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CHARACTERIZATION OF APOLIPOPROTEIN A-I FUNCTIONAL DOMAINS

by

Philippe Guy Frank

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

Philosophiæ Doctor (Ph.D.)

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ABSTRACT

CHARACTERIZATION OF APOLIPOPROTEIN A-I
FUNCTIONAL DOMAINS

by Philippe Guy Frank

The plasma concentration of high-density lipoprotein (HDL) is inversely correlated to the development of coronary heart disease. This inverse relationship has been attributed to the role that HDL and its major constituent, apolipoprotein A-I (apoA-I), play in reverse cholesterol transport. This pathway allows HDL to acquire excess cellular cholesterol from peripheral cells and mediate its transport to the liver for degradation and removal from the body. ApoA-I has three major functions in this process. Due its lipid-binding capability, apoA-I promotes cellular cholesterol efflux and form HDL particles. ApoA-I activates lecithin:cholesterol acyltransferase (LCAT), an enzyme that esterifies free cholesterol and further promotes the flux of cholesterol through the HDL pool by the formation of a neutral lipid core in this lipoprotein. ApoA-I has also been demonstrated to interact with the receptor SR-BI. In order to identify the different domains of human apoA-I involved in these functions, a bacterial expression system was designed. Recombinant apoA-I, with an N-terminal extension, was shown to have similar physicochemical properties to native apoA-I and to be identical to the native protein in its ability to interact with phospholipids and to promote cellular cholesterol efflux and cholesterol esterification.

This expression system was used to express apoA-I mutants and to identify the specific domains responsible of its functions. Three apoA-I mutants, corresponding to deletions of apoA-I residues 100-143, 122-165, 144-186, have been characterized for their

ability to bind phospholipids and to form reconstituted apoA-I-containing lipoproteins, as well as for their structural and physical properties. The results show that the amphipathic α-helices within residues 100-186 are directly involved in the interactions with phospholipids and determine the lipid binding capacity of the protein. The helical region 100-121 appears to be important to the stabilization of the lipid-apoprotein complex formed, whereas helices within residues 122-186 appear to be critical to the initial rates of association of the apoprotein with phospholipids.

We have also examined the contribution of these amphipathic α-helices to the ability of apoA-I to promote cholesterol efflux from human skin fibroblasts. All central deletion mutants in reconstituted HDL could acquire cellular cholesterol from normal fibroblasts with similar affinity and binding capacity for cholesterol. These results indicate that no specific sequence in the central domain of apoA-I is required for efficient acquisition of cellular cholesterol by HDL from normal fibroblasts.

The contribution of amphipathic α-helices (central and C-terminal helices) to the ability of lipid-free apoA-I to promote cellular cholesterol efflux was examined with cholesterol-loaded fibroblasts and macrophages. ApoA-I interaction and cholesterol efflux from cells such as fibroblasts, which accumulate moderate amounts of cholesterol, do not require any specific sequence between residues 100 and 243. In contrast, cholesterol and phospholipid efflux from cholesterol-loaded macrophages involves a pathway that is dependent upon the binding of apoA-I to macrophages and appears to be mediated by the C-terminal amphipathic α-helices (residues 187-243). This domain may be necessary for initial binding of lipids and/or for interaction with a macrophage-specific cell surface-binding site.

The implication of the central domain of apoA-I in the activation of LCAT has been proposed by several studies. We have therefore examined the ability of the central domain deletion mutants in reconstituted lipoproteins to activate the reaction mediated by LCAT. While deletion of residues 100-143 has little effect on LCAT activation, deletion of residues 122-165 or 144-186 results in an inability of the mutant apoproteins to promote cholesterol esterification. This domain (144-186) is therefore critical for optimum LCAT activation and cholesteryl ester accumulation.

This study provides evidence for a different role of the central and C-terminal domains of apoA-I. The central domain appears to give a certain plasticity to apoA-I, allowing this protein to accumulate varying amounts of lipid. The region corresponding to residues 144-186, is necessary for efficient activation of cholesterol esterification by LCAT. It may therefore play a major role in the formation of large HDL. On the other hand, the C-terminal domain appears crucial for the initial lipid binding of this apoprotein and is involved in the elimination of excess cellular cholesterol from peripheral cells. This study and others suggest that apoA-I contains three functionally distinct domains: the N-terminal domain plays an important role in the stabilization of the lipid-free protein; the C-terminal domain is involved in the initial binding of lipids; the central domain mediates the maturation of high density lipoproteins.

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DEDICATION

I dedicate this thesis to my family.

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GLOSSARY

ApoA-I: Apolipoprotein A-I.

ApoA-II: Apolipoprotein A-II.

ApoA-IV: Apolipoprotein A-IV.

ApoB-48: Apolipoprotein B-48.

ApoB-100: Apolipoprotein B-100.

ApoC-I: Apolipoprotein C-I.

ApoC-II: Apolipoprotein C-II.

ApoC-III: Apolipoprotein C-III.

ApoE: Apolipoprotein E.

CD: Circular dichroism.

CE: Cholesterol ester.

CETP: Cholesterol ester transfer protein.

CHD: Coronary heart disease.

CLA-I: CD36 and LIMP II Analogous-I , the human homologue of rodent SR-BI.

CM: Chylomicrons.

DMPC: Dimyristoyl phosphatidylcholine.

FC: Free cholesterol.

GdnHCl: Guanidine hydrochloride.

GGE: Gradient gel electrophoresis.

HDL: High-density lipoproteins.

HDL-C: Plasma cholesterol (free and esterified) associated with high-density lipoproteins.

HL: Hepatic lipase.

HSPG: Heparin sulfate proteoglycan.

ICAM: Intercellular adhesion molecule 1.

LCAT: Lecithin:cholesterol acyltransferase.

LDL: Low density lipoprotein.

LDL-R: LDL receptor.

LPL: Lipoprotein lipase.

LRP: LDL-R related protein.

MTP: Microsomal triglyceride transfer protein.

NCEH: Neutral cholesterol ester hydrolase.

PKC: Protein kinase C.

PL: Phospholipid.

PLTP: Phospholipid transfer protein.

POPC: 1-palmitoyl 2-oleyl phosphatidylcholine.

RAP: Receptor associated protein.

RCT: Reverse cholesterol transport.

SR-BI: Scavenger receptor class B type I.

TG: Triglyceride.

VCAM: Vascular cell adhesion molecule 1.

VLDL: Very low-density lipoproteins.

Chapter 1: INTRODUCTION

Lipoproteins and Atherosclerosis

Coronary heart disease (CHD), and more specifically atherosclerosis is one of the leading causes of death in industrialized countries. Atherosclerosis is characterized by the deposition of lipids (both intracellularly and extracellularly), cell proliferation, and deposition of connective tissues (1). The first step consists of the accumulation and modification (oxidation, glycation) of LDL and other lipoproteins in the subendothelial space. This process causes endothelial cells to produce monocyte adhesion molecules and cytokines that will lead to the transfer of monocyte into the subendothelial space and their differentiation into macrophages. Macrophages will subsequently produce several growth factors (PDGF, TGF-α, TGF-β, IL-1) responsible for the progression of the fatty streak to fibrous plaques (2). Macrophages accumulate lipids owing to the presence, at their surface, of receptors for modified lipoproteins (termed scavenger receptors). This uptake generally leads to the eventual formation of foam cells and to the synthesis by macrophages of cellular factors that mediate the proliferation of smooth muscle cells and the formation of an atheromatous plaque. The early stage, termed the fatty streak, contains T-lymphocytes, macrophages, and smooth muscle cells. The last two cell types contain lipid deposits. A more advanced lesion can eventually develop (termed fibrous plaque) and is characterized by the migration and proliferation of smooth muscle cells in the arterial intima. These cells form a fibrous cap with new connective tissue matrix and deposit of extracellular lipids (2). Fibrous plaque formation leads to decreased blood flow but can also break or tear and thrombi can form on the surface of this plaque, a process which can further narrow the diameter of the artery.

Atherosclerosis is a polygenic disorder. Amongst other factors, disorders of plasma lipid metabolism appear to play a major role in the progression of CHD and cholesterol is known to accumulate in atherosclerotic lesions. Several epidemiological studies have indicated a strong positive correlation between plasma low-density lipoprotein-cholesterol (LDL-C) levels and the occurrence of these lesions (3). On the other hand, there is a strong inverse correlation between the development of CHD and cholesterol levels in high density lipoproteins (HDL) (3-5). This protective role of HDL has been attributed to its function in the reverse cholesterol transport (RCT) pathway, which allows the transfer of excess peripheral cell cholesterol via HDL to the liver for degradation and removal from the body (6). Apolipoprotein A-I (apoA-I), the major protein component of HDL, is directly involved in several steps of this pathway. This study was therefore designed to investigate the structure-function relationship of apoA-I with respect to its role in RCT.

I. STRUCTURE AND METABOLISM OF LIPOPROTEINS

The concept of discrete lipoprotein structure was first described at the beginning of the century, as a means to explain how nonpolar lipids are solubilized in the extracellular milieux (7). High-density lipoprotein (HDL) was the first lipoprotein identified (8). At that time, investigators mainly used solvents and salts to identify and separate the different components of lipoproteins. As new techniques appeared, a better characterization was possible. Electrophoresis, for example, allowed the identification of different lipoproteins according to their charge, and facilitated a classification of lipoproteins according to their associated proteins (called apolipoproteins, apolipoprotein A with α–HDL, apolipoprotein B with β-LDL, see Table I-1). This classification was based on the original description of the

electrophoretic mobility of plasma globulins (α, β, and γ). Another important technique developed and utilized for lipoprotein analysis was the analytical ultracentrifugation. This technique permitted the characterization of lipoproteins according to their density (Table I-1) (9) and formed the basis for the lipoprotein separation methods that are still in use. These studies further defined the relationships between certain lipoprotein fractions and CHD risk (10).

A. General structure of lipoproteins

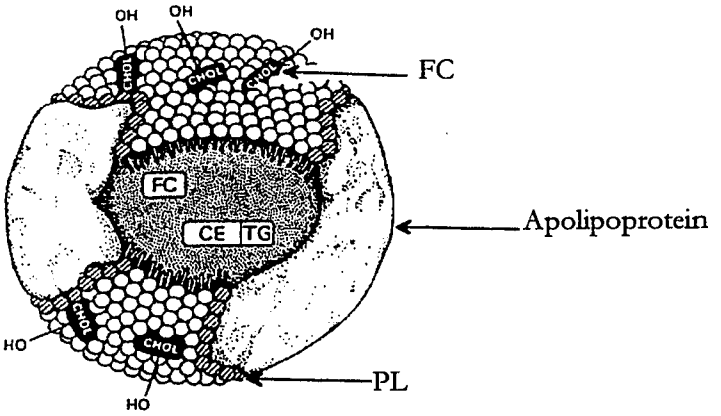

Figure I-1: Model of a Spherical Lipoprotein.
Apolipoproteins can interact with the phospholipid (PL) monolayer [also containing free cholesterol(FC)] surrounding a neutral lipid core composed of free cholesterol, triglycerides (TG), and cholesterol ester (CE). In this model, apolipoprotein(s) stabilize the lipid microemulsion (11).

Lipoproteins, generally spherical in structure, are the major carriers of lipid molecules, including phospholipids, glycerides, free and esterified cholesterol, as well as lipophilic hormones and vitamins. They are stabilized by hydrophobic proteins termed apolipoproteins

or apoproteins. They are found in peripheral fluid and in the circulatory system. Lipoproteins increase the solubility of these lipids by sequestering them in a lipophilic environment created by the surrounding apolipoproteins. Their structure therefore reflects this property, with a neutral lipid (triglycerides and cholesterol ester) in the core of the sphere surrounded by a phospholipid and cholesterol monolayer that is stabilized by the interaction of apolipoproteins (Fig. I-1). Several classes of lipoproteins have been identified. They were first classified according to their hydrated density, which reflects mostly their lipid to protein ratio. Their size is also inversely correlated to their density. Each class is also characterized by specific apolipoproteins (Table I-1).

Table I-1: Classification of Human Lipoproteins According to Density, Size and Apoprotein Composition.
[from (12)].

Lipoprotein	Density (g/ml)	Electrophoretic Mobility[1]	Diameter (nm)[2]	Associated Apolipoprotein
Chylomicron	0.93	Remains at the origin	75-1200	A-I, A-II, B-48, C-I, C-II, C-II, C-III, E
VLDL	0.93-1.006	Preβ-migration	30-80	A-I, B-100, C-I, C-II, C-III, E
IDL	1.006-1.019	Slow preβ-migration	25-35	B-100, C-I, C-II, C-III, E
LDL	1.019-1.063	β-migration	18-25	B-100
HDL_2	1.063-1.125	α-migration	9-12	A-I, A-II, A-IV, C-I, C-II, C-III, D, E
HDL_3	1.125-1.210	α-migration	5-9	A-I, A-II, A-IV, C-I, C-II, C-III, D, E

[1] As determined by agarose gel electrophoresis.
[2]: Hydrodynamic diameter.

Apolipoproteins have specific functions, not only in the transport of lipid but also in the regulation of enzymes involved in lipid metabolism and in the interaction with cellular receptors involved in the uptake of lipoproteins (Table I-2).

Table I-2: Properties of the Major Apolipoproteins Associated with Human Plasma Lipoproteins.
[from (12; 13)].

Apolipoprotein	Molecular Weight (Da)	Aminoacids	Major Tissue Source	Function[b]
ApoA-I	28,078	243	Liver and Intestine	Activation of LCAT SR-BI Ligand
ApoA-II	17,400[a]	77	Liver	Structural?
ApoA-IV	42,500	391	Intestine	Activation of LCAT
ApoB-48	246,000	2152	Intestine	Structural
ApoB-100	513,000	4,536	Liver	LDL receptor ligand
ApoC-I	6,605	57	Liver	Inhibits chylomicron uptake
ApoC-II	8,824	79	Liver	LPL activator
ApoC-III	8,750	79	Liver	LPL inhibitor
ApoD	19,000	169	Many sources	Transports steroid, bilirubin, myelinization of nerves[c]
ApoE	34,200	299	Many tissues	LDL receptor ligand, LRP ligand

[a]: Homodimer form (monomer linked by a disulfide bond).
[b]: By definition, each apoprotein serves in lipid binding and transport.
[c]: Potential functions of apoD.

There are two classes of apolipoproteins. The first class is defined by the presence of non-exchangeable apoproteins, apoB-48 or apoB-100, which, in their lipid-free forms, are insoluble in aqueous solutions. All the other apolipoproteins are termed exchangeable because

of their solubility in aqueous solution and capacity to transfer between lipoproteins. This characteristic appears very important for the function of these proteins. ApoA-I can, for example, dissociate from large HDL complexes before they are metabolized, and enter another cycle of the reverse cholesterol transport (See part II for more details). TG-rich lipoproteins acquire apoE, allowing for their cellular uptake through the LDL-R and other receptors. These specific functions will be reviewed in detail in the following sections.

B. Metabolism of apoB-containing lipoproteins

ApoB-containing lipoproteins transport newly absorbed dietary lipids and cholesterol and reabsorbed biliary cholesterol (chylomicrons, part of the exogenous pathway) as well as newly synthesized triglycerides (VLDL, LDL in the endogenous pathway).

1. Metabolism of chylomicrons

Chylomicrons (CM) are synthesized by intestinal cells following dietary fat intake (Fig. I-2). This is termed the exogenous pathways due to the entry of lipids from the diet, an exogenous source. Following gastric digestion, emulsification with bile acids and alkalinization of the milieu (after gastric secretion) permit the hydrolysis of dietary lipids by lipases secreted by the pancreas. Almost all the cholesterol in the diet is in the form of free cholesterol and some is partially esterified within intestinal cells. However, dietary triglycerides (TG) and phospholipids (PL) are hydrolyzed to form monoglycerides, fatty acids, and lysophospholipids. After absorption by intestinal mucosal cells, new TG are synthesized through a process that involves sequential addition of fatty acid to monoglycerides (14). In these epithelial cells, TG, PL and other lipids are assembled with apoB-48 in the endoplasmic reticulum. Although

apoB-48 and apoB-100 are the products of the same gene (15; 16), intestinal apoB-48 mRNA is synthesized by editing apoB-100 mRNA: Codon 2153, CAA, encoding Gln, is edited and changed to UAA, Stop (17). One of the major differences between apoB-48 and apoB-100 is that the former does not bind the LDL-receptor and association with apoE or lipoprotein lipase (LPL) is therefore required for endocytosis of CM remnants.

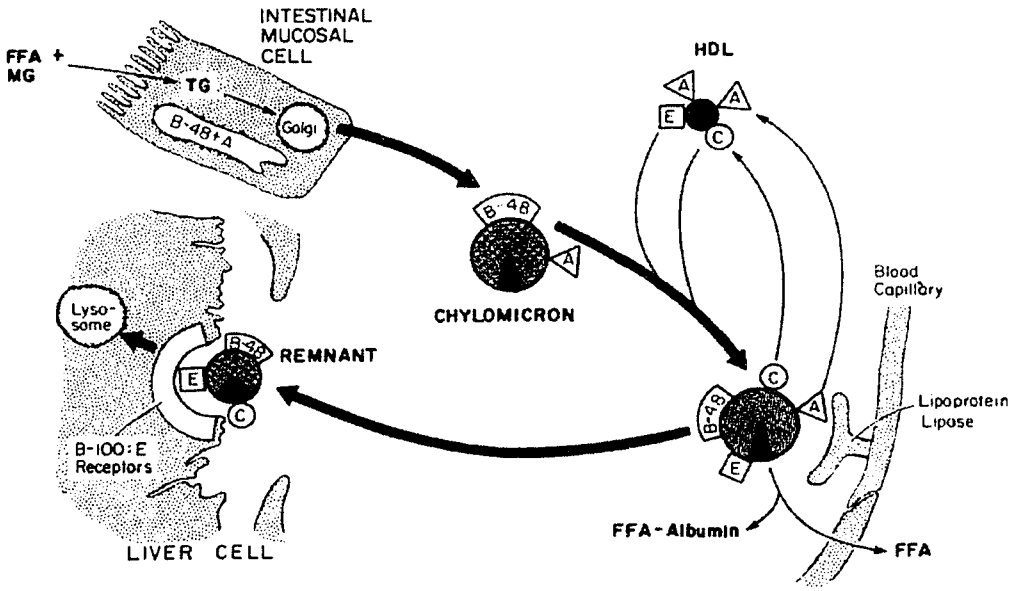

Figure I-2: The Exogenous Pathway or Metabolism of Human CM Lipoproteins.
Dietary lipids are assembled into CM and secreted by intestinal mucosal cells. After having reached their target organ (muscle, adipose tissue), these TG-rich lipoproteins are catabolized by LPL bound to the endothelium surface. Their degradation leads to the formation of free fatty acid (FFA) that are transferred to tissues. Concomitant with this degradation, apoAs transfer to the HDL pool whereas apoCs and apoE do the reverse. The resulting CM remnants, enriched with apoE, can be endocytosed and catabolized through the LDL-R and LRP pathway (B-100 : E receptors) at the surface of liver cells (from (12)).

It is important to note that absence of apoB-48 production leads to the accumulation of TG and other lipids in the cells. On the other hand, apoB-48 (like apoB-100) secretion is strictly dependent on the lipid availability. Absence of a lipid supply leads to the degradation of

the protein by cellular proteases. Addition of lipids to apoB-48 is accomplished by the microsomal triglyceride transfer protein (MTP) (18).

Each CM particle contains a single molecule of apoB-48 (19) and multiple surface molecules of apoA-I, apoA-II, and apoA-IV. After secretion, CM enter the lymph and the circulatory system where they are metabolized. Apolipoprotein C-II, C-III, and E, as well as CE are transferred from HDL to CM in the circulatory system. For many tissues (adipose tissue and muscle being the primary targets), CM serve as a major source of fatty acids, via hydrolysis of TG, the main CM constituent (Table I-3). One of the major contributors to TG hydrolysis is LPL, associated with the surface of endothelial cells, on the luminal side of arteries. It is attached by a heparin-binding site that allows its association with endothelial surface proteoglycan (20). LPL activity requires the presence of an activator apoC-II (21) and its transfer from HDL is therefore essential for efficient LPL activity. Absence of LPL or apoC-II leads to hyperchylomicronemia and increased VLDL, which demonstrates the important role for these proteins in the catabolism of TG-rich lipoproteins (1; 21; 22). Degradation of TG by LPL eventually leads to the formation of 2-monacylglycerol and unesterified fatty acids. Surface PL dissociate forming sheets, vesicles, and/or discs, probably associated with apoA-I, apoA-II, and apoA-IV(14). These poorly characterized structures are significant contributors to the HDL pathway (23). This reaction appears to occur at the surface of endothelial cells where LPL is bound. ApoE transferred from HDL could play a very important role in the anchorage of the remnant lipoprotein. Indeed, its heparin-binding site allows CM (and VLDL) to associate with endothelial cells and hence facilitates hydrolysis of TG by LPL (24). Fatty acids transfer through the endothelium to target cells (muscle cells or adipocytes) where they are used for energy production or storage.

Hydrolysis of the CM core by LPL results in a dramatic reduction in the diameter of these macromolecular complexes and is accompanied by an increased cholesterol content. This process leads to the formation of CM remnants, which eventually acquire the apoE molecules necessary for their elimination by the liver (25). These processes permit the entrance of remnants into the liver, more specifically into the space of Disse, separated from the circulation by the fenestrated hepatic sinusoidal endothelium (26).

Table I-3: Chemical Composition of Normal Human Plasma Lipoproteins (12)

	Surface Components			Core Lipids	
	Free Cholesterol	Phospholipids	Apolipoprotein	Triglycerides	Esterified Cholesterol
Chylomicrons	2	7	2	86	3
VLDL	7	18	8	55	12
IDL	9	19	19	23	29
LDL	8	22	22	6	42
HDL_2	5	33	40	5	17
HDL_3	4	35	55	3	13

Composition is given as percentage of dried mass.

Clearance of CM remnants from the plasma appears to be mediated through several pathways. This process occurs mostly in the liver, although the bone marrow may also play a role (25). Anchorage of CM remnants is generally accomplished through proteoglycans, which can associate with newly bound apoE or LPL released in peripheral tissues. This interaction can also occur through the action of hepatic lipase (HL), another lipase that is bound to

proteoglycan and found almost exclusively in the liver (27). This association can be even further enhanced by apoE secreted by hepatocytes in the space of Disse. The apoE that is secreted binds to CM remnants and facilitates their catabolism (Secretions-capture role of apoE; (28)). Finally, uptake of CM remnants is accomplished by receptor-mediated endocytosis, mainly through two receptors of the LDL-receptor superfamily. The LDL-receptor (LDL-R) was the first lipoprotein receptor to be described (29). The other important receptor in this family is the LDL receptor related protein (LRP) (30). The LDL-R, which is the best-characterized lipoprotein receptor, contains 839 residues and can bind apoE or apoB-100 (apoB-48 does not bind the LDL-R) present at the surface of remnant lipoproteins (31). After binding, receptors cluster and are internalized through coated pit vesicles. This process precedes the intracellular dissociation of the complex and individual components of the lipoprotein are degraded in the lysosomal compartment. LRP contains 4,525 residues and has numerous ligands, among them, apoE, LPL, RAP, α2-macroglobulin, and other protease inhibitors (32). CM remnants appear to be cleared by both LDL-R and LRP(33; 34).

2. Metabolism of VLDL

The metabolism of human VLDL (Fig. I-3) presents some similarities to that of CM. VLDL are assembled and secreted exclusively in hepatocytes (35). They are part of the endogenous TG pathway (transport of hepatic TG produced endogenously). Contrary to CM, they are assembled with apoB-100, which is essential for their secretion. Their secretion only occurs when the lipid supply is adequate; low levels of lipid generally lead to apoB-100 intracellular degradation (36). VLDL are enriched in TG (Table I-3), contain apoB-100 and

apoE and are secreted with apoA-I that is rapidly transferred to HDL in the circulation (14). LPL is again the major lipolytic enzyme involved in the hydrolysis of VLDL-TG.

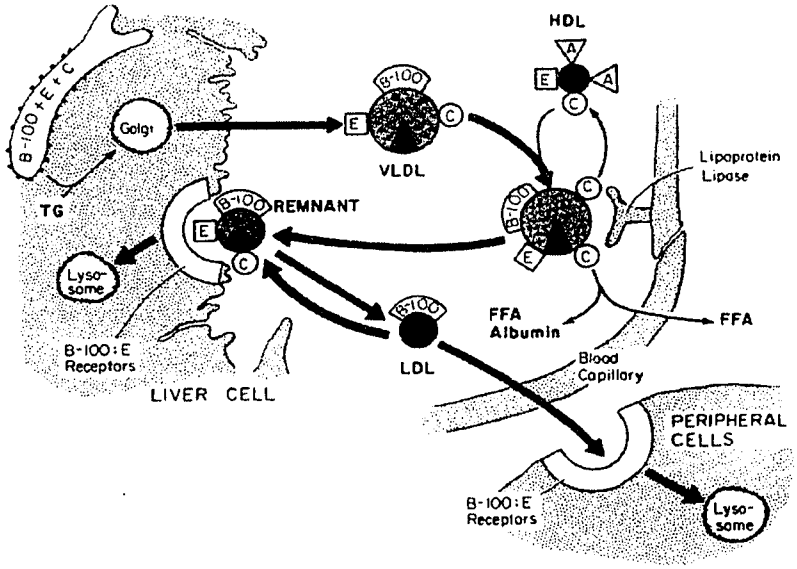

Figure I-3: The Endogenous Pathway or Metabolism of Human VLDL Lipoproteins.
VLDL are assembled and secreted by the liver. Contrary to CM, they contain apoB-100. Like CM, these TG-rich lipoproteins are catabolized at the site of their target organs, through the lipolytic action of LPL bound to the endothelium. This step yields remnant particles (IDL followed by LDL, in increasing order of degradation). Apoprotein transferred between HDL pools and remnants occurs similarly to that observed with CM remnants. In addition, CE transfers from large HDL to LDL in exchange for TG (mediated by CETP). LDL-derived cholesterol is a major source of cholesterol in peripheral cells (mediated by the LDL-receptor pathway). Other remnants appear to be degraded by the LDL-R LRP pathway (apoB-100 : E receptors) [from (12)].

The progressive delipidation and remodeling of VLDL leads to the formation of lipoproteins of higher density (IDL, LDL). The remodeling of the latter lipoproteins also requires the action of HL, which hydrolyzes TG and surface lipids and cholesterol ester transfer protein (CETP), which exchanges CE and TG between apoB-containing lipoproteins and HDL (37). This process results in a net CE enrichment of IDL and LDL and TG enrichment of large HDL (Table I-3). This reaction has led to the classification of "LDL-cholesterol" animals (plasma cholesterol mainly associated with LDL, e.g., in human and

rabbit) as opposed to "HDL-cholesterol" animals that lack CETP activity (HDL carries most of the plasma cholesterol, e.g., in rodents).

Whereas VLDL remnants are also taken up by the liver following a mechanism similar to that of CM remnants, IDL and LDL appear to be mainly endocytosed through the LDL-R. Defects in the LDL-receptor lead to hypercholesterolemia, with levels of LDL-cholesterol levels usually above 7 mmol/l in heterozygous or over 15 mmol/l if the patient is homozygous for the defect (38). This disease leads to accumulation of LDL in connective tissue and in the arterial wall, leading to xanthoma formation and atherosclerosis. The delayed clearance of LDL results in their accumulation in plasma with entrapment in the subendothelial space where oxidation or other chemical transformations can occur. These LDL modifications promote their uptake through scavenger receptors found at the surface of macrophages and leads to the formation of foam cells in atherosclerotic lesions (32).

Lp(a), an LDL-like lipoprotein, is a minor lipoprotein found in plasma and represents another independent risk factor for the development of atherosclerosis (39). It is formed by the interaction of apoB-100 and apolipoprotein (a). Apo(a)'s sequence is highly variable (different numbers of a well-defined motif, termed a kringle). Lp(a)'s function is unknown but due to the homology between apo(a) and plasminogen, Lp(a) has been implicated in the delivery of cholesterol in injured blood vessels and in thrombosis (40).

C. Metabolism of HDL and reverse cholesterol transport

The metabolism of HDL (Fig. I-4) is closely associated with that of plasma cholesterol and apoA-I, its major protein. The importance of HDL in the removal of cholesterol has been illustrated by several epidemiological studies that show an inverse correlation between the

development of coronary artery disease and HDL-cholesterol levels (3-5; 41; 42). This property has been attributed to their role in the reverse cholesterol transport, a process by which HDL can remove excess peripheral cholesterol and transfer it to the liver for degradation and removal from the body (6).

1. Heterogeneity of HDL lipoproteins

In human, HDL is a very heterogeneous population of lipoproteins (43) that can be found in peripheral fluids, lymph, and plasma. Their only common denominator is their high density, which by definition ranges from 1.063 to 1.210 g/ml. HDL can be separated according to several criteria. As mentioned above, the first is the hydrated density, which distinguishes three major subclasses (HDL_2, HDL_3, Table I-1, and VHDL with a density ranging from 1.210 to 1.250 g/ml) and a minor one (HDL_1) that is apoE-enriched and that is found at high levels in certain pathological conditions (e.g., CETP deficiency). This subpopulation is more important in the rat and mouse possibly because they lack CETP activity. The densities of HDL subclasses reflect both their size and composition (Table I-3).

Characterization of the size of lipoproteins has been accomplished by nondenaturing polyacrylamide gradient gel electrophoresis. This technique has been useful to further resolve HDL size heterogeneity and identify new subpopulations of HDL complexes. HDL_2 was therefore subdivided into HDL_{2b} (8.8-9.7 nm) and HDL_{2a} (9.7-12 nm) whereas three subpopulations of HDL_3 were identified (HDL_{3c}: 7.2-7.8 nm; HDL_{3b}: 7.8-8.2 nm; HDL_{3a}: 8.2-8.8 nm) (44). The bimodal distribution of HDL appears to be specific to human apolipoprotein A-I (45). These subpopulations are thought to correspond to different steps in the maturation of HDL, in which the small HDL_3 appears to be the precursor of HDL_2 (23).

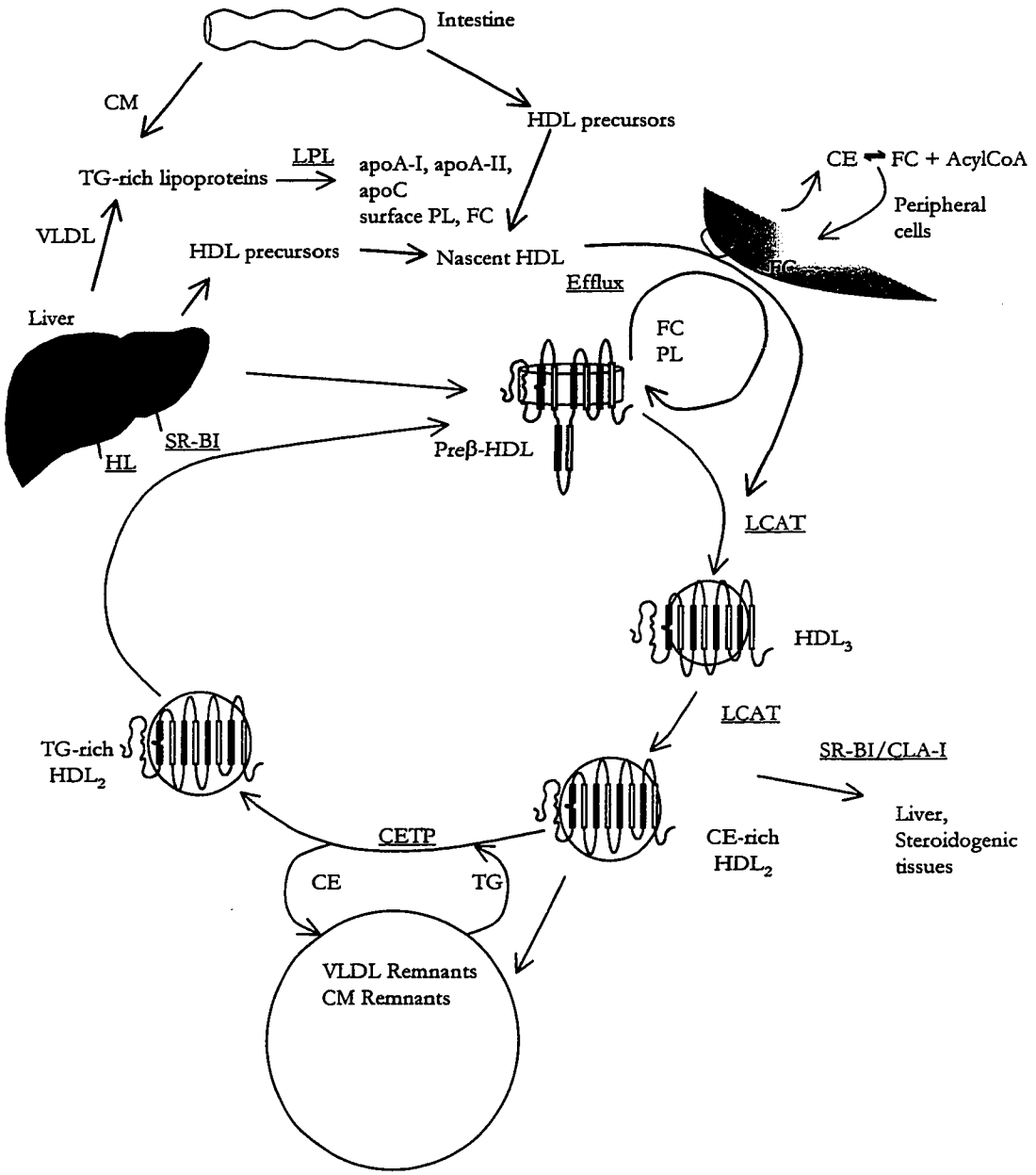

Figure I-4: Reverse Cholesterol Transport and the Metabolism of HDL.

ApoA-I is secreted by the liver and the intestine. Interaction of small nascent HDL (or preβ–HDL) is thought to initiate cholesterol efflux from peripheral cells. Their enrichment in cholesterol and PL allows for their interaction with LCAT and promotes the formation of larger HDL (in order, HDL_3 and HDL_2). The largest HDL are not capable of promoting a very efficient cholesterol efflux but can interact with CETP and apoB-containing lipoproteins. CE-enriched HDL may also interact with the SR-BI receptor and mediate the delivery of cholesterol to steroidogenic tissues and the liver. The transfer mediated by CETP leads to the formation of TG-rich HDL that can be acted upon by HL and converted into smaller HDL.

Immunoaffinity has been utilized for the separation of HDL subclasses based on their apoprotein composition. Investigators have shown that immunosorbents coupled with antibodies against apoA-I or apoA-II could separate HDL in two major subclasses, lipoproteins containing apoA-I but not apoA-II (LpA-I), and apoA-I and apoA-II-containing lipoproteins (LpA-I:A-II) (46). Other minor apolipoprotein-containing lipoprotein subclasses may also exist (such apoA-IV- or apoE-containing lipoprotein, LpA-IV and LpE, respectively). ApoC-containing HDL have also been isolated (47). Even after isolation, these complexes remain heterogeneous in sizes (46).

Electrophoresis, the first method applied to the separation of lipoproteins, has allowed the separation of HDL subclasses according to their charge. After electrophoresis in 0.5 % agarose gel, variations of lipoprotein mobility are directly related to particle charge. A quantitative classification of lipoprotein electrophoretic behavior based on their surface potential (proportional to the electrophoretic mobility) has been proposed by Sparks & Phillips (48). The beta mobility region is associated with a surface potential that ranges between –4.5 to –7.0 mV, the pre-beta region ranges between –7.0 to –10.5 mV, the alpha mobility ranges between –10.5 to –12.5 mV, and the pre-alpha mobility ranges between –12.5 to –14.5 mV. The bulk of HDL is usually associated with an α-migration. However, in normolipidemic plasma, a minor subpopulation of HDL (preβ-HDL) is also present with a preβ–migration. Preβ-HDL were first discovered after isolation of HDL lipoprotein by immunoaffinity purification (49). This labile subpopulation of HDL was shown to be absent after purification of HDL by ultracentrifugation or precipitation with polyanions. It was initially characterized by Kunitake *et al.* (50) who showed that it contains 10 % lipids (PL, CE, FC) and 90% protein (mainly apoA-I and no apoA-II nor apoB). The α-helicity of the protein in this complex was

reduced as compared to α-HDL. Preβ-HDL have subsequently been identified in mice (51), monkeys (52), and dogs (53). Preβ-HDL form 2-14% of total plasma HDL (50; 54; 55).

2. Origin of nascent HDL

Since apoA-I is the major protein component of HDL, much attention has been focused on apoA-I synthesis, secretion, and assembly with lipids. Nascent HDL are secreted by both the liver and the intestine, probably at equivalent levels (56) although their relative contribution may be dependent on dietary fat intake. ApoA-I may be synthesized as lipid-free protein or as small HDL. Nascent HDL, secreted by both liver and intestine, have been described as discoidal or spherical particles depending on the experimental conditions (i.e., lecithin:cholesterol acyltransferase (LCAT) active or not) (57-60). However, the discoidal HDL structure is not believed to represent a physiological form of secretion since only the spherical structures have been observed in the cellular secretory pathways (57; 58; 61). Discoidal HDL rather appear to be produced by hydrolysis of TG-rich lipoproteins, which results in the formation of a discoidal structure in the presence of surface material (PL and apoproteins) and fatty acids (62-64). LPL and HL may therefore play a major role in the formation of HDL. Indeed, a very strong relationship between LPL activity and HDL levels has been demonstrated (65). Another important mechanism by which these nascent HDL arise may be through specific interaction of apoA-I with membrane lipids (57; 58; 61; 66; 67). The relative contribution of these different pathways is not yet known and remains controversial and can certainly vary in the fasting and postprandial states (23).

3. Cholesterol transfer to HDL

Mechanism of HDL-Cell Interaction

One of the major functions of HDL is to remove excess peripheral cell cholesterol and transport it to the liver for elimination or recycling (Fig. I-4). Therefore, the metabolism of HDL reflects that of cholesterol in the circulation and in peripheral fluid. Nascent HDL is capable of interacting with the cellular plasma membrane, which results in the subsequent removal of excess cholesterol. The mechanism by which apoA-I operates is still a matter of controversy, however, two pathways have been defined. In the first, apoA-I in association with PL can accept cellular cholesterol from the plasma membrane. In this case, cholesterol, which has first to transfer from the cytofacial leaflet of the plasma membrane (only 3-5 % of the total cholesterol is in the exofacial leaflet of the plasma membrane; (68)), desorbs from the plasma membrane and is transferred passively by aqueous diffusion to HDL. The rate-limiting step of this process is the desorption from the plasma membrane since flip-flop is very rapid within the membrane and cholesterol is poorly soluble in aqueous solution (69). This net transfer is also a function of the respective cholesterol contents of the donor (i.e., plasma membrane) and acceptor (i.e., HDL). This process has also been termed diffusional or nonspecific efflux, because any type of apolipoprotein associated with lipid can promote this efflux, with a possible limitation concerning the number of amphipathic α-helices required (6). It is distinct from the specific interaction of apoA-I with cell-surface binding sites (Table I-4). The second pathway proposed by several investigators involves the binding of apoA-I or HDL to specific cellular binding sites at the plasma membrane, which allows the translocation of intracellular cholesterol to the plasma membrane and cholesterol efflux by aqueous diffusion (70). Several groups have attempted to identify a specific HDL-receptor (71-79). Results have been

inconsistent with a candidate protein having a molecular mass varying between 80 and 180 kDa. These observations were made in a number of different cell lines including fibroblasts, hepatocytes, adipocytes, and macrophages, which may explain the variability of the binding sites. Nevertheless, it is interesting to note that some of these putative HDL binding proteins were shown to be upregulated upon cholesterol loading of the cells. This may suggest a possible involvement of these proteins in the elimination of excess cellular cholesterol (71). However, some investigators have suggested that these interactions are rather the reflection of the association of apoA-I with specific lipid domains of the plasma membrane (80; 81). Other researchers have concluded that no relationship existed between apoA-I binding and cellular cholesterol efflux (82; 83). In support of the latter concept, it is important to note that apoA-I can interact much more strongly with cholesterol-enriched vesicles than with non-enriched ones (84). However, these investigations were not always performed under similar or comparable experimental conditions: loading of the cells with cholesterol was performed using various techniques (pure cholesterol, cholesterol-enriched dispersions, modified LDL) and time-courses of efflux varied over a wide range in these reported studies.

Table I-4: Comparison of the Characteristics of Specific and Nonspecific Cholesterol Efflux (from (6))

Specific Efflux	Nonspecific Efflux
Fast cholesterol efflux pool ($t_{½} \approx 1$ h)	Slow pool ($t_{½} \approx 10$ h)
Protease-sensitive	Protease resistant
LpA-I-dependent	LpA-I ≈ LpA-I:A-II; also to albumin
Maintains preβ1-HDL	Does not maintain preβ1-HDL
Linked to LCAT-mediated esterification	LCAT-independent

The murine scavenger receptor class B type I (SR-BI, identified as CLA-I in human) has recently been demonstrated to be involved in the cholesterol efflux process (85). Levels of SR-BI mRNA in different cell lines were correlated with the ability of HDL to promote cholesterol efflux. SR-BI is a putative transmembrane glycoprotein from the scavenger receptor family (86; 87). It has been shown to bind HDL, LDL, modified LDL, and anionic PL as well as reconstituted lipoproteins containing apoA-I, apoA-II, and apoC-III (88-91). The importance of PL in the process mediated by SR-BI was recently demonstrated by a study of Jian *et al.* (92). In this work, the authors have shown that increased PL concentrations in the extracellular milieu enhances cholesterol efflux in cells expressing SR-BI but not in others. Nonetheless, the mechanism that involves SR-BI in cholesterol efflux is still poorly understood.

