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The Interplay Between Apolipoproteins and ATP-Binding Cassette Transporter A1

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A dissertation presented to the faculty of the University of Cincinnati College of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pathobiology and Molecular Medicine from the department of Pathology and Laboratory Medicine

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ABSTRACT

High density lipoprotein (HDL) plasma levels are inversely correlated with the risk of developing cardiovascular disease. HDL is formed when lipid-free apolipoproteins in the plasma accept excess phospholipids and cholesterol from cells such as hepatocytes, enterocytes, and macrophages. This process is mediated by a cell membrane protein known as ATP-binding cassette transporter A1 (ABCA1). It is unknown what structural elements in apolipoproteins allow them to participate in ABCA1-mediated cholesterol efflux. The hypothesis tested in this work is that amphipathic and charged helical structural elements of exchangeable apolipoproteins allow these proteins to facilitate ABCA1-mediated cholesterol efflux at the cell surface. Recently, it was proposed that a negatively charged and a hydrophobic surface patch on apolipoprotein (apo) A-I were important in this process (1). Our data shows that neither of these surface patches plays an important functional role in apoA-I promoted cholesterol efflux via ABCA1. It has also been proposed that a linear array of acidic amino acids aligned along the junction of the hydrophobic and hydrophilic faces of two amphipathic α-helices is the critical element for this process (2). However, studies using apoC-I point mutants demonstrated that this element was also functionally unnecessary. Instead, our studies with peptides modeling the amphipathic α-helices of apoA-II and apoC-I have shown that the minimal structural unit in apolipoproteins which allows them to serve as cholesterol acceptors in ABCA1-mediated efflux is a bihelical peptide.
composed of an amphipathic non-lipid binding helix joined to an amphipathic fast lipid binding helix. In apoA-I, apoA-II, apoC-I, and likely apoE this structural element is found at the extreme C-terminus of the protein with the fast lipid binding helix being closest to the C-terminus. It was found that the non-lipid binding helix altered the phospholipid binding preference of the fast lipid binding helix, perhaps directing the fast lipid binding helix towards areas of the cell membrane with more tightly-packed phospholipids.

Having identified the minimal apolipoprotein structure necessary for ABCA1-mediated cholesterol efflux, it was important to next identify the location of the lipid transfer to apolipoproteins. Recently it was proposed that this lipidation process occurs through a retroendocytosis pathway (3). In this pathway apolipoproteins are endocytosed into the cell with ABCA1, lipidated intracellularly, and exocytosed as nascent HDL. Using fluorescently-labeled and radiolabeled apoA-I, we found that in non-lipid loaded cells, a majority of the endocytosed apoA-I is resecreted into the media in a degraded form. Indeed, only 11% of the HDL produced in a three hour period could be accounted for by ABCA1-mediated retroendocytosis of apoA-I. This data clearly demonstrates that, at least in non-lipid loaded cells, retroendocytosis of apoA-I is not the main pathway of HDL biogenesis.

These studies further our understanding of the mechanism of ABCA1-mediated cholesterol efflux by demonstrating that apolipoproteins utilize a bihelical non-lipid binding/fast lipid binding motif to serve as cholesterol acceptors, and that this lipidation process occurs at the cell surface.
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ABBREVIATIONS

a.a., amino acid; ABCA1, ATP-binding cassette transporter A1; Ac-LDL, acetylated low density lipoprotein; ANS, 1-anilinonaphthalene-8-sulfonate; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II; apoC-I, apolipoprotein C-I; apoE, apolipoprotein E; ATR-FTIR, attenuated total reflectance fourier transform infrared spectroscopy; BS3, Bis(sulfosuccinimidyl) suberate; BSA, bovine serum albumin; cAMP, 8-bromoadenosine 3',5'-cyclic monophosphate; CD, circular dichroism; CHO, Chinese hamster ovary cell; CVD, cardiovascular disease; DMEM, Dulbecco’s Modified Eagle Medium; DMPC, dimyristoyl-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DPPE, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; DTT, dithiothreitol; E. coli, Escherichia coli; ESR, electron spin resonance; FBS, fetal bovine serum; FC, free cholesterol; FITC, fluorescein isothiocyanate; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GFP-ABCA1, green fluorescent protein labeled ATP-binding cassette transporter A1; HCl, hydrochloric acid; HDL, high density lipoprotein; HIC, hydrophobic interaction column; His-tag, histidine-tag; Igase, IgA protease; IMAC, nickel affinity column; IPTG, isopropyl-beta-D-thiogalactoside; LB, Luria broth; LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoprotein; MLV, multilamellar vesicles; MW, molecular weight; NBD-OLPC, nitrobenzoxadiazole 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] dodecanoyl]-sn-glycero-3-phosphocholine; OD, optical density; OD0, initial optical density; PAGE, polyacrylamide gradient gel electrophoresis; PBS, phosphate buffered saline; POPC, 1-palmitoyl 2-oleoyl phosphatidylcholine; rHDL, reconstituted HDL; SD, standard deviation; SE, standard
error; STB, standard Tris buffer; TCA, trichloroacetic acid; TCEP, Tris-(2-carboxyethyl) phosphine; TEV, Tobacco Etch virus; WMF, wavelength of maximum fluorescence; WT, wild-type; \( \lambda \), wavelength.
Cardiovascular disease (CVD) is the number one killer in the United States, and is increasing in prevalence as the population ages. One of the body’s most important protective mechanisms against the development of CVD is high density lipoproteins (HDL) in the plasma. These protein and lipid particles transport excess cholesterol from the peripheral cells of the body back to the liver for excretion. High concentrations of HDL in the plasma are strongly correlated with a decreased risk of developing CVD. HDL is formed when lipid-poor apolipoproteins in the plasma emulsify cholesterol from cells expressing the integral membrane protein ATP-binding cassette transport A1 (ABCA1). It is unclear which structural elements allow apolipoproteins to serve as acceptors of cholesterol from ABCA1-expressing cells. It is also unclear if this cholesterol transfer occurs at the cell surface or intracellularly. In the following work, these questions will be experimentally addressed. Chapter II provides the background necessary to frame these questions within the current model of atherosclerosis, and reviews the current data regarding the process of HDL biogenesis. In Chapter III, surface patches of negative charge and hydrophobicity recently identified on apolipoprotein A-I (apoA-I) will be investigated to determine if they are necessary structural elements for HDL biogenesis. Chapter IV describes a series of peptide studies used to define the minimal structure within natural apolipoproteins which can elicit ABCA1-mediated cholesterol efflux from macrophages. The location of cholesterol transfer to
apolipoproteins will be investigated in Chapter V. Finally, in Chapter VI, the scientific findings of Chapters III through V will be incorporated into the current hypothesized mechanism for HDL biogenesis, and future work in this area will be discussed. It is our hope that this work will help direct the development of ABCA1-mediated HDL biogenesis enhancement therapies in the future, providing a powerful new tool in the fight against cardiovascular disease.
The American Heart Association estimates that as of 2006, one out of every three adults in the United States suffers from some form of CVD. CVD encompasses a number of diseases including stroke, coronary artery disease, heart failure, and hypertension. CVD is estimated to have cost 475.3 billion dollars in health expenditures and loss of productivity in 2009. More importantly, CVD is costly in lives. In 2005, more than 864,000 people died as a consequence of CVD, making it the leading cause of death in the United States. In other words, on average, CVD kills one American every 37 seconds. And this extreme mortality is not a new development. This set of diseases has been the leading killer in our country since the turn of the last century, every year except 1918, when it was the influenza pandemic (4). It is clear that these diseases pose a significant threat to the health and well-being of our populous. It is also clear that with so many people affected by these diseases, if we are to keep our health care costs under control, we must decrease our dependence on expensive surgical treatments such as arterial stents and bypasses, and develop additional, easily-administered pharmacotherapies. In order to do this however, a deeper understanding of the etiology of CVD and also of the body’s innate protective systems is needed.

*Atherosclerosis and Cardiovascular Disease* - One of the key factors leading to the development of CVD is the development of a pathological condition known as
Atherosclerosis is a disease of the large and medium-sized muscular and elastic arteries of the body. Associated lesions are termed atheromas, and consist of a fibroinflammatory lipid plaque representing an accumulation of lipids, smooth muscle cells, connective tissue, and inflammatory cells within the vessel’s intimal layer. Although not entirely established, it is commonly believed that atheromas develop from small fatty streaks which appear throughout the arterial tree in early childhood. However, the widespread distribution of childhood fatty streaks does not explain the predilection of atheromas for specific branching points within the arterial tree, such as at the cervical carotid bifurcation, the first branch of the coronary arteries, and the aortic bifurcation into the common iliac arteries. Atheromas are thought to be the result of damaged endothelial cells and/or accumulation of smooth muscle cells in that location. Lipids, mostly derived from oxidized low density lipoprotein (LDL), a plasma particle made of proteins and lipids, begin to accumulate in these areas, furthering cellular damage and expanding the lesion. This damage attracts macrophages. As macrophages within the developing atheroma begin to phagocytose the lipids deposited in these early plaques by LDL, their cytoplasm become engorged with lipid droplets, causing them to take on a foamy appearance. Such “foam cells” are a characteristic sign of atherosclerosis. In a short time, these macrophages begin to secret fibroblast growth factors and platelet derived growth factor, signaling smooth muscle immigration and proliferation, thus expanding the lesion. Eventually the lesion becomes so large that cells at the interior of the plaque cannot obtain adequate nourishment, despite increased angiogenesis, and a necrotic core forms. This necrotic debris augments the inflammatory process, leading to further leukocyte immigration and plaque expansion. Such plaques
can grow insidiously within the body for decades undetected. Often outward signs and
symptoms suggestive of plaque build-up do not occur until a vessel lumen is over 75%
occluded or until an advanced, unstable plaque spontaneously ruptures and bleeds,
resulting in a thrombus which occludes the vessel lumen. For the past sixty years, a
vigorous search has ensued to find factors which would allow for the detection of such
dangerous plaques before they rupture. One of the factors that has proven most helpful in
predicting which patients are at risk of developing atherosclerosis is the measurement of
cholesterol-containing lipid particles, such as LDL, in the blood.

*Lipoproteins and Atherosclerosis- A correlation between CVD, and later atherosclerosis,
and lipoprotein levels was first notably established in the Framingham Heart Study,
which began in 1948. In this study a CVD-free cohort of over 5,000 men and women age
30-62 was examined every two years over the course of their lifetimes. As the study
progressed, it became clear that LDL was positively correlated with coronary artery
disease, while another plasma lipid particle, HDL was strongly inversely correlated with
coronary artery disease (5,6). This group of cholesterol-containing lipid particles is
referred to as lipoproteins, and proteins found on these particles are referred to as
apolipoproteins. Due to the strong correlation between these lipoproteins and CVD, the
National Heart, Lung, and Blood Institute designed the Multiple Risk Factor Intervention
Trial (MRFIT), a randomized control trial which showed that the absolute level of
cholesterol in the plasma was strongly correlated with the risk of developing
cardiovascular disease (7). These early trials made it clear that plasma lipid particles
such as LDL and HDL and total plasma cholesterol levels are important diagnostic
measurements of a patient’s risk of developing CVD. From these trials it was hypothesized that treatments aimed at favorably altering these lipid levels might result in a decreased risk of CVD. This hypothesis was tested in the Coronary Primary Prevention trial (CPT), which treated 1,900 middle-aged hypercholesterolemic men with cholestyramine, a bile acid sequestrant which primarily lowers plasma LDL levels. Compared to a matched control group, the men who received cholestyramine had a 19% reduction in CVD mortality over a nine year follow-up period (8). This was one of the first demonstrations of the dramatic reduction in CVD which can be accomplished by manipulation of plasma levels of lipoproteins. It was still unclear however, what physiological changes accompanying the favorable lipid profiles were associated with this decreased mortality. To address this, the Program on the Surgical Control of the Hyperlipidemias, or POSCH trial, enrolled patients with a recent history of myocardial infarct, divided them into two groups, and performed partial ilial bypass surgery on one group. This surgery hinders normal cholesterol absorption in the gut, and therefore decreases plasma cholesterol levels. Angiograms were then used to correlate lower plasma cholesterol levels with a significant size regression of existing atherosclerotic plaques (9). Amazingly, this plaque regression, which occurred over a three year period, was accompanied by a 35% decrease in mortality compared to the control group (p<0.01). Such dramatic results suggested that increased plasma LDL levels caused or supported the growth of atherosclerotic plaques, and that medical treatments which decreased LDL could slow or even reverse the growth of these plaques.
Low Density Lipoprotein: A risk factor for cardiovascular disease- LDL is a core of esterified cholesterol and triglycerides surrounded by phospholipids, unesterified cholesterol, apolipoprotein B-100, and apolipoprotein E (apoE). The apolipoproteins in conjunction with the phospholipid head groups help to sequester the particle’s hydrophobic core from the aqueous plasma environment, allowing for the transport of hydrophobic cholesterol esters and triglycerides throughout the body. It is currently believed that LDL is the body’s main shuttle for cholesterol from the liver to the peripheral tissues. Peripheral cells expressing high-affinity LDL receptors, such as smooth muscle cells, fibroblasts, and adrenal cells endocytose the LDL and catabolize it to release its cholesterol. This delivered cholesterol is vital for processes such as synthesizing new cellular membranes and in endocrine organs for synthesizing various hormones. However, there is reason to believe that the delivery of cholesterol to the periphery by LDL is not always beneficial. As previous mentioned, it is known that the cholesterol in atherosclerotic plaques is mostly derived from oxidized or otherwise modified LDL, and that increased LDL levels are correlated with CVD. Thus, an overabundance of LDL may be linked to CVD by contributing to the dangerous accumulation of cholesterol in atherosclerotic plaques in the peripheral arteries. Currently, statin drugs are available which can drastically lower plasma LDL levels. As such, this class of drugs has been a powerful tool in the fight against CVD. Still, a large percentage of CVD mortality does not appear to be affected by statins. Additional compatible treatment options are needed. One such option might be the therapeutic increase of plasma HDL levels.
HDL and the Reverse Cholesterol Transport System- HDL is a much smaller, denser lipoprotein than LDL and is composed of an esterified cholesterol and triglyceride core surrounded by phospholipids, unesterified cholesterol, apolipoprotein A-I (apoA-I), and apolipoprotein A-II (apoA-II). Often small numbers of other exchangeable apolipoproteins are associated with HDL such as apolipoprotein C-I (apoC-I) and apoE. Since the Framingham study it has been known that HDL plays a cardioprotective role in the body, but the mechanism of this protection is still not entirely known. Currently evidence suggests that HDL may play anti-inflammatory and anti-oxidative roles in atherosclerosis, slowing plaque growth and preventing the formation of oxidized LDL. In addition, HDL may derive some of its cardioprotective qualities from its role in the reverse cholesterol transport system, in which HDL shuttles excess cholesterol from peripheral cells of the body, in particular macrophages, back to the liver. In the liver, the cholesterol can be used to synthesize bile acids which are secreted into the digestive system. Small amounts of these bile acids are excreted each day. This daily loss of bile acids and associated biliary cholesterol is the body’s main method for disposing of excess cholesterol. Presumably, on a population basis, a dose-response exists such that the higher the HDL concentration, the greater the shuttling of excess cholesterol from the peripheral arteries back to the liver. Thus, high levels of plasma HDL would result in low levels of cholesterol in the periphery of the body which can be incorporated into developing atherosclerotic plaques. This connection between HDL and reverse cholesterol transport has led to speculations that a drug which effectively increased the plasma level of HDL might increase the flux of cholesterol from the periphery to the liver, thus providing protection from atherosclerosis. Currently, the most effective drug
for increasing HDL levels is niacin. Niacin decreases the catabolism of HDL, and inhibits hepatic secretion of very low density lipoprotein (VLDL), a precursor of LDL. At pharmacological doses, niacin can increase HDL levels by 15-35% (10,11), and has been repeatedly shown to decrease cardiovascular disease mortality when combined with statin drugs (12,13). However, considering the impressive cardioprotection that combined niacin-statin therapy provides, surprisingly few patients are compliant with this therapy. This is due to the unpleasant side effects such as flushing and pruritus (itching) associated with niacin. Many formulations of niacin have been developed with the hope of reducing these off-target effects, but no widely accepted formulation has been found. Thus, a need for well-tolerated, HDL-targeted pharmacotherapy remains.

*ATP-Binding Cassette Transporter A1-* Another potential strategy for raising HDL levels is to increase the anabolic rate of HDL production. The rate-limiting step of HDL biogenesis is the first step, the transfer of cholesterol and phospholipids to lipid-free or lipid-poor apolipoproteins, such as apoA-I, via the integral membrane protein known as ATP-binding cassette transporter A1 (ABCA1). No orthologs of ABCA1 have been identified in bacteria or yeast, suggesting that ABCA1’s function addresses homeostatic challenges present only in multicellular organisms (14). High levels of ABCA1 protein are expressed in hepatocytes, enterocytes, Purkinje and cortical pyramidal neurons, kidney proximal convoluted tubule cells, and macrophages. A discrepancy between mRNA levels and protein expression levels suggest that ABCA1 is highly post-translationally regulated (15). ABCA1 is a large 240 KDa protein made of 2201 amino acids which form two transmembrane domains, each with an ATP-binding domain. It is
predicted that each transmembrane domain is composed of six membrane-spanning $\alpha$-helices, and that each ATP-binding domain contains a Walker A and B motif (See Figure 2-1).

Figure 2-1

**ABCA1 structure and regulation by calpain.**
Here phospholipids, represented by yellow spheres, are effluxed through ABCA1 to apoA-I, represented by a red cylinder. Notice the dimer configuration of ABCA1 with 6 transmembrane helices in each monomer. Figure taken from (85).
These transmembrane domains may form a pathway for translocation of substrates across the plasma membrane. Although incompletely understood, it is thought that ABCA1 shuttles phospholipids across the cell membrane, thus providing phospholipids to lipid-poor apolipoproteins in the plasma. Although ABCA1 activity is necessary for the net efflux of excess cholesterol to lipid-poor apolipoproteins in macrophages, evidence exists which suggests that ABCA1 does not directly bind cholesterol (16). It is known that ABCA1 forms dimers and tetramers in the cell membrane, and that apoA-I will associate with both these forms but not with the monomeric form of the transporter. In fact, when mutant forms of ABCA1 are expressed which do not self-associate, cholesterol efflux to apoA-I is severely impaired (17). Co-immunoprecipitation experiments have demonstrated that this ABCA1 protein complex in the cell membrane does not contain any other proteins (17). Therefore any strategy for the regulation or modulation of cholesterol efflux to apoA-I will have to focus on expression levels or functional efficiency of ABCA1 and/or apoA-I. Cell surface binding of apoA-I is greatly enhanced in the presence of functional ABCA1, and it is known that the C-terminal domain sequence VFVNFA of ABCA1 is necessary for this increased binding (18). The location of ABCA1-dependent apoA-I lipidation is still controversial. There is some thought that apoA-I may be endocytosed and then lipidated by ABCA1 intracellularly. ABCA1 has been observed to shuttle from the cell membrane to early endosomes, late endosomes, and lysosomes, and it is known that this shuttling process is carefully regulated (19). It is possible that the purpose of this pathway is to contact and transfer lipids to endocytosed apoA-I. One regulator of ABCA1 movement is cathepsin D, a lysosomal protease necessary for the efficient transport of ABCA1 from late endosomes to the plasma
membrane (20). In the absence of lipid-poor apolipoproteins, ABCA1 is rapidly removed from the cell membrane and degraded by calpain, a thiol protease (21,22). This calpain-mediated degradation is dependent on the phosphorylation of a PEST sequence found on the cytoplasmic portion of ABCA1. Extracellular lipid-poor apolipoproteins signal for the dephosphorylation of this PEST sequence, thereby blocking the degradation of ABCA1 and extending its lifetime at the cell surface (22,23). While not yet well understood, it is thought that the fourth extracellular loop of ABCA1 is necessary for apolipoprotein stabilization (24). The signaling pathways responsible for the stabilizing effect of apolipoproteins on ABCA1 are highly debated, with some research showing that PKCα pathways are stimulated by apoA-I, leading to phosphorylation of ABCA1, possibly due to the enhanced production of diacylglycerol stimulated by the removal of sphingolipid (25). Other studies have suggested that apoA-I increases the concentration of cAMP in stimulated cells, resulting in ABCA1 phosphorylation via a PKA dependent pathway (26).

Although more research will need to be done before the cellular shuttling and stabilization of ABCA1 by apolipoproteins is understood, it is clear that ABCA1 is heavily regulated at multiple steps in the cholesterol efflux pathway, from protein expression, to transport to the cell membrane, to degradation. This is a logical consequence of the fact that ABCA1-dependant efflux can save a cell such as a macrophage from the toxic effects of excess cholesterol, but would damage cell membranes if too much cholesterol were removed from the cell. Indeed, we now know that mutations in ABCA1 affecting expression, transport, or stabilization are sometime strongly correlated with CVD risk.
The Influence of ABCA1 Variants on CVD Risk- As ABCA1 transports phospholipids to the outer leaflet of a membrane, the nearby lipid-poor apolipoproteins which stabilize ABCA1 gradually become lipidated, forming particles composed primarily of phospholipid and apolipoprotein. These small discoidal particles are known as nascent HDL. Very low levels of this nascent HDL are found circulating in human plasma because very quickly, a plasma enzyme known as lecithin-cholesterol acyl-transferase (LCAT) esterifies the cholesterol in these particles. This hydrophobic, esterified cholesterol sequesters itself in the center of the particle, forming the core of spherical, mature HDL.

The physiological impact of the lipid transport activity of ABCA1 is demonstrated when the transporter is absent or dysfunctional, which occurs in diseases such as Tangier disease (TD) and certain forms of familial hypoalphalipoproteinemia. These diseases have been linked to mutations in the ABCA1 gene. In these patients, cholesterol efflux via ABCA1 is markedly reduced. The magnitude of ABCA1 impairment varies, and is correlated with a decreased plasma HDL level (27). A study of 77 ABCA1 variant heterozygotes by Clee et al. showed there was a three-fold increase in the likelihood of developing coronary artery disease in this set of subjects compared to unaffected relatives. A study of TD homozygotes found that 20% of these patients had some form of CVD, while only 5% of matched controls suffered from CVD (p<0.05) (28,29). Further studies have demonstrated that ABCA1 dysfunction is not isolated to these rare diseases. Multiple variants of ABCA1 have now been demonstrated in the population as a whole, and many of these variants have been associated with a change in
the risk of CVD. Some variants have been shown to be protective while others seem to exacerbate the cardiovascular disease process (30-33), and still other variants of ABCA1 appear to have no affect on CVD development, perhaps because they have no affect on ABCA1 function (34,35). Recently, Kyriakou et al. demonstrated that variants which affect the expression of ABCA1, in this case a mutation in the promoter region of the ABCA1 gene, can significantly affect the atherosclerotic plaque burden (36). Patients with this promoter variant had decreased expression of ABCA1 in their atherosclerotic plaques and a significant increase in the mean number of diseased coronary arteries compared to controls. Another study showed that in four kindreds possessing ABCA1 variants with decreased fibroblast cholesterol efflux ability, heterozygotes had lower plasma HDL levels and increased intimal medial thickness in their carotid arteries compared to control subjects (37). Indeed, a strong correlation was found between a subject’s HDL plasma levels and his/her fibroblast cholesterol efflux ability \( r=0.9, p<0.001 \), suggesting that the efflux of cholesterol via ABCA1 may be an important factor in the development of atherosclerosis. Indeed, some studies suggest that the magnitude of cholesterol flux through ABCA1 for eventual deposition in the liver is a more direct measure of HDL cardioprotection than the plasma level of HDL, since some ABCA1 variants associated with altered CVD risk are not associated with changes in HDL plasma levels (38-40).

In Vivo Evidence For Potential of ABCA1-based Therapeutics- All these human studies suggest that ABCA1 activity plays an important role in protecting the body from atherosclerosis, and that enhancement of this activity may provide a powerful means of
preventing or reversing atherosclerosis. To test this hypothesis in vivo a wide array of mouse models have been developed in which ABCA1 levels are manipulated. In early transgenic models with human ABCA1 it was shown that overexpression of human ABCA1 led to increased apoA-I and HDL levels, an increase in efflux of cholesterol from macrophages to lipid-free apoA-I, and a 1.5 fold increase in delivery of cholesterol to the liver. This increased hepatic cholesterol was associated with an increased cholesterol level in the bile, suggesting that a greater flux of cholesterol from the periphery of the body into the feces for excretion was occurring (41). Further testing in these transgenic overexpression models established that, as predicted, these mice were dramatically protected from the development of atherosclerosis (42). For example, in one study, a 65% decrease in aortic atherosclerosis was observed in the transgenic mice compared to control mice (43). As a proof of concept, these studies were ground breaking, as they provided the first in vivo evidence that manipulation of ABCA1 can have a direct and profound impact on CVD.

Further studies, however, have made it clear that the relationship between ABCA1 and atheroprotection is not as simple as originally hoped. Recent data suggests that ABCA1’s role in reverse cholesterol transport and cardioprotection is tissue-specific. For example, Singaraja et al. performed a study comparing ABCA1 total knock-out mice with liver-specific ABCA1 knock-out mice. Their findings suggest that extra-hepatic ABCA1 plays a critical role in generating simple, phospholipid-rich nascent HDL-like particles, while hepatic ABCA1 is critical for maintaining plasma HDL levels (44). This association between hepatic ABCA1 levels and HDL plasma levels is now well established, both in studies where hepatic ABCA1 has been increased and HDL levels
have risen (45,46), and in studies where hepatic ABCA1 has been specifically knocked down and HDL levels have dropped (47,48). Perhaps more interestingly, however, are the studies regarding macrophage ABCA1. Slight overexpression of macrophage ABCA1 in mice fed a Western diet led to smaller atherosclerotic lesion sizes, particularly as the mice aged (49). Conversely, selective macrophage ABCA1 knock-out models demonstrate lesion progression (50). Fortunately, even though it has become clear that ABCA1 activity is tissue-specific, an overall increase in ABCA1 still appears to be beneficial, as the individual effects of each tissue’s ABCA1 additively provide CVD protection.

