Studies on the intracellular mechanisms of hepatic apolipoprotein B degradation.

Dora. Cavallo

University of Windsor

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Studies on the Intracellular Mechanisms of Hepatic Apolipoprotein B Degradation

by

Dora Cavallo

A Dissertation
Submitted to the College of Graduate Studies and Research Through the School of Physical Science Chemistry and Biochemistry Program for Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

Windsor, Ontario, Canada
1999
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ABSTRACT

Studies on the Intracellular Mechanisms of Hepatic Apolipoprotein B Degradation

by

DORA CAVALLO

Acute modulation of apolipoprotein B (apoB) biogenesis is essential for the proper assembly and secretion of the hepatic apoB-containing lipoproteins. There is substantial evidence suggesting that translocation of apoB is a major regulatory factor influencing the rate of apoB and apoB-containing lipoprotein secretion and intracellular stability.

Studies were conducted to examine the relationship between the length of apoB and its intracellular translocation and stability using McA-RH7777 cells expressing recombinant human apoB variants, ranging in size from B15 to B100. Translational status of apoB was assessed based on trypsin sensitivity of apoB using isolated permeabilized McA-RH7777 cells. Shorter apoB variants (B15 to B72) were relatively resistant to exogenous trypsin (percent trypsin-resistant apoB ranged from 69-79%) in contrast to recombinant human B100 which was only 42% trypsin resistant. Thus, an inverse correlation between the length of apoB and its sensitivity to exogenous trypsin was established. An inverse relationship was also observed between the size of apoB and its co-translational (during the pulse) and post-translational (over a 2 h chase) resistance to proteasomal degradation. As the size of the nascent apoB polypeptide increases there appears to be a higher sensitivity to proteasomal degradation.

It has been well established that the biogenesis of apoB is mediated by the cytosolic proteasome. Here, the roles of both the cytosolic proteasome as well as non-proteasome mediated degradation systems were investigated. Although post-translational apoB degradation in intact HepG2 cells was sensitive to the proteasome inhibitor lactacystin, in permeabilized cells there was no post-translational protection of apoB by lactacystin, nor clasto-lactacystin β-lactone. Further investigations of proteasomal activity in HepG2 cells revealed that, following permeabilization, there was a dramatic
loss of the 20S proteasomal subunits, and consequently the cells exhibited no detectable lactacystin-inhibitable activity. Interestingly, apoB fragmentation and the generation of the 70 kDa apoB degradation fragment characteristic to permeabilized cells, continued to occur in these cells despite the absence of functional cytosolic proteasome. These data thus suggest that although the cytosolic proteasome appears to be involved in the post-translational turnover of apoB in intact cells, the specific post-translational fragmentation of apoB generating the 70 kDa fragment observed in permeabilized cells occurs independent of the cytosolic proteasome. Secretion of apoB was restored in permeabilized HepG2 cells supplemented with cytosol and an ATP generating system, and interestingly, apoB secretion was also sensitive to MG132, thus suggesting a role for the proteasome in the post-translational regulation of the 70 kDa apoB fragment.

Studies were also conducted to explore the potential role of retrograde translocation of apoB from the secretory pathway, as a targeting mechanism for proteasomal degradation. Subcellular fractionation of HepG2 cells revealed apoB in both the cytosolic and microsomal fractions of these cells. The accumulation of cytosolic apoB in comparison to microsomal apoB appeared to increase significantly post-translationally, and was also sensitive to MG132, thus suggesting a role for the cytosolic proteasome in the post-translational degradation of apoB. Further characterization of cytosolic apoB revealed that this pool of apoB was ubiquitinated and glycosylated. Ubiquitination indicated that the cytosolic apoB was targeted for proteasomal degradation. Glycosylation implied that cytosolic apoB had been previously exposed to the ER lumen. Inhibition of glycosylation increased the early accumulation of apoB in the cytosol. In the presence of Brefeldin A the rate of apoB accumulation in the cytosol was reduced and the degradation of apoB in the microsomes appeared to be enhanced. Thus, it is postulated that apoB which accumulates in the cytosol of HepG2 cells may have been retrograde translocated from the secretory pathway (endoplasmic reticulum) to the cytosol. A model for the potential role of retrograde translocation was also developed in an attempt to elucidate a general mechanism to explain how luminal apoB may be targeted to the cytosol for proteasomal degradation.
ACKNOWLEDGEMENTS

I would like to begin by expressing my gratitude to my research supervisor Dr. Khosrow Adeli for the opportunity to conduct my graduate research in his laboratory. His support, advice and friendship are greatly appreciated.

I would also like to thank and acknowledge my committee members, Dr. D.A. Cotter, Dr. B. Mutus and Dr. K. Taylor for their academic and personal advice as well as their constant support.

In addition, I would like to express my sincere gratitude to my entire family and friends for their constant love, encouragement and humour.
DEDICATION

To the four most special people in my life:
David, Antonella, Mom and Dad

Your encouragement, faith and love has given me
the strength to achieve one of my greatest dreams.
It is with great honour that I now dedicate this accomplishment to you.
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<tr>
<td>α-MEM</td>
<td>Eagle’s minimum essential medium (alpha) powder</td>
</tr>
<tr>
<td>ABC1</td>
<td>ATP cassette-binding transporter 1</td>
</tr>
<tr>
<td>ACAT</td>
<td>acyl coenzyme A: cholesterol acyltransferase</td>
</tr>
<tr>
<td>ALLN</td>
<td>N-acetyl-leucyl-leucyl-norleucinal</td>
</tr>
<tr>
<td>APO</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CERP</td>
<td>cholesterol efflux regulatory protein</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CMR R</td>
<td>chylomicron remnant receptor</td>
</tr>
<tr>
<td>CPM</td>
<td>counts per minute</td>
</tr>
<tr>
<td>CSK</td>
<td>cytoskeletal buffer</td>
</tr>
<tr>
<td>CYS</td>
<td>cysteine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium powder</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E64</td>
<td>trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EGTA</td>
<td>ethylene Glycol-bis (β-aminooethyl Ether) N,N,N’,N’-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>F.U.</td>
<td>fluorescent unit</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
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<tr>
<td>HDL R</td>
<td>high density lipoprotein receptor</td>
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<tr>
<td>HEPES</td>
<td>fluorescamine, glycerol, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>β-hydroxy-β-methylglutaryl coenzyme A</td>
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<tr>
<td>HL</td>
<td>hepatic lipase</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>LCAT</td>
<td>lecithin-cholesterol acyltransferase</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<td>LDL R</td>
<td>low density lipoprotein receptor</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
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<td>MET</td>
<td>methionine</td>
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<tr>
<td>MG115</td>
<td>N-carbobenzyoxy-leucyl-leucyl-norvalinal</td>
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<tr>
<td>MG132</td>
<td>N-carbobenzyoxy-leucyl-leucyl-leucinal</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MTP</td>
<td>microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>NADPH</td>
<td>α-Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PDI</td>
<td>protein disulfide isomerase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>PIPES</td>
<td>piperazine-N,N'-bis[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PSI</td>
<td>N-carbobenzoxy-isoleucyl-glu(O-t-Bu)-alanyl-leucinal</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage activating protein</td>
</tr>
<tr>
<td>S.D.</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>S.E.</td>
<td>standard error</td>
</tr>
<tr>
<td>SER</td>
<td>smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N,N’-methylene-bis-acrylamide</td>
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<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)-aminomethane glycine</td>
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<tr>
<td>Triton X-100</td>
<td>t-octylphenoxy-polyoxyethylene</td>
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<tr>
<td>TWEEN 20</td>
<td>polyoxyethylenesorbitan</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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CHAPTER 1

GENERAL INTRODUCTION

1.1 Lipoprotein and Apolipoproteins

Plasma lipoproteins are water-soluble aggregates composed of lipids and specialized proteins. The basic composition of lipoproteins is an inner core of neutral lipids, namely triglycerides and cholesterol esters surrounded by an outer membrane composed of phospholipids, unesterified cholesterol and apolipoproteins (Figure 1.1). The major function of lipoproteins is to transport hydrophobic lipids through the aqueous environment of the plasma to cells and tissues that will utilize the lipids as a source of energy. Accordingly, the assembly and secretion of lipoproteins and the regulation of their accompanying apolipoproteins (apo) are crucial processes that participate in the metabolic homeostasis of the organism.

Lipoproteins are divided into five main classes based on their buoyant nature (density). The densities of the lipoprotein groups have been identified by their banding pattern following isopycnic gradient ultracentrifugation. Table 1.1 outlines the general characteristics of the five major classes of lipoproteins based on size, density and composition (reviewed in Davis and Vance, 1996 and Ginsberg, 1994). Chylomicrons are the largest lipoproteins (density <0.95 g/mL) and are mainly rich in triglycerides. They are synthesized and secreted by the intestinal mucosal cells, and are responsible for the transport of dietary (exogenous) lipids. The metabolism of chylomicrons is directed through the exogenous lipid metabolic pathway. Very low density lipoproteins (VLDL) are mainly synthesized and secreted by the liver, although some secretion from the intestines does occur. These lipoprotein particles are also rich in triglycerides and their
Figure 1.1 Schematic Diagram of a Lipoprotein Particle. Lipoprotein particles are comprised of a neutral lipid core containing triglycerides and cholesterol esters. In addition, lipoproteins contain an outer shell composed of non-neutral lipids including phospholipids (⊙) and free cholesterol (■). Lipoproteins also contain amphipathic apolipoproteins (⌒⌒) that function to maintain the integrity of the lipoprotein particle.
FIGURE 1.1
densities are very low (0.95-1.006 g/mL). In the plasma, VLDL particles transport endogenously synthesized triglycerides and cholesterol. The metabolism of hepatic-derived VLDL and its derivatives is directed through the endogenous lipid metabolic pathway. As a result of VLDL metabolism, intermediate and low density lipoproteins (IDL and LDL, respectively) are generated. Production of IDL occurs within the plasma following the lipolysis of VLDL triglycerides by plasma lipoprotein lipase (LPL). IDL contain relatively similar amounts of both cholesterol and triglycerides and are characteristically denser than VLDL (1.006-1.019 g/mL). Further delipidation of IDL by hepatic lipase (HL) leads to the generation of LDL, which are dense, cholesterol-rich particles (1.019-1.063 g/mL). Finally, high density lipoproteins (HDL) are the smallest and most dense lipoprotein group (1.063-1.210 g/mL). Although the metabolism of HDL also occurs via the endogenous lipid metabolic pathway, the assembly of HDL is distinct from that of other lipoproteins. HDL, unlike the other lipoproteins is also believed to have antiatherogenic properties. Further characterization of these lipoproteins and the mechanisms involved in their assembly and metabolism are discussed below.

Apolipoproteins, which constitute the protein portion of lipoproteins, are generally divided into two main classes: the non-exchangeable lipoproteins (apoB100 and apoB48) and the exchangeable lipoproteins (apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II and apoE). Non-exchangeable apolipoproteins are highly insoluble in aqueous solution and therefore, in the plasma they remain associated with their respective lipoprotein particle. The exchangeable apolipoproteins, however, are soluble in aqueous solution thus allowing them to be transferred between different lipoproteins. Various other differences also exist among the apolipoproteins with respect to their individual size, function and
**TABLE 1.1**

Classification of Human Plasma Lipoproteins Based on Density, Size and Composition

<table>
<thead>
<tr>
<th></th>
<th>Chylomicrons</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density (g/mL)</td>
<td>&lt; 0.95</td>
<td>0.95-</td>
<td>1.006</td>
<td>1.019</td>
<td>1.063</td>
</tr>
<tr>
<td></td>
<td>1.006</td>
<td></td>
<td>1.019</td>
<td>1.063</td>
<td>1.210</td>
</tr>
<tr>
<td>Diameter (nm)</td>
<td>75-1200</td>
<td>30-80</td>
<td>25-35</td>
<td>18-25</td>
<td>5-12</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>83</td>
<td>50</td>
<td>31</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>(% weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8</td>
<td>22</td>
<td>29</td>
<td>45</td>
<td>30</td>
</tr>
<tr>
<td>(% weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phospholipid</td>
<td>7</td>
<td>18</td>
<td>22</td>
<td>21</td>
<td>29</td>
</tr>
<tr>
<td>(% weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>1-2</td>
<td>10</td>
<td>18</td>
<td>25</td>
<td>33</td>
</tr>
<tr>
<td>(% weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reviewed in Davis and Vance (1996).
TABLE 1.2

Properties and Functions of Apolipoproteins

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Molecular Weight (kDa)</th>
<th>Lipoprotein</th>
<th>Metabolic Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo A-I</td>
<td>28.3</td>
<td>HDL</td>
<td>Cholesterol Efflux; Activate LCAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chylomicrons</td>
<td></td>
</tr>
<tr>
<td>ApoA-II</td>
<td>18.6</td>
<td>HDL</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chylomicrons</td>
<td></td>
</tr>
<tr>
<td>ApoA-IV</td>
<td>43.3</td>
<td>HDL</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chylomicrons</td>
<td></td>
</tr>
<tr>
<td>ApoB48</td>
<td>240</td>
<td>Chylomicrons</td>
<td>Necessary for assembly and secretion of chylomicrons</td>
</tr>
<tr>
<td>ApoB100</td>
<td>512</td>
<td>VLDL, LDL</td>
<td>Necessary for assembly and secretion of chylomicrons; Structural component of VLDL, IDL and LDL; Ligand for LDL receptor</td>
</tr>
<tr>
<td>ApoC-I</td>
<td>6.6</td>
<td>All lipoproteins</td>
<td>Activator of LPL</td>
</tr>
<tr>
<td>ApoC-II</td>
<td>8.8</td>
<td>All lipoproteins</td>
<td>Activator of LPL</td>
</tr>
<tr>
<td>ApoC-III</td>
<td>8.8</td>
<td>All lipoproteins</td>
<td>Inhibitor of LPL</td>
</tr>
<tr>
<td>ApoE</td>
<td>34.2</td>
<td>All lipoproteins</td>
<td>Cholesterol Efflux; Ligand to LDL receptor; Ligand to LRP</td>
</tr>
</tbody>
</table>

Reviewed in Fielding and Fielding (1996)
lipoprotein particle formation (Table 1.2). Despite, the differences between the classes of apolipoproteins, there are specific structural features that remain common among all of them. One common feature is their amphipathic α-helical structures, which contain both hydrophobic and hydrophilic regions. These regions allow the proteins the ability to bind neutral lipids while remaining water-soluble (Segrest et al., 1974; Segrest et al., 1994). In all lipoproteins, with the exception of apoB, the hydrophobic regions of the α-helical structures allow them to self-associate within the plasma and promote their exchange between different lipoproteins. The amphipathic nature of all apolipoproteins also functions to determine and stabilize the size and structure of the lipoprotein particle.

1.2 Lipoprotein Metabolism

Intracellular production of lipoproteins occurs predominantly in the intestines and the liver. In the intestines dietary lipids are mainly used in the assembly of chylomicrons. In the liver, however, endogenous lipids are used in the assembly of VLDL and LDL. Endogenous and dietary lipids in the plasma both contribute to the assembly of HDL particles. The two systems that participate in the metabolism of lipoproteins are the exogenous and endogenous lipid metabolic pathways (Figure 1.2). The exogenous metabolic pathway is utilized in the metabolism of dietary lipids, and therefore involves the catabolism of chylomicrons. In the lumen of the intestines, dietary lipids such as triglycerides are degraded into their constitutive molecules (i.e. fatty acids) which are absorbed by the enterocytes. Following absorption, these complex lipids are re-synthesized, and along with apolipoproteins (apoB48, apoA and apoC), phospholipids and free cholesterol, chylomicrons are assembled (Green and Glickman, 1980). These
Figure 1.2 A Schematic Diagram of the Endogenous and Exogenous Metabolic Pathways of Plasma Lipoproteins. The exogenous pathway metabolizes lipoproteins that are composed of dietary lipids. The main lipoprotein involved is triglyceride-rich chylomicrons, which are produced in the intestines. The endogenous pathway metabolizes lipoproteins that are comprised of lipids synthesized \textit{in vivo}. These lipoproteins include hepatic VLDL and its degradation intermediates IDL and LDL. The endogenous pathway also metabolizes plasma HDL. CETP, cholesterol ester transfer protein; CMR R, chylomicron remnant receptor; HDL R, HDL receptor; HL, hepatic lipase; LCAT, lecithin-cholesterol acyltransferase; LDL R, LDL receptor; LPL, lipoprotein plasma lipase (reviewed in Schneider, 1996).
FIGURE 1.2
chylomicrons are then transported throughout the lymphatic vessels and into the bloodstream where they acquire more apoC proteins. ApoC is an essential component of chylomicron metabolism and activates the hydrolysis of chylomicron triglycerides by LPL. Chylomicron metabolism also involves the removal and transfer of apoA-I, apoA-II and apoC along with cholesterol and phospholipids to plasma HDL particles (Fielding and Fielding, 1996). This process results in the generation of cholesterol-rich chylomicron remnants (Redgrave, 1970). These remnants are cleared from the plasma via the apoE receptor mediated pathway, which involves the interactions of apoE with the LDL receptor-related protein (LPR) (Beisiegal et al., 1989). In mice overexpressing apoC-I, the level of plasma cholesterol is increased (Shachter et al., 1996). It is believed that apoC-I either masks or alters the conformation of apoE, and may directly delay the removal of chylomicron remnants from the plasma.

In humans, the endogenous lipid metabolic pathway is a more complex system that has been strongly associated with the development of atherosclerosis. The endogenous lipid metabolic pathway is subdivided into two main processes: the apoB100 lipoprotein pathway involving VLDL, IDL and LDL particles and the apoA lipoprotein pathway involving HDL particles.

The synthesis and assembly of VLDL predominantly occurs in the liver (Janero et al., 1984; Olofsson et al., 1987). Once assembled, VLDL is transported through the secretory pathway and secreted into the plasma where it is metabolized into smaller, denser intermediates via the endogenous lipid metabolic pathway. Once secreted into the plasma, apoB-containing VLDL acquires apoC and apoE, and becomes a substrate for LPL. LPL hydrolyzes the triglycerides on the VLDL and following hydrolysis surface
cholesterol, phospholipids and apoC proteins are transferred to HDL. This process results in the generation of IDL, a VLDL remnant particle. IDL particles are then either cleared from the plasma via the apoE receptor mediated pathway or further hydrolyzed to produce LDL. Hydrolysis of IDL is believed to require hepatic lipase (HL), located on the surface of the vascular endothelium of the liver. As a result triglycerides and apoE are removed from IDL, thus forming a LDL particle that is rich in cholesterol ester (reviewed Davis and Vance, 1996). The physiological function of LDL is then to transport endogenously derived cholesterol ester to the peripheral tissues via LDL receptor mediated endocytosis (Goldstein and Brown, 1977). However it has been shown that approximately 50% of LDL are also cleared by the liver (reviewed in Ginsberg, 1994). The apoB molecule on the surface of LDL, which acts as a ligand, mediates the binding of LDL to the LDL receptor. However, it has been shown that subfractions of LDL, notably small, dense LDL are poor ligands for the hepatic LDL receptor (Swinkels et al., 1990). The prolonged appearance of small, dense LDL in the plasma is associated with its reduced rate of clearance. Furthermore, it has been suggested that small, dense LDL are more likely to be oxidized and lead to the generation of atherosclerotic plaques (Anber et al., 1996).

The second endogenous lipid metabolic pathway involves the metabolism of apoA containing lipoproteins, namely HDL. In the plasma, HDL is assembled with components that are acquired by the metabolism of other lipoproteins, thus classifying HDL as a cholesterol scavenger. The functional complexity of HDL has been attributed to its heterogeneous size, density, lipid and apolipoprotein compositions. There are two major subclasses of HDL, HDL2 and HDL3 that are separated based on their size and
presence of a secondary apolipoprotein, apoA-II (Patsch et al., 1980; Cheung and Albers, 1984). All classes of HDL contain apoA-I, a major apolipoprotein that acts as an activator of LCAT. An HDL3 subtype, preβ HDL, is thought to be the initial acceptor of cholesterol from the peripheral tissue in a process termed reverse cholesterol transport (Fielding and Fielding, 1996). As these particles continue to collect cholesterol they become larger in size and eventually acquire apoC-II and apoC-III, resulting in the formation of HDL2 (McCall et al., 1989). Free cholesterol is esterified by LCAT to cholesterol ester that is inserted into the core of HDL, the primary determinant for the increase in HDL size. The HDL2 then transports the cholesterol ester to the liver and other tissues, and is used either in the synthesis of gonadal and adrenal steroid hormones or it is excreted into the bile acids. In addition, cholesterol esters can also be exchanged for triglycerides on other lipoproteins (i.e. VLDL) by the actions of cholesterol ester transfer protein (CETP).

HDL metabolism is thought to occur by one of two pathways. The first pathway involves the transfer of cholesterol esters from HDL to LDL or chylomicrons, which are cleared from the plasma through the interactions with the LDL receptor or LRP, respectively. The second pathway involves any number of HDL binding proteins that may have an involvement in HDL metabolism. It has been established that the scavenger receptor, SR-BI, on the surface of the liver and steriodogenic tissues act as “docking” site for HDL (Acton et al., 1996). Once bound to SR-BI, cholesterol esters can be removed from the HDL. The HDL is then released and re-circulated in the plasma to acquire more cholesterol. Another candidate HDL receptor is HB2, one of a pair of liver HDL binding proteins that shows high sequence homology with adhesion molecules, particularly
activated leukocyte-cell adhesion molecule. Studies have shown that cells overexpressing HB2 revealed a 2 fold increase in the binding of HDL (Kurata et al., 1998; Fidge, 1999). Furthermore, cubilin, an endocytic receptor for the intrinsic factor-vitamin B(12) was suggested to also play a role in the acquisition and catabolism of HDL in embryonic and the renal tissues (Hammad et al., 1999). Another mechanism in the liver believed to be participating in the clearance of HDL from the plasma is the binding of HDL to a receptor that may mediate the clearance of the whole HDL particle (reviewed in Steinberg, 1996). However, the mechanisms in which these proteins may function in the catabolism of HDL remains to be elucidated.

1.3 Lipoprotein Disorders

Fredrickson et al. (1967) originally classified lipoprotein disorders (dyslipoproteinemias) based on their electrophoretic migration pattern. Today dyslipoproteinemias, are based on a number of criteria including the etiology of the disorder and the alteration in the levels of lipoproteins and plasma lipids (Havel, 1982). Dyslipoproteinemias are categorized into primary or secondary lipid disorders. Primary dyslipoproteinemias are of genetic (familial) origin and are not as common as secondary dyslipoproteinemias (Kostner, 1991), which originate as a result of diet, substance abuse, hormonal or metabolic diseases, and infectious or malignant causes. Table 1.3 outlines different forms of dyslipoproteinemias and briefly describes their characteristics. Here the focus will be on lipid disorders that are characterized by the abnormal regulation of apoB-containing lipoproteins, namely LDL and VLDL, and their potential involvement in the development of atherosclerosis.
## TABLE 1.3

**Classification of Primary Dyslipidemias**

<table>
<thead>
<tr>
<th>Disease</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperchylomicronemia</td>
<td>Deficiency in lipoprotein lipase and elevated levels of TG</td>
</tr>
<tr>
<td></td>
<td>Deficiency in apoCII</td>
</tr>
<tr>
<td>Familial Hypercholesterolemia</td>
<td>Deficiency in LDL receptor and elevated levels of LDL-cholesterol</td>
</tr>
<tr>
<td>Familial Combined Hypercholesterolemia</td>
<td>Elevated levels of VLDL-TG and VLDL-cholesterol</td>
</tr>
<tr>
<td>Familial Dys-β-lipoproteinemia</td>
<td>Defect in apoE and elevated levels of TG and cholesterol</td>
</tr>
<tr>
<td></td>
<td>Deficiency in hepatic lipase</td>
</tr>
<tr>
<td>Hypertriglyceridemia</td>
<td>Elevated levels of VLDL-TG</td>
</tr>
<tr>
<td>Hypo-α-lipoproteinemia</td>
<td>Reduced levels of HDL-cholesterol</td>
</tr>
</tbody>
</table>

Elevated levels of plasma LDL and cholesterol have been strongly correlated to the development of atherosclerosis (Mahley et al., 1991; Ginsberg, 1994). These conditions may result from the impaired clearance of plasma LDL and/or the overproduction of apolipoprotein B-containing lipoproteins (O'Brien and Chait, 1994). Considerable research has focused on the mechanisms that contribute to the elevated plasma LDL levels, including alterations in the LDL and mutations in the LDL receptor.

Impairment in the clearing of plasma LDL has been shown to occur in patients with familial hypercholesterolemia (FH) (reviewed in Goldstein and Brown, 1989). This disease is inherited as an autosomal dominant gene, and FH patients are either heterozygotes or homozygotes for the nonsense mutation or deletion in the gene encoding for the LDL receptor (Goldstein and Brown, 1989). Consequently, cells that are involved in both the uptake of apoB and apoE-containing lipoproteins are either absent or defective in LDL receptors (Mahley et al., 1991). The absence of any immunochemically detectable LDL receptor on the surface of the cell membrane is the result of a large number of mutations. A second class of mutations is characterized by the normal expression of LDL receptor on the surface of the cell membrane but a defect in the binding of apoB or apoE-containing lipoproteins. Interestingly, a recent study reported a form of inherited hypercholesterolemia that is not characterized by direct modulation of LDL receptor gene expression (Zuliani et al., 1999). This disorder was termed familial recessive hypercholesterolemia and appeared to cause an increase in plasma LDL, likely due to a selective reduction in hepatic LDL uptake. Moreover, it was speculated that suppression of hepatic LDL receptor activity, by a recessive defect in the regulation of cellular cholesterol metabolism, may be responsible for this selective reduction in LDL.
clearance. Nevertheless, the levels of plasma LDL and cholesterol are dramatically increased, thus making the LDL particles susceptible to oxidative modifications. Oxidized LDL particles are not recognized by the LDL receptor but instead are ligands for scavenger receptors on macrophages (Goldstein et al., 1979; Nagelkerke et al., 1983). The uptake of oxidized LDL particles by macrophages in the subendothelial layer of the arterial wall stimulates the development of foam cells. In addition, a cascade of events that is induced by oxidatively modified LDL may initiate the formation of an atherosclerotic plaque.

Extensive research has also focused on the relationship between various subpopulations of LDL and the development of atherosclerosis. Increased plasma levels of small, dense LDL have been demonstrated in patients with coronary heart disease. Elevated levels of plasma LDL have also been shown in patients with disorders associated with coronary heart disease (CHD) such as diabetes mellitus, familial combined hyperlipidemia, hyperapobetalipoproteinemia and familial dyslipidemic hypertension (Kwiterovich and Sniderman, 1983; Feingold et al., 1992; Austin, 1991). A number of studies have suggested an inverse relationship between LDL particle diameter and its susceptibility to oxidation (Tribble et al., 1992; de Uraaf et al., 1991). Smaller, dense LDL particles have higher ratio of free cholesterol on the surface of the particle than esterified cholesterol in the core of the particle than larger LDL. In addition, there appears to be a strong correlation between the rate of lipid peroxidation and the level of free cholesterol on the surface of the LDL particle (Tribble et al., 1992).

An increasing body of evidence is also revealing an important role for VLDL in the development of CHD. Identification of VLDL as an independent risk factor is an
issue that remains controversial. The difficulty is due in part to the observation that an increase in plasma triglycerides are often associated with a reduction in HDL levels. In addition, daily variation in the fasting plasma levels of triglycerides may distort the results of epidemiological studies thus making assessments difficult (Ginsberg, 1994). However, increasing evidence has revealed that hypertriglycerideremia, with and without associated hypercholesterolemia, occurs frequently in patients with coronary heart disease (reviewed in Ooi and Ooi, 1998). Characteristics of hypertriglycerideremia include excess triglyceride-rich lipoproteins such as chylomicrons and VLDL and reduced levels of HDL in the plasma. Furthermore, it is believed that a deficiency in the production of apoC-II may be a contributing factor to the disease by reducing LPL activity and decreasing the rate of triglyceride hydrolysis. Although the direct role of hypertriglycerideremia on CHD remains unclear, several hypotheses have been developed. One hypothesis is that triglyceride-rich lipoproteins, namely VLDL, have the potential to cross the arterial wall and directly contribute to the formation of foam cells. A second possibility is that VLDL are used in the generation of small, dense LDL particles that have a slower catabolic rate, undergo oxidative modification and contribute to the formation of atheromas (reviewed in Ooi and Ooi, 1998).

In addition to high levels of LDL and VLDL, low levels of plasma HDL have also been shown to be a common lipoprotein disorder in patients with premature atherosclerosis. Tangier disease is a rare and severe form of HDL deficiency and is characterized by a biochemical defect in cellular cholesterol efflux. A major contribution to the cause of Tangier disease was the recent discovery of mutations in the gene encoding the ATP cassette-binding transporter 1 (ABC1) (Brooks-Wilson et al., 1999a).
This protein was found to influence intracellular cholesterol transport and was named the cholesterol efflux regulatory protein (CERP). The mutations in the gene encoding ABC1 created either a stop codon leading to truncated forms of CERP or alterations in the amino acid residues (Brooks-Wilson et al., 1999b). Studies by Remaley et al. (1999) also revealed that a deletion in the ABC1 gene resulted in a frameshift mutation and a premature stop codon leading to the production of a nonfunctional protein of approximately half the size of full-length ABC1. The data presented by these investigators thus suggests that mutations in ABC1 are major contributors to the cause of familial HDL deficiency. This deficiency is associated with a defect in cholesterol efflux and a reduction in the formation of HDL particles commonly seen in Tangier disease.

Diabetes mellitus has been shown to be a cause of secondary dyslipoproteinemias, and has been associated with a 3-4 fold increase in the risk of atherosclerosis compared to nondiabetic populations (Krolewski et al., 1990). The lipid abnormalities in diabetic patients are likely to play an important role in the development of atherogenesis. More specifically, non-insulin dependent diabetes mellitus (NIDDM, Type II) dyslipidemia, has been characterized by an increase in plasma triglycerides and small, dense LDL particles, and a reduction in plasma HDL-cholesterol (reviewed in Verges, 1999). The increase in plasma triglyceride-rich lipoproteins is correlated with higher VLDL production by the liver and a decrease in their clearance. The reduction in HDL-cholesterol is believed to be the result of an increase in HDL catabolism. Moreover, the atherogenicity of small, dense LDL particles is attributed to their increased susceptibility to oxidation (reviewed in Kreisberg, 1998). Increased lipid peroxidation in diabetes has been suggested not to be the result of an increase in free radical generation and/or the
degeneration of antioxidant systems (Julier et al., 1999). The physiopathology of lipid disorders in diabetes mellitus is multi-factorial and remains to be elucidated however such factors as hyperglycemia and insulin resistance (in NIDDM) are likely to play an important role.

The treatment of dyslipoproteinemia and CHD has commonly been by two major approaches, population-based and individual-based therapies. The population-based approach for CHD is comprised of guidelines for the lifestyle of an entire population with respect to the regulation of daily intake of dietary cholesterol and saturated fat as well as weight control and physical activity. On the other hand, the individual-based approach involves the management of a high risk CHD individual by assessing their overall CHD factors including lipid and lipoprotein profiles, history of clinical evidence of CHD and identification of major risk factors associated with CHD. The treatment regimen in this approach includes both dietary and drug therapies (Hunningshake et al., 1994).

