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AN *IN VIVO* APPROACH TO THE STUDY OF APOLIPOPROTEIN A-I STRUCTURE-FUNCTION RELATIONSHIPS

by

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A thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of

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ABSTRACT

An *In Vivo* Approach to the Study of Apolipoprotein A-I Structure-Function Relationships

By Daniel Charles McManus

Apolipoprotein A-I (apoA-I) is a non-glycosylated 243 amino acid (aa) circulating plasma protein and the major constituent of high density lipoproteins (HDL). To study apoA-I in a physiologically relevant *in vivo* model, recombinant adenoviruses carrying cDNAs encoding for native human apoA-I (hapoA-I) and different apoA-I mutations were created. This is the first reported use of recombinant adenoviruses to study structure-function relationships of human apoA-I. In the first study, two functional domains within the central region of the protein (aa 100-186) were identified. The results establish that helix 6 (aa 144-165) is primarily responsible for activation of the enzyme lecithin:cholesterol acyltransferase (LCAT) while the more N-terminal half of the central domain, specifically the class Y helix 4 (aa 100-121), is important for lipid binding and/or stabilizing apoA-I on the surface of lipoproteins.

A recombinant adenovirus was also produced encoding for a naturally occurring apoA-I point mutation with a Leu → Arg substitution at aa 159. This apoA-I variant, known as apoA-I Finland (apoA-I_{FIN}), causes an unexpected 80% reduction in HDL cholesterol (HDL-C) and apoA-I concentrations in heterozygous carriers. A combined *in vitro*, *in vivo* and *ex vivo* study demonstrated that this mutant has impaired activation of LCAT, is susceptible to proteolysis and has a decreased rate of secretion from primary hepatocytes. These dysfunctions appear to be responsible for the dominantly inherited hypoalphalipoproteinemia in heterozygous carriers of the apoA-I_{FIN} mutation.

Lastly, secretion of nascent HDL was monitored from primary hepatocytes. The recombinant adenovirus expressing hapoA-I was used to infect apoA-I deficient murine

hepatocytes. In contrast to some previous studies, a significant pool of apoA-I was secreted with phospholipids (PL) and was heterogeneous in size. The role of this nascent HDL pool in determining plasma HDL-C concentrations is not known nor is the importance of other proteins in this process. The recent discovery of a major role for ATP binding cassette transporter A1 (ABCA1) in phospholipid (PL) and cholesterol efflux to apoA-I has led to the speculation that this protein may also shuttle lipids onto newly secreted apoA-I. The model described here will be useful to address this possibility.

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I could not have reached this point in my research career without the support of my parents, Murray and Barb McManus. Their love and guidance throughout my life is beyond anything I could have possibly hoped for. This thesis is dedicated to their many sacrifices.

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ABBREVIATIONS

1. **apoA-I**: apolipoprotein A-I
2. **aa**: amino acid
3. **HDL**: high density lipoprotein(s)
4. **hapoA-I**: native human apoA-I
5. **LCAT**: lecithin:cholesterol acyltransferase
6. **apoA-I_{FIN}**: apoA-I Finland
7. **HDL-C**: high density lipoprotein cholesterol
8. **mapoA-I**: murine apoA-I
9. **ABCA1**: ATP-binding cassette transporter A1
10. **CHD**: coronary heart disease
11. **LDL**: low density lipoprotein(s)
12. **LDL-C**: LDL cholesterol
13. **EC(s)**: endothelial cell(s)
14. **NO•**: nitric oxide
15. **SMC(s)**: smooth muscle cell(s)
16. **oxLDL**: oxidized LDL
17. **MMP(s)**: matrix metalloproteinases
18. **TF**: tissue factor
19. **PL**: phospholipids
20. **FC**: free cholesterol
21. **CE**: cholesteryl esters
22. **TG**: triglycerides
23. **VLDL**: very low density lipoprotein(s)
24. **IDL**: intermediate density lipoprotein(s)
25. **apoB48**: the N-terminal 48% of mature apolipoprotein B100
26. **ER**: endoplasmic reticulum
27. **apoA-II**: apolipoprotein A-II
28. **apoA-IV**: apolipoprotein A-IV
29. **apoB100**: apolipoprotein B100
30. **apoE**: apolipoprotein E
31. **apoC-I**: apolipoprotein C-I
32. **apoC-II**: apolipoprotein C-II
33. **apoC-III**: apolipoprotein C-III
34. **LPL**: lipoprotein lipase
35. **HSPG(s)**: heparan sulphate proteoglycan(s)
36. **LRP**: LDL receptor related protein
37. **MTP**: microsomal triglyceride transfer protein
38. **HL**: hepatic lipase
39. **FH**: familial hypercholesterolemia
40. **AGEs**: advanced glycation end-products
41. **SRA-I/II**: scavenger receptor class A type I, type II
42. **LpA-I**: lipoproteins containing apoA-I and no apoA-II
43. **LpA-I/A-II**: lipoproteins containing apoA-I and apoA-II
44. **M_r**: molecular mass (Da or kDa)
45. **PON1**: paraoxonase-1
46. **FHD**: familial HDL deficiency

47. **PPAR**: peroxisome proliferator-activated receptor
48. **PLTP**: phospholipid transfer protein
49. **CETP**: cholesteryl ester transfer protein
50. **BPI**: bactericidal permeability-increasing protein
51. **Lp2A-I**: reconstituted lipoproteins containing two molecules of apoA-I
52. **SDS**: sodium dodecyl sulphate
53. **FLD**: familial LCAT deficiency
54. **FED**: fish eye disease
55. **CD**: circular dichroism
56. **SR-BI**: scavenger receptor class B, type I
57. **TC**: total cholesterol
58. **HNF-4**: hepatocyte nuclear factor-4
59. **HRE**: hormone response element
60. **RXR**: retinoid X receptor
61. **GdnHCl**: guanidine hydrochloride
62. **DMPC**: dimyristoylphosphatidylcholine
63. **MLVs**: multilamellar vesicles
64. **Ad5**: recombinant adenovirus serotype 5
65. **ppapoA-I**: prepro apoA-I
66. **CMV**: cytomegalovirus
67. **SV40**: simian virus 40
68. **EMEM**: Earle's Minimal Essential Medium
69. **pfu**: plaque forming units
70. **luc**: firefly luciferase
71. **rec.apoA-I_{FIN}**: recombinant apoA-I_{FIN}
72. **rec.hapoA-I**: recombinant hapoA-I
73. **Δ4-5A-I**: apoA-I with deletion of aa 100-143
74. **Δ5-6A-I**: apoA-I with deletion of aa 122-165
75. **Δ6-7A-I**: apoA-I with deletion of aa 144-186
76. **FBS**: fetal bovine serum
77. **SDS-PAGE**: SDS polyacrylamide gel electrophoresis
78. **HRP**: horseradish peroxidase
79. **PAGGE**: polyacrylamide gradient gel electrophoresis
80. **U**: unit(s)
81. **moi**: multiplicity of infection
82. **DMEM**: Dulbecco's Minimal Essential Medium
83. **POPC**: 1-palmitoyl-2-oleoyl-phosphatidylcholine
84. **BSA**: bovine serum albumin
85. **Lp2A-I_{WT}**: reconstituted lipoproteins containing two molecules of hapoA-I
86. **Lp2A-I_{FIN}**: reconstituted lipoproteins containing two molecules of apoA-I_{FIN}
87. **Lp2A-$I_{WT/FIN}$**: reconstituted lipoproteins prepared with equimolar amounts of hapoA-I and apoA-I_{FIN}
88. **appK_m**: apparent K_m
89. **SE**: standard error of the mean
90. **FER**: fractional cholesterol esterification rate
91. **FHA**: familial hypoalphalipoproteinemia
92. **HuA-I Tg**: human apoA-I transgenic

CHAPTER 1 – INTRODUCTION

There is an intimate but yet complex relationship between the plasma concentrations of lipid and protein complexes known as lipoproteins and atherosclerosis-related diseases, particularly coronary heart disease (CHD). On one hand, high concentrations of low density lipoproteins (LDL) are positively correlated with the incidence of CHD while a second lipoprotein class, high density lipoproteins (HDL) are inversely correlated with CHD. For these reasons, HDL and LDL cholesterol (HDL-C and LDL-C) are known as the good and bad cholesterol, respectively.

In the introduction, the atherosclerosis disease process is discussed with respect to lesion initiation, progression, and plaque rupture. Lipoproteins play an important role in atherosclerosis and both genetic and environmental factors influence the concentrations of circulating lipoproteins. The metabolism of the triglyceride rich lipoproteins and the role of LDL in atherosclerosis are addressed. However, emphasis is placed on the factors that regulate the metabolism of HDL and in particular the role of the major structural protein apolipoprotein A-I (apoA-I) in the formation of these anti-atherogenic lipoproteins. Structure-function relationships of apoA-I have been studied extensively but there are many unresolved issues regarding the importance of different regions or domains of this protein in the metabolism of HDL. It is this latter point and specifically the lack of an informative *in vivo* model to study structure-function relationships of apoA-I that is the basis of this thesis.

1.1 - Atherosclerosis: A Disease Process of Lipid Accumulation and Inflammation

1.1.1 - Overview

Atherosclerosis is a disease process and a form of arteriosclerosis (general term applied to the narrowing and hardening of arteries) (1) that results from the accumulation of lipids and

fibrous elements in the walls of large arteries (2), such as the aorta, epicardial coronary, femoral, and carotid arteries (3). In Western societies, atherosclerosis is the principal cause of myocardial infarction (heart attack), stroke, and peripheral vascular disease, accounting for nearly half of all mortalities (4). This has prompted extensive studies of the factors responsible for and the mechanisms underlying the development of atherosclerosis. Injury to the single layer of endothelial cells (ECs) lining the arterial wall is an early step in the development of atherosclerosis. Lipids, primarily in the form of LDL, accumulate at these sites of injury and infiltrate into the underlying intima. This leads to lipoprotein modifications including oxidation, formation of lipid-laden macrophages (foam cells), chronic inflammation, smooth muscle cell proliferation and a diverse number of biochemical changes that all contribute to the pathogenesis of atherosclerosis (5). Acute thrombosis following rupture of a complicated atherosclerotic plaque (section 1.1.4) accounts for 98% of all myocardial infarctions and strokes (6).

1.1.2 - Blood Flow Hemodynamics and Atherosclerosis

Atherosclerotic lesions (section 1.1.3) form predominantly at sites of bifurcations (branch points) or curvatures in the large arteries. Interestingly, these regions also co-localize with areas of the arterial vasculature under low and/or non-uniform laminar shear stress (disturbed blood flow) (7). In contrast, regions of the arterial wall exposed to uniform shear stress (unidirectional blood flow) are resistant to the development of atherosclerosis. The reasons for this are poorly understood at the molecular level, although important clues are beginning to become unraveled and it appears that the variations in blood flow hemodynamics affect the ECs lining the arterial wall differently.

The ability to mimic the arterial hemodynamic forces both *in vivo* (8) and in cell culture settings (9,10) has shed light on the effects of steady laminar versus non-uniform shear stress on ECs. ECs exposed to steady laminar shear stress are ellipsoidal, aligned in the direction of the blood flow, and have intact tight junctions (11). In contrast, ECs exposed to non-uniform shear stress have a cuboidal morphology with leaky tight junctions and are randomly orientated (7). Many of these differences can be accounted for by the effects of shear stress on the regulation of genes in ECs. For example, nitric oxide synthase in ECs is preferentially upregulated by steady laminar shear stress but not by non-uniform shear stress (12). This enzyme is responsible for the production of nitric oxide (NO·), the most potent of all endogenous vasodilators (13). NO· also has many localized anti-atherogenic effects that are important for proper functioning of the endothelium [reviewed in (14)]. Therefore, reduced NO· production in ECs exposed to non-uniform shear stress can impair endothelium function by leading to increased surface expression of vascular cell adhesion molecule-1 and a potent smooth muscle cell (SMC) proliferator known as platelet derived growth factor-1 (9). Consequently, there is increased lipid uptake, monocyte adhesion, and SMC proliferation at these sites within the arterial wall (11), important events in the atherosclerotic disease process (see below). These are but a few examples of genes regulated by shear stress mechanisms. Current research detailing the relationship between blood flow hemodynamics and atherosclerosis will provide important insights into the development of atherosclerosis and why there is preferential uptake of LDL at certain sites within the arterial wall.

1.1.3 - The Arterial Wall and Atherosclerotic Lesions

The coronary artery consists of three distinct layers known as the intima (innermost), media (middle), and adventitia (outermost) that all undergo a variety of changes during the

atherosclerotic disease process (Fig. 1.1) (5). The intima is bound by ECs that line the lumen of the artery and by the internal elastic lamina on its external side. Between the ECs and the internal elastic lamina is an area of loose connective tissue components including collagen type IV and proteoglycans that act as a basement membrane for the ECs (15). The media layer is composed primarily of SMCs. These multipotent cells are capable of migration, proliferation, and cellular synthesis and play an important role in the pathogenesis of atherosclerosis (see below). The outermost layer of the artery or adventitia is composed of both loose and dense connective tissue and is inter-dispersed with fibroblasts and some SMCs. In the past, atherosclerosis had been considered to be a disease primarily of the intima, although important changes occur in the media and adventitia as well during the progression of this disease. Therefore, an understanding of the atherosclerosis disease process requires studying biochemical and morphological changes that occur throughout the arterial wall.

Atherosclerotic lesions are heterogeneous and have been classified into three main types: the fatty streak, fibrous plaque, and complicated lesion (15). The fatty streak is the earliest lesion to form and is often present in infants and children (16,17). The fatty streaks form at sites of predilection (see above) due to an increased permeability of the EC to macromolecules such as LDL (11). As will be discussed (see section 1.2.5), LDL modified by oxidation, known as oxidized LDL (oxLDL), (and by other processes) promotes further injury to the endothelium. Macrophages, T-lymphocytes and SMCs accumulate and persist at these sites (chronic inflammation), and lipids are delivered to macrophages from oxLDL through scavenger receptor-mediated processes (section 1.2.5). Lipid-laden foam cells form and give rise to the signature yellow fatty streak due to localized cholesterol accumulation within the intima. Fatty streaks are small (1-3 mm in diameter) and only minimally elevated above the surface of the surrounding intima (3).

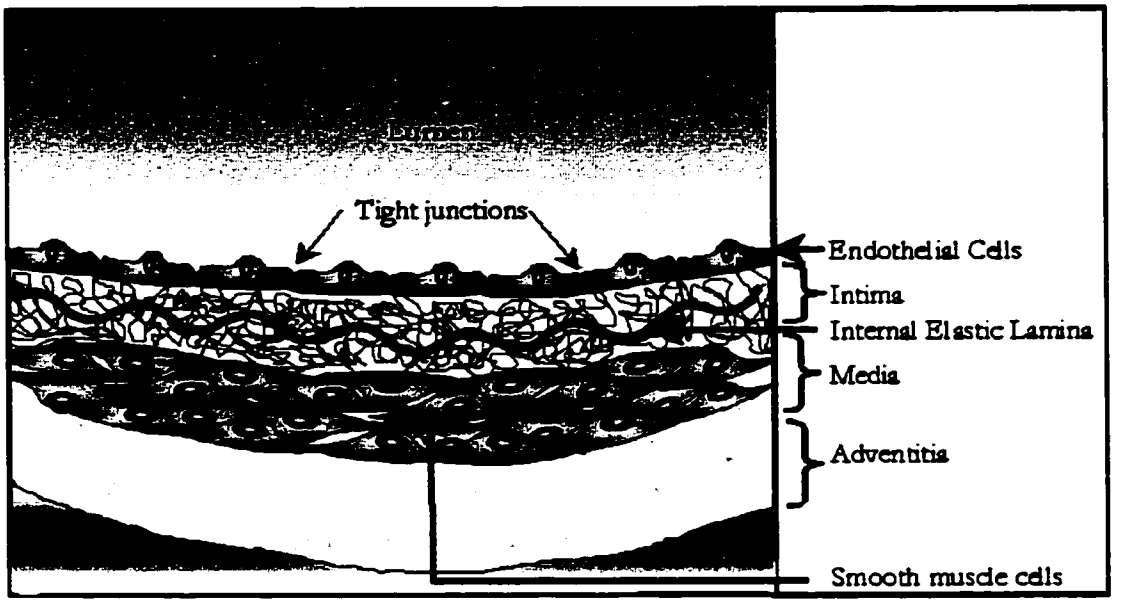


Figure 1.1: Structure of a Normal Large Artery

A single layer of ECs lines the lumen of the artery and through tight junctions maintains a selectively permeable barrier of the arterial wall. The intima composed of connective tissue including collagen IV and proteoglycans acts as a basement membrane for the ECs. The internal elastic lamina separates the intima from the media layer, which consists primarily of SMCs. The outermost layer of the artery called the adventitia is composed of both loose and dense connective tissue and very few cells. Oxygen and nutrients are supplied to the adventitia by a microvasculature called the vasa vasorum (18) [adapted from (2)].

The progression of the fatty streak into the fibrous plaque is the hallmark of well-developed atherosclerosis and is controlled by many environmental and genetic factors [reviewed in (2)]. Fibrous plaques are larger than fatty streaks (3-15 mm in diameter) and protrude into the arterial lumen, which causes a weakening of the underlying media. At the center of the plaque is a necrotic core produced by the continued accumulation of lipids from oxLDL. Cytokines and growth factors released by macrophages and T-lymphocytes promote the proliferation and migration of SMCs from the media into the intima through a compromised internal elastic lamina (4). The SMCs accumulate within the atherosclerotic lesion and secrete connective tissue proteins including type-I and type-III collagens that form

with other extracellular matrix components to produce a fibrous cap that surrounds the necrotic lipid core (3). In this manner, the fibrous cap provides a protective barrier to the atherosclerotic lesion by sequestering the thrombogenic necrotic core from mediators of coagulation in the bloodstream (section 1.1.4).

The progression of the fibrous to the complicated or advanced lesion is characterized by necrosis (especially of SMCs), hemorrhage (of blood vessels), formation of calcified deposits, and proteolysis of extracellular matrix components (Fig. 1.2) (19). Many of these changes produce a weak fibrous cap that makes the atherosclerotic plaque susceptible to rupture (20). For one, necrosis of SMCs impairs their ability to replenish the extracellular matrix proteins of the fibrous cap. Collagens, the normally stable proteins of the extracellular matrix, are degraded by matrix metalloproteinases (MMPs), which are upregulated by interactions between macrophages and T-lymphocytes within the lesions. These enzymes are therefore major contributors to the destabilization of the plaque (21-24). Type I collagen, the main protein component of the fibrous cap, is degraded by MMP-1 (also known as collagenase) whose expression in ECs is stimulated upon exposure to oxLDL (25). Immune responses also regulate expression of MMPs (22). CD40 and its ligand known as CD40L (or CD154) are expressed together on inflammatory cells ($CD4^+$ T-lymphocytes and macrophages) as well as on some non-immune cells (ECs and SMCs). Expression of either recombinant or native CD40L stimulates expression of MMPs in cells associated with atherosclerotic lesions (26-29). Furthermore, disruption of CD40-CD40L interactions by either genetic (30) or immunological approaches (31) produces a more stable plaque phenotype in atherosclerosis susceptible mice.

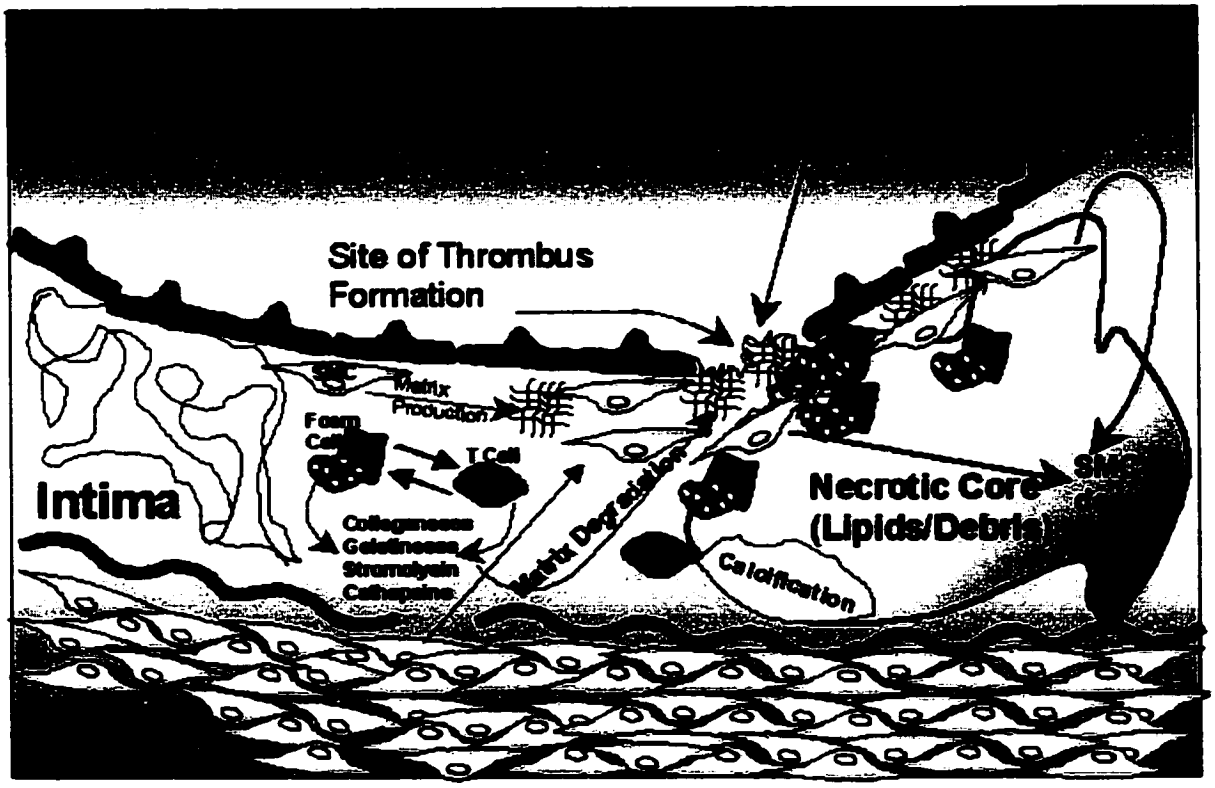


Figure 1.2: The Complicated Atherosclerotic Lesion

The complicated or advanced atherosclerotic lesion is characterized by instability in the plaque due to a weak fibrous cap. There is a greater preponderance of T-lymphocytes at this stage of the disease. Cross talk between T-lymphocytes and macrophages stimulates expression and secretion of MMPs (collagenases, gelatinases, stromolysins, and cathepsins) that degrade the extracellular matrix components that make up the fibrous cap. Failure to replenish these components is due in part to death of the SMCs that is increased at this stage of the atherosclerotic disease [adapted from (2)].

1.1.4 - Plaque Rupture and Thrombosis

Complete blockage of a major artery following rupture of an atherosclerotic lesion is the most clinically important event in the atherosclerosis disease process. Occlusion of the artery is due to the formation of a massive thrombus that arises through multiple and complex pathways. The initiating event is the release of tissue factor (TF) from within the lesion into the bloodstream where it forms a complex with VIIa (TF.VIIa) (32,33). First, TF.VIIa activates factors X and IX to Xa and IXa, respectively, which in turn catalyzes the conversion

of prothrombin to thrombin (34). Thrombin has a central role in the blood coagulation cascade, and most importantly it converts fibrinogen to fibrin and activates factor XIII that crosslinks and stabilizes the fibrin clot (35). Normally, thrombin production does not produce a massive clot within the arterial lumen due to the presence of anticoagulation factors (*i.e.* antithrombin) produced and secreted by ECs lining the arterial wall. However, in the vicinity of the ruptured atherosclerotic lesion the ECs are either lost or dysfunctional, and the concentrations of anticoagulation factors are usually not sufficient to prevent the formation of a massive thrombus. The thrombus generated can effectively block blood flow to the heart or brain and produce a myocardial infarction (*i.e.* coronary artery) or stroke (*i.e.* carotid artery), respectively.

1.2 – Metabolism of Triglyceride-Rich Lipoproteins and their Remnants

Lipoproteins have an important role in the development of atherosclerosis, although many factors contribute to the progression of this disease [reviewed in (2)]. An initiator of atherosclerosis is the accumulation of LDL within the subendothelium of the arterial wall (2). Here, the LDL become modified (section 1.2.5) and are taken up by macrophages producing foam cells, which accumulate and form the fatty streak lesion. HDL, on the other hand, can remove lipids from macrophages within the intima of the artery and thereby reduce lipid accumulations (primarily of cholesterol) at these sites. This anti-atherogenic property was first proposed by Glomset (36) and is an important function of HDL. HDL delivers these lipids (mainly cholesterol and phospholipids [PL]) either directly or indirectly to the liver for storage and/or secretion in the form of bile; a process known as reverse cholesterol transport. In this manner, HDL and LDL act antagonistically in the transport of lipids (primarily cholesterol) to

and from the arterial wall and the plasma concentrations of these lipoproteins ultimately influences the progression of the atherosclerotic lesion.

1.2.1 - General Structure and Function of Lipoproteins

Plasma lipoproteins are spherical microemulsions of proteins and lipids. They have an outer surface of proteins and polar lipids with detergent like properties (amphipathic) that solubilize nonpolar lipids that make up the core of the lipoprotein particle (Fig. 1.3) (37). The surface proteins are known as apolipoproteins and are classified as either exchangeable or nonexchangeable. Only the exchangeable apolipoproteins move freely within the circulation from one lipoprotein to another. PL and to a lesser extent free cholesterol (FC) are the primary polar lipids on the surface of lipoproteins while the core neutral lipids are cholesteryl esters (CE) and triglycerides (TG). In addition, small amounts (1-3%) of these nonpolar lipids are found at the surface of lipoproteins.

Despite sharing similar features, plasma lipoproteins are very heterogeneous and differ from one another in size, buoyancy, and lipid and protein compositions (37) (Fig 1.3.). Chylomicrons (section 1.2.2) and very low density lipoproteins (VLDL) (section 1.2.3) are secreted from the intestine (enterocytes) and liver (hepatocytes), respectively, and are collectively referred to as TG-rich lipoproteins [reviewed in (38)]. Chylomicron remnants are formed following hydrolysis of chylomicrons, while both intermediate density lipoproteins (IDL) (1.2.2) and LDL (section 1.2.3) are formed by lipolysis of VLDL. LDL are smaller, denser, and more cholesterol enriched than are IDL. The smallest and least buoyant of all lipoproteins are HDL. HDL are not secreted as mature lipoprotein particles per se but are formed mainly within the circulation following association of its constituent proteins with

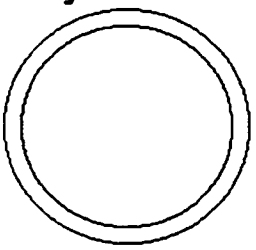
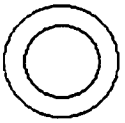
Lipoprotein Class (Relative Sizes)	Physical Properties				
	Diameter, nm	Major Core Lipid (% of mass)	Density (g/ml)	Electrophoretic Mobility	Molecular Weight (Da)
Chylomicrons 	75 - 400	Triglycerides (85%)	0.93	Remains at Origin	50 -1000 × 10^6
VLDL 	30 - 80	Triglycerides (55%)	0.93 - 1.006	Pre-β Migrating	10 - 80 × 10^6
IDL 	25 - 35	Triglycerides (29%) Cholesteryl Esters (23%)	1.006 - 1.019	Pre-β Migrating (Slow)	5 - 10 × 10^6
LDL 	18 - 25	Cholesteryl Esters (42%)	1.019 - 1.063	β Migrating	2.3 × 10^6
HDL_2	9 - 12	Cholesteryl Esters (17%)	1.063 - 1.125	α Migrating	360,000
HDL_3	5 - 9	Cholesteryl Esters (13%)	1.125 - 1.21	α Migrating	175,000

Figure 1.3: Relative Sizes and Physical Properties of the Different Lipoproteins

Chylomicrons and VLDL have a large neutral lipid core enriched primarily in TG (yellow). IDL contain a large proportion of TG (yellow) and CE (gray). LDL, which are more extensively hydrolyzed VLDL remnants than are IDL, have a lipid core composed primarily of CE (gray). The smallest of the lipoproteins, HDL, contain a proportionally smaller core of neutral lipids that is mainly CE.

lipids. The metabolism of HDL is complex and depends on many factors including interactions with the other lipoproteins (section 1.3).

Chylomicrons and VLDL function to deliver TG, in the form of free fatty acids, to peripheral tissues. This enables cells to meet their energy demands (for immediate or future use) and provides for the proper storage of excess TG (*i.e.* adipose tissue). However, chylomicron remnants and LDL formed during this process are enriched in cholesterol and are potentially atherogenic. Receptors on hepatocytes function to clear these remnant particles from the circulation but there are conditions in which these atherogenic lipoproteins (mostly LDL) accumulate within the circulation. As alluded to, HDL serve a useful function by returning excess lipids from the arterial wall to the liver for proper disposal as bile acids.

1.2.2 - Chylomicrons and Chylomicron Remnants

The old adage "you are what you eat" is partly true in the context of atherosclerosis and a diet high in saturated fats and cholesterol is a major environmental risk factor in the progression of this disease. Chylomicrons are secreted in response to these dietary fats, which in a Western diet consists of on average 100-150 g/day of TG and 1-2 g/day of cholesterol (39). In the postprandial (fed) state, the lipids are hydrolyzed by pancreatic lipase in the intestinal lumen and the products (fatty acids, monoacylglycerols, lysophosphatidylcholine, and cholesterol) are subsequently taken up by the small intestine (mainly in the duodenum and jejunum) (40). TG regenerated from the newly absorbed fatty acids are assembled with apolipoprotein B48 (apoB48) into chylomicrons in the endoplasmic reticulum (ER) of the enterocyte. ApoA-I, apolipoprotein A-II (apoA-II), and apolipoprotein A-IV (apoA-IV) are also incorporated into these lipoproteins, but only apoB48 is essential for the production of

chylomicrons (41). The assembly of chylomicrons is still not well understood, and it is often modeled after the assembly of VLDL in the hepatocyte (section 1.2.3).

ApoB48 represents the N-terminal 48% of apolipoprotein B100 (apoB100). In the hepatocyte, full-length apoB100 is produced and becomes lipidated to form VLDL (section 1.2.3). However, in the enterocyte the mRNA for apoB100 undergoes a specific deamination reaction. The enzyme APOBEC-1 catalyzes the conversion of cytosine to uracil and a stop codon at aa 2153 (of the mature protein) is generated (normally codes for Gln) (42,43). Therefore, in humans, apoB48 is produced only in the enterocyte and represents the first 2152 amino acids (aa) of apoB100 (4536 aa). Despite this editing, apoB48 is still sufficiently large and hydrophobic to bind and package the large TG load into chylomicrons. The chylomicrons vary in size from 75 to 400 nm in diameter and estimates indicate that TG make up between 75-85% (41) or 85-92% of the total lipid mass (39). The surface lipids are mainly PL (7-20%) with some FC (0.5-2%) that surrounds the neutral lipid core that, in addition to TG, consists of small amounts of CE (0.5-2%). Chylomicrons are not only large but are very buoyant ($\rho < 0.93$ g/ml) due to the fact that proteins make up only 3% of the lipoprotein mass (38).

Chylomicrons are secreted into the mesenteric lymph and enter the blood via the subclavian vein after passage through the thoracic duct (44). Once in the blood, chylomicrons rapidly exchange apoA-I and apoA-II for apolipoproteins E (apoE), C-I (apoC-I), C-II (apoC-II), and C-III (apoC-III) on HDL (45). The newly transferred apoC-II on chylomicrons activates lipoprotein lipase (LPL) that is bound on the capillary endothelium in adipose tissue, the heart, and skeletal muscle via interactions with heparan sulphate proteoglycans (HSPGs) (46). LPL, as a homodimer, hydrolyzes the TG in chylomicrons and multiple LPL dimers bind to a chylomicron to enhance this lipolysis (46). This results in the

rapid clearance of between 70-90% of the TG in chylomicrons within 5 to 10 minutes of their transport in the blood. The delipidated lipoproteins are called chylomicron remnants (see below). This lipolysis liberates free fatty acids and sn-2 monoacylglycerols due to the preference of LPL for the sn-1 and sn-3 ester bonds in the TG (46). The free fatty acids are taken up by skeletal muscle and adipose tissue and used for oxidation (energy production) or storage. Alternatively, the fatty acids can bind albumin for transport to other tissues including the liver (37). PL and apolipoproteins are removed from the chylomicrons during lipolysis and are incorporated into HDL. It is estimated that as much as 50% of all the apoA-I present in HDL is derived from the enterocyte (38,47).

Lipolysis of chylomicrons depletes these lipoproteins of PL and TG but not cholesterol. As such, cholesterol makes up 8% of the lipid mass in chylomicron remnants compared to 1-2% in chylomicrons. This relative enrichment in cholesterol is thought to be responsible for the reduced affinities of apolipoproteins for chylomicron remnants (48). In fact, apoA-I and apoA-II are absent from these lipoproteins. A typical chylomicron (> 100 nm diameter), which also acquires greater than 20 molecules of apoE following transfer from HDL, contains only about 4 molecules after lipolysis by LPL. The remaining apoE molecules (> 4/lipoprotein), together with perhaps apoE on the hepatocyte cell surface, are responsible for clearance of these remnant lipoproteins (see below).

The liver parenchymal cells or hepatocytes account for the majority of chylomicron remnant uptake and clearance. Chylomicron remnants filter from the blood through the hepatic sinusoids (too small for entry of chylomicrons) and bind to hepatocytes through multiple interactions that are mediated by apoE. HSPGs trap the chylomicron remnants within the Space of Disse to facilitate binding to the hepatocyte cell surface (49). There have also been reports that HSPGs are directly responsible for the uptake of chylomicron remnants

by hepatocytes (49) but this hypothesis has been difficult to address. As well, cell surface apoE added to chylomicron remnants may enhance their uptake, a process that has been coined secretion-capture (50) or secretion-recapture (51). However, the importance of this apoE enrichment in chylomicron remnant clearance has been difficult to prove.

The internalization of chylomicron remnants involves at least two receptors on hepatocytes that have some overlapping specificity [reviewed in (49)]. The LDL receptor, which also binds to apoB100 on LDL and IDL, mediates the uptake of chylomicron remnants by binding to apoE on these lipoproteins (52,53). However, it became apparent that individuals with "non-functional" LDL receptors (section 1.2.5) had only slightly delayed clearance of chylomicron remnants (54). Subsequently, the LDL receptor related protein (LRP), also known as the α2-macroglobulin receptor, was shown to bind apoE-containing lipoproteins and represented a putative second chylomicron remnant receptor (55-57). However, it was only recently that a physiological role was established for this large multifunctional receptor in chylomicron remnant clearance. LRP deficient mice die in embryogenesis (58,59) but mice with targeted deletion of LRP in the hepatocytes are viable. Herz and colleagues demonstrated that deletion of LRP in mice already deficient in the LDL receptor produces large increases in plasma apoB48 concentrations as result of the accumulation of chylomicron remnants in the plasma (60). However, deletion of the LRP in a wild-type mouse background does not have this phenotypic effect illustrating that the LDL receptor is also efficient in clearing these lipoproteins. Therefore, these studies suggest that either receptor alone can account for hepatocyte uptake of chylomicron remnants. However, under certain conditions (*i.e.* a high dietary fat load) both receptors may be necessary to provide for efficient removal of these lipoproteins.

The role of chylomicron remnants in atherosclerosis has been difficult to address for a number of reasons. It was originally hypothesized that chylomicron remnants were too large to enter the subendothelium of the arterial wall [reviewed in (61)]. This would preclude a direct involvement of chylomicron remnants in the formation of atherosclerotic lesions. Recent data, however, suggest that this is not the case and chylomicron remnants can be retained in the arterial wall, which is consistent with a role of postprandial lipemia in atherosclerosis (62). Yet, most plasma cholesterol is carried in LDL (>60%) and only a small percentage (1-3%) is found in chylomicron remnants, even in the postprandial state (41). Therefore, a direct involvement of chylomicron remnants in atherosclerosis requires elevated plasma concentrations of these lipoproteins. Such elevated concentrations of chylomicron remnants are observed in type-III hyperlipidemia (elevated plasma concentrations of both cholesterol and TG). Chylomicron and VLDL remnants enriched in cholesterol collectively referred to as β-VLDL, accumulate in the plasma of individuals with this condition. This complex condition is caused by environmental and genetic (*i.e.* obesity, diabetes, and age) interactions with apoE mutants that are defective in binding to the hepatocyte receptors. Homozygosity for the most common apoE variant responsible for this condition, apoE2 (Arg^{158} → Cys), occurs in approximately 1% of the population and of these individuals 1% develops type-III hyperlipidemia. Premature atherosclerosis is observed in one-third to one-half of individuals with type III hyperlipidemia (63). Therefore, at high concentrations chylomicron remnants are associated with atherosclerosis, although their actions remain less well characterized than those of LDL (63).

1.2.3 - Synthesis and Secretion of VLDL

The synthesis of endogenous TG occurs in the liver by either a *de novo* pathway (breakdown of carbohydrates) or from the uptake of free fatty acids and chylomicron remnants. It is this latter pathway that links the metabolism of chylomicrons to the production of TG in the hepatocyte. VLDL are assembled and secreted from hepatocytes to provide tissues with TG for energy production (*i.e.* skeletal muscle) or storage (*i.e.* adipose tissue). At the same time, lipids are removed from hepatocytes that would otherwise accumulate and interfere with normal liver function (37). VLDL synthesis and secretion is important, especially under conditions of limited food availability, but is also associated with atherosclerosis, diabetes, and obesity (64). Increased synthesis rates of very buoyant VLDL often correlate with high concentrations of TG and LDL-C in the plasma (65). For these reasons, much effort has been put forth to try to understand the factors that regulate the production of VLDL.

The hepatic assembly of VLDL is centered on apoB100 that serves as the major structural protein [reviewed in (66)]. ApoB100 is synthesized mainly in the liver [small amounts also produced in the heart (67)] and like many other secretory proteins has a signal sequence, is N-linked glycosylated, and does not contain a classical transmembrane domain (68). ApoB100 is very large (4536 aa mature protein) and hydrophobic making it suitable for its role in solubilizing lipids and assembling VLDL particles. However, since apoB48 is capable of assembling larger chylomicrons in the enterocyte it appears that the important hydrophobic sequences within apoB100 lie in the N-terminal 50% of the protein. The absolute sequence requirements in apoB100 or apoB48 for lipid association have not been fully elucidated.

VLDL assembly and secretion is not regulated at the transcriptional level of apoB100 since this protein is constitutively expressed in hepatocytes (69). Rather, the assembly and

subsequent secretion of mature VLDL particles depends on two important components. This first of these is lipid availability. Of the four major VLDL lipids (TG, phosphatidylcholine, FC, and CE), phosphatidylcholine and TG availability are rate limiting and most important for VLDL secretion (64). VLDL secretion is impaired from primary hepatocytes when isolated from rats fed a choline deficient diet (70). In this same study, VLDL secretion was normalized upon supplementation of choline to the growth medium. The specificity for phosphatidylcholine in VLDL secretion is strengthened by studies showing that VLDL assembly and secretion is impeded with other PL (71,72). However, since PL made in the ER have a long half-life (73), it has been suggested that under most physiological conditions PL availability is not rate limiting for VLDL assembly and secretion. Oleic acid (oleate), a monounsaturated fatty acid (18:1 ω9 fatty acid) increases VLDL secretion from hepatocytes in culture. There has been some debate on whether this is due to an effect on CE or TG availability or both [reviewed in (65)]. However, most evidence indicates that this is due to an increase in TG availability. Triacsin D, a potent fatty acyl-CoA synthetase inhibitor, blocked TG synthesis and decreased VLDL secretion in the presence of oleate, whereas an inhibitor of CE synthesis, a compound known as 58-035, had no effect on VLDL production (74).

The second important component in VLDL assembly and secretion is the heterodimeric microsomal triglyceride transfer protein (MTP). It consists of a 97 kDa subunit and a 55 kDa subunit. Interestingly, the 55 kDa subunit is the multifunctional protein disulphide isomerase that catalyzes the oxidation, reduction, and isomerization of protein disulphide bonds in the ER when not associated with the 97 kDa subunit (75). MTP is responsible for transferring lipids to the nascent apoB100, although the mechanism by which this is achieved has still not been fully elucidated (76). The lipidation of apoB100 is thought to occur in two steps. In the first step, MTP via the 97 kDa subunit (77) transfers lipids to the newly synthesized apoB100

as it enters the ER (co-translational lipidation). This lipidation is believed to promote proper folding of apoB100 and is supported by studies showing inhibitors of MTP increase apoB100 ubiquitination and degradation via the proteasome pathway (78,79). In the second step, the bulk core of TG (lipid droplet) moves into the ER by an MTP-dependent mechanism, which then becomes incorporated into the nascent VLDL particle (80). This hypothesis is supported by studies of gene-targeted and hepatocyte specific MTP-deficient mice (81). These mice are unable to assemble and secrete apoB100 VLDL, and large sized lipid droplets are absent in the ER and Golgi apparatus.

1.2.4 - Lipolysis of VLDL and Production of IDL/LDL

The fate of VLDL upon entry into the blood is complex and shares both similarities and differences to the metabolism of chylomicrons. VLDL are hydrolyzed by both LPL and hepatic lipase (HL) bound to the lumenal surfaces of endothelial cells (82). VLDL secreted from hepatocytes contains various apolipoproteins including apoA-I and apoA-II in addition to apoE, apoC-III, and apoC-II (the LPL cofactor). Therefore, VLDL do not acquire apoC-II from exchange with HDL, unlike chylomicrons. Lipolysis by LPL and HL causes the release of free fatty acids, which are delivered to the adipose tissue (storage) or skeletal muscles (energy) in a manner similar to that described for the metabolism of chylomicrons (section 1.2.2). The exchangeable apolipoproteins released, particularly apoA-I, enter the HDL pool. Therefore, the collective and efficient lipolysis of the TG-rich lipoproteins (VLDL and chylomicrons) is an important contributing factor to the plasma concentrations of HDL (section 1.3).

VLDL size, lipid, and apolipoprotein composition influences the extent of lipolysis and determines in part the distribution and size of IDL and LDL in the plasma. VLDL are

secreted from hepatocytes as larger and more buoyant $VLDL_1$ (S_f 60-400) and smaller and less buoyant $VLDL_2$ (S_f 20-60), where S_f is the Svedberg units of flotation (83). Both $VLDL_1$ and $VLDL_2$ are converted to IDL under normal circumstances (*i.e.* functional LPL activity). IDL are removed from the circulation following the association of apoE on these lipoproteins with LRP or the LDL receptor. Alternatively, IDL can be further processed by HL into LDL. Estimates suggest that $VLDL_2$ are converted more efficiently to LDL than are $VLDL_1$, although LDL derived from $VLDL_1$ are smaller and denser (84). IDL and LDL differ not only in lipid composition and density (Fig. 1.3) but also in apolipoprotein composition. Importantly, IDL retain apoE on the surface, which is removed during HL-mediated conversion of IDL to LDL (85). During this hydrolysis an epitope on apoB100 becomes exposed that enables LDL to bind to LDL receptors. This region comprises aa 3359 to 3369 and contains positively charged amino acids (RLTRKRGLKLA) (86) that interacts with negatively charged residues on the LDL receptor (87). Binding of LDL to the LDL receptor is complex and is regulated by the conformation of apoB100 in addition to these electrostatic interactions (88).

1.2.5 – LDL Modifications, Scavenger Receptors and Atherosclerotic Lesions

LDL is the major carrier of cholesterol in the plasma (between 60-70% of total) and is arguably one of the most important components in the development of atherosclerosis. LDL-C concentrations are determined by a combination of genetic and environmental factors and epidemiological studies have shown a direct correlation between LDL-C concentrations and the incidence of atherosclerosis (89-91). Individuals who lack exercise and/or consume diets high in saturated fats and cholesterol have increased plasma LDL-C concentrations and in many cases premature atherosclerosis (90). Age and sex are also important determinants of

LDL-C concentrations. Older individuals and men over women tend to have higher LDL-C concentrations (92). Other variations in LDL-C concentrations are less well understood and result from more subtle and complex interactions between environmental and genetic factors.

The most striking genetic cause for elevated LDL-C concentrations is a disease known as familial hypercholesterolemia (FH), which demonstrates, in the absence of other confounding factors, the casual link between high plasma LDL-C concentrations and atherosclerosis (93). Pioneering work by Brown and Goldstein showed that cells (*i.e.* skin fibroblasts) from these individuals had impaired (heterozygotes) or completely defective (homozygotes) uptake of LDL [reviewed in (93)], which led to the cloning of the LDL receptor (94,95). FH heterozygotes (1 in 500 incidence) inherit a mutant copy of the LDL receptor gene that codes for a non-functional protein (many different mutations have been identified) (96). These individuals have a two-fold increase in the circulating number of LDL particles and an increased risk of CHD. FH homozygotes (1 in a million incidence) inherit two mutant copies of the LDL receptor gene (often different mutations) and are unable to remove LDL from the plasma via the LDL receptor pathway (96). Consequently, these individuals develop heart disease early in childhood and frequently die from myocardial infarctions before the age of twenty due to extremely elevated plasma LDL-C concentrations, which are six to ten times the normal concentrations (650 to 1000 mg/dl) (93).

Delivery of LDL-C to the arterial wall is involved in most stages of the atherosclerotic disease process and is the initiator of the atherosclerotic disease process (97,98). The accumulation of LDL at sites prone to develop atherosclerotic lesions (section 1.1.2) is mediated in part by apoB100 binding to the extracellular matrix of the vascular wall, particularly to proteoglycans (88,99). It is well established that LDL is modified within the arterial wall by a number of processes including aggregation, oxidation, and glycation prior to

foam cell formation [reviewed in (100)]. Derivatization of LDL by glucose can give rise to advanced glycation end-products (AGEs) following complex rearrangements and condensation reactions under oxidative conditions (101). Macrophages express receptors for AGEs that bind and promote the internalization of AGE-modified LDL by scavenger receptors (see below), which contributes to the number of foam cells and lipid deposits within the atherosclerotic lesion (101). Diabetics are particularly prone to AGE modifications of LDL (and other proteins), which partly explains why these individuals are at an increased risk of developing CHD [reviewed in (102)].