Overall, these ABCA1 transgenic and knock out animal models have allowed us to move beyond human studies which could only show that ABCA1 dysfunction led to disease and demonstrate that modulation of ABCA1-mediated cholesterol efflux to lipid-poor apolipoproteins can significantly impact CVD. In this way, these animal studies have opened a new therapeutic avenue for CVD treatment. It is now reasonable to hypothesize that a treatment which increases ABCA1 expression might cause regression or at least a slowing in the growth of atherosclerotic plaques.

*Liver X Receptor Agonists*- One group of compounds which is known to increase the expression of ABCA1 is liver X receptor (LXR) agonists. Liver X receptors are ligand-activated transcription factors which bind LXR response elements located in the promoter region of many genes involved in cholesterol transport and metabolism and carbohydrate metabolism. When a LXR is activated it forms a heterodimer with a retinoic-acid X receptor. This dimerization is necessary for binding the LXR response element. In
humans there are two isoforms of LXR, designated LXRα and LXRβ. Both forms act in a similar manner, although LXRα appears to play a larger role in cholesterol homeostasis under excess cholesterol conditions. It is believed that a wide variety of oxysterols, such as 22(R)-hydroxycholesterol, are the endogenous agonists of LXRs. These endogenous agonists allow LXRs to serve as a sensor for excess cholesterol within the cell (51).

Endogenous LXR activation has a plethora of effects including increasing the net amount of cholesterol transported to the liver via the reverse cholesterol transport system, increasing bile acid synthesis for enhanced cholesterol excretion, decreasing intestinal absorption of cholesterol, and decreasing de novo synthesis of cholesterol. One of the most interesting consequences of LXR activation is the upregulation of ABCA1. Costet et al. showed that LXR/RXR heterodimers activate the ABCA1 promoter in a sterol-dependent manner (52). Because of this research, synthetic LXR agonists have been developed over the past few years with the hope of utilizing this physiological system to expel excess cholesterol from the body’s arterial walls. Early tests with these drugs were very promising. In multiple mouse models, these compounds have been shown to increase ABCA1 expression and increase the transport of cholesterol from the periphery to the liver (53,54). This enhanced cholesterol transport has also been demonstrated in hamster models, where macrophages labeled with tritiated cholesterol were injected into the peritoneal cavity, and counts were collected in the feces (53,55).

Joseph et al. were the first to show that LXR agonists could protect against developing CVD. They showed that LDL receptor knock-out mice treated with LXR agonists suffered significantly lower atherosclerotic burden than placebo mice. In fact, the total lesion area in male mice treated with LXR agonist was 53% less than that of
controls (56). These findings were confirmed by Terasaka et al. who noticed that LXR agonist-treated mice also possessed significantly higher plasma HDL levels than controls (57). Furthermore, Levin et al. showed that administering LXR agonist to LDL receptor knock-out mice with established advanced atherosclerosis produces a large (62%) reduction in lesion area compared to controls. This lesion reduction was associated with a 48% decrease in macrophage number, 67% increase in ABCA1 mRNA, and 34% increase in collagen mass within the remaining plaques. Interesting, the additional collagen found in these plaques was located above the necrotic core in the fibrous cap, suggesting that not only had the plaques shrunk, but they become increasingly stable upon LXR agonist treatment (58).

Such dramatic findings produced great excitement over the development of LXR agonists for CVD prevention and treatment. However, problems with this strategy have become readily apparent. Since these receptors regulate many pathways involved in systemic lipid metabolism, undesirable side effects have been observed, and eliminating these side effects through increased selectivity has so far proven difficult. One study performed in mouse and hamster models demonstrated that LXR agonists increased lipogenesis and thus plasma triglyceride and phospholipids levels. These increases may be due to sterol regulatory element binding protein 1c induction through LXR (59). Such pleotropic effects of LXR agonism were less apparent in the early in vivo trials due to a natural absence of cholesterol ester transfer protein (CETP) in mice. In animal models with CETP such as hamsters and cynomolgus monkeys, an increase in LDL and no increase in plasma HDL were observed upon LXR agonist treatment (60). Since humans possess CETP, these off-target effects pose a problem for the therapeutic use of LXR
agonists for CVD treatment. Currently, multiple pharmaceutical companies are attempting to develop a second generation of LXR agonists meant to address these unfavorable actions of early agonists. It remains to be seen if this newest generation of selective LXR agonists can overcome these problems.

ApoA-I Mimetics- While LXR agonists represent one strategy for therapeutically modulating ABCA1, other strategies have also been explored. One of the most promising alternatives to increased ABCA1 expression as a means of increasing reverse cholesterol transport is increased plasma levels of apoA-I or apoA-I mimetic peptides. In 2003, Zhang et al. first showed in vivo evidence that, compared to wild-type mice, mice overexpressing human apoA-I had increased flux of cholesterol from peripheral macrophages to the liver and into the feces (61). This began the search for methods of raising plasma apoA-I levels. The Medicines Company has acquired the patent to a variant of apoA-I known as apoA-I Milano, and conducted a brief clinical trial with this protein. Human subjects with atherosclerotic disease were injected with apoA-I Milano combined with phospholipids, a product similar to nascent HDL, once a week for five weeks. At the end of this five week period, intravascular ultrasound was used to assess atheroma volume, which was found to be 4.2% lower than the initial baseline measurement (62). This study is an exciting demonstration of the potential of apoA-I based therapeutics. However, since apoA-I Milano is not readily bioavailable and requires intravenous injection, and since the cost of protein therapeutics production is often high, this avenue is unlikely to become a widespread treatment for CVD. It has been recognized that this problem might be solved with a synthetic peptide capable of
stimulating ABCA1-specific cholesterol efflux or with a synthetic peptide capable of displacing apoA-I from endogenous HDL, generating a larger pool of lipid-poor apoA-I. Using apoA-I as a template, a variety of synthetic peptides has been developed for this purpose. One of the earliest apoA-I mimetic peptides is referred to as 4F. It is an 18 amino acid peptide designed to form a single class A amphipathic alpha helix, which is an α-helix with distinct hydrophobic and hydrophilic faces and with positively charged residues at the border of these two faces. This design was meant to mimic apoA-I, which is composed of ten amphipathic alpha helices, most of which are class A. In vitro testing showed that 4F bound lipid in an apoA-I-like manner, suggesting it might have other biological activities similar to apoA-I (63,64). Unfortunately, when orally administered, 4F showed little bioavailability.

To overcome its bioavailability problem, a version of 4F made entirely of d-amino acids was formulated. When orally administered to LDL receptor knock-out mice fed a Western diet, D4F decreased atherosclerotic lesion size by 79% (p<0.05), and when placed in the drinking water of apoE knock-out mice on chow-diet, lesion area was decrease by 75% (p<0.05) (65). Further study showed that this impressive reduction in lesion area was associated with an increase in preβ HDL, a cholesterol poor nascent-like HDL, probably due to redistribution of apoA-I from large, mature HDL to these smaller particles. It was also noted that as a whole population, the HDL in D4F treated animals had increased anti-inflammatory properties (66). Later studies have suggested that this anti-inflammatory effect might be due to the fact that these single helix mimetic peptides have an enhanced affinity for oxidized phospholipids compared to apoA-I (67). Perhaps the most interesting finding though, was that in D4F treated animals an increased mass of
cholesterol was being transported from the peripheral macrophages to the liver for excretion into the feces. This was the first suggestion that synthetic apoA-I mimetic peptides could stimulate the reverse cholesterol transport system. With such a mechanism, it was thought that D4F might be used to safely regress atherosclerotic plaques in humans. To demonstrate this principle, Li et al. used a bypass vein graft model in apoE knock out mice with established atherosclerotic lesions in the aortic sinus. This allowed for the study of evolving and established atherosclerotic plaques within the same animal. Surprisingly, while the D4F treatment decreased the evolving plaque size by 42%, it had no significant effect on the established plaques in the aortic sinus (68). This suggested that while apoA-I mimetic peptides such as D4F might be an efficient preventative treatment for atherosclerosis, they would not be useful for regressing plaques already formed in patients. To overcome this weakness in D4F treatment, a combination treatment of D4F and pravastatin was administered to both young and old apoE knock out mice. The dose of both drugs was too low for either drug to be independently effective. This combined treatment resulted in increased HDL and apoA-I plasma levels and increased anti-inflammatory properties of HDL, suggesting that the drugs worked synergistically. Most importantly, atherosclerotic plaque formation was prevented in the young mice, and lesion area was found to significantly decrease in the old mice (69). Since statins are already wildly used clinically and well tolerated by most patients, such a combination might prove to be an important paradigm for future CVD treatment.

Since the smallest natural apolipoprotein able to efflux cholesterol in an ABCA1 dependent manner is apoC-I, which is composed of two amphipathic α-helices, it is
reasonable to hypothesize that a synthetic peptide composed of two helices might have functional advantages over D4F. To test this hypothesis Wool et al. evaluated the peptide 4F and three different bihelical peptides for cholesterol efflux activity, HDL remodeling ability, and LDL oxidation protection (70). The bihelical peptides were two 4F helices joined by varying linkers. One of the bihelical peptides, designated 37pA, used proline as a linker between the two helices. This might be the most physiological linker, since apoA-I and apoA-II, the two most abundant proteins in HDL are both proline punctuated between their individual α-helices. Interestingly, data from this study showed that all the bihelical peptides were more effective at remodeling HDL and releasing lipid-poor apoA-I than 4F, as shown by a significantly increased potency. These bihelical peptides also showed a greater ability to efflux cholesterol from cholesterol loaded macrophages in an ABCA1-dependent manner than 4F. Both these results suggest that bihelical peptides are better able to stimulate reverse cholesterol transport compared to 4F. Also, 4F was a more effective LDL anti-oxidant than the bihelical peptides, suggesting that single helical peptides might still be an interesting therapeutic option for the prevention of LDL modification.

The development of bihelical peptides as a means of pharmacologically increasing reverse cholesterol transport has been hindered by the observation that these peptides not only remove cholesterol from macrophage and fibroblast cells in an ABCA1-dependent manner, but also in an ABCA1 independent manner (71). This suggests that although they possess some of the lipid accepting functionality of natural apolipoproteins, they also behave like detergents, readily accumulating cellular lipids in the absence of ABCA1 to efflux them. This detergent behavior raises the concern that
these peptides might have dangerous, cytotoxic side effects from non-specific cell
membrane solubilization. Since natural apolipoproteins do not show significant amounts
of non-specific lipid-solubilization, it is reasonable to believe that such side-effects could
be avoided. However, understanding what structural differences exist between the
bihelical peptide 37pA and small apolipoproteins such as apoC-I and apoA-II, which are
composed of two and three α-helices respectively, has proven difficult. A better
understanding of the structural element or elements necessary for ABCA1-specific
cholesterol efflux is needed in order to advance these peptides into clinical trials. This is
a goal of the present work, and will be addressed more fully in Chapters III and IV.

The Amphipathic α-helix as the Necessary Element for Cholesterol Efflux via ABCA1-
When searching for the structural elements of an apolipoprotein necessary for efflux via
ABCA1 it is wise to first examine all apolipoproteins known to be capable of
participating in this process. It has been shown that ABCA1 can transfer lipids to many
apolipoproteins, including apoA-I, A-II, C-I, and E (72). Unfortunately, no consensus
sequence exists between these many apolipoproteins and peptides which would suggest a
common binding domain within these proteins. In fact, the structure of these proteins
varies widely, making a common structural element difficult to identify. ApoA-I and
ApoE are large proteins with a N-terminal four helix bundle, an interconnecting hinge
region, and a C-terminal two helix bundle (73-75). These bundles sequester hydrophobic
resides of their constituent helices from the aqueous environment. It is thought that the
rate-limiting step of apoA-I lipid binding involves the opening of the four helix bundle.
This process is driven by the formation of α-helical structure from random coil,
particularly in the C-terminal region of the protein (76). In contrast, apoA-II and apoC-I are much smaller proteins. In fact, apoC-I is the smallest exchangeable apolipoprotein in the human body, made of only 57 amino acids, while apoA-II is only slightly larger, composed of 77 amino acids. Both circular dichroism spectroscopy and nuclear magnetic resonance measurements predict that apoC-I is composed of two α-helices connected by a short linker region, while modeling studies suggest apoA-II is composed of three α-helices punctuated by prolines (77-79). As with apoA-I, these proteins are thought to increase their α-helical structure upon lipid binding (77,79). In this case, little opening of the structure can occur upon lipid binding as these proteins are basically short strings of α-helices with little tertiary structure when lipid-free. However, one structural similarity is readily apparent within this group of apolipoproteins. All of these proteins are composed almost exclusively with amphipathic α-helices, usually punctuated with prolines to form various numbers of 11-mers and 22-mers along their length. Furthermore, of the three types of amphipathic α-helices found in exchangeable apolipoproteins, class G, Y, and A, most of the helices within these proteins are class A. Class G helices have a random pattern of charge on their polar face, while class A amphipathic α-helices have positively charged residues at the polar-nonpolar interface and negatively charged residues toward the center of the polar face. Class Y helices are similar to class A helices except that a third region of positive charge splits the negatively charged polar face roughly in half. It has been hypothesized that each of these classes of helices plays a different functional role in apolipoproteins (See Figure 2-2).
Classes of amphipathic α-helices.
The grey shaded region represents the hydrophobic face.

Class G helices may represent regions of the apolipoprotein which are better able to interact with proteins, such as receptors and enzymes. In contrast, class A helices are thought of as being the primary lipid binding helices in apolipoproteins. It is thought that the juxtaposition of positively charged residues to the edge of the hydrophobic face is what allows class A helices to bury themselves deeply into a phospholipid bilayer, such as a cell membrane. This arrangement allows the hydrophobic portions of the positively charged residues to remain within the bilayer while the charged atoms themselves snorkel out beyond the bilayer to the aqueous extracellular environment (79). It has been speculated that class Y helices serve as low affinity lipid binding helices, perhaps allowing apolipoproteins to be exchangeable, i.e. to sometimes detach from lipid surfaces.

These observations have led to the hypothesis that the common element needed for cholesterol efflux via ABCA1 is simply the amphipathic α-helix, or perhaps the class A amphipathic helix. This hypothesis is attractive because it explains why such a variety of proteins and peptides can all serve as cholesterol acceptors, and why replacement of
the L-amino acids in these proteins and peptides with D-amino acids does not affect their ability as cholesterol acceptors. This hypothesis also explains the strong association which has been identified between lipid binding capacity of a protein or peptide and its ability to participate in ABCA1-mediated cholesterol efflux (80). However, the existence of peptides and proteins composed of amphipathic α-helices which cannot elicit lipid transfer via ABCA1, and the observation that the ability to bind lipid and the ability to serve as a cholesterol acceptor for ABCA1 do not always correlate (see Figure 2-3) suggests that in the common structural element needed to participate in ABCA1-dependent cholesterol efflux, more than simply the amphipathic α-helix is necessary (81,82).

![Figure 2-3](image)

**Incomplete correlation between ability to participate in ABCA1-mediated cholesterol efflux and lipid binding ability.**

Here lipid binding ability is estimated using a DMPC liposome clearance assay. All samples are apoA-I mutants. While the ability of most mutants to accept cholesterol from ABCA1 expressing cells positively correlates with their ability to clear DMPC liposomes, for helix swap mutants H4@H1 and H10@H7, this is not the case. Figure taken from (81).
Natarajan et al. attempted to identify the minimal element necessary for ABCA1-dependent cholesterol efflux by generating peptides representing helices within apoA-I. They identified two 22mer α-helices within apoA-I, helix 1 and helix 10, which were very efficient lipid binders (2). Unfortunately, neither helix could stabilize ABCA1 on the cell surface, nor efflux cholesterol via ABCA1. However, when they added helix 9 onto either helix 1 or helix 10, they generated a peptide which could both stabilize and efflux via ABCA1. Upon examination of these two peptides, they noticed that both possess a linear array of acidic amino acids spanning 32 Å along the interface between the hydrophobic and hydrophilic faces of two amphipathic helices (see Figure 2-4).
Peptides formulated by Natarajan et al. showing a linear array of acid amino acids. This linear array was proposed to be the important element in apoA-I for ABCA1-mediated cholesterol efflux by these authors. A) Helical wheel diagrams of apoA-I derived peptides known to efflux cholesterol via ABCA1 B) Helical net diagrams of apoA-I peptides with proposed element highlighted. Acidic residues shaded grey. This figure was taken from (2).
To test the hypothesis that this was the critical element, they generated a third peptide, helix 10/9, which had the same amphipathicity and hydrophobicity as helix 9/10, but had a disrupted acidic residue distribution. As predicted, this peptide could neither stabilize ABCA1 on the cell surface, nor efflux cholesterol via ABCA1. They concluded that indeed, this was the minimal element necessary for ABCA1-mediated efflux.

This conclusion appears to have been premature, however. Since these findings, this linear array of acidic amino acids has not been shown to be important in any of the other apolipoproteins or peptides which efflux lipid via ABCA1. Indeed, in a later paper published by the same group, it was concluded that in apoE an element composed of roughly four 22-mer helices was critical for ABCA1-mediated cholesterol efflux (83). In this case the first two helices contained a linear array of acidic residues, while the second two helices were efficient lipid binders. However, this 4 helix structure was never shown to be dependent on the linear array of acidic amino acids for its efflux ability. Indeed, this search for the structural elements necessary for ABCA1-mediated cholesterol efflux is ongoing, with many ideas still in need of evaluation. The newest structural element proposed was based on a new synthetic peptide, called 5A, which showed a high level of ABCA1-selectivity compared to other standard peptides composed of amphipathic α-helices (84). This peptide is composed of a poor lipid binding amphipathic α-helix joined with a strong lipid binding amphipathic α-helix, as defined by each helix’s overall hydrophobicity. The authors suggest that this motif might be important for ABCA1-specificity. Like many of the other theories on the important structural element for ABCA1-mediated cholesterol efflux, however, the importance of such a motif has not been demonstrated in any other proteins or peptides known to participate in this reaction.
Contributions of This Work

The two most pressing gaps in our knowledge of ABCA1-mediated cholesterol efflux involve the structural requirements necessary for an apolipoprotein to serve as an acceptor in this process, and the location of the lipid exchange. Delineation of the basic structural elements necessary for apolipoproteins to serve as acceptors in this process allows for development of therapeutic peptides and possible small molecule drugs to enhance reverse cholesterol transport. This addresses treatment from the apolipoprotein side of the process. Determining if this exchange occurs extracellularly at the surface of the cell membrane or intracellularly will dictate which methods of regulation of ABCA1 might be effectively manipulated to increase cholesterol efflux in a specific manner for lipid-laden cells in atherosclerotic plaques. This addresses treatment from the ABCA1 side of the process. It is currently impossible to predict which side of the reaction will be more amenable to modulation for therapeutic purposes, and therefore both approaches must be evaluated. The following work contributes answers to both questions. First, a negatively charged and a hydrophobic surface patch on apoA-I are assessed for functional importance in ABCA1-mediated cholesterol efflux. Then a number of novel peptides which model the individual amphipathic α-helices in apoA-II and apoC-I are utilized to determine the smallest naturally-occurring structural element necessary for ABCA1-specific efflux. Finally, the location of ABCA1-dependent apolipoprotein lipidation is determined.
CHAPTER III – THE ROLE OF HYDROPHOBIC AND NEGATIVELY CHARGED SURFACE PATCHES OF LIPID-FREE APOLIPOPROTEIN A-I IN LIPID BINDING AND ABCA1-MEDIATED CHOLESTEROL EFFLUX

Introduction

In order to try and identify the common structural motif necessary for apolipoproteins to participate in ABCA1-mediated cholesterol efflux, a hydrophobic and negatively-charged surface patch on apoA-I were evaluated for functional significance. These experiments were based on a long history of studies, starting with the crystal structure of a N-terminal truncation mutant of apoA-I solved by Borhani et al. in 1997. This model showed lipid-free apoA-I to be a horseshoe-shaped protein composed of a string of amphipathic \( \alpha \)-helices which was broken by prolines (86). The hydrophobic faces of these successive helices seem to rotate around the helical axis along the length of the protein. This crystal structure ignited new research interest in the lipid-free structure of apoA-I. Unfortunately, this research began to show that N-terminal truncation mutants such as the one crystallized had significantly different tertiary structures than wild-type apoA-I. For example, such mutants were shown to be significantly less resistant to urea denaturation and to have increased 1-anilinonaphthalene-8-sulfonate (ANS) binding suggesting a partly unfolded or destabilized structure with a greater exposure of hydrophobic residues to the aqueous environment (87). To complicate matters further,
also in 1997, Roberts et al. published a limited proteolysis study of lipid-free apoA-I which proposed that lipid-free apoA-I formed a helical bundle which did not include the extreme C-terminus, making this terminus susceptible to proteolytic cleavage (88). This model was not particularly compatible with the horseshoe-shaped protein model Borhani et al. had proposed.

In the following years, studies were designed to try and distinguish between these models. One such study used five cysteine mutants of proapoA-I labeled with acrylodan, a fluorophor, and anisotropy measurements to estimate the shape of lipid-free apoA-I (89). These measurements suggested that lipid-free apoA-I was an elongated ellipsoid with an axial ratio of 5:1. When combined with previous experimental data, this data led to the development of a helical hairpin model of lipid-free apoA-I, in which the N and C terminus of the protein were close to each other and the remainder of the protein formed two rows of helices tightly folded together.

Thus a conflict between a helix bundle model and a helical hairpin model of lipid-free apoA-I existed. To try and distinguish between these models, Brouillette et al. preformed a series of fluorescence resonance energy transfer (FRET) experiments (90). Probes were incorporated into apoA-I mutants in such a way that energy transfer would only occur if apoA-I formed a helical bundle but not if the helices were stretched out in a helical hairpin formation. When energy transfer occurred, it was concluded that lipid-free apoA-I is folded into a globular helix bundle in solution. Rogers et al. utilized analytical ultracentrifuge measurements to model lipid-free apoA-I in aqueous solution, but came to a slightly different conclusion. Models formed from their measurements suggested that lipid-free apoA-I was in a dynamic equilibrium between a loosely folded
helical bundle and a helical hairpin monomer (91). They suggested that this helical hairpin might actually be an intermediate structure for apoA-I as it converts from a lipid-free to lipid-bound conformation. In support of this hypothesis was prior work which had shown that monoclonal antibodies had very different immunoreactivity to lipid-free and lipid-bound apoA-I, suggesting the protein underwent large structural changes upon lipid binding (92). This opening of the helix bundle is consistent with observations that apoE, which is composed of a N-terminal four helix bundle, is known to have increased receptor binding upon opening of its bundle (93).

Other studies suggested that although unfolding of an N-terminal bundle may be an important step in apoA-I lipid binding, that the C-terminal helices of apoA-I were also necessary for this activity. Minnich et al. began establishing the importance of these helices when they showed that C-terminal deletion mutants showed decreased binding to HDL (94). Later, limited proteolysis studies showed that residues 1-192, presumably the N-terminal helix bundle, were a very stable structure, but were slow to initiate lipid binding, suggesting this function might be performed by the extreme C-terminal helices (95). Davidson et al. suggested that these C-terminal helices might energetically drive the lipid binding process through an increase in α-helicity (96).

These studies and models culminated in a paper published by Saito et al. in 2003, where scanning mutagenesis, CD spectroscopy, and tryptophan fluorescence measurements were used to model lipid-free apoA-I as a two domain structure with a N-terminal helix bundle and a less organized C-terminal domain (97). This model was noted to be very similar to that of apoE. Using this model and past experimental observations, a two-step lipid binding mechanism was proposed. First, the C-terminal
region of the protein binds to available lipid surfaces, followed by an unfolding and binding of the N-terminal helical bundle.

Until recently, this model and mechanism of apoA-I lipid binding was little changed or amended. Then, an X-ray crystallography model of full length lipid-free apoA-I was published (1). This model generally resembles an earlier model of lipid-free apoA-I developed using a combination of chemical cross-linking followed by mass spectroscopy and *in silico* sequence threading (98). Upon analysis of the crystal structure, Ajees et al. identified clusters of both charged and hydrophobic residues on the protein surface. The first is a hydrophobic surface patch composed of leucine (Leu) residues 42, 44, 46, and 47. These residues are located at the turn between the first and second helix in the N-terminal four helix bundle in both the Ajees crystal structure and in the Silva cross-linking model. This arrangement is unusual in that such a high concentration of hydrophobic residues might be expected to be sequestered in the interior of the helical bundle. Ajees et al. suggested that the solvent exposed hydrophobic patch might be the initial lipid interaction site that triggered the structural transition of apoA-I from its compact lipid-free state to an open, lipid-bound state. The second domain is a negatively charged patch composed of glutamic acid residues 179, 191, and 198 (and possibly 183). This patch is located between the helical bundle and the C-terminal lipid binding domain in both models, although the patch appears more localized in the crystal structure, owing to the higher state of organization of the C-terminus in the crystal. It has been speculated that the charged surface patch might be important for a direct interaction between apoA-I and ABCA1, which has been speculated to occur prior to the lipid
transfer event (99,100). The functional significance of these apoA-I surface patches for HDL formation is unknown.

We hypothesized that these patches may play an important role in ABCA1-mediated cholesterol efflux. To test this hypothesis, three point mutants of apoA-I were designed to disrupt these patches, as shown in Figure 3-1.

Figure 3-1

**Structural models showing apoA-I surface patches.**
A) Hydrophobic surface patch of apoA-I displayed on the X-ray crystal structure model (left) and lipid-free apoA-I homology model (right) (1,98). Models depict residues 42 and 44, which composed mutant L42,44D as space-filled light (teal) and residues 46 and 47, which composed mutant L46,47D as space-filled dark (red). B) Negatively charged surface patch of apoA-I displayed on X-ray crystal structure model (left) and lipid-free apoA-I homology model (right), with residue 183 included for completion (1,98). The models depict residues 179, 183, and 191 as space-filled light (teal), and residue 198 as space-filled dark (red).
The mutants apoA-I L(42,44)D and apoA-I L(46,47)D each have two of the four Leu residues comprising the putative hydrophobic patch replaced by a negatively charged Asp of relatively similar volume. For functional studies of the negatively charged surface patch, the central glutamic acid (198) was changed to a lysine residue, thus separating the remaining two negatively charged residues and in effect eliminating this surface patch. We found that none of the mutations introduced in this study altered cholesterol efflux via ABCA1 from murine macrophages. Interestingly, and in contrast to previous predictions, introduction of negative charges into the hydrophobic patch actually increased the ability of apoA-I to solubilize synthetic dimyristoyl phosphatidylcholine liposomes.(1).