It has been well established that inhibitors of HMG-CoA reductase, known as statins, are effective agents in the treatment of primary hypercholesterolemia. The first commercially available statin was lovastatin and since its debut several other statins including atorvastatin, pravastatin, simvastatin, fluvastatin and cerivastatin have been developed and made available as therapeutic agents. In adjunct to dietary therapies, these statins have been shown to reduce plasma LDL cholesterol concentrations by 20-40%. Statins have also been shown to upregulate the expression of hepatic LDL receptors and reduce the hepatic synthesis of apoB-VLDL (Thompson et al., 1996). They have been demonstrated to directly interfere with the synthesis of hepatic cholesterol resulting in the reduction of intracellular cholesterol available for VLDL assembly. This reduction also
leads to the enhanced expression of LDL receptors on the cell surface, and in turn increases the rate of VLDL metabolism. Simvastatin (Raal et al., 1997) and atorvastatin (Marais et al., 1997) have also been shown to reduce LDL in patients with homozygous familial hypercholesterolemia, thus suggesting that LDL reduction by the inhibitors may occur even in the absence of LDL receptors.

Other pharmacological agents have also been shown to be effective in the treatment of dyslipoproteinemias. One class includes the bile acid sequestrants such as cholesteryramine and cholestipol. These agents act by binding bile acids in the lumen of the intestines, which prevents their absorption and leads to increased excretion of steroids. The increased excretion of steroids results in the depletion of hepatic cholesterol. Consequently, cholesterol synthesis and the expression of hepatic LDL receptors and the rate of plasma LDL uptake are enhanced (Shepherd et al., 1980).

Nicotinic acid (niacin) has also been shown to decrease plasma LDL levels and has widely been used to treat atherosclerosis (Brown et al., 1990). Early studies reported that niacin acts to lower plasma LDL levels by decreasing the production and transport of VLDL. It has also been proposed that niacin may reduce lipolysis in adipose tissue, reduce the synthesis of lipoprotein (a), and alter the metabolism of HDL (Drood et al., 1991). A recent study by Jin et al. (1999) revealed that niacin also increases the intracellular rate of apoB degradation by selectively decreasing hepatic synthesis of triglycerides.

Therapeutic potential has also been revealed with inhibitors of the ACAT enzyme. Several ACAT inhibitors including CI-976 (Krause et al., 1992), FCE27677 (Musanti et al., 1996), CL 277,082 (Graham et al., 1996), and CI-1011 (Wilcox et al., 1999) were
shown to reduce plasma total cholesterol, apoB and LDL cholesterol. Recently, NTE-122, the newest and strongest selective inhibitor of ACAT was shown to substantially reduce the secretion of cholesterol esters in HepG2 cells (Azuma et al., 1999). It was suggested that the effects produced by NTE-122 were through the inhibition of VLDL secretion and the stimulation of bile acid production by the liver.

Treatment of the dyslipoproteinemia in diabetic patients is currently conducted by the administration of statins. Although gemfibrozil, a fibric acid derivative, is more effective in decreasing triglycerides and increasing HDL cholesterol in diabetic patients than the statins, LDL-cholesterol levels are not altered in the presence of the drug. Instead the levels of LDL-cholesterol may be enhanced in the presence of gemfibrozil. Thus, the change in LDL-cholesterol-to-HDL-cholesterol ratio is better directed with the statins (reviewed in Kriesberg et al., 1998). In theory, nicotinic acid can also restore or improve all lipid or lipoprotein abnormalities in patients with type II diabetes, however nicotinic acid also causes insulin resistance and may enhance hyperglycemia. The effects of improved glycemic control in potentially reducing the risk of coronary heart disease in such patients are currently being examined (reviewed in Kreisberg et al., 1998).

Although the therapeutic agents described above have shown to be effective in the treatment of primary and secondary dyslipoproteinemia, future research on the precise mechanisms of actions of these hyperlipidemic agents is essential in order to maximize the efficacy of these drugs while minimizing their inherent side effects.
1.4 The Role of Lipoproteins in Atherosclerosis

The development of atherosclerosis and CHD involves many genetic and environmental factors including dyslipoproteinemia, hyperlipidemias and cigarette smoking (Stein, 1987; Steinberg et al., 1989; Kostner, 1991). Others factors including diabetes, obesity, and physical inactivity are also believed to be important contributors to the disease. There is considerable evidence to suggest that lipoprotein disorders, in particular elevated levels of LDL and reduced levels of HDL, are central etiological factors in the development atherosclerosis (Frick et al., 1987; Assman and Sculte, 1993).

1.6 Atherosclerosis

In most developed countries, cardiovascular diseases have been the leading cause of death in both men and women. Coronary heart disease is the most common form of cardiovascular disease, which leads to the premature deaths of millions of people, particularly in Western societies. CHD is the result of an abnormality in the flow of blood through the coronary arteries leading to the onset of a myocardial ischemia and/or a myocardial infarction. Although many factors can contribute to CHD such as a coronary embolism, Kawasaki disease and coronary arteritis, the main cause of CHD is atherosclerosis.

Atherosclerosis is defined as the thickening and hardening of the arterial wall due to the development of specific lesions termed atheromas. Atheromas are composed of cholesterol, cholesterol esters, lipid-laden macrophages and smooth muscles cells, collagen and elastic fibers. The development of atheromas are believed to begin with the deposition of lipids in the macrophages of the intima layer of the arterial wall at the site
of an endothelial injury. Lipids enter the site of injury as LDL particles, and once in the subendothelial space the LDL undergo oxidative modification (reviewed in Schneider, 1996). Oxidized LDL stimulates the expression of chemotactic factors and adhesion molecules that in turn attract inflammatory cells such as monocytes (Quinn et al., 1987) and T lymphocytes (Hansson, 1993) into the subendothelial space. Colony stimulating factors, which are secreted from the endothelial cells, induce the differentiation of monocytes into macrophages (Liao et al., 1991). Cytokines secreted by the T lymphocytes then activate the macrophages to release several growth factors that stimulate the proliferation and migration of smooth muscles to the site of injury (Nomoto et al., 1988). Stimulation of these smooth muscle cells by the cytokines also induces the synthesis of a number of matrix proteins such as collagen and elastin (Navab et al., 1991). Oxidized LDL are subsequently removed by macrophages via scavenger receptor-mediated endocytosis. Macrophage uptake of modified LDL transforms these cells into cholesterol ester loaded foam cells. Any residual modified LDL not engulfed by the macrophages aggregate together to form an extracellular cholesterol core. Foam cells, the cholesterol core and fibrous tissues together form a deposit, which may eventually lead to the formation of an atheroma (reviewed in Schneider, 1996).

Although several factors may contribute to the atherosclerotic process, current research appears to suggest that abnormalities in lipoprotein metabolism may be a major factor that predispose an individual to the development of atherosclerosis.
CHAPTER 2

INTRACELLULAR MECHANISMS THAT REGULATE THE SYNTHESIS, ASSEMBLY AND SECRETION OF APOLIPOPROTEIN B

2.1 General Introduction

Apolipoprotein B100 (apoB) is a large hydrophobic glycoprotein (~550 kDa) that is involved in the assembly of lipoproteins for the transport of lipids namely triglycerides and cholesterol (reviewed in Schumaker et al., 1994). ApoB can be synthesized in two forms: apoB100 and apoB48. In humans, apoB100 is predominantly secreted by the liver whereas apoB48 is exclusively secreted by the intestines with some apoB100 (Krishnaiah et al., 1980; Chen et al., 1987; Yao and McLeod, 1994). More specifically, apoB is a major protein component (30-40%) in the assembly of VLDL and has the ability to bind both the hydrophobic core and hydrophilic shell of the lipoprotein (Gruffat et al., 1996; Dixon and Ginsberg, 1993). The properties of apoB allow it to maintain the structural integrity of the lipoprotein while facilitating the transport of these particles through the aqueous environment (Olofsson et al., 1987). The apoB molecule also contains an LDL receptor binding site (Knott et al., 1986; Yang et al., 1986) that is central for the uptake of plasma LDL by the liver and peripheral tissues (Brown and Goldstein, 1986).

The assembly and secretion of apoB-containing lipoproteins such as VLDL is dependent upon the synthesis of apoB. Increasing evidence appears to suggest that the overproduction of apoB-containing lipoproteins may be a major contributor to the development of hyperlipidemia in humans (Janus et al., 1980; Linton et al., 1993; McCormick et al., 1996). Regulation of apoB biogenesis therefore is important in maintaining lipid homeostasis. Attention has focused on the multiple mechanisms
involved in apoB production including gene transcription, mRNA editing, apoB translation and intracellular degradation (reviewed in Davis and Vance, 1996).

2.2 The Human Apolipoprotein B Gene

In humans, the apoB gene is located on chromosome 2p (Mehrabian et al., 1986; Knott et al., 1986; Cann and Guyer, 1992) unlike other apolipoproteins which have been mapped to chromosomes 1, 11 and 19 (Jackson et al., 1984; Knott et al., 1986). The apoB gene is 43 kb long and contains 28 introns and 29 exons with the mature protein sequence starting at exon 2 (Blackhart et al., 1986; Carlsson et al., 1986; Higuchi et al., 1987; Wagener et al., 1987; Ludwig et al., 1987). ApoB is constitutively expressed and tissue specific with hepatocytes and enterocytes being the main sites of expression (Knott et al., 1986). Regulatory sequences in the 5' region of the apoB gene include a classical TATA box and a CAAT box (Blackhart et al., 1986). ApoB expression is controlled by two positive elements in the liver that bind liver nuclear proteins such as BRF-1, BRF-2 and C/EBO (Das et al., 1988; Zhuang et al., 1992). Tissue-specific transcriptional enhancer sequences (Brooks and Levy-Wilson, 1992) and negative regulatory sites (Das et al., 1988; Paulweber et al., 1991) also influence the expression of apoB.

There are a number of polymorphisms identified with the human apoB gene, although there has been no direct evidence that these polymorphisms alter the function of apoB (Linton et al., 1993; Yao and McLeod, 1994). Various nonsense or framshift mutations of the apoB gene have been revealed to cause truncations in the apoB protein, and the production of these truncated apoB proteins results in a decrease in the concentration of plasma apoB, LDL and cholesterol (Linton et al., 1993; Xu et al., 1990).
The production of truncated forms of apoB appears to results in the onset of hypobetalipoproteinemia (Linton et al., 1993). In addition to alterations in the size of the apoB molecule, a point mutation in the amino acid residue 3500 of apoB has been shown to cause impaired binding of apoB to the LDL receptor which in turn contributes to hypercholesterolemia (Innerarity et al., 1990).

2.3 Apolipoprotein B mRNA

The transcriptional product of the apoB gene in the liver is a 14 kb mRNA that has a half-life of 16 hours. This apoB mRNA can be translated to produce a protein of 4536 amino acids (Scott, 1989; Pullinger et al., 1989). Differences in post-transcriptional processing of apoB mRNA has been shown to occur in most animal species. The synthesis of apoB48 is known to be the result of apoB mRNA editing. This process involves an enzymatic deamination of a single cytidine at human nucleotide cytosine 6666. This in turn converts the glutamine codon 2153 (CAA) to a stop codon (UAA) (Powell et al., 1987; Chen et al., 1987), and consequently, translation of this edited mRNA is halted earlier and apoB48 is produced. ApoB48 is a truncated form of apoB100 that is comprised of the first N-terminal 48% of the full-length apoB100 (Chen et al., 1987). The molecular weight of apoB48 is 240 kDa, approximately half of the full-length apoB100. In humans, the main function of apoB48 is to direct the assembly and transport of chylomicrons (Schumaker et al., 1994).

The mRNA editing enzyme has been a major focus of attention in the study of apoB48 biogenesis. APOBEC-1, the catalytic subunit of the apoB mRNA editing cytidine deaminase enzyme has been cloned from a number of species, and has been shown to be
a homodimer zinc-containing cytidine deaminase (Navaratnam et al., 1998). Protein factors have been revealed to be essential for the competent editing activity of this enzyme, possibly by acting as RNA-binding components of the holoenzyme or as RNA chaperones in the recognition of sequence and structural motifs within the apoB mRNA (Navaratnam et al., 1993; Giannoni et al., 1994; Driscoll and Zhang, 1994; Nakamuta et al., 1995). In a computer modeling analysis, the apoB mRNA was predicted to have a highly conserved stem loop secondary structure, which contained the edited cytidine residue (Shah et al., 1991; Navaratnam et al., 1993). A recent study by Richardson et al. (1998) demonstrated the formation of this stem loop structure and proposed that it is used in the presentation of the edited cytidine to the active site of the APOBEC-1 enzyme.

2.4 The Structural and Functional Domains of Apolipoprotein B

ApoB100 is one of the largest single polypeptide proteins, and has a molecular weight of 513 kDa (Segrest et al., 1994). The translated apoB product contains a signal sequence of 24 to 27 amino acids followed by the mature apoB protein of 4536 amino acids (Figure 2.1) (Knott et al., 1986; Yang et al., 1989; Cladaras et al., 1986; Olofsson et al., 1987). The primary sequence of apo100 contains 25 cysteine residues with a majority of these residues located within the N-terminal region of the protein. Sixteen of these cysteine residues form disulfide linkages, which are believed to bridge together hydrophobic regions of the apoB molecule (Yang et al., 1990). Folding of the amino terminal disulfide domain has been revealed to be essential in the initiation of apoB-containing lipoprotein assembly (Shelness and Thornburg, 1996; Huang and Shelness, 1997). In addition, two of the reduced cysteine residues 3734 and 4190 that are exposed
at the surface of the LDL particle, are believed to be involved in the formation of disulfide bonds with apolipoprotein (a) (Guervara et al., 1993).

ApoB also contains 19 N-linked glycosylation sites of which 4 are located in the β-turn structures of the apoB protein. Sixteen of these N-linked glycosylation sites are linked to carbohydrates (Yang et al., 1989), and the total carbohydrate component of apoB is 8-10% of the protein. Secreted apoB has been shown to contain both mannose and complex carbohydrate moieties, and the addition of these carbohydrates onto the apoB protein increases its molecular weight to approximately 550 kDa. It has been suggested that the carbohydrates of apoB, particularly those located in the LDL receptor-binding site, contribute to the binding of LDL to the receptor (Schumaker et al., 1994).

Further post-translational modifications of apoB include fatty acid acylation and phosphorylation. The acylation of secreted apoB from HepG2 cells with both stearic and palmitic acids have been observed, and it has been suggested that the physiological role of this modification is to facilitate the assembly of lipoproteins by anchoring the apoB molecule to the ER membrane (Hoeg et al., 1988). Phosphorylation of apoB on its serine and tyrosine residues has been identified in rat hepatocytes (Sparks and Sparks, 1990). An increase in the number of phosphorylated sites has also been observed in diabetic rats in comparison to non-diabetic rats (Sparks and Sparks, 1990).

ApoB is thought to contain a pentapartite structure with a globular N-terminal domain followed by alternating amphipathic β-strands and α-helices (NH₂-α₁-β₁-α₂-β₂-α₃-COOH) (Segrest et al., 1994). The location of these α-helices and β-strands within human apoB100 are illustrated in Figure 2.1. The β-strands are believed to be the primordial apoB molecules since they are similar to the ones that bind lipids in
vitellogenin (Raag et al., 1988). These β-strands are spread throughout apoB, however, there are some clustering of the β-strands in the proline-rich repeats of the protein (Knott et al., 1986). It is well accepted that the β-strands are the major lipid binding domains in apoB, and that β-strands are also responsible for the association of newly synthesized apoB with the endoplasmic reticulum (ER) membrane. Amphipathic α-helices of apoB, which are also common secondary structures among proteins, are believed to be too short to have any significant interactions with the ER membrane (Segrest et al., 1994). Instead it has been proposed that the α1-helix is involved in the globular domain of the N-terminus and α2- and α3-helices may represent flexible, hinged domains that may contribute to particle size variability (Segrest et al., 1994). The unusual length of apoB100 is also believed to be responsible for its ability to recruit lipids in the assembly of lipoprotein particles (Yao et al., 1991; Spring et al., 1992). Furthermore, the length of apoB appears to play a role in the metabolic fate of apoB-containing lipoproteins and may influence the intracellular biosynthesis and extracellular secretion of these particles (Sniderman, 1988, Liang et al., 1998).

In addition to the α and β-domains, apoB also contains several heparin-binding domains and an LDL receptor-binding domain. The heparin binding domains, which are localized to the basic regions (lysine and arginine) of apoB (Segrest et al., 1994), are believed to participate in the binding of LDL particles to the capillary endothelium surfaces acted upon by lipoprotein lipases. The LDL receptor-binding domain, which comprises three of the heparin-binding domains, is localized at amino acid residues 3100-3600 of the apoB. This region of the apoB molecule is also homologous to the LDL receptor-binding region of apoE (residues 136-155) (Wilson et al., 1991). The functional
Figure 2.1. The Location of the $\alpha$-helix and $\beta$-strand Domains within Human Apolipoprotein B. A schematic diagram of the primary structure of apoB100 outlining the position of the $\alpha$-helix (\textit{\begin{alltt}0000\end{alltt}}) and $\beta$-stand (\textit{\begin{alltt}\wedge\wedge\end{alltt}}) domains within apoB100 and various C-terminally truncated apoB constructs (reviewed in Segrest \textit{et al.}, 1998).
ApoB100 Polypeptide

N-terminal

ApoB15  675 a.a.

ApoB29  1305 a.a.

ApoB48  2160 a.a.

ApoB72  3240 a.a.

C-Terminal

FIGURE 2.1
role of the LDL receptor-binding domain is to regulate the uptake of plasma LDL by the LDL receptors on the membrane of liver and peripheral tissues (Knott et al., 1986; Yang et al., 1986; Brown and Goldstein, 1986). Binding between LDL and LDL receptor is believed to occur through positively charged residues within the LDL receptor-binding domain of apoB and the negatively charged residues on the LDL receptor (Brown and Goldstein, 1986).

The hydrophobic and hydrophilic nature of the apoB domains thus allows the protein to both bind the neutral lipid core and interact with the aqueous environment (Olofsson et al., 1987). The conformation of apoB molecule to the lipid composition is described as a ribbon and bow model (Schumaker et al., 1994; Chatterton et al., 1995). In this model, the first 89% of the apoB molecule is wrapped around the LDL particle in a belt-like fashion. The C-terminal 11% of the apoB molecule is stretched back towards the LDL-receptor binding domain forming a bow-like structure that may act as a negative regulator of LDL-LDL receptor-binding interactions (Chatterton et al., 1995).

2.5 Mechanisms that Acutely Modulate the Biosynthesis and Secretion of Apolipoprotein B-containing Lipoproteins

2.5.1 Transcriptional Regulation of Apolipoprotein B Production

Several studies have revealed that hepatic secretion of apoB does not appear to be regulated at the transcriptional level (Dashti et al., 1989; Pullinger et al., 1989; Moberly et al., 1990; Kaptein et al., 1991). ApoB mRNA levels have been shown to remain relatively constant under various conditions that alter apoB production. Such conditions include the addition of free fatty acids (i.e. oleate) to HepG2 cells, which stimulates apoB
secretion without altering apoB mRNA levels (Pullinger et al., 1989; Dashti et al., 1989; Moberly et al., 1990). Insulin treatment of HepG2 cells results in a decrease in apoB secretion without a change in the steady-state of apoB mRNA (Pullinger et al., 1989; Dashti et al., 1989). There are certain conditions, however, that do modulate the levels of hepatic apoB mRNA. In HepG2 cells, apoB mRNA levels have been shown to increase in the presence of 25-hydroxycholesterol (Dashti et al., 1992) and thyroid hormone (Theriault et al., 1992).

2.5.2 Translational Regulation of Apolipoprotein B Production

Regulation of apoB at the translational level has been demonstrated in several studies using various cell systems. These studies have revealed that apoB synthesis can be regulated by alterations in the translation process. An increase in apoB secretion was observed in HepG2 cells treated with thyroid hormone (Theriault et al., 1992) and also with HepG2 cells treated with exogenous VLDL (Wu et al., 1994). It was revealed that an increase in the rate of apoB synthesis contributed to the increase in apoB secretion under these conditions (Theriault et al., 1992). In addition, CaCo-2 human colon adenocarcinoma cells revealed an increase in apoB production when the cells were incubated with phosphatidylcholine. ApoB mRNA translation can also be downregulated as seen by a decrease in apoB secretion in rat hepatocytes treated with insulin (Sparks and Sparks, 1990). An in vitro translation system in HepG2 cells directly revealed that insulin can inhibit apoB mRNA translation (Adeli and Theriault, 1992). In addition, streptozotocin-induced diabetic rats also revealed a decrease in apoB production, primarily due to a decrease in apoB synthesis (Sparks et al., 1992).
2.5.3 Co-translational and Post-translational Regulation of Apolipoprotein B

Production

Regulation of apoB production appears to occur either co-translationally and/or post-translationally. The translocation of apoB across the ER is thought to be a key regulatory event in the assembly and secretion of apoB-containing lipoproteins. Translocational efficiency may determine whether apoB is secreted from the hepatocytes or intracellularly degraded (Borchardt and Davis, 1987; Davis et al., 1990). ApoB molecules that are not efficiently translocated across the ER membrane and assembled into a lipoprotein particle are then subject to intracellular degradation (Du et al., 1994). Current research is focusing on elucidating the precise mechanisms involved in apoB translocation and degradation of and the regulatory factors that modulate these events.

2.5.3.1 Apolipoprotein B Translocation Across the Endoplasmic Reticulum Membrane

ApoB synthesis occurs on the ER membrane bound ribosomes, and is co-translationally translocated across the ER membrane. Although there is an agreement among investigators that apoB associates with the ER membrane, the controversy concerns whether or not apoB acts as a transmembrane protein on the surface of the ER and/or throughout the secretory pathway. Knott et al., (1986) and Yang et al., (1986) originally reported that apoB does not contain any classical membrane spanning domains. However, early studies by Bostrom et al., (1986) revealed that over 60% of apoB in the ER of HepG2 cells was associated with the ER membrane. It was later revealed through pulse chase studies that apoB remained associated with the ER membrane for
approximately 15 minutes prior to being transferred into the ER lumen. While some laboratories have revealed that ER membrane associated apoB is not exposed to the cytosol (Pease et al., 1995; Ingram and Shelness, 1996; Shelness et al., 1994), evidence from most other laboratories suggest that apoB does contain regions that are cytosolically exposed (Davis et al., 1990; Thrift et al., 1992; Macri and Adeli, 1997a; Chuck and Lingappa, 1992; McLeod et al., 1996; Liang et al., 1998; Du et al., 1998).

*In vitro* translation and translocation studies using truncated variants of apoB revealed that although apoB was associated with the ER membrane it was not exposed to the cytosol (Pease et al., 1991). Similar results were observed by Shelness et al. (1994) indicating complete translocation of apoB50 (first 50% N-terminal region of apoB100) in COS-I cells, a non-hepatic cell line, transfected with human apoB. Furthermore, Pease et al. (1995) also revealed no cytosolic exposure of apoB30 in the presence of dog pancreas microsomes. Studies examining translocational efficiency (percent trypsin-resistant) of endogenous apoB in HepG2 demonstrated that as much as 80% of these apoB molecules were protected from trypsin digestion (Ingram and Shelness, 1996; Leiper et al., 1996).

On the other hand, many investigators have suggested that apoB contains cytosolic exposure. Early reports by Davis et al. (1990) identified apoB as a cytosolically exposed protein by revealing that approximately 56% of apoB100 and 70% of apoB48 isolated in the ER of rat hepatocytes were sensitive to exogenous trypsin. Further studies using isolated microsomes in conjunction with protease protection assays have supported the findings reported by Davis and coworkers (Chuck et al., 1990; Dixon et al., 1992; Furukawa et al., 1992; Boren et al., 1993; Rusiñol et al., 1993; Verkade et al., 1993; Wilkinson et al., 1993; Du et al., 1994; Bonnardel and Davis, 1995; Wang et al., 1995;
McLeod et al., 1996). Macri and Adeli (1997a) also revealed using a digitonin-permeabilized HepG2 cell system that apoB was approximately 60-80% sensitive to exogenous trypsin. More recently, Du and coworkers (1998), using permeabilized HepG2 cells, identified two regions of ER membrane-bound apoB100 that were exposed to the cytosol at the site of apoB synthesis and throughout most of the remaining secretory pathway. They further suggested that a transmembrane orientation may be important in the efficient apoB lipidation by microsomal triglyceride transfer protein (MTP).

The association between apoB and the ER membrane has been suggested to be the result of complex structural features within the apoB molecule. Like other secretory proteins, apoB also contains a cleavable signal sequence (Dixon and Ginsberg, 1993; Innerarity et al., 1996). However, unlike other secretory proteins, apoB slowly crosses the ER membrane (Chuck et al., 1990). It was described that apoB translocation proceeded in a step-wise manner resulting from specific ‘pause transfer sequences’ thought to mediate the transient arrest of apoB translocation (Chuck and Lingappa, 1992, 1993). Further studies revealed 41 potential pause transfer sequences in apoB100 that are asymmetrically distributed throughout apoB (Chuck and Lingappa, 1993; Kivlen et al., 1997). It was determined that these sequences function independently of each other and independent of protein synthesis (Chuck and Lingappa, 1993). Hedge and Lingappa (1996) also revealed that during the translocational pausing, the ribosome membrane junction opens and exposes the apoB molecule to the cytosol. An alternative mechanism for the appearance of transmembrane forms of apoB was suggested by Pease et al. (1995) using COS I cells expressing the amino portion of apoB. They speculated that the transient pausing of apoB was the result of translational pausing. More specifically,
translational pausing was attributed to ribosomal pausing caused by tRNA persistence on the emerging apoB polypeptide (Pease et al., 1995). Once apoB translation resumes it becomes uncoupled from translocation generating cytoplasmically exposed apoB. Although there is convincing evidence for both translational and translocational pausing processes, the differences observed between various groups may reflect variation in experimental approaches.

There are a number of reports revealing that the translocation of apoB across the ER membrane is an important regulatory event in the secretion of apoB-containing lipoproteins (Bonnardel and Davis, 1995; Davis et al., 1990; Davis et al., 1989; Thrift et al., 1992; Bostrom et al., 1988). Several factors are known to contribute to the efficiency of apoB translocation, including lipid availability, structural and functional domains within the nascent polypeptide and the presence of chaperone proteins.

Molecular chaperones have been described as a class of proteins which function to ensure the proper conformation and assembly of maturing proteins by preserving the correct folding of the nascent polypeptide (Ellis, 1987). Translocation of most secretory proteins involves the aid of ER chaperone proteins such as protein disulfide isomerase (PDI), calnexin and BiP. ApoB has been found to be associated with these ER chaperone proteins, and growing evidence suggest that these chaperone proteins are involved in the translocation and maturation of apoB and apoB-containing lipoproteins (reviewed in Yao et al., 1997; Ginsberg, 1997). Calnexin is an integral membrane protein which has been shown to be associated with nascent glycoproteins, including apoB (David et al., 1993; Ou et al., 1993). Chen et al. (1998) demonstrated that the translocation of apoB decreases when the interaction between apoB and calnexin is inhibited. Other molecular chaperones
including Hsp70 (Fisher et al., 1997; Zhou et al., 1995) and BiP, an ER homologue of Hsp70 (Plemer et al., 1997) have also been found to associate with apoB. The binding of Hsp70 to apoB was linked to the ubiquitin-dependent proteasomal degradation of apoB. BiP on the other hand was suggested to be involved in the movement of apoB through the translocational channel. Another group of molecular chaperones associated with apoB are ERp72, ER60 and PDI, members of the thioredoxin family of proteins (Ou et al., 1993; Adeli et al., 1997a). ER60 was suggested to be involved in the post-translational degradation of apoB (Adeli et al., 1997a). However, ERp72 is thought to participate in the disulfide bond formation of apoB (Linnik and Herscovitz, 1998). The formation and arrangement of disulfide bonds within apoB is also believed to be catalyzed by PDI, a molecular chaperone shown to strongly associate with apoB.

2.5.3.2 Intracellular Degradation of Apolipoprotein B

Inefficient translocation of apoB and inadequate assembly and secretion of apoB-containing particles has been shown to be a determinant of intracellular apoB degradation. Degradation of apoB was first observed by Borchardt and Davis (1987) and believed to occur in the ER and post-ER compartments (i.e. Golgi) (Wang et al., 1995; Adeli, 1994; Verkade et al., 1993; Furukawa et al., 1992; Davis et al., 1990; Davis et al., 1989). It has been suggested that newly synthesized apoB molecules that do not efficiently translocate into the ER lumen are subsequently diverted for intracellular degradation. Evidence to date support the notion of a two step degradation process involving early, co-translational degradation of nascent apoB chains and post-translational degradation of fully-translated apoB molecules (reviewed in Yao et al.,
1997). Current evidence appears to suggest that the cytosolic proteasome may be involved in both stages of the degradation process (Sakata et al., 1999; Chen et al., 1998; Fisher et al., 1997; Benoist and Grand-Perret, 1997; Yeung et al., 1996). Details of our current understanding of the mechanisms mediating apoB degradation are discussed later in this chapter.

2.5.4 Assembly of Apolipoprotein B-containing Lipoproteins

VLDL is the main carrier for the transport of endogenous triglyceride, and its metabolic product, LDL, is the main carrier of cholesterol ester. Modulation of VLDL assembly and secretion has been revealed to alter both plasma triglyceride and cholesterol ester levels. An increase in plasma triglyceride-rich lipoproteins such as VLDL has also been shown to potentially play a role in the atherogenesis. The synthesis and assembly of VLDL predominantly occurs in the liver via a two-step process. The assembly of VLDL begins with apoB synthesis and translocation into the rough ER where apoB associates with intracellular lipids such as triglycerides, cholesterol esters and phospholipids to form a primordial-VLDL particle (Borchardt and Davis, 1987; Bostrom et al., 1986; Cartwright et al., 1997). In the second step, large triglyceride-rich globules are added to the primordial-particle to form mature VLDL that is rich in triglycerides (Boren et al., 1990; Cartwright and Higgins, 1995; Cartwright et al., 1997). In a recent report by Wang et al. (1999), it was reported that assembly of all apoB molecules, regardless of size, followed a similar process. It was further revealed that the MTP activity is essential in facilitating the accumulation of triglycerides in the microsomes and incorporating this triglyceride pool during the final stages of apoB100 VLDL assembly (Wang et al., 1999).
2.5.4.1 The Microsomal Triglyceride Transfer Protein as a Regulator of Apolipoprotein B-VLDL Assembly

MTP is a heterodimer consisting of a large catalytic subunit (97kDa) and PDI (55 kDa). The expression of MTP is limited to the lumen of the smooth ER of the liver and the intestines (Gordon et al., 1994) and possibly the yolk sac (Farese et al., 1996). MTP is thought to catalyze the transfer of lipids such as triglycerides and cholesterol esters to phospholipid surfaces of the ER (Wetterau et al., 1985; Gordon et al., 1994; Benoist et al., 1996). Several studies have suggested a role for MTP in the proper conformation and assembly of apoB-containing lipoproteins (Gordon et al., 1995; Gordon et al., 1996). In cells devoid of MTP activity, such as Chinese hamster ovary, the translocation and secretion of transfected apoB is inhibited, and thus it is suggested that MTP activity is required for the efficient translocation of apoB (Thrift et al., 1992). Co-expression of apoB with MTP results in the synthesis and secretion of apoB-containing lipoprotein particles (Gordon et al., 1994; Leiper et al., 1994; Gretch et al., 1996).