Another pathway for cholesterol efflux may involve the retroendocytosis of HDL, as suggested by earlier work (78; 93). Elucidation of the pathways leading to the translocation of intracellular cholesterol has provided evidence for a role of protein kinase C (PKC) pathway in lipid efflux (94-97). This pathway is termed translocation-dependent cholesterol efflux or PKC-dependent cholesterol efflux. In this pathway, intracellular CE may be hydrolyzed by the enzyme neutral cholesterol ester hydrolase (NCEH) permitting transfer of FC from intracellular stores to the plasma membrane where it becomes available for efflux by passive aqueous diffusion to extracellular acceptors (70). ApoA-I association with cell surface binding sites appears to promote the translocation of intracellular sterol from the ER to the plasma membrane in several cell lines (98-100). Substrates of PKC, that may have a role in the translocation of intracellular stores of cholesterol, have been identified and include an unknown phosphoprotein termed pp18 (101). Activation of PKC by acute treatment with phorbol ester can stimulate translocation of intracellular cholesterol to the plasma membrane

(95; 102). Cholesterol efflux from smooth muscle cells, mediated by apoA-I but not HDL, can only be observed after treatment with growth factors and PKC activators, which indicates the absence of a cellular factor involved in specific efflux from these cells (103). These treatments lead to the transformation of the cells into a macrophage-like phenotype. The opposite was observed when the ability of apoA-I to promote cellular cholesterol efflux from macrophages treated with PKC inhibitors was examined. In that case, cholesterol efflux was reduced with no change in PL efflux, which indicates a very specific effect on the metabolism of cholesterol and no effect on the non-specific efflux (i.e., from the plasma membrane) (104). Insight into the mechanism of cholesterol efflux has also been made available through the study of Tangier disease (described in more detail in the next section). Recently, two independent studies have identified a specific defect in the metabolism of cholesterol in these patients: apoA-I and HDL-mediated cholesterol efflux of intracellular cholesterol from Tangier fibroblasts are reduced (105; 106). This defect appears to affect an upstream effector of PKC (107; 108). Therefore, the PKC mediated-pathway appears to be of major importance, if not essential, for an efficient removal of intracellular excess cholesterol from the cells.

Beside these different pathways of cholesterol efflux, cellular cholesterol exist in association with cellular pools (e.g., intracellular esterified cholesterol, in different membrane, in different domain of the plasma membrane such as caveolae) (68; 109; 110). Depending on the conditions, cholesterol effluxed may represent one or several of these pools.

Importance of Preβ-HDL in the Initial Steps of the Reverse Cholesterol Transport

Fielding and colleagues have documented the existence of minor subpopulations of HDL that act as an initial acceptor of cellular cholesterol, and which have been termed preβ-

HDL by virtue of their preβ-migration upon electrophoresis on an agarose gel (111; 112). Interestingly, these complexes have been observed not only in plasma but also in peripheral lymph (113) where early steps of the reverse cholesterol transport process are believed to occur (114).

Preβ-HDL most probably have multiple origins. ApoA-I secreted by hepatic cells or incubated with cells can generate several subpopulations of HDL, among them, preβ-HDL, which can accumulate if LCAT is not active (67; 115; 116). Other reactions have also been shown to promote the formation of preβ-HDL. HDL modifications induced by HL (117), CETP (118; 119), or PLTP (phospholipid transfer protein) (120) lead to the dissociation of lipid-poor apoA-I. Studies with transgenic animals have recently underlined the role of these proteins in the formation of preβ-HDL (121-124). Although the presence of lipid-poor apoA-I may enhance cholesterol efflux, it is also rapidly catabolyzed [by increased renal clearance (125)].

A functional role for preβ-HDL was proposed by Castro & Fielding (111). In this report, the authors showed that incubation of ^{3}H-cholesterol-labeled fibroblasts with serum resulted in the appearance of the label in a fraction of preβ-HDL termed preβ_1-HDL (at very early time-point, 1 min). Subsequent transfer toward other HDL subpopulations, from the smaller preβ-HDL particles to preβ_2-HDL and preβ_3-HDL successively, occurred, with the tracer eventually appearing in large spherical α-HDL (Fig. I-5). Lipoprotein separation was performed using a 2-dimensional electrophoresis system, where the first dimension is based on separation according to the charge of the complexes (agarose gel), and the second based on separation according to the size of the complexes (non-denaturing polyacrylamide gradient gel).

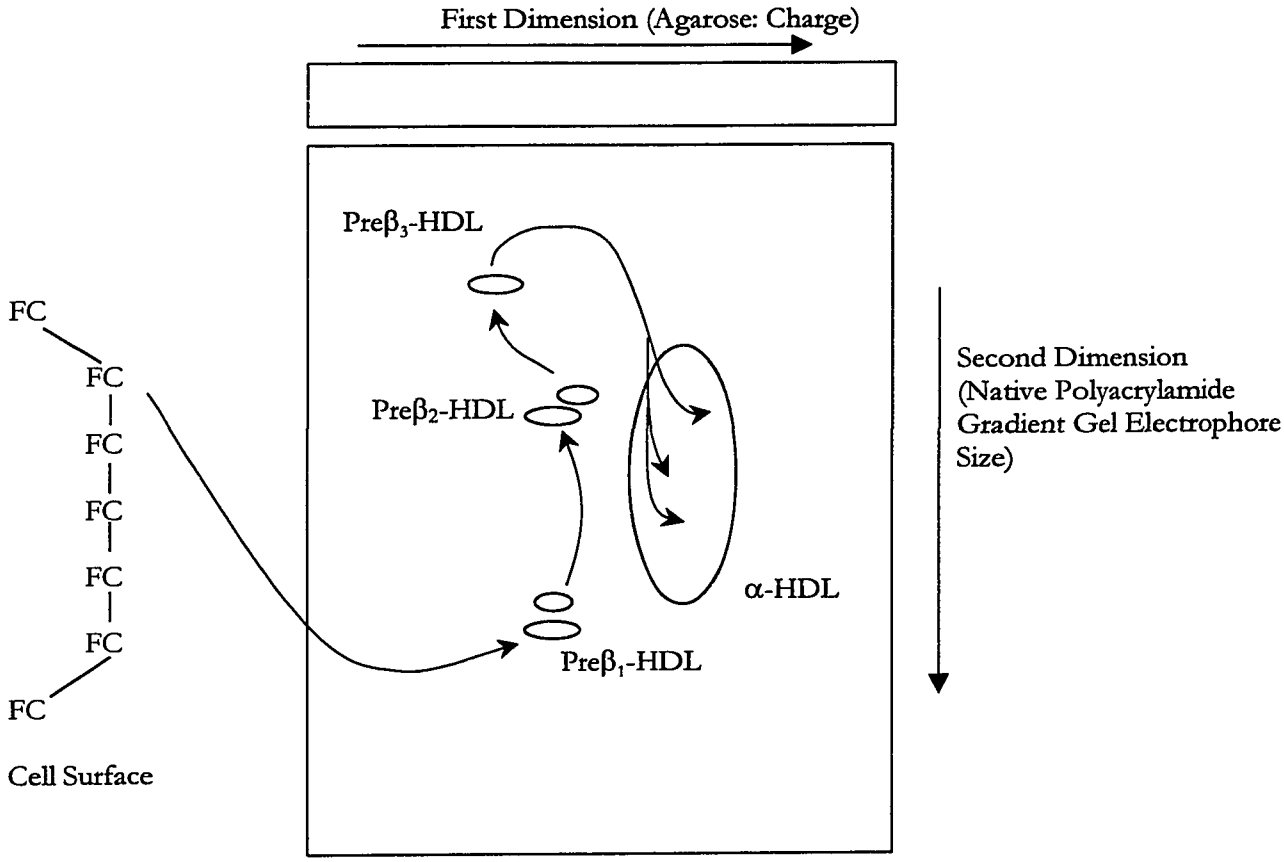

Figure I-5: Sequential Transfer of ^{3}H-cholesterol from Fibroblasts to Different HDL Subfractions.
Upon incubation of ^{3}H-cholesterol-labeled fibroblasts, cellular free cholesterol (FC) from fibroblasts first appears in the preβ$_1$-HDL fraction (within less than 1 min of incubation) followed in order by preβ$_2$-HDL (≈1 min), preβ$_3$-HDL (≈2 min), and α-HDL (<15 min) (from (112)).

Although the shape of these complexes has not yet been clearly defined, they may have a globular structure (lipid-poor apoA-I) since the formation of a disc would not be possible, due to physical/structural constraints (6). In the second step of this efflux pathway, cholesterol can rapidly transfer to discoidal preβ$_2$-HDL, which contain 2 molecules of apoA-I and are enriched in FC (112; 126). One possible mechanism by which these Lp2A-I and larger HDL

subpopulations are produced may be by acquisition of PL or by fusion of the small lipid-poor preβ$_1$-HDL through interactions with PLTP. PLTP has been recently characterized and shown to promote the transfer of PL and enlargement of HDL populations (127; 128). Another simpler possibility is that the lipidation of apoA-I with PL and FC generates preβ$_1$-HDL or Lp1A-I, thermodynamically unstable (i.e., more unstable than lipid-free apoA-I) (Sparks *et al.*, manuscript in preparation) and which, we propose, would spontaneously associate to generate preβ$_2$-HDL or larger HDL subpopulations. The third step requires the reaction mediated by LCAT, which esterifies FC and leads to the formation of a neutral lipid core in preβ$_3$-HDL (2 A-I molecules, 40 FC molecules , 10 CE molecules, and 1 LCAT molecule). This step appears to occur in plasma, as apoA-I-associated with preβ$_2$-HDL in lymph may have a unique structure, which may prevent its ability to activate LCAT (129). After maturation of preβ$_3$-HDL, esterification of FC continues and progressively leads to the formation of larger α-HDL subspecies (in order HDL_3 followed by HDL_2, see Fig. I-4).

Composition of preβ-HDL

The physico-chemical properties of preβ$_1$-HDL have been characterized. Their apparent molecular mass varies between 70-80 kDa, with a Stokes' diameter, depending on their origin, of 6.8 nm in human follicular fluid and 7.0 nm in plasma, although such a difference may not be significant. In plasma, their concentration is ≈74 μg/ml and they form about 6 % of apoA-I-containing lipoproteins (130). Plasma preβ$_1$-HDL were initially described by Castro & Fielding (111) and preβ$_1$-HDL from follicular fluid, which contains HDL as the sole lipoprotein, have been analyzed in detail (131). Their composition is indicated in Table I-5

and compared to that of α-HDL. All these compositions were determined after electroelution of the complexes from agarose gels, a technique that may result in variable recovery of the different lipids (no apolar lipid is observed in plasma preβ$_1$-HDL).

Table I-5: Composition of α-HDL and preβ$_1$-HDL from Human Plasma and Follicular Fluid. (expressed as a percentage, w/w).

	FC	CE	TG	PL	A-I	Reference
Preβ$_1$-HDL (Follicular fluid)	1.28	7.16	1.46	22.46	67.64	(131)
Preβ$_1$-HDL (plasma)	7.6	nd	nd	44.6	47.5	(111)
α-HDL (plasma)	1.57	28.53	1.82	20.58	47.50	(131)

nd: not detected

Nevertheless, calculation of the molecular composition of this preβ$_1$-HDL would give 1-2 apoA-I, 18 PL, 2 FC, 7 CE, and 1 TG molecules/ particle (113). Among the PL, the sphingomyelin composition may be very similar to that of the outer leaflet of the plasma membrane (6). A direct interaction of lipid-free or –poor apoA-I with the plasma membrane may therefore give rise to preβ$_1$-HDL. However, studies with *in vitro* reconstituted apoA-I-containing lipoproteins did not permit identification of any specific HDL with a markedly enhanced ability to remove cellular cholesterol (132-134). One possible explanation may be that this subpopulation is very labile and/or that it is generated only after incubation of apoA-I with cells (135). Another possibility is that preβ$_1$-HDL or lipid-poor apoA-I can act as a shuttle between cells and α-HDL, with a high affinity but a poor capacity for cellular cholesterol (136; 137), which may indicate that both preβ$_1$-HDL and α-HDL are required for efficient cholesterol removal from cells. The role of preβ-HDL has been underlined in studies using

transgenic mice or rabbits. Overexpression of human apoA-I in rabbits (138)or apoA-I and CETP in mice (123) leads to an enhanced ability of the plasma to promote cholesterol efflux from cells, which could be related to an increase in preβ-HDL levels. However, high levels of $preβ_1$-HDL have also been observed in patients with evidence of CHD (139). In this case, a defect downstream of $preβ_1$-HDL metabolism may cause their accumulation in this particular pathology.

Other investigators have identified preα-HDL as the first lipoprotein formed upon interaction of lipid-free apoA-I with cells (140). However, a different technique was employed for the characterization of the lipoprotein charge in this study (agarose gel with no albumin, believed to modify the lipoprotein migration profile), which may probably explain the different results obtained.

Finally, two other cholesterol efflux pathways, which involve other apoproteins, have been described. Huang *et al.* (141) have identified a minor fraction of apoE-containing lipoproteins (γ-LpE) as a potent acceptor of cellular cholesterol. Another specific subpopulation of preβ-HDL containing apoA-IV but not apoA-I has also been proposed to mediate the initial steps of the reverse cholesterol transport pathway (142).

Transfer of Free Cholesterol from LDL to HDL

FC is almost exclusively associated with LDL and HDL. Although cholesterol esterification by LCAT has been demonstrated on LDL (143), this pathway appears relatively minor in comparison with CE formation within HDL. Therefore, FC must transfer from LDL to HDL in order to be efficiently esterified. The mechanism by which FC transfers from LDL to HDL has been studied in detail by several groups (144-149). Initial studies suggested that

the kinetics of exchange are consistent with a mechanism in which FC desorbs passively from the donor (LDL) and transfers to the acceptor (HDL). However, more recent studies using reconstituted HDL complexes have indicated a more complicated mechanism. Meng *et al.* (149) have shown that the rate of transfer from HDL is a function of their size (smaller HDL being more efficient as an acceptor of LDL-derived FC) and their lipid composition. They have proposed that a transient fusion between donor and acceptor occurs during the transfer and that apoA-I conformation, which is affected by the lipid environment, may play a major role in this process. Indeed, they have also shown that mAbs binding to apoA-I, which affects its conformation, can significantly decrease FC transferred from LDL to HDL while enhancing this process in the opposite direction (148).

4. Maturation of HDL: Roles of LCAT, CETP, and HL

Free cholesterol acquired by HDL is esterified by lecithin:cholesterol acyltransferase (LCAT), at the surface of HDL. This glycoprotein contains 416 amino acids and has two enzymatic activities: LCAT hydrolyses the fatty acid at the *sn*-2 position of lecithin (phospholipase A_2 activity) and transfers it to cholesterol, a reaction that leads to the formation of esterified cholesterol (acyltransferase activity). Free cholesterol, which is predominantly found at the surface of lipoproteins (70% at the surface vs. 30% in the core), is then transferred into the lipoprotein core after esterification. This interfacial enzyme is therefore believed to promote the formation of spherical HDL (150). In patients with no LCAT activity, HDL levels are markedly reduced and discoidal HDL complexes can be detected in plasma (151). This enzyme is secreted by the liver and requires the presence of FC and PL and also apoA-I as a cofactor. In the absence of apoA-I, only 1% of LCAT activity remains. The

mechanisms by which apoA-I activates LCAT will be discussed in more detail in the next section (apoA-I and LCAT activation).

Glomset (152) proposed that LCAT can maintain the concentration gradient of free cholesterol between cells and HDL. *In vitro* and *in vivo* studies have shown that, after cellular cholesterol efflux, LCAT could prevent a back-transfer of cholesterol from HDL to cells (153; 154). The first HDL complex with which LCAT is associated is preβ_3-HDL (155). Cholesterol esterification may lead to the formation of HDL_3 and eventually HDL_2 (larger than HDL_3 and enriched in CE as shown in Table I-1). The maturation of HDL_3 to HDL_2 requires that the HDL_3 complex increases its CE content (from 40-50 to 100-120 molecules/complex), accommodates another molecule of apoA-I (from 3 to 4 molecules/complex), and also increases its PL and FC content (from ≈10 to ≈50 and ≈100 to ≈190/complex, respectively) (23). An increase in the TG content of HDL_2 may be due to the activity of cholesterol ester transfer protein (CETP). The core of HDL_2 is about 50 % larger than that of HDL_3 (60 and 40 Å, respectively), but, the large increase in the lipid content is not fully accommodated by a parallel increase in the apoA-I content or that of other apoproteins (apoCs appear in HDL_2 but are a minor constituent of these lipoproteins). This observation suggests an important ability of apoA-I to accommodate varying amounts of lipid. The source of PL and apolipoproteins appears to be TG-rich lipoprotein remnants and depends therefore on the activity of LPL (156-158). HDL_3, but not HDL_2, is also good acceptor of cellular FC (159).

CETP is an extremely hydrophobic glycoprotein that contains 476 amino acids and catalyzes the exchange of CE from HDL to TG-rich lipoproteins for TG (160). Large HDL_2 are the best HDL donor for CETP. Lipid transfer activity by CETP is elevated in humans and rabbits whereas it is almost absent in rodents (161; 162). CETP appears to associate with

apoA-I-containing lipoproteins, where it may be the most active (163). Studies with transgenic mice have indicated a species specificity of this binding, since HDL formed with human apoA-I appears to interact better with human CETP than HDL formed with mouse apoA-I (164).

CETP deficiency has been observed in several Japanese populations and is characterized by high HDL levels (165), but whether CETP deficiency is proatherogenic or protective against atherosclerosis is still a matter of controversy (166). Recent studies have indicated that, in humans, CETP may be necessary for efficient reverse cholesterol transport (167). Insight into the *in vivo* role of CETP has also been obtained from studies of CETP expression in transgenic mice. Although expression of human CETP leads to reduced HDL in transgenic mice (168), it also reduces their FC/CE ratio, which may enhance the reverse cholesterol transport pathway (122; 123; 167). On the other hand, CETP deficiencies may alter other steps of HDL metabolism, such as the reaction mediated by LCAT, which may explain an increased CHD in CETP deficient patients (169). In subjects with functional apoB/E receptors (31), CE-enriched LDL are catabolized by the liver for removal from the body or serve as a cholesterol source for organs that require high levels of cholesterol such as steroidogenic tissues. This uptake represents the last step of reverse cholesterol transport. Another pathway leading to the removal of cholesterol from HDL or its transfer to cells is the selective uptake of HDL-CE at the surface of liver or steroidogenic tissues (170). This non-endocytotic mediated pathway has been recently suggested to be mediated by the cell surface receptor SR-BI (or CLA-I) (89). Selective uptake appears to require the presence of apoA-I at the surface of HDL to be fully active (171) and occurs without any cellular degradation of apoA-I (selective uptake), in contrast to the delivery of cholesterol mediated by the LDL-receptor (31). In humans, where most of the cholesterol is associated with LDL, it is still

unclear whether the pathway mediated by SR-BI is important in the efflux or uptake of cholesterol or possibly both.

The transfer mediated by CETP, which leads to the formation of TG-rich HDL, modifies the physico-chemical properties of HDL. They become a good substrate for HL, which hydrolyzes PL and TG into fatty acid and monoglycerides. HL is a 472-amino acid protein with 50 % sequence identity to LPL, also bound to endothelial cell surface proteoglycans in the liver where it is produced. HDL_2 is a good substrate for HL, which is a functional monomer and does not require any cofactor unlike LPL, which is a functional dimer and requires apoC-II for activation (172). HL reduces the PL/protein ratio in HDL (forming smaller HDL subpopulations such as HDL_3) and also promotes the shedding of apoA-I from HDL (formation of preβ-HDL) as suggested by both *in vitro* and *in vivo* studies (117; 173). The dissociation of apoA-I is thought to enhance its recirculation in HDL metabolism. However, previous work has suggested that the dissociation of apoA-I may also promote HDL clearance by enhancing the renal filtration of apoA-I (125). In support of this hypothesis, an inverse correlation has been observed between HDL levels and HL activity in human populations (174; 175) and in transgenic animals overexpressing human HL (121; 176). Nonetheless, HL overexpression in mice appears to reduce aortic cholesterol levels, which may indicate an antiatherogenic role (176). This reduction may be the consequence of two different pathways. HL, like the receptor SR-BI, may mediate a non-endocytotic pathway for the removal of HDL-cholesterol by the liver (177-179). Another possible mechanism, which may explain the antiatherogenic effect of HL, is the production of the small preβ-HDL important in the removal of excess peripheral cell cholesterol (180). HDL remnants may be the ligand of a possible liver receptor (77; 181), which catabolizes HDL through a pathway similar to that of

the LDL-receptor (182). Alternatively, apoE may associate with large HDL_2 and facilitate their catabolism through the LDL-receptor.

5. Role of LpA-I versus LpA-I:A-II

The role of apoA-II in the metabolism of HDL remain unclear. ApoA-II is primarily found associated with apoA-I containing lipoproteins, with which it forms LpA-I:A-II particles. They originate mostly from the liver whereas LpA-I (lipoproteins containing apoA-I without apoA-II) are produced by both the liver and the intestine (43). LpA-I and LpA-I:A-II can easily be separated by immunoaffinity techniques (46). Turn-over studies have demonstrated that LpA-I are catabolized more rapidly than LpA-I:A-II (183). Both subpopulations appear heterogeneous when separated according to their size on a native polyacrylamide gradient gel electrophoresis or by gel-filtration (46; 184). They also appear to contain differing ratios of other proteins, LpA-I:A-II being enriched in apoD, and apoE, (46). LCAT preferentially associates with LpA-I (185) but significant amounts of LCAT are associated with LpA-I:A-II since they form the major part of the alpha-migration HDL fraction (6).

Increased LpA-I levels are usually associated with a decreased risk of CHD whereas no relationship has been observed with LpA-I:A-II levels (46; 184; 186; 187). The functional and metabolic difference between these two HDL subpopulations has been extensively studied. Several groups have studied the respective ability of LpA-I and LpA-I:A-II lipoprotein complexes to stimulate cholesterol efflux from cells (188-192). However, results have not always been consistent. Barbaras *et al.* found that LpA-I but not LpA-I:A-II had the capacity to promote cholesterol efflux from Ob1771 adipocyte cells (188). Johnson *et al.* (189) and Oikawa

et al. (190) have found no difference in the ability of LpA-I and LpA-I:A-II to promote cellular cholesterol efflux. Huang *et al.* (191) and Lagrost *et al.* (192) have observed that LpA-I are better acceptors of cellular cholesterol. These discrepancies may relate to the different cell types used in these studies (adipocytes (188), fibroblasts (189-191), endothelial cells (190), or hepatocytes (192)). Another aspect to consider in the interpretation of these studies is the composition of the different HDL subpopulations, which may have varied and which is known to affect cellular cholesterol efflux (133; 193; 194). The study of Lagrost *et al.* (192), in which LpA-II were prepared by displacing apoA-I from HDL by incubation with apoA-II is the only one that compares LpA-I and LpA-I:A-II with similar lipid contents. In agreement with this result, De la Llera Moya *et al.* (195) have shown that the serum concentration of LpA-I is a good indicator of the ability of plasma to stimulate cellular cholesterol efflux. Other studies have demonstrated that LpA-I:A-II may preferentially accumulate FC from LpA-I complexes (191) or LDL (196). LpA-I:A-II, which also bind LCAT, could then promote FC esterification (6).

The presence of apoA-II in HDL appears to affect other enzymes. Whereas apoA-II was shown to enhance HL activity (197), it may inhibit CE transfer mediated by CETP (198), possibly because of a reduced affinity for this transfer protein (199).

ApoA-II may play a regulatory role in the metabolism of HDL but does not seem to be essential since its absence is not associated with profound changes in HDL-C levels or with increased CHD (200). Studies with transgenic mice have shown that overexpression of human apoA-II with human apoA-I affects the quality of HDL produced and increases the susceptibility to atherosclerosis as compared to mice overexpressing human apoA-I only (201).

6. Other roles of HDL

Protection of LDL against oxidation

The antiatherogenic role of HDL may not only be mediated by its function in reverse cholesterol transport. A study by Parthasarathy *et al.* (202) have suggested that HDL may protect LDL from oxidation and decrease foam cell formation. In recent years, the HDL components responsible for this effect have been more precisely analyzed. Vitamin E (tocopherol), an important lipid-soluble chain breaking (i.e., peroxyl radical-trapping) antioxidant in human plasma (203), is associated with TG-rich lipoproteins and, upon lipolysis, is subsequently transferred to HDL (204). Plasma tocopherol levels appear to be positively correlated with the levels of HDL-cholesterol and proteins (205). Epidemiological studies have also demonstrated its protective effect against atherosclerosis (206). Enzymes associated with HDL have also been involved in this process. These include paraoxonase (207), lecithin:cholesterol acyl transferase (208; 209), and platelet activating factor acetylhydrolase (210). These enzymes were shown to detoxify lipid peroxides or prevent their appearance. ApoA-I and apoA-II may also have a direct role in the protection against lipid oxidation since they were shown to eliminate lipid peroxides in HDL (211).

Inhibition of cell adhesion during the early steps leading to atherosclerosis

Exposure of endothelial cells to atherogenic lipoproteins is also thought to induce the expression of several factors involved in the adherence of monocytes to the endothelium (212). These factors include intercellular adhesion molecule 1 (ICAM-1), which promotes the adhesion of monocytes, neutrophils, and lymphocytes, and endothelium leukocyte adhesion molecule 1 (ELAM-1), which enhances monocyte and T-lymphocyte interactions. The vascular

cell adhesion molecule 1 (VCAM-1) is responsible for the binding of monocytes and T-lymphocytes (213). In a recent report from Cockerill *et al.* (214), it was found that HDL could inhibit the expression of E-selectin (another adhesion molecule), VCAM-1, and ICAM-1, which demonstrates other potential antiatherogenic effects of HDL (215).

7. Disorders of HDL metabolism

Since epidemiological studies have demonstrated an inverse correlation between HDL and the development of coronary artery disease (216), the focus of many studies has been to examine the factors that regulate HDL-cholesterol (HDL-C) levels. In humans, HDL-C levels are highly variable, and are dependent on age, gender, genetic and environmental factors such as diet and physical exercise. For North American men (age 40-44), the average level of HDL-C is 1.1 mmol/l whereas for women in the same group, the average is 1.4 mmol/l (217). The identification and characterization of specific HDL deficiency genetically based have been shown to be useful in identifying the key events intervening in the metabolism of HDL. Several defects have been identified but not all of them have been fully characterized (217; 218).

Mutations in genes affecting HDL-C levels

A summary of the phenotypes that are associated with disorders of HDL metabolism is given in Table I-6. As noted in this table, low levels of HDL are not always related to the development of CHD, but it appears that when other disorders are present (e.g., hypertriglyceridemia), there is an increase in the risk of CHD.

In most cases, however, when mutations in the apoA-I gene lead to the absence of apoA-I, increased incidence of CHD has been observed. The apoA-I gene-knock-out

(elimination of apoA-I gene by gene targeting) mouse failed to show increase in susceptibility to CHD (220). This may be related to the higher resistance of the mouse to atherosclerosis as compared to the human. Only certain strains of mice (i.e., C57BL/6) are sensitive to atherosclerosis, and only when fed a high fat, high cholesterol diet. Nevertheless, when crossed with human apoB-100 transgenic mice (increased LDL levels), apoA-I knock-out-mice have demonstrated an increased susceptibility to atherosclerosis, which indicates the importance of multiple defects in this disease (221). Despite low HDL, patients with Tangier disease and LCAT' deficiencies show a limited susceptibility to atherosclerosis (Tangier disease reviewed below). However, increased HDL levels in CETP deficient patients can be associated with CHD.

Table I-6: Lipid and Lipoprotein Levels in Disorders of HDL Metabolism.
(adapted from (217-219)

	TG	CM	VLDL	LDL	HDL	CHD
ApoA-I deficiency	_	_	_	_	↓↓↓	↑↑
HL deficiency	↑↑	↑↑	↑↑	↓	↑↑	↑
LCAT deficiency	_	_	_	↓↑	↓↓↓	_↑
CETP deficiency	↑	↑	↑	_↑	↑↑↑	_↑
Tangier disease	_↑	_	_	_↑	↓↓↓	_↑
LPL deficiency	↑↑↑	↑↑↑	↑	↓↓↓	↓↓↓	?
ApoC-II deficiency	↑↑	↑↑	↑	↓↓↓	↓↓↓	?

The number of arrows indicates the magnitude of the decrease or increase. _ indicates levels in the normal range.

Tangier disease

Tangier disease is characterized by severe deficiency or absence of HDL in plasma and by the accumulation of CE in several tissues (tonsils, liver, spleen, lymph nodes, thymus, intestinal mucosa, peripheral nerves, and cornea) (218). CE storage is observed in different cell types, including histiocytes (long-lived resident macrophages found within tissues), Schwann cells, neurons, smooth muscles cells, and fibroblasts (218). Decreased apoA-I levels in heterozygous (50 % of controls) and homozygous patients (less than 3 % of controls) appears to be related to their enhanced catabolism (222; 223). No defect in apoA-I sequence or in any known protein involved in the metabolism of lipoproteins has been identified (218). Nevertheless, at the cellular level, the metabolism of cholesterol seems to be particularly affected. HDL incubated with monocytes from Tangier patients were shown to be internalized and degraded while incubation with normal monocytes resulted in their re-secretion as cholesterol-enriched HDL (224). In addition, a defect in the lipid metabolism has been observed in Tangier macrophages, which synthesize five times more TG, two times more PL, and three times more CE than normal cells (225). This last defect may explain the accumulation of CE observed in several tissues. In plasma from these patients, preβ_1-HDL is the only HDL subpopulation present (226). More recently, two separate studies have identified another specific defect in the metabolism of cholesterol: ApoA-I and HDL-mediated removal of cellular cholesterol and PL are reduced in fibroblasts from Tangier patients (105; 106). In addition, defective lipid transfer was also observed with other apoproteins (apoA-II, A-IV, C-I, C-II, and C-III) but not with PL vesicles or cyclodextrin (a very efficient acceptor of cellular cholesterol) (227). The defect appears to affect an upstream effector of PKC. Recently, Von Eckardstein *et al.* (228) have observed that not only cannot the cells transfer PL to apoA-I but

the plasma of these patients is also defective for this transfer. Moreover, Huang *et al.* (229) have shown that plasma from Tangier patients lacked a factor required for conversion of preβ$_1$-HDL into α-HDL. Taken together, these data indicate that a defect in a PL transfer activity is responsible for impaired cellular lipid efflux to apoA-I and the maturation of preβ$_1$-HDL into α-HDL. When the main(s) defect(s) of this disease is identified, it should shed some light on the mechanisms regulating cellular cholesterol homeostasis.

II. APOLIPOPROTEIN A-I AND HDL

Apolipoprotein A-I was one of the first apoproteins to be identified and characterized. It is still one of the best-characterized and most studied proteins on lipoprotein particles. Nevertheless, its full structural characterization, notably in the lipid-associated form, has not been achieved and the domains involved in its multiple functions are not yet fully elucidated.

A. Structure of apolipoprotein A-I

1. Primary structure

The sequence of apoA-I was determined by Brewer *et al.* (230). After the isolation of the cDNA (231-234), the genomic DNA was isolated and characterized (235; 236). The gene encoding apoA-I is a member of the apolipoprotein multigene superfamily, which includes genes encoding exchangeable apolipoproteins (apoA-I, A-II, Cs, and E) (237). They are thought to have evolved from a common ancestor by duplication/deletion of a 33-mer motif. Moreover, they present similar genomic organization with three introns at the same location for all of them (Fig. I-6A). *APOA-I* gene is localized to 11q23 (238), on the same chromosome as apoA-IV and apoC-III. Interestingly, apoA-I and apoC-III genes are translated in opposite

directions, with the 3' end of apoA-I being 2.5 kb upstream of the 3' end of apoC-III (Fig. I-6B). These localizations explain the considerable effect observed on lipoprotein metabolism in the case of major chromosomal alterations of chromosome 11. These genes also appear to share regulatory elements (liver and intestine specific, Fig. I-6B), which suggests that change at the transcriptional level of one gene is associated with a variation in the transcription of the others (239).

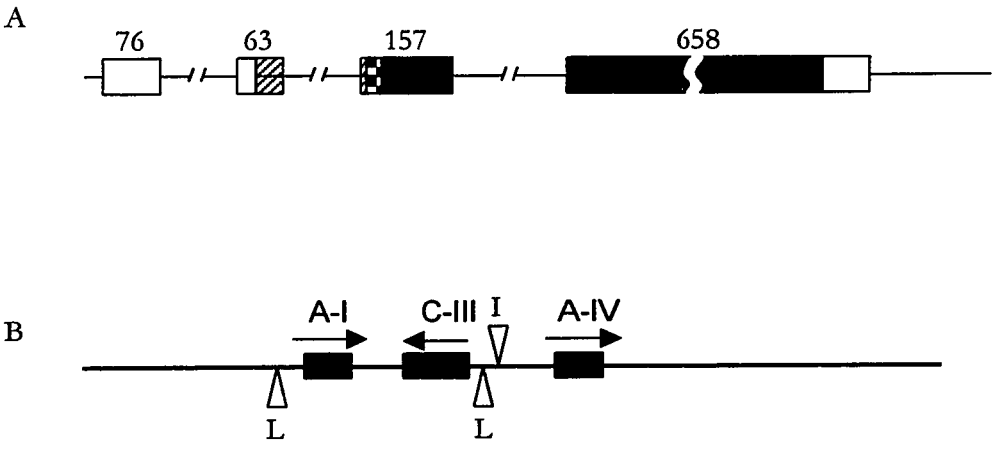

Figure I-6: Genomic Organization of the Human Apolipoprotein A-I Gene and Apolipoprotein Gene Cluster on Chromosome 11q23.
A. Genomic organization of human apoA-I gene. The wide bars represent the exons while the thin lines represent the introns. The bars are divided into 4 domains: the empty bar represents the 5' and 3' untranslated region of apoA-I while the hatched bar encodes the signal sequence and the dotted bar the prosequence. Finally, the black bar encodes the mature protein. The numbers above each exon indicate their number of nucleotides. (from (237))
B. Apolipoprotein gene cluster on chromosome 11q23. The arrows indicate the direction of transcription and the triangles the position of the regulatory elements (L: liver specific transcription element, I: intestine specific transcription element) (217).

Like all exchangeable apoproteins, the analysis of the amino acid sequence of mature apoA-I (243 aa, 28078 Da) has allowed the identification of 11- and 22-mer repeats (240; 241).

The 22-mer repeats, usually separated by Pro residues, have been associated with the formation of amphipathic α-helices (242). These amphipathic α-helices are thought to be the major structures that allow the interaction of the protein with PL through its hydrophobic domain while the hydrophilic face of the helix interacts with the aqueous phase.

ApoA-I sequences from a number of species have been determined (Fig. I-7), with sizes ranging between 258 and 267 amino acids. Comparison of sequences amongst mammals indicates that the N-terminal domain of apoA-I is highly conserved while the central and C-terminal domains show conservative substitutions between species. This result is consistent with the study of Collet *et al.* (243), who showed using mAbs to human apoA-I, that the antigenicity of this protein is better conserved between mammalian species in the N-terminal domain (residues 1 to 98 of the mature protein). These observations are in accord with the major role this domain plays in the structure and/or function of human apoA-I. As described in the latter sections, the N- and C-terminal domains of apoA-I have been involved in the binding of lipids, an essential function of this apoprotein (244). The central domain of the protein, which has been involved in LCAT activation, may have evolved in parallel with the LCAT sequence. However, in species in which this domain has evolved faster than in humans (mouse and rat), the ability of apoA-I to activate LCAT is reduced as compared to human apoA-I (245; 246). Despite changes in the primary structure of apoA-I amongst different species, the secondary structure appears unmodified, as shown by the Edmundson-wheel representation (243). Studies by Januzzi *et al.* suggest that *APOA-I* has evolved about 25 % faster than an average gene in mammalian lineage (247). However, all portions of the protein have evolved at similar rates, which suggests a global conservation of the structure.

Figure I-7: Part 1:

```
Human  -24-MKAAVLTLAVLFLTGSQARHFWQQDEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGS-36
Baboon      T                                T        V    EA     K
Dog        ________________________________  -              AV          A   A
Pig         V                             D -         F     AI          A   A
Rabbit      V                           R    -R S   KI F     TV      E  A   A
Cow         V                             D -  S      F     EAI         A   A
Hedgehog                                D A-K Y DQI  ML      TA     K  LTSLDT
Mouse       V  AV LV       W V           -  Q   K    F N     AV                S
Rat            AV LV    C  WE            -  Q        F       AV                S
chicken   RGVLV         T    S     H     - T L   IR MVD   LETV A   K AIA      S
Duck      RVV V    L    T    Y     H     - A L   LR   VD  LETV A   K AIA      A
Salmon      L  A TI LAA T  -FPM A A ---  QLEH  AALNM  IAQV LTAQRSIDLLDDT

Human  37-ALGKQLNLKLLDNWDSVTSTFSKLREQLGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAK-96
Baboon                         V
Dog                        LS  VT                              V             Q
Pig          H             LG   T V                            A             K
Rabbit    F                SLS  V   Q                              E  N   Q  RQ
Cow                        TLA  L  V                           AS     H      Q
Hedgehog  F                LS   V   Q                              E  N   Q  RQ
Mouse    S  Q    N  E     TLG  VSQ Q R    L RD                DWV     N      Q
Rat      T       N        TLG  VGR Q               A          DW  N   N   N  Q
Chicken   V    D   A  L TLSAAAA      DMA YYK VREMWL D  A   A LT             E
Duck      V    D   A  L TLGAAAA      DMA YYK VREMWL D  S   A LT             E
Salmon   EY- EYKMQ SQSL NLQQFADSTSKSWPPTPRSS-APSCDA ATV A VM   V D RTQ

Human  97-VQPYLDDFQKKWQEEMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHV-156
Baboon                                     H  T        H          V
Dog                        V      A  GS  R       Q                L     T
Pig               N          T    MA  G  FR      VQ          A    L   L
Rabbit          E          V R        G    R S     T          A    L   S  T
Cow             E     H    V I    A   GE FR      VQ    D     AQ L
Hedgehog        S    VE    L      A    S  WR Q    AQ    Q AGE  QQH     VRT
Mouse           E      K DV     K A   G      S     Q    GR     VA    F     M T
Rat       M  H  E  E  N   V A     L   GT  HKN -----K M RH KVVA     F     M VNA
Chicken   IR F  Q SA   T   L Q   RLT VAQ  K LTK   VELM A   T VA     A     L G
Duck      IR F  Q SA   T   L Q   RLA VAE  K LTK   VELM Q   T VA     A     L G
Salmon    LE KRAELTEVLNKHIDE  K L   IKQHI LR TEMDAFRA ID VV     AKVAVN

Human157-DALRTHLAPYSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQ-216
Baboon                                              S
Dog           AQ      D  E      Q    G   S      R S Q  A G    R    Q
Pig       E   Q V     D    M    F    G  -S    Q   Q Q KA G
Rabbit     T   K     N  Q        SI  G   S    Q   R     V      R
Cow       ET  QQ       D       T     G  -S        S Q KA G       V
Hedgehog       D     GE A KL LQ  QDI AKS -D   QT LS    KSFG    Q T Q    H
Mouse      S   Q   H EQM ES   QR AE  S P--T N  TR KT   K  G    R        H
Rat           AKFGL   QM EN   Q  TEIRNHP--T I  T  GD   R  G         D  G
Chicken   EE  KN         K SQK  EIR K IPQAS   Q  VM Q   N R   MT LVQEF E
Duck      EE  KN         K SQK  EIR K IPQA    Q  VV Q   N R   MT LVQ FKE
Salmon    EETK K M IVEIV AK TE   E RTLAAPYAE   KEQMFKAVGEVR   VA LS   FKA
```

Figure I-7: Part 2:

```
Human   217-GLLPVLESFKVSFLSALEEYTKKLNTQ-243
Baboon                                S
Dog                       L A  D A     A
Pig                  NL   I A ID AS    A
Rabbit                  A VQNV D A
Cow                  E L   I A ID AS    A
Hedgehog       E LW GI AGAM -M  LG    S
Mouse       S M M  TL TKAQ VIDKASET TA
Rat           M    AW AKIM MID AK    A-
Chicken     R T YA NL NRLI  F D LQ SVA—
Duck        R T YA NL TR I  L D LQ TVA—
Salmon      RWA PPRRPSK SWLSTRPSARP———
```

Figure I-7: Sequence Comparison Between ApoA-I from 11 Species.
Human (231), baboon (248), dog (249), pig (250), rabbit (251), cow (252), hedgehog (253), mouse (254), rat (255) chicken (256), duck (Swiss-Prot accession number, O42296), and salmon (257) apoA-I sequence are presented in decreasing order of their similarity to human apoA-I. Blanks indicate identity to human apoA-I; dashes (-) indicate deletions inserted to maximize homology between sequences. The prepro sequence of dog apoA-I has not been determined. Colors indicates proline (purple, P), aspartic acid or glutamic acid (red, D and E), arginine or lysine (blue, R or K), and phenylalanine, isoleucine, leucine, methionine, valine, tryptophane, or tyrosine (green, F, I, L, M, V, W, and Y). The remaining amino acids, alanine, cysteine, glycine, histidine, asparagine, glutamine, serine, and threonine (A, C, G, H, N, Q, S, and T) are uncolored and called indifferent (237). The numbering of human apoA-I starts at –24 (signal peptide of apoA-I), residue # 1 being the first one of mature apoA-I.

2. Expression and physico-chemical properties of apoA-I

Human apolipoprotein A-I is produced by the liver and the intestine, as a 267 amino acid-preproprotein (Fig. I-8) with a signal sequence (18 residues), which is post-translationaly cleaved and allows for its secretion in the circulation. The propeptide (6 residues) is removed by a plasma factor not yet identified (258; 259). The function of this propeptide remains unclear but may improve the exit of nascent apoA-I from the endoplasmic reticulum (260). Human apolipoprotein A-II is the only other apolipoprotein to possess a prosequence.

ApoA-I may be myristoylated but the importance of this post-translational event has not been evaluated (233; 234). After ultracentrifugation of HDL from human plasma, apoA-I can be purified by delipidation with organic solvents and gel-filtration chromatography to

remove other HDL apoproteins (261). A renaturation step is necessary to obtain the native form of the protein after purification. Isoforms of apoA-I have been identified based on their modified charge (normal pI of apoA-I is 5.6-5.7). They appear to be the result of deamidation of asparagine and glutamine residues (262). Oxidation of methionine residues of this protein, which may affect the conformation of the protein (263), has been reported (264).

ApoA-I is also known to self-associate, a process which may stabilize the lipid-free protein. However, at low concentrations (below 0.2 mg/ml) (265), or in the presence of guanidine hydrochloride (GdnHCl) (266), only the monomeric form is detected. Denaturation studies performed with the protein have indicated a midpoint of denaturation close to 1 M GdnHCl for the lipid-free form as assessed by the change in the ellipiticity determined at 222 nm (reflecting the α-helical content of a protein) (266; 267), but a decrease in the efficiency of the unfolding in the presence of PL (267; 268). In GdnHCl, urea, or calorimetric denaturation experiments, a low free energy of denaturation about 2-3 kcal/mol has been determined (268; 269). This value is much lower than the value determined for globular proteins (in the range of 5-10 kcal/mol) (270; 271). This observation is in agreement with a loosely folded and relatively flexible structure of the protein in the lipid-free form. This loosely folded conformation may allow rapid lipid-interaction of exposed hydrophobic portions of the protein. In support of this view, thermal denaturation experiments by Gursky & Atkinson have suggested a molten globular-like state for lipid-free apoA-I that may explain its lipid-binding properties *in vivo* (272). The N-terminal domain of apoA-I, apparently not required for lipid-binding, may be important in the stabilization of lipid-free apoA-I in solution (273; 274).