The most well characterized modification of LDL is oxidation. This occurs within the intima of arteries by different reactive oxygen species. Scavenger receptors on macrophages subsequently bind and internalize oxLDL (103). Interestingly, unlike gene regulation of the LDL receptor, the cholesterol content in macrophages does not regulate the expression of the scavenger receptors. Therefore, macrophages continue to accumulate cholesterol in the presence of oxLDL. These receptors include the class B receptor CD36 (104) and the scavenger receptor class A types I/II (SR-AI/II), the two most studied macrophage scavenger receptors. The most compelling evidence for a role of these receptors in atherosclerosis comes from studies in which mice deficient in SRA-I/II or CD36 were crossed with atherosclerosis susceptible mice (*i.e.* apoE-deficient or LDL receptor-deficient mice). Atherosclerotic lesion size on average was reduced by 77% in apoE deficient mice when crossed with CD36-deficient mice (105). The macrophages isolated from the CD36/apoE-deficient mice have greatly reduced binding and uptake of oxLDL compared to macrophages isolated from apoE-deficient mice. In a slightly different approach, Linton and colleagues injected irradiated LDL receptor-deficient mice with fetal liver cells from either SR-AI/II-positive or SR-AI/II-deficient embryos (106). The mice receiving the SR-AI/II-deficient fetal

liver cells have no macrophage SRAI/II expression and a lesion area in the proximal aorta that is reduced 60% compared to the same mice receiving the SRAI/II-positive fetal liver cells. Combined, these studies demonstrate that CD36 and SRAI/II are two key scavenger receptors responsible for the uptake of oxLDL leading to foam cell.

Foam cell formation is only one process that occurs as a result of LDL modifications [reviewed in (2,103)]. For one, oxLDL enhances the expression of cell adhesion molecules on the surface of the ECs (107). This allows monocytes and T-lymphocytes to adhere to the arterial wall and penetrate the underlying intima. OxLDL has also been shown to inhibit the production of $NO^{\cdot}$ by ECs, a molecule with many important anti-atherogenic functions such as vasodilatation of the arteries and maintenance of a non-adhesive cell surfaces (section 1.1.2) (108). These processes induced by modified forms of LDL lead to the continued accumulation of monocytes and T-lymphocytes from the blood into the arterial wall and progression of the fatty streak into the advanced lesion (section 1.1.3).

1.3 – HDL Metabolism

The inverse correlation between the plasma concentrations of HDL and the incidence of atherosclerosis (109) has generated much scientific research interest into understanding the factors that regulate and control HDL metabolism. This dense lipoprotein population ($1.063\ g/ml < \rho < 1.21\ g/ml$) consists of a heterogeneous mixture of particles that differ in size, shape, composition, and density (section 1.3.1). Various exchangeable apolipoproteins (section 1.3.2) contribute to HDL structure and function but the synthesis of these lipoproteins is centered on the major structural protein apoA-I. HDL metabolism is complex and therapeutic interventions to raise HDL-C concentrations have been difficult to achieve. In addition to apoA-I, the maturation of HDL in the bloodstream requires many accessory

proteins that interact either directly or indirectly with apoA-I for lipid efflux and exchange (section 1.3.3), CE production (section 1.3.4), remodeling (section 1.3.5), and delivery of cholesterol to cells (*i.e.* hepatocytes) (section 1.3.6).

1.3.1 - HDL Subclasses and Anti-Atherogenic Functions

HDL display the most heterogeneity of all the lipoproteins and can be classified according to density (47), size (110), charge (111), and apolipoprotein composition (112). Initially, HDL were characterized by their floatation within a density gradient of 1.063-1.21 g/ml (113) in which two main subclasses known as HDL_2 and HDL_3 were identified. HDL_2 are larger (9-12 nm diameter) and more buoyant (ρ = 1.063-1.125 g/ml) than HDL_3 (6-9 nm diameter, ρ = 1.125-1.21 g/ml). HDL_2 are further separated into HDL_{2b} (10.6 nm mean diameter) and HDL_{2a} (9.2 nm mean diameter) by gradient gel electrophoresis whereas separation of HDL_3 by this technique yields 3 distinct populations; HDL_{3a} (8.44 nm), HDL_{3b} (7.97 nm), and HDL_{3c} (7.62 nm) (114). The largest HDL, HDL_{2b} have the highest lipid to protein ratio due to a relatively large neutral lipid core of CE and TG and represent the most mature HDL species within the circulation. In contrast, the smaller HDL (*i.e.* HDL_{3c}) contain fewer lipids (low lipid/protein mass ratio) and a small neutral lipid core.

Cheung and Albers used immunoaffinity chromatography to isolate HDL according to apolipoprotein composition (112). The two main particles identified were HDL containing only apoA-I (LpA-I) and HDL containing both apoA-I and apoA-II (LpA-I/A-II) (112). In normolipidemic subjects, 25-30% of apoA-I is found in LpA-I and 65-70% of apoA-I and all of the plasma apoA-II is found in LpA-I/A-II (115). The respective contributions of these two HDL populations to protection against atherosclerosis remains to be established. However, epidemiological studies tend to support a more important role for LpA-I over

LpA-I/A-II in protection against CHD [reviewed in (115)]. In one study, normolipidemic subjects with CHD were found to have lower LpA-I but not LpA-I/A-II concentrations compared to asymptomatic individuals (116). Recently, Asztalos and colleagues confirmed and extended these previous results to show that compared with control subjects, CHD patients have major rearrangements in their HDL subpopulations with proportionally lower LpA-I and higher LpA-I/A-II concentrations (117).

Another important classification of HDL is based on surface charge. This is achieved using agarose gel electrophoresis at pH 8.6 (111). HDL are separated into two main populations by this technique known as α-migrating and pre-β migrating HDL (111), although a less well-characterized third pre-α HDL class has been identified (118). Most plasma HDL are α-migrating (85-95% of total apoA-I) and only a small percentage are present as pre-β migrating species (5-15% of total apoA-I) (119). The basis for the difference in electrophoretic migrations is due primarily to the neutral lipid core present in α-migrating HDL that is not found in pre-β HDL (120). The neutral lipid core in α-migrating HDL causes a conformational change in apoA-I that results in a greater electronegative surface potential on these particles (120). The minor pre-β HDL are apoA-I particles that are poorly lipidated and represent nascent HDL (section 1.3.3) or a component of mature HDL that has undergone extensive remodeling (section 1.3.5). Three forms of pre-β HDL can be distinguished by two-dimensional non-denaturing electrophoresis, which separates lipoproteins on the basis of both charge and size (121). Pre-β_1 HDL are the smallest particles and contain only apoA-I (one or two molecules) in association with PL (molecular mass [M_r] ≈ 71 kDa compared to apoA-I with a M_r ≈ 28 kDa). Pre-β_1 particles are converted to larger pre-β_2 and pre-β_3 HDL following the accumulation of additional polar lipids (including FC)

and apoA-I molecules (two to four apoA-I molecules per particle). Pre-β_3 HDL are larger than pre-β_2 particles (121) and both subclasses are efficiently converted to α-migrating HDL by the actions of the enzyme lecithin:cholesterol acyltransferase (LCAT) (section 1.3.4).

There is overlap between the different classification systems used to identify HDL. First, the majority of LpA-I exists as HDL_2 while LpA-I/A-II are found primarily, but not exclusively, in the HDL_3 density range (114). HDL_2 and HDL_3 contain neutral lipid cores of different sizes and are sometimes collectively classified as α-migrating or mature HDL species. The classification of pre-β HDL is reserved primarily for nascent HDL (lipid-poor apoA-I particles). This subclass exists at low concentrations in the plasma (122), as mentioned above, and at higher concentrations in lymph (123). Pre-β_1 HDL are most important in regulating removal or efflux of cellular lipids (122,124) (section 1.3.3), a key step in the subsequent production of α-migrating HDL and the first step in reverse cholesterol transport (see below). It has been suggested that the distribution of the different HDL subclasses (*i.e.* LpA-I vs. LpA-I/A-II distribution) rather the total plasma HDL-C concentrations is a more important measure of the ability of HDL to confer protection against atherosclerosis (119,125). However, more studies are needed to clearly define the anti-atherogenic potentials of these different HDL subclasses.

Epidemiological studies have demonstrated an inverse correlation between HDL-C (and apoA-I) concentrations and the incidence of developing CHD (126). Furthermore, inborn errors of metabolism and genetic manipulations of animals (mice and other species) have helped establish a causal relationship between HDL-C/apoA-I concentrations and atherosclerosis. Many individuals with apoA-I null alleles have premature CHD when confounded with other risk factors, such as elevated LDL-C concentrations (127). Somewhat

surprisingly, mice deficient in apoA-I do not develop atherosclerosis (128). This is attributed to low levels of atherogenic LDL particles in these animals that are normally resistant to atherosclerosis. However, both transgenic mice (129) and transgenic rabbits (130) for human apoA-I have reduced diet-induced atherosclerosis compared to control littermates. This suggests that in the presence of atherogenic lipoproteins such as LDL and β-VLDL that apoA-I (or HDL) confers direct protection against atherosclerosis. This hypothesis is supported by the findings that human apoA-I suppresses atherosclerosis in apoE deficient mice, a strain that normally develops large and advanced atherosclerotic lesions (131,132).

The mechanisms by which HDL protects against the development of atherosclerosis and the roles of the different HDL subclasses in this process are still much debated. To date, the most widely accepted role of HDL in the plasma is a process termed reverse cholesterol transport, first coined by Glomset (36), in which cholesterol from peripheral tissues is removed by HDL and transported to the liver for proper secretion as bile acids. Nonetheless, direct evidence for this process *in vivo* has been difficult to establish [reviewed in (119)]. Studies with apoA-I deficient mice do suggest that reverse cholesterol transport plays an important, but not exclusive, role in protection against CHD. Plump *et al.* used these mice that have greatly reduced HDL to show that apoA-I is required for the delivery of CE to steroidogenic tissues such as the adrenal glands, ovaries, and testes (133). In another study, Stein and colleagues demonstrated that there is delayed removal of cholesterol from peripheral tissues in apoA-I deficient mice compared to control mice (134), the important first step in reverse cholesterol transport. However, more in-depth kinetic analyses by Dietschy *et al.* showed that the flux of cholesterol from peripheral tissues to the liver may not be rate-limiting with respect to plasma apoA-I or HDL-C concentrations (135,136). These findings are supported by the observation that high levels of human apoA-I in transgenic mice do not

increase further the efflux of cholesterol from cells *in vivo* compared to control mice expressing physiological concentrations of apoA-I (137). Therefore, these latter studies suggest that HDL exerts anti-atherogenic effects by means other than by solely increasing the movement of cholesterol from peripheral tissues to the liver.

One such function of HDL is to act as an antioxidant. HDL can prevent the oxidation of LDL *in vitro* (138). These antioxidant functions are primarily attributed to enzyme paraoxonase-1 (PON1) that associates with HDL and hydrolyzes oxidized PL on LDL (139,140). Moreover, PON1 deficient mice are more susceptible to diet-induced atherosclerosis compared to wild-type littermates (141), and when crossed to the apoE deficient background have increases in the size and number of atherosclerotic lesions compared to apoE deficient mice alone (142). However, other proteins on HDL have antioxidant capabilities and can similarly reduce LDL oxidation, but perhaps not to the same extent as PON1. These include platelet activating factor acetyl hydrolase (143), LCAT (144), and apoA-I itself (145). Therefore, there is growing evidence that the protein constituents of HDL protect against atherosclerosis by reducing oxidation of LDL *in vivo*.

HDL can also inhibit the inflammatory response associated with atherosclerosis. The cytokine-induced expression of EC adhesion molecules that allows monocytes to adhere to the endothelium is reduced significantly by HDL in cell culture (146) and *in vivo* (147). These same researchers also report that HDL make ECs less susceptible to platelet and leukocyte activation by increasing the production of prostacyclin, a potent inhibitor of platelet and leukocyte functions (148). More recently, apoA-I on HDL was shown to inhibit T-lymphocyte contact-mediated activation of monocytes and limit the production of cytokines tumor necrosis factor-α and interleukin-1β by these cells (149). The production of these

inflammatory cytokines is an important component in the pathogenesis of atherosclerosis [reviewed in (150)].

1.3.2 - Exchangeable Apolipoproteins in HDL Metabolism

The exchangeable apolipoproteins of HDL (Table 1.1) have evolved from a common ancestral gene and have characteristic and repetitive amphipathic α-helices (151). The two major structural proteins of HDL are apoA-I and apoA-II that account for 70% and 20% of the total protein mass, respectively. Above all, the synthesis of apoA-I is the major

Table 1.1: The Exchangeable Apolipoproteins of HDL

Protein	Plasma Concentration (mg/dl) (% on HDL)	Major Tissue Source	Amino Acids (aa) and (M_r)
apoA-I	**130 *(90-100%)***	**Liver and small intestine**	**243 aa (28.1 kDa)**
apoA-II	40 *(90-100%)*	Liver and small intestine	77 aa (17.4 kDa as a dimer)
apoA-IV	Not defined	Small intestine	376 aa (44.5 kDa)
ApoC-I	6 *(97%)*	Liver	57 aa (6.6 kDa)
ApoC-II	3 *(60%)*	Liver	79 aa (8.9 kDa)
ApoC-III	12 *(60%)*	Liver	79 aa (8.8 kDa)
ApoE	5 *(50%)*	Liver	299 aa (34.2 kDa)

* The table was adapted from (37,119).

driving force in the production of HDL [reviewed in (152)]. In fact, apoA-II do not form HDL as evident from the lack of HDL containing only apoA-II (*i.e.* LpA-II). As well, the

incidence of atherosclerosis is inversely correlated with the plasma concentrations of apoA-I but not with apoA-II levels (129). Therefore, the role of apoA-II in HDL metabolism and atherosclerosis is unclear and studies suggest that apoA-II can be either pro-atherogenic (153,154) or anti-atherogenic (155). Recent evidence also suggests that apoA-II may be a physiological inhibitor of HL on HDL (156) (see section 1.3.5).

Both apoA-I and apoA-II are synthesized and secreted by hepatocytes and enterocytes as either lipid poor proteins or as components of TG-rich lipoproteins (157). ApoA-I is a 28.1 kDa non-glycosylated 243 aa protein in the plasma (158). ApoA-II is smaller (79 aa) and found predominantly as an 18 kDa homodimer joined by a single cystine bond. The plasma concentrations of apoA-I but not apoA-II correlate with HDL-C concentrations in humans (159) and in mice transgenic for these proteins (160). Therefore, it appears that apoA-I but not apoA-II is necessary for maintaining the steady-state concentrations of HDL. However, apoA-II deficient mice have HDL-C concentrations that are reduced by 50 to 70% depending on the dietary conditions (161), which suggests that at least in the mouse apoA-II is important in HDL metabolism. This finding may be due to increased lipolysis and clearance of HDL in mice lacking apoA-II since, as mentioned above, it has been suggested that murine apoA-II inhibits HL activity (162) (section 1.3.5). Whether or not these findings from apoA-II deficient mice can be related to humans remains to be addressed. Sequence comparisons reveal that the human and mouse apoA-II are 61% identical but despite their similarities the two proteins appear to function differently in many aspects [reviewed in (163)]. One important difference is that human apoA-II circulates as a dimer whereas murine apoA-II is monomeric.

ApoA-IV, apoE, apoC-I, apoC-II, and apoC-III account for the remaining 10% of the HDL protein mass (37). ApoA-IV is secreted as a 46 kDa glycoprotein (164) from enterocytes

as a component of chylomicrons (165) and shares significant homology to apoA-I (24% identity and 48% positive). ApoA-IV, like apoA-I and apoA-II, is displaced from the surface of chylomicrons during lipolysis by LPL and exchanged for apoE and apoCs on HDL. However, the concentrations of apoA-IV in the plasma are low making it difficult to establish a direct role for this protein in the metabolism of HDL. It has been suggested that possible functions of apoA-IV are to increase intestinal fat and fat-soluble vitamin absorption and to act as a satiety factor (166). Apolipoproteins E, C-I, C-II, C-III are also present at low concentrations on HDL. These proteins are important for lipolysis of the TG-rich lipoproteins and the subsequent uptake of remnant particles, as discussed (section 1.2.2). Therefore, HDL serves as a reservoir for these minor apolipoprotein components so they can be readily transferred onto the TG-rich lipoproteins for their efficient metabolism.

1.3.3 - ABCA1, Caveolae and PLTP

It is generally accepted that apoA-I is secreted by enterocytes or hepatocytes in a lipid-free or poor state often referred to as nascent or pre-β_1 HDL (section 1.3.1). As well, significant amounts of pre-β_1 HDL are produced following the removal of apoA-I and PL from the surface of the TG-rich lipoproteins during lipolysis. Therefore, the first step in the maturation of HDL is the acquisition of additional lipids by this nascent apoA-I/HDL pool. This can occur by different mechanisms (see below) but perhaps the most important physiologic process involves the efflux of cholesterol and PL from peripheral cells. Individuals with Tangier disease have HDL-C concentrations that are less than 3% of normal values (167) despite having regular synthesis and secretion of apoA-I [reviewed in (168)]. It was subsequently found that fibroblasts from these patients have defective efflux of cholesterol to lipid-poor apoA-I (169) and other apolipoproteins (170). Recently, three independent groups

of researchers demonstrated that mutations in the ATP binding cassette transporter A1 (ABCA1) gene are responsible for Tangier disease (171-173). Heterozygotes for mutations in ABCA1 have half the normal HDL-C concentrations, a condition that has been described as familial HDL deficiency (FHD) (174,175). This latter finding demonstrates that mutations in ABCA1 are co-dominant with respect to apoA-I and HDL concentrations and represent an important rate-limiting step in the production of HDL (168).

ABCA1 is an integral membrane protein of the ABC superfamily that is found at the plasma membrane (176) and within intracellular organelles [reviewed in (177)]. It is classified in the subgroup A on account of its amino acid sequence and contains two nucleotide binding (Walker motifs A and B) and integral membrane (each containing six putative transmembrane helices) domains with an interposing regulatory domain unique to this subgroup. The two functional domains define ABCA1 as full size transporter. Based on its cell surface localization it has been suggested that apoA-I may interact directly with ABCA1, and two research groups were able to cross-link apoA-I to ABCA1 on the surfaces of cells (176,178). However, a recent study has questioned whether apoA-I binds directly to ABCA1 (179). In this study, cells containing structurally intact but ATP-binding defective forms of ABCA1 could not associate with apoA-I. It was suggested that, as previously demonstrated (180), the active process of moving phosphatidylserine from the inner to outer leaflet by ABCA1 (flippase activity) modifies the cell surface and enables apoA-I to interact with the plasma membrane. ApoA-I cross-linked to ABCA1 could reflect apoA-I that is only close proximity to ABCA1 and not directly bound to it. Future studies will be needed to address in greater detail the mechanism(s) by which ABCA1 transfers lipids to apoA-I and to identify whether ABCA1 contains a binding site for apoA-I. Interestingly, ABCA1 expression can be upregulated by peroxisome proliferator-activated receptor-γ (PPAR-γ) (181) and oxysterol

receptor agonists (182) and treatment with these drugs in the near future may provide therapeutic control of HDL-C concentrations in FHD individuals.

Caveolae are discrete bottlenecked cell surface membrane invaginations on the order of 50-100 nm in diameter (183) that are generated by the oligomerization of a protein known as caveolin (three isoforms: cav-1, cav-2, cav-3) (184). They are enriched in cholesterol, sphingomyelin, and glycophospholipids and represent microdomains on the cell surface that are resistant to detergent solubilization (185). Caveolae have many putative functions [reviewed in (186)], and may represent sites on the cell surface that preferentially efflux cholesterol to apoA-I (187). A recent study proposes a two-step mechanism for FC and PL efflux to apoA-I from SMCs (188). In this model, functional ABCA1 is required for the delivery of PL (primarily phosphatidylcholine) to apoA-I to form an apoA-I enriched PL particle that subsequently promotes the removal of cholesterol from the caveolae microdomains. Chemical inhibitors, vanadate and okadaic, decrease cellular caveolae and caveolin concentrations and in this study prevented cholesterol efflux to apoA-I (188). Therefore, it was suggested that caveolae are important cell surface microdomains responsible for cholesterol efflux to apoA-I and that this process is independent of ABCA1-mediated PL efflux. Yokoyama and colleagues reported similar findings using a different approach (189). Treatment of differentiated THP-1 macrophages with anti-sense against caveolin-1 mRNA decreased cholesterol but not PL efflux to apoA-I. Together, these new observations suggest that ABCA1 may not be directly involved in cholesterol efflux to apoA-I. Furthermore, ABCA1 is apparently not localized to caveolae (190), which suggests that efflux of PL and cholesterol may occur at separate regions of the plasma membrane. However, Frank *et al.* reported recently that caveolae may actually inhibit cholesterol efflux since a reduction in caveolin-1 was associated with an increase in cholesterol efflux from 3T3 murine fibroblasts to

HDL (191). This finding is opposite to that proposed by Fielding (188) and Yokoyama (189). Therefore, additional experiments are needed to test this hypothesis further. In fact, caveolin-1 deficient mice are currently being studied (Dr. Frank, personal communication) which should help define a role of caveolae (caveolin), if any, in cholesterol efflux to apoA-I.

Phospholipid transfer protein (PLTP) is also important for normal HDL concentrations. This protein is a member of the lipopolysaccharide binding/lipid transfer protein family that includes cholesteryl ester transfer protein (CETP) (section 1.3.5), the lipopolysaccharide-binding protein, and the bactericidal/permeability-increasing protein (BPI) (192). PLTP transfers PL between lipoproteins in the plasma and under physiological conditions mediate the net transfer of PL from TG-rich lipoproteins into the nascent HDL pool (193). However, the physiological significance of PLTP in HDL metabolism has been difficult to address (194). For one, CETP mediates the exchange of PL between lipoproteins *in vitro* (195), and CETP deficient individuals were reported to retain 50% of PL exchange activity in the plasma [reviewed in (196)]. These two findings suggest CETP and PLTP have some overlapping functions. Secondly, no human PLTP deficient states have been identified which makes it difficult to establish a role for this protein in HDL metabolism. Only recently, with the generation of PLTP deficient mice, has a crucial role of PLTP-mediated lipid transfer in the maintenance of HDL levels been established (197). There is a 60-70% reduction in HDL-C concentrations in mice with targeted knockout of PLTP that results from a complete loss in PL transfer activity (197). Mice, unlike humans, do not possess CETP (section 1.3.5). Therefore, CETP in humans, as discussed above, might also facilitate the transfer of PL onto nascent HDL species. To address this possibility, PLTP deficient mice were crossed with human CETP transgenic mice (198). Mice transgenic for human CETP but deficient in PLTP have very low HDL levels and no plasma PL transfer activity. This provides the first *in vivo*

evidence that CETP and PLTP do not have overlapping functions and under physiologic conditions CETP does not transfer PL between lipoproteins. Subsequent analysis has demonstrated that HDL is susceptible to hypercatabolism in PLTP deficient mice (199) supporting the hypothesis that PL transfer to apoA-I during lipolysis of TG-rich lipoproteins is necessary for HDL maturation and stability.

PLTP also appears to have a role in HDL remodeling. Studies have shown that in addition to PL transfer activity, PLTP promotes the fusion of HDL *in vitro* (200,201). This results in larger HDL as well as an increase in the production of pre-β_1 HDL due to the release of lipid-poor apoA-I in this process. Whether PLTP mediates HDL remodeling *in vivo* has been difficult to address. The best supporting evidence comes from human PLTP transgenic that have between 3- to 5-fold increases in PLTP activity (202). Pre-β HDL concentrations are increased in these mice compared to control mice (when LCAT is inactivated) even though total HDL concentrations are decreased significantly. Despite the lower HDL concentrations it was suggested that moderate increases in PLTP might be anti-atherogenic because pre-β HDL (*i.e.* lipid-poor apoA-I) are efficient acceptors of cellular derived cholesterol and PL. As well, studies by Oram and colleagues showed that PLTP can enhance the efflux of cholesterol and PL from cholesterol-loaded fibroblasts to HDL (203). This process, presumably intracellular lipid transfer to acceptor particles, could be another mechanism by which this lipid transfer protein regulates the concentrations of HDL-C in the plasma.

In summary, recent evidence indicates that the combined actions of ABCA1, caveolae, and PLTP facilitate the efficient transfer of lipids onto nascent HDL, the first step in reverse cholesterol transport and HDL maturation (Fig. 1.4). In this process, small pre-β_1 HDL are converted to larger pre-β_2 and pre-β_3 HDL that are acted upon by the enzyme LCAT for the

subsequent maturation of HDL (section 1.3.4). In addition, PLTP also appears to promote HDL fusion and the production of pre-β_1 HDL at the expense of total HDL concentrations. There are, however, many questions that remain to be addressed regarding the initial lipidation of apoA-I. First, what is the role of hepatocyte ABCA1 in this process since it is established that the liver is a tissue with perhaps the highest expression of ABCA1? We have found that human apoA-I is secreted from primary murine hepatocytes in three different lipoprotein fractions (VLDL, $HDL_{2/3}$, and lipid poor) (see Chapter 5 – The Heterogeneity and Lipidation of ApoA-I Secreted by Primary Hepatocytes). Therefore, it is tempting to suggest that hepatocyte ABCA1 might be important for secreting nascent HDL. If so, what role does hepatocyte ABCA1 have in regulating the plasma HDL-C and apoA-I concentrations? Second, do caveolae mediate the efflux of cholesterol to apoA-I or is this process carried out by ABCA1? The mechanisms of lipid transfers to apoA-I by ABCA1 and PLTP are also not well understood. Do ABCA1 and PLTP bind apoA-I and, if so, are there specific sequences within apoA-I that are important for these interactions? One study reports the PLTP interacts with the N-terminus of apoA-I (204) but the exact sequence is not known. As well, there may be other proteins involved in the lipidation of apoA-I and nascent HDL at both the cellular level or within the plasma that have not yet been identified.

1.3.4 - The Role of LCAT in HDL Maturation

The association of apoA-I (and other apolipoproteins) with PL and cholesterol, as detailed above, is required for the subsequent conversion of FC to CE in HDL by LCAT (EC 2.3.1.43). LCAT catalyzes a transesterification reaction (acyltransferase) that proceeds by a substitution or "ping-pong" type mechanism (205). The sn-2 fatty acyl chains of HDL are

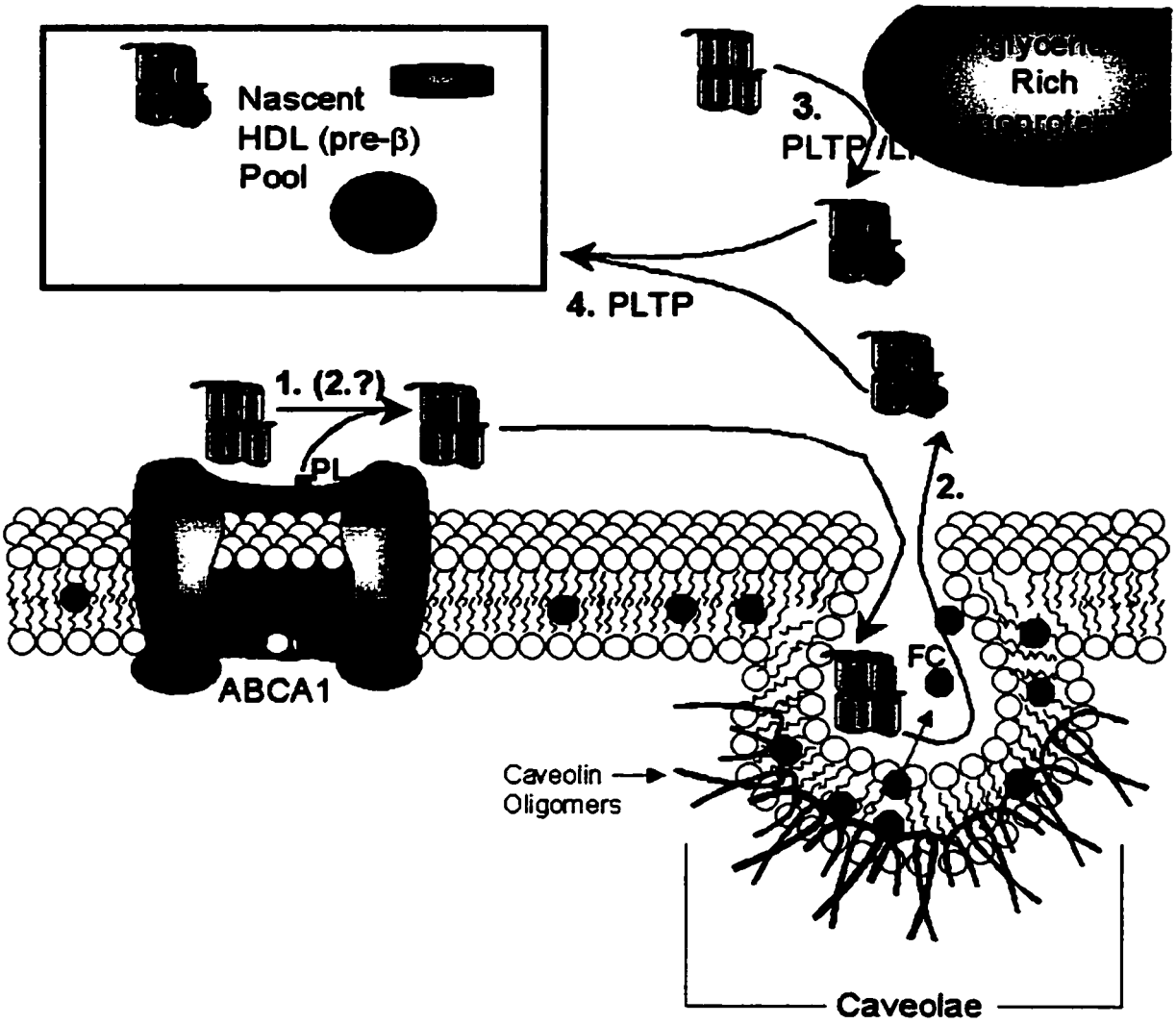


Figure 1.4: Formation of Nascent HDL – Roles of ABCA1, Caveolae and PLTP

Lipid-poor or lipid-free apoA-I acquires PL and possibly FC in a process that is mediated by ABCA1 (step 1.). Caveolae on the surface of cells are cholesterol-enriched membrane invaginations that may be important for cholesterol efflux to apoA-I/PL complexes (step 2.). PLTP facilitates the transfer of PL, and perhaps also cholesterol, to apoA-I in the plasma following lipolysis of TG-rich lipoproteins (step 3.). The apoA-I/lipid complexes (LpA-I) associate to form nascent HDL particles that contain two molecules of apoA-I (Lp2A-I) or alternatively remain as LpA-I (step 4.). This step may also be facilitated by PLTP. The nascent HDL pool formed is itself heterogeneous and consists of discoidal and small spherical Lp2A-I and LpA-I particles (shown in box). This Figure was adapted from (177,199).

first hydrolyzed generating a fatty acyl chain and lysophosphatidylcholine. In the second step, LCAT mediates the transfer of fatty acyl chains onto the free hydroxyl groups of cholesterol molecules producing CE (205). The CE formed by LCAT serve two important functions in HDL metabolism. First, this reaction promotes the additional flux of FC from cells into HDL, an important component of the reverse cholesterol transport process. Secondly, the CE partition into the HDL and form a neutral lipid core converting nascent pre-β-migrating discoidal particles (pre-β_2) into larger and more buoyant α-migrating spherical HDL. These HDL have longer residence times in the circulation and thus are considerably more stable, which is important for maintaining the steady-state concentrations of HDL in the plasma.

Glomset first identified LCAT in 1962 (206), and since this discovery considerable research has been devoted to the study of this important enzyme in lipoprotein metabolism. LCAT is synthesized primarily in the liver (207) and to lesser extents in brain and testes (208). This hydrophobic enzyme has a predicted M_r of 47 kDa and migrates on sodium dodecyl sulphate (SDS) polyacrylamide gels as a 63 kDa protein due to N- and O-linked glycosylation (25% of total mass). LCAT is secreted into the plasma and resides primarily on HDL where apoA-I acts as its primary activator (209,210) (section 1.4.5). The active site of LCAT contains a Gly-X-Ser-X-Gly motif that was identified by comparison to other serine esterases (211) and confirmed by site-directed mutagenesis (212). LCAT may adopt an α/β-hydrolase fold based on molecular modeling of the enzyme against proteins with known three-dimensional structures (213). Based on this structure, Ser^{181} in the Gly-X-Ser-X-Gly motif forms a catalytic triad with Asp^{345} and His^{377}.

Inborn errors of metabolism have provided critical insights into the role of LCAT in lipoprotein metabolism. Two disorders known as familial LCAT deficiency (FLD) and fish-eye disease (FED) occur as a result of recessive mutations in the LCAT gene (214).

Individuals with FLD have no plasma LCAT activity and severely reduced plasma CE and LCAT mass (215). The concentrations of HDL-C are only 20-30% of normal values in these individuals (216) and the HDL pool consists of discoidal and unusually small spherical particles (217). Interestingly, atherosclerosis does not usually accompany LCAT deficiency, although early atherosclerosis has been reported in some FLD individuals (218). Furthermore, and most clinically relevant, FLD patients have corneal opacifications, anemia, proteinuria (protein in urine), and renal dysfunction (218). Renal dysfunction often occurs in the fourth decade of life due to the accumulation lipid deposits within the glomerular cells, a condition known as glomerulosclerosis. The etiology is not well understood but may be related to a unique group of lipid particles formed in these individuals termed LpX. LpX are large discoidal structures derived during lipolysis of TG-rich lipoproteins that are rich in FC and PL and relatively poor in apolipoproteins (218). Studies with LCAT deficient mice are currently being undertaken to evaluate the mechanisms responsible for the accumulation of lipids in the kidney and the roles of LpX and LDL-C in this process (219).

FED differs from FLD in that there is only partial and selective functional loss of LCAT activity within the plasma. FED individuals have low HDL levels, similar to FLD patients, but possess some LCAT activity and have near normal plasma CE concentrations. On the surface this appears to be a paradoxical finding. However, FED individuals retain LCAT activity against LDL and VLDL (β-LCAT activity) but not against HDL (loss of α-LCAT activity) (220,221). These and other studies demonstrate that LCAT esterifies cholesterol not only in HDL but on LDL as well (and perhaps VLDL), and that this is an important physiological reaction with apoB-containing lipoproteins. In contrast to FLD patients, FED individuals present with a much better clinical outcome. They have age-dependent corneal opacities but show no evidence of hematological (anemia) or renal abnormalities. Therefore, the renal

dysfunction observed in FLD patients appears to be related to the changes in the apoB-containing lipoproteins (↑ FC mass) and/or the production of LpX since the HDL compositions and concentrations are similar in FLD and FED patients.

Structure-function relationships of LCAT have been difficult to address. For one, the three-dimensional structure of LCAT has not been solved even though a structural model for this enzyme has been proposed as described above. Also, it is clear that the mutations that give rise to either FLD or FED are not localized to specific regions within the enzyme. In fact, all naturally occurring LCAT point mutations identified affect enzyme activity and no silent mutations in the form of single nucleotide polymorphisms have been identified (218). This suggests that LCAT function is sensitive to subtle conformational changes, which could be related to the diverse activities of this enzyme in lipoprotein metabolism. This is supported by the observation that two LCAT mutants Thr^{123} → Ile (T123L) and Asn^{228} → Lys (N228K) that cause FED and FLD, respectively, are structurally indistinguishable from each other and native LCAT when analyzed by circular dichroism (CD) and fluorescence spectroscopy (222).

Many studies have addressed the structural requirements (*i.e.* size, charge, protein and lipid compositions) of lipoproteins that are important for LCAT reactivity [reviewed in (205)]. Phosphatidylcholine is the preferred and most physiologically important acyl donor for LCAT. However, phosphatidylethanolamine is also an effective substrate, whereas phosphatidylserine and diacylglycerol show no or very little reactivity with LCAT (223). Sphingomyelin incorporated into lipoproteins interferes with the LCAT reaction and may be an important modulator of cholesterol esterification *in vivo* (224,225). In addition to head group specificity and lipid backbone (*i.e.* glycerol vs. sphingosine), the PL acyl chain length can also affect the efficiency of the LCAT reaction on HDL. It has been reported that an acyl chain length of 14-18 carbons at the sn-2 position of phosphatidylcholine is ideal (226) while longer fatty acyl

chains interfere with CE production by LCAT (227). The degree of unsaturation of the fatty acyl chain also influences LCAT reactivity under experimental conditions where the chain length is kept constant. For example, oleic (18:1) or linoleic (18:2) acid containing one and two double bonds, respectively, are utilized much more efficiently by the human form of LCAT as the sn-2 acyl donor than is stearic acid (18:0) (226).

ApoA-I is the most potent activator of LCAT on HDL (228) and functional domains within the protein have been localized to the central domain of apoA-I (section 1.4.5). Less, however, is known of the sequence requirements in LCAT that interact with apoA-I (205). ApoA-II, on the other hand, interferes with LCAT reactivity on HDL. This may explain why HDL with both apoA-I and apoA-II (LpA-I/A-II) are denser and contain fewer CE relative to HDL with only apoA-I (LpA-I). Apolipoproteins A-IV, C-I, and E also activate LCAT [reviewed in (229)] but under normal physiological conditions are less important than apoA-I.

The mechanism of LCAT activation by apoA-I remains poorly understood. The initial binding of LCAT to HDL may not involve apoA-I or specific lipids since the on-rate constants for the interfacial binding of LCAT to reconstituted HDL, LDL, and phosphatidylcholine vesicles are essentially equal as determined by surface plasmon resonance (222,230). As well, poorly lipidated apoA-I is an effective substrate of LCAT, at least *in vitro*, which suggests that the association of LCAT with lipids is not necessary for the activation of this enzyme on these smaller particles (231). ApoA-I enhances catalysis likely through protein-protein interactions with LCAT following binding of the enzyme to the surface of HDL. This has been suggested to position the active site of LCAT in an orientation that is favourable for hydrolysis of the PL fatty acyl chains (205). This activation by apoA-I increases the dissociation rate of the enzyme, which is reflected by a decrease in the Michaelis-Menten constant K_m. Using surface plasmon resonance Jonas and colleagues have recently shown that

the LCAT dissociation rate constant and catalytic turnover rate are similar, which suggests that LCAT leaves the surface of HDL after each catalytic cycle (230). This is in contrast to LPL that remains bound on the surface of TG-rich lipoproteins for several rounds of lipolysis (232).

The understanding of LCAT functions in lipoprotein metabolism has been quite extensive over the last decade. Studies of transgenic animals, not reviewed in detail here, have helped illustrate the important physiological role of LCAT in lipoprotein metabolism. Particularly, LCAT deficient mice provide an excellent model for the future study of renal dysfunction that occurs in FLD patients. Yet, there is much to be learned about structure-function relationships of this enzyme. A clearer picture of the macromolecular interactions of LCAT with lipid surfaces and apolipoproteins, particularly apoA-I, is necessary. This will further help define the roles of LCAT in lipoprotein metabolism and possibly lead to the identification of novel functions for this enzyme. In fact, it was recently demonstrated that LCAT possesses antioxidant capabilities and can reduce the LDL oxidation *in vitro* by inhibiting the formation of copper induced conjugated dienes and lipid hydroperoxides on these lipoproteins (144).

1.3.5 - Remodeling of HDL by CETP and HL

CETP acts in conjunction with HL to remodel the HDL pool and effectively lower the apoA-I and HDL-C concentrations (233). CETP, like PLTP, belongs to the lipid transfer/ lipopolysaccharide binding protein gene family (section 1.3.3) and is responsible for the TG-enrichment of HDL by facilitating the net transfer of HDL-CE to VLDL, IDL, and LDL in exchange for TG (234). These HDL are preferred substrates for the enzyme HL. Lipolysis of HDL by HL lowers HDL-C concentrations (235) and generates pre-β apoA-I (236) that is either cleared from the circulation or re-enters the HDL pool for subsequent maturation

including additional cycles of lipid efflux (237). At the same time, the CE delivered to IDL and LDL by CETP are removed from the circulation following uptake of these lipoproteins by hepatocytes (section 1.2.4). Therefore, there is debate as to whether CETP is anti-atherogenic because of its role in reverse cholesterol transport (delivery of cholesterol to liver) or pro-atherogenic because it decreases plasma HDL-C concentrations and/or increases the CE content of apoB-containing lipoproteins which could be harmful under conditions where they are cleared poorly from the circulation [reviewed in (238)]. As such, there is a complex relationship between CETP and atherosclerosis that is not yet fully appreciated and depending on one's own metabolic state (*i.e.* the activity of the LDL receptor) CETP may act as an anti-atherogenic or pro-atherogenic protein.

CETP was first isolated and purified from human plasma by Tall and colleagues in the late 1980's (239) and antibodies against this protein were generated shortly thereafter (240). One particular antibody, TP2, inhibits CETP function and was used to demonstrate that CETP accounts for all the neutral lipid transfer activity in the plasma (240,241). During the same period, the cDNA (242) and genomic structure (243) of human CETP were solved. The gene resides on chromosome 16 (16q12-21) (244) and regulation of CETP expression has been studied extensively over the last decade [reviewed in (234)]. CETP mRNA levels correlate directly with its plasma protein concentrations and both are increased in hyperlipidemic states (245).

The tissue distributions and expression levels of CETP vary amongst different species [reviewed in (245)]. Rabbits express high concentrations of CETP, humans intermediate levels, while rats and mice have very little or no detectable plasma CETP (246). Many tissues including the liver (different cell types), small intestines, spleen, adipose, adrenal glands, kidney, heart, and skeletal muscles synthesize CETP. In primates, including humans,

hepatocytes and sinusoidal cells of the liver appear to be the major sources of CETP (247) while in hamsters adipose tissue, skeletal muscle, and small intestine show the highest levels of CETP expression (248). CETP is secreted in humans as a 476 aa protein with an apparent M_r of 53 kDa but is present in the plasma as a 68 or 74 kDa protein due to variable glycosylation states (245). Most of the CETP is loosely associated with HDL in plasma (249,250) but can also be found on the cell surface of adipocytes where it has been shown to be involved in the selective uptake of CE (251). The interactions of CETP with HDL, particularly with apoA-I on these lipoproteins, or with cell surfaces have not been well characterized (discussed below).

The elevated plasma concentrations of HDL-C and apoA-I in CETP deficient subjects firmly established an important role of CETP in lipoprotein metabolism (252). The increased HDL-C concentrations are attributed to delayed plasma clearance of apoA-I and not from increases in the apoA-I production rates (253). The HDL in these individuals are on average larger (254), enriched in CE and FC (255), as well as apoE (256) compared to HDL from control subjects. Similarly, mice that normally lack CETP activity have larger and more CE-enriched HDL (*i.e.* HDL_1). However, introduction of either human (257) or simian (258) CETP transgenes into mice decreases apoA-I and HDL-C concentrations substantially. The HDL are enriched in TG and there is more pre-β apoA-I in the plasma compared to HDL from wild-type mice (259). Furthermore, fatty acids accumulate in the plasma of these mice suggesting that the TG enrichment of HDL by CETP results in subsequent lipolysis by HL and increased catabolism of poorly lipidated HDL that are generated during this remodeling.

Structure-function relationships have provided important insights into the mechanisms of CETP-mediated neutral lipid transfer between lipoproteins. Importantly, CETP shares a similar structure to BPI whose crystal structure was solved recently at 2.4 Å (260). Modeling of CETP on BPI is based on sequence homologies between the two proteins (261),

conservation of key amino acids (261,262), epitope-mapping (263,264), and similarities in secondary structures (265). However, although similar, CETP has an extra 12 aa at its C-terminus. Deletion of this epitope or binding of antibody TP2 to this region prevents the CETP-mediated transfer of neutral lipids suggesting that this domain is responsible for the neutral lipid transfer activity of the protein. The mechanism of neutral lipid transfer however is not clear. It has been suggested that the C-terminus of CETP forms an amphipathic helix that interacts with and disrupts the PL acyl chains of lipoproteins (262,266). This process is believed to facilitate the transfer of neutral lipids out of the lipoprotein core and into the neutral lipid-binding pocket of CETP. It is not clear whether interactions with apoA-I or other apolipoproteins are required during CETP-mediated neutral lipid transfer. There is some indirect evidence that CETP associates with apoA-I and is species specific. In mice transgenic for both human CETP and apoA-I most if not all of the CETP is found associated with HDL (267). In contrast, mice expressing CETP and no human apoA-I (but still expressing murine apoA-I) have only one-fifth of the total CETP associated with HDL (267). As a consequence, there is a greater reduction in HDL-C concentrations in CETP transgenic mice also expressing human apoA-I. This is presumably due to increased binding of CETP to human apoA-I on HDL, which facilitates a more efficient TG-enrichment of the lipoproteins and subsequent lipolysis by HL. However, it can not be ruled out that other factors including the size and lipid composition of the HDL account for these differences.

HL is synthesized and secreted from hepatocytes as a mature 476 aa glycoprotein (M_r 53.4 kDa) with an apparent M_r of 65 kDa as a consequence of N-linked glycosylation (268). Most of the enzyme is found bound via ionic interactions to HSPGs on the lumenal surface of ECs lining the liver sinusoids (269). There is also evidence HL is present on the surface of hepatocytes (270). HL belongs to the lipase gene family that includes LPL and pancreatic

lipase (271), and its newest member endothelial lipase (272). HL, LPL, and pancreatic lipase are thought to share similar three-dimensional structures that are based on amino acid sequence similarities and conservation of critical disulphide bonds (273). Modeling of HL on the crystal structure of pancreatic lipase (274) together with numerous studies over the past decade has helped define functional domains within the enzyme (275).

As alluded to above, lipolysis of HDL lipids by HL generates smaller and less stable particles that are cleared more rapidly from the circulation (276). HL hydrolyzes TG, diglycerides, and PL but not CE on lipoproteins (46). Epidemiological studies have determined that polymorphisms within the promoter of HL can affect enzyme activity within the body and may be important in the development of atherosclerosis (277-282). Increased HL activity is often associated with the higher concentrations of small dense LDL and low plasma HDL-C concentrations (283). At the same time, HL deficiency results in defective lipolysis of TG-rich lipoproteins and accumulation of remnant particles that are themselves atherogenic (82). Therefore, finely regulated HL activity is necessary for maintaining an anti-atherogenic phenotype.