Experimental Procedures

*Cells and Reagents-* Isopropyl β-D-1-thiogalactopyranoside (IPTG) was purchased from Fisher (Pittsburgh, PA). IgA protease was obtained from Mobitech (Gottingen, Germany). His bind resin was obtained from Novagen (Madison, WI). Dimyristoyl-phosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were acquired from Invitrogen (Carlsbad, CA). [1,2-3H(N)]-cholesterol was supplied by Amersham Biosciences (Piscataway, NJ). 8-bromoadenosine 3’,5’-cyclic monophosphate sodium salt (8-bromo-cAMP) and gentamicin were purchased from Sigma (St. Louis, MO). The Bis(sulfosuccinimidyl) suberate (BS₃) cross-linker was obtained from Pierce (Rockford, IL). The RAW264.7 macrophages used in the
cholesterol efflux assay were obtained from the American Type Culture Collection (Manassas, VA), and were maintained in DMEM supplemented with 10% FBS and 10 μg/mL gentamicin. Radiolabeling and efflux measurements were performed in DMEM supplemented with 0.2% fatty-acid free bovine serum albumin (Calbiochem, Gibbstown, NJ). All other reagents were the highest quality available.

**Point Mutagenesis of Human apoA-I**- DNA manipulation of apoA-I cDNA was performed in a PET30 construct contains an N-terminal histidine-tag (his-tag) separated from the human apoA-I cDNA by an IgA protease cleavage site (101). Point mutations were generated using PCR-based site-directed mutagenesis (Quick-Change, Stratagene, La Jolla, CA). Complementary primers were synthesized which matched the target sequence except at the codon they were intended to change. Additionally, all the primers were designed to add a silent restriction enzyme site to the construct for screening purposes. When completed, all mutant sequences were verified by the Cincinnati Children’s Hospital Sequencing Core on an Applied Biosystems 3730 DNA Analyzer.

**ApoA-I Expression and Purification**- ApoA-I was expressed and purified in *Escherichia coli* (*E. coli*) as previously described (101). Briefly, BL-21 *E. coli* cells transformed with our construct of choice were grown to an absorbance of 0.7-0.8 at 600 nm. IPTG was added to a final concentration of 0.5 mM. After 2 h, cells were pelleted, and stored dry at -80°C. Cells were lysed using probe sonication, and the apoA-I was purified using His bind resin followed by a phenyl sepharose hydrophobic interaction column (GE Healthcare). The his-tag was cleaved from the apoA-I with IgA protease, and the apoA-I
was purified away from the his-tag using a Superdex 200 size exclusion chromatography column (GE Healthcare). Later sets of proteins were expressed with a tabacco etch virus cleavable his-tag. This purification protocol omitted the phenyl sepharose column, as previously described (102). All protein samples remained in solution during the entire purification procedure and when purified were stored in standard Tris buffer (STB: 150 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.02% sodium azide). When used for a control, human plasma apoA-I was isolated as previously described (103), and stored in STB.

Far UV Spectral Analysis by Circular Dichroism- All samples were freshly dialyzed against 20 mM phosphate buffer (pH 7.4), and diluted to 100 μg/ml. All measurements were made on a Jasco J-715 spectropolarimeter. A background scan of phosphate buffer was subtracted from each sample scan. The mean residual ellipticity of each sample was calculated as described by Woody (104), and the formula of Chen et al. (105) was used to calculate the fractional helical content using the mean residual ellipticity at 222 nm. This measurement was performed on two independently prepared sets of proteins on different days. Percent helicity for each mutant was normalized to WT apoA-I for each set of proteins. Normalized trial values were averaged, and compared to WT apoA-I using an unpaired, two-tailed Student’s t-test, with a p-value of 0.05 or less indicating a significant change in percent helicity compared to WT apoA-I.

Tertiary Structural Analysis Using Tryptophan Fluorescence and BS3 Cross-linking Analysis- All fluorescence measurements were performed on a Photon Technology
International Quantamaster spectrometer. The four tryptophan residues in apoA-I were excited at 295 nm to minimize tyrosine fluorescence interference. The emission spectra were collected from 305 to 380 nm at room temperature. The protein samples were 100 μg/ml in 20 mM phosphate buffer (pH 7.4), and a phosphate buffer blank was measured and subtracted from each sample scan. Each sample was measured three times, and the wavelength of maximum fluorescence determined was the average of the maximum wavelength of each scan. We performed this measurement on two independently prepared protein sets and the average wavelength of maximum fluorescence of each mutant was compare to that of WT apoA-I using an unpaired, two-tailed Student’s t-test, with a p-value of 0.05 or less indicating a significant change in tertiary structure compared to WT apoA-I.

In order to assess the self-association characteristics of each mutant compared to WT apoA-I, BS₃ cross-linking analysis was used. Each mutant was incubated for 24 h at 4°C with the cross-linker BS₃ at a mole:mole ratio of 10:1 (BS₃:protein) in phosphate buffered saline (pH=7.4). The final protein concentration was 1 mg/mL. Self-associated complexes were assessed with SDS-PAGE.

Assessing Lipid Binding using the DMPC Clearance Assay- The dimyristoyl-phosphatidylcholine (DMPC) lipid clearance assay was performed as previously described (81). Proteins in STB were added to lipid vesicles at a mass:mass ratio of 2.5:1 (DMPC:protein) and the absorbance at 325 nm was recorded every 30 s for 20 min on an Amersham Biosciences Ultraspec 4000 UV/visible spectrophotometer. Samples were measured three times and absorbances averaged. The solution temperature was held at
24.5°C during the duration of the assay using a temperature controlled cuvette. Although lipid binding and solubilization is a complex, multistep process, this assay is widely accepted in the field as a means of approximating the lipid binding ability of an apolipoprotein. Two independently prepared protein sets were measured using different preparations of multilamellar vesicles. The average rate constant of each mutant was compared to that of WT apoA-I using an unpaired, two-tailed Student’s t-test, with a p-value of 0.05 or less indicating a significant change in the rate of lipid binding.

**ABCA1-dependent Cholesterol Efflux Assay**- RAW 264.7 macrophages were grown to 80% confluency, then incubated with media containing 1.0 μCi/mL [1,2-3H(N)]-cholesterol +/- 0.3 mM 8-bromo-cAMP. After a thorough wash, media containing a given concentration of apoA-I +/- 0.3 mM 8-bromo-cAMP was applied for 8 h. Media alone and with 10 μg/mL of human plasma isolated apoA-I were included as experimental controls. After 8 h the media was filtered and sampled to determine the amount of labeled cholesterol transferred to the media by scintillation counting. Three trial wells were treated with each protein sample, with the percent efflux calculated for each sample as the average of its three wells. Percent efflux was calculated by dividing the counts in the media by the total internalized counts per well. The total internalized counts were determined by dissolving the media-only treated cells with isopropanol at the end of the experiment, drying down this solution, resolubilizing the sample in toluene, and counting. Two sets of independently prepared protein samples were measured using cells prepared on separate days. The percent efflux of each mutant was normalized to WT apoA-I. In dose response experiments, the percent efflux of each mutant was
normalized to the highest dose of WT apoA-I. Each mutant’s average normalized percent efflux from the two trials was compared to that of the corresponding WT apoA-I dose using an unpaired, two-tailed Student’s t-test, with a p-value of 0.05 or less indicating a significant change in the ability of the mutant to stimulate cholesterol efflux compared to WT apoA-I.

Results

*Structural Characterization of ApoA-I Variants*- To understand the effect on secondary structure of sequence changes associated with the mutant apoA-I protein, each protein was analyzed using circular dichroism spectroscopy. As seen in Figure 3-2, each mutant generated a spectrum with minima in the mean residual ellipticity at 208 and 222 nm, the hallmark of a predominantly α-helical structure.
Representative circular dichroism spectra of apoA-I surface patch mutants. WT apoA-I (●), apoA-I E198K (○), apoA-I L42,44D (▲), and apoA-I L46,47D (▽). Far UV (195-260 nm) spectra of each protein at 0.1 mg/mL in 20mM phosphate buffer, pH 7.0. Every fifth data point is plotted. Points are the mean of three replicate scans from 260 to 190 nm at 100 nm/min with a 0.5 nm step size and 0.5 s response, using a bandwidth of 1 mm and a slit width of 500 μm. Error bars represent 1 SD.
Table 3-1 shows the calculated percent helical contents of the various mutants.

<table>
<thead>
<tr>
<th>ApoA-I Mutant</th>
<th>Normalized % α-helical content</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.000</td>
<td>0.000</td>
</tr>
<tr>
<td>E198K</td>
<td>1.079</td>
<td>0.179</td>
</tr>
<tr>
<td>L42,44D</td>
<td>0.8443</td>
<td>0.057</td>
</tr>
<tr>
<td>L46,47D</td>
<td>0.9484*</td>
<td>0.020</td>
</tr>
</tbody>
</table>

Table 3-1

**Helical Content of WT and mutant apoA-I.**

1 Average of two sets of triplicate measurements made on independent preparations of proteins. Values normalized to WT.

*p<0.05 compared to WT by unpaired, 2-tailed Student’s t-test

Most mutants exhibited helical contents that were statistically indistinguishable from WT apoA-I, with the exception of the L46,47D mutant, which showed a slightly lower value. This may indicate that disruption of these residues in the hydrophobic surface patch leads to small changes in the secondary structure of the protein. Since the difference in percent helicity and thus secondary structure of the L46,47 mutant was small compared to WT apoA-I, this mutant was included in the functional studies discussed below. To further evaluate any structural changes associated with the mutations, the wavelength of maximum tryptophan fluorescence (WMF) was measured, as shown in Table 3-2.
The WMF varies depending on the chemical environment of the four tryptophan residues in apoA-I. If these residues are buried within the hydrophobic core of the protein, the WMF is characteristically blue shifted to about 333 nm compared to tryptophan in an aqueous environment, which fluoresces around 355 nm (106). All the mutants displayed a tryptophan fluorescence pattern similar to that of WT apoA-I except L46,47D, which showed a slightly higher wavelength of maximum tryptophan fluorescence, again suggesting that the average tryptophan environment in the L46,47D mutant is more exposed to the aqueous environment than WT apoA-I. Together the CD and tryptophan fluorescence data indicate that disruption of the hydrophobic patch at residues 46 and 47
leads to small changes in the lipid-free structure of apoA-I, suggesting these residues may play a minor role in the overall stability of this protein. Finally, to assess the extent of self-association of each mutant compared to WT apoA-I, each mutant was cross-linked in solution with BS$_3$, and the extent of self-association analysed by SDS-PAGE. At 1 mg/mL, all mutants show comparable proportions of monomer and dimer in solution as WT apoA-I (data not shown).

*Lipid Clearance by ApoA-I Variants-* The rate at which each apoA-I variant can bind to and solubilize multilamellar DMPC vesicles (MLV) was measured. Figure 3-3 shows the decrease in turbidity over time as the protein binds and emulsifies the MLVs.
Figure 3-3

Representative curves from liposome clearance assay with apoA-I surface patch mutants.
WT apoA-I (●), apoA-I E198K (○), apoA-I L42,44D (▼), and apoA-I L46,47D (△).
The experiment was performed as described in Chapter III Materials and Methods. All points are the mean of three replicates. The fractional absorbance was calculated as optical density (OD) divided by the initial optical density (OD₀). A rate constant, k (min⁻¹), was calculated for each mutant by fitting the fractional absorbance at 325 nm vs time to a monoexponential decay equation for the first 5 min of the reaction using Microsoft Excel (see Table 3-3).
Table 3-3 presents the rate constants of lipid clearance for the first 5 minutes of the reaction assuming the binding event occurs under pseudo-first order kinetics with excess MLV present. All k-values are normalized to WT apoA-I.

<table>
<thead>
<tr>
<th>ApoA-I Mutant</th>
<th>k (min⁻¹)</th>
<th>Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>E198K</td>
<td>0.767</td>
<td>0.23</td>
</tr>
<tr>
<td>L42,44D</td>
<td>1.59*</td>
<td>0.07</td>
</tr>
<tr>
<td>L46,47D</td>
<td>1.77*</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Table 3-3

**Rate constants for pseudo-first order reaction of DMPC clearance assay.**
Rate constants calculated by fitting first 5 min of clearance time courses to pseudo-first order equation. All k values normalized to WT. The k-values shown are an average of two trials of independently prepared protein sets.
*p<0.02 compared to WT by unpaired- 2-tailed Student’s t-test

It is clear that apoA-I E198K cleared liposomes similarly to WT apoA-I. However, introduction of negatively charged residues into the putative hydrophobic surface patch in apoA-I L(42,44)D and apoA-I L(46,47)D resulted in a significant increase in the rate of liposome clearance compared to WT apoA-I.
ABCA1-dependent Cholesterol Efflux by ApoA-I Variants - The ability of each apoA-I variant to promote ABCA1-dependent cholesterol efflux from RAW 264.7 macrophages labeled with ³H-cholesterol was measured. In this cell system, treatment with cAMP leads to increased expression of ABCA1 on the cell surface (107-109). In these cells, the increased cellular cholesterol effluxed to apoA-I with cAMP treatment is due solely to this increase in ABCA1 on the cell surface. As seen in Figure 3-4A, in the presence of ABCA1, all the lipid-free apoA-I mutants promoted comparable magnitudes of cholesterol efflux as the WT apoA-I when compared at 10 μg/ml. To ensure that the cholesterol efflux was not saturated at this concentration, a range of apoA-I concentrations was investigated (Fig. 3-4B).
Efflux of cholesterol to lipid-free apoA-I surface patch mutants.
A) Cholesterol efflux to lipid free acceptors. B) Dose curve of cholesterol efflux to lipid-free acceptors. Percent efflux is normalized to dose of WT. Error bars represent mean ± SE from two independent trials of three replicates. WT apoA-I (●), apoA-I E198K (○), apoA-I L42,44D (▼), and apoA-I L46,47D (△).
It is clear that all mutants showed a concentration-dependent promotion of cholesterol efflux leading to saturation that was similar to WT apoA-I. Together, these data suggest that, despite the disruption of the putative hydrophobic or negatively charged surface patches, apoA-I can still fully participate in cholesterol efflux, and thus in HDL biogenesis.

Discussion

Due to its highly dynamic nature, the determination of a detailed three-dimensional structure of lipid-free apoA-I has proven difficult. Only in the last few years have attempts been successful in producing reasonably detailed models. The first of these was generated by using a fragment sequence threading approach guided by 21 experimental distance constraints provided by chemical cross-linking (98,101). This structure depicted a loosely associated four helical bundle reminiscent of higher resolution structures determined for a fragment of apoE (110) and intact lipophorin III (111-113). The C-terminus was predicted to be almost completely lacking in stable secondary structure, consistent with assertions that stabilization of an amphipathic helix in this domain triggers lipid binding. Ajees et al were successful at producing an X-ray crystal structure of full length lipid-free apoA-I under conditions of high concentration and with the additive chromium tris-acetylacetonate (1). This structure also depicted an N-terminal four helical bundle and a separate C-terminal domain. However, the C-terminus was more highly organized than predicted in the homology model. Although an important advance, this structure probably represents a highly stabilized conformation.
that does not reflect its in-solution conformation, as it is not consistent with a significant number of previous experimental observations. For a detailed discussion of both the homology and X-ray crystal structures, please see (114).

Although both models of lipid-free apoA-I have their limitations, a striking feature of both is the relatively exposed patch of hydrophobic Leu residues at the turn of the first and second helix in the N-terminal bundle. Palgunachari et al. have speculated that apoA-I binds to lipid surfaces by first anchoring its first and tenth amphipathic helix into lipid surface defects followed by subsequent cooperative binding of the central helices (115). It is clear from both the homology and crystal structures that helix 10 (the most C-terminal helix) could be positioned to participate in such a reaction. However, the availability of the N-terminal helix, associated with the helical bundle in both models, is less clear. One could speculate, as Ajees et al. did, that the unusual exposure of the Leu surface patch might act as an initial interaction site which triggers an opening of the bundle to allow the N-terminal sequence to bind lipid. Following this logic, we hypothesized that disrupting this putative patch with negative charges should decrease the tendency of apoA-I to interact with lipids. Our results with apoA-I L(42,44)D and apoA-I L(46,47)D, as shown in Figure 3-3, clearly do not support this hypothesis. In fact, we saw the opposite result, an apparently enhanced ability to bind lipids for both mutants. Given that the introduction of such polar residues should have disrupted the hydrophobic nature of the patch, we conclude that this site is not likely to participate in initial interactions with lipid. One possible explanation for the increase in lipid affinity is that our introduced mutations destabilized the already tenuous thermodynamic stability of the apoA-I helical bundle. It has been suggested that apoA-I lipid binding may be driven by
a decrease in free energy provided by lipid contact (97). Thus, factors that increase the thermodynamic stability of the lipid-free apoA-I are expected to decrease the free energy savings gained by lipid binding, i.e. the protein is less likely to bind lipid. It follows that the decrease the thermodynamic stability of the lipid-free protein would have the opposite effect and tend to drive the equilibrium toward the lipid-bound state. Thus, the hydrophobic patch may play a role in overall stability of the lipid-free protein.

It is interesting to note that, despite clear changes in lipid affinity, the surface patches did not exhibit differences in ABCA1-mediated cholesterol efflux. Phillips et al. have proposed that apoA-I solubilizes membrane domains that are generated by ABCA1 activity in addition to direct binding and stabilization of ABCA1 at the cell surface (99). They demonstrated a distinct correlation between solubilization of membrane-like lipid preparations and the degree of cholesterol efflux. In earlier work, we suggested a similar relationship, though this only held for mutations made in the C-terminal lipid binding helix of apoA-I. Indeed, a “helix-swap” mutant was produced in other regions of apoA-I that exhibited normal ABCA1 cholesterol efflux, but impaired DMPC clearance. Again in this work it is shown that mutations made in the N-terminal bundle can significantly affect DMPC lipid clearance, but have no affect on ABCA1-mediated cholesterol efflux. Clearly, further work is needed to address the relationship of lipid affinity and cholesterol efflux under control of ABCA1.

Our results unambiguously showed that introduction of a positive charge into the putative negative charge patch had no effect on lipid binding or cholesterol efflux. In contrast to the hydrophobic patch described above, the existence of such a charged patch is less clear. It is readily apparent in the crystal structure (Fig. 3-1), as the residues are
oriented by the relatively organized C-terminal helical domains apparent in the model. In
the homology model, these residues are predicted to exist in a region of random coil and
it seems unlikely that such a charge cluster could occur in such a dynamic part of the
molecule. Nevertheless, we introduced a positive charge in the middle of the “patch” as
indicated in the crystal structure. Our structural and functional experiments were unable
to detect any consequence of this substitution.

After this work was performed and published, however, the crystal structure of
apoA-I came under question due to the discovery that this and other crystal structures
published by this group were likely falsified (116). Therefore, the lack of functional
significance found for the negatively charged surface patch in these studies might point to
the fact that this patch may not exist, since it only appeared in the crystal structure, and
not in the homology model of apoA-I. Regardless, even if such a patch truly exists in
lipid-free apoA-I, our data suggests that it does not play a major role in lipid binding or
ABCA1-mediated cholesterol efflux.
CHAPTER IV – IDENTIFICATION OF APOLIPOPROTEIN STRUCTURAL ELEMENT THAT DETERMINES ABCA1-SPECIFIC CHOLESTEROL EFFLUX AND ABCA1 CELL SURFACE STABILIZATION

Introduction

High density lipoprotein (HDL) is a plasma particle composed primarily of phospholipids, cholesterol, and proteins known as apolipoproteins. For over half a century, it has been repeatedly demonstrated that high levels of HDL cholesterol in the plasma are strongly associated with a reduced risk of cardiovascular disease (CVD) (5,117). However, therapies to raise the level of plasma HDL have been difficult to develop. The rate limiting step of HDL biogenesis is the first step, the assembly of phospholipids and cholesterol to lipid-poor apolipoproteins produced in the liver and intestine. This process is dependent on a plasma membrane protein known as ATP-binding cassette transporter A1 (ABCA1) (118). Although abundant in tissues that produce HDL, ABCA1 also plays important roles in reverse cholesterol transport from the periphery to the liver in cells such as macrophages when excess intracellular cholesterol is present. When this transporter is dysfunctional, such as in Tangier Disease, the removal of excess cholesterol from sites like the arterial wall is impaired. This predisposes the vasculature to the development of atherosclerosis, plaques strongly associated with ischemic heart disease and strokes. A wide variety of exchangeable apolipoproteins can mediate this process, including apolipoprotein (apo) A-I, A-II, C-I, and E, though they must be lipid-unassociated prior to the interaction (72). In addition,
synthetic peptides have been shown to promote ABCA-I specific cholesterol efflux from a variety of cell types (70,71). Although, these acceptors share the common trait of amphipathic helical repeats, no consensus sequence has yet been identified that exclusively mediates the interaction. Thus, the structural elements of apolipoproteins necessary for stimulation of ABCA1-mediated lipid efflux are currently unknown. Identification of these elements would greatly add to our understanding of the physiological mechanism of HDL biogenesis, and thus to the body’s main defense against the development of cardiovascular disease.

Several investigators have suggested that the structural element necessary for interaction with ABCA1 may relate to the amphipathic α-helix, a structure common to all exchangeable apolipoproteins, rather than a specific sequence of amino acids. However, the existence of peptides and proteins composed of amphipathic α-helices which fail to elicit lipid transfer via ABCA1 imply that these domains must contain specific features that are required for ABCA1 interaction. The problem is further clouded by recent findings that acceptors of ABCA1 effluxed lipid may not have to directly interact with the transporter (99). Phillips et al. have suggested that a small subset of apoA-I may bind to and activate/stabilize ABCA1 prior to removal of ABCA1-translocated lipid by membrane solubilization via apoA-I bound to lipid sites. This is consistent with prior studies showing that apoA-I can be cross-linked to ABCA1 and that apoA-I binding can prolong the surface residence time of ABCA1 at the cell surface (22,23,119). Therefore, a search for the necessary structural elements for ABCA1-mediated cholesterol efflux should begin with a thorough characterization of the amphipathic α-helical constituents of
proteins known to mediate ABCA1 lipid transfer. In this way, the minimal structural element necessary to serve as an acceptor of ABCA1-effluxed lipids might be identified.

Sequence analysis and an NMR structure (78,79) shows that that apoC-I, the smallest exchangeable apolipoprotein, is composed of two \( \alpha \)-helices. ApoA-II is composed of only three. Like their larger relative, apoA-I, both proteins can mediate ABCA1-mediated cholesterol efflux (72). To study the structural components necessary for ABCA1-mediated cholesterol efflux to apolipoproteins, we chose to study the properties of each helical domain in these two small proteins. Utilizing this approach, we have identified a potential structural motif possessed by apolipoproteins that allows them to participate in HDL biogenesis. This is an important first step in understanding the parameters necessary for cholesterol efflux via ABCA1, and may help make it possible to enhance this mechanism for therapeutic purposes in the future.

Experimental Procedures

**Materials**- Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were acquired from Invitrogen (Carlsbad, CA). \([1,2-^3\text{H(N)}]\)-cholesterol was supplied by Amersham Biosciences (Piscataway, NJ). 8-bromoadenosine 3’,5’-cyclic monophosphate sodium salt (cAMP) and gentamicin were purchased from Sigma (St. Louis, MO). The RAW264.7 macrophages used in the cholesterol efflux assay were obtained from the American Type Culture Collection (Manassas, VA), and were maintained in DMEM supplemented with 10% FBS and 10 \( \mu\text{g/mL} \) gentamicin.
Radiolabeling and efflux measurements were performed in DMEM supplemented with 0.2% fatty-acid free bovine serum albumin (Calbiochem, Gibbstown, NJ). All phospholipids, including 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), and NBD 1-oleoyl-2-[12-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] dodecanoyl]-sn-glycero-3-phosphocholine (NBD-OLPC) were purchased from Avanti Polar Lipids (Birmingham, AL). Human plasma apoC-I was purchased from Athens Research and Technology (Athens, GA). All other reagents were the highest quality available.

**Peptide Synthesis**- Peptides were synthesized by GenScript Corp (Scotch Plains, NJ), 21st Century Biochemicals (Marlboro, MA), or Peptide 2.0 (Chantilly, VA). All peptides were greater than 95% pure as determined by high performance liquid chromatography. Peptides were stored lyophilized at -80°C until use. When needed, peptides were solubilized in ultrapure water +/- 3M guanidine, then dialyzed into Standard Tris Buffer (STB, 10 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide, pH 7.4), and stored under N₂ until use. Secondary structure content was estimated by circular dichroism spectroscopy on a Jasco J-715 spectropolarimeter. Samples were dialyzed into 20 mM phosphate buffer, pH 7.4 and diluted to a concentration of 100 μg/mL. A background scan of phosphate buffer was subtracted from each sample scan. Plasma apoA-II from normolipidemic patients was isolated and purified as previously described for apoA-I (120).
DMPC Liposome Clearance Assay- Peptides or plasma proteins were added to DMPC liposomes at a specified mole lipid: mole α-helix ratio and the absorbance of the solution at 325nm was recorded every 30 s for 20 min. Samples were measured in triplicate and absorbances were averaged. Data is expressed as a normalized optical density (OD) calculated by dividing the sample OD by the initial OD (OD₀). Two trials were measured on different days using fresh preparations of multilamellar vesicles for each set of peptides. All measurements were made on an Amersham Biosciences Ultraspec 4000 UV/Visible spectrophotometer within a temperature controlled cuvette at a temperature of 24.5°C.