A.

MKAAVLTLAVLFLTGSQA *RHFWQQ*

DEPPQSPWDRVKDLATVYVDVLKDSGRDYVSQFEGSALGKQLNLKLLDNWDSVTS

TFSKLREQEGPVTQEFWDNLEKETEGLRQEMSKDLEEVKAKVQPYLDDFQKKWQE

EMELYRQKVEPLRAELQEGARQKLHELQEKLSPLGEEMRDRARAHVDALRTHLAP

YSDELRQRLAARLEALKENGGARLAEYHAKATEHLSTLSEKAKPALEDLRQGLLPV

LESFKVSFLSALEEYTKKLNTQ

B.

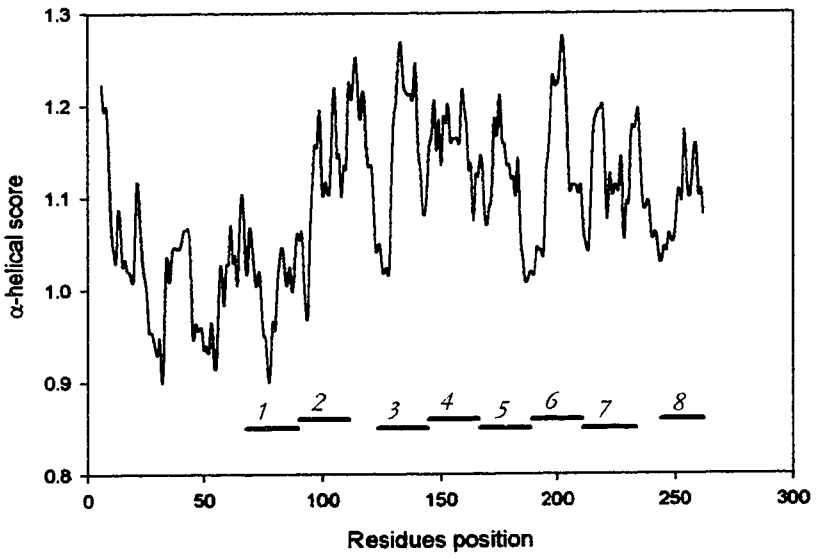

Figure I-8: Amino Acid Sequence of Human Apolipoprotein A-I and α-Helix Prediction According to the Algorithm Developed by Chou & Fasman.

A. Amino acid sequence of apoA-I. The presequence (signal peptide) is underlined and the pro segment is identified in italics. The 8 putative α-helices are numbered and colored.

B. Predicted α-helical content of apoA-I. Helices identified in the sequence (Fig. I-8 A) are identified with the corresponding colored bars (prediction with a window of 11 residues, according to (275)). Colored bars indicate helices identified in A.

B. Role of apoA-I amphipathic α-helices in the interaction with lipids

1. Secondary structure of apoA-I

Several methods are available to correlate the secondary structure of a protein to its primary sequence. Methods based on the known secondary structure of other proteins have been initially used but experimental data have also been obtained through circular dichroic (CD) and infrared (IR) spectroscopy. Using algorithms based on the known structure of proteins, such as those defined by Chou and Fassman (275) or Garnier *et al.* (276), predictions have proposed that apoA-I may be composed of 8-9 α-helices formed essentially by the 22-mer repeats, with a less well-defined N-terminal secondary structure (Fig. I-9). These predictions were partially confirmed by experimental data using CD and IR spectroscopy to determine the total α-helicity of apoA-I (277-279). Similar conclusions were drawn by investigators using Trp fluorescence to examine the change in apoA-I conformation observed upon association with lipids (277; 280).

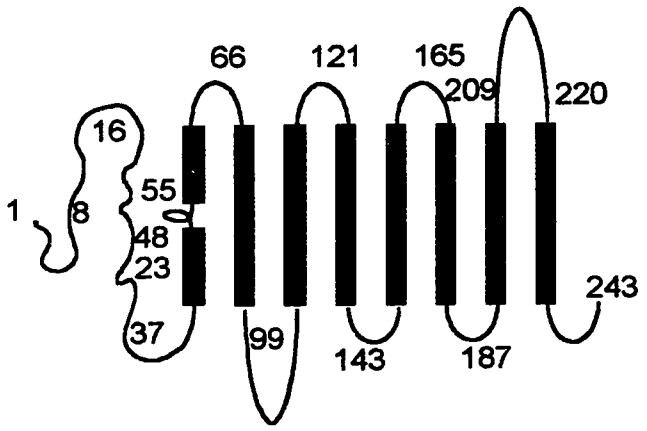

Figure I-9: Secondary Structure Model of Apolipoprotein A-I.
Amphipathic α-helices, β-turns and random coils are respectively represented by boxes, curvilinear sections and curves. Colors correspond to the helices identified in Fig. I-8.

2. Mode of interaction with lipids

Early studies have shown that isolated apoA-I can interact spontaneously with dimyristoyl phosphatidylcholine (DMPC) at its transition temperature (281-283) and form discoidal complexes (284). Several groups have also demonstrated that the binding of apoA-I to egg phosphatidylcholine [mostly palmitoyloleyl phosphatidylcholine (POPC), one the major glycerophospholipids found *in vivo*], in the presence of the detergent sodium cholate, resulted in the formation of discoidal lipoproteins (285; 286). The formation of spherical complexes could be obtained by incubation of LCAT and LDL, as a FC donor, with discoidal complexes (150; 150; 280; 287) or by co-sonication of a lipid-apoA-I mixture (288; 289).

Investigations have been conducted to determine the affinity of apoA-I and other apoproteins for lipid interfaces (290). These interactions can be monitored by employing a lipid monolayer surface balance. Apoproteins have been demonstrated to be highly surface active and to bind readily to the lipid-water interface: they can penetrate a PL monolayer, thereby increasing the surface pressure in the interface. Affinity of apoproteins for hydrophobic surfaces are in the range of 10^{-6} to 10^{-7} M depending on the lipid used (290-292). The surface affinity of apoA-II has also been demonstrated to be higher than that of apoA-I, which explains the displacement of apoA-I by apoA-II in HDL (293; 294).

A mechanism to explain the mode of apoprotein association with the lipid surface was first proposed by Segrest *et al.* (295), who described the existence of amphipathic α-helices in apolipoproteins. An amphipathic α-helix has been defined by an amino acid sequence, which contains regularly distributed hydrophobic residues every three or four positions. Such helices have a hydrophobic face that interacts with the PL acyl chains while the other, hydrophilic, face is in close contact with the aqueous phase (295). The involvement of these α-helices in

the binding of PL was demonstrated by measuring the change in the ellipiticity at 222 nm after binding of the apoprotein to PL. Lipid-free apoA-I typically exhibits an α-helix content of 40-50 % while in association with PL the α-helicity increases to ≈ 75 % depending on the type of lipids and complexes formed (268; 277). This property was also demonstrated for other apolipoproteins and suggested a very similar mechanism of association with lipids for all exchangeable apoproteins. ApoA-I immunoreactivity toward mAbs has also been shown to be altered by the presence of lipids (296; 297).

The amphipathic character of an α-helix is defined by two major parameters. One measure of the affinity of an α-helix for lipids can be obtained by determination of its hydrophobic moment (298). This parameter is represented by the vector sum of the hydrophobicity values associated with each residue, taking into account its orientation in the helix (one residue every 100° or 3.6 residues per helical turn). This value has been well correlated with the surface activity of a protein. In that context, helices of apoproteins have a high hydrophobic moment but average hydrophobic index, while those of transmembrane proteins have a high hydrophobic index and low hydrophobic moment, and most helices of globular proteins have both an average hydrophobic moment and hydrophobic index (298). The graphical representation of a helix proposed by Shiffer and Edmundson (299) in the form of a wheel projection illustrates this property (Fig. I-10). In this representation, two domains in the helix can be identified, one hydrophobic that forms the hydrophobic angle, while the other is mostly composed of hydrophilic residues that form the hydrophilic angle. In a more precise manner, Brasseur (300) has used a three-dimensional representation to define the hydrophobic contour around each helix. The relative proportion of the two angles is usually in favor of the hydrophilic angle in the case of apoA-I and other exchangeable apoproteins. These

observations suggest that the contact area with the aqueous phase is larger than the area in contact with lipids, therefore favoring the formation of a lipidic structure surrounded by apoA-I such as discoidal complexes. These properties are in contrast with those of transmembrane proteins such as pore-forming or ion channel proteins, that have a larger hydrophobic angle and allow a small domain within the plasma membrane to be hydrophilic (300).

The Edmundson wheel representation also gives other hints as to how charged residues affect the affinity of an α-helix. Segrest *et al.* (242) proposed that α-helices of apoproteins are not all equivalent for their affinity towards lipids. The differences in affinity do not appear to be simply related to their hydrophobic moment but also to the distribution of charge residues along the axis of the helix. Segrest *et al.* (242) have used computer analysis to identify different classes of amphipathic helices, which led them to characterize the physico-chemical properties associated with each class. Amphipathic α-helices of apolipoproteins form the class A. Class L, H, M, and G have been used to identify lytic, hormone, transmembrane, and globular amphipathic α-helices, respectively. Class A is characterized by a high mean hydrophobic moment and by its unique charge distribution: positively charged residues are clustered at the polar-nonpolar interface, whereas negatively charged residues are found at the center of the polar face (Fig. I-10). For apoA-I, 6 helices have been identified with this specific amino acid distribution (helices 44-65, 66-87, 121-142, 143-164, 166-186, 187-208). Two other types of helices have been identified: class G^* (helix 8-33) and class Y (helices 88-98, 99-120, 209-219, 220-241) (Fig. I-10). They are all characterized by a specific charge distribution (Fig. I-11). These different helices may have different structural functions and lipid-binding properties but we still have a poor understanding of these relationships in apoA-I. Segrest *et al.*

(242) had proposed that class Y had a reduced lipid affinity but, as it will be discussed below, helix 220-241 has the highest affinity for lipids amongst apoA-I α-helices. In class A amphipathic α-helices, the importance of the charge distribution has been examined with synthetic peptides with positively charged residues clustered at the polar-nonpolar interface and negatively charged residues at the center of the polar face. A decreased affinity for lipids was observed for peptides with a reverse charge distribution (301-303). The snorkel model of Segrest *et al.* (242) proposes that the positively charged residues, which are also amphipathic and have their long alkyl chain buried in the hydrophobic interior, extend ("snorkel") toward the polar face of the helix to insert their positive moieties into the aqueous phase.

The properties of synthetic peptides that correspond to each of the predicted apoA-I helical segments (helices 44-65, 66-87, 99-120, 121-142, 143-164, 166-186, 187-208, 220-241) have been recently examined (244). Only peptides corresponding to helices 44-65 and 220-241 have been found to associate with lipids with a significant affinity. This result has been attributed to a deeper penetration of these two helices into the lipid interface, as compared to other helices. Helices 44-65 and 210-241 also present a higher total hydrophobicity on the nonpolar face. Although these isolated helices had faster kinetics of association with DMPC than apoA-I as a whole molecule, apoA-I could however reduce the enthalpy of the gel-to-liquid crystalline phase transition much more effectively, which suggests a cooperativity between central α-helices (244). It has therefore been suggested that the two end helices of apoA-I may initiate the binding to PL, followed by a cooperative binding of the other helices (244).

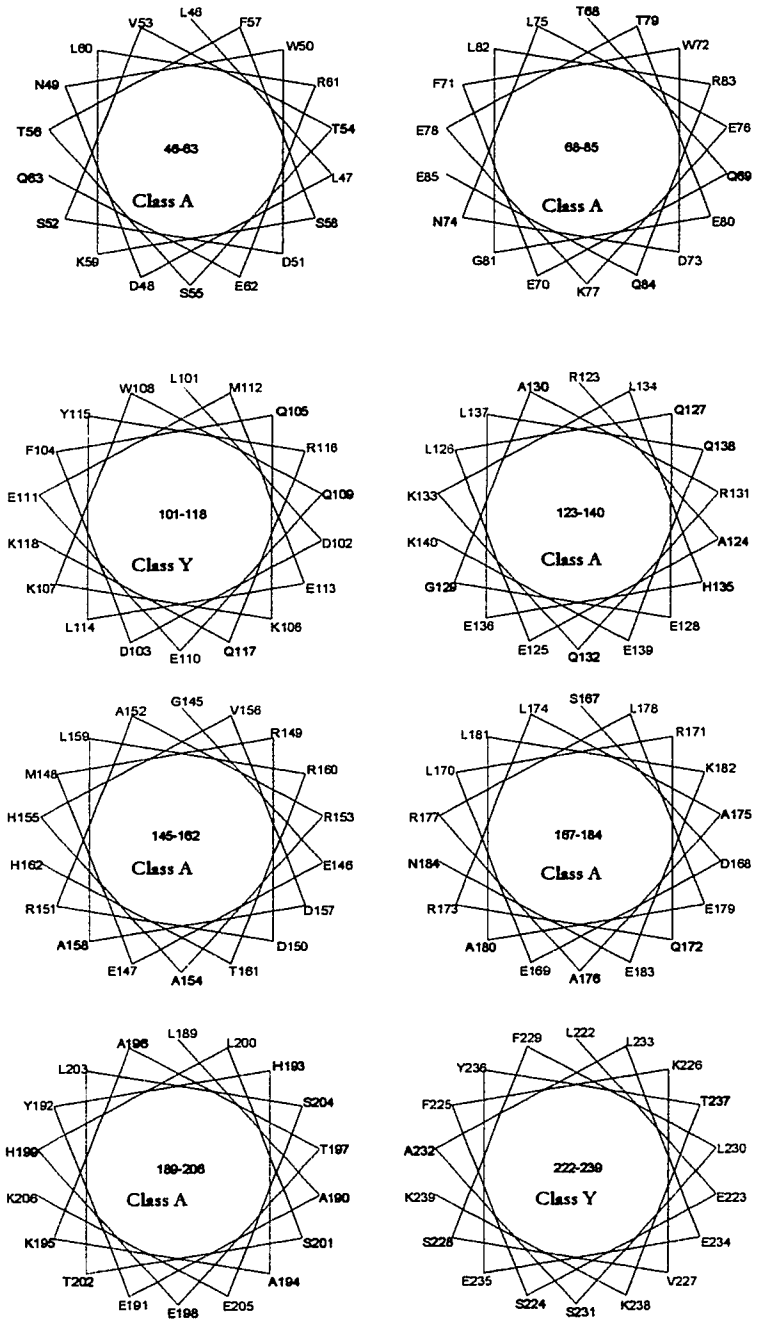

Figure I-10: Edmundson Wheel Representation of ApoA-I α-Helices.
Colors indicates proline (purple, P), aspartic acid or glutamic acid (red, D and E), arginine or lysine (blue, R or K), and phenylalanine, isoleucine, leucine, methionine, valine, tryptophan, or tyrosine (green, F, I, L, M, V, W, and Y). The remaining amino acids, alanine, cysteine, glycine, histidine, asparagine, glutamine, serine, and threonine (A, C, G, H, N, Q, S, and T) are uncolored and called indifferent (237). The class of amphipathic helices (A, Y, see Fig. I-11) to which each helix belongs to is also indicated.

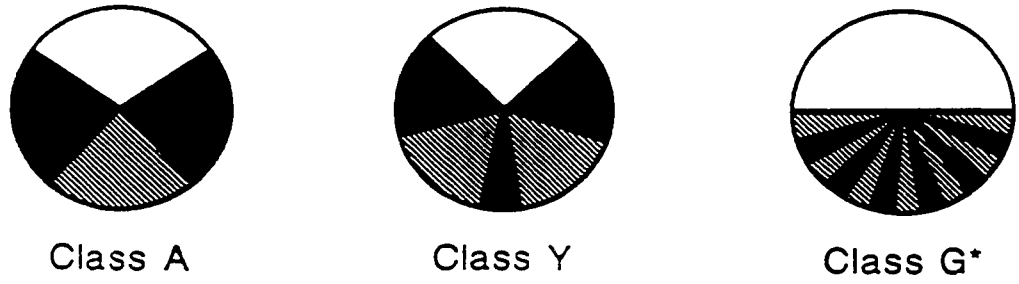

Figure I-11: Amphipathic α-Helix Classes Identified in Human Apolipoprotein A-I.
The classification presented in this figure, is based on the distribution of charged residues (304). The charge distribution of each class of helices is represented in an Edmunson wheel representation of an helix. Class A helices are characterized by positively charged residues (represented by dark shading) cluster at the polar –nonpolar interface and negatively charged residues (represented by light shading) cluster at the center of the polar face. Class Y helices are characterized by the presence of positively charged residues at the polar nonpolar interface and at the center of the polar face, so as to form the shape of the letter Y. Class G* helices are characterized by a random distribution of positively charged residues on the polar face. In all helices, the nonpolar face is represented with no shading (from (304)).

The observation on cooperativity has confirmed other studies that have demonstrated that two monomers of a putative amphipathic α-helices linked by a Pro (to introduce a β-turn and form antiparallel α-helices) bind better to PL than the corresponding monomer or two monomers linked by Ala (290; 301; 305). This process may be facilitated by antiparallel helix-helix salt-bridges and hydrogen bond interactions (306; 307). Pro, present at the first position of almost every 22-mer (308)(11 residues form 3 complete turns of an α-helix) often form β-turns between helices and are therefore essential for the secondary structure of apoproteins. The presence of a Pro residues at almost every 22 amino acids (size of a lipid bilayer) appears necessary in defining the structural and functional properties of apoA-I, which associates

mostly with small lipoproteins (HDL). In contrast, the apoE lipid-binding domain contains a 65-mer helix and, consequently, associates with larger lipoproteins (242).

3. Factors affecting the binding of apoA-I to lipids

Several groups have focussed their research on the effect of different lipids on apoA-I structural and functional properties. The first lipids studied were phosphatidylcholine (usually egg phosphatidylcholine which contains mostly POPC). As already indicated, increasing the POPC content of LpA-I leads to an increase in the α-helical content of apoA-I (277; 279). It also leads to reduced protease accessibility suggesting a major conformational change upon association with lipids. ApoA-I may form complexes with one, two, three or more molecules per lipoprotein particle (277). *In vitro* reconstituted discoidal LpA-I complexes usually have a preβ-HDL migration on agarose gel electrophoresis. They change to an α-mobility following incubation with plasma factors (d>1.21 g/ml) or LCAT alone (150). Several studies have shown a direct effect of the surface charge of LpA-I on the structure and function of lipoproteins (268; 289; 309-312). From these studies, it appears that apoA-I conformation is affected by HDL lipid composition and is the major determinant of HDL charge, even if charged lipids (phosphatidylinositol, phosphatidylserine) can directly contribute to the negative charge of HDL. Different lipids can also have different effects on the stability of apoA-I in HDL (268; 289; 309; 312). NMR and mAb immunoreactivity studies have also implicated a specific domain of apoA-I in the binding of FC in HDL (279; 313). This binding, which occurs around residue 144 of apoA-I, may also be responsible for the specific changes in the charge of HDL containing FC (309).

ApoA-II, the second major protein of HDL, has also been shown to affect apoA-I conformation and function (314). Combining fluorescence and protease sensitivity studies, Durbin & Jonas have shown that apoA-II appears to replace 4 central α-helices (possibly residues 99-187) upon binding to HDL. Binding of apoA-II to these lipoproteins may lead to the displacement of apoA-I from the HDL surface and clearance from the plasma. Indeed, previous work has suggested that this dissociation of apoA-I may promote HDL clearance by enhancing the renal filtration of the apoprotein (125). It may therefore explain the proatherogenic role of human apoA-II (201).

4. Structure of HDL

ApoA-I has been shown to form different types of lipoprotein complexes, depending on the conditions of association. It can form discoidal as well as spherical complexes. The best-characterized model of apoA-I interaction with lipids remains the discoidal complex that will be discussed here. A secondary structure model of this interaction has been suggested by several groups (279; 281; 297; 304; 315-317). In almost all these models, eight antiparallel amphipathic α-helices connected by β-turns have been proposed to interact in parallel to the PL acyl chains (Fig. I-12). These models were derived from investigations that combined secondary structure prediction methods and biophysical methods (279; 316; 317) or mAb immunoreactivity studies (297). Several of these models suggest the existence of a hinge domain that may be responsible for the ability of apoA-I to associate with lipoprotein complexes of different size (277). This domain (aa 100-143), which may have a lower affinity for PL, may be excluded from the interaction with lipid in small discoidal LpA-I whereas it may participate at the interaction with PL in large discoidal LpA-I (Fig. I-12).

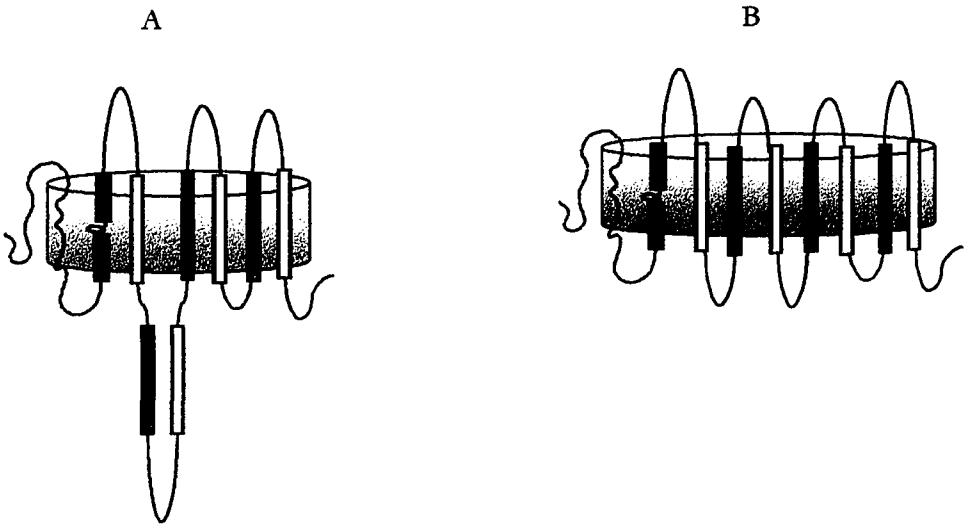

Figure I-12: Mode of Interaction of ApoA-I with Phospholipids: Discoidal Model.
The disc formed by a phospholipid bilayer can accommodate apoA-I amphipathic α-helices, parallel to the phospholipid acyl chains. In small discoidal LpA-I (apoA-I containing lipoproteins), two helices do not interact with the lipid interface, possibly because of a reduced affinity for lipids (A). However, in large discoidal LpA-I (B), these helices, which have been termed a hinge domain, interact with lipids and can therefore allow one molecule of apoA-I to associate with varying amounts of lipid.

Protease accessibility studies performed with lipidated apoA-I have provided data that are consistent with this view and have identified a domain that is more sensitive to protease digestion in the central region of apoA-I in small LpA-I complexes (318). Another interesting feature of some of these models is that the N-terminal domain of apoA-I may interact with the central domain of the protein, especially in small LpA-I (279; 297; 313).

Bergeron *et al.* (313) suggested that the two apoA-I molecules are organized in a head-to-tail configuration, based on mAb competition for binding to a discoidal Lp2A-I. In contrast, a computer generated model from Phillips *et al.* (319) suggested that both conformations may coexist, since no particular configuration is favoured. However, Phillips *et al.* (319) could not integrate residues 1-47 in their model, and this region has been shown to

contribute significantly to the overall structure of apoA-I (279; 297; 313). Borhani *et al.* (320) have recently determined the crystal structure of a truncated lipid-free form of apoA-I (lacking residues 1-43) at 4-Å resolution. The mutant protein exhibits a conformation similar in its lipid-free form to that of apoA-I in its lipid-bound form (274). This protein appears as an elliptical ring-like structure consisting of four molecules of the apoprotein. These four molecules associate via their hydrophobic faces to form a four-helix bundle (Fig. I-13). In this model, the monomeric mutant apoA-I is formed by 10 helices, interrupted as expected by Pro and Gly residues. However, the predicted punctuations are not β-turns but rather kinks occurring at these residues. In this paper, the authors also proposed a model of a spherical HDL containing four apoA-I molecules (Fig. I-14). The HDL has an ellipsoidal shape and apoA-I binds lipid in a belt mode. Crystallization of the 22 kDa N-terminal fragment of apoE also showed that this fragment adopts a helical bundle conformation that comprises 4 α-helices arranged with their hydrophobic faces brought together towards the center of the molecule (321) similar to the structure observed for apolipophorin III, an insect apolipoprotein (322). According to this model, apoA-I on discoidal HDL may wrap around the edge of the disc with its α-helices oriented perpendicular to the PL acyl chains. This model is not, however, consistent with the results obtained by attenuated total reflection infra red spectroscopy that have shown that, in a disc, apoA-I α-helices are, rather, oriented parallel to the PL acyl chain (278; 323). This structure may, however, be similar to that observed in spherical HDL (320), the N-terminal may in that case modulate apoA-I conformation by directly interacting with the central domain of the protein (268; 297).

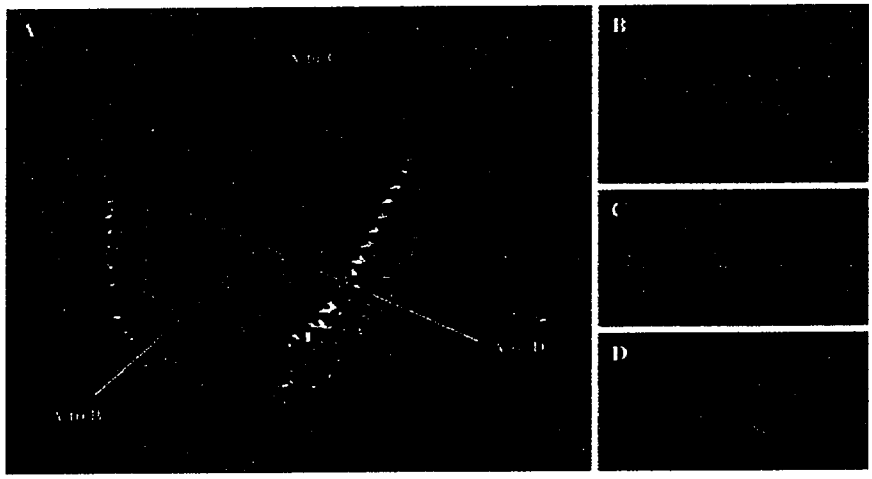

Figure I-13: Ribbons Representation of the Crystallized Tetramer of ApoA-I Δ(1-43).
The elliptical and curved shape of the protein is evident from this structure. a, b, c, and d represent three different views of the same structure along different axis. Molecule A is gold, B is purple, C is pink, and D is green. Note the antiparallel orientation of A and B, and C and D. The A/B dimer and the C/D dimer are also oriented in an antiparallel manner (320).

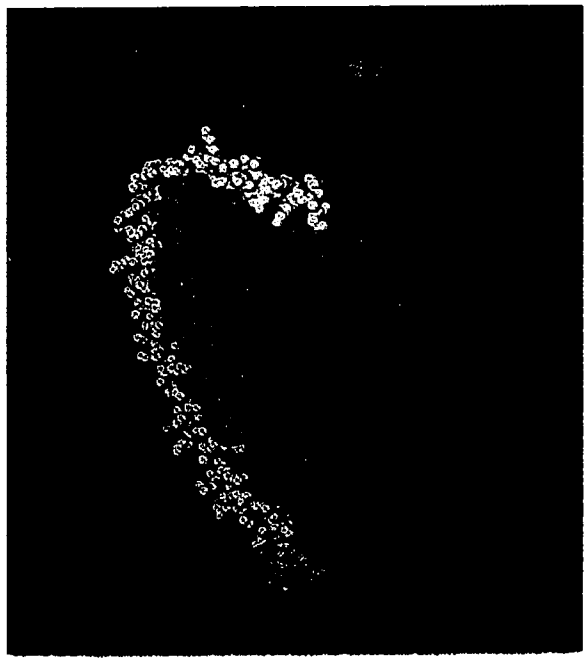

Figure I-14: Hypothetical Model of ApoA-I Bound to a Spherical HDL.
Two dimers of apoA-I are represented in this figure and are colored as indicated in Fig. I-13. The lipid head groups are represented by blue spheres (320).

5. Mutations affecting the phospholipid binding association

As described in the previous section, the C-terminal domain of apoA-I has been shown to be essential for efficient lipid binding. The first study to illustrate this point was performed by Holvoet *et al.* (324) who presented evidence that deletion of residues 190-243 reduced the initial binding of apoA-I for PL. *In vivo* studies later confirmed this view and showed that the carboxyl-terminal domain (residues 190-243) of apoA-I is essential for apoA-I association with HDL (325; 326). In rabbits, injection of apoA-I mutants with deletion of residues 201-243, 217-243, or 226-243 (325) and 190-243 (326) revealed markedly increased rates of catabolism as compared to the wild-type protein. Furthermore, the C-terminal truncated apoA-I proteins were mostly associated with very high density lipoprotein (VHDL, poorly lipidated apoprotein), which suggests a poor ability to bind lipid and form HDL. Similar results have been obtained by Holvoet *et al.* (326) with transgenic mice overexpressing an apoA-I mutant with deletion of residues 190-243. A more detailed study has been performed by Laccotripe *et al.* (327) who demonstrated that Phe and Leu (Leu 211, 214, 218, 219, 222, Phe 225 and 229) residues located in the C-terminal domain, but not other residues in this domain or Leu_{159}, were essential for efficient *in vitro* binding to DMPC. This confirmed the crucial role of this domain, at least in the initial binding of PL. Increases in α-helical content of apoA-I upon binding to lipid therefore appear to be associated with an increase of the helicity in the C-terminal domain (324; 328; 329). As previously suggested, other domains may contribute to the association of apoA-I with PL in a cooperative manner. Deletions of helices that have been involved in lipid binding apparently result in a reduced PL binding capacity, at least *in vitro* (324; 328; 329).

ApoA-I appears to contain three major domains. However, the role of central α-helices in the formation of a hinge domain and their ability to modulate the stability and structure of apoA-I have not yet been studied. It may be the ability of this domain to interact with lipids and other proteins that constitutes an essential function of apoA-I. This function may be crucial for the ability of apoA-I to activate LCAT and promote cellular cholesterol efflux.

C. Interaction of apoA-I with cellular cholesterol

1. Regulation of cholesterol homeostasis

Cholesterol is required by all mammalians cells to maintain the integrity of their membranes. Cells can acquire exogenous cholesterol from several different sources. It can be obtained through *de novo* synthesis but cholesterol may also be derived from LDL through the LDL-receptor pathway, which has been extensively described by Brown & Goldstein (31). FC can also be selectively taken up from plasma LDL (330). Finally, selective uptake of CE from LDL (331) and HDL (89) can also occur, a process possibly mediated by SR-BI (or CLA-I). CE acquired by these mechanisms is always hydrolyzed in the lysosomal compartment. If the supply exceeds the need, FC downregulates its own entry into the system by inhibiting enzymes required for its own synthesis and also by inhibiting the production of proteins involved in its entry into the cell, such as the LDL-receptor. On the other hand, FC activates the enzyme acyl-coenzyme A:cholesterol acyltransferase (ACAT), responsible for the conversion of FC into CE and which is located in the rough endoplasmic reticulum (332). This mechanism of storage is very important since excess FC appears to be toxic to the cell (333). CE can accumulate in several cell types such as macrophages, hepatocytes, intestine cells, or

steroidogenic cells. In others cells, very little CE is detectable. CE also accumulates in certain disease states, such as atherosclerosis, because of a lipid metabolism disorder. To prevent any excessive accumulation, cholesterol from peripheral tissues could be removed from the cells and transferred to the liver for degradation. This elimination is mostly performed by HDL and apoA-I.

2. Mechanisms of cellular cholesterol efflux

ApoA-I is the major protein involved in the process of cellular cholesterol efflux. Three possible mechanisms can be distinguished. Free cholesterol can dissociate from the plasma membrane and be acquired by an acceptor (i.e. HDL). ApoA-I may interacts directly with specific sites at the cell surface and promotes the transfer of intracellular cholesterol to the plasma membrane, which becomes available for transfer by aqueous diffusion to HDL. There is also evidence (78; 93) for a retroendocytotic pathway but this mechanism has still not been well documented.

In the first process (termed diffusional efflux, since it is mediated by aqueous diffusion), the rate-limiting step is the desorption of FC from the plasma membrane. Desorption may depend on the properties of the cell membrane as noted by several investigators, who showed that under similar experimental conditions, the rate of cholesterol efflux varies widely and depends on the cell type examined (334-336). In addition, the presence of different pools of cholesterol and their inter-exchange within the plasma membrane may affect this process (68; 335; 337). As discussed above, the protein composition of HDL (apoA-I/apoA-II ratio) has also been shown to affect this transfer. Lipid composition of acceptors may be a key factor in this process since several studies have shown that the PL

(133; 194), and FC (193; 338) content of HDL could affect cholesterol flux between cells and lipoproteins. This process appears to depend mostly on the presence of lipid in HDL since their modification by tetranitromethane or limited proteolysis do not affect this diffusional transfer (339; 340). Large PL vesicles are also less efficient than HDL when compared on a PL basis (341).

Overall, these observations suggest a major role for apoA-I in cholesterol efflux mediated by passive aqueous phase diffusion. The role of different apoA-I domains in this process has been examined using mAbs to apoA-I or by site-directed mutagenesis. The ability of apoA-I to interact with lipid surfaces suggests a possible role for its amphipathic α-helices to promote cellular cholesterol efflux from the plasma membrane. The first studies performed with mAbs to apoA-I have indicated the involvement of a central domain in this process (342-345). Banka *et al.* (342) found that antibodies binding to residues 74-110 could inhibit cholesterol efflux to HDL. Similarly, Luchoomun *et al.* and Sviridov *et al.* (344; 345) demonstrated the importance of domains around residues 165 and 140-150, respectively. In a different study, Fielding *et al.* (343) showed that an epitope of apoA-I (region 137-144) was more exposed in preβ1-HDL than other subspecies of HDL. The central domain was suggested to be much more labile than other domains and, therefore, could interact more easily with the plasma membrane to promote cellular cholesterol efflux (337). However, the effects of mAbs on apoA-I are complex and not only interfere sterically with other domains (313) but can also modify the secondary structure of the protein (148).

Mutagenesis studies performed by Gillotte *et al.* and Sviridov *et al.* (329; 346) have, however, shown that mutations of apoA-I corresponding to deletions of residues 44-126, 139-

170, and 190-243 (329) or residues 222-243, 210-243, 150-243, and 135-243 (346) had no effect its ability to promote cellular cholesterol efflux.

The second mechanism involves the direct binding of apoA-I to the cell membrane, which causes the transfer of intracellular cholesterol to the plasma membrane (also called binding- and translocation-dependent cholesterol efflux). Whether this transfer is specifically due to apoA-I-cell interaction *per se* or to a transfer of cholesterol between intracellular pools and the plasma membrane as a response to the removal of FC from the plasma membrane, remains to be established. Nevertheless, cell binding of HDL and intracellular cholesterol transfer are abolished upon tetranitromethane modification or limited proteolysis of HDL (339; 340), which demonstrates the importance of intact apoA-I. Mendez recently demonstrated that cholesterol efflux to lipid-free apoA-I occurs only with quiescent (growth-arrested) or cholesterol enriched cells (347). Several groups have reported the existence of a specific receptor for apoA-I (see section on HDL cholesterol efflux), one recent candidate being SR-BI (85). The interesting feature of this receptor is that efflux to acceptors appears to be dependent on their PL content (92). This suggests that the ability of apoA-I (C-terminal domain) to bind PL is required for efficient cholesterol efflux and was suggested by several earlier studies (348). Recently, Sviridov *et al* (346), using C-terminal deletion mutants, have shown that the C-terminal domain (deletion of residues 222-243, 210-243, 150-243, and 135-243) may be important in intracellular cholesterol efflux mediated by apoA-I. However, they did not observe any effect of these deletions on the binding to HepG2 cells. These surprising findings can not be simply explained by the presence of a receptor that mediates cholesterol efflux. Alternatively, the presence of other non-specific cell surface binding sites for apoA-I on HepG2 cells may mask the specific binding to the low affinity binding sites involved only in the transfer of intracellular cholesterol to the plasma membrane. Other cell lines, such as

macrophages, which appear more relevant to the development of atherosclerosis, should be examined as well.

ApoA-I interaction with cells may also be mediated through cholesterol-enriched micro-domains of the plasma membrane since it has been demonstrated that apoA-I associates better with lipid emulsions containing cholesterol (84). This possibility is confirmatory of the work of Fielding & Fielding (349) who showed that caveolae represent a major site for cellular cholesterol efflux to HDL. Caveolae are clathrin-free, flask shaped, cell surface organelles, characterized by a high FC and sphingolipid content (350). Expression of caveolin, the major structural protein of caveolae, is in fact under the positive control of cellular cholesterol levels (351) and transfers to the plasma membrane of the cell only in the presence of cholesterol (352). Therefore, one of the functions of this organelle may be to eliminate excess cellular cholesterol and distribute it to preβ-HDL (109).

From this overview, it appears that the mechanisms by which apoA-I can bind to cells and promote cellular cholesterol efflux are multiple and that the contribution of specific apoA-I domains to the pathway remain to be elucidated. Are binding and cholesterol efflux related? Is it necessary to have a high affinity cell surface binding site to promote cholesterol efflux or would it, on the contrary, prevent an efficient efflux?

D. Activation of Lecithin:cholesterol acyl transferase

Initial work from Glomset (152) suggested that LCAT might play an important role in reverse cholesterol transport. Cholesterol esterification appears to promote the flux of cholesterol through the HDL pool by stimulating the formation of cholesterol esters that are

transferred to apoB-containing lipoproteins, by CETP, and then cleared via the LDL-receptor pathway in the liver (6).

1. Mechanism of reaction

The LCAT glycoprotein (Enzyme Classification 2.3.1.43), consisting of 416 amino acids, has a molecular mass of ≈62 kDa. This enzyme displays two activities: a phospholipase A_2 activity, since it can hydrolyze the *sn*-2 fatty acid from phosphatidylcholine, and an acyltransferase activity, since it can transfer the fatty acid to FC and form CE.

The α-helical content of LCAT has been estimated to be 25 % (353). The N-terminal domain may be glycosylated and does not appear to share any homology with other reported protein sequences. A domain corresponding to residues 150-225 has been identified as a potential lipid-binding domain. This sequence, Pro_{150}-Pro_{195}, contains potential amphipathic α-helices with some homology to the apoE C-terminal domain, known to be responsible for the binding of apoE to lipid. Interestingly, this domain is also homologous to an amino acid sequence found in LPL and HL, other interfacial enzymes. Studies of a synthetic peptide corresponding to residues 154-171 have shown that it could, by itself, associate with lipids (354). These results are consistent with a role for this domain in the interfacial binding of LCAT. The existence of a catalytic triad (or proton relay) in LCAT was first proposed by Jauhiainen and Dolphin (355). Based on homologies with other lipases, the catalytic site appears to be centered around a Ser [Ser_{181} (356)] residue which participates with a His [His_{377} (357)] and most likely an Asp residue [probably Asp_{345}, based on the LCAT model proposed by Peelman et al. (358)]. Cys_{31} or Cys_{184} in LCAT may be associated with a transesterification role (21) although mutagenesis studies appear to have disproved this hypothesis (359).

Numerous mutations of this enzyme have been described and they have been shown to segregate into two distinct phenotypes, termed familial LCAT deficiency and Fish Eye Disease (360). The former phenotype is caused by a complete absence of LCAT activity and is associated with a marked corneal opacity, anemia, proteinuria, and renal failure. Fish eye disease patients are characterized by corneal opacities (151). Patients carrying any of these defects present markedly reduced HDL levels. HDL appear in both cases as discoidal and small spherical particles (151). However, plasma from patients with Fish Eye Disease still contains some residual LCAT activity but it is associated with LDL- and VLDL-cholesterol (β-LCAT activity, as opposed to α-LCAT associated with HDL).

2. LCAT activation

Although LCAT may be able to bind lipids directly, optimum reaction requires activation by one of the exchangeable apoproteins. ApoA-I was the first described activator of LCAT (361) and most potent *in vivo* activator (362). This reaction requires at least three steps. First, LCAT binds to the substrate (HDL) and/or the activator, and in a second step, LCAT hydrolyzes the fatty acid at the *Sn-2* position of a PL. The third and final step is the transesterification of cholesterol and concomitant release CE.

Study of the mechanism of LCAT activation by apoproteins requires the utilization of specific substrates. Different substrate have been characterized, including reconstituted spherical or discoidal lipoprotein, native lipoprotein, or synthetic vesicles. The problem with synthetic vesicles is that the activator requires initial binding to the lipid interface. In that case, if the protein tested does not possess a significant high affinity for lipids, its ability to activate LCAT is reduced even compared with a protein that has a high affinity for lipid but a low

ability to activate LCAT. Therefore, the use of a reconstituted HDL, with which the potential activator is already associated, provides a better way to identify the activation potential of a protein or peptide (363).

Another important point is the species of PL used, which can affect the packing and fluidity of the lipid interface. Several studies have demonstrated the preference of LCAT towards specific acyl chains and PL head groups. The presence of oleic acid at the sn-2 position and PC or PE appears to give the best results (364; 365). Increased fluidity of a lipoprotein complex appears to increase the reactivity with LCAT (364). Some PL, such as DMPC, are very poor substrates and at the same time not physiologically relevant since, for example, DMPC is not present in human plasma (365).

The lipid composition of HDL can also affect the ability of apoA-I to activate cholesterol esterification by LCAT. In several studies, it has been suggested that this effect was directly related to a modification of apoA-I stability and/or conformation or to the ability of LCAT to bind substrates (277; 280; 366-369). In particular, Sparks *et al.* (289; 312) have suggested a role for the surface charge properties of apoA-I in its interaction and ability to activate LCAT.

The apoprotein composition of HDL can also affect its reactivity with LCAT. Apoproteins such as apoA-II can directly affect the reaction by interacting with apoA-I or by displacing the protein from lipid interfaces because of its higher affinity for lipids (314; 370). The use of reconstituted HDL complexes eliminates such complications. The morphology of the HDL substrate is also known to affect this reaction. Indeed, discoidal lipoprotein complexes are much better substrates than spherical complexes (280).