HL requires no protein cofactors for lipolysis. By contrast, LPL requires apoC-II for effective lipolysis of the TG-rich lipoproteins and is inhibited by apoC-III (section 1.2.2). Nonetheless, there is mounting evidence HL activity is also affected by apolipoproteins. An early *in vitro* study showed that most apolipoproteins, with the exception of apoE, could inhibit hydrolysis of lipid monolayers by HL (284). However, the physiological functions of these proteins in modulating HL activity could not be addressed from this study. More recently, uremic subjects with hypertriglyceridemia were found to have increased concentrations of pre-β HDL (lipid poor apoA-I) that had lipase inhibitor activity (285). This past year, a detailed *in vitro* analysis demonstrated that lipid-poor apoA-I could inhibit HL at high concentrations and

displace the enzyme from HSPGs (286). These data suggest that lipid poor apoA-I but not apoA-I associated with large HDL modulates the activity of HL. An interesting conclusion that can be drawn from these studies is that the pre-β HDL produced during HL-mediated lipolysis of HDL regulates the activity of the enzyme by feedback (product) inhibition.

ApoA-II on HDL also appears to inhibit HL activity and may be more effective than lipid-poor apoA-I. However, there is some conflicting data as to the role of apoA-II in HL-mediated hydrolysis of HDL. Initially, it was suggested that apoA-II was an activator of HL on HDL (46,287,288). More recent studies, perhaps more convincingly, have now demonstrated that apoA-II inhibits to varying degrees the activity of HL on HDL. Mice transgenic for human apoA-II and CETP have increases in HDL TG compared to the same mice that also express human apoA-I (46,289). HDL isolated from these mice could inhibit HL activity in an emulsion-based assay, which correlated to the apoA-II/apoA-I ratio of the HDL. Other groups have supported these findings. ApoA-II deficient mice have markedly lower HDL concentrations compared to wild-type (161). This is attributed to an increase in HDL lipolysis by HL that occurs in the absence of apoA-II. HDL from apoA-II transgenic mice are also found to be poor substrates for HL (156) as are reconstituted lipoproteins containing apoA-II (290). It was suggested that apoA-II may be the physiological inhibitor of HL on HDL and function in similar manner to the inhibition of LPL by apoC-III (156).

The concentrations and compositions of HDL in the plasma are determined to a large extent by the combined actions of CETP and HL. The regulation of these enzymes by different apolipoproteins, particularly apoA-I and apoA-II, has provided new insights into the metabolism of HDL. Future work is required to address the mechanisms by which apoA-I and apoA-II modulate CETP and HL activity in the plasma. As well, methods to determine

the optimal activities of CETP and HL within the body may lead to therapeutic interventions and ultimately a reduction in the incidences of atherosclerosis.

1.3.6 - SR-BI: An HDL Receptor

Many studies have reported high affinity and saturable binding of HDL to different cells including hepatocytes (291-296). Nonetheless, characterization of putative HDL receptors has been hindered for a number of reasons including complications that result from non-specific binding of HDL to cell surfaces (297). Only recently was the scavenger receptor, class B, type I (SR-BI) identified as a definitive HDL receptor (298). This multifunctional ligand receptor [reviewed in (299)] binds HDL and, at least in rodents (mice and rats), plays an important role in the metabolism of HDL (see below). Other proteins may bind HDL but their physiological significance is less certain. These include HDL binding protein or vigilin (300), HDL binding protein-2 (301), and a newly identified 95 kDa HDL binding protein (302). As well, cubilin appears to be important for the whole HDL particle (holoparticle) uptake by the kidney (303,304), a major site of apoA-I catabolism (305). However, the physiologic role of cubilin in HDL metabolism is not yet well established and needs to be addressed further.

Previous studies demonstrated that HDL-CE are delivered to cells in a manner distinct from the LDL receptor pathway since it does not apparently involve HDL holoparticle uptake and is independent of apoE (306-313). In a seminal report by Krieger and colleagues SR-BI was shown to bind HDL and mediate the selective transfer of lipids (*i.e.* CE) into cells, identifying it as the potential HDL receptor in this process (298). Consistent with this role, SR-BI is expressed most abundantly in tissues that are actively involved in the selective uptake of HDL-CE such as the liver, ovary, and adrenal gland (314). SR-BI can also interact with

anionic PL (315), native LDL (316,317), and maleylated serum albumin (316) but the physiologic significance of these interactions is not as well defined.

Mice with altered expression levels of SR-BI were produced as a means to establish the importance of SR-BI in the *in vivo* metabolism of HDL. Over-expression of SR-BI by either adenovirus (318) or transgenic (319) approaches resulted in significantly lower HDL-C concentrations compared to control mice. Peak expression of SR-BI following adenovirus-mediated gene transfer in mice drastically lowers plasma HDL concentrations and increases the biliary cholesterol output two-fold (318). Similar findings were reported for SR-BI transgenic mice (319). The reduced HDL-C and apoA-I concentrations are attributed to the formation of small CE-depleted HDL that are cleared more rapidly from the circulation. In contrast, mice with gene-targeted deletion of the SR-BI gene have elevated plasma total cholesterol (TC) (2.2-fold increase) and HDL-C concentrations and accumulate larger CE-rich HDL compared to control SR-BI expressing mice (320).

Hamster (316), mouse (298), rat (321), bovine (322), and human SR-BI (or CLA-1) (323) have been cloned. The proteins are highly conserved and share 75 to 80% homology over their 509 aa lengths. SR-BI is glycosylated (299), palmitoylated at two cysteine residues (324) and has an apparent M_r of 82 kDa. The SR-BIs belong to the CD36 superfamily and share approximately 30% aa identity to CD36 (316). SR-BI and CD36 are thought to have similar structures (325). Each has two putative transmembrane domains, short N- and C-terminal cytoplasmic tails, and a large extracellular loop with highly conserved cysteine residues. Interestingly, CD36 binds HDL with an affinity similar to SR-BI (326) but does not mediate the selective uptake of HDL-CE (325). This suggests that selective uptake of CE by SR-BI requires more than just efficient binding of HDL to the cell surface. The mechanism(s) of

lipid transfer and the involvement of apoA-I and other apolipoproteins in this process have yet to be fully elucidated (see below).

ApoA-I deficient mice are unable to deliver CE to the adrenal gland for the production of steroid hormones (133). This provided the first indirect evidence that SR-BI may bind to apoA-I on HDL. Yet, *in vitro* studies show that SR-BI can also bind LDL (316) and anionic PL (315), which raises the question of whether SR-BI recognizes lipids on the surface of lipoproteins instead of the apolipoprotein constituents. This possibility is unlikely since reconstituted lipoproteins containing either apolipoprotein A-I, A-II, or C-III can compete with HDL for binding to SR-BI whereas PL and cholesterol vesicles alone can not (327). In addition, high plasma concentrations of LDL in hamsters do not interfere with the delivery of HDL-CE to either the liver or adrenal glands, which raises the question as to the physiological significance of SR-BI/LDL interactions (328). Therefore, these and other data suggest that the most important interactions *in vivo* are between SR-BI and HDL and that binding to SR-BI is likely mediated by apoA-I and apoA-II, the two major HDL structural apolipoproteins. ApoE might also participate in this process since it has been reported that apoE deficient mice have reduced HDL CE uptake by the liver and adrenal gland (329). However, this study is complicated by the fact that HDL in apoE deficient mice have altered compositions (very large and TG-enriched) and apoA-I domains that might normally interact with SR-BI could be hidden on these particles.

The amphipathic α-helices of the exchangeable apolipoproteins (see section 1.4.1) may represent the common structural motif on HDL that binds to SR-BI. Williams and colleagues showed that reconstituted lipoproteins prepared with cyanogen bromide cleavage fragments of apoA-I, containing amphipathic α-helical repeat units, as well as a synthetic model peptide of class A amphipathic α-helices (known as 37pA) bind effectively to SR-BI (330). In this same

study, apoA-I cross-links to SR-BI indicating that HDL/SR-BI interactions may be mediated by direct protein-protein interactions between the amphipathic α-helical segments of apoA-I and an unidentified region on SR-BI. However, it is not clear whether the HDL subclasses have different affinities for SR-BI. In one study, HDL_2 (most buoyant HDL) binds to cells expressing SR-BI with a higher affinity than does HDL_3, as do larger reconstituted lipoproteins containing two molecules of apoA-I (Lp2A-I) (9.6 nm diameter) compared to smaller Lp2A-I (7.8 nm diameter) (331). This finding conflicts with data from Williams and colleagues who show that lipid-free apoA-I is as effective as HDL in binding to SR-BI expressing cells (330). Therefore, while most studies agree that amphipathic α-helices of apoA-I and apoA-II, interact with SR-BI (332) there is still some controversy as to the affinities of the various HDL subclasses for this HDL receptor.

The mechanism of selective CE uptake by SR-BI is also not clearly understood. Most studies suggest that HDL-CE are delivered to cells without whole particle uptake and apolipoprotein degradation [reviewed in (299,325)]. These conclusions are drawn from the observations that only the ^{3}H-CE (or cholesteryl ether) and not the ^{125}I-apolipoprotein component of doubly-labeled HDL accumulates in SR-BI expressing cells over time (298,317,333). There are also no TCA ^{125}I-soluble counts in the cells or medium in these studies suggesting that apoA-I is not degraded during selective uptake of CE. However, using alternative approaches (confocal microscopy, cell surface biotinylation, and SR-BI neutralizing antibodies) Tall and colleagues have shown recently that HDL are internalized by primary murine hepatocytes and then re-secreted in a cholesterol-depleted form (334), a process previously described as retroendocytosis (335). Subsequent studies confirmed that this process is mediated by SR-BI (336). Moreover, Reaven reported recently that heterologous expression of SR-BI in *Spodoptera frugiperda* insect cells results in the formation of microvillar

channels to which HDL localize (337). This may provide the means for whole particle uptake of HDL. Therefore, these data suggest that selective CE uptake occurs by a retroendocytosis pathway whereby SR-BI is involved in holoparticle uptake and subsequent re-secretion of CE-depleted HDL.

Rodents (*i.e.* mice and rats) do not possess CETP and require SR-BI for efficient delivery of cholesterol to the liver. In this manner, SR-BI may offer protection against atherosclerosis. Overexpression of SR-BI has been reported to decrease the extent of atherosclerosis in susceptible strains of mice. Increased expression of SR-BI decreases the extent of atherosclerosis in heterozygous LDL receptor-deficient mice (338) and in mice transgenic for human apoB (339). In contrast, SR-BI deficiency can exacerbate atherosclerosis in susceptible mouse strains. SR-BI deficient mice crossed with apoE deficient mice ($SR\text{-}BI^{-/-}/apoE^{-/-}$) have more pronounced atherosclerosis compared to apoE deficient ($apoE^{-/-}$) mice alone (340). Therefore, at least in rodents SR-BI is important in HDL metabolism and as with CETP in humans, there likely exists optimal SR-BI expression necessary for protection against atherosclerosis. Moreover, the similar expression pattern in HepG2 and other human cell lines suggests that SR-BI may also be important for lipoprotein metabolism in humans [reviewed in (325)]. However, as discussed above, CETP deficiency results in hyperalphalipoproteinemia in humans (high HDL concentrations) suggesting that SR-BI cannot fully compensate for the lack of CETP in this way. Therefore, a greater understanding is required of the mechanism of SR-BI-mediated lipid transport, affinities for different HDL subclasses, and the role of apoA-I as a ligand for this HDL receptor, especially in humans.

1.4 - Structure-Function Relationships of Human Apolipoprotein A-I

ApoA-I serves a diverse number of functions in HDL metabolism as detailed above (Fig. 1.5). These include lipid binding, activation of LCAT, modulation of HL activity, and possible interactions with receptors such as SR-BI and ABCA1 and lipid transfer proteins PLTP and CETP. Structure-function studies of apoA-I have been instrumental in defining functional domains within this protein and providing important insights into the metabolism of HDL. Nonetheless, there are many unresolved issues regarding the involvement of particular regions of apoA-I in different aspects of HDL metabolism. Of note, there have been relatively few *in vivo* experiments to corroborate and expand upon previous *in vitro* studies. The current understanding of apoA-I structure-function relationships is reviewed as well as the rationale for the work detailed in this thesis.

1.4.1 - ApoA-I Gene Expression, Evolution and Conservation of Secondary Structural Motifs

ApoA-I is a member of a multigene family that includes at least seven other exchangeable apolipoproteins (apoC-I, apoC-II, apoC-III, apoC-IV, apoA-II, apoA-IV, and apoE) (341). The primary amino acid (158), genomic (342-344) and cDNA sequences (342,345) of human apoA-I have been determined. ApoA-I sequences from a number of different species have been solved and are found to be highly homologous to human apoA-I (see below) [reviewed in (346)]. In humans, the apoA-I gene (*apoA-I*) is located on the long arm of chromosome 11 (11q23) in close proximity to the genes for apoC-III (*apoC-III*) (347) and apoA-IV (*apoA-IV*) (Fig. 1.6) (348).

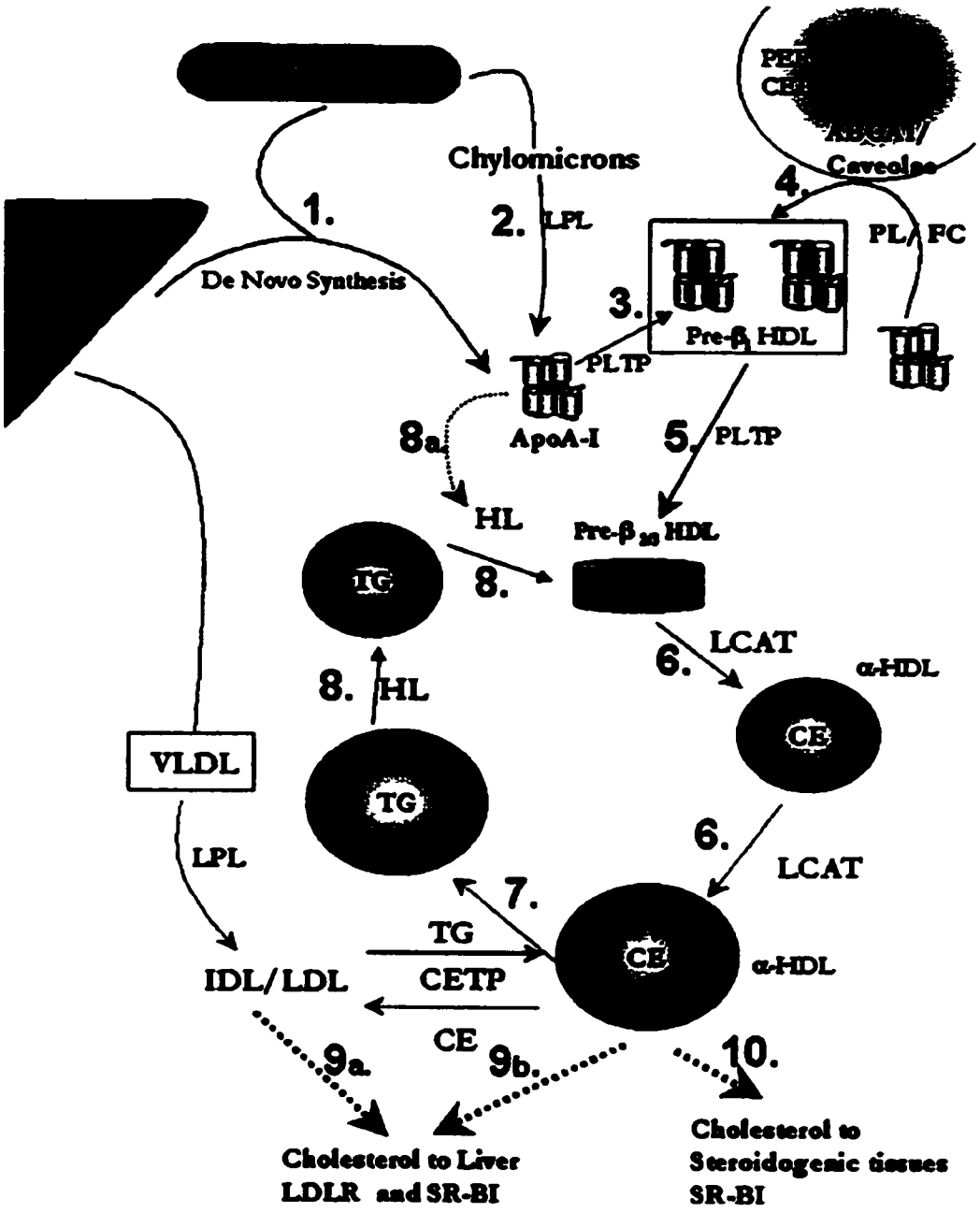


Figure 1.5: The Metabolism of ApoA-I and HDL

ApoA-I and HDL metabolism involves many discrete pathways. Lipid poor apoA-I is derived from *de novo* synthesis by the liver and intestine [1.] or from lipolysis of chylomicrons by LPL [2.]. The nascent pre-β_1 HDL pool is generated by the combined actions of PLTP in the plasma [3.] and ABCA1 (+caveolae) mediated efflux of lipids (FC and PL) from peripheral cells [4.]. Larger pre-β HDL (pre-β_2 and pre-β_3) formed following self-association, which may require PLTP [5.], are efficiently converted to CE-enriched α-migrating HDL by LCAT following activation by apoA-I [6.]. HDL become enriched in TG through the actions of CETP [7.], which are preferred substrates for HL that hydrolyzes the lipids to regenerate pre-β

HDL [8.]. ApoA-I liberated in this process may also act to inhibit HL and prevent further hydrolysis of HDL lipids [8a.]. CEs are delivered to the liver [9.] following uptake of IDL/LDL by the LDL receptor [9a.] or selective uptake of HDL-CE by SR-BI [9b.], the last step in reverse cholesterol transport. HDL also binds to SR-BI on steroidogenic tissues in an apoA-I dependent fashion for the delivery of CE [10.]. A balance between *de novo* synthesis [1.] and clearance of smaller HDL by the liver and kidney [11.] determine the steady-state concentrations of HDL and apoA-I.

ApoA-I is a small gene of 1863 bp that contains four exons and three introns (342). In mammals, the major sites of apoA-I synthesis are the liver and intestine. Transcriptional control of *apoA-I* and other apolipoprotein genes is complex and involves many transcription factors and enhancers [reviewed in (349)]. The apoA-I promoter contains three regulatory elements known as AIB (-128 to -17 bp), AIC (-175 to -148 bp), and AID (-220 to -190 bp) that are required for transcription of *apoA-I* (350). These regions, particularly AIB and AID, contain hormone response elements (HREs) that bind orphan and ligand-dependent nuclear receptors. In fact, mutations in AIB, AID, or both results in promoter activity that is only 3-7% that of the normal (351). An important orphan receptor in apoA-I expression is hepatocyte nuclear factor-4 (HNF-4) that binds to sites AIB and AID in the promoter (as a homodimer) and activates transcription of the gene (352). HNF-4 is expressed primarily in the liver, kidney and intestine, which helps explain the tissue distribution of apoA-I expression. Interestingly, the effect of HNF-4 on apoA-I transcription is greater in cell lines derived from the intestinal epithelium than in hepatoma cells (*i.e.* HepG2 and Hep3B), which could be related to the finding that an enhancer region of apoC-III (see Fig.1.6), which binds tightly to HNF-4, is required for expression of apoA-I in the intestine but not in the liver (353).

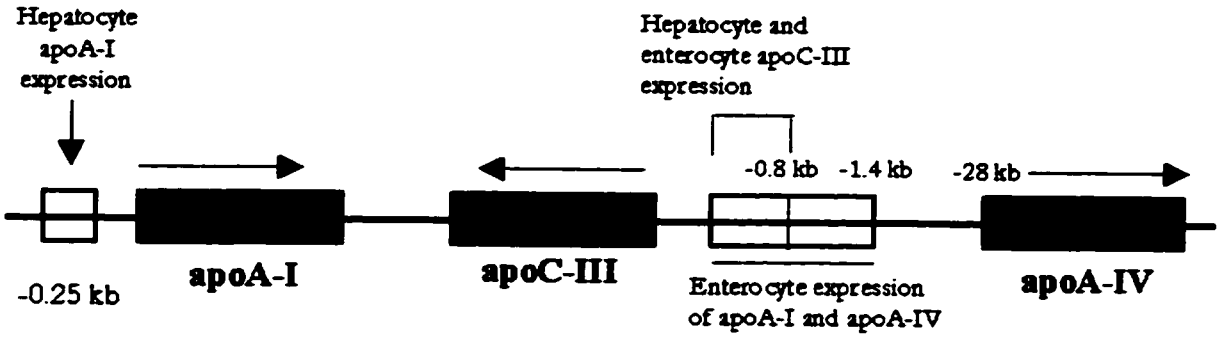


Figure 1.6: The ApoA-I, ApoC-III, and ApoA-IV Gene Cluster

The apoA-I, apoC-III and apoA-IV gene cluster spans approximately 22 kb on chromosome 11 (11q23). The genes for apoA-I and apoA-IV are transcribed in the same direction opposite to that of apoC-III. The promoters and regulatory elements are shown (open boxes). The hepatocyte and enterocyte expression of apoA-I are controlled by different regulatory elements as indicated and discussed below. This figure was adapted from (349).

Other transcription factors are also involved in the regulation of apoA-I gene expression [reviewed in (349)]. An orphan receptor known as ARP-1 binds to the same HRE as HNF-4 and acts as a repressor of apoA-I gene transcription (354). The ligand depend receptor retinoid X receptor α (RXRα) activates apoA-I transcription (as a RXRα/RXRα homodimer) in the presence of its ligand 9-cis-retinoic acid (355). These transcriptional regulators, including HNF-4, as well as some not discussed here, belong to the steroid/thyroid receptor superfamily. Fibrates such as gemfibrozil and rosiglitazone, known activators of PPARα, can influence the transcription of apoA-I. The activated PPARα forms a heterodimer with RXRα (PPARα/RXRα) and induces the transcription of genes including apoA-I [reviewed in (356)]. Such drugs, working through the ligand-dependent receptors such as PPARα and RXRα, appear to provide beneficial effects on lipoprotein metabolism, which is thought to be related in part to the upregulation of apoA-I gene expression.

Effective transcription of the apoA-I gene in the intestine and liver gives rise to a mRNA of 893 nucleotides. This mRNA encodes for a 267 aa protein of which the first 18 aa comprise a signal sequence (prepeptide) cleaved in the endoplasmic reticulum and the next 6 aa (19-24) make up a prosequence that is cleaved extracellularly (357,358), most likely in the plasma and/or lymph (359,360). The function of the prosequence or the identity of the protease responsible for cleaving these 6 residues is not known. It has been suggested that the prosequence facilitates the secretion of apoA-I from cells (361,362). Following this processing, apoA-I circulates in the plasma as a mature 243 aa non-glycosylated protein (M_r - 28.1 kDa).

The amino acid sequences of the mature exchangeable apolipoproteins, including apoA-I, are defined by repetitive 33 or 22 aa units derived from a basic 11-mer repeat (341). These proteins evolved over time by duplication of either 11 or 22 codon repeats and/or deletion of 11 codon repeats from a common primordial gene, believed to most closely resemble the gene for apolipoprotein C-I (*apoC-I*) (341). The multiple repeats of 22 (or 11) aa in apoA-I were also evident from partial amino acid sequences prior to solving the genomic structures (363). These sequences form amphipathic α-helical repeats in apoA-I (364) that are ideally suited for binding lipids due to their high hydrophobic moment (section 1.4.3). However, not all α-helices have equal lipid binding capacities in apoA-I. As well, there are highly conserved Pro residues between the helical segments that likely form β-turns or kinks in the protein structure (Fig. 1.7). The amphipathic α-helices are encoded by exon 4 and represent the carboxyl 200 aa of the protein (aa 44-243). In this region, there are eight 22-mer and two 11-mer amphipathic α-helical segments [reviewed in (365)] (Fig. 1.7). The remaining N-terminal portion (aa 1-43) is encoded by exon 3 and has a less-well defined secondary structure,

resembling a globular domain (366) (Fig. 1.7). Nolte and Atkinson proposed, using a molecular modeling approach, that this region (including up to aa 57) has a complex tertiary structure built from amphipathic β-sheets and random coils, in contrast to the largely α-helical nature of the remaining C-terminal region of apoA-I (367). More recently, NMR spectroscopy of a C-terminal apoA-I truncation mutant (deletion of aa 187-243) showed that residues 8-32 form an amphipathic α-helix in a lipid mimetic environment (368). The interpretation of this recent finding and the function of this region in HDL metabolism await further analysis.

The primary amino acid sequences of apoA-I from a number of species (nineteen in total) have been reported in the Swiss-Prot and TrEMBL databases (http:// ca.expasy.org/sprot/). Most of the proteins are homologous to human apoA-I [reviewed in (369)], which ranges from 95% identity (97% positive) for cynomolgus monkey apoA-I to 24% identity (47% positives) for salmon apoA-I. The highest conservation is in the N-terminal regions of the proteins, although there are significant homologies in the central and C-terminal regions as well (346). Conservative substitutions (*i.e.* Glu for Asp) account for many of the individual amino acid differences between species. As well, the overall secondary structure of hapoA-I (Fig. 1.7) appears to be conserved throughout evolution as determined from Edmunson-wheel representations (346).

1.4.2 - Physico-chemical and Structural Properties of ApoA-I

ApoA-I is easily purified from HDL (following delipidation) by various chromatography techniques (*i.e.* size exclusion or ion exchange). ApoA-I has a pI of 5.2, and like the other exchangeable apolipoproteins, is soluble in aqueous solutions, a property that has aided in the characterization of this protein. Purified apoA-I is a monomer in aqueous solutions at concentrations below 0.2 mg/ml and self-associates (into dimers, tetramers, and

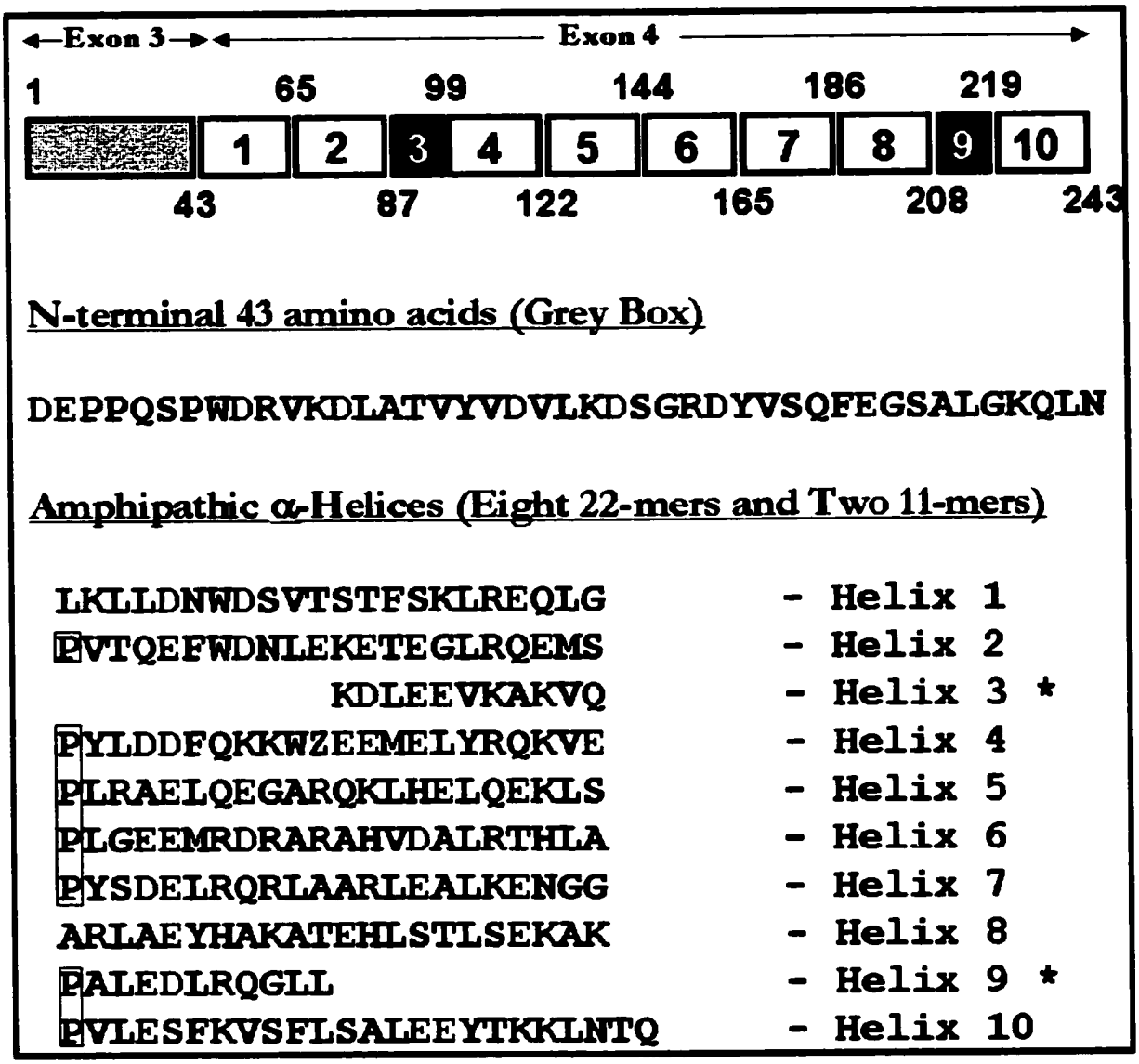


Figure 1.7: Secondary Structure and Amino Acid Sequence of Mature Human ApoA-I

Exon 3 encodes for the N-terminal 43 aa of apoA-I (gray box) that has an amino acid sequence more reminiscent of globular proteins (sequence shown). Exon 4 encodes the eight 22-mer and two 11-mer amphipathic α-helical segments (number 1-10). Note the characteristic Pro residues present in most of the amphipathic α-helices (boxed purple). Helix 3 and Helix 9 (black box with white text) comprise the two smaller putative 11-aa amphipathic α-helices. The distribution of negative and positive charged residues are illustrated in red and blue text, respectively.

octamers) at higher concentrations (370). CD spectroscopy measurements in the near UV-range (222 nm) show that lipid-free apoA-I is largely α-helical (50-60%) [reviewed in (371)] and has a denaturation midpoint in guanidine hydrochloride (GdnHCl) at 1.08M (372). This is the concentration of GdnHCl required for a 50% reduction in the α-helicity of the protein. From these results, the free energy of apoA-I in water (ΔG_D^{H2O}) was found to equal to 3.7 kcal/mol, much lower than that reported for typical globular proteins (ΔG_D^{H2O} between 9-16 kcal/mol) (372). Similar findings (2-3 kcal/mol) have been reported in other studies (373,374). Together, these data suggest that in a monomeric and lipid-free state apoA-I is unfolded and less stable compared to other proteins. More recently, Gursky and Atkinson used similar denaturation and calorimetry techniques to study apoA-I and hypothesized based on their results that that lipid-free apoA-I is in a molten-globule state (375). The molten-globule state is defined as compact folding intermediate with well-defined secondary structure but poor tertiary structure (376), although no crystal structure has been solved for the full-length protein to confirm this structure for lipid-free apoA-I. The crystal structure of apoA-I missing the first 43 aa [apoA-I(Δ1-43)] has been solved to 4 Å (377). Nonetheless, this structure is thought to more closely resemble a lipid-bound conformation of apoA-I rather than that of the lipid-free state (see below).

The transition of apoA-I from lipid-free to lipidated states (*i.e.* nascent HDL) is accompanied by structural changes in the protein that have yet to be fully characterized (addressed below). The α-helical content of apoA-I increases upon lipid binding and accounts for between 70-80% of the secondary structure motifs (371,378). Deletion mutants of apoA-I, purified from *E. coli*, were used to show that the N-terminal portion of the protein (aa 44-126)

accounts for the majority of the α-helical segments in the lipid-free state (379). In contrast, the C-terminal half (aa 139-243) undergoes substantial conformational change and contributes to the increase in the α-helical content upon lipid binding (379). This domain (particularly aa 210-243) corresponds to a region of the protein proposed to have the highest lipid binding affinity (section 1.4.3) (380). The greater α-helical content of apoA-I also correlates with an enhanced stability of apoA-I on a nascent discoidal-type HDL that increases with the size of the particle (374). This increase in stability favours the association of apoA-I with lipids as well as the conversion of smaller pre-β_1 to larger pre-β_2 and pre-β_3 HDL. Therefore, an unfolded and less thermodynamically stable structure for lipid-free apoA-I (*i.e.* molten-globule state) creates a favourable environment for its association with lipids.

The crystal structure of apoA-I(Δ1-43), solved by Borhani and colleagues, has been the catalyst behind rethinking the structure of apoA-I on HDL (377). They proposed that apoA-I(Δ1-43) mimics the lipid-bound conformation of apoA-I, as mentioned above. This proposal is based on findings that, unlike native apoA-I, the apoA-I(Δ1-43) mutant does not show any structural changes upon lipid binding (*i.e.* no increases in α-helicity). Second, apoA-I(Δ1-43) adopts a distinct tertiary structure from lipid-free apoA-I and displays a proteolytic cleavage pattern identical to lipid-bound apoA-I. Therefore, based on the assumption that apoA-I(Δ1-43) adopts a lipid-bound conformation in the absence of lipid, a model for the structure of apoA-I on HDL has been proposed (377,381). In this model, two molecules of apoA-I form a pair of continuous amphipathic α-helices that wrap around a discoidal lipoprotein (*i.e.* pre-β_2 HDL) perpendicular to the PL acyl chains (Fig. 1.8A). This is the so-called "belt model" for apoA-I on HDL (377,381,382). Furthermore, the most energetically favourable conformation is where the two molecules of apoA-I are arranged anti-

parallel to one another (so-called LL orientation) (381). Subsequently, other studies have provided support for the belt-model of apoA-I on discoidal HDL (383-385).

Prior to solving the crystal structure of the apoA-I(Δ1-43) mutant, it was generally accepted that apoA-I was arranged with its α-helices arranged parallel to the PL acyl chains on discoidal HDL (Fig. 1.8B). This arrangement, termed the "picket-fence model", was based largely on the studies of attenuated total reflection spectroscopy (386,387). More recently, Axelsen and colleagues performed similar studies, however this time under native conditions, to show that apoA-I exists in a belt orientation in model membrane bilayers (388). It was suggested that this method has an advantage because the lipoprotein complexes were not dried on film, as was done in previous work (386), which could alter the structure or denature apoA-I in lipoprotein complexes. Therefore, this study suggests that apoA-I exists in the belt topology around the surface of nascent reconstituted discoidal HDL particles, and it may adopt a similar conformation on HDL *in vivo*.

There are still many questions as to the structure of apoA-I in both a lipid-free state and on HDL. For one, it is intriguing how removal of aa 1-43 transforms apoA-I into a lipid-bound conformation, and raises the question as to the role of this globular-type domain in the structure and function of apoA-I? Recent NMR studies in a lipid mimetic environment suggest that aa 8-32 form an amphipathic α-helix in an apoA-I mutant lacking the C-terminal 56 aa (deletion aa 187-243) (368). This study indicates that this region may have lipid-binding capabilities that were not previously appreciated (see section 1.4.3). As well, even though the belt model is a favoured conformation for apoA-I on discoidal HDL, it is not clear how apoA-I is arranged on spherical α-migrating HDL. Most HDL contain either four molecules

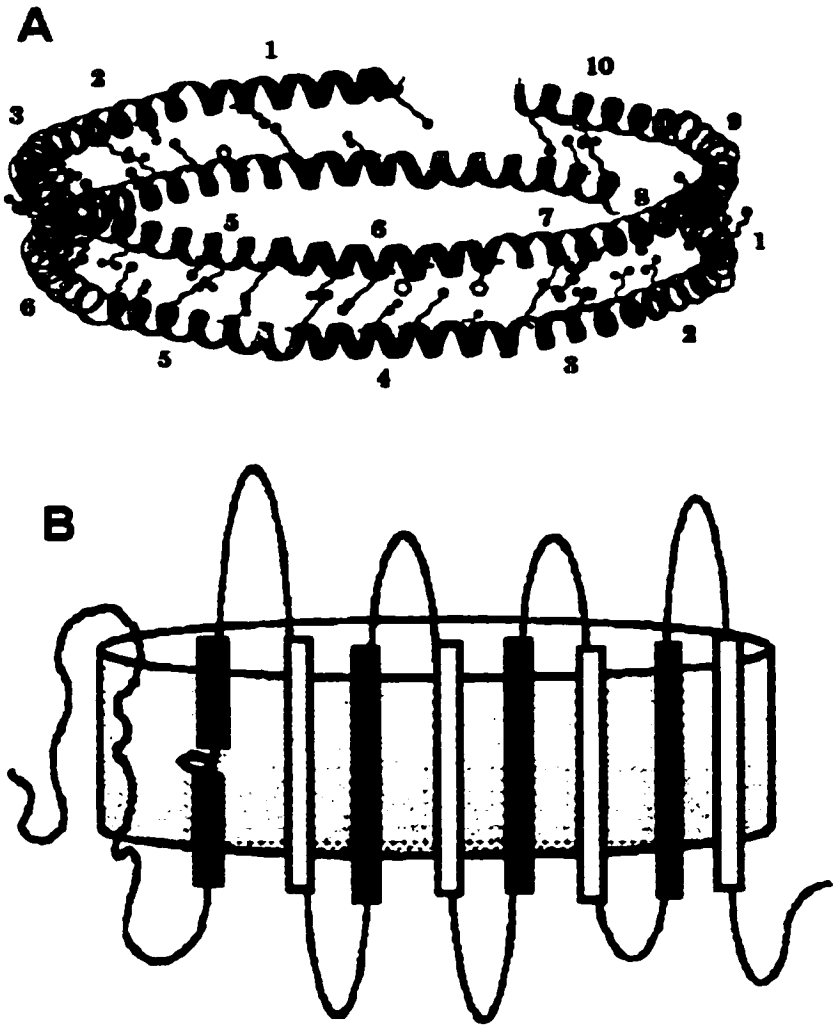


Figure 1.8: The "Belt" and "Picket-Fence" Orientations of ApoA-I on Discoidal HDL

A. In the "belt" model, each apoA-I molecule forms an extended amphipathic α-helix around the nascent HDL perpendicular to the PL acyl chains. The hydrophobic amino acids are buried with the apolar acyl chains while the polar residues are on the exterior. The two apoA-I molecules are in an anti-parallel orientation and are stabilized by both intra and inter-helical salt bridges. The Pro residues provide kinks (green) in the chain and allow the apoA-I molecules to curve around the periphery of the HDL. Adapted from (381).

B. In the "picket-fence" model, the two apoA-I molecules are arranged around the disk with the α-helices parallel to the PL acyl chains and anti-parallel to one another. Only one apoA-I on the front side of the disk is shown for clarity. The Pro are found at the center of β-turns (loops). Adapted from (369).

of apoA-I (LpA-I) or two molecules of apoA-I and two molecules of apoA-II (LpA-I/A-II), as documented above (1.3.1). No models have been proposed that approximate the structure of these two major apolipoproteins on these more physiologically relevant HDL species. Furthermore, as reviewed recently, neither the belt nor picket-fence models present with a thermodynamically favourable topology for apoA-I even on simpler discoidal HDL [reviewed in (389)]. For example, a hinge domain in apoA-I has been proposed that enables apoA-I to adopt different conformations and accommodate more or less PL molecules on the surface of HDL (390,391). Epitope mapping with a panel of monoclonal antibodies, directed against apoA-I, suggests the hinge domain is located within the central region of apoA-I (392). Yet, it is not apparent how the belt model for apoA-I allows for the formation of this structure within the protein or discoidal HDL that contain greater than two molecules of apoA-I. Recently, Jonas and colleagues propose that the structure of apoA-I on discoidal HDL does not fit with either of the two proposed conformations (393). Therefore, although the crystal structure of apoA-I(Δ1-43) has been an exciting development in the study of the structure of apoA-I on HDL, it is evident that more work is required in this area. A clearer picture of the topology of apoA-I on the various HDL species should aid future functional studies of the protein. These functions, which are addressed below, include lipid binding, LCAT activation, lipid efflux, and interactions with other proteins that regulate HDL metabolism.

1.4.3 - Lipid Binding Domains of ApoA-I

The amphipathic α-helix, as first reported by Segrest and colleagues (364), is the structural lipid-binding motif in apoA-I. The opposing polar and apolar (or nonpolar) faces are oriented along the long axis of the α-helix such that the nonpolar face associates with lipids on the surface of the lipoproteins while the polar face is exposed to the aqueous

environment. Seven different types of amphipathic α-helices have been identified [reviewed in (394)], three of which are present in apoA-I. The class A amphipathic α-helix occurs most frequently (Fig. 1.9) and is distinguished from other amphipathic α-helices by a clustering of positively charged residues at the polar-nonpolar interface and negatively charged amino acids at the center of the polar face. The class Y motif is a variation of the class A helix but contains an additional cluster of positively charged amino acids at the center of the polar face (Fig. 1.9). Two 22-mer and two 11-mer amphipathic α-helices share the class Y motif (Fig. 1.9). The third amphipathic α-helix motif in apoA-I is known as class G*. It resembles but is not identical (hence the * designation) to class G amphipathic α-helices found in globular proteins. Only one G* helix is present in apoA-I (aa 8-33), which was recently shown to form in a lipid mimetic environment by NMR studies (368). This helix, however, is not designated as such in the nomenclature used to identify the amphipathic α-helices in this thesis (see Figs. 1.7 and 1.9). In contrast to the class A and Y amphipathic α-helices, the class G* helix has a random distribution of negative and positive charges around the perimeter of the polar face and a slightly lower hydrophobic moment (394).

Segrest and colleagues first proposed that the positively charged amino acids Lys and Arg at the polar-nonpolar interface are important for the lipid binding affinities of class A amphipathic α-helices (366). In this "snorkel hypothesis" the alkyl chains of Arg and Lys penetrate into the PL bilayer and make favourable hydrophobic contacts with the acyl chains while the positive charges extend outward to make electrostatic interactions with the negative charges in the PL head groups. As well, the arrangements of positively and negatively charged residues in the class A amphipathic α-helix are complementary to the zwitterionic charge distribution in the polar head group region of PL monolayers (*i.e.* for phosphatidylcholine).

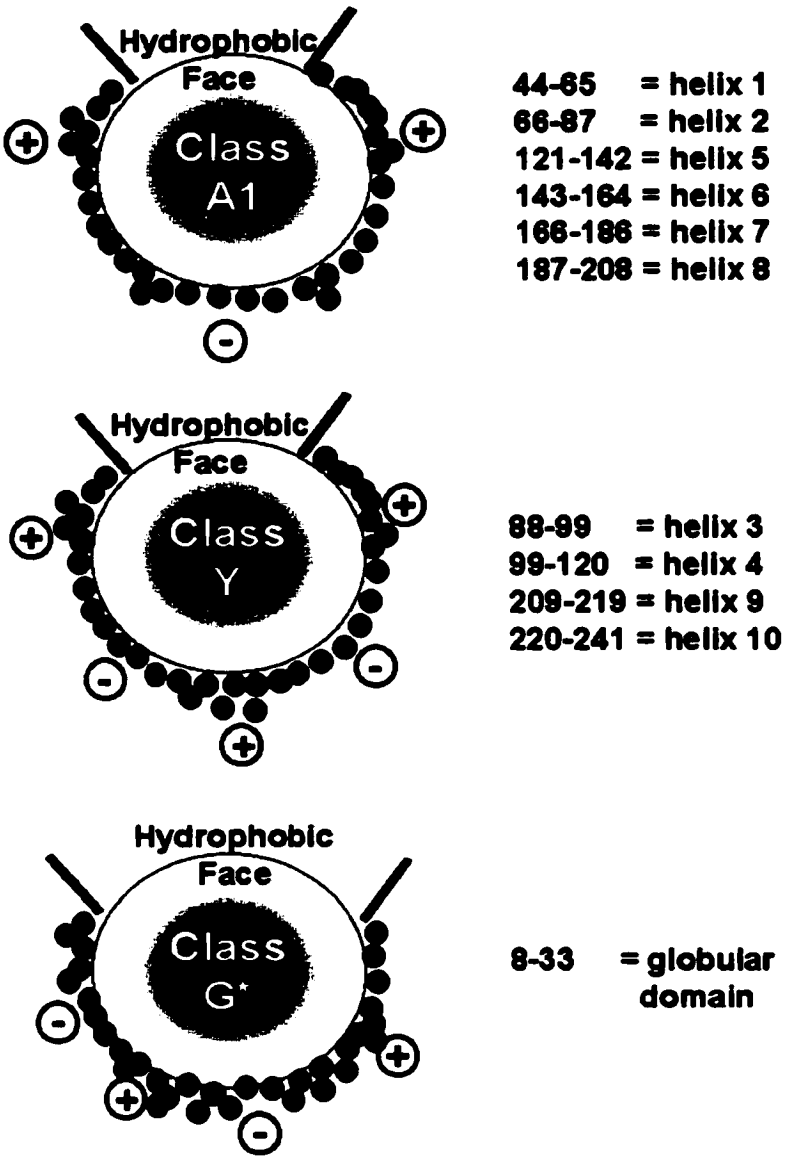


Figure 1.9: The Amphipathic α-Helices of ApoA-I

The distribution of positively and negatively charged amino acids around nonpolar faces of the three amphipathic α-helices in apoA-I is illustrated (looking into the helix). The class G* helix (aa 8-33) is more reminiscent of amphipathic α-helices found in globular proteins that have average hydrophobicity and hydrophobic moment characteristics. The class A helix, the primary amphipathic α-helix in apoA-I, has characteristic distribution of positive charges at the nonpolar-polar interface and of negatively charged amino acids at the polar face. The class Y amphipathic α-helix is similar to the class A motif in the distribution of positively charged residues at the nonpolar-polar interface but also has a cluster of positively charged residues at the polar face. Both the class Y and class A amphipathic α-helices have a high mean hydrophobic moment.

This is believed to be important for initiating association of apolipoproteins with the surface of PL monolayers (394). The class G* helix does not have this charge distribution to initiate and form stable contacts with PL. This fits with the observation that apoE has five class G* amphipathic α-helices in the N-terminus, a region of the protein that contains the receptor binding domain but has low lipid binding affinity (395,396). Furthermore, synthetic peptides corresponding to amino-terminal fragments of apoA-I, apoA-I[1-33] and apoA-I[8-33], only associate weakly with lipids (397). Therefore, these studies suggest that the N-terminal amino acids encoded by exon 3 (aa 1-43) do not contribute significantly to the lipid binding properties of apoA-I, although this might not be true in all instances (see below).