Free Cholesterol Efflux Assay- RAW 264.7 macrophages were grown to 80% confluency, then incubated with media containing 1.0 μCi/mL [1,2-3H(N)]-cholesterol for labeling +/- 0.3 mM cAMP. In these cells, the presence of cAMP leads to expression of ABCA1 on the cell surface (108). After washing away any radiolabel not internalized by the cells, media containing the peptide or plasma protein of interest +/- 0.3 mM cAMP was incubated with the cells for 8 h. At the beginning of this incubation, a t₀ control plate was washed with PBS and the labeled cells in each well were dissolved in isopropanol. This solution was then dried under air, resolubilized in toluene, and counted on a 1900CA Packard liquid scintillation analyzer to determine the total amount of labeled cholesterol internalized by the cells at the beginning of the efflux experiment. After 8 h, a sample of media was filtered to remove dead cells and debris and counted to determine how much labeled cholesterol was transferred to the peptide or plasma protein in the media during the incubation. Each protein or peptide was tested in triplicate wells, and the percent
efflux calculated was the average of these wells. Percent efflux is the counts in the media divided by the total internalized counts per well calculated using the t₀ control plate, with background efflux to STB subtracted. The percent efflux of each peptide was compared to baseline using a two-tailed Student’s t-test, with a p-value less than 0.05 indicating an ability of the peptide to stimulate free cholesterol (FC) efflux. In the competition assay the percent efflux was compared to A-II helix 2+3 alone using a Student’s one-tailed t-test, with a p value less than 0.05 indicating competition between the peptides. For each set of peptides, at least two independent assays were performed using cells prepared on separate days.

\textit{ABCA1 Cell Surface Stabilization Experiment}- RAW264.7 cells were incubated for 16 h +/- 0.3 mM 8-bromo-cAMP in 0.2% BSA DMEM to upregulate ABCA1 on the cell surface. The cells were then washed and incubated with equal molar \( \alpha \)-helical amounts of peptides and plasma proteins in 0.2% BSA DMEM and incubated at 37°C for 6 h. Cells were then quickly chilled to 4°C on ice and washed with chilled PBS. All procedures from this point on were carried out at 4°C unless otherwise noted. Cell surface proteins were biotinylated with 0.5 mg/mL sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in PBS on a platform orbital rotator for 20 min. This biotinylation process was repeated once. The biotinylation reaction was quenched with 50mM Tris in PBS, pH 7.4 with an incubation of 5 min, again on a platform rotator. The quenching reaction was also repeated once. Cells were washed with chilled PBS, scraped into 1 mL PBS, and pelleted with centrifugation. The supernatant was discarded and the cells were lysed for 30 min with RIPPA buffer (50mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% Na
deoxycholate, 0.1% SDS) + 1 μg/mL pepstatin A, 1 μM leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride. Cell debris was pelleted by centrifugation and the supernatant was transferred to a new tube. The protein concentration of this supernatant was determined by a Lowry assay. 100 μg protein was loaded onto 85 μL Ultralink Plus immobilized streptavidin gel in a column (Pierce, Rockford, IL), and incubated with shaking for 16 h. The elutant, containing non-biotinylated proteins, was removed from beads by centrifugation. The beads were washed five times with RIPPA buffer, then the biotinylated proteins were eluted by incubation at 37°C for 30 min with 50μL 2-mercaptoethanol enriched SDS-PAGE loading dye (60 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 350 mM 2-mercaptoethanol), followed by centrifugation. Equal volumes of each sample were loaded onto a 4-15% tris HCl ready gel (Bio-Rad, Hercules, CA) and run at 200V for 60 min. Proteins were transferred to a PVDF membrane and blotted with 1:500 rabbit anti-ABCA1 (Novus Biologicals, Littleton, CO) in a 5% milk solution for 2 h, washed, and blotted with 1:5000 ECL anti-rabbit, horseradish peroxidase linked antibody (GE healthcare, Piscataway, NJ) in a 5% milk solution for 2 h. Protein was detected with supersignal west pico chemiluminescent substrate (Thermo Scientific, Rockford, IL) and blots were developed on a Konica SRX-101A developer. A chemidoc XRS imaging system (Bio-Rad, Hercules, CA) was used to measure band density for densitometry comparisons. For each set of peptides, at least two independent experiments were preformed using cells prepared on separate days.

DPPE or DPPS Enriched DPPC Vesicle Protein Binding Assay- DPPC vesicles with 5% DPPE, 10% DPPE, 15% DPPE, 5% DPPE+10% DPPS, and 5% DPPE+20% DPPS (by
mass) were generated by drying down appropriate amounts of lipids under N₂,
resolubilizing the lipids in PBS, and sonicating on a 1 sec on/1 sec off cycle at 50%
amplitude for 1 h. All vesicles contained 5% biotinylated DPPE, included in the masses
listed above, and 0.5% NBD-OLPC for detection. To ensure equally sized vesicles, each
vesicle was applied to a superose 6 10/300 GL size exclusion FPLC column (GE
healthcare Piscataway, NJ) equilibrated in PBS. Equivalent fractions from each run were
combined and quantified with an elemental phosphorus assay as previously described
(121). Vesicles were applied to Ultralink Plus immobilized streptavidin gel on a column
(Pierce, Rockford, IL) and incubated with shaking for 1 h at room temperature. The
column was then spun in a centrifuge at 4000 rpm for 2 min to elute unbound vesicles.
Preliminary experiments demonstrated that, as expected due to equal amounts of biotin,
each vesicle bound to the streptavidin gel with equal affinity. 10 μg of peptide was then
applied to the columns, excluding the vesicle only control columns. Peptides were mixed
and incubated with the vesicles for 10 min at room temperature. The column was again
spun to elute unbound protein. The CBQCA assay (Invitrogen, Carlsbad, CA) was used
to determine the quantity of unbound protein which flowed through the column, as it has
a high tolerance for phospholipids. Fluorescent measurement (ex. 485 nm, emit. 538nm)
was used to quantify unbound vesicles, both after first wash and in the final flowthrough.
Normalized % protein bound was calculated as the amount of protein bound divided by
the % protein bound by the 5% DPPE vesicles. Normalized % binding was compared
using a Student’s two-tailed t-test with a p-value less than 0.05 indicating a difference in
peptide binding behavior. For each series of vesicles, at least two independent assays
were preformed on different days.
Results

*Design of Peptides Modeling the Individual α-helices of ApoC-I and ApoA-II* - To examine the α-helices of human apoC-I and apoA-II for important structural elements with respect to ABCA1-mediated efflux, we took the approach of synthesizing short peptides represented each of the individual α-helices of these apolipoproteins. The determination of the beginning and end of each α-helix in apoC-I are based on the analyses of Gursky et al. (77) who determined helices of lipid-free apoC-I with proline scanning and circular dichroism measurements, Rozek et al. who used NMR on lipid-bound apoC-I to estimate helices (78), and Segrest et al. (79) who used computer modeling to estimate helical regions within lipid-free apoC-I. During this design process, helical breakpoints were favored if they resulted in peptide with a strong amphipathic nature and were roughly 22 amino acids in length in order to model the 22-mer helical pattern observed in a wide range of exchangeable apolipoproteins (122). The sequences of the apoC-I peptides are shown in Table 4-1.
<table>
<thead>
<tr>
<th></th>
<th>Residues of Mature Sequence</th>
<th>Hydrophobic Face Angle&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ApoC-I</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helix 1</td>
<td>7-33</td>
<td>160°</td>
</tr>
<tr>
<td>Helix 2</td>
<td>34-57</td>
<td>200°</td>
</tr>
<tr>
<td><strong>ApoA-II</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helix 1</td>
<td>8-31</td>
<td>180°</td>
</tr>
<tr>
<td>Helix 2</td>
<td>38-53</td>
<td>160°</td>
</tr>
<tr>
<td>Helix 3</td>
<td>51-73</td>
<td>220°</td>
</tr>
</tbody>
</table>

Table 4-1

Sequence and hydrophobic face angle of individual α-helixes of apoC-I and apoA-II

<sup>1</sup> Angles were estimated with helical wheel diagrams, with lysine residues included in the hydrophobic face angle if located at junction of hydrophobic and hydrophilic face due to snorkle effect (136).
In a similar manner, by observing the proline punctuation within apoA-II, modeling the protein with the Predictprotein program (123), and combining these findings with the computer modeling of lipid free apoA-II by Segrest et al. (79), designations for our apoA-II helical peptides were obtained (Table 4-1). Due to some ambiguity in determining the boundaries of apoA-II helix 2 we produced a short (residues 38-53) and a long form (residues 32-50) to ensure that any functional changes were not due to inappropriate truncation. We found that the long form was functionally identical to the shortened peptide (data not shown) and therefore refer to the short form throughout the manuscript. All peptides were N-terminally acylated and C-terminally amidated to aid in helical formation in solution, as previously described (124).

**DMPC Liposome Clearance Assay** - Given the tight association between the ability of apolipoproteins to solubilize lipid surfaces on one hand and reactivity with ABCA1 on the other (80,125), we first assessed the ability of the individual peptides to clear DMPC multilamellar liposomes. In this assay, the protein or peptide of interest is added to large turbid DMPC liposomes at the transition temperature of the DMPC, and the binding and emulsification of the lipid by the protein is indicated by a decrease in the solution turbidity as the liposomes are converted to small, non-light scattering lipoproteins. For these experiments, each helix of apoC-I was compared to human plasma isolated apoC-I on an equimolar helical basis (i.e. 2:1 molar ratio, the same number of α-helices was in each sample solution). Similarly, each helix of apoA-II was compared to human plasma isolated apoA-II on an equimolar helical basis. Figure 4-1A shows that intact plasma apoC-I rapidly cleared DMPC liposomes within about 5 min under these conditions. By
contrast apoC-1_helix 1 failed to significantly modify liposome light scattering, exhibiting a similar clearance profile to DMPC liposomes incubated with buffer alone (not shown). This indicates that this helix either a) failed to bind to the lipids or b) was unable to rearrange the bilayer structure. ApoC-1_helix 2 however, exhibited markedly increased clearance with the reaction being effectively over by the second time point. These data suggest that the majority of the ability of intact apoC-I to bind liposomes lies in its C-terminal helix. Figure 4-1B shows a similar distribution of lipid binding abilities among the helices of apoA-II.
Figure 4-1

**DMPC liposome clearance assay.**
A) Peptides modeling the individual α-helices of apoC-I were added to DMPC liposomes at a 23 mole lipid: 1 mole α-helix ratio. The solution’s turbidity over time is plotted with ● representing plasma apoC-I, ○ C-I helix 1, and ▼ C-I helix 2. B) Peptides modeling the individual α-helices of apoA-II were added to DMPC liposomes at a 10 mole lipid: 1 mole α-helix ratio, with ● representing plasma apoA-II, ○ A-II helix 1, ▼ A-II helix 2, and ▲ A-II helix 3. These experiments were performed as described in Experimental Procedures. All points are the mean of three replicates, and bars represent mean +/- SD. The fractional absorbance was calculated as optical density (OD) divided by the initial optical density (OD₀).
While intact apoA-II was quite effective, apoA-I helix 2, was almost entirely ineffective. However apoA-I helix 1 and apoA-I helix 3 were highly effective. Therefore, it is clear that both apoC-I and apoA-II contain both "fast" lipid emulsifying and poor emulsifying helices.

*ABCA1*-mediated FC Efflux- We next compared each peptide to its matched plasma protein with respect to promotion of ABCA1-specific cholesterol efflux. Again, individual helices were compared to plasma proteins on an equal molar α-helical basis. Figure 4-2A demonstrates that neither helix of apoC-I can serve as an acceptor of cholesterol from ABCA1, although the intact protein is highly capable. Figure 4-2B demonstrates that the same is true for the individual helices of apoA-II with the intact protein exhibiting robust activity.
**Figure 4-2**

**ABCA1-dependent efflux of cholesterol to lipid-free plasma proteins and individual helical peptides.**
A) Cholesterol efflux to lipid free human plasma apoC-I and individual C-I helical peptides.  B) Cholesterol efflux to lipid free human plasma apoA-II and individual A-II helical peptides. Bars represent mean ± SD of three replicates.
This suggests that neither a fast-lipid emulsifying helix nor a non-lipid emulsifying helix is sufficient for cholesterol efflux via ABCA1.

With the knowledge that a physiological pair of these two different types of helices, i.e. full length apoC-I, can serve as an effective acceptor for ABCA1-mediated cholesterol efflux, we hypothesized that perhaps the physiological minimal structural element for cholesterol acceptors is a pair of helices, one that is a fast emulsifier and one that is poor. To test this, two bihelical peptides were synthesized representing the two naturally occurring pairs of fast-emulsifying and non-emulsifying helices in apoA-II, apoA-II helix 1+2 which spanned residues 8-53 of the mature sequence, and apoA-II helix 2+3 which spanned residues 38-73. The function of these bihelical peptides with regards to ABCA1-specific cholesterol efflux was compared to full-length apoA-II. We found that apoA-I helix 2+3, while not as effective as full-length apoA-II, was able to promote ABCA1-mediated cholesterol efflux (Figure 4-3).
ApoA-I helix 1+2, which contained both a fast and poor helix (in order N to C), failed to promote significant cholesterol efflux. This data supports the idea that at least two helices are required for specific cholesterol efflux via ABCA1, with a poor and fast lipid emulsifying helix arranged N to C, respectively.

To determine if the requirement for the poor lipid emulsifying helix to be N terminal to the fast lipid emulsifying helix was related to a dipole moment of the helical motif or perhaps due to the orientation of the amino acid side chains to the long axis of
the helix motif, these characteristics were altered by making a reverse apoC-I mutant composed of residues 57-1 of apoC-I. A helix switch mutant of apoC-I was also made composed of residues 1-6:34-57:7-33. These mutants were analyzed with the cholesterol efflux assay. Both mutants served as cholesterol acceptors as efficiently as a wild-type apoC-I peptide, as seen in Figure 4-7. Although this arrangement of helices is not seen in nature, this suggests that neither the dipole nor the orientation of amino acid side chains plays a key role in the apolipoprotein element which determines ABCA1-specific cholesterol efflux.

ABCA1 Cell Surface Stabilization - It is known that apoA-I stabilizes ABCA1 on the cell surface through intracellular signaling pathways which decrease the degradation of ABCA1 residing on the cell surface (21-23). To verify that these bihelical peptide constructs interact with ABCA1 via a mechanism that is similar to intact apolipoproteins, the ability of these peptide pairs to stabilize ABCA1 on the cell surface was examined. Macrophages expressing ABCA1 on their surface were incubated with each peptide, then after 6 h all cell surface proteins were biotinylated and collected using a streptavidin column (as described in Methods). Figure 4-4 and Table 4-2 show a representative Western blot and the relative quantification of this blot by densitometry.
Figure 4-4

**ABCA1 cell surface stabilization by lipid-free human plasma apolipoproteins and bihelical A-II peptides.**

As described in Experimental Procedures, RAW cells were incubated +/- cAMP for 18 h, and then washed. Lipid free apoA-II or bihelical peptides on an equal molar \( \alpha \)-helical basis were incubated with cells for 6 h without cAMP. Cells were then chilled and surface proteins biotinylated and collected with streptavidin columns. Equal volumes of biotinylated cell surface protein were loaded onto an SDS-PAGE gel. The western blot shown is against ABCA1. Lane 1) –cAMP, plasma apoA-I; Lane 2) +cAMP, plasma apoA-II; Lane 3) +cAMP, A-II helix 1+2; Lane 4) +cAMP, A-II helix 2+3. All lanes taken from the same exposure.
### Table 4-2

**Densitometric analysis of ABCA1 cell surface stabilization by plasma apoA-II and bihelical A-II peptides.**

1. Average of three Western blots representing three independent cell surface stabilization experiments. All percentages normalized to human plasma lipid-free apoA-II.
The bihelical peptide A-II helix 2+3 was the only peptide able to stabilize ABCA1 on the cell surface with equal efficiency as plasma apoA-II. This strongly supports the conclusion that A-II helix 2+3 interacts with ABCA1-expressing cells in a similar manner as natural apolipoproteins. To further investigate this behavior, the experiment was repeated with the individual α-helices of apoC-I and apoA-II, and A-II helix 3 showed partial ABCA1 cell surface stabilization, while A-II helix 2 did not (data not shown). This suggests that this stabilization behavior is facilitated by helix 3 and not by helix 2.

**ABCA1-mediated Cholesterol Efflux Competition Assay**- Since neither the non-lipid binding nor the lipid binding helix of an ABCA1-mediated cholesterol efflux active bihelical pair can participate in cholesterol efflux itself, it is clear that something about the joining of the two endows them with this ability. It is easy to understand why the non-lipid binding helix needs to be joined to a lipid binding helix in order participate in efflux, since an essential component of the efflux process is the solubilization of phospholipids and cholesterol. However, it is less easy to understand what the non-lipid binding helix contributes to the pair that enables ABCA1-specific cholesterol efflux. One hypothesis is that the non-lipid binding helix directly binds ABCA1, perhaps stimulating ABCA1 or simply tethering the peptide to area of the cell membrane around ABCA1. To test this hypothesis, a competition efflux assay was performed with A-II helix 2+3, an efflux active bihelical peptide, and A-II helix 2, the non-lipid binding helix. If A-II helix 2 does bind ABCA1, then high levels of A-II helix 2 in solution should inhibit cholesterol efflux to A-II helix 2+3. However, as seen in Figure 4-5, no competition was observed between these...
peptides. Therefore, it is unlikely that A-II helix 2 is directly binding ABCA1 in a mechanistically meaningful way.

Figure 4-5

**Competition assay between A-II helix 2 and A-II helix 2+3 in ABCA1-mediated free cholesterol efflux.**
Note, in this efflux proteins are competing on an equal molar, not an equal molar α-helical basis. Bars represent mean ± SD of three replicates.
Vesicle Lipid Binding- In another attempt to understand what role the non-lipid bind helix was playing in these important helix pairs, a vesicle binding assay was developed. Although the non-lipid binding helix cannot emulsify DMPC liposomes on its own, it is still possible that it might affect the lipid binding behavior of the lipid binding helix when they are joined. To test this hypothesis, the binding behavior of A-II helix 3 was compared to that of A-II helix 2+3. Figure 4-6A shows that there is no change in the lipid binding behavior of either peptide over increasing concentrations of DPPS, demonstrating that DPPS is not an attractant for these helix pairs. Figure 4-6B shows that as the concentration of DPPE increases in the vesicles, a statistically significant increase in the percentage of A-II helix 3 bound to the vesicles was observed. No increase in the binding of A-II helix 2+3 was observed.
Figure 4-6

**Phospholipid binding behavior of A-II helix 3 and A-II helix 2+3.**
Peptides were incubated with DPPC vesicles containing an increasing mass percent of A) DPPS and B) DPPE. The graphs display the percent of total protein mixed with the vesicles that bound to the vesicles, normalized to the percent bound to the 5% DPPE vesicles, with ● representing A-II helix 3 and ○ A-II helix 2+3. Bars represent mean ± SD of three replicates.

* indicates a p value < 0.02 compared to 5% DPPE vesicle binding by a two-tailed Student’s t-test.
Since DPPC is a cylindrically shaped phospholipid capable of forming tightly packed bilayers and DPPE is a conically shaped phospholipid which loosens the packing of a lipid bilayer, it is possible that the fast-lipid binding helix is attracted to loosely packed lipid bilayers.

Discussion

For many years, a positive correlation between the ability of a given protein to bind lipid and its ability to promote ABCA1-specific cholesterol efflux has been observed (80,125). However, this correlation does not hold true in all cases. For example, Panagotopulos et al. reported two helix swap mutants of apoA-I, apoA-I(helix 10 at helix 7) and apoA-I(helix 4 at helix 1), which displayed low rates of lipid binding and high levels of ABCA1-mediated cholesterol efflux (81). The studies in the current report suggest an explanation for these discrepancies. Although an ability to bind lipid is necessary for ABCA1-specific efflux, a peptide with this quality alone is not sufficient. Our data suggests that in apolipoproteins, the naturally necessary and sufficient structural element for this behavior is a bihelical peptide composed of a non-lipid binding and a fast lipid binding helix. Although this pattern was not recognized at the time, previous studies have shown that other apolipoproteins also contain this pattern in regions known to be of functional importance for ABCA1-mediated cholesterol efflux. Natarajan et al. published data demonstrating that helix 9 and 10 of apoA-I compose the structural element which mediates cholesterol efflux via ABCA1 in this protein (2). Previous work by Mishra et al. measured the reduction in enthalpy of the gel to liquid-crystalline phase
transition of DMPC, an indication of lipid binding, when apoA-I helix 9 and apoA-I helix 10 were incubated with DMPC (126). ApoA-I helix 9 did not reduce this enthalpy, suggesting it is a non-lipid binding helix, while apoA-I helix 10 was associated with the greatest reduction in this enthalpy among all the individual helices of apoA-I, suggesting it is a very strong lipid binding helix. Thus, the important element for ABCA1 mediated efflux in apoA-I again appears to be a non-lipid binding helix paired with a strong lipid binding helix at the C-terminus of the protein. Moreover, close examination of the literature suggests this motif likely exists in apoE. Vedhachalam et al. showed that the C-terminal lipid binding region of apoE was the important region for ABCA1-mediated efflux in this protein. The region identified spanned residues 222-299, a span much larger than two helices (83). However, it was shown that a peptide composed of residues 216-248 was a non-lipid binder, while a peptide composed of residues 238-270 was a strong lipid binder. Neither of these regions alone was able to participate in ABCA1-mediated cholesterol efflux. Most importantly, a peptide spanning both regions, residues 216-268, was found to be capable of some ABCA1-mediated efflux, similar to our A-II helix 2+3 peptide. These observations make a strong case that a pair of amphiphatic α-helices, one of which is a non-lipid binder, and the other of which is a fast lipid binder at the C-terminus of the apolipoprotein is likely the minimal structural element necessary for intact apolipoproteins to serve as a cholesterol acceptor during ABCA1-mediated efflux. Indeed, a similar observation has been made in synthetic peptides that are not based on any natural apolipoprotein sequence (84). When these synthetic bihelical peptides were composed of two fast lipid binding helices, they removed cholesterol from a cell in a mostly non-specific manner. However, when one of the helices in these
synthetic peptides was altered such that it became a poor lipid binding helix, the majority of the cholesterol removed from cells by the bihelical peptide was removed in an ABCA1-specific manner. Thus, the non-lipid binding helix conferred ABCA1-specificity on the bihelical peptide.

Interestingly, if the naturally necessary and sufficient structural element for ABCA1-mediated cholesterol efflux is a bihelical peptide composed of a non-lipid binding and fast lipid binding helix, then apoC-I represents the quintessential apolipoprotein structure for this mechanism. Indeed, apoC-I is a proficient cholesterol acceptor in this process, effluxing more FC than apoA-I on a mass basis (data not shown). It is important to realize that although this bihelical motif represents the minimal structural element of apolipoproteins which allows for ABCA1-specific cholesterol efflux, additional structural elements may enhance the ability of an apolipoprotein to serve as a cholesterol acceptor. For example, Figure 4-3 demonstrates that although A-II helix 2+3 can accept FC from ABCA1, full-length apoA-II accepts a greater mass of FC when compared on an equal molar α-helical basis. This difference is significant, with a p-value < 0.01. Therefore, structures outside this motif must also be considered when the extent of an apolipoprotein’s ability to serve as a cholesterol acceptor is being considered.

The most interesting and mysterious aspect of this bihelical structural motif is the role of the non-lipid binding helix. The competitive cholesterol efflux assay (Figure 4-5) demonstrates that this helix in apoA-II cannot inhibit the flux of cholesterol to the bihelical peptide A-II helix 2+3, even at a 10-fold molar excess. This strongly suggests that the role of the non-lipid binding helix is not binding to an efflux stimulating site on ABCA1, if such a site exists. Since ABCA1 has floppase activity and is thought to alter
the cell membrane in its vicinity, it was important to consider the possibility that this non-
lipid binding helix might somehow direct the binding of the lipid binding helix to areas
around ABCA1, which are presumably regions of the plasma membrane particularly
suited for microsolubilization by apolipoproteins (127). Unfortunately our understanding
of the effects of ABCA1 on the cell membrane is still very incomplete. It has been
proposed that ABCA1 transports phosphotidylserine (PS) from the inner leaflet of the
plasma membrane to the outer leaflet during cholesterol efflux (128-130), and that this PS
might stimulate apolipoprotein binding to the cell surface around ABCA1. Using a
vesicle binding assay (Figure 4-6A), we demonstrated that increased concentrations of PS
in the vesicles did not alter the lipid binding behavior of either a fast lipid binding helix
(A-II helix 3) or an example of the bihelical structural element (A-II helix 2+3). This agrees
with the data of Smith et al. who showed that PS increase on the cell surface did not
adequately account for the increased apoA-I binding observed with ABCA1 expression
(131). Thus the non-lipid binding helix does not redirect the lipid binding behavior of the
fast lipid binding helix toward PS or toward lipid patches with an overall negative charge.

The majorities of phospholipids in nascent HDL, however, are not negatively
charged and are not in the PS class. Numerous studies have demonstrated that nascent
HDL is primarily composed of phosphotidylcholines (PC) and sphingomyelin (SM)
(132,133). Therefore it is possible that the membrane around ABCA1 which is
accessible to microsolubilization by apolipoproteins is composed primarily of PC and
SM. Indeed, studies have shown that ABCA1 activity at the cell surface increases the
concentration of SM outside lipid-rafts (134), and that the solubilization rate of PC
vesicles by apoA-I is increased with increasing amounts of SM (135). Since both PC and
SM are primarily cylindrical in shape, it is likely that they would form a tightly packed membrane around ABCA1. Therefore, perhaps the non-lipid binding helix directs or facilitates the binding of the fast lipid binding helix to more tightly packed membranes. Figure 4-6B shows that indeed the non-lipid binding helix does alter the lipid binding preference of the lipid binding helix. Without the non-lipid binding helix, the fast lipid binding helix prefers vesicles with increasing amounts of DPPE, vesicles with presumably less and less tightly packed membranes due to the conical shape of this phospholipid. However, the addition of the non-lipid binding helix to the fast lipid binding helix serves to inhibit this preference for loosely packed membranes. This might allow some of the bihelical peptide to bind to more tightly packed regions of the plasma membrane, such as regions containing high concentrations of PC and SM. Unfortunately, without a better understanding of ABCA1’s affect on the cell membrane’s phospholipid composition, this assertion cannot be fully supported. Until this information is available, we can only clearly conclude that the non-lipid binding helix does indeed modify the lipid binding behavior of the fast lipid binding helix, possibly directing or enabling the fast lipid binding helix to bind to certain advantageous areas of the plasma membrane.