The mechanism of activation is not simple, as LCAT can hydrolyze small soluble substrates in the absence of apolipoproteins and can directly interact with lipid interfaces,

although with reduced affinity as compared to apoproteins (371). Numerous investigations have demonstrated the effect of varying the HDL apoprotein-lipid composition on the LCAT reaction (277; 289; 312; 314; 366; 367; 369). These changes have been in part attributed to modification of the charge and the conformation of apoA-I, which suggests that *in vivo*, the apoprotein and lipid composition of HDL can modulate the interaction between apoA-I and LCAT (277; 289; 312). In this context, several studies have shown that small complexes (low PL/A-I molar ratio) were more efficient in activating LCAT (312; 366). In that case, the increased reactivity was, in part, associated with a change in the accessibility of the N-terminal and central domains of apoA-I that may be in close contact in the tertiary structure (312; 313). Other lipids such as cholesterol (increases LCAT activity with increased cholesterol content) or sphingomyelin (reduces LCAT activation like phosphatidylcholine) may affect differently these domains of apoA-I (289; 312; 314; 369). The conformational changes of apoA-I that are associated with very specific lipid compositions appear to allow the interaction of LCAT, either directly with the lipid phase or through apoA-I.

ApoA-I is known to be the best activator of LCAT. Although apoA-IV can also significantly activate LCAT (372), it exhibits about 20 % of apoA-I's ability to activate the enzyme. Other apoproteins (E>C-I>C-III>C-II>A-II) can also activate the LCAT reaction but below 20 % of that observed for apoA-I (373; 374). Since the presence of amphipathic α-helices is required but not sufficient to activate LCAT efficiently, a specific domain of apoA-I may be responsible for this function. Studies designed to examine the sequence involved have been performed using synthetic peptides, CNBr fragments of apoA-I and, finally, mutagenesis. Studies with peptides suggested that despite a high affinity for lipids, the C-terminal domain could not activate LCAT whereas the region 148-185 could bind and activate the reaction

(375). Other synthetic peptides with a high amphipathic character, but no homology to the apoA-I sequence could also activate LCAT (376; 377). Anantharamaiah *et al.* (378) have constructed a peptide corresponding to a dimer of the consensus amino acid sequence obtained for the eight 22-mer predicted α-helices of apoA-I. This peptide gave maximal LCAT activation only when residue 13 of the helix was replaced with a glutamic acid. Since only helices within residues 66-121 contain a Glu at position 13 of each helix (residues 78 and 111 of apoA-I), it was concluded that this region should be the LCAT activating domain. Other studies have also been performed using mAbs to apoA-I. Inhibition of cholesterol esterification in the presence of these mAbs suggested that the epitopes with which they are interacting are responsible for this function. Overall, these results indicate a role for a rather large domain corresponding to residues 95-175. Moreover, Meng *et al.* (379) demonstrated that a mAb recognizing the N-terminal domain of apoA-I could enhance cholesterol esterification under certain conditions, suggesting that this domain may regulate the interaction with LCAT, similar to its role in the binding of PL. However, the effects of mAbs on apoA-I are complex as mAbs not only interfere sterically with other domains (313) but can also modify the structure of the protein (148).

Interestingly, the central region of apoA-I may also be involved in the specific binding of cholesterol in HDL. The domain could therefore allow the presentation of cholesterol to LCAT.

3. Mutations of apoA-I affecting LCAT activation

Mutagenesis studies have also been performed and these results will be discussed in more detail in Chapter 4. Several natural mutants have been described, some of which are

associated with a reduced LCAT activation. However, until recently, only two missense mutations have been associated with this defect. These, however, also correspond to major modifications of the structure of the mutant apoprotein ($Lys_{107}\rightarrow 0$, disruption of the helix, $Pro_{143}\rightarrow Arg$, disruption of the β-turn).

In summary, it appears that the central region of apoA-I is a good candidate for LCAT activation but this domain may require adjacent α-helices of apoA-I (C-terminal domain), still capable of binding lipids, in order to efficiently activate LCAT.

III. RATIONALE AND SPECIFIC AIMS

The involvement of apoA-I in reverse cholesterol transport and HDL metabolism is now clearly demonstrated. However, as discussed above, it is apparent that the role of apoA-I in reverse cholesterol transport and the metabolism of HDL is still not fully understood. A study of the structure-function relationships of apoA-I would therefore shed some light in understanding the mechanisms by which apoA-I exerts its functions in the different steps of the reverse cholesterol transport pathway.

The initial goal of the project was to develop an expression system, which would yield high levels of protein to study apoA-I mutants. This system was optimized as indicated by initial studies with apoA-I, which showed that, in *E. coli*, the expression was difficult (324; 325; 380-383), due to degradation of the recombinant apoprotein or, in certain cases, no expression.

The recombinant apoprotein was first compared to plasma apoA-I for its physico-chemical and functional properties (Chapter 2). In this work, the ability of recombinant apoA-I (Rec. apoA-I) to associate with PL and form lipoprotein complexes (LpA-I), which is essential for its function, was examined. The conformation of apoA-I in these LpA-I was studied by

circular dichroism and immunochemistry as previously described (309; 313). The major functional properties of apoA-I (activation of lecithin:cholesterol acyl transferase (LCAT) and ability to promote cellular cholesterol efflux) were also examined.

This expression system was then used to express three apoA-I mutants [Δ(100-143), Δ(122-165), Δ(144-186)]. The rationale for the selection of these mutants was that the central domain of apoA-I has been involved in apoA-I function, including lipid binding, cholesterol efflux, and LCAT activation. The sequential deletion of a pair of (putative) α-helices at the site of the Pro punctuation was selected as the least disruptive for the global secondary and tertiary structure. We hypothesized that the deletion would moderately affect the inter-helix interactions if they exist at the deletion site. These proteins are the first apoA-I mutants that have been fully characterized for their PL binding properties (Chapter 3). They have been studied for their ability to form reconstituted apoA-I-containing lipoproteins (LpA-I) with PL, and for their structural and physical properties.

These mutant proteins were subsequently studied for their functional properties in cholesterol efflux and LCAT activation (Chapter 4 and 5). The first cholesterol efflux experiments were performed with well-characterized Lp2A-I (reconstituted HDL containing 2 apoA-I molecules per particle). As others suggested, the central domain may be involved in the removal of cellular cholesterol (342; 343; 345).

Another part of this study dealt with the ability of these mutants to activate LCAT. ApoA-I has been shown to be the best activator of LCAT. Some studies have suggested the importance of region 100-121 for this function (378). In contrast, preliminary studies with apoA-I mutants have shown different results (382; 383) with the implication of both the central and C-terminal domains in the activation of LCAT. However, these studies were done

with poorly characterized complexes which is not appropriate in light of previous works (289) that have demonstrated the importance of the type and the amount of lipid associated with apoA-I in this process. These studies therefore rely on the use of very well characterized reconstituted lipoproteins.

Experiments to measure the ability of lipid-free proteins to promote cholesterol efflux from cholesterol-loaded cells were also performed. Under these conditions, it is thought that apoA-I first associates with cellular PL before it promotes cholesterol efflux. Since the carboxyl-terminal domain has been involved in lipid binding, we decided to add a new mutant corresponding to the deletion of residues 187-243 [Δ(187-243)] to this study. This type of experiment was performed with the four lipid-free mutants (Chapter 5). This study was designed to clarify the role of apoA-I central and carboxyl-terminal domains in the binding of cellular PL and cholesterol.

Chapter 2: CHARACTERIZATION OF HUMAN APOLIPOPROTEIN A-I EXPRESSED IN ESCHERICHIA COLI

SUMMARY

Human apolipoprotein A-I (apoA-I), with an additional N-terminal extension (Met-Arg-Gly-Ser-$(His)_6$-Met) (Rec.-apoA-I), has been produced in *Escherichia coli* with a final yield after purification of 10 mg protein/l of culture medium. We have characterized the conformation and structural properties of Rec.-apoA-I in lipid-free form, and in reconstituted lipoproteins containing two apoA-I per particle (Lp2A-I) by both immunochemical and physicochemical techniques. The lipid-free forms of native and Rec.-apoA-I present very similar secondary structure and stability, and have very similar kinetics of association with dimyristoyl phosphatidylcholine. Rec.-apoA-I and native apoA-I can be complexed with 1-palmitoyl-2-oleyl phosphatidylcholine (POPC) to form similar, stable, discoidal or spherical (sonicated) Lp2A-I particles. Lipid-bound native and Rec.-apoA-I also showed very similar α-helical content (in discoidal Lp2A-I 69 and 66%, respectively, and in spherical Lp2A-I 54 and 51%, respectively). The conformation of Rec.-apoA-I in lipid-free form and in discoidal or spherical Lp2A-I also appears to be similar to native apoA-I by immunochemical measurements using 13 monoclonal antibodies recognizing distinct apoA-I epitopes. In the lipid-free protein and in reconstituted Lp2A-I, the N-terminal extension has no effect on the affinity of any of the monoclonal antibodies and minimal effect on immunoreactivity values. Small differences in the exposure of some apoA-I epitopes are evident on discoidal particles, while no difference is apparent in the expression of any epitope of apoA-I on spherical Lp2A-I. The presence of the N-terminal

extension also has no effect on the reaction of LCAT with the discoidal Lp2A-I or on the ability of complexes to promote cholesterol efflux from fibroblasts in culture. In conclusion, we show that Rec.-apoA-I expressed in *E. coli* exhibits similar physicochemical properties to native apoA-I and is also identical to the native apoprotein in its ability to interact with phospholipids and to promote cholesterol esterification and cellular cholesterol efflux. The expression system, described in this chapter, will be used to characterize apoA-I mutants and their structural and functional properties in the next chapters.

INTRODUCTION

Human apoA-I is a nonglycosylated polypeptide of 243 amino acid residues (230). Synthesized as a precursor protein of 267 a.a. (preproapoA-I), the prepeptide (18 a.a.) is cleaved during secretion and the propeptide (6 a.a.) is converted to the mature form by a plasma protease (258; 384). ApoA-I has been expressed in several eukaryotic cell lines (66; 67; 260; 381-383; 385-391). In these expression systems, under appropriate conditions, the propeptide is processed by an, as yet unidentified proteolytic mechanism and mature apoA-I is secreted, a portion of which is associated with lipid in the form of particles that resemble nascent HDL (66). Unfortunately, expression levels of apoA-I in eukaryotic cells are low and, consequently, it is difficult to obtain enough purified protein for the preparation and characterization of reconstituted HDL particles.

Initial attempts to express human apoA-I in *E. coli* have proven to be difficult due to the instability of the apoprotein, and, particularly, the susceptibility of the protein to intracellular degradation during bacterial growth (381; 392). Even when expressed as a fusion protein, the apoA-I moiety is still sensitive to degradation. Furthermore, the recombinant apoA-I must be

proteolytically cleaved to produce the mature protein (325; 381; 392; 393). To circumvent these problems, the efficiency of expression of the protein has been optimized by keeping the pro-segment or adding a bacterial signal peptide to apoA-I (324; 380; 394-397). Silent mutations in the first eight codons also result in a significantly increased expression without altering the amino acid sequence of the secreted protein (324; 380; 394; 397; 398). These results suggest a critical role for the human apoA-I mRNA secondary structures, which, presumably, greatly influence the efficiency of initiation of translation in this prokaryotic expression system. Nevertheless, since these reports, only one successful large-scale production of an apoA-I mutant in *E. coli* has been reported. Unfortunately, it was not applied to the production of native apoA-I but only to apoA-I_{Milano} (397). As a result of these difficulties, the information available from apoA-I mutants produced by site-directed mutagenesis using recombinant DNA techniques remains limited (324; 325; 382; 383; 398).

In this chapter, we show that large amounts of human apoA-I can be produced in *E. coli* as a stable and undegraded molecule by the inclusion of silent mutations in the N-terminal domain for efficient expression and an N-terminal extension of eleven residues (including six histidines) to facilitate purification. This recombinant apoA-I (Rec.-apoA-I) has been tested in the formation of reconstituted HDL (with 1-palmitoyl 2-oleoyl phosphatidylcholine and dimyristoyl phosphatidylcholine) *in vitro* and the structural properties of the reconstituted lipoproteins have been evaluated. Detailed characterization of the physicochemical properties and abilities of these proteins to stimulate cholesterol esterification by LCAT and to promote cellular cholesterol efflux shows that Rec.-apoA-I is both structurally and functionally similar to native apoA-I isolated from human plasma.

EXPERIMENTAL PROCEDURES

Materials

Cholesterol and sodium cholate were purchased from Sigma Chemical Co. (St.-Louis, MO). 1-palmitoyl 2-oleoyl phosphatidylcholine (POPC), dimyristoyl phosphatidylcholine (DMPC) and guanidine HCl (GdnHCl) were obtained from Avanti Polar Lipids (Birmingham, AL) and from Bethesda Research Laboratories (Bethesda, MD) respectively. [1α,2α-^{3}H]cholesterol was purchased from DuPont NEN (Boston, MA). All other reagents were analytical grade.

Methods

Construction of cDNA for expression of human apoA-I

The expression vector described in this section (pXL2116) was obtained through a collaboration with Dr. Patrice Denèfle (Rhône-Poulenc Rorer, Vitry sur Seine, France). A plasmid clone, pMDB1408, which contains a PstI-PstI fragment corresponding to human apoA-I sequence, from codons -10 to +243 (399), was used for the construction of plasmids directing the expression of this protein and its derivative with adjacent histidine residues. The PstI-PstI fragment was introduced in M13mp18 and a site-directed mutagenesis was performed using a synthetic oligonuclotide 5'-GCATTTCTGGCATATGGACGAGCCACCGCAGA-GTCCATTGG-GATCGAG-3' in order to create an NdeI site and a translation initiation codon immediately upstream of the first codon of mature apoA-I. Moreover, the same oligonucleotide was used to modify the first eight codons according to Isacchi et al. (380). The DNA sequence of the resulting vector, named pXL1773, was verified. An 853-bp fragment from the NdeI/BamHI-cut pXL1773 was then inserted into the NdeI/BamHI-cut vector pET-

3a (400). The resulting expression vector was named pXL1791. In order to fuse a polyhistidine tail to the N-terminal of recombinant apoA-I, an 853-bp fragment from the NdeI/BamHI-cut pXL1791 was inserted into the NdeI/BamHI-cut vector pXL2102 (vector containing the N-terminal coding sequence for Met-Arg-Gly-Ser-$(His)_6$, (401). The resulting expression vector was named pXL2116.

Expression and purification of Rec.-apoA-I

This expression system, under the control of T7 promoter, has already been described (401). The vector pXL2116 was introduced by a simple transformation procedure in *E. coli* (BL21(DE3) pLys S) and used for expression of Rec.-apoA-I under control of the *Lac* promoter. Overnight culture (10 ml at 37°C) in LB medium (bacto-tryptone, 10 g/l; bacto-yeast extract, 5 g/l; NaCl, 10 g/l) supplemented with ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml) was used to inoculate a 1 l flask (M9 supplemented with 0.4% glucose, 0.4% casaminoacids, 10 μg/ml of thiamin, 100 μg/ml ampicillin and 50 μg/ml chloramphenicol, (402)). The culture was performed at 37°C, up to an absorbance (610 nm) of about 0.5; isopropyl-β-D-thiogalactoside was then added at a final concentration of 1 mM to induce T7 RNA polymerase expression. After a 20 min. induction, rifampicin (100 μg/ml) was finally added and the culture was carried on for a further 60 min. The cells were then harvested by centrifugation and frozen at -20°C. The bacterial culture pellet was suspended in 10 ml buffer/g of bacteria (containing 100 mM phosphate, 2 mM EDTA, 1 mM PMSF, 6 M guanidine HCl pH 7.4). The cells were disrupted by sonication (5 min. cycles x 3, 250 watts mode pulsed, 50 % duty cycle, 60 output control) using a Branson sonicator. The bacterial debris were removed by centrifugation for 1 h at 11,000 g in a Beckman J2-21 MIE centrifuge. Nucleic acids in the

supernatant were removed by the addition of streptomycin sulfate 10% (w/v) (10 ml/g of protein), incubated 1h at 4°C. After centrifugation (1 h at 11,000 g) the supernatant was dialyzed against 100 mM phosphate, 6 M guanidine HCl, pH8. It was then applied on a Ni-nitriloacetic acid agarose (NTA, Qiagen) column equilibrated with dialysis buffer. The column was then washed with the same buffer and weakly bound proteins were removed by eluting with 100 mM phosphate/citrate, 6 M guanidine HCl, pH6. Finally, the recombinant apoprotein was eluted with 100 mM phosphate/citrate, 6 M guanidine HCl, pH5. Fractions collected were immediately neutralized with 1 M NaOH (30 µl/ml) and supplemented with 20 µl/ml 0.1 M EDTA and 5 µl/ml 0.2 M PMSF. They were analyzed on SDS-PAGE 14 %. Fractions containing the expected product were pooled and incubated with His powder (50 mM final) for 1h at 4°C. Ni and His were removed by dialyzing against PBS pH7.4 containing 2 mM EDTA. Purified Rec.-apoA-I was subjected to SDS-PAGE gel, blotted onto nitrocellulose and tested for the presence of fragments and full-length apoA-I with mAbs raised against human apoA-I as previously described (403).

Isolation of human apoA-I and preparation of reconstituted LpA-I

Human HDL was isolated from pooled plasma from normolipidemic volunteers by sequential ultracentrifugation as described previously (404). HDL was delipidated and apoA-I was prepared according to the method of Brewer *et al.* (261).

Discoidal reconstituted LpA-I were prepared by the cholate dispersion / Bio-Beads removal technique using a starting POPC/apoA-I ratio of 100:1 as published by Sparks *et al.* (279; 289). Spherical LpA-I particles were prepared by co-sonication of phospholipids and apoA-I as recently reported by Sparks *et al.* (289). Final compositions of all LpA-I particles were

evaluated after isolation by gel filtration on a Superose 6 column (2.5 cm x 100 cm) and concentration (Centriprep 30, MW cut off 30 kDa, Amicon) under low speed centrifugation.

Determination of LpA-I structural characteristics

The homogeneity and hydrodynamic diameters of LpA-I particles were determined by non-denaturing gradient gel electrophoresis using precast 8-25% polyacrylamide gels (Pharmacia LKB Biotechnology Inc.) and reference globular proteins (thyroglobulin, 17.0 nm; ferritin, 12.2 nm; catalase, 10.4 nm; lactate dehydrogenase, 8.2 nm and albumin, 7.1 nm) (high molecular weight calibration kit, Pharmacia) as described (405). The number of apoA-I molecules per LpA-I was determined by cross-linking the apoA-I with dimethyl suberimidate (406), and the products of the reaction were analyzed by SDS-PAGE on 8-25% polyacrylamide gel using the Phast System (Pharmacia LKB Biotechnology Inc.). Phospholipid and cholesterol concentrations in purified isolated LpA-I particles were determined using enzymatic assays (Boehringer Mannheim, Gmbh, Mannheim). The concentration of apoA-I was measured by the Lowry method as modified by Markwell *et al.* (407) using bovine serum albumin as standard.

Kinetics of association with dimyristoyl phosphatidylcholine

The ability of native and recombinant apoA-I to associate with DMPC was determined as previously described (282). Briefly, the desired amount of DMPC was dried under nitrogen and solubilized in Tris buffer (1 mg/ml) above its transition temperature. Before the assay, the apoprotein sample and the DMPC sample (DMPC/protein molar ratio = 50/1) were separately preincubated at 24°C for 10 min. The rate of lipid-protein association was followed at 24°C by monitoring the reduction in turbidity at 325 nm. Turbidity clearance curves were fit to a double exponential equation and $t_{½}$ values were determined.

Monoclonal antibodies and solid-phase radioimmunoassay of apoA-I

Antibodies 4H1, 2F1, 3G10 and 5F6, were prepared and previously characterized in our laboratory (408). Antibodies 2G11 and 4A12, first characterized by Petit *et al.* (409), were purchased from SANOFI Inc. (Paris, France) while A05, A51, A16, A11, A03, A07 and A44, also previously described (403) were produced by the Institut Pasteur, Lille. All mAbs were murine IgG, purified on protein G- or protein A-Sepharose (Pharmacia) and proven free of murine apoA-I.

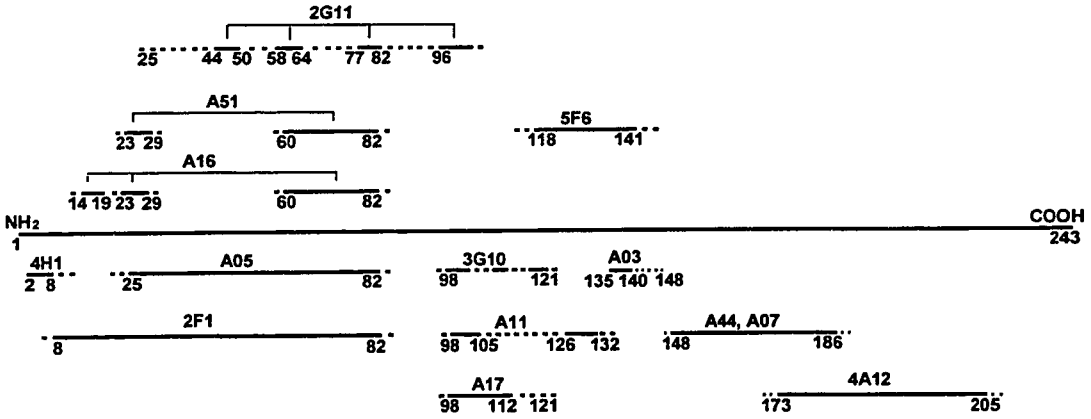

Figure II-1: Epitope Map of Apolipoprotein A-I.
The positions of epitopes recognized by these mAbs have been previously defined (403). The names of mAbs used in this study are placed above the *solid bars*, which represent the sequences recognized by these mAbs. The *dashed lines* on either side of the bars indicate that the antigenic recognition at this site may extend further.

The location of epitopes recognized by all these mAbs is summarized in Fig. II-1. Solid-phase RIA were done as previously described (297). Briefly, Immulon II Removawells were coated with apoHDL, washed, and saturated with gelatin. Anti-apoA-I mAb was mixed with serial dilutions of the competitive antigen (LpA-I or the lipid-free apoprotein), and incubated in the coated and saturated wells for 1 h. at room temperature. After washing, ^{125}I-labeled rabbit anti-mouse IgG was added and the wells incubated for another 1 h. at room temperature. The wells were washed and counted; B/Bo were then calculated where B and Bo represent the cpm

bound in the presence and absence of competitive antigen. The plot $B / Bo = f(antigen\ concentration)$ was used to calculate the ED_{50} and the affinity of the antibody.

Circular dichroism measurements

Circular dichroism (CD) spectroscopy was performed using a Jasco J41A spectropolarimeter. CD spectra were measured at 24°C in a 0.1-cm path length quartz cell, 6 scans from 260 to 184 nm were collected and averaged. The percentage of α-helix content was calculated from the molar ellipticity at 222 nm and using a mean residue weight of 115.3 for native apoA-I and 116.0 for Rec.-apoA-I as already described (268). The change in molar ellipticity was followed to determine the effect of GdnHCl concentration on the secondary structure of apoA-I and recombinant apoprotein. In these experiments, the free energy of unfolding of the protein was calculated according to Sparks *et al.* (268).

Lecithin: cholesterol acyltransferase (LCAT) assay

Lp2A-I were prepared with [1α,2α-^{3}H]cholesterol and either Rec.- or native apoA-I and then were tested for their ability to stimulate the LCAT reaction. LCAT was purified as previously reported (289). The LCAT reaction mixture consisted of varying amounts of Lp2A-I, 1.5 mg of fatty acid free BSA (Sigma), 5 mM β-mercaptoethanol and reaction buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA and 1 mM NaN_3, pH 8.0) to 450 μl final volume. Conditions for assay were generally as described before (289). Under these conditions, initial rates were estimated with minimal substrate conversion.

Cellular cholesterol efflux studies

Human skin fibroblasts were cultured and maintained at 37°C in a CO_2 incubator as described by others (343) and in DMEM supplemented with 10 % calf serum (GibcoBRL), glutamine (4 mM) and antibiotics (100 u/ml penicillin, 100 μg/ml streptomycin). After the cells were grown to confluence in 24-wells plate (initial seeding at $2.75x10^4$ cells per well in 500 μl of media), usually for 48 h, they were washed twice with PBS containing 0.2 % BSA and once with PBS. The cells were then labeled to a high cholesterol specific activity by incubation in DMEM containing 5% calf serum and 20 μCi/ml [1α,2α-^{3}H]cholesterol for 48 h. After the labeling, cells were incubated with DMEM (with supplements) containing 1% fatty acid-free BSA for 24 h. After incubation, the cell layers were rinsed twice with DMEM containing 0.2% BSA and twice with DMEM alone, and incubated at 37°C in CO_2 incubator with DMEM containing Lp2A-I (varying concentrations). Aliquots of 50 μl were taken between 2 and 120 min., then placed in Eppendorf tubes containing 100 μl PBS (with 0.2% BSA), vortexed, and centrifuged at 10,000 rpm for 5 min. Finally, a sample of 100 μl from these tubes was taken for counting. After removal of the last aliquot, cell layers were washed twice with PBS (0.2 % BSA) and twice with PBS alone. Then, they were dissolved in 0.1 M NaOH, and aliquots were taken to quantify radioactivity and protein concentration. Labeled cholesterol efflux was calculated as the label appearing in the media relative to the total cell label and as a function of time.

RESULTS

Production of Rec.-apoA-I

Efficient production of soluble Rec.-apoA-I has been obtained (>10 mg/l of culture media before purification and 10 mg/l after purification) which accounted for about 10% of the

total bacterial proteins. The protein remained stable during purification by affinity chromatography according to the protocol previously described with this amino-terminal tag of six histidines (410). After purification, Rec.-apoA-I showed a single band on SDS-PAGE with a molecular weight of 29.5 kDa, corresponding to human apoA-I (28 kDa) but taking in account the eleven extra amino acid residues (Fig. II-2).

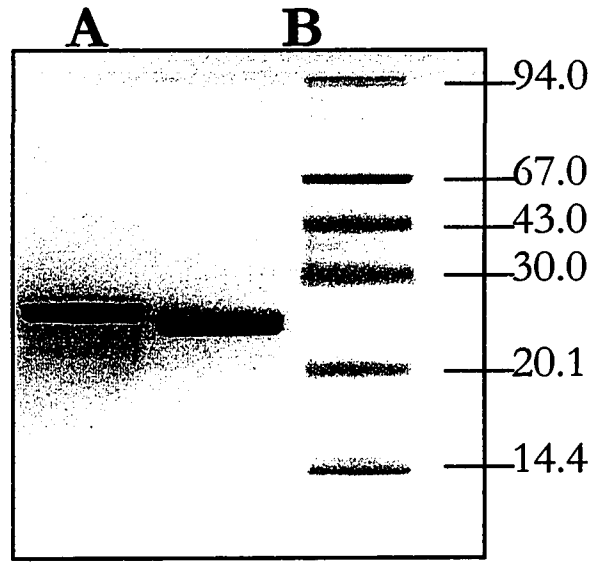

Figure II-2. Comparison of Native and Recombinant Human ApoA-I by SDS-PAGE Analysis. Purified native and recombinant apoA-I subjected to electrophoresis in 0.1% SDS/12% polyacrylamide gel. Proteins were stained with Coomassie Brilliant Blue. Lane A: Rec.-apoA-I; lane B: native apoA-I isolated from plasma; lane C: protein calibration kit consisting of phosphorylase b (94.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa).

No fragment or incomplete translated proteins have been detected by immunoblotting when several mAbs recognizing epitopes distributed along the apoA-I sequence were used to check the purity of Rec.-apoA-I. The N-terminal sequence of the expressed Rec.-apoA-I was verified by microsequencing up to the third residue in apoA-I and found to be as expected for the histidine tag sequence.

Characterization of LpA-I prepared with native and Rec.-apoA-I

Two different methods have been used to produce discoidal (sodium cholate dispersion) and spherical (sonicated) LpA-I particles, respectively. Rec.-apoA-I was able to form discoidal complexes similar to those obtained with native apoA-I as observed by electron microscopy (data not shown). Discoidal particles containing native or Rec.-apoA-I have been prepared using starting POPC/apoA-I ratios of 100:1 as previously described (279). After isolation, all complexes exhibited only one band on non-denaturing gradient gel electrophoresis, which correspond to homogeneous LpA-I preparations (Fig. II-3). Their sizes ranged from 9.8 to 10.2 nm with equivalent POPC compositions, close to the starting ratios for both proteins, suggesting similar lipid binding properties (Table II-1).

Chemical cross-linking of apoA-I showed that all reconstituted LpA-I contained 2 molecules of apoA-I per particle (Lp2A-I). For each paired preparation, we consistently observed a larger size for Lp2A-I containing Rec.-apoA-I, which corresponds to the additional mass of two extension tails. Discoidal Lp2A-I particles containing cholesterol in addition to POPC were also prepared as described above, using a POPC/cholesterol/apoA-I ratio of 80:8:1. After purification, these homogeneous complexes showed sizes varying from 9.7 to 10.3 nm, contained two apoA-I per particle and had essentially the same composition when made with either Rec.- or native-apoA-I (Table II-1). The N-terminal extension sequence contributed an increased positive charge that added 0.6 e/mole to the molecular valence of Rec.-apoA-I as determined by electrophoretic migration and this added charge was the same in lipid-free Rec.-apoA-I and Lp2A-I containing Rec.-apoA-I.

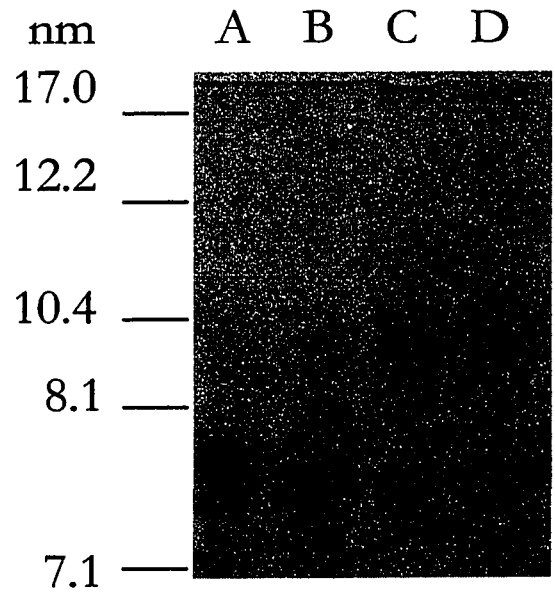

Figure II-3. Non-Denaturing Polyacrylamide GGE of Purified Lp2A-I.
Discoidal (A, Rec. apoA-I; B, apoA-I with both a POPC/apoA-I ratio of 94:1) and sonicated-spherical (C, apoA-I; D, Rec.-apoA-I with POPC/apoA-I ratio of 23:1 and 21:1 respectively) Lp2A-I subjected to electrophoresis in 8-25% polyacrylamide gels under non-denaturing conditions and run in adjacent lanes of the same gel. Hydrodynamic diameters were determined by comparison to high molecular weight standards (S) as described under "Experimental procedures" with a standard deviation of 0.3 nm for discoidal particle and of 0.1 nm for sonicated particles (see Table II-1 for actual values).

This added charge had no effect on the observed molecular weight for any of the LpA-I tested that contained Rec.-apoA-I, as shown when non-denaturing gradient gel electrophoresis was run to the limit of pore size exclusion.

Two spherical (sonicated) LpA-I have been prepared with native and Rec.-apoA-I, respectively, using 30 to 1 POPC/apoA-I ratios as recently reported (289). The isolated complexes exhibit a major homogeneous band on gradient gel electrophoresis (Fig. II-3). No difference was observed between particles prepared with native and recombinant apoA-I: both complexes contained two apoA-I per particle, showed similar molar POPC content, and had very similar sizes (Table II-1). Both proteins also presented a similar yield of complexation with

phospholipids (around 80 % of the proteins bind lipids). Evidence for a spherical structure was reported by Sparks *et al.* (309). In this report, it was clearly shown that sonicated LpA-I particles (including those devoid of a neutral lipid core) are most similar (in morphology and physical properties) to native, spherical HDL and differ significantly from discoidal LpA-I.

Table II-1: Size and Composition of Reconstituted Lp2A-I prepared with Native or Recombinant apoA-I.

ApoA-I type	Size[a] (nm)	POPC / chol / apoA-I[b] (mol/mol/mol)
Discoidal Lp2A-I[c]		
Native apoA-I	**9.8** (0.3)	**97.1** (3.9): **0**: **1**
Rec.-apoA-I	**10.2** (0.3)	**96.6** (15.1): **0**: **1**
Native apoA-I	**9.7** (0.3)	**76.0** (2.5): **6.7** (1.0): **1**
Rec.-apoA-I	**10.3** (0.3)	**73.2** (3.0): **6.4** (1.3): **1**
Spherical (sonicated) Lp2A-I[c]		
Native apoA-I	**7.3** (0.1)	**23.0** (5.0): **0**: **1**
Rec.-apoA-I	**7.5** (0.1)	**20.6** (1.9): **0**: **1**

Results are means (± SD) from three different experiments.

[a] Hydrodynamic diameter from non-denaturing gradient gel electrophoresis.

[b] Discoidal complex prepared by a cholate/Bio-Bead removal technique and spherical complex prepared by sonication; for both, composition after purification by Superose 6 chromatography is indicated.

The number of apoA-I molecules per particle obtained by cross-linking with dimethyl suberimidate was 2 for each preparation.

Kinetics of association with DMPC

The graph presented in Fig. II-4 demonstrates the absence of any significant effect of the N-terminal extension on the kinetics of association with DMPC. The $t_{½}$ values calculated

are 4 min. for both native apoA-I and Rec.-apoA-I. The complexes formed are also similar in size although a slight increase was observed for Rec.-apoA-I, a result that is very similar to what has been observed with the POPC-containing LpA-I (not illustrated).

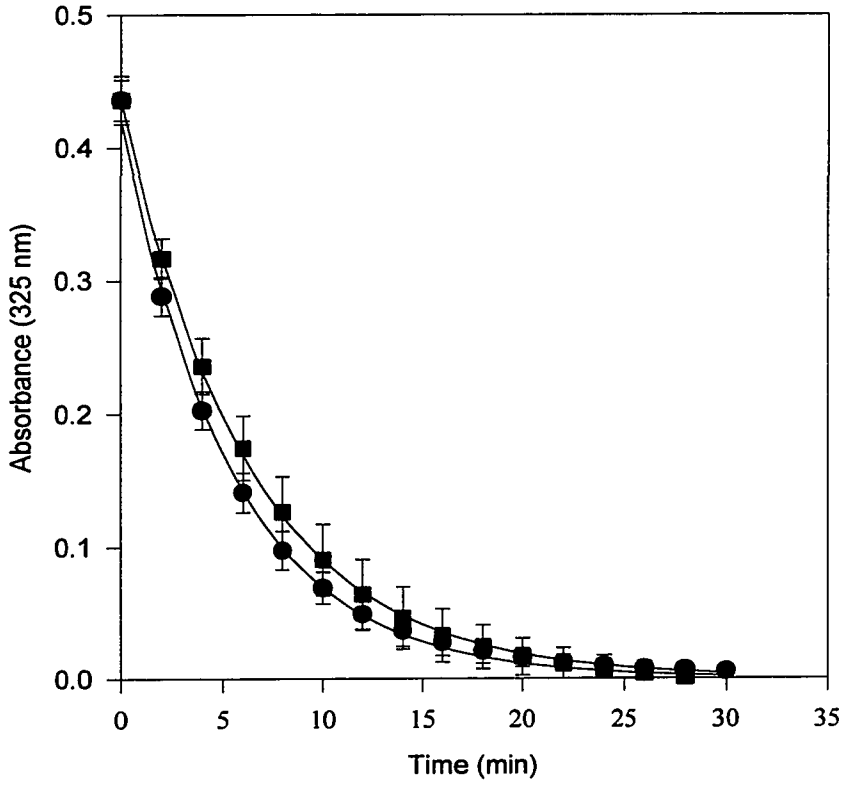

Figure II-4: Kinetic of DMPC Association with DMPC.
The plot illustrates the reduction of turbidity of DMPC vesicles induced by native apoA-I (●) or Rec.-apoA-I (■). The decrease in the absorbance was measured at 24°C and 325 nm. Results represent means of 3 assays ±SD.

Comparison of the immunoreactivity of native and recombinant apoA-I in lipid-free forms and within Lp2A-I particles (POPC-containing Lp2A-I)

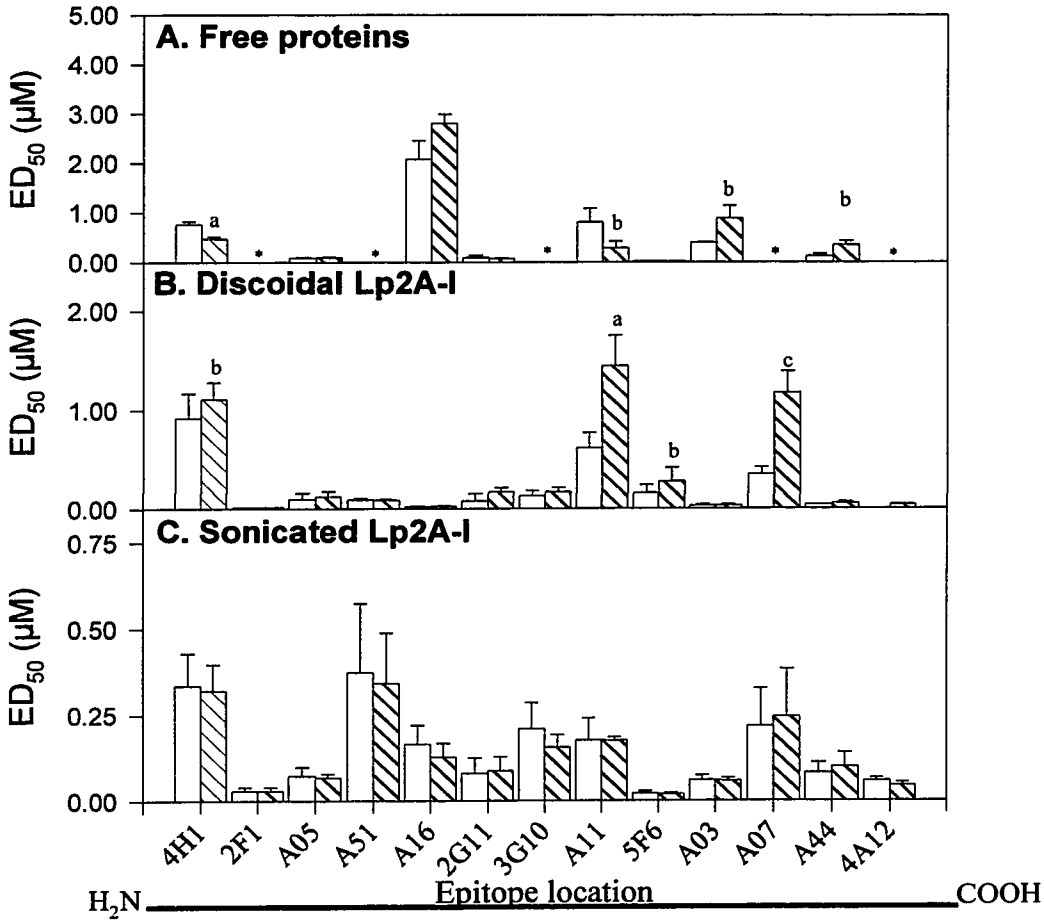

Figure II-5: Immunoreactivity of 13 Epitopes of ApoA-I in Lipid-Free Form (A) and Lp2A-I (Discoidal (B) or Sonicated (C)).
Each complex preparation is given in insets and the epitopes are positioned on the x axis as they appear in apoA-I sequence. The ED_{50} (inversely proportional to immunoreactivity) represents an antigen concentration needed to bind half of an antibody dilution. Monoclonal antibodies are separated in three groups representing the N-terminal region (4H1 to 2G11), the central region (3G10 to A03), and the C-terminal region (A07 to 4A12). Results are means (±SD) of three independent experiments where [a], [b], and [c] indicate the degree of significance relative to ED_{50} for native apoA-I. [a] $p< 0.05$, [b] $p<0.01$, [c] $p< 0.005$, *$ED_{50}>5$ µM.

Using a panel of thirteen mAbs reacting with well-defined apoA-I epitopes (Fig. II-1), we have examined the reactivity of recombinant and native apoA-I, in order to evaluate their

conformation. The results of the immunoreactivity in lipid-free form, discoidal (containing POPC only) and sonicated Lp2A-I are shown in Table II-2 and Fig. II-5.

Table II-2: Antibodies Affinity for Native and Recombinant ApoA-I in Lp2A-I.

Mabs[a]	Discoidal Lp2A-I		Spherical Lp2A-I	
	Native apoA-I	Rec.-apoA-I	Native apoA-I	Rec.-apoA-I
4H1	-1.86 ± 0.06	-1.92 ± 0.27	-2.03 ± 0.52	-2.19 ± 0.53
2F1	-4.55 ± 0.35	-4.45 ± 0.65	-5.09 ± 0.95	-5.14 ± 1.34
A05	-1.47 ± 0.52	-1.20 ± 0.11	-1.12 ± 0.20	-0.98 ± 0.13
A51	-0.88 ± 0.27	-0.85 ± 0.09	-0.69 ± 0.24	-0.77 ± 0.36
A16	-1.15 ± 0.21	-1.06 ± 0.21	-0.67 ± 0.06	-0.66 ± 0.05
2G11	-0.93 ± 0.16	-1.05 ± 0.24	-0.97 ± 0.28	-0.82 ± 0.15
3G10	-1.89 ± 0.93	-1.27 ± 0.43	-2.43 ± 0.64	-2.17 ± 0.18
A11	-1.34 ± 0.05	-1.08 ± 0.05	-1.43 ± 0.46	-1.36 ±0.10
5F6	-1.62 ± 0.46	-1.22 ± 0.28	-1.52 ± 0.42	-1.55 ± 0.50
A03	-1.69 ± 0.13	-1.63 ± 0.09	-1.20 ± 0.30	-1.22 ± 0.24
A07	-1.62 ± 0.33	-1.67 ± 0.33	-2.01 ± 0.27	-1.97 ± 0.59
A44	-1.27 ± 0.03	-1.34 ± 0.03	-1.30 ± 0.21	-1.47 ± 0.62
4A12	-1.50 ± 0.11	-1.64 ± 0.16	-1.74 ± 0.19	-1.90 ± 0.12

Results are means (± SD) of the slope from three different RIA experiments.
[a] mAbs are separated in three groups representing the N-terminal region (4H1 to 2G11), the central region (3G10 to A03), and the C-terminal region (A44 and 4A12).