An important question with regards to the remainder of the protein encoded by exon 4 (aa 44-243) is whether specific lipid binding domains exist within this region or do all amphipathic α-helices contribute equally and cooperatively in lipid binding? Different techniques including the use of synthetic peptides, proteolytic fragments of apoA-I, and molecular biological approaches have been employed to address this question [reviewed in (369,394)]. Initially, Segrest and colleagues proposed that the class Y motif was not as effective as the class A helix in PL binding despite having a similar hydrophobic moment (394). This was based mainly on theoretical calculations (not as efficient in "snorkeling" [see above]) and the fact that apoA-IV, an apolipoprotein with reduced binding affinity compared to apoA-I (398), contains numerous class Y amphipathic α-helices. In contrast, a number of studies (reviewed here) indicated that the C-terminus of apoA-I, containing the class Y amphipathic α-helices, is most important for lipid binding. First, Minnich *et al.* showed that deletion of the C-terminus (aa 213-243) prevented the association of apoA-I with HDL, whereas deletion of central regions of the protein was less detrimental to lipid binding (399). Studies by Holvoet *et al.* (400) and Ji and Jonas (401) corroborated this finding and showed that deletions in the C-terminus significantly

decreased the ability of apoA-I to solubilize dimyristoyl-phosphatidylcholine (DMPC) in aqueous solution (measured as a reduction in turbidity at 325 nm). This DMPC clearance assay is often used to assess the lipid-binding kinetics of apolipoproteins (see Chapter 2 – Experimental Model and Methods). Furthermore, three ^{125}I-labeled apoA-I carboxyl terminal deletion mutants were found to be associated with smaller denser HDL and cleared faster from the circulation when injected into mice compared to native apoA-I (402). This illustrates the importance of the C-terminal class Y amphipathic α-helices in lipid binding and in maintaining normal plasma apoA-I and HDL concentrations.

A panel of synthetic peptides were also created to evaluate the lipid affinities of the individual or adjacent apoA-I amphipathic α-helices (380,403). In the first study (380), synthetic peptides corresponding to the six 22-mer amphipathic α-helices between aa 44-243, but not to the two class Y 11-mer helices (aa 88-99 and aa 209-219), were created. Interestingly, only the two terminal amphipathic α-helices (aa 44-65 and aa 220-243) were found to have significant lipid binding affinity with the most important contribution provided by the C-terminal class Y helix (aa 220-243). This substantiates previous findings (documented above) and is compatible with a study showing that a class Y model synthetic peptide has a greater affinity for lipids than does an apoA-I class A model synthetic peptide (404), a reversal of Segrest's original hypothesis (394). In the follow up study (403), larger synthetic peptides were produced (either 33-, 44-, or 55-mers in length) corresponding to adjacent amphipathic regions within apoA-I. It was found that the peptide corresponding to helices 9-10 (peptide 9-10) had the highest lipid binding affinity, which was greater than the affinity of peptide 10 alone. Yet, a shortcoming of these synthetic peptide studies was the failure to show cooperation, amongst and the involvement of, other apoA-I helices in lipid binding (with the possible exception of helix 1). This certainly occurs since full-length apoA-I

is ten times more efficient (on a molar basis) than is peptide 9-10 in reducing the gel to liquid-crystalline phase transition of DMPC multilamellar vesicles (MLVs) (403).

Our laboratory has gone on to show that a central domain and possibly the N-terminus of apoA-I also contributes to the overall lipid binding by apoA-I. Recombinant apoA-I proteins were produced in *E. coli* containing deletions within the central domain of apoA-I (405). The three mutants referred to as Δ4-5A-I, Δ5-6A-I, and Δ6-7A-I contain deletions 100-143, 122-165, and 144-186, respectively. The Δ4-5A-I mutant has a decreased stability on reconstituted lipoproteins compared to the other two mutants and native human apoA-I (hapoA-I) as assessed by GdnHCl denaturation studies. This implies that helix 4, a class Y amphipathic α-helix, is important for the stability of apoA-I on HDL. The work outlined in this thesis substantiates and expands upon these initial results (Chapter 3 – The Role of the ApoA-I Central Domain in the Maturation of HDL). Particularly, the *in vivo* experimental model (described in Chapter 2) I developed allows us to address the effect of these central domain mutations on the lipid composition and morphology of HDL in a physiologically relevant system. Also, not to be overlooked, is a potential role of the class G* helix corresponding to aa 8-33 in lipid binding by apoA-I. Previous results suggest that the extreme N-terminus does not contribute to lipid binding of apoA-I (as detailed above). However, studies carried out in our laboratory (manuscript in preparation) have shown that removal of this domain can affect the DMPC clearance by apoA-I and the maturation of HDL in mice. Recently, the G* motif was found to have moderate lipid affinity (403) and form an amphipathic α-helix in a lipid mimetic environment (368). Therefore, although the C-terminus of apoA-I is essential for lipid binding other domains in the protein are also important. As will be outlined, the central lipid-binding domain of apoA-I does not co-localize with the region of the protein responsible

for the activation of LCAT, and the implications of these findings will be addressed in Chapter 3.

1.4.4 - Lipid Efflux and the Amphipathic α-Helix

The net movement of cellular PL and FC to acceptor particles such as apoA-I is referred to as lipid efflux. With the exception of a few tissues, such as the liver, intestine, and endocrine glands, the efflux of FC is the sole mechanism by which cells can remove this potentially atherogenic lipid [reviewed in (406)]. As discussed above (section 1.3.3), this process is also important for HDL maturation and represents the first step in reverse cholesterol transport. The newly ascribed role for ABCA1 in this process (section 1.3.3) has been an exciting development and has raised many questions. Does apoA-I or other exchangeable apolipoproteins bind directly to ABCA1 on the cell surface and if so are there specific efflux domains required for these interactions? Furthermore, what are the roles of the different classes of amphipathic α-helices found within the exchangeable apolipoproteins (section 1.4.3) in this process?

The efflux of lipids can occur by two independent mechanisms. Diffusional efflux is a passive process in which lipids desorb from the cell membrane and are transferred to acceptor particles such as α-migrating HDL. This process is independent of ABCA1 and can also occur with other acceptors such as PL vesicles and whole serum. Fielding and Fielding have reviewed the process of diffusional efflux (407). This is a slow efflux pool that is not affected by protease-treatment of cells (*i.e.* not receptor dependent). It is also bi-directional in that acceptor particles in turn can deliver cholesterol and PL to the cells. Therefore, the net flux (*i.e.* net efflux versus net influx) depends on the lipid compositions of both the cell membranes and acceptor particles. The process of diffusional efflux is increased by the presence of

apoA-I or amphipathic α-helical peptides in acceptor particles. Davidson *et al.* showed that that DMPC vesicles containing either apoA-I or class A synthetic peptides had significantly increased efflux compared to DMPC vesicles alone (408). There are mixed results as to whether any apoA-I domains are involved in this process. Antibodies to an epitope in apoA-I covering aa 74-111 inhibited cholesterol efflux suggesting that this region of the protein is involved (408,409). Other studies using antibodies directed against distinct epitopes suggest that a region around residue 165 (410) and between aa 140-150 (411) are also important for efflux. Nonetheless, these studies are complicated by potential steric hindrance of the antibodies that might cause a nonspecific reduction in cholesterol efflux. In contrast, apoA-I deletion mutants covering the entire sequence of apoA-I are equally as effective as native apoA-I in promoting the efflux of cellular cholesterol to reconstituted lipoproteins (412-414). These latter studies, including those from our laboratory, suggest that no specific sequences within apoA-I are responsible for the diffusional efflux of cholesterol.

The second lipid efflux mechanism involves the energy-dependent transfer of lipids to lipid-free apolipoproteins, primarily apoA-I. In contrast to diffusional efflux, this efflux is both saturable and unidirectional [reviewed in (406)]. It also requires at least one functional copy of ABCA1, which appears to be the rate-limiting protein in this process (see section 1.3.3). At first glance there does not appear to be specific sequence requirements in apoA-I that stimulate the efflux of cellular cholesterol and PL. ApoA-II (415), apoA-IV (416), apoCs (417), and apoE (418) in addition to amphipathic synthetic peptides (419,420) can all promote the efficient efflux of cholesterol and PL from cells. Furthermore, Remaley and colleagues found recently that apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE were all equally effective as lipid-free proteins in promoting lipid efflux from HeLa cells stably-expressing an ABCA1-green fluorescent protein (ABCA1-GFP) fusion protein (421).

However, studies by Phillips and colleagues showed that only synthetic peptide mimetics of the N-terminal helix (aa 44-65) and more importantly the C-terminus class Y amphipathic α-helices (aa 209-241) of apoA-I could promote efflux of FC and PL from human skin fibroblasts (422). This suggests that important lipid binding α-helices within apoA-I, and possibly in other apolipoproteins, are also required for lipid efflux; a process that Phillips and colleagues have coined "microsolubilization".

Different cell types efflux lipids at different rates [reviewed in (406)]. Furthermore, it appears that not all cell types interact with lipid-free apoA-I in the same manner. Human skin fibroblasts were shown to have a decreased affinity for apoA-I compared to THP-1 macrophages (human transformed monocyte-derived cell line) (423). It was found that efflux of cholesterol to an apoA-I C-terminal deletion mutant missing aa 187-243 (Δ187-243A-I) was reduced by 50-70% compared to native apoA-I, whereas no difference in efflux for the two proteins was observed with human fibroblasts. Similar findings have been observed with a C-terminal mutant lacking only the last two class Y amphipathic α-helices (Δ210-243A-I) (Marcel lab unpublished observations). Therefore, this is one of the first demonstrations that the C-terminus of apoA-I is important for lipid efflux and that it is cell specific. Given the role of macrophages in atherosclerosis it is important to elucidate the mechanisms of lipid removal by apoA-I from these cells. Preliminary results suggest that apoA-I interacts via its C-terminal class Y amphipathic α-helices with a protein that is not ABCA1 in macrophages. The identification and localization of this putative binding site is ongoing.

1.4.5 - Activation of LCAT by ApoA-I

The activation of LCAT by apoA-I is a key step in HDL maturation and reverse cholesterol transport. Numerous studies have attempted to define the minimal sequence

requirements within apoA-I that are necessary for the efficient conversion of FC to CE on HDL. ApoA-I was first shown to activate LCAT by Fielding and colleagues in the early 1970s (209). However, it was close to another twenty years before mutagenesis studies were performed to localize the LCAT activation domain within apoA-I. Prior to mutagenesis studies, monoclonal antibodies and synthetic peptides were employed to map the apoA-I LCAT activation domain [reviewed in (369)]. In the last ten years there is growing evidence to suggest that the major LCAT activation domain within apoA-I is centered on helix 6 (aa 143-164) [reviewed in (369)]. This has come principally from *in vitro* studies performed with reconstituted lipoprotein particles and purified LCAT.

Deletion mutagenesis studies performed by Minnich *et al.* (399) and Sorci-Thomas *et al.* (424) first proposed that different regions within apoA-I were involved in the activation of LCAT. The greatest effects were deletions that removed residues between 148-186 (helices 6 and 7) in apoA-I, although deletions in the C- and N-terminus also affected LCAT function. However, the lipoprotein substrates prepared in these studies varied in lipid composition, size and the number of apoA-I proteins per particle or were not characterized at all. These complications therefore did not allow for an accurate assessment of regions within apoA-I that activated this enzyme. Subsequent studies from our laboratory (412) and others (400,412,425-428) using better defined lipoprotein substrates and various deletion mutagenesis strategies suggested that helix 6 (aa 144-165) is required for efficient activation of LCAT. Yet, none of these studies addressed the maturation of HDL *in vivo* and were limited by the use of non-physiological and artificial lipoprotein substrates. Clearly, confirmation of these *in vitro* findings in an *in vivo* model will aid in characterization of the apoA-I LCAT activation domain. Therefore, three deletion mutants used previously in our laboratory for *in vitro* studies were analyzed for their abilities to activate this enzyme *in vivo* (Chapter 3 – The Role of the Central

Domain of ApoA-I in the Maturation of HDL). The implications of these findings and how they relate to previous *in vitro* studies is addressed in Chapter 3.

Some naturally occurring apoA-I mutants are associated with significant decreases in plasma HDL-C concentrations. These mutations are interesting from a structure-function viewpoint since their location implies an important functional domain within the protein. Many apoA-I mutations have been identified, most of which result from single amino acid substitutions [reviewed in (369,429)]. The study of these mutants also overcomes some of the potential complications that could arise from the study of larger deletions in apoA-I, many of which have been used previously to study LCAT activation (see above). Interestingly, most of the naturally occurring apoA-I mutations that are associated with low HDL-C concentrations occur within helix 6, a region of the protein that also shows high conservation amongst species (346). These include point mutants apoA-I Finland (apoA-I_{FIN} – Leu^{159} → Arg) (430), apoA-I Oslo (apoA-I_{Oslo} Arg^{160} → Leu) (431), apoA-I Oita (apoA-I_{Oita} Val^{156} → Glu) (432), apoA-I Paris (apoA-I_{Paris} Arg^{151} → Cys) (433), and a deletion mutant known as apoA-I Seattle (apoA-$I_{Seattle}$ Δ146-160A-I) (434). All of these mutants have impaired LCAT activation and all but apoA-$I_{Seattle}$ have normal lipid binding capabilities (369). Therefore, studies of these mutants have provided further support that helix 6 within apoA-I is the major LCAT activation domain. Moreover, replacement of three highly conserved Arg residues (R149, R153, R160) for Gln within helix 6 decreases LCAT activation by apoA-I without altering lipid binding (383). Therefore, the positively charged Arg residues in helix 6 may be distributed in such a manner so as to make important electrostatic interactions with negatively charged amino acids in LCAT (*i.e.* aa 152-169) (369).

Most of the naturally occurring apoA-I mutants that do affect HDL levels are co-dominant resulting in a 40-50% reduction in HDL-C and apoA-I concentrations in

heterozygous carriers [reviewed in (369)]. However, both apoA-I_{FIN} and apoA-$I_{Seattle}$ are dominant negative mutants that result in 80-90% reductions in HDL-C and apoA-I concentrations in heterozygous carriers. The mechanisms by which these two mutants lower HDL-C and apoA-I concentrations are not clear. ApoA-$I_{Seattle}$ has both reduced LCAT activation and altered lipid-binding properties that contribute to its dominant negative phenotype (435). ApoA-I_{FIN} has only impaired LCAT activation *in vitro* (436), which alone does not appear to account for the drastic lowering of HDL-C levels in heterozygous carriers. One possibility is that both these mutations interfere with LCAT activation of native apoA-I as well and that this could account for their dominant negative effects. In this thesis, the effects of the apoA-I_{FIN} mutation on HDL metabolism are addressed further, and the possibility that this mutation interferes with LCAT activation by native apoA-I is tested. A combined *in vivo*, *ex vivo*, and *in vitro* approach is used to carry out studies on this mutant. (Chapter 4 – Metabolic Studies of an ApoA-I Mutant Associated with Dominantly Inherited Hypoalphalipoproteinemia).

In summary, the identification of the major LCAT activation domain within apoA-I has been localized to helix 6. Studies from work described within this thesis provide the first data that deletion of this helix (Chapter 3) and a point mutation within it (Chapter 4) drastically alters the maturation of HDL *in vivo*. However, it should be noted that mutations in adjacent amphipathic α-helices (helices 5 and 7) and within the N-terminus (437) (and B. Scott unpublished observations) can also affect LCAT function. More careful analysis of these other domains within apoA-I is therefore required. Nonetheless, it is apparent that the distribution of positively charged Arg residues within helix 6 of apoA-I is important for LCAT function, since either an increase (*i.e.* apoA-I_{FIN}) or decrease (*i.e.* apoA-I_{Oslo}) in the net positive charge affects the activation of this enzyme. Future studies need to address the nature of

these electrostatic interactions and identify the LCAT domain that interacts with helix 6 of apoA-I.

1.4.6 – Rationale

ApoA-I lies at the center of HDL metabolism and there is still much to be learned about structure-function relationships of this protein. Importantly, prior to this work there were few if any *in vivo* studies that addressed the effect of apoA-I mutations on HDL metabolism *in vivo*. Recombinant adenoviruses lend themselves nicely to the study of apoA-I structure-function relationships and are the basis for the *in vivo* and *ex vivo* studies described in this thesis (section 2.1). These studies not only confirm previous *in vitro* data but also provide new information on apoA-I structure-function relationships in a physiologically relevant animal model. We have shown that the central domain of HDL has two distinct functions in HDL maturation, which supports and extends previous *in vitro* studies (Chapter 3). The infection of primary hepatocytes with recombinant adenoviruses has provided novel insights into the effects of the apoA-I_{FIN} mutation on apoA-I secretion from hepatocytes (Chapter 4). Furthermore, we have developed an elaborate metabolic system during the course of studying apoA-I_{FIN} that incorporates *in vivo* and *ex vivo* results with results obtained from *in vitro* studies of this mutant (section 2.1). This approach will be useful in studying other apoA-I mutations associated with hypoalphalipoproteinemia and should further our understanding of apoA-I structure-function relationships.

Another aspect of HDL metabolism that is not clearly understood is the nature of apoA-I particles secreted by hepatocytes. Most studies suggest that apoA-I is secreted as lipid-free or as lipid-poor particles (so-called nascent HDL). The composition and heterogeneity of these nascent pre-β HDL are not known. I have utilized recombinant adenoviruses to look at

secretion of human apoA-I from primary murine hepatocytes and have discovered that apoA-I is secreted in three different lipoprotein pools (Chapter 5 – Heterogeneity and Lipidation of Human ApoA-I Secreted by Primary Hepatocytes). Interestingly, ABCA1 is expressed at extremely high levels in hepatocytes (438). Others (439) as well as ourselves, propose that ABCA1 might be important for the lipidation of nascent HDL that are secreted by hepatocytes. The methods currently developed in the lab (Chapter 5) should enable us to address this issue, and determine the importance of hepatocyte ABCA1 in the metabolism of HDL.

CHAPTER 2 – EXPERIMENTAL MODEL AND METHODS

2.1 – Experimental Model

2.1.1 - Utility of Recombinant Adenoviruses for In Vivo and Ex Vivo Studies

Recombinant (or replication-deficient) adenoviruses hold promise as gene-therapy vectors. While progress has been made in modifying adenoviruses to make them more amenable to gene therapy there are still many inherent problems with their use in this area [reviewed in (440)]. Major obstacles have been the development of a host cellular immune response triggered by viral proteins and a lack of tissue specificity for the viruses [reviewed in (441)]. Nonetheless, recombinant adenoviruses have a number of other useful applications. They have been employed successfully to infect cells that are normally resistant to transfection (442,443). Furthermore, in cardiovascular research, recombinant adenoviruses have been used to study the effects of transgenes on the progression and/or reversion of atherosclerosis in mice (444-446). Here, we have utilized recombinant adenoviruses to study structure-function relationships of human apoA-I in mice. This approach has a number of advantages over transgenic mice that express human apoA-I mutations. For one, the construction of transgenic mice is time-consuming and costly. Second, a number of recombinant adenoviruses carrying various apoA-I mutations have been created (point and deletion) in our laboratory. This allows for a more comprehensive evaluation and comparison of the effects of the different apoA-I mutations on HDL maturation *in vivo*. We have also taken advantage of apoA-I deficient mice for most of our injections to circumvent potential complications that could arise due to the presence of native murine apoA-I. As well, adenoviruses target the liver (>95%) when injected intravenously into mice (section 2.1.3), a major tissue for apoA-I synthesis and secretion. But perhaps most importantly, the maturation of HDL *in vivo* is compatible with the time course of expression following gene transfer by 1st generation

recombinant adenoviruses (section 2.1.3). Therefore, this approach has allowed us to provide one of the first in-depth studies of apoA-I structure-function relationships in a physiologically relevant animal model.

2.1.2 - Production of 1st Generation Recombinant Adenoviruses

The 1st generation recombinant adenoviruses (Ad) were produced as outlined by Graham and colleagues and are based on the adenovirus type 5 serotype (Ad5) (447). Briefly, the replication of recombinant adenoviruses requires a human embryonic kidney cell line known as 293 (448). This cell line contains 12% of the Ad5 genome known as E1 (early genes) and complements the growth of recombinant adenoviruses deficient in this region (see below). The apoA-I cDNAs including the region encoding for the prepro sequence (ppapoA-I) are incorporated into a plasmid known as pCA13 (447) by standard molecular biological techniques (section 2.2.1). The plasmid contains a constitutively active cytomegalovirus (CMV) promoter and a simian virus 40 (SV40) polyadenylation signal flanking the 5' and 3' ends of the apoA-I cDNAs, respectively. Importantly, the plasmid also contains the flanking sequences of the E1 region surrounding the promoter and polyadenylation sites. These E1 sequences are required for homologous recombination in 293 cells with plasmid pJM17 that contains the adenovirus genome (449). pJM17 was constructed with the sequences from plasmid pBR322 (4.3 kbp) inserted into the E1 region. Effectively, this makes the DNA (40.2 kbp) too large to be packaged into adenovirus capsids (38 kbp is maximum). Recombination in 293 cells between pJM17 and pCA13 via the E1 flanking sequences removes pBR322 and much of the E1 region from pJM17. At the same time, the apoA-I cDNA (either native or mutant) is inserted into the adenovirus genome. The resulting DNA is smaller by approximately 3 kbp ($\approx$ 37 kbp) and of a size that can be packaged into virus capsids. In this

manner, only the DNA that has recombined to include the apoA-I cDNA can form viable adenovirus particles in 293 cells (Fig. 2.1).

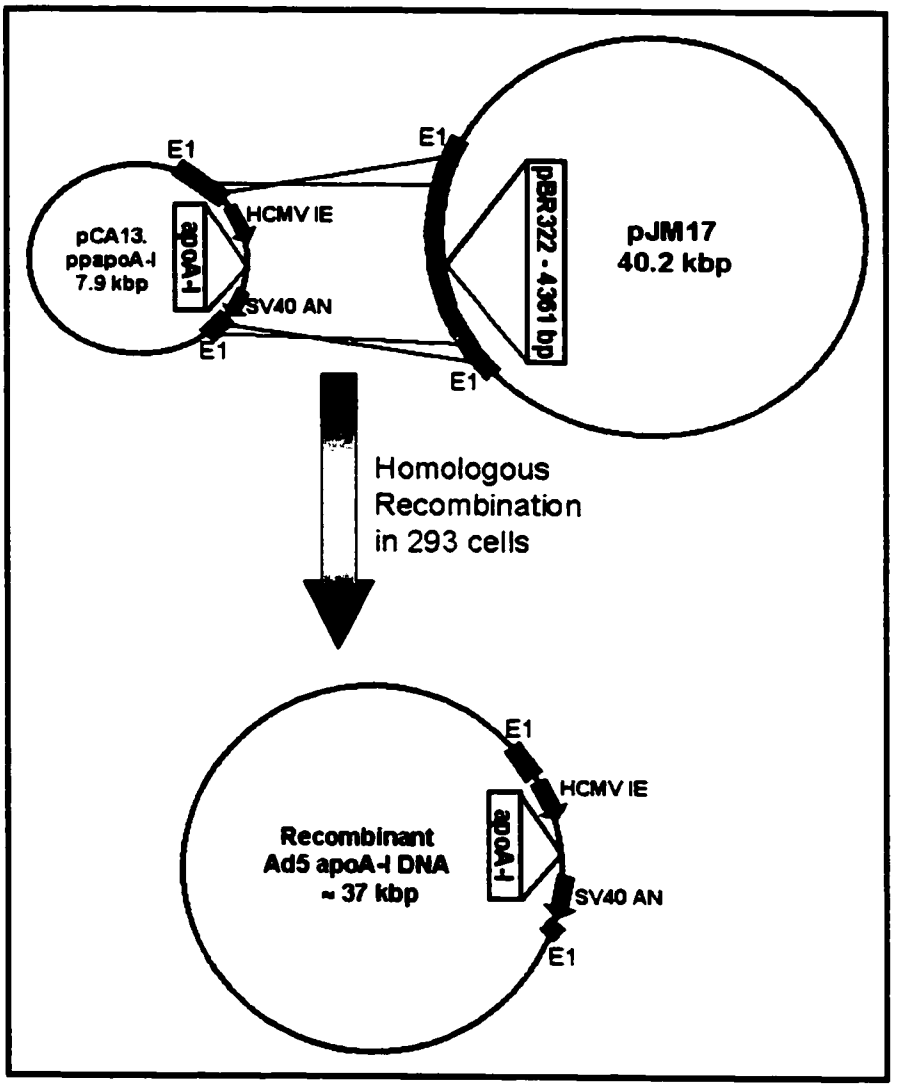


Figure 2.1: Construction of Replication-Deficient Adenoviruses by Homologous Recombination in 293 cells.

The cDNAs for native human and mutant apoA-I are cloned into pCA13 (pCA13.ppapoA-I). Each pCA13.ppapoA-I is co-transfected into 293 cells with pJM17. Homologous recombination between flanking E1 sequences in pCA13.ppapoA-I and the E1 region gene in pJM17 (top) generates a smaller adenovirus genome deleted of most of the E1 region and the pBR322 sequences (bottom). In place, the apoA-I cDNA flanked by CMV promoter (HCMV IE) and the SV40 polyadenylation sequences (SV40 AN) at the 5' and 3' ends, respectively, is inserted. The DNA is approximately 3 kbp smaller and can now be efficiently packaged in 293 cells into virus particles

2.1.3 - Recombinant Adenoviruses for In Vivo and Ex Vivo Studies

The *in vivo* and *ex vivo* structure-function studies of apoA-I are made possible because the recombinant adenoviruses infect hepatocytes very efficiently in mice and in culture, respectively. In fact, Herz and Gerard showed that greater than of 99% of recombinant adenoviruses injected into mice target the liver (450). The cellular uptake is mediated following binding of adenoviruses to the coxsackievirus receptor (451), which is highly expressed in murine hepatocytes (452,453). The adenovirus DNA is unpacked in the nucleus following internalization and subsequent movement of the virus through the nuclear pore complex. The DNA rarely integrates into the host chromosome and is usually maintained as a linear episome (454). Recombinant adenoviruses that are deleted in E1 are not packaged into new viruses (requires 293 cells for this) and the apoA-I transgenes are transcribed efficiently in murine hepatocytes by RNA polymerase II acting at the CMV promoter. The apoA-I mRNA is translated and the proteins are secreted from hepatocytes into the circulation (*in vivo* studies) or medium (*ex vivo* studies) where HDL maturation is monitored.

Transgene expression following injection of 1st generation recombinant adenoviruses typically lasts three to four weeks (455). Although transient, this time course is sufficient for studying structure-function relationships of proteins *in vivo*. This approach was used to make HL and LPL hybrids and compare structure-function aspects of these two lipolytic enzymes in mice (456). On this note, I have established that native human apoA-I circulates for greater than thirty days in mice following injections of a standard adenovirus dose (2×10^9 plaque forming units [pfu]) (see Chapter 3). This time course of expression also establishes the times of peak apoA-I expression and when apoA-I reaches physiological concentrations. This is the basis for comparisons to adenoviruses carrying different apoA-I mutations (Chapters 3 and 4).

One possible drawback of 1st generation recombinant adenoviruses is that they may illicit nonspecific effects on HDL metabolism not directly attributable to the apoA-I transgene. This could result from expression of the viral proteins and the associated immune response that follows. To minimize and account for these non-specific effects, a firefly luciferase reporter adenovirus (luc.Ad5) was created (section 2.2.1). Mice injected with luc.Ad5 were used to establish the baseline lipid and HDL concentrations in the mice. Furthermore, most comparisons were made at early time points after the adenovirus injections (0-6 days) when the immune response would be less significant.

Isolated murine hepatocytes are also easily infected with the recombinant adenoviruses and were used for *ex vivo* studies (Chapters 4 and 5). Primary hepatocytes from apoA-I deficient mice were prepared and used to establish an effect of the apoA-I_{FIN} mutation on apoA-I secretion (Chapter 4) following infection with the recombinant adenoviruses. This complements the *in vivo* (see above) and *in vitro* studies (section 2.1.4) of this apoA-I point mutation. The hepatocytes were also used to study secretion of hapoA-I (Chapter 5). The purpose of this study was to determine if apoA-I is secreted as a uniform population or as a heterogeneous nascent HDL pool. This study lays the foundation for future work to address the factors responsible for the lipidation of nascent HDL secreted by hepatocytes and the importance of this to HDL metabolism. This to our knowledge is the first study to report on the secretion of both native and mutant apoA-I from primary hepatocytes following infection with recombinant adenoviruses.

2.1.4 - Recombinant Proteins for In Vitro Studies

Recombinant His-tagged apoA-I proteins, purified from *E. coli*, were used to study the effects of the apoA-I_{FIN} mutation on lipid binding, cholesterol efflux, and LCAT activation *in*

vitro (Chapter 4). Recombinant apoA-I_{FIN} (rec.apoA-I_{FIN}) was generated according to methodologies established in our laboratory (405) (section 2.2), and compared in these assays to recombinant wild-type apoA-I (rec.hapoA-I) previously produced in our laboratory. The His-tag 11 aa extension at the N-terminus (MRGSHHHHHHM) facilitates purification of recombinant apoA-I but does not interfere with either structural or functional properties of the protein (405). Lipid binding was assessed by DMPC clearance (kinetics of binding) and GdnHCl denaturation (thermodynamics of binding) studies. Studies were also performed to address the effect of apoA-I_{FIN} on both diffusional and specific cholesterol efflux from macrophages. Lastly, Lp2A-I prepared with rec.hapoA-I, rec.apoA-I_{FIN}, or both were prepared for LCAT activation experiments. These *in vitro* studies when combined with the *in vivo* and *ex vivo* studies provide a more detailed analysis of apoA-I_{FIN} and insights into the mechanisms responsible for the hypoalphalipoproteinemia in heterozygous carriers of this mutation.

2.2 - Methods

2.2.1 - Production and Screening of First Generation Recombinant Adenoviruses

The cDNAs for firefly luciferase (luc), hapoA-I, Δ4-5A-I (deletion of aa 100-143), Δ5-6A-I (deletion of aa 122-165), and Δ6-7A-I (deletion of aa 144-186) were subcloned from pBlueScript (SK^+) into the vector pCA13 under the control of the CMV promoter (Microbix Biosystems Inc., Toronto, ON, Canada). The sequences for all cDNAs were confirmed by sequencing following subcloning into pCA13. Recombinant adenovirus (Ad5) constructs carrying these cDNAs were prepared following co-transfection of 293 cells (Microbix Biosystems Inc.) with the plasmid pJM17 and the appropriate pCA13 plasmid. Lipofectamine (Life Technologies, Burlington, ON, Canada) was used as the transfection reagent. After an

8 h incubation with the transfection mixture in OptiMeM medium (Life Technologies), the 293 cells were incubated overnight with Eagle's Minimal Essential Medium (EMEM) containing 10% fetal bovine serum (FBS) and then overlaid with 0.65% SeaPlaque agar (FMC BioProducts, Rockland, ME) in EMEM media containing FBS (5%), penicillin (100 U/ml) and streptomycin sulphate (100μg/ml). Plaques were picked (1-2 weeks later) from the agar and resuspended in sterile phosphate buffered saline (PBS, 137mM NaCl, 8.2mM Na_2HPO_4, 1.5mM KH_2PO_4, 2.7mM KCl) containing $CaCl_2$ (0.68mM), $MgCl_2$ (0.50mM) (PBS^{2+}), and glycerol (10%). These plaques were used to infect subsequent confluent monolayers of 293 cells in order to amplify the recombinant adenoviruses. PCR of DNA prepared from SDS (0.5%) and pronase (0.05%) lysed 293 cells infected with the appropriate Ad5 construct was performed to detect the apoA-I cDNAs or luc cDNA.

The apoA-I_{FIN} cDNA was generated by the QuikChange™ mutagenesis protocol from Stratagene (La Jolla, CA) with sense 5'-GTGGACGCGC***GG***CGCACGCATC-3' and antisense 5'-GATGCGTGCG***CC***GCGCGT-3' primers (Life Technologies, Inc.). The bold and italicized sequence indicates the mutagenic bases required for the Leu to Arg conversion at aa 159 within apoA-I. For generation of the Ad5 carrying the apoA-I_{FIN} cDNA, mutagenesis was carried out on plasmid pCA13 harboring the hapoA-I cDNA. Positive clones were determined by the loss of the *FspI* restriction site that results from a T to G substitution for this mutation as documented previously (430). The full-length apoA-I_{FIN} cDNA was sequenced prior to generation of the apoA-I_{FIN}-Ad5 as described above.

Secretion of the different apoA-I proteins was confirmed in culture by infecting COS-7 cells with the adenoviruses prior to large-scale purification. Medium was analyzed 2-3 d after infection by 12 % SDS polyacrylamide gel electrophoresis (SDS-PAGE) performed under reducing conditions, which was then subjected to Western blot analysis following transfer to

nitrocellulose membrane. The membrane was probed with anti-hapoA-I monoclonal antibodies 4H1 (against the extreme N-terminus – epitopes 1 to 8) and 5F6 (against the central region – epitopes 118-148) and the apoA-I proteins in the medium were detected by chemiluminescence (WestPico SuperSignal Substrate, Pierce) following treatment with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Pharmacia Amersham Biotech).

All adenoviruses were then subjected to several rounds of amplification prior to a final purification on two successive CsCl gradients. The purified virus stocks were dialyzed extensively against PBS^{2+} containing 10% glycerol, aliquoted through sterile 0.22 μm filters (Millipore Corporation, Bedford, MA), and pfu per ml were determined by incubating fresh monolayers of 293 cells (60 mm dishes) with the appropriate dilution (usually 10^{-9}) of adenovirus stock. The viral particle number was also determined by the $A_{260\ nm}$ according to standard protocols (457). Comparisons show that 18-20 virus particles equate to a single pfu.

2.2.2 - Animals

ApoA-I deficient (*ApoA1*^{tm1Unc}) C57BL/6J (458) and wild-type C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and Charles River Laboratories (Wilmington, MA), respectively. Mice were maintained on a 12 h light/12 h dark schedule and were fed either a normal chow (Charles River rodent diet 5075 – 18% protein and 4.5% fat) or high fat Western (Harlan Teklad TD 88137 – 19.5% protein, 42% fat, 0.15% cholesterol) diet as indicated. All experiments were performed in accordance with protocols approved by the University of Ottawa Animal Care Committee. Mice used for these studies were 3-8 month old females, except where indicated.

2.2.3 - Adenovirus Injections and Lipid Analysis

Lipid measurements were performed on the plasma of fasted (8-10 h) and non-fasted mice (fed state). Briefly, blood was collected into EDTA(K3) microcapillary tubes (Sarstedt Inc., St-Leonard, Québec, Canada) following clipping of the tail vein and plasma was subsequently isolated by centrifugation (5 min at 4000 ×g). The plasma samples were immediately placed on ice and incubated with a protease inhibitor mixture consisting of aprotinin (0.15 μM), leupeptin (1 μM), and a protease inhibitor cocktail (complete EDTA-free®) (Roche Molecular Biochemicals). TC and FC levels were measured with enzymatic kits (Roche Diagnostics, Laval, Québec, Canada) as was PL (Wako Chemicals, Neuss, Germany) using freshly isolated plasma samples. CE levels were determined by subtracting FC from TC. These values were used for pre-adenovirus injection lipid levels. Mice were allowed to recover at least two days prior to the injection of the recombinant adenoviruses. For the central domain deletion mutants (Chapter 3), all adenoviruses were injected into the tail vein of apoA-I deficient mice at a final dosage of either 2×10^9 or 1×10^{10} pfu as indicated. For the apoA-I_{FIN} studies (Chapter 4), wild-type and apoA-I deficient C57BL/6J mice were injected via the tail vein with either the hapoA-I.Ad5 or the apoA-I_{FIN}.Ad5 at a dose of 2×10^9 pfu. ApoA-I deficient mice were also co-injected with 1×10^9 pfu of each of the hapoA-I.Ad5 and the apoA-I_{FIN}.Ad5. To monitor the efficiency of each tail vein injection, 2×10^7 pfu of *luc*.Ad5 was included in the injections of the Ad5 carrying the apoA-I cDNAs. For the time course studies, blood was collected via the tail as described above. For mice that were anesthetized, blood was collected into EDTA(K3) vacutainer tubes following a laparotomy and subsequent clipping of the descending aorta. The liver was removed, weighed (typically 1.0 to 1.3 g wet weight), and homogenized on ice in luciferase lysis buffer (1 ml). Luciferase activity was measured in a

Lumat LB9507 luminometer (EG&G Berthold) following addition of 10 µl luciferase assay reagent (Promega Corporation). Plasma was isolated by centrifugation at 3000 xg for 13 min and TC, FC, CE, and PL levels were determined as described above.

2.2.4 - Western Blot and Radioimmunoassay Assays of ApoA-I

Western blots for human (native or mutant forms) or mapoA-I were performed following separation of plasma or isolated lipoprotein fractions (see below) by 12% SDS-PAGE. Human apoA-I was probed, following transfer to nitrocellulose (BioRad electroelution apparatus), with monoclonal antibodies 4H1 and 5F6 that were biotinylated with Sulpho-NHS-Biotin (Pierce Chemical Co.). This was required to circumvent the use of HRP-conjugated anti-mouse IgG that would react strongly with mouse IgG in the plasma samples. ApoA-I was visualized by chemiluminescence, as described above, following treatment with Streptavidin-conjugated HRP (Amersham Pharmacia Biotech). MapoA-I was detected with an anti-mouse apoA-I antibody (BIODESIGN International, Kennebunk, ME) and chemiluminescence was carried out as above following treatment with anti-rabbit HRP-conjugated IgG (Amersham Pharmacia Biotech).

The concentrations of the different apoA-I variants in the plasma or isolated lipoprotein samples (see below) were determined by a solid-phase radioimmunoassay as described previously (392). The antibody used for the radioimmunoassay, A03, recognizes an epitope in the N-terminus of the protein that is present in all mutants. Plasma from non-injected apoA-I deficient mice was added to the human apoA-I standards to account for any cross-reactivity between monoclonal antibody A03 and potential antigens in the plasma. No cross-reaction was detected.

2.2.5 - Analysis of Murine Apolipoprotein E Expression

The effect of expressing the apoA-I central domain mutants on murine apoE levels in plasma was measured (Chapter 3). Briefly, plasma (1.5 µl) isolated from fasted mice prior to or following injection of luc.Ad5, Δ4-5A-I.Ad5, or Δ5-6A-I.Ad5 (2×10^9 pfu of each) was subjected to 12% SDS-PAGE. Following transfer to nitrocellulose (BioRad electroelution apparatus), the membrane was probed with a polyclonal anti-mouse apoE antibody (BIODESIGN International, Kennebunk, ME) and visualized by chemiluminescence after incubation with HRP-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Baie d'Urfé, Québec, Canada). The relative intensities of the apoE signals were determined with software from BioRad (Quantity One® – version 4.10).

2.2.6 – Isolation of Lipoproteins by FPLC

Plasma (500 µl) isolated following the different Ad5 injections (96 h) was loaded on two Superdex 200 columns (analytical grade - Amersham Pharmacia Biotech) connected in series with a total bed volume of approximately 400 ml and void volume of 100 ml. The columns were standardized with a mixture of high and low molecular weight markers of known Stokes' diameters (Amersham Pharmacia Biotech). Plasma was passed down the columns at a flow rate of 0.1 ml per min and 5 ml fractions were collected. The VLDL and LDL component of the plasma appeared in the void volume (fractions 9 to 12) on these columns. Large size HDL_2 particles were localized in fractions 15 to 19 (12.1 to 10.2 nm), HDL_3 particles in fractions 20 to 23 (9.7 to 8.2 nm) and smaller HDL fractions (*i.e.* pre-β HDL) containing albumin were in fractions 24 to 28. TC concentrations were measured in the various fractions. Briefly, samples were concentrated (5-fold by SpeedVac), resuspended in PBS, and cholesterol levels were quantified by standard enzymatic kits (section 2.2.3). Aliquots (100 µl) from each

fraction were analyzed for apoA-I by slot blot (BioRad Bio-Dot SF unit) analysis as described for the Western blots above (section 2.2.4). No background signal could be detected as indicated by analysis of plasma fractions isolated following the luc.Ad5 injections. Lipid levels (TC, FC, CE, and PL) in the HDL_2/HDL_3 FPLC fractions were measured as described above (section 2.2.3).

2.2.7 – Isolation of Lipoproteins by Discontinuous Gradient Density Ultracentrifugation

Plasma prepared from mice following the Ad5 injections were subjected to discontinuous gradient density ultracentrifugation. Briefly, each sample (0.5 to 1.0 ml) was brought to a final volume of 3 ml (1 mM EDTA) with the addition of dried potassium bromide (1 g KBr) and sucrose (50 mg). The discontinuous gradient consisted of plasma (3 ml on bottom) layered successively with ρ=1.21 g/ml KBr (2 ml), ρ=1.08 g/ml KBr (3 ml), and ρ=1.00 g/ml (3 ml top). The tubes were centrifuged for 18 h at 35,000 rpm on a Beckman L8-70M ultracentrifuge at 8°C and fractions (1 ml) were collected from top to bottom. The densities of the fractions were determined by comparing the readings measured with a refractometer (Fisher Scientific, Nepean, ON, Canada) to values obtained for solutions with known densities.

Aliquots of each of the fractions were dialyzed against PBS (0.025 µM filter disks – Millipore Corporation), placed in SDS sample buffer, and analyzed by 12% SDS-PAGE. The gels were either stained for protein (GELCODE Blue Stain – Pierce Chemical Co.) or transferred to nitrocellulose for Western blot analysis probing for either human apoA-I or mouse apoE. The apoA-I concentrations (wild-type and mutant) in the different lipoprotein fractions from VLDL to lipid-poor HDL were measured by radioimmunoassay as described (section 2.2.4).

2.2.8 - Agarose and Non-denaturing Gradient Gel Electrophoresis

To determine the charge of apoA-I HDL species, plasma was separated into α- and pre-β migrating HDL by agarose gel electrophoresis (Beckman Lipogel®, Beckman Coulter, Fullerton CA). The gels were transferred to nitrocellulose and then subjected to Western blot analysis as described above (section 2.2.4). HDL size was determined by non-denaturing polyacrylamide gradient gel electrophoresis (PAGGE) followed by Western blot analysis. Plasma or discontinuous gradient density samples (dialyzed against PBS) were applied to the 4-20% gradient gels (Novex®) that were run for 2200 volt.h and included high molecular weight markers (Amersham Pharmacia Biotech) that were biotinylated with Sulpho-NHS-Biotin (Pierce Chemical Co.). The gels were transferred at 4°C for 4 h with one change of transfer buffer. The Western blot was performed as described above (section 2.2.4).

2.2.9 - Electron Microscopy

HDL fractions were analyzed for their morphology by negative staining electron microscopy as described previously (459). The HDL were concentrated to 100-200 μg/ml as determined by the Markwell Lowry method (460) and mixed 1:1 (vol/vol) with 2% sodium phosphotungstate pH 7.4 (previously filtered through 0.22 μm membrane) before applying (10 μl) to carbon-coated Formvar grids (200 mesh). After 30 seconds on the grid, the samples were dried and analyzed in a Hitachi H-7100 electron microscope at 50,000 X or 70,000 X magnifications.

2.2.10 - Plasma Cholesterol Esterification Studies

Cholesterol esterification rates of plasma samples from apoA-I deficient mice expressing hapoA-I or Δ4-5A-I were analyzed as described (461) with the following modifications. The

apoA-I deficient plasma samples (30 µl) containing no apoA-I (luc. Ad5 injected mice), hapoA-I (10 µg), or Δ4-5A-I (10 µg) were diluted in PBS (final volume 250 µl) and incubated with ^{3}H-cholesterol on filter disks (5 µCi ^{3}H-FC) at 4°C overnight. Two aliquots (50 µl each) were removed and used as the time zero point and two aliquots were placed at 37°C for 30 min. Ethanol (1 ml) was added (2 h room temperature incubation) to the time zero and 30 min samples. The supernatant collected following centrifugation (10 min at 10,000 × g) was incubated with hexane carrier (3 ml containing 50 µg/ml CE and 100 µg/ml FC) and then evaporated to dryness under N_2. The samples were solubilized in chloroform (200 µl), spotted (50 µl) on thin layer chromatography (TLC) plates (Silica Glass Fiber – Gelman Science, Ann Arbor, MI), run in a hydrophobic solvent system (89% hexane, 10% diethyl ether, and 1% acetic acid), and FC and CE were visualized with iodine staining. Bands corresponding to FC and CE were cut and radioactivity was determined. The fractional cholesterol esterification rate (FER) is expressed as the percentage of FC converted to CE per h.

2.2.11 – In Vivo Clearance of Recombinant ApoA-I

Rec.hapoA-I, rec.Δ4-5A-I, rec.Δ5-6A-I, and Δ6-7A-I proteins were purified from *E. coli* as described previously (405). The proteins (150 µg of each) were iodinated with ^{125}I using the IODO-BEAD iodination reagent (Pierce Chemical Co.), dialyzed against PBS, and 1×10^{6} cpm of each was injected into tail veins of apoA-I deficient mice together with 2.5 µg of the corresponding unlabeled protein (100 µl final volume). Blood was collected via the saphenous vein during a period from 5 min to 24 h, and plasma was isolated as described (section 2.2.3). The radioactivity (cpm) remaining in plasma (10 µl) as a function of time following the

injections was determined by γ-counting. The percentage of the initial radioactivity was plotted as a function of time using the 3-5 min sample as time zero (not shown).

2.2.12 - Secretion of HapoA-I and ApoA-I_{FIN} from Primary Mouse Hepatocytes.

Primary hepatocytes were prepared from apoA-I deficient mice according to an established protocol (462,463). Briefly, the cells were seeded in fibronectin-coated (25 μg/well) 6-well plates at an initial density of 1-2 × 10^6 cells per well in William's medium containing penicillin (100 U/ml), streptomycin sulfate (100 U/ml), Fungizone® (250 ng/ml) (Life Technologies), and 10% FBS (Sigma). Cells were infected the following day (24 h) with either the hapoA-I.Ad5 or the apoA-I_{FIN}.Ad5 at a multiplicity of infection (moi) of 75:1 (pfu:cell) or with a mixture of hapoA-I.Ad5 and apoA-I_{FIN}.Ad5 each at a moi of 37.5:1 (total moi = 75:1). After 24 h of infection, the William's medium was removed and the hepatocytes were incubated Dulbeco's Minimal Essential Medium (DMEM) without methionine and cysteine containing 10% FBS (pre-labeling medium) for 1 h. Following this, the cells were pulse-labeled for 1 h with labeling medium (pre-labeling medium plus 150 μCi/well [^{35}S]-methionine). The labeling medium was then removed and growth medium (DMEM plus 10% FBS) was added. Cells and medium were collected at the various time points up to 4 h after the initial pulse and harvested as described by McLeod *et al.* (464). ApoA-I was immunoprecipitated from the media and cell lysates with anti-human apoA-I antisera (Roche Molecular Biochemicals) and protein G-sepharose (Amersham Pharmacia) and then subjected to 12% SDS-PAGE. The radioactivity associated with apoA-I at each time point was determined by phosphorimager analysis (Personal Molecular Imager® FX, Bio-Rad Laboratories, Mississauga) and is represented as a percentage of the initial cell apoA-I associated radioactivity counts (t=0). The

data presented is the mean of triplicate measurements plus and minus the standard error of the mean (± SE) and is representative of three independent experiments.

