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Cellular mechanisms regulating CETP mediated selective uptake of HDL derived cholesteryl esters in mammalian cells

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by

Meena Na

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

Department of Biochemistry, Microbiology & Immunology
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Abstract

Cellular mechanisms regulating CETP mediated selective uptake of HDL derived cholesteryl esters in mammalian cells

by Meena Na

Cholesteryl ester transfer protein (CETP) is a hydrophobic glycoprotein that mediates the transfer of neutral lipids between lipoproteins. Recently, we have demonstrated a novel role for CETP in directly mediating the selective acquisition of CE from HDL by hepatocytes, indicating a direct and potentially anti-atherogenic function in reverse cholesterol transport. Further studies have been carried out to address the cellular mechanisms of CETP-mediated selective uptake of HDL-CE. Using biochemical plasma membrane isolation followed by detergent extraction and sucrose gradient membrane fractionation, we demonstrate that CETP localizes in the low density, detergent-resistant membrane fractions in both COS-7 and primary murine hepatocytes over-expressing CETP. In an attempt to dissect the intracellular events following the selective uptake of HDL-derived CE mediated by CETP, immunofluorescence confocal microscopy was used. By incubating HeLa cells and primary hepatocytes overexpressing CETP with fluorescently labeled HDL and CE, we demonstrate that CETP colocalizes with both CE and HDL both on the cell surface and intracellularly consistent with internalization of a CETP/HDL complex. We also note that the CETP/HDL complex colocalizes with a subset of early endosomes. The mechanism of internalization is unknown but could involve raft-mediated endocytic route. At 1h post-treatment, HDL and CETP were seen to separate from the early endosomes and segregate in perinuclear structures that are identified as a Rab11 positive endocytic recycling compartment. Based on these findings, we speculate that HDL and CETP may be recycled through a retroendocytic pathway, during which CE is removed from the HDL particle and directed to the lipid droplets for storage. These studies

provide new insight into CETP membrane localization and intracellular trafficking, relevant to its role in CE selective uptake.

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List of Abbreviations

Acyl CoA:Cholesterol Acyltransferase (ACAT)
Adenovirus (Ad)
Amino terminus (N-terminus)
Apolipoprotein AI (apoAI)
Apolipoprotein AII (apoAII)
Apolipoprotein AIV (apoAIV)
Apolipoprotein B (apoB)
ATP binding cassette (ABC)
ATP Binding Cassette transporter AI (ABCAI)
ATP Binding Cassette transporter GI (ABCGI)
ATP Binding Cassette transporter G4 (ABCG4)
ATP Binding Cassette transporter G5 (ABCG5)
ATP Binding Cassette transporter G8 (ABCG8)
Bactericidal/permeability increasing protein (BPI)
Base pairs (bp)
Bovine serum albumin (BSA)
Cholesteryl 4,4-difluoror-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate (BODIPY-CE)
Carboxy terminus (C-terminus)
Carboxyl ester lipase (CEL)
Cardiovascular disease (CVD)
Caveolin-1 (Cav-1)
CD-36, LIMPII analogous-1 (Cla-1)
Cholesteryl ester or cholesteryl oleate (CE)
Cholesteryl ester transfer protein (CETP)
Cholesteryl ester transfer protein transgenic mouse (CETPtg)
Chylomicrons (CM)
Coronary artery disease (CAD)
Cyan fluorescent protein (CFP) or enhanced Cyan fluorescent protein (ECFP) used interchangeably to described the enhanced protein
Cycloheximide (CHX)
Dulbecco's Modified Eagle's Medium (DMEM)
Dynamin-2 lysine (K) 44 to alanine (A) mutation (dyn2K44A)
Early endosome antigen-1 (EEA1)
Electron microscopy (EM)
Endocytic recycling compartment (ERC)
Endoplasmic reticulum (ER)
Endothelial nitric oxide synthase (eNOS)
Fetal bovine serum (FBS)
Fatty acids (FA)
Free or unesterified cholesterol (FC)
Green fluorescent protein (GFP)
Hanks Balanced Salt Solution (HBSS)
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