By comparison of lipid free and lipid-bound (Lp2A-I) apoA-I, we have previously shown that major changes in the conformation of the apoprotein occur upon binding to lipids and that a condensed structure for soluble lipid-free apoA-I could be responsible for the lack of immunoreactivity of several epitopes (297). In agreement with these previous studies, we have observed that many epitopes of native apoA-I are completely masked in the lipid-free form as seen in the N-terminal region (2F1 and A51), the middle region (3G10) and the C-terminal region (A07 and 4A12). The very similar results obtained with Rec.-apoA-I in terms of masked epitopes demonstrate the same folding of the two proteins in the absence of lipids. In general, lipid-free, native and Rec.-apoA-I were similar in their respective affinities as determined by RIA and calculated affinity from the curves $B / B_0 = f(\textit{antigen concentration})$ (data not shown). Nevertheless, differences between native and Rec.-apoA-I were seen for certain epitopes (Fig. II-5). First, the extreme N-terminal epitope for mAb 4H1, located at residues 2-8 in the apoA-I sequence and close to the extension tail (polyhistidine) of the recombinant apoprotein, was more reactive than in native apoA-I. In the middle region of the protein, we observed an increase of immunoreactivity for one epitope (A11, residues 99-132) and a decrease for another (A03, residues 135-148), as compared to native apoA-I. Finally, in the C-terminal region, the only reactive epitope in free form apoA-I, A44 (149-186) showed a lower immunoreactivity. All of these slight differences in epitope exposure on Rec.-apoA-I are consistent with a slight change in the protein conformation resulting from small residual lipids associated with the recombinant apoprotein. Inclusion of a small amount of cholesterol or POPC with native apoA-I causes essentially the same differences in epitope immunoreactivity (Frank P.G., Bergeron J., Marcel Y.L. unpublished results).

When we studied the two apolipoproteins within spherical (sonicated) Lp2A-I, there was no variation in immunoreactivity between recombinant and native apoA-I. In discoidal Lp2A-I however, the presence of the N-terminal extension caused a small but significant decrease of immunoreactivity for the N-terminal epitope 4H1; other changes occurred in the middle of the molecule where the immunoreactivity decreased significantly for the overlapping epitopes 5F6 (residues 118-141) and A11 (99-132). An epitope of the C-terminal domain of apoA-I, A07, located between residues 149-86, also close to the central domain, showed a decrease of immunoreactivity. Table II-2 further shows that no significant difference in the binding affinity for any of the mAbs is apparent between native and recombinant apoA-I on both spherical and discoidal Lp2A-I particles.

Circular dichroism measurements

Circular dichroic spectroscopy performed with the two lipid-free proteins did not present any evidence for a change in the secondary structure (Fig. II-6). Similarly, circular dichroic measurements done on both kinds of Lp2A-I complexes (presented in Table II-1) also revealed no significant difference in the α-helix content of recombinant vs. native apoA-I (66 % vs. 69 % respectively for discoidal Lp2A-I without cholesterol, 51 % vs. 54 % respectively for spherical Lp2A-I and 50 vs. 56 % respectively for the lipid-free proteins).

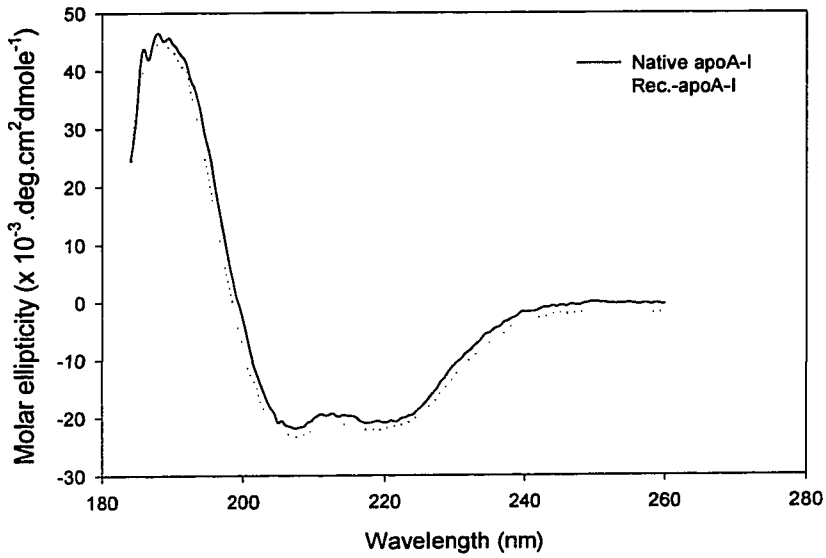

Figure II-6: Circular Dichroism Spectra of Lipid-Free Native and Rec.-apoA-I.
Spectra were recorded between 184 and 260 nm and represent the average of 6 acquisitions. Samples were analyzed at a protein concentration of 67 µg/ml in 0.05 M phosphate buffer pH7.4.

Isothermal denaturations of the lipid-free protein were also performed as described by Sparks *et al.* ((268), Fig. II-7). This technique allows the characterization of the structural stability of the protein. The midpoints of denaturation calculated from the curve Fig. II-7 ($D_{½}$) were identical (1.1 ± 0.03 M for both proteins). The free energies of denaturation (ΔG_D°) although slightly higher for native apoA-I (2.7 ± 0.2 kcal/mol) as compared to Rec.-apoA-I (2.4 ± 0.2 kcal/mol) remain similar and comparable to values already published (268).

Activation of Lecithin:cholesterol acyltransferase (LCAT)

Discoidal Lp2A-I containing either Rec.-apoA-I or native apoA-I and similar in terms of POPC/Chol/A-I molar ratios (Table II-1) have been characterized as substrates for LCAT. Equal amounts of discoidal Lp2A-I were incubated with purified LCAT as described under Methods. Using increasing amounts of discoidal Lp2A-I, no significant difference between native and recombinant apoA-I for LCAT activation was observed (Fig. II-8).

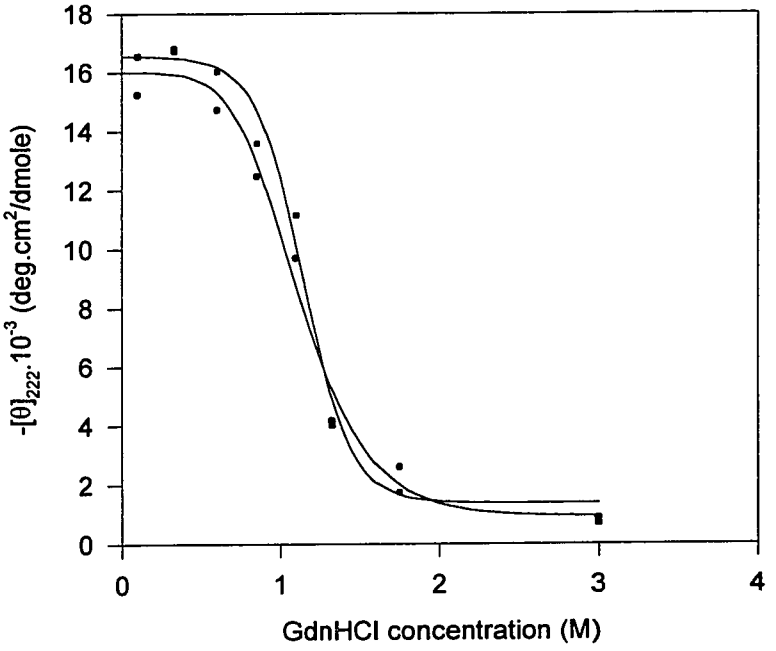

Figure II-7: Effect of GdnHCl Concentration on the α-Helix Stability of Lipid-Free ApoA-I and Rec.-apoA-I.
Aliquots of each protein (apoA-I (●) and Rec.-apoA-I (■) at 33.3 μg/ml) were preincubated with 0 to 3 M GdnHCl in 0.05 M phosphate buffer for 72 h. at +4°C and then the molar ellipticity at 222 nm was determined. Values are the average of duplicate determinations.

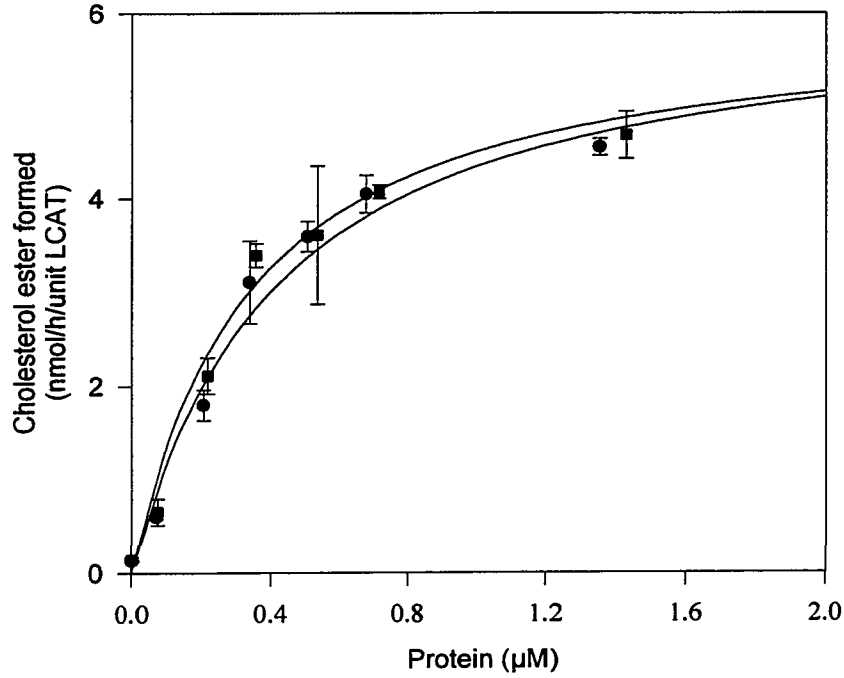

Figure II-8: LCAT Reaction with Lp2A-I.
Reconstituted discoidal particles containing native (●) and Rec.-apoA-I (■) have been tested for their ability to act as substrates for purified human LCAT reaction. The results are the mean of triplicate determinations and are representative of three different experiments.

Cholesterol efflux studies

We also compared the ability of native and Rec.-apoA-I to promote cholesterol efflux from cultured human fibroblasts. This study was performed with varying concentrations of each Lp2A-I (discoidal Lp2A-I without cholesterol described in Table II-1). Cholesterol efflux was measured by the appearance of radiolabeled cholesterol in the medium after incubation of prelabeled cells with Lp2A-I. As shown in Fig. II-9A and B, both discoidal Lp2A-I were able to promote cholesterol efflux as a function of time.

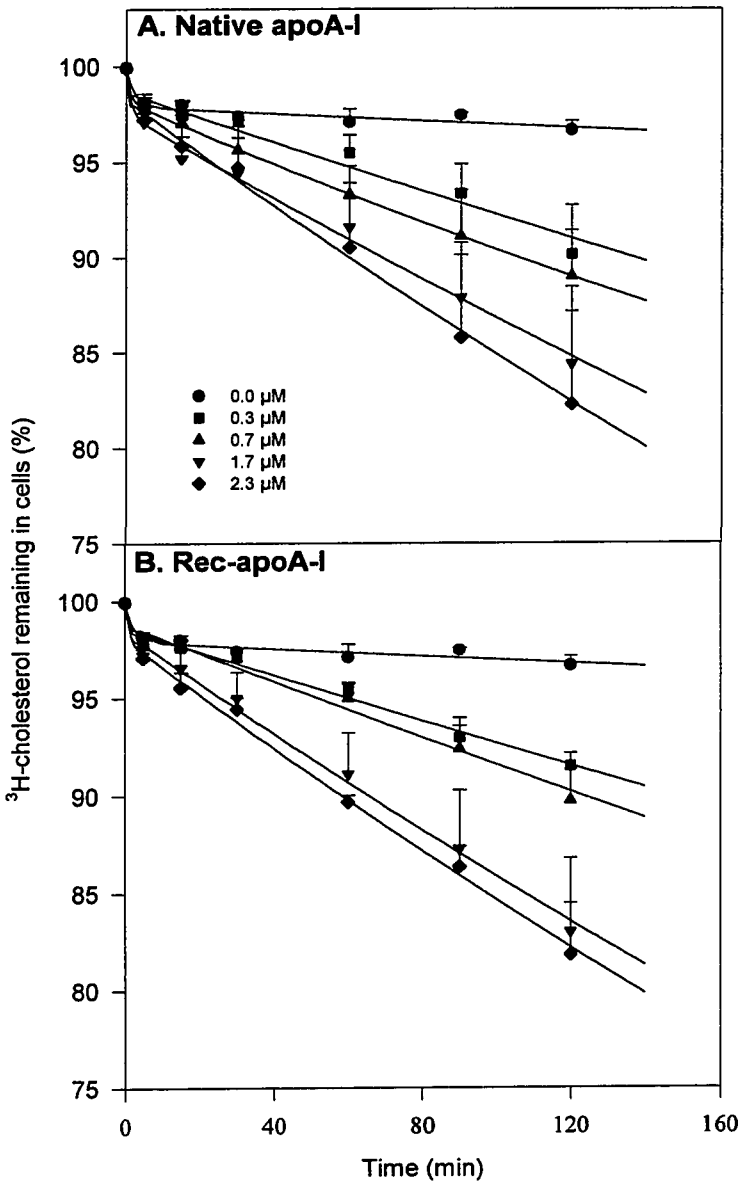

Figure II-9: Cholesterol Efflux from Fibroblasts to Discoidal Lp2A-I.
Cholesterol efflux from human fibroblasts induced by different concentrations of Lp2A-I (discoidal complexes with similar POPC content and without cholesterol, characterized in Table II-1) containing native (Panel A) or Rec.-apoA-I (Panel B) was determined over a 120 min. period at 37°C and compared to the medium (DMEM) without Lp2A-I as described under "Experimental procedures". The results are expressed in percentage of the labeled removed from the cells as a function of time. Each result represents the mean (± SD) for three experiments. The curves were obtained by fitting the time-course to a double exponential equation.

Comparisons of the effect of the concentration on the initial rate of cholesterol efflux (5 min.) show no differences between native and Rec.-apoA-I (not illustrated). In addition, no significant difference in the rate of cholesterol efflux from fibroblasts was observed when comparing the two proteins in spherical Lp2A-I (100 μg/ml, data not shown).

DISCUSSION

Unfortunately, high level of expression of human apoA-I has until now proven to be very difficult. Several groups have described methods for recombinant apoA-I production using different eukaryotic expression systems (66; 67; 260; 381-383; 385-391). The levels of the unpurified form measured in the culture medium were often encouraging even as recently reported (325), but the final recoveries were usually too low for use in reconstitution of well-defined lipoproteins (382; 383). Recently baculovirus-infected insect cells provided high levels of immunoreactive apoA-I (35 mg/l of culture) in some cases without preparative purification (391) and more recently in a system yielding proapoA-I (411). Our own experience has shown that often the initial yields estimated by immunoassay were grossly overestimated due to greatly increased immunoreactivity of apoA-I fragments present in the cell culture media. This, we believe, explains, at least in part, the disappointing yields after purification reported by a number of investigators.

In parallel, other groups have expressed apoA-I in *E. coli* (324; 325; 380; 381; 392-398). However, production of the protein in *E. coli* has been limited by high intracellular proteolytic activity. Indeed, expression as fusion protein with glutathione-S-transferase or β-galactosidase has been limited by degradation of the apoA-I moiety (381; 392). The production of truncated forms at the N-terminal end of the apoA-I could be also due to mRNA instability or inefficient

translation (380; 394). We also observed the same problem with the expression of apoA-I fused to glutathione-S-transferase (381). In addition, the cleavage of the fusion protein (using activated Factor X) was usually partial, leading to low recovery of the full-length purified form (Bergeron J., Marcel YL unpublished data). It has been recently suggested that the problems could be circumvented by keeping the pro segment of the protein (or adding a signal peptide) and/or by optimizing the first codons (silent mutations) of the apoA-I protein in order to increase greatly the expression efficiency (324; 380; 394; 397; 398). Based on these reports, we developed an approach where the sequence coding for the N-terminal part of the protein was modified by silent mutations and where the presence of an N-terminal extension provided an easy purification of only full-length proteins. This method results in a very efficient expression system which yields 10 mg of purified apoA-I per l of *E. coli* culture medium. The recombinant apoA-I produced is stable with physical characteristics similar to the native form isolated from plasma. This final yield is among the highest reported. Recently, another prokaryotic expression system was described which provided very high level of an apoA-I mutant, apoA-I_{Milano} in the culture medium (397). Although the industrial size-bioreactor used gave 2 g/L of immunoreactive apoA-I, the presence of proteolytic cleavage products imposed four complex purification steps and no final yield was provided making comparison difficult. The system described here is much easier in that it avoids the loss of material due to many steps of purification and can be performed in a 1-liter flask without bioreactor. The same system has also been successfully applied to the expression of native and mutant apoA-IV (401). Many investigators have also reported the successful expression of heterologous proteins in *E. coli* using this His-tag system, without any apparent effect on the function of the protein (412-420).

In this investigation, we have performed a detailed characterization of the physicochemical and functional properties of Rec.-apoA-I. While we observed a small increase

in the size and surface charge of the recombinant apoprotein, consistent with the additional residues of the N-terminal extension, all other physicochemical parameters were essentially identical to that of native apoA-I. Both the content of α-helix in Rec.-apoA-I and the stability of these helices to guanidine denaturation were not different from that observed for native apoA-I. This indicates that the N-terminal extension does not significantly alter the conformational stability of apoA-I, a parameter that may be central to its ability to form lipoprotein particles and their properties (268; 362; 421). The fact that the helical structure was not disrupted in the recombinant apoprotein may explain why Rec.-apoA-I was able to form reconstituted discoidal and spherical particles equally as well as native apoA-I. The proteins both appeared to form LpA-I that were equivalent in homogeneity, size and in overall lipid/protein stoichiometry. Detailed DMPC studies further showed that both the rate of association and the capacity of lipid binding were identical for the two proteins.

Immunochemical studies confirmed the conformational similarity of Rec.- and native apoA-I. Only small differences in the conformation of N-terminal and central regions of Rec.-apoA-I are evident. However, for Rec.-apoA-I in solution, these differences are primarily due to a small amount of lipid associated with recombinant apoprotein. In contrast, when Rec.-apoA-I is complexed with POPC, essentially no difference in antibody binding affinity is evident with any of the antibodies, for native and Rec.-apoA-I on spherical or discoidal LpA-I. While a small difference in ED_{50} values between the two proteins on discoidal Lp2A-I was also noted for 3 central epitopes, ED_{50} values were essentially identical for both native and recombinant proteins on the spherical LpA-I complexes. Taken together, these data suggest that both the secondary and tertiary structure of native and Rec.-apoA-I are similar.

When we assessed the specific functional properties of Lp2A-I formed with recombinant apoA-I, as substrates for LCAT reaction, it was clear that the N-terminal histidine extension did not modify the functional properties of recombinant Rec.-apoA-I as an activator of LCAT (Fig. II-8). This is all the more significant given that other studies have shown that differences in the conformation of the central domain of apoA-I, induced by modifying Lp2A-I lipid composition, could modulate LCAT reaction (362; 379). Finally, native and Rec.-apoA-I in both discoidal and spherical Lp2A-I are equivalent in their ability to promote cholesterol efflux from fibroblasts. This again indicates that the overall conformation of Rec.-apoA-I is functionally comparable to the native form.

In conclusion, recombinant apoA-I with the N-terminal Rec. extension shows similar physicochemical and functional properties when compared to the native form. Taking into account the efficiency and the facility of this expression system to produce large amounts of purified recombinant apoA-I without major modifications of its structural and functional properties, this represents a suitable expression system for apoA-I mutagenesis studies. The expression system, described in this chapter, was therefore used to characterize apoA-I mutants and their structural and functional properties as described in the following chapters.

Chapter 3: DELETION OF CENTRAL α–HELICES IN HUMAN APOLIPOPROTEIN A-I: EFFECT ON PHOSPHOLIPID ASSOCIATION

SUMMARY

In order to understand better the structure/function properties of apolipoprotein A-I, we have constructed and expressed three apoA-I mutants using a system described in Chapter 2 for the expression of human apolipoprotein A-I (Rec.-apoA-I). These mutants (corresponding to deletion of apoA-I residues 100-143, 122-165, and 144-186) have been studied for their ability to form reconstituted apoA-I-containing lipoproteins (LpA-I) with POPC and DMPC, and for their structural and physical properties. Rec.- and native apoA-I can form homogenous discoidal Lp2A-I over a wide range of POPC/apoA-I ratios (20 to 130/1) and exhibit sizes ranging from 9.5 to 10.5 nm. When recombined with varying POPC content (20 to 130/1, POPC/A-I), the three mutants produce homogeneous discoidal Lp2A-I that contain a low POPC/A-I molar ratio (20 to 40/1 for all mutants) and exhibit a nearly constant size [7.5-7.6 nm for Δ(100-143) and 7.9-8.0 nm for the other two mutants]. Kinetics of association of these proteins with DMPC are similar for Δ(100-143) and Rec.-apoA-I ($t_{½}$ of 4.0 and 4.4 min., respectively) but appear significantly reduced for Δ(122-165) and Δ(144-186) ($t_{½}$ of 7.5 and 6.9 min, respectively). In the lipid-free form, all proteins have a similar thermodynamic stability with very comparable free energy of unfolding (ΔG_D^0) for the α-helical structure, as determined by isothermal denaturation studies. However, Δ(100-143) has a significantly lower α-helical content (33 %) as compared to the other proteins (40, 41 and 45

% for Rec.-apoA-I, Δ(122-165) and Δ(144-186), respectively). When associated with POPC, Δ(122-165) and Δ(144-186) have a higher α-helicity (63 and 63 %) and an enhanced stability (2.5 and 2.3 kcal/mol respectively) as compared to Δ(100-143) (49 % and 1.8 kcal/mol) and Rec.-apoA-I (52 % and 1.9 kcal/mol). These results suggest that the amphipathic α-helices within residues 100-186 of apoA-I are directly involved in interactions with PL. The helical region 100-121 appears to be more important to the stabilization of the lipid-apoprotein complex formed whereas helices within residues 122-186 appear to be critical to the initial rates of association of the apoprotein with DMPC. These data suggest that an important role of the central domain 100-186 may be to maintain the plasticity of apoA-I and its ability to form different classes of HDL particles. Therefore, it is likely that this region may also play an important role in the functional properties of this apoprotein.

INTRODUCTION

The lipid binding properties of apoA-I are central to the other functional properties of this protein since the amount and the type of lipid associated can affect both apoA-I structural (268; 277; 297; 313; 318) and functional properties (192; 289).

The use of several algorithms for the prediction of apoA-I secondary structure has identified eight consecutive amphipathic antiparallel 22-mers α-helices interrupted by β-turn at Pro or Gly residues. These helices are thought to be important mediators of the association with PL (242) and synthetic model peptides with comparable amino acid sequences have been shown to associate with PL (244; 302; 422). Segrest *et al.* (242) have proposed a classification of the different helices (reviewed in the introduction). For apoA-I, 6 helices have been identified with an amino acid distribution characteristic of a class A amphipathic α-helix (helices 44-65,

66-87, 121-142, 143-164, 166-186, 187-208). Two other types of helices have been identified: class G* (helix 8-33) and class Y (helices 88-98, 99-120, 209-219, 220-241) (Fig. I-10). Segrest *et al.* (242) have proposed that, compared to class A helices, class Y helices have a reduced lipid affinity. Moreover, proteolysis and immunoreactivity studies using well-characterized mAbs to apoA-I have shown that a central domain of apoA-I (possibly corresponding to helix 100-121) was more accessible in small discoidal HDL than in the larger particles (297; 318). The central domain of apoA-I may therefore be involved in the binding of varying amount of PL and may form a hinge domain as previously suggested (297; 316). While these different helices may all be implicated in binding lipid, individual helices appears to have distinct properties and/or functions (382; 383). Previous mutagenesis studies have not fully analyzed the properties of the constructed mutant apoproteins in their lipid-free and -associated forms and very little is known about the structural requirements necessary for the binding of apoA-I to PL.

To analyze the role and importance of the central helices in apoA-I association with lipids, we have produce three mutants corresponding to deletions of central α-helices Δ(100-143), Δ(122-165), Δ(144-186) using a previously characterized system (Chapter 2). These deletions have been designed to characterize the lipid-binding properties of the central helices and to test the hypothesis of a possible hinge domain in human apoA-I. In the resulting mutants, the periodicity of the helices and the overall secondary structure found in native human apoA-I were maintained.

EXPERIMENTAL PROCEDURES

Materials

1-palmitoyl 2-oleyl phosphatidylcholine and 1, 2-dimyristoyl phosphatidylcholine were obtained from Avanti Polar Lipids (Birmingham, AL). Sodium oleate was purchased from Sigma Chemical Co. (St. Louis, MO).

Construction, expression and purification of the mutant protein

Wild-type apoA-I with an N-terminal Met-Arg-Gly-Ser-$(His)_6$ extension (Rec-apoA-I) was produced using an expression vector under the control of the T7 promoter as already described (pXL2116, Chapter 2). All cDNA encoding apoA-I mutants were constructed in Dr. Eric Rassart laboratory (University du Québec à Montréal, Québec, Montréal). Three mutants each lacking two contiguous 22-mers α-helices: 100-143, 122-165, and 144-186 were constructed by site-specific deletion on the cDNA. Briefly, the cDNA encoding the entire apoA-I sequence was cloned in the PstI site of pBluescript SK^+ vector. Three 36-mer oligonucleotides were synthesized (Gene assembler II, Pharmacia) so that each oligo was complementary to two portions of 18 nucleotides each located upstream and downstream, respectively of the DNA sequence to be deleted. The sequences are as follows: for oligo 100-143: 5' GCGCATCTCCTCGCCCAGGGGCTGCACCTTGGCCTT 3'; for oligo 122-165: 5' GCGCAGCTCGTCGCTGTACGGCTCCACCTTCTGGCG 3'; and for oligo 144-186: 5' GTACTCGGCCAGTCTGGCTGGGCTCAGCTTCTCTTG 3'. Each oligo was annealed to the denatured plasmid and served as a primer in the repair reaction that was performed according to the method of Kunkel (423). The DNA of each mutant was completely sequenced prior to its transfer into the expression vector.

Expression was performed with some modifications of the initial procedures (Chapter 2). Briefly, an overnight culture (LB medium) of each mutant or recombinant apoA-I (Rec.-apoA-I) was used to inoculate 1 L of culture in M9 (supplemented with ampicillin, 100 μg/mL and chloramphenicol, 50 μg/mL). The culture was grown at 37 °C until it reached an OD at 600 nm of 0.5, and expression was induced by adding IPTG (1 mM final). The expression was continued for 90 min, and the cells were then harvested by centrifugation and frozen at -20 °C. The pellet was then resuspended in 5 mL/g cells of buffer A (100 mM Na_2HPO_4, 1 mM PMSF, 6 M GdnHCl, pH 8). Each step of the purification was performed in 6 M GdnHCl. The solution obtained was then sonicated on ice (5 min. cycles x 3, 250 watts mode pulsed, 50 % duty cycle, 60 output control). Bacterial debris were removed by centrifugation (1 h at 11000*g*). Nucleic acids were removed by incubation of the supernatant with 10 mL/g protein of streptomycin (10 %), at + 4°C for 1 h. After centrifugation, the supernatant was applied on a nitrilotriacetic acid-agarose (Ni-NTA, Qiagen) column equilibrated with buffer A. The column was washed with the same buffer. Weakly bound proteins were eluted by washing the column with 100 mM phosphate/citrate, 6 M GdnHCl, pH 6. Finally, the recombinant protein was eluted with the latter buffer at pH 5. Fractions collected were immediately neutralized with 1 M NaOH (30 μL/mL) and supplemented with 0.1 M EDTA (20μL/mL) and 0.2 M PMSF (5 μL/mL). They were analyzed on SDS-PAGE 14 % and the most concentrated fractions were pooled and incubated with His powder (50 mM final), for 1h at +4°C. The sample was then dialyzed against TBS pH8. Each sample was analyzed on SDS PAGE 4-20 % and transferred onto nitrocellulose to test for the presence of possible degradation with well-characterized monoclonal antibodies (403).

Preparation and characterization of Reconstituted lipoproteins

Discoidal LpA-I were produced using the cholate dispersion/ Biobeads removal method originally described by Sparks *et al.* (268). The reconstituted lipoproteins were generated with either native Rec.-apoA-I or the mutant proteins and POPC (POPC/protein molar ratios are indicated for each experiment). LpA-I complexes were filtered through a 0.22 μm syringe tip filter. For analysis, all complexes were reisolated by gel filtration on a Superose 6 column (Pharmacia). LpA-I were characterized as described in Chapter 2. LpA-I sample were also submitted to electrophoresis on precast 0.5 % agarose gel (LipoGel, Beckman) under a constant electric field for 30 min. at 25 °C. After protein staining, electrophoretic mobility, surface potential, density of surface charge and valence per protein were determined with the method described by Sparks & Phillips (48).

Electron microscopy

Electron microscopy was performed as described by Forte *et al.* (424). Briefly, samples to be analyzed were dialyzed against $(NH_4)OAc$ before each experiment. The samples were mixed with 2 % sodium phosphotungstate (1/2 dilution) and examined using a Hitachi H-7100 electron microscope.

DMPC kinetics analysis

The ability of the different proteins to clear a DMPC solution was determined as previously described in Chapter 2.

Circular dichroic measurements

Circular dichroic spectroscopy was performed using a Jasco J41A spectropolarimeter. The percentage of α-helix content was calculated from the molar ellipticity at 222 nm and using a mean residue weight of 115.2 for native apoA-I, 116 for Rec.-apoA-I, 116.2 for Δ(100-143), 116.2 for Δ(122-165) and 116 for Δ(144-186). The change in molar ellipticity was followed to determine the effect of GdnHCl concentration on the secondary structure of apoA-I and recombinant proteins. In this experiment, the free energy of unfolding ($\Delta G_d^{(')}$) of the protein on the surface of reconstituted lipoproteins was calculated as described by Sparks *et al.* (268).

RESULTS

Secondary structure analysis of Rec.-apoA-I and the mutants

Three apoA-I mutants corresponding to the elimination of residues (100-143), (122-165) and (144-186) have been constructed (Fig. III-1). These mutants were selected to avoid any disruption of the secondary structure and keep the periodicity of the resulting protein. The periodicity marked by the Pro or Gly residues was previously shown to be an important feature that allows apoA-I to associate PL (425). To demonstrate the absence of any effect, the secondary structure of the protein was determined using the algorithm proposed by Garnier *et al.* (276). The results showed no significant difference in the overall secondary structure in the deleted mutants as compared to native apoA-I, especially around the deleted regions.

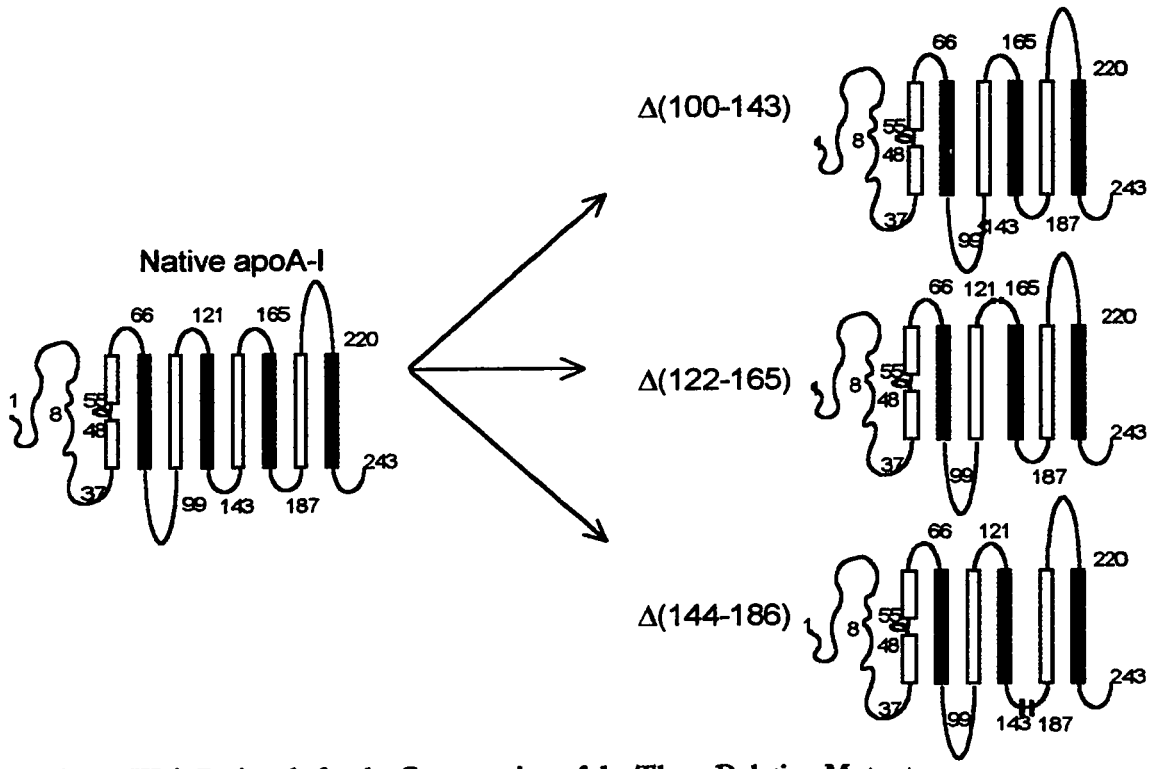

Figure III-1: Rationale for the Construction of the Three Deletion Mutants.
The predicted secondary structure of apoA-I illustrates the central α-helical region studied and the predicted maintained periodicity in each of the three constructed mutants. Amphipathic α-helices, β-turns and random coils are respectively represented by boxes, curvilinear sections and curves.

Expression of the recombinant apoproteins

The recombinant wild-type protein (Rec.-apoA-I) and the site directed mutants have been produced in *Escherichia coli* using the pET expression system already described (Chapter 2). The expression of the T7 RNA polymerase was induced by adding IPTG (1 mM final) to a growing culture (Abs_{600}=0.5). In this system, we were able to produce Rec.-apoA-I (10.5 mg of pure protein/l of culture) and three mutants (Δ(100-143) 2.6 mg/l, Δ(122-165) 14 mg/l, Δ(144-186) 9.9 mg/l).

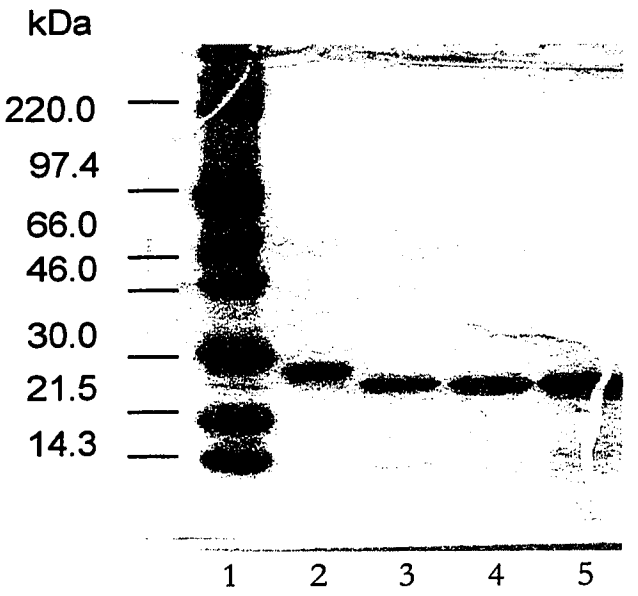

Figure III-2: SDS olyacrylamide Gradient Gel Electrophoresis of the Recombinant Proteins. (1: Molecular weight markers; 2: Rec. apoA-I; 3: Δ(100-143); 4: Δ(122-165); 5: Δ(144-186)). The purity of each protein was shown to be greater than 90 % as demonstrated by densitometric scan analysis.

However, this expression system was slightly modified in that it did not include rifampicin (after the induction of the T7 RNA polymerase) that would have specifically directed the expression of genes under the control of the T7 promoter. This modification resulted in a highly improved expression especially for one mutant [Δ(100-143)] that was hardly expressed in the classical system. The purified proteins have been shown to be pure at greater than 90 % as observed on SDS polyacrylamide gel (Fig. III-2).

Determination of the ability to form discoidal reconstituted lipoproteins (Lp2A-I)

The first study was performed to test the ability of each mutant to associate with PL. The objective was first to evaluate the optimal POPC/A-I molar ratio for each mutant that would produce homogeneous complexes. In a first attempt, 20, 30, 40, 50, 60, 80, 100, 130/1

ratios were tested. These complexes were prepared with the cholate dispersion / biobead removal method described by Sparks *et al.* (268). The resulting complexes were analyzed directly by non-denaturing gradient gel electrophoresis (8-25%) to determine their size and homogeneity (Fig. III-3). Whereas Rec.-apoA-I could form stable complexes containing two apoA-I molecules (Lp2A-I) with POPC over a wide range (20 to 130/1) and with a size varying between 9.5 and 10.5 nm, (Fig. III 3A), the three mutants formed stable and homogeneous Lp2A-I complexes over a lower and narrower POPC/apoA-I ratio range (20 to 40/1, Fig. III 3B, C, D). The size of the corresponding Lp2A-I was also reduced (7.5-7.6 nm for Δ(100-143) and 7.9-8.0 nm for the other two mutants). Above 40/1, each of the 3 mutants had a tendency to form large and heterogeneous complexes that contain 3 or more molecules of the protein per complex. Overall, these results suggest that the mutant apoproteins have a reduced capacity to bind PL. At the highest ratios, Δ(100-143) formed the largest and the most heterogeneous complexes, even larger than those formed by Rec.-apoA-I. It is also noteworthy that this mutant formed a diffuse band, while the other two mutants produced narrower bands.

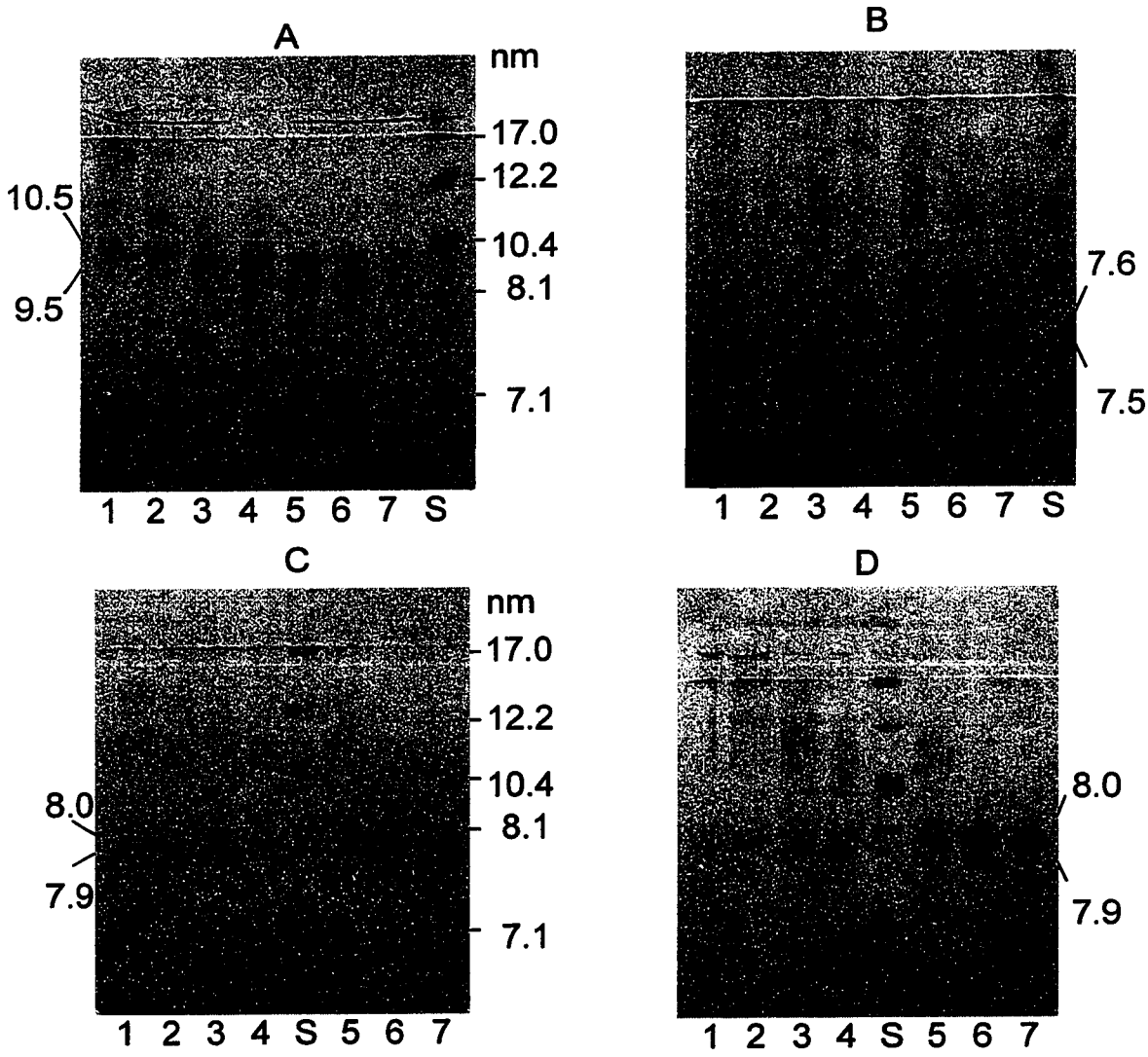

Figure III-3: Polyacrylamide Gradient Gel Electrophoresis (8-25%) of LpA-I Complexes Obtained with the Different Proteins and with Varying POPC Content.
Molecular weight markers were used to calculate the size of the resulting complexes. (complexes obtained with Panel A: Rec. apoA-I, Panel B: Δ(100-143), Panel C: Δ(122-165), Panel D: Δ(144-186)). Complexes with varying POPC/A-I molar ratio were loaded on the GGE (1. 130/1; 2. 100/1; 3. 60/1; 4. 50/1; 5. 40/1; 6. 30/1; 7. 20/1).

These results suggest that Δ(100-143) is the most affected in its lipid binding properties. The reduced size of the complexes formed by the mutants did not appear to be due

to a change in the morphology of the complex. All the mutants and Rec.-apoA-I (with POPC/A-I 30/1) were shown to form discoidal complexes as observed by electron microscopy (Fig. III-4).