2.2.13 - Purification of Recombinant His-tagged Proteins.

To generate the purified rec.apoA-I_{FIN}, mutagenesis was carried out on plasmid pXL2116 as described above (section 2.2.1). This plasmid is a modified pET3a vector (Stratagene) which contains the apoA-I cDNA (minus the region coding for the prepro sequence) and an upstream region coding for an 11 aa N-terminal extension containing a 6X His tag as described previously (405). The recombinant His-tagged hapoA-I (rec.hapoA-I) and rec.apoA-I_{FIN} used for these studies were prepared as described previously (405) with some minor changes. *E. coli* strain BL21(DE3) (Stratagene) was used instead of strain BL21(DE3)pLysS (Stratagene), LB broth and not M9 minimal medium was used for expression of the proteins, and rifampicin was omitted from the bacterial cultures. Following purification on Ni-nitriloacetic acid agarose (NTA, Qiagen) columns, the proteins were dialyzed against sodium bicarbonate (5 mM - pH 8) containing EDTA (1 mM) and azide (0.02%), lyophilized and stored at -20 °C. Prior to the *in vitro* studies, the lyophilized recombinant proteins were solubilized in 6 M GdnHCl and dialyzed extensively against PBS or tris buffered saline as required.

2.2.14 - Preparation of Reconstituted Lipoproteins (Lp2A-I)

Discoidal reconstituted lipoproteins containing two apoA-I molecules per particle (Lp2A-I) were prepared by the cholate dispersion/Bio-Beads removal technique using a starting 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) / FC / apoA-I ratio of 80:10:1 as published by Sparks *et al.* (374). The homogeneity and hydrodynamic diameters of the Lp2A-I were determined by non-denaturing PAGGE as described previously (405). The reconstituted

lipoproteins were shown to contain two molecules of recombinant apoA-I by cross-linking with dimethyl suberimidate according to an established protocol (465). The Lp2A-I PL compositions were determined with an enzymatic kit (Wako Chemicals, Neuss, Germany) as was FC (Roche Diagnostics, Laval, Québec). The apoA-I concentrations were measured by the Markwell Lowry method (460) using bovine serum albumin (BSA) as the standard.

2.2.15 - Kinetics and Stability of Association with Lipids

The association of rec.apoA-I_{FIN} with DMPC was compared to native and rec.hapoA-I as described by Pownall *et al.* (466). Briefly, the $A_{325\,nm}$ was measured for a turbid solution of DMPC (1 mg/ml) at 24°C. The apoA-I proteins were then added (DMPC/protein molar = 50:1) and the reduction in turbidity was followed over a period of 30 min.

The stability of association of rec.hapoA-I and rec.apoA-I_{FIN} on the Lp2A-I was measured by CD spectroscopy using a Jasco J41A spectropolarimeter. The change in molar ellipticity at 222 nm in the presence of increasing amounts of GdnHCl was used to calculate the standard free energy of denaturation (ΔG_D^0) as described previously (374).

2.2.16 - Lecithin:cholesterol Acyltransferase Assay

LCAT was purified according to Albers *et al.* (467) and the cholesterol esterification studies were performed as outlined previously (468). The Lp2A-I used in these studies (initial POPC / FC / apoA-I ratio of 80:10:1) were labeled with [1α,2α-^{3}H] cholesterol (NEN Life Science Products, Boston, MA) and contain either rec.hapoA-I alone (Lp2A-I_{WT}), rec.apoA-I_{FIN} alone (Lp2A-I_{FIN}), or an equimolar mixture of the two (Lp2A-$I_{WT/FIN}$). The Lp2A-$I_{WT/FIN}$ is a hybrid population that was prepared to most closely resemble the nascent lipoproteins formed in heterozygous carriers of the apoA-I_{FIN} mutation. Since rec.hapoA-I and apoA-I_{FIN} have equal lipid binding capabilities and given that all proteins were incorporated into the particles, the

Lp2A-$I_{WT/FIN}$ represent a heterogeneous lipoprotein population, similar to those that might form *in vivo*. Approximately 50% of these Lp2A-I contain one molecule of each, 25% two molecules of rec.hapoA-I, and 25% two molecules of rec.apoA-I_{FIN}. Two different types of experiments were performed. The initial rate constants apparent K_m (appK_m) and V_{max} were calculated by incubating the Lp2A-I at the concentrations indicated (given as μM of apoA-I) with the enzyme for 10 min at 37°C and terminating the reaction with the addition of 2 ml of ethanol. Under these conditions, there is minimal substrate conversion as previously documented (468). In the second experiment, the time course of CE formed was followed over 5 h by incubating Lp2A-I particles at a final apoA-I concentration of 2.0 μM with 3.5 units (U) of LCAT. For both sets of experiments, the values are the mean (± SE) of triplicate measurements and represent the average of two independent experiments. One U of LCAT is defined as the amount required to convert 1 nmol of FC to CE per h using a standard Lp2A-I particle (prepared as described above) at a final apoA-I concentration of 2.0 μM.

2.2.17 - Cholesterol Efflux Studies

Efflux of cholesterol from mouse J774 macrophages (ATCC TIB-67) to rec.hapoA-I or rec.apoA-I_{FIN} with or without stimulation by cAMP was performed as outlined previously (469). Briefly, J774 macrophages in 12-well plates were loaded with [1α,2α-^{3}H] cholesterol (10 μCi/well) using acetylated low density lipoproteins (75 μg/ml) in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml). After 36 h, the loading medium was removed and replaced with serum-free DMEM containing 0.2% (w/v) BSA (DMEM-BSA) with or without the membrane permeable cAMP analog 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP) (0.15 mM final) (Sigma). Cholesterol was allowed to equilibrate with the cells for 10-12 h at which time the medium was removed and replaced

with DMEM-BSA with or without pCPT-cAMP containing rec.hapoA-I or rec.apoA-I_{FIN} as lipid-free proteins or as Lp2A-I (1.7 μM final apoA-I concentration). Efflux was measured over 3 h after subtracting the basal ^{3}H-FC efflux to medium of cells without apoA-I (DMEM-BSA control), which was less than 10% of the apoA-I specific efflux.

2.2.18 - Heterogeneity and Lipidation of ApoA-I Secreted from Primary Hepatocytes

Primary hepatocytes were prepared from apoA-I deficient mice as described above (section 2.2.12), except 5 h after plating, the William's complete medium was replaced with serum-free Hepatozyme® medium (Life Technologies) containing 5μCi per ml of ^{3}H-choline (Hepatozyme-choline) (Amersham Pharmacia). The next day, the cells were infected for 1 h with hapoA-I.Ad5 (moi = 75 to 1 pfu:cell) in William's medium containing no FBS. The adenovirus medium was removed, and the cells were incubated for another 24 h in Hepatozyme-choline medium. The labeled medium was then removed and the cells were washed gently but extensively in 2 × 2 ml of unlabeled ("cold") Hepatozyme medium. Cold Hepatozyme medium was added to the cells (1 ml per well in a 6-well plate) for 3.5 h. After 3.5 h the medium was removed, concentrated (Amicon 10K M_r cutoff Centripreps®) to 2 ml, and separated by FPLC as described above (section 2.2.6). ApoA-I present in the different lipoprotein fractions was determined by slot blot analysis (section 2.2.6). The VLDL/LDL (fractions 10-14), $HDL_{2/3}$ (fractions 19-23), and lipid-poor HDL species (fractions 25-29) were pooled, concentrated (30K M_r cutoff concentrators), and subjected to 4-20% non-denaturing PAGGE followed by Western Blot analysis to size apoA-I within the different lipoprotein fractions (section 2.2.8). In addition, equal volumes of the three fractions were immunoprecipitated for apoA-I with a polyclonal anti-human apoA-I antibody raised in sheep (Roche Molecular Biochemicals) and protein G-sepharose. An equal volume of an anti-human

apoB antibody raised in sheep, which does not cross-react with murine apoB, was used a control. The immunoprecipitates were washed twice with 5 ml PBS under non-denaturing conditions and resuspended in PBS (1 ml). The immunoprecipitates were subjected to β-counting to determine the ^{3}H-phosphatidylcholine associated with apoA-I.

2.2.19 – Statistical Analysis

Statistical analysis was performed using Student's t-test (paired two sample for means) where indicated. A value of $p \leq 0.05$ is considered highly significant and a value of $p \leq 0.10$ is considered moderately significant.

CHAPTER 3 – THE ROLE OF THE APOA-I CENTRAL DOMAIN IN THE MATURATION OF HDL

3.1 – Summary

Recombinant adenoviruses with cDNAs for hapoA-I and three mutants, referred to as Δ4-5A-I, Δ5-6A-I, and Δ6-7A-I that have deletions removing regions coding for aa 100-143, 122-165, and 144-186, respectively, were created to study structure-function relationships of apoA-I *in vivo*. All mutants were expressed at lower concentrations than hapoA-I in plasma of fasting apoA-I deficient mice. The Δ5-6A-I mutant was found primarily in the lipid poor HDL pool and at lower concentrations than Δ4-5A-I and Δ6-7A-I that formed more buoyant $HDL_{2/3}$ particles. At an elevated adenovirus dose and earlier blood sampling from fed mice, both Δ5-6A-I and Δ6-7A-I increased HDL-FC and PL but not CE. In contrast, hapoA-I and Δ4-5A-I expression produced significant increases in HDL-CE in these mice. Further analysis showed that Δ6-7A-I and hapoA-I could bind similar amounts of PL and cholesterol that was reduced slightly for Δ5-6A-I and greatly for Δ4-5A-I. We conclude from these findings that aa 100-143, specifically helix 4 (aa 100-121), contributes to the maturation of HDL through a role in lipid binding and that the downstream sequence (aa 144-186) centered around helix 6 (aa 144-165) is responsible for the activation of LCAT.

3.2 – Background

Epidemiological studies have shown that the levels of HDL-C and apoA-I, the major protein of HDL, are inversely correlated with the incidence of CHD (470,471). ApoA-I circulates as a 243 aa protein (158) and plays an important role in determining the concentrations of plasma HDL-C. This is accomplished through the ability of apoA-I to promote the removal of cholesterol and PL from cells and subsequently activate LCAT

(209,472) that catalyzes the formation of CE on HDL. Additional interactions of apoA-I with the cell surface SR-BI (section 1.3.6) (473) and possibly with CETP (section 1.3.5) may also influence circulating HDL-C levels.

Domains of apoA-I required for lipid association and the activation of LCAT have been the focus of many *in vitro* investigations. The initial binding of apoA-I to lipids is thought to occur via the extreme N- (aa 44-65) and C-terminal (aa 220-243) amphipathic α-helices that may or may not require additional interactions with other regions of the protein (380,400,403,474). In the lipid bound state the central helices (aa 100 to 186) have been proposed to contain a putative hinge domain (392,475) thought to enable apoA-I to adopt distinct conformations on HDL, as well as the major LCAT activating domain (412,425,427,435,476-478).

We have previously generated a series of mutants in which a pair of adjacent helices were deleted sequentially within the central domain of apoA-I (405). These mutants denoted here as Δ4-5A-I, Δ5-6A-I, and Δ6-7A-I contain deletions in which aa 100-143, 122-165, and 144-186 have been removed, respectively. Our rationale in choosing these deletions was that removing a pair of helices would minimally modify the periodicity of the amphipathic α-helices and their putative interactions. Subsequent characterization of these mutants *in vitro* confirmed that the amphipathic α-helices within residues 100-186 were involved in interactions with PL and may contribute to the overall lipid binding capacity of the apoA-I in the formation of HDL (479). Removal of helices 4 and 5 (aa 100-143) caused a slight decrease in LCAT activation *in vitro* whereas deletion of either helices 5 and 6 (aa 122-165) or 6 and 7 (aa 144-186) almost abolished cholesterol esterification (412). This finding is in agreement with previous work suggesting that helix 6 (aa 144-165) is necessary for optimum LCAT activation (427) although other domains of apoA-I may also be involved (400,424,480).

Despite the insights gained from these *in vitro* studies, the roles of apoA-I central domain amphipathic α-helices in HDL maturation *in vivo* are still not fully understood.

In this study, we generated recombinant adenoviruses containing cDNAs for hapoA-I and the three central domain deletion mutants for the purpose of structure-function analysis of apoA-I *in vivo*. HapoA-I and the mutants were analyzed following injections of the recombinant adenoviruses into apoA-I deficient mice. These studies demonstrate that specific amphipathic α-helices within the central domain of apoA-I contribute to the *in vivo* maturation of HDL through roles in either lipid binding or the activation of LCAT.

3.3 - Results

3.3.1 - Plasma ApoA-I Concentrations Following Adenovirus-Mediated Gene Transfer

We first established that hapoA-I reached physiological levels (170 ± 40 mg/dl) in the plasma of fasted apoA-I deficient mice 96 h post Ad5 injection at a dose of 2×10^9 pfu. Under the same conditions, all apoA-I mutants were expressed at significantly lower levels. The highest expression was observed for Δ4-5A-I, followed by Δ6-7A-I, and lowest for Δ5-6A-I. No impairment in secretion rates of the apoA-I central domain deletion mutants was detected following infection of COS-7 cells in culture (>95% of all apoA-I forms were secreted in the media as measured by Western blot analysis 3 days post-infection – data not shown). This suggests that all apoA-I mutants are folded normally and secreted equally. When we increased the Ad5 doses and analyzed plasma in the fed state at earlier times post-injection (within 40 h), the levels of the apoA-I mutants obtained were similar to or greater than those found for hapoA-I (Table 3.1 - 40 h fed column). The time course of expression showed that all mutants had early peak levels that decreased markedly by day 6 post-injection

whereas hapoA-I reached a peak concentration 10 d post-injection and remained circulating for over 30 days (Fig. 3.1).

3.3.2 – The Effects of ApoA-I Expression on Fasting Plasma Lipid Levels

Expression of the apoA-I mutants in plasma taken from fasted apoA-I deficient mice (96 h post-injection) was not accompanied by any increase in plasma lipid levels but rather by moderate decreases (Table 3.2). In contrast, hapoA-I produced significant increases in plasma cholesterol and PL. This increase in plasma cholesterol was due exclusively to an increase in the HDL-CE concentration. The control luc.Ad5 injected mice had slightly decreased lipid levels compared to non-injected mice but this did not reach statistical significance. Therefore, the decrease in plasma lipid levels following expression of the apoA-I mutants, and in particular Δ5-6A-I (Table 3.2), was significant ($p<0.05$) and not explained by an effect of the adenovirus vector alone.

3.3.3 - Effect of Δ4-5A-I and Δ5-6A-I Expression on Mouse Plasma ApoE Levels

We tested the possibility that the reduced concentration of lipids in plasma of apoA-I deficient mice following expression of the apoA-I mutants was correlated with decreases in the concentrations of other apolipoproteins normally found in these mice. It was previously reported that apoA-I deficient mice have increased HDL apoE levels compared to control C57BL/6 mice (481). The HDL apoE was barely detectable (by Western blot analysis) in control mice whereas apoE comprised approximately 25% of the total HDL protein in apoA-I deficient mice. We observed that plasma apoE levels (primarily in the HDL pool) were significantly reduced following expression of either Δ4-5A-I (Fig. 3.2A - lanes 6 and 7) or Δ5-6A-I (Fig. 3.2A. - lanes 8 to 10) 96 h post-injection with 2×10^9 pfu of each recombinant

Table 3.1: Plasma ApoA-I Concentrations following Adenovirus-Mediated Gene Transfer

Ad5 Construct	ApoA-I Concentrations (mg/dl)	
	96 hour fasted (n)	40 hour fed (n)
luc	0.32 ± 0.22 (2)	N.D.
hapoA-I	172 ± 43 (4)[a]	178 ± 130 (3)[a]
Δ4-5A-I	27 ± 6 (4)[a]	643 ± 206 (3)[b]
Δ5-6A-I	3.6 ± 2.3 (7)[a]	405 ± 230 (3)[b]
Δ6-7A-I	9.3 ± 4.2 (3)[a]	198 ± 90 (4)[b]

Each Ad5 construct was administered via the tail vein of apoA-I deficient mice at either one of the two doses as described in the Methods (section 2.2.3). The number of mice (n) used for each mutant and either treatment is indicated. There are significant increases in the plasma protein concentrations of the apoA-I mutants when a higher Ad5 dose is combined with earlier sampling (40 h post-injection) and maintenance of the fed state.
[a] adenovirus dose = 2×10^9 pfu
[b] adenovirus dose = 1×10^{10} pfu
N.D. = not determined

adenovirus. However, injection with luc.Ad5 had no effect on the circulating apoE levels in apoA-I deficient mice (Fig. 3.2A - lanes 1 and 2 compared to lanes 3 to 5 for non-injected mice). On average, the plasma apoE levels were reduced by 50% following expression of Δ4-5A-I and Δ5-6A-I as shown in Fig. 3.2B and this occurred primarily in the HDL pool (data not shown). Similar results were obtained upon injection of either hapoA-I or Δ6-7A-I, which also promoted significant lowering of apoE plasma levels (not shown).

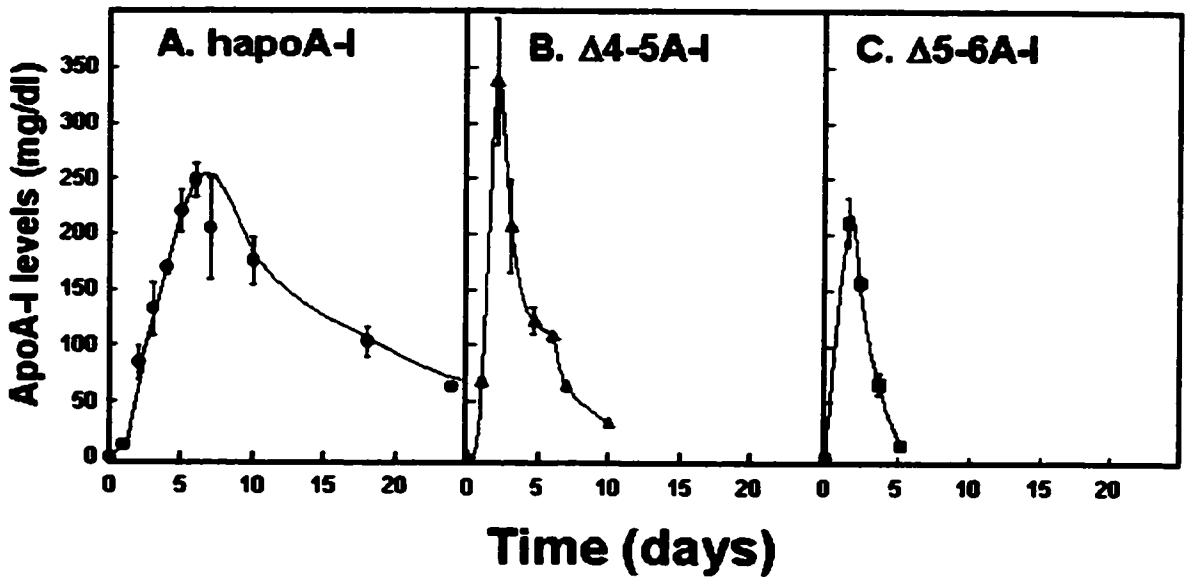


Figure 3.1: Time Course of ApoA-I Expression.

Pairs of apoA-I deficient mice were injected with either 2×10^9 pfu of hapoA-I.Ad5 (panel A.), 1×10^{10} pfu of Δ4-5A-I.Ad5 (panel B.), or 1×10^{10} pfu of Δ5-6A-I.Ad5 (panel C.). Plasma was sampled at the times indicated and the concentrations of the apoA-I proteins were determined by a solid-phase radioimmunoassay as described (section 2.2.4). The time course of expression of Δ6-7A-I was similar to Δ5-6A-I and is not shown.

3.3.4 - FPLC Analysis of ApoA-I Deficient Mouse Plasma Lipoproteins

Plasma samples isolated from fasted apoA-I deficient mice 96 h following injections of the various Ad5 constructs (2×10^9 pfu) were subjected to FPLC analysis. The lipoprotein TC profiles observed following expression of Δ5-6A-I and Δ6-7A-I at levels between 3 to 10 mg/dl were similar (not shown) to the profile observed with luc.Ad5 (Fig. 3.3, ▲). In contrast, Δ4-5A-I expressed at much higher levels (Fig. 3.3, ○; 43 mg/dl of Δ4-5A-I) resulted in a significant increase in HDL cholesterol. HDL-C was further increased with expression of hapoA-I (Fig. 3.3, ●; 168 mg/dl of hapoA-I protein). Native apoA-I was found

predominantly in the HDL_2 size range (fractions 14 to 19) whereas Δ4-5A-I was located in a smaller HDL pool comprising both HDL_2 (fractions 17 to 19) and HDL_3 (fractions 20 to 22) as shown by slot blot analysis below and above Fig. 3.3, respectively.

Table 3.2: Fasting Plasma Lipid Levels following Adenovirus-Mediated Gene Transfer

Ad5 Injection* (n)	Plasma Lipid Levels (mg/dl)				CE/TC
	TC	FC	CE	PL	
None (8)	53 ± 5	26 ± 5	27 ± 6	66 ± 12	0.51 ± 0.09
luc (3)	44 ± 3	23 ± 2	21 ± 5	46 ± 6	0.47 ± 0.08
hapoA-I (4)	115 ± 14[a]	34 ± 11	81 ± 26[a]	181 ± 25[a]	0.70 ± 0.06[a]
Δ4-5A-I (3)	41 ± 14	24 ± 12	17 ± 4	44 ± 10	0.43 ± 0.10
Δ5-6A-I (4)	27 ± 4[a]	15 ± 3[a]	12 ± 1[a]	28 ± 5[a]	0.44 ± 0.03
Δ6-7A-I (3)	47 ± 4	33 ± 5	14 ± 2[a]	46 ± 3	0.29 ± 0.05[a]

* All adenovirus constructs were injected at a final titer of 2×10^9 pfu. Plasma was sampled 96 h post-injection from a given number (n) of fasted (8-10 h) apoA-I deficient mice. The lipid and CE/TC ratio values for hapoA-I and the mutants are shown and those that are statistically significant at $p < 0.05$ (Student's t-test) from non-injected apoA-I deficient mice are indicated ([a]). Low concentrations of Δ5-6A-I and Δ6-7A-I (see Table 3.1) decrease the already low plasma lipid levels found in apoA-I deficient mice while hapoA-I increases plasma CE and PL as well as the CE/TC ratio.

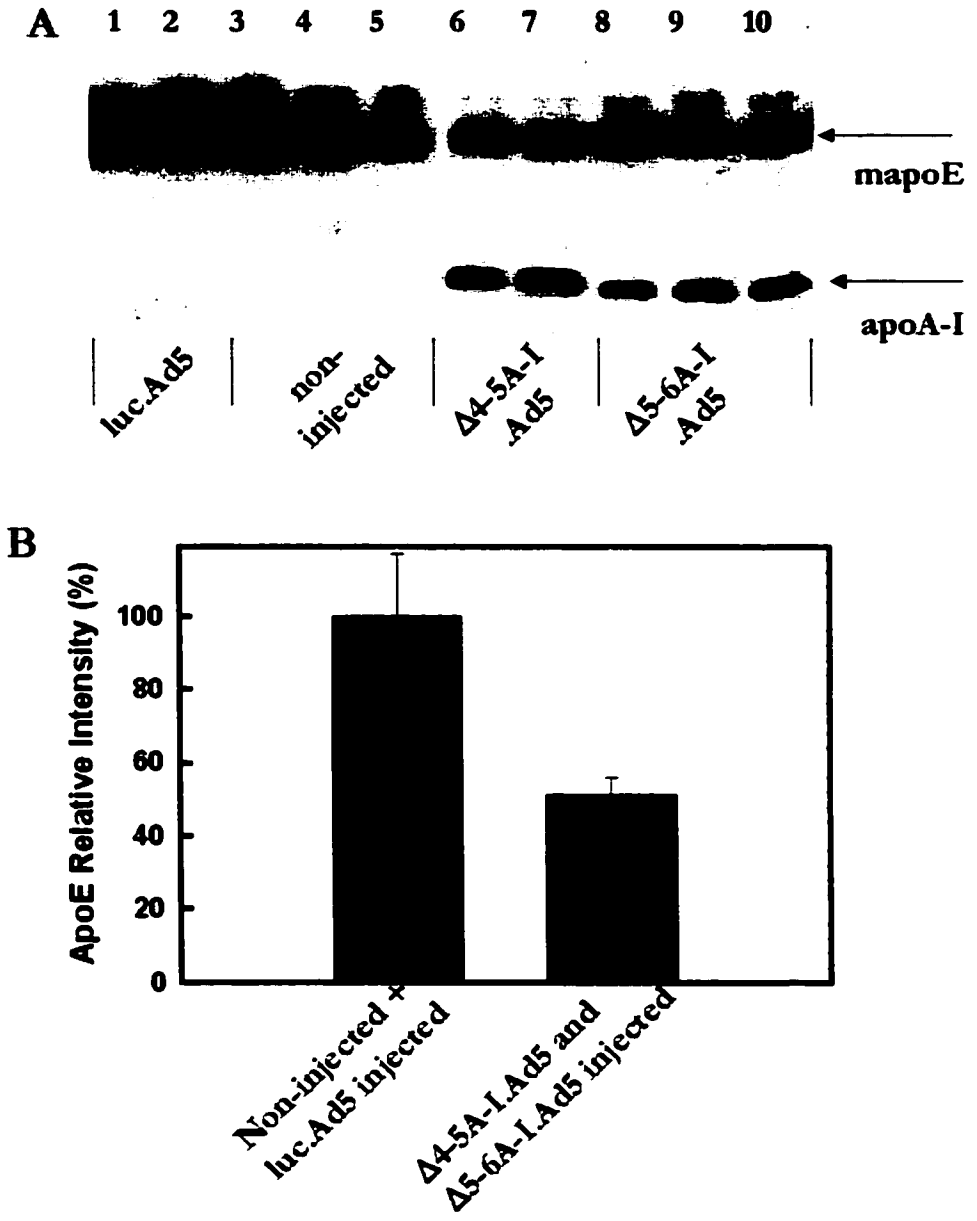


Figure 3.2: Δ4-5A-I and Δ5-6A-I Expression Decreases Murine ApoE Levels

Panel A - Western blot of a 12% SDS-reducing polyacrylamide gel of whole fasted mouse plasma. Each lane contains 1.5μL of plasma from either luc.Ad5 injected apoA-I deficient mice, non-injected apoA-I deficient mice, or apoA-I deficient mice injected with Δ4-5A-I.Ad5 or Δ5-6A-I.Ad5 as indicated. The gels were transferred to nitrocellulose (BioRad mini trans-blot transfer cell) and probed with a polyclonal anti-mouse apoE antibody or a combination of biotinylated monoclonal anti-human apoA-I antibodies as described (section 2.2.4). *Panel B* - The average intensity (± SE) of the apoE signals following Δ4-5A-I and Δ5-6A-I expression (96 h post-injection with 2×10^9 pfu.) are given as a percentage of the average signal obtained for the combined luc.Ad5 injected and non-injected mice.

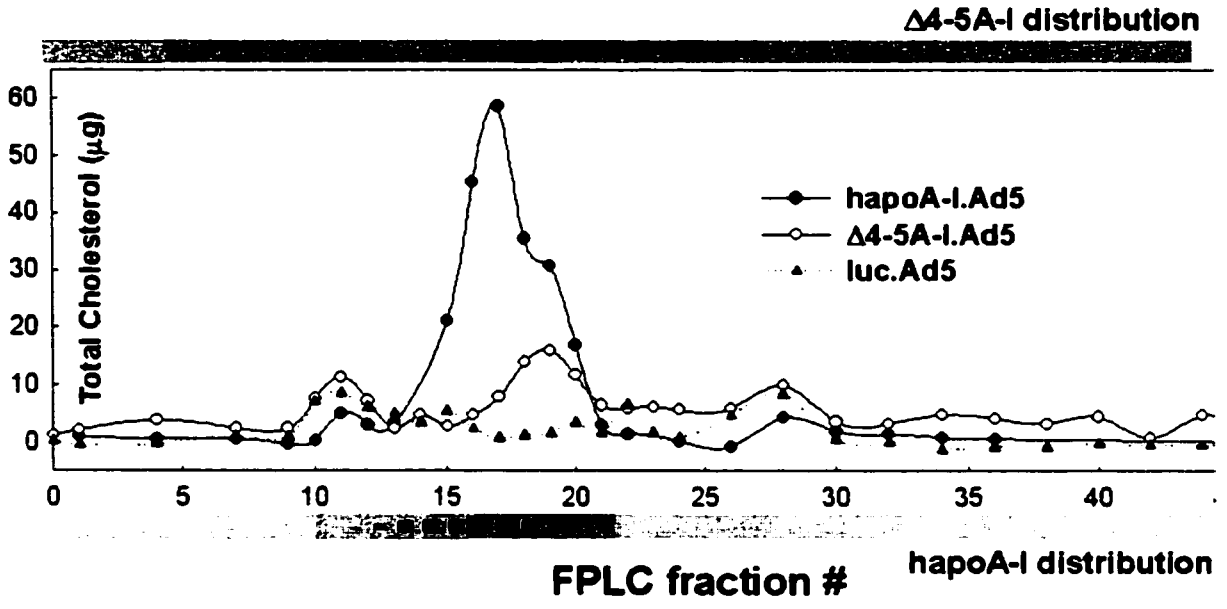


Figure 3.3: FPLC Analysis of Plasma Lipoproteins following Expression of Either HapoA-I or Δ4-5A-I.

Plasma pooled from three apoA-I deficient mice (500 μl – fasted) following injections of luc.Ad5 (▲), Δ4-5A-I.Ad5 (○), or hapoA-I.Ad5 (●) were separated on two consecutive Superdex 200 columns as described (section 2.2.6). The levels of hapoA-I and Δ4-5A-I in the plasma samples loaded on the columns were 168 mg/dl and 43 mg/dl, respectively, as determined by radioimmunoassay. The location of hapoA-I and Δ4-5A-I are shown below and above the figure, respectively, as analyzed by slot blot analysis (BioRad Bio-Dot SF Apparatus).

To compare the HDL lipid profiles obtained following expression of hapoA-I and Δ4-5A-I, the distributions of HDL TC, FC, CE, and PL were normalized per mg of apoA-I loaded on the columns and are expressed as μg of lipid per fraction (Fig. 3.4). From these data it is clear that Δ4-5A-I formed mature HDL but appeared to bind proportionally less lipid (cholesterol and PL) than did hapoA-I in the HDL pool.

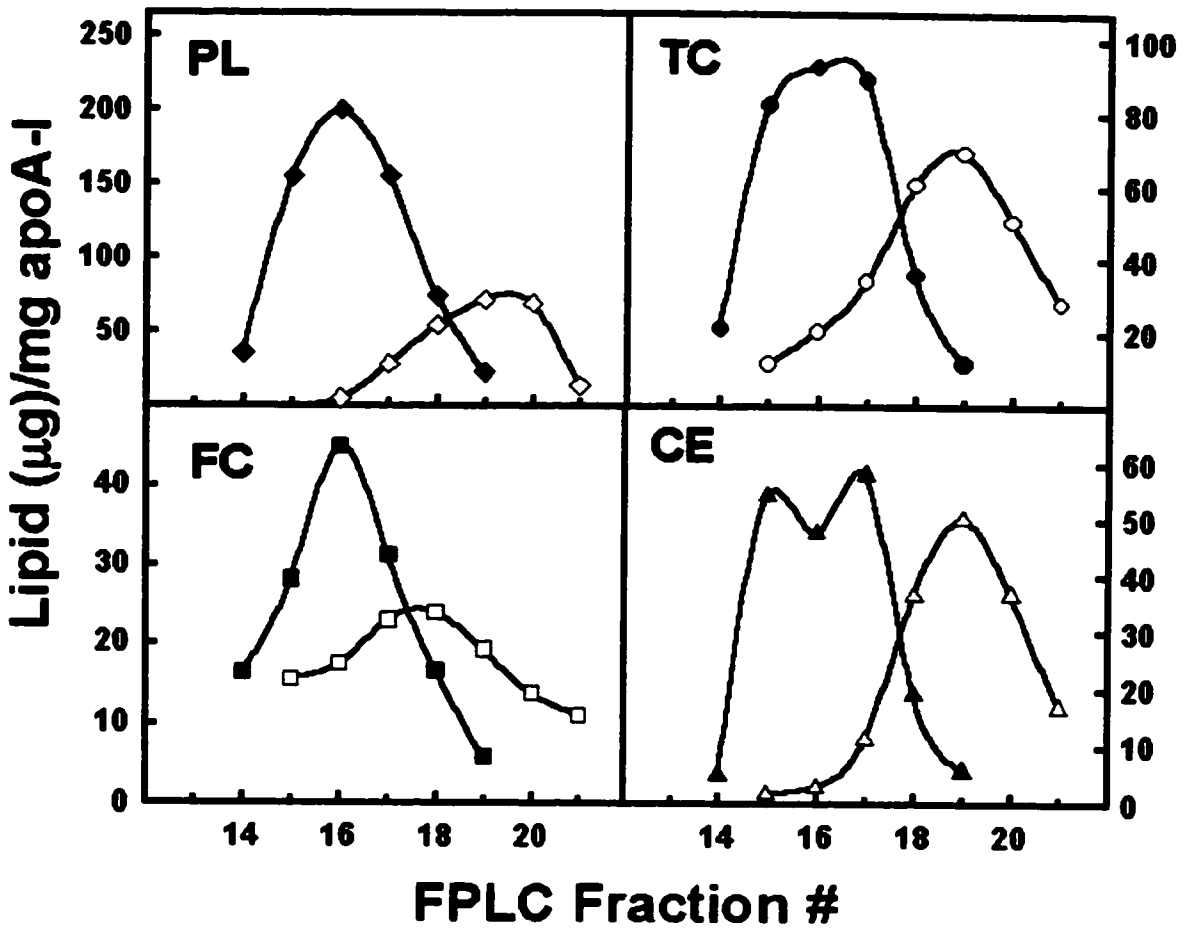


Figure 3.4: The HDL Lipid Profiles following Expression of HapoA-I and Δ4-5A-I

The amounts of PL, TC, FC and CE were determined in each of the hapoA-I (solid symbols) or Δ4-5A-I (open symbols) HDL FPLC fractions and are expressed as µg of lipid/fraction. The lipid mass was normalized to mg of apoA-I (either hapoA-I or Δ4-5A-I) loaded on the Superdex 200 columns. The lipid measurements were carried out with enzymatic kits as described (section 2.2.3).

3.3.5 - Fractional Cholesterol Esterification Rate of ApoA-I Deficient Mouse

Despite the overall reduction in lipids found in the Δ4-5A-I HDL pool, a significant proportion of CE was found. This prompted us to compare the ability of apoA-I deficient mouse plasma expressing either hapoA-I or Δ4-5A-I to esterify cholesterol. The FER of

apoA-I deficient mouse plasma without apoA-I (luc.Ad5 injected mice), or following expression of either hapoA-I or Δ4-5A-I was compared to control mouse plasma (wild-type C57BL/6 mice) (Fig. 3.5). The levels of hapoA-I and Δ4-5A-I obtained following

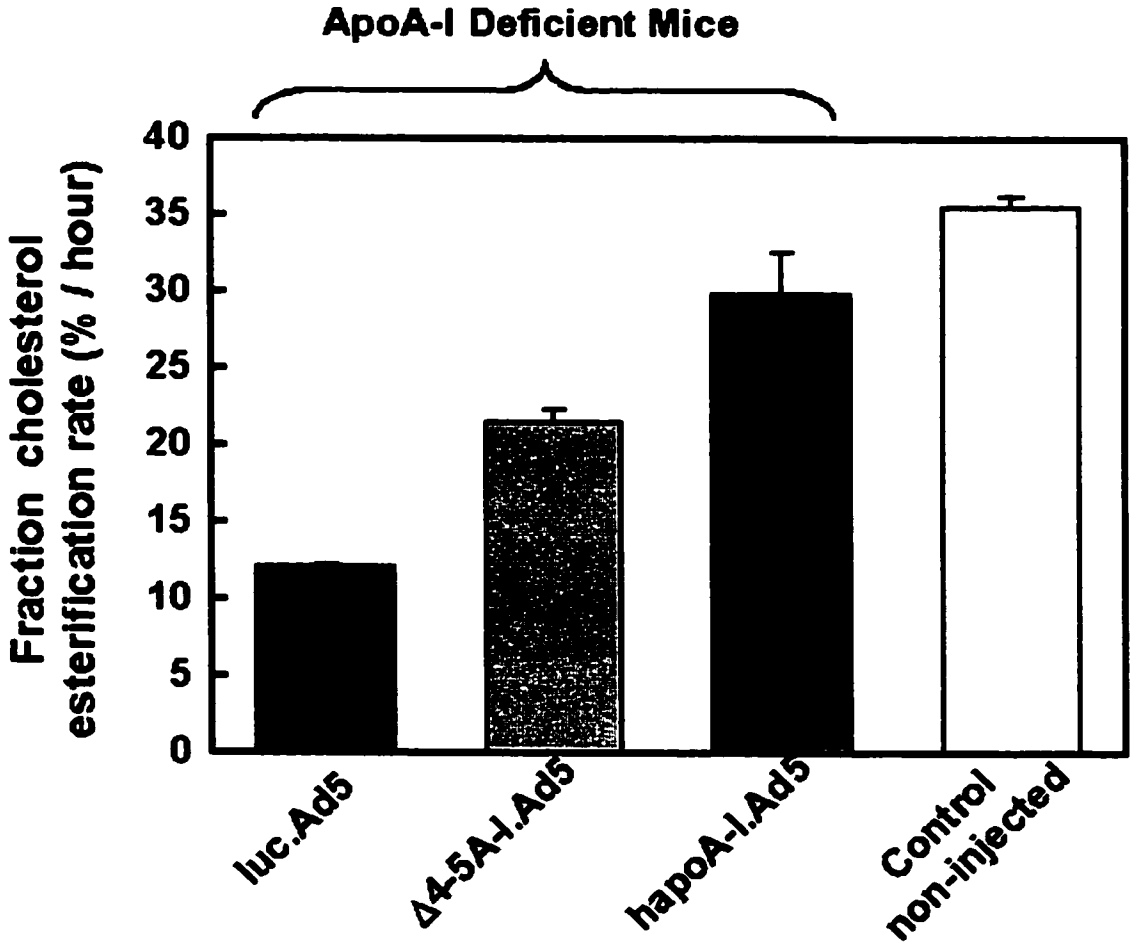


Figure 3.5: Fractional Cholesterol Esterification Rate of Mouse Plasma Samples

Plasma was pooled from three apoA-I deficient fasted female mice either injected with luc.Ad5, hapoA-I.Ad5, and Δ4-5A-I.Ad5 (section 2.2.10). Radioimmunoassays were performed on plasma following injections of hapoA-I.Ad5 and Δ4-5A-I.Ad5 and plasma samples containing a total of 10 μg of either hapoA-I or Δ4-5A-I was used in the experiments. All samples were all brought to the same final volume (30 μl) with plasma from apoA-I deficient mice infected with luc.Ad5, and compared to the same volume of non-injected wild-type C57BL/6J mouse plasma (*i.e.* control non-injected sample).

Ad5 injections were quantified by radioimmunoassay and equal amounts of expressed protein (10 μg) in equal plasma volumes were used for the experiment. Compared to control mouse plasma, apoA-I deficient plasma had a 65% reduction in FER of exogenously added ^{3}H-FC (Fig. 3.5) in agreement with a previous report (482). The latter study also demonstrated that the marked decrease of CE concentration observed in apoA-I deficient plasma resulted primarily from a decrease in apoA-I activator protein. We observed significant increases in the FER of apoA-I deficient mice plasma expressing Δ4-5A-I and hapoA-I (Fig. 3.5), although Δ4-5A-I was not as effective as hapoA-I.

3.3.6 - Distribution of the ApoA-I Mutants in HDL Density Subclasses

The lipoprotein density distribution profiles of the three apoA-I mutants were compared to hapoA-I following separation of the lipoproteins from plasma expressing the various apoA-I proteins. All injections were with 2×10^9 pfu of Ad5 and plasma from fasted mice was collected 96 h post-injection. Native apoA-I formed very buoyant lipoprotein particles that extended to a density lower than 1.063 g/ml (down to 1.04 g/ml) with the majority of the protein found in the $HDL_{1/2}$ subclasses (Fig. 3.6). Interestingly, both Δ4-5A-I and Δ6-7A-I exhibited similar density distribution profiles and were found associated with the smaller HDL_2 and HDL_3 subclasses. However, only approximately 20% of Δ5-6A-I was found in the buoyant $HDL_{2/3}$ pool and the remaining protein was located in the very high density subclass (VHDL – lipid poor or lipid free fractions).

3.3.7 - Size of HDL Formed by the ApoA-I Central Domain Deletion Mutants

We observed that each of hapoA-I, Δ4-5A-I, and Δ6-7A-I formed normal HDL sized particles, either HDL_2 (Fig. 3.7 – lanes A, D, and F, respectively) or HDL_3 (Fig. 3.7, lanes B, E,

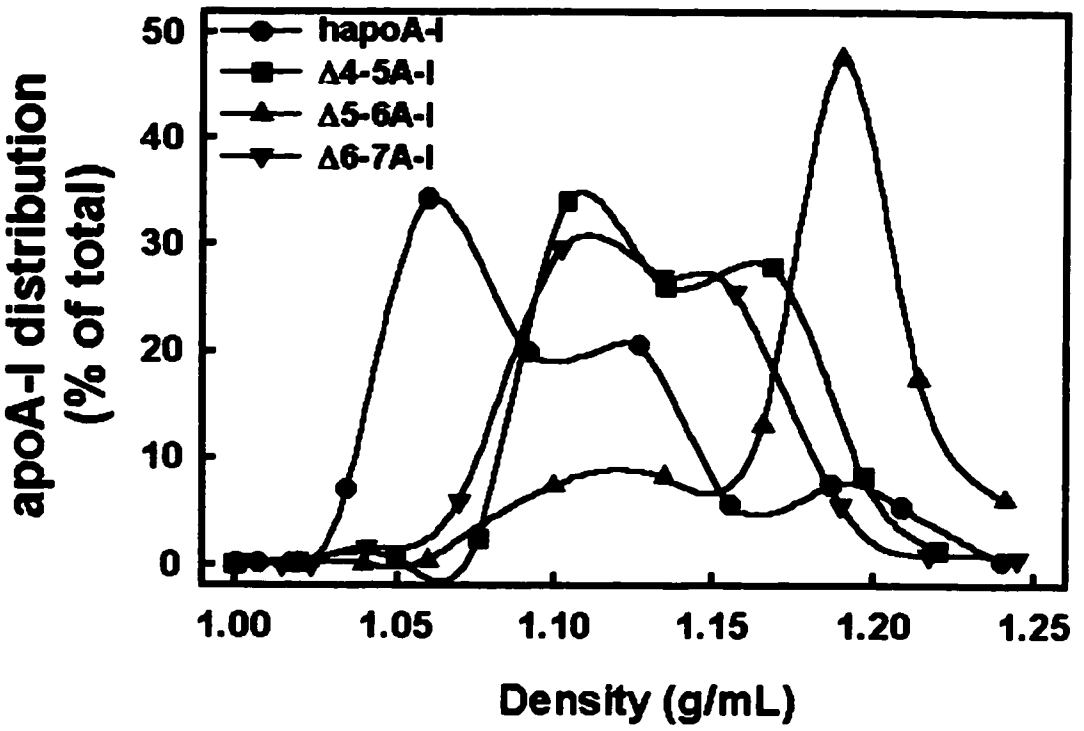


Figure 3.6: The HDL Distributions of HapoA-I and the Central Domain Mutants

Plasma isolated from fasted apoA-I deficient mice expressing either hapoA-I (●), Δ4-5A-I (■), Δ5-6A-I (▲), or Δ6-7A-I (▼) were subjected to discontinuous gradient density ultracentrifugation as described (section 2.2.7). The concentrations of each apoA-I variant in the lipoprotein fractions from VLDL (#1) to lipid-poor (#11) were determined by radioimmunoassay. The amount of apoA-I in each fraction is expressed as a percentage of the total amount of apoA-I found in all fractions combined.

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and G, respectively). However, these two central domain deletion mutants were found in greater amounts as HDL_3 compared to hapoA-I, confirming quantification by radioimmunoassay (Fig. 3.6). Very little Δ5-6A-I was detectable by Western blot analysis in the $HDL_{2/3}$ subclasses but it was found predominantly in lipid-poor (d > 1.16 g/ml) HDL fractions of much smaller size (Stokes' diameter between 7 to 8 nm, Fig. 3.7, lane H).