Finally, the possibility that the non-lipid binding helix may also play a role in modifying the lipid penetration ability of our bihelical structural motif should be considered. Table 4-1 shows the hydrophobic face angles of each individual helical peptide designed for these studies, as estimated from helical wheel diagrams. Note that each of the fast lipid binding helices associated with biologically active bihelical pairs, C-I helix 2 and A-II helix 3, have large hydrophobic face angles, taking into account the snorkel affect of lysine residues at the hydrophobic face edge (136), while the non-lipid binding
helices, C-I helix 1 and A-II helix 2, have very small hydrophobic face angles. Presumably, such wide hydrophobic faces cause the fast lipid binding helices to embed themselves deeply in the plasma membrane, while the narrow hydrophobic faces of the non-lipid binding helices hold these helices at the lipid surface among the phospholipid headgroups. Thus, the non-lipid binding helix may physically tether the pair close to the surface of the lipid bilayer. This controlled submersion of the lipid binding helix may facilitate the microsolubilization process. A similar mechanism has been demonstrated to facilitate the displacement of apoA-I from HDL by apoA-II. It was shown that A-II helix 3 deeply embeds itself into lipid bilayers, assuming an angle of insertion of roughly 35° due to a gradient of increasing hydrophobicity in the N to C terminal direction (137). This deep lipid embedment was shown to be critical for apoA-I displacement, since a mutant peptide lacking this angle of insertion was unable to displace apoA-I from native or reconstituted HDL, presumable due to its superficial contact with the phospholipids. This superficial contact prevents the disruption of the phospholipid acyl chains, thereby preventing the destabilization of the lipid-bound apoA-I. A similar acyl chain destabilization process may be important for the microsolubilization of the cell membrane around ABCA1. Such a mechanism would explain why the bihelical pair A-II helix 1+2 was unable to efflux cholesterol from cells when ABCA1 was expressed on the cell surface. Due to its smaller hydrophobic face angle compared to C-I helix 2 and A-II helix 3, A-II helix 1 may not be able to burrow deeply enough into the lipid bilayer to sufficiently disrupt the phospholipid acyl chains and initiate the process of membrane microsolubilization. Such a mechanism would also explain why these important bihelical peptides are all found at the extreme C-terminal end of natural apolipoproteins, the region
thought to be the most structurally flexible, particularly in the large apolipoprotein A-I and E which possess N-terminal four helix bundles (73-75). It would also explain why in natural apolipoproteins the fast lipid binding helix is always found to be more C-terminal than the non-lipid binding helix. This arrangement would allow the fast-lipid binding helix to bury itself without hindrance. If this is true, then apoC-I, since it is composed of only two helices, should retain its biological ABCA1-related activity even if its helices were reversed. A reverse apoC-I mutant composed of residues 57-1, and a helix swapped apoC-I bihelical peptide, composed of residues 1-6:34-57:7-33, was generated to test this hypothesis. Both mutants showed ABCA1-specific cholesterol efflux activity (Figure 4-7). Therefore, although in nature the fast-lipid binding helix is always found C-terminal to the non-lipid binding helix, this arrangement is not necessary for biological activity in the minimal structural element. This suggests that the fast lipid binding helix is located at the extreme end of natural apolipoproteins in order to be more available for membrane and/or ABCA1 interaction, and not due to any intrinsic structural requirement within the minimal bihelical functional unit itself.
ABCA1-dependent efflux of cholesterol to bihelical C-I peptides.
Bars represent mean ± SD of three replicates.
CHAPTER V – AN ANALYSIS OF THE ROLE OF A RETROENDOCYTOSIS PATHWAY IN ATP-BINDING CASSETTE TRANSPORTER-A1-MEDIATED CHOLESTEROL EFFLUX FROM MACROPHAGES

Introduction

The location of apoA-I lipidation and HDL formation by ABCA1 is controversial. Evidence exists suggesting that the lipidation of apoA-I via ABCA1 may occur through a retroendocytosis pathway where lipid-poor apolipoprotein is taken into the cell, is lipidated, and is then resecreted as nascent HDL (3,138). One of the first papers to propose this pathway used transmission electron microscopy to show that apoA-I appears to associate with clathrin-coated pits when ABCA1 is present at the cell surface, and that of all the apoA-I taken into a cell, after a 90 minute chase period, 58% of it is resecreted intact, while 32% is degraded and 10% remains in the cell (70). It was speculated that while within the cell, apoA-I might be lipidated via ABCA1. In support of this idea, Neufeld et al. and Smith et al. have both published results showing that fluorescently-labeled ABCA1 and apoA-I colocalize within human fibroblasts and RAW macrophage cell lines respectively (131,139). However, in these studies the colocalization is actually quite minimal. The confocal images show intracellular spaces almost completely filled with GFP-tagged ABCA1, and only small areas of colocalization with labeled apoA-I. This could be due to either a pathologic level of overexpression of ABCA1 leading to accumulation in a wide variety of non-physiological compartments, or might simply demonstrate that the GFP fluorescence signal does not accurately locate the position of
ABCA1 within the cell. To bolster the claim of a retroendocytosis pathway, Smith et al. demonstrated that induction of ABCA1 expression at the cell surface was correlated with increased apoA-I uptake, suggesting a role for uptake in ABCA1-mediated cholesterol efflux (88). Neufeld et al. added to this by using time-lapse confocal microscopy to demonstrate that endocytic vesicles stained for both ABCA1 and apoA-I are rapidly cycled between the cell surface and endosomal or lysosomal compartments (89). It has been shown that these intracellular compartments are the source of a majority of the cholesterol effluxed to apoA-I in an ABCA1-dependent manner (19,140). In further support of the claims of a retroendocytosis pathway, Lorenzi et al. treated RAW macrophage cells with cyclosporin A, a treatment which inhibits intracellular uptake of apoA-I, and showed that this treatment correlated with decreased ABCA1-mediated cholesterol efflux (141). However, this experiment is complicated by the fact that cyclosporin A treatment not only inhibits apoA-I uptake, but also inhibits ABCA1 endocytic trafficking, trapping ABCA1 at the cell surface (110). Indeed, it is known that treatment with cyclosporin can dramatically reduce HDL levels in mice (110).

Unfortunately, with cyclosporin treatment, it is difficult to determine if the decreased cholesterol efflux observed is due to inhibition of apoA-I uptake, ABCA1 trafficking, or both.

To address this shortcoming with cyclosporin A treatment, Verghese et al. recently performed a study using brefeldin A, a chemical known to inhibit ABCA1 trafficking (142). Unlike cyclosporin A however, brefeldin A does not affect apoA-I uptake. When adipocyte cells were treated with brefeldin A, apoA-I uptake remained intact while total cholesterol efflux was decreased more than 80% (p<0.001), suggesting
that apoA-I uptake is not a critical part of the cholesterol efflux pathway. Thus, conflicting evidence exists regarding the retroendocytosis pathway for ABCA1-mediated cholesterol efflux.

In the following set of experiments, the existence of a retroendocytosis pathway, and its importance for ABCA1-mediated cholesterol efflux was assessed. It is hypothesized that if retroendocytosis plays an important function in the lipidation of apoA-I, then the following should be true: a) apoA-I should be specifically internalized into the cell upon activation of ABCA1, b) the mass of apoA-I cycled though the cell should account for most of the nascent HDL particles formed in a given period of time, and c) the degree of apoA-I internalization should correlate with the degree of cholesterol efflux promoted.

To test these predictions, both fluorescent and radiolabeled apoA-I were generated so the movement and possible degradation of apoA-I could be studied throughout the retroendocytosis pathway. Using these tools, the intracellular location of internalized apoA-I was again assessed, and the contribution of internalized apoA-I to overall HDL formation was estimated. Finally, a wide variety of endocytosis inhibitors were tested in an attempt to chemically uncouple the internalization of apoA-I from ABCA1-mediated efflux. These studies address both the existence and necessity of a retroendocytosis pathway for ABCA1-mediated efflux.

Experimental Procedures
Materials- Enterokinase was purchased from Novagen (Madison, WI). IgA protease was obtained from Mobitec (Marco Island, FL). Isopropyl-β-D-thiogalactoside (IPTG) was from Fisher Scientific (Pittsburgh, PA). 1-Palmitoyl, 2-oleoyl phosphatidylcholine (POPC) was acquired from Avanti Polar lipids (Birmingham, AL). Fatty acid-free bovine serum albumin (BSA) was from Calbiochem (San Diego, CA). 8-bromoadenosine 3′, 5′-cyclic monophosphate (cAMP) was from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and phosphate buffered saline (PBS) were from Invitrogen (Carlsbad, CA). Dithiothreitol (DTT) was from Amresco (Solon, OH). Tris-(2-carboxyethyl) phosphine (TCEP) was obtained from Molecular Probes (Eugene, OR). All chemical reagents were of the highest quality available.

Construction, Expression and Labeling of Cysteine Mutants of ApoA-I- The human apoA-I Cys mutants used in this study were expressed in the bacterial pET30 expression vector (Novagen, Madison, WI) which encodes a N-terminal histidine tag sequence that can be cleaved from the protein by enterokinase. The cysteine point mutants shown in Figure 5-1 were generated directly in this vector with PCR-based techniques using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA).
Figure 5-1

Effect of the introduction of a Cys residue and attachment of an Alexa Fluor probe on the ability of apoA-I to promote apolipoprotein-mediated cholesterol efflux from cultured macrophages.

RAW264.7 cells were labeled for 24 h with $^3$H-cholesterol as described in Chapter V Experimental Procedures. After removal of labeling media and 3 washes, medium containing 10 µg/mL of lipid-free acceptor with 0.3 mM cAMP, to upregulate ABCA1, was added to the cells for 24 h. All Cys mutants represented as acceptors in this figure have the Alexa Fluor 546 probe attached. The cholesterol efflux data are expressed as percentage of total cell $^3$H-cholesterol for cells whose lipids were extracted immediately after the washes that followed labeling ($t_0$). Error bars represent 1 SD. of triplicate samples from one of three representative experiments. A one-way analysis of variance (ANOVA) was performed and the * indicates a significant difference from human plasma apoA-I (p<0.001) by a Tukey-Kramer multiple comparison test.
Human apoA-I ordinarily lacks a cysteine. The sequence of each construct was verified on an Applied Biotechnology System DNA sequencer at the University of Cincinnati DNA Core. The proteins were expressed in BL-21 *E. coli* cells according to established methods (143). The proteins were then purified over Zn$^{2+}$ chelating columns, (Roche Molecular Biochemicals, Indianapolis, IN) according to manufacturer instructions. The use of Zn$^{2+}$ vs. the more commonly used Ni$^{2+}$ chelation approach was required because DTT, which is necessary to keep the Cys reduced during purification, tended to precipitate out of solution when Ni$^{2+}$ was used. Fractions containing the pure proteins were dialyzed against standard Tris buffer (STB: 10 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 0.2% NaN$_3$, pH 8.2) with 1 gram DTT per liter. Before labeling, the DTT was removed by dialysis against STB. TCEP and sodium-cholate were added to a final concentration of 0.35 μM and 0.1%, respectively. To 7.5 mgs of apoA-I mutant, 1 mg of solid Alexa Fluor 546, C5-maleimide, sodium salt (Molecular Probes, Eugene, OR) in 1 mL STB, pH 7.1, was added. The label was added to the protein 30 μL at a time with one minute between each addition. The reaction was then incubated at room temperature for 2 h. The labeled protein was reconstituted into HDL particles for cleavage with enterokinase (apoA-I is non-specifically cleaved by enterokinase if it is lipid-free (144)). Reconstituted HDL (rHDL) particles were prepared using POPC at a lipid to protein molar ratio of 110:1 according to the method of Matz and Jonas (145). The histidine tag was cleaved with enterokinase (2.2 U/mg apoA-I) and passed over a Superdex 200 gel filtration column (Amersham-Pharmacia, Piscataway, NJ), to separate the labeled protein from the cleaved histidine tag and unreacted fluorescent label. The fractions containing labeled protein were combined, lyophilized and subjected to a chloroform/methanol
delipidation to remove lipid and lipopolysaccharide (101). The dried protein was dissolved in 3 M guanidine HCl and dialyzed into STB for use.

Expression and Purification of $^{35}$S-labeled ApoA-I- WT (wild-type) apoA-I cDNA in the pET30 vector containing the Igase cleavage site (101) was transfected into a methionine auxotroph line of *E. coli* (b834-DE3) (Novagen, Madison, WI). A single colony was used to generate 100 mL LB cultures which were pelleted and washed with M9A medium (1.0 g/L ammonium chloride; 5.8 g/L dibasic sodium phosphate; 3.0 g/L monobasic potassium phosphate; 2 mM MgCl$_2$; 0.1 mM CaCl$_2$; 2% glucose (v:v); 0.1 mg/mL Thr; 0.1 mg/mL Leu; 0.2 mg/mL Pro; 0.2 mg/mL Arg; and 0.0001% (v:v) thiamine). The cells were grown in fresh M9A medium for 1 h at 37°C. Then $^{35}$S-Met (Amersham-Pharmacia, Piscataway, NJ) and IPTG were added at a concentration of 5 μCi/mL and 0.5 mM respectively for 1 h at 37°C. The apoA-I was isolated as described above with the exception that IgA protease was used to remove the His-tag.

Cholesterol Efflux Studies- The transformed mouse macrophage cell line, RAW264.7 (ATCC, Manassas, VA) was maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA) with 10% FBS and 50 μg/mL gentamycin. Cells were grown to 75% confluence in a 48 well plate then this maintenance media was removed and $^3$H-cholesterol labeling media was added for 24 h (DMEM, 10% FBS, 50 μg/mL gentamycin, $^3$H-cholesterol 1.0 uCi/mL (Amersham-Pharmacia, Piscataway, NJ); 0.5 mL per well). After 24 h, the labeling media was removed and cells were washed twice with phosphate buffered saline (PBS) with 0.2% BSA and once with DMEM
containing 0.2% BSA. Efflux media (DMEM, 0.2% BSA, 10 μg/mL acceptor (apoA-I or specified mutant)) was added, with or without 0.3 mM 8-bromo-cAMP. For all instances in this chapter, the term “cAMP” refers to this analog. After 24 h, a 100 μL sample of efflux media was passed through a 0.45 μm filter to remove any floating cells and then measured by liquid scintillation counting. Percent efflux was calculated by dividing the counts in the media by total counts in the cells at time 0 h (146). In experiments using the endocytosis inhibitors, the cells were pre-incubated for 1.5 h with the inhibitor alone in DMEM, 0.2% BSA. This was removed and the cells were incubated with inhibitor and 10 μg/mL apoA-I acceptor in DMEM, 0.2% BSA for 1.5 or 6 h as indicated in figure legends. Otherwise, the cholesterol efflux studies were performed as above and apoA-I uptake studies were performed as below.

Confocal Microscopy Studies- RAW264.7 macrophages were grown on slide well plates (#138121, Nalge Nunc International, Rochester, NY) in the maintenance media described above. Once the cells were 60% confluent, they were incubated in DMEM containing 0.2% BSA with or without 0.3 mM cAMP for 16 h. Media containing the Alexa Fluor labeled apoA-I and 0.2% BSA was then added with or without 0.3 mM cAMP for the appropriate length of time. Cells were washed three times with PBS (no BSA). Then 0.75 mL of a 3% paraformaldehyde, 2% sucrose solution was added to the cells. The cells were fixed on ice for 45 minutes. Fixed cells were washed in PBS with 1% BSA (to block non-specific binding). In some experiments, a rat, anti-mouse, monoclonal antibody to the macrophage membrane protein CD11b (also known as MAC-1) (Research Diagnostics, Flanders, NJ), 10 μg/mL in PBS with 1% BSA was added to the
cells. The cells were incubated for 40 minutes on ice and washed in PBS with 1% BSA. An Oregon Green labeled secondary antibody, which excites at the same wavelength as fluorescein isothiocyanate (FITC) (Oregon Green 488 conjugated goat anti-rat IgG; Molecular probes, Eugene, OR), was added to the cells at a concentration of 20 µg/mL in PBS with 1% BSA. The cells were then incubated for an additional 40 minutes on ice, washed with PBS with 1% BSA, and sealed with cover slips using melted paraffin. For colocalization experiments, LysoTracker Green (Molecular Probes, Eugene, OR), was added to cells at a final concentration of 200 nM in the Alexa Fluor labeled apoA-I media described above. Once the cells were labeled, confocal imaging was performed on a Leica TCS 4D microscope/SCANware system (Heidelberg, Germany) equipped with an Omnichrome krypton-argon laser (Chino, CA). Excitation was performed using the FITC/TRITC channel, which excites at 488 nm and 568 nm simultaneously. Emission was collected using an RSP580 beam splitter and BP530 filter at detector #1 and a BP600 filter at detector #2. The data was analyzed using the image analysis software on the SCANware system. The amount of apoA-I within the cell was calculated by determining the number of pixels in the TRITC channel per cell and subtracting background. For control studies performed using dextran, the dextran conjugate-Alexa Fluor 546 (Molecular Probes, Eugene OR) was dissolved in STB to a final concentration of 2 mg/mL. The dextran conjugate was added to the cells in DMEM with 0.2% BSA ± 0.3 mM cAMP at a final dextran concentration of 18 µg/mL for 2 h. The cells were washed, fixed, and the membranes labeled with CD11b as described above. Confocal studies using CHO cells were performed in a similar manner but the base medium was Ham’s F-12 (Invitrogen, Carlsbad, CA) instead of DMEM. The media for the CHO cells
transfected with ABCA1-GFP additionally contained 3 mg/mL of the antibiotic G-418 sulfate (USB Corporation, Cleveland, OH). Similar to the macrophage experiments, cells were incubated in Ham’s F-12 media with 0.2% BSA for 16 h.

$^{35}$S-apoA-I Uptake, Resecretion, and Degradation- Pulse-chase apoA-I uptake and resecretion experiments were performed in RAW264.7 cells plated in 24 well plates and grown until 85% confluent in DMEM maintenance media. Cells were washed twice with 1 mL PBS with 0.2% BSA and once with 1 mL DMEM with 0.2% BSA, and pre-incubated for 16 h in DMEM with 0.2% BSA ± 0.3 mM cAMP. Then they were pulsed with 3 μg/mL $^{35}$S-radiolabeled apoA-I ± 0.3 mM cAMP in 0.75 mL DMEM with 0.2% BSA at 37°C for 1 h. The cells were washed five times with 0.75 mL cold PBS with 1 mM CaCl$_2$, 0.2% BSA and 5 μg/mL human HDL to remove adherent apolipoprotein (3). The original media was sampled to determine total counts added to the cells. The chase incubation containing 0.5 mL DMEM, 0.2% BSA, and 50 μg/mL human HDL ± 0.3 mM cAMP was incubated with the cells at 37°C for the indicated time period. The media was collected to find total secreted activity and then subjected to a TCA precipitation in which 50% w/v TCA was added in a 1:4 ratio to media, incubated 30 min at 4°C, and centrifuged 20 min at 15000 RCF. 100 μl of supernatant was counted to determine the amount of degraded protein present. The cells were washed once with 1 mL PBS with 0.2% BSA, twice with 1 mL PBS, and dissolved in 0.5 mL 0.2 M NaOH. 100 μL of this solution was counted on the scintillation counter to determine residual cell label. Total cell uptake was calculated as the sum of the medium and residual cell-associated radioactivity. The degraded protein was expressed as the TCA soluble fraction of the
chase medium, and the resecreted (intact) protein was calculated as total counts in the chase medium minus the TCA soluble counts (3). To determine the rate of particle formation, $^{35}$S-apoA-I was added to cells as described above. The media was collected at 3 h and run over a Superdex 200 gel filtration column (Amersham-Pharmacia, Piscataway, NJ). These fractions were analyzed by liquid scintillation counting. The same experiment was performed with $^3$H-cholesterol labeled cells with or without 10μg unlabeled apoA-I as the acceptor protein to determine which column fractions contained cholesterol.

**Miscellaneous**- Human plasma apoA-I was purified as previously described (147). The ABCA1-GFP construct (a gift of Dr. Richard Lawn) in the mammalian expression vector pEGFP-N1 (Clontech, Palo Alto, CA) was stably expressed in Chinese hamster ovary (CHO) cells using the Lipofectamine Plus kit (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions.

**Results**

If a retroendocytosis pathway plays a key role in apolipoprotein-mediated cholesterol efflux, then apoA-I should be detectable within macrophages soon after its introduction to cells expressing ABCA1. To test this, we constructed a panel of apoA-I mutants each containing a single cysteine to which a thiol reactive probe was attached. Previous work has shown that the presence of this probe does not have a major effect on the secondary structure content of these proteins as determined by circular dichroism.
Absorbance spectroscopy confirmed that each apoA-I molecule contained a single molecule of fluorescent probe. Figure 5-1 shows that the label introduced at amino acids 9, 93, and 124 did not affect the ability of apoA-I to participate in apolipoprotein-mediated cholesterol efflux in RAW macrophages stimulated with 0.3 mM cAMP. However, when the probe was introduced at position 232 in helix 10, a 40% decrease in ABCA1-mediated cholesterol efflux was observed. This is not surprising as helix 10 has been shown to be critical to apoA-I’s ability to stimulate ABCA1-mediated cholesterol efflux (81). The V93C mutant was chosen for further studies because it exhibited the highest levels of expression in our recombinant system.

Next, the ability to visualize fluorescent apoA-I (V93C) within RAW264.7 macrophages treated with cAMP to induce expression of ABCA1 was assessed. Panels 1 and 2 of Figure 5-2A show an optical slice taken through the center of the macrophage by confocal microscopy after incubation with fluorescent apoA-I (V93C) for 2 h. The red fluorescing Alexa Fluor probe on apoA-I was clearly identifiable within the confines of the CD11b membrane marker (green) in cells pre-incubated with cAMP. Within each cell, the apoA-I label was excluded from a large area that appeared to be the cell nucleus. Much less of the label was apparent within the cells in the absence of cAMP pretreatment (compare Panels 1 and 2). This is illustrated quantitatively by computer image analysis in Figure 5-2B.
The specificity of apoA-I uptake by RAW264.7 macrophages.

RAW264.7 cells were incubated for 16 h ± cAMP to upregulate ABCA1. Then either 10 μg/mL Alexa Fluor labeled apoA-I (V93C) ± 400 μg/mL unlabeled apoA-I competitor, or 18 μg/mL Alexa Fluor labeled dextran beads were added ± cAMP for 2 h. A) Representative confocal images (magnification of 63x) taken through the approximate center of fixed cells from the various treatments. The apoA-I and dextran labels appear red whereas the CD11b outer membrane marker is green. The labels on the left edge of the figure refer to treatment with or without cAMP for the entire row of images. The labels across the top identify the internalized component for the entire column of images. B) Computer image analysis in which the red pixels were quantified on a per cell basis from 6 fields (at least 75 cells) per sample after background subtraction. Bars that have the same symbol are significantly different from each other, $ (p<0.0001)$ and * (p < 0.001) by an unpaired two-tailed Student’s t-test. The error bars represent 1 SD of triplicate samples from one of two representative experiments.
The cells shown in Figure 5-2 were fixed in paraformaldehyde (see Methods), but similar results were obtained when live cells were imaged (Figure 5-3).

Live cell confocal images of apoA-I uptake by RAW264.7 macrophages.
RAW264.7 cells were incubated for 16 h ± cAMP to upregulate ABCA1. Then, 10 μg/mL Alexa Fluor-labeled apoA-I (V93C) was added ± cAMP for 2 h. These representative confocal images (magnification of 63x) were taken through the approximate center of the cells. The apoA-I label appears red whereas the CD11b outer membrane marker is green.

We also characterized the time dependence of apoA-I uptake. ApoA-I was detected within the cells 15 min after its addition to the media. However, before 30 min, most of the label was associated with, or just beneath, the cell surface (data not shown). After 30 min, most of the label appeared to be inside the cells as shown in Fig. 5-2A.
A trivial explanation for the cellular internalization of apoA-I is that treatment with cAMP, a second messenger known to exert a multitude of effects on macrophage metabolism, simply stimulates non-specific endocytosis in macrophages. To evaluate this possibility, the effect of cAMP treatment on the uptake of dextran polymers labeled with the identical Alexa Fluor 546 probe as that present on our apoA-I (V93C) was studied. This method has been used extensively to study phagocytic processes in macrophages (148). Panels 3 and 4 in Figure 5-2A show that the uptake of dextran was similar regardless of cAMP treatment. To further determine the specificity of fluorescent apoA-I internalization, a 40-fold excess of unlabeled apoA-I was added to the treatment. Figure 5-2 shows that the presence of excess unlabeled apoA-I competitively inhibited the internalization of apoA-I label (compare Panels 2 and 6), indicating significant specificity in apoA-I internalization. To gain insight into the identity of the intracellular compartments into which the apoA-I label was sequestered, we performed additional confocal studies using the Lysotracker reagent. Figure 5-4 shows that a substantial fraction of the internalized apoA-I label colocalized with Lysotracker when cAMP was present, indicating that some of the internalized apoA-I is trafficked to lysosomal or late endosomal compartments (149). This colocalization was not observed in the absence of cAMP (data not shown).
Colocalization of Alexa Fluor apoA-I (V93C) with Lysotracker green in RAW cells. Fluorescent apoA-I was incubated with RAW cells, as described in Fig. 5-2, with the addition of lysotracker green to the labeling media at a final concentration of 200 nM. The cells were incubated in labeling media for 2 h. A) Representative confocal image (magnification of 60x) taken through the approximate center of fixed cells showing Alexa Fluor signal (marking apoA-I location). B) Identical confocal image as Panel A, viewed through lysotracker green channel. Panel C) Merged image of Panel A and Panel B. All images representative of two independent experiments.

Finally, we studied the ability of the label to exit the cell after a thorough wash and chase incubation in clean media. Figure 5-5 shows a non-significant trend toward the release of a small amount of label, hinting that a portion of the label might be resecreted. However, the majority of the fluorescence signal stayed associated with the cells for the duration of the experiment.
Pulse-chase incubation of Alexa Fluor apoA-I (V93C) in RAW cells.