The binding of MTP to apoB appears to be regulated by the length of the apoB polypeptide and the degree of lipidation (Hussain et al., 1997). Hussain et al. (1998) identified amino acids 430-570, in the α1-globular domain of apoB, as a critical region for the binding of MTP. It was suggested that the role of MTP binding to α1-globular domain may involve initiation and maturation of apoB-containing lipoproteins (Hussain et al., 1998). More specifically, the lysine and arginine residues in the N-terminal 18% of apoB were shown to be crucial for the interaction of apoB with MTP (Bakillah et al., 1998). Nicodeme et al. (1999) recently identified a region between the N-terminal 48 and 53% of apoB100 that also shows a high requirement for MTP. This region contains an α-
helical domain flanked on either side by β-sheet domains. Although the precise function of these domains is unknown, it was speculated that they may stabilize the apoB molecule during its lipidadation by MTP. Secretion of apoB molecules smaller than B48 were found to be resistant to an MTP inhibitor whereas secretion of larger apoB (greater than B48) were shown to be highly sensitive to an MTP inhibitor. It was further suggested that during apoB polypeptide elongation the α-helical domain may switch the apoB molecule from one stable conformation (lipid-poor apoB48) to another stable conformation (lipid-rich apoB100) (Nicodeme et al., 1999).

Evidence appears to suggest that MTP is required for the efficient secretion of apoB-containing lipoproteins. Modulation of MTP expression and activity has also been shown to influence the rate of apoB assembly and secretion (Gordon et al., 1994; Leiper et al., 1994; Jamil et al., 1996; Patel and Grundy, 1996; Wang et al., 1996; Wang et al., 1997; van Greevenbroek et al., 1998). Recently, the sterol regulatory element-binding protein (SREBP) was found to negatively regulate the expression of the gene encoding MTP by binding to the sterol response element site in the promoter region (Sato et al., 1999). Furthermore, it was suggested that the mechanism by which SREBP modulates MTP gene expression may be distinct from that of the positive upregulation of sterol-regulated genes that encode proteins involved in lipid metabolism. The concerted action of SREBPs along with co-regulators have been identified as upregulators of the expression of the human LDL receptor gene (Briggs et al., 1993; Wang et al., 1993). Transgenic mice overexpressing the nuclear form of SREBP were also shown to overproduce cholesterol and fatty acids and to accumulate massive amounts of cholesterol and triglycerides in the hepatocytes (Horton et al., 1999).
2.5.4.2 Core Lipoprotein Lipids as Regulators of VLDL Assembly and Secretion

ApoB-containing lipoprotein secretion is dependent upon several factors, including lipid availability, post-translational regulation of apoB, and the regulation of functional LDL receptors. The availability of lipids including cholesterol esters (Thompson et al., 1996), triglycerides (Cartwright and Higgins, 1995) and phospholipids (Yao and Vance, 1988; Gruffat et al., 1996) are initial regulatory factors in the assembly of apoB-containing lipoprotein particles. Although all of these lipids contribute to the biogenesis of VLDL, debate currently remains over which of the neutral lipids, cholesterol esters or triglycerides, has the most influential role in VLDL secretion.

Cholesterol ester is one of the two main components of the neutral lipid core of lipoproteins. The synthesis of cholesterol ester is dependent upon the actions of two rate limiting enzymes: hydroxymethylglutaryl-CoA reductase (HMG-CoA reductase) and acyl-CoA:cholesterol acyltransferase (ACAT). HMG-CoA reductase and ACAT both reside in the ER and function to synthesize and esterify cholesterol, respectively. The amount of cholesterol ester available for lipoprotein assembly appears to be regulated by several factors, including the requirement for cholesterol in the synthesis of bile acids (Lin et al., 1996) and the nutritional state of the organism (Tarugi et al., 1996). In the liver, receptor-mediated endocytosis of LDL is a major contributing process in the recycling of cholesterol and cholesterol ester (Brown and Goldstein, 1986). As the hepatic intracellular level of free cholesterol rises by LDL endocytosis, HMG-CoA reductase activity is inhibited. This in turn suppresses the de novo synthesis of hepatic cholesterol. Free cholesterol also acts directly on the LDL receptor gene to reduce the
expression of receptor, which in turn reduces the uptake of plasma LDL. Esterification of cholesterol by ACAT decreases the intracellular levels of free cholesterol, and alleviates the negative control over HMG-CoA reductase activity and LDL receptor expression (reviewed in Goldstein and Brown, 1990). The esterified cholesterol pool is stored as lipid droplets and is eventually used in the synthesis and assembly of VLDL. Studies have also shown a role for SREBP in the synthesis of cholesterol and the assembly and secretion of VLDL (Wang et al., 1997). SREBP is an ER transmembrane protein, and under conditions of low intracellular sterol levels, the N-terminal DNA binding domain of SREBP is cleaved (Yokoyama et al., 1993; Sakai et al., 1996). This DNA binding domain then enters the nucleus and activates the transcription of genes that are involved in the de novo synthesis of sterol lipids, including HMG-CoA reductase (Vallet et al., 1996). Consequently, cholesterol synthesis is stimulated and apoB-VLDL assembly and secretion are enhanced. Cleavage of the SREBP is regulated by the transmembrane SREBP cleavage activating protein (SCAP). SCAP and SREBP interact in the ER membrane through their cytoplasmic C-terminal domains, a complex essential for the cleavage of SREBP (Sakai et al., 1998). It has also been shown that SCAP contains a sterol-sensing domain, like other transmembrane sterol-sensing proteins such as HMG co-A reductase (Nohturfft et al., 1998). As such in sterol-overloaded cells the activity of SCAP is downregulated and thus the cleavage of SREBP is inhibited. It has been suggested that the sterols regulate the cleavage of SREBP by decreasing the ability of SCAP to transport SREBP to post-ER compartments which house Site-1 protease that are responsible for SREBP cleavage (Nohturfft et al., 1999).
There has been controversy over the regulatory importance of cholesterol ester in the assembly and secretion of VLDL. Several studies in HepG2 cells (Sato et al., 1990; Furukawa and Hirano, 1993; Wu et al., 1994; Benoist et al., 1996) have revealed that a reduction in cholesterol ester synthesis does not decrease the rate of apoB-VLDL secretion. Several other investigators, however, have used both HMG-CoA reductase and ACAT inhibitors, in several different cell models, to demonstrate a significant regulatory role for cholesterol ester in apoB-VLDL assembly and secretion (Cianflone et al., 1990; Tanaka et al., 1993; Huff et al., 1994; Musanti et al., 1996; Avramoglu et al., 1995; Carr et al., 1995; Thompson, et al., 1996; Burnett et al., 1997; Zhang et al., 1999). Recently, Zhang et al. (1999) examined HMG-CoA reductase and ACAT inhibitors in primary hamster hepatocytes. Their studies revealed a close correlation between the mass of cholesterol ester in hamster hepatocytes and the secretion of apoB100-VLDL. Inhibitors to HMG-CoA reductase were also shown to reduce in vivo both the apoB-VLDL and LDL production in miniature pigs (Burnett et al., 1997). A similar effect on apoB secretion was observed in studies using HMG-CoA reductase inhibitors in HepG2 cells (Mohammadi et al., 1997). Comparative studies between two inhibitors of HMG-CoA reductase, atorvastatin and simvastatin, in HepG2 cells revealed that although both drugs inhibited cholesterol synthesis and decreased cellular cholesterol mass, only atorvastatin reduced the rate of apoB secretion (Wilcox et al., 1999). The effects of atorvastatin on apoB secretion was shown to be the result of increased apoB degradation, an affect not observed with simvastatin. In addition, atorvastatin was also shown to decrease the mRNA level for MTP which may account for the increase in the rate of apoB degradation (Wilcox et al., 1999b). Inhibitors of ACAT were also shown to decrease hepatic apoB
secretion in miniature pigs (Huff et al., 1994) and in a recent report by Burnett et al. (1999) it was revealed that the ACAT inhibitor avasimibe (CI-1011) decreased both the plasma concentrations of total triglycerides, VLDL-triglycerides and VLDL-cholesterol in these animals. In addition, avasimibe also reduced the VLDL apoB pool size and hepatic secretion of VLDL apoB thus revealing an association between cholesterol esterification and hepatic VLDL apoB secretion (Burnett et al., 1999). Further studies by Wilcox et al. (1999) revealed that inhibition of ACAT in HepG2 cells decreased intracellular cholesterol mass levels and apoB secretion, and enhanced intracellular apoB degradation. These in vivo and in vitro studies therefore suggest that the synthesis of both cholesterol and cholesterol esters is a major regulatory factor in the intracellular stability of apoB and consequently the assembly and secretion of apoB-containing lipoproteins.

Triglycerides comprise the second major constituent of the neutral lipid core of lipoproteins, and its regulation is thought to strongly influence VLDL production (Wu et al., 1996a). It has been revealed that in hepatocytes triglycerides synthesized from free fatty acids are stored in the cytosol and as well in the lumen of the ER. Triglycerides that enter the lumen of the ER via a hydrolysis re-esterification pathway are the major source of triglyceride in the assembly of VLDL (Wu et al., 1996a). It was suggested that the rate of triglyceride synthesis, and not the mass of triglycerides, determine the secretion of apoB. Traesin D, an inhibitor of fatty acid synthetase, and consequently triglyceride production, was shown to be associated with a decrease in apoB secretion (Wu et al., 1994). Conversely, oleic acid treatment of HepG2 cells has been shown to stimulate, in vitro, the production of VLDL triglyceride and apoB (Pullinger et al., 1989; Byrne et al., 1992; Dixon et al., 1991; Gibbons et al., 1992; White et al., 1992). More specifically, it
has been suggested that oleate may facilitate the transport of nascent apoB across the ER membrane, which in turn increases the pool of apoB available for VLDL assembly (Boren et al., 1992; Sakata et al., 1993). There is also a progressing interest in the role of insulin in the production of triglyceride and the secretion of hepatic VLDL. *In vitro* studies revealed that insulin inhibits VLDL triglyceride and apoB production (reviewed in Sparks and Sparks, 1994). The interactions of oleate and insulin have also been examined in cultured hepatocytes, and results revealed that insulin appeared to reduce the oleate-induced stimulation of VLDL secretion (Dashti et al., 1987; Pullinger et al., 1989; White et al., 1992). Studies *in vivo* have revealed a suppression of VLDL apoB and triglyceride production in humans with acute hyperinsulinemia (Lewis et al., 1995). On the other hand, insulin-resistance in both humans and cultured hepatocytes appears to be insensitive to the effects of insulin on VLDL production (Sparks and Sparks, 1994; Wiggins et al., 1995; Bourgeois et al., 1996), thus indicating an association between insulin-resistance and hypertriglyceridemia.

2.6 Current Understanding of the Mechanisms Mediating Intracellular Apolipoprotein B Degradation

2.6.1 Proteasome Mediated Intracellular Degradation of Apolipoprotein B

There is considerable evidence suggesting that nascent apoB molecules undergo early, rapid degradation by the cytosolic proteasome (Fisher et al., 1998; Benoist and Grand-Perret, 1977; Yeung et al., 1996; Chen et al., 1998). The identification of the proteasome as a major system for the degradation of apoB stemmed from the observation that early degradation of apoB was reduced in the presence of proteasomal inhibitors.
such as ALLN and MG115 (Yeung et al., 1996). Studies have also revealed that nascent apoB chains appear to be ubiquitinated during apoB mRNA translation, a modification linked to the activities of the proteasome (Fisher et al., 1997; Benoist and Grand-Perret, 1997; Yeung et al., 1996; Chen et al., 1998). A recent study revealed that in HepG2 cells ubiquitinated apoB is associated with the Sec61 complex of the translocon (Mitchell et al., 1998). Addition of oleic acid and the proteasomal inhibitor, lactacystin, appeared to disrupt the interactions between ubiquitinated apoB and the Sec61 complex, resulting in the de-ubiquitination of apoB and the formation of apoB-containing lipoprotein particles (Mitchell et al., 1998). These findings suggested that apoB associated with the ER membrane remains in close proximity to the translocon and is accessible to both the proteasome degradation system and the lipoprotein-assembly pathway.

It has been revealed that MTP is also involved in the degradation of apoB. MTP binds nascent apoB molecules and assists in the translocation of these nascent polypeptides across the ER membrane (Thrift et al., 1992). ApoB molecules that are not completely translocated become associated with the ER membrane and eventually degraded (Sakata et al., 1993; Bonnerdal and Davis, 1995). In the absence of functional MTP, the accumulation of nascent apoB in the ER is decreased, an effect that is prevented by the presence of proteasomal inhibitors (Benoist and Grand-Perret, 1997). Thus, co-translational degradation of apoB by the proteasome may represent an early quality control mechanism for apoB.

Recent evidence, however, has suggested that the cytosolic proteasome may also mediate the degradation of apoB, post-translationally (Mitchell et al., 1998; Zhou et al., 1998). The mechanism(s) of apoB degradation by the cytosolic proteasome is unclear.
Recent studies have hypothesized that apoB may be transported from the ER back to the cytosol via a process termed retrograde translocation in order to be accessible to the cytosolic proteasome (Sakata and Dixon, 1999; Sakata et al., 1999; Chen et al., 1998; Liao et al., 1998). Retrograde translocation is a relatively novel concept in the field of apoB biogenesis and is a mechanism in which secretory and transmembrane proteins are transported from the ER to the cytosol whereby they are ultimately degraded by the cytosolic proteasome. Some investigators have argued against the hypothesis of complete retrograde translocation of apoB and instead have suggested that post-translational apoB that is not completely translocated into the ER may be degraded by the proteasome near and around the ER membrane (Mitchell et al., 1998; Chen et al., 1998).

2.6.2 Proteasome-Independent Mediated Intracellular Degradation of Apolipoprotein B

Post-translational degradation of apoB following its translocation into the ER is also believed to occur in the lumen of the ER (Adeli, 1994; Sato et al., 1990; Furukawa et al., 1992; Wu et al., 1997; Cartwright and Higgins, 1996) or other subcellular compartments (Wang et al., 1995; Verkade et al., 1993; Cartwright and Higgins, 1996; Sparks and Sparks, 1994). Wu and coworkers (1997) have hypothesized that in HepG2 cells, the post-translational degradation of apoB100 may occur in at least two distinct steps. In the first step, translocation arrested apoB that is associated with the ER membrane is degraded by an ALLN-sensitive process. The second step of apoB degradation however occurs in the lumen of the ER and is DTT sensitive (Wu et al., 1997). Post-translational degradation of apoB in the lumen of the ER appears to generate
a number of distinct degradation intermediates (Adeli, 1994; Sallach and Adeli, 1995; Du et al., 1994; Adeli et al., 1995; Adeli et al., 1997b). One degradation intermediate generated by post-translational degradation of apoB in digitonin-permeabilized HepG2 cells is an N-terminal 70 kDa apoB fragment (Adeli, 1994; Sallach and Adeli, 1995; Adeli et al., 1997b). The degradation of apoB and generation of the 70 kDa fragment involves a protease that was also ALLN-sensitive and temperature and pH-dependent (Adeli, 1994). Adeli et al. (1997a) identified an ER60 protease homologue in HepG2 cells that is associated with apoB and may be involved in its post-translational degradation. ER60 is a cysteine protease that was first identified in the ER of rat liver (Urade and Kito, 1992), and it is homologous in amino acid sequence to rat phosphoinositide-specific phospholipase C (Urade et al., 1992). ER60 was revealed to cross-link with misfolded mutant lysozyme and has been implicated in the degradation of reduced and denatured forms of lysozyme (Otsu et al., 1995)

2.7 The Proteasome

The major nonlysosomal proteolytic pathway in eukaryotes involves the degradation of proteins via the cytosolic 26S proteasome (Coux et al., 1996; Hochstrasser, 1995). The 26S proteasome is a large protein complex that constitutes up to 1% of the total protein in mammalian cells. The functional role of the 26S proteasome is to remove abnormal or misfolded nonspecific proteins from the cytosol of the cell. Many studies in the field of apoB biogenesis have revealed that the proteasome is also involved in the degradation of apoB, however, the precise mechanism by which apoB is targeted to the cytosolic proteasome for degradation remains to be elucidated.
2.7.1 The Structure of the 26S Proteasome

The 26S proteasome is a large (2 MDa) complex that is comprised of a 20S proteasome barrel shaped core (700 kDa) flanked by two asymmetric 19S caps (reviewed in Coux et al., 1996).

The 20S proteasome core has a 7-fold symmetrical structure that is conserved from archaeabacteria to higher eukaryotes. Although most prokaryotic proteasomes are composed of two subunits, α and β, eukaryotic proteasomes contain up to 14 different but related subunits also classified as α and β types (21-30 kDa) (Tanaka, 1995; Coux et al., 1996). The α and β-subunits are both comprised of a central five-stranded β-sheet sandwich flanked on either side by α-helices. The barrel shaped core of the 20S proteasome is comprised of 4 stacked rings where the two outer rings each consist of 7 α-subunits and the two inner rings each consist of 7 β-subunits (α7β7β7α7) (Grziwa et al., 1991; Schauer et al., 1993; Kopp et al., 1995). Electron tomographic studies revealed that within the barrel shaped core there are three cavities. The central cavity is between the two inner β-subunit rings whereas the two outer cavities lie between each α and β-subunit rings. It is speculated that proteasomal activity occurs within the cavities of the barrel-shaped core. Access to the inner cavity is controlled by α and β-ring constrictions (Wenzel and Baumeister, 1995). The dimensions of the constrictions are less than 2.2 nm and thus suggest that complete unfolding of the protein substrate is required before entry into the catalytic core (Wenzel and Baumeister, 1995).

The 19S caps are comprised of approximately 15 different subunits with molecular weights ranging between 25 and 100 kDa (Chu-Ping et al., 1994; DeMartino et al., 1996; Tanaka, 1995). Six of these subunits are ATPases and it has been speculated
that these ATPases unfold proteins prior to their translocation into the 20S proteasome core (Lupas et al., 1993). The other subunits are believed to participate in the initial recognition of the protein substrate (Baumeister and Lupas, 1997).

2.7.2 Assembly of the 26S Proteasome

In *Thermoplasma*, the assembly of the 26S proteasome may occur in conjunction with the autocatalytic activation of inactive β-subunits (Chen and Hochstrasser, 1996; Schmidtke et al., 1996; Seemuller et al., 1996). However, in eukaryotic systems such as yeast and *Dictyostelium*, the proteasome can only assemble in the presence of inactive β subunits (Schauer et al., 1993; Chen and Hochstrasser, 1996). It has been proposed that the propeptide regions of the inactive β-subunit act as intramolecular chaperones in the assembly of the proteasome (Baumeister et al., 1997). In eukaryotic systems, the seven α-subunits initially self-associate to form a primary ring-like backbone structure in which the seven β subunits then assemble. Once the four ringed cylinder of the 20S proteasomal complex is formed then regulatory components such as ATPases (19S caps) (Tanahashi et al., 1993) or PA28 can bind (Mott et al., 1994). Binding of the 19S caps leads to the formation of the 26S proteasome that is capable of ubiquitin-mediated proteasomal degradation (Rechsteiner et al., 1993).

2.7.3 The Activity of the 26S Proteasome

Initial studies examining the proteasome concluded that the activities of the proteasome were likely serine-like. Eventually, it was reported that the catalytic nucleophile of the 20S proteasome was the N-terminal threonine (Thr1) (reviewed in
Coux et al., 1996). This then led to the classification of a new family of enzymes: the threonine proteases. Mutations at the Thr1 site of the proteasome was shown to completely abolish proteasomal activity whereas substitution of the Thr1 with a serine allowed the proteasome to remain fully active (Dahmann et al., 1992). Further studies revealed that several residues close to the active site in the β subunits including Glu17, Lys33 and Asp166 are essential to the activation of the enzyme (Dahmann et al., 1992).

2.7.3.1 The Ubiquitin-Proteasome Proteolytic Pathway

In most cases, activity of the proteasome is concomitant to the ubiquitin pathway. Two distinct steps are involved in degradation of proteins via the ubiquitin pathway: signaling of the protein by the covalent attachment of multiple ubiquitin molecules and degradation of the protein with the release of reusable ubiquitin molecules (reviewed in Ciechanover, 1994). The binding of ubiquitin to proteins destined for degradation follows three events and involves three distinct enzymes (E1, E2 and E3). The initial step involves the activation of the C-terminal glycine of the ubiquitin molecule by ATP. The ubiquitin molecule is converted to a high-energy thiol ester intermediate in a reaction catalyzed by E1, the ubiquitin-activation enzyme. Secondly, the activated ubiquitin molecule is transferred to the protein substrate by E2, the ubiquitin carrier protein. Finally the activated ubiquitin is bound to the protein substrate by the ubiquitin-protein ligase, E3. An isopeptide bond is formed between the activated C-terminal glycine of ubiquitin and the ε-NH₂ group of the lysine residue of the protein substrate. The process of ubiquitin binding onto the protein substrate is repeated, and a ubiquitin chain linked to the Lys48 of the previous ubiquitin molecule is produced. Once the protein is ‘tagged’ by
ubiquitin, it is transported to the 26S proteasome where it is recognized and unfolded by the 19S caps and translocated into the 20S proteasome core for degradation (Hershko and Ciechanover, 1992).

2.7.4 Inhibitors of the 26S Proteasome

Studies in several systems identifying the proteasome as a major contributor to protein degradation have utilized a number of general and specific proteasomal inhibitors. General inhibitors, including ALLN (N-acetyl-leucyl-leucyl-norleucinal), MG132 (N-carbobenzoxy-leucyl-leucyl-leucinal) and MG115 (N-carbobenzoxy-leucyl-leucyl-norvalinal) are synthetic peptide aldehydes that have been shown to have reversible inhibitory effects on proteasomal degradation (Rock et al., 1994; Lee and Goldberg, 1996). MG132 has been shown to reversibly inhibit the degradation of short lived proteins, and more interestingly, MG132 was also shown to inhibit the slower degradation of long-lived proteins (Rock et al., 1994). Another effective peptide aldehyde inhibitor is PSI (N-carbobenzoxy-isoleucyl-glu(O-t-Bu)-alanyl-leucinal) which has been shown to block the proteolytic activity of the 26 S proteasome without affecting its ATPase or isopeptidase activities (Figueiredo-Pereira et al., 1994). A further advantage of the peptide aldehydes is that they generally do not reduce cell viability and growth under limited exposure (10-20 h) (Rock et al., Lee and Goldberg, 1996). On the other hand, the major disadvantage of the use of these agents is their nonspecific protease inhibitory nature (Rock et al., 1994; Coux et al., 1996). It has been revealed that peptide aldehydes can also inhibit both calcium-activated proteases such as the calpains and certain lysosomal cysteine proteases including cathepsin B, H and L (Rock et al., 1994).
Two approaches can be taken to ensure that inhibition of proteolytic activity by peptide aldehydes is in fact proteasomal. One approach is to show that selective inhibitors of lysosomal cysteine proteases and calpains (i.e. E64 (trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane)) do not exert the same inhibitory effects as the peptide aldehydes. The second approach is to use specific inhibitors of the proteasome such as lactacystin to show similar effects as those caused by the peptide aldehydes.

Lactacystin is a natural and potent inhibitor of the proteasome (Omura et al., 1991) that covalently modifies the active site of the β-subunit of the proteasome (Fenteany et al., 1995) (Figure 2.2). The active inhibitory species of lactacystin is its derivative, clasto-lactacystin β-lactone (Dick et al., 1996). In an aqueous environment, lactacystin undergoes a spontaneous intramolecular reaction to form the active inhibitory species, clasto-lactacystin β-lactone (Dick et al., 1996). Clasto-lactacystin β-lactone will modify the two β-catalytic subunits on the side chain of the threonine residue in the active site of the proteasome (Fenteany et al., 1995). In turn, lactacystin can inhibit three of the characteristically distinct peptidase activities of the proteasome, chymotrypsin-like, trypsin-like and caspase-like (Fenteany et al., 1995). Lactacystin has also been used to study the degradation of ER resident proteins including cystic fibrosis transmembrane conductance regulator (Rock et al., 1994; Jensen et al., 1995; Xiong et al., 1999), HMG-CoA reductase (Roitelman and Simoni, 1992; Edwards et al., 1983; Hampton et al., 1996) and apoB (Fisher et al., 1997; Chen et al., 1998; Liao et al., 1998; Sakata et al., 1999; Sakata and Dixon, 1999).
Figure 2.2. Mechanism of action of lactacystin. The process by which lactacystin is activated and inhibits the proteasome is outlined. Activation of lactacystin follows its hydrolysis converting it to its active species, clasto-lactacystin β-lactone. Clasto-lactacystin β-lactone covalently binds to the threonine 1 residue of the proteasome active site thus inactivating the catalytic proteasome (Fenteany et al., 1995).
FIGURE 2.2
2.7.5 Retrograde Translocation in Relation to Proteasomal Degradation

The proteasome involvement in the degradation of ER resident proteins has uncovered a novel mechanism for the clearance of aberrant or misfolded polypeptides. In eukaryotic cells, the assembly, folding and membrane integration of secretory proteins follows their translocation into the rough ER (Rapoport et al., 1996). The ER acts as the quality control compartment of the cell for the recognition and clearance of defective secretory proteins. Degradation of these proteins has traditionally been thought to occur within the secretory pathway (ER and Golgi), however, data revealed by many researchers has introduced a novel, complex system for the degradation of these proteins. This novel system involves the translocation of these proteins out of the ER and back into the cytosol. This method of translocation against the traditional flow of proteins is termed retrograde translocation.

Retrograde translocation has been implicated as the preliminary step in the degradation of many secretory and transmembrane proteins by the cytosolic proteasome. Once localized in the cytosol, these proteins are ubiquitinated and degraded by the proteasome (Plemer et al., 1997; Wiertz et al., 1996; Pilon et al., 1997). Certain substrates such as major histocompatibility complex class I heavy chains (Wiertz et al., 1996; Hughes et al., 1997; Wiertz et al., 1996) and unglycosylated prepro-α factor (McCracken and Brodsky, 1996) undergo complete retrograde translocation into a soluble, cytosolic form. Other proteins including the mutant forms of carboxypeptidase Y and unassembled T cell receptor α subunits remain associated with the ER membrane while having exposure to the cytosol (Yu et al., 1997; Hiller et al., 1996). In all cases,
retrograde transport of these proteins is believed to occur through the Sec61 translocon complex (Plemer et al., 1997; Wiertz et al., 1996; Pilon et al., 1997).

It has been hypothesized that apoB may also undergo retrograde translocation from the ER to the cytosol prior to its degradation by the cytosolic proteasome (Sakata et al., 1999; Liao et al., 1998; Chen et al., 1998). Ubiquitinated apoB that are bound to the ER membrane have been shown to be associated with the secα61 (Mitchell et al., 1998; Chen et al., 1998) and secβ61 (Mitchell et al., 1998) proteins of the translocon. Ubiquitinated apoB has been shown to bind molecular chaperone proteins including calnexin (Chen et al., 1998) and the heat shock protein 70 (Fisher et al., 1997). These chaperone proteins have been implicated as factors that are involved in the potential retrograde translocation and proteasomal degradation of aberrant or misfolded proteins. Although there is evidence to suggest that apoB undergoes retrograde translocation from the ER to the cytosol, the mechanism(s) by which this process occurs remains to be elucidated.

2.8 Objectives and Experimental Approach

Objective 1

There is substantial evidence suggesting that translocation of apoB is a major regulatory factor influencing the rate of apoB and apoB-containing lipoprotein secretion. Many reports have speculated that the length of apoB and more specifically, domains within the C-terminal portion of apoB polypeptide influence the efficiency of apoB translocation. It is hypothesized that there exists an inverse correlation between the length of the apoB polypeptide and its translocational efficiency. It should be noted that
translocational efficiency of apoB in this dissertation refers to the resistance of apoB to exogenous trypsin due to a lack of cytosolic exposure. Intracellular stability (resistance to endogenous degradation) of apoB also appears to be associated its translocational efficiency. It is believed that there may also be a relationship between length of apoB polypeptide and its intracellular stability during synthesis and translocation, following apoB translation. It is further hypothesized that the intracellular degradation of apoB as a result of inefficient translocation is mediated by the cytosolic proteasome.

**Approach 1**

McA-RH7777 cells transfected with human apoB cDNA of various lengths ranging from 15-100% of full-length apoB100 (hB15-hB100) were used to examine translocational status and intracellular stability of these various sized apoB molecules. Although, most studies on the translocation of apoB have been performed using isolated microsomes, here an alternative translocation protocol that was applied. This protocol was first used in HepG2 cells (Adeli, 1994), and involves the semi-permeabilization of McA-RH7777 cells with digitonin. The translocational efficiency of each newly synthesized apoB construct was determined using a trypsin protease protection assay. The percent trypsin-resistance of each apoB construct was correlated to length of the apoB polypeptide. Degradation studies involved assessing the amount of nascent apoB present in the intact McA-RH7777 cells both co- and post-translationally in the presence and absence of the proteasomal inhibitor, MG132. Hence, a correlation between the length of the nascent apoB polypeptide and its intracellular stability as well as its sensitivity to proteasomal degradation was determined.
Objective 2

Post-translational degradation of apoB appears to involve more than one proteolytic system. It is hypothesized that both a non-proteasome as well as a proteasome mediated system are involved in the post-translational degradation of apoB. In a permeabilized cell system however, post-translational degradation of apoB and the generation of the distinct 70 kDa degradation intermediate is believed to occur independent of the cytosolic proteasome.

Approach 2

Intact and permeabilized HepG2 cells were examined with respect to the intracellular stability of newly synthesized apoB in the presence and absence of the proteasomal inhibitor, lactacystin. Furthermore, both the intact and permeabilized cell lysates were used in proteasome assays to assess the amount of proteasomal activity in permeabilized cells compared to intact cells. In addition, immunoblotting experiments were conducted to determine the relative loss of functional proteasomal subunits following permeabilization of these cells. The generation of the 70 kDa fragment was also observed in the presence and absence of lactacystin and comparisons were made to the general protease inhibitor, ALLN and the more specific proteasome inhibitor, clasto-lactacystin β-lactone. Finally, permeabilized HepG2 cells were reconstituted with cytosolic components following the generation of degradation intermediates in an attempt to follow the fate of the newly generated 70 kDa fragment.
Objective 3

The main objective of this study was to explore the role of the cytosolic proteasome in the post-translational (and post-translocational) degradation of apoB. It is hypothesized that apoB may accumulate in the cytosol whereby it is susceptible to ubiquitination and proteasomal degradation. More specifically, the potential role of retrograde translocation of apoB from the secretory pathway as a targeting mechanism for proteasomal degradation was investigated.

Approach 3

HepG2 cells were used to examine the cellular distribution of apoB under various experimental conditions. HepG2 cells were homogenized, and the cytosol and crude microsomal fractions were separated by ultracentrifugation. Each subcellular fraction was examined for the presence of total newly synthesized apoB and ubiquitinated apoB. Modulation of apoB expression in both the cytosolic and microsomal fractions was conducted and assessed under various conditions including the presence of the proteasomal inhibitor, MG132 and the presence of the brefeldin A, an inhibitor of the protein secretion. To determine whether the cytosolic pool of apoB had at some point been exposed to the ER lumen, endoglycosidase H experiments were conducted to reveal the presence of carbohydrate moieties on cytosolic apoB. In turn, partial inhibition of glycosylation was also examined with respect to the cellular distribution of apoB. Finally, a model for the potential retrograde translocation of apoB from the ER to the cytosol was developed in an attempt to elucidate a general mechanism to explain how luminal apoB may be targeted to the cytosol for proteasomal degradation.
CHAPTER 3
MATERIALS AND METHODS

3.1 Cell Cultures

Human hepatoma cell line, HepG2, (ATCC HB 8065) were obtained from American Type Culture Collection. Rat hepatoma cell line, McArdle 7777 (McA-RH7777) stably transfected with human apoB15 (hB15), apoB29 (hB29), apoB48 (hB48), apoB72 (hB72) and apoB100 (hB100) cDNA were generously provided by Dr. Zemin Yao (University of Ottawa Heart Institute). These rat hepatoma cells also express endogenous apoB48 (rB48) and apoB100 (rB100).

