translocation into the lumen of the ER for eventual retrograde translocation (Chen *et al.*, 1998). The present work demonstrated that sequences downstream of apoB-39 increase the susceptibility of apoB to be polyubiquitinated by increasing cytosolic exposure of the polypeptide. Decreases in stability were found in both the truncated apoB proteins (e.g., apoB-42) and the apoB

fusion proteins that contain apoB-29 as the reporter. Degradation was remarkably rapid in fusion proteins containing the segment apoB39-42 (apoB29/B34-42 and apoB29/B37-42) and was partly blocked by either ALLN or MG132 implicating the proteasome in a significant portion of the degradation of these unstable apoB proteins. The predicted hydrophobic nature of sequences between apoB-39 and apoB-42 may be readily recognized by the ubiquitin-proteasome system if exposed to the cytosol. Because ALLN and MG132 do not prevent all of the degradation of the model proteins, these studies also suggest that other ALLN-insensitive pathways in McA-RH7777 hepatoma cells are involved in the posttranslational degradation of these model apoB proteins, as previously described for apoB-100 in other systems (Adeli *et al.*, 1997a; Adeli *et al.*, 1997b; Cardozo *et al.*, 2002).

The studies of intracellular stability and secretion efficiency of the apoB fusion proteins indicate that not all sequences from the β1 domain are involved in apoB degradation. A significant decrease in apoB stability was observed when either the apoB-34–42 or the apoB-37–42 segment was fused to apoB-29, but fusion to apoB-34–37 did not markedly affect secretion or stability and fusion to FABP had little effect on the stability of the resulting model protein. Further development of a model structure based on the homology to FABP may provide additional evidence for a structural relationship between this region of apoB and FABP, but the FABP homology in apoB cannot be functionally replaced by FABP itself. It is also important to note that even apoB-29 contains a portion of the β1 domain but that this protein is not rapidly degraded. This suggests that the instability of apoB is not a function of all regions of the β1 domain and that a proven β-sheet domain does not necessarily introduce apoB instability. On the

contrary, unique features of the apoB protein sequence, such as the acquisition of the ability to recruit neutral core lipids, may also be associated with translocation arrest and a markedly increased susceptibility to intracellular degradation.

5.2 The Role of the AAA-ATPase p97 in the Retro-translocation of Apolipoprotein B

The present work evaluated the role of p97 in the retro-translocation of apoB-100 to the cytosol for proteasomal degradation. To study the role of different cellular components involved in the degradation of apoB, a digitonin permeabilised cell system was utilized (Adeli, 1994; Winitz *et al.*, 1996; Macri and Adeli, 1997b; Sakata *et al.*, 1999). Digitonin-permeabilisation of HepG2 cells indicated that in the absence of cytosol, degradation of newly synthesized apoB-100 was minimal (R.S. McLeod, unpublished data) an observation that is supported by previous work (Sakata *et al.*, 1999) suggesting that the cytosolic proteasomal proteolysis is the dominant mechanism for apoB degradation in this cell line. However, other studies of digitonin-permeabilised HepG2 cells showed that post-translational degradation of the apoB protein may also involve non-proteasomal degradation pathways, such as the lumenal ER protease ER-60 (Adeli, 1994; Adeli *et al.*, 1997a; Qiu *et al.*, 2004).

During ERAD, retro-translocation of ER proteins to the cytosol and degradation by the proteasome are two dissociable steps. Inhibitors of the proteolytic activity of the 20S proteasome have allowed the demonstration of the accumulation of various ERAD substrates in the cytosol suggesting that the proteolytic activities of the proteasome and retro-translocation can be uncoupled (Wiertz *et al.*, 1996b; Yu *et al.*, 1997; VanSlyke

and Musil, 2002; Oberdorf *et al.*, 2006). Similarly, proteasome inhibition in HepG2 cells resulted in the accumulation of polyubiquitinated apoB-100 in the cytosol. This finding extends previous observations in microsomes (Liao *et al.*, 2003a) and in intact cells (Pariyarath *et al.*, 2001) suggesting that ubiquitinated apoB may accumulate in the cytosol during proteasome inhibition. Experiments with proteasome inhibitors affecting the proteolytic activity of β-subunits of the 20S core do not exclude the possibility that activities of the 19S subunit are involved in the retro-translocation of ERAD substrates. A recent *in vitro* study of CFTR degradation has suggested that the accumulation of full length ERAD substrates in the cytosol during proteasome inhibition may be the result of the uncoupling of the peptidase activities of the 20S core from the unfolding and delivery by the ATPase activities of the 19S subunit (Oberdorf *et al.*, 2006). Evidence showing the proximity of the proteasome to the site of apoB-100 translocation suggested that retro-translocation and delivery of apoB-100 to the proteasome may be coupled and may involve the same membrane pore as anterograde translocation (Sakata *et al.*, 1999; Pariyarath *et al.*, 2001). This is further supported by the observation that the proteasome interacts with the translocation channel during proteolysis (Kalies *et al.*, 2005).

The AAA-ATPase p97 has been shown to be involved in the recognition and delivery of several ERAD substrates for delivery to the proteasome [reviewed in (Tsai *et al.*, 2002)]. Reducing the level of p97 in HepG2 cells substantially impaired the retro-translocation of apoB-100 indicating that the 19S subunit of the proteasome may not be sufficient to release apoB-100 to the cytosol. The distribution of p97 in HepG2 cells demonstrated the presence of two pools of p97, a cytosolic and a membrane-associated pool. The membrane-associated pool was removable with high salt suggesting

electrostatic interactions between p97 and the membrane. This is consistent with the presence of a retro-translocation complex associated with ER and containing p97 (Ye *et al.*, 2004). Using a crosslinking approach, p97 was also found to associate with apoB-100 supporting the role of p97 in recognizing apoB for ERAD.

Polyubiquitinated apoB proteins accumulated in proteasome inhibitor-treated HepG2 cells suggesting that, similar to other ERAD substrates, polyubiquitination of the apoB polypeptide signals p97-mediated retro-translocation (Flierman *et al.*, 2003). While the possibility that apoB-100 becomes polyubiquitinated as it is released into the cytosol cannot be excluded, a noticeable amount of apoB that remained associated with the permeabilised cells was polyubiquitinated, suggesting that polyubiquitination may precede apoB release to the cytosol. Previous work has also suggested that ubiquitinated apoB proteins remained associated with the Sec61 translocon and may be deubiquitinated and secreted if LpB assembly is stimulated by exogenous OA (Mitchell *et al.*, 1998). The retro-translocation analysis presented in this work was not extended to further characterize possible fates of cytosolic ubiquitinated apoB-100, although it seems unlikely that such a large polyubiquitinated polypeptide would be deubiquitinated, reinserted in the translocon for translocation into the ER and assembled into an LpB. Instead, proteasome inhibition may present a unique situation where uncoupling of delivery and degradation leads to the release of cytosolic ubiquitinated apoB proteins as intermediates in the proteasomal degradation process.

Findings in the present work provided additional evidence for the role of the AAA-ATPase p97 in the retro-translocation of ERAD substrates (Plemper and Wolf, 1999; Ye *et al.*, 2001; Jarosch *et al.*, 2002; Bays and Hampton, 2002; Tsai *et al.*, 2002;

Meyer *et al.*, 2002; Ye *et al.*, 2004). Reducing cellular levels of p97 impaired the proteasomal degradation of apoB by reducing the efficiency of apoB-100 retro-translocation. The role of p97 appeared to be limited to the co-translational degradation of apoB-100 supporting the presence of temporally and spatially regulated degradation systems in the secretory pathway of hepatic cells. There may only be a window of time during which p97 can recognize cytosolically exposed apoB such that proteasomal degradation may serve as the initial quality control for translating and newly synthesized apoB. Moreover, the presence of full length and fragmented polyubiquitinated apoB proteins in the cytosol suggests that targets for p97-mediated retro-translocation include partially or completely translated apoB-100. Further assembly and completion of apoB-100 translocation across the ER membrane may render the polypeptide inaccessible to p97 for retro-translocation. However, since the role of the proteasome in apoB degradation has been shown to include fully translocated apoB (Cavallo *et al.*, 1998), the role of p97 in the retro-translocation of fully translocated apoB remains to be clarified. There is recent evidence that the ER lumenal chaperone Bip is able to recognize and send apoB for retro-translocation and proteasomal degradation (Qiu *et al.*, 2005). One could argue that during the radiolabeling analysis of siRNA-transfected cells, p97 expression is reinitiated, causing post-translational proteasomal degradation of apoB to be reconstituted. Complete and continuous knockdown of p97 during radiolabeling analyses may be required to address this issue. However, complete knockdown of p97 may be detrimental for the endomembrane system and the proliferation of mammalian cells as it is has been shown to be lethal in yeast (Latterich *et al.*, 1995). Further analysis of the post-translational role of p97 on apoB may provide clues and support for the

proposed retro-translocation mechanism of fully translocated apoB (Ingram and Shelness, 1996).

Although its mechanism of action remains unclear, p97 has been shown to interact with both polyubiquitinated and non-ubiquitinated proteins (Ye *et al.*, 2003). Polyubiquitination may trigger retro-translocation powered by the ATPase activity of p97 (Flierman *et al.*, 2003). Therefore, an appealing possibility is that apoB degradation occurs in a similar fashion as that of other ERAD substrates **(figure 5.1)**. In this manner, p97 may initiate apoB ERAD by interacting with apoB as it becomes exposed to the cytosol during translocation arrest. While p97-apoB complex is formed, apoB may become polyubiquitinated by E3 ligases (Liang *et al.*, 2003). One possibility is that the E3 ligase gp78 associates with p97 to facilitate apoB polyubiquitination (Zhong *et al.*, 2004). Polyubiquitination of apoB may trigger the p97-mediated retro-translocation of the polypeptide in the cytosol guided by Hsp70 and Hsp90 (Gusarova *et al.*, 2001), chaperones that have been suggested to deliver ubiquitinated targets to the 19S subunit of the proteasome. During retro-translocation, continuous delivery of apoB to the 20S core may require the combined ATPase activities of both p97 and proteins in the 19S lid. Hence, the proximity of p97 and 26S proteasome at the site of apoB translocation may explain how apoB is rapidly directed for degradation co-translationally (Mitchell *et al.*, 1998; Zhou *et al.*, 1998).

Some aspects of the retro-translocation of apoB-100 remain to be clarified. Protease protection assays have shown that the majority of intracellular apoB-100 is accessible to trypsin suggesting that apoB-100 translocation is inefficient (Cavallo *et al.*, 1998). For translocation arrested apoB-100, the role of p97 in the retro-translocation

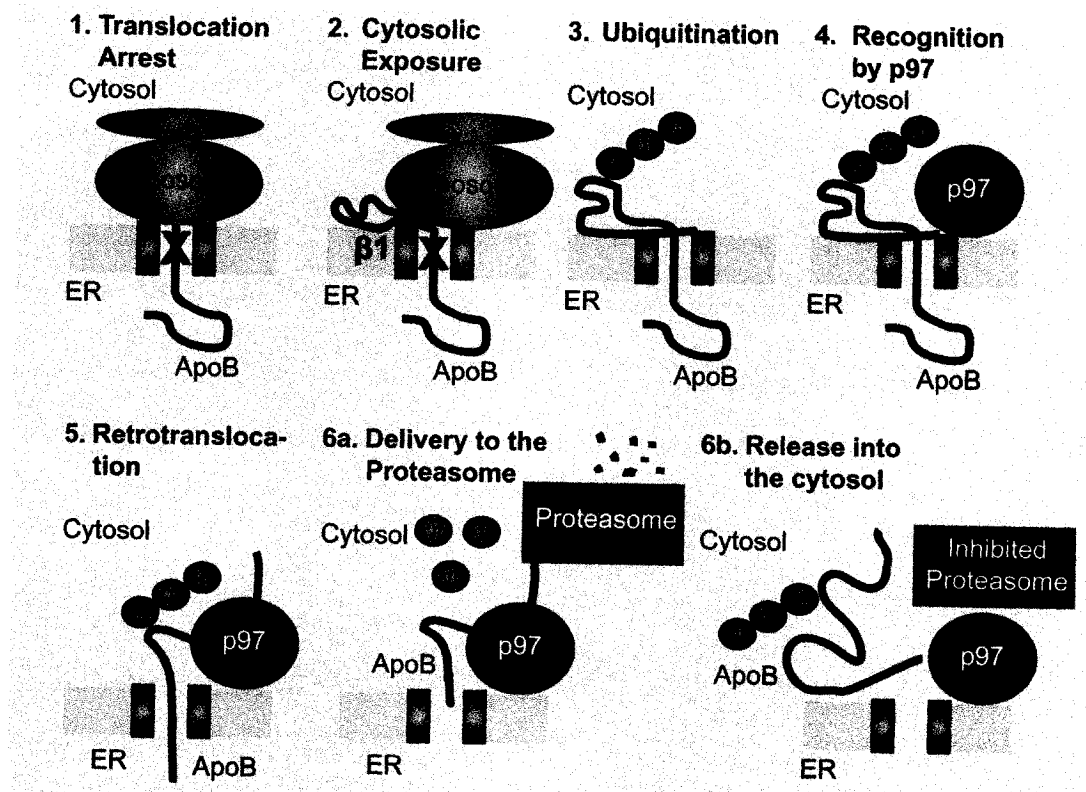

Figure 5.1 Model for the ER-associated degradation of apoB. Schematic representation of the steps leading to proteasomal degradation of apoB. 1. Arrest of the translocation by sequences in the β1 domain. 2. Opening of the Ribosome-Translocon junction and escape of a portion of the translating apoB polypeptide in the cytosol. 3. Polyubiquitination of the apoB polypeptide. 4. Recognition of apoB by p97. 4. Initiation of the retro-translocation of apoB across the ER membrane. 5. Coupled delivery of apoB to the proteasome during retro-translocation. 6. Release of the polyubiquitinated apoB polypeptide in the cytosol during proteasome inhibition.

may be especially important for the elimination of N-terminal domains of apoB-100 as the C-terminal domains are already in the cytosol. Conversely, p97 may completely extract fully translocated apoB-100 to the cytosol for proteasomal degradation. In the case where apoB-100 is being assembled into a lipoprotein, it is conceivable that disassembly of the lipid-protein complex must occur to allow the polypeptide to transit back through the translocation channel toward the cytosol. ER lumenal chaperones such as Bip are potential partners in this process. The presence of apoB-100 in the cytosol, apparently associated with little or no lipid supports the disassembly hypothesis. The analysis of the density of cytosolic apoB may be interpreted differently as the density of apoB has been suggested to increase when chaperones are bound to the particle (Zhang and Herscovitz, 2003). In this view, the apoB observed in the cytosol may then become associated with cytosolic lipid droplets as suggested recently (Ohsaki *et al.*, 2006). Analysis of the lipid content of cytosolic apoB may be important to clarify the steps required to release apoB into the cytosol. The presence of cytosolic apoB associated with a large quantity of lipids is unlikely since immunofluorescence microscopy studies have shown that apoB accumulates in punctate structures in the cytosol when the proteasome is inhibited, suggesting the formation of dense aggresomes (Pariyarath *et al.*, 2001). It is likely, however, that chaperones such as Hsp70 and Hsp90 are maintaining apoB solubility in the cytosol (Gusarova *et al.*, 2001), but further crosslinking experiments of cytosolic apoB are needed to confirm this possibility. Proteasome inhibition has been shown to induce the expression of Hsp70, in response to the accumulation of unfolded protein in the cytosol (Bush *et al.*, 1997). Further investigations of the partners in ERAD such as chaperones Bip, Hsp70 and Hsp90 and

lectin-like chaperones calnexin and EDEM may clarify the mechanism by which apoB is selectively taken from the ER to the cytosol for delivery to the proteasome. Analysis of potential components of the retro-translocation machinery such as Derlin-1 and VIMP may also reveal the presence of regulatory mechanism for signaling apoB degradation. A proteomic approach of cytosolic apoB, such as the one performed for microsomal apoB (Rashid *et al.*, 2002), is the next logical step in characterizing potential factors involved in apoB release into the cytosol and elucidating the mechanism of apoB proteasomal degradation.