Heparin sulfate proteoglycans (HSPG)
Hepatic lipase (HL)
High-density lipoproteins (HDL)
High-density lipoprotein cholesterol (HDL-C)
High-density lipoprotein cholesterol ester (HDL-CE)
Human scavenger receptor BI (hSR-BI)
Intermediate-density lipoproteins (IDL)
Kilo base pairs (kb)
Low density lipoproteins (LDL)
LDL receptor (LDLr)
LDLr related protein (LRP)
Lecithin-cholesterol acyltransferase (LCAT)
Lipoprotein deficient serum (LPDS)
Lipoprotein lipase (LPL)
Liver X receptor (LXR)
Methyl beta cyclodextrin (MßCD)
Microtubule organizing center (MTOC)
Multiplicity of infection (MOI)
Nitric oxide (NO)
Oxidized LDL (oxLDL)
Paraforlmaldehyde (PFA)
Phosphate buffered saline (PBS)
Phospholipid (PL)
Phospholipid transfer protein (PLTP)
Reverse cholesterol transport (RCT)
Scavenger Receptor BI (SR-BI)
Scavenger Receptor BI knockout mouse (SR-BI KO)
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
Standard error of the mean (SEM)
Sterol regulatory element binding protein (SREBP)
Sterol regulatory elements (SRE)
Trans Golgi network (TGN)
Transferrin (Tf)
Trichloroacetic acid (TCA)
Triglycerides (TG)
Very-low density lipoproteins (VLDL)
Wild type (wt)

1 – Introduction

1.1 – Cardiovascular disease and atherosclerosis

Cardiovascular disease (CVD) is the major cause of mortality in industrialized nations and includes coronary artery disease (CAD) and stroke. Currently established risk factors for cardiovascular disease include elevated plasma low-density lipoprotein-cholesterol (LDL-C) levels, low levels of high-density lipoprotein-cholesterol (HDL-C), smoking, abdominal obesity, hypertension and diabetes. Atherosclerosis is the common pathological condition underlying cardiovascular diseases [1]. Atherosclerosis is initiated by the build-up of cholesterol-rich deposits called plaques and the rupture of these plaques and the subsequent formation of blood clots is the main precipitant of acute cardiovascular events [2]. To name a few, the rupture and clotting of atherosclerotic plaques cause stroke (in the carotid arteries to the brain), myocardial infarction and CAD (in coronary arteries to the heart) and peripheral vascular disease (all peripheral arteries). As such, a vast majority of the mortality due to CVD stems from atherosclerosis and research pertaining to this disease is critical from both medical and economic perspective.

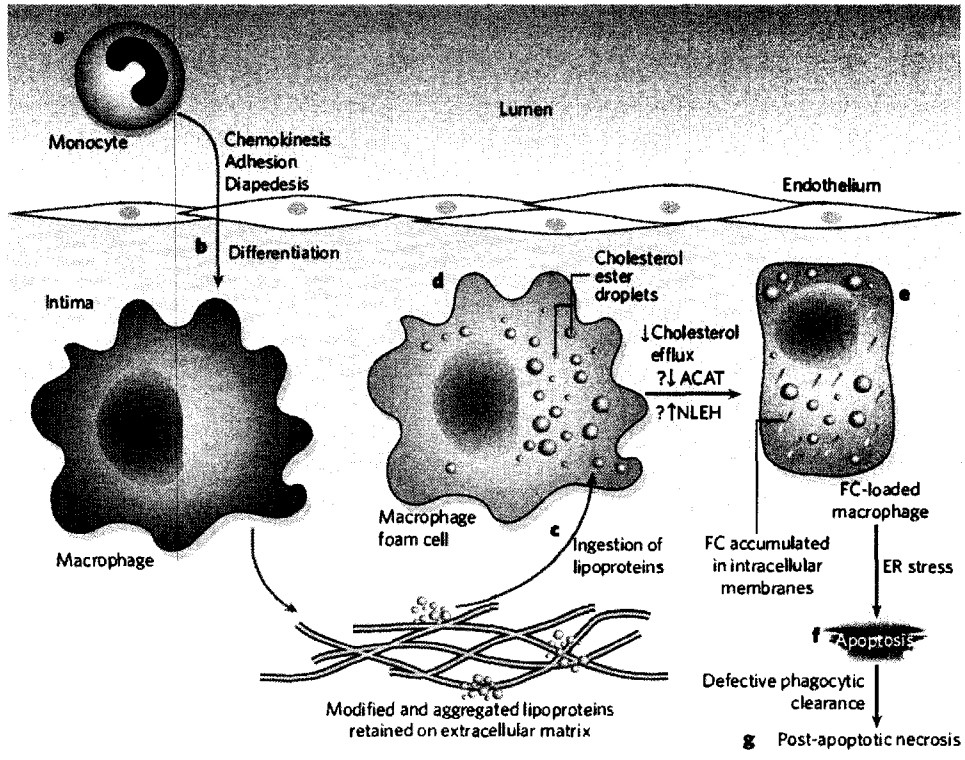
1.1.1 – Atherosclerosis

The pathological hallmark of atherosclerosis is the excessive buildup of cholesterol in macrophages trapped in the arterial wall, leading to their conversion to foam cells which trigger a series of inflammatory responses that eventually render the artery wall vulnerable to plaque formation and rupture [1;3].