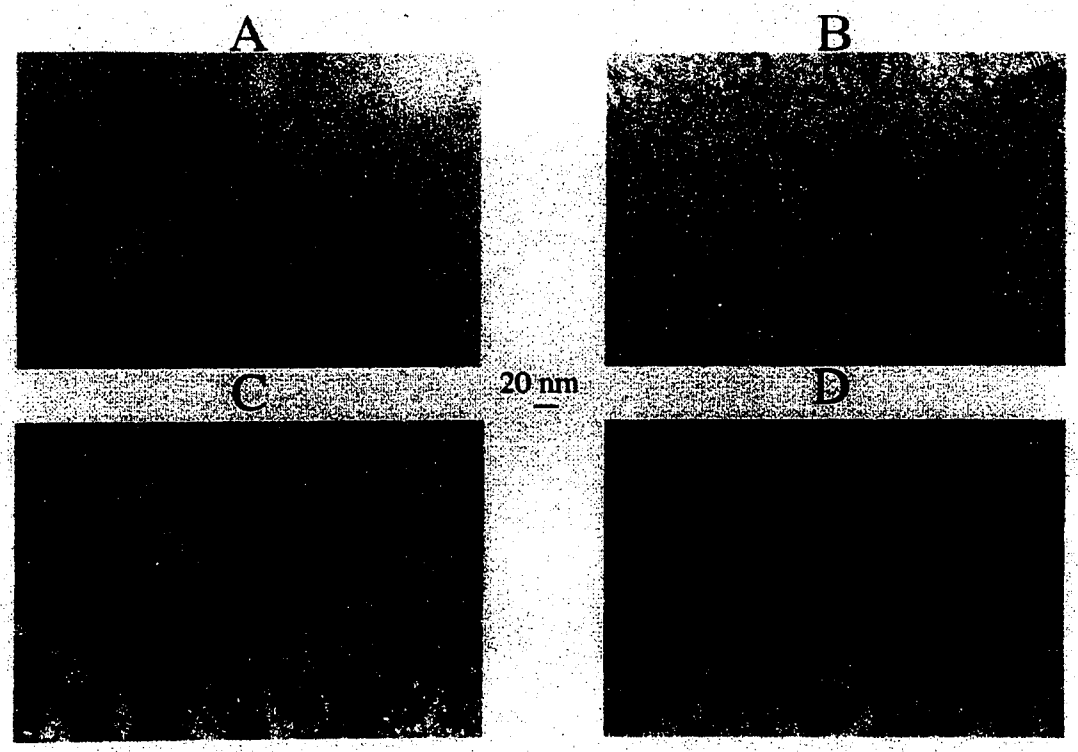

Figure III-4: Electron Microscopy of Negatively Stained POPC-Complexes Obtained with the Different Proteins.
All complexes were prepared using the cholate dispersion/biobeads removal method. Immediately before the experiment, they were dialyzed against 0.125 M ammonium acetate, 2.6 mM ammonium carbonate, 0.26 mM EDTA, then diluted at ≈0.2 mg/ml then diluted with sodium phosphotungstate (1% final w/v). The sample were applied on Formvar carbon coated grids and visualized on the microscope. [Panel A. Rec. apoA-I, Panel B. Δ(100-143), Panel C. Δ(122-165), Panel D. Δ(144-186)].

DMPC kinetic assays

The kinetics of association with DMPC were performed at 24°C and followed by the decrease in the turbidity at 325 nm, which reflects the formation of discoidal complexes. The results of the kinetics of association with DMPC are represented on Fig. III-5.

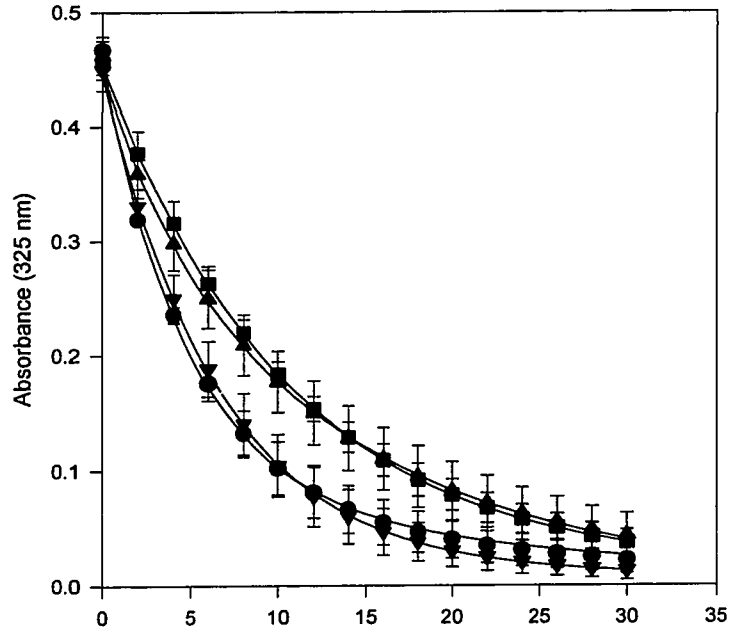

Figure III-5: DMPC Kinetics of Association with the Different Recombinant Apoproteins. DMPC was solubilized in TBS pH8 and the proper amount diluted in the same buffer (DMPC/A-I: 50/1) was added to the protein after a 10 min preincubation at 24°C. The reaction was followed for 30 min at 24°C for 30 min, at 325 nm in a thermo-controlled cell (▼: Rec. apoA-I, ●: Δ(100-143); ■: Δ(122-165); ▲: Δ(144-186)).

Rec.-apoA-I and mutant Δ(100-143) presented very similar kinetic properties. However, mutants Δ(122-165) and Δ(144-186) displayed very similar properties but their kinetics of association were reduced when compared to the other two proteins. All the complexes obtained exhibited a discoidal shape as demonstrated by the presence of rouleaux of stacked

disks by electron microscopy (not illustrated). When submitted to a native polyacrylamide GGE, at the ratio tested (50/1), the mutants produced slightly larger complexes as compared to Rec.-apoA-I. However, whereas the control protein produced Lp2A-I, the mutants formed predominantly Lp3A-I, as shown by cross-linking experiments, therefore explaining the difference in the size of the complexes formed. The results are therefore similar to those obtained with the POPC experiments. The decreased PL/apoA-I ratio, which is indicative of a reduced PL capacity, results in the formation of multi-apoA-I complexes (Lp3A-I with DMPC, in some cases Lp4A-I at the highest POPC/A-I ratios).

Circular dichroic analysis and stability of the mutants in lipid-free and -associated forms

The mutants were analyzed for their α-helical content and stability in discoidal Lp2A-I formed with POPC. The compositions of the complexes are presented in Table III-1. As expected by the preliminary experiments presented in Fig. III-3, the complexes obtained with the mutants were smaller than those obtained with Rec.-apoA-I complexes.

Table III-1: Composition and Size of Lp2A-I Complexes.

Protein	POPC/A-I* *(mol/mol)*	size *(nm)*
Rec. apoA-I	42 (10)	10.7 (0.6)
Δ(100-143)	24 (2)	7.9 (0.3)
Δ(122-165)	45 (6)	8.3 (0.5)
Δ(144-186)	35 (5)	8.0 (0.1)

*Average of three different preparations (±SD), Lp2A-I complexes as determined by cross-linking experiments.

Complexes incubated with increasing GdnHCl concentrations were analyzed by circular dichroism at 222 nm (Fig. III-6).

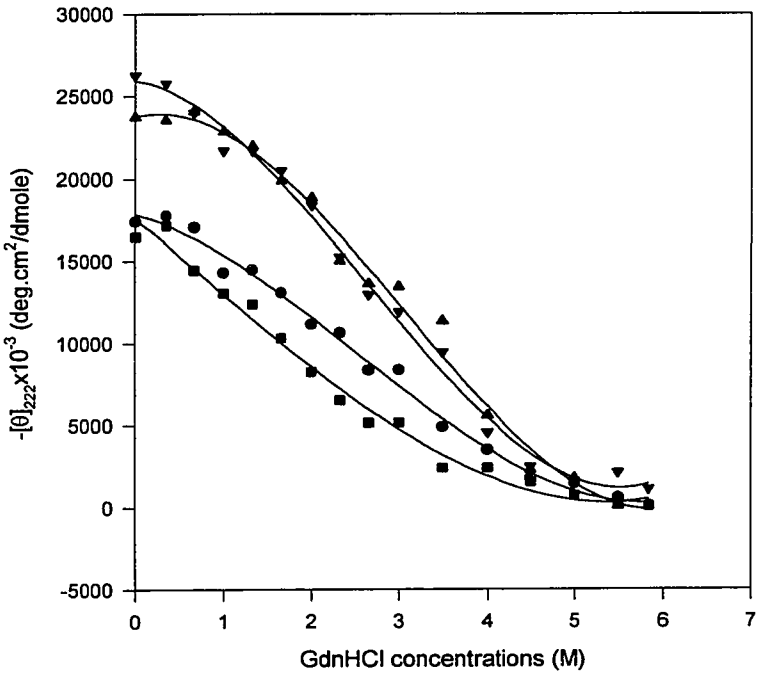

Figure III-6: Effect of GdnHCl on the α-Helix Stability of Lipid-Associated Wild-Type and Mutant ApoA-I.
(●: Rec-apoA-I; ■: Δ(100-143); ▲: Δ(122-165); ▼: Δ(144-186)). Aliquots of each protein (33 μg/ml) were preincubated with 0 to 6 M in phosphate buffer pH7.4 for 72 h. at 4°C. The molar ellipticity at 222 nm was then recorded on a Jasco J41A spectropolarimeter. Values are average of duplicate determination.

The stability of the α-helices was estimated according to the method developed by Sparks *et al.* (268). The results are presented in Table III-2. For the lipid-free form, no significant difference in the stability was evident for any of the mutant when compared to Rec.-apoA-I. However, when the stability of the Lp2A-I were compared, the mutant Δ(100-

143) was significantly less stable than the other two mutants which were significantly more stable than Rec.-apoA-I.

Table III-2: Denaturation Characteristics of Lipid-Free and -Bound Apoproteins.

Complex	$D_{½}$	ΔG_D^0	Δn
	(M Gdn HCl)	*(kcal / mol apoprotein)*	*(mol Gdn HCl/ mol apoprotein)*
-Lipid-free apoproteins			
Rec.-apoA-I	0.83 (0.1)	2.0 (0.3)	13.5 (1.9)
Δ(100-143)	0.74 (0.1)	2.1 (0.3)	15.9 (1.9)
Δ(122-165)	0.82 (0.1)	2.3 (0.1)	16.7 (0.9)
Δ(144-186)	0.79 (0.1)	2.2 (0.2)	15.9 (1.0)
Lipid associated apoproteins[a]			
Rec.-apoA-I	1.6 (0.5)	1.9 (0.2)	8.5 (3.8)
Δ(100-143)	2.1 (0.1)	1.8 (0.6)	6.7 (1.9)
Δ(122-165)	3.0 (0.1)	2.5 (0.2)*	6.5 (0.4)
Δ(144-186)	2.7 (0.1)	2.3 (0.2)*	6.7 (0.6)

[a] POPC complexes (composition indicated in Table III 1)
$D_{½}$: Midpoints of Gdn HCl denaturation.(±SD)
ΔG_D^0: Free energy of denaturation at zero Gdn HCl concentration. (±SD)
Δn: Gdn HCl bound during denaturation. (±SD)
* $p<0.05$, comparison vs. Rec. apoA-I.

When the α-helical content of the proteins was determined, similar trends were obtained (Table III-3). These results show that the stability is not necessarily correlated to the α-helix content of a protein, but may be affected by other parameter such as its charge properties. In both the lipid-free and -associated forms, Δ(100-143) has a significantly lower α-helical content than the other two mutants.

Table III-3: Calculated and Predicted Secondary Structures of Wild-Type and Mutant Apoproteins.

Complex	α-helix content[a] (%)	Number of helices predicted[b]	Number of helices calculated[c]
Lipid-free proteins			
Rec.-apoA-I	40 (1)	nd	nd
Δ(100-143)	33 (1)*		
Δ(122-165)	41 (3)		
Δ(144-186)	45(5)		
Lipid-associated proteins[d]			
Rec.-apoA-I	52 (4)	8	8
Δ(100-143)	49 (1)	6	6
Δ(122-165)	63 (2)*	6	8
Δ(144-186)	63 (2)*	6	8

[a] α-helicity determined by the measurement of the ellipticity at 222 nm.
[b] according to the model described (297).
[c] assuming that 17 residues participate in the formation of an α-helix.
[d] POPC complexes as described in Table III 1.
* $p<0.05$ comparison vs. Rec.-apoA-I.
nd: not determined.

Electrophoretic properties of the mutants

The electrokinetic analysis of the different proteins was performed on both the lipid-free and lipid-associated proteins by electrophoresis on agarose gel. The mobility, surface charge, charge density and valence per protein were calculated and are presented in Table III-4. This type of analysis allows for the determination of surface charge properties related to very fine conformational changes of a protein such as apoA-I in association with various lipids (48) These results need to be compared to the calculated p*I* of each protein [Rec.-apoA-I: 5.76; Δ(100-143): 5.91; Δ(122-165): 5.43; and Δ(144-186): 5.54]. Rec. -apoA-I exhibited an increased molar valence (-2.9/mol) as expected with the additional six His as already reported (Chapter

2). For all proteins, we also observed a decreased charge density when the proteins are associated with POPC. The mutant Δ(100-143) presents different properties compared to those of Rec.-apo-A-I, with, as expected a lower valence. Like Rec.-apoA-I, its charge density decreases in association with POPC but contrary to Rec.-apoA-I its valence decreases.

Table III-4: III Electrokinetic Analysis of Lipid-Free and Lipid-Associated Apoproteins.

Complex	Mobility[a]	Surface potential[b]	Charge Density[c]	Valence[d]
	(-μm.s^{-1}.cm.V^{-1})	*(-mV)*	*(X 10^3 esu / cm^2)*	*(/ mol protein)*
Lipid-free apoproteins				
Rec.-apoA-I	0.36	7.0	1.30	2.9
Δ(100-143)	0.27	5.2	1.05	1.6
Δ(122-165)	0.39	7.6	1.55	2.3
Δ(144-186)	0.38	7.3	1.48	2.2
Lipid associated apoproteins[e]				
Rec.-apoA-I	0.32	6.2	1.07	2.9
Δ(100-143)	0.31	6.1	0.95	1.9
Δ(122-165)	0.41	7.9	1.25	2.7
Δ(144-186)	0.42	8.1	1.28	2.8

[a] Corrected electrophoretic mobility (Agarose 0.5 %, pH8.6) ± 0.04 (SD).
[b] Potential at the complex surface ±0.6 (SD).
[c] Density of surface charge ±0.1 (assuming a globular morphology for the lipid-free proteins).
[d] Number of negative charge in electronic unit ±0.2 (SD).
[e] POPC complexes as described in Table III 1.

Compared to Rec.-apoA-I, Δ(122-165) and Δ(144-186) have greater surface potentials and charge densities in both lipid-free and lipid-associated forms. The predicted p*I* would also suggest an increased negative charge at pH 8.6 for the last two mutants. In the lipid-free form, they are, as expected, less charged than Rec.-apoA-I but in the lipid-associated form they have

a similar charge. We also observed that the p*I* observed for the mutants are not always well correlated with the charge properties determined in both the lipid-free and -associated forms. Thus p*I* is not the only factor that influences the electrokinetic properties; the conformation of the protein that is affected by the lipidated state is also important. Whereas Rec.-apoA-I formed complexes of ≈10.7 nm in size, the other proteins form much smaller complexes. This size difference could explain the decreased charge density of the complexes formed with Rec.-apoA-I compared to the complexes with Δ(122-165) and Δ(144-186). However, lipidation of Δ(100-143) did not significantly decrease its charge density, suggesting a different conformation in keeping with a reduced stability compared to the other mutants.

DISCUSSION

ApoA-I sequence was first determined by Brewer *et al.* (230). It is formed by repetitive segments of 11 residues (237), with six 22-mers and two 33-mers that form α-helices as proposed by several models of secondary structure (268; 278; 315; 317; 403). These helices would interact with different affinities with the PL (242). The different models proposed agree well on the secondary structure of apoA-I between residues 100-186 (represented in Fig. III-1). This domain would be formed by six amphipathic antiparallel α-helices interrupted by Pro and Gly residues. The orientation of these helices would be parallel to the PL acyl chain (278; 323). Among these helices, domain (100-121) was proposed by several groups to be important in the formation of a hinge domain and also found to be sensitive to limited proteolysis (297; 316; 318).

In this study, we clearly showed that the region between residues 100-186 is involved in the interaction with PL. We also present evidence that these helices have different structural

and physical properties. When the pair of helices 100-143 is eliminated, there is no effect on the initial association with PL. In contrast, when other pairs of helices (122-165 and 144-186) are deleted, the initial rate of association with PL is reduced. This suggests that helix 100-121 is not important for that step in keeping with the results of Palgunachari *et al.* (244), which indicate that the terminal amphipathic helices 44-65 and 220-241 are the most important for the initial binding of apoA-I to lipids. However, these authors also showed that helices 99-120 and 143-164 have a higher hydrophobic moment and affinity for PL than helices 121-142 and 165-186 (Table III-5).

Table III-5: Hydrophobic Moment and Hydrophobicity Properties of the Four Central Helical Segments of ApoA-I.

Helix	Hydrophobic Moment *(/Residue[a])*	Lipid Affinity *(kcal/mol[b])*
99-120	0.42	3.5
121-142	0.23	1.4
143-164	0.39	3.5
165-186	0.23	1.0

[a] As reported by Brouillette & Anantharamaiah (304).
[b] As reported by Palgunachari *et al.* (244).

The above study and ours are not directly comparable, and the results may reflect the intrinsic difference between studies based on single helices versus our own approach with pairs of adjacent helices which are intended to minimize disruption of interhelix salt bridges that play a role in the interaction with PL and/or the stabilization of the secondary structure as also suggested by others (300; 307; 316). In support of this view, Vanloo *et al.* (305) were able to obtain complexes with synthetic peptides formed by two adjacent helices of apoA-I, even with certain helices that according to Palgunachari *et al.* (244) do not form by themselves any

complex with lipids. Our observations on the charge density showing an increased charge density for Δ(122-165) and Δ(144-186) (Table III 4) are compatible with a more compact and more stable structure for Δ(122-165) and Δ(144-186), possibly by stabilization with interhelix salt bridges or hydrogen bonds. We therefore conclude that the decreased stability of mutant Δ(100-143) is due to the loss of helix 100-121 that may not be important for initial PL association but for interaction with adjacent helices. The association of apoA-I with lipids usually enhances the stability of the protein. However, this is not the case with the small discoidal complexes described here for either Rec.-apoA-I or any of the mutants. The association of Δ(122-165) and Δ(144-186) with lipids increases their stability compared to Rec.-apoA-I. We propose that the deletion of two helices in this case generates a mutant protein more adaptable to the small discoidal complexes formed.

The central α-helices of apoA-I have been proposed by several groups to be important in the formation of discoidal complexes with varying PL content (277; 297; 316). Some of these helices may interact with the PL only in larger LpA-I, therefore allowing apoA-I to associate with varying amounts of PL. In this study, we have observed that variation in the initial POPC/apoA-I ratio was associated with a 1 nm size variation of the complexes formed with Rec.-apoA-I. In contrast, no change in size was evident for any of the three mutants (≈0.1 nm, value that is below the accuracy of the method). Thus, deletion of any pair of central helices in apoA-I abolishes the ability to bind varied amounts of PL implying that the process of LpA-I size variation involves more than one pair of helices and maybe also cooperativity between these helices. The denaturation study demonstrated that elimination of helix 100-121 results in a reduced stability of the α-helices. This property may be explained either by the interaction with adjacent helices with salt-bridge formation (as explained above) or by a

specific conformation of this domain that would allow the helix to form a hinge between two domains. Helix 100-121 is also a class Y amphipathic α-helix as opposed to the class A amphipathic α-helix formed by the other helices examined in this study (Segrest *et al.*, 1994). It is characterized by the presence of positively charged residues at the polar-nonpolar interface as well as at the center of the polar face, a charge distribution particularly adapted to interhelix salt bridges and in keeping with the abundance of predicted salt bridges (307). More recently, we suggested (313) the existence of an interaction between the central and N-terminal domains of apoA-I that may be important in the stabilization of the LpA-I complex formed. The N-terminal domain 8-22 was classified as a class G* amphipathic α-helix that is characterized by a random distribution of positively and negatively charge residues on the polar face (242). Therefore, the elimination of helix 100-121 may abolish an important tertiary motif in apoA-I that stabilizes the conformation and the association with PL due to the presence of salt bridges and hydrogen bonds. Possible interactions between pairs of helices via putative salt bridges between, may include for example, Lys_{107}-Lys_{108} and acidic residues such as Glu_{91}-Glu_{92}, or Lys_{94}-Lys_{96} and Asp_{103}-Asp_{104}. Another possible ionic interaction could be between Lys_{107}-Lys_{108} and the acidic residues located in the N-terminal domain (Asp_1-Glu_2): these two domains have been shown to exhibit similar changes of immunoreactivity when exposed to different lipid environments (313). It is also noteworthy that the absence of Lys_{107} has been associated with significantly altered lipid-binding properties compared to wild-type apoA-I (426).

Another interesting result of this study is presented in Table III-3 where we compared the predicted (according to the model) and the calculated number of helices (based on the α-helix content and assuming an average helix length of 17 residues, (307)) for the different mutants. The result obtained for Δ(122-165) and Δ(144-186) are unexpected and show the

presence of two supplementary helices as compared to the model whereas Δ(100-143) behaves accordingly to the model. When domain 122-165 or 144-186 is deleted, it appears that new helices are formed, probably in the N-terminal domain. These newly formed α-helices contribute to the increase in the overall stability of the protein associated with PL. However, these helices neither would directly interact with PL nor have an important role in the association with PL since these mutants, Δ(122-165) or Δ(144-186), do not produce significantly larger or PL enriched Lp2A-I as compared to Δ(100-143). This is even more clear when the number of helix associated with the PL acyl chains for each complex was calculated. Based on the size of the lipoprotein formed, we calculated [according to (277)] that all mutants have a very similar number of associated helices (7), as compared to 9.5 helices for Rec. apoA-I. These results suggest that the new helices observed with the last two mutants do not directly interact with PL. The slight differences with the calculations obtained for the α-helix content may be explained by an expanded structure of the helices in association with PL.

The ability of these mutants to activate LCAT, one of the major functions of apoA-I, will also be of special interest since several authors have assigned the domain responsible of the activation of LCAT to the central region of apoA-I. Well-characterized and defined (homogeneous) preparations of complexes including cholesterol will be required. However obtaining homogeneous particle preparations with these mutants has been difficult. Since they cannot bind high levels of POPC, addition of only a few molecules of cholesterol per molecules of mutants may lead to a reorganization of the complexes and result in the formation of heterogeneous preparations. A similar result has also been observed for wild-type apoA-I at high cholesterol/POPC molar ratio where the complexes formed were more heterogeneous. Preliminary experiments with the mutants and Rec.-apoA-I show that addition

of as little as 5 molecules of cholesterol with 30 POPC results in the formation of heterogeneous preparations for all apoA-I mutants with central deletions (Frank P.G. & Marcel Y.L., unpublished results), suggesting a critical role for the POPC/cholesterol ratio in the formation of these discoidal complexes.

In conclusion, this work is consistent with the existence of a PL-binding domain located between residues 100-186, which has an important role in the ability of apoA-I to associate with varying PL contents. Within this domain, the sequence 122-186 appears more important in the initial association with PL whereas helix 100-121 is more important in the stabilization of the complex formed possibly through interhelix interactions. Experiments with these interesting mutants will indicate whether the central domain of apoA-I is important in determining apoA-I ability to activate LCAT and to promote cellular cholesterol efflux (Chapter 4 and 5).

Chapter 4: IMPORTANCE OF CENTRAL α-HELICES OF HUMAN APOLIPOPROTEIN A-I IN THE MATURATION OF HIGH-DENSITY LIPOPROTEINS

SUMMARY

Three central apoA-I deletion mutants [Δ(100–143), Δ(122–165), and Δ(144–186)], which have been shown to exhibit altered lipid-binding properties in the previous chapter, were tested for their ability to activate LCAT and promote cellular cholesterol efflux. When recombined with phospholipids to form homogeneous LpA-I containing equivalent amounts of POPC and tested for their ability to promote diffusional cholesterol efflux from normal ^{3}H-cholesterol labeled fibroblasts, each mutant and the wild-type recombinant protein (Rec.-apoA-I) promoted cholesterol efflux with very similar rates at all the concentrations tested. These experiments showed that all apoA-I mutant particles could acquire cellular cholesterol with similar affinity and binding capacity. However, when the cell-incubated LpA-I were incubated with purified LCAT, two mutants, Δ(122-165) and Δ(144-186), appeared incapable of activating the enzyme. To directly determine their ability to activate LCAT, each mutant and the control apoproteins were recombined with equivalent amounts of cholesterol and phospholipid and incubated with the purified enzyme. The results show that whereas deletion of residues 100-143 has little effect on LCAT activation, deletion of residues 122-165 or 144-186 results in an inability of the mutants to promote cholesterol esterification. In conclusion, our results show that no specific sequence in the central domain of apoA-I is required for efficient diffusional cholesterol efflux from normal fibroblasts, however, residues 144-186 appear critical for optimum LCAT activation and cholesteryl ester accumulation. Since

deletion of residues 144-186 also perturbs phospholipid association and prevents the formation of large LpA-I particles (Chapter 3), the data suggests that this pair of α-helices plays an important role in the maturation of HDL.

INTRODUCTION

One of the mechanisms by which apoA-I acquires cellular cholesterol efflux involves the dissociation of free cholesterol from the plasma membrane and its acquisition by an acceptor (i.e., HDL). In this process, which will be examined in this chapter, (termed diffusional efflux, since it is mediated by aqueous diffusion), the rate-limiting step is the desorption of FC from the plasma membrane. Lipid composition of acceptors may be a key factor in this process since several studies have shown that the PL (133; 194), and FC (193; 338) content of HDL could affect cholesterol flux between cells and lipoproteins. The ability of apoA-I to interact with lipid surfaces suggests a possible role for its amphipathic α-helices to promote cellular cholesterol efflux from the plasma membrane. Several studies using mAbs against apoA-I have implicated the central domain of apoA-I as being important in this process (342-345). Banka *et al.* (342) found that antibodies binding to residues 74-110 could inhibit cholesterol efflux to HDL. Similarly, Luchoomun *et al.* and Sviridov *et al.* (344; 345) demonstrated the importance of domains around residues 165 and 140-150, respectively. In a different study, Fielding *et al.* (343) showed that an epitope of apoA-I (region 137-144) was more exposed in preβ_1-HDL than in other subspecies of HDL. The central domain was suggested to be much more labile than other domains and, therefore, could interact more easily with the plasma membrane to promote cellular cholesterol efflux (337).

Cholesterol esterification, mediated by LCAT, appears to promote the flux of cholesterol through the HDL pool by stimulating the formation of cholesterol esters that are transferred to apoB-containing lipoproteins, by CETP, and then cleared via the LDL-receptor pathway in the liver (6). LCAT activation was previously shown to be affected by HDL lipid composition (312; 367; 369) and apoA-I conformation (289; 312; 314). To identify the domain(s) of apoA-I involved in LCAT activation, Anantharamaiah *et al.* (378) constructed a peptide corresponding to a dimer of the consensus amino acid sequence obtained for the eight 22-mer predicted α-helices of apoA-I. This peptide gave maximal LCAT activation only when residue 13 of the helix was replaced with a glutamic acid. Since only helices within residues 66-121 contain a Glu at position 13 of each helix (residues 78 and 111 of apoA-I), it was concluded that this region is the LCAT activating domain. Studies using mAbs against apoA-I to inhibit LCAT activation have suggested the involvement of the central domain of apoA-I corresponding to residues 95-175 (379; 427; 428).

In the present work, we have analyzed the role of the central domain of apoA-I in both diffusional cholesterol efflux from ^{3}H-cholesterol-labeled fibroblasts and in LCAT activation. To this end, three characterized apoA-I mutants were produced, each by deletion of two consecutive α-helices, Δ(100–143), Δ(122–165), and Δ(144–186) (Chapter 3). These mutant apoA-I molecules were previously shown to retain their ability to form lipoprotein particles, even though 2 of the mutants, Δ(122–165) and Δ(144–186), showed reduced kinetics of association with phospholipids (Chapter 3).

EXPERIMENTAL PROCEDURES

Materials

1-palmitoyl 2-oleyl phosphatidylcholine (POPC) and cholesterol were obtained from Avanti Polar Lipids (Birmingham, AL). [1α, 2α-^{3}H]cholesterol was purchased from DuPont NEN (Boston, MA). PMA was obtained from Sigma. All other reagents were analytical grade. Human skin fibroblasts (GM00038B) were obtained from the Coriell Institute for medical research (Camden, NJ).

Production of the Mutant Proteins

Wild-type apoA-I with an N-terminal extension Met-Arg-Gly-Ser-$(His)_6$-Met (Rec.-apoA-I) was expressed in a bacterial system as described in Chapter 3.

Preparation and Characterization of Reconstituted Lipoproteins

Reconstituted discoidal lipoproteins were produced using the cholate dispersion / Biobeads removal method as described in Chapter 2. The initial POPC/A-I molar ratio used for all proteins was 25/1 (Cholesterol was also added for LCAT activation studies). All complexes were reisolated by gel-filtration on a Superdex 200 column (Pharmacia). Final composition of the complexes after purification was determined as described in Chapter 2 and 3.

Cell Culture and Cholesterol efflux

Human skin fibroblasts were cultured in a CO_2 incubator at 37°C as previously described (Chapter 2). They were maintained between passage 15-25 in DMEM low-glucose, 10% FBS, 4 mM glutamine, and antibiotics (100 u/ml penicillin, 100 μg/ml streptomycin). For

the efflux experiments, cells were grown in 24-well plates (seeded at 2.75×10^4 cells per well in 500 µl of media) for 48 h. After two washes with PBS containing 0.2 % fatty acid-free BSA (Sigma) and once with PBS only, cells were labeled with 20 µCi/ml [1α,2α-^{3}H]cholesterol in DMEM (with supplements) containing 5% FBS for 48 h. After labeling, cells were incubated with DMEM (with supplements) containing 1 mg/ml fatty acid-free BSA for 24 h. The efflux experiment was started by washing the cells twice with DMEM, 0.2% BSA, and once with DMEM alone. Appropriate concentrations of lipoprotein complexes in media were added to each well and the experiment started. Aliquots were taken at different times and treated as previously described (Chapter 2). At the end of the experiment, cells were solubilized in 1 N NaOH, protein and the radioactivity were determined. Results were expressed as the percentage of labeled cholesterol removed from the cells as a function of time.

Lecithin:Cholesterol Acyl Transferase Assay

LCAT was purified and cholesterol esterification experiments were conducted as previously described (289). In these assays, two types of substrate were used. [1α,2α-^{3}H]cholesterol-labeled LpA-I prepared by incubation of LpA-I (Table IV-1, series 1) with [1α,2α-^{3}H]cholesterol-labeled fibroblasts were re-isolated by gel-filtration on Superdex 200. A second series of [1α,2α-^{3}H]cholesterol-labeled LpA-I were prepared by the cholate dispersion / Biobeads removal methods with POPC and cholesterol. LpA-I prepared with [1α,2α-^{3}H]cholesterol and either Rec.-apoA-I or the mutant proteins were tested for their ability to stimulate the LCAT reaction. The LCAT reaction mixture consisted of varying amounts of Lp2A-I, 1.5 mg of fatty acid free BSA, 5 mM β-mercaptoethanol and reaction buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA and 1 mM NaN_3, pH 8.0) to 450 µl final volume. Conditions

for assay were generally as described before (289). Under these conditions, initial rates were estimated with minimal substrate conversion.

RESULTS

Preparation of Reconstituted Lp2A-I with Rec.-apoA-I and the Mutant Proteins

To analyze the importance of apoA-I domains in diffusional efflux, we prepared reconstituted lipoproteins with very similar phospholipid to protein molar ratios. This experimental design eliminates compositional effects on efflux due to differing lipid content, an interpretative concern with other studies (329; 346). Stable, homogeneous Lp2A-I were therefore produced using the cholate dispersion / Biobeads removal method (268). For each recombinant protein, we have utilized the optimum POPC/apoA-I molar ratio of 25/1, which gives rise to a completely homogeneous preparation of Lp2A-I, as we have previously shown (Chapter 3). Under these conditions, Rec.-apoA-I formed larger complexes as compared to the mutant proteins. After re-isolation by gel filtration, the final composition of each Lp2A-I was essentially the same (Table IV-1, series 1).

For LCAT studies, proteins were recombined with POPC and cholesterol (including ^{3}H-cholesterol) as described above. Re-isolated complexes contained an average of one molecule of cholesterol and a comparable phospholipid content per apoprotein (Table IV-1, series 2). In these complexes, the recovery of cholesterol was low as compared to other studies, apparently due to the low phospholipid content (289). Electron microscopy showed that all complexes appear as rouleaux of stacked discs, indicative of a discoidal structure (data not shown).

Table IV-1: Properties of the Discoidal Lp2A-I used in Efflux with ^{3}H-Cholesterol-labeled Fibroblasts.

	Protein	Initial Composition POPC/Chol/apoA-I (mol/mol/mol)	Final Composition POPC/Chol/apoA-I (mol/mol)
Series 1	Rec.-apoA-I[1]	25/0/1	30/0/1
	Δ(100-143)[1]	25/0/1	27/0/1
	Δ(122-165)[1]	25/0/1	28/0/1
	Δ(144-186)[1]	25/0/1	29/0/1
Series 2	Rec.-apoA-I[2]	35/5/1	43/1/1
	Δ(100-143)[2]	35/5/1	34/1/1
	Δ(122-165)[2]	35/5/1	26/1/1
	Δ(144-186)[2]	35/5/1	30/1/1

[1]Values represent the average of three different preparations. These complexes were used in all cholesterol efflux experiments (including the preparation LpA-I containing cell-derived ^{3}H-cholesterol used for LCAT assays described in Fig. IV-3).
[2]Values represent the average of two different preparations. These complexes were used in LCAT activation assays described in Fig. IV-4.

Ability of ApoA-I Mutants to Promote Cholesterol Efflux from Fibroblasts

The ability of Rec.-apoA-I and the three central deletion mutants to promote cholesterol efflux from human skin fibroblasts was first tested at the same molar concentration (1.70 μM). As shown in Fig. IV-1, the efflux measured between 5 and 90 min to the different LpA-I was linear and essentially identical.

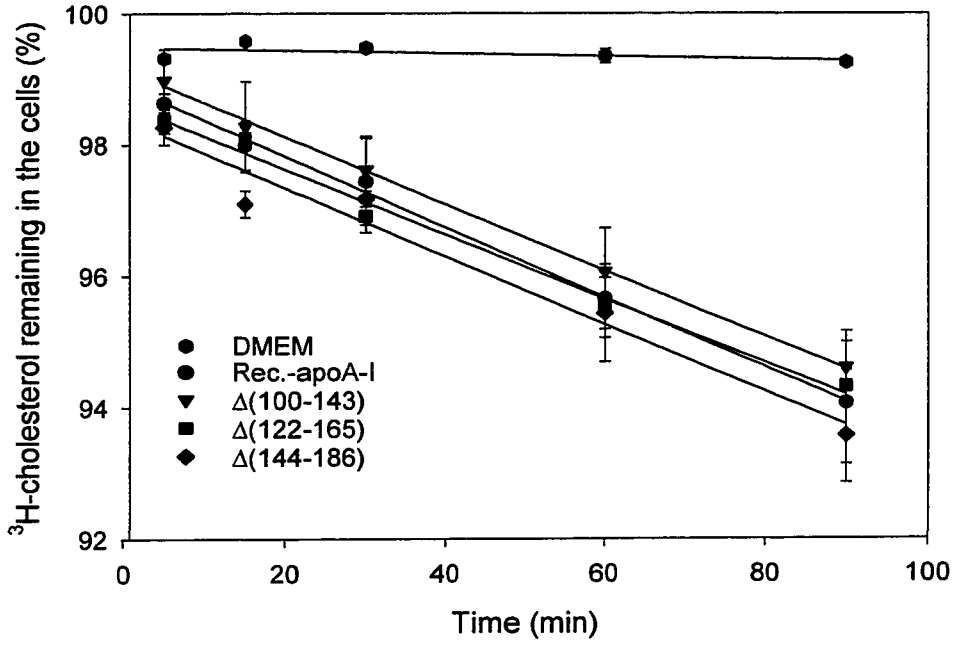

Figure IV-1: Cholesterol Efflux from Normal ^{3}H-Cholesterol-Labeled Fibroblasts in the Presence of Lp2A-I Complexes.
All complexes were incubated at the same concentration of apoA-I protein (1.70 μM). Aliquots of media were removed at different times of incubation and counted. The efflux was expressed as the % of ^{3}H-cholesterol removed from the cells at different times of incubation. Experiments were performed in triplicate and are representative of three independent experiments.

The effect of the acceptor concentration on efflux was determined using LpA-I concentrations varying between 0 and 4 μM. The rate of efflux was plotted as a function of LpA-I concentration with the efflux curves fitted using a single compartment model as described by others (335). When the reciprocal of the rates of efflux (k) was plotted against the reciprocal of the concentrations of each LpA-I used (Fig. IV-2), linear regressions demonstrated a correlation coefficient of 0.99.

The plots obtained with Rec.-apoA-I and each of the mutants proteins had very similar slope and intercept, suggesting that all complexes had very similar "affinity" (K_d) for cholesterol and similar "binding capacity" (B_{max}) (Table IV-2).

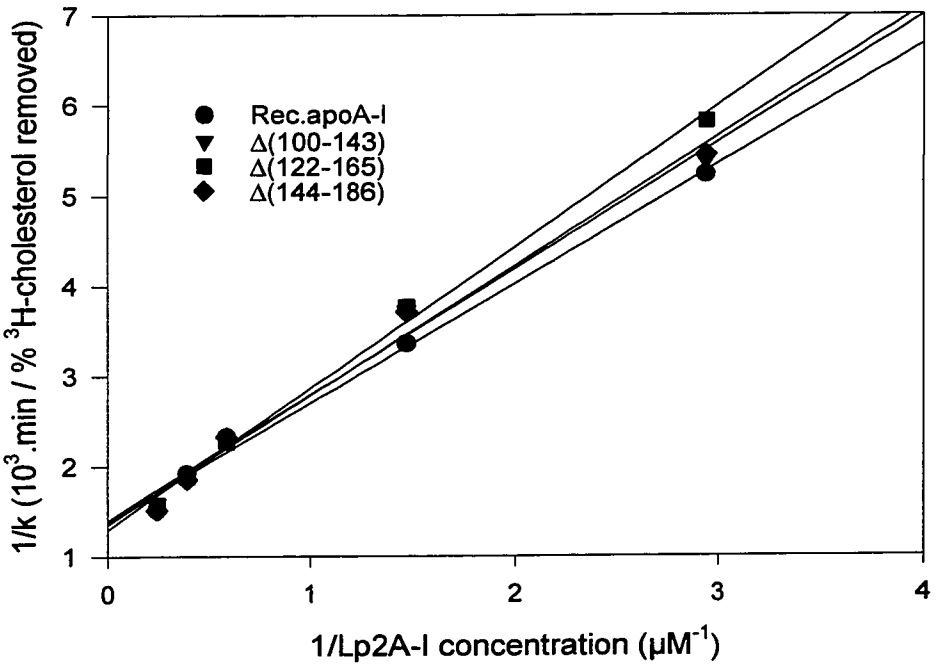

Figure IV-2: Double-Reciprocal Plot of the Rate Cholesterol Efflux versus the Lp2A-I Concentration used for each Protein.
Efflux curves at every concentration were fitted with a monoexponential model ($y = A\exp(-kx) + B$) and the reciprocal of the rate k of efflux was plotted against the reciprocal of the protein concentration.

Table IV-2: Kinetics Parameters of Cholesterol Efflux to Lp2A-I Complexes Formed with the Different Proteins.

Protein in the complex	B_{max} (10^{-4}.% of ^{3}H-cholesterol removed/min)	K_d (μM)
Rec.-apoA-I	7.26	0.96
Δ(100-143)	7.19	1.01
Δ(122-165)	7.73	1.21
Δ(144-186)	7.42	1.06

Apparent Kd and B_{max} were after determination of the x and y intercepts of a linear regression of the double-reciprocal plots presented in Fig. IV-2.

Reaction of Lecithin: Cholesterol Acyltransferase with LpA-I Labeled with Cell-derived ^{3}H-Cholesterol

To determine if LpA-I containing cell-derived ^{3}H-cholesterol could efficiently activate LCAT, reconstituted LpA-I (Table IV-1) were incubated with ^{3}H-cholesterol-labeled fibroblasts for 2 h. Cell-incubated LpA-I were re-purified by gel-filtration on Superdex 200 and then characterized for size and composition. No significant change in size and composition was observed after incubation with cells, other than enrichment in cholesterol (≈0.8 μg FC/mmol of protein). Assays were then performed to characterize their ability to activate LCAT (Fig. IV-3). Rec.-ApoA-I and Δ(100-143) had very similar ability to promote cholesterol

esterification whereas Δ(122-165) and Δ(144-186) could only activate LCAT at a level of ≈10 % of the control.