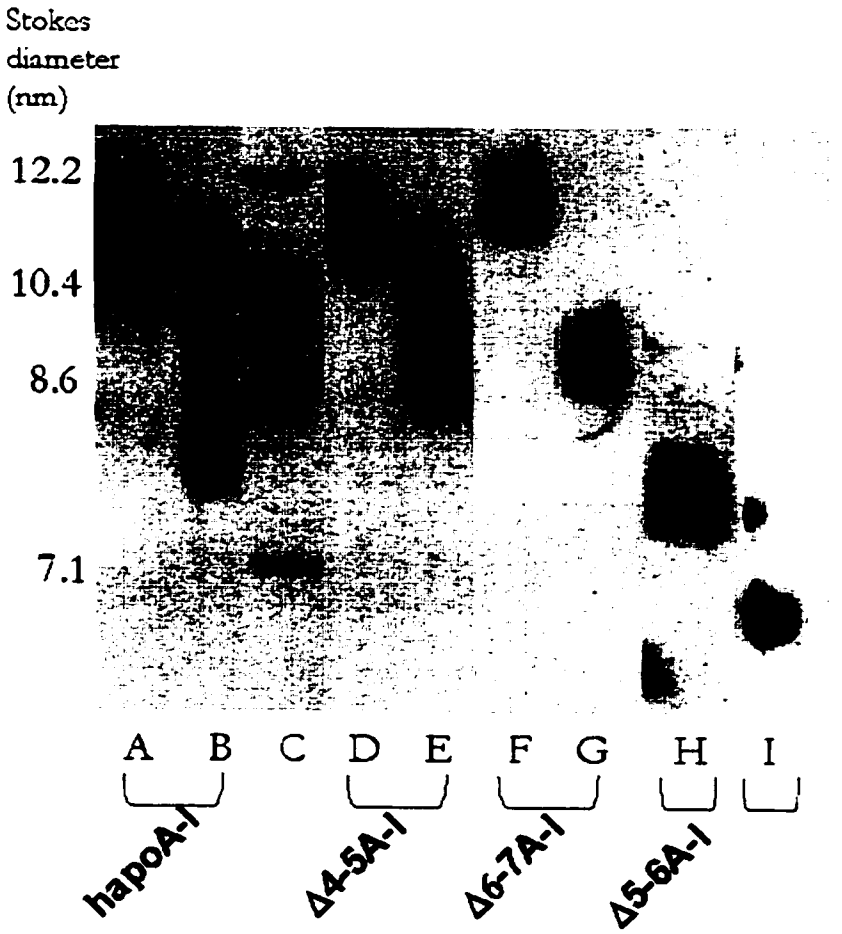

Figure 3.7: The HDL Size is Affected Differently by the ApoA-I Mutants

Western blot of a 4-20% nondenaturing polyacrylamide gradient gel of isolated HDL fractions obtained by discontinuous gradient density ultracentrifugation is shown. Each lane contains 10μL of dialyzed HDL fractions. Gels were run for 2200 volt.h, transferred to nitrocellulose, and probed for human apoA-I proteins as described (section 2.2.8). Lanes A (ρ = 1.07 g/ml) and B (ρ = 1.11 g/ml) contain hapoA-I HDL fractions, lanes D (ρ = 1.07 g/ml) and E (ρ = 1.11 g/ml) Δ4-5A-I HDL fractions, lanes F (ρ = 1.07 g/ml) and G (ρ = 1.11 g/ml) Δ6-7A-I HDL fractions, lane H is a Δ5-6A-I HDL fraction (ρ = 1.16 g/ml), and lane I is lipid free plasma apoA-I. HDL size was determined from biotinylated HMW markers (lane C) with known Stokes' diameters: ferritin – 12.2 nm, catalase –10.4 nm, lactate dehydrogenase 8.6 nm, and bovine serum albumin 7.1 nm.

3.3.8 - Morphology of HDL Formed by the ApoA-I Central Domain Deletion Mutants

The isolated HDL fractions (from 1.06 g/ml to 1.12 g/ml) were also examined by negative staining electron microscopy (Fig. 3.8). The HDL isolated from apoA-I deficient mice injected with the control luc.Ad5 vector (panel A) were spherical in shape as judged by the characteristic hexagonal packing array. This confirmed the result of an earlier study that detected only spherical HDL in the plasma of apoA-I deficient mice (481). Analysis of HDL proteins from luc.Ad5 injected mice by 12% SDS-PAGE and either Western blotting or Coomassie demonstrated that apoE was the major HDL protein in the apoA-I deficient background (data not shown). The HDL (1.06 g/ml) isolated following injections of hapoA-I.Ad5 were also spherical (panel B) but larger than those normally found in apoA-I deficient mice. Greater than 95% of the protein in this HDL fraction was hapoA-I (Colloidal blue staining and Western blot analysis of SDS-PAGE– data not shown). Panels C and D depict HDL isolated following expression of Δ4-5A-I. In the more buoyant fraction (d = 1.07 g/ml – panel C) rouleaux of stacked HDL indicative of discoidal particles were present. However, the majority of Δ4-5A-I formed spherical HDL at a higher density (1.11 g/ml – panel D) in which very few discoidal HDL were observed. In contrast, both Δ5-6A-I and Δ6-7A-I formed predominantly discoidal particles in the HDL_3 density range as shown in panels E and F, respectively (d = 1.11 g/ml). These HDL did not exhibit the tight hexagonal packing that HDL containing hapoA-I and Δ4-5A-I showed at this same density. Therefore, Δ5-6A-I and Δ6-7A-I induced the greatest population of discoidal HDL *in vivo* when compared to HDL formed with either hapoA-I (no discoidal HDL) or Δ4-5A-I (small population of discoidal HDL).

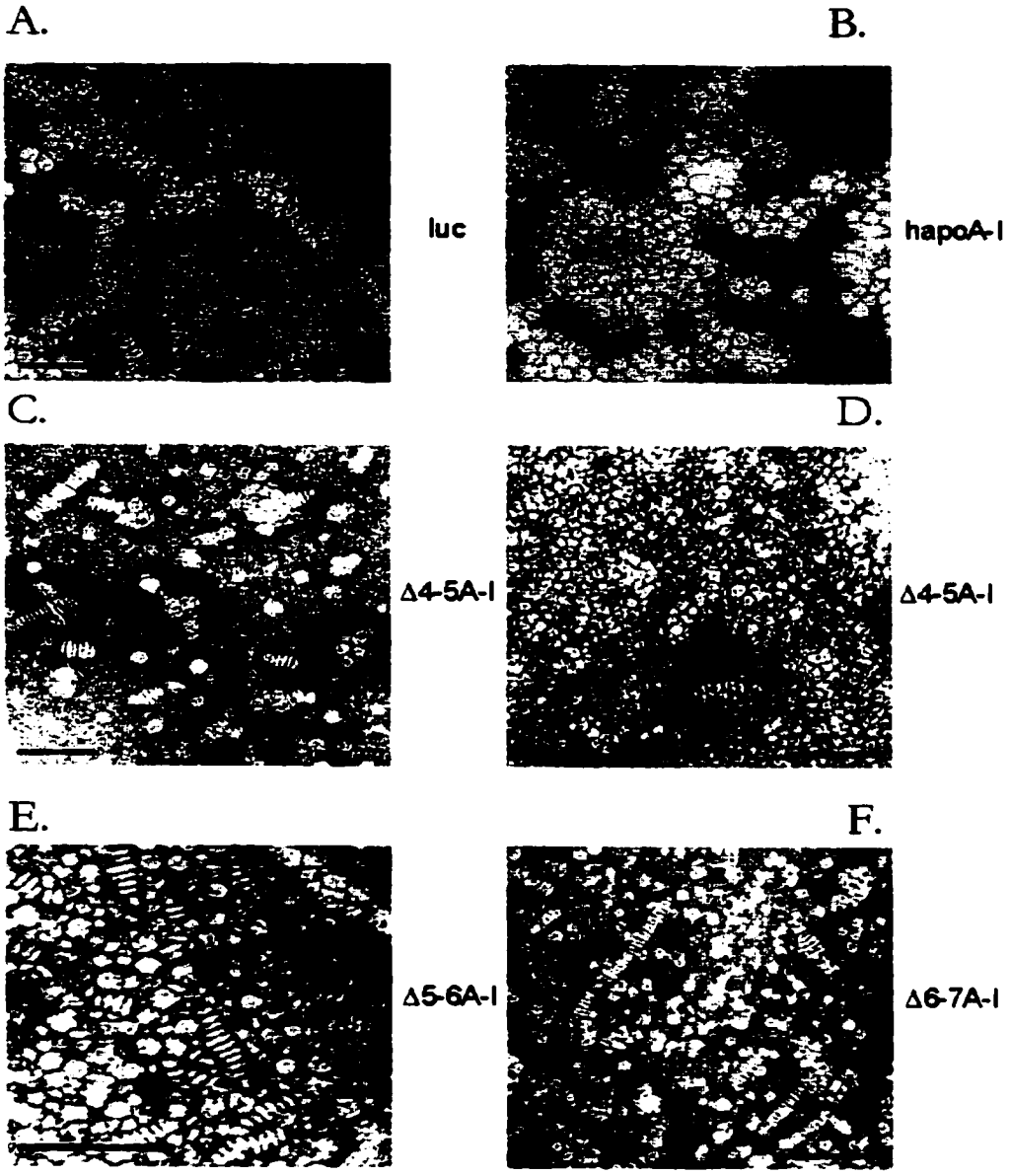

Figure 3.8: Effect of ApoA-I Central Domain Deletion Mutants on HDL Morphology

Electron microscopy of negatively stained HDL fractions isolated by discontinuous gradient density ultracentrifugation. The HDL were prepared for electron microscopy as outlined (section 2.2.9). The bar (—) in each panel represents 50 nm. All images were taken at 50,000X magnification with the exception of panel E that was taken at 70,000X magnification. Panel A is HDL (ρ = 1.076 g/ml) isolated from apoA-I deficient mice injected with the control luc.Ad5 construct. Panels B to F represent HDL formed following injections of the indicated human apoA-I Ad5 constructs. Panel B, hapoA-I HDL (ρ = 1.06 g/ml); panel C, Δ4-5A-I HDL (ρ = 1.07 g/ml); panel D, Δ4-5A-I (ρ = 1.11 g/ml); panel E, Δ5-6A-I HDL (ρ = 1.11 g/ml); panel F, Δ6-7A-I HDL (ρ = 1.11 g/ml).

3.3.9 - Effect of the ApoA-I Mutants on Plasma Lipid Levels in the Fed-State

High concentrations of the apoA-I mutants were obtained when earlier sampling (40 h post Ad5 injection) of plasma from mice maintained in the fed state (chow diet) was combined with an increase in the adenovirus dose (to 1×10^{10} pfu - Table 3.1 - last column). These modifications enabled us to compare the ability of each mutant to transport plasma lipids relative to hapoA-I. The hapoA-I adenovirus dose was kept at 2×10^{9} pfu since the protein concentration was already at or slightly above physiological levels. Sets of either 3 or 4 mice (all females between 3 to 8 months of age) were used to study each apoA-I variant under these conditions. The increased plasma lipid mass was confined to the HDL pool upon expression of all apoA-I forms (data not shown). Mice expressing hapoA-I had the greatest increase in HDL-CE mass 40 h after Ad5 injection (Table 3.3). There was also a significant increase in the HDL-CE concentration accompanying expression of Δ4-5A-I. In contrast, Δ5-6A-I and Δ6-7A-I did not increase the plasma CE concentrations although they both produced large increases in PL and FC. Interestingly, at these higher concentrations the normal plasma CE levels in apoA-I deficient mice decreased even further upon expression of Δ6-7A-I and even more dramatically upon expression of Δ5-6A-I (Table 3.3).

To evaluate the ability of each mutant to associate with HDL lipids, we determined the mass ratio of lipids (TC, FC, CE, and PL) to apoA-I for each mutant in comparison to hapoA-I. As shown in Fig. 3.9, Δ4-5A-I associated with significantly fewer of each lipid on a mass basis than did hapoA-I (*$p \leq 0.05$ or **$p \leq 0.1$ in comparison to hapoA-I) or the other two mutants confirming earlier results observed by FPLC analysis (Fig. 3.4). In contrast, Δ6-7A-I was as efficient as hapoA-I in associating with total amount of lipid (TC or PL), but produced a HDL pool with a much greater proportion of FC (** $p \leq 0.1$) to CE (* $p \leq 0.05$) as

shown in Table 3.3. Similarly, Δ5-6A-I expression also formed HDL with negligible CE (*$p \leq 0.05$ compared to hapoA-I). However, expression of Δ5-6A-I was also associated with a slight reduction in the TC/A-I ratio (** $p \leq 0.1$) that was not seen for Δ6-7A-I as well as a trend towards reduced PL association (though not statistically significant).

Table 3.3: Effect of High Expression Levels of ApoA-I on Plasma Lipids

Cholesterol (TC, FC, and CE) and PL levels in plasma sampled from fed apoA-I deficient mice (on a chow diet) prior to and 40 hours post-injection with recombinant apoA-I adenovirus constructs

Ad5 construct (n)	TC*	FC*	CE*	PL*	CE/TC
	mg/dl				
None (4)	53 ± 6.8	24 ± 7.5	30 ± 6.9	70 ± 14	0.56 ± 0.11
hapoA-I (3)	151 ± 51[a]	72 ± 28[a]	80 ± 23[a]	250 ± 100[a]	0.53 ± 0.02
Δ4-5A-I (3)	167 ± 20[a]	99 ± 23[a]	67 ± 16[a]	310 ± 62[a]	0.40 ± 0.10
Δ5-6A-I (3)	190 ± 95[a]	205 ± 99[a,b]	-15 ± 30[a,b]	344 ± 180[a]	-0.07 ± 0.04[a]
Δ6-7A-I (4)	165 ± 50[a]	148 ± 59[a,b]	17 ± 9[a,b]	256 ± 90[a]	0.13 ± 0.12[a]

*Mean ± SE All adenovirus injections are at a dose of 1×10^{10} pfu with the exception of hapoA-I.Ad5 that is injected at a dose of 2×10^{9} pfu. All plasma lipid values following high expression levels (see Table 3.1) of hapoA-I or each mutant are statistically different at $p<0.05$([a]) from non-injected mice. In addition, lipid values for the mutants that are different from hapoA-I at $p<0.05$([b]) are shown (Student's t-test). At these high concentrations, both hapoA-I and Δ4-5A-I increase the circulating CE concentrations in the plasma while Δ5-6A-I and Δ6-7A-I have the opposite effects and cause further reductions in the CE concentrations.

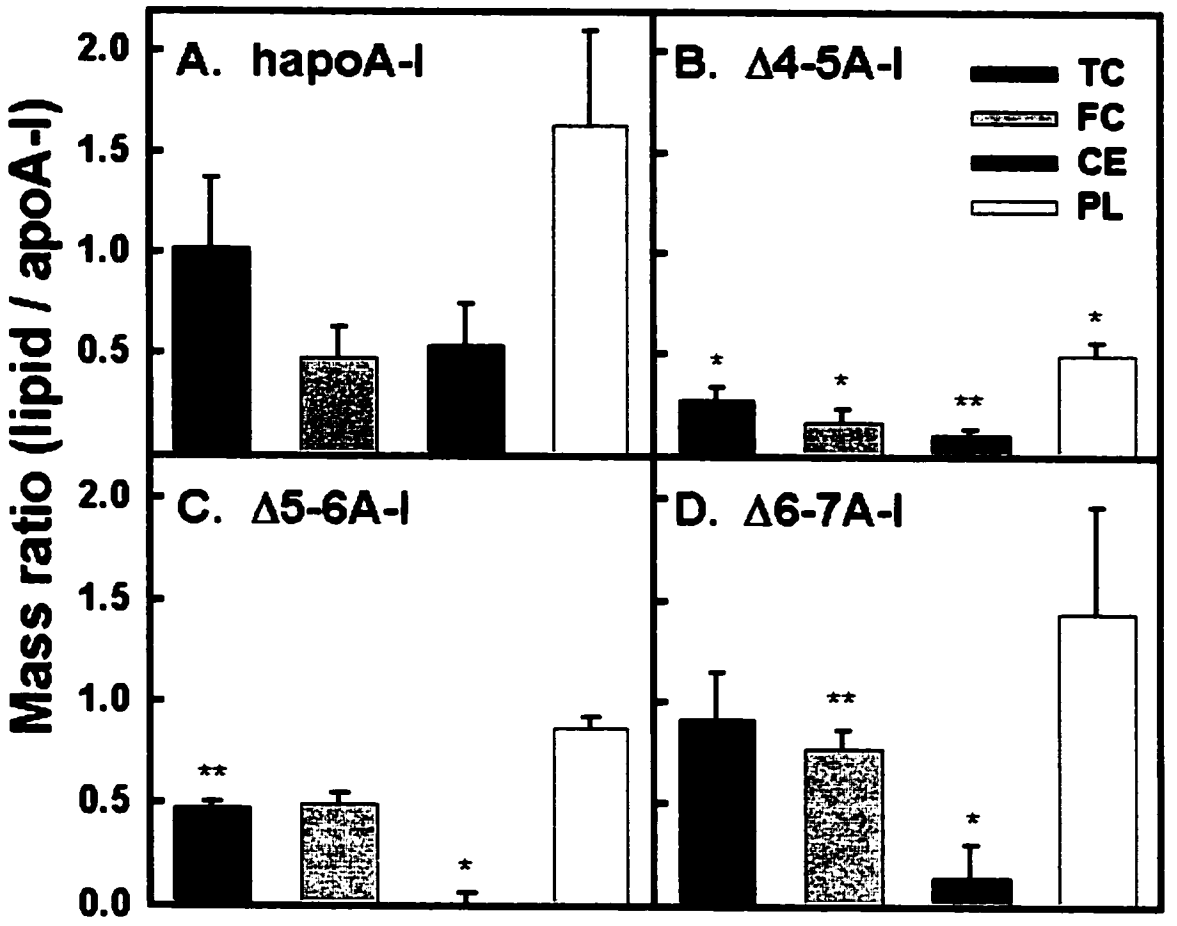

Figure 3.9: HDL lipid to apoA-I protein mass ratio for the different injections

The HDL lipid to apoA-I mass ratio was determined following injections of the recombinant human apoA-I adenoviruses. Plasma was sampled 40 h post-injection from mice maintained in the fed state. All adenovirus constructs were injected at a dose of 1×10^{10} pfu with the exception of hapoA-I.Ad5 that was injected at 2×10^{9} pfu. Panel A, hapoA-I; panel B, Δ4-5A-I; panel C, Δ5-6A-I; panel D, Δ6-7A-I. The lipid values (TC, FC, CE, or PL) for each mutant were compared to the corresponding hapoA-I value in panel A for statistical significance (Student's t-test) at either *$p \leq 0.05$ or **$p \leq 0.1$. Bars with no symbols are not statistically different from the corresponding hapoA-I value ($p > 0.1$).

3.4 - Discussion

Recombinant adenoviruses containing native and mutant cDNAs were generated to study structure-function relationships of apoA-I *in vivo* in apoA-I deficient mice. This approach provides new insights into the roles of apoA-I central domain amphipathic α-helices in the maturation of HDL, and shows that, between residues 100 and 186, two distinct functional domains can be distinguished that are primarily responsible for either lipid binding or stability and the activation of LCAT. Our results also clarify discrepancies between previous *in vitro* studies addressing the LCAT activation domain of apoA-I and provide new information on amino acids within this region that are important to the overall lipid binding abilities of apoA-I. This work was published in the Journal of Biological Chemistry (483).

We have observed that all three apoA-I central domain deletion mutants are expressed at lower levels than native apoA-I in the plasma of apoA-I deficient mice 96 h post-injection when the mice are fasted (Table 3.1). Our results suggest that this is a consequence of their enhanced clearance from the plasma and not a result of impaired synthesis and secretion. Indeed, all mutants are secreted as efficiently as hapoA-I from COS-7 cells following infection with recombinant adenoviruses in culture (not shown) and reach high concentrations, similar to and in some cases exceeding hapoA-I levels, at early times post-injection in plasma sampled from fed mice (Table 3.1). Furthermore, the time course of expression (Fig. 3.1) shows that the plasma concentrations of the apoA-I mutants decrease rapidly between 3-6 days whereas hapoA-I levels increase up to day 10 before starting a more gradual decline. Therefore, these data suggest that the low concentrations in plasma samples taken from fasted mice (96 h post-injection) resulted primarily from their enhanced clearance due to deficiencies in the abilities of these mutants to accumulate CE or bind lipids, and not from impaired production by the liver.

The amphipathic α-helices are responsible for the lipid binding properties of apoA-I (394) and the class Y helix as compared to classes G* and A_I appears to have the highest affinity for lipids (404). Two class Y helices are localized to the C-terminus (aa 209-241) and are predicted to be important components for the interaction of apoA-I with lipids (379,403). This is supported by the enhanced clearance of various apoA-I C-terminal truncation mutants when injected as lipid-free apoA-I into mice (484) or rabbits (402). While it has been suggested by Mishra *et al.* that all helical domains of apoA-I contribute to lipid binding, their data indicate that the central region of the protein exhibits a much weaker affinity for lipids than the C-terminal domain (aa 209-241) (403).

The present *in vivo* study, however, demonstrates that helix 4 (aa 100 to 121) within the central domain also contributes significantly to the lipid binding abilities of apoA-I. According to the FPLC profile (Fig. 3.4), Δ4-5A-I associates with proportionally fewer lipids in the HDL pool compared to hapoA-I and the HDL are shifted to a smaller size (Fig. 3.3). The apparently reduced lipid binding of Δ4-5A-I becomes more evident at higher expression levels. The concomitant increases in plasma lipids in the HDL pool (Table 3.3) observed at high apoA-I mutant concentrations shows that, although Δ4-5A-I increases significantly the plasma CE concentrations (discussed later), it has a decreased ability to associate with lipids compared to hapoA-I (Fig. 3.9). Since lipid binding is significantly reduced for Δ4-5A-I and only slightly reduced for Δ5-6A-I and normal for Δ6-7A-I (Fig. 3.9), we conclude that helix 4 (aa 100 to 121) accounts for the majority of the lipid binding mediated by the central domain of apoA-I. This is the first direct *in vivo* evidence that helix 4 (aa 100-121), a class Y helix, is required for efficient lipid binding of apoA-I, leading to the formation of circulating plasma lipoproteins.

There is evidence in the literature to support our conclusion that helix 4 is required for efficient lipid binding by apoA-I while downstream sequences within the central domain are not as critical. One study demonstrated that a 44-mer synthetic peptide corresponding to helices 4-5 but not peptides containing helices 5-6 or 6-7 could lower the enthalpy of the gel to liquid crystalline phase transition of DMPC MLVs (403). As well, *in vitro* studies from our laboratory support a role for helix 4 in binding lipids. First, an epitope comprising aa 100-143 is sensitive to the PL and cholesterol environment in well-defined Lp2A-I (485), suggesting an interaction between this domain and lipids (either cholesterol or PL). Second, Sparks *et al.* showed that this epitope in apoA-I (aa 98-121) becomes less exposed during the conversion of LpA-I (one molecule of apoA-I) to Lp2A-I, suggesting an important role in lipid association (231). Third, Lp2A-I containing Δ4-5A-I are less stable (based on GdnHCl studies) than Lp2A-I prepared with hapoA-I, Δ5-6A-I, or Δ6-7A-I, supporting the hypothesis that helix 4 stabilizes apoA-I conformation and binds with PL (479). This is also consistent with our turnover studies of lipid-free ^{125}I-apoA-I proteins where we find that Δ4-5A-I has a shorter half-life than either hapoA-I or the other two mutants following tail vein injections into apoA-I deficient mice (not shown).

Interestingly, in contrast to helix 4, the downstream sequence (aa 122-186) of the central domain contributes very little to the lipid binding as discussed above. This observation is consistent with the notion that this sequence contains the hinge domain previously suggested to exist (392,475,485) and that its loosely lipid-bound amphipathic α-helices are important for the efficient activation of LCAT (discussed below). The low lipid binding affinity of this region also explain why apoA-II is able to displace these central helices on lipoprotein particles (486). The sequential deletions of helix pairs have allowed us to distinguish the contribution of each helix to either or both lipid binding and LCAT activation. At high concentrations,

Δ5-6A-I and Δ6-7A-I increase markedly the concentrations of plasma HDL-FC and PL but not CE (Table 3.3). The HDL formed by these two mutants are predominantly discoidal (Fig. 3.8, panels E and F) and migrate exclusively to the pre-β position on agarose gels (not shown). On the other hand, despite a lower binding capacity for lipids, Δ4-5A-I significantly increases the plasma CE mass, forms spherical HDL particles (Fig. 3.8, panel D.) and migrates to both the pre-β and α positions on agarose gels (similar to hapoA-I). Together, these data demonstrate that the C-terminal half (aa 144-186) of the central domain is critical for the conversion of FC to CE *in vivo* while the N-terminal half (aa 100-143) is not.

Many *in vitro* experiments have been performed to identify the minimal sequence requirements for apoA-I activation of LCAT. Some studies suggested a broad region of apoA-I between aa 96-186, and a smaller region between aa 95-125 (476). Other studies have showed that aa 144-186 (399,412,424,427) are necessary, while additional reports have implicated contributions by N-terminal (437) and C-terminal sequences (474). In contrast to previous results (477,478), our *in vivo* observations show that the N-terminal portion (aa 100-143) of the central domain which contains an important lipid binding helix (aa 100-121) is not essential for the activation of LCAT. Our data also demonstrate that helix 6 (aa 144-165), a helix absent from Δ5-6A-I and Δ6-7A-I, is required for the conversion of pre-β particles to mature CE-rich α-migrating HDL. This is the first *in vivo* evidence that helix 6 contributes little to lipid binding but appears to be essential for LCAT activation. Subsequently, other *in vivo* studies confirmed our initial findings (487,488). Our findings are also consistent with previous *in vitro* studies showing that the major LCAT activation domain is contained within aa 144-186 (399,412,424,427). The reduced lipid association of this domain (aa 144-186) may enable apoA-I to interact directly with LCAT *in vivo* and/or allow LCAT to more easily access

the underlying PL fatty acid acyl chains. This concept is supported by the study of Sorci-Thomas *et al.* where replacement of helix 6 (aa 143-165) with helix 10 (aa 220-241) produced an apoA-I variant that has a 5-fold decrease in its ability to activate LCAT despite a higher affinity for lipids (426).

Plasma lipoproteins from mice expressing Δ4-5A-I (Fig. 3.5) as well as reconstituted lipoproteins formed with this mutant (412) could activate LCAT with 50% efficiency compared to hapoA-I. Based on the reduced lipid association of Δ4-5A-I compared to hapoA-I, we propose that the lower FER for this mutant relative to the native protein is mostly a consequence of lower concentrations of lipid substrates (*i.e.* PL fatty acyl chains) available for the LCAT-dependent reaction in the HDL pool. However, secondary interactions of LCAT with residues 100-143 on apoA-I cannot be ruled out. Also, the deletion of helices 4 and 5 may produce an apoA-I variant that cannot accommodate the same size of CE core as hapoA-I during HDL maturation. Nonetheless, aa 100-143 contributes only minimally to LCAT activation by apoA-I when compared to the more C-terminal region (aa 144-168) in this reaction.

Analysis of plasma samples isolated from fasted apoA-I deficient mice 96 h post-injection with the recombinant adenoviruses has also demonstrated the specific contributions of central helices to the formation and stability of HDL. Interestingly, despite its inability to incorporate CE into HDL, Δ6-7A-I forms buoyant lipoprotein particles similar to Δ4-5A-I in both density distribution (Fig. 3.6, ▼ and ■) and size (Fig. 3.7 lanes E, F and C, D), although both mutants are shifted to a higher density relative to hapoA-I (Fig. 3.6, ●). In contrast, Δ5-6A-I is found predominantly in lipid-poor, less buoyant (Fig. 3.6, ▲) and much smaller HDL (Fig. 3.7 panel H). Therefore, removal of helices 5 and 6 has the biggest impact not only on the circulating apoA-I levels but as well on the ability to maintain a stable conformation on HDL. We

postulate that the slight decrease in lipid binding of this mutant compared to hapoA-I (Fig. 3.9), combined with its inability to accumulate any CE (even less than Δ6-7A-I), generates an unstable apoA-I protein that is subsequently cleared more rapidly in fasted apoA-I deficient mice.

Low plasma lipids are observed when the apoA-I mutants, in particular Δ5-6A-I, are expressed at low concentrations (Table 3.2). As well, the CE/TC ratios, normally between 0.4 and 0.6 in the plasma of apoA-I deficient mice, are reduced to values that approach zero (Table 3.3) when Δ5-6A-I and Δ6-7A-I are expressed at high concentrations. These dominant negative effects are likely explained by the observed reduction in mouse apoE levels (Fig. 3.2) and possibly other apolipoproteins, in the HDL pool that normally contribute to plasma lipid levels in apoA-I deficient mice. Therefore, these mutants provide useful tools to investigate the potential mechanisms that could underlie the dominant negative effects on HDL-C produced by some apoA-I variants. Future studies are planned to address this more thoroughly.

In summary, the *in vivo* results presented here demonstrate that the domain containing helices 4 and 5, and within it the class Y helix 4 (aa 100-121), is necessary for the efficient lipid binding of apoA-I and stabilization of lipoprotein particles. Therefore, this domain together with the C-terminal class Y amphipathic α-helices (aa 209-241) and helix 1 in the N-terminal (aa 44-65) is important for the association of apoA-I with lipids. At the same time, helices 4 and 5 are not essential for LCAT activation while the downstream sequence (aa 144-186) is required for the activation of this enzyme. This is also the first *in vivo* demonstration that aa 144 to 186 contributes very little to apoA-I lipid binding. Based on these observations, we conclude that two distinct functional domains exist within the central region (aa 100-186) of

apoA-I. The N-terminal sequence (aa 100-143) contains an important lipid binding domain while the C-terminal sequence (aa 144-186) contains the major LCAT activation domain.

CHAPTER 4 – METABOLIC STUDIES OF AN APOA-I MUTANT RESPONSIBLE FOR DOMINANTLY INHERITED HYPOALPHALIPOPROTEINMEMIA

4.1 - Summary

We have devised a combined *in vivo*, *ex vivo*, and *in vitro* approach to elucidate the mechanism(s) responsible for the hypoalphalipoproteinemia in heterozygous carriers of a naturally occurring apoA-I variant (Leu^{159} to Arg) known as apoA-I_{FIN}. Adenovirus-mediated expression of apoA-I_{FIN} decreased apoA-I and HDL-C concentrations in both wild-type C57BL/6J mice and in apoA-I deficient mice expressing hapoA-I. Interestingly, apoA-I_{FIN} was degraded in the plasma and the extent of proteolysis correlated with the most significant reductions in mapoA-I concentrations. ApoA-I_{FIN} also had impaired activation of LCAT *in vitro* compared to hapoA-I but in a mixed lipoprotein preparation consisting of both hapoA-I and apoA-I_{FIN} there was only a moderate reduction in the activation of this enzyme. Importantly, secretion of apoA-I was also decreased from primary apoA-I deficient hepatocytes when hapoA-I was co-expressed with apoA-I_{FIN} following infection with recombinant adenoviruses, a condition that mimics secretion in heterozygotes. Thus, this is the first demonstration of an apoA-I point mutation that decreases LCAT activation, impairs hepatocyte secretion of apoA-I and makes apoA-I susceptible to proteolysis leading to dominantly inherited hypoalphalipoproteinemia.

4.2 - Background

The complex and often poorly understood etiology for variations in HDL-C concentrations within the general population has made the therapeutic control of HDL levels an elusive target to date. This is attributed to the intricate nature of HDL metabolism that involves many components including the major HDL structural protein apoA-I and multiple

factors required for CE formation, lipolysis, lipid transfer, cellular lipid efflux, and cell surface interactions (section 1.3 and 1.4) [reviewed in (47,365)].

Nascent HDL that are derived from the liver and intestine are poorly lipidated (47,489) and must acquire additional lipids for their maturation into the more stable α-migrating HDL in plasma (section 1.3.3). Defective clearance of TG-rich lipoproteins is recognized as a major determinant of HDL-C concentrations (section 1.2.4). Recessive mutations in LPL and its major activator protein apoC-II, which result in impaired hydrolysis of TG-rich lipoproteins, also contribute to low HDL-C concentrations. As well, the efficient conversion of FC to CE on HDL by LCAT is necessary for HDL maturation and depends on apoA-I as its physiological activator [reviewed in (47,365,369)]. Recent work has also highlighted the importance of both the ABCA1 and PLTP in maintaining normal HDL-C concentrations. PLTP deficient mice have HDL-C levels that are reduced by 60 to 70% (197) as a result of enhanced catabolism of HDL apparently due to defective transfer of lipids from TG-rich lipoproteins to nascent HDL (199). Mutations in the ABCA1 cause Tangier disease (171-173) in which affected individuals have less than 5% of the normal HDL-C concentrations due to impaired cellular lipid efflux to nascent HDL.

Isolated familial hypoalphalipoproteinemia (FHA) is more common than Tangier disease and heterozygous mutations in ABCA1 can give rise to FHA (172,490,491). However, mutations in apoA-I also contribute significantly to FHA in the general population. A recent study has shown that in a group of 1264 Japanese school children, 6% of FHA cases defined as HDL-C concentrations below the 5th percentile matched for age and sex were related to apoA-I mutations (492). This would represent an incidence of 0.3% for apoA-I mutations in the general population, which is significant and warrants mechanistic studies on the relationships between these mutations and hypoalphalipoproteinemia. Many apoA-I

mutations have been identified [reviewed in (369,429)], but particularly interesting amongst them are those associated with a dominant FHA phenotype, such as apoA-I_{FIN}. This mutation was originally identified in a Finnish kindred and results from a Leu to Arg substitution at aa 159 (430). These individuals are heterozygous carriers of this mutation and yet have HDL-C and apoA-I concentrations that are 20% and 25% of the normal plasma concentrations, respectively (430). The cause for this dominant negative effect on HDL-C concentrations remains largely unknown. ApoA-I_{FIN} was shown to have reduced LCAT activation compared to hapoA-I (436) but it has not been established whether this mutant can inhibit LCAT activation by hapoA-I and confer its dominant negative phenotype in this manner. Here, we present the results of a combined *in vivo*, *ex vivo*, and *in vitro* study of the mechanisms responsible for the dominant negative effects on HDL-C brought about by apoA-I_{FIN}, and demonstrate that the mutation exerts its effects at multiple levels.

4.3 - Results

4.3.1 – Effect of HapoA-I and ApoA-I_{FIN} Expression on Murine ApoA-I Levels

Since apoA-I_{FIN} causes hypoalphalipoproteinemia in carriers of this mutation, we first sought to reproduce this phenotype in a mouse model by de novo expression of apoA-I_{FIN}. This was accomplished by two different approaches. First, wild-type C57BL/6J mice were injected with either the apoA-I_{FIN}.Ad5 or the hapoA-I.Ad5 and their effects on mapoA-I (Fig. 4.1) and HDL-C concentrations were compared. The concentrations of mapoA-I were decreased significantly by apoA-I_{FIN} (Fig. 4.1B lower right panel – lanes 1 to 4) but not by hapoA-I (Fig. 4.1A lower right panel – lanes 1 to 4). Interestingly, evidence of apoA-I_{FIN} proteolysis was seen in the plasma (Fig. 4.1B – upper right panel) and the greatest decreases in mapoA-I concentrations correlated with plasma samples that had the most significant amounts

of apoA-I_{FIN} degradation (Fig. 4.1B lower right panel – lanes 3 and 4). Overall, low concentrations of circulating apoA-I_{FIN} (50 ± 4.6 mg/dl) caused a statistically significant decrease ($p < 0.03$) in mapoA-I concentrations (to 32 ± 22% of pre-injected values) that was not found for higher and more physiological concentrations (141 ± 27 mg/dl) of hapoA-I ($p = 0.14$) (Fig. 4.1C). Furthermore, apoA-I_{FIN} decreased the HDL-C concentrations and caused a remodeling of HDL in these mice converting the large HDL_2 to smaller HDL_3 (not shown). This is similar to what was observed when hapoA-I and apoA-I_{FIN} were co-expressed in apoA-I deficient mice (below).

4.3.2 – ApoA-I and Plasma Lipid Concentrations following Expression of HapoA-I, ApoA-I_{FIN}, or Both in ApoA-I Deficient Mice

To confirm these results and more closely mimic the human heterozygote state, apoA-I deficient mice were co-injected with the hapoA-I.Ad5 and the apoA-I_{FIN}.Ad5 and the circulating apoA-I and TC concentrations were compared to mice injected with either the hapoA-I.Ad5 or the apoA-I_{FIN}.Ad5 alone (Fig 4.2). Mice injected with the hapoA-I.Ad5 had high circulating concentrations of hapoA-I that reached physiological concentrations by 4 d and peaked at high levels between 7 and 9 d before returning to lower concentrations at 12 d (Fig. 4.2A,●). In contrast, co-expression of hapoA-I and apoA-I_{FIN} (Fig. 2A, ▲) resulted in only moderate concentrations of circulating apoA-I (50- 70 mg/dl) that were 3 to 8-fold lower in these mice throughout the time course of expression compared to hapoA-I. In fact, the apoA-I concentrations following co-expression of the two proteins were similar to mice expressing apoA-I_{FIN} alone (Fig. 4.2A, ■), except that the apoA-I levels were sustained longer in the plasma when both proteins were present. The increases in TC concentrations were confined to the HDL pool ($\rho > 1.06$ g/ml) and paralleled the expression of the human apoA-I

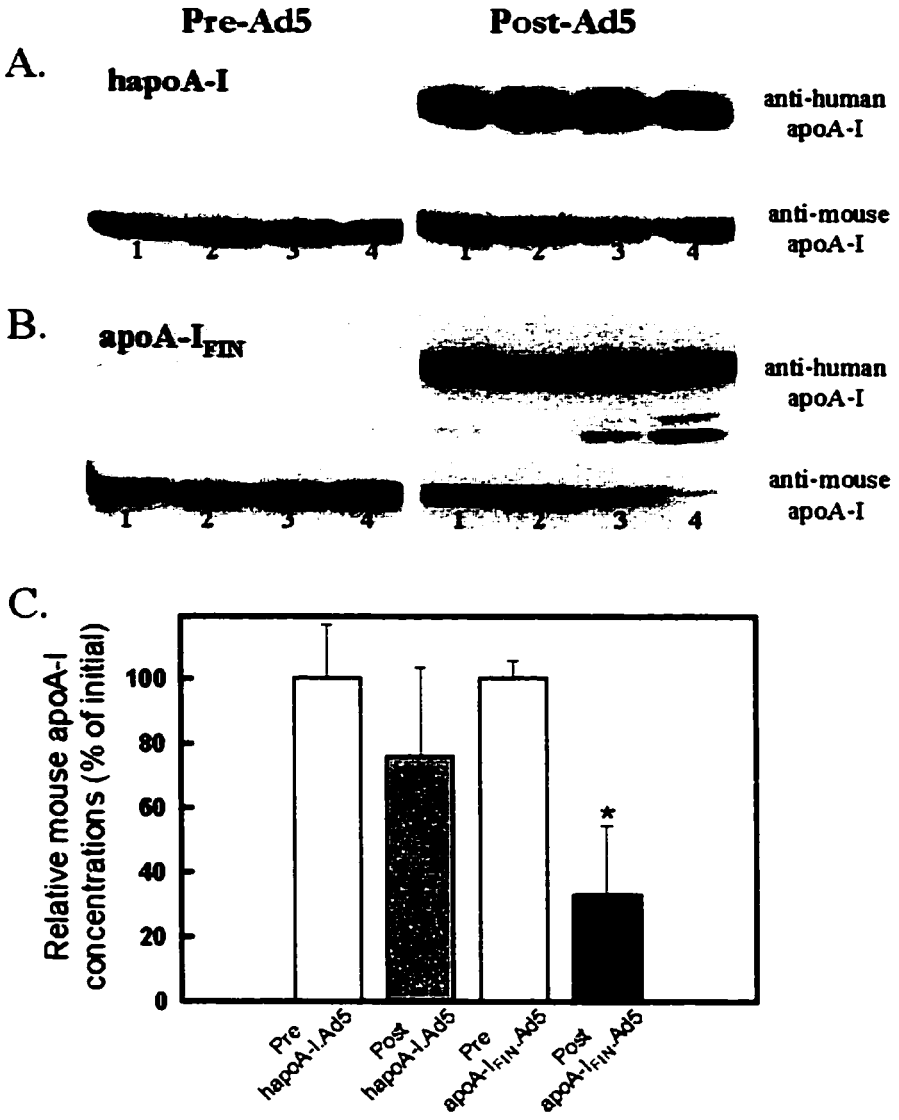

Figure 4.1: Proteolysis of ApoA-I$_{FIN}$ Is Correlated with Decreases in Murine ApoA-I

Plasma (1.5 µl) was isolated from mice and subjected to 12 % SDS-PAGE and then Western blot analysis following transfer to nitrocellulose. The blots were probed with either a mixture of biotinylated anti-human apoA-I monoclonal antibodies (upper panels) or a polyclonal anti-mouse apoA-I antibody (lower panels) as described (section 2.2.4). It should be noted that the hapoA-I and apoA-I$_{FIN}$ blots were exposed for different times and no quantitative comparison can be determined from these blots. ***A.***, plasma was isolated from 4 mice prior to (left panels) or 4 d after injections of 2 × 10^9 pfu of the hapoA-I.Ad5 (right panels). ***B.***, plasma was isolated from 4 mice prior to (left panels) or 4 d after injections of 2 × 10^9 pfu of the apoA-I$_{FIN}$.Ad5 (right panels). ***C.***, densitometric scanning of the mapoA-I bands before (white bars) or following injections of the hapoA-I.Ad5 (gray bar) or the apoA-I$_{FIN}$.Ad5 (black bar). There is a statistically significant reduction ($p < 0.03$) in mapoA-I concentrations (to 33 ± 22 % of normal values) following expression of apoA-I$_{FIN}$ (50 ± 4.6 mg/dl) but with hapoA-I ($p = 0.14$) at higher and more physiological concentrations (141 ± 27 mg/dl).

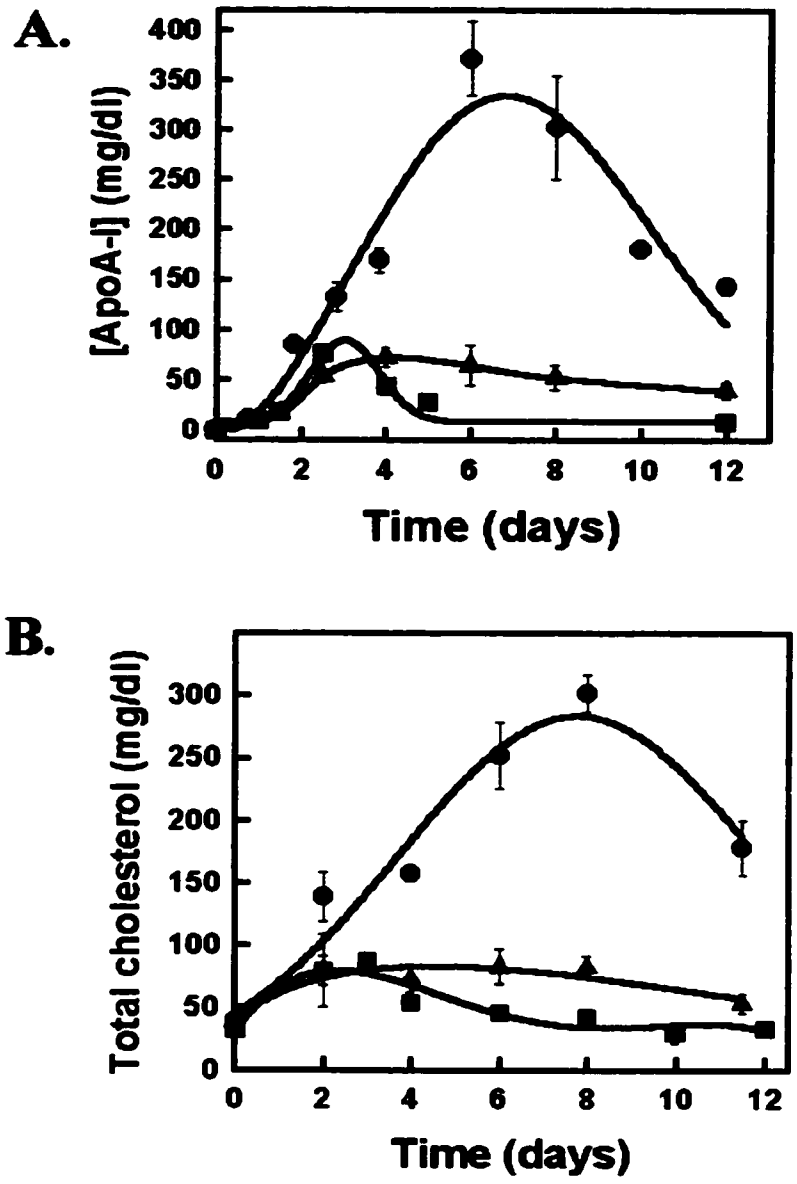

Figure 4.2: Dominant Effect of ApoA-I_{FIN} on HDL-C and ApoA-I Concentrations

ApoA-I deficient mice were pre-bled and then injected with either 2×10^9 pfu of the hapoA-I.Ad5 alone (3 males) (●), 1×10^9 pfu of each of the hapoA-I.Ad5 and the apoA-I_{FIN}.Ad5 (2 males and 1 female) (▲), and for comparison 2×10^9 pfu of the apoA-I_{FIN}.Ad5 alone (3 females) (■) as described (section 2.2.3). ***A.***, the apoA-I concentrations in mouse plasma samples were measured with a standard radioimmunoassay as described previously (392) (section 2.2.4). ***B.***, the plasma TC concentrations were determined as (section 2.2.3) and the increases following apoA-I expression are confined to the HDL pool. The apoA-I and TC concentrations are 3-8 fold lower throughout the time course regardless of whether apoA-I_{FIN} is expressed alone or in the presence of hapoA-I compared to mice expressing only hapoA-I.

proteins in these mice (Fig. 4.2B). Native hapoA-I increased the HDL-TC concentrations reaching a maximum between 7 and 9 d (Fig. 4.2B, ●), whereas co-expression of hapoA-I and apoA-I_{FIN} had only a moderate effect on the HDL-TC concentrations (2 to 3-fold increase) (Fig 4.2B, ▲) throughout the time course (Table 4.1). ApoA-I_{FIN} (Fig. 2B, ■) produced a similar effect but the return to baseline plasma cholesterol concentrations occurred more rapidly (by 6 to 8 d) (Table 4.1) and followed the more transient expression of this protein in apoA-I deficient mice.

A more detailed analysis of the effects of the different adenovirus injections on plasma lipid concentrations in these mice is provided in Table 4.1. Shown are the changes at three different days (2 d, 4 d, and 8 d) and are representative of early, intermediate, and late time points following injections of the adenoviruses. Statistically significant increases ($^{a}p < 0.05$) in all pre-injected plasma lipid concentrations occur following expression of hapoA-I by 4 d post-injection and are maintained at day 8 and beyond. Expression of apoA-I_{FIN} or co-expression of hapoA-I and apoA-I_{FIN}, on the other hand, did not produce these large changes in plasma lipid concentrations. Therefore, by 4 d post-injections there were significant differences in most plasma lipid concentrations between mice expressing apoA-I_{FIN} alone or co-expressing hapoA-I and apoA-I_{FIN} when compared to mice expressing hapoA-I ($^{b}p < 0.05$). Co-expressing hapoA-I and apoA-I_{FIN} produced significant changes by 8 d post-injections compared to mice expressing apoA-I_{FIN} alone ($^{c}p < 0.05$) due to the return to baseline plasma lipid concentrations in mice expressing the mutant protein.