Fluorescent apoA-I was incubated with cells as described for Fig. 5-2. However, instead of immediately fixing the cells after the 2 h incubation, the cells were extensively washed. Then chase medium (0.5 mL DMEM, 0.2% BSA) lacking fluorescent apoA-I was placed on the cells for the indicated period of time. At each time point, the cells were fixed, visualized by confocal microscopy, and then quantified by image analysis as described for Fig. 5-2. A one-way ANOVA failed to detect a significant difference between uptake at $t_0$ and the subsequent time points.
The confocal microscopy approach, while well suited for measuring fluorescent apoA-I internalization, gives little information on the fate of the label after internalization. Did the protein remain intact or undergo immediate lysosomal degradation? To address this question, $^{35}$S-methionine was used to endogenously label wild-type apoA-I expressed in bacteria. This method was chosen because studies have shown that radioiodination of apoA-I can have significant effects on its structure and metabolism (150). The purified $^{35}$S-apoA-I performed at levels similar to plasma apoA-I in a cholesterol efflux assay using RAW macrophages (data not shown). Figure 5-6A shows the results of a pulse-chase experiment in which RAW cells were incubated with $^{35}$S-apoA-I for 1 h, extensively washed, then chased with clean media for 1.5 h according to the protocol of Takahashi et al. (3). At the end of the pulse incubation, approximately 66 ng of apoA-I/mg cell protein was associated with the cells when cAMP was present. This represents about 0.9% of the total apoA-I mass initially added to the media. During the 90 min chase period, 70% (or about 46 ng/mg cell protein) of the cellular $^{35}$S label appeared in the medium in cAMP treated cells. However, 29 ng/mg cell protein of this protein was found to be degraded. This left 17 ng/mg cell protein (about 37% of the total secreted label) of presumably intact apoA-I. The remaining label stayed associated with the cells. In the absence of cAMP, the amounts of label in each category were substantially smaller, except the amount associated with the cell at the end of the experiment, which was similar to the cAMP treatment level. The effect of the length of the chase incubation on the amount of intact apoA-I resecreted vs. that degraded was determined next. For chase times > 90 min, it was found that little additional label came out of the cells (data not shown), indicating that the resecretion process was largely
complete by 90 min. In addition, Figure 5-6B shows that the ratio of intact to degraded resecreted apoA-I was consistent over time.
A pulse-chase quantification of uptake, resecretion (intact) and degradation of endogenously labeled $^{35}$S-apoA-I from macrophages.

A) RAW cells were incubated (pulse) with 3 μg/mL $^{35}$S-apoA-I $\pm$ cAMP for 1 h. They were washed thoroughly according to Chapter V Experimental Procedures to remove residual surface $^{35}$S-apoA-I and then incubated with clean medium containing 50μg/mL human HDL for 1.5 h (chase) to assess resecretion. Total uptake is defined as the sum of the residual cell label and the label in the chase medium at the end of the experiment. The amount of secreted, degraded label was determined from a TCA precipitation performed on the chase medium. Resecreted (intact) apoA-I was defined as the difference between the total label in the chase medium and the amount that was degraded. Mass values were determined from the specific activity of the initial $^{35}$S-apoA-I. B) The pulse-chase experiment was performed exactly as stated above except the length of the chase incubation was varied as shown and cAMP was included in all samples.
To measure the mass of lipid-free apoA-I incorporated into nascent, discoidal HDL particles, we pre-incubated macrophages ± cAMP for 8 h, added $^{35}$S-apoA-I to the media for an additional 3 h ± cAMP, and then fractionated the media by gel filtration chromatography. The chromatographic profile in Figure 5-7A shows two major peaks occurring at about 21 and 34 mL, respectively, in samples lacking cAMP. Western blot analysis identified peak #1 as intact, lipid-free $^{35}$S-apoA-I. Peak #2 was completely TCA soluble indicating that it contained degraded apoA-I. With the addition of cAMP (Fig. 5-7B), a broad third peak appeared which contained intact $^{35}$S-apoA-I in the typical size range of discoidal HDL particles for this column. Figure 5-7C shows the chromatograph of a similar experiment using unlabeled apoA-I and RAW macrophages prelabeled with $^3$H-cholesterol and treated with cAMP. Activity measurements identified cholesterol in peak #3, indicating that it represents nascent HDL particles.
Figure 5-7

Gel filtration analysis of cholesterol efflux medium.

For the experiments shown in Panels A and B, RAW264.7 macrophages were pre-incubated 16 h ± cAMP to upregulate ABCA1. Medium containing 3 μg/mL \( ^{35} \text{S}\)-apoA-I ± cAMP was added to the cells and incubated for 3 h at 37°C. The medium was collected, floating cells were removed, and then it was applied to a Superdex 200 gel filtration column. The fractions were analyzed for \( ^{35} \text{S}\)-activity. Panel A shows the resulting profile in the absence of cAMP while Panel B shows the result in the presence of cAMP. For the experiment shown in Panel C, RAW cells were incubated 16 h with 1 μCi/mL \( ^{3} \text{H}\)-cholesterol and cAMP, washed and incubated in medium containing cAMP ± 10 μg/mL unlabeled WT apoA-I. The medium was collected and passed over the sizing column as in A and B. Peak 1: lipid-free \( ^{35} \text{S}\)-apoA-I, Peak 2: degraded \( ^{35} \text{S}\)-apoA-I. Peak 3: nascent HDL particles containing \( ^{35} \text{S}\)-apoA-I and \( ^{3} \text{H}\)-cholesterol (see Table 5-1).
Table 5-1 shows the percent distribution of the $^{35}$S-apoA-I and the mass of apoA-I associated with each peak in Figure 5-7. The data in Figures 5-6, 5-7 and Table 5-1 were used to approximate the flux of intact apoA-I through a retroendocytosis pathway (see Discussion).

<table>
<thead>
<tr>
<th>Peak #</th>
<th>Identity</th>
<th>Distribution of $^{35}$S apoA-I label$^a$</th>
<th>(-) cAMP</th>
<th>(+) cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Intact $^{35}$S-apoA-I</td>
<td></td>
<td>73% (164 ng)</td>
<td>24% (54 ng)</td>
</tr>
<tr>
<td>2</td>
<td>Degraded (TCA soluble) $^{35}$S-label</td>
<td></td>
<td>15% (34 ng)</td>
<td>27% (61 ng)</td>
</tr>
<tr>
<td>3</td>
<td>Intact $^{35}$S-apoA-I in cholesterol-containing particles</td>
<td></td>
<td>8% (18 ng)</td>
<td>48% (108 ng)</td>
</tr>
<tr>
<td>Total$^c$:</td>
<td></td>
<td></td>
<td>96%</td>
<td>99%</td>
</tr>
</tbody>
</table>

Table 5-1

**Gel filtration distribution of $^{35}$S apoA-I label in media after a 3 h incubation with RAW macrophages.**

$^a$ The percent distribution of label from the column runs in Fig. 5-7 was determined by summing the total counts in each fraction. For each experiment, peak 1 was defined as fractions 19-24, peak 2 was 30-36, and peak 3 was 10-18. The percent distribution is shown along with the approximate mass of apoA-I that would be associated with each peak given that 2250 ng of apoA-I was initially used in the incubation (see Chapter V Discussion).

$^b$ The state of apoA-I (intact, degraded, lipidated, etc.) was determined by western blot analysis, TCA precipitation, and co-fractionation with cholesterol as described in Chapter V Results.

$^c$ The total did not add up to 100% because there were small amounts of counts present in fractions that were not included in the three major peaks.
To test our third prediction that the degree of apoA-I internalization should correlate with the degree of cholesterol efflux promoted if a retroendocytosis pathway is the main method of apoA-I lipidation, we studied the effects of various endocytosis inhibitors on apoA-I uptake and cholesterol efflux. We tested cytochalasin D, which blocks cellular endocytosis by disassembling actin microfilaments and preventing endocytotic vesicle formation, amiloride, a sodium channel inhibitor that disrupts endocytic vesicle formation, and monensin, an ion transport inhibitor that blocks transfer from endosome to lysosome by increasing pH of intracellular vesicles thus inhibiting trafficking from Golgi to plasma membrane (151). Using initial inhibitor concentrations found in the literature, monensin and amiloride could disrupt apoA-I internalization to various extents (Figure 5-8). Although cholesterol efflux decreased slightly from untreated, there was no correlation between the degree of apoA-I uptake and cholesterol efflux inhibition. Cytochalasin D did not appear to affect apoA-I uptake or cholesterol efflux under these conditions.
Effects of endocytosis inhibitors on apolipoprotein-mediated 3H-cholesterol efflux and Alexa Fluor-labeled apoA-I cellular uptake. For cholesterol efflux studies, RAW264.7 macrophages were labeled with 3H-cholesterol and pretreated with 0.3 mM cAMP as described in Methods. Then cells were incubated with the appropriate concentration of inhibitor for 1.5 h. Medium containing 10 μg/ml plasma isolated apoA-I, 0.3 mM cAMP and the appropriate concentration of inhibitor were added to the cells for 1.5 h. The cholesterol efflux and cellular uptake measured by confocal microscopy are shown as a fraction of activity in the absence of any inhibitor. Panel A) Amiloride (in DMSO) was used at 1 mM. Monensin (in DMSO) was present at 25 μM. Panel B) Cytochalasin D (in DMSO) was added at 1 μM. For the cholesterol efflux studies, * represents a significant difference compared to the untreated cells determined by an unpaired, two-tailed student’s t-test (p<0.05). For the cellular uptake studies, ** represents a significant difference from the untreated cells determined by an unpaired, two-tailed student’s t-test (p<0.0005). Error bars represent 1 S.D. of at least triplicate samples from one of two representative experiments.
We further probed the monensin effect by performing a dose response experiment. Figure 5-9 shows that, with the exception of very low concentrations, the degree of cholesterol efflux did not change substantially with increasing monensin concentration. In contrast, a clear dose-dependent decrease in apoA-I internalization was observed. At the highest monensin concentration, apoA-I label was almost completely excluded from the cell, yet cholesterol was still effluxed at nearly normal levels.
Effect of monensin concentration on apolipoprotein-mediated cholesterol efflux and apoA-I uptake.

A) For cholesterol efflux studies, RAW264.7 macrophages were labeled with $^3$H-cholesterol and pretreated with cAMP as described in Chapter V Experimental Procedures. The cells were incubated with the appropriate concentration of monensin for 1.5 h, and then incubated with medium containing 10 μg/mL apoA-I + cAMP, and the appropriate concentration of monensin for 6 h. Cholesterol efflux and cellular uptake are displayed as a fraction of activity in the absence of monensin. For the apoA-I uptake studies, the * represents a significant difference from the untreated cells (p<0.01) by one-way ANOVA followed by a Tukey-Kramer comparison. The error bars represent 1 SD of triplicate samples from one of two representative experiments. B) Representative confocal images taken through the center of the cell. The images were taken within 1 h of each other during the same experiment using identical instrument settings. The concentration of monensin used is indicated under each image.
Finally, using Chinese hamster ovary (CHO) cells, the effect of overexpression of ABCA1 on the internalization of fluorescent apoA-I was determined. CHO cells exhibit higher basal levels of ABCA1 expression than RAW macrophages and do not require (nor respond to) cAMP pretreatment (152). Expression of an ABCA1 construct containing a green fluorescent protein (GFP) tag increased cholesterol efflux in these cells by about 240% (Fig. 5-10). Despite the increase in cholesterol efflux in the transfected cells, the degree of internalization of the fluorescent apoA-I was actually decreased by about 50% vs. untransfected cells.
Effect of ABCA1 overexpression on apolipoprotein-mediated cholesterol efflux and apoA-I uptake in Chinese hamster ovary cells.

CHO cells were transfected with an ABCA1-GFP construct and compared to untransfected cells in terms of cholesterol efflux and apoA-I uptake over a 6 h incubation as described in Chapter V Experiment Procedures and presented as in Fig. 5-9. The * and # symbols represent significant (p<0.0001) differences from untransfected cells for apoA-I uptake and cholesterol efflux, respectively, by unpaired two-tailed Student’s t-test.

**Figure 5-10**

**Effect of ABCA1 overexpression on apolipoprotein-mediated cholesterol efflux and apoA-I uptake in Chinese hamster ovary cells.**

CHO cells were transfected with an ABCA1-GFP construct and compared to untransfected cells in terms of cholesterol efflux and apoA-I uptake over a 6 h incubation as described in Chapter V Experiment Procedures and presented as in Fig. 5-9. The * and # symbols represent significant (p<0.0001) differences from untransfected cells for apoA-I uptake and cholesterol efflux, respectively, by unpaired two-tailed Student’s t-test.
Discussion

A retroendocytosis pathway for apolipoprotein-mediated cholesterol efflux is an attractive idea because it would offer numerous sites of regulation for potential pharmacological exploitation. We reasoned that if such a pathway plays a major role in cholesterol efflux from macrophages then, a) apoA-I should be specifically internalized upon activation of ABCA1, b) the mass of apoA-I cycled though the cell should account for most of the nascent HDL particles formed in a given period of time, and c) the degree of apoA-I internalization should correlate with the degree of cholesterol efflux promoted. Each of these predictions is discussed below with respect to our data, then we present a model of apolipoprotein-mediated cholesterol efflux in which retroendocytosis plays, at most, a minor role.

To address the cellular uptake of apoA-I, we used a recombinant form of apoA-I that had been labeled with a fluorescent probe at a single and highly targeted site in the molecule. It has been shown previously that the C-terminal helix of apoA-I is critical for the ability of apoA-I to promote cholesterol efflux from these cells (81,153). Consistent with this, cholesterol efflux capacity was negatively impacted when we tried to place a label in this helix at position 232 (Fig. 5-1). This demonstrates that a targeted approach to fluorescent labeling of apoA-I is a crucial component of any study designed to follow the cell biology of apoA-I by fluorescent microscopy. Thus, fluorescent labeling
techniques that rely on random labeling of lysine residues, such as the Cy5 reagent, should be interpreted with caution.

As predicted, the fluorescent apoA-I was indeed taken into RAW macrophages when ABCA1 was upregulated using a cAMP analog (Fig. 5-2). This uptake could not be attributed to nonspecific uptake or recognition of the probe because upregulation of ABCA1 with cAMP did not affect the uptake of dextran beads labeled with the same Alexa-Fluor probe. In addition, the uptake of fluorescent apoA-I was competitively inhibited by unlabeled apoA-I, indicating that the uptake was specific for the sequence of apoA-I. The uptake occurred rapidly and the label appeared to be targeted to endosomal/lysosomal compartments within the cell. These observations are consistent with the idea of a retroendocytic pathway for apoA-I lipidation, but are also consistent with a degradation pathway in which apoA-I would also be transported to the lysosome. The fact that a large portion of the fluorescent label could not come back out of the cell during extended chase periods in media lacking labeled apoA-I supports the latter explanation (Fig. 5-3). Indeed, when we studied the fate of the internalized protein using the endogenously $^{35}$S-Met labeled apoA-I in the presence of cAMP, the total amount of apoA-I associated with the cells was about 0.9% of that present in the medium, and although the majority of the cell-associated label reappeared in the chase medium, much of it had already been degraded. Our results differed from those of Takahashi et al. (3) discussed above, who showed that intact apoA-I represented the majority of the resecreted species (58%). These investigators used apoA-I that had been non-specifically radioiodinated whereas our studies used endogenously labeled protein which contained no covalent modifications and was demonstrated to be equally capable of stimulating

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cholesterol efflux as WT apoA-I. It is possible that the labeling method may affect the cellular trafficking of apoA-I or its susceptibility to normal degradation processes. The finding of a relatively small pool of resecreted apoA-I does not preclude the ability of such a pathway to account for the majority of apolipoprotein-mediated cholesterol efflux, especially if this small pool is rapidly turned over. To evaluate this possibility, we devised an estimate of the total flux of apoA-I through the putative retroendocytosis pathway. The estimation uses the degree of apoA-I degradation as a marker for the amount of apoA-I passed through the cells. This estimate depends on the following three assumptions, the merits of which will be discussed below: 1) degradation of apoA-I primarily occurs inside the cell. 2) the ratio of degraded apoA-I to intact apoA-I resecreted from the cell is constant over time, and no further degradation occurs once apoA-I is lipidated (see Fig. 5-6B), and 3) all apoA-I that goes through the cell is lipidated. From the pulse-chase study shown in Figure 5-5, a ratio of intact apoA-I to degraded apoA-I of about 0.60 was measured and was consistent over a wide range of chase incubation lengths. As seen in Table 5-1, in the presence of cAMP, the macrophages converted 108 ng of apoA-I to a lipidated HDL particle in 3 h, giving an approximate rate of conversion of 90 ng of apoA-I/mg cell protein/h (using an average of 0.4 mg cell protein/well). During that time, 61 ng of apoA-I was degraded. If assumption 2 is correct, then 61 ng * 0.60 (the ratio of intact to degraded apoA-I) = 37 ng of intact apoA-I came out of the cells over 3 h, giving a rate of about 30.5 ng apoA-I/mg cell protein/h. Therefore, we estimate that about one third of the total lipidated apoA-I product can be accounted for by retroendocytosed apoA-I. If one accounts for the nonspecific degradation of apoA-I evident in the non-cAMP treated macrophages, then
only about 11% of the product HDL can be explained by ABCA1-mediated retroendocytosis. It is also likely that a percentage of the “resecreted” apoA-I was never actually internalized. Although much care was taken to wash off the apoA-I present in the pulse incubation, it is highly likely that some of the apoA-I recovered intact in the chase medium was apoA-I that simply stuck to the outside of the cell. Thus, our estimation of the contribution of endocytosed apoA-I to lipidated particle formation is likely an overestimation.

If most of apoA-I is lipidated at the cell surface, as follows from the arguments above, then the cholesterol effluxed from macrophages should not correlate with the amount of apoA-I internalized. From the experiments in Fig. 5-8 and 5-9, it is clear that the uptake of apoA-I and resultant cholesterol efflux can be pharmacologically decoupled. The fact that monensin treatment affected cholesterol efflux was not unexpected because it has been shown that compounds which affect Golgi trafficking can have profound effects on apolipoprotein-mediated lipid efflux (154). However, we were surprised at the relatively modest effect that we observed compared to the work of others (155). Although the reason for this discrepancy is not immediately clear, perhaps differences in cell type and degree of cholesterol loading may affect monensin-sensitive pathways. The experiments performed in the ABCA1 transfected CHO cells provide further support for the claim that a direct correlation between the degree of internalized apoA-I and the degree of cholesterol efflux does not exist (Fig. 5-10), at least in these cell types.

Based on our data, we propose that although retroendocytosis of intact apoA-I may occur at low levels, the process is likely not critical for the overall efflux of
cholesterol from macrophages, i.e. the majority of lipid transfer events likely occur at the cell surface. Based on the work of Vedhachalam et al. (127) and Denis et al. (156) a general model has emerged in which lipid-free apoA-I first binds to ABCA1 and then associates with specialized lipid domains that have been created/modified by ABCA1 translocase activity. Indeed, most of the apoA-I that binds to cells in an ABCA1-dependent manner is bound to lipid rather than ABCA1 itself (127). Although the time frame of this apoA-I to membrane association is not clearly known, we propose that the observation of apoA-I retroendocytosis may be less of a pathway and more of a side-effect of macrophage membrane turnover. Since macrophages are professional phagocytes, the high degree of endocytic vesicle formation on the cell surface could be expected to trap some of this membrane-associated apoA-I and drag it into the cell. Depending on the intracellular targeting of the particular vesicle, it is conceivable that much of the trapped apoA-I would be delivered to lysosomal compartments and degraded whereas a small fraction might find its way back to the membrane without degradation. This idea has been suggested previously by Magnusson et al. (157) who concluded that retroendocytosis of certain glycoproteins in liver cells occurs mainly because of incomplete dissociation of ligands from receptors before receptor recycling to the cell surface. They demonstrated a positive relationship between a ligand’s affinity for its receptor and the degree of retroendocytosis. The case of apoA-I in macrophages is similar if one thinks of an ABCA1-generated patch of lipid as the apoA-I “receptor”. It has been shown that ABCA1 is endocytosed during cholesterol efflux (134,158,159), and it is possible that during this process apoA-I is occasionally internalized as well. This would explain our observation of the specific nature of the apoA-I uptake and its
relationship to ABCA1 activity. The relatively efficient degradation of this internalized apoA-I is consistent with our assertion that the intracellular apoA-I does not account for a significant fraction of apoA-I that is converted to lipidated HDL. The CHO cells used in the transfection experiment in Figure 5-10 are likely much less active in terms of endocytosis than macrophages and likely took up much less apoA-I for that reason. In this same cell line, we have shown that exogenous treatment with ceramide modulates ABCA1 trafficking such that it becomes enriched at the cell surface (160). The fact that this cell surface enrichment of ABCA1 corresponds to a several-fold increase in cholesterol efflux to apoA-I supports the idea that the plasma membrane may be the key site for apolipoprotein-mediated cholesterol efflux.

Further support for our hypothesis that the cell surface is the primary site for apolipoprotein lipidation via ABCA1 was published shortly after our own work. Denis et al. demonstrated in both macrophages and fibroblasts that fluorescently-labeled apoA-I colocalized with fluorescent dextran, a bulk phase uptake marker, suggesting non-specific uptake (161). Consistent with our studies, they found colocalization with lysosomes, suggesting apoA-I internalization was a degradative pathway. These authors noted poor colocalization between internalized apoA-I and ABCA1. They estimated that only about 27% of apoA-I which bound to ABCA1-expressing cells was internalized, and of this internalized apoA-I, only 20% was resecreted intact. Therefore, they estimated that internalized apoA-I accounted for only about 1.4% of nascent HDL production. This estimate is significantly lower than our estimate, perhaps because of differences in mathematical assumptions, and also because they used a cell surface protein stripping method before measuring internalized apoA-I. This cell surface stripping method
decreases the chance of overestimating the amount of internalized apoA-I, but can also introduce further experimental error, as the sucrose stripping media also affects endocytosis pathways. Finally, in contrast to our study, this study found that apoA-I internalization could be uncoupled from ABCA1-mediated cholesterol efflux using cytochalasin D. This discrepancy might be due to the fact that they used six-fold higher concentration of inhibitor than we did, and may also be the result of cell line differences, as we used RAW macrophages and they used baby hamster kidney cells. Overall, however, Denis et al. strongly corroborated our findings, and the conclusion that apoA-I lipidation occurs primarily at the cell surface.

Coincident with the publication of our work, a commentary on it was published in the same journal (162). While overall a positive assessment of the experimental design and results, the editor did point out one limitation in our study. Our tritiated cholesterol labeling technique used with the RAW macrophages leads to a non-physiological labeling of the plasma membrane cholesterol, and no excess cholesterol in the cells. *In vivo*, foam cells are formed when excess cholesterol accumulates in the cell as the cell continually endocytoses lipoproteins in an unregulated manner via scavenger receptor A. Thus, it is possible that macrophages do not utilize a retroendocytosis pathway under normal cholesterol homeostasis, but do utilize such a pathway under the stress of cholesterol excess. Such a pathway would be missed with our experimental design. Unfortunately, Denis et al. utilized the same tritiated cholesterol labeling technique in their retroendocytosis studies, as it is a very common method for performing cholesterol efflux measurements (161). Therefore, further studies need to be conducted to assess the
possibility that a retroendocytosis pathway may be active only under cholesterol loaded conditions in macrophages.

Currently, a preponderance of evidence points to our rejection of an endocytic pathway playing a major role in apolipoprotein-mediated cholesterol efflux. It is important to realized, however, that we do not preclude the possibility that the internalization of apoA-I may play an important biological role. For example, it is possible that the small amount of apoA-I that is internalized and degraded might function as a signaling agent, perhaps mediating some aspect of macrophage lipid or ABCA1 metabolism such as those elucidated by the elegant studies of Tang et al. (163). In addition, this process may be more important in other cell types. For example, Rohrer et al. have demonstrated that lipid-free apoA-I may be transcytosed across endothelial cells, demonstrating a unique intracellular trafficking pattern (138). In terms of therapeutically targeting ABCA1-mediated efflux from macrophages in atherosclerotic plaques, however, it appears that intracellular retroendocytosis is not necessary for the generation of nascent HDL, and that therapeutic strategies should focus on ABCA1 cell surface stabilization, cholesterol acceptor cell surface binding, and possibly on ABCA1 intracellular trafficking, although this last strategy needs further evaluation.

1 In using the term “specific” we do not mean to imply that apoA-I is the only apolipoprotein that can interact with these domains. The other exchangeable apolipoproteins that are capable of promoting apolipoprotein-mediated cholesterol efflux can undoubtedly compete for these same sites.
CHAPTER VI – MECHANISTIC REVIEW AND FUTURE WORK

Although the studies presented here provide insight into the process of HDL biogenesis, much of this mechanism still remains a mystery. Future studies to further delineate the mechanism can be built upon this work. For example, the retroendocytosis studies discussed in Chapter V suggest that this lipid exchange likely occurs at the cell surface. However, Oram’s criticism (162) that the macrophages used in our experiments were not cholesterol loaded must be addressed. This could be done by repeating the pulse-chase experiments performed in chapter V with macrophages that are pre-incubated with acetylated-LDL (ac-LDL). This will allow us to determine if excess intracellular cholesterol can rescue internalized apoA-I from the degradation we previously observed in the non-loaded cells. A range of loading doses of ac-LDL should be tested so that if a rescue effect is found, a threshold effect could be distinguished from a gradual increase in the utilization of a retroendocytosis pathway. The macrophages should be incubated +/- cAMP the night before the pulse-chase experiment so that total and non-specific internalization, resecretion, and degradation can be measured respectively and thus ABCA1-specific internalization, resecretion, and degradation can be calculated. By quantifying the mass of radiolabeled apoA-I internalized, resecreted, and degraded in an ABCA1-specific manner, a retroendocytosis pathway utilized under cholesterol loaded conditions would be readily apparent.
Assuming we do not find a retroendocytosis pathway utilized under conditions of increased cholesterol loading, future studies should focus on developing a complete model of HDL biogenesis in which apolipoproteins participate in ABCA1-mediated cholesterol efflux at the cell surface. There are three general models which describe lipid transfer to apolipoproteins at this site. The first predicts that apolipoproteins and ABCA1 directly interact, allowing ABCA1 to directly transfer lipids to these bound apolipoproteins. The second predicts that apolipoproteins and ABCA1 never directly interact. Instead, ABCA1 is proposed to modify the proximal plasma membrane, allowing apolipoproteins to bind and emulsify these membrane lipids by a microsolubilization mechanism. The third model is a combined model, involving both a direct interaction between apolipoproteins and ABCA1 and a direct interaction between apolipoproteins and the plasma membrane. In this third model it is thought that HDL biogenesis begins when apolipoproteins directly bind ABCA1. This binding stimulates ABCA1 to alter the cell membrane in such a way that phospholipids and possibly cholesterol in the membrane around ABCA1 become soluble to apolipoproteins. Apolipoproteins then directly bind to the cell membrane and emulsify portions of it, forming nascent HDL particles (see Figure 6-1).
Evidence of a direct protein-protein interaction between apolipoproteins and ABCA1 would support model one or three. One of the most compelling pieces of evidence for a direct interaction between these proteins was reported in a study by Chroni et al. in which apoA-I was cross-linked to ABCA1 on the cell surface (119). The cross-linker used in this study has an arm length of 3 Å. Since the headgroups of phospholipids are roughly 7-10 Å in length, it is clear that this cross-linking has captured a population
of apoA-I which is extremely close to ABCA1. If a direct protein-protein interaction was not occurring, and apoA-I was simply binding the membrane around ABCA1 and occasionally drifting within 3 Å of ABCA1, it would be highly unlikely to reproducibly crosslink enough of these two proteins together for detection. Another strong piece of evidence for a direct protein-protein interaction between apolipoproteins and ABCA1 is contained in a study by Fitzgerald et al. In this study, an ABCA1 mutant called W509S, which is incapable of participating in cholesterol efflux but is known to be expressed at the cell surface to an equal extent as wild-type ABCA1, was cross-linked to apoA-I. The efficiency of this cross-linking was equal to that of wild-type ABCA1 (164). Since the reason for dysfunction in W509S is unknown, the following possibilities should be considered: A) this point change might prevent ABCA1 from being stabilized by apolipoproteins or B) this point change might prevent ABCA1 from altering the cell membrane. If possibility A were correct, we would expect to see reduced, but not absent efflux activity, since some of the mutant ABCA1 would be transported to the cell surface and efflux cholesterol to apolipoproteins before being endocytosed and degraded. Since mutant W509S shows almost a complete absence of cholesterol efflux activity, it is more likely that possibility B is correct. Still, if B is correct, then it is difficult to explain why apoA-I is consistently close enough to W509S ABCA1 to produce detectable levels of cross-linked proteins unless the proteins are undergoing a protein-protein interaction. Therefore, taken together Chroni et al. and Fitzgerald et al.’s findings support the idea of a direct protein-protein interaction between apolipoproteins and ABCA1.