3.2 Chemicals and Reagents

Adenosine 5'-triphosphate (ATP), bovine serum albumin (BSA), β-mercaptoethanol, brefeldin A, bromophenol blue (sodium salt), casein-fluoroscein isothiocyanate (Type II: from Bovine Milk), creatine phosphate, creatine phosphokinase, deoxycholic acid (disodium salt), digitonin (50% purity), dithiothreitol (DTT), ethylene Glycol-bis (β-aminoethyl Ether) N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA) (disodium salt), fluorescamine, glycerol, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid (Hepes), imidazole, iodoacetamide, α-Nicotinamide adenine dinucleotide phosphate (NADPH), polyoxyethylenesorbitan (Tween 20), phenylmethylsulfonylfluoride (PMSF), piperazine-N,N'-bis (PIPES), potassium acetate, rabbit anti-goat immunoglobulin (IgG), sodium carbonate (monohydrate), sucrose, t-octylphenoxypolyethoxyethylene (Triton X-100), trypsin
(tissue-culture grade) and soybean trypsin inhibitor (tissue-culture grade) were purchased from Sigma Chemical Company (St. Louis, MO).

Goat anti-mouse antibodies conjugated to peroxidase and all electrophoresis reagents (ultra pure quality) including 40% acrylamide/bis acrylamide (37.5:1 solution (2.67% C)), tris(hydroxymethyl)-aminomethane (TRIS), glycine, sodium dodecyl sulfate (SDS), ammonium persulfate and N,N,N,N′-methyl-bis-acrylamide (TEMED) were purchased from BioRad Laboratories (Richmond, CA).

Acetic acid (glacial), dimethylsulphoxide (DMSO), EDTA (Na-free), ethanol (anhydrous), hydrochloric acid, hydrogen peroxide, isopropanol, magnesium chloride, methanol, perchloric acid, potassium chloride, potassium cyanide, potassium hydroxide, potassium phosphate (dibasic), potassium phosphate (monobasic), sodium acetate, sodium chloride, sodium hydroxide, sodium phosphate (dibasic) and trichloroacetic acid (TCA) were obtained from British Drug House (BDH, Toronto, ON).

Antibiotic-antimycotic (100X), Eagle’s minimum essential medium (alpha) powder (α-MEM), Dulbecco’s modified Eagle’s medium powder (DMEM), fetal bovine serum (FBS) (certified grade), α-MEM without L-methionine, L-leucine and L-lysine, Geneticin™, L-leucine, L-lysine, L-methionine, trypsin-EDTA (1X liquid), ultra pure EDTA, and ultra pure sucrose were obtained from Life Technologies (Toronto, ON).

[^35S]protein labeling mixture (Easy Tag EXPRE[^35S]^35STM, specific activity of >1000 Ci/mmol) was from Mandel Scientific (Guelph, ON). Prestained protein standards (rainbow markers), fluorographic enhancer Amplify™, ECL™ Western Blotting detection reagents and Hyperfilm were from Amersham Life Sciences (Amersham, UK). Digitonin of a higher purity (100%), clasto-lactacystin β-lactone, and N-Leu-Leu-
norleucine (calpain inhibitor I) (ALLN) were obtained from Calbiochem (LaJolla, CA). 
Z-Leu-Leu-Leu-CHO (MG132) was obtained from Biomol (Plymouth Meeting, PA). 
Tunicamycin, cytochrome C and Endoglycosidase H were from Roche Molecular 
Biochemicals Canada (Montreal, PQ). Rabbit anti-rat transferrin antibodies and 
Universol scintillation cocktail were purchased from ICN Pharmaceuticals, Inc. (Aurora, 
Ohio). Rabbit anti-human ubiquitin antibodies were obtained from Dako Diagnostics Inc. 
(Mississauga, ON), Trasylol (aprotinin) was purchased from Bayer (Leverkusen, FRG) 
and N-ethylmaleimide (NEM) was obtained from Pierce Chemical (Rockford, IL). 
Filter/blotting paper was obtained from VWR Scientific Co. (Media, PA) and perchloric 
acid was from Aldrich Chemical Co. (Milwaukee, WI).

Goat anti-human apoB antibodies were obtained from Genzyme. Rabbit anti-rat 
20S proteasome antibody was a kind gift from Dr. Walter Ward (University of Texas). 
Monoclonal antibodies to human apoB (1D1) were a kind gift from Drs. Ross Milne and 
Yves Marcel (University of Ottawa Heart Institute). Polyclonal antibodies to rat apoB 
(LRB220) were a kind gift from Dr. L. Wong (Louisiana State University). Rabbit anti- 
rat ER60 was kindly provided by Dr. Makito Kito (Kyoto University).

3.3 Laboratory Supplies

Tissue culture flasks (25 cm², 75 cm²) and tissue culture dishes (35 mm, 100mm) 
were purchased from Sarstedt (Newton, NC) whereas tissue culture plates (6 well, 96 
well) were from Falcon (Plymouth, UK). Disposable, sterile polystyrene conical tubes 
(15 mL and 50 mL), disposable, sterile serological pipettes (5 mL, 10 mL, 25 mL), 
disposable syringe filters (0.25 μm and 0.45 μm), cell scrapers, micropipette tips (10-100
μL and 100-1000 μL), ultramicropipette tips (0.5-10 μL), conical bottom polypropylene graduated microcentrifuge tubes (1.5 mL and 2.0 mL), sterile cryovials and scintillation vials (10 mL) were purchased from Sarstedt (Newton, NC).

Disposable syringes (3 mL with a 21G1.5 needle and 10 mL with a 18G1.5 needle) were obtained from Becton Dickinson & Co (Franklin Lakes, NJ). Nalgene Lab VI autoclavable tubing was from Nalge Company (Rochester, NY) and VacuCap 90 0.2-μm high flow filters were obtained from Gelman Sciences (Ann Arbor, MI). Scientific imaging film (Kodak X-Omat Film), X-ray exposure cassettes, screens, photographic GBX developing and fixing solutions were purchased from Mandel Scientific (Guelph, ON).

All laboratory glassware including beakers, funnels, Erlenmeyer flasks, volumetric flasks, graduated cylinders and vacuum flasks were from Corning (Corning, NY). Tissue wipers were from Kimberly-Clark Inc. (Mississauga, ON), parafilm laboratory film was from American National Co. (Greenwich, CT) and President's Choice small latex gloves (non-sterile) were from Bionuclear Diagnostics Inc. (ON, CA).

3.4 Apparata

Preparation of tissue culture reagents, subculturing of cell lines and performance of experiments involving cell cultures were all conducted under a Nuaire class II type A/B3 flowhood cabinet (Plymouth, MN). Cell lines were maintained in a Nuaire Auto Flow CO₂ incubator (Plymouth, MI). Cells were viewed under a Nikon TMS inverted phase-contrast microscope (Nikon Inc., Melville, NY). A Haake type W19 water bath
(Haake Circulators, Dieselstrasse, Germany) was used to warm media and buffers to required temperatures.

Micropipettes were purchased from Eppendorf (0.5-10 µL, 10-100 µL, 200-1000 µL, (Germany)), and from Gilson (1.0-20 µL, 10-200 µL, Mandel Scientific, Ville St. Pierre, PQ). Repetitive pipettors were from Eppendorf (Germany) and pipette aids were from Drummond Scientific Co. (Broomall, PA).

A Corning pH meter (model 240) (Corning Science products, Corning New York) was used for all pH measurements. The electrodes were calibrated using pH standard solutions (pH 4, pH 7 and pH 10) from BDH (Toronto, ON). Mass measurements were performed using an A&D electronic ER-60A balance from Johns Scientific Inc. (Toronto, ON) and a Sartorius CT series electronic balance from Ohaus Corporation (Florham, NJ).

Microcentrifuges were from Fisher Scientific (Model 235-C) (Toronto, ON) and Sorvall (Model 24S) (Dupont Co., Mississauga, ON). Ultracentrifugation was performed on a Sorvall® Ultra Pro™ 80 ultracentrifuge (Dupont) in conjunction with SW55Ti (Beckman Instruments Inc., Palo Alto, CA). Ultracentrifuge tubes were also purchased from Beckman Instruments Inc. (Palo Alto, CA). The orbital shaker and the incubator shaker were from Lab Line Instruments Inc (Melrose Park, IL). Rotators were from Mallinckrodt Chemical Works (St. Louis, MO). Maxim I Mixers was obtained from Thermolyne (Dubuque, Iowa) and the Vortex Genie-2 from VWR Scientific Co. (Media, PA). The glass dounce homogenizer was from Kontes Glass Co. (Vineland, NJ). Proteins were heated to required temperatures using a Thermolyne Dri-Bath.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using SE400 Sturdier Vertical Slab Unit from Hoefer Scientific Instruments (San Francisco,
CA) or the PROTEAN® II XI from Bio-Rad Laboratories (Richmond, CA). Mini-gels were prepared in a Bio-Rad Mini-PROTEAN® Cell system, and electrophoretic transfer for immunoblotting experiments was performed on a Mini-Trans-Blot Electrophoretic Transfer Cell from Bio-Rad Laboratories (Richmond, CA). The power supplies used were VWR-105 from VWR Scientific Co. (Media, PA) and Power Pac 300 from Bio-Rad Laboratories (Richmond, CA). Polyacrylamide gels were dried on a SE540 Slab Gel Dryer (Hoefer Scientific Instruments, San Francisco, CA).

Radiolabeled proteins were quantitated using the LS 7500 Liquid Scintillation System (Beckman Instruments Inc., Palo Alto, CA). Non-radiolabeled proteins were quantitated on an Imaging Densitometer Model GS-670 (Bio-RAD Laboratories, Richmond, CA), fluorometric plate reader was used for the protein assays and the Labsystems Fluorskan II (Labsystems, Finland) was used in the proteolytic assays. A UV-visible Recording Spectrophotometer UV-160 (Shimadzu Corp., Japan) was used in the NADPH cytochrome C reductase assay. Analysis of quantified proteins and/or enzymatic reactions was performed using Microsoft Office Excel version 5.0 and Excel 98.

3.5 Preparation of Tissue Culture Reagents

*Alpha modification of Eagle's minimal essential medium (α-MEM):* One package (1X, 10.1 g) of the anhydrous form of minimum essential medium alpha medium was dissolved into 800 mL of ddH₂O along with 2.2 g of NaHCO₃. The pH of the solution was adjusted to 7.3 using 1 M NaOH or 1 M HCl, and the final volume was raised to 1 L using ddH₂O.
The medium was sterilized by filtration using a membrane with a porosity of 0.22 microns.

**Methionine-free α-MEM:** One package (1X, 9.4 g/L) of the anhydrous form of α-MEM devoid of L-methionine, L-lysine and L-leucine was dissolved into 800 mL of ddH2O along with 52 mg of L-leucine, 58 mg of L-lysine and 2.2 g NaHCO3. The pH of the solution was adjusted to 7.3 using 1 M HCl and the final volume was adjusted to 1 L using ddH2O. The medium was sterilized by filtration using a membrane with a porosity of 0.22 microns.

**Heat-Inactivated Fetal Bovine Serum (FBS):** The FBS solution was thawed at 37°C and then heat inactivated by incubation in a 52°C water bath for 30 minutes. The heat-inactivated FBS solution was then stored at -20°C.

**Complete Culture Media:** α-MEM containing 10% FBS and 1% antibiotic-antimycotic mix.

**Methionine-rich Media (Chase media):** α-MEM supplemented with 10 mM of L-methionine.

**Dulbecco’s Modified Eagle’s Medium (DMEM):** One package (1X, 8.3 g/L) of the anhydrous form of Dulbecco’s modified eagle’s medium was dissolved into 800 mL of ddH2O along with 3.7 g of NaHCO3. The pH of the solution was adjusted to 7.3 using 1 M NaOH or 1 M HCl, and the final volume was raised to 1 L using ddH2O. The medium was sterilized by filtration using a membrane with a porosity of 0.22 microns.
**Complete DMEM:** DMEM containing 20% FBS and 250 μM Geneticin in order to select for cells stably transfected with neomycin resistance genes.

### 3.6 Preparation and Maintenance of HepG2 and McA-RH7777 Cell Lines

The human hepatoma cell line, HepG2, was prepared according to instructions by the supplier (American Type Culture Collection, Rockville, MA). A 1 mL aliquot of frozen HepG2 cells maintained in DMSO was thawed for 1-2 min at 37°C. Once thawed, the cells were transferred into a 15 mL sterile tube, and 2 mL of complete α-MEM, warmed to 37°C, was added dropwise to the tube. The cell suspension was mixed twice with a 10 mL 18G1.5 gauge sterile syringe and seeded into a T-25 flask containing 2 mL of complete α-MEM medium, warmed to 37°C. The cells were maintained in a Nuaire incubator at 37°C, 95% air/5%CO2, and the media was replaced with fresh complete α-MEM every day for the following 3 days. After the third day, the media was changed every three days until the cells had reached 90-95% confluency. The cells in the T-25 flask were considered the first generation of cells. Once the first generation of cells had developed a confluent monolayer in the T-25 flask, as determined by microscopic examination, the cells were subcultured into a T-75 flask and the media was changed every 3 days. These cells were considered a second generation of cells. Once a 90-95% confluent monolayer was achieved in the T-75 flask, the cells were subcultured once again to form a third generation of cells. A portion of the third generation of HepG2 cells were frozen at -80°C to establish a stock of cells for future use and the remainder were used for subculturing into tissue culture dishes for experimental purposes.
The McA-RH7777 cells were prepared and maintained as described for the HepG2 cells with the only difference being that the medium used with these cells was complete DMEM instead of complete α-MEM.

The freezing of third generation HepG2 and McA-RH7777 cells was conducted as follows. The medium from a T-75 flask of cells was removed and 8 mL of trypsin-EDTA was added to the flask. HepG2 cells were incubated in the trypsin-EDTA for 1 min at room temperature, and then following the removal of trypsin, the cells were incubated, with caps closed, at 37°C for 5-10 minutes. McA-RH7777 cells were incubated with trypsin-EDTA for 30 seconds at room temperature and then following the removal of trypsin, the cells were incubated, with caps closed, 5-10 min at room temperature. The cells are resuspended in either complete α-MEM, for HepG2 cells, or complete DMEM, for McA-RH7777 cells, at 1mL of medium per 10% confluence of cells. The cells were then transferred to a 15 mL sterile tube and mixed three times with either a disposable sterile syringe with a 10 mL 18G1.5 gauge needle, for HepG2 cells, or a sterile disposable pipette, for McA-RH7777 cells. Cells were then centrifuged and the media was removed. The cells were then resuspended in freezing media to a final concentration of 4 x 10⁶ cells/mL. Then 1 mL aliquots of the cell suspension were prepared in sterile cryovials and stored in a Styrofoam box at −80°C.

*Freezing Medium for HepG2 Cells:* α-MEM containing 10% (v/v) DMSO and 20% (v/v) FBS, and sterilized by filtration using a membrane with a porosity of 0.22 microns.
Freezing Medium for McA-RH7777 Cells: DMEM containing 5% (v/v) DMSO and 20% (v/v) FBS, and sterilized by filtration using a membrane with a porosity of 0.22 microns.

3.7 Subculturing of HepG2 and McA-RH7777 Cells

The confluency of both HepG2 and McA-RH7777 cells maintained in T-75 flasks were determined by microscopic examination. Once cells had reached over 60% confluency they were subcultured into new T-75 flasks for the growth of a new generation of cells and/or they were subcultured into tissue culture dishes for experimental purposes. The media in which the cells were maintained was removed and approximately 8 mL of trypsin-EDTA was added to the cells. HepG2 cells were incubated in the trypsin for 1 min at room temperature and then following the removal of the trypsin they were incubated, without aeration, at 37°C for 5-10 min. The McA-RH7777 cells on the other hand were incubated in the trypsin for 30 seconds at room temperature and then following removal of the trypsin they were incubated, without aeration, for 5-10 min at room temperature. HepG2 and McA-RH7777 cells were resuspended in complete α-MEM and complete DMEM, respectively at 1 mL of medium per 10% confluency of cells. The cells were then transferred to 50 mL sterile tubes and mixed three times with either a disposable sterile syringe with a 10 mL 18G1.5 gauge needle, for HepG2 cells, or a sterile disposable pipette, for McA-RH7777 cells. Approximately 1.5 mL of cells were added to T75 flasks containing 8.5 mL of complete α-MEM, for HepG2 cells, or 8.5 mL of complete DMEM, for McA-RH7777 cells and mixed thoroughly. The remainder of the cells were diluted to the desired concentration.
and transferred to either 35 mm or 100 mm tissue dishes at a final volume of 2 mL and 10 mL, respectively. Cells were then maintained in a Nuaire incubator at 37°C, 95% air/5% CO₂ and the media was replenished every 3-4 days. Cultures were allowed to reach approximately 80% confluence before experiments were carried out.

3.8 General Pulse Chase Protocol

Near confluent HepG2 cultures, grown in tissue culture dishes, were washed 2 times with methionine-free MEM and then pre-incubated with methionine-free MEM for 60 min at 37°C. Cells were then pulsed-labeled with 100-200 μCi/mL [³⁵S] methionine for 15 min at 37°C. Following the pulse, the cells were washed 2 times with α-MEM and chased for various time periods (10-120 min) with α-MEM supplemented with 10 mM non-radiolabeled (cold) methionine. At the end of each chase period, the media was collected and the cells were washed once with phosphate-buffered saline (PBS) (8 g/L of NaCl, 0.2 g/L of KCl, 1.44 g/L Na₂HPO₄ and 0.24 g/L KH₂PO₄, pH 7.4). The cells were harvested at 4°C in 500 μL of solubilizing buffer (PBS containing 1% Nonidet P-40, 1% deoxycholate, 5 mM EDTA, 1 mM EGTA, 1 mM PMSF, 100 KIU/mL Trasylol, 0.5 μM ALLN) and mixed with a 3 mL syringe containing a 21G1.5 gauge needle. The cell lysates were centrifuged at 14,000 g for 10 min at 4°C, and the supernatants were collected. An additional 500 μL of solubilizing buffer was added to each supernatant collected, and the samples were subjected to immunoprecipitation, SDS-PAGE and fluorography. The media samples were centrifuged at 8,000 g for 2 min and 500 μL of solubilizing buffer was added to the supernatants. The media samples were
also subjected to immunoprecipitation, SDS-PAGE and fluorography. The same procedure was employed for McA-RH7777 cells with DMEM used in place of α-MEM.

3.9 Pulse-Chase Protocol for Determining Proteasome-Mediated Degradation of Apolipoprotein B

Pulse-chase protocols were performed as described above with the addition of proteasome inhibitors during various stages of the procedure. In the studies involving the proteasome inhibitor lactacystin, 25 μM of the inhibitor was added throughout the pre-pulse incubation, pulse and chase periods. In the case of the proteasome inhibitor MG132, 25 μM of the inhibitor was added during the final 15 min of the pre-pulse incubation period as well as during the pulse and chase periods. In the studies involving ALLN and clasto-lactacystin β-lactone, 40 μg/mL and 10 μM of the inhibitors, respectively, were added during the pulse and chase periods only. The concentrations of the proteasomal inhibitors used were based on dose dependent assays in which the effects of the various inhibitors on apoB degradation were maximal at the various concentrations described above (data not shown).

3.10 Preparation and Pulse-Chase Analysis of Permeabilized Cells

To examine apoB degradation in permeabilized cells, intact cells were washed 2 times with methionine-free MEM and then incubated with methionine-free MEM for 60 min at 37°C. Cells were then pulse-labeled for 15 min with 100 μCi/mL at 37°C. Following the pulse, the cells were washed 2 times with α-MEM and chased for 10 min with α-MEM supplemented with 10 mM non-radiolabeled methionine. The media was then removed and the cells were washed once with cytoskeletal (CSK) buffer (0.3 M
sucrose, 0.1 M KCl, 2.5 mM MgCl₂, 1 mM Na-free EDTA, 10 mM PIPES, pH 6.8). The cells were permeabilized with CSK buffer containing 50 μg/mL digitonin for 10 min at room temperature (optimized by Adeli, 1994). Permeabilized cells were washed three times in CSK buffer and incubated under various conditions as described in the figure legends. The cells were harvested in solubilizing buffer as described in section 3.8 and the cell extracts were subjected to immunoprecipitation, SDS-PAGE and fluorography.

3.11 Analysis of the Translocational Efficiency of Newly-Synthesized Apolipoprotein B in HepG2 and McA-RH7777 Cells

In the translocation studies, HepG2 cells were incubated with 25 μM MG132 during the pre-pulse incubation period with methionine free α-MEM. Following a 15 min pulse with 100μCi/mL [³⁵S]methionine, the radioactivity was chased for 10 minutes in α-MEM containing 10 mM cold methionine. The cells were then washed and permeabilized by incubating in CSK buffer containing 75 μg/ml digitonin for 5 min at room temperature. Permeabilized cells were washed once in CSK buffer and were then incubated in CSK buffer for 10 min at room temperature, in the presence and absence of 200 μg/mL trypsin, 150 μM puromycin, 50 μg/mL cyclohexamide and 1.25 μM ALLN (Macri and Adeli, 1997a). An equal volume of CSK buffer containing 2 mg/ml soybean trypsin inhibitor, 1 mM PMSF, 1.25 μM ALLN and 100 KIU/ml Trasylol was added to all dishes and cells were incubated for 10 min at room temperature. The cells were then incubated on ice for an additional 10 min. Finally, the cells were collected, solubilized and centrifuged as described in section 3.8, and supernatants were subjected to immunoprecipitation, SDS-PAGE and fluorography. The translocational efficiency of
newly synthesized apoB in McA-RH7777 cells was determined using a similar protocol with DMEM used as the culture medium instead of α-MEM.

3.12 Analysis of the Translational Status of the Total Mass of ApoB in HepG2 and McAR-H7777 Cells

HepG2 cells were incubated in complete αMEM containing 25 μM MG132 for 60 min. The cells were washed once and incubated with CSK containing 75 μg/mL digitonin for 5 min at room temperature. Permeabilized cells were then subjected to trypsin treatment as described in section 3.11. Cells were collected and solubilized as described in section 3.8 with the following exception: cells were collected and solubilized in 250 μL of solubilizing buffer and following the 10 min centrifugation no further solubilizing buffer was added to the samples. Cell lysates were then subjected to chemiluminescent immunoblotting. The same protocol was employed for McA-RH7777 cells with the use of complete DMEM in place of αMEM.

3.13 Reconstitution of ApoB Secretion in Permeabilized HepG2 Cells

The initial step in the regeneration of function in permeabilized cells was the isolation and preparation of cytosol. PBS (1 mL) was added to a 100 mm dish containing near confluent HepG2 cells. The cells were collected and centrifuge at 5,500 g for 5 min. The supernatants were removed and the cell pellets were resuspended in 400 μL of 25 mM Hepes, pH 7.2, containing 125 mM potassium acetate. The cells were transferred to a glass dounce homogenizer placed on ice and the cells were homogenized (20 strokes with a glass pestle). The cell lysate was transferred to a 10 mL ultraclear centrifuge tube and
centrifuged at 100,000 g for 1 h at 4°C. The cytosol fraction (supernatant) was collected and one third of the cytosol volume was used for each 35 mm dish used in the reconstitution experiments.

HepG2 cells grown in 35 mm dishes that were approximately 60-70% confluent were washed twice and incubated for 1 h with methionine free α-MEM. The cells were then pulsed for 15 min with 100 μCi/mL [³⁵S]methionine, washed twice and chased for 10 minutes with α-MEM supplemented with 10 mM cold methionine. Cells were then permeabilized as described in section 3.10 and incubated with CSK buffer for 2 h at 37°C to allow for the generation of the 70 kDa fragment. After the 2 hour incubation period the CSK buffer is removed and replaced with reconstitution buffer consisting of CSK buffer, pH 7.1, one third the volume of cytosol obtained from a 100 mm dish and an ATP generating system (1 mM ATP, 0.5 units/mL creatine phosphokinase, 5 mM creatine phosphate, and 10% FBS). The cells were then incubated for 0, 1 and 2 hours at 37°C.

Following the incubation period, CSK buffer was collected from each dish and 500 μL of solubilizing buffer was added to each dish. Cells were collected and solubilized as described in section 3.8 and cell lysates were immunoprecipitated for apoB. Finally, all samples were subjected to SDS-PAGE, fluorography and scintillation counting.

3.14 Subcellular Fractionation

Subcellular fractionation was conducted in some experiments directly following the chase period of intact pulsed cells. The media was collected, centrifuged at 8,000 g for 2 min, and 500 μL of solubilizing buffer was added to the supernatants. The cells were washed once with 250 mM sucrose and then washed a second time with 50 mM
sucrose. The cells were then collected in 500 μL of 50 mM sucrose containing 3 mM Imidazole, pH 7.4, and a cocktail of protease inhibitors (1.25 μM ALLN, 100 KIU/mL Trasylol and 1 mM PMSF). The cells were homogenized in a dounce glass homogenizer (20 strokes of a glass pestle) and then 50 μl of a 49% sucrose solution was added to the cell lysate and further homogenized (5 strokes of a glass pestle). The cell lysate was then transferred to 1.5 mL microcentrifuge tube. Approximately, 500 μL of 250 mM sucrose containing 3 mM Imidazole, pH 7.4, and a cocktail of protease inhibitors (1.25 μM ALLN, 100 KIU/mL Trasylol and 1 mM PMSF) was added to the homogenizer and used to collect the residual cell debris. The cell lysate was centrifuged at 2,200 x g for 10 min at 4°C and the supernatant was collected and placed into an ultraclear ultracentrifuge tube. The pellet was resuspended in the 250 mM sucrose solution containing the residual cell debris and centrifuged at 2,200 g for 10 min at 4°C. This supernatant was added to the cell lysate supernatant in the ultraclear centrifuge tubes. The tubes were then weighed and balanced to the nearest 1/100 of a gram using 250 mM sucrose. The samples were centrifuged at 100,000 g for 1 h at 4°C in a Sorvall® UltraPro80™ ultracentrifuge using a SW55Ti rotor. Following centrifugation, the supernatants which represented the cytosol fractions (~ 1 ml) were collected and 500 μl of solubilizing buffer was added to each sample. The pellets which represented the crude microsome fractions were resuspended in 500 μl of solubilizing buffer and transferred to a 1.5 ml microcentrifuge tubes. The samples were mixed with a 3 mL syringe with a 21G1.5 gauge needle 10 times to further solubilize the microsomal fractions. Following solubilization, an additional 500 μl of solubilizing buffer was added to each microsome fraction. All subcellular fractions and media samples were subjected to immunoprecipitation, SDS-PAGE and fluorography. In
some cases, these samples were subjected to protein assays, chemiluminescent immunoblotting and/or NADPH cytochrome C reductase assays.

3.15 Preparation of Immunoprecipitin

*Staphylococcus aureus* was the organism used in isolating protein A, the major component of immunoprecipitin. A single colony of *S. aureus* was isolated and used to inoculate 50 mL of Penassay medium. The culture was shaken (aerated) overnight at 37°C to allow for saturated growth of *S. aureus*. The 50 mL culture was then used to inoculate 500 mL of Penassay medium where the culture of *S. aureus* was allowed to grow at 37°C with aeration. Once saturated, the culture was transferred into two 250 mL centrifuge bottles and centrifuged in a Sorvall RC-5B centrifuge with a GSA rotor for 10 min at 6,500 x g at 15°C. The supernatant was discarded carefully into a 2 L flask containing 100 mL of bleach. Centrifugation was repeated with the remaining culture and the supernatant was discarded as described. The pellet of the first bottle containing *S. aureus* was resuspended into 40 mL of PBS containing 0.02% sodium azide, and this suspension was then transferred to the second bottle containing an *S. aureus* pellet. The second pellet was also resuspended in the PBS containing 0.02% azide. A second 40 mL of PBS containing 0.02% azide was added to the first bottle to collect residual bacteria and then transferred to the second bottle. The final volume of the suspension was then raised to 125 mL with additional PBS containing 0.02% sodium azide and this suspension of cells was centrifuged again at 6,500 rpm for 10 min at 15°C. The supernatant was again discarded as described, and the pellet was resuspended into 100 mL of PBS containing 0.02% sodium azide. Again the suspension was centrifuged at
6,500 x g for 10 min at 15°C and the supernatant was discarded. The pellet was then resuspended to 10% (w/v) in PBS containing 0.02% sodium azide and transferred to a 250 mL Erlenmeyer flask. The cell suspension was then stirred at room temperature while formaldehyde was slowly added to give a final concentration of 1.5%. The cells were then stirred for 90 minutes at room temperature and then centrifuged for 10 min at 5,500 x g at 20°C. The supernatant was discarded and the pellet was resuspended to 10% (w/v) in PBS containing 0.02% sodium azide. The suspension was then transferred again to the same 250 mL Erlenmeyer flask and a thermometer was placed into the suspension. The flask was then submerged into an 80°C waterbath and constantly swirled. Once the temperature of the cell suspension reached 75°C, it was incubated for an additional 5 min. The flask was then transferred to ice and the cell suspension was cooled. Once cooled the cells were transferred to a 50 mL tube and centrifuged at 6,500 x g for 10 min at 15°C. The supernatant was discarded and the pellet was resuspended in PBS containing 0.02% sodium azide, centrifuged again and the supernatant was discarded. The pellet was then resuspended in approximately 35 mL of boiling buffer (0.14 M NaCl, 0.02 M Na-di-hydrogen phosphate, pH 7.5, 1.5 % SDS and 5 % β-mercaptoethanol) and boiled for 30 min. The cells were then centrifuged as described above and the pellet was resuspended in 35 mL of boiling buffer and boiled for an additional 30 minutes. Cells were then resuspended in approximately 40 mL wash buffer (0.14 M NaCl, 0.02 M Na-di-hydrogen phosphate, pH 7.5) and centrifuged at 6,500 x g for 10 min at 15°C. The supernatant was discarded and the centrifugation procedure was repeated an additional three times. After the final centrifugation, the pellet was resuspended to 10% (w/v) in immunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 % Triton
X-100 and 1% BSA). This solution (immunoprecipitin) was then stored at 4°C and used during immunoprecipitation of specific proteins.