4.3.3 – Effect of ApoA-I_{FIN} on HDL Size and Charge

HDL size and charge were also monitored 4 d following injections of the different adenoviruses (Fig. 4.3). Interestingly, only small HDL formed (8-9 nm) in apoA-I deficient

Table 4.1: The Changes in ApoA-I Deficient Plasma Lipid Concentrations at Different Days following Expression of HapoA-I, ApoA-I_{FIN}, or Both

ApoA-I Expressed	Lipids (mg /dl)											
	TC			FC			CE			PL		
	d2	d4	d8	d2	d4	d8	d2	d4	d8	d2	d4	d8
hapoA-I (n=3)	138 ±20	157 ±4[a]	302 ±15[a]	56 ±9	58 ±5[a]	119 ±11[a]	82 ±12	99 ±7[a]	182 ±6[a]	268 ±43	374 ±49[a]	612 ±28[a]
apoA-I_{FIN} (n=3)	79 ±12	53 ±4[b]	42 ±1[b]	48 ±17	30 ±8	26 ±2[b]	31 ±5	23 ±4[b]	16 ±3[b]	164 ±20	160 ±22	120 ±23[b]
hapoA-I/ apoA-I_{FIN} (n=3)	79 ±28	73 ±11[b]	82 ±8[b,c]	41 ± 8	33 ±4[a]	41 ±6[a]	38 ±20	39 ±8[b]	41 ±6[b,c]	174 ±33	170 ±24[a]	221 ±26[a,b]
Pre-injected (n =9)	40 ±6			23 ±3			18 ±4			102 ± 13		

* The increases in plasma lipid concentrations were found in HDL fractions ($\rho > 1.06$ g/ml)

Mice (n=3 for each group) were injected with either 2×10^9 pfu of either the hapoA-I.Ad5 or the apoA-I_{FIN}.Ad5, or co-injected with 1×10^9 pfu of each. Plasma was collected and lipids were measured the same day as outlined (section 2.2.3).

Plasma lipid concentrations are shown for 2 (d2), 4 (d4), and 8 d (d8) following injections of the apoA-I recombinant adenoviruses. The values are compared for statistical significance (Student's t-test) as outline below.

[a] Statistically significant lipid concentrations ($p < 0.05$) from pre-injected mice

[b] Statistically significant lipid concentrations ($p < 0.05$) from the hapoA-I.Ad5 injected mice

[c] Statistically significant lipid concentrations ($p < 0.05$) for hapoA-I/apoA-I_{FIN} mice compared to apoA-I_{FIN} mice

mice injected with both the hapoA-I.Ad5 and the apoA-I_{FIN}.Ad5 (Fig 4.3A, lanes 3 and 5) similar in size although somewhat larger than in mice injected with the apoA-I_{FIN}.Ad5 (lane 6). The absence HDL_2 in these mice is consistent with that found in heterozygous carriers of the apoA-I_{FIN} mutation (436). In contrast, hapoA-I formed mostly large HDL_2 in these mice (Fig 4.3A, lanes 2 and 4). Despite the decrease in HDL pool size and concentrations, both α and pre-β migrating HDL were present in mice co-expressing apoA-I_{FIN} and hapoA-I (Fig 4.3B – lanes 4 and 5) as was found for mice expressing only hapoA-I (Fig 4.3B – lanes 2 and 3). In contrast, apoA-I_{FIN} was found predominantly as pre-β HDL with a minor population that migrated between the pre-β and α-migrating species. Negative staining electron microscopy also revealed that discoidal HDL were formed by apoA-I_{FIN} (not shown) similar to what we observed previously for apoA-I central domain deletion mutants (Chapter 3). Of note here, the antibodies used (which were generated against lipid free apoA-I) reacted much more strongly with pre-β HDL on transferred agarose gels and no quantitative information on HDL charge distribution is implied or can be determined from this figure (Fig. 4.3B). As expected, most of the hapoA-I was α-migrating as demonstrated by lipid and protein staining (not shown), large (Fig. 4.3A) and very buoyant (Fig. 4.4). The pre-β HDL containing the human apoA-I proteins in these mice were predominantly larger pre-β_2 HDL that co-migrated with the smaller α-HDL on native gels (Fig. 4.3A). There were also smaller amounts of lipid poor pre-β_1 HDL that appeared on the native gels (bottom of Fig. 4.3A.) and when HDL were isolated by discontinuous gradient ultracentrifugation (Fig. 4.4).

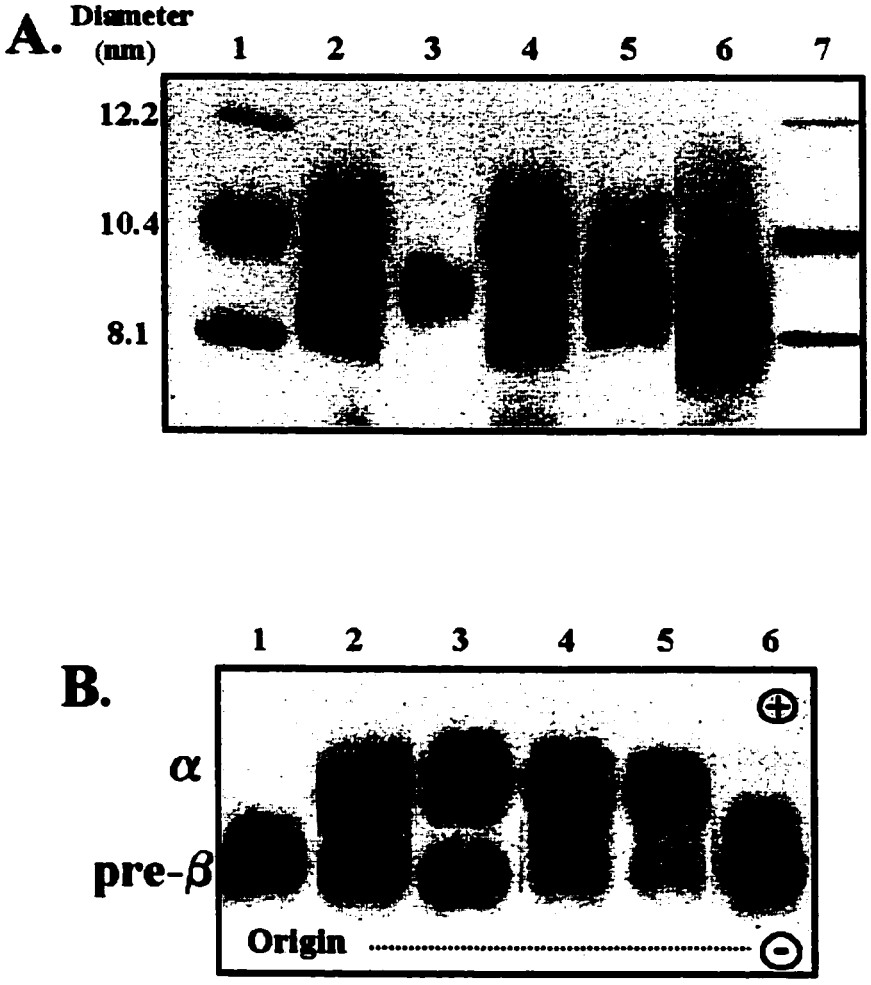

Figure 4.3: Differences in HDL Size and Charge Resulting from the ApoA-I_{FIN} Mutation

Plasma was isolated from apoA-I deficient mice 4 d following injections with the hapoA-I.Ad5, the apoA-I_{FIN}.Ad5, or both as described for Fig. 4.2. ***A.***, plasma was subjected to 4-20% nondenaturing PAGGE and then to Western blot analysis following transfer to nitrocellulose (section 2.2.4). Lanes 1 and 7 contain biotinylated high molecular weight markers of known hydrodynamic diameters as indicated on the left, lanes 2 and 4 hapoA-I plasma (0.33 µl), lanes 3 and 5 hapoA-I/apoA-I_{FIN} plasma (1 µl), and lane 6 apoA-I_{FIN} plasma (1 µl). ***B.***, the charge of the apoA-I lipoproteins was determined by Western blot analysis of an agarose gel electrophoresis. The antibodies react much more strongly with the pre-β HDL and no quantitative information on HDL charge distribution is implied from this figure. However, of note, apoA-I circulates as a mixture of α- and pre β-migrating HDL following expression of hapoA-I alone (1µl, lanes 2 and 3) or following co-expression of hapoA-I and apoA-I_{FIN} (5µl, lanes 4 and 5). In contrast, apoA-I_{FIN} (1 µl, lane 6) is found predominantly as pre-β HDL with minor population that migrates in between the α and pre-β species. Lipid-free apoA-I (0.2 µg) prepared from human plasma (lane 1) is run as a standard.

4.3.4 – Proteolysis of ApoA-I_{FIN} is Confined to HDL_3 and Lipid-poor ApoA-I Species

Proteolysis of apoA-I_{FIN} was also evident following adenovirus-mediated expression in apoA-I deficient mice (Fig. 4.4), similar to that observed in C57BL/6J mice (Fig. 4.1). For these experiments, female apoA-I deficient mice between 4 to 6 months of age were maintained on a Western diet 3 weeks prior to injection of the recombinant adenoviruses. Under these dietary conditions, apoA-I_{FIN} reached significantly higher levels (>100 mg/dl) allowing direct detection of the degradation products in the plasma by both Ponceau staining (not shown) and Western blot analysis (Fig. 4.4A). The monoclonal antibodies used for Western blot analysis recognize epitopes N-terminal to the Leu^{159} → Arg mutation (4H1 and 5F6 epitopes map to residues 1-8 and 118-148 of human apoA-I, respectively). Therefore, the major 18.5 kDa fragment represents an N-terminal degradation product and corresponds to the size expected if cleavage occurred at or near the site of the mutation. HDL fractions were isolated by centrifugation of pooled plasma collected from fasted (8-10 h) apoA-I deficient mice on the Western diet 4 d after injection with either the hapoA-I or the apoA-I_{FIN} recombinant adenoviruses (Fig. 4.4B). The apoA-I_{FIN} degradation products were confined to the small HDL and lipid-poor fractions where the majority of this mutant was located (ρ > 1.13 g/ml). In contrast, no degradation products were found for hapoA-I, which existed mainly as buoyant HDL_2 (ρ= 1.07 g/ml).

4.3.5 –Impaired Hepatocyte Secretion of ApoA-I_{FIN}

Hepatocyte secretion of apoA-I was monitored in primary apoA-I deficient murine hepatocytes infected with either the hapoA-I.Ad5 or the apoA-I_{FIN}.Ad5 (moi=75:1 pfu:cell). As well, in order to simulate hepatic secretion of this mutant in heterozygous individuals, cells were co-infected with equal amounts of both recombinant adenoviruses

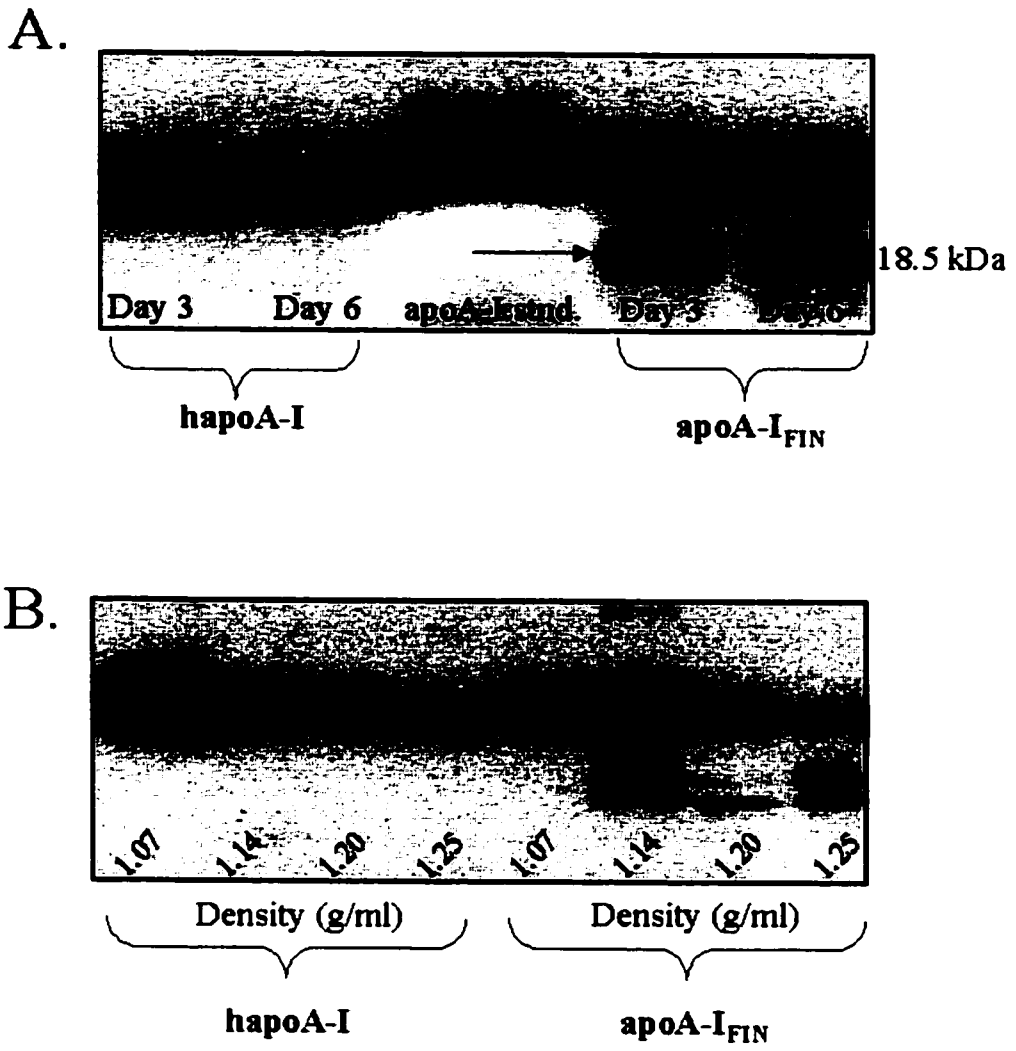

Figure 4.4: Proteolysis of ApoA-I$_{FIN}$ Is Confined to HDL$_3$ and Lipid Poor Species

ApoA-I deficient mice were maintained on the Teklad Western diet for 3 weeks prior to injection of either the hapoA-I or the apoA-I$_{FIN}$ recombinant adenoviruses (2×10^9 pfu). ***A.***, plasma isolated from apoA-I deficient mice at the given days following injections with either the hapoA-I.Ad5 or the apoA-I$_{FIN}$.Ad5 was subjected to 12 % SDS-PAGE under reducing conditions followed by Western blot analysis. Proteolysis of apoA-I$_{FIN}$ is detected in the plasma and the size of the major fragment (←) was found to be 18.5 kDa. ***B.***, selected HDL fractions were isolated from the plasma of fasted (9-11 h) apoA-I deficient mice 4 d following injections of the recombinant adenoviruses and were subjected to 12 % SDS-PAGE and Western blot analysis as described (section 2.2.4). Degradation products for apoA-I$_{FIN}$ are confined to the smaller HDL (HDL$_3$) and lipid-poor species and none are detected for hapoA-I.

at a half dose (moi=37.5 each) in which the combined titer was equivalent to that used to study secretion independently (Fig. 4.5). These results are representative of three separate experiments and significant differences in the amounts of secreted and cell-associated ^{35}S-apoA-I were detected under the different conditions during the initial chase period (t = 1h) (* $p < 0.05$). Less apoA-I was secreted in cells co-infected with the two adenoviruses (Fig. 4.5A, ▲) compared to hepatocytes infected with the hapoA-I.Ad5 alone (Fig. 4.5A, ●). The apoA-I secretion rate was further decreased in cells expressing only the mutant (Fig. 4.5A, ■). Interestingly, there was also less ^{35}S-apoA-I associated with the hepatocytes expressing both apoA-I proteins (Fig 4.5B, Δ) at 1 h compared to hepatocytes expressing only hapoA-I (Fig. 4.5B, ○), and between 20 % to 30 % of the ^{35}S-apoA-I is unaccounted for in these cells during the chase. Likewise, there was also less cell-associated ^{35}S-apoA-I than expected in apoA-I_{FIN} expressing hepatocytes throughout the chase even though this mutant accumulated in the hepatocytes (Fig. 4.5B, □) as anticipated from its impaired rate of secretion.

4.3.6 – Physico-chemical Properties of ApoA-I_{FIN}

The physico-chemical properties of rec.hapoA-I and rec.apoA-I_{FIN} purified from *E. coli* were also compared and found to be very similar. The two proteins have identical kinetics of association with DMPC and behaved similarly to apoA-I purified from human plasma in this assay (not shown). When recombined with lipids *in vitro*, there were also no significant differences in the final molar compositions of Lp2A-I_{WT}, Lp2A-I_{FIN} or Lp2A-$I_{WT/FIN}$ (Table 4.2). All reconstituted lipoproteins contained two molecules of apoA-I as demonstrated by cross-linking with dimethyl suberimidate, and were homogeneous in size (single band between10.0-10.6 nm in diameter) and all proteins were incorporated into the lipoproteins as

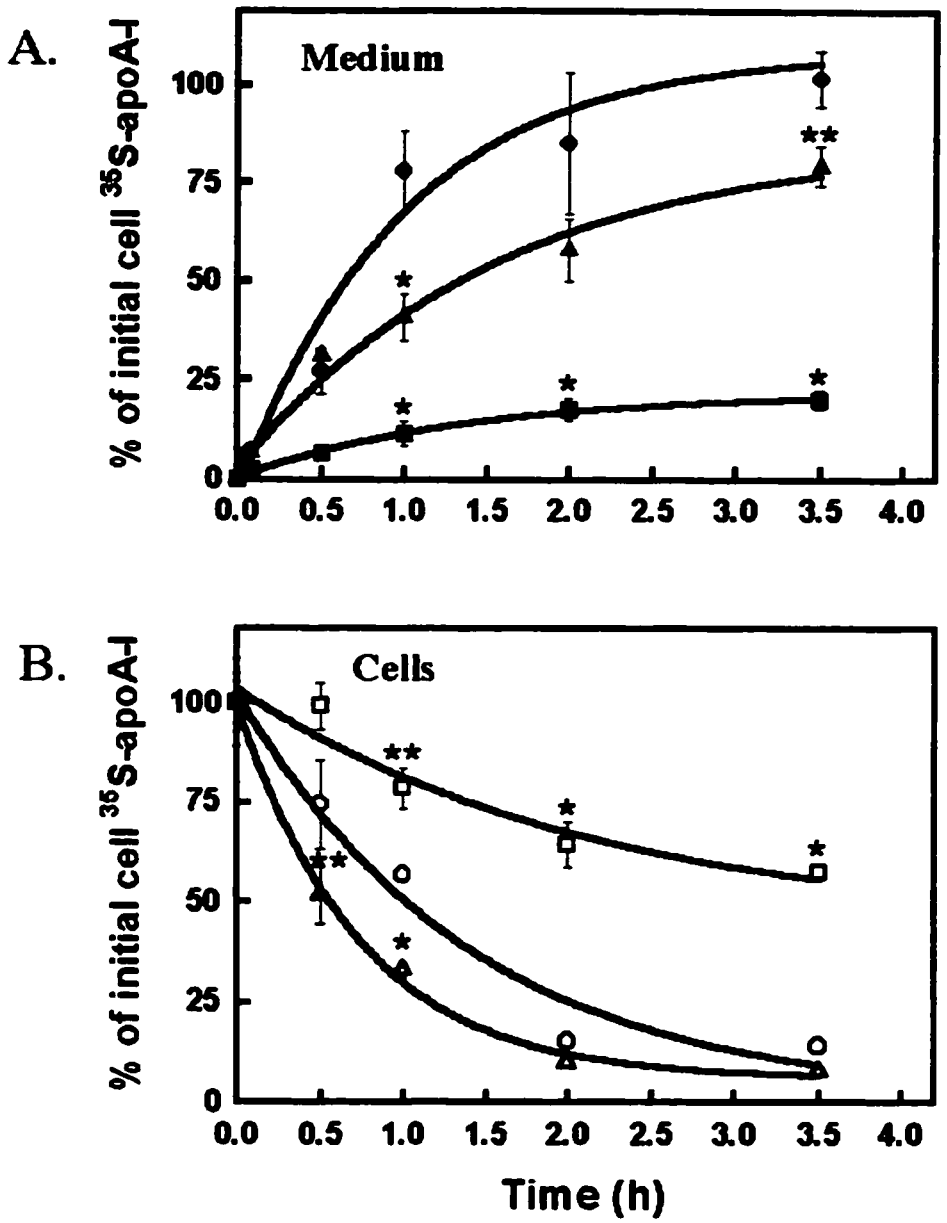

Figure 4.5: Impaired Secretion of ApoA-I_{FIN} from Primary Mouse Hepatocytes

Primary hepatocytes from apoA-I deficient mice were prepared as described previously (462,463) and infected with the recombinant adenoviruses as described (section 2.2.12) ***A.***, the accumulation of ^{35}S-apoA-I (given as the percentage of initial cell associated apoA-I) in the medium is reported as a function of chase time following expression of hapoA-I (●), apoA-I_{FIN} (■) or both (▲). The initial rates of apoA-I secretion at the 1 h time point during the chase (before the plateaus are reached) shows that hepatocytes co-expressing hapoA-I and apoA-I_{FIN} (▲) have impaired apoA-I secretion compared to hepatocytes expressing hapoA-I (●) ($p < 0.05$, Student's t-test). ApoA-I secretion is reduced further in hepatocytes expressing apoA-I_{FIN} (■) ($p < 0.05$ at 1 h). ***B.***, the amount of ^{35}S-apoA-I remaining in the cells (as a

percentage of initial cell associated apoA-I) is reported as a function of chase time following expression of hapoA-I (○), apoA-I_{FIN} (□) or both (Δ). Interestingly, at the 1 h time point less cell associated apoA-I is found in hepatocytes co-expressing hapoA-I and apoA-I_{FIN} (Δ) compared to hepatocytes expressing hapoA-I (○) ($p < 0.05$) despite the fact these cells also secrete less apoA-I into the medium. Statistically significance differences in medium and cell-associated apoA-I between hepatocytes co-expressing the two proteins or expressing apoA-I_{FIN} alone and hepatocytes expressing hapoA-I are shown (* $p < 0.05$ or ** $p < 0.10$).

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assessed by native 8-25 % PAGGE (not shown). In addition, the stability of association (ΔG_D^0) of rec.apoA-I_{FIN} with lipids was similar to rec.hapoA-I (Table 4.2).

4.3.7 - Effect of ApoA-I_{FIN} Mutation on LCAT activation

The effect of the apoA-I_{FIN} mutation on LCAT activation was assessed (Fig. 4.6). As mentioned (section 2.2.16), the Lp2A-$I_{WT/FIN}$ were prepared to represent the nascent lipoprotein population that would most likely form in heterozygotes for the apoA-I_{FIN} mutation. They comprise a heterogeneous mixture of reconstituted lipoproteins that contain one molecule of each recombinant protein (≅ 50% of total), two molecules of rec.hapoA-I (≅ 25% of total), or two molecules of rec.apoA-I_{FIN} (≅ 25% of total). The Michaelis Menten constants, appK_m and V_{max} (Table 4.2) were determined for the three sets of Lp2A-I particles from the double reciprocal Lineweaver-Burke plot (Fig. 4.6C). There was both a large increase in appK_m and decrease in V_{max} for Lp2A-I_{FIN} (■) over Lp2A-I_{WT} (●) (Fig. 4.6A, Table 4.2). Interestingly, the Lp2A-$I_{WT/FIN}$ (Δ) exhibited only a small increase in appK_m over the Lp2A-I_{WT} and no difference in V_{max} (Fig 4.6A, Table 4.2). In addition, the Lp2A-$I_{WT/FIN}$ activated LCAT more efficiently than a 1:1 mixture of preformed Lp2A-I_{WT} and Lp2A-I_{FIN} (▽), which had an expected activation of LCAT that was intermediate between that found for Lp2A-I_{WT} (●) and Lp2A-I_{FIN} (■) (Fig. 4.6B). There were also only small differences in the LCAT activation of

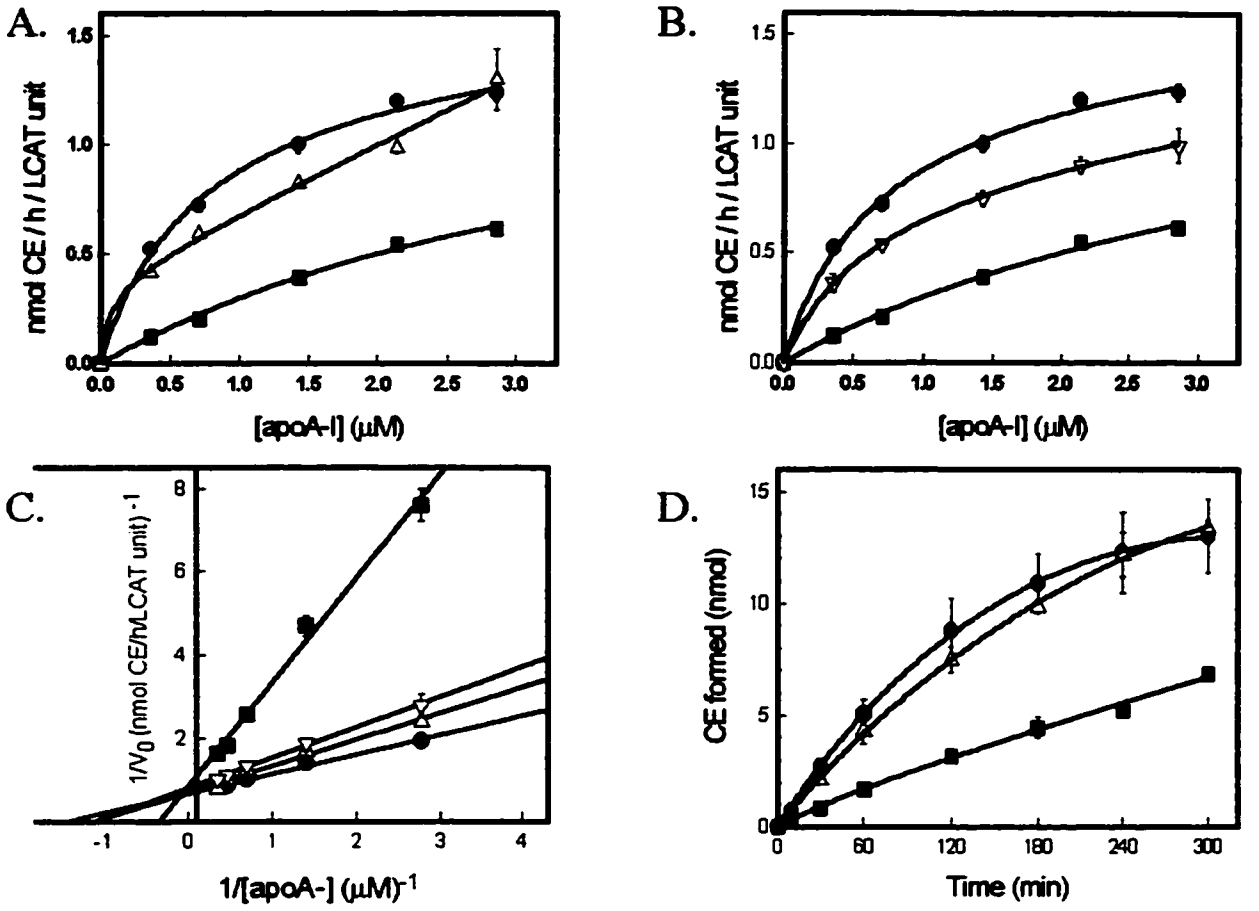

Figure 4.6: ApoA-I_{FIN} has Impaired LCAT Activation but Does Not Negatively Affect the Activation by HapoA-I

The three sets of reconstituted lipoproteins (Lp2A-I) were prepared as described (section 2.2.16). ***A.***, the esterification of [^{3}H-cholesterol] in three different reconstituted Lp2A-I particles by LCAT is shown. The Lp2A-I prepared with rec.hapoA-I alone (Lp2A-I_{WT}) (●), rec.apoA-I_{FIN} alone (Lp2A-I_{FIN}) (■), or a 1:1 molar ratio of two (Lp2A-$I_{WT/FIN}$) (Δ) was incubated with purified LCAT. Values are the mean (± SE) of duplicate experiments with triplicate measurements at each data point. ***B.***, as in *A.*, except LCAT activation of a 1:1 molar mixture of pre-formed Lp2A-I_{WT} and Lp2A-I_{FIN} (▽) instead of the hybrid particles is compared to Lp2A-I_{WT} (●) and Lp2A-I_{FIN} (■). *C.*, the double-reciprocal plot of cholesterol esterification for the 4 sets of Lp2A-I preparations is shown. The reciprocals of the initial velocities (V_O) are plotted against the reciprocals of apoA-I concentration (μM) for each of the Lp2A-I preparations and are used to calculate the rate constants appK_m and V_{max} for three sets of Lp2A-I (excluding the mixture of the two Lp2A-I) given in Table 4.2. ***D.***, the nmol of CE formed over a 5 h period with 3.5 U of LCAT was determined for the Lp2A-I_{WT} (●), Lp2A-I_{FIN} (■), and Lp2A-$I_{WT/FIN}$ (Δ) particles each at a final apoA-I concentration of 2.0μM.

Lp2A-I_{WT} (●) and the Lp2A-$I_{WT/FIN}$ (Δ) (measured as total CE formed) over an extended period of time at a set lipoprotein concentration (2.0 μM apoA-I) (Fig. 4.6D). However, the Lp2A-I_{FIN} (■) had a greatly reduced LCAT activation, similar to what was found during the shorter incubation at varying substrate concentrations.

4.3.8 – Cholesterol Efflux and the ApoA-I_{FIN} Mutation

Lastly, the effect of the apoA-I_{FIN} mutation on the ability of apoA-I, both as lipid-free and as Lp2A-I, to promote efflux of cholesterol from macrophages was also determined (Fig. 4.7). There were no differences between rec.apoA-I_{FIN} and rec.hapoA-I in their abilities to promote cholesterol efflux from J774 macrophages as either lipid-free proteins or when reconstituted as Lp2A-I. Therefore, these data are consistent with the initial observations that the apoA-I_{FIN} mutation does not negatively affect cholesterol efflux from cells in culture (436).

4.4 - Discussion

This study offers new insights into the multiple and complex effects of apoA-I_{FIN} on HDL metabolism. The cause for the dominant hypoalphalipoproteinemia induced by this mutation in heterozygous carriers has remained poorly understood since it was first identified by Miettinen *et al.* (430). The mutation was found to impair the ability of apoA-I to activate LCAT but it was suggested that this could not account for the 4 to 5-fold reduction in HDL-C and apoA-I concentrations in these individuals (436). Therefore, we have expanded on these initial *in vitro* LCAT studies and designed informative *in vivo* experiments using recombinant adenoviruses to study the effect of the apoA-I_{FIN} mutation on HDL metabolism. Our results show that while impaired LCAT activation may contribute to the dominant hypoalphalipoproteinemia in carriers of apoA-I_{FIN} (discussed below), it is clear that other

Table 4.2: Physical Properties and LCAT Kinetic Data for Lp2A-I Prepared with Recombinant HapoA-I, ApoA-I_{FIN}, or a 1:1 Molar Mixture of the Two Proteins.

Reconstituted [a] Lipoprotein	ApoA-I per [b] particle	POPC: FC: apoA-I ratio (n = 6)	$appK_m$ [c] (μM)	V_{max} [c] (nmol CE/h/ LCAT unit)	ΔG_D^0 [d] (kcal / mol apoA-I)
Lp2A-I_{WT}	2	86 : 10 : 1 ± 6 ±0.8	0.70 ± 0.02	1.57 ± 0.07	2.49 ± 0.43
Lp2A-I_{FIN}	2	90 : 11 : 1 ±8 ±0.9	3.28 ± 0.24[e]	1.26 ± 0.07[e]	2.18 ± 0.47
Lp2A-$I_{WT/FIN}$	2	85 : 10 : 1 ± 8 ±0.6	1.00 ± 0.12	1.54 ± 0.15	2.30 ± 0.14

[a] All reconstituted lipoproteins were prepared by the cholate dispersion/Bio-Beads removal technique as described (section 2.2.14).

[b] All reconstituted lipoproteins contain 2 molecules of rec.apoA-I as determined by cross-linking with dimethyl suberimidate.

[c] $appK_m$ and V_{max} values were derived from the double reciprocal Lineweaver-Burke plot (Fig. 4.6 C).

[d] The stability of association of the proteins with the Lp2A-I was determined in the presence of GdnHCl using a Jasco J4 CD spectropolarimeter.

[e] Statistically significant ($p < 0.05$) from Lp2A-I_{WT} as determined by Student's t-test (paired two sample for means).

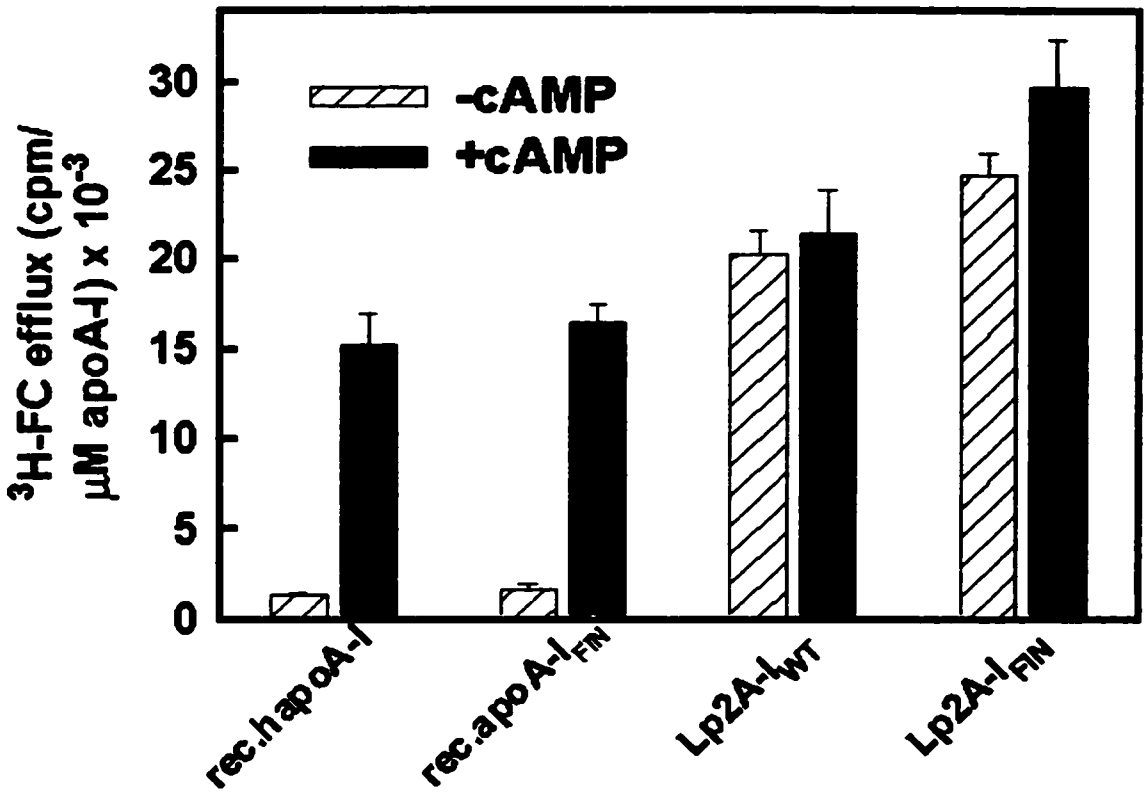

Figure 4.7: Cholesterol Efflux to ApoA-I_{FIN} from Macrophages is Normal in Cell Culture

Cholesterol efflux from J774 macrophages to lipid-free or lipidated rec.apoA-I or rec.apoA-I_{FIN} was performed as outlined (section 2.2.17). There is no difference in cholesterol efflux to rec.hapoA-I or rec.apoA-I_{FIN} as either lipid-free proteins or when reconstituted as Lp2A-I in the presence (solid bars) or absence (hatched bars) of cAMP.

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mechanisms are also responsible for conferring this dominant negative phenotype. Furthermore, this study illustrates the advantages of using recombinant adenoviruses for expression of apoA-I variants in mice and primary hepatocytes when *in vitro* studies alone are not sufficiently informative for the study of HDL metabolism.

The apoA-I_{FIN} mutation (Leu^{159} to Arg) occurs on the hydrophobic face of helix 6 (aa 143-165), a region of the protein that has been shown by both *in vitro* studies (383,412,425,427,428,435,476-478) and more recently by *in vivo* studies (483,487,488) to be important for LCAT activation. This helix is highly conserved amongst species (346) and therefore non-conserved amino substitutions in this region might be expected to interfere with LCAT activation by apoA-I. This was demonstrated for apoA-I_{FIN} (436) and more recently in a study where three conserved Arg residues in this region were mutated (383). However, it remains unclear how lipoproteins containing both apoA-I_{FIN} and hapoA-I activate LCAT compared to lipoproteins that contain only hapoA-I (see below). Also, the effect of apoA-I_{FIN} on LCAT activity has not been analyzed *in vivo* in the homozygous state. In this study apoA-I_{FIN} generates HDL with predominantly pre-β migration in apoA-I deficient mice (Fig. 3B, lane 6) and analysis of plasma lipoproteins from these mice by negative-staining electron microscopy demonstrates that this mutant but not hapoA-I forms discoidal HDL (not shown). As well, even during peak expression of this mutant (2-3 d) the CE/TC ratio in the plasma samples average only 0.39 and is lower than found for pre-injected values (derived from Table 4.1). Therefore, these *in vivo* findings support previous and current (see below) *in vitro* data that apoA-I_{FIN} has impaired LCAT activation in the absence of hapoA-I.

Detailed *in vitro* LCAT experiments were next performed to address the possibility that the apoA-I_{FIN} mutation might inhibit LCAT activation in lipoproteins containing both apoA-I_{FIN} and hapoA-I and contribute to its dominant negative effect on HDL-C and apoA-I concentrations in this manner (Fig. 4.6). Lp2A-I of similar size, lipid composition, and stability are formed with rec.hapoA-I (Lp2A-I_{WT}), rec.apoA-I_{FIN} (Lp2A-I_{FIN}) or both (Lp2A-$I_{WT/FIN}$) (Table 4.2). This ensures that we are studying an effect of the mutation on LCAT activation and not secondary effects due to differences in lipid composition that can

independently alter the activity of this enzyme (468,493). The Lp2A-$I_{WT/FIN}$ are important because they most closely represent the nascent HDL that would form in heterozygotes for this mutation. This heterogeneous lipoprotein population comprises Lp2A-I with either one molecule each of rec.hapoA-I and rec.apoA-I_{FIN} or Lp2A-I containing two molecules of rec.hapoA-I or two molecules of rec.apoA-I_{FIN}. As such, the majority (70-80%) of the Lp2A-I would contain at least one molecule of rec.hapoA-I and one molecule of rec.apoA-I_{FIN}. Therefore, if apoA-I_{FIN} is dominant over hapoA-I with respect to LCAT activation, the Lp2A-$I_{WT/FIN}$ should behave similarly to the Lp2A-I_{FIN} in this assay. If not, the LCAT activation of the Lp2A-$I_{WT/FIN}$ should more closely resemble that of the Lp2A-I_{WT}. The latter is observed here. We find that the Lp2A-I_{FIN} (Fig 4.6A, ■ and Table 4.2) does have a marked reduction in the affinity for the enzyme (large increase in appK_m), which is consistent with our *in vivo* data (above), whereas the Lp2A-$I_{WT/FIN}$ (Fig 4.6A, Δ) have only slightly impaired LCAT activation compared to Lp2A-I_{WT}. There is a small increase in the appK_m and no difference in V_{max} for the Lp2A-$I_{WT/FIN}$ compared to Lp2A-I_{WT} (Table 4.2). Furthermore, the Lp2A-$I_{WT/FIN}$ are more efficient at activating LCAT than is a 1:1 mixture of preformed Lp2A-I_{WT} and Lp2A-I_{FIN} (Fig. 4.6B, ∇). These data clearly demonstrate that the apoA-I_{FIN} mutation does not negatively affect LCAT activation of hapoA-I and hapoA-I even appears to overcome some of the inhibition of apoA-I_{FIN}. Our *in vivo* data supports these *in vitro* results. Both pre-β and mature α-migrating HDL are present in apoA-I deficient mice expressing both proteins (Fig. 4.3B, lanes 4 and 5), but not in mice expressing only apoA-I_{FIN} (Fig. 4.3B, lane 6). Taken together, these data indicate that LCAT activation is only moderately impaired in the heterozygote state and can not fully account for the hypoalphalipoproteinemia in carriers of apoA-I_{FIN}.

Since many prohormones are cleaved at paired basic amino acids (494) it was suggested that the presence of two consecutive arginines within apoA-I produced by the $Leu^{159} \rightarrow Arg$ substitution may make the mutant protein susceptible to proteolytic cleavage (430). Of note, the pro sequences of both human and apoA-II are cleaved in the plasma at a paired basic sequences immediately downstream (–1,–2) of the first amino acid in the mature proteins. Nonetheless, proteolysis of apoA-I_{FIN} was not detected by Western blot analysis of plasma samples isolated from heterozygotes in the initial studies of Miettinen *et al.* and the possibility that apoA-I_{FIN} was degraded was not explored further in these studies (430). However, it is clear in this study that even with precautions to reduce degradation artifacts, that apoA-I_{FIN} but not hapoA-I undergoes proteolysis in both wild-type (Fig. 4.1) and apoA-I deficient mice (Fig. 4.4) following adenovirus-mediated expression. The higher concentrations of apoA-I_{FIN} obtained following injections of the recombinant adenoviruses have made possible the detection of these proteolytic cleavage products of apoA-I_{FIN}, which under normal circumstance are likely cleared rapidly from the circulation and escape detection. This proteolysis appears to play an important role in reducing HDL-C and apoA-I concentrations. In our *in vivo* system, we show that there is a direct correlation between the extent of apoA-I_{FIN} proteolysis and the decrease in mapoA-I concentrations (Fig. 4.1). In fact, mapoA-I is barely detectable in the plasma sample where there is greatest amount of detectable apoA-I_{FIN} proteolysis (Fig. 4.1B, lane 4 – lower right panel). This reduction in mapoA-I levels occurs with only low to moderate concentrations of apoA-I_{FIN} (50 ± 4.6 mg/dl) and is specific for the mutant protein. Higher and more physiological concentrations of hapoA-I (147 ± 26 mg/dl) do not have this statistically significant effect (Fig. 4.1). This finding appears different from that reported previously with human apoA-I transgenic (HuA-I Tg) mice, in which high expression of hapoA-I was shown to reduce mapoA-I concentrations (495). However, the

two systems are not directly comparable. Two major differences between this study and the previous one is that in the prior study the HuA-I Tg mice were fasted overnight and had higher circulating concentrations of human apoA-I. In the present study, the C57BL/6J mice were not fasted and hapoA-I is found only to slightly reduce mapoA-I plasma concentrations without reaching statistical significance ($p = 0.14$). In contrast, sub-physiological concentrations of apoA-I_{FIN} significantly reduce mapoA-I levels ($p < 0.03$) (Fig. 4.1C). Furthermore, we observe a similar and dominant effect of apoA-I_{FIN} on apoA-I and HDL-C concentrations when this mutant is co-expressed with hapoA-I in apoA-I deficient mice (Fig. 4.2), a system that more closely mimics the heterozygous state. ApoA-I concentrations are 3 to 8-fold lower in apoA-I deficient mice expressing both proteins compared to mice expressing hapoA-I alone throughout the time course of expression (Fig. 4.2A). Consequently, the HDL-C (Fig 4.2B) and plasma lipid (Table 4.1) concentrations are greatly reduced and large HDL_2 are absent (Fig. 4.3A - lanes 3 and 5) in these mice, similar findings to those reported previously for heterozygous carriers of this mutation (436).

The dramatic reductions in apoA-I and HDL-C concentrations caused by apoA-I_{FIN} proteolysis should not come as a surprise. Numerous studies have demonstrated that proteolytic degradation of apoA-I can have a major impact on HDL metabolism. ApoA-I degradation by elastase was shown to enhance the binding and intracellular clearance of HDL by macrophages (496). Limited proteolysis of apoA-I by matrix metalloproteinases has also shown to decrease cholesterol efflux from cholesterol-loaded macrophages (497) and similarly, mild trypsinization of HDL effectively abolishes apolipoprotein-mediated cholesterol efflux from cholesterol-loaded fibroblasts (498). Therefore, even though the apoA-I_{FIN} mutation does not affect cholesterol efflux from cells in culture as we (Fig. 4.7) and Miettinen *et al.* (436) have found, it is likely that proteolysis of apoA-I_{FIN} *in vivo* interferes with HDL-mediated efflux

in mice expressing this mutant. In support of this, apoA-I_{FIN} is selectively degraded on smaller HDL and lipid poor species (Fig. 4B), the most important acceptors of cell-derived PL and cholesterol [reviewed in (119)]. Furthermore, another study has shown that proteolysis leads to dissociation of apoA-I from HDL, especially on the less buoyant HDL_3, and produces an unstable HDL population (499). We also find that apoA-I_{FIN} is preferentially degraded on HDL_3 and lipid-poor species and this is likely to promote a more rapid clearance of native apoA-I and other HDL apolipoproteins and in the process interfere with their maturation into larger and more buoyant HDL. This hypothesis is consistent with our findings that mice co-expressing apoA-I_{FIN} and hapoA-I (Fig. 4.2B, lanes 3 and 5) and wild-type mice expressing this mutant are devoid of HDL_2. It also provides an explanation as to why other HDL apolipoproteins, such as apoA-II, are also found at lower than normal concentrations in heterozygotes for this mutation (430).