In support of model three, equally compelling evidence exists for the direct interaction between apolipoproteins and the plasma membrane. Mukhamedova et al.
showed that ABCA1-dependent cholesterol efflux and apolipoprotein binding to ABCA1 can be uncoupled (24). This was demonstrated using monoclonal antibodies against ABCA1. One monoclonal antibody, designated 3F9, when preincubated with THP-1 macrophages showed no effect on cholesterol efflux, but did inhibit total apoA-I binding by roughly 10-20%. This antibody was also capable of stabilizing ABCA1 on the cell surface to a similar extent as apoA-I. Oddly, in the presence of this antibody, no change in the extent of apoA-I/ABCA1 cross-linking was observed. However, this final observation should be considered with care, since it appears that the authors only ran one sample and only performed one experiment to obtain these results. Also, the western blot used to draw this conclusion was over-developed, making it possible that decreased apoA-I/ABCA1 cross-linking would be undetectable due to density saturation of the bands.

Further evidence for apolipoproteins directly binding the cell surface when ABCA1 is expressed can be found in a study by Vedhachalam et al. who showed that of all the apoA-I which binds to a J774 macrophage, only 10% of it can be cross-linked to ABCA1 (127). While it is likely that some of the protein-protein interactions were not captured by the cross-linking agent, it is highly unlikely that 90% of the interactions were missed. Additionally, this study showed that C-terminal deletion mutants of apoA-I, which are known to have reduced lipid binding abilities, had substantially lower overall rates of cell surface binding, yet can be cross-linked to ABCA1 with equal efficiency as wild-type apoA-I. Unfortunately, this experiment did lack one important control. To validate the conclusion that there must be a cell membrane binding site since only 10% of cell surface bound apoA-I can be cross-linked to ABCA1, the authors needed to utilize a
positive control. In this control a protein ligand and a cell surface receptor should have been cross-linked in a parallel experiment. The observation that a high percentage of all the cell surface-bound positive control ligand was captured with its receptor in the cross-linking assay would have demonstrated the ability of this cross-linking assay to capture a majority of protein-protein interactions occurring at the cell surface.

Further evidence for a specific cell membrane binding site for apolipoproteins is found in the observation that cell surface-bound apoA-I is not equally distributed across the cell membrane, but clusters in certain regions of the plasma membrane, as shown by electron microscopy and immunostaining with colloidal gold (99). Since apoA-I can be readily cross-linked to ABCA1 as previously discussed, it is safe to assume that the areas of the plasma membrane to which apoA-I binds surround ABCA1. And finally, perhaps the most compelling reason to reject model one is the observation that photoreactive cholesterol analogs do not directly bind ABCA1 (16), strongly suggesting that part of the cholesterol efflux process involves apolipoproteins emulsifying cholesterol from the plasma membrane.

Taken together, the studies above provide strong evidence for the existence of a plasma membrane binding domain for apolipoproteins separate from ABCA1 and also for a direct protein-protein interaction between apolipoproteins and ABCA1. Therefore, most researchers can agree that a combined model for apolipoprotein/ABCA1 interaction is most likely. However, many questions remain to be resolved about this interaction. Firstly, if such a protein-protein binding interaction does occur and is necessary for ABCA1-mediated cholesterol efflux, then how do so many different apolipoproteins and peptides access the same binding site on ABCA1? It is well-established that no sequence
homology exists between all these proteins and peptides. In fact, studies we performed comparing mouse (mixed genetic background), African green monkey, and human plasma apoA-II demonstrated that all these proteins were equally able to stimulate ABCA1-specific cholesterol efflux from RAW macrophages, which express murine ABCA1 (see Figure 6-2).

Figure 6-2

**ABCA1-mediated cholesterol efflux of plasma apoA-II from three different species.** RAW264.7 cells were labeled for 18 h with tritiated cholesterol in media +/- cAMP to upregulate ABCA1. After removal of labeling media and washing of the cells, medium containing lipid-free acceptor, again +/- cAMP, was added to the cells for 8 h. Error bars represent mean +/- SD of three replicates.

Assuming that all proteins which interact with ABCA1 utilize the same mechanism, it seems that a general structural motif and not a specific sequence must facilitate this interaction. The assumption that all apolipoproteins interact with ABCA1 in the same
manner is supported by data showing that the cross-linking of apoA-I to ABCA1 can be competitively inhibited by the presence of other apolipoproteins in the media (165). Additionally, this study showed that cross-linking apoA-I or apoA-II to ABCA1 produced similar molar saturation rates and that apoA-II competitively inhibited cross-linking of apoA-I to ABCA1 in a 1:1 molar ratio, suggesting a similarity in their interaction with ABCA1. One of the most recent general apoA-I structural motifs suggested to be critical to ABCA1-mediated cholesterol efflux was that of Natarajan et al. This group suggested that a linear array of acidic amino acids which were aligned along the junction between the hydrophobic and hydrophilic faces of two joined amphipathic α-helices was the necessary structure in apoA-I (2). If all apolipoproteins interact with ABCA1 in the same manner, and if these acidic residues allow apoA-I to bind to ABCA1 and induce or enhance cholesterol efflux, then the same pattern must be present and functionally necessary in all the other apolipoproteins and peptides capable of ABCA1-specific cholesterol efflux. To test if the linear array of acidic residues proposed by Natarajan et al. is present and necessary in other apolipoproteins known to interact with ABCA1, we searched for this element in apoC-I. A linear array of acidic residues matching the description of Natarajan et al. was identified in the glutamic acid residues 33, 40, 44, and 51 of the mature sequence. Using a recombinant apoC-I system and site-directed mutagenesis, each of these residues was individually mutated to a lysine residue. Recombinant proteins were compared using the DMPC liposome clearance assay and FC efflux assays previously described. As shown in Figure 6-3, only the mutant E51K showed a significantly lower ability to participate in ABCA1-mediated cholesterol efflux.
ABCA1-dependent cholesterol efflux assay of apoC-I acidic residue mutants. Assay was performed in RAW264.7 cells, and the buffer was STB. * represents p<0.05 compared to wild type apoC-I. Error bars represent mean ± SD of three replicates.

This mutant was also found to be a slightly faster lipid binder in the DMPC assay (data not shown). Since only one residue was found to have an effect, we concluded that in apoC-I, a linear array of acidic amino acids is not functionally necessary for ABCA1-mediated cholesterol efflux. Since apoC-I can participate in FC efflux via ABCA1, and all apolipoproteins are assumed to use the same mechanism to interact with ABCA1, this finding shows that a linear array of acidic residues is not a necessary structural element for apolipoproteins to stimulate HDL biogenesis. As described in Chapter IV, further studies using peptides modeling the helices of apoC-I and apoA-II allowed us to conclude that instead, the minimal structural unit necessary for apolipoproteins to elicit cholesterol
efflux from ABCA1 was a helical peptide segment composed of a non-lipid binding helix joined to a fast lipid binding helix. In apoA-I, apoA-II, apoC-I and possibly apoE, this element was located at the C-terminus of the protein with the non-lipid binding helix preceding the fast lipid binding helix. This helps to explain how so many different apolipoproteins and peptides can all participate in cholesterol efflux via ABCA1 without sequence homology. It also suggests that the protein-protein interaction occurring between these apolipoproteins and ABCA1 is not a highly specific, lock-and-key type binding event.

While our data identifies which region of apolipoproteins is important for their role in cholesterol efflux, it is still unclear which region of ABCA1 is participating in this interaction, and if the binding of apolipoproteins affects the activity of ABCA1. *In vitro* experiments could be used to determine which portion of ABCA1 is participating in this binding event. Macrophages grown in media lacking methionine and leucine, and containing photoreactive cross-linking L-photoleucine and L-photomethionine could be utilized to incorporated cross-linkers into the macrophage ABCA1. Since ABCA1 typically has a half-life of 1-2 h, an overnight incubation in this media should be more than sufficient for ensuring that most of the cell’s ABCA1 has the photoactivatable cross-linkers incorporated (166). Next, any unincorporated cross-linkers should be washed away, and apoA-I +/- cAMP should be added to the media. After 30 min at 37°C, a period of time which allows significant quantities of apoA-I to bind to the surface of the cells (167), the photoreactive amino acid cross-linkers can be activated by exposing the cell culture dishes to UV light. ApoA-I and any cross-linked ABCA1 can be collected using immunoprecipitation. These proteins can then be digested with trypsin and
analyzed with an electrospray ionization mass spectroscopy machine. This would allow us to identify which regions of apoA-I and ABCA1 were typically in close proximity. This method is unlikely to cross-link other protein partners to ABCA1, since coimmunoprecipitation experiments suggest that ABCA1 does not have other protein partners (17). However, if the experimental conditions of these previously reported coimmunoprecipitations were too harsh to preserve another protein interaction ABCA1 might have, this experimental system might identify this unknown protein partner. Using this method it is likely that ABCA1 will also be cross-linked to itself, since it is known that ABCA1 forms dimers and tetramers in the plasma membrane (17).

One potential problem that may be encountered with the cross-linking experiment above is an over-abundance of ABCA1-derived peptides which are not cross-linked to apoA-I. Since ABCA1 is such a large protein, many of these peptides may be generated. It is possible that these peptides could be in such high abundance in the experimental sample that they obscure the detection of lower-abundance cross-linked peptides. The use of the amino acid cross-linkers as opposed to externally applied cross-linkers is one answer to this problem, as it should substantially increase the efficiency of the cross-linking reaction. However, if this method does not produce enough cross-linked peptides to allow for detection among the non-cross-linked peptides, a cross-linking compound with an attached affinity tag could be utilized. Again the cross-linked proteins could be immunopurified and digested with trypsin. However, before analysis, these peptides would be applied to an affinity column to isolate cross-linked peptides from non-cross-linked peptides. This would allow only cross-linked peptides to be analyzed, preventing their signal from being drowned out. Unfortunately, no photoreactive amino acid cross-
linkers with affinity tags are currently commercially available, allowing an affinity tag cross-linker to be incorporated into the ABCA1 protein. Therefore, an external cross-linking agent with an affinity tag would have to be placed into the media to cross-link the cell surface proteins.

Once potential regions of interaction between apolipoproteins and ABCA1 were identified with this mass spectroscopy method, site-directed mutagenesis in ABCA1 could be used to more precisely define the residues or chemical properties of importance in these regions. Vectors with mutant or wild-type ABCA1, as well as the empty vector for a control, could be transfected into Hela cells, which do not normally express ABCA1. All ABCA1 mutants would need to undergo preliminary studies to ensure their expression levels and transport to the cell surface were not drastically altered by their mutation. This can be accomplished with RT-PCR measurements, comparing the number of cycles to threshold to wild-type ABCA1, and a simple cell surface biotinylation assay. A method for cell surface biotinylation is described in Chapter IV. These point mutants could then be used to strengthen the argument that the direct interaction between apolipoproteins and ABCA1 is necessary for cholesterol efflux by disrupting the interaction and studying the effect on efflux. More importantly, further experimentation to determine what step(s) of the mechanism these regions and residues are necessary for would be informative. For example, does disruption of this interaction affect ABCA1 cell surface stabilization by apolipoproteins, a process thought to be important for ABCA1-mediated efflux (22,23)? This possibility could be assessed by again using a cell surface biotinylation assay to quantify the amount of mutant and wild-type ABCA1 stabilized on the cell surface by apoA-I.
It is equally possible that the protein-protein interaction between apolipoproteins and ABCA1 affects ABCA1 ATPase activity. To measure this process accurately, a slightly more complex system should be used. A protocol for preparing liposomes containing recombinant ABCA1 has previously been published, and these proteoliposomes have been shown to possess ATPase activity (168). In order to determine if the interaction between apolipoproteins and ABCA1 stimulates increased ATPase activity, ABCA1 proteoliposomes should be made with wild-type ABCA1 and each of the mutants previously discussed. Then a standard amount of ATP should be added, and the baseline ATPase rate of the particular batch of proteoliposomes measured. Next, the ATPase rate can be measured in these same proteoliposomes when apolipoprotein is added to the solution. Each mutant should only be compared within its own batch of proteoliposomes, with and without apolipoprotein, with different batches of proteoliposomes only being compared on relative terms. In this manner, any effect of the protein-protein interaction between apolipoproteins and ABCA1 on ATPase activity of ABCA1 could also be assessed.

Finally, it is possible that the protein-protein interaction between apolipoproteins and ABCA1 energetically favors a tertiary or quaternary structure for ABCA1 not favorable in the absence of the apolipoprotein. Using polarized attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) and proteoliposomes similar to those discussed above, apolipoprotein induced changes in the secondary structure and orientation of ABCA1 in the lipid bilayer can be assessed. Proteoliposomes with only ABCA1 or apolipoprotein should be compared to proteoliposomes with both ABCA1 and apolipoprotein to detect changes in the ABCA1 signal in the presence of apolipoprotein.
Different combinations of phospholipids and cholesterol concentrations can be tested to see if these variables affect any structural changes observed in ABCA1 upon apolipoprotein binding.

An equally important, and seldom studied part of the ABCA1-mediated cholesterol efflux mechanism is the alterations ABCA1 is thought to make to the plasma membrane, and the mechanism apolipoproteins use to microsolubilize this portion of the membrane. As discussed in Chapter IV, some studies have suggested that ABCA1 flops PS from the inner leaflet of the plasma membrane to the outer leaflet, and that this allows apolipoproteins to bind to the cell surface (128-130). However, other data has suggested that the increased PS on the cell surface cannot account for the increased apoA-I binding which results from ABCA1 expression (131). Still more studies have suggested that ABCA1 on the cell surface results in a redistribution of cholesterol and SM from the lipid raft areas of the cell membrane to the non-raft areas, possibly providing accessible cholesterol for apolipoprotein emulsification (134). More information is needed on the lipid environment around active ABCA1 if a complete understanding of apolipoprotein membrane solubilization is to be obtained.

One way to address this problem is to pre-incubate green fluorescent protein-tagged ABCA1 (GFP-ABCA1) inducible expression cells with a fluorescently-labeled phospholipid or sphingolipid in the media. A preliminary timecourse experiment will determine the incubation period which allows for detectable amounts of the fluorescently-labeled lipid to exchange into the cell membrane without excessive internalization. Since it is unknown which features of the lipid patch generated by ABCA1 are important for apolipoprotein binding, phospholipids labeled on their acyl
chains and on their headgroup should be tested to avoid unknowingly disrupting the important portion of the lipid under study. Also, fluorescent labels outside the spectral range of GFP should be selected so that colocalization studies with ABCA1 can be preformed. Once the incubation time is established, cells +/- induced GFP-ABCA1 expression can be prelabeled with the fluorescent phospholipid or SM of interest, and then monitored with time-lapse fluorescent microscopy. Phospholipid distribution should be studied with and without apolipoprotein in the media. The time lapse microscopy can be used to look for changes in the distribution of fluorescently-labeled lipid in the membrane, as well as colocalization with cell surface ABCA1. Any distribution change seen in cells expressing ABCA1 and not seen in cells without ABCA1 could provide important clues to ABCA1’s effects on the plasma membrane. Further, if uneven distribution of the fluorescent lipids is observed, an analysis of the nascent HDL particles formed when apolipoprotein is present in the media could yield important information on which area of the plasma membrane is being emulsified by apolipoproteins.

While fluorescently-labeled phospholipids are great tools for studying overall membrane distribution, they are not easily used to study ABCA1’s floppase activity. To try and understand if ABCA1 is altering the population of phospholipids on the outer leaflet of the plasma membrane, spin-labeled phospholipids should be incorporated into the plasma membrane of RAW macrophages. Then, after washing away the unincorporated spin-labeled phospholipids, media +/- cAMP and +/- apolipoprotein should be incubated with the cells. Finally, ascorbate should be added to the media and the electron spin resonance (ESR) spectrum recorded over time. Any spin-labeled phospholipid in the outer leaflet will be chemically reduced, eliminating its signal. This
will allow for an accurate measure of any ABCA1-related effect on phospholipid leaflet distribution, and any dependence of this effect on the presence of apolipoproteins.

ESR spectroscopy combined with site-directed spin-labeling of peptides can also be used to test the lipid bilayer tethering hypothesis proposed in Chapter IV. If the poor lipid binding helix is serving as a tether to the bilayer surface, then a spin-label incorporated into this helix would be susceptible to ascorbate reduction. A second peptide with the spin label on the fast lipid binding helix would not be susceptible to ascorbate reduction if the helix were deeply buried within the bilayer. If this tethering hypothesis is found to be correct, its functional importance can be tested by measuring additional peptides in which the hydrophobic face angle on each helix is modified. Using ESR spectroscopy, effects of these changes on the interaction of the peptide with the membrane bilayer can be detected. By combining this data with FC efflux results, any correlations between FC efflux activity and membrane bilayer penetration could be identified.

If the experiments discussed above are performed, a better understanding of the molecular mechanism of ABCA1-mediated cholesterol efflux to apolipoproteins will be obtained. The role of both the protein-protein interaction between apolipoproteins and ABCA1 and the direct binding of apolipoproteins to specific regions of the plasma membrane will be elucidated. Additionally, the localization of this lipidation event to the plasma membrane will be ascertained under cholesterol-loaded conditions. Since the interplay between apolipoproteins and ABCA1 is the rate-limiting step in HDL biogenesis, it is possible that these mechanistic details could be used to develop well-
tolerated treatments to increase HDL levels, a therapy with the potential to improve the lives of millions of patients here and around the world.
Treatments for CVD have improved dramatically over the past fifty years. With the aid of drug-eluting stents, coronary bypass surgery, carotid angioplasty, statins, and β-blockers, patients with CVD are living longer than ever. Unfortunately, the atherosclerotic plaques which cause many of the dangerous sequela of CVD in these patients rarely regress, and thus our current treatments only forestall the inevitable. Therapies which enhance the reverse cholesterol transport system could complement our current CVD treatments. Numerous animal studies and some human trials, discussed in Chapter II, have demonstrated that increased apoA-I levels can cause plaque regression. However, increasing endogenous apoA-I expression levels has proven difficult, and the administration of recombinant apoA-I is unfeasible at a population scale. Ideally, a small molecule drug should be found which could enhance the interaction between apolipoproteins and ABCA1, thereby increasing HDL production in the body. This could be done through a variety of mechanisms, such as enhanced ABCA1 cell surface stability, enhanced ABCA1 ATPase activity, enhanced apolipoprotein cell surface and/or ABCA1 binding, etc. However, before these therapeutical avenues can be assessed and developed, a better understanding of the interplay between apolipoproteins and ABCA1 must be obtained. This work contributes important, new pieces to this understanding. It was shown that previously hypothesized important structural elements such as a linear array of acidic amino acids aligned along the junction between the hydrophobic and
hydrophilic faces of two joined amphipathic α-helices and a hydrophobic and negatively charged surface patch on apoA-I were not necessary for ABCA1-dependent efflux. Instead, the minimal structural unit of apolipoproteins which can mediate ABCA1-dependent cholesterol efflux was shown to be a bihelical pair of amphipathic α-helices composed of a poor lipid binding helix and a fast lipid binding helix. In natural apolipoproteins, this fast lipid binding helix possesses a wide hydrophobic face angle and is always located at the extreme C-terminus of the protein, suggesting that perhaps the helix buries itself deep within the lipid bilayer, while the poor lipid binding helix tethers the pair to the bilayer surface. Lastly, studies performed to determine the location of apolipoprotein lipidation by ABCA1 found that this lipid transfer occurred at the cell surface in non-lipid loaded cells, and not via a retroendocytosis pathway as previously suggested. This data provides a firm platform on which future work can be based, allowing for continued building of a mechanistic understanding of ABCA1-mediated cholesterol efflux. It is with the hope that each new set of experiments contributed to this search might bring us closer to improved treatment options that this research has been performed. And it is with the hope that others will continue the search that this work is completed.
APPENDIX 1 – REAGENT DEVELOPMENT: PURIFICATION OF RECOMBINANT APOLIPOPROTEINS A-I AND A-IV AND EFFICIENT AFFINITY TAG CLEAVAGE BY TOBACCO ETCH VIRUS PROTEASE

Introduction

The development of recombinant systems for producing apoA-I and apoA-IV has allowed the use of site-directed mutagenesis to narrow down regions important for particular functions (8-10). Functional forms of apoA-I and apoA-IV have both been bacterially expressed for some time (11-13). However, the flexible nature of apolipoproteins has made determining the correct protease for affinity tag cleavage rather difficult. Enterokinase, which is often used for cleavage of tags from recombinant proteins, cleaves lipid-free apoA-I non-specifically near its C-terminus (14). IgA protease (15) has been shown to specifically cleave tags from both apoA-I and apoA-IV (16), but the yield of this reaction is relatively low, particularly for apoA-IV (17). Additionally, the protease is expensive from commercial sources so that it is cost prohibitive to use the enzyme near the recommended ratio of protease to target protein, at least when expressing milligram quantities of multiple mutants for comparison studies. Sviridov and colleagues have used a unique baculovirus expression system in insect cells with relatively low yields of 6 mg/L of culture (169). Others have achieved much higher yields using the baculovirus system (170). Unfortunately, bacculovirus systems are somewhat unwieldy for generating large numbers of mutants in a short period of time and require several days of purification including preparative HPLC and anion exchange
chromatography. Bergeron and colleagues expressed apoA-I in a bacterial system and achieved 10 mg/L yield (171). More recently, Ryan and colleagues achieved 100 mg/L yield using a bacterial expression system by optimizing the cDNA codon usage for bacteria (172). This construct contains an altered N-terminus that allows for acid cleavage of the histidine tag, a process that requires incubation in 45% formic acid at 55°C. Feng and colleagues used a yeast expression system for apoA-I yielding 91 mg/L (173). Their system used a large scale fermenter and required five days for expression alone. Thus, there are numerous expression systems available for apoA-I, and fewer for apoA-IV, each with their strengths (high yield) and weaknesses (time considerations, cost of enzyme, or caustic tag cleavage procedures).

For these reasons, we began looking into other protease systems to cleave the histidine-tag (His-tag) from recombinant apoA-I and apoA-IV proteins expressed in *Escherichia coli* (*E. coli*). The Tobacco Etch Virus (TEV) protease has been used by numerous investigators to produce recombinant proteins (18-20). One group has worked extensively to produce a stable, highly efficient TEV protease which can be easily expressed in *E. coli* (21) by any laboratory equipped for recombinant protein production. This S219V mutant of TEV protease is resistant to self-cleavage and auto-inactivation. Furthermore, this construct also contains a non-cleavable His-tag for purification away from the cleaved target protein product.

In the current report, we show that the TEV protease can specifically and efficiently remove a His-tag from human apoA-I and apoA-IV, resulting in structurally and functionally identical protein as compared to either plasma apoA-I or our previously used recombinant apoA-IV. Compared to our previous IgA protease system, TEV
protease cleavage is less costly, faster, carried out at a lower temperature, removed easily, and results in a recombinant protein with only a single non-native amino acid, glycine, on the N-terminus.

**Experimental Procedures**

*Materials-* Dimyristoyl-phosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was obtained from Fisher. His bind resin was purchased from Novagen. YM30 centricon concentrators were obtained from Amicon. Dulbecco’s Modified Eagle Medium (DMEM) was obtained from Invitrogen. [1,2-³H(N)]-cholesterol was supplied by Amersham Biosciences. 8-bromoadenosine 3’,5’-cyclic monophosphate sodium salt (8-bromo-cAMP) was purchased from Sigma. The RAW264.7 macrophages used in the cholesterol efflux assay were purchased from the American Type Culture Collection. All other reagents were analytical grade.