Initiation of atherosclerosis is characterized by the accumulation of plasma low density lipoprotein (LDL) in the arterial wall and its subsequent modification by oxidation, lipolysis, proteolysis and aggregation [4]. This initiates an inflammatory process resulting in the recruitment

Figure 1.1

Initiation and progression of atherosclerotic lesion formation. **a.** Monocytes are attracted to areas of arterial intimal layer where lipoproteins have been retained. Trapped lipoproteins are further modified by oxidation and signals to the endothelium to express chemokines and adhesion molecules. **b.** The monocytes migrate through the endothelial layer and differentiate into macrophages. **c.** The macrophages ingest the retained lipoproteins by endocytic and phagocytic mechanisms and accumulate large amount of lipoprotein-derived cholesterol. **d.** In early lesions, the cholesterol is stored in macrophages as ACAT-derived cholesteryl esters and thus gives the macrophage a foamy appearance. **e.** In advanced lesions, unesterified or 'free' cholesterol (FC) further accumulates, leading to macrophage apoptosis **(f)**, and necrosis **(g)**. (Figure adapted from Maxfield and Tabas, *Nature*, 2005.)


Lumen
Monocyte
Chemokinesis
Adhesion
Diapedesis
Endothelium
b
Differentiation
Intima
Macrophage
d
Cholesterol ester droplets
Macrophage foam cell
↓Cholesterol efflux
?↓ ACAT
?↑NLEH
e
FC-loaded macrophage
c
Ingestion of lipoproteins
FC accumulated in intracellular membranes
ER stress
f
Apoptosis
Defective phagocytic clearance
g
Post-apoptotic necrosis
Modified and aggregated lipoproteins retained on extracellular matrix

of circulating monocytes (Fig. 1.1a). Monocytes trapped in the arterial intima undergo differentiation into macrophages, thus enabling them to engulf modified LDL, resulting in extensive cholesterol accumulation (Fig. 1.1b). The differentiated macrophages are stimulated to express scavenger receptors by the modified lipoproteins which then mediate rapid internalization of large amounts of modified LDL by phagocytosis (Fig. 1.1c). When the macrophages are no longer able to efficiently dispose of the accumulated oxLDL-cholesterol, they become engorged and undergo morphological change, leading to formation of foam cells (Fig. 1.1e)[5]. These foam cells can also become activated and eventually trigger a chronic inflammation. Resulting cytokines and chemokines promote adhesion molecule expression and further attract immune and inflammatory cells, especially in regions of turbulent blood flow. Smooth muscle cells are also recruited and induced to proliferate in this region. These series of events lead to the fatty-fibrous early atherosclerotic lesion formation.

Throughout the progression of atherosclerosis, foam cells accumulate significant amounts of free cholesterol that trigger apoptosis and are efficiently scavenged by other macrophages. In the early lesions, where phagocytic clearance of apoptotic cells appears to be efficient, macrophage apoptosis is associated with diminished lesion cellularity and decreased lesion progression (Fig. 1.1f). However, when the phagocytic cells in the lesion can no longer efficiently dispose of the increasing amounts of apoptotic cells, the apoptotic cells lyse and become necrotic leading to harmful lesion formation (Fig. 1.1g) [6]. The necrotic core in these late or advanced lesions, in concert with pro-atherogenic effects of residual surviving macrophages, promote further inflammation, plaque instability, and thrombosis [6]. Thus, the ability of lesional phagocytes to safely dispose of apoptotic macrophages is an important factor in determining the progression of atherosclerosis.

1.2 – Lipoproteins

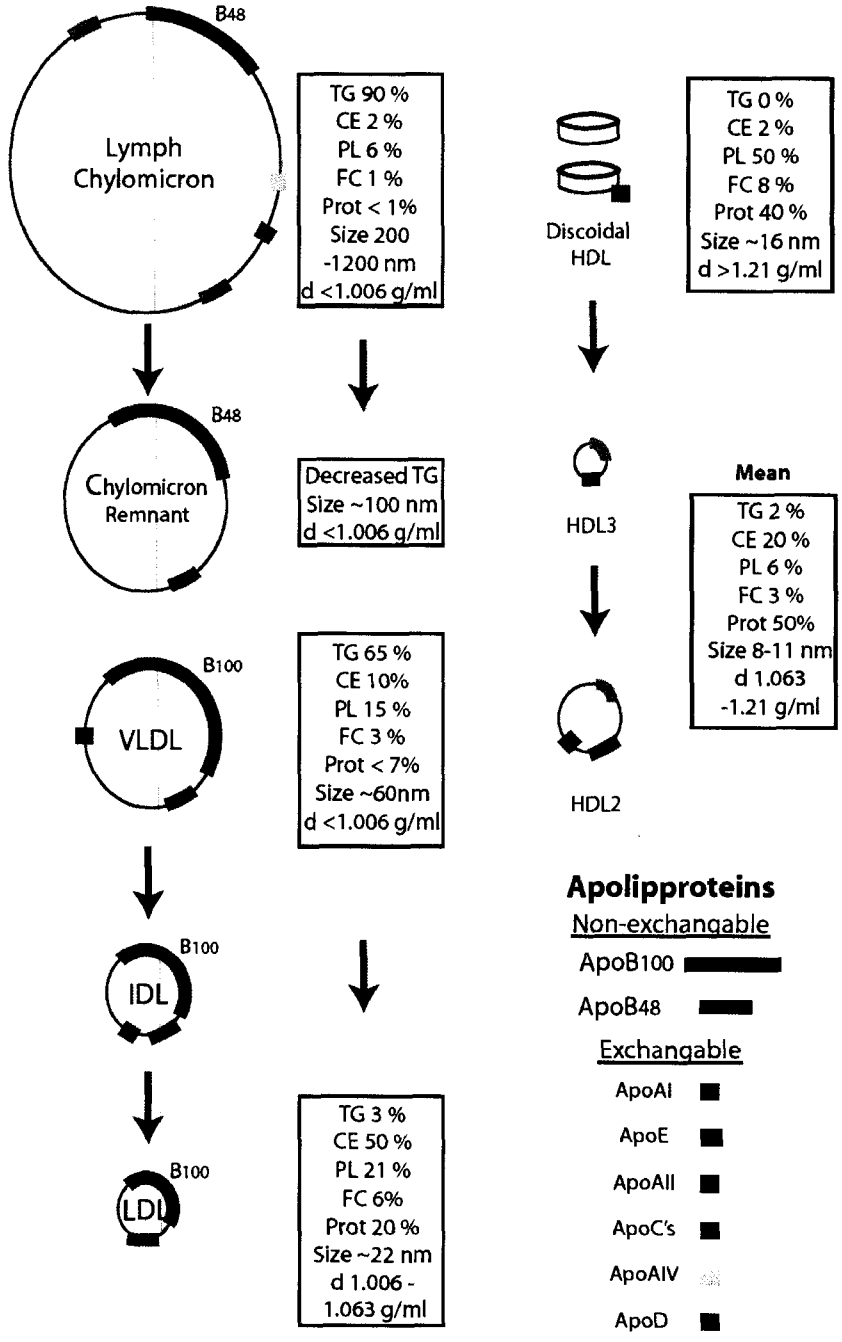
1.2.1 – General classification of lipoproteins

The main function of plasma lipoproteins is to act as vehicles transporting water-insoluble lipids from the organs where the lipids are synthesized to the target organs where the lipids can be used as energy source or for manufacturing new molecules, hence maintaining body cholesterol and lipid homeostasis.

Typical lipoproteins are composed of a monolayer of amphipathic phospholipids (embedded with free or unesterified cholesterol (FC) and surface proteins termed apolipoproteins) surrounding a hydrophobic core of neutral lipids, mainly cholesteryl esters (CE) and triacylglycerides (TG). Plasma lipoproteins are remarkably heterogeneous in terms of size, lipid and protein content, surface electrical charge, and apolipoprotein composition. Lipoproteins with a high lipid-to-protein ratio have a lower buoyant density compared to lipoproteins with a low lipid-to-protein ratio. As such, plasma lipoproteins can be separated into distinct classes according to their characteristic buoyant densities using density gradient ultracentrifugation. The buoyant density of lipoproteins is inversely related to their size; the smaller the lipoproteins, the denser the particles. The lipoprotein particles can exist in both spherical and discoidal forms. The majority of plasma lipoproteins are spherical microemulsions that are stabilized by apolipoproteins. Most of the apolipoproteins are amphipathic in nature, thus allowing them to associate with lipids as well as rendering lipoproteins soluble in aqueous solutions. The surface apolipoproteins are categorized into exchangeable and non-exchangeable groups. The non-exchangeable apolipoprotein B (apoB), is a key identifier of the lower density more buoyant lipoprotein particles including chylomicrons (CM), very-low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL) and low-density lipoproteins (LDL) (Fig. 1