The role of p97 in recognizing particular regions of apoB exposed to the cytosol may be clarified by using truncated and fusion model proteins that contain section of the β1 domain. One could speculate that silencing of p97 in McA-RH7777 cells may protect apoB proteins containing the β1 domain from early degradation. The characterization of the role of p97 in degrading β1 domain-containing apoB proteins may also provide clues in the mechanism of recognition of ERAD substrate by p97. Thus, these experiments need to be performed to determine the role of p97 in a different hepatic cell model. Work in primary hepatocytes may also be pertinent in corroborating the findings in HepG2 and McA-RH7777 cells.

Evidence presented in this work suggested that p97 is a central component in both recognizing and actively extracting apoB for delivery to the proteasome. This work provides insight on the mechanism of apoB proteasomal degradation by demonstrating a key role for the AAA-ATPase p97 in the recognition and retro-translocation process. Therefore, p97 may serve as a pivotal protein in the assembly/degradation tug-of-war that LpB is subjected to very early in its biosynthesis.

CHAPTER 6

CONCLUSIONS

Recent advances in ERAD research and the need for a better understanding of the mechanism of the regulatory apoB proteasomal degradation prompted this investigation. This thesis provides clarification of the role of sequences involved in VLDL assembly in the translocation of the apoB polypeptide in the ER and consequently impact presecretory apoB stability. These findings indicate that apoB assembly and degradation may arrive at a checkpoint during the assembly of the β1 domain to decide the fate of the newly assembling apoB. The role of the proteasome in the rapid degradation of β1 domain containing apoB proteins suggests that co-translational and early post-translational degradation are important quality control pathways for apoB in both McA-RH7777 and HepG2 cells. Notably, it is important to consider these cell lines as models for hepatocytes.

The distribution of ERAD factors in the cytosol and on the membrane of the ER supports a tightly associated system of degradation surrounding the translocating apoB polypeptide. This thesis has presented evidence that the AAA-ATPase p97 is involved in the proteasomal degradation pathway by recognizing and retro-translocating apoB into the cytosol. ER-associated proteasomes in proximity to the bitopic apoB may also be coupled to p97 in the retro-translocation/delivery process leading to the proteolysis of apoB. The contribution of p97 in the retro-translocation of apoB is consistent with the paradigm of the role of p97 in ERAD. Currently, the list of p97 substrates is expanding and new methods to assay retro-translocation in different cell lines are being developed (Carlson *et al.*, 2005; Lee *et al.*, 2005). The use of mutated or deleted proteins in yeast

can now be complemented with the use of siRNA in mammalian cells to characterize the role of different factors in apoB ERAD. Further use of this technology will prove to be extremely useful in the elucidation of various aspects surrounding ERAD.

Studies in yeast and mammalian cells have supported the role of ERAD in governing the secretory capacity of cells [reviewed in (Molinari and Sitia, 2005; Meusser *et al.*, 2005)]. Defects in the efficiency of ERAD have been proposed to contribute to the development of various age-related human diseases (Plemper and Wolf, 1999). ERAD of apoB may be particularly relevant in the progression of diseases such as FCH where the genetic mutation(s) of the disease have not been described. One could speculate that a causative factor for the increase in VLDL production in this prevalent disorder is the decrease in proteasomal function. The efficiency of the proteasome to degrade proteins has been shown to decrease with aging (Chondrogianni and Gonos, 2005) and the activation of proteasomal function has been linked with increased lifespan in yeast and mammalian cells (Chondrogianni *et al.*, 2005; Chen *et al.*, 2006). Thorough knowledge of the partners involved in the proeasomal degradation of apoB may provide new insight in the presecretory regulation of hepatic VLDL. In addition, understanding the molecular mechanisms involved in the decline of proteasomal efficiency will be useful in defining solutions to prevent the development of hyperlipidemia, cardiovascular diseases and aging.

REFERENCES

Abujarour,R.J., Dalal,S., Hanson,P.I., Draper,R.K. (2005). p97 is in a complex with cholera toxin and influences the transport of cholera toxin and related toxins to the cytoplasm. J.Biol.Chem. *280*, 15861-15871.

Accad,M., Smith,S.J., Newland,D.L., Sanan,D.A., King,L.E., Jr., Linton,M.F., Fazio,S., Farese,R.V., Jr. (2000). Massive xanthomatosis and altered composition of atherosclerotic lesions in hyperlipidemic mice lacking acyl CoA:cholesterol acyltransferase 1. J.Clin.Invest *105*, 711-719.

Adeli,K. (1994). Regulated intracellular degradation of apolipoprotein B in semipermeable HepG2 cells. J.Biol.Chem. *269*, 9166-9175.

Adeli,K., Macri,J., Mohammadi,A., Kito,M., Urade,R., Cavallo,D. (1997a). Apolipoprotein B is intracellularly associated with an ER-60 protease homologue in HepG2 cells. J.Biol.Chem. *272*, 22489-22494.

Adeli,K., Wettesten,M., Asp,L., Mohammadi,A., Macri,J., Olofsson,S.O. (1997b). Intracellular assembly and degradation of apolipoprotein B-100-containing lipoproteins in digitonin-permeabilized HEP G2 cells. J.Biol.Chem. *272*, 5031-5039.

Afshar,N., Black,B.E., Paschal,B.M. (2005). Retrotranslocation of the chaperone calreticulin from the endoplasmic reticulum lumen to the cytosol. Mol.Cell Biol. *25*, 8844-8853.

Albring,J., Koopmann,J.O., Hammerling,G.J., Momburg,F. (2004). Retrotranslocation of MHC class I heavy chain from the endoplasmic reticulum to the cytosol is dependent on ATP supply to the ER lumen. Mol.Immunol. *40*, 733-741.

Anant,S., Davidson,N.O. (2001). Molecular mechanisms of apolipoprotein B mRNA editing. Curr.Opin.Lipidol. *12*, 159-165.

Anderson,C.M., Townley,R.R., Freeman,M., Johansen,P. (1961). Unusual causes of steatorrhoea in infancy and childhood. Med.J.Aust. *48*, 617-622.

Anderson,T.A., Levitt,D.G., Banaszak,L.J. (1998). The structural basis of lipid interactions in lipovitellin, a soluble lipoprotein. Structure. *6*, 895-909.

Anitschkow,N. (1933). Experimental atherosclerosis in animals. A Survey of the Problem.E.V.Cowdry, editor.Macmillan, New York. 271-322.

Aridor,M., Balch,W.E. (1999). Integration of endoplasmic reticulum signaling in health and disease. Nat.Med. *5*, 745-751.

Asp,L., Claesson,C., Boren,J., Olofsson,S.O. (2000). ADP-ribosylation factor 1 and its activation of phospholipase D are important for the assembly of very low density lipoproteins. J.Biol.Chem. *275*, 26285-26292.

Atkinson,D., Deckelbaum,R.J., Small,D.M., Shipley,G.G. (1977). Structure of human plasma low-density lipoproteins: molecular organization of the central core. Proc.Natl.Acad.Sci.U.S.A *74*, 1042-1046.

Avramoglu,R.K., Cianflone,K., Sniderman,A.D. (1995). Role of the neutral lipid accessible pool in the regulation of secretion of apoB-100 lipoprotein particles by HepG2 cells. J.Lipid Res. *36*, 2513-2528.

Babin,P.J., Bogerd,J., Kooiman,F.P., Van Marrewijk,W.J., Van der Horst,D.J. (1999). Apolipophorin II/I, apolipoprotein B, vitellogenin, and microsomal triglyceride transfer protein genes are derived from a common ancestor. J.Mol.Evol. *49*, 150-160.

Babin,P.J., Deryckere,F., Gannon,F. (1995). Presence of an extended duplication in the putative low-density-lipoprotein receptor-binding domain of apolipoprotein B. Cloning and characterization of the domain in salmon. Eur.J.Biochem. *230*, 45-51.

Bakillah,A., Jamil,H., Hussain,M.M. (1998). Lysine and arginine residues in the N-terminal 18% of apolipoprotein B are critical for its binding to microsomal triglyceride transfer protein. Biochemistry *37*, 3727-3734.

Bamberger,M.J., Lane,M.D. (1990). Possible role of the Golgi apparatus in the assembly of very low density lipoprotein. Proc.Natl.Acad.Sci.U.S.A *87*, 2390-2394.

Barlowe,C., Orci,L., Yeung,T., Hosobuchi,M., Hamamoto,S., Salama,N., Rexach,M.F., Ravazzola,M., Amherdt,M., Schekman,R. (1994). COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell *77*, 895-907.

Barrett,P.H., Watts,G.F. (2002). Shifting the LDL-receptor paradigm in familial hypercholesterolemia: novel insights from recent kinetic studies of apolipoprotein B-100 metabolism. Atheroscler.Suppl *2*, 1-4.

Bays,N.W., Gardner,R.G., Seelig,L.P., Joazeiro,C.A., Hampton,R.Y. (2001a). Hrd1p/Der3p is a membrane-anchored ubiquitin ligase required for ER-associated degradation. Nat.Cell Biol. *3*, 24-29.

Bays,N.W., Hampton,R.Y. (2002). Cdc48-Ufd1-Npl4: stuck in the middle with Ub. Curr.Biol. *12*, R366-R371.

Bays,N.W., Wilhovsky,S.K., Goradia,A., Hodgkiss-Harlow,K., Hampton,R.Y. (2001b). HRD4/NPL4 is required for the proteasomal processing of ubiquitinated ER proteins. Mol.Biol.Cell *12*, 4114-4128.

Beckmann,R., Bubeck,D., Grassucci,R., Penczek,P., Verschoor,A., Blobel,G., Frank,J. (1997). Alignment of conduits for the nascent polypeptide chain in the ribosome-Sec61 complex. Science *278*, 2123-2126.

Benn,M., Nordestgaard,B.G., Jensen,J.S., Nilausen,K., Meinertz,H., Tybjaerg-Hansen,A. (2005). Mutation in apolipoprotein B associated with hypobetalipoproteinemia despite decreased binding to the low density lipoprotein receptor. J.Biol.Chem. *280*, 21052-21060.

Benoist,F., Grand-Perret,T. (1997). Co-translational degradation of apolipoprotein B100 by the proteasome is prevented by microsomal triglyceride transfer protein. Synchronized translation studies on HepG2 cells treated with an inhibitor of microsomal triglyceride transfer protein. J.Biol.Chem. *272*, 20435-20442.

Bertolotti,A., Zhang,Y., Hendershot,L.M., Harding,H.P., Ron,D. (2000). Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat.Cell Biol. *2*, 326-332.

Biederer,T., Volkwein,C., Sommer,T. (1996). Degradation of subunits of the Sec61p complex, an integral component of the ER membrane, by the ubiquitin-proteasome pathway. EMBO J. *15*, 2069-2076.

Biederer,T., Volkwein,C., Sommer,T. (1997). Role of Cue1p in ubiquitination and degradation at the ER surface. Science *278*, 1806-1809.

Boerwinkle,E., Chan,L. (1989). A three codon insertion/deletion polymorphism in the signal peptide region of the human apolipoprotein B (APOB) gene directly typed by the polymerase chain reaction. Nucleic Acids Res. *17*, 4003.

Bonnardel,J.A., Davis,R.A. (1995). In HepG2 cells, translocation, not degradation, determines the fate of the de novo synthesized apolipoprotein B. J.Biol.Chem. *270*, 28892-28896.

Borchardt,R.A., Davis,R.A. (1987). Intrahepatic assembly of very low density lipoproteins. Rate of transport out of the endoplasmic reticulum determines rate of secretion. J.Biol.Chem. *262*, 16394-16402.

Bordallo,J., Plemper,R.K., Finger,A., Wolf,D.H. (1998). Der3p/Hrd1p is required for endoplasmic reticulum-associated degradation of misfolded lumenal and integral membrane proteins. Mol.Biol.Cell *9*, 209-222.

Boren,J., Graham,L., Wettesten,M., Scott,J., White,A., Olofsson,S.O. (1992). The assembly and secretion of ApoB 100-containing lipoproteins in Hep G2 cells. ApoB 100 is cotranslationally integrated into lipoproteins. J.Biol.Chem. *267*, 9858-9867.

Boren,J., Lee,I., Zhu,W., Arnold,K., Taylor,S., Innerarity,T.L. (1998). Identification of the low density lipoprotein receptor-binding site in apolipoprotein B100 and the modulation of its binding activity by the carboxyl terminus in familial defective apo-B100. J.Clin.Invest *101*, 1084-1093.

Boren,J., Rustaeus,S., Olofsson,S.O. (1994). Studies on the assembly of apolipoprotein B-100- and B-48-containing very low density lipoproteins in McA-RH7777 cells. J.Biol.Chem. *269*, 25879-25888.

Boren,J., Rustaeus,S., Wettesten,M., Andersson,M., Wiklund,A., Olofsson,S.O. (1993). Influence of triacylglycerol biosynthesis rate on the assembly of apoB-100-containing lipoproteins in Hep G2 cells. Arterioscler.Thromb. *13*, 1743-1754.

Boren,J., Wettesten,M., Sjoberg,A., Thorlin,T., Bondjers,G., Wiklund,O., Olofsson,S.O. (1990). The assembly and secretion of apoB 100 containing lipoproteins in Hep G2 cells. Evidence for different sites for protein synthesis and lipoprotein assembly. J.Biol.Chem. *265*, 10556-10564.

Bostrom,K., Wettesten,M., Boren,J., Bondjers,G., Wiklund,O., Olofsson,S.O. (1986). Pulse-chase studies of the synthesis and intracellular transport of apolipoprotein B-100 in Hep G2 cells. J.Biol.Chem. *261*, 13800-13806.

Bouma,M.E., Beucler,I., Aggerbeck,L.P., Infante,R., Schmitz,J. (1986). Hypobetalipoproteinemia with accumulation of an apoprotein B-like protein in intestinal cells. Immunoenzymatic and biochemical characterization of seven cases of Anderson's disease. J.Clin.Invest *78*, 398-410.

Bradbury,P., Mann,C.J., Kochl,S., Anderson,T.A., Chester,S.A., Hancock,J.M., Ritchie,P.J., Amey,J., Harrison,G.B., Levitt,D.G., Banaszak,L.J., Scott,J., Shoulders,C.C. (1999). A common binding site on the microsomal triglyceride transfer protein for apolipoprotein B and protein disulfide isomerase. J.Biol.Chem. *274*, 3159-3164.

Braun,S., Matuschewski,K., Rape,M., Thoms,S., Jentsch,S. (2002). Role of the ubiquitin-selective CDC48(UFD1/NPL4)chaperone (segregase) in ERAD of OLE1 and other substrates. EMBO J. *21*, 615-621.

Brodsky,J.L., Gusarova,V., Fisher,E.A. (2004). Vesicular Trafficking of Hepatic Apolipoprotein B100 and Its Maturation to Very Low-Density Lipoprotein Particles; Studies from Cells and Cell-free Systems. Trends Cardiovasc.Med. *14*, 127-132.

Brodsky,J.L., Werner,E.D., Dubas,M.E., Goeckeler,J.L., Kruse,K.B., McCracken,A.A. (1999). The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. J.Biol.Chem. *274*, 3453-3460.

Brown,M.S., Goldstein,J.L. (1983). Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu.Rev.Biochem. *52*, 223-261.

Brown,M.S., Goldstein,J.L. (1986). A receptor-mediated pathway for cholesterol homeostasis. Science *232*, 34-47.

Buhman,K.K., Accad,M., Novak,S., Choi,R.S., Wong,J.S., Hamilton,R.L., Turley,S., Farese,R.V., Jr. (2000). Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. Nat.Med. *6*, 1341-1347.

Buhman,K.K., Smith,S.J., Stone,S.J., Repa,J.J., Wong,J.S., Knapp,F.F., Jr., Burri,B.J., Hamilton,R.L., Abumrad,N.A., Farese,R.V., Jr. (2002). DGAT1 is not essential for intestinal triacylglycerol absorption or chylomicron synthesis. J.Biol.Chem. *277*, 25474-25479.