Circular Dichroism measurements were made on a Jasco J-715 spectropolarimeter. The fluorescence measurements were performed on a Photon Technology International Quantamaster spectrometer. A 1900CA Packard liquid scintillation analyzer was used to count the radioactive cholesterol counts associated with the cholesterol efflux assay. All absorbance measurements were made on an Amersham Biosciences Ultraspec 4000 UV/visible spectrophotometer.
**TEV Protease Expression and Purification** - TEV protease vector (pRK793) along with accessory plasmid pRIL (Stratagene), originating from the laboratory of David Waugh, were obtained from addgene.org (Addgene plasmid 8827). The construct has a maltose binding protein fused with the catalytic domain of TEV protease with a self-cleavage site between the two proteins. After self-cleavage, the TEV protease retains an N-terminal His-tag, allowing for easy removal from the sample solution after cleavage. The TEV protease domain contains the mutation S219V (21) which confers resistance to auto-inactivation and is about 2x as active as wild-type TEV protease. The TEV protease was expressed in BL21 (DE3) *E. coli* cells. Briefly, 10 ml Luria broth (LB) cultures with 100 μg/ml ampicillin (pRK792) and 30 μg/ml chloramphenicol (pRIL) were inoculated with a single colony from an LB agar plate and grown overnight at 37°C. In the morning, 100 ml LB cultures were supplemented with the same selection agents and inoculated with 1 ml of the overnight culture. At OD600 ~ 0.5, cultures were induced with 1 mM IPTG and the temperature was reduced to 30°C for 4 hours. The cells were pelleted, resuspended in 10 ml of 20 mM Tris-HCl (pH 7.9) + 500 mM NaCl + 10% glycerol + 5 mM imidazole (lysis buffer) per 1 gram of wet cell paste. The cells were lysed by probe sonication for 10 min at 50% duty cycle. 5% polyethyleneimine (pH 7.9) was added to a final concentration of 0.1% and the sample was mixed by inversion and centrifuged at 15,000 x g for 30 min. The supernatant containing the TEV protease was applied to a nickel affinity (IMAC) column equilibrated with lysis buffer. After 7 washes with lysis buffer and 3 additional washes with wash buffer (lysis buffer with 50 mM total imidazole), the protease was eluted with 20 mM Tris-HCl (pH 7.9) + 500 mM NaCl + 10% glycerol + 1 M imidazole. EDTA and DTT were added to the eluted sample at a
final concentration of 1 mM each and the sample was concentrated by ultrafiltration using YM30 centricons. The sample was dialyzed against 25 mM K₂HPO₄ (pH 8.0) + 200 mM NaCl + 5% glycerol + 2 mM EDTA + 10 mM DTT. The protease was aliquoted into 1 mg samples, flash frozen in liquid nitrogen and stored at -80°C until use. In a subsequent purification, standard Tris salt buffer (STB: 10 mM Tris HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.02% sodium azide) + 5 mM DTT + 10% glycerol was used for storage with no difference in protease activity.

Expression and purification of apoA-I. ApoA-I was expressed in E. coli and purified using IMAC columns as described previously (16) (see Results for construct generation). In some experiments, apoA-I was then applied to a phenyl sepharose hydrophobic interaction column (HIC). In others, this step was omitted. The His-tag was then cleaved from the protein by TEV protease at a 20:1 protein to protease ratio for 30 min at room temperature (22°C). Cut apoA-I was purified using Superdex 200 (Amersham) size exclusion chromatography. Finally, the protein was dialyzed into STB and stored at 4°C until use.

**Expression and Purification of ApoA-IV**- ApoA-IV was also expressed in E. coli and purified using IMAC columns as described previously (10). An appropriate protein:protease ratio was determined using TEV G apoA-IV and TEV protease at ratios from 2:1 to 1000:1 and it was determined that cleavage at room temperature overnight (16 h) with a 20:1 ratio was sufficient to achieve complete cleavage of the His-tag (data not shown). After dialysis into STB, TEV cut apoA-IV was reapplied to the IMAC
column in 1x Bind buffer to capture the cleaved tag, the protease, and any uncut protein. Purified protein was stored in STB at 4°C until use.

Circular Dichroism- For circular dichroism (CD) studies, proteins were freshly dialyzed against 20 mM phosphate buffer (pH 7.4), and relative concentration was determined by the Markwell-Lowry method (22). Proteins were then diluted to 100 μg/ml and spectra were collected in a 1 mm cell as an average of three scans. The scans were from 260 to 190 nm at 100 nm/min with a 0.5 nm step size and 0.5 s response. Bandwidth was set to 1 mm, and slit width was 500 μm. To confirm the accuracy of dilution, protein concentration was verified by A280 and mean residual ellipticity was calculated based on this value. Mean residual ellipticity was calculated as described by Woody (23) using 115.3 as the mean residual weight for apoA-I and apoA-IV. Fractional helical content was calculated using the formula of Chen et al. (24) and the mean residual ellipticity at 222 nm. Each experiment was repeated on two independent protein preparations.

Tryptophan fluorescence. Protein samples from the above CD experiments were used to monitor tryptophan (Trp) fluorescence. Fluorescence emission spectra of plasma apoA-I, TEV G-apoA-I, IgA protease apoA-IV and TEV G-apoA-IV were recorded at room temperature. The emission spectra were collected from 305 to 380 nm using the Trp excitation wavelength of 295 nm chosen to minimize the tyrosine fluorescence. The appropriate buffer controls were also included.

DMPC Liposome Solubilization Assay- The rate of lipid association and reorganization was determined using the DMPC (dimyristoyl-phosphatidylcholine) liposome clearance
assay (25;26). Briefly, DMPC multilamellar vesicles (MLVs) at 5 mg/ml were prepared in STB by brief probe sonication. MLVs and STB were mixed, so that once protein was added, the final DMPC concentration was 0.425 mg/ml. Protein was quickly added to the above mixture to a final concentration of 0.17 mg/ml (2.5:1 DMPC/protein, w/w). Proteins for all experiments were in STB. Absorbance at 325 nm was measured at 24.5°C at 30 s intervals for 20 min. Samples were run in triplicate, and plots were normalized to the initial absorbance of the sample (OD$_0$). The clearance of the reaction is due to apolipoprotein binding to, and solubilizing the MLVs to small discoidal lipoproteins that do not scatter light at 325 nm. The assay was carried out at 24.5°C, the gel/liquid crystal transition temperature of DMPC, where an optimal number of lattice defects exist on the MLV surface. Each result was verified by an additional DMPC experiment on a separate day from an independent protein expression. The DMPC liposome clearance assay is a multistep reaction that involves binding to the lipid surface followed by an unknown reorganization step. Thus, this assay is not strictly a measure of lipid binding. However, it has been proposed that the initial binding steps are rate-limiting and that once a critical number of apolipoproteins bind to the lattice defects on the MLV surface, the rate of reorganization is similar for all proteins (26).

**Cholesterol Efflux-** RAW264.7 macrophages were maintained in Dulbecco’s Modified Eagle Medium (DMEM) with 10% FBS and 50 μg/ml gentamycin. Cells were grown to 70% confluency, then washed twice with a minimal media (DMEM and 0.22% BSA). Next 0.5 ml labeling media (DMEM, 0.22% BSA, +/- 0.3 mM 8-bromo-cAMP, and 1.0 μCi/ml [1,2-$^3$H(N)]-cholesterol was added to the cells for 18 h. Cells were washed twice
with minimal media, then 0.5 ml of efflux media (DMEM, 0.22% BSA, +/- 0.3 mM 8-bromo-cAMP, and 10 μg/ml of acceptor protein) was incubated with cells for 8 h. Percent efflux was calculated by dividing the efflux media counts by the total counts (calculated as the efflux media counts plus the intracellular counts after cells were solubilized in isopropanol). Efflux media was 0.45 μm filtered before counting to remove any floating cells.

Results

**TEV Protease Purification**- We obtained the TEV protease expression construct from the laboratory of Dr. David S. Waugh via Addgene.org, a nonprofit site dedicated to the dispersal of plasmids to the scientific community. The expression and purification of the protease was performed as described in Methods. After purification, we obtained a single band on SDS-PAGE at a molecular weight (MW) of 30 kDa along with some minor bands that may represent autoproteolysis (data not shown).

We first tested the protease to make sure that it lacked the ability to non-specifically cleave lipid-free recombinant apoA-I or apoA-IV (produced by our IgA protease method (16;17)). SDS-PAGE analysis showed no evidence of non-specific cleavage of either apolipoprotein even at high ratios of apoA-IV protein to TEV protease (2:1 mol:mol) for 16 h at room temperature (data not shown). This indicates that TEV protease should be a suitable protease for specific cleavage of His-tags from apoA-I and apoA-IV, provided that they contain an optimal recognition sequence.
Design and Cleavage of Recombinant Apolipoproteins- Site-directed mutagenesis was used to replace the IgA protease recognition (PRPP/TP) site in apoA-I and apoA-IV, both in the pET30 vector (Novagen) (16;17) (Figure A1-1), with the cleavage site for TEV protease (ENLYFQ/X). Ideally, position X represents the first amino acid of the mature target protein. However, Kapust et al. (27) have shown that the identity of the amino acid at position X can profoundly affect the efficiency of the TEV cleavage reaction, with G, A or S found to be the most permissive. Therefore, we generated two different TEV protease recognition constructs for both proteins in this study. The first was a so called “clean cutting” construct in which position X was the naturally occurring first amino acid of either human apoA-I(D) or human apoA-IV(E). This leaves no non-native amino acids on the N-terminus of either apolipoprotein. The second set of constructs placed a G, A, or S at position X, resulting in one additional amino acid on the N-terminus of each apolipoprotein after TEV protease cleavage. ApoA-I constructs were generated with G, A, and S, and apoA-IV was generated with G only. The mutants were sequenced to verify their identity and expressed and purified from E.coli as described in Methods. We found no significant difference in protein expression level for any of the above described constructs (data not shown).

To determine the efficiency of recombinant protein cleavage by the TEV protease, we incubated the various apoA-I and apoA-IV mutants with TEV protease at a 20:1 molar ratio (apolipoprotein:protease) for 30 min (apoA-I) or 16 h (apoA-IV) at room temperature. As shown in Figure A1-2A, the “clean cutting” apoA-I was minimally cleaved after 30 min as indicated by a band of about 34 kDa with no mature apoA-I (28 kDa) visible. By contrast A-apoA-I, G-apoA-I and S-apoA-I were all cleaved completely
to the 28 kDa band by the TEV protease. In some preparations, we occasionally observe some non-specific C-terminal cleavage of our recombinant apoA-I, presumably due to proteases released from the bacterial cells during lysis (i.e. the band at about 30 kDa in Fig. 2A lane 2). Additional bands apparent in the lane represent protein that had been clipped at the C-terminus and then again, specifically, by TEV at the N-terminus. Figure A1-2B shows that “clean-cutting” apoA-IV displayed relatively more cleavage than “clean cutting” apoA-I, exhibiting a band with a MW of the intact fusion protein (47 kDa) as well as a band at 43 kDa corresponding to mature apoA-IV. Similarly to G-apoA-I, G-apoA-IV was completely cleaved by the protease as indicated by the appearance of the 43 kDa band. Because the cleavage efficiencies of the “clean cutting” constructs were poor, we elected to further study the apolipoproteins containing a G on the N-terminus. We reasoned that the neutral charge and low side-chain volume afforded the least chance of perturbing protein function.

Apolipoprotein Purification- G-apoA-I and G-apoA-IV mutants containing the TEV protease site were expressed and purified with regard to amounts of protein at each step in order to track the yield of mature apolipoprotein after purification. Figure A1-3 shows the purification scheme of apoA-I and apoA-IV. As shown in Figure A1-4, our purification schemes resulted in preparations that were about 90% pure by densitometry for both apolipoproteins. Protein yield was 26 mg/L (9.6x10^-7 moles/L) for apoA-I and 84 mg/L (2.0x10^-6 moles/L) for apoA-IV.
Structural and Functional Characterization of Apolipoproteins generated by TEV Cleavage- To determine if the presence of the N-terminal glycine perturbed the structure or function of either apoA-I or apoA-IV, we compared G-apoA-I to isolated human plasma apoA-I and G-apoA-IV to our IgA protease-cleaved recombinant apoA-IV (10;28). CD spectroscopy was used to estimate the overall secondary structural content of the lipid-free proteins. As shown in Figure A1-5A, the CD spectra of G-apoA-I and human plasma apoA-I revealed the characteristic shape of a predominantly α-helical protein with two minima at 208 and 222 nm. The curves were essentially superimposable. Calculations revealed that the proteins were about 44% helical, which is consistent with previous studies (29). Similarly, as shown in Figure A1-5B, the spectra for G-apoA-IV and IgA protease-cleaved apoA-IV were of similar shape to apoA-I and were also superimposable with an estimated helical content of about 77%. We followed up the secondary structure analysis with fluorescence measurements to determine the chemical environment of the four Trp residues in apoA-I or the single residue in apoA-IV. Figures A1-6A and A1-6B show the tryptophan fluorescence spectra of the apoA-I and apoA-IV proteins, respectively. After excitation at 295 nm, G-apoA-I emitted at a maximal wavelength of 331.3 +/- 0.6 nm, whereas plasma apoA-I emitted at 331.7 +/- 1.5 nm. These values are significantly blue shifted relative to free tryptophan in solution (352 nm) and indicate that the Trp residues are in a relatively non-polar environment within the folded protein (30). Similarly, the single tryptophan of TEV G-apoA-IV emitted at 335.8 +/- 1.6 nm whereas IgA protease apoA-IV emitted at 337.2 +/- 2.0 nm (Figure A1-6B). We noted a small change in fluorescence intensity between human plasma and recombinant apoA-I. The reason for this is unclear, however the intensity
measurement is highly dependent on small buffer differences (i.e. pH, salt concentration) and likely does not reflect differences in protein structure. Taken together, these data indicate that the apolipoproteins generated by TEV protease cleavage are structurally similar to either plasma purified protein or that generated by previously validated recombinant methodologies.

To assess the functionality of our TEV protease-cleaved proteins, we performed lipid solubilization and cholesterol efflux comparisons. Figure A1-7 shows the results of a DMPC clearance assay, which determines the ability of a given protein to bind and reorganize multilamellar vesicles. Figure A1-7A shows that G-apoA-I and plasma apoA-I both solubilized DMPC liposomes with similar kinetics, though the plasma protein tended to drive the reaction to a higher degree of completion. Similarly, Figure A1-7B shows no difference in lipid binding between IgA protease- and TEV protease-generated apoA-IV. Since a major function of apoA-I is to promote cholesterol efflux via the ATP binding cassette transporter A1 (ABCA1), we incubated the apoA-I proteins with RAW macrophages that had been stimulated to produce ABCA1 with exogenous cAMP (31). In 8 h, it is clear from Fig. 8 that G-apoA-I and human plasma apoA-I promoted similar degrees of cholesterol efflux in this system at a concentration of the protein at which cholesterol efflux saturation has not been reached (32). Taken together, we found no evidence that apoA-I or apoA-IV cleaved with TEV protease is structurally or functionally different than either plasma purified apoA-I or apoA-IV generated by other strategies.
Stability of TEV Protease-generated Apolipoproteins- To determine the suitability of these proteins for crystallography studies, we tested the stability of the purified TEV-generated proteins at room temperature for extended periods of time. Figure A1-9 shows an SDS-PAGE analysis of TEV G-apoA-I and TEV G-apoA-IV proteins that had been placed at 4°C or room temperature for 2 weeks in STB (see Methods). There was no additional degradation of either protein in the room temperature samples, indicating that the purified samples contained no additional protease activity capable of degrading the protein.

Discussion

The modified TEV protease used in this study has several advantages over other proteolytic systems that have been utilized for apolipoproteins. Perhaps chief among these is the fact that the protease can be easily expressed in the laboratory, eliminating the need to purchase expensive enzymes commercially. Second, the TEV protease contained an integral His-tag, allowing it to be purified away from our recombinant proteins. Since our target proteins also contain a His-tag, this allowed the simultaneous removal of the cleaved tag and the protease from our target products. This typically saves at least one additional chromatography step that can lower final product yield. Third, TEV protease is highly active at room temperature. Many other proteases require elevated cleavage temperatures that can also activate minor contaminating proteases that can degrade the target protein. Furthermore, shorter, lower temperature incubations minimize oxidation and other protein modifications that can affect target protein function.
Another advantage of the TEV protease is the possibility of generating native proteins that lack extraneous amino acids on the N-terminus. We attempted to produce such so-called “clean-cutting” apoA-I and apoA-IV. Unfortunately, the N-terminal residues of apoA-I and apoA-IV did not allow for efficient processing by the TEV protease. The clean-cutting apoA-I, containing an aspartic acid in the X position, was not cut at all in 16 h, and clean-cutting apoA-IV, with a glutamate in the X position, cut with only about 30% efficiency. This was unexpected because the same amino acids, aspartic acid and glutamate, in a NusG-maltose binding protein fusion allowed for around 90% and 60% cleavage, respectively (27). Even though we could not achieve completely native proteins in these cases, the addition of a small and flexible Gly residue, would seem to be less obtrusive than the Thr-Pro addition required by the IgA protease or Gly-Pro for the PreScission® protease. Indeed, the structure and functional characterizations performed here bear this out.

ApoA-I and ApoA-IV cDNAs were previously subcloned into the pET30 bacterial expression vector with an IgA protease recognition site between the histidine tag and the apolipoprotein cDNA. We used mutagenesis to replace the IgA protease site with the recognition site of the tobacco etch virus (TEV) protease. The arrow represents the transcription start site. The restriction endonucleases used for 5’ and 3’ cloning are shown (*mutagenesis removed the NcoI splice site from the apoA-IV plasmid). The “G” at the beginning of the native apolipoprotein sequence represents the glycine that dramatically improves cleavage of the tag from both apoA-I and apoA-IV. The sequence below shows the exact construction of the TEV cleavage site in relation to the protein cDNA.
Histidine tag cleavage from apolipoprotein constructs by TEV protease.
A) Samples of “clean cutting” apoA-I as well as apoA-I containing an N-terminal A, G, or S were incubated at room temperature for 30 min without (lanes 2, 4, 6, and 8, respectively) or with (lanes 3, 5, 7, and 9, respectively) TEV protease. B) Samples of apoA-IV either clean cutting or containing an N-terminal G were incubated at room temperature for 16 hours without (lanes 2 and 4, respectively) and with (lanes 3 and 5, respectively) TEV protease. The apolipoprotein:protease ratio was 20:1 for all samples. 4-15% SDS-PAGE analysis performed on 6 μg of each reaction mixture and visualized with Coomassie blue. Lane 1 of both gels contains low molecular weight protein standards.
Expression and purification scheme for TEV protease and apolipoproteins A-I and A-IV.

Both the TEV protease and both apolipoproteins were expressed in liquid LB broth after transformation into the BL21 strain of *Escherichia coli*. The TEV protease was captured from cell lysate using its non-cleavable histidine tag and immobilized metal affinity chromatography (IMAC). Crude apolipoproteins were captured using IMAC. After dialysis, the apolipoprotein histidine tag was cleaved with TEV protease at a 20:1 protein:protease ratio at room temperature. After cleavage was complete, pure cut apolipoprotein was isolated using IMAC (* or gel filtration chromatography for some experiments with apoA-I). The protein was dialyzed into STB and stored at 4°C until use.
Purification of recombinant apolipoproteins.
6 μg of each sample (total protein) was analyzed by 4-15% SDS-PAGE and visualized with Coomassie blue. A) apoA-I: lane 1, low molecular weight protein standards; lane 2, cleared lysate; lane 3, uncut after IMAC; lane 4, TEV-cut after size exclusion. B) apoA-IV: lane 1, low molecular weight protein standards; lane 2, cleared lysate, lane 3, uncut after IMAC; lane 4, TEV-cut after IMAC.
Figure A1-5

Far UV circular dichroism spectra of apolipoproteins.
A) apoA-I: recombinant apoA-I cut with IgA protease (▼) and TEV protease (●) as well as plasma apoA-I (○). B) apoA-IV: recombinant apoA-IV cut with IgA protease (Igase) (○) or TEV protease (●). Samples were run at room temperature in triplicate with each being the mean of three accumulations. Mean residual ellipticity for all samples was calculated as described in Appendix 1 Experimental Procedures section.
Figure A1-6

Tryptophan fluorescence spectra of apolipoproteins.
A) apoA-I; recombinant apoA-I cut with TEV protease (●) as well as plasma apoA-I (○).
B) apoA-IV; recombinant apoA-IV cut with TEV protease (●) or IgA protease (○).

Samples were analyzed in triplicate at room temperature, excited with 295 nm light.
DMPC clearance assay of apolipoproteins.
A) apoA-I; recombinant apoA-I cut with TEV protease (●) as well as plasma apoA-I (○).
B) apoA-IV; recombinant apoA-IV cut with TEV protease (●) or IgA protease (○) along with IgA protease-cut, fast-lipid binding, F334A apoA-IV (▼). All samples were run in triplicate at 24.5°C, the transition temperature of DMPC, for 20 min, readings every 30 sec. Error bars represent one SD.
Figure A1-8

Cholesterol efflux of apoA-I samples.
Plasma-derived and recombinant TEV G-apoA-I samples were used as the cholesterol acceptors from cholesterol-loaded RAW264.7 macrophages. Proteins were added to the media at 10 μg/mL for 8 hrs. Percent efflux was calculated by dividing the efflux media counts by the total counts. Points represent mean of three triplicate samples and error bars represent one SD.
Long-term stability of recombinant apolipoproteins.

ApoA-I and apoA-IV cut with TEV protease were incubated for 2 weeks at 4 °C (lanes 2 and 4, respectively) or room temperature (lanes 3 and 5, respectively). 6 μg of each sample was analyzed by 4-15% SDS-PAGE and visualized with Coomassie blue. Lane 1 contains low molecular weight protein standards.
From the studies described in Chapter IV, a hypothesis was formed that perhaps the two helices of the bihelical functional unit could be rearranged without affecting the ABCA1-specific activity of the bihelical peptide. To test this hypothesis, two additional peptides were made, A-II$_{helix\ 2+1}$ composed of residues 32-53/8-31 and A-II$_{helix\ 3+2}$ composed of residues 51-73/32-50. Upon testing, it was found that A-II$_{helix\ 2+1}$ did not efflux cholesterol from RAW cells, which was to be expected. However, surprisingly this peptide also did not clear DMPC liposomes (see Table A2-1). Equally surprising, the A-II$_{helix\ 3+2}$ also did not efflux cholesterol from RAW cells despite the fact that it clears DMPC liposomes slightly faster than A-II$_{helix\ 2+3}$. These results demonstrate that the two amphipathic $\alpha$-helices which compose the minimal structural unit necessary for ABCA1-mediated efflux in apoA-II and apoC-I cannot be treated as building blocking and rearranged at will while maintaining biological function. Clearly, by switching the order of these helical pairs, an important natural transition was disrupted. We can be certain that simply switching the order of the helices or the orientation in the N to C terminal direction does not always affect a peptide’s ability to participate in ABCA1-mediated cholesterol efflux due to experiments discussed in Chapter IV in which both a helix swapped and a reverse apoC-I peptide were found to have ABCA1-dependent cholesterol efflux activity. It is unclear at this time what the critical transition characteristic in A-II$_{helix\ 2+1}$ and A-II$_{helix\ 3+2}$ which was disrupted is. Possibilities which should be considered include disruption of the alignment of the hydrophobic faces of the individual
amphipathic α-helices shown in Figure A2-1, alteration of the structural flexibility between the two helices, and alteration in the length of the hinge region between the two helices. Further study will be needed determine which of these changes resulted in the alteration of activity seen in these peptides.
Helical wheel diagrams of individual apoA-II and apoC-I helical peptides. Residues of mature sequence designated in red numbers. Hydrophobic residues are shown as blue, hydrophilic residues are shown as red, and glycine, proline, and tyrosine are shown as grey. All diagrams made with Antheprot program (174).
Table A2-1

<table>
<thead>
<tr>
<th>Protein</th>
<th>DMPC</th>
<th>FC Efflux</th>
<th>ABCA1 stabilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma A-II</td>
<td>++ (4)</td>
<td>++++ (3)</td>
<td>++++ (5)</td>
</tr>
<tr>
<td>A-II helix 1</td>
<td>++++ (3)</td>
<td>- (3)</td>
<td>+++ (2)</td>
</tr>
<tr>
<td>A-II helix 2</td>
<td>- (4)</td>
<td>- (3)</td>
<td>+ (2, variable)</td>
</tr>
<tr>
<td>A-II helix 2 extended</td>
<td>- (1)</td>
<td>- (2)</td>
<td>+ (2, variable)</td>
</tr>
<tr>
<td>A-II helix 3</td>
<td>+++ (3)</td>
<td>-</td>
<td>++++ (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(2 out of 3 trials showed no significant efflux above buffer)</td>
<td></td>
</tr>
<tr>
<td>A-II helix 1+2</td>
<td>++++ (4)</td>
<td>- (2)</td>
<td>++ (3)</td>
</tr>
<tr>
<td>A-II helix 2+1</td>
<td>- (4)</td>
<td>- (2)</td>
<td>++ (3)</td>
</tr>
<tr>
<td>A-II helix 2+3</td>
<td>++++ (3)</td>
<td>+ (2)</td>
<td>++++ (3)</td>
</tr>
<tr>
<td>A-II helix 3+2</td>
<td>++++ (3)</td>
<td>-</td>
<td>++ (3)</td>
</tr>
<tr>
<td></td>
<td>Better than A-II helix 2+3</td>
<td>(2 trials, one showed significant efflux above buffer while the other did not. The counts were lower on the non-significant trial)</td>
<td></td>
</tr>
<tr>
<td>Plasma C-I</td>
<td>++ (2)</td>
<td>++++ (2)</td>
<td>+++ (1)</td>
</tr>
<tr>
<td>C-I helix 1</td>
<td>- (2)</td>
<td>- (2)</td>
<td>++ (1)</td>
</tr>
<tr>
<td>C-I helix 2</td>
<td>++++ (2)</td>
<td>- (2)</td>
<td>++ (1)</td>
</tr>
<tr>
<td>Reverse C-I</td>
<td>N/A</td>
<td>+++ (3, 2 trials show slightly lower efflux, dose curve shows no difference)</td>
<td>N/A</td>
</tr>
<tr>
<td>(57-1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helix switch C-I</td>
<td>N/A</td>
<td>++++ (3, 1 trial shows slightly lower ability than plasma C-I; dose curve shows no difference at any concentration)</td>
<td>N/A</td>
</tr>
<tr>
<td>(2+1)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Summary of apoA-II and apoC-I peptide experiments.**

Pluses indicate efficiency in reaction, with ++++ being very good and – being no reaction. Numbers in parentheses represent number of trials performed.


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