3.16 Immunoprecipitation

Cell lysates, media and/or subcellular fractions were pre-immunoprecipitated by the addition of 2 µL of goat IgG serum and incubation for 15 min at room temperature. Then 30 µl of immunoprecipitin was added to each sample and allowed to mix for 30 min. All samples were then centrifuged for 3 min at 14,000 g, and the supernatants were collected. The supernatants were subjected to immunoprecipitation with an overnight incubation at 4°C in the presence of 5 µL of goat anti-human apoB antibodies. The following day, 10 µL of a 1/10 dilution of rabbit anti-goat IgG anti-serum was added to each sample and mixed for 1 hour at room temperature. Then 100 µL of immunoprecipitin was added to each sample and mixed for 1 hour at room temperature. The immunoprecipitates were centrifuged for 2 min at 14,000 g, the supernatants were discarded and 1.0 mL of wash buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 0.1% SDS, 1% Triton X-100) was added to all pellets. The pellets were resuspended in the wash buffer and allowed to mix for 15-30 min. This step was repeated two more times. After the final wash, the immunoprecipitates were centrifuged at 14,000 g for 2 min and the pellets were resuspended in 120 µL of Laemmli electrophoresis sample buffer (dH2O containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.1 mg Bromphenol Blue). Samples were then boiled at 100°C for 5 min, centrifuged at 14,000 g for 3 min and the supernatants were loaded onto SDS-PAGE gels. The same procedure was followed when using antibodies against proteins other than apoB. In the
case of antibodies that were raised in rabbits, the pre-clearing stage involved 2 μL of rabbit IgG serum instead of goat IgG serum, and the addition of rabbit anti-goat IgG anti-serum was eliminated.

3.17 Serial Immunoprecipitation

In some cases, serial immunoprecipitation of apoB and ubiquitin from the cell lysates was also conducted. Initially, cell lysates were immunoprecipitated for apoB as described in section 3.16 with the addition of 5 mM of NEM to both the solubilizing and wash buffers. Following the final wash, the immunoprecipitates were centrifuged at 14,000 g for 3 min. The pellets were resuspended in 100 μL of serial immunoprecipitation sample buffer (ddH₂O containing 125 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 15.43 mg/mL DTT, 5 mM NEM) and boiled for 10 min at 100°C. The samples were then centrifuged for 5 min at 14,000 g and the supernatants were diluted with 1.0 mL immunoprecipitation buffer (ddH₂O containing 45% (v/v) 5XC (250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 25 mM EDTA, 5 mM PMSF, and 5 % (v/v) Triton X-100), 51.3% (v/v) PBS, 250 KIU/mL Trasylol, 2.5 mM PMSF, 5 mM NEM). Then 5 μL of rabbit anti-human ubiquitin antibodies was added to each sample and incubated overnight at 4°C. The remainder of the immunoprecipitation procedure was performed as described in section 3.16 with the addition of 5 mM NEM to both the wash and Laemmli sample buffers. Following immunoprecipitation the samples were subjected to SDS-PAGE and fluorography.
3.18 Endoglycosidase H Treatment

Once immunoprecipitates have undergone the final wash with wash buffer and prior to the addition of Laemmli sample buffer, they were centrifuged at 14,000 g for 2 min and the supernatants were discarded. The pellets were resuspended in 100 µL of 0.1 M sodium acetate solution that contains 0.2% SDS and 0.05 M β-mercaptoethanol and boiled for 5 min at 100°C. The samples were then centrifuged for 1 min and the supernatants were transferred to a 1.5 mL microcentrifuge tube. Then 100 µL of 0.1 M sodium acetate solution, pH 5.4 was added to all supernatants and mixed gently. Once mixed, 100 µL of the mixtures were transferred to new 1.5 mL microcentrifuge tube containing 1 milliunit of endoglycosidase H and mixed gently. All samples, with and without Endoglycosidase H, were incubated at 37°C overnight. Following incubation, 20 µL of a 5 X Laemmli sample buffer (250 µL/ml of β-mercaptoethanol, 500 µL/mL of glycerol, 125 mM Tris-HCl, pH 6.8, 10% SDS, 0.04 mg/mL of Bromophenol Blue) was added to all samples, and the samples were boiled for 5 min at 100°C. All samples were then subjected to SDS-PAGE and fluorography.

3.19 SDS-PAGE

SDS-PAGE was performed essentially as described (Laemmli, 1970). The main variation between experiments was the percentage of acrylamide in both the resolving and stacking gels. The percentage of acrylamide in the gels was dependent upon the size of the protein(s) being analyzed as indicated in the figure legends. The resolving gels were prepared with either 4.5%, 6%, 7% or 10% (w/v) acrylamide and the stacking gels were prepared with either 3% or 4% (w/v) acrylamide. The following outlines the composition of the major buffers used to perform SDS-PAGE:
**Resolving Gel:** 4.5%, 6%, 7%, or 10% (w/v) acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.07% (v/v) TEMED.

**Stacking Gel:** 3% or 4% (w/v) acrylamide, 0.125 M tris-HCl, pH 6.8, 0.05% (w/v) SDS, 0.1% (w/v) ammonium persulfate and 0.05% (v/v) TEMED.

**Laemmli Sample Buffer:** 0.125 M Tris-HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 4.1% (w/v) SDS and 0.02% (w/v) bromophenol blue.

**Running Buffer:** 0.02 M Tris, 0.192 M glycine and 0.01% (w/v) SDS.

Once prepared the resolving gel was poured into a gel casting system and overlaid with ddH2O and allowed to polymerize for 45 to 60 min. Once polymerized the overlaid water was removed and the resolving gel was washed with ddH2O. Then a 15-well Teflon comb was place into the gel casting system above the resolving gel and the prepared stacking gel was added. The stacking gel was allowed to polymerize for 30 to 45 min and following polymerization the comb was removed and the wells of the stacking gel were washed with running buffer.

Samples that were prepared for SDS-PAGE were loaded into separate wells of the stacking gels. In addition, a solution of standard molecular weight markers (4 μL of the rainbow markers and 10 μL of Laemmli sample buffer) ranging in size from 30 to 260 kDa was added to one of the wells. Once the samples were loaded into the wells, they were gently overlaid with running buffer. Both the upper and lower chambers of the gel system were filled with running buffer and an electrical current was applied. In the case of the large gels containing radiolabeled samples, SDS-PAGE was conducted at 60 V for
approximately 16 h or until the dye front reached the bottom of the gel. Following SDS-PAGE, the gels were removed from the glass plates and incubated in a fixer solution (ddH₂O containing 40% (v/v) methanol and 10% (v/v) glacial acetic acid) for at least 30 min at room temperature. The gels were then fluorographed.

3.20 Fluorography and Scintillation Counting

Gels were fluorographically enhanced by incubation in Amplify™ for 15-20 min at room temperature. The gels were then washed in distilled water for 1 min and placed onto filter paper. A sheet of plastic wrap was placed on top of the gel and air bubbles were removed. The gels were dried onto the filter paper under vacuum for 2 h on a slab gel dryer at 80°C. The plastic wrap was then removed and the dried gels were exposed to Dupont autoradiographic film at -80 °C for 1-7 days. Following exposure, the autoradiographic films were developed for 1-3 min in Kodak developer solution (H₂O containing 21.8% (v/v) developer), neutralized for 1 min in 3% (v/v) glacial acetic acid and fixed for 2-3 min in Kodak fixer solution (H₂O containing 21.8% (v/v) fixer). The films were then thoroughly washed with water and air dried. Once films were dried the bands of interest were excised from the gels and placed into scintillation tubes. Hydrogen peroxide (400 μL) and perchloric acid (200 μL) was added to each vial, mixed and incubated overnight at 60°C. The following day 4.0 mL of Universol® scintillation cocktail was added to each vial and mixed thoroughly to ensure that the cut gel pieces were completely dissolved. The samples sat at room temperature for 1 hour prior to scintillation counting. The radioactivity of each sample was measured for 2 min on a Beckman scintillation counter and quantitated as counts per minute (CPM).
3.21 Chemiluminescent Immunoblotting

Immunoblotting was performed by SDS-PAGE analysis of non-radiolabeled cell lysates on mini-gels (8 x 5 cm) with a resolving gel of either 4.5%, 7%, 10% or 12% polyacrylamide gels. SDS-PAGE was conducted at 200 V for 45-60 min at 4°C. Following SDS-PAGE, the gels as well as the nitrocellulose membranes were equilibrated in transfer buffer (ddH₂O containing 2.92 g/mL glycine, 5.82 g/mL Tris, 3.75% SDS, 20% methanol) for 20 min and 10 minutes, respectively. The proteins from the gels were then transferred electrophoretically overnight at 4°C onto the nitrocellulose membranes using a Bio-Rad Wet Transfer System completely filled with transfer buffer. The membranes were then blocked with 5% (w/v) solution of fat-free dry milk powder for 1 hour at room temperature. Membranes were then incubated in a 1:1000 fold dilution of a primary antibody specific to the protein of interest with TBST buffer (ddH₂O containing 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20) for 1 h. The membranes were then subjected to three washes with TBST buffer for 15 min, 5 min and 5 min, respectively. After the washes, the membranes were then incubated in a 1:3000 or 1:8000 fold dilution of a secondary antibody conjugated to peroxidase (specific to the primary antibody) with TBST buffer for 1 h. The wash steps, as described above, were repeated. Membranes were then incubated in an ECL detection reagent for 60 seconds, wrapped in plastic wrap and exposed to Hyperfilm for various time periods ranging from 15 sec to 30 minutes. Films were then developed, neutralized and fixed as described above and air dried. Quantitative analysis of the bands of interest was performed using an Imaging Densitometer and measurements were assessed as optical density per square mm.
3.22 Fluorescamine Protein Assay

*Fluorescamine solution:* 1.08 mM Fluorescamine in 20 µL of acetone.

*K₃PO₄ buffer, pH 8.0:* 0.8218 g/L of potassium phosphate monobasic (KH₂PO₄) was mixed with 6.127 g/L of potassium phosphate dibasic (K₂HPO₄). The pH of the solution was adjusted to pH 8.0 with 5 M NaOH or 6 M HCl.

**BSA standard:** BSA (20 mg) was dissolved into 1 mL of solubilizing buffer used during the experiment. The 20 mg/mL BSA solution was then diluted with solubilizing buffer at a ratio of 1:1. Using the diluted BSA, a second 1:1 dilution with solubilizing buffer was prepared. This procedure was repeated until 6 BSA standard samples were processed.

A 25 µL aliquot of each protein sample, each BSA standard sample and the blank (solubilizing buffer) was added in triplicate to a 96 well microtitre dish. Then 50 µL of K₃PO₄ buffer, pH 8.0, and 25 µL of fluorescamine solution were added to each protein sample, BSA standard and blank well. The solutions were mixed for 5 min by gentle shaking in the absence of light. All samples were then read in a fluorometric microtitre plate reader at an excitation wavelength of 400 nm and emission wavelength of 460 nm. The fluorescent units obtained for the protein samples and BSA standard was subtracted by the fluorescent units obtained from the blank. A standard curve was prepared by plotting the fluorescent units of each BSA standard (minus the blank) against the protein concentration of the BSA, respectively. An equation of the line (y=mx+b) was determined for the standard curve and this equation was used in determining the protein concentration of the samples within the microtitre plate well, where y represents the fluorescent units and x represents the protein concentration in each well. Once the protein
concentration within each well was determined, this value was then multiplied by the dilution factor used in preparing the protein assay in order to assess the concentration of protein in the entire sample.

3.23 Fluorogenic Proteasome Assays

Cells were incubated in complete α-MEM in the presence and absence of 25 μM lactacystin for 1 hour. Some cells were then washed in CSK buffer and permeabilized as described in section 3.10 while other cells were left intact. Both intact and permeabilized cells were collected in 250 μL of PBS in the presence of 0.1% Triton X-100 and homogenized in a dounce glass homogenizer (20 strokes with a glass pestle). Cell homogenates were centrifuged at 14,000 g for 10 min at 4°C, and the supernatants were subjected to fluorogenic protease assays. The protease assays were performed in 1.5 mL centrifuge tubes according to Twining (1984) with modifications to assess proteasomal activity. To each reaction tube the following components were added: 20 μL of the respective sample, 25 μL of a 100 mM Tris pH 7.8 buffer, 5 μL of 5 mg/mL FITC-casein and 5 μL of 250 μM lactacystin only to those samples already pre-treated with lactacystin. The reaction was then carried out at 37°C in the absence of light for 1 hour in a shaking incubator. Reactions were terminated by the addition of 120 μL of 5% trichloroacetic acid followed by a 2 h incubation at room temperature in the absence of light to precipitate out all insoluble proteins. Samples were then centrifuged for 5 minutes and 60 μL of the supernatants were added to 400 μL of 500 mM Tris, pH 8.8 buffer and mixed thoroughly. A 250 μL aliquot of each sample solution was transferred into microtitre plate wells and read in a Fluoroskan at an excitation wavelength of 485 nm and
an emission wavelength of 538 nm. The data was then quantitated as fluorescent units (FU) per mg of protein.

3.24 NADPH Cytochrome c Reductase Assay

*Cocktail solution:* The cocktail solution consisted of 500 µL of 0.2 g/mL of Triton X-100, 5.125 mL of 0.2 M phosphate buffer, pH 7.5 (27.2 mg/mL of KH₂PO₄ combined with 28.4 mg/mL of Na₂HPO₄), 750 µL of 5 mg/mL of cytochrome C, and 300 µL of 50 mM KCN.

In a 1 mL glass cuvette, 266 µL of cocktail solution and 244 µl of the sample or blank (solubilizing buffer used in experiment) was added. Then 120 µL of 0.5 mM NADPH was immediately added to the cuvette, mix with a fine glass rod and the cuvette was placed in a spectrophotometer. The enzymatic reaction was read every 15 seconds from 0 to 3 minutes at a wavelength of 548 nm. The absorbance for every time interval was plotted against the time of the reaction for all samples including the blank and the equation of the line for each curve (y=mx+b) was determined. Finally, the specific activity for each sample was assessed by dividing the slope of the line (represented by m) of the sample curve by the protein concentration of each sample as obtained through fluorometric protein assays.
CHAPTER 4

INTRACELLULAR TRANSLOCATION AND STABILITY OF APOLIPOPROTEIN B ARE INVERSELY PROPORTIONAL TO THE LENGTH OF THE NASCENT POLYPEPTIDE

4.1 Results

4.1.1 Trypsin Sensitivity of Newly Synthesized Human Apolipoprotein B Variants in Permeabilized Cells

The translocational efficiency of apoB was investigated using a protease protection assay in McA-RH7777 cells expressing various sized human apoB constructs. This assay, which was previously characterized and reported in HepG2 cells (Macri and Adeli, 1997a), involved a pulse chase protocol to establish a radiolabeled (newly synthesized) apoB pool followed by permeabilization of the cells, trypsin treatment, and immunoprecipitation of apoB. In examining the trypsin sensitivity of various sized apoB constructs, the protease protection assays were conducted in the presence of the proteasomal inhibitor, MG132, to ensure inhibition of endogenous proteasomal degradation of apoB. Figure 4.1 shows the effects of trypsin treatment of cells expressing recombinant human apoB15, apoB29, apoB72 (hB15, hB29 and hB72, respectively) and the endogenous rat apoB100 (rB100). It appeared that hB15 and hB29 showed little susceptibility to trypsin digestion indicating that these constructs were relatively inaccessible to trypsin as indicated by the high percentage of apoB recovered over control (% trypsin resistant apoB: 72±5.0% and 79±5.0%, respectively) (Fig. 4.1A and 4.1B). Compared to the smaller apoB constructs, hB72 and the endogenous rB100 were highly sensitive to trypsin digestion (% trypsin resistant, 59.13±12.46% and 52±2.9%, respectively) (Fig. 4.1C and 4.1D).
Figure 4.1 Trypsin sensitivity of newly synthesized C-terminally truncated apolipoprotein B variants expressed in permeabilized McA-RH7777 cells. McA-RH7777 cells transfected with various apoB constructs were incubated in the presence of 25 μM MG132 for 15 min. Intact cells were then pulsed for 15 min with 100 μCi of [35S]methionine and chased for 20 min with 10 mM cold methionine. Following the chase time, cells were permeabilized with digitonin (75 μg/ml) for 5 min, and then incubated in the presence or absence of trypsin (100 μg/ml) for 10 min. Trypsin digestion was halted by the addition of soybean trypsin inhibitor (1 mg/ml), and the cells were subjected to immunoprecipitation with a monospecific anti-human apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography, and apoB radioactivity was quantitated by cutting and scintillation counting the apoB band. The autoradiographs shown depict representative experiments for each apoB variant performed in the presence and absence of trypsin. In addition, quantitative analysis was performed to determine the percent apoB resistant to trypsin digestion compared to control. Graphs shown are the mean±S.E. of several experiments for each apoB variant. A, hB15 (n=6); B, hB29 (n=6); C, hB72 (n=2); D, endogenous rB100 (n=3)
FIGURE 4.1
The employment of permeabilized cells in the protease protection assay was advantageous in maintaining the integrity of the microsomal membranes. A previous report (Adeli, 1994) has documented the trypsin inaccessibility of control proteins such as albumin and NADPH cytochrome c reductase thus verifying the intactness of the microsomal membranes in permeabilized HepG2 cells. In this study, transferrin was used as a control protein in the trypsin protection assays, and results revealed that transferrin was highly resistant to trypsin digestion (% trypsin resistant, 96±12%) in permeabilized McA-RH7777 cells (Fig. 4.2).

4.1.2 Translocational Studies of the Total Mass of Human Apolipoprotein B Variants

In addition to examining the trypsin accessibility of newly-synthesized apoB variants, we also investigated the trypsin accessibility of the total mass of apoB variants using a modification of the protease protection assay. Prior to permeabilization, McA-RH7777 cells were pre-incubated for 1 h in the presence of MG132. Unlabeled McA-RH7777 cells were then permeabilized, digested with trypsin and solubilized. Cell lysates were then subjected to SDS-PAGE, and chemiluminescent immunoblotting was used to assess the total apoB mass. Cell lysates were immunoblotted with either monoclonal anti-human apoB (1D1) or polyclonal anti-rat apoB antibodies. The monoclonal human apoB antibody (1D1) distinguished the recombinant hB48 and hB100 from the endogenous rB48 and rB100, respectively, and thus allowed for the assessment of trypsin sensitivity of recombinant hB48 and hB100. The immunoblotting data revealed that the total mass of
Figure 4.2 The integrity of intracellular organelles is maintained following permeabilization of McA-RH7777 cells. McA-RH7777 cells were incubated in the presence of 25 µM MG132 for 15 min. Intact cells were then pulsed with 100 µCi of [35S]methionine and chased for 20 min with 10 mM cold methionine. Cells were then permeabilized with digitonin (75 µg/ml) for 5 min, and then incubated in the presence or absence of trypsin (100 µg/ml) for 10 min. The addition of soybean trypsin inhibitor (1 mg/ml) terminated the trypsin digestion. Cells were subjected to immunoprecipitation with a polyclonal anti-rat transferrin antibody, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. The autoradiograph shown depicts a representative experiment performed in the presence and absence of trypsin. The radioactivity of transferrin in the presence and absence of trypsin treatment was quantitated by cutting and scintillation counting of the transferrin bands. The percentage of trypsin resistant transferrin (as a percentage of untreated control) was determined. The graphs show the mean±S.E. of several experiments (n=3).
FIGURE 4.2
Figure 4.3 Trypsin accessibility of total intracellular mass of C-terminally truncated apolipoprotein B variants in permeabilized McA-RH7777 cells. McA-RH7777 cells transfected with various apoB constructs were incubated in the presence of 25 μM MG132 for 1 h. Cells were then permeabilized with digitonin (75 μg/ml) for 5 min, and permeabilized cells were incubated in the presence or absence of trypsin (100 μg/ml) for 10 min. Trypsin digestion was halted by the addition of soybean trypsin inhibitor (1 mg/ml). The cells were then solubilized and the cell lysates were resolved by SDS-PAGE. ApoB was visualized by immunoblot analysis using the monoclonal antibody 1D1. The immunoblots shown depict representative experiments performed in the presence and absence of trypsin. Quantitation of the apoB bands was performed using densitometer analysis and percent trypsin resistant apoB (as a percentage of untreated control) was determined. Graphs shown are the mean±S.E. of several experiments for each apoB variant. A, hB15 (n=6); B, hB29 (n=3); C, hB48 (n=3); D, hB72 (n=3); E, hB100 (n=3).
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**FIGURE 4.3**
hB15 and hB29 were minimally sensitive to trypsin treatment (74±5.4 % and 70±3.0 % trypsin resistant, respectively) (Fig. 4.3A and 4.3B). Human B48 and hB72 exhibited a greater sensitivity to exogenous trypsin (58±5.3 and 55±4.2 % trypsin resistant, respectively) compared to the shorter apoB variants (Fig. 4.3C and 4.3D). However, hB100 showed the highest sensitivity to trypsin digestion of all the apoB variants (42±7.5 % trypsin resistant) (Fig. 4.3E). Overall, the immunoblotting data was consistent with the data from protease protection experiments in radiolabeled permeabilized cells. Thus, an inverse relationship between length of apoB polypeptide and its resistance to exogenous trypsin was very well defined.

4.1.3 Comparison of Trypsin Sensitivities between Recombinant Apolipoprotein B100 and Endogenous Apolipoprotein B100

The translocational status of recombinant hB100 expressed in McA-RH7777 cells was compared with those of the endogenous rB100 of the same cell line as well as the endogenous hB100 in HepG2 cells. The results revealed that the different species of human and rat apoB100 had comparable trypsin sensitivities (Fig. 4.4). The percent trypsin-resistant apoB was 42±7.5% for recombinant hB100 expressed in McA-RH7777 cells, 52±2.9% for endogenous rB100 in McA-RH7777 cells, and 46±6.3% for endogenous hB100 in HepG2 cells (Fig. 4.4). Thus, the high trypsin sensitivity of the recombinant hB100 appeared to be consistent with the trypsin sensitivity of the endogenous B100 of both rat and human origin.
Figure 4.4 Comparison of trypsin sensitivity of various apolipoprotein B100 species.

Trypsin sensitivity of the human B100 construct expressed in McA-RH7777 cells was assessed by immunoblotting with monoclonal IDI antibody. The results were compared to the trypsin sensitivity of the endogenous McA-RH7777 rat apoB100. In addition, the trypsin sensitivity of the human B100 construct expressed in McA-RH7777 cells was compared to that of endogenous HepG2 human apoB100 which was assessed using the pulse chase protocol outlined in Figure 4.1. The figures show representative experiments performed in the presence and absence of trypsin. In addition, quantitative analysis was performed to determine the percent apoB resistant to trypsin digestion compared to control. Graphs shown are the mean±S.E. of several experiments for each apoB variant. A, exogenous hB100 construct expressed in McA-RH7777 cells (n=3); B, endogenous rB100 (n=15) expressed in McA-RH7777 cells; C, endogenous hB100 expressed in HepG2 cells (n=3).
### FIGURE 4.4

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<th>Construct</th>
<th>Traspin</th>
<th>Percent Resistant</th>
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<td>B100</td>
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<tr>
<td>Rat ApoB100</td>
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<td>Human ApoB100</td>
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4.1.4 Correlation between the Length of the Apolipoprotein B Polypeptide and Trypsin Sensitivity

A correlation was established between the apoB size and its trypsin sensitivity for both human apoB variants and endogenous rat apoB species based on data obtained from protease protection experiments. The percent trypsin resistant apoB plotted against the length of apoB protein (Fig. 4.5). ApoB variants ranging in size from B15 to B48 were relatively insensitive to trypsin digestion whereas the larger apoB variants were increasingly trypsin-sensitive. Hence, an inverse correlation appears to exist between the size of the apoB polypeptide and its translocational efficiency with the full length apoB100 exhibiting a significantly higher sensitivity to exogenous trypsin.

4.1.5 Co-Translational Stability of Human Apolipoprotein B Variants in McA-RH7777 Cells

It has been previously established that the translocation of apoB across the ER membrane occurs simultaneously with the synthesis of the polypeptide. As such, the co-translational stability of apoB is likely affected by the efficiency of its translocation into the ER lumen. In this study, the co-translational stability of apoB variants expressed in McA-RH7777 cells was investigated using a pulse-labeling protocol. Cells were pretreated with or without MG132 for 15 min prior to the pulse. Control cells were pretreated with 0.1% DMSO (the solvent used to dissolve MG132). Following the pulse, cells were collected, solubilized, and immunoprecipitated for apoB using monospecific anti-human and anti-rat antibodies. The immunoprecipitable apoB recovered after a 15 min pulse in the absence or presence of MG132 is shown in Fig. 4.6A. Quantitatively, the
Figure 4.5 An inverse correlation between translocational efficiency and the size of apolipoprotein B polypeptide. Data from pulse chase translocation studies were analyzed to establish a correlation between the size of the apoB variant and its translocational efficiency. In addition, the results were compared to the translocational efficiency of full length endogenous rat apoB. The graph shown is the mean±S.E. of several experiments: hB15 (n=6), hB29 (n=6), rB48 (n=8), hB72 (n=2), rB100 (n=15) and HepG2 B100 (n=3). *Closed circles*, human apoB constructs; *open circles*, endogenous rat apoB; *open triangles*, endogenous human apoB of HepG2 cells.
FIGURE 4.5
Figure 4.6 Co- translational sensitivity to proteasomal degradation of apolipoprotein B variants expressed in McA-RH7777 cells and endogenous rat apolipoprotein B. Cells were incubated in the presence and absence of 25 μM MG132 for 15 min. Intact cells were then labeled for 15 min with 100 μCi of [35S]methionine, washed and subjected to immunoprecipitation with either a monospecific anti-human apoB antibody or anti-rat apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. A, shows representative experiments performed in the presence and absence of MG132. ApoB radioactivity was quantitated by cutting and scintillation counting the apoB band. B, shows the correlation between the size of the apoB variants and their co-translational sensitivity to proteasomal degradation. Results were also compared to the co-translational MG132-sensitivity of endogenous rB48 and rB100. The graph shown is the mean±S.E. of several experiments: hB15 (n=4); hB29 (n=6); rB48 (n=2); hB72 (n=6); rB100 (n=9). Closed circles, exogenous human apoB constructs; open circles, endogenous rat apoB.
FIGURE 4.6
percentage of apoB recovered in the presence of MG132 compared to control were 130±7.0 % for hB15, 94±8.0 % for hB29, 110±0.1 % for rB48, and 140±6.0 % for hB72 (Fig. 4.6B). Among the truncated apoB constructs, hB15 and hB72 were slightly sensitive to the MG132 as seen by the accumulation of these constructs in the presence of the proteasome inhibitor. There was no noticeable change in stability of hB29 and rB48 with MG132 treatment. In contrast, the presence of the proteasome inhibitor caused a significant increase in recovery of the endogenous rB100 (280±17% of the control; p<0.01) (Fig. 4.6B) during the pulse period, suggesting substantial co-translational instability of full-length apoB. Overall, the data suggest that co-translationally, apoB100 has a much greater sensitivity to proteasomal degradation compared to truncated apoB variants. In summary, as the size of the newly-synthesized apoB nascent chain increases its intracellular stability during translation decreases principally due to increased sensitivity to the proteasomal degradation.

4.1.6 Post-translational Stability of Human Apolipoprotein B Constructs in McA-RH7777 Cells

The stability of human apoB variants following translation was also assessed using a pulse-chase labeling protocol. Post-translational degradation was assessed by comparing the amount of apoB recovered after a 2 h chase (cellular and media), with that recovered at time 0 (following the pulse and before the chase), in the presence and absence of MG132. In the absence of MG132, approximately half of all newly synthesized apoB constructs, regardless of size, were degraded after a 2 h chase. As expected, endogenous rB48 and rB100 were also degraded over a 2 h chase with rB100
Figure 4.7 Post-translational sensitivity to proteasomal degradation of exogenous apolipoprotein B variants expressed in McA-RH7777 cells and endogenous rat apolipoprotein B. Cells were incubated in the presence and absence of 25 μM MG132 for 15 min. Intact cells were then labeled for 15 min with 100 μCi of [35S]methionine and chased for 2 h with 10 mM cold methionine in the presence or absence of 25 μM MG132. Cells were washed and subjected to immunoprecipitation with either a monospecific anti-human apoB antibody or anti-rat apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography, and apoB radioactivity was quantitated by cutting and scintillation counting the apoB band. In addition, percent apoB remaining over a 2 h chase in the presence of MG132 was compared to controls. Figures show representative experiments for each apoB variant and the graphs shown are the mean±S.E. of several experiments. A, hB15 (n=6); B, hB29 (n=8); C, endogenous rB48 (n=2), D, hB72 (n=7); E, endogenous rB100 (n=16).
FIGURE 4.7
revealing a greater rate of degradation (48±8.5% and 39±4.5% apoB remaining after a 2 h chase, respectively) (Fig. 4.7B and 4.7C, respectively).

Post-translational turnover of all apoB variants as well as the endogenous apoB species was sensitive to the presence of MG132 although to varying degrees. In the presence of MG132, the recovery of hB15, hB29 and endogenous rB48 (in the cell lysate plus media) increased 1.5±0.10, 1.7±0.06, and 1.3±0.10 fold, respectively, after a 2 h chase compared to untreated control (Fig. 4.7A, 4.7B, and 4.7C), whereas hB72 showed a 1.8±0.12 fold increase (Fig. 4.7D). The most striking affect was observed with the amount of endogenous rB100 recovered in the presence of MG132 (2.5±0.19 fold increase over untreated control, Fig. 4.7E). Thus, the presence of a proteasome inhibitor resulted in significant increases in recovery of both expressed apoB variants as well as endogenous apoB species, suggesting the involvement of the proteasome in their post-translational turnover, regardless of size. Furthermore, there appeared to be an inverse correlation between the size of the apoB polypeptide and the degree of susceptibility to post-translational proteasomal degradation (Fig. 4.8), with full-length apoB100 being the most sensitive to proteasomal degradation.