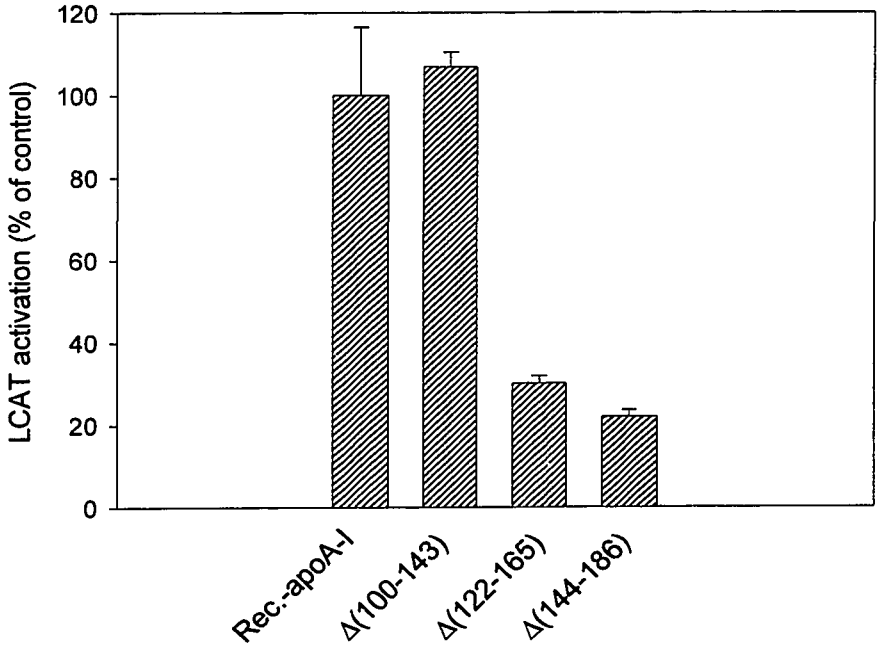

Figure IV-3: Reaction of LCAT with LpA-I re-isolated after Incubation with ^{3}H -Cholesterol-Labeled Fibroblasts.
LpA-I were prepared as indicated in the cholesterol efflux experiments (composition indicated in Table IV-1, series 1). After a 2-h incubation with ^{3}H-cholesterol labeled fibroblasts, LpA-I were re-isolated by gel-filtration on Superdex 200. After analysis of the purified LpA-I, they were used in an LCAT activation experiment as described under *Material and Methods*. Percentage of LCAT activation (as compared to control Rec.-apoA-I) is indicated at a concentration of 1.21 μM for all proteins.

Activation of LCAT by ApoA-I Deletion Mutants

Reconstituted LpA-I were prepared with ^{3}H-cholesterol (composition shown in Table IV-1) and were then incubated with purified LCAT. Saturable substrate curves were obtained for all LpA-I particles (Fig. IV-4).

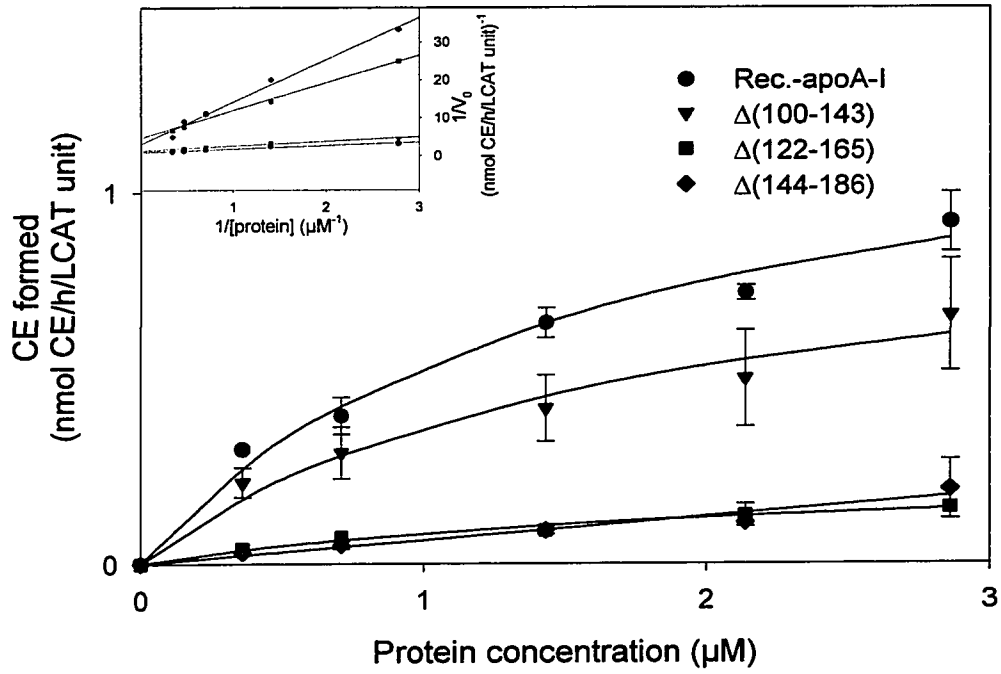

Figure IV-4: Effect of ApoA-I Central Domain Deletions on LCAT Activation.
Experiments were performed with LpA-I (composition indicated in Table IV-1, Series 2) as described in *Material and Methods. Inset,* double-reciprocals plots are shown where reciprocal of initial velocities are plotted against reciprocal of protein concentrations.

These plots indicate that Rec.-apoA-I and Δ(100-143) have a similar ability to activate LCAT whereas Δ(122-165) and Δ(144-186) show markedly reduced ability to promote cholesterol esterification. Double reciprocal plots were derived from these graphs (Fig. IV-4, inset), and appK_m and appV_{max} values were estimated (Table IV-3) and shown to be similar to previously reported values for similar discoidal Lp2A-I (289; 312). Kinetic values are consistent with the curves shown in Fig. IV-3 and indicate markedly reduced rates of cholesterol

esterification (appV_{max} and k_{cat}) and increased appK_m for both Δ(122-165) and Δ(144-186) whereas Δ(100-143) could still promote a very efficient LCAT activation.

Table IV-3: LCAT Activation Properties of ApoA-I and Central Deletion Mutants.

Protein	K_m (μM apoA-I)	V_{max} (nmol CE/h)	k_{cat} (V_{max}/K_m)
Rec.-apoA-I	0.91	1.05	1.15
Δ(100-143)	0.84	0.71	0.84
Δ(122-165)	1.59	0.22	0.14
Δ(144-186)	3.76	0.34	0.09

Estimated values are representative of three separate experiments.

DISCUSSION

In this study, we have examined the ability of LpA-I formed with central deletion mutants of apoA-I to promote cholesterol efflux from normal fibroblasts. This efflux is thought to be mediated through a passive aqueous diffusion process and is dependent on the lipoprotein properties (69). Previous studies have demonstrated the importance of LpA-I-associated phospholipid in diffusional efflux (133; 194). The cholesterol content of lipoproteins has also been shown to influence efflux by modifying the cholesterol gradient between cells and lipoproteins in the medium (193; 338). To eliminate compositional effects, we have used homogeneous Lp2A-I complexes with similar POPC/A-I ratios (Table IV-1, series 1). Despite changes in the apoprotein stability (Chapter 2), apoA-I mutant-containing

lipoproteins have maintained their ability to interact with and retain cellular cholesterol. Other studies, in which efflux was determined on the basis of the phospholipid content of the LpA-I, have also reported that the deletion of selected domains of apoA-I has no effect on diffusional efflux of cholesterol from mouse L-cell fibroblasts (329) or from HepG2 cells (346) (Table IV-4). It should be noted, however, that in the study of Gillotte et al. (329), reconstituted lipoproteins that were compared, differed in their lipid content, and contained between 2 and 4 apoA-I molecules per complex. Likewise, in the study of Sviridov et al. (346), evaluation of apoA-I mutants for their relative abilities to promote cholesterol efflux from HepG2 cells was confounded by the comparison of LpA-I that differed in lipid and apolipoprotein composition. As both the phospholipid and cholesterol content of LpA-I can influence the ability to mediate cellular cholesterol efflux, it is difficult, in these two studies, to dissociate the effects that can be specifically attributed to deletions in apoA-I primary structure from those that are due to the lipid composition of the LpA-I particles. Furthermore, in each study, very different types of mutations have been examined. The three mutants that were evaluated by Gillotte et al. (329) have deletions that are distributed throughout apoA-I primary structure (residues 44-126, 139-170, and 190-243, respectively) whereas Sviridov et al. (346) have used a series of carboxy-terminally truncated apoA-I variants (deletion of residues 222-243, 210-243, 150-243, and 135-243, respectively). However, despite these interpretative concerns, our data appears consistent with the conclusion derived from these two studies; the deletion of any helices within apoA-I has little effect on the ability of the lipidated protein to promote cellular diffusional cholesterol efflux.

Table IV-4: Effect of ApoA-I Mutation or Neutralizing Antibodies on Cellular Cholesterol Efflux.

Domain examined	Cell type	Basis of Comparison[1]	Results[2] (% control)	Reference
Mutagenesis studies				
44-126	L-fibroblasts	phospholipid	77 (NS)	(329)
139-170	L-fibroblasts	phospholipid	95 (NS)	(329)
190-243	L-fibroblasts	phospholipid	87 (NS)	(329)
223-243	HepG2 cells	phospholipid	≈80-90 (NS)	(346)
		particle number	≈90 (NS)	(346)
151-243	HepG2 cells	phospholipid	≈70 (S)	(346)
		particle number	≈100 (NS)	(346)
136-243	HepG2 cells	phospholipid	≈65 (S)	(346)
		particle number	130 (S)	(346)
Antibodies inhibition studies				
74-105	THP-1 monocytes	NA	<50 (S)	(342)
96-111	THP-1 monocytes	NA	<50 (S)	(342)
137-144	Human skin Fibroblasts	NA	≈60 (S)	(343)
28-82	Ob1771 Adipocytes	NA	≈75 (S)	(344)
149-186	Ob1771 Adipocytes	NA	≈75 (S)	(344)
140-147	HepG2 cells	NA	≈60 (S)	(345)
149-150	HepG2 cells	NA	≈60 (S)	(345)

1. For mutagenesis studies. Apo-A-I-containing lipoprotein were compared based on phospholipid concentration or particle number.
NA: Not applicable.
2. S: Efflux significantly different from that of control.
NS: Efflux not different from control.

Several studies using mAbs against apoA-I have implicated the central region of apoA-I as being important in cholesterol efflux. (summarized in Table IV-4). Binding of mAbs to apoA-I has, however, been shown to induce conformational changes in apoA-I that can affect its ability to interact with lipids (148). In addition, amino acid residues that are widely separated within the primary structure of a protein may be close together in the native protein. Thus, the ability of an anti-apoA-I mAb to block LpA-I-mediated cellular cholesterol efflux may reflect steric hindrance rather than the proximity in apoA-I primary structure of the

corresponding epitope and an apoA-I functional domain. Indeed, we have reported in an earlier study with discoidal Lp2A-I that the mAb 3G10 (epitope 98-121) could compete with mAb 4A12 (epitope 173-205) for binding to apoA-I (313). This observation is of interest since mAbs binding close to the epitope recognized by 3G10 have been shown to affect cellular cholesterol efflux (342-345). Therefore, the observed effects of anti-apoA-I mAbs on cholesterol efflux may not be related to the function of specific domains in the apoA-I molecule, but may be due to secondary indirect effects resulting from the mAb-apoA-I interaction.

The K_d values of Rec.-apoA-I and other mutants in Lp2A-I for cellular cholesterol with normal fibroblasts are in the range of 1 μM (Table IV-2). Lower values were reported for lipid-free apoA-I in the case of cholesterol-loaded fibroblasts (0.04 μM), in contrast to a K_d of 1.6 μM obtained for HDL. Thus, our reconstituted Lp2A-I and HDL interact similarly with cellular cholesterol (429). In our experiments, the calculated K_d are slightly lower than the dissociation constant of apoA-I for lipid (291). This indicates that, under these conditions, the efflux to Lp2A-I reflects association of apoA-I with directly accessible membrane lipids, with little or no contribution of intracellular pools. Therefore, central deletion mutants identified in this study can still interact properly with cholesterol from the plasma membrane of normal ^{3}H-cholesterol-labeled fibroblasts.

Several groups have attempted to determine the structural requirements for efficient activation of LCAT by apoA-I. We have summarized these complex and, in some cases, contradictory results in Table IV-5.

Table IV-5: Effect of ApoA-I Mutation or Neutralizing Antibodies on LCAT activation.

Technique	Domain examined	Substrate	Results[1] (% control)	Reference
Mutagenesis	146-186	Reconstituted LpA-I	90 (NS)	(398)
	113-124	Unilamellar vesicle	47 (S)	(382)
	148-186	Unilamellar vesicle	0.5 (S)	(382)
	Pro_{99}→His	Unilamellar vesicle	93 (NS)	(382)
	Pro_{121}→His	Unilamellar vesicle	77 (NS)	(382)
	99-120	Unilamellar vesicle	15 (S)	(383)
	121-142	Unilamellar vesicle	25 (S)	(383)
	143-164	Unilamellar vesicle	2 (S)	(383)
	165-186	Unilamellar vesicle	2 (S)	(383)
	44-126	Reconstituted LpA-I	45 (S)	(324)
	139-170	Reconstituted LpA-I	9 (S)	(324)
	190-243	Reconstituted LpA-I	11 (S)	(324)
	123-166	Reconstituted LpA-I	5 (S)	(430)
Proteolysis	193-243	Reconstituted LpA-I	100 (NS)	(431)
Antibodies inhibition	95-121	Reconstituted LpA-I	20 (S)	(427)
	96-122	Reconstituted LpA-I	2-25 (S)	(379)
	135-148	Reconstituted LpA-I	10-45 (S)	(379)
	149-186	Reconstituted LpA-I	10-60 (S)	(379)
	96-174	Reconstituted LpA-I	20 (S)	(428)

1. S: Esterification rates significantly different from that of control.
NS: Esterification rates not different from control.

Anti-apoA-I mAbs have been tested for their ability to inhibit the interaction between enzyme and substrate. These studies identified a region of apoA-I composed of residues 95-186 that may participate in LCAT activation. As discussed above and elsewhere (379), anti-apoA-I mAbs could potentially modulate LCAT activation by several different mechanisms. ApoA-I deletion mutants have also been used to identify domains of apoA-I that are responsible for activation of LCAT. Some of the earlier studies identified, not only the same large central domain, but also the C-terminal domain of apoA-I as being important for LCAT activation. However, many studies used vesicles incubated with lipid-free apoA-I mutants as substrates. With this approach, a decreased LCAT reaction may reflect decreased lipid-binding properties of apoA-I mutants with C-terminal deletions (324; 325).

To determine the LCAT activation properties of the three central deletion mutants, homogeneous reconstituted LpA-I were prepared with very similar POPC/cholesterol/A-I molar ratio. This ensures that we are primarily studying a protein effect and not secondary effects due to differing lipid composition that may affect apoA-I conformation (312). Two of the mutants examined, Δ(122-165) and Δ(144-186), were found to have significantly lower LCAT activation. This would suggest that helix 144-164, which is common to the two deletion mutants, may participate in the activation of LCAT. Importantly, helix 144-164 of apoA-I has been the most conserved of the apoA-I helices during evolution, which would be consistent with this domain having a functional role. As previously hypothesized (6; 312), this helix may interact with residues 151-174 of LCAT and allow access to free cholesterol in discoidal LpA-I. This model of interaction, however, remains to be confirmed. Immunoreactivity and NMR studies suggested a possible involvement of this apoA-I domain in the binding of cholesterol (309; 313). Mutagenesis study performed on LCAT by Wang et al. (432) have

shown that Glu 154, 155, and 155 are not important for LCAT activity but may be involved in the binding of cholesterol. The region 151-174 may therefore indirectly interact with apoA-I through the binding with HDL cholesterol, a process that may result in a conformational change of LCAT and its activation.

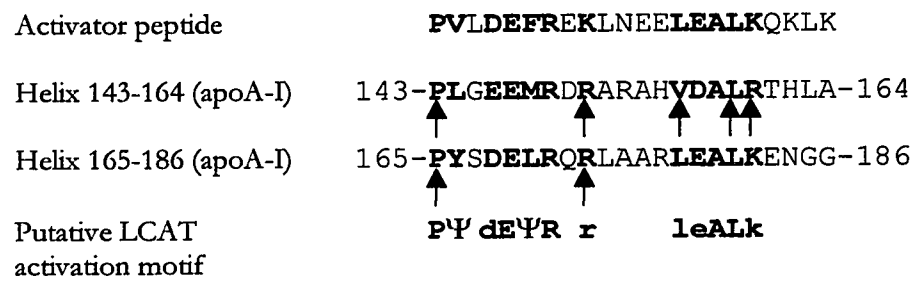

Figure IV-5: Alignment of the LCAT activator peptide with amino acids 143-164, 165-186 of apoA-I.
The residues highlighted "in bold" reflect common or conserved amino acids that may be required for LCAT activation. An LCAT activation motif, which reflects common or conserved residues between the three aligned sequences, is proposed. Lowercase letters (d, r, l, e, and k) indicate conserved residues (the preponderant residue is shown) and the symbol Ψ denotes the presence of hydrophobic residues in the three aligned sequences. Mutations of residues identified by arrows have been identified in patients with reduced HDL levels and may result in lower LCAT activity (see text for details).

Interestingly, deletion of residues 100-143 has little effect on the ability apoA-I to activate LCAT, a finding that is in contrast with the results of others (378). In this work, the presence of a Glu residue at position 13 of helices between aa 66-121 (residues 78 and 111 of apoA-I), was shown to be essential for efficient LCAT activation. Sequence alignment of this activator peptide with apoA-I helices indicates that region 143-164 has only 27.3 % identity and shows that Glu at position 13 of an α-helix is not essential for LCAT activation. Surprisingly, when helix 165-186 is aligned with the activator peptide, an identity of 45.5 % is observed (Fig. IV-5). Such homology could suggest that helix 165-186 may be important in

LCAT activation. We propose that residues of the activator peptide that are identical or homologous to residues of helix 143-164 and 165-186 may be essential for LCAT interaction. Alignment of helices 143-164, 165-186, and the LCAT activator peptide indeed reveals a common motif, particularly with respect to hydrophobic and charged residues, that may be critical to the activation of LCAT by apoA-I (identified as an LCAT activating domain in Fig. IV5).

NMR and X-ray crystallography suggest that an interaction between helices 143-164 and 165-186 is unlikely (320; 433). It therefore appears that rather than interacting together, these two helices may facilitate the association of LCAT with LpA-I, possibly through electrostatic interactions between these two helices (charged residues identified) and residues 151-174 of LCAT. Hydrophobic residues identified in Fig IV-5 could stabilize this interaction with the lipid interface. The N-terminal domain of apoA-I, which has been shown to affect LCAT activation (312; 379) and to interact with the central domain of apoA-I (313) may also affect this interaction. In small discoidal LpA-I, interactions between the N-terminus and central domain of apoA-I may prevent the interaction of LCAT with apoA-I and lipid. In large LpA-I, this access is facilitated since the interaction between these two apoA-I domains is reduced. Other studies from Minnich et al. (382), Sorci-Thomas et al. (383), Holvoet et al. (324), and Dhoest et al. (430) have also suggested an important role for residues 144-186 of apoA-I in the activation of LCAT.. Sorci-Thomas et al. have recently produced apoA-I variants in which residues 143-164 and 220-241 were exchanged (434) or in which the sequence of helix 143-164 was altered so that the hydrophobic face of this domain was rotated by 80° (435). Both variants are defective in their ability to activate LCAT. Therefore, helix 143-164 of apoA-I not only appears to be essential for LCAT activation but may also require

adjacent domains such as residues 164-186. This is supported by the present study and others (382; 383; 430).

In support of this view, mutations of the essential amino acids shown in Fig. 5 have been associated with reduced LCAT activation properties of the resulting variants [Pro_{143}→Arg (436), Δ(146-160) (437), Leu_{151}→Cys (438), Val_{156}→Glu (439), Leu_{159}→Arg (440), Arg_{160}→Leu (441), Arg_{173}→Cys (442)]. A recent report from Miettinen et al. showed that a Leu_{159}→Arg substitution in apoA-I results in a 40 % reduction of LCAT activation (440) and also absence of large plasma HDL_2 in patients carrying this mutation.. Other mutations (Arg_{160}→Leu, (441); Val_{156}→Glu, (439) are associated with reduced HDL-cholesterol levels, HDL size and LCAT activation for Val_{156}→Glu (not demonstrated for Arg_{160}→Leu but suggested by the authors). Mutations of residues within the domain 144-186 of apoA-I appear to affect both its interaction with lipid and its ability to activate LCAT. The reduced HDL size that is associated with inheritance of these mutant alleles may reflect a reduced ability of the variants to activate LCAT. The region of apoA-I composed of residues 144-186 may help HDL to accumulate CE and form large HDL and could therefore be an important regulator of HDL maturation.

Chapter 5: APOLIPOPROTEIN A-I C-TERMINAL DOMAIN MEDIATES CELL BINDING AND LIPID EFFLUX IN MACROPHAGES BUT NOT IN FIBROBLASTS

SUMMARY

In this chapter, we have studied the role of specific amphipathic α-helices in apoA-I on the ability of this apoprotein to promote cholesterol efflux from cholesterol-loaded human skin fibroblasts and macrophages. Four apoA-I mutants were designed, each by deletion of a pair of predicted adjacent helices, and expressed in *Escherichia coli.* The first three mutants lacked two consecutive central α-helices [Δ(100-143), Δ(122-165), Δ(144-186)] whereas the final mutant lacked the C-terminal domain [Δ(187-243)]. The central deletion mutants have been previously shown to exhibit altered phospholipid binding properties (Chapter 3). However, when recombined with phospholipids, their ability to promote cholesterol efflux from unloaded fibroblasts was not affected (Chapter 4). The ability of each of the four mutants, as lipid-free apoprotein, to promote cholesterol efflux from cholesterol-loaded fibroblasts was also similar. When the mutant apoproteins were compared at equivalent molar concentrations, the rate of efflux that each mutant promoted was very similar to that observed with Rec.-apoA-I. Therefore, domain 100-243 of apoA-I does not contain any specific sequence required for efficient lipid efflux from cholesterol-loaded fibroblasts. When efflux ability was tested with a different cell line (THP-1 monocyte-derived macrophages), which stores higher levels of cholesterol, the mutant Δ(187-243) exhibited a markedly reduced ability (70 % reduction) to promote cholesterol efflux compared to Rec.-apoA-I and the central domain deletion mutants. This reduced capacity to bind and retain cellular cholesterol was

associated with a similarly decreased ability (70 %) of this mutant to associate with cellular phospholipids and form lipoproteins. Competitive binding studies with cholesterol-loaded macrophages showed that only Δ(187-243) could not compete effectively with ^{125}I-labeled Rec.-apoA-I. The binding of this mutant to cholesterol-loaded macrophages was reduced by 6-fold, as compared to Rec.-apoA-I. Overall, these results show that cholesterol efflux from cells such as fibroblasts, which accumulate moderate amounts of free cholesterol and no or little cholesterol ester, does not require any specific sequence between residues 100 and 243 of apoA-I. In contrast, cholesterol and phospholipid efflux from cholesterol-loaded macrophages involves a pathway that is dependent on the binding of apoA-I to macrophages and appears to be mediated by the C-terminal amphipathic α-helices. This domain may be necessary for the initial binding of cellular lipids and/or for interaction with a macrophage-specific cell surface-binding site.

INTRODUCTION

Two different mechanisms of cholesterol efflux from cells have been described. The first one is the non-specific, aqueous diffusion pathway (69), in which cholesterol molecules desorb from the cell membrane to transfer by passive diffusion through the aqueous phase to phospholipid-containing acceptors, principally lipoprotein particles. This pathway has been examined in Chapter 4. The second pathway is specific and involves an acceptor, which interacts with a cell surface binding site and promotes cholesterol efflux (70). Only lipid-free or lipid-poor apoA-I appears to promote specifically this type of pathway, whereas HDL can

promote both kind of efflux. In the present chapter, we have addressed the role of apoA-I domains in the second, specific, cholesterol efflux pathway.

Four apoA-I mutants were prepared to examine the role of the central and C-terminal domains of apoA-I. These mutants were previously shown to maintain the ability of apoA-I to form lipoproteins, albeit with a generally reduced phospholipid binding capacity for the central deletions (Chapter 3) and the C-terminal deletion (324; 325) and with reduced kinetics of association with phospholipids for mutants Δ(122–165), Δ(144–186). We have also shown that, despite their altered lipid-binding properties, the central deletion mutants, associated with lipids in the form of LpA-I, could still promote a very efficient cholesterol efflux from normal, quiescent fibroblasts (Chapter 4).

In the present study, we have analyzed the ability of the lipid-free apoA-I mutants to elicit specific lipid efflux from intracellular sterol stores. For this purpose, we have examined both cholesterol and phospholipid efflux from cholesterol-loaded fibroblasts or monocyte-derived macrophages (THP-1) to the lipid-free mutants. We have also measured the ability of the mutant apoproteins to bind to the cells. The results demonstrate that, in contrast to previous studies (342; 345), no particular sequence within the central domain of apoA-I is required for cholesterol efflux from either cholesterol-loaded fibroblasts or macrophages. There are however important differences between human skin fibroblasts and macrophages in the effect of a C-terminal deletion in apoA-I on interaction with the cells and lipid efflux. Deletion of the C-terminal domain of apoA-I is associated with reduced binding of the protein to THP-1 macrophages, and decreases in both phospholipid and cholesterol efflux.

EXPERIMENTAL PROCEDURES

Materials

1-palmitoyl 2-oleyl phosphatidylcholine and cholesterol were obtained from Avanti Polar Lipids (Birmingham, AL). [1α, 2α-^{3}H]cholesterol and [methyl-^{3}H]choline chloride were purchased from DuPont NEN (Boston, MA). PMA was obtained from Sigma. All other reagents were analytical grade. Human skin fibroblasts (GM00038B) and human THP-1 monocytes were obtained from the Coriell Institute for medical research (Camden, NJ) and from the American Type Culture Collection (# ATCC TIB-202), respectively.

Construction of the carboxyl-domain deletion mutant cDNA

All cDNA constructs were prepared in the laboratory of Dr. Eric Rassart (Université du Québec à Montréal, Montréal, Québec). Construction of the central deletion mutants was previously described (Chapter 3). Deletion of nucleotides encoding residues 187-243 was performed in a similar manner using a 36-base oligonucleotide (5'-GCGGCGGCGGGCGCCTCA / GCCGCCGTTCTCCTTGAG-3'). This oligo was complementary to 2 portions of 18 nucleotides each located upstream and downstream respectively of the DNA sequence to be deleted. This oligo was annealed to the denatured plasmid and served as a primer in the repair reaction that was performed according to the method of Kunkel (423). The DNA of the corresponding mutant was completely sequenced prior to its transfer into the expression vector.

Production of the Mutant Proteins

Wild-type apoA-I with an N-terminal extension Met-Arg-Gly-Ser-$(His)_6$-Met (Rec.-apoA-I) was expressed in a bacterial system as previously described. Four mutants, Δ(100-143), Δ(122-165), Δ(144-186), and Δ(187-243) were produced as previously described (Chapter 3). After purification on Nitriloacetic acid agarose (NTA, Qiagen), the purified samples were dialyzed against 5 mM NH_4HCO_3, 1 mM EDTA, 0.02 % NaN_3, and lyophilized. Proteins were then stored at -20°C.

Cell Culture and Cholesterol efflux

Human skin fibroblasts were cultured in a CO_2 incubator at 37°C as previously described (Chapter 2). They were maintained between passage 15-25 in DMEM low-glucose, 10% FBS, 4 mM glutamine, and antibiotics (100 u/ml penicillin, 100 μg/ml streptomycin).

For experiments performed with human skin fibroblasts, cells were seeded in 6-well plates at a density of 6×10^4 cells/well in DMEM with supplements and 10 % FBS. After 48 h, media was replaced with DMEM containing the supplements, 5 % FBS, 200 μg/ml cholesterol from a cholesterol-rich dispersion, and 50 μg/ml LDL. The lipid dispersion was prepared as described by Arbogast *et al.* (443), with a cholesterol/phospholipid molar ratio of 3. After 3 days of loading, which resulted in a doubling of the cholesterol content of the cells, media was changed to DMEM containing the supplements, 5 % FBS, and 15 μCi/ml ^{3}H-cholesterol dispersed in 0.1 % ethanol (% final volume of media) for 24 h. Before each efflux experiment, cells were washed 3 times with DMEM and then incubated with DMEM containing 2 mg/ml fatty acid-free BSA (Sigma) for 4 hours. For the efflux experiments, cells were washed 3 times with DMEM and then incubated with DMEM containing the lipid-free apoprotein and 0.2 %

fatty acid-free BSA. Media aliquots were taken at different times of incubation and treated as previously described (Chapter 2). At the end of the experiment, cells were solubilized in isopropanol, and their cholesterol content was determined by gas chromatography (444) after extraction of cellular lipids following the method of Bligh & Dyer (445). Results were expressed as the percentage of labeled cholesterol remaining in the cells as a function of time.

Human THP-1 monocytes were grown in RPMI 1640 containing 10 % FBS, 100 u/ml penicillin and 100 μg/ml streptomycin, 5.10^{-5} M β-mercaptoethanol, 2 mM glutamine. To differentiate these cells into macrophages, monocytes were seeded in 6-well plates at a density of 2×10^{6} cells/well in the same medium containing 100 nM phorbol 12-myristate 13-acetate (Sigma). After 72 h of differentiation, THP-1 macrophages (adherent cells) were washed with RPMI 1640 and incubated with complete media containing 75 μg/ml acetylated LDL (prepared as described by Basu *et al.* (446)), 10 μCi/well ^{3}H-cholesterol or 10 μCi/well ^{3}H-choline, 100 nM PMA, and 0.2 % fatty acid free BSA for 48 h. After the loading period, cells were incubated in RPMI 1640 containing 0.2 % fatty acid free BSA, 100 nM PMA for 24 h. Before efflux experiments, cells were washed with RPMI 1640 and media containing the lipid-free proteins was added (in 0.2 % fatty acid free BSA). Efflux experiments (cholesterol or phospholipid) were conducted as previously described for the fibroblasts. In the case of phospholipid efflux, media was extracted to remove any free ^{3}H-choline. Results were also expressed as % ^{3}H-cholesterol or ^{3}H-phospholipid remaining in the cells as a function of the incubation time. At the end of the experiment, cells were solubilized in isopropanol for phospholipid, free and total cholesterol analyses (Boehringer Mannheim enzymatic kit) or in 0.5 N NaOH for protein determination.

Characterization of the complexes formed during efflux

After efflux, media was recovered and concentrated using Centricon concentrators (Amicon, Beverly, MA). Samples of media were loaded on native 8-25% gradient gel electrophoresis or on Beckman Spe Agarose gel. Gels were transferred to nitrocellulose and were detected using a combination of mAbs against apoA-I (2F1, 5F6, and A44; (297)), followed by an anti-mouse IgG HRP-conjugated polyAb. Presence of apoA-I was revealed by BM Chemiluminescence Blotting Substrate (Boehringer Mannheim).

DMPC kinetics analysis

The ability of the different proteins to clear a DMPC solution was determined as previously described in Chapter 3.

Cell Binding Studies

Macrophages were prepared and cholesterol-loaded as described above. After a 24h incubation with RPMI 0.2 % BSA, cells were washed with RPMI and incubated for 3 h with ^{125}I-labeled Rec.-apoA-I (500,000 cpm/well, 24-well plate) and varying concentration of the cold competing protein. Cells were then washed 3 times with RPMI and solubilized in 1N NaOH. Radioactivity and protein content of the cell extract were determined and the percentage of ^{125}I-Rec.-apoA-I bound to the cells was determined and expressed as a function of the concentration of cold competing protein. In experiments where the affinity of apoproteins for macrophages was determined, experiments were conducted as followed. Macrophages were loaded with acetylated-LDL as indicated above. After one day of incubation in RPMI 0.2 % fatty acid free BSA, 100 nM PMA, cell were washed and incubated

with varying concentrations of ^{125}I-labeled apoprotein [Rec.-apoA-I or Δ(187-243)] for 2 h. At the end of the incubation, cells were washed three time with PBS and solubilized in 0.5N NaOH. Radioactivity and protein were subsequently determined.

RESULTS

Cholesterol efflux from cholesterol-loaded fibroblasts

To study efflux to lipid-free apoA-I, human skin fibroblasts were loaded with cholesterol using LDL and cholesterol-enriched phospholipid dispersions as described by others (443). In these assays, cell cholesterol content was doubled following cholesterol loading as previously described (189); 15.3 ± 4.0 μg FC / mg cell protein in control cells as compared to 26.9 ± 2.2 μg/ml in cholesterol-loaded cells).

As reported by Bielicki *et al.* (447), cholesterol loading of fibroblasts significantly enhanced efflux to lipid-free apoA-I (i.e., 5 % of the cellular cholesterol removed from the cells in 24 h). In contrast, experiments performed with normal (non-loaded) fibroblasts showed that lipid-free apoA-I could promote a very low cholesterol efflux, similar to that of media devoid of apoprotein (versus 5 % after 1 h for Lp2A-I, see Chapter 4). It should also be noted that the rate of cholesterol efflux to lipid-free apoA-I studied here is significantly lower than that to lipid-associated apoA-I (i.e., Lp2A-I) studied in Chapter 4.

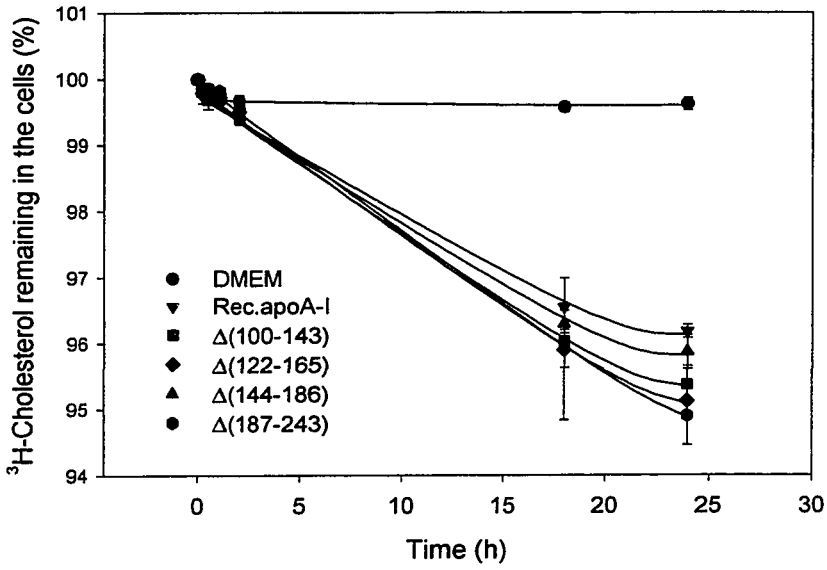

Figure V-1: Cholesterol Efflux from Cholesterol-loaded Fibroblasts to Lipid-Free Proteins. After cholesterol loading and labeling (see Methods), cells were incubated with the lipid-free apoprotein (1.70 μM). Aliquots of media were removed at different times of incubation and counted. The efflux was expressed as the percentage of ^{3}H-cholesterol removed from the cells at the different times of incubation. Experiments were performed in triplicate and are representative of three independent experiments.

The experiments with lipid-free mutants were therefore conducted over a 24-h period (Fig. I-1). When the cholesterol-loaded cells were incubated with the same molar concentrations of Rec.-apoA-I or each of the central or C-terminal deletion mutants, no significant difference in the rate of efflux promoted by each apoprotein was observed.

Cholesterol efflux from cholesterol-loaded macrophages

The interaction of lipid-free apoA-I with macrophages was previously shown to permit the mobilization of cholesterol from a PKC-sensitive pool (104). To analyze the domain-

specificity of this interaction with apoA-I, human THP-1 monocytes were differentiated in the presence of phorbol ester and loaded with cholesterol using acetylated-LDL. This procedure resulted in a significant increase in the cellular cholesterol content of the cells as compared to the non-loaded cells (97.0 ± 9.7 vs. 48.1 ± 4.1 μg total cholesterol/mg of cell protein respectively). It is important to note that these cells also showed a much higher cholesterol content as compared to the cholesterol-loaded fibroblasts (97.0 ± 9.7 vs. 26.9 ± 2.2 μg total cholesterol/mg of cell protein respectively). For cholesterol-loaded macrophages, cholesterol was composed of 40 % CE and 60 % FC, on average, a result similar to previously reported values (448), whereas no CE was detected in cholesterol-loaded fibroblasts. Interestingly, when these cholesterol-loaded cells were incubated with the lipid-free apoproteins, the mutant with a deletion of the C-terminal domain had a significantly lower ability to promote cholesterol efflux. As compared to Rec.-apoA-I and the other mutants, Δ(187-243) showed a 70% decrease in its ability to remove cholesterol from macrophages after 24 h of incubation (Fig. V-2).

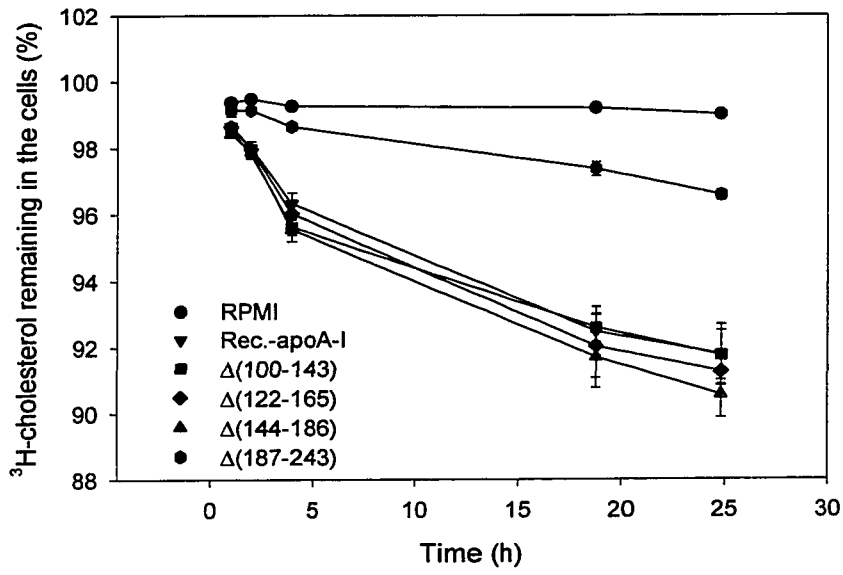

Figure V-2: Cholesterol Efflux from Cholesterol-loaded and ^{3}H-Cholesterol-labeled Macrophages in the Presence of Lipid-Free Proteins.
After cholesterol loading and labeling (see Methods), cells were incubated with the lipid-free apoprotein (1.70 μM). Aliquots of media were removed at different time of incubation and counted. The efflux was expressed as the percentage of ^{3}H-cholesterol removed from the cells as a function of time. Experiments were performed in triplicate and are representative of three independent experiments.

Phospholipid efflux from cholesterol-loaded macrophages

To determine if the decreased ability of Δ(187-243) to promote cholesterol efflux was due to or accompanied by a decrease in ability to associate with phospholipids, we also determined the efflux of phospholipid from cholesterol-loaded macrophages (Fig.V-3). Similar to the cholesterol efflux experiments, the C-terminal deletion mutant demonstrated a 70% reduction in its ability to promote phospholipid efflux after 24h of incubation when compared to Rec.-apoA-I and the other deletion mutants.

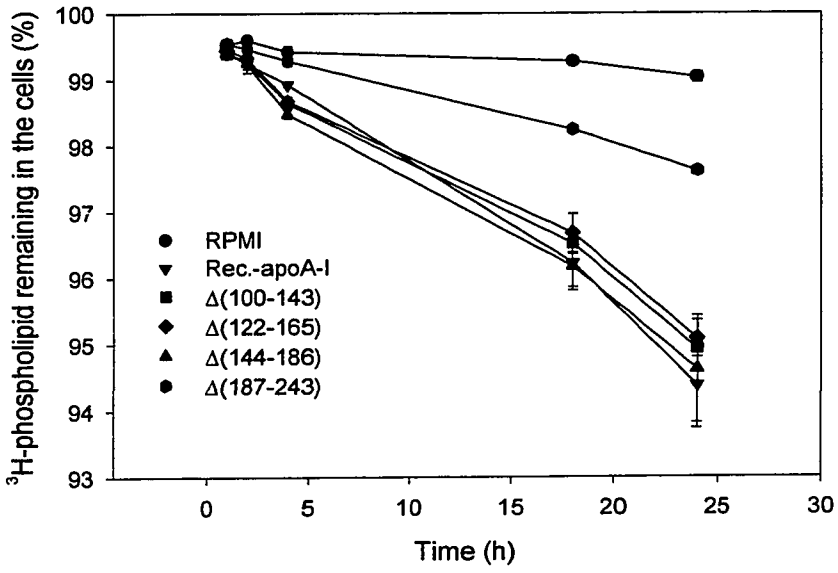

Figure V-3: Phospholipid Efflux from Cholesterol-loaded and Phosphatidyl ^{3}H-Choline-labeled Macrophages in the Presence of Lipid-Free Proteins.
After cholesterol loading and ^{3}H-choline labeling (see Methods), cells were incubated with the lipid-free apoprotein (1.70 μM). Aliquots of media were removed at different times of incubation, and the lipids extracted by the method of Bligh and Dyer (445) and counted. The efflux was expressed as the % of ^{3}H-phospholipid removed from the cells at the different times of incubation. Experiments were performed in triplicate and are representative of three independent experiments.

Properties of the re-isolated complexes after incubation with cholesterol-loaded macrophages

Media obtained before and after incubation of native and mutant apoproteins with macrophages were concentrated and electrophoresed on either 8-25% native gradient gel to determine changes in particle size (Fig. V-4A,B) or on agarose gels to detect changes in charge (Fig. V-4C).

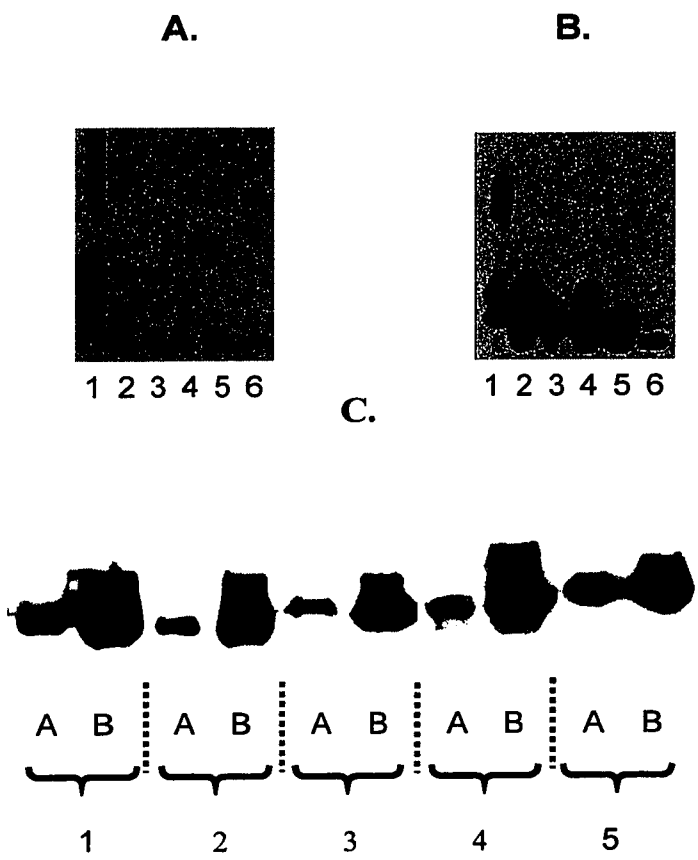

Figure V-4: Characterization of the Complexes formed after Incubation with Cholesterol-loaded Macrophages.
Samples of media obtained before and after incubation with cholesterol-loaded macrophages were loaded on a native 8-25% gradient gel electrophoresis (gel A. before efflux, no incubation with cells, gel B. after efflux) [lanes 1 to 6 represent total HDL, Rec.-apoA-I, Δ(100-143), Δ(122-165), Δ(144-186), and Δ(187-243) respectively] or on an agarose gel [gel C, lanes A before efflux and lanes B. after efflux and lanes 1 to 5 represent Rec.-apoA-I, Δ(100-143), Δ(122-265), Δ(144-186), and Δ(187-243)] respectively). In both cases, gels were transferred on a nitrocellulose membrane and the proteins were detected using mAbs against apoA-I (2F1, 5F6, and A44). The enhanced signal observed with samples after efflux may be due to an increased immunoreactivity of the apoproteins after lipidation.