Bukau,B., Horwich,A.L. (1998). The Hsp70 and Hsp60 chaperone machines. Cell *92*, 351-366.

Burch,W.L., Herscovitz,H. (2000). Disulfide bonds are required for folding and secretion of apolipoprotein B regardless of its lipidation state. J.Biol.Chem. *275*, 16267-16274.

Bush,K.T., Goldberg,A.L., Nigam,S.K. (1997). Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. J.Biol.Chem. *272*, 9086-9092.

Cardozo,C., Wu,X., Pan,M., Wang,H., Fisher,E.A. (2002). The inhibition of microsomal triglyceride transfer protein activity in rat hepatoma cells promotes proteasomal and nonproteasomal degradation of apoprotein b100. Biochemistry *41*, 10105-10114.

Carlson,E., Bays,N., David,L., Skach,W.R. (2005). Reticulocyte lysate as a model system to study endoplasmic reticulum membrane protein degradation. Methods Mol.Biol. *301*, 185-205.

Carr,A., Samaras,K., Burton,S., Law,M., Freund,J., Chisholm,D.J., Cooper,D.A. (1998). A syndrome of peripheral lipodystrophy, hyperlipidaemia and insulin resistance in patients receiving HIV protease inhibitors. AIDS *12*, F51-F58.

Carraway,M., Herscovitz,H., Zannis,V., Small,D.M. (2000). Specificity of lipid incorporation is determined by sequences in the N-terminal 37 of apoB. Biochemistry *39*, 9737-9745.

Cartwright,I.J., Higgins,J.A. (1992). Quantification of apolipoprotein B-48 and B-100 in rat liver endoplasmic reticulum and Golgi fractions. Biochem.J. *285 (Pt 1)*, 153-159.

Cavallo,D., McLeod,R.S., Rudy,D., Aiton,A., Yao,Z., Adeli,K. (1998). Intracellular translocation and stability of apolipoprotein B are inversely proportional to the length of the nascent polypeptide. J.Biol.Chem. *273*, 33397-33405.

Chatterton,J.E., Phillips,M.L., Curtiss,L.K., Milne,R., Fruchart,J.C., Schumaker,V.N. (1995). Immunoelectron microscopy of low density lipoproteins yields a ribbon and bow model for the conformation of apolipoprotein B on the lipoprotein surface. J.Lipid Res. *36*, 2027-2037.

Chen,G.C., Chapman,M.J., Kane,J.P. (1983). Secondary structure and thermal behavior of trypsin-treated low-density lipoproteins from human serum, studied by circular dichroism. Biochim.Biophys.Acta *754*, 51-56.

Chen,Q., Thorpe,J., Dohmen,J.R., Li,F., Keller,J.N. (2006). Ump1 extends yeast lifespan and enhances viability during oxidative stress: central role for the proteasome? Free Radic.Biol.Med. *40*, 120-126.

Chen,S.H., Yang,C.Y., Chen,P.F., Setzer,D., Tanimura,M., Li,W.H., Gotto,A.M., Jr., Chan,L. (1986). The complete cDNA and amino acid sequence of human apolipoprotein B-100. J.Biol.Chem. *261*, 12918-12921.

Chen,Y., Le Caherec,F., Chuck,S.L. (1998). Calnexin and other factors that alter translocation affect the rapid binding of ubiquitin to apoB in the Sec61 complex. J.Biol.Chem. *273*, 11887-11894.

Chen,Z., Fitzgerald,R.L., Schonfeld,G. (2002). Hypobetalipoproteinemic mice with a targeted apolipoprotein (Apo) B-27.6-specifying mutation: in vivo evidence for an important role of amino acids 1254-1744 of ApoB in lipid transport and metabolism of the apoB-containing lipoprotein. J.Biol.Chem. *277*, 14135-14145.

Chen,Z., Saffitz,J.E., Latour,M.A., Schonfeld,G. (1999). Truncated apo B-70.5-containing lipoproteins bind to megalin but not the LDL receptor. J.Clin.Invest *103*, 1419-1430.

Chondrogianni,N., Gonos,E.S. (2005). Proteasome dysfunction in mammalian aging: steps and factors involved. Exp.Gerontol. *40*, 931-938.

Chondrogianni,N., Tzavelas,C., Pemberton,A.J., Nezis,I.P., Rivett,A.J., Gonos,E.S. (2005). Overexpression of proteasome beta5 assembled subunit increases the amount of proteasome and confers ameliorated response to oxidative stress and higher survival rates. J.Biol.Chem. *280*, 11840-11850.

Chuck,S.L., Lingappa,V.R. (1992). Pause transfer: a topogenic sequence in apolipoprotein B mediates stopping and restarting of translocation. Cell *68*, 9-21.

Chuck,S.L., Lingappa,V.R. (1993). Analysis of a pause transfer sequence from apolipoprotein B. J.Biol.Chem. *268*, 22794-22801.

Cianflone,K.M., Yasruel,Z., Rodriguez,M.A., Vas,D., Sniderman,A.D. (1990). Regulation of apoB secretion from HepG2 cells: evidence for a critical role for cholesteryl ester synthesis in the response to a fatty acid challenge. J.Lipid Res. *31*, 2045-2055.

Cladaras,C., Hadzopoulou-Cladaras,M., Nolte,R.T., Atkinson,D., Zannis,V.I. (1986). The complete sequence and structural analysis of human apolipoprotein B-100: relationship between apoB-100 and apoB-48 forms. EMBO J. *5*, 3495-3507.

Clemons,W.M., Jr., Menetret,J.F., Akey,C.W., Rapoport,T.A. (2004). Structural insight into the protein translocation channel. Curr.Opin.Struct.Biol. *14*, 390-396.

Corsi,A.K., Schekman,R. (1996). Mechanism of polypeptide translocation into the endoplasmic reticulum. J.Biol.Chem. *271*, 30299-30302.

Cortner,J.A., Coates,P.M., Bennett,M.J., Cryer,D.R., Le,N.A. (1991). Familial combined hyperlipidaemia: use of stable isotopes to demonstrate overproduction of very low-density lipoprotein apolipoprotein B by the liver. J.Inherit.Metab Dis. *14*, 915-922.

Dai,R.M., Chen,E., Longo,D.L., Gorbea,C.M., Li,C.C. (1998). Involvement of valosin-containing protein, an ATPase Co-purified with IkappaBalpha and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IkappaBalpha. J.Biol.Chem. *273*, 3562-3573.

Dashti,N., Williams,D.L., Alaupovic,P. (1989). Effects of oleate and insulin on the production rates and cellular mRNA concentrations of apolipoproteins in HepG2 cells. J.Lipid Res. *30*, 1365-1373.

Davidson,N.O., Shelness,G.S. (2000). APOLIPOPROTEIN B: mRNA editing, lipoprotein assembly, and presecretory degradation. Annu.Rev.Nutr. *20*, 169-193.

Davis,R.A. (1999). Cell and molecular biology of the assembly and secretion of apolipoprotein B-containing lipoproteins by the liver. Biochim.Biophys.Acta *1440*, 1-31.

Davis,R.A., Thrift,R.N., Wu,C.C., Howell,K.E. (1990). Apolipoprotein B is both integrated into and translocated across the endoplasmic reticulum membrane. Evidence for two functionally distinct pools. J.Biol.Chem. *265*, 10005-10011.

de Virgilio,M., Weninger,H., Ivessa,N.E. (1998). Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. J.Biol.Chem. *273*, 9734-9743.

Deak,P.M., Wolf,D.H. (2001). Membrane topology and function of Der3/Hrd1p as a ubiquitin-protein ligase (E3) involved in endoplasmic reticulum degradation. J.Biol.Chem. *276*, 10663-10669.

Demmer,L.A., Levin,M.S., Elovson,J., Reuben,M.A., Lusis,A.J., Gordon,J.I. (1986). Tissue-specific expression and developmental regulation of the rat apolipoprotein B gene. Proc.Natl.Acad.Sci.U.S.A *83*, 8102-8106.

Dixon,J.L., Chattapadhyay,R., Huima,T., Redman,C.M., Banerjee,D. (1992). Biosynthesis of lipoprotein: location of nascent apoAI and apoB in the rough endoplasmic reticulum of chicken hepatocytes. J.Cell Biol. *117*, 1161-1169.

Dixon,J.L., Furukawa,S., Ginsberg,H.N. (1991). Oleate stimulates secretion of apolipoprotein B-containing lipoproteins from Hep G2 cells by inhibiting early intracellular degradation of apolipoprotein B. J.Biol.Chem. *266*, 5080-5086.

Dorner,A.J., Wasley,L.C., Kaufman,R.J. (1992). Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. EMBO J. *11*, 1563-1571.

Du,E.Z., Kurth,J., Wang,S.L., Humiston,P., Davis,R.A. (1994). Proteolysis-coupled secretion of the N terminus of apolipoprotein B. Characterization of a transient, translocation arrested intermediate. J.Biol.Chem. *269*, 24169-24176.

Du,E.Z., Wang,S.L., Kayden,H.J., Sokol,R., Curtiss,L.K., Davis,R.A. (1996). Translocation of apolipoprotein B across the endoplasmic reticulum is blocked in abetalipoproteinemia. J.Lipid Res. *37*, 1309-1315.

Du,X., Stoops,J.D., Mertz,J.R., Stanley,C.M., Dixon,J.L. (1998). Identification of two regions in apolipoprotein B100 that are exposed on the cytosolic side of the endoplasmic reticulum membrane. J.Cell Biol. *141*, 585-599.

Ebert,D.L., Maeda,N., Lowe,S.W., Hasler-Rapacz,J., Rapacz,J., Attie,A.D. (1988). Primary structure comparison of the proposed low density lipoprotein (LDL) receptor binding domain of human and pig apolipoprotein B: implications for LDL-receptor interactions. J.Lipid Res. *29*, 1501-1509.

Fang,S., Ferrone,M., Yang,C., Jensen,J.P., Tiwari,S., Weissman,A.M. (2001). The tumor autocrine motility factor receptor, gp78, is a ubiquitin protein ligase implicated in degradation from the endoplasmic reticulum. Proc.Natl.Acad.Sci.U.S.A *98*, 14422-14427.

Fewell,S.W., Travers,K.J., Weissman,J.S., Brodsky,J.L. (2001). The action of molecular chaperones in the early secretory pathway. Annu.Rev.Genet. *35*, 149-191.

Fiebiger,E., Story,C., Ploegh,H.L., Tortorella,D. (2002). Visualization of the ER-to-cytosol dislocation reaction of a type I membrane protein. EMBO J. *21*, 1041-1053.

Fisher,E.A., Ginsberg,H.N. (2002). Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. J.Biol.Chem. *277*, 17377-17380.

Fisher,E.A., Pan,M., Chen,X., Wu,X., Wang,H., Jamil,H., Sparks,J.D., Williams,K.J. (2001). The triple threat to nascent apolipoprotein B. Evidence for multiple, distinct degradative pathways. J.Biol.Chem. *276*, 27855-27863.

Fisher,E.A., Zhou,M., Mitchell,D.M., Wu,X., Omura,S., Wang,H., Goldberg,A.L., Ginsberg,H.N. (1997). The degradation of apolipoprotein B100 is mediated by the ubiquitin-proteasome pathway and involves heat shock protein 70. J.Biol.Chem. *272*, 20427-20434.

Flierman,D., Ye,Y., Dai,M., Chau,V., Rapoport,T.A. (2003). Polyubiquitin serves as a recognition signal, rather than a ratcheting molecule, during retrotranslocation of proteins across the endoplasmic reticulum membrane. J.Biol.Chem. *278*, 34774-34782.

Ford,E.S., Giles,W.H., Dietz,W.H. (2002). Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. JAMA *287*, 356-359.

Fuller,W., Cuthbert,A.W. (2000). Post-translational disruption of the delta F508 cystic fibrosis transmembrane conductance regulator (CFTR)-molecular chaperone complex with geldanamycin stabilizes delta F508 CFTR in the rabbit reticulocyte lysate. J.Biol.Chem. *275*, 37462-37468.

Furukawa,S., Sakata,N., Ginsberg,H.N., Dixon,J.L. (1992). Studies of the sites of intracellular degradation of apolipoprotein B in Hep G2 cells. J.Biol.Chem. *267*, 22630-22638.

Gaffney,D., Pullinger,C.R., O'Reilly,D.S., Hoffs,M.S., Cameron,I., Vass,J.K., Kulkarni,M.V., Kane,J.P., Schumaker,V.N., Watts,G.F., Packard,C.J. (2002). Influence of an asparagine to lysine mutation at amino acid 3516 of apolipoprotein B on low-density lipoprotein receptor binding. Clin.Chim.Acta *321*, 113-121.

Ganji,S.H., Tavintharan,S., Zhu,D., Xing,Y., Kamanna,V.S., Kashyap,M.L. (2004). Niacin noncompetitively inhibits DGAT2 but not DGAT1 activity in HepG2 cells. J.Lipid Res. *45*, 1835-1845.

Gantz,D.L., Walsh,M.T., Small,D.M. (2000). Morphology of sodium deoxycholate-solubilized apolipoprotein B-100 using negative stain and vitreous ice electron microscopy. J.Lipid Res. *41*, 1464-1472.

Gardner,R.G., Swarbrick,G.M., Bays,N.W., Cronin,S.R., Wilhovsky,S., Seelig,L., Kim,C., Hampton,R.Y. (2000). Endoplasmic reticulum degradation requires lumen to cytosol signaling. Transmembrane control of Hrd1p by Hrd3p. J.Cell Biol. *151*, 69-82.

Gibbons,G.F., Bartlett,S.M., Sparks,C.E., Sparks,J.D. (1992). Extracellular fatty acids are not utilized directly for the synthesis of very-low-density lipoprotein in primary cultures of rat hepatocytes. Biochem.J. *287 (Pt 3)*, 749-753.

Gibbons,G.F., Wiggins,D., Brown,A.M., Hebbachi,A.M. (2004). Synthesis and function of hepatic very-low-density lipoprotein. Biochem.Soc.Trans. *32*, 59-64.

Gilham,D., Alam,M., Gao,W., Vance,D.E., Lehner,R. (2005). Triacylglycerol Hydrolase Is Localized to the Endoplasmic Reticulum by an Unusual Retrieval Sequence where It Participates in VLDL Assembly without Utilizing VLDL Lipids as Substrates. Mol.Biol.Cell *16*, 984-996.

Gilham,D., Ho,S., Rasouli,M., Martres,P., Vance,D.E., Lehner,R. (2003). Inhibitors of hepatic microsomal triacylglycerol hydrolase decrease very low density lipoprotein secretion. FASEB J. *17*, 1685-1687.

Gillece,P., Luz,J.M., Lennarz,W.J., de La Cruz,F.J., Romisch,K. (1999). Export of a cysteine-free misfolded secretory protein from the endoplasmic reticulum for degradation requires interaction with protein disulfide isomerase. J.Cell Biol. *147*, 1443-1456.

Gillian-Daniel,D.L., Bates,P.W., Tebon,A., Attie,A.D. (2002). Endoplasmic reticulum localization of the low density lipoprotein receptor mediates presecretory degradation of apolipoprotein B. Proc.Natl.Acad.Sci.U.S.A *99*, 4337-4342.

Goldberg,A.L. (2003). Protein degradation and protection against misfolded or damaged proteins. Nature *426*, 895-899.

Goldstein,J.L., Brown,M.S. (1977a). Atherosclerosis: the low-density lipoprotein receptor hypothesis. Metabolism *26*, 1257-1275.

Goldstein,J.L., Brown,M.S. (1977b). The low-density lipoprotein pathway and its relation to atherosclerosis. Annu.Rev.Biochem. *46*, 897-930.

Goormaghtigh,E., De Meutter,J., Vanloo,B., Brasseur,R., Rosseneu,M., Ruysschaert,J.M. (1989). Evaluation of the secondary structure of apo B-100 in low-density lipoprotein (LDL) by infrared spectroscopy. Biochim.Biophys.Acta *1006*, 147-150.

Gordon,D.A., Jamil,H. (2000). Progress towards understanding the role of microsomal triglyceride transfer protein in apolipoprotein-B lipoprotein assembly. Biochim.Biophys.Acta *1486*, 72-83.