Although, a relationship between apoB length and rate of apoB secretion was not examined in this study, several reports have suggested a relationship between the size of the nascent apoB polypeptide and its secretion rate (Parhofer et al., 1996; Welty et al., 1997). In vivo studies examining familial hypobetalipoproteinemia, showed that secretion of characteristically truncated apoB molecules into the plasma appeared to be linearly correlated to the size of apoB (Parhofer et al., 1996). It was further revealed that apoB variants smaller than apoB25 were not secreted and instead intracellularly degraded.
Figure 4.8 A correlation between post-translational susceptibility to proteasomal degradation and the size of the apolipoprotein B polypeptide. Data from pulse chase proteasomal inhibitor studies were analyzed to explore a correlation between the size of the apoB variant and its post-translational sensitivity to proteasomal degradation. In addition, the results were compared to the proteasomal sensitivity of endogenous McA-RH7777 apoB. The graph shown is the mean±S.E. of several experiments (n≥3). *Closed circles*, human apoB constructs expressed in McA-RH7777 cells; *open circles*, endogenous rat apoB.
FIGURE 4.8
CHAPTER 5

STUDIES ON THE DEGRADATIVE MECHANISMS MEDIATING POST-
TRANSLATIONAL FRAGMENTATION OF APOLIPROTEIN B AND THE
GENERATION OF THE 70 kDa FRAGMENT

5.1 Results

5.1.1 Effects of Lactacystin on the Co- and Post-Translational Degradation of
Apolipoprotein B in Intact Cells

In an attempt to distinguish the role of the proteasome in the degradation of apoB,
intact HepG2 cells were first pretreated with the proteasome inhibitor, lactacystin, and
then pulsed and chased over a 120 min period. Lactacystin pretreatment of cells 30 min
before the pulse induced a significant increase in the amount of apoB accumulated at 0 h
(2.6 fold increase over control) which remained high during the 120 min chase (Fig.
5.1A). To assess post-translational sensitivity to lactacystin, cells were chased for 60 and
120 min in the presence and absence of the inhibitor (Fig. 5.1B). In control cells, 53% of
apoB was recovered after a 60 min chase whereas in lactacystin treated cells 78% were
recovered. This difference was also observed after 120 min of chase time with only a
48% recovery of apoB in control cells as opposed to a 76% recovery in lactacystin treated
cells. Hence, even after a 2 h chase, degradation of apoB remained sensitive to lactacystin
thus suggesting a degradative role for the proteasome after translation of apoB.
Nevertheless, there was still an approximate 24% loss in $^{35}$S labeled apoB during the
chase of lactacystin-treated cells, despite the presence of the inhibitor (Fig. 5.1B).
Figure 5.1 Lactacystin affects co- and post-translational turnover of apolipoprotein B in intact HepG2 cells. A. Near confluent cells in 35 mm dishes were pre-incubated for 60 min in methionine free medium, pulsed for 15 min with $^{35}$S] methionine and chased for 120 min with excess cold methionine. Lactacystin (25 μM) was added to some dishes 60 min before the pulse and also during both pulse and chase periods. After the chase, media was collected and cells were solubilized. Cell lysates were immunoprecipitated with anti-apoB antibody, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. B. The percent apoB remaining after 60 and 120 min was assessed by determining the amount of radioactivity in the apoB100 bands (recovered from media and lysates of control and lactacystin treated cells) as a percentage of the amount recovered at 0 chase time (n=3). Open circles, control cells; closed circles, lactacystin treated cells.
FIGURE 5.1
5.1.2 Effect of Lactacystin on Post-Translational Fragmentation of Apolipoprotein B in Permeabilized Cells

A permeabilized cell system was used in conjunction with lactacystin, to further examine the role of the proteasome in the specific fragmentation of apoB and the generation of the 70 kDa degradation fragment. A permeabilized cell system is ideal for the study of apoB degradation since protein synthesis and secretion are abolished following permeabilization of cells. Intact HepG2 cells were briefly pulsed, chased and then permeabilized with 50 µg/mL digitonin. Permeabilized cells were then incubated in CSK buffer with and without lactacystin to monitor degradation of the newly synthesized radiolabeled apoB pool during a 120 min chase period. The inhibitory function of lactacystin demonstrates a lag period (Dick et al., 1997); thus, the addition of this drug 30 min before the pulse was necessary to ensure that the inhibitor was present in its active form during the pulse. Figs. 5.2A and 5.2B show that a significantly higher level of apoB was present in lactacystin-treated permeabilized cells at 0 time, in comparison to cells not treated with lactacystin (3.4 fold increase over control). Over the 120 min chase following permeabilization, a major proportion of [35S] labeled apoB was degraded in both control and lactacystin-pretreated cells (Fig. 5.2B). Furthermore, in the presence of lactacystin there was a dramatic accumulation of the major degradation intermediates of apoB, including the 70 kDa fragment (Fig. 5.2A). Enhanced generation of the fragment appeared to result from a greater pool of [35S] labeled apoB100 at 0 time in lactacystin-pretreated cells.

We also examined the possibility that the digitonin treatment of the cells may have resulted in the leakage of apoB from the secretory pathway (ER-Golgi system) into
Figure 5.2 In permeabilized HepG2 cells lactacystin inhibits co-translational turnover of apolipoprotein B but does not block post-translational degradation of apolipoprotein B, nor the generation of the 70 kDa fragment. A. Near confluent HepG2 cells were pulsed for 15 min with $[^{35}\text{S}]$methionine and chased for 10 min with excess cold methionine. The cells were then permeabilized with digitonin (50 μg/mL, 10 min), and permeabilized cells were incubated in CSK buffer. In some dishes, lactacystin (25 μM) was included in the pre-incubation media, the pulse, the chase, as well as during the incubation of permeabilized cells in CSK buffer. Permeabilized cells were solubilized and then immunoprecipitated with a polyclonal anti-apoB antibody. Immunoprecipitates were analyzed by SDS-PAGE and fluorography. The arrowheads indicate the 550 kDa apoB100 and its 70 kDa degradation intermediate. B. For the experiment in (A), the turnover of apoB in permeabilized cells in the presence and absence of lactacystin was assessed by plotting the total apoB radioactivity recovered (in the apoB100 bands) from permeabilized cells at various times of incubation in CSK buffer (0 time, 60 min, and 120 min). Open circles, control cells; closed circles, lactacystin treated cells.
**FIGURE 5.2**

A.

![Image of gel electrophoresis with markers at 550 kDa and 70 kDa.]

<table>
<thead>
<tr>
<th>Chase Time (h)</th>
<th>0</th>
<th>0</th>
<th>1</th>
<th>1</th>
<th>2</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactacystin</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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</tr>
</tbody>
</table>

B.

![Graph showing immunoprecipitable ApoB remaining in permeabilized cells vs. chase time (h).]

**Immunoprecipitable ApoB Remaining in Permeabilized Cells**

- **(CPM x 10^3)** (35 mm dish)

<table>
<thead>
<tr>
<th>Chase Time (h)</th>
<th>0</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>CPM x 10^3</td>
<td>45</td>
<td>30</td>
<td>15</td>
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<td>CPM x 10^3</td>
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<td>CPM x 10^3</td>
<td>20</td>
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the CSK buffer. After permeabilization, we collected both the cells and CSK (plus digitonin) buffer at 0 h and 2 h chase periods of chase and probed for apoB. The amount of intact apoB in permeabilized cells declined over the chase period, concomitant with an increase in the accumulation of the 70 kDa fragment in the cells (Fig. 5.3A). Little or no radiolabeled full length apoB or the 70 kDa fragment could be detected in the CSK buffer at different chase times (Fig. 5.3B and 5.3C). There was also no continued accumulation of the intact apoB or its 70 kDa fragment in the CSK buffer over time (Fig. 5.3B and 5.3C, respectively). Overall, the data suggest that there is minimal non-specific loss of radiolabeled apoB following permeabilization of HepG2 cells, and that intracellular degradation rather than cell leakage can account for temporal disappearance of intact apoB in permeabilized cells.

5.1.3 Immunoblotting Reveals that the 300 kDa Band Is Not a Fragment of Apolipoprotein B100

A protein band of approximately 300 kDa was detected in the autoradiographs represented in Figure 5.3, as well as other autoradiographs depicting immunoprecipitated radiolabeled apoB. It was suspected that this band may represent a heavily glycosylated protein that binds non-specifically to the immunoprecipitin used to isolate apoB. To confirm that this band was in fact not a fragment of apoB, HepG2 cells were collected, solubilized and immunoblotted for apoB using goat ant-human apoB antibodies. This method was advantageous in that it eliminated the use of the immunoprecipitin. The results revealed that the 300 kDa band detected in the autoradiographs were not detected in the immunoblots, which did reveal the presence of the apoB100 and its fragmented
Figure 5.3 Permeabilization of HepG2 cells does not cause leakage of radiolabeled apolipoprotein B or the 70 kDa degradation fragment into the surrounding medium.

Near confluent HepG2 cells were pulsed for 15 min with $[^{35}\text{S}]$methionine, chased for 10 min, permeabilized with digitonin (50 µg/mL, 10 min), and permeabilized cells were incubated in CSK buffer for 0 and 2 h. Cells and CSK buffer were immunoprecipitated for apoB and immunoprecipitates were analyzed by SDS-PAGE and fluorography. A. Representative experiment showing the distribution of apoB$_{100}$ and the 70 kDa fragment in permeabilized cells and CSK buffer over a 2 h chase period. B. A graph comparing the amount of immunoprecipitable radiolabeled full-length apoB detected in permeabilized cells (open circles) and the CSK buffer (closed circles) for each incubation period (mean±SD, n=2). C. A graph comparing the amount of immunoprecipitable radiolabeled 70 kDa apoB degradation fragment detected in permeabilized cells (open circles) and the CSK buffer (closed circles) for each incubation period (mean±SD, n=2).
FIGURE 5.3
intermediates (Fig. 5.4). Thus, the immunoblotting data confirmed the fact that the 300 kDa band detected in the autoradiographs was not a degradative intermediate of apoB.

To confirm that the 300 kDa protein was non-specifically binding to the immunoprecipitin, radiolabeled HepG2 cell lysates were immunoprecipitated for either apoB or non-immune serum and subjected to SDS-PAGE and fluorography (Fig. 5.4B). The results revealed that the 300 kDa protein was detected in both samples immunoprecipitated for apoB and non-immune serum. ApoB however was only detected in samples immunoprecipitated for apoB. Thus, it is evident that the 300 kDa band detected in gels representing immunoprecipitated apoB is an artifact of the experiment and represents a protein that is not antigenitically related to apoB.

5.1.4 Comparison of Proteasomal Activity in Both Intact and Permeabilized Cells

In order to assess any effects permeabilization may have on the proteasomal activity of HepG2 cells, we measured protease activity of cell lysates prepared from intact and permeabilized HepG2 cells that were pre-treated with and without lactacystin. In this particular assay, all detectable proteolytic activity was measured using FITC-labeled casein as the proteolytic substrate. We initially examined the difference in total proteolytic activity between intact and permeabilized cells without lactacystin treatment and found that there was a decrease in the amount of proteolytic activity after permeabilization of the cells (0.23±0.16 F.U. detected in permeabilized cells in comparison to 5.97±1.2 F.U. detected in intact cells) (Fig. 5.5A). In the presence of
Figure 5.4 Evidence that the approximate 300 kDa band recovered in the apolipoprotein B immunoprecipitates is not antigenically related to apolipoprotein B. Near confluent HepG2 (\(^{35}S\)radiolabeled or non-radiolabeled) were collected and solubilized. A. Non-radiolabeled cell lysates were subjected to SDS-PAGE (4.5% (v/v) acrylamide resolving gel). The proteins were then transferred overnight at 4°C onto nitrocellulose membranes, and the membranes were blocked with a 5% solution of fat-free dry milk powder. Immunoblotting was performed to detect apoB by using the primary antibody goat anti-human apoB (1:1000 for 1 h) followed by the secondary antibody rabbit anti-goat conjugated to peroxidase (1:8000 for 1 h). Membranes were then incubated in an ECL detection reagent for 60 seconds, exposed to Hyperfilm and developed (n=3). B. \(^{35}S\) radiolabeled cell lysates were immunoprecipitated with either apoB antibodies or non-immune serum and subjected to SDS-PAGE and fluorography.
FIGURE 5.4
Figure 5.5 Comparison of total proteolytic activity and proteasomal activity in intact and permeabilized cells using a fluorogenic protease assay. Near confluent cells were pre-incubated in the presence or absence of lactacystin (25 μM) for 1 h. Some cells were then permeabilized and others were left intact. All cells were collected in PBS plus 0.1% Triton X100, homogenized and centrifuged. Cell lysates were then used in performing fluorogenic protease assays with FITC-casein as the proteolytic substrate. Following a 1 h incubation time, samples were treated with 5% TCA to precipitate insoluble proteins and centrifuged. Then 60 μL of the supernatant was diluted with 400 μL of 500 mM Tris, pH 8.8 and read in a Fluoroskan at an excitation wavelength of 485 nm and emission wavelength of 538 nm. A. Comparison of total proteolytic activity between intact (open bars) and permeabilized cells (closed bars) (n=4). B. Comparison of lactacystin-inhibitable proteolytic activity assessed by the difference in fluorescent units between control and lactacystin treated intact cells (open bars) and permeabilized cells (closed bars).
A.

![Graph A: Total Proteolytic Activity](image)

B.

![Graph B: Total Lactacystin-Inhibitible Activity](image)

FIGURE 5.5
lactacystin there was a considerable decrease in detectable proteolytic activity in intact cells (lactacystin-inhibitable activity, 2.84±0.75 F.U.) (Fig. 5.5B). The loss in proteolytic activity was considered to be proteasomal in nature since the difference between the two conditions was the presence of the proteasomal inhibitor, lactacystin. We then examined permeabilized cells and found no detectable lactacystin-inhibitable proteolytic activity (Fig. 5.5B). The absence of any appreciable lactacystin-inhibitable protease activity in permeabilized cells suggests a significant loss of proteasomal activity in these cells.

5.1.5. Detection of the 20S Proteasomal Subunits in Intact and Permeabilized Cells

To assess whether the loss of proteasomal activity following permeabilization was due to the loss of cytosolic proteasome, we immunoblotted intact and permeabilized cells for several of the subunits of the 20S proteasome which have both structural and functional roles in the proteasome complex. Immunoblotting of intact HepG2 cell lysates with a polyclonal antibody revealed several of the 20S subunits ranging in size from 25-35 kDa (Fig. 5.6A). However, immunoblotting of permeabilized cell lysates revealed a dramatic reduction in the detection of the 20S subunits (~68% reduction compared to intact cells), thus indicating a significant loss of the cytosolic proteasome upon permeabilization (Fig. 5.6B). Furthermore, a significant amount of proteasomal subunits were detected in CSK plus digitonin buffer used in the permeabilization of the cells (~49% detected in comparison to intact cells) (Fig. 5.6B).
Figure 5.6 Immunoblotting of the 20S proteasomal subunits in intact and permeabilized HepG2 cells. A. Cell lysates from the intact and permeabilized cells used in Figure 5.5 were subjected to SDS-PAGE (10% (v/v) acrylamide resolving gel) and proteins were then transferred overnight at 4°C onto nitrocellulose membranes. Membranes were blocked with a 5% solution of fat-free dry milk powder. Immunoblotting was performed to detect 20S proteasomal subunits by using the primary antibody rabbit anti-rat 20S proteasome (1:1000 for 1 h) followed by the secondary antibody goat anti-rabbit IgG conjugated to peroxidase (1:8000 for 1 h). Membranes were then incubated in an ECL detection reagent for 60 seconds, exposed to Hyperfilm and developed. B. Bands were quantitated by densitometry and the amount of total 20S subunits in permeabilized cells and CSK plus digitonin buffer were determined as a percentage of that found in intact cells.
FIGURE 5.6
5.1.6 Differential Sensitivity of Apolipoprotein B Degradation in Permeabilized Cells to ALLN, Lactacystin and clasto-lactacystin β-Lactone

We compared the inhibitory effects of ALLN and lactacystin on post-translational degradation of apoB in permeabilized cells. In contrast to lactacystin, ALLN increased the amount of apoB remaining after a 2 h chase (Fig. 5.7A). The apoB remaining (as a percentage of total apoB at time 0) in lactacystin-treated and ALLN-treated permeabilized cells was 30.7±5.2% and 75.1±6.7%, respectively (p<0.05, n=3) (Fig. 5.7B). Thus, unlike lactacystin, ALLN appeared to inhibit post-translational apoB degradation. In addition, ALLN also inhibited apoB fragmentation, which generates the distinct apoB intermediates in permeabilized cells, including the 70 kDa fragment (Fig. 5.7A).

The inhibitory effect of clasto-lactacystin β-lactone, the active species of the lactacystin inhibitor (Dick et al., 1997), was also examined. The purpose was to ensure that the insensitivity of apoB fragmentation to lactacystin was not a result of the inability of the inhibitor to convert to its active form in a permeabilized cell system. Addition of clasto-lactacystin β-lactone, either before or after permeabilization, did not prevent the loss of apoB in permeabilized cells nor did it interfere with the appearance of the 70 kDa fragment (Fig. 5.8). Interestingly, the addition of the inhibitor before the pulse resulted in an increase in the abundance of the 70 kDa fragment, most likely as a result of an increased initial pool of the full length apoB (Fig. 5.8). Thus, the data suggest that the generation of the 70 kDa fragment may involve a degradation system independent of the cytosolic proteasome.
Figure 5.7 Comparative effects of ALLN and lactacystin on apolipoprotein B degradation and the generation of the 70 kDa fragment. Near confluent HepG2 cells were pulsed for 15 min with [\(^{35}\)S]methionine and were chased for 10 min with excess cold methionine. The cells were then permeabilized with digitonin (50 \(\mu\)g/mL, 10 min), and permeabilized cells were incubated in CSK buffer. Either lactacystin (25 \(\mu\)M) or ALLN (40 \(\mu\)g/mL) was included in the pre-incubation media, the pulse, the chase, as well as during the incubation of permeabilized cells in CSK buffer. Permeabilized cells were solubilized and immunoprecipitated for apoB, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. A. A representative experiment is shown with arrowheads indicating the 550 kDa apoB100 and its 70 kDa degradation intermediate. B. The amount of radioactivity in the apoB100 bands were quantitated by scintillation counting (n=3). Open bars (no inhibitors), dotted bars (lactacystin treated cells), and closed bars (ALLN treated cells).
FIGURE 5.7
Figure 5.8 Effects of clasto-lactacystin β-lactone on the generation of the 70 kDa fragment. Near confluent HepG2 cells were pulsed for 15 min with [35S] methionine and chased for 10 min with excess cold methionine. The cells were then permeabilized with digitonin (50 μg/mL, 10 min), and permeabilized cells were incubated in CSK buffer in the presence and absence of either clasto-lactacystin β-lactone (10 μM). Permeabilized cells were solubilized, immunoprecipitated for apoB, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography. A. The amount of radioactivity in the 70 kDa bands was quantitated by scintillation counting (n=2). Open bars (no inhibitor), closed bars (clasto-lactacystin β-lactone treated cells).
FIGURE 5.8
5.1.7 Detection of an ER60 Homologue in HepG2 cells

In an attempt to detect an alternative non-proteosomal degradation system responsible for apoB degradation, intact HepG2 cells were collected, solubilized and cell lysates were immunoblotted for the ER60 protease. The ER60 protease has been previously detected in the ER of rat livers (Urade et al., 1992; and Urade and Kito, 1992). Figure 5.9A represents an immunoblot of HepG2 cell lysates probed with anti-rat ER60 antibody. Despite the fact that HepG2 cells are of human origin, a 58 kDa protein, representing the ER60 protease, was readily detectable with this antibody. In addition, this antibody was also used to detect ER60 in primary hamster hepatocytes of Golden Syrian hamsters (Fig.5.9B). Thus, the immunoblotting data suggest the presence of an ER resident protease that is homologous to the rat ER60 in size and antigenecity in both HepG2 cells as well as primary hamster hepatocytes.

5.1.8 Fate of the 70 kDa Fragment in Permeabilized HepG2 Cells

Supplemented with Cytosolic Factors

The fate of the 70 kDa fragment was examined by attempting to reconstitute intracellular protein transport in HepG2 cells following permeabilization. Cells at approximately 60% confluency were briefly pulsed with \([^{35}\text{S}]\)methionine, chased and permeabilized with 50 \(\mu\text{g/mL}\) of digitonin. Following permeabilization, the cells were incubated in CSK buffer for 2 h to allow for the generation of the specific 70 kDa apoB fragment. After a 2 h incubation, the CSK buffer was removed and the cells were incubated with and without fresh reconstitution buffer comprised of fresh CSK buffer, pH 7.1, an ATP generating system and fresh cytosol. In addition, some cells were incubated in the presence of
Figure 5.9 Detection of an ER60 protease homologue in HepG2 and primary hamster hepatocytes. A. Near confluent HepG2 and primary hamster hepatocytes were collected, solubilized and subjected to SDS-PAGE (10% (v/v) acrylamide resolving gel). The proteins were then transferred overnight at 4°C onto nitrocellulose membranes, and the membranes were blocked with a 5% solution of fat-free dry milk powder. Immunoblotting was performed to detect the ER60 protease by using the primary antibody rabbit anti-rat ER60 (1:1000 for 1 h) followed by the secondary antibody goat anti-rabbit conjugated to peroxidase (1:8000 for 1 h). Membranes were then incubated in an ECL detection reagent for 60 seconds, exposed to Hyperfilm and developed (n=2).
FIGURE 5.9
MG132 to observe any effects the cytosolic inhibitor may have on apoB within reconstituted cells. The cells and the CSK buffer were collected following 0, 1 and 2 h of incubation with the reconstitution buffer and immunoprecipitated for apoB. Fig. 5.10 reveals the effects on the fate of apoB and the 70 kDa fragment in permeabilized cells supplemented with cytosolic components and an ATP generating system. The results show that even in the presence of the reconstitution buffer and MG132 there was still a decrease in intracellular apoB over a 2 h incubation period (Fig. 5.10A). However, the levels of the 70 kDa fragment within the cells did not appear to change over this 2 h incubation period, in the presence or absence of the reconstitution buffer and/or MG132 (Fig. 5.10A). After a 2 h incubation period, in the presence of fresh CSK with cytosol and an ATP generating system, apoB molecules could be detected in the CSK buffer, and the levels of apoB were further increased in the presence of MG132 (Fig. 5.10B). The presence of the 70 kDa fragment could also be observed in the CSK buffer only in the presence of the reconstitution buffer (Fig. 5.10B). Thus, the data suggests that the addition of the reconstitution buffer to permeabilized HepG2 cells had allowed for the transport of both apoB100 and the 70 kDa fragment out of the ER. Furthermore, it appeared that the cytosolic proteasome affected the amount of apoB secreted.
Figure 5.10 Fate of the apolipoprotein B100 and the 70 kDa fragment following supplementation of permeabilized HepG2 cells with cytosolic components. Near confluent HepG2 cells were pulsed for 15 min with $[^{35}\text{S}]$methionine and chased for 10 min with excess cold methionine. The cells were then permeabilized with digitonin (50 μg/mL, 10 min), and permeabilized cells were incubated in CSK buffer for 2 h. Following the 2 h incubation, cells were incubated in CSK buffer, pH 7.1, in the absence or presence of fresh HepG2 cytosol and an ATP generating system (reconstitution buffer) for 0, 1 and 2 h. Permeabilized cells and CSK buffers were collected, solubilized, immunoprecipitated for apoB. The immunoprecipitates were analyzed by SDS-PAGE and fluorography. A. A representative experiment showing the fate of the apoB100 and the 70 kDa fragment in the permeabilized cells during the 0, 1 and 2 h incubation period in the presence and absence of the reconstitution buffer. B. The apoB100 and 70 kDa fragment immunoprecipitated from the CSK buffer surrounding the permeabilized cells depicted in (A).
FIGURE 5.10
CHAPTER 6

RETROGRADE TRANSLOCATION AND THE INVOLVEMENT OF THE CYTOSOLIC PROTEASOME IN THE POSTTRANSLATIONAL DEGRADATION OF APOLIPOPROTEIN B

6.1 Results

6.1.1 Identification of Newly Synthesized Apolipoprotein B in the Cytosol of HepG2 cells.

The primary objective in this study was to explore the potential retrograde translocation of apoB from the secretory pathway to the cytosol. To examine this possibility, pulse-chase labeling experiments were performed and the distribution of apoB was probed in both microsomes and cytosol. In these experiments, the cells were pulsed with 100 μCi/ml of [35S]methionine for 15 min and then chased with 10 mM cold methionine for 0 and 2 h. Following each chase period the cells were collected, homogenized and separated into cytosolic and crude microsomal fractions. Figure 6.1A reveals the presence of apoB in both the microsomal and cytosolic fractions of HepG2 cells. The distribution of apoB in each fraction was assessed as a percentage of radiolabeled apoB in each fraction compared to the total (cytosolic and microsomal) radiolabeled apoB pool for each chase period. The results revealed that at 0 hour the percent distribution of apoB in the cytosol and microsomal fraction was 7.1% and 92.9%, respectively (Fig. 6.1B). Interestingly, the percent distribution of apoB shifted over a 2 h chase period with the percent apoB in the cytosol rising to 27.5% and the percent apoB in the microsome decreasing to 72.5% (Fig. 6.1B). Thus, it appears that the increase in

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Figure 6.1 Identification and intracellular distribution of newly synthesized apolipoprotein B in the microsomal and cytosolic fractions of HepG2 cells. Near confluent HepG2 cells were pulsed with 100 μCi/ml of [\textsuperscript{35}S]methionine for 15 min and then chased with 10 mM cold methionine for 0 and 2 h. Following each chase period, the cells were collected, homogenized and separated into cytosolic and crude microsomal fractions. A. A representative experiment showing the pattern of radiolabeled apoB in both the microsomal and cytosolic fractions during the 0 and 2 h chase times in the presence and absence of MG132. B. The distribution of apoB in both the microsomal (\textit{shaded bars}) and cytosolic (\textit{open bars}) fractions in the absence of MG132 was assessed as a percentage of radiolabeled apoB in each fraction compared to the total (cytosolic and microsomal) radiolabeled apoB pool for each chase period (mean\pm S.E., n≥3). C. The distribution of apoB in both the microsomal (\textit{shaded bars}) and cytosolic (\textit{open bars}) fractions in the presence of MG132 was assessed as a percentage of radiolabeled apoB in each fraction compared to the total (cytosolic and microsomal) radiolabeled apoB pool for each chase period (mean\pm S.E., n≥3).
FIGURE 6.1
cytosolic apoB may have been a result of apoB molecules being translocated from the ER into the cytosol.

In addition, we assessed the percent distribution of apoB at 0 and 2 h in the presence of MG132 and the results revealed that at 0 h is 8.0 % of radiolabeled apoB was localized in the cytosol and 92.0 % in the microsomes (Fig. 6.1C). Following a 2 h chase, the percent apoB in the cytosol increased by 20.2 % and in the microsomal fraction the percent apoB decreased by 79.8 % (Fig. 6.1C). Although the amount of apoB in both the cytosolic and microsomal fractions increased in the presence of MG132 compared to controls, the percent distribution of apoB within these fractions remained relatively constant.

6.1.2 Proteasomal Inhibitor, MG132, Increases the Post-translational Pool of Cytosolic Apolipoprotein B

To assess the effect of inhibiting the cytosolic proteasome on post-translational accumulation of newly synthesized apoB, HepG2 cells were pulsed briefly and chased for 0 and 2 h in the presence and absence of the proteasomal inhibitor, MG132. Cells were collected, homogenized and the cytosolic fractions were isolated by ultracentrifugation. Cytosolic fractions were immunoprecipitated for apoB and subjected to SDS-PAGE. Quantitation of newly synthesized apoB revealed that over a 2 hour chase period the amount of apoB accumulated in the cytosol was increased by approximately 1.5 fold in the presence of MG132 compared to the controls (Fig. 6.2). This suggested that the cytosolic apoB was sensitive to MG132 and thus likely degraded by the cytosolic proteasome.
Figure 6.2 Cytosolic apolipoprotein B was sensitive to post-translational proteasomal degradation. HepG2 cells were pulsed with 100 μCi/mL [35S]methionine for 15 min in the presence and absence of 25 μM MG132. The cells were then chased for 0 and 2 h with excess cold methionine. The cells were collected, homogenized and ultracentrifuged to isolate the cytosolic fractions. ApoB was immunoprecipitated from both fractions and subjected to SDS-PAGE and fluorography. Cutting and scintillation counting determined the amount of radiolabeled apoB isolated from the cytosol. The amount of apoB recovered over a 2 h chase (as a percentage of the pool of cytosolic apoB at time 0) is shown. Control cells (open circles) and MG132 treated cells (closed circles) (mean±S.E., n≥3).
FIGURE 6.2
6.1.3 Preparation of Subcellular Fractions Does Not Cause Appreciable Leakage of Microsomal Components into the Cytosol

To address the possibility that the apoB present in the cytosol was a result of leakage from the ER, NADPH cytochrome c reductase assays (an ER marker), using the cytosolic and microsomal fractions as well as the whole cell lysates, were performed. Our results indicated that both the whole cell lysates as well as the microsomal fractions possessed high NADPH cytochrome c reductase activity whereas the cytosolic fractions revealed minimal activity for this ER associated enzyme (Fig. 6.3). The specific activity of the enzyme was measured for each subcellular fraction and compared to the specific activity of the total cell lysate ($2.1 \times 10^{-4}$ absorbance per mg of protein). The specific activity within the microsomal fractions was $3.1 \times 10^{-4}$ absorbance per mg of protein, whereas the specific activity within the cytosolic fractions was $1.7 \times 10^{-5}$ absorbance per mg of protein. Thus, the specific activity within the cytosolic fractions was approximately 5% of that observed in the microsomal fractions. This in turn suggested that there was minimal leakage of microsomal components into the cytosol as a result of subcellular fractionation procedures. In addition, it should be noted that if the observed cytosolic apoB (Fig. 6.1A) were due to leakage of the ER, following homogenization of the cells, then the percent distribution of apoB over a 2 h chase would likely have remained constant. The fact that the percent distribution of cytosolic apoB increased after 2 h even though the total pool of radiolabeled apoB was reduced would indicate that active transport of apoB from the ER to the cytosol is likely occurring.
Figure 6.3 Preparation of subcellular fractions did not cause appreciable leakage of microsomal components into the cytosol. HepG2 cells were collected, homogenized in 250 mM sucrose, and centrifuged for 1 h at 100,000 g. The cytosolic and crude microsomal fractions were collected and solubilized. The samples were then subjected to NADPH cytochrome c assays and the absorbance of the reactions were measured every 15 seconds for 3 min on a spectrophotometer, at a wavelength of 548 nm. A graph of the measured absorbance over time was plotted for both the cytosolic and microsomal fractions as well as for the entire cell lysate. In addition, dividing the slope of the curve by the amount of protein in the respective sample determined the specific activity of each reaction. The protein concentration was determined using a fluorogenic protein assay. Whole cell lysates (closed triangles), microsomal fraction (closed squares), and cytosolic fraction (closed circles).
FIGURE 6.3
6.1.4 Identification of Ubiquitinated Cytosolic and Microsomal Apolipoprotein B

HepG2 cells were pulsed with 200 μCi/ml of [35S]methionine and chased for 0 and 2 h in the presence and absence of 25 μM MG132. In order to identify ubiquitinated apoB, both the cytosolic and microsomal fractions isolated from these cells were immunoprecipitated for apoB and further immunoprecipitated for ubiquitin. Immunoprecipitates were analyzed by SDS-PAGE and fluorography and the gels revealed that in the absence of MG132, ubiquitinated apoB is identified principally in the microsomal fraction of the cells (Fig. 6.4). However, in the presence of MG132 ubiquitinated apoB was also detected in the cytosol, and there also appeared to be an increase in the pool of ubiquitinated microsomal apoB (Fig. 6.4). Interestingly, this pool of cytosolic ubiquitinated apoB decreased over a 2 h chase in the presence of proteasome inhibitor even though the pool of total cytosolic apoB appeared to increase (as observed in Fig. 6.1A and 6.2). Thus, despite inhibition of proteasomal degradation of apoB by MG132, the results suggested that this pool of ubiquitinated apoB might have been de-ubiquitinated over a 2 h chase in the presence of the proteasomal inhibitor.