All proteins except Δ(187-243) could form lipoprotein complexes upon incubation with cholesterol-loaded macrophages as shown by the increase in the size of the molecule. It is interesting to note that complexes obtained with Rec.-apoA-I presented a similar charge pattern to that previously observed by Forte *et al.* (449) with CHO cells expressing human apoA-I. After incubation with the cells, the mutant Δ(187-243) was unique in that it still

migrated as a single band on an agarose gel, in contrast to the other proteins, which all exhibited a second band with a faster electrophoretic mobility (Fig. V-4C).

DMPC clearance assays

To determine if the ability of Δ(187-243) to bind PL was impaired, DMPC clearance experiments were performed. The kinetics of association with DMPC were carried out at 24°C and followed by the decrease in the turbidity at 325 nm, which reflects the formation of discoidal complexes. The results of the experiments are represented in Fig. V-5. We have previously shown that Rec.-apoA-I and Δ(100-143), which have similar kinetics of association with DMPC, present slightly enhanced rates of association with DMPC compared to Δ(122-165) and Δ(144-186) (Chapter 3). In the present study, we observed that Δ(187-243) displayed a significantly reduced kinetics of association with DMPC ($t_{½}$ of 60.3 min for Δ(187-243) versus 11.3 min for Rec.-apoA-I) This is also significantly lower than those observed for Δ(122-165) and Δ(144-186) (Chapter 3).

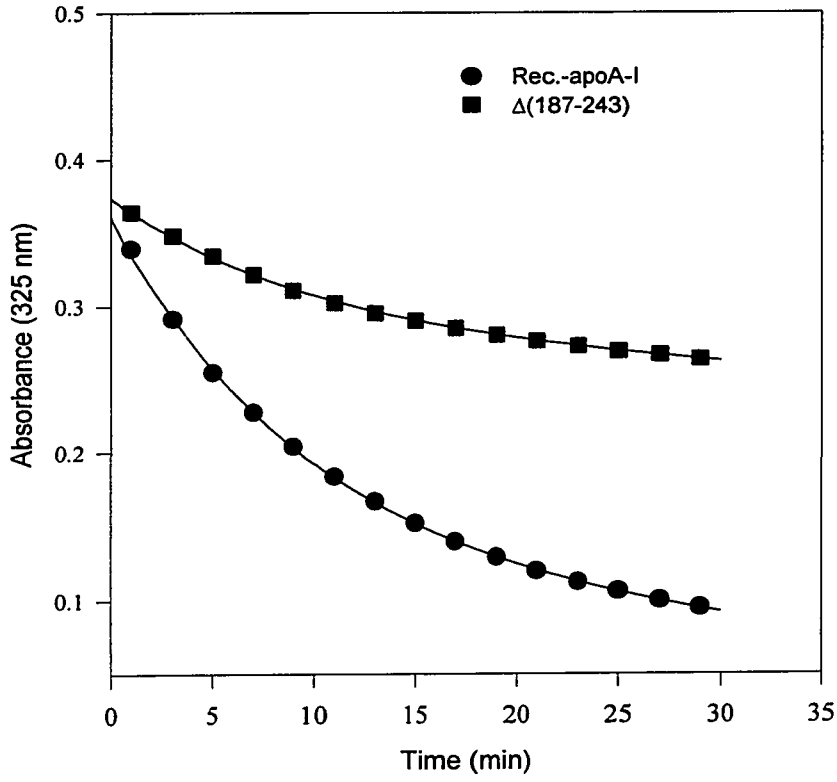

Figure V-5: DMPC Kinetics of Association with the Different Recombinant Apoproteins. DMPC were solubilized in TBS pH8 and the appropriate amount diluted in the same buffer (DMPC/A-I: 50/1) was added to the apoprotein after a 10 min preincubation at 24°C. The reaction was followed for 30 min at 24°C for 30 min, at 325 nm in a thermo-controlled cell.

Binding of lipid-free apoA-I mutants to macrophages

In order to determine if the decreased efflux observed with Δ(187-243) was related to a decreased binding to the cells, competitive cell-binding studies were performed. In the first experiment, cholesterol-loaded cells were incubated in the presence of ^{125}I-Rec-apoA-I and increasing concentrations of cold competing protein. As indicated in Fig. V-6, all central deletion mutants could compete as efficiently as cold Rec.-apoA-I with ^{125}I-Rec-apoA-I for the binding to the cells. However, at the highest concentration tested (40-fold excess over ^{125}I-Rec-

apoA-I, a concentration equivalent to the concentrations used for the efflux), Δ(187-243) showed a significant decrease (2-fold) in its ability to compete with ^{125}I-Rec-apoA-I as compared to the other proteins.

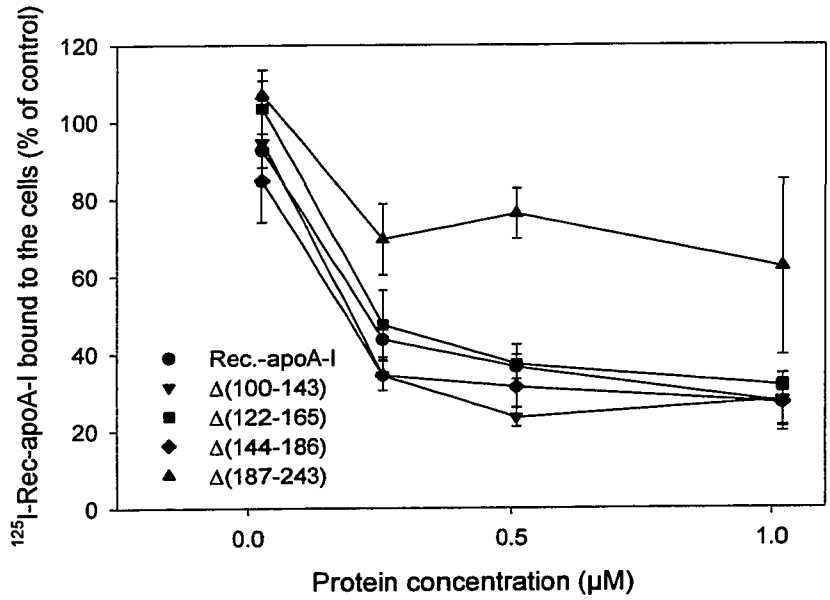

Figure V-6: Competition between ^{125}I-Rec.-apoA-I and other Proteins for the Binding of Cholesterol-loaded Macrophages.
After differentiation and cholesterol loading of macrophages, cells were incubated with ^{125}I-labeled Rec.-apoA-I (500,000 cpm/well, 24-well plate) and varying concentration of control and mutant proteins. Results are expressed as the percent of bound ^{125}I-labeled Rec.-apoA-I as a function of the competing protein concentration (100 %: no competitor). The results are the average of quadruplicate wells and representative of three assays.

In a second experiment, the binding properties of Δ(187-243) and Rec.-apoA-I for cholesterol-loaded macrophages were quantified. From the binding curves shown on Fig. V-7, it appears evident that Δ(187-243) can not bind to these cells as efficiently as Rec.-apoA-I. Determination of the binding affinity of both proteins indicates that Δ(187-243) present a 6-

fold reduction in the affinity for cholesterol-loaded macrophages compared to the wild-type apoprotein (K_D of 1.9 and 12.1 μM for Rec.-apoA-I and Δ(187-243), respectively).

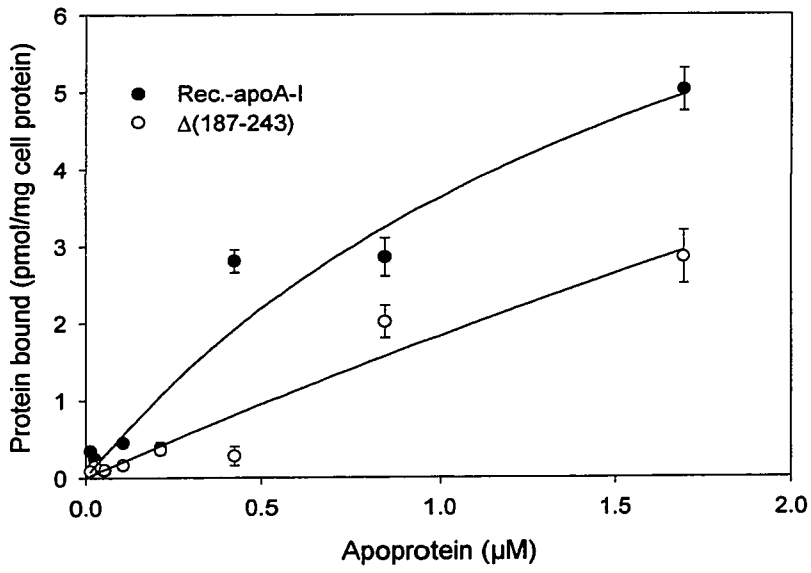

Figure V-7: Binding of Apoproteins to Cholesterol-loaded Macrophages.
Macrophages were loaded with acetylated-LDL as described in Materials and Methods. After one day incubation in RPMI 0.2 % BSA, 100 nM PMA, cell were washed and incubated with varying concentrations of ^{125}I-labeled apoprotein [Rec.-apoA-I or Δ(187-243)] for 2 h. At the end of the incubation, cells were washed three time with PBS and solubilized in 0.5N NaOH. The radioactivity and protein content were determined.

DISCUSSION

Understanding of the specific cellular cholesterol efflux pathway is complicated by the apparent and unresolved cell specificity of this pathway. In the present study, we have demonstrated that deletion of the carboxyl-terminal domain of apolipoprotein A-I results in a

dramatic decrease in efflux promoted by the lipid-free apoprotein from cholesterol-loaded macrophages but not from cholesterol-loaded fibroblasts. This altered function appears to be the result of a reduced ability of Δ(187-243) to bind these cells as shown in Fig.V-7. This reduced association may reflect either reduced lipid-binding properties, i.e. to a lipid domain of the membrane, or reduced binding to a specific receptor. The decreased initial binding of this mutant to phospholipids is demonstrated in Fig. V-5 and by other studies (324; 382; 431). Moreover, since the last helix of apoA-I has the highest affinity for phospholipid (244), it is reasonable to propose that the C-terminal domain of apoA-I may directly interact with cell lipids and therefore promote lipid efflux (phospholipid and cholesterol) by direct interaction with the plasma membrane. The difference between macrophages and fibroblasts could reflect the different ability of the two cell types to accumulate or localize excess cholesterol (103; 336; 348). It has been proposed that apoA-I associates better with a lipid emulsion when it contains cholesterol (84). The increased cholesterol content of cholesterol-loaded macrophages may lead to the formation cholesterol-enriched plasma membrane domains that might have a higher affinity for apoA-I, particularly its C-terminal domain, than other plasma membrane domains. The absence of caveolin in THP-1 macrophages (450) could also induce changes in the plasma membrane lipid distribution and a different mechanism of efflux than that has been described for fibroblasts (109).

Two studies using apoA-I mutants have provided conflicting results. The first study reported no difference in the ability of apoA-I mutants in reconstituted LpA-I to promote cholesterol efflux from plasma membrane of mouse L-cell fibroblasts (diffusional efflux)(329). A second study with HepG2 cells showed that a lipid-free mutant, with a deletion of region 210-243, had a decreased ability to promote cholesterol and phospholipid efflux from either

plasma or intracellular pools but binding of the mutant to HepG2 cells was not affected.(346). The normal binding of Δ(210-243) to HepG2 cells suggests that the sequence 187-210 is important for cell interaction whereas residues 210-243 are important for lipid binding. The difference in the cell type as well as the cholesterol-loading status of THP-1 macrophages may explain the difference between the two studies. The model used for cholesterol efflux therefore appears to be crucial to determine the specific function of apoA-I in the removal of the cellular cholesterol. Macrophages, which have been involved in the early development of atherosclerosis (1) and should be the target of future research in this field, have certainly all the prerequisites to be a good model. They can accumulate high levels of cholesterol, particularly in the form of CE, and their "foam cell'-like phenotype is reversible as is the development of an atheromateous plaque.

Multiple mechanisms of cell-apoA-I interaction have been proposed but the specific nature of the interaction is still not clearly understood. Several groups have suggested a direct interaction between apoA-I and a cellular receptor (85; 451; 452). It has been postulated that interaction of apoA-I with its receptor permits the transfer of cholesterol from intracellular stores to the plasma membrane. Membrane-associated cholesterol would then be available for transfer to lipoproteins by passive aqueous diffusion. The murine scavenger receptor class B type I (SR-BI, shown to be CLA-I in human) has recently been proposed to be involved in this process (85). Ji *et al.* have also shown expression of SR-BI in atherosclerotic lesions, possibly in foam cells. Cholesterol loading of macrophages might lead to an upregulation of SR-BI transcription similarly to that observed for CD36, another scavenger receptor (453). SR-BI has been shown to bind HDL (89) and other apolipoproteins including apoA-I (91). In its lipid–poor form, apoA-I may promote cholesterol efflux whereas bound to CE-enriched HDL, it

may instead mediate selective CE uptake. Several putative phosphorylation sites have been identified in SR-BI amino-acid sequence and therefore, different phosphorylation states could also be responsible for distinct functions. Expression of SR-BI mRNA in different cell lines has been correlated with the ability of HDL to promote cholesterol efflux (85). However, as recent studies have indicated (92), this receptor may be especially efficient with lipoproteins containing large amounts of phospholipid, which may promote a more effective binding to its ligand, SR-BI (88).

The importance of macrophage apoE in plaque development has been demonstrated in studies with mice lacking apoE. These mice can readily develop atherosclerotic lesions (454). However, specific expression of apoE by macrophages in apoE-deficient mice can reduce hypercholesterolemia and atherosclerosis (455; 456), while the absence of apoE secretion by macrophages in normal mice is associated with increased atherosclerosis (457). We have recently shown in this laboratory that apoA-I can associate with apoE bound to glycosaminoglycans (Burgess, J, and Marcel, Y.L, unpublished results). Studies with macrophages secreting apoE have also shown that apoE can interact with cell surface proteoglycans (458). ApoE could therefore also play a role in the interaction between cell surface and apoA-I, and allow the transfer of cholesterol to apoA-I. This hypothesis is consistent with the data reported by Lin *et al.* (459) who suggested that apoE can modulate the binding of HDL_3 to macrophages. They were however unable to demonstrate any effect of this binding on cholesterol efflux mediated by HDL_3. In the case of lipid-free apoA-I, the situation may be very different, as the lipid-free apoprotein, like apoE, has a high affinity for lipid. Since apoE secreted by macrophages can also mediate efficient cholesterol efflux (460), the interaction of apoA-I and apoE may exert a synergistic effect on the cholesterol efflux process. In support of this view, we observed that cholesterol efflux to lipid-free apoA-I is

more efficient with cholesterol-loaded macrophages than with cholesterol-loaded fibroblasts, which do not secrete apoE (Fig. V-1 and V-2).

In summary, we have demonstrated that the carboxyl-terminal domain of apoA-I is crucial for its ability to promote cellular lipid from cholesterol-loaded macrophages. This reduced efflux also appears to be related to a reduced ability of the apoprotein to associate with these cells. Further studies with this mutant will clarify whether efflux is related to a direct apolipoprotein-membrane lipid interaction, a protein binding site or a specific receptor.

Chapter 6: DISCUSSION

ApoA-I may be composed of three domains that are not structurally well defined but are associated with specific functions. In the following section, we will analyze and compare the properties of natural apoA-I variants that have been described in specific patients and for which, at least, a partial lipoprotein phenotype and/or apoA-I structure-function characterization has been performed (Table VI-1). This discussion will also attempt to correlate the *in vivo* observations with the *in vitro* functional apoA-I characterizations, including those described as part of this thesis. The three major functions of apoA-I, namely lipid binding, cellular cholesterol efflux, and LCAT activation will be discussed.

Lipid binding properties and structure analysis of apoA-I

Several mutations in the N-terminal domain have been identified and these mutations are often associated with amyloidosis (Table VI-1). This disease is a disorder of protein metabolism in which autologous proteins or their fragments associate with amyloid precursor proteins and are deposited as fibers in the tissues. Small N-terminal fragments of apoA-I variants have been found in some individual with amyloidosis (Table VI-1). The presence of an extra positive charge in the N-terminal domain may be responsible for the formation of amyloid deposits. Defects in the metabolism of HDL resulting from mutations in the N-terminal domain have not yet been fully elucidated.

Table VI-1: Mutation of apolipoprotein A-I observed in patients.
Phenotype is described as well as the properties observed *in vitro*. Only mutations with small modification (mutants with additional residues or with less the 90 % of apoA-I sequence are not included) and for which a phenotype or functional data is available are described.

Mutation	Functional and Clinical Consequences					
	Lipid-binding	Cholesterol efflux	LCAT activation	HDL/A-I levels	Comments	References
$Pro_3 \rightarrow Arg$	Normal			Normal	Impaired ProapoA-I conversion	(426; 461)
$Pro_3 \rightarrow His$			Normal	Normal	Impaired ProapoA-I conversion	(426; 462)
$Pro_4 \rightarrow Arg$	Normal		Normal	Normal	No apparent effect	(426; 426; 461; 462)
$Arg_{10} \rightarrow Leu$				Normal	(?)	(463)
$Asp_{13} \rightarrow Tyr$				Reduced A-I levels		(464)
$Gly_{26} \rightarrow Arg$					Amyloidosis	(465)
$Ala_{37} \rightarrow Thr$				Normal	No apparent effect	(466)
$Trp_{50} \rightarrow Arg$					Amyloidosis	(467)
$Leu_{60} \rightarrow Arg$					Amyloidosis	(468)
60-71→Val-Thr					Amyloidosis	(469)
Δ(70-72)					Amyloidosis	(470)
$Ala_{95} \rightarrow Asp$				Normal	No apparent effect	(471)
$Tyr_{100} \rightarrow His$				Normal	No apparent effect	(472)
$Asp_{103} \rightarrow Asn$				Normal	No apparent effect	(462)
$Lys_{107} \rightarrow 0$	Impaired		Reduced	Reduced HDL-C LpA-I:A-II	Possible Amyloidosis	(426; 473-475)
$Lys_{107} \rightarrow Met$	Normal		Normal	Normal		(426; 476)
$Trp_{108} \rightarrow Arg$				Normal	No apparent effect	(471)
$Glu_{110} \rightarrow Lys$			Normal			(477)
$Glu_{136} \rightarrow Lys$			Normal	Normal	$ApoE_2$ phenotype	(478)
$Leu_{141} \rightarrow Arg$		Reduced (Plasma)	Reduced	Reduced α-HDL	CHD (other risk factors)	(479; 480)

Mutation	Functional and Clinical Consequences					
	Lipid-binding	Cholesterol efflux	LCAT activation	HDL/A-I levels	Comments	References
Pro_{143}→Arg			Reduced	Reduced A-I		(436)
Δ(146-160)	Impaired		Reduced	Reduced A-I + HDL	Dominant negative phenotype	(437; 481)
Arg_{151}→Cys			Reduced	Reduced A-I + HDL	Similar to apoA-I_{Milano}	(438)
Val_{156}→Glu			Reduced	Reduced	Corneal opacity	(439)
Ala_{158}→Glu				Reduced A-I levels		(482)
Leu_{159}→Arg	Normal	Normal	Reduced	Reduced A-I + HDL	Dominant negative phenotype	(440; 483)
Arg_{160}→Leu		?		Reduced HDL, A-I, LpA-I:A-II		(441)
His_{162}→Gln					No apparent effect	(472)
Pro_{165}→Arg	Normal	Reduced	Reduced	Reduced A-I + HDL		(426; 461)
Arg_{173}→Cys	Impaired	Reduced	Reduced	Reduced A-I + HDL	Increased TG Longevity (?)	(397; 442; 484-486)
Glu_{198}→Lys	Normal	Normal	Normal	Normal		(426; 476; 487; 488)

(?) indicates that reduced HDL-C levels or CHD has been associated in some patients with the mutation.

We have studied a mutant of apoA-I lacking the domain 1-43. We have observed that the mutant apoprotein remains oligomerized in the presence of cholate (used to prepared discoidal complexes), whereas this was not the case for apoA-I and other mutants, including those of the C-terminal domain (Frank, P.G., Brouillette, C.G., and Marcel, Y.L., unpublished results). Our data show that the N-terminal domain is not only important in the stabilization of the lipid-free protein (274) but may also favor the formation of lipid-protein complexes by maintaining a certain accessibility to the lipid-binding domains, N- (44-65) and C-terminal

(220-241), and possibly to the central domain. Although the domain 44-65 has not yet been fully characterized, studies by Palgunachari *et al.* (244) have indicated that it may bind lipids almost as efficiently as the domain 220-241.

Several mutations in the central domain of apoA-I have been described (Table VI-1). However, very few of them have been associated with clear defects in lipid binding properties. Only when the secondary structure of the protein was clearly modified (Lys_{107}→0, Δ(146-160)), impaired lipid-binding properties were observed (426; 437). Of importance, when incubated with cells, mutant Δ(146-160) was predominantly associated with large HDL (9-20 nm) whereas the native protein could also form smaller complexes (7.5 nm) (437). ApoA-I_{Milano} (Arg_{173}→Cys), which has been extensively studied (397; 442; 484-486; 489-493) also appears to be associated with size restricted HDL lipoproteins (smaller HDL and reduced number of HDL subpopulations) as observed *in vitro* and *in vivo* compared to the wild-type apoprotein (485; 491). Apparently, this size restriction is the result of the dimerization of apoA-I induced by the presence of a Cys residue in the mutant protein. Consistent with these observations, we have shown that deletion in the central domain of apoA-I has little effect on the kinetics of association with DMPC as compared to a deletion mutant of the C-terminal domain (Chapter 3 and 5). Moreover, these central deletion mutants can still associate with cellular lipids and form lipoprotein particles (Chapter 5). These results therefore suggest that these helices may not play an important role in the initial binding of lipid. However, they appear to have a reduced lipid binding capacity (Chapter 5), which may indicate that they are be capable of accommodating varying amounts of PL. Taken together, these observations support the hypothesis that the central domain of apoA-I (100-186) is actually responsible for the plasticity of apoA-I and allows the binding of varying amounts of lipid.

Very few mutations have been described in the C-terminal domain of apoA-I (Table VI-1). Funke *et al.* (494) have described a mutant in which a frameshift mutation leads to a modification of residues 203-229 and a smaller mature protein (229 residues instead of 243). Examination of this patient revealed corneal opacity and reduced plasma LCAT activity. However, the presence of Cys in the modified C-terminal domain of the protein may also explain the phenotype since this protein was found to form hetero-oligomers with apoA-II. A Glu_{198}→Lys mutation has been described by Strobl *et al.* (487) but it was not possible to firmly establish whether this mutation caused reduced HDL-C levels. In this case, half of the subjects carrying the mutation had normal HDL-C levels whereas the other half had HDL-C below the fifth percentile for age and sex. This mutant was further characterized *in vitro* and no particular impairment in its functional properties could be demonstrated (426; 488). Studies with rabbits (325; 495) and with transgenic mice (326) have confirmed the importance of the C-terminal domain of apoA-I in the formation of HDL lipoproteins. In rabbit studies, deletion of residues 201-243, 217-243, or 226-243 (325) and 190-243 (495) markedly increased the rates of apoA-I catabolism. Furthermore, the C-terminal truncated apoA-I proteins were mostly associated with very high-density lipoprotein (VHDL), which suggests a poor ability of the mutant apoproteins to associate with lipids and form mature and fully lipidated HDL. Holvoet *et al.* (495) have also shown that replacement of domain 190-243 with helices of apoA-II (residues 12-77), which is believed to have a higher lipid-binding affinity than that of apoA-I, could not improve this association. Slightly different results were observed in transgenic mice where the same chimeric apoprotein, apoA-I(1-189)-apoA-II(12-77), could associate very well with lipids and form large HDL particles (326). These differences may be related to the genesis of HDL: Rabbit apoA-I that is already associated with rabbit HDL has to be displaced by the injected

apoprotein whereas in transgenic mice, preformed HDL containing the protein of interest were being studied. Moreover, studies from Laccotripe *et al* (327) suggested that mutants, in which hydrophobic residues of the last helix were modified, had reduced initial binding to phospholipids. These observations are consistent with our data showing that the C-terminal domain 187-243 is necessary for the initial association with lipids (kinetics of association with DMPC, Chapter 5). Moreover, we have also shown that the mutant Δ(187-243) could associate with cellular lipids and form lipoprotein particles (Chapter 5). The C-terminal domain, probably residues 220-241, is involved in the initial association with lipids and in the formation of lipoprotein particles. The absence of natural mutations in the C-terminal domain of apoA-I may indicate that this domain is essential for the function of apoA-I (496).

The presence of amphipathic α-helices as an essential structural motif in the binding of lipids is now well recognized (242; 304). However, the role and contribution of the different apoA-I helices in this process have not been clearly addressed until recently. The varying lipid affinity of these helices (discussed in the introduction and Chapter 3) appears to regulate apoA-I binding with lipids. For example, the fact that central helices have a reduced affinity for lipid as compared to the N- (44-65) and C-terminal (220-241) helices may explain the ability of apoA-I to associate with varying amounts of lipid (244). The central helices can form a stable structure in the absence of large amounts of lipids, allowing the formation of a "hinge domain", whereas the C-terminal domain may require the presence of the N-terminal domain (residue 1-43) to maintain a stable structure, accessible for lipid interaction (274). In the model of apoA-I Δ(1-43) derived from the crystal structure presented by Borhani *et al.* (320), four apoprotein molecules are associated via their hydrophobic faces to form a tetramer. Among these four molecules, two dimers with identical tertiary structure are observed but the

monomers of each dimer have different conformations. Interestingly, in this model (320), the interhelical angle can vary such that different interhelix angles are observed in the two constituents of the dimer (Fig VI-1), suggesting that the plasticity of apoA-I is in part due to the cooperativity between helices. The tetramer model does not appear to be relevant for the structure of apoA-I in a lipidated state since most of the Leu and Val residues are buried in this model. The dimer model may be more relevant with hydrophobic residues in contact with the lipid phase and residues 1-43, absent of this model, may favor the formation of a dimer complex in lipoprotein particles.

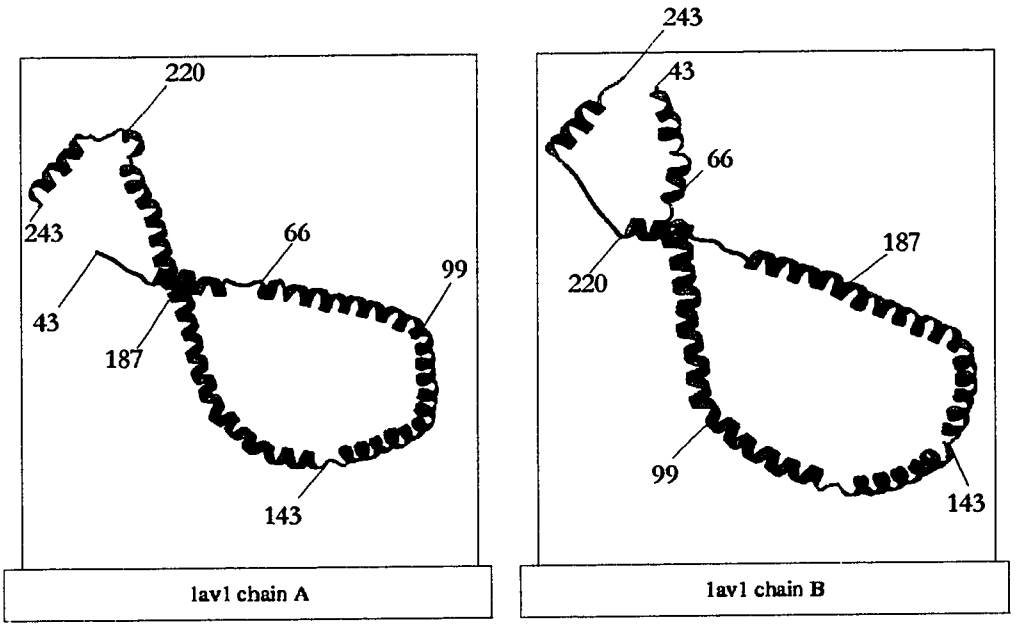

Figure VI-1: Ribbon representation of two monomers of apoA-I Δ(1-43).
The two basic constituents of a dimer of apoA-I Δ(1-43) are represented. The N- and C-termini of each monomer are labeled as well as the first residues of each identified helices (320). 1AV1 A and B refer to the PDB identification codes given by the Brookhaven protein structure database (address on the world wide web: http://pdb.pdb.bnl.gov/).

It is interesting to note that the N- and C-terminal domains have not only the highest affinity for lipids (244) but are also very close to each other in the model of apoA-I Δ(1-43) (Fig. VI-1). The organization of these domains may enhance the initial binding of apoA-I to lipids.

Cellular cholesterol efflux mediated by apoA-I

Only a few studies have characterized the efflux capability of naturally occurring apoA-I mutants (440; 480; 486; 488). No mutants with a mutation in the N-terminal domain have been examined for this ability.

Most of the naturally occurring apoA-I mutants that have been examined correspond to alteration of residues in the central domain of apoA-I. Three natural variants of apoA-I have been shown to have a reduced ability to promote cellular cholesterol efflux. These mutations (Pro_{165}→Arg, (488); Arg_{173}→Cys, (486)) may affect the secondary and/or tertiary structure of the C-terminal domain of apoA-I. The substitution Pro_{165}→Arg results in the elimination of a β-turn which could modify the accessibility of the C-terminal domain to lipids and/or cells, in the resulting mutant. The Arg_{173}→Cys mutation induces the dimerization of apoA-I, which may alter the conformation of its C-terminal domain and modify the lipid binding properties of the mutant apoprotein (497). In our studies (Chapter 4 and 5), we have not observed any effect of the deletion of central α-helices on the ability of apoA-I to promote cellular cholesterol efflux from normal and cholesterol-loaded fibroblasts, or from cholesterol-loaded macrophages. However, all these results were obtained in the absence of LCAT and other plasma factors involved in the metabolism of HDL. In particular, mutants Δ(122-165)

and Δ(144-186) may display a reduced ability to promote cellular cholesterol efflux in plasma or in the presence of LCAT alone since they cannot activate the cholesterol esterification reaction as efficiently as Rec.-apoA-I and Δ(100-143) (Chapter 4). In support of this hypothesis, the ability of a naturally occurring apoA-I mutant (Leu_{141}→Arg) to promote cellular cholesterol efflux was examined with plasma from subjects carrying this mutation (hemizygote patients) (480). In this system, a reduced cellular cholesterol efflux to plasma was observed and may be due to a reduced transfer of cholesterol from preβ-HDL to α-HDL, which probably resulted from a reduced ability of the mutant apoprotein to activate LCAT (480). Cholesterol esterification may therefore promote the flux of cholesterol through the HDL pool by stimulating the formation of cholesteryl esters that are transferred to apoB-containing lipoproteins, by CETP, and then cleared via the LDL-receptor pathway in the liver (6).

Only one natural mutant in the C-terminal domain of apoA-I (Glu_{198}→Lys) has been examined for its ability to promote cellular cholesterol efflux (488). This mutant was not associated with reduced cholesterol efflux. In our study (Chapter 5), we have observed that deletion of residues 187-243 of apoA-I was associated with a markedly reduced ability of the mutant apoprotein to promote cellular cholesterol efflux from cholesterol-loaded macrophages and that this property was associated with a reduced kinetic of association with DMPC. The natural apoA-I mutant Glu_{198}→Lys did not appear to have a reduced ability to associate with lipids (426), which may explain the absence of any effect of this mutation on the ability of the mutant apoprotein to promote cellular cholesterol efflux.

As indicated in the introduction, cellular cholesterol efflux mediated by apoA-I is believed to occur through two different pathways. Simple diffusional transfer between the

plasma membrane and the apoA-I-containing lipoprotein acceptor (i.e., HDL) appears to require the presence of amphipathic α-helices. The ability of an amphipathic peptide to promote cellular cholesterol efflux depends on its lipid affinity and ability to retain cholesterol molecules (341; 348). In agreement with these models, we have shown that apoA-I mutants (deletion of the central domain 100-143, 122-165, 144-186) that could associate with lipid (Chapter 3) could still promote cellular cholesterol efflux (Chapter 4 and 5).

The second mechanism involves the interaction of lipid-free apoA-I with specific cell surface binding sites, followed by the transfer of intracellular cholesterol from an ACAT-accessible pool to the plasma membrane and subsequent transfer to the donor. The first step also appears to be mediated by amphipathic α-helices since synthetic peptides with affinity for lipids can mediate this transfer (348; 498). In this process, apoA-I may interact with specific plasma membrane domains such as caveolae (enriched in FC and sphingomyelin) and subsequent transfer of FC and PL to the apoprotein may generate preβ-HDL (109). In agreement with this mechanism, the reduced affinity of peptides for the lipid surface is associated with a reduced ability to promote cellular cholesterol efflux (348; 498). The C-terminal domain of apoA-I may therefore play a major role in this interaction of the lipid-free apoprotein with the cell. Our data showing that Δ(187-243) displays a reduced ability to promote cellular cholesterol efflux from cholesterol-loaded macrophages agree with this model (Chapter 5) although the interaction of this mutant with cholesterol-loaded fibroblasts does not show a reduced efflux. The latter observation may be due to the reduced cholesterol content of cholesterol-loaded fibroblasts as compared to cholesterol-loaded macrophages (Chapter 5). The N-terminal domain (44-65) may also play an important role in this function since it may also influence the initial binding of apoA-I to lipids (244). Further studies of

apoA-I with mutations in this domain would help to determine if this domain is also involved in this important function of apoA-I.

ApoA-I mediated LCAT activation

LCAT activation mediated by apoA-I has been the subject of investigation for more than two decades (371; 499). Numerous groups have investigated the relationship between LCAT activation and apoA-I structure and/or conformation (289; 312; 366; 371). Recent studies have identified the central and C-terminal domains of apoA-I in the activation of LCAT.

Among the natural mutants of apoA-I corresponding to modification of residues in the N-terminal domain, only a few have been studied for their ability to activate LCAT and none were shown to negatively affect cholesterol esterification (426). A study of apoA-I Δ(1-43) suggested that this mutant had a reduced ability to activate the LCAT reaction (274). Our data suggest that the central domain 144-186 is involved in this reaction (Chapter 4). Since this central domain may interact with the N-terminal domain (297; 313), this observation may indicate that a specific interaction between these two domains is required for efficient esterification of FC by LCAT.

Natural variants with defective LCAT activation properties (Lys_{107}→0, Leu_{141}→Arg, Δ(146-160), Arg_{151}→Cys, Val_{156}→Glu, Leu_{159}→Arg, Pro_{165}→Arg, Arg_{173}→Cys) have been identified and most of them correspond to mutations in the central domain 144-186. Other mutations (Lys_{107}→0, Leu_{141}→Arg) may have a direct effect on the conformation of this central domain and indirectly affect the ability of apoA-I to activate LCAT (312; 379; 427). Our results (Chapter 4) and those of others (382; 383; 430; 434) are consistent with these

observations and suggest that the LCAT activating domain in apoA-I may reside between residues 144 and 186.

The mechanism by which apoA-I activates LCAT is still unknown but it may involve a direct interaction between these helices and residues 152-169 of LCAT. As observed in Fig. VI-2, positively charged residues present in both helices 144-165 and 166-186 may interact with negatively charged residues found on this LCAT helix. Mutagenesis study performed on LCAT by Wang et al. (432) have shown that Glu 154, 155, and 155 are not important for LCAT activity but may be involved in the binding of cholesterol. The region 151-174 may therefore indirectly interact with apoA-I through the binding with HDL cholesterol, a process that may result in a conformational change of LCAT and its activation.

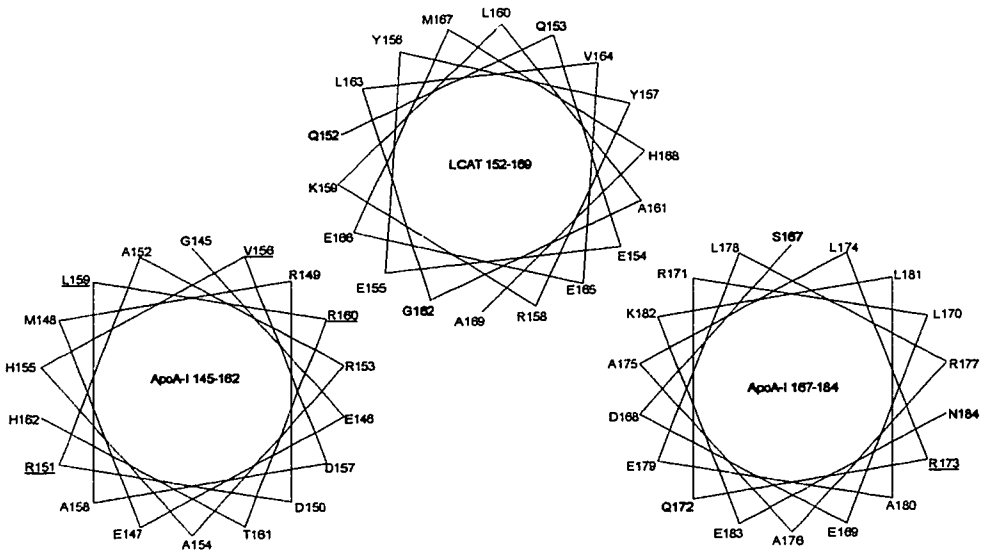

Figure VI-2: Putative Mechanism of Interaction between ApoA-I and LCAT.
Edmunson wheel representation of apoA-I helices 145-162 and 167-184 suggesting a possible mechanism of interaction with helix 152-169 of LCAT. Residues underlined in apoA-I helices have been associated with LCAT deficiency. Colors indicates proline (purple, P), aspartic acid or glutamic acid (red, D and E), arginine or lysine (blue, R or K), and phenylalanine, isoleucine, leucine, methionine, valine, tryptophan, or tyrosine (green, F, I, L, M, V, W, and Y). The remaining amino acids, alanine, cysteine, glycine, histidine, asparagine, glutamine, serine, and threonine (A, C, G, H, N, Q, S, and T) are uncolored and called indifferent (237).

Interestingly, mutations of positively charged residues in these helices usually result in decreased LCAT activation (438; 442). In small discoidal LpA-I where these central helices may not interact completely with lipids, LCAT activation appears to be most efficient because of an enhanced accessibility of the lipid interface and to the central domain of apoA-I (312). In support of this model, an increase in the negative surface charge of the Lp2A-I is associated with increased LCAT reactivity (312). In HDL complexes, where the lipid species have little effect on the lipoprotein surface charge, it appears that a modification of the surface charge properties is due only to a change in apoA-I conformation (268; 309; 310; 312). LCAT activity may therefore be a function of the exposure of apoA-I charged residues in these lipoproteins.

The C-terminal domain of apoA-I was suggested to be involved in LCAT activation in several studies (327; 382; 383) but it now appears that this observation was merely due to defective lipid-binding properties of the mutants. In these assays, lipid vesicles were incubated with apoA-I mutants and since the C-terminal mediates the initial binding, these mutants could not interact properly with lipids and therefore activate LCAT. This has been confirmed by Dhoest *et al.* (430) who showed that replacement of the carboxyl terminal (190-243) by residues 12-75 of apoA-II restores the lipid binding properties of the protein but does not change its ability to activate cholesterol esterification. ApoA-II is not as efficient as apoA-I in activating the reaction mediated by LCAT (449).

Physiological significance

The N-terminal domain (residues 1-43) may be important for the stability of the protein in the lipid-free state and possibly in the modulation of its interaction with LCAT. The central domain (100-186) may have a function in the regulation of the interaction with PL and

be responsible for the plasticity of the molecule by allowing its association with varying amounts of PL. Beside this role, part of the central domain, which includes residues 144-186, forms the site involved in LCAT activation (Chapter 4). However, efficient LCAT activation also requires a certain affinity for PL that is contributed by the C-terminal domain (187-243), and within it, probably by the last helix (220-241). In addition, the lipid binding affinity of the C-terminal domain appears crucial for the ability of apoA-I to promote cholesterol efflux from cholesterol-loaded macrophages, a process, which may require the efficient binding of the apoA-I C-terminal domain to the cell.

As indicated in Table VI-1, mutations in the LCAT activating domain of apoA-I result in reduced HDL-C levels but are not necessarily associated with increased risk of CHD, possibly because LCAT can still esterify LDL-derived cholesterol. Additional risk factors (e.g., elevated LDL-C, obesity) may be required to observe an increased in CHD as observed by Miccoli *et al.* (479). However, cholesterol esterification may promote the flux of cholesterol through the HDL pool by stimulating the formation of cholesterol esters that are transferred by CETP to apoB-containing lipoproteins (480). Nevertheless, it is intriguing that almost all identified mutations that affect the LCAT activating domain, are associated with reduced HDL-C. However, the technique used in screening for apoA-I mutations has relied, in most cases, on the presence of a charge modification of the mutant protein. Therefore, the screening procedure may not have detected mutations that did not affect the protein charge.

Future Plans

A refined strategy to identify the specific functions of apoA-I domains is required. It appears necessary to examine the different functions of apoA-I using, for example, a specific mutagenesis strategy. The mechanism, by which apoA-I associates with PL, has to be

examined in more detail and studies with apoA-I that contains point mutations of hydrophobic and charged residues in helices, will provide new insights into the mechanism of apoA-I association with lipids. The role of the N-terminal domain (residues 1-43) has not yet been clearly identified, point mutations of charged residues may resolve this question. The mechanism of LCAT activation by apoA-I has to be further characterized and the identification of specific residues (charged and hydrophobic residues) remains to be done. *In vivo* studies of apoA-I mutants (natural mutants in human, or in transgenic or adenovirus-infected animals) may also help to identify the mechanisms by which apoA-I participates in reverse cholesterol transport.

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