This is also the first demonstration that the apoA-I_{FIN} mutation decreases the rate of apoA-I secretion from primary hepatocytes (Fig. 4.5). The effect is greatest under conditions that mimic apoA-I_{FIN} homozygosity (infection with the apoA-I_{FIN} adenovirus alone) but it is also observed in the heterozygous state (co-infection with the two adenoviruses). This is particularly true early on in the chase (t = 1 h) before the plateaus in the secretion time course are reached. In fact, hepatocytes expressing both apoA-I_{FIN} and hapoA-I have statistically significant decreases (* $p < 0.05$) in secreted ^{35}S-apoA-I (Fig 4.5A, ▲) as well as cell-associated ^{35}S-apoA-I (Fig. 4.5B, Δ) at the 1 h time point compared to hepatocytes expressing only hapoA-I (Fig 4.5A, ● and Fig 4.5B, ○). This suggests that the apoA-I_{FIN} mutation interferes with apoA-I secretion and causes apoA-I to be degraded intracellularly given that between 20-30% of the initial cell associated apoA-I cannot be accounted for throughout the chase. This is also found in hepatocytes expressing apoA-I_{FIN} alone. The decreased secretion of apoA-I_{FIN}

(Fig. 4.5A, ■) into the medium cannot be fully accounted for by its accumulation within the cell (Fig 4.5B, □). Therefore, this is the first data to suggest that heterozygous carriers of the apoA-I_{FIN} mutation also have impaired apoA-I secretion in addition to enhanced apoA-I clearance from the plasma. Some studies have suggested that HDL-C levels are inversely correlated with the fractional catabolic rate of apoA-I and not with the apoA-I secretion rate (500). However, a more recent study utilizing endogenously labeled apoA-I has shown that both the fractional catabolic rate and secretion rate contribute to plasma HDL-C levels (501). Therefore, the reduced secretion rate of apoA-I in hepatocytes expressing both apoA-I_{FIN} and hapoA-I most likely contributes to the low apoA-I and HDL-C concentrations in heterozygous carriers of this mutation. We also propose that there is an increased intracellular clearance of hapoA-I in the presence of apoA-I_{FIN}, since a proportion of apoA-I secreted from these primary hepatocytes is in the form of lipid-associated multimeric complexes (Chapter 5). Proteolysis of apoA-I_{FIN} inside the hepatocytes could prevent efficient secretion of this nascent HDL population, similar to its effect on the clearance of the lipid-poor HDL pool in the plasma.

There are at least 7 other mutations identified within the central domain of apoA-I that are also associated with reduced plasma HDL-C and apoA-I concentrations (433,434,502,502-508). We have shown in this study that apoA-I_{FIN} has a greatly reduced ability to activate LCAT but this alone cannot account for the hypoalphalipoproteinemia in heterozygous carriers of this mutation. In contrast, a recent *in vivo* study suggests that deletion of aa 143 to 164 within apoA-I may negatively affect LCAT activation by native apoA-I (488). It was proposed that this might explain the dominant negative effect on HDL-C concentrations seen in heterozygotes for the apoA-$I_{Seattle}$ mutation (deletion of aa 146 to 160), although it should be noted that the two mutations are structurally different. In addition, no *in vitro* LCAT studies

have been reported to confirm this hypothesis. Conversely, it has been suggested that the low HDL-C concentrations in heterozygotes for apoA-I mutations such as apoA-$I_{Seattle}$, apoA-I_{FIN}, and apoA-$I_{Zavalla}$ (Leu^{159} to Pro) results from hypercatabolism of apoA-I that is only partially due to or independent of a decrease in LCAT activation (503). This latter viewpoint is consistent with the results obtained from this study in which we show that proteolysis of apoA-I_{FIN} and impaired secretion of apoA-I from hepatocytes caused by this mutation are more likely to account for the low HDL-C and apoA-I concentrations than is dysfunctional LCAT activation.

In summary, this extensive metabolic study of a naturally occurring apoA-I variant known as apoA-I_{FIN} should also prove valuable in the study of other apoA-I mutations contributing to hypoalphalipoproteinemia, and further our understanding of the roles of apoA-I domains in the metabolism of HDL. We have shown that the apoA-I_{FIN} mutation does not affect the ability of apoA-I to associate with lipids and form stable reconstituted lipoproteins. ApoA-I_{FIN} does have reduced LCAT activation (5-fold increase in appK_m) compared to hapoA-I but in a heterogeneous lipoprotein preparation with hapoA-I there is only a slight decrease in the affinity for the enzyme (1.4-fold increase in appK_m). Importantly, this mutation impairs apoA-I secretion from primary hepatocytes and leads to proteolysis of apoA-I. These effects appear to be primarily responsible for the remodeling of and decrease in the HDL pool size in both wild-type C57BL/6J mice expressing this mutant and in apoA-I deficient mice co-expressing apoA-I_{FIN} and hapoA-I. Therefore, we propose the combination of these defects (including the decrease in LCAT activation) account for the 4 to 5-fold reductions in HDL-C and apoA-I concentrations in heterozygous carriers of the apoA-I_{FIN} mutation.

CHAPTER 5 - THE HETEROGENEITY AND LIPIDATION OF HUMAN APOA-I SECRETED FROM PRIMARY HEPATOCYTES

5.1 - Summary

ApoA-I deficient mouse primary hepatocytes were infected with the rec.hapoA-I adenovirus to study the speciation of apoA-I secreted by these cells. The cells were maintained in serum-free medium to avoid complications that would arise due to lipoproteins in the medium and were labeled with ^{3}H-choline in order to measure PL secreted with apoA-I. Following a 3.5 h chase in cold medium, apoA-I associated with three different lipoprotein fractions isolated by FPLC. A small proportion (5-10%) of apoA-I was secreted with larger VLDL/LDL sized species, while the majority (60-80%) of apoA-I was secreted as lipid-free or lipid-poor species that co-localized with albumin. Interestingly, the remaining apoA-I (15-25%) was secreted as larger HDL ($HDL_{2/3}$ size) of pre-β migration. Importantly, this fraction was not associated with apoB-containing lipoproteins (*i.e.* apoB48 HDL) suggesting that in humans a significant amount of newly secreted apoA-I may be of pre-β_2 size. Immunoprecipitations of apoA-I under native conditions were performed on the three lipoprotein pools and the majority of ^{3}H-choline containing PL were present with immunoprecipitated apoA-I in the $HDL_{2/3}$ pool. The HDL in this pool were heterogeneous in size and varied from 7-11 nm in hydrodynamic diameter. In contrast, the lipid-poor apoA-I was smaller as expected (less than 7 nm) and of higher concentrations. This model will be useful to further characterize apoA-I secreted from primary hepatocytes and to establish if ABCA1 contributes to the lipidation of this nascent HDL pool.

5.2 – Background

There is still some uncertainty as to the size and heterogeneity of apoA-I lipid complexes secreted from enterocytes and hepatocytes. Over thirty years ago, Windmueller and Levy showed that HDL were secreted from rat hepatocytes as distinct products from VLDL (509). In this study, α-migrating HDL were found in the re-circulating perfusates of rat livers in which VLDL secretion was inhibited by orotic acid. Subsequently, Hamilton *et al.* showed that discoidal particles accumulate in the perfusates of rat liver when LCAT is inhibited with 5,5'-dithionitrobenzoic acid (510). These discoidal particles are not derived from TG-rich lipoproteins as they were also found following treatment with orotic acid (511). However, liver perfusion studies cannot differentiate between discoidal HDL as newly secreted particles or as lipoproteins that have been remodeled and/or formed within the circulation.

Some of the complications inherent in liver perfusion studies are overcome by studying apoA-I secretion from cultured cells. Transformed cell lines (some which do not normally secrete apoA-I), primary hepatocytes and intestinal mucosa cells have been used for this purpose. In the absence of serum, many studies have reported that apoA-I is secreted from cells predominantly as a lipid-free or poor form using density gradient ultracentrifugation techniques (512-515). In all cases, supplementation of the medium with lipids (*i.e.* 10% FBS) caused apoA-I to associate with more buoyant HDL sized species. Studies by Castle *et al.* using cynomolgus monkey hepatocytes restored to culture from cryopreservation showed that apoA-I is secreted into serum-free medium as a lipid-poor 50-70 kDa pre-β migrating protein (516). This confirms the result from other studies (above) and suggests that larger pre-β_2 and pre-β_3 HDL are produced in the circulation prior to their conversion to spherical α-migrating HDL.

Secretion of apoA-I from hepatocytes is revisited in this work. As mentioned, most previous studies isolated apoA-I by ultracentrifugation techniques. These methods may cause dissociation of apoA-I from newly secreted HDL and increase the amount of apoA-I present in lipid-poor fractions (see discussion). This is supported by studies showing that apoA-I is often displaced from larger HDL during ultracentrifugation spins (517). In this work, apoA-I deficient hepatocytes were infected with the hapoA-I.Ad5 to study secretion of native human apoA-I from a primary cell line. The lipoproteins secreted into serum-free medium were separated on calibrated Superdex 200 columns as a means to circumvent the use of density gradient isolation techniques. As outlined (section 2.2.6) these columns enable effective separation of VLDL/LDL, $HDL_{2/3}$ and lipid-poor apoA-I pools. In addition, the cells were labeled with choline to evaluate the association of newly synthesized PL with secreted apoA-I. While the results from this study support previous work that the majority of apoA-I is secreted as a lipid poor species (< 70 kDa), we find that as much as 20% of the total apoA-I secreted can be secreted as larger pre-β migrating HDL. The implications of these findings and future studies to further characterize this nascent HDL pool are addressed.

5.3 - Results

5.3.1 – Newly Secreted HapoA-I has Pre-β Electrophoretic Migration

First, the migration of newly secreted apoA-I on agarose gels from primary hepatocytes was assessed (Fig. 5.1). In this experiment, medium was collected following 24 h of infection of the hepatocytes with the hapoA-I.Ad5. Similar to previous studies (section 5.2), apoA-I was found to have exclusively pre-β migration and no α-migrating HDL was found secreted by primary hepatocytes expressing hapoA-I.

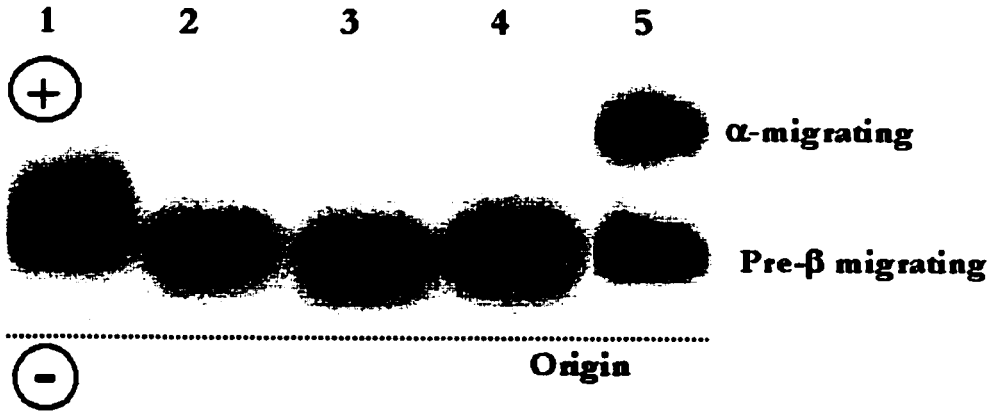

Figure 5.1: HapoA-I Secreted from Primary Hepatocytes has Pre-β Migration

Twenty-four hours after initial infection of apoA-I deficient primary mouse hepatocytes with the hapoA-I.Ad5, medium samples were collected and spun down to pellet any cell debris (10 min 2,000 xg). The medium (Hepatozyme®) from different wells were concentrated 50-fold (10 K M_r cutoff) and 1 µl of the concentrated medium was loaded on the Lipogel: lane 1: plasma apoA-I standard (1 µg), lanes 2-4: hapoA-I secreted into Hepatozyme medium from three independent samples, lane 5 – human HDL standard (1.5 µg). The gels were transferred to nitrocellulose and subjected to Western blot analysis as described (section 2.2.4)

5.3.2 – ApoA-I is Secreted in Different Lipoprotein Pools

To evaluate the lipoprotein distribution of apoA-I secreted over a short period of time, Hepatozyme medium labeled with ^{3}H-choline (see section 2.2.18) was removed from the hepatocytes expressing hapoA-I. ApoA-I secretion into unlabeled serum-free medium was monitored over a 3.5 h chase period (below). This time was chosen so there would be ample secretion of hapoA-I for analysis, yet relatively short to prevent the formation of artifacts within the medium (*i.e.* interaction with and removal of lipids from cell membrane surfaces). Free choline was removed following concentration of the medium (Centripep-10). Care was also taken to analyze the medium by FPLC soon after collection (< 2 h) to minimize the loss of potentially unstable nascent HDL populations. Equal volumes of the FPLC fractions were

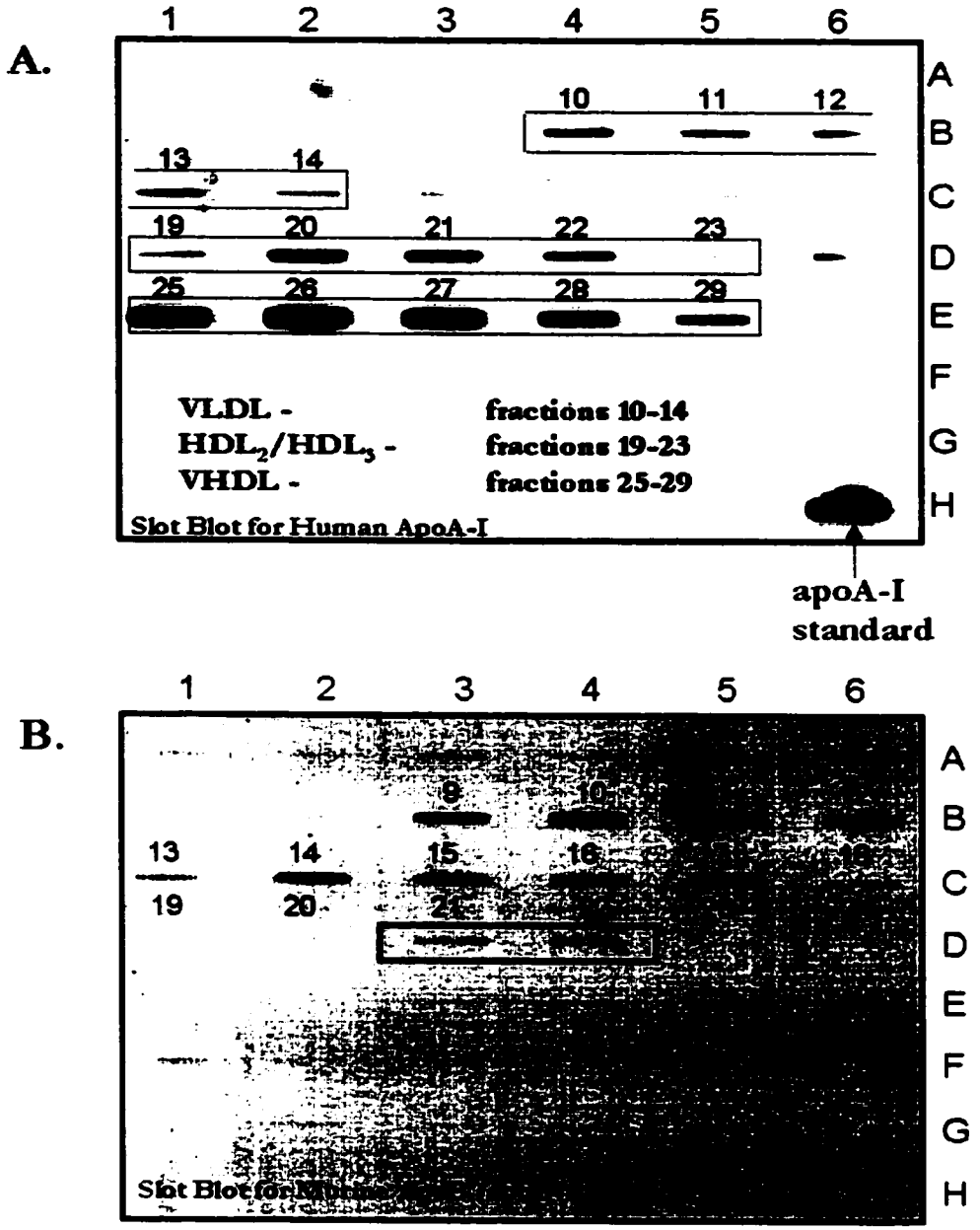

Figure 5.2: Slot Blot Analysis of HapoA-I and MapoB in FPLC Fractions

Concentrated medium from hepatocytes labeled with choline and infected with the hapoA-I.Ad5 was separated by FPLC and fractions (100 µl) obtained were analyzed by slot blot analysis by probing for either hapoA-I (Panel A.) or mapoB (Panel B.). FPLC fractions were loaded in order beginning in lane 1A going from left to right and down. *Panel A.* - there is good separation of the VLDL (fractions 10-14), $HDL_{2/3}$ (fractions 19-23) and lipid poor species (25-29) containing hapoA-I. *Panel B.* – MapoB is localized to large VLDL (lanes 9-14) as expected and very little apoB is found secreted in the $HDL_{2/3}$ lanes (faint expression in fractions 22 and 23 – boxed). Note the good separation of apoB lipoproteins (panel B.) from the apoA-I lipoproteins (panel A..)

analyzed by slot blot analysis for apoA-I (Fig. 5.2A) and murine apoB (mapoB [both apoB100 and apoB48] – Fig. 5.2B). There is good separation of the three lipoprotein fractions containing hapoA-I (Fig. 5.2A.). Furthermore, there was very little apoB found in the $HDL_{2/3}$ fraction and most is contained within the VLDL/LDL pool (fractions 9-14). Three separate experiments were performed to analyze the distribution of hapoA-I in the three different lipoprotein pools during the 3.5 h chase into serum-free medium. The intensity of the apoA-I bands, at non-saturating levels, was measured by densitometric scanning. The percent distribution (± SE) of apoA-I in the different lipoprotein fractions is shown in Fig. 5.3. Interestingly, approximately 20% of the total apoA-I secreted was found in $HDL_{2/3}$ sized fractions. As well, a smaller but significant percentage of secreted apoA-I was also found associated with the VLDL/LDL pool.

5.3.3 – ApoA-I in the $HDL_{2/3}$ Pool is Heterogeneous in Size

The different apoA-I populations secreted by the primary hepatocytes were sized by nondenaturing 4-20% PAGGE and Western blot analysis (Fig. 5.4). ApoA-I is found at various sizes in concentrated medium (lane 1) although the majority of the protein migrated to the bottom of the gel and was less than 7.1 nm in diameter when compared to biotinylated high molecular weight markers of known hydrodynamic diameters (lane 5). The apoA-I present in VLDL (lanes 2A, 2B), $HDL_{2/3}$ (lanes 3A, 3B), and lipid-poor fractions (lanes 4A, 4B) are separated very well from one another. The lipid-poor apoA-I is somewhat homogeneous in size (although less exposure yields three distinct bands suggesting various degrees of lipidation of this pool). ApoA-I was also found on VLDL, although some previous studies suggest that hapoA-I is not secreted with these lipoproteins (see discussion). Strikingly, significant amounts of hapoA-I are secreted as $HDL_{2/3}$ sized species of pre-β

migration. As well, there is significant heterogeneity with these lipoproteins and apoA-I in this pool can reach 12 nm in size.

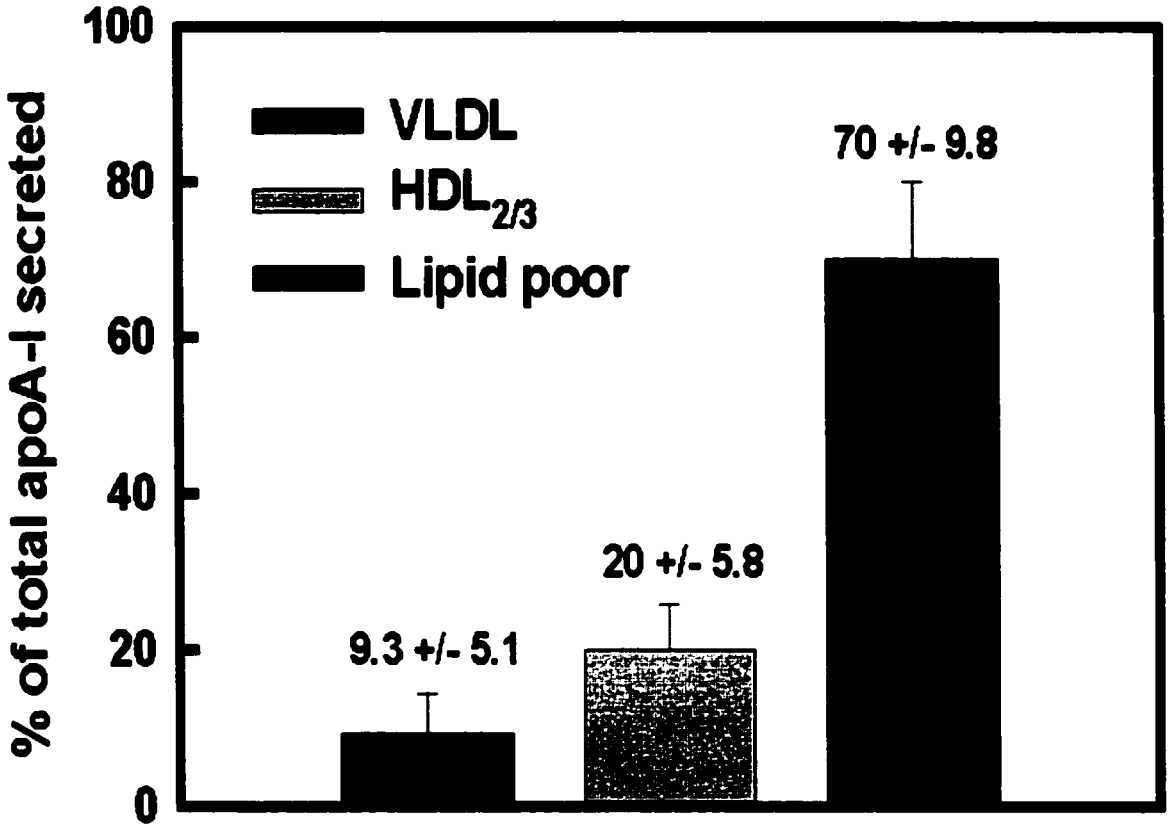

Figure 5.3: HapoA-I Is Secreted as a Heterogeneous Lipoprotein Mixture

From Fig. 5.2, the distribution of apoA-I in the different FPLC fractions is shown. The VLDL (fractions 10-14), $HDL_{2/3}$ (fractions 19-23) and lipid poor species (25-29) were subjected to densitometric scanning for hapoA-I (Fig. 2A.). The percentage of total hapoA-I secreted (± SE) from primary apoA-I deficient hepatocytes into medium over 3.5 h is shown (three independent experiments). In addition to the majority (70 ± 9.8%) of apoA-I that is secreted as lipid-poor lipoproteins, significant amounts of secreted apoA-I associate with VLDL (9.3 ± 5.1%) and $HDL_{2/3}$ (20 ± 5.8%) as well.

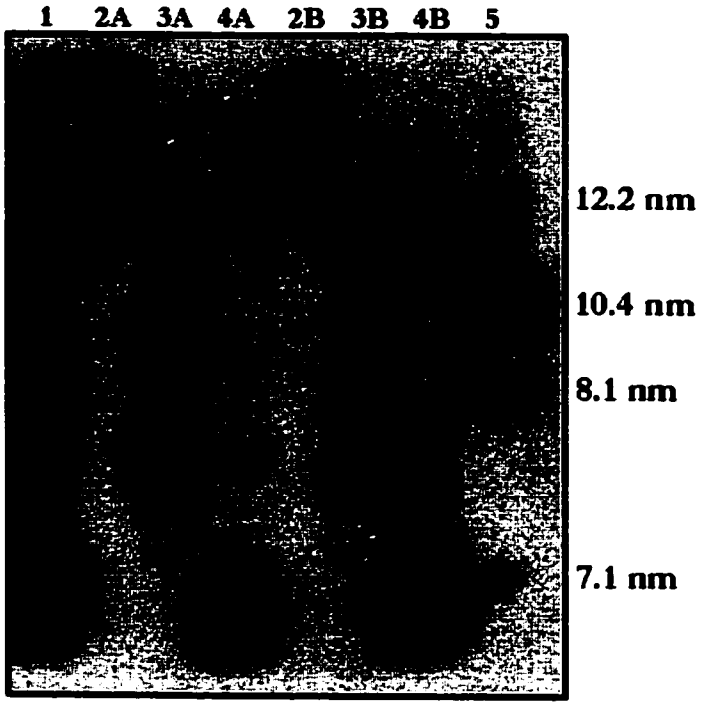

Figure 5.4: Heterogeneous Mixture of Secreted Lipoproteins Containing HapoA-I

Concentrated medium (lane 1) and pooled and concentrated FPLC lipoprotein fractions VLDL (lane 2A, 2B), $HDL_{2/3}$ (lane 3A, 3B), and lipid-poor apoA-I (lanes 4A, 4B) were separated by nondenaturing 4-20% PAGGE. The lipid-poor apoA-I population contains the majority of hapoA-I and is homogeneous. In contrast the VLDL and $HDL_{2/3}$ contain less hapoA-I and are more heterogeneous in size.

5.3.4 – Association of Phospholipids with Secreted ApoA-I

The association of ^{3}H-choline PL with hapoA-I in the three lipoprotein pools was estimated by immunoprecipitations of hapoA-I under native conditions. Equal volumes of the FPLC lipoproteins were immunoprecipitated with an anti-hapoA-I antibody raised in sheep. To control for non-specific binding an anti-hapoB antibody raised in the same species (sheep), which does not react against mapoB, was used. The results show that while the majority of hapoA-I is in the lipid-poor fraction, most of the PL are associated with apoA-I in the $HDL_{2/3}$

lipoprotein pool (Fig. 5.5). If the values are normalized to the total amount of apoA-I present in each fraction, then there is an approximately 15-fold increase in the number of PL molecules associated per apoA-I molecule in the $HDL_{2/3}$ pool compared to the lipid-poor species. Therefore, this demonstrates that apoA-I can be secreted with significant quantities of PL, which is consistent with the size and heterogeneity of hapoA-I in the $HDL_{2/3}$ pool as determined by 4-20% nondenaturing PAGGE (Fig. 5.4).

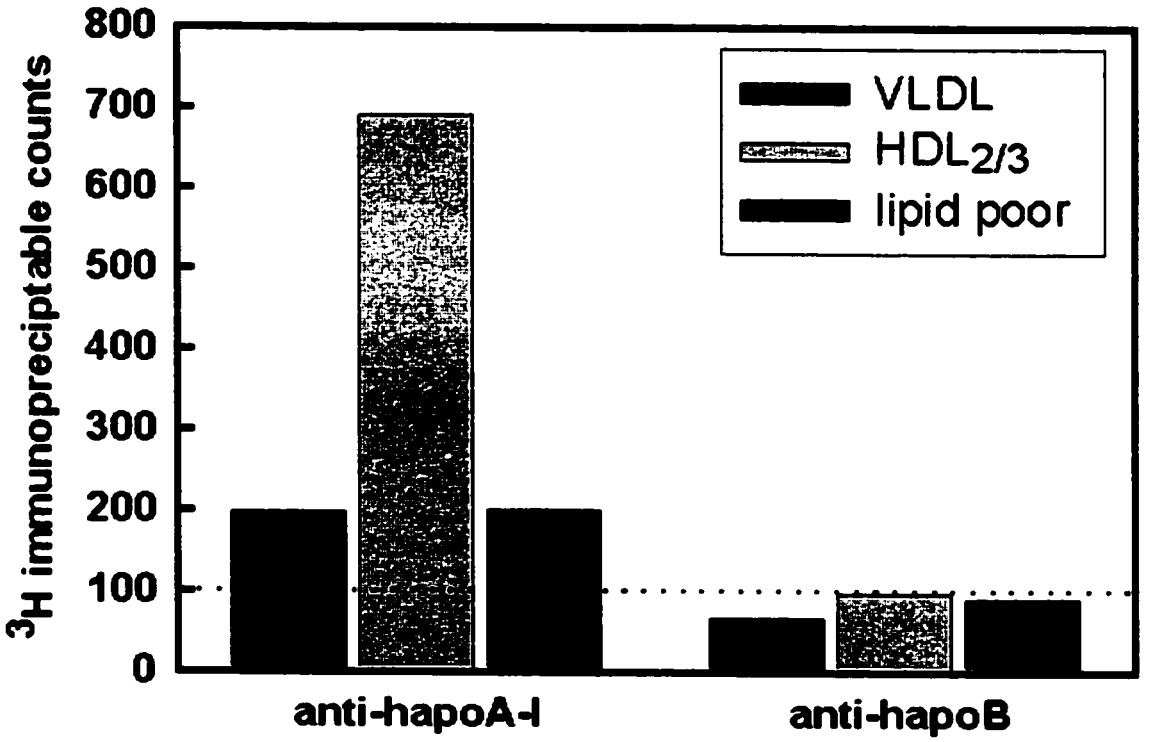

Figure 5.5: ApoA-I Phospholipid Association in the Different Lipoprotein Pools

HapoA-I was immunoprecipitated in equal volumes (250 µl) of the three lipoprotein size pools as described (section 2.2.18). To control for non-specific binding by sheep antiserum, an anti-hapoB antibody raised in the same species was used (anti-hapoB). The majority of PL are pulled down with hapoA-I in the $HDL_{2/3}$ pool. The background (non-specific counts) for the three lipoprotein pools is shown on the right (anti-hapoB antibody).

5.4 - Discussion

This study has accounted for most possible sources of artifacts (see below) and used the most physiologically relevant cell model, primary hepatocytes, to address the notoriously difficult problems associated with HDL secretion. The difficulties are centered on the instabilities of nascent HDL that may be secreted into the medium. To minimize disruptions of the nascent HDL pool and artifacts that might arise the following steps were taken. The hepatocytes were maintained in serum-free medium (plus ^{3}H-choline) for 48 hours prior studying apoA-I secretion. As well, apoA-I secreted into the medium was evaluated over a short period of time (3.5 h) so as to minimize the possibility that hapoA-I could form HDL sized species extracellularly (*i.e.* solubilizing cell membranes or associating with cell debris). Finally, the lipoproteins secreted into the medium were separated by FPLC on calibrated Superdex 200 columns as opposed to ultracentrifugation techniques used in most previous studies (see below). With these modifications, a significant amount ($\approx$ 20% of total) of hapoA-I is found secreted as mature sized HDL particles with pre-β migration. The implications of this finding are discussed here.

Ultracentrifugation is the most traditional and common method employed to isolate lipoproteins, although other methods have been established which include size exclusion chromatography (*i.e.* Superose 6 and Superdex 200), affinity chromatography, and capillary electrophoresis. In the early 1980's Kunitake and Kane showed that a significant amount of apoA-I is lost from the HDL pool during ultracentrifugation (517). It is conceivable therefore that apoA-I on larger and less stable nascent HDL are removed from these particles during isolation by this technique. This could explain in part why many previous studies (see section 5.2) found that the majority, if not all, of the newly secreted apoA-I from cells is found in the >1.21 g/ml density fraction. This issue was addressed by Havel and colleagues and it was

suggested that non-dissociating techniques may be required to define the process by which nascent HDL are assembled and secreted (511) (see below).

The cell types used to study apoA-I secretion also have to be considered. Cells that normally do not secrete apoA-I may not express proteins that are required for lipidation of the nascent HDL pool. Therefore, similar to the role of MTP in apoB secretion, hepatocytes and enterocytes might also express proteins that facilitate the lipidation of newly secreted apoA-I (*i.e.* ABCA1 – discussed below). Therefore, it is difficult to interpret studies from 3T3 cells (512), polarized MDCK cells (514), and mouse C127 cells (515) which have all been transfected with apoA-I cDNAs and were found to secrete lipid-poor apoA-I containing particles. Furthermore, most of these studies isolated apoA-I by ultracentrifugation, which adds another level of complication as discussed above.

Hepatocytes (primary and transformed) and enterocytes are better models to study apoA-I secretion. Thrift *et al.* studied lipoprotein secretion from transformed HepG2 (hepatocyte) cells into serum-free medium (518). Similar to the results found in this study, analysis of concentrated HepG2 cell medium by PAGGE revealed a broad apoA-I band between 7.1-12.2 nm with the majority of apoA-I in the lipid-free or lipid-poor form (< 8 nm). However, HepG2 cells do not secrete normal VLDL sized particles and not surprisingly no apoA-I was detected in this lipoprotein pool (518). The group of Melchior also analyzed secretion of nascent apoA-I particles from cynomolgus monkey hepatocytes in culture (519). Unlike the results presented here (Figs. 5.2-5.5), very little heterogeneity in secreted apoA-I was observed. After 3 d in culture, only a small 47 kDa apoA-I complex was detected in addition to a larger complex (> 1 million Da). The results from this study are however difficult to interpret due to the extended period of time that secreted apoA-I was in the medium and perhaps due to the lack of sensitivity in the assay required for detection of minor subpopulations of apoA-I.

The discovery of a major role for ABCA1 in PL and cholesterol efflux to nascent HDL (lipid-poor apoA-I) has established a renewed interest in the processes by which apoA-I is lipidated. While most reports have studied ABCA1 in peripheral cells (*i.e.* fibroblasts and macrophages) and show that this transporter is responsible for efflux of cholesterol and PL to apoA-I, no studies have yet evaluated the role of hepatocyte ABCA1 in this process. Furthermore, Rubin and colleagues have reported that ABCA1 is most abundant in the liver (presumably hepatocytes) (438). This suggests that ABCA1 could have a role in lipidating newly secreted apoA-I. This was part of the reason for re-examining the production of nascent HDL by hepatocytes here. We find that a significant pool of apoA-I is secreted with PL (Fig. 5.5) from primary apoA-I deficient mouse hepatocytes infected with the hapoA-I.Ad5. Therefore, is ABCA1 or another protein responsible for shuttling the lipids onto apoA-I as it is secreted? If so, what contribution does this make to the plasma concentrations of HDL PL, cholesterol and apoA-I? The methods developed in my research, will allow others to carry out future studies to evaluate the role of ABCA1 in the lipidation of newly secreted apoA-I and to address the specific role of hepatocyte ABCA1 in regulating HDL-C concentrations.

CHAPTER 6 – CONCLUSIONS AND FUTURE PERSPECTIVES

A novel approach to study apoA-I structure-function relationships was developed during the course of my Ph.D. The studies detailed in this thesis have been subjected to peer review and published in a well-respected journal (483,520). We first showed that deletions within the central domain of apoA-I have different effects on HDL metabolism. Our data suggest that helix 4 (aa 100-121) is important for stabilizing the association of apoA-I with lipids, and supports the current notion that class Y amphipathic amphipathic α-helices are most important for lipid binding within apoA-I. To further assess the role of helix 4 in association with lipids, future studies should address more subtle mutations within this helix. For example, site-directed mutagenesis could be utilized to alter the charge distribution and change helix 4 into a more typical class A helix. Both *in vitro* and *in vivo* studies could be performed as outlined in this thesis to determine how such mutations affect lipid binding and HDL morphology.

The *in vivo* studies outlined here have also established that helix 6 (aa 144-165) of apoA-I is the critical LCAT activation domain but does not contribute significantly to the lipid binding (Chapter 4). It is also clear from the study of apoA-I_{FIN} (Chapter 5) and other cited work that the charge distribution within helix 6 is critical for the LCAT reaction. This suggests that electrostatic interactions between helix 6 of apoA-I and LCAT are important for CE formation and HDL maturation. Future studies will need to focus on the nature of the electrostatic interactions to further understand the mechanism of LCAT activation by apoA-I. Mutations within LCAT that give rise to FED (*i.e.* retain β-LCAT activity) might be useful to study from this perspective. Recombinant adenoviruses carrying human LCAT (hLCAT) and hapoA-I mutations could be used to study this important reaction in HDL metabolism.

Furthermore, the ability to cross LCAT deficient and apoA-I deficient mice will allow study of the human proteins in the absence of the corresponding murine ortholog.

The study of apoA-I_{FIN} has also demonstrated how a combined *in vivo*, *ex vivo*, and *in vitro* approach can provide important insights into the mechanisms by which some apoA-I mutations drastically affect HDL metabolism. The proteolysis of apoA-I_{FIN} detected in the plasma is most interesting and appears responsible in part for the lowering of HDL-C concentrations. This raises some important questions. Is the same protease responsible for processing the pro-sequence of apoA-II the enzyme that degrades apoA-I_{FIN}? Furthermore, would diarginine mutations generated elsewhere result in proteolysis? This would serve a few purposes. For one, if proteolysis was detected *in vivo* but a new mutant had normal LCAT activation it might establish how much of the hypoalphalipoproteinemia in heterozygous carriers of apoA-I_{FIN} is contributed by this reaction. If there is no proteolysis, then sequences within helix 6, in addition to the diarginine motif, are also likely important for degradation of apoA-I_{FIN}.

Finally, the methods developed to study secretion of hapoA-I from primary hepatocytes will be used in the laboratory to address the role of ABCA1 in secreting nascent HDL. To accomplish this, an anti-sense recombinant adenovirus directed against murine ABCA1 will be created. This should be feasible as other studies have reported the successful generation of recombinant adenoviruses expressing anti-sense against other proteins (521,522). If successful, the effect of down-regulating ABCA1 on the formation of nascent HDL secreted by hepatocytes will be studied. Furthermore, since adenoviruses target the liver preferentially when injected into mice (section 2.1.3) this construct will be useful to study specifically the role of hepatocyte ABCA1 on the circulating plasma HDL-C and apoA-I levels. Complimentary approaches will include compounds that stimulate production of ABCA1 or

chemical inhibitors of the ATP-binding cassette transporter family members (*i.e.* glyburide and DIDS).

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- B.Sc. Honours Biochemistry, University of Guelph, 1992
- M.Sc. Biochemistry, University of Guelph, 1994
- Ph.D. Biochemistry, University of Ottawa, 2001

SKILLS AND ASSETS

1. Molecular Biology:

a) Bacteriology – growth characteristics, genetic manipulation, transformations, large-scale cultures

b) Mutagenesis – point, deletion and insertional mutagenesis based on both PCR and non-PCR methodologies.

c) DNA – generation of highly purified Maxi-prep DNA suitable for cell culture transfection and intracellular recombination. Also familiar with automated sequencing of DNA.

d) RNA – teaching assistant in a undergraduate molecular biology course that included the isolation of RNA, preparation of polyA RNA, reverse transcription, and Northern blot analysis.

2. *Cell Culture:*

a) Growth and handling of various transformed cell lines including 293, J774, CHO, COS-7.

Isolation and growth properties of primary mouse hepatocytes

b) Growth of suspension cultures in large spinner flasks. Transfection by various methods including calcium phosphate and cationic liposome-mediated approaches.

c) Well-versed in the generation and selection of stably-transfected cell lines

3. *Adenovirus Production:*

a) Preparation of 1st generation recombinant adenoviruses by homologous recombination of 293 cells (12+ constructs generated)

b) Large-scale CsCl purification of recombinant adenoviruses suitable for *in vivo* studies in mice

4. *Animal Experience:*

a) Extensive handling of C57BL/6J mice including proper holding techniques, ear tagging, and breeding protocols.

b) Blood collection techniques via the tail and saphenous veins.

c) Expertise in the injection of recombinant adenoviruses and ^{125}I-labeled proteins (lipoproteins) via the tail vein (non-anesthetic approach) of mice.

d) Laparotomies performed routinely for tissue and organ removal.

5. *Cell Biology:*

a) Cholesterol efflux studies to lipid-free and lipidated apoA-I

b) Continuous labeling studies with ^{3}H-choline to determine the lipids associated with secreted apoA-I

c) Pulse-chase labeling with ^{35}S-methionine/cysteine for secretion studies

6. *Biochemical and Lipoprotein Techniques:*

a) Protein Purification – His-tagged recombinant proteins generated in *E. coli* and purified via NTA resin

b) Denaturing (+/- reducing) and native polyacrylamide gel electrophoresis (PAGE) analysis of proteins

c) Western blot analysis of various proteins (primarily human and murine apolipoproteins) expressed both *in vitro* and *in vivo* systems

d) Lipoprotein isolation by continuous or discontinuous gradients and by size exclusion chromatography

e) Reconstituted lipoproteins by cholate dispersion method

f) *In vitro* LCAT enzymatic studies

g) Protein secondary structure analysis by CD spectropolarimetry

h) TLC and lipid analysis

7. *Work Ethic:*

a) A strong team player confident in my own abilities' yet willing to take suggestions and constructive criticism from others.

8. *Communication and Computer Skills:*

a) I have given many oral and poster presentations including those listed at international conferences.

b) Extensive experience with Word, Excel, and PowerPoint. Very knowledgeable with the Windows 98 environment and associated programs as well as the different hardware components of PCs.

AWARDS AND DISTINCTIONS

B.Sc.:	1991-1992	•	Held a National Sciences and Engineering Research Council (NSERC) of Canada scholarship for two consecutive summers.
B.Sc.:	1991	•	University of Guelph Soap Company Scholarship for the highest combined grades in 3rd year Biochemistry and Organic chemistry
B.Sc.:	1992	•	Honour role distinction for maintaining a cumulative 80% average during my B.Sc.
Ph.D.:	1996	•	Selected for a Medical Research Council of Canada Studentship by Dr. Marcel to work in his laboratory
Ph.D.:	1997-2001	•	Awarded and successfully maintained a Ph.D. research scholarship from the Heart and Stroke Foundation of Canada
Ph.D.:	1997-1998	•	Honourable mentions for basic science research presentation at the University of Ottawa Heart Institute Research Day
Ph.D.:	1999-2000	•	Awarded best basic science research presentation at the University of

			Ottawa Heart Institute Research Day two consecutive years
Ph.D.	2000	•	Awarded best Ph.D. poster presentation at the Department of Biochemistry Research Day
Postgraduate	2001	•	Awarded a NSERC Industrial Research Fellowship to work for Aegera Oncology Inc. (2 years – current)

RESEARCH TRAINING:

Summers 1991-1992:	•	Summer research assistant – utilized genetically modified bacterial strains to monitor redox-cycling capabilities of various compounds. *Laboratory of P.D. Josephy*
Sept. 1991- April 1992	•	Honours research project – site-directed mutagenesis on bacteria pore-forming protein colicin E1 for structure function analysis. *Laboratory of A.R. Merrill*
1992-1994	•	M.Sc. studies - The roles of superoxide dismutase and catalase in bacterial defense against phagocytic killing by human neutrophils. *Laboratory of P.D. Josephy*
1995-1996	•	Research Technician – McMaster University. *Laboratory of V.S. Ananthanarayanan*
1996 – 2001	•	Ph.D. Studies – An *in vivo* approach to the study of apolipoprotein A-I structure-function relationships *Laboratory of Yves L. Marcel*

PUBLICATIONS:

a) Published peer-reviewed articles:

1. **McManus DC** and Josephy PD. A new approach to measurement of redox-cycling activity in *Escherichia coli*. *Arch. Biochem. Biophys.* 1993. **304**: 367-370.

2. **McManus DC** and Josephy PD. Superoxide dismutase protects *Escherichia coli* from killing by human serum. *Arch. Biochem. Biophys.* 1995. **317**: 57-61.

3. Burgess JW, Frank PG, Franklin V, Liang P, **McManus DC**, Desforges M, Rassart E, and Marcel YL. Deletion of the C-terminal domain of apolipoprotein A-I impairs cell surface binding and lipid efflux in macrophage. *Biochemistry*. 1999. **38**: 14524-33.

4. **McManus DC**, Scott BR, Frank PG, Franklin V, Schultz JR, and Marcel YL. Distinct central amphipathic α-helices in apolipoprotein A-I contribute to the *in vivo* maturation of high density lipoprotein by either activating lecithin-cholesterol acyltransferase or binding lipids. *J. Biol. Chem.* 2000. **275**: 5043-51.

5. **McManus DC**, Scott BR, Franklin V, Sparks DL, and Marcel YL. Proteolytic degradation and impaired secretion of an apolipoprotein A-I mutant associated with dominantly inherited hypoalphalipoproteinemia. *J. Biol. Chem.* 2001. **276**: 21292-21302.

6. Scott BR, **McManus DC**, Franklin V, McKenzie AG, Neville T, Sparks DL, and Marcel YL. The N-terminal globular domain and the first class A amphipathic helix of apolipoprotein A-I are important for lecithin:cholesterol acyltransferase activation and the maturation of high density lipoproteins in vivo. *J. Biol. Chem.* In press.

b) Published peer-reviewed communications:

1. **McManus DC**, Frank PG, Franklin VI, Schultz J, and Marcel YL. The *in vivo* maturation of HDL requires central domains of apolipoprotein A-I but not the C-terminus. *Circulation* 1998, **98** suppl 1.

c) Unpublished communications:

1. **McManus DC**, and Marcel YL. Generation of human wild-type apoA-I containing recombinant adenovirus. Canadian Lipoprotein Conference, Ste-Adele, Quebec, September 19-22, 1997.

2. **McManus DC**, Frank PG, Schultz J, and Marcel YL. *In vivo* structure function studies of human apolipoprotein A-I following adenovirus-mediated gene transfer into apoA-I deficient mice. Canadian Lipoprotein Conference, Minett (Muskokas), Ontario, October 16-19, 1998.

d) Accepted Presentations at International Conferences:

1. **McManus DC**, Frank PG, Franklin VI, Schultz J, and Marcel YL. The *in vivo* maturation of HDL requires central domains of apolipoprotein A-I but not the C-terminus. Oral presentation at the American Heart Association 71st Scientific Sessions, Dallas, Texas, November 8-11, 1998 (less than 35% of submitted abstracts accepted for presentation).

2. **McManus DC**, Scott B, Frank PG, Franklin V, Schultz JR, and Marcel YL. Distinct amphipathic α-helices within the central domain of apolipoprotein A-I contribute to the *in vivo* maturation of HDL by either activating LCAT or binding lipids. Accepted for an oral presentation at the American Heart Association 72nd Scientific Sessions, Atlanta, Georgia, November 7-10, 1999 (less than 30% of submitted abstracts accepted for presentation).

3. **McManus DC**, Scott B, Frank PG, Franklin V, Schultz JR, and Marcel YL. Apolipoprotein A-I Finland ($Leu^{159} \rightarrow Arg^{159}$) has normal phospholipid binding and LCAT activation but exhibits a modified conformation on HDL with an enhanced susceptibility to proteolytic degradation *in vivo*. Accepted for a poster presentation at the American Heart Association 72nd Scientific Sessions, Atlanta, Georgia, November 7-10, 1999 (less than 30% of submitted abstracts accepted for presentation).

REFERENCES:

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