In the microsomal fraction, the levels of ubiquitinated apoB also appeared to decrease over a 2 h chase (Fig. 6.4) even in the presence of MG132, however, this was concomitant with a similar decrease in total microsomal apoB observed in Fig. 6.1A. This decrease in both ubiquitinated and total apoB is likely due to either a transport of apoB from the ER to the cytosol and/or de-ubiquitination and degradation of apoB via a non-proteasome mediated system.
Figure 6.4 Cytosolic and microsomal apolipoprotein B were both ubiquitinilated. Near confluent HepG2 cells are pulsed for 15 min with 200 μCi/mL of [35S]methionine and then chased for 0 and 2 h. In some cells MG132 was added throughout the experiment. Cells were collected after each chase period, homogenized in 250 mM sucrose and ultracentrifuged for 1 h at 100,000 g to isolate both cytosolic and crude microsomal fractions. N-ethylmaleimide (5 mM) was added to the sucrose solutions and all buffers following homogenization of the cells. Both the cytosolic and microsomal fractions were then immunoprecipitated for apoB and then serial immunoprecipitated for ubiquitin using rabbit anti-human antibodies. The immunoprecipitates were then subjected to SDS-PAGE and fluorography. A representative gel depicting the ubiquitinilated apoB isolated from the microsomal and cytosolic fractions in the absence and presence of MG132 (25 μM).
FIGURE 6.4
6.1.5 Cytosolic Apolipoprotein B is sensitive to Endoglycosidase H Treatments

ApoB isolated from the cytosolic and microsomal fractions were subjected to endoglycosidase H (Endo H) treatments in order to determine whether the cytosolic and/or microsomal pools of newly synthesized apoB were glycosylated. Glycosylated apoB would indicate that this molecule had at some point been exposed to the lumen of the ER, where the enzyme necessary for N-glycosylation resides. It should be noted that all cells were treated with MG132 to obtain a greater pool of apoB to facilitate Endo H treatments. Following immunoprecipitation of newly synthesized radiolabeled apoB from both the cytosolic and microsomal fractions, the samples were incubated in the presence and absence of 1 milliunit of Endo H overnight at 37°C. All samples were then subjected to SDS-PAGE and fluorography. The results revealed that apoB isolated from the cytosol was sensitive to Endo H treatments as observed by a downward shift in the banding pattern apoB following Endo H treatment (Fig. 6.5A). Since the amount of full-length apoB in the cytosol at 0 h was barely detectable, the effects of Endo H treatment were only clearly visible after the 2 h chase. This downward shift in banding patterns indicates a decrease in molecular weight of the protein due to the removal of N-linked oligosaccharide chains. Detection of glycosylated apoB in the cytosol suggests that this pool of apoB had likely entered the ER during translation where it was glycosylated before being released from the ER into the cytosol. As an internal control, the pool of microsomal apoB was also shown to be glycosylated at both 0 and 2 h chase times (Fig. 6.5B).
Figure 6.5 Cytosolic and microsomal apolipoprotein B were sensitive to endoglycosidase H treatments. HepG2 cells were pulsed with 200 μCi/mL of [\textsuperscript{35}S]methionine and chased for 0 and 2 hours. Cells were collected at the end of each chase period and separated by ultracentrifugation into cytosolic and microsomal fractions. Subcellular fractions were then solubilized and immunoprecipitated for apoB. Immunoprecipitated apoB was then incubated with and without 1 milliunit of endoglycosidase H, overnight at 37°C. The samples were then subjected to SDS-PAGE and fluorography. Representative gels showing the effects of endoglycosidase H treatment on apoB isolated from the cytosol (A) and from the microsomal fractions (B).
FIGURE 6.5
6.1.6 Effects of Brefeldin A on the Cytosolic and Microsomal Distribution of Apolipoprotein B

HepG2 cells were briefly pulsed and chased for 0 and 2 h in the presence and absence of brefeldin A and in the presence and absence of MG132. Subcellular fractionation of the cells was performed and the fractions were immunoprecipitated for apoB. The effects of brefeldin A treatments were analyzed by SDS-PAGE and fluorography. The gels showed that brefeldin A slightly increased the levels of cotranslational apoB, particularly in the microsomal fractions (Fig. 6.6A and 6.6B). As an internal control, secretion of apoB was halted in the presence of brefeldin A (Fig. 6.6C). The effects of brefeldin A were also examined in the presence of MG132, in order to eliminate any proteasomal degradation. At the end of the pulse, in the presence of MG132, there did not appear to be any significant alteration in the distribution of apoB between the cytosol and the microsomes. Following the 2 h chase, the amount of apoB recovered in the cytosol and microsomes appeared to decrease in the presence of brefeldin A and MG132 in comparison to cells treated with MG132 only. This decrease in recovered apoB is not likely a result of Brefeldin A having an effect on the inhibitory nature MG132. Evidence for this was observed at 0 h where Brefeldin A did not alter the amount of apoB in MG132 treated cells in comparison to cells treated only with MG132. In fact, if Brefeldin A did block the inhibitory effect of MG132, then the amount of apoB present in MG132 and Brefeldin A treated cells would likely reach the levels observed in control cells (no treatments). Thus, the loss in accumulated cytosolic apoB over a 2 h chase in the presence of MG132 and Brefeldin A may be due to an effect Brefeldin A has
Figure 6.6 Brefeldin A altered the distribution pattern of apolipoprotein B between the microsomal and cytosolic fractions. Near confluent HepG2 cells were pretreated with and without brefeldin A (1 μg/mL) and with and without MG132 (25 μM) prior to the pulse. Cells were pulsed with 100 μCi/mL of $[^{35}S]$methionine for 15 min and then chased for 0 and 2 h in the presence and absence of brefeldin A and MG132. Cells were collected at the end of each chase period and separated by ultracentrifugation into microsomal and cytosolic fractions. The subcellular fractions were solubilized, immunoprecipitated for apoB and subjected to SDS-PAGE and fluorography. The gels represent the distribution pattern of apoB in the presence and absence of MG132 and/or brefeldin A in the cytosol (A), the microsomes (B), and the media fractions (C).
FIGURE 6.6
on the transport of apoB into the cytosol. As a result, apoB that remains associated with the ER may be degraded via a non-proteasome mediated pathway.

6.1.7 Brefeldin A Decreases the Rate of Apolipoprotein B Accumulation in the Cytosol and Increases the Rate of Apolipoprotein B Degradation in the Microsomes

To assess the effects of brefeldin A on the accumulation of apoB in the cytosol, HepG2 cells were pretreated with and without brefeldin A (1 μg/mL) prior to the pulse. Cells were then pulsed with 100 μCi/mL of [³⁵S]methionine for 15 min and then chased for 0 and 2 h in the presence and absence of brefeldin A. MG132 (25 μM) was added to all cells during the pretreatment, pulse and chase periods to eliminate any proteasomal degradation of apoB. Quantitation of these results revealed that in the absence of brefeldin A, the accumulation of apoB in the cytosol, over a 2 h chase, was approximately 3.0 fold greater than the amount of apoB present at 0 h (Fig 6.7A). In the presence of brefeldin A, the accumulation of cytosolic apoB was 1.8 fold greater than the amount of apoB present at 0 h (Fig. 6.7A). Thus, the data suggested that brefeldin A decreased the rate of appearance of apoB in the cytosol. Since MG132 was present throughout the experiment, it is not likely that the reduction in cytosolic apoB accumulation is the result of cytosolic degradation of apoB.

The reduction in apoB over a 2 h chase by approximately 30 % was also observed in the microsomal fractions of cells treated with brefeldin A (Fig. 6.7B). Considering apoB secretion was abolished in the presence of Brefeldin A and the accumulation of apoB in the cytosol was reduced, then the loss in microsomal apoB would have likely
Figure 6.7 Brefeldin A decreased the rate of apolipoprotein B accumulation in the cytosol and increased the rate of apolipoprotein B degradation in the microsomes. HepG2 cells were pretreated with and without brefeldin A (1 µg/mL) prior to the pulse. Cells were pulsed with 100 µCi/mL of [35S]methionine for 15 min and then chased for 0 and 2 h in the presence and absence of brefeldin A. MG132 (25 µM) was added to all cells during the pretreatment, pulse and chase periods. Cells were collected at 0 and 2 h and subjected to subcellular fractionation by ultracentrifugation. The cytosolic and microsomal fractions subcellular fractions were solubilized, immunoprecipitated for apoB and subjected to SDS-PAGE and fluorography. Cutting and scintillation counting assessed the amount of radiolabeled apoB. A. A graph showing the percentage of cytosolic apoB recovered over a 2 h chase in the presence (closed circles) and absence of brefeldin A (open circles) in comparison to the amount of apoB present at 0 h (mean±S.E., n≥3). B. A graph showing the percentage of microsomal plus media apoB recovered over a 2 h chase in the presence (closed circles) and absence of brefeldin A (open circles) in comparison to the amount of apoB present at 0 h (mean±S.E. n≥3).
FIGURE 6.7
been due to degradation of the protein. Furthermore, apoB degradation was likely mediated by a non-proteasome system since the experiments were conducted in the presence of MG132.

6.1.8 Partial Inhibition of Glycosylation Enhances the Accumulation of Apolipoprotein B in Both the Cytosol and Microsomes

In order to assess the effects glycosylation may have on the subcellular distribution of apoB, HepG2 cells were pretreated with 5 µg/mL of tunicamycin. Cells were then briefly pulsed and then chased for 0 and 2 hours in the presence and absence of MG132 and immunoprecipitated for apoB. The results revealed that in the absence of MG132, the amount of cytosolic and microsomal apoB detected in tunicamycin treated cells appeared to increase at 0 h in comparison to control cells (Fig. 6.8A and 6.8B). However, after a 2 h chase, the levels of cytosolic and microsomal apoB both appeared to decrease to levels similar to that detected in control cells. In MG132 and tunicamycin treated cells, the increase in both cytosolic and microsomal apoB again was observed at 0 h (Fig. 6.8A and 6.8B). Following a 2 h chase, there did not appear to be any significant difference between the presence and absence of tunicamycin in MG132 treated cells (Fig. 6.8A and 6.8B).

6.1.9 Time Course Effects of Tunicamycin on Cytosolic Apolipoprotein B

The effects of tunicamycin on cytosolic apoB were examined through a time course experiment. All cells were incubated in the presence of MG132 during the pre-pulse, pulse and chase periods. Tunicamycin was added to some of the cells, and the cells
Figure 6.8 Partial Inhibition of glycosylation enhanced accumulation of apolipoprotein B in both the cytosol and the microsomes. Near confluent HepG2 cells were pretreated with and without tunicamycin (5 μg/mL) and with and without MG132 (25 μM) prior to the pulse. Cells were pulsed with 100 μCi/mL of [35S]methionine for 15 min and then chased for 0 and 2 h in the presence and absence of tunicamycin and MG132. Cells were collected at the end of each chase period and separated by ultracentrifugation into microsomal and cytosolic fractions. The subcellular fractions were solubilized, immunoprecipitated for apoB and subjected to SDS-PAGE and fluorography. The gels represent the distribution pattern of apoB in the presence and absence of MG132 and/or tunicamycin in both the cytosolic (A), and the microsomal fractions (B).
A. Cytosolic

550 kDa →

Chase (h) 0 0 0 0 2 2 2 2
MG132 - + - + - + - +
Tunicamycin - - + + - - + +

B. Microsomal

Chase (h) 0 0 0 0 2 2 2 2
MG132 - + - + - + - +
Tunicamycin - - + + - - + +

FIGURE 6.8
were chased for 0, 15, 30, 60, 90 and 120 min. Cells were collected from each chase period and the cytosolic fractions were isolated and immunoprecipitated for apoB and serial immunoprecipitated for ubiquitin. Figure 6.9 shows the effects of tunicamycin on cytosolic apoB at various times of chase. As previously observed, tunicamycin appeared to enhance the accumulation of apoB at the end of the pulse period (0 h). The time course experiment revealed that this increase in cytosolic apoB in the presence of tunicamycin was observed up to 60 min of chase. At this point the level of cytosolic apoB appeared to approach the levels observed for control cells (MG132 only treated cells). In fact, at approximately 90 min into the chase the amount of apoB in tunicamycin treated cells began to drop below the amount observed in control cells. The data suggested that the early increase in cytosolic apoB was likely due to the presence of tunicamycin. Furthermore, after a 2 h chase, the level of cytosolic apoB was reduced to that observed in the controls. The fact that the cytosolic proteasome was inhibited by MG132 would suggest that degradation of apoB was likely conducted via non-proteasome mediated degradation system(s) under these conditions.
Figure 6.9 Time Course Effects of Tunicamycin on Cytosolic Apolipoprotein B. HepG2 cells were pretreated with and without tunicamycin (5 µg/mL) prior to the pulse. Cells were pulsed with 200 µCi/mL of $[^{35}\text{S}]$methionine for 15 min and then chased for 0, 15, 30, 60, 90, 120 min in the presence and absence of tunicamycin. MG132 (25 µM) was added to all cells during the pretreatment, pulse and chase periods. Cells were collected at 0, 15, 30, 60, 90 and 120 min and the cytosolic fractions of the cells were isolated by ultracentrifugation. The cytosolic fractions were immunoprecipitated for apoB and the immunoprecipitates were then subjected to SDS-PAGE and fluorography.
FIGURE 6.9

<table>
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<th>Chase (min)</th>
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<th>0</th>
<th>15</th>
<th>15</th>
<th>30</th>
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<tr>
<td>Tunicamycin</td>
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550 kDa →
CHAPTER 7
DISCUSSION AND CONCLUSIONS

Intracellular regulation of apoB biogenesis is essential for the proper assembly and secretion of the hepatic apoB-containing lipoproteins. The regulation of hepatic apoB secretion appears to occur post-transcriptionally (Dashti et al., 1989; Pullinger et al., 1989; Moberly et al., 1990; Kaptein et al., 1991). ApoB is constitutively expressed (Pullinger et al., 1989), and apoB mRNA levels do not change under conditions that modulate its hepatic secretion (Kosykh et al., 1988; Pullinger et al., 1989). Post-transcriptional regulation of apoB secretion appears to depend on the intracellular lipid availability and the rate of apoB translocation into the ER (reviewed in Dixon and Ginsberg, 1992 and in Ginsberg, 1995). Inefficient translocation of apoB due to insufficient lipid availability or misfolding of the apoB protein leads to the intracellular degradation of the apoB molecule (reviewed in Yao et al., 1997). The mechanisms of apoB translocation and degradation have been under intense investigation over the past decade, and many important components of these processes have recently been identified. In the studies presented in this dissertation, translocational and degradative mechanisms have been further investigated in both human and rat hepatoma cultured cells, HepG2 and McA-RH7777, respectively. As discussed below, these studies have made important contributions to our understanding of the intracellular biogenesis of apoB and the mechanisms regulating its hepatic secretion.
7.1 The Relationships between the Translocational Efficiency, Intracellular Stability and the Size of the Nascent Apolipoprotein B Polypeptide

Translocation of apoB across the ER membrane has been proposed as an important step regulating the assembly and secretion of apoB-containing lipoproteins (Davies et al., 1989; Davies et al., 1990; Thrift et al., 1992; Sakata et al., 1993; Bonnardel and Davis, 1995). This process appears to be dependent upon both the lipid availability (Pullinger et al., 1989; Dixon et al., 1991; Sakata et al., 1993; Boren et al., 1993; Boren et al., 1994) and the conformation of the nascent apoB polypeptide (Shelness and Thornburg, 1996; Macri and Adeli, 1997b). Whether apoB forms a transmembrane conformation and its translocation is regulated has been subject to considerable controversy in the field. Although it was originally reported that apoB did not contain any classical transmembrane domains (Knott et al., 1986; Yang et al., 1986), many investigators have since reported that regions within apoB may associated with the ER membrane and become exposed to the cytosol (Davis et al., 1990; Thrift et al., 1992; Macri and Adeli, 1997a; Chuck and Lingappa, 1992; McLeod et al., 1996; Liang et al., 1998; Du et al., 1998). Protease protection assays have been used to assess the translocational efficiency of apoB however conflicting results have been reported from numerous studies. For example, Ingram and Shelness (1996) have reported that as much as 80% of newly synthesized apoB100 in HepG2 cells were resistant to trypsin digestion whereas other groups (Chuck et al., 1990; McLeod et al., 1994) have revealed a lower resistant (0-25%) of apoB to trypsin digestion. In rat hepatocytes, apoB48 has been reported to be 100% trypsin-resistant by McLeod et al. (1996) and 78% trypsin-resistant by Rusiñol et al. (1993). On the other hand, Davis et al. (1990) and Verkade et al. (1993)
reported that apoB48 in rat hepatocytes was approximately 30% trypsin-resistant. Conflicting observations in apoB translocational efficiency has also been shown in studies using truncated apoB constructs expressed in various cell lines (Thrift et al., 1992; Plutner et al., 1992; Rusiñol and Vance, 1995). Shelness and coworkers (1994) have reported that apoB50 expressed in COS-1 cells (not expressing MTP activity) was efficiently translocated across the ER membrane and was minimally sensitive to exogenous trypsin. On the other hand, Thrift et al. (1992) reported that the apoB53 construct in Chinese hamster ovary (CHO) fibroblasts cells was found to be associated with the ER membrane and thus sensitive to exogenous trypsin digestion. However, Thrift et al. (1992) found that apoB15 constructs expressed in these cells were capable of efficient translocation and secretion. Wang et al. (1996) observed that transfection of COS-7 cells with MTP catalytic subunit allowed these cells to translocate apoB constructs larger than apoB53. In addition, Rusiñol and Vance (1995) reported that human B28 and B18 expressed in McAR-H7777 cells were 84% and 99% resistant to trypsin digestion, respectively. It is thus apparent that considerable variation exists in the degree of trypsin sensitivity of both endogenous apoB as well as truncated apoB variants.

Differences in experimental conditions used in the isolation of microsomes and the protease protection assays may account for at least some of the variability in reported apoB translocational efficiency and thus in the present study an attempt was made to circumvent some of these variables. A permeabilized cell system was employed to examine the translocational efficiency of both full-length apoB and various apoB constructs. The most beneficial advantage of using a permeabilized cell system is the maintenance of both the morphology and integrity of intracellular organelles (ER and
Golgi) in addition to their accessibility to exogenous trypsin (Plutner et al., 1992). Protease protection assays using a permeabilized cell system has previously been employed in determining the translocational efficiency of newly synthesized apoB polypeptides (Macri and Adeli, 1997a). Hence, similar protease protection assays were conducted in this study on permeabilized McA-RH7777 cells transfected with human apoB cDNAs expressing various sized apoB constructs. Subsequently, a relationship between the length of the nascent apoB polypeptide and its translocational efficiency was explored. To confirm the integrity of the intracellular organelles of the McA-RH7777 cells following permeabilization, transferrin, a secretory protein, was found to be completely resistant to trypsin digestion. These findings were also reported in permeabilized HepG2 cells where control secretory proteins such as α1-antitrypsin, transferrin and albumin were also resistant to trypsin digestion (Macri and Adeli, 1997a). Analogous to the control proteins, the shorter truncated apoB constructs, including hB15 and hB29 in permeabilized McA-RH7777 cells, were also found to be relatively insensitive to exogenous trypsin suggesting minimal exposure to the cytosol. However, a significant increase in trypsin sensitivity was observed for newly synthesized full-length endogenous apoB100. A similar relationship was observed when investigating the trypsin sensitivity of the total steady state mass of apoB. Intracellular mass of the hB100 construct exhibited the greatest sensitivity to trypsin digestion, compared to the mass of shorter apoB variants, which generally showed significantly lower trypsin sensitivities. The results observed in the protease protection assays using a permeabilized cell system were also comparable to the data obtained from protease protection assays using isolated microsomes (Cavallo et al., 1998). These experiments also revealed a correlation between
the length of the apoB polypeptide and its trypsin sensitivity. Overall, various data from protease protection assays in different cell model systems appear to suggest that sequences within the extreme C-terminal domain of the full-length apoB (the region absent in these truncated apoB variants) may be responsible for the increase in trypsin sensitivity of apoB100. This increase in trypsin sensitivity may in turn reflect increased membrane association of nascent apoB and therefore higher cytosolic exposure. In general, a pattern exhibiting an increase in trypsin sensitivity with an increase in apoB polypeptide length was postulated.

ApoB100 consists of three amphipathic α-helix domains and two amphipathic β strand domains. Although the α-helix domains are able to associate with lipids, the β-strand domains are considered the major lipid-associating motif in apoB (Segrest et al., 1994; McLeod et al., 1996). The two β-strand domains, β1 and β2, are located within the 18-43% and 58-85% regions of apoB, respectively (Segrest et al., 1994). It is believed that these β strand domains are responsible for the association of the apoB polypeptide with the ER membrane, thus inhibiting apoB from complete translocation (Davis et al., 1989; Liang et al., 1998; Du et al., 1998;). The C-terminally truncated apoB constructs used in the present study either lacked part or all of the β1 and/or β2 domains. The deletion of part or all of the β domains may be the underlying factor for the differences observed in the trypsin sensitivities of various apoB constructs. Based on the data from the protease protection studies in permeabilized cells, the constructs that did not contain the complete β2 domain (hB15, hB29, hB48, hB72) had the highest resistance to exogenous trypsin whereas hB100 (containing the complete β2 domain) was most sensitive to trypsin digestion. Inefficient translocation of these apoB100 polypeptides
across the ER membrane may have resulted in an increase in accessibility to exogenous trypsin. Recent studies by Liang et al. (1998) clearly demonstrated that the translocational efficiency of chimeric apoB constructs was determined by the presence of lipid binding β sheet domains. These chimeric apoB constructs consisted of the first 13 and 16 % N-terminal amino acids of full-length apoB (containing α globular domains) with and without the addition of the β1 domain of the apoB protein. In the absence of the β1 domain, the chimeric apoB constructs were efficiently translocated across the ER membrane. However the translocational efficiencies of these chimeric apoB constructs were reduced with the addition of the β1 domain (Liang et al., 1998). However, our data appears to suggest that the β1 domain does not directly contribute to the rate of translocation of these apoB constructs. A role may however exist for the α3-domain in the translocational efficiency of apoB considering hB100 was the only construct containing the α3-domain and was the most sensitive to trypsin treatment. However, studies using apoB constructs greater than apoB72 in the presence and absence of the α3-domain are needed to clearly define a role for this C-terminal domain in the translocational efficiency of apoB.

Early studies suggested that incomplete translocation may result in the formation of an apoB pool that is associated with the ER membrane (Davis et al., 1990). In addition, numerous studies have revealed that regions of membrane bound apoB are exposed to the cytosolic face of the ER (Davis et al., 1990; Furukawa et al., 1992; Verkade et al., 1993; Wilkinson et al., 1993; Boren et al., 1993; Rusiñol et al., 1993; Du et al., 1994; Wang et al., 1995; Bonnardeau and Davis, 1995; McLeod et al., 1996; Liang et al., 1998; Du et al., 1998). Du et al. (1998) have reported the presence of at least two
cytosically exposed apoB regions, CC3.4 and B4. These regions include amino acids 690-797 (region CC3.4) and 3221-3240 (region B4), which were identified as strongly hydrophilic domains juxtaposed next to a highly hydrophobic domain (Du et al., 1998). Furthermore, it has also been suggested that these ER membrane apoB are susceptible to degradation, presumably by the cytosolic proteasome (Yeung et al., 1996; Fisher et al., 1997; Benoist and Grand-Perret, 1997; Wu et al., 1997; Chen et al., 1998). The proteasome has been established as one of the main mechanisms involved in the degradation of hepatic apoB (Yeung et al., 1996; Fisher et al., 1997; Benoist and Grand-Perret, 1997; Wu et al., 1997; Chen et al., 1998; Zhou et al., 1998; Liao et al., 1998; Liang et al., 1998; Sakata and Dixon, 1999; Sakata et al., 1999; Cavallo et al., 1999).

Hence, in this study MG132, a proteasomal inhibitor, was used in assessing the intracellular stability of both endogenous and exogenous apoB variants, and a correlation between apoB size and its intracellular stability was also explored. In the presence of MG132, the intracellular stability of most truncated apoB variants (hB15, hB72 and rB48) was slightly increased at the end of the pulse period. However, there was almost a three-fold increase in the amount of endogenous rat apoB100 recovered at 0 h in the presence of the inhibitor. Thus, the data revealed a substantial difference between truncated apoB variants and the full-length apoB100 with respect to their co-translational stability. It is suggested that truncated apoB variants were much less accessible to proteasomal degradation, most likely as a result of their efficient translocation.

It is hypothesized, based on current observations, that the β2 domain in the C-terminal region of the polypeptide may be responsible for incomplete translocation, and moreover co-translational instability of full-length apoB. The β1 domain has also been
shown to be responsible for incomplete translocation of chimeric apoB constructs across the ER membrane which in turn leads to the proteasomal degradation of these constructs (Liang et al., 1998). Thus, there appears to be a correlation between the β domains of the apoB molecule and its susceptibility to proteasomal degradation. For example, apoB100 in HepG2 cells have been shown to be associated with cytosolic Hsp70 (Fisher et al., 1997), a chaperone protein apparently involved in the proteasomal degradation process (Mayer et al., 1998). On the other hand, the shorter apoB15, which does not contain β domains and is efficiently translocated, exhibited minimal if any association with this chaperone protein (Zhou et al., 1995). It is speculated that cytosolic Hsp70 may specifically associate with the cytosolically exposed hydrophobic domains of apoB, such as the β2 domain in the C-terminal region (Fisher et al., 1997; Zhou et al., 1995). An apoB50 construct expressed in CHO cells was also found to efficiently associate with Hsp70 indicating its sensitivity to proteasomal degradation (Zhou et al., 1995). The interaction of apoB50 with Hsp70 may have resulted from the fact that this construct was expressed in a non-hepatic cell line devoid of MTP activity and thus inefficiently translocated across the ER membrane (Thrift et al., 1992). In the present study, expression of a similarly sized construct (hB48) in a hepatic cell line resulted in efficient translocation and thus a lower sensitivity to proteasomal degradation.

As suggested the translocational efficiency and intracellular stability of apoB may be dependent on the activity of MTP (Leiper et al., 1994; Wang et al., 1996; Wu et al., 1996b; Fisher et al., 1997; Zhou et al., 1998). It has been shown that MTP facilitates the association of apoB with lipids, and furthermore, inhibition of this association results in the rapid degradation of apoB by the proteasome (Gordon et al., 1994; Leiper et al.,
1994; Benoist and Grand-Perret, 1997). Moreover, inhibition in MTP activity in HepG2 cells has been shown to block the secretion of apoB (Benoist et al., 1996). In McA-RH7777 cells, MTP was found to participate in the early stages of apoB48 VLDL assembly, however MTP activity was not found to be essential for the later stages of VLDL assembly in which the major bulk of lipids are added to the particle (Gordon et al., 1996). It was also reported in HepG2 cells that the early stages of VLDL assembly are MTP-dependent whereas the later stages are independent of MTP activity (Rustaeus et al., 1998). Bakillah et al. (1998) and Hussain et al. (1997) have demonstrated that the binding sites for MTP was in the first 16% of apoB100 polypeptide. The interactions between MTP and apoB occur between the first 264 residues of apoB and the predicted β-barrel of MTP (residues 22-303) (Mann et al., 1999), as well as between residues 512 and 721 of apoB and residues 517-603 of MTP (Bradbury et al., 1999). It was also recently reported that MTP activity is essential for the efficient translocation of chimeric apoB constructs containing the β1 sheet (Liang et al., 1998). It was suggested however that MTP does not bind the β1 sheet domain directly but instead facilitates the translocation of β1 domain containing constructs (Liang et al., 1998). Hence, it was hypothesized that as the apoB polypeptide increases in size, there is a greater accumulation of lipophilic β sheets in the protein (Segrest et al., 1994), and therefore an increase in the need for MTP activity in the translocation of apoB (Liang et al., 1998). Inhibition and/or reduction of MTP activity has been implicated as the main cause for proteasomal degradation of these inefficiently translocated apoB polypeptides.

Previous studies (Sato et al., 1990; Furukawa et al., 1992; Adeli, 1994) appeared to suggest that following translation and translocation, apoB may also be subject to post-
translational degradation. Here, all truncated apoB variants as well as full-length apoB were found to be unstable with 40-60% newly synthesized apoB degraded over a 2 h chase. Furthermore, the data revealed that all apoB constructs, independent of size, were susceptible to proteasomal degradation, post-translationally. Studies using the proteasomal inhibitor, MG132, revealed that full-length apoB100 was more susceptible to post-translational proteasomal degradation than the shorter constructs. This may be due to a higher degree of ER membrane association of apoB100 and thus a greater accessibility to the cytosolic proteasome. The degradative mechanisms involved in such post-translational instability of truncated apoB constructs remains unclear. It is interesting to note that the post-translational degradation of shorter apoB constructs was partially sensitive to MG132. This is somewhat intriguing since short apoB constructs were found to be efficiently translocated across the ER membrane and thus inaccessible to cytosolic components such as the proteasome (Palmer et al., 1996). However, sensitivity of these constructs to the proteasome inhibitor, MG132, would suggest that the proteasome is in fact involved in the degradation of these efficiently translocated proteins. One possibility that may explain the above findings is the retrograde translocation of luminal ER proteins from the secretory pathway into the cytosol. Several recent studies have shown that retrograde translocation acts as a form of quality control where secretory proteins may be transported back out of the ER and into the cytosol (Wiertz et al., 1996; Hiller et al., 1996; Werner et al., 1996). Once in the cytosol, ubiquitination of these proteins acts as a signal for their destruction via a proteasome mediated process (Wiertz et al., 1996; Hiller et al., 1996; Werner et al., 1996). Thus, it is possible that under certain conditions, such as limited availability of lipids, apoB though fully translocated into the ER, may be
shipped back out of the ER for degradation. Retrograde translocation of apoB has been speculated to occur in HepG2 cells (Chen et al., 1998; Liao et al., 1998; Du et al., 1998; Linnik and Herscovitz, 1998). Chen et al. (1998) have suggested that retrograde translocation may be involved in the degradation of ubiquitinated apoB identified in the translocon of HepG2 cells. In addition, preliminary studies in our laboratory in McA-RH7777 cells expressing human B29 have identified a pool of hB29, which appears in the cytosol early during pulse chase labeling experiments. This pool of cytosolic hB29 appears to be protected from degradation in the presence of MG132. In addition, post-translational degradation of apoB constructs was only partially sensitive to the proteasome inhibitor indicating that other degradative mechanisms, independent of the proteasome, may also modulate post-translational stability of apoB.

7.2 The Degradative Mechanisms Mediating Post-Translational Fragmentation of Apolipoprotein B and the Generation of the 70 kDa Fragment

Several reports have revealed that hepatic apoB is synthesized in excess of that secreted, and that a substantial amount of newly synthesized apoB is degraded intracellularly (Boren et al., 1990; Davis et al., 1990; Sato et al., 1990; Dixon et al., 1991; White et al., 1992; Boren et al., 1993; Dixon and Ginsberg, 1993; Cartwright and Higgins, 1996). Hence, the intracellular mechanisms responsible for degradation of nascent apoB chains have been the subject of intense investigation over the past several years. It has been well established that the proteasome is involved in the co-translational, rapid degradation of apoB in HepG2 cells. This evidence includes the sensitivity of apoB
degradation to various proteasome inhibitors (Yeung et al., 1996; Fisher et al., 1997; Benoist and Grand-Perret, 1997; Chen et al., 1998; Sakata et al., 1999), the detection of ubiquitinated apoB (Zhou et al., 1995; Yeung et al., 1996; Chen et al., 1998; Sakata and Dixon, 1999), the association of ubiquitinated apoB with the Sec61 complex of the translocon (Chen et al., 1998; Mitchell et al., 1998) and the association of apoB with both the cytosolic chaperone protein, Hsp70 (Zhou et al., 1995; Fisher et al., 1997) and ER molecular chaperones (Patel and Grundy, 1996; Chen et al., 1998; Linnik and Herscovitz, 1998). Moreover, recent reports (Mitchell et al., 1998; Liao et al., 1998) have also provided evidence for the involvement of the cytosolic proteasome in the post-translational degradation of apoB. The data in the present study supports the hypothesis that the proteasome may be involved in the post-translational degradation of apoB, however the data also reveals the participation of a non-proteasomal degradative system.