Gordon,D.A., Jamil,H., Gregg,R.E., Olofsson,S.O., Boren,J. (1996). Inhibition of the microsomal triglyceride transfer protein blocks the first step of apolipoprotein B lipoprotein assembly but not the addition of bulk core lipids in the second step. J.Biol.Chem. *271*, 33047-33053.

Gordon,D.A., Jamil,H., Sharp,D., Mullaney,D., Yao,Z., Gregg,R.E., Wetterau,J. (1994). Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. Proc.Natl.Acad.Sci.U.S.A *91*, 7628-7632.

Graham,D.L., Knott,T.J., Jones,T.C., Pease,R.J., Pullinger,C.R., Scott,J. (1991). Carboxyl-terminal truncation of apolipoprotein B results in gradual loss of the ability to form buoyant lipoproteins in cultured human and rat liver cell lines. Biochemistry *30*, 5616-5621.

Groll,M., Ditzel,L., Lowe,J., Stock,D., Bochtler,M., Bartunik,H.D., Huber,R. (1997). Structure of 20S proteasome from yeast at 2.4 A resolution. Nature *386*, 463-471.

Gusarova,V., Brodsky,J.L., Fisher,E.A. (2003). Apolipoprotein B100 exit from the endoplasmic reticulum (ER) is COPII-dependent, and its lipidation to very low density lipoprotein occurs post-ER. J.Biol.Chem. *278*, 48051-48058.

Gusarova,V., Caplan,A.J., Brodsky,J.L., Fisher,E.A. (2001). Apoprotein B degradation is promoted by the molecular chaperones hsp90 and hsp70. J.Biol.Chem. *276*, 24891-24900.

Haglund,K., Dikic,I. (2005). Ubiquitylation and cell signaling. EMBO J. *24*, 3353-3359.

Hamman,B.D., Chen,J.C., Johnson,E.E., Johnson,A.E. (1997). The aqueous pore through the translocon has a diameter of 40-60 A during cotranslational protein translocation at the ER membrane. Cell *89*, 535-544.

Hampton,R.Y. (2002). ER-associated degradation in protein quality control and cellular regulation. Curr.Opin.Cell Biol. *14*, 476-482.

Hebbachi,A.M., Gibbons,G.F. (2001). Microsomal membrane-associated apoB is the direct precursor of secreted VLDL in primary cultures of rat hepatocytes. J.Lipid Res. *42*, 1609-1617.

Hebert,D.N., Foellmer,B., Helenius,A. (1995). Glucose trimming and reglucosylation determine glycoprotein association with calnexin in the endoplasmic reticulum. Cell *81*, 425-433.

Hegde,R.S., Lingappa,V.R. (1996). Sequence-specific alteration of the ribosome-membrane junction exposes nascent secretory proteins to the cytosol. Cell *85*, 217-228.

Hegde,R.S., Voigt,S., Rapoport,T.A., Lingappa,V.R. (1998). TRAM regulates the exposure of nascent secretory proteins to the cytosol during translocation into the endoplasmic reticulum. Cell *92*, 621-631.

Helenius,A., Aebi,M. (2004). Roles of N-linked glycans in the endoplasmic reticulum. Annu.Rev.Biochem. *73*, 1019-1049.

Herscovitz,H., Derksen,A., Walsh,M.T., McKnight,C.J., Gantz,D.L., Hadzopoulou-Cladaras,M., Zannis,V., Curry,C., Small,D.M. (2001). The N-terminal 17% of apoB binds tightly and irreversibly to emulsions modeling nascent very low density lipoproteins. J.Lipid Res. *42*, 51-59.

Herscovitz,H., Hadzopoulou-Cladaras,M., Walsh,M.T., Cladaras,C., Zannis,V.I., Small,D.M. (1991). Expression, secretion, and lipid-binding characterization of the N-terminal 17% of apolipoprotein B. Proc.Natl.Acad.Sci.U.S.A *88*, 7313-7317.

Hershey,J.W. (1991). Translational control in mammalian cells. Annu.Rev.Biochem. *60*, 717-755.

Hershko,A., Ciechanover,A. (1998). The ubiquitin system. Annu.Rev.Biochem. *67*, 425-479.

Hevonoja,T., Pentikainen,M.O., Hyvonen,M.T., Kovanen,P.T., Ala-Korpela,M. (2000). Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim.Biophys.Acta *1488*, 189-210.

Hicke,L. (2001). Protein regulation by monoubiquitin. Nat.Rev.Mol.Cell Biol. *2*, 195-201.

Higgins,J.A. (1988). Evidence that during very low density lipoprotein assembly in rat hepatocytes most of the triacylglycerol and phospholipid are packaged with apolipoprotein B in the Golgi complex. FEBS Lett. *232*, 405-408.

Hill,K., Cooper,A.A. (2000). Degradation of unassembled Vph1p reveals novel aspects of the yeast ER quality control system. EMBO J. *19*, 550-561.

Hiller,M.M., Finger,A., Schweiger,M., Wolf,D.H. (1996). ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. Science *273*, 1725-1728.

Hirsch,C., Ploegh,H.L. (2000). Intracellular targeting of the proteasome. Trends Cell Biol. *10*, 268-272.

Hitchcock,A.L., Krebber,H., Frietze,S., Lin,A., Latterich,M., Silver,P.A. (2001). The conserved npl4 protein complex mediates proteasome-dependent membrane-bound transcription factor activation. Mol.Biol.Cell *12*, 3226-3241.

Hoppe,T. (2005). Multiubiquitylation by E4 enzymes: 'one size' doesn't fit all. Trends Biochem.Sci. *30*, 183-187.

Horwich,A.L., Weber-Ban,E.U., Finley,D. (1999). Chaperone rings in protein folding and degradation. Proc.Natl.Acad.Sci.U.S.A *96*, 11033-11040.

Hosokawa,N., Wada,I., Hasegawa,K., Yorihuzi,T., Tremblay,L.O., Herscovics,A., Nagata,K. (2001). A novel ER alpha-mannosidase-like protein accelerates ER-associated degradation. EMBO Rep. *2*, 415-422.

Huang,X.F., Shelness,G.S. (1997). Identification of cysteine pairs within the amino-terminal 5% of apolipoprotein B essential for hepatic lipoprotein assembly and secretion. J.Biol.Chem. *272*, 31872-31876.

Huang,X.F., Shelness,G.S. (1999). Efficient glycosylation site utilization by intracellular apolipoprotein B. Implications for proteasomal degradation. J.Lipid Res. *40*, 2212-2222.

Humphries,S.E., Talmud,P.J. (1995). Hyperlipidaemia associated with genetic variation in the apolipoprotein B gene. Curr.Opin.Lipidol. *6*, 215-222.

Huppa,J.B., Ploegh,H.L. (1997). The alpha chain of the T cell antigen receptor is degraded in the cytosol. Immunity. *7*, 113-122.

Hussain,M.M. (2000). A proposed model for the assembly of chylomicrons. Atherosclerosis *148*, 1-15.

Hussain,M.M., Bakillah,A., Jamil,H. (1997). Apolipoprotein B binding to microsomal triglyceride transfer protein decreases with increases in length and lipidation: implications in lipoprotein biosynthesis. Biochemistry *36*, 13060-13067.

Hussain,M.M., Bakillah,A., Nayak,N., Shelness,G.S. (1998). Amino acids 430-570 in apolipoprotein B are critical for its binding to microsomal triglyceride transfer protein. J.Biol.Chem. *273*, 25612-25615.

Hussain,M.M., Shi,J., Dreizen,P. (2003). Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. J.Lipid Res. *44*, 22-32.

Hussain,M.M., Strickland,D.K., Bakillah,A. (1999). The mammalian low-density lipoprotein receptor family. Annu.Rev.Nutr. *19*, 141-172.

Hussain,M.M., Zhao,Y., Kancha,R.K., Blackhart,B.D., Yao,Z. (1995). Characterization of recombinant human apoB-48-containing lipoproteins in rat hepatoma McA-RH7777 cells transfected with apoB-48 cDNA. Overexpression of apoB-48 decreases synthesis of endogenous apoB-100. Arterioscler.Thromb.Vasc.Biol. *15*, 485-494.

Huyer,G., Piluek,W.F., Fansler,Z., Kreft,S.G., Hochstrasser,M., Brodsky,J.L., Michaelis,S. (2004). Distinct machinery is required in Saccharomyces cerevisiae for the endoplasmic reticulum-associated degradation of a multispanning membrane protein and a soluble luminal protein. J.Biol.Chem. *279*, 38369-38378.

Huyton,T., Pye,V.E., Briggs,L.C., Flynn,T.C., Beuron,F., Kondo,H., Ma,J., Zhang,X., Freemont,P.S. (2003). The crystal structure of murine p97/VCP at 3.6A. J.Struct.Biol. *144*, 337-348.

Imamura,T., Haruta,T., Takata,Y., Usui,I., Iwata,M., Ishihara,H., Ishiki,M., Ishibashi,O., Ueno,E., Sasaoka,T., Kobayashi,M. (1998). Involvement of heat shock protein 90 in the degradation of mutant insulin receptors by the proteasome. J.Biol.Chem. *273*, 11183-11188.

Ingram,M.F., Shelness,G.S. (1996). Apolipoprotein B-100 destined for lipoprotein assembly and intracellular degradation undergoes efficient translocation across the endoplasmic reticulum membrane. J.Lipid Res. *37*, 2202-2214.

Ingram,M.F., Shelness,G.S. (1997). Folding of the amino-terminal domain of apolipoprotein B initiates microsomal triglyceride transfer protein-dependent lipid transfer to nascent very low density lipoprotein. J.Biol.Chem. *272*, 10279-10286.

Innerarity,T.L., Young,S.G., Poksay,K.S., Mahley,R.W., Smith,R.S., Milne,R.W., Marcel,Y.L., Weisgraber,K.H. (1987). Structural relationship of human apolipoprotein B48 to apolipoprotein B100. J.Clin.Invest *80*, 1794-1798.

Jamil,H., Chu,C.H., Dickson,J.K., Jr., Chen,Y., Yan,M., Biller,S.A., Gregg,R.E., Wetterau,J.R., Gordon,D.A. (1998). Evidence that microsomal triglyceride transfer protein is limiting in the production of apolipoprotein B-containing lipoproteins in hepatic cells. J.Lipid Res. *39*, 1448-1454.

Jamil,H., Dickson,J.K., Jr., Chu,C.H., Lago,M.W., Rinehart,J.K., Biller,S.A., Gregg,R.E., Wetterau,J.R. (1995). Microsomal triglyceride transfer protein. Specificity of lipid binding and transport. J.Biol.Chem. *270*, 6549-6554.

Jamil,H., Gordon,D.A., Eustice,D.C., Brooks,C.M., Dickson,J.K., Jr., Chen,Y., Ricci,B., Chu,C.H., Harrity,T.W., Ciosek,C.P., Jr., Biller,S.A., Gregg,R.E., Wetterau,J.R. (1996). An inhibitor of the microsomal triglyceride transfer protein inhibits apoB secretion from HepG2 cells. Proc.Natl.Acad.Sci.U.S.A *93*, 11991-11995.

Janus,E.D., Nicoll,A., Wootton,R., Turner,P.R., Magill,P.J., Lewis,B. (1980a). Quantitative studies of very low density lipoprotein: conversion to low density lipoprotein in normal controls and primary hyperlipidaemic states and the role of direct secretion of low density lipoprotein in heterozygous familial hypercholesterolaemia. Eur.J.Clin.Invest *10*, 149-159.

Janus,E.D., Nicoll,A.M., Turner,P.R., Magill,P., Lewis,B. (1980b). Kinetic bases of the primary hyperlipidaemias: studies of apolipoprotein B turnover in genetically defined subjects. Eur.J.Clin.Invest *10*, 161-172.

Jarosch,E., Taxis,C., Volkwein,C., Bordallo,J., Finley,D., Wolf,D.H., Sommer,T. (2002). Protein dislocation from the ER requires polyubiquitination and the AAA-ATPase Cdc48. Nat.Cell Biol. *4*, 134-139.

Jiang,X.C., Li,Z., Liu,R., Yang,X.P., Pan,M., Lagrost,L., Fisher,E.A., Williams,K.J. (2005a). Phospholipid transfer protein deficiency impairs apolipoprotein-B secretion from hepatocytes by stimulating a proteolytic pathway through a relative deficiency of vitamin E and an increase in intracellular oxidants. J.Biol.Chem. *280*, 18336-18340.

Jiang,Z.G., Carraway,M., McKnight,C.J. (2005b). Limited proteolysis and biophysical characterization of the lipovitellin homology region in apolipoprotein B. Biochemistry *44*, 1163-1173.

Johs,A., Hammel,M., Waldner,I., May,R.P., Laggner,P., Prassl,R. (2006). Modular structure of solubilized human apolipoprotein B-100: Low resolution model revealed by small angle neutron scattering. J.Biol.Chem.

Jones,B., Jones,E.L., Bonney,S.A., Patel,H.N., Mensenkamp,A.R., Eichenbaum-Voline,S., Rudling,M., Myrdal,U., Annesi,G., Naik,S., Meadows,N., Quattrone,A., Islam,S.A., Naoumova,R.P., Angelin,B., Infante,R., Levy,E., Roy,C.C., Freemont,P.S., Scott,J., Shoulders,C.C. (2003). Mutations in a Sar1 GTPase of COPII vesicles are associated with lipid absorption disorders. Nat.Genet. *34*, 29-31.

Jones,H.B., Gofman,J.W., Lindgren,F.T., Lyon,T.P., Graham,D.M., Strisower,B., Nichols,A.V. (1951). Lipoproteins in atherosclerosis. Am.J.Med. *11*, 358-380.

Kalies,K.U., Allan,S., Sergeyenko,T., Kroger,H., Romisch,K. (2005). The protein translocation channel binds proteasomes to the endoplasmic reticulum membrane. EMBO J. *24*, 2284-2293.

Karaveg,K., Siriwardena,A., Tempel,W., Liu,Z.J., Glushka,J., Wang,B.C., Moremen,K.W. (2005). Mechanism of class 1 (glycosylhydrolase family 47) {alpha}-mannosidases involved in N-glycan processing and endoplasmic reticulum quality control. J.Biol.Chem. *280*, 16197-16207.

Kikkert,M., Hassink,G., Barel,M., Hirsch,C., van der Wal,F.J., Wiertz,E. (2001). Ubiquitination is essential for human cytomegalovirus US11-mediated dislocation of MHC class I molecules from the endoplasmic reticulum to the cytosol. Biochem.J. *358*, 369-377.

Kivlen,M.H., Dorsey,C.A., Lingappa,V.R., Hegde,R.S. (1997). Asymmetric distribution of pause transfer sequences in apolipoprotein B-100. J.Lipid Res. *38*, 1149-1162.

Knop,M., Finger,A., Braun,T., Hellmuth,K., Wolf,D.H. (1996). Der1, a novel protein specifically required for endoplasmic reticulum degradation in yeast. EMBO J. *15*, 753-763.

Knott,T.J., Pease,R.J., Powell,L.M., Wallis,S.C., Rall,S.C., Jr., Innerarity,T.L., Blackhart,B., Taylor,W.H., Marcel,Y., Milne,R., . (1986). Complete protein sequence and identification of structural domains of human apolipoprotein B. Nature *323*, 734-738.

Kondo,H., Rabouille,C., Newman,R., Levine,T.P., Pappin,D., Freemont,P., Warren,G. (1997). p47 is a cofactor for p97-mediated membrane fusion. Nature *388*, 75-78.

Kothe,M., Ye,Y., Wagner,J.S., De Luca,H.E., Kern,E., Rapoport,T.A., Lencer,W.I. (2005). Role of p97 AAA-ATPase in the retrotranslocation of the cholera toxin A1 chain, a non-ubiquitinated substrate. J.Biol.Chem. *280*, 28127-28132.

Krauss,R.M., Burke,D.J. (1982). Identification of multiple subclasses of plasma low density lipoproteins in normal humans. J.Lipid Res. *23*, 97-104.

Kuge,O., Dascher,C., Orci,L., Rowe,T., Amherdt,M., Plutner,H., Ravazzola,M., Tanigawa,G., Rothman,J.E., Balch,W.E. (1994). Sar1 promotes vesicle budding from the endoplasmic reticulum but not Golgi compartments. J.Cell Biol. *125*, 51-65.

Kulinski,A., Rustaeus,S., Vance,J.E. (2002). Microsomal triacylglycerol transfer protein is required for lumenal accretion of triacylglycerol not associated with ApoB, as well as for ApoB lipidation. J.Biol.Chem. *277*, 31516-31525.

Kulinski,A., Vance,D.E., Vance,J.E. (2004). A choline-deficient diet in mice inhibits neither the CDP-choline pathway for phosphatidylcholine synthesis in hepatocytes nor apolipoprotein B secretion. J.Biol.Chem. *279*, 23916-23924.

Kushwaha,R.S., McMahan,C.A., Mott,G.E., Carey,K.D., Reardon,C.A., Getz,G.S., McGill,H.C., Jr. (1991). Influence of dietary lipids on hepatic mRNA levels of proteins regulating plasma lipoproteins in baboons with high and low levels of large high density lipoproteins. J.Lipid Res. *32*, 1929-1940.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Latterich,M., Frohlich,K.U., Schekman,R. (1995). Membrane fusion and the cell cycle: Cdc48p participates in the fusion of ER membranes. Cell *82*, 885-893.

Law,A., Scott,J. (1990). A cross-species comparison of the apolipoprotein B domain that binds to the LDL receptor. J.Lipid Res. *31*, 1109-1120.

Law,S.W., Grant,S.M., Higuchi,K., Hospattankar,A., Lackner,K., Lee,N., Brewer,H.B., Jr. (1986). Human liver apolipoprotein B-100 cDNA: complete nucleic acid and derived amino acid sequence. Proc.Natl.Acad.Sci.U.S.A *83*, 8142-8146.

Ledford,A.S., Weinberg,R.B., Cook,V.R., Hantgan,R.R., Shelness,G.S. (2006). Self-association and lipid binding properties of the lipoprotein initiating domain of apolipoprotein B. J.Biol.Chem.

Lee,C., Schwartz,M.P., Prakash,S., Iwakura,M., Matouschek,A. (2001). ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. Mol.Cell *7*, 627-637.

Lee,R.J., Liu,C.W., Harty,C., McCracken,A.A., Latterich,M., Romisch,K., DeMartino,G.N., Thomas,P.J., Brodsky,J.L. (2004). Uncoupling retro-translocation and degradation in the ER-associated degradation of a soluble protein. EMBO J. *23*, 2206-2215.

Lee,R.J., McCracken,A.A., Brodsky,J.L. (2005). Reconstitution of endoplasmic reticulum-associated degradation using yeast membranes and cytosol. Methods Mol.Biol. *301*, 175-184.

Leiper,J.M., Bayliss,J.D., Pease,R.J., Brett,D.J., Scott,J., Shoulders,C.C. (1994). Microsomal triglyceride transfer protein, the abetalipoproteinemia gene product, mediates the secretion of apolipoprotein B-containing lipoproteins from heterologous cells. J.Biol.Chem. *269*, 21951-21954.

Leung,G.K., Veniant,M.M., Kim,S.K., Zlot,C.H., Raabe,M., Bjorkegren,J., Neese,R.A., Hellerstein,M.K., Young,S.G. (2000). A deficiency of microsomal triglyceride transfer protein reduces apolipoprotein B secretion. J.Biol.Chem. *275*, 7515-7520.

Levy,E., Stan,S., Delvin,E., Menard,D., Shoulders,C., Garofalo,C., Slight,I., Seidman,E., Mayer,G., Bendayan,M. (2002). Localization of microsomal triglyceride transfer protein in the Golgi: possible role in the assembly of chylomicrons. J.Biol.Chem. *277*, 16470-16477.

Li,Z., Kako,Y., Pang,L., Freeman,M.W., Glick,J.M., Wang,X., Goldberg,I.J. (2000). Effects of overexpression of the amino-terminal fragment of apolipoprotein B on apolipoprotein B and lipoprotein production. J.Lipid Res. *41*, 1912-1920.

Liang,J., Ginsberg,H.N. (2001). Microsomal triglyceride transfer protein binding and lipid transfer activities are independent of each other, but both are required for secretion of apolipoprotein B lipoproteins from liver cells. J.Biol.Chem. *276*, 28606-28612.

Liang,J., Wu,X., Jiang,H., Zhou,M., Yang,H., Angkeow,P., Huang,L.S., Sturley,S.L., Ginsberg,H. (1998). Translocation efficiency, susceptibility to proteasomal degradation, and lipid responsiveness of apolipoprotein B are determined by the presence of beta sheet domains. J.Biol.Chem. *273*, 35216-35221.

Liang,J.J., Oelkers,P., Guo,C., Chu,P.C., Dixon,J.L., Ginsberg,H.N., Sturley,S.L. (2004). Overexpression of human diacylglycerol acyltransferase 1, acyl-coa:cholesterol acyltransferase 1, or acyl-CoA:cholesterol acyltransferase 2 stimulates secretion of apolipoprotein B-containing lipoproteins in McA-RH7777 cells. J.Biol.Chem. *279*, 44938-44944.

Liang,J.S., Distler,O., Cooper,D.A., Jamil,H., Deckelbaum,R.J., Ginsberg,H.N., Sturley,S.L. (2001). HIV protease inhibitors protect apolipoprotein B from degradation by the proteasome: a potential mechanism for protease inhibitor-induced hyperlipidemia. Nat.Med. *7*, 1327-1331.

Liang,J.S., Kim,T., Fang,S., Yamaguchi,J., Weissman,A.M., Fisher,E.A., Ginsberg,H.N. (2003). Overexpression of the tumor autocrine motility factor receptor Gp78, a ubiquitin protein ligase, results in increased ubiquitinylation and decreased secretion of apolipoprotein B100 in HepG2 cells. J.Biol.Chem. *278*, 23984-23988.

Liang,S., Wu,X., Fisher,E.A., Ginsberg,H.N. (2000). The amino-terminal domain of apolipoprotein B does not undergo retrograde translocation from the endoplasmic reticulum to the cytosol. Proteasomal degradation of nascent apolipoprotein B begins at the carboxyl terminus of the protein, while apolipoprotein B is still in its original translocon. J.Biol.Chem. *275*, 32003-32010.

Liao,W., Chan,L. (2001). Tunicamycin induces ubiquitination and degradation of apolipoprotein B in HepG2 cells. Biochem.J. *353*, 493-501.

Liao,W., Chang,B.H., Mancini,M., Chan,L. (2003a). Ubiquitin-dependent and - independent proteasomal degradation of apoB associated with endoplasmic reticulum and Golgi apparatus, respectively, in HepG2 cells. J.Cell Biochem. *89*, 1019-1029.

Liao,W., Hui,T.Y., Young,S.G., Davis,R.A. (2003b). Blocking microsomal triglyceride transfer protein interferes with apoB secretion without causing retention or stress in the ER. J.Lipid Res. *44*, 978-985.

Liao,W., Yeung,S.C., Chan,L. (1998). Proteasome-mediated degradation of apolipoprotein B targets both nascent peptides cotranslationally before translocation and full-length apolipoprotein B after translocation into the endoplasmic reticulum. J.Biol.Chem. *273*, 27225-27230.

Lilley,B.N., Ploegh,H.L. (2004). A membrane protein required for dislocation of misfolded proteins from the ER. Nature *429*, 834-840.

Lindquist,J.A., Jensen,O.N., Mann,M., Hammerling,G.J. (1998). ER-60, a chaperone with thiol-dependent reductase activity involved in MHC class I assembly. EMBO J. *17*, 2186-2195.

Lingappa,V.R., Rutkowski,D.T., Hegde,R.S., Andersen,O.S. (2002). Conformational control through translocational regulation: a new view of secretory and membrane protein folding. Bioessays *24*, 741-748.

Linnik,K.M., Herscovitz,H. (1998). Multiple molecular chaperones interact with apolipoprotein B during its maturation. The network of endoplasmic reticulum-resident chaperones (ERp72, GRP94, calreticulin, and BiP) interacts with apolipoprotein b regardless of its lipidation state. J.Biol.Chem. *273*, 21368-21373.

Luders,J., Demand,J., Hohfeld,J. (2000). The ubiquitin-related BAG-1 provides a link between the molecular chaperones Hsc70/Hsp70 and the proteasome. J.Biol.Chem. *275*, 4613-4617.

Lyon,T.P., Yankley,A., Gofman,J.W., Strisower,B. (1956). Lipoproteins and diet in coronary heart diseases; a five-year study. Calif.Med. *84*, 325-328.

Macri,J., Adeli,K. (1997a). Conformational changes in apolipoprotein B modulate intracellular assembly and degradation of ApoB-containing lipoprotein particles in HepG2 cells. Arterioscler.Thromb.Vasc.Biol. *17*, 2982-2994.

Macri,J., Adeli,K. (1997b). Studies on intracellular translocation of apolipoprotein B in a permeabilized HepG2 system. J.Biol.Chem. *272*, 7328-7337.

Macri,J., Kazemian,P., Kulinski,A., Rudy,D., Aiton,A., Thibert,R.J., Adeli,K. (2000). Translocational status of ApoB in the presence of an inhibitor of microsomal triglyceride transfer protein. Biochem.Biophys.Res.Commun. *276*, 1035-1047.

Mancini,R., Aebi,M., Helenius,A. (2003). Multiple endoplasmic reticulum-associated pathways degrade mutant yeast carboxypeptidase Y in mammalian cells. J.Biol.Chem. *278*, 46895-46905.

Mann,C.J., Anderson,T.A., Read,J., Chester,S.A., Harrison,G.B., Kochl,S., Ritchie,P.J., Bradbury,P., Hussain,F.S., Amey,J., Vanloo,B., Rosseneu,M., Infante,R., Hancock,J.M., Levitt,D.G., Banaszak,L.J., Scott,J., Shoulders,C.C. (1999). The structure of vitellogenin provides a molecular model for the assembly and secretion of atherogenic lipoproteins. J.Mol.Biol. *285*, 391-408.

Marcel,Y.L., Hogue,M., Theolis,R., Jr., Milne,R.W. (1982). Mapping of antigenic determinants of human apolipoprotein B using monoclonal antibodies against low density lipoproteins. J.Biol.Chem. *257*, 13165-13168.

Marcel,Y.L., Hogue,M., Weech,P.K., Milne,R.W. (1984). Characterization of antigenic determinants on human solubilized apolipoprotein B. Conformational requirements for lipids. J.Biol.Chem. *259*, 6952-6957.

Mayer,T.U., Braun,T., Jentsch,S. (1998). Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein. EMBO J. *17*, 3251-3257.

McCracken,A.A., Brodsky,J.L. (1996). Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. J.Cell Biol. *132*, 291-298.

McCracken,A.A., Brodsky,J.L. (2003). Evolving questions and paradigm shifts in endoplasmic-reticulum-associated degradation (ERAD). Bioessays *25*, 868-877.

McLeod,R.S., Wang,Y., Wang,S., Rusinol,A., Links,P., Yao,Z. (1996). Apolipoprotein B sequence requirements for hepatic very low density lipoprotein assembly. Evidence that hydrophobic sequences within apolipoprotein B48 mediate lipid recruitment. J.Biol.Chem. *271*, 18445-18455.

McLeod,R.S., Zhao,Y., Selby,S.L., Westerlund,J., Yao,Z. (1994). Carboxyl-terminal truncation impairs lipid recruitment by apolipoprotein B100 but does not affect secretion of the truncated apolipoprotein B-containing lipoproteins. J.Biol.Chem. *269*, 2852-2862.

McNamara,J.R., Small,D.M., Li,Z., Schaefer,E.J. (1996). Differences in LDL subspecies involve alterations in lipid composition and conformational changes in apolipoprotein B. J.Lipid Res. *37*, 1924-1935.

Menetret,J.F., Neuhof,A., Morgan,D.G., Plath,K., Radermacher,M., Rapoport,T.A., Akey,C.W. (2000). The structure of ribosome-channel complexes engaged in protein translocation. Mol.Cell *6*, 1219-1232.

Meusser,B., Hirsch,C., Jarosch,E., Sommer,T. (2005). ERAD: the long road to destruction. Nat.Cell Biol. *7*, 766-772.

Meyer,H.H., Shorter,J.G., Seemann,J., Pappin,D., Warren,G. (2000). A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. EMBO J. *19*, 2181-2192.

Meyer,H.H., Wang,Y., Warren,G. (2002). Direct binding of ubiquitin conjugates by the mammalian p97 adaptor complexes, p47 and Ufd1-Npl4. EMBO J. *21*, 5645-5652.

Millar,J.S., Maugeais,C., Fuki,I.V., Rader,D.J. (2002). Normal production rate of apolipoprotein B in LDL receptor-deficient mice. Arterioscler.Thromb.Vasc.Biol. *22*, 989-994.

Millar,J.S., Maugeais,C., Ikewaki,K., Kolansky,D.M., Barrett,P.H., Budreck,E.C., Boston,R.C., Tada,N., Mochizuki,S., Defesche,J.C., Wilson,J.M., Rader,D.J. (2005). Complete Deficiency of the Low-Density Lipoprotein Receptor Is Associated With Increased Apolipoprotein B-100 Production. Arterioscler.Thromb.Vasc.Biol. *25*, 560-565.

Mimnaugh,E.G., Xu,W., Vos,M., Yuan,X., Isaacs,J.S., Bisht,K.S., Gius,D., Neckers,L. (2004). Simultaneous inhibition of hsp 90 and the proteasome promotes protein ubiquitination, causes endoplasmic reticulum-derived cytosolic vacuolization, and enhances antitumor activity. Mol.Cancer Ther. *3*, 551-566.

Mitchell,D.M., Zhou,M., Pariyarath,R., Wang,H., Aitchison,J.D., Ginsberg,H.N., Fisher,E.A. (1998). Apoprotein B100 has a prolonged interaction with the translocon during which its lipidation and translocation change from dependence on the microsomal triglyceride transfer protein to independence. Proc.Natl.Acad.Sci.U.S.A *95*, 14733-14738.

Moberly,J.B., Cole,T.G., Alpers,D.H., Schonfeld,G. (1990). Oleic acid stimulation of apolipoprotein B secretion from HepG2 and Caco-2 cells occurs post-transcriptionally. Biochim.Biophys.Acta *1042*, 70-80.

Molinari,M., Calanca,V., Galli,C., Lucca,P., Paganetti,P. (2003). Role of EDEM in the release of misfolded glycoproteins from the calnexin cycle. Science *299*, 1397-1400.

Molinari,M., Sitia,R. (2005). The secretory capacity of a cell depends on the efficiency of endoplasmic reticulum-associated degradation. Curr.Top.Microbiol.Immunol. *300*, 1-15.

Morris,J.A., Dorner,A.J., Edwards,C.A., Hendershot,L.M., Kaufman,R.J. (1997). Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. J.Biol.Chem. *272*, 4327-4334.

Muresan,Z., Arvan,P. (1998). Enhanced binding to the molecular chaperone BiP slows thyroglobulin export from the endoplasmic reticulum. Mol.Endocrinol. *12*, 458-467.

Musliner,T.A., Krauss,R.M. (1988). Lipoprotein subspecies and risk of coronary disease. Clin.Chem. *34*, B78-B83.

Nakahara,D.H., Lingappa,V.R., Chuck,S.L. (1994). Translocational pausing is a common step in the biogenesis of unconventional integral membrane and secretory proteins. J.Biol.Chem. *269*, 7617-7622.

Nassir,F., Xie,Y., Patterson,B.W., Luo,J., Davidson,N.O. (2004). Hepatic secretion of small lipoprotein particles in apobec-1-/- mice is regulated by the LDL receptor. J.Lipid Res. *45*, 1649-1659.

Navaratnam,N., Morrison,J.R., Bhattacharya,S., Patel,D., Funahashi,T., Giannoni,F., Teng,B.B., Davidson,N.O., Scott,J. (1993). The p27 catalytic subunit of the apolipoprotein B mRNA editing enzyme is a cytidine deaminase. J.Biol.Chem. *268*, 20709-20712.

Nicodeme,E., Benoist,F., McLeod,R., Yao,Z., Scott,J., Shoulders,C.C., Grand-Perret,T. (1999). Identification of domains in apolipoprotein B100 that confer a high requirement for the microsomal triglyceride transfer protein. J.Biol.Chem. *274*, 1986-1993.

Nielsen,L.B., Veniant,M., Boren,J., Raabe,M., Wong,J.S., Tam,C., Flynn,L., Vanni-Reyes,T., Gunn,M.D., Goldberg,I.J., Hamilton,R.L., Young,S.G. (1998). Genes for apolipoprotein B and microsomal triglyceride transfer protein are expressed in the heart: evidence that the heart has the capacity to synthesize and secrete lipoproteins. Circulation *98*, 13-16.

Nishikawa,S., Brodsky,J.L., Nakatsukasa,K. (2005). Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). J.Biochem.(Tokyo) *137*, 551-555.

Nishikawa,S.I., Fewell,S.W., Kato,Y., Brodsky,J.L., Endo,T. (2001). Molecular chaperones in the yeast endoplasmic reticulum maintain the solubility of proteins for retrotranslocation and degradation. J.Cell Biol. *153*, 1061-1070.

Nishimaki-Mogami,T., Suzuki,K., Takahashi,A. (1996). The role of phosphatidylethanolamine methylation in the secretion of very low density lipoproteins by cultured rat hepatocytes: rapid inhibition of phosphatidylethanolamine methylation by bezafibrate increases the density of apolipoprotein B48-containing lipoproteins. Biochim.Biophys.Acta *1304*, 21-31.

Nishimaki-Mogami,T., Yao,Z., Fujimori,K. (2002). Inhibition of phosphatidylcholine synthesis via the phosphatidylethanolamine methylation pathway impairs incorporation of bulk lipids into VLDL in cultured rat hepatocytes. J.Lipid Res. *43*, 1035-1045.

Noga,A.A., Zhao,Y., Vance,D.E. (2002). An unexpected requirement for phosphatidylethanolamine N-methyltransferase in the secretion of very low density lipoproteins. J.Biol.Chem. *277*, 42358-42365.

Oberdorf,J., Carlson,E.J., Skach,W.R. (2001). Redundancy of mammalian proteasome beta subunit function during endoplasmic reticulum associated degradation. Biochemistry *40*, 13397-13405.

Oberdorf,J., Carlson,E.J., Skach,W.R. (2006). Uncoupling proteasome peptidase and ATPase activities results in cytosolic release of an ER polytopic protein. J.Cell Sci. *119*, 303-313.

Oda,Y., Hosokawa,N., Wada,I., Nagata,K. (2003). EDEM as an acceptor of terminally misfolded glycoproteins released from calnexin. Science *299*, 1394-1397.

Ohsaki,Y., Cheng,J., Fujita,A., Tokumoto,T., Fujimoto,T. (2006). Cytoplasmic Lipid Droplets Are Sites of Convergence of Proteasomal and Autophagic Degradation of Apolipoprotein B. Mol.Biol.Cell.

Olofsson,S.O., Bostrom,K., Carlsson,P., Boren,J., Wettesten,M., Bjursell,G., Wiklund,O., Bondjers,G. (1987). Structure and biosynthesis of apolipoprotein B. Am.Heart J. *113*, 446-452.

Olofsson,S.O., Stillemark-Billton,P., Asp,L. (2000). Intracellular assembly of VLDL: two major steps in separate cell compartments. Trends Cardiovasc.Med. *10*, 338-345.

Osborne,A.R., Rapoport,T.A., van den,B.B. (2005). Protein translocation by the Sec61/SecY channel. Annu.Rev.Cell Dev.Biol. *21*, 529-550.

Otsu,M., Urade,R., Kito,M., Omura,F., Kikuchi,M. (1995). A possible role of ER-60 protease in the degradation of misfolded proteins in the endoplasmic reticulum. J.Biol.Chem. *270*, 14958-14961.

Pan,M., Cederbaum,A.I., Zhang,Y.L., Ginsberg,H.N., Williams,K.J., Fisher,E.A. (2004). Lipid peroxidation and oxidant stress regulate hepatic apolipoprotein B degradation and VLDL production. J.Clin.Invest *113*, 1277-1287.

Pan,M., Liang Js,J.S., Fisher,E.A., Ginsberg,H.N. (2002). The late addition of core lipids to nascent apolipoprotein B100, resulting in the assembly and secretion of triglyceride-rich lipoproteins, is independent of both microsomal triglyceride transfer protein activity and new triglyceride synthesis. J.Biol.Chem. *277*, 4413-4421.

Pan,M., Liang,J., Fisher,E.A., Ginsberg,H.N. (2000). Inhibition of translocation of nascent apolipoprotein B across the endoplasmic reticulum membrane is associated with selective inhibition of the synthesis of apolipoprotein B. J.Biol.Chem. *275*, 27399-27405.

Parhofer,K.G., Barrett,P.H., Aguilar-Salinas,C.A., Schonfeld,G. (1996). Positive linear correlation between the length of truncated apolipoprotein B and its secretion rate: in vivo studies in human apoB-89, apoB-75, apoB-54.8, and apoB-31 heterozygotes. J.Lipid Res. *37*, 844-852.

Pariyarath,R., Wang,H., Aitchison,J.D., Ginsberg,H.N., Welch,W.J., Johnson,A.E., Fisher,E.A. (2001). Co-translational interactions of apoprotein B with the ribosome and translocon during lipoprotein assembly or targeting to the proteasome. J.Biol.Chem. *276*, 541-550.

Parodi,A.J. (2000). Protein glucosylation and its role in protein folding. Annu.Rev.Biochem. *69*, 69-93.

Patel,S., Latterich,M. (1998). The AAA team: related ATPases with diverse functions. Trends Cell Biol. *8*, 65-71.

Patel,S.B., Grundy,S.M. (1996). Interactions between microsomal triglyceride transfer protein and apolipoprotein B within the endoplasmic reticulum in a heterologous expression system. J.Biol.Chem. *271*, 18686-18694.

Patil,C., Walter,P. (2001). Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Curr.Opin.Cell Biol. *13*, 349-355.

Pease,R.J., Harrison,G.B., Leiper,J., Scott,J. (1992). Apolipoprotein B intermediates. Nature *356*, 115-116.

Pease,R.J., Harrison,G.B., Scott,J. (1991). Cotranslocational insertion of apolipoprotein B into the inner leaflet of the endoplasmic reticulum. Nature *353*, 448-450.

Pease,R.J., Leiper,J.M., Harrison,G.B., Scott,J. (1995). Studies on the translocation of the amino terminus of apolipoprotein B into the endoplasmic reticulum. J.Biol.Chem. *270*, 7261-7271.

Phillips,M.L., Schumaker,V.N. (1989). Conformation of apolipoprotein B after lipid extraction of low density lipoproteins attached to an electron microscope grid. J.Lipid Res. *30*, 415-422.

Pilon,M., Schekman,R., Romisch,K. (1997). Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. EMBO J. *16*, 4540-4548.

Plemper,R.K., Bohmler,S., Bordallo,J., Sommer,T., Wolf,D.H. (1997). Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature *388*, 891-895.

Plemper,R.K., Wolf,D.H. (1999). Retrograde protein translocation: ERADication of secretory proteins in health and disease. Trends Biochem.Sci. *24*, 266-270.

Powell,L.M., Wallis,S.C., Pease,R.J., Edwards,Y.H., Knott,T.J., Scott,J. (1987). A novel form of tissue-specific RNA processing produces apolipoprotein-B48 in intestine. Cell *50*, 831-840.

Proctor,S.D., Vine,D.F., Mamo,J.C. (2002). Arterial retention of apolipoprotein B(48)- and B(100)-containing lipoproteins in atherogenesis. Curr.Opin.Lipidol. *13*, 461-470.

Pullinger,C.R., Hennessy,L.K., Chatterton,J.E., Liu,W., Love,J.A., Mendel,C.M., Frost,P.H., Malloy,M.J., Schumaker,V.N., Kane,J.P. (1995). Familial ligand-defective apolipoprotein B. Identification of a new mutation that decreases LDL receptor binding affinity. J.Clin.Invest *95*, 1225-1234.

Pullinger,C.R., North,J.D., Teng,B.B., Rifici,V.A., Ronhild de Brito,A.E., Scott,J. (1989). The apolipoprotein B gene is constitutively expressed in HepG2 cells: regulation of secretion by oleic acid, albumin, and insulin, and measurement of the mRNA half-life. J.Lipid Res. *30*, 1065-1077.

Qiu,W., Kohen-Avramoglu,R., Mhapsekar,S., Tsai,J., Austin,R.C., Adeli,K. (2005). Glucosamine-Induced Endoplasmic Reticulum Stress Promotes ApoB100 Degradation. Evidence for Grp78-Mediated Targeting to Proteasomal Degradation. Arterioscler.Thromb.Vasc.Biol. *25*, 571-577.

Qiu,W., Kohen-Avramoglu,R., Rashid-Kolvear,F., Au,C.S., Chong,T.M., Lewis,G.F., Trinh,D.K., Austin,R.C., Urade,R., Adeli,K. (2004). Overexpression of the endoplasmic reticulum 60 protein ER-60 downregulates ApoB100 secretion by inducing its intracellular degradation via a nonproteasomal pathway: evidence for an ER-60-mediated and pCMB-sensitive intracellular degradative pathway. Biochemistry *43*, 4819-4831.

Raabe,M., Veniant,M.M., Sullivan,M.A., Zlot,C.H., Bjorkegren,J., Nielsen,L.B., Wong,J.S., Hamilton,R.L., Young,S.G. (1999). Analysis of the role of microsomal triglyceride transfer protein in the liver of tissue-specific knockout mice. J.Clin.Invest *103*, 1287-1298.

Rabinovich,E., Kerem,A., Frohlich,K.U., Diamant,N., Bar-Nun,S. (2002). AAA-ATPase p97/Cdc48p, a cytosolic chaperone required for endoplasmic reticulum-associated protein degradation. Mol.Cell Biol. *22*, 626-634.

Rape,M., Hoppe,T., Gorr,I., Kalocay,M., Richly,H., Jentsch,S. (2001). Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. Cell *107*, 667-677.

Rapoport,T.A., Goder,V., Heinrich,S.U., Matlack,K.E. (2004). Membrane-protein integration and the role of the translocation channel. Trends Cell Biol. *14*, 568-575.

Rashid,K.A., Hevi,S., Chen,Y., Le Caherec,F., Chuck,S.L. (2002). A proteomic approach identifies proteins in hepatocytes that bind nascent apolipoprotein B. J.Biol.Chem. *277*, 22010-22017.

Rava,P., Ojakian,G.K., Shelness,G.S., Hussain,M.M. (2006). Phospholipid transfer activity of microsomal triglyceride transfer protein is sufficient for the assembly and secretion of apoB-lipoproteins. J.Biol.Chem.

Ravid,T., Doolman,R., Avner,R., Harats,D., Roitelman,J. (2000). The ubiquitin-proteasome pathway mediates the regulated degradation of mammalian 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J.Biol.Chem. *275*, 35840-35847.

Richardson,P.E., Manchekar,M., Dashti,N., Jones,M.K., Beigneux,A., Young,S.G., Harvey,S.C., Segrest,J.P. (2005). Assembly of Lipoprotein Particles Containing Apolipoprotein-B: Structural Model for the Nascent Lipoprotein Particle. Biophys.J. *88*, 2789-2800.

Richly,H., Rape,M., Braun,S., Rumpf,S., Hoege,C., Jentsch,S. (2005). A series of ubiquitin binding factors connects CDC48/p97 to substrate multiubiquitylation and proteasomal targeting. Cell *120*, 73-84.

Rodighiero,C., Tsai,B., Rapoport,T.A., Lencer,W.I. (2002). Role of ubiquitination in retro-translocation of cholera toxin and escape of cytosolic degradation. EMBO Rep. *3*, 1222-1227.

Rouiller,I., Butel,V.M., Latterich,M., Milligan,R.A., Wilson-Kubalek,E.M. (2000). A major conformational change in p97 AAA ATPase upon ATP binding. Mol.Cell *6*, 1485-1490.

Rouiller,I., DeLaBarre,B., May,A.P., Weis,W.I., Brunger,A.T., Milligan,R.A., Wilson-Kubalek,E.M. (2002). Conformational changes of the multifunction p97 AAA ATPase during its ATPase cycle. Nat.Struct.Biol. *9*, 950-957.

Roy,C.C., Levy,E., Green,P.H., Sniderman,A., Letarte,J., Buts,J.P., Orquin,J., Brochu,P., Weber,A.M., Morin,C.L., . (1987). Malabsorption, hypocholesterolemia, and fat-filled enterocytes with increased intestinal apoprotein B. Chylomicron retention disease. Gastroenterology *92*, 390-399.

Rusinol,A.E., Chan,E.Y., Vance,J.E. (1993). Movement of apolipoprotein B into the lumen of microsomes from hepatocytes is disrupted in membranes enriched in phosphatidylmonomethylethanolamine. J.Biol.Chem. *268*, 25168-25175.

Rusinol,A.E., Hegde,R.S., Chuck,S.L., Lingappa,V.R., Vance,J.E. (1998). Translocational pausing of apolipoprotein B can be regulated by membrane lipid composition. J.Lipid Res. *39*, 1287-1294.

Rusinol,A.E., Jamil,H., Vance,J.E. (1997). In vitro reconstitution of assembly of apolipoprotein B48-containing lipoproteins. J.Biol.Chem. *272*, 8019-8025.

Rusinol,A.E., Vance,J.E. (1995). Inhibition of secretion of truncated apolipoproteins B by monomethylethanolamine is independent of the length of the apolipoprotein. J.Biol.Chem. *270*, 13318-13325.

Rustaeus,S., Lindberg,K., Stillemark,P., Claesson,C., Asp,L., Larsson,T., Boren,J., Olofsson,S.O. (1999). Assembly of very low density lipoprotein: a two-step process of apolipoprotein B core lipidation. J.Nutr. *129*, 463S-466S.

Rustaeus,S., Stillemark,P., Lindberg,K., Gordon,D., Olofsson,S.O. (1998). The microsomal triglyceride transfer protein catalyzes the post-translational assembly of apolipoprotein B-100 very low density lipoprotein in McA-RH7777 cells. J.Biol.Chem. *273*, 5196-5203.

Sacchettini,J.C., Gordon,J.I. (1993). Rat intestinal fatty acid binding protein. A model system for analyzing the forces that can bind fatty acids to proteins. J.Biol.Chem. *268*, 18399-18402.

Sakata,N., Stoops,J.D., Dixon,J.L. (1999). Cytosolic components are required for proteasomal degradation of newly synthesized apolipoprotein B in permeabilized HepG2 cells. J.Biol.Chem. *274*, 17068-17074.

Sakata,N., Wu,X., Dixon,J.L., Ginsberg,H.N. (1993). Proteolysis and lipid-facilitated translocation are distinct but competitive processes that regulate secretion of apolipoprotein B in Hep G2 cells. J.Biol.Chem. *268*, 22967-22970.

Sallach,S.M., Adeli,K. (1995). Intracellular degradation of apolipoprotein B generates an N-terminal 70 kDa fragment in the endoplasmic reticulum. Biochim.Biophys.Acta *1265*, 29-32.

Sasak,W.V., Lown,J.S., Colburn,K.A. (1991). Human small-intestinal apolipoprotein B-48 oligosaccharide chains. Biochem.J. *274 (Pt 1)*, 159-165.

Sato,R., Imanaka,T., Takatsuki,A., Takano,T. (1990). Degradation of newly synthesized apolipoprotein B-100 in a pre-Golgi compartment. J.Biol.Chem. *265*, 11880-11884.

Scapin,G., Gordon,J.I., Sacchettini,J.C. (1992). Refinement of the structure of recombinant rat intestinal fatty acid-binding apoprotein at 1.2-A resolution. J.Biol.Chem. *267*, 4253-4269.

Schmidtke,G., Holzhutter,H.G., Bogyo,M., Kairies,N., Groll,M., de Giuli,R., Emch,S., Groettrup,M. (1999). How an inhibitor of the HIV-I protease modulates proteasome activity. J.Biol.Chem. *274*, 35734-35740.

Schmitz,A., Herrgen,H., Winkeler,A., Herzog,V. (2000). Cholera toxin is exported from microsomes by the Sec61p complex. J.Cell Biol. *148*, 1203-1212.

Schonfeld,G. (1995). The hypobetalipoproteinemias. Annu.Rev.Nutr. *15*, 23-34.

Schumaker,V.N., Phillips,M.L., Chatterton,J.E. (1994). Apolipoprotein B and low-density lipoprotein structure: implications for biosynthesis of triglyceride-rich lipoproteins. Adv.Protein Chem. *45*, 205-248.

Segrest,J.P., Jackson,R.L., Morrisett,J.D., Gotto,A.M., Jr. (1974). A molecular theory of lipid-protein interactions in the plasma lipoproteins. FEBS Lett. *38*, 247-258.

Segrest,J.P., Jones,M.K., De Loof,H., Brouillette,C.G., Venkatachalapathi,Y.V., Anantharamaiah,G.M. (1992). The amphipathic helix in the exchangeable apolipoproteins: a review of secondary structure and function. J.Lipid Res. *33*, 141-166.

Segrest,J.P., Jones,M.K., De Loof,H., Dashti,N. (2001). Structure of apolipoprotein B-100 in low density lipoproteins. J.Lipid Res. *42*, 1346-1367.

Segrest,J.P., Jones,M.K., Mishra,V.K., Anantharamaiah,G.M., Garber,D.W. (1994). apoB-100 has a pentapartite structure composed of three amphipathic alpha-helical domains alternating with two amphipathic beta-strand domains. Detection by the computer program LOCATE. Arterioscler.Thromb. *14*, 1674-1685.

Segrest,J.P., Jones,M.K., Mishra,V.K., Pierotti,V., Young,S.H., Boren,J., Innerarity,T.L., Dashti,N. (1998). Apolipoprotein B-100: conservation of lipid-associating amphipathic secondary structural motifs in nine species of vertebrates. J.Lipid Res. *39*, 85-102.

Shamu,C.E., Flierman,D., Ploegh,H.L., Rapoport,T.A., Chau,V. (2001). Polyubiquitination is required for US11-dependent movement of MHC class I heavy chain from endoplasmic reticulum into cytosol. Mol.Biol.Cell *12*, 2546-2555.

Shamu,C.E., Story,C.M., Rapoport,T.A., Ploegh,H.L. (1999). The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. J.Cell Biol. *147*, 45-58.

Shelness,G.S., Hou,L., Ledford,A.S., Parks,J.S., Weinberg,R.B. (2003). Identification of the lipoprotein initiating domain of apolipoprotein B. J.Biol.Chem. *278*, 44702-44707.

Shelness,G.S., Morris-Rogers,K.C., Ingram,M.F. (1994). Apolipoprotein B48-membrane interactions. Absence of transmembrane localization in nonhepatic cells. J.Biol.Chem. *269*, 9310-9318.

Shelness,G.S., Thornburg,J.T. (1996). Role of intramolecular disulfide bond formation in the assembly and secretion of apolipoprotein B-100-containing lipoproteins. J.Lipid Res. *37*, 408-419.

Shen,J., Chen,X., Hendershot,L., Prywes,R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Dev.Cell *3*, 99-111.

Singh,S., Lee,D.M. (1986). Conformational studies of lipoprotein B and apolipoprotein B: effects of disulfide reducing agents, sulfhydryl blocking agent, denaturing agents, pH and storage. Biochim.Biophys.Acta *876*, 460-468.

Sivaram,P., Vanni-Reyes,T., Goldberg,I.J. (1996). Endothelial cells synthesize and process apolipoprotein B. J.Biol.Chem. *271*, 15261-15266.

Sniderman,A.D., Cianflone,K. (1993). Substrate delivery as a determinant of hepatic apoB secretion. Arterioscler.Thromb. *13*, 629-636.

Sniderman,A.D., Rosenbloom,M. (2005). If apoB is so good, why isn't everybody measuring it? One reason why we need The Netherlands Journal of Medicine! Neth.J.Med. *63*, 232-235.

Sniderman,A.D., Zhang,X.J., Cianflone,K. (2000). Governance of the concentration of plasma LDL: a reevaluation of the LDL receptor paradigm. Atherosclerosis *148*, 215-229.

Sommer,T., Jentsch,S. (1993). A protein translocation defect linked to ubiquitin conjugation at the endoplasmic reticulum. Nature *365*, 176-179.

Sorci-Thomas,M., Hendricks,C.L., Kearns,M.W. (1992). HepG2 cell LDL receptor activity and the accumulation of apolipoprotein B and E in response to docosahexaenoic acid and cholesterol. J.Lipid Res. *33*, 1147-1156.

Spring,D.J., Chen-Liu,L.W., Chatterton,J.E., Elovson,J., Schumaker,V.N. (1992). Lipoprotein assembly. Apolipoprotein B size determines lipoprotein core circumference. J.Biol.Chem. *267*, 14839-14845.

Steinberg,D. (2004). Thematic review series: the pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy: part I. J.Lipid Res. *45*, 1583-1593.

Steinberg,D. (2005a). Thematic review series: the pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy: part II: the early evidence linking hypercholesterolemia to coronary disease in humans. J.Lipid Res. *46*, 179-190.

Steinberg,D. (2005b). Thematic review series: the pathogenesis of atherosclerosis: an interpretive history of the cholesterol controversy, part III: mechanistically defining the role of hyperlipidemia. J.Lipid Res. *46*, 2037-2051.

Steinberg,D. (2006). Thematic review series: the pathogenesis of atherosclerosis. An interpretive history of the cholesterol controversy, part IV: The 1984 coronary primary prevention trial ends it--almost. J.Lipid Res. *47*, 1-14.

Stillemark,P., Boren,J., Andersson,M., Larsson,T., Rustaeus,S., Karlsson,K.A., Olofsson,S.O. (2000). The assembly and secretion of apolipoprotein B-48-containing very low density lipoproteins in McA-RH7777 cells. J.Biol.Chem. *275*, 10506-10513.

Stone,S.J., Myers,H.M., Watkins,S.M., Brown,B.E., Feingold,K.R., Elias,P.M., Farese,R.V., Jr. (2004). Lipopenia and skin barrier abnormalities in DGAT2-deficient mice. J.Biol.Chem. *279*, 11767-11776.

Swift,L.L. (1995). Assembly of very low density lipoproteins in rat liver: a study of nascent particles recovered from the rough endoplasmic reticulum. J.Lipid Res. *36*, 395-406.

Swift,L.L., Valyi-Nagy,K., Rowland,C., Harris,C. (2001). Assembly of very low density lipoproteins in mouse liver: evidence of heterogeneity of particle density in the Golgi apparatus. J.Lipid Res. *42*, 218-224.

Taghibiglou,C., Rudy,D., Van Iderstine,S.C., Aiton,A., Cavallo,D., Cheung,R., Adeli,K. (2000). Intracellular mechanisms regulating apoB-containing lipoprotein assembly and secretion in primary hamster hepatocytes. J.Lipid Res. *41*, 499-513.

Taghibiglou,C., Van Iderstine,S.C., Kulinski,A., Rudy,D., Adeli,K. (2002). Intracellular mechanisms mediating the inhibition of apoB-containing lipoprotein synthesis and secretion in HepG2 cells by avasimibe (CI-1011), a novel acyl-coenzyme A: cholesterol acyltransferase (ACAT) inhibitor. Biochem.Pharmacol. *63*, 349-360.

Taniguchi,T., Ishikawa,Y., Tsunemitsu,M., Fukuzaki,H. (1989). The structures of the asparagine-linked sugar chains of human apolipoprotein B-100. Arch.Biochem.Biophys. *273*, 197-205.

Tardieu,A., Mateu,L., Sardet,C., Weiss,B., Luzzati,V., Aggerbeck,L., Scanu,A.M. (1976). Structure of human serum lipoproteins in solution. II. Small-angle x-ray scattering study of HDL3 and LDL. J.Mol.Biol. *105*, 459-460.

Tatu,U., Helenius,A. (1999). Interaction of newly synthesized apolipoprotein B with calnexin and calreticulin requires glucose trimming in the endoplasmic reticulum. Biosci.Rep. *19*, 189-196.

Taxis,C., Hitt,R., Park,S.H., Deak,P.M., Kostova,Z., Wolf,D.H. (2003). Use of modular substrates demonstrates mechanistic diversity and reveals differences in chaperone requirement of ERAD. J.Biol.Chem. *278*, 35903-35913.

Terasawa,Y., Cases,S.J., Wong,J.S., Jamil,H., Jothi,S., Traber,M.G., Packer,L., Gordon,D.A., Hamilton,R.L., Farese,R.V., Jr. (1999). Apolipoprotein B-related gene expression and ultrastructural characteristics of lipoprotein secretion in mouse yolk sac during embryonic development. J.Lipid Res. *40*, 1967-1977.

Thompson,G.R., Naoumova,R.P., Watts,G.F. (1996). Role of cholesterol in regulating apolipoprotein B secretion by the liver. J.Lipid Res. *37*, 439-447.

Thrift,R.N., Drisko,J., Dueland,S., Trawick,J.D., Davis,R.A. (1992). Translocation of apolipoprotein B across the endoplasmic reticulum is blocked in a nonhepatic cell line. Proc.Natl.Acad.Sci.U.S.A *89*, 9161-9165.

Thrower,J.S., Hoffman,L., Rechsteiner,M., Pickart,C.M. (2000). Recognition of the polyubiquitin proteolytic signal. EMBO J. *19*, 94-102.

Tirosh,B., Furman,M.H., Tortorella,D., Ploegh,H.L. (2003). Protein unfolding is not a prerequisite for endoplasmic reticulum-to-cytosol dislocation. J.Biol.Chem. *278*, 6664-6672.

Tran,K., Boren,J., Macri,J., Wang,Y., McLeod,R., Avramoglu,R.K., Adeli,K., Yao,Z. (1998a). Functional analysis of disulfide linkages clustered within the amino terminus of human apolipoprotein B. J.Biol.Chem. *273*, 7244-7251.

Tran,K., Boren,J., Macri,J., Wang,Y., McLeod,R., Avramoglu,R.K., Adeli,K., Yao,Z. (1998b). Functional analysis of disulfide linkages clustered within the amino terminus of human apolipoprotein B. J.Biol.Chem. *273*, 7244-7251.

Tran,K., Thorne-Tjomsland,G., DeLong,C.J., Cui,Z., Shan,J., Burton,L., Jamieson,J.C., Yao,Z. (2002). Intracellular assembly of very low density lipoproteins containing apolipoprotein B100 in rat hepatoma McA-RH7777 cells. J.Biol.Chem. *277*, 31187-31200.

Travers,K.J., Patil,C.K., Wodicka,L., Lockhart,D.J., Weissman,J.S., Walter,P. (2000). Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. Cell *101*, 249-258.

Tsai,B., Rodighiero,C., Lencer,W.I., Rapoport,T.A. (2001). Protein disulfide isomerase acts as a redox-dependent chaperone to unfold cholera toxin. Cell *104*, 937-948.

Tsai,B., Ye,Y., Rapoport,T.A. (2002). Retro-translocation of proteins from the endoplasmic reticulum into the cytosol. Nat.Rev.Mol.Cell Biol. *3*, 246-255.

Tsiodras,S., Mantzoros,C., Hammer,S., Samore,M. (2000). Effects of protease inhibitors on hyperglycemia, hyperlipidemia, and lipodystrophy: a 5-year cohort study. Arch.Intern.Med. *160*, 2050-2056.

Twisk,J., Gillian-Daniel,D.L., Tebon,A., Wang,L., Barrett,P.H., Attie,A.D. (2000). The role of the LDL receptor in apolipoprotein B secretion. J.Clin.Invest *105*, 521-532.

Unno,M., Mizushima,T., Morimoto,Y., Tomisugi,Y., Tanaka,K., Yasuoka,N., Tsukihara,T. (2002). The structure of the mammalian 20S proteasome at 2.75 A resolution. Structure. *10*, 609-618.

Urade,R., Nasu,M., Moriyama,T., Wada,K., Kito,M. (1992). Protein degradation by the phosphoinositide-specific phospholipase C-alpha family from rat liver endoplasmic reticulum. J.Biol.Chem. *267*, 15152-15159.

Valyi-Nagy,K., Harris,C., Swift,L.L. (2002). The assembly of hepatic very low density lipoproteins: evidence of a role for the Golgi apparatus. Lipids *37*, 879-884.

van den Berg,B., Clemons,W.M., Jr., Collinson,I., Modis,Y., Hartmann,E., Harrison,S.C., Rapoport,T.A. (2004). X-ray structure of a protein-conducting channel. Nature *427*, 36-44.

van Nocker,S., Sadis,S., Rubin,D.M., Glickman,M., Fu,H., Coux,O., Wefes,I., Finley,D., Vierstra,R.D. (1996). The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in Saccharomyces cerevisiae and plays a nonessential, substrate-specific role in protein turnover. Mol.Cell Biol. *16*, 6020-6028.

VanSlyke,J.K., Musil,L.S. (2002). Dislocation and degradation from the ER are regulated by cytosolic stress. J.Cell Biol. *157*, 381-394.

Verkade,H.J., Fast,D.G., Rusinol,A.E., Scraba,D.G., Vance,D.E. (1993). Impaired biosynthesis of phosphatidylcholine causes a decrease in the number of very low density lipoprotein particles in the Golgi but not in the endoplasmic reticulum of rat liver. J.Biol.Chem. *268*, 24990-24996.

Verma,R., Chen,S., Feldman,R., Schieltz,D., Yates,J., Dohmen,J., Deshaies,R.J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol.Biol.Cell *11*, 3425-3439.

Vermeulen,P.S., Lingrell,S., Yao,Z., Vance,D.E. (1997). Phosphatidylcholine biosynthesis is required for secretion of truncated apolipoprotein Bs from McArdle RH7777 cells only when a neutral lipid core is formed. J.Lipid Res. *38*, 447-458.

Vilas,G.L., Berthiaume,L.G. (2004). A role for palmitoylation in the quality control, assembly and secretion of apolipoprotein B. Biochem.J. *377*, 121-130.

Voges,D., Zwickl,P., Baumeister,W. (1999). The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu.Rev.Biochem. *68*, 1015-1068.

Vukmirica,J., Nishimaki-Mogami,T., Tran,K., Shan,J., McLeod,R.S., Yuan,J., Yao,Z. (2002). The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL. J.Lipid Res. *43*, 1496-1507.

Vukmirica,J., Tran,K., Liang,X., Shan,J., Yuan,J., Miskie,B.A., Hegele,R.A., Resh,M.D., Yao,Z. (2003). Assembly and secretion of very low density lipoproteins containing apolipoprotein B48 in transfected McA-RH7777 cells. Lack of evidence that palmitoylation of apolipoprotein B48 is required for lipoprotein secretion. J.Biol.Chem. *278*, 14153-14161.

Walter,J., Urban,J., Volkwein,C., Sommer,T. (2001). Sec61p-independent degradation of the tail-anchored ER membrane protein Ubc6p. EMBO J. *20*, 3124-3131.

Wang,C.N., Hobman,T.C., Brindley,D.N. (1995). Degradation of apolipoprotein B in cultured rat hepatocytes occurs in a post-endoplasmic reticulum compartment. J.Biol.Chem. *270*, 24924-24931.

Wang,H., Chen,X., Fisher,E.A. (1993). N-3 fatty acids stimulate intracellular degradation of apoprotein B in rat hepatocytes. J.Clin.Invest *91*, 1380-1389.

Wang,L., Fast,D.G., Attie,A.D. (1997a). The enzymatic and non-enzymatic roles of protein-disulfide isomerase in apolipoprotein B secretion. J.Biol.Chem. *272*, 27644-27651.

Wang,L., Walsh,M.T., Small,D.M. (2006). Apolipoprotein B is conformationally flexible but anchored at a triolein/water interface: A possible model for lipoprotein surfaces. Proc.Natl.Acad.Sci.U.S.A.

Wang,S., McLeod,R.S., Gordon,D.A., Yao,Z. (1996). The microsomal triglyceride transfer protein facilitates assembly and secretion of apolipoprotein B-containing lipoproteins and decreases cotranslational degradation of apolipoprotein B in transfected COS-7 cells. J.Biol.Chem. *271*, 14124-14133.

Wang,Y., McLeod,R.S., Yao,Z. (1997b). Normal activity of microsomal triglyceride transfer protein is required for the oleate-induced secretion of very low density lipoproteins containing apolipoprotein B from McA-RH7777 cells. J.Biol.Chem. *272*, 12272-12278.

Wang,Y., Tran,K., Yao,Z. (1999). The activity of microsomal triglyceride transfer protein is essential for accumulation of triglyceride within microsomes in McA-RH7777 cells. A unified model for the assembly of very low density lipoproteins. J.Biol.Chem. *274*, 27793-27800.

Ward,C.L., Omura,S., Kopito,R.R. (1995). Degradation of CFTR by the ubiquitin-proteasome pathway. Cell *83*, 121-127.

Wei,C.F., Chen,S.H., Yang,C.Y., Marcel,Y.L., Milne,R.W., Li,W.H., Sparrow,J.T., Gotto,A.M., Jr., Chan,L. (1985). Molecular cloning and expression of partial cDNAs and deduced amino acid sequence of a carboxyl-terminal fragment of human apolipoprotein B-100. Proc.Natl.Acad.Sci.U.S.A *82*, 7265-7269.

Weisgraber,K.H., Rall,S.C., Jr. (1987). Human apolipoprotein B-100 heparin-binding sites. J.Biol.Chem. *262*, 11097-11103.

Weissman,A.M. (2001). Themes and variations on ubiquitylation. Nat.Rev.Mol.Cell Biol. *2*, 169-178.

Welty,F.K., Seman,L., Yen,F.T. (1995). Purification of the apolipoprotein B-67-containing low density lipoprotein particle and its affinity for the low density lipoprotein receptor. J.Lipid Res. *36*, 2622-2629.

Wenham,P.R., Henderson,B.G., Penney,M.D., Ashby,J.P., Rae,P.W., Walker,S.W. (1997). Familial ligand-defective apolipoprotein B-100: detection, biochemical features and haplotype analysis of the R3531C mutation in the UK. Atherosclerosis *129*, 185-192.

Wetterau,J.R., Aggerbeck,L.P., Bouma,M.E., Eisenberg,C., Munck,A., Hermier,M., Schmitz,J., Gay,G., Rader,D.J., Gregg,R.E. (1992). Absence of microsomal triglyceride transfer protein in individuals with abetalipoproteinemia. Science *258*, 999-1001.

Wetterau,J.R., Aggerbeck,L.P., Laplaud,P.M., McLean,L.R. (1991a). Structural properties of the microsomal triglyceride-transfer protein complex. Biochemistry *30*, 4406-4412.

Wetterau,J.R., Combs,K.A., McLean,L.R., Spinner,S.N., Aggerbeck,L.P. (1991b). Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. Biochemistry *30*, 9728-9735.

Wetterau,J.R., Gregg,R.E., Harrity,T.W., Arbeeny,C., Cap,M., Connolly,F., Chu,C.H., George,R.J., Gordon,D.A., Jamil,H., Jolibois,K.G., Kunselman,L.K., Lan,S.J., Maccagnan,T.J., Ricci,B., Yan,M., Young,D., Chen,Y., Fryszman,O.M., Logan,J.V., Musial,C.L., Poss,M.A., Robl,J.A., Simpkins,L.M., Biller,S.A., . (1998). An MTP inhibitor that normalizes atherogenic lipoprotein levels in WHHL rabbits. Science *282*, 751-754.

White,A.L., Graham,D.L., LeGros,J., Pease,R.J., Scott,J. (1992). Oleate-mediated stimulation of apolipoprotein B secretion from rat hepatoma cells. A function of the ability of apolipoprotein B to direct lipoprotein assembly and escape presecretory degradation. J.Biol.Chem. *267*, 15657-15664.

Whitfield,A.J., Barrett,P.H., van Bockxmeer,F.M., Burnett,J.R. (2004). Lipid disorders and mutations in the APOB gene. Clin.Chem. *50*, 1725-1732.

Wiertz,E.J., Jones,T.R., Sun,L., Bogyo,M., Geuze,H.J., Ploegh,H.L. (1996a). The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell *84*, 769-779.

Wiertz,E.J., Tortorella,D., Bogyo,M., Yu,J., Mothes,W., Jones,T.R., Rapoport,T.A., Ploegh,H.L. (1996b). Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature *384*, 432-438.

Wiggins,D., Gibbons,G.F. (1996). Origin of hepatic very-low-density lipoprotein triacylglycerol: the contribution of cellular phospholipid. Biochem.J. *320 (Pt 2)*, 673-679.

Wilcox,L.J., Barrett,P.H., Newton,R.S., Huff,M.W. (1999). ApoB100 secretion from HepG2 cells is decreased by the ACAT inhibitor CI-1011: an effect associated with enhanced intracellular degradation of ApoB. Arterioscler.Thromb.Vasc.Biol. *19*, 939-949.

Wilkinson,J., Higgins,J.A., Groot,P., Gherardi,E., Bowyer,D. (1993). Topography of apolipoprotein B in subcellular fractions of rabbit liver probed with a panel of monoclonal antibodies. J.Lipid Res. *34*, 815-825.

Williams,K.J., Brocia,R.W., Fisher,E.A. (1990). The unstirred water layer as a site of control of apolipoprotein B secretion. J.Biol.Chem. *265*, 16741-16744.

Winitz,D., Shachar,I., Elkabetz,Y., Amitay,R., Samuelov,M., Bar-Nun,S. (1996). Degradation of distinct assembly forms of immunoglobulin M occurs in multiple sites in permeabilized B cells. J.Biol.Chem. *271*, 27645-27651.

Wirth,A., Jung,M., Bies,C., Frien,M., Tyedmers,J., Zimmermann,R., Wagner,R. (2003). The Sec61p complex is a dynamic precursor activated channel. Mol.Cell *12*, 261-268.

Wishart,D.S., Boyko,R.F., Sykes,B.D. (1994a). Constrained multiple sequence alignment using XALIGN. Comput.Appl.Biosci. *10*, 687-688.

Wishart,D.S., Boyko,R.F., Willard,L., Richards,F.M., Sykes,B.D. (1994b). SEQSEE: a comprehensive program suite for protein sequence analysis. Comput.Appl.Biosci. *10*, 121-132.

Wojcik,C., Yano,M., DeMartino,G.N. (2004). RNA interference of valosin-containing protein (VCP/p97) reveals multiple cellular roles linked to ubiquitin/proteasome-dependent proteolysis. J.Cell Sci. *117*, 281-292.

Wong,L., Torbati,A. (1994). Differentiation of intrahepatic membrane-bound and secretory apolipoprotein B by monoclonal antibodies: membrane-bound apolipoprotein B is more glycosylated. Biochemistry *33*, 1923-1929.

Wu,X., Sakata,N., Dixon,J., Ginsberg,H.N. (1994a). Exogenous VLDL stimulates apolipoprotein B secretion from HepG2 cells by both pre- and post-translational mechanisms. J.Lipid Res. *35*, 1200-1210.

Wu,X., Sakata,N., Lui,E., Ginsberg,H.N. (1994b). Evidence for a lack of regulation of the assembly and secretion of apolipoprotein B-containing lipoprotein from HepG2 cells by cholesteryl ester. J.Biol.Chem. *269*, 12375-12382.

Wu,X., Shang,A., Jiang,H., Ginsberg,H.N. (1996a). Low rates of apoB secretion from HepG2 cells result from reduced delivery of newly synthesized triglyceride to a "secretion-coupled" pool. J.Lipid Res. *37*, 1198-1206.

Wu,X., Zhou,M., Huang,L.S., Wetterau,J., Ginsberg,H.N. (1996b). Demonstration of a physical interaction between microsomal triglyceride transfer protein and apolipoprotein B during the assembly of ApoB-containing lipoproteins. J.Biol.Chem. *271*, 10277-10281.

Yamaguchi,J., Gamble,M.V., Conlon,D., Liang,J.S., Ginsberg,H.N. (2003). The conversion of apoB100 low density lipoprotein/high density lipoprotein particles to apoB100 very low density lipoproteins in response to oleic acid occurs in the endoplasmic reticulum and not in the Golgi in McA RH7777 cells. J.Biol.Chem. *278*, 42643-42651.

Yamazaki,T., Sasaki,E., Kakinuma,C., Yano,T., Miura,S., Ezaki,O. (2005). Increased very low density lipoprotein secretion and gonadal fat mass in mice overexpressing liver DGAT1. J.Biol.Chem. *280*, 21506-21514.

Yang,C.Y., Chen,S.H., Gianturco,S.H., Bradley,W.A., Sparrow,J.T., Tanimura,M., Li,W.H., Sparrow,D.A., DeLoof,H., Rosseneu,M., Lee,F.S., Gu Z.W., Gotto,A.M., Chan,L. (1986). Sequence, structure, receptor-binding domains and internal repeats of human apolipoprotein B-100. Nature *323*, 738-742.

Yang,C.Y., Gu,Z.W., Weng,S.A., Kim,T.W., Chen,S.H., Pownall,H.J., Sharp,P.M., Liu,S.W., Li,W.H., Gotto,A.M., Jr., . (1989a). Structure of apolipoprotein B-100 of human low density lipoproteins. Arteriosclerosis *9*, 96-108.

Yang,C.Y., Kim,T.W., Pao,Q., Chan,L., Knapp,R.D., Gotto,A.M., Jr., Pownall,H.J. (1989b). Structure and conformational analysis of lipid-associating peptides of apolipoprotein B-100 produced by trypsinolysis. J.Protein Chem. *8*, 689-699.

Yang,L.Y., Kuksis,A., Myher,J.J., Steiner,G. (1995). Origin of triacylglycerol moiety of plasma very low density lipoproteins in the rat: structural studies. J.Lipid Res. *36*, 125-136.

Yang,L.Y., Kuksis,A., Myher,J.J., Steiner,G. (1996). Contribution of de novo fatty acid synthesis to very low density lipoprotein triacylglycerols: evidence from mass isotopomer distribution analysis of fatty acids synthesized from [2H6]ethanol. J.Lipid Res. *37*, 262-274.

Yang,M., Omura,S., Bonifacino,J.S., Weissman,A.M. (1998a). Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. J.Exp.Med. *187*, 835-846.

Yang,Y., Turner,R.S., Gaut,J.R. (1998b). The chaperone BiP/GRP78 binds to amyloid precursor protein and decreases Abeta40 and Abeta42 secretion. J.Biol.Chem. *273*, 25552-25555.

Yao,T., Cohen,R.E. (2002). A cryptic protease couples deubiquitination and degradation by the proteasome. Nature *419*, 403-407.

Yao,Z., Tran,K., McLeod,R.S. (1997). Intracellular degradation of newly synthesized apolipoprotein B. J.Lipid Res. *38*, 1937-1953.

Yao,Z.M., Blackhart,B.D., Linton,M.F., Taylor,S.M., Young,S.G., McCarthy,B.J. (1991). Expression of carboxyl-terminally truncated forms of human apolipoprotein B in rat hepatoma cells. Evidence that the length of apolipoprotein B has a major effect on the buoyant density of the secreted lipoproteins. J.Biol.Chem. *266*, 3300-3308.

Yao,Z.M., Vance,D.E. (1988). The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. J.Biol.Chem. *263*, 2998-3004.

Ye,Y., Meyer,H.H., Rapoport,T.A. (2001). The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature *414*, 652-656.

Ye,Y., Meyer,H.H., Rapoport,T.A. (2003). Function of the p97-Ufd1-Npl4 complex in retrotranslocation from the ER to the cytosol: dual recognition of nonubiquitinated polypeptide segments and polyubiquitin chains. J.Cell Biol. *162*, 71-84.

Ye,Y., Shibata,Y., Yun,C., Ron,D., Rapoport,T.A. (2004). A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature *429*, 841-847.

Yeung,S.J., Chen,S.H., Chan,L. (1996). Ubiquitin-proteasome pathway mediates intracellular degradation of apolipoprotein B. Biochemistry *35*, 13843-13848.

Yu,H., Kaung,G., Kobayashi,S., Kopito,R.R. (1997). Cytosolic degradation of T-cell receptor alpha chains by the proteasome. J.Biol.Chem. *272*, 20800-20804.

Yu,H., Kopito,R.R. (1999). The role of multiubiquitination in dislocation and degradation of the alpha subunit of the T cell antigen receptor. J.Biol.Chem. *274*, 36852-36858.

Yu,Y.H., Ginsberg,H.N. (2004). The role of acyl-CoA:diacylglycerol acyltransferase (DGAT) in energy metabolism. Ann.Med. *36*, 252-261.

Zammit,V.A., Lankester,D.L. (2001). Oleate acutely stimulates the secretion of triacylglycerol by cultured rat hepatocytes by accelerating the emptying of the secretory compartment. Lipids *36*, 607-612.

Zhang,J., Herscovitz,H. (2003). Nascent lipidated apolipoprotein B is transported to the Golgi as an incompletely folded intermediate as probed by its association with network of endoplasmic reticulum molecular chaperones, GRP94, ERp72, BiP, calreticulin, and cyclophilin B. J.Biol.Chem. *278*, 7459-7468.

Zhang,K., Kaufman,R.J. (2004). Signaling the unfolded protein response from the endoplasmic reticulum. J.Biol.Chem. *279*, 25935-25938.

Zhang,X., Shaw,A., Bates,P.A., Newman,R.H., Gowen,B., Orlova,E., Gorman,M.A., Kondo,H., Dokurno,P., Lally,J., Leonard,G., Meyer,H., van Heel,M., Freemont,P.S. (2000). Structure of the AAA ATPase p97. Mol.Cell *6*, 1473-1484.

Zhang,Y., Nijbroek,G., Sullivan,M.L., McCracken,A.A., Watkins,S.C., Michaelis,S., Brodsky,J.L. (2001). Hsp70 molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. Mol.Biol.Cell *12*, 1303-1314.

Zhang,Z., Cianflone,K., Sniderman,A.D. (1999). Role of cholesterol ester mass in regulation of secretion of ApoB100 lipoprotein particles by hamster hepatocytes and effects of statins on that relationship. Arterioscler.Thromb.Vasc.Biol. *19*, 743-752.

Zhao,Y., McCabe,J.B., Vance,J., Berthiaume,L.G. (2000). Palmitoylation of apolipoprotein B is required for proper intracellular sorting and transport of cholesteroyl esters and triglycerides. Mol.Biol.Cell *11*, 721-734.

Zhong,X., Shen,Y., Ballar,P., Apostolou,A., Agami,R., Fang,S. (2004). AAA ATPase p97/valosin-containing protein interacts with gp78, a ubiquitin ligase for endoplasmic reticulum-associated degradation. J.Biol.Chem. *279*, 45676-45684.

Zhou,M., Fisher,E.A., Ginsberg,H.N. (1998). Regulated Co-translational ubiquitination of apolipoprotein B100. A new paradigm for proteasomal degradation of a secretory protein. J.Biol.Chem. *273*, 24649-24653.

Zhou,M., Wu,X., Huang,L.S., Ginsberg,H.N. (1995). Apoprotein B100, an inefficiently translocated secretory protein, is bound to the cytosolic chaperone, heat shock protein 70. J.Biol.Chem. *270*, 25220-25224.

Zhu,D., Ganji,S.H., Kamanna,V.S., Kashyap,M.L. (2002). Effect of gemfibrozil on apolipoprotein B secretion and diacylglycerol acyltransferase activity in human hepatoblastoma (HepG2) cells. Atherosclerosis *164*, 221-228.