To explore the role of the proteasome in the post-translational degradation of apoB, a permeabilized HepG2 cell system was used in conjunction with the proteasome inhibitor, lactacystin. The data revealed that although lactacystin inhibited the early rapid degradation of apoB, post-translational fragmentation of apoB, which normally results in the generation of specific apoB intermediates, was not inhibited by lactacystin. Furthermore, the results from the proteasome assays indicated that, in contrast to intact cells, permeabilized cells contained no detectable lactacystin-inhibitable protease activity, suggesting the lack of proteasomal activity in these cells. The deficiency in proteasomal activity was determined to be a result of a major loss of both functional and structural 20S proteasomal subunits following permeabilization of these cells. It is important to note that the possibility of some ER-bound proteasome remaining associated with
permeabilized cells could not be excluded. In fact, a recent report by Sakata et al., (1999) identified an association of both α and β-type subunits of the proteasome with the ER membranes in the perinuclear regions of digitonin-permeabilized cells. However, it appeared that the lack of cytosolic factors in these permeabilized cells rendered these residual proteasomal subunits inactive. Furthermore, the absence of proteasomal activity in permeabilized cells and the lack of sensitivity to lactacystin in this study appeared to rule out the possibility that a potential membrane-associated proteasome pool is responsible for the specific fragmentation of apoB.

ApoB degradation, independent of the cytosolic proteasome, has previously been suggested by a number of researchers (Adeli et al., 1997b; Wu et al., 1997; Chen et al., 1998). One candidate for proteasome independent apoB degradation is ER60, an ER resident cysteine protease that also acts as a molecular chaperone (Urade and Kito, 1992). ER60 was initially identified in the ER of rat hepatocytes (Urade and Kito, 1992), and was also found to degrade in vitro the reduced and denatured forms of lysozyme (Otsu et al., 1995). Recently, an ER60 homologue was identified in HepG2 cells and was also shown to be associated with apoB (Adeli et al., 1997a). In this study, an ER60 homologue was also identified within primary hamster hepatocytes. In these cells, it is suggested that this protein may play a similar role in the degradation of apoB. In addition to ER60, ERp72 has also been reported to associate with apoB (Linnik and Herscovitz, 1998). ERp72, like ER60, is a member of the ER resident thioredoxin family of proteins that also includes protein disulfide isomerase, a subunit of MTP (Hirano et al., 1995; Luz and Lennarz, 1996). Although the exact function of ERp72 remains unclear, it has been speculated that ERp72 may exhibit limited PDI-like activity (Rupp et al., 1994).
Moreover, ERp72 has been found to associate with misfolded proteins and is believed to be involved in the ER degradation of these aberrant proteins (Urade et al., 1993). Analogous to ER60, ERp72 also exhibits ALLN-sensitive proteolytic activity (Urade et al., 1993). Thus, it is plausible that in permeabilized cells these ER-resident proteases may participate in the post-translational degradation of apoB within the ER. Furthermore, proteasome-independent apoB degradation may not be limited only to permeabilized cells but instead may also participate in the post-translational degradation of apoB in intact cells. The fact that the degradation of apoB in intact cells was not completely inhibited in the presence of lactacystin indicates that in addition to proteasome-mediated degradation, an alternative degradation system may be involved in the post-translational and post-translocaitional turnover of apoB. Moreover, it appears that this non-proteasome mediated degradation pathway can be unmasked following permeabilization of these cells resulting in the loss of functional cytosolic proteasome.

A major characteristic of apoB degradation in permeabilized HepG2 cells is the accumulation of an N-terminal 70 kDa apoB fragment (Adeli, 1994; Sallach and Adeli, 1995; Adeli et al., 1997b). Here, an attempt was made to explore the potential role of the proteasome in the generation of this 70 kDa apoB fragment. Two potent proteasome inhibitors, lactacystin and clasto-lactacystin β-lactone as well as a more general inhibitor, ALLN, were employed to examine their effects on the accumulation of the 70 kDa fragment. Interestingly, pretreatment with lactacystin and clasto-lactacystin β-lactone caused an increase in the initial pool of radiolabeled apoB that was fragmented in permeabilized cells, resulting in an increased accumulation of the 70 kDa intermediate. This accumulation was likely due to an increase in apoB at the co-translational level as a
result of early proteasome inhibition. In contrast to lactacystin and clasto-lactacystin β-lactone, ALLN completely inhibited the generation of the 70 kDa fragment. ALLN is known not only as a proteasome inhibitor, but also as an inhibitor of calpains and other ER cysteine proteases (Saksi et al., 1990). ALLN is thus not as specific for the proteasome in comparison to lactacystin and clasto-lactacystin β-lactone, the most selective proteasome inhibitors presently known (Rock et al., 1994; Coux et al., 1996; Mellgran, 1997). Thus, these data appear to suggest that apoB fragmentation in permeabilized cells was independent of the proteasome and may instead have been dependent upon other ER cysteine protease(s).

In a recent study, our laboratory further characterize the degradative process operating in permeabilized HepG2 cells by examining the effects of inhibiting MTP on the generation of the 70 kDa fragment (Cavallo et al., 1999). Inhibition of MTP increased the pool of secretion incompetent apoB, and the results established an inverse relationship between the concentration of the MTP inhibitor and the generation of the 70 kDa fragment. This suggested that the pool of apoB accumulated in the presence of the MTP inhibitor was not accessible to the degradation machinery responsible for the generation of the fragment. Instead, it seemed likely that a large percentage of this apoB pool was co-translationally and rapidly degraded by the proteasome (Benoist and Grand-Perret, 1997; Cavallo et al., 1999) thus decreasing the pool of apoB available for the generation of the 70 kDa fragment. On the other hand, treatment with the reducing agent, DTT, resulted in acceleration of the post-translational degradation of apoB in permeabilized HepG2 cells. Previous work (Shelness and Thornburg, 1996; Macri and Adeli, 1997b) has shown that disruption of disulfide bond formation within the apoB
molecule can inhibit the secretion of apoB and induce its intracellular degradation. Wu et al. (1997) also reported the generation of an N-terminal 70 kDa fragment in the ER lumen of intact HepG2 cells by an DTT-sensitive proteolytic system. Although our results revealed a loss in the appearance of the 70 kDa fragment in the presence of DTT, physiological differences between intact and permeabilized cells may account for the observed variations. The observation that apoB degradation in DTT-treated cells occurred without the generation of the 70 kDa apoB fragment suggested that the apoB degradation pathway characteristic to permeabilized cells may have been altered in the presence of DTT.

It has been established that permeabilization of HepG2 cells abolishes extracellular secretion of apoB (Adeli, 1994), and thus the fate of the 70 kDa fragment in permeabilized HepG2 cells remains unknown. However, in this study, an attempt was made to restore the mechanisms of apoB secretion in permeabilized HepG2 cells by supplementing these cells with cytosolic components. As a result, secretion of both full length apoB and turnover of the 70 kDa fragment was induced in permeabilized HepG2 cells following the addition of cytosol (isolated from HepG2 cells) and an ATP generating system. Furthermore, secretion of apoB and the 70 kDa degradation intermediate was enhanced in the presence of MG132, thus suggesting a role for the proteasome in the regulation of the 70 kDa fragment in reconstituted cells. This finding was indicative of a relationship between the cytosolic proteasome and the proteasome-independent system responsible for the generation of the 70 kDa fragment. Sakata and coworkers (1999) have also examined the effects on apoB stability in permeabilized cells following the addition of cytosolic components including an ATP generating system.
They reported that incubation of permeabilized HepG2 cells with rabbit reticulocyte lysate, as a source of proteasomal components including the ubiquitin-conjugating enzymes, and an ATP generating system resulted in the rapid proteasomal degradation of full length apoB. Furthermore, the proteasome inhibitor, clasto-lactacystin β-lactone, and the ubiquitin K48R mutant protein reduced the proteasomal degradation of apoB, in these reconstituted permeabilized cells (Sakata et al., 1999).

There is precedence for the involvement of proteases functioning in conjunction with the proteasome in the regulated degradation of proteins in eukaryotic cells (Jensen et al., 1995). The data presented here suggest that in intact cells, if newly synthesized apoB chains are rescued from early, rapid proteasome-mediated degradation, they are still sensitive to the proteasome (based on data in intact cells and reconstituted permeabilized cells) and other non-proteasomal degradative system(s) (based on data in permeabilized and intact cells), post-translationally. Presumably there may be an association between proteasomal and non-proteasomal degradation systems. This is particularly apparent from the observation that inhibition of co-translational proteasomal degradation by pretreatment with lactacystin increased the abundance of the 70 kDa fragment apparently by providing more substrate for the post-translational non-proteasome mediated fragmentation process. Therefore, it is apparent that the interrelationship between proteasomal and non-proteasomal degradative systems that acts to destabilize the secretion incompetent apoB is complex and needs to be further elucidated.
7.3 The Potential Role of Retrograde Translocation in the Post-translational Proteasomal Degradation of Apolipoprotein B

Recent studies have suggested that the cytosolic proteasome may be involved in the post-translational degradation of apoB (Chen et al., 1998; Liao et al., 1998; Cavallo et al., 1998; Mitchell et al., 1998; Linnik and Herscovitz, 1998; Sakata and Dixon, 1999; Cavallo et al., 1999; Sakata et al., 1999). Our studies using truncated apoB variants in McA-RH7777 cells revealed that following a 2 h chase all apoB constructs, regardless of size and translocational efficiency were sensitive to MG132. Interestingly, post-translational degradation of full-length apoB100 in intact HepG2 cells also revealed the involvement of the proteasome. It remains unclear how the cytosolic proteasome mediates the degradation of apoB, however recent reports have speculated that retrograde translocation of apoB from the ER to the cytosol may be a mechanism that delivers these apoB molecules to the proteasome (Liao et al., 1998; Chen et al., 1998; Sakata et al., 1999).

In the present study, an attempt was made to further characterize the mechanisms surrounding the proteasomal degradation of apoB. Considering that the proteasome is a resident of the cytoplasm, apoB from two subcellular fractions, cytosol and crude microsomes (containing the ER) of HepG2 cells were examined. Newly synthesized apoB molecules were identified within both the microsomal and cytosolic fractions of HepG2 cells. Although the percent distribution of newly synthesized apoB in the cytosol was relatively low compared to the microsomes (less than 10%), the abundance of cytosolic apoB increased significantly over a 2 h chase (greater than 25 %). Moreover, this increase in cytosolic apoB coincided with a decrease in microsomal apoB. The post-
translational shift in percent distribution of apoB between cytosolic and microsomal fractions suggested that the accumulation of cytosolic apoB following translation may have resulted from the transport of apoB from the ER to the cytosol. Recently, Mitchell et al. (1998) reported that over a 60 min chase, there was no significant accumulation of apoB within the cytosol of HepG2 cells. However, in the present study the newly synthesized apoB molecules were chased for 2 h, and an accumulation of cytosolic apoB was clearly evident. Furthermore, the accumulation of apoB in the cytosol was greatly enhanced in the presence of the proteasomal inhibitor, MG132, therefore suggesting a role for the proteasome in the degradation of cytosolic apoB.

To further characterize this pool of MG132-sensitive, cytosolic apoB, immunoprecipitation with ubiquitin antibodies revealed that in the presence of MG132 a portion of this cytosolic apoB is ubiquitinated. The fact that the cytosolic pool of apoB contained ubiquitin modifications further supports the involvement of the proteasome in the degradation of cytosolic apoB. In the absence of the proteasome inhibitor, this pool of ubiquitinated apoB was not detectable, likely due to its rapid degradation by the proteasome. Ubiquitinated apoB has been previously identified in whole cell lysates (Yeung et al., 1996; Fisher et al., 1997, Benoist and Grand-Perret, 1997; Zhou et al., 1998; Liao et al., 1998; Mitchell et al., 1998; Chen et al., 1998), however, this is the first study to identify ubiquitinated apoB molecules from the cytosolic fraction of HepG2 cells. Ubiquitination is a primary method of ‘tagging’ misfolded or aberrant proteins for proteasomal degradation (Ciechanover, 1994; Weissman, 1997). Moreover, the enzymes involved in the ubiquitination process are localized within the cytoplasm (Hochstrasser, 1996), which further supports the hypothesis that apoB has cytosolic exposure.
Microsomal apoB was also found to be ubiquitinated, and this pool of ubiquitinated apoB appeared to increase in the presence of MG132. Hence, it is likely that ubiquitinated microsomal apoB are ER membrane bound polypeptides that are cytosolically exposed, and thus susceptible to proteasomal degradation. A recent report by Du et al. (1998) revealed that at least two regions of the apoB polypeptide that are exposed to the cytosol. Mitchell et al. (1998) and Chen et al. (1998) have revealed ubiquitinated apoB associated with the Sec61 complex of the translocon. A reduction in the interaction between ubiquitinated apoB and the Sec61 complex appeared to decrease the proteasomal degradation of these apoB molecules. Thus, it is plausible that in this study ubiquitinated apoB detected in the microsomal fractions in the present study may be accessible to the cytosolic proteasome and thus susceptible to proteasomal degradation.

Upon further examination, it was surprising to note that over a 2 h chase period (post-translationally) the pool of cytosolic ubiquitinated apoB was reduced despite the fact that the total pool of cytosolic apoB increased during this time period. The loss in ubiquitinated apoB however was not a result of proteasomal degradation, since the presence of MG132 would have abolished any potential proteasomal activity. Furthermore, any degradation of ubiquitinated apoB by the proteasome would have also resulted in a loss of total cytosolic apoB. Thus, it is likely that due to the inhibition of the proteasome, ubiquitinated apoB that was originally destined for proteasomal degradation was de-ubiquitinated and possibly diverted to an alternative degradative pathway. In examining microsomal fraction, there was also a decrease in ubiquitinated apoB over a 2 h chase in the presence of MG132, however, the loss in ubiquitinated microsomal apoB was concomitant with a decrease in total microsomal apoB. The reduction in microsomal
apoB was probably due to a combination of apoB secretion, apoB degradation within the ER lumen and/or transport of apoB molecules out of the ER compartment. The decrease in ubiquitinated microsomal apoB, however, was likely due to the de-ubiquitination of this apoB pool and degradation via a non-proteasome mediated system. De-ubiquitination of apoB molecules in HepG2 cells has been reported both in vitro (Sakata and Dixon, 1999) and in vivo (Mitchell et al., 1998). Sakata and Dixon (1999) demonstrated, in vitro, that de-ubiquitination of apoB was a prerequisite for proteasomal degradation. However, Mitchell et al. (1998) revealed, in vivo, that in the presence of oleic acid and lactacystin, ubiquitinated apoB molecules that were associated with the ER membrane were de-ubiquitinated and recruited into the secretory pathway. Thus, it appears that de-ubiquitination of apoB may either lead to apoB proteasomal degradation or, under favourable conditions, route apoB into the secretory pathway thereby rescuing it from degradation.

It is hypothesized that the pool of apoB that accumulates in the cytosol is characteristically secretion-incompetent, likely due to a lack of intracellular lipids required for the assembly of lipoprotein particles. To examine this further, HepG2 cells in the presence of the proteasomal inhibitor, MG132, were treated with brefeldin A. Brefeldin A is an inhibitor of protein secretion by causing the Golgi to collapses back into the ER thus blocking the transport of proteins from the ER to the Golgi. In turn, this does not allow for the secretion of apoB-containing lipoproteins. In presence of brefeldin A the distribution of apoB between the cytosol and the microsomal fractions did not initially (0 h) change in comparison to control cells (cells treated only with MG132). This suggested that brefeldin A did not affect the early stages of apoB biogenesis nor did
brefeldin A appear to affect the early subcellular distribution of apoB. Brefeldin A did however appear to reduce the accumulation of both cytosolic and microsomal apoB over a 2 h chase. Since MG132 would have abolished all proteasomal degradation then the decrease in cytosolic apoB accumulation may have been attributed to the presence of brefeldin A. Considering that brefeldin A blocks the extracellular transport of proteins through the secretory pathway, it is plausible that brefeldin A may have had an inhibitory effect on the transport of proteins from the secretory pathway into the cytosol. As a result, apoB may have been confined to the ER and eventually degraded by ER resident proteases. In fact, the results revealed an increase in the rate of ER-associated apoB degradation in the presence of brefeldin A. Stimulation of apoB degradation in the presence of brefeldin A has been previously reported (Sato et al., 1990; Furukawa et al., 1992; Adeli, 1994), however, here it is suggested that brefeldin A may also be functioning to inhibit the transport of apoB into the cytosol.

The proper folding and intracellular transport of some proteins appears to require N-linked glycosylation of these proteins (reviewed in Helenius, 1994). Glycosylation is a post-translational modification of proteins that occurs in the lumen of the ER and normally signals the exposure of these proteins to the lumen of the ER where enzymes for protein glycosylation reside. To confirm whether or not cytosolic apoB was glycosylated, endoglycosidase H assays were performed. As a positive control, endoglycosidase H assays were also performed on microsomal apoB, and the results showed that both cytosolic and microsomal apoB were sensitive to endoglycosidase H treatments. The effect of endoglycosidase H treatment was more obvious on cytosolic apoB accumulated over 2 h since the amount of cytosolic apoB accumulated at 0 time
was relatively low. Nonetheless, the data clearly suggested that apoB accumulated in the cytosol during the chase was at some point exposed to the lumen of the ER.

Inhibition of glycosylation results in the improper folding of secretory proteins thus rendering them secretion-incompetent. Therefore, it was suspected that inhibition of apoB glycosylation by tunicamycin would result in a change in the subcellular distribution of apoB molecules within HepG2 cells. Partial inhibition of apoB glycosylation resulted in an increase in cytosolic apoB during the pulse however, over a 2 h chase the amount of apoB present in the cytosol in tunicamycin treated cells was consistent with that found in control cells. Tunicamycin treatment in MG132 treated cells however did not appear to alter the distribution of apoB in both the cytosol and microsomes in comparison to cells treated only with MG132. A recent experiment from our laboratory also revealed that complete inhibition of apoB glycosylation in the presence of MG132 did not significantly change the 0 and 2 h apoB distribution pattern between the cytosol and microsomal fractions (unpublished data).

There are several possible explanations for the effect of tunicamycin on apoB subcellular distribution. One possibility is that the translocation of apoB may be altered in the presence of tunicamycin. As a result, unglycosylated apoB may be directly shunted to the cytosol during its translation instead of being translocated into the ER lumen. This possibility would at least account for the increase in cytosolic apoB at 0 h in the presence of tunicamycin. However, previous data in permeabilized HepG2 cells have shown that apoB translocation was unaffected by the inhibition of N-linked glycosylation (Macri and Adeli, 1997b). Moreover, the production and assembly of apoB-containing lipoproteins was also found to be unaffected by the inhibition of apoB glycosylation (Struck et al.,
Tunicamycin however was shown to decrease the amount of intracellular apoB, most likely as a result of apoB degradation and not inhibition of protein synthesis (Struck et al., 1978; Adeli, 1994; Marci and Adeli, 1997b). Thus, a second possible scenario is that inhibition of apoB glycosylation by tunicamycin may promote the intracellular degradation of apoB. Glycosylated apoB has been shown to interact with calnexin (Ou et al., 1993; Chen et al., 1998), which in turn promotes the proper folding and assembly of apoB. In a study where the proper trimming of glycosylated apoB molecules was inhibited, ubiquitination and proteasomal degradation of apoB was significantly increased (Chen et al., 1998). Inhibition of calnexin-apoB interactions has been shown to enhance the association of apoB with the Sec61 complex of the translocon which in turn promotes apoB degradation by the proteasome (Liao et al., 1998; Chen et al., 1998). More interestingly, calnexin has recently been shown to bind to ubiquitinated apoB molecules thought to be cytosolically exposed (Liao et al., 1998), and tunicamycin treatment of HepG2 cells completely abolished this association suggesting that this pool of ubiquitinated apoB was also glycosylated (Liao et al., 1998). Inhibition of the interaction between calnexin and apoB also appeared to stimulate the association of ubiquitinated apoB with the Sec61 complex of the translocon (Chen et al., 1998). It is possible that in the presence of tunicamycin, the interaction between apoB and calnexin is prevented (Liao et al., 1998), therefore promoting both the ubiquitination and proteasomal degradation of apoB. Tunicamycin was also shown to alter the degradation pattern of apoB in permeabilized HepG2 cells (Macri and Adeli, 1997b), however this process was found to be largely ALLN-insensitive. Thus, this may suggest that degradative pathways independent of the proteasome are also involved in the turnover of unglycosylated apoB.
It should be noted that the possibilities outlined here are mere speculations and conclusive evidence requires more extensive experimentation. Research in our laboratory is continuing to explore the role of N-linked glycosylation on intracellular apoB distribution and stability.

The relationship between calnexin and proteasomal degradation does not appear to be limited to apoB. In fact, a correlation between the association of nascent polypeptides with calnexin and proteasomal degradation has been reported for several proteins such as the nicotinic acetylcholine receptors (Chang et al., 1997), the major histocompatibility complex class I and II (Romagnoli and Germain, 1995; Vassilakos et al., 1996), the influenza hemagglutinin (Hebert et al., 1996), and the subunits of the T-cell receptors (Kearse et al., 1994). Besides calnexin, other ER resident molecular chaperone proteins have been identified in association with newly synthesized apoB. Such chaperone proteins include ERp72 (Chen et al., 1998), ER60 (Adeli et al., 1997a), BiP (Ou et al., 1993; Chen et al., 1998), Hsp70 (Zhou et al., 1995; Fisher et al., 1997), calreticulin (Chen et al., 1998), GRP94 (Chen et al., 1998), GRP78 (Chen et al., 1998) and PDI (Wu et al., 1996b). It is hypothesized that several of these molecular chaperones act in concert to facilitate either the proper folding and assembly of apoB into secretion competent lipoprotein particles and/or participate in the degradation of misfolded, lipid-poor apoB (Wu et al., 1996b; Fisher et al., 1997; Chen et al., 1998; Linnik and Herscovitz, 1998). Furthermore, it is speculated that the interactions of apoB with the molecular chaperones may also facilitate the transport of apoB into the cytosol via retrograde translocation thereby making these apoB molecules accessible to the cytosolic proteasome.
Retrograde translocation is a process identified in both mammalian and yeast systems in which secretory proteins are transported from the ER back to the cytosol (Wiertz et al., 1996; Brodsky and McCracken, 1997; Sommer and Wolf, 1997; Pilon et al., 1997; Plember et al., 1997; Xiong et al., 1999). It has been revealed that misfolded proteins are transported from the ER back to the cytosol whereby they are ubiquitinated and degraded by the cytosolic proteasome (Wiertz et al., 1996; Pilon et al., 1997; Plember et al., 1997). In some cases, the proteins undergo complete retrograde translocation and become soluble, cytosolic proteins that are eventually degraded. These proteins include the major histocompatibility complex class I heavy chains (Wiertz et al., 1996; Hughes et al., 1997) and the unglycosylated prepro-α factor (McCraek and Brodsky, 1996). On the other hand, other proteins such as the mutant form of the carboxypeptidase Y and unassembled T cell receptor α subunits are not completely retrograde translocated into the cytosol, and thus maintain an association with the cytoplasmic face of ER membrane (Hiller et al., 1996; Yu et al., 1997). In addition, there are polytopic ER proteins that are also degraded by the cytosolic proteasome, and these proteins include HMG CoA reductase (Edwards et al., 1983; Roitelman and Simoni, 1992; Hampton et al., 1996), the cystic fibrosis transmembrane conductance regulator (Lukacs et al., 1994; Jensen et al., 1995; Qu et al., 1997; Zhang et al., 1998) and mutant forms of the yeast Sec61p protein (Biederer et al., 1996).

Retrograde translocation of newly synthesized apoB molecules from the ER to the cytosol has been suggested in a number of reports (Liao et al., 1998; Cavallo et al., 1998; Chen et al., 1998; Sakata et al., 1999). This process has been suggested to be a potential mechanism in the delivery of secretion-incompetent apoB molecules to the cytosolic
proteasome. Liao et al. (1998) suggested that following translation and translocation into
the ER, apoB may be targeted for proteasome-mediated degradation via retrograde
translocation from the lumen of the ER to the cytosol. Mitchell et al. (1998), on the other
hand, argued against complete retrograde translocation of apoB from the lumen of the ER
to the cytosol. Instead they revealed that following translocation, apoB may be
ubiquitinated and remains associated with the translocon whereby it becomes susceptible
to proteolytic degradation by the cytosolic proteasome (Mitchell et al., 1998). Although
complete retrograde translocation of apoB is a controversial issue our data supports the
hypothesis that apoB may be translocated from the ER to the lumen. The results revealed
that both glycosylated and ubiquitinated apoB are present in the cytosol of HepG2 cells,
and that this pool of apoB is sensitive to the cytosolic proteasome. Furthermore, our data
revealing the post-translational (2 h) accumulation of ubiquitinated and glycosylated
apoB in the cytosol in HepG2 cells would suggest that this pool of cytosolic apoB was
transported from the ER via retrograde translocation. It is also possible that following
translocation into the cytosol apoB may remain loosely associated with the ER
membrane, and due to the conditions of the experiment the interactions between cytosolic
apoB and the ER membrane may be disrupted. Thus, our data at this point cannot state
with certainty whether or not apoB is partially or completely translocated back into the
cytosol.

Although, the precise mechanism(s) for apoB degradation by the cytosolic
proteasome currently remains unclear, Figure 7.1 illustrates a model based on the data
presented here and reports from other laboratories, for the potential mechanisms involved
in this event. Figure 7.1A shows the synthesis of apoB and assembly of apoB-containing
lipoproteins in the ER under ideal conditions where the availability of lipids is unlimited. Under these conditions, apoB is efficiently translocated across the ER membrane, glycosylated and readily becomes associated with intracellular lipids such as cholesterol esters and triglycerides. As a result, a secretion-competent apoB-containing lipoprotein particle is assembled and secreted. However, as currently and previously shown, a significant amount of apoB is intracellularly degraded, and this process is known to involve the cytosolic proteasome. Under conditions where lipid availability is limited and/or the transport of lipids to the nascent apoB molecule is inhibited, newly synthesized apoB polypeptides are inefficiently translocated, forming a transmembrane orientation involving the Sec61 complex of the translocational channel (Figure 7.1B) (Mitchell et al., 1998). Thus as apoB is being synthesized and translocated into the ER it is glycosylated, ubiquitinated and may be associated with molecular chaperone proteins. It is speculated that this pool of ER-membrane associated apoB may undergo early, rapid degradation of apoB by the proteasome. However, in the presence of sufficient amounts of intracellular lipids apoB may be rescued from proteasomal degradation and routed towards the lipoprotein assembly pathway, thus resulting in the production of secretion-competent apoB-containing lipoproteins. The proteasome has also been implicated in the post-translational turnover of apoB. Figure 7.1C attempts to illustrate the involvement of the proteasome in the post-translational degradation of apoB. This model suggests that in the event of limited intracellular lipids, apoB may become associated with the ER membrane possibly through interactions with the β sheet domains of the apoB polypeptide. This ER membrane-associated apoB is glycosylated and ubiquitinated and may remain associated with ER-membrane until intracellular lipids become readily available. In the event that
Figure 7.1 A model depicting the potential mechanisms of apolipoprotein B degradation by the cytosolic proteasome. A. Under conditions of unlimited lipid availability, apoB is efficiently translocated across the ER membrane and assembles into a secretion-competent apoB-containing lipoprotein particle. B. In the event of limited amounts of lipid, apoB remains associated with the ER membrane and is degraded by the cytosolic proteasome during its synthesis. However, if lipids become available, then apoB may be rescued from degradation and routed towards the lipoprotein assembly pathway. C. Following complete apoB mRNA translation, apoB may still be degraded by the cytosolic proteasome. Degradation may occur near the ER while apoB remains associated with the ER membrane. Alternatively, apoB may be completely retrograde translocated from the ER to the cytosol where it is degraded by the cytosolic proteasome. As well, apoB may be routed towards the ER lumen and eventually degraded by a non-proteasome mediated degradation system.
FIGURE 7.1
lipids do not become accessible to the nascent apoB polypeptide, the protein is ubiquitinated on the regions exposed to the cytosolic face of the ER. As well, Hsp70 may associate with ER-membrane apoB, which in turn may facilitate apoB proteasomal degradation. Hence, this pool of membrane-associated apoB is likely transported from the ER to the cytosol. It has been suggested that this process may involve retrograde translocation. Although unclear, apoB may be transported from the ER to the cytosol through the translocon, possibly through the interactions of apoB with the Sec61 complex and with the aid of molecular chaperone proteins. Once transported into the cytosol, apoB may be de-glycosylated and ultimately degraded by the proteasome. It is not clear however whether apoB is completely translocated into the cytosol or remains loosely associated with the ER membrane and degraded by the proteasome. In addition, it should be noted that ER associated apoB may also be degraded via a non-proteasome mediated pathway, probably localized within the lumen of the ER or other secretory compartments. This degradation pathway likely involves proteases localized within the ER such as ER60. It is hypothesized that proteasomal and non-proteasomal degradation systems function co-operatively to maintain intracellular apoB homeostasis.

7.4 Summary and Future Perspectives

Hepatic secretion of apoB-containing lipoproteins is a complex process involving the interaction of newly synthesized apoB with a number of components including lipoprotein lipids and many other cellular factors and processes. The objective of this study was to provide further insight into the possible regulatory factors influencing apoB intracellular translocation and stability. Initially, the translocation of
apoB was examined and an inverse relationship between the length of nascent apoB polypeptide and its translocational efficiency was demonstrated. These studies suggested that specific β sheet domains within the apoB polypeptide were likely responsible for the inefficient translocation of full-length apoB100. This study was the first to use a permeabilized cell system concomitant with various sized apoB constructs in an attempt to provide insight into the structural elements influencing apoB translocation. Further investigation into the interactions between the specific apoB domains and the ER membrane are required in order to clearly elucidate the translocation process of nascent apoB polypeptides and factors that regulate its efficiency.

Inefficient translocation appears to result in the intracellular instability and ultimate degradation of nascent apoB polypeptides. Degradation of apoB has been established to occur in two stages: early, rapid degradation and slower post-translational degradation. The cytosolic proteasome has been implicated as the main proteolytic system involved in the early, rapid degradation of apoB. In this study, a correlation was established between the size of the nascent apoB polypeptide and its susceptibility to proteasomal degradation. As well, the proteasome was also shown to play a role in the post-translational degradation of apoB regardless of its size. Using a permeabilized cell system, it was also demonstrated that post-translational degradation of apoB and the generation of specific apoB intermediates occurred independent of the cytosolic proteasome. Thus, an alternative non-proteasome mediated degradation system was also shown to be involved in the degradation of apoB. Future studies will involve exploring this non-proteasome mediated degradation pathway in an attempt to uncover the
protease(s) and influencing factors, such as ER resident chaperone proteins, participating in the degradation of apoB and generation of specific apoB intermediates.

The potential role of the proteasome in the post-translational degradation of apoB is currently being examined by many researchers. Recently, it has been suggested that retrograde translocation of apoB from the ER to the cytosol may be required for the proteasomal degradation of luminal apoB. This study was the first to reveal the presence of apoB in the cytosol of HepG2 cells. Interestingly, these apoB molecules were found to be ubiquitinated and sensitive to a proteasomal inhibitor. Furthermore, cytosolic apoB was also found to contain carbohydrate modifications providing evidence for their exposure to the ER lumen. Future studies are needed to unravel the mechanisms by which apoB becomes exposed and degraded by the proteasome. It is likely that retrograde translocation of apoB from the ER to the cytosol is required for its disposal. This study has provided some direct evidence to support the potential involvement of retrograde translocation in the regulation of intracellular apoB biogenesis. Future studies on the role of glycosylation, phosphorylation and molecular chaperone proteins will provide further insight into the mechanisms controlling apoB degradation. Such studies will benefit from the in vitro reconstitution of apoB retrograde translocation in permeabilized cells. A better understanding of the translocational and degradative mechanisms can potentially lead to the development of pharmaceutical measures that can be used to beneficially alter the assembly and secretion of atherogenic lipoproteins from the human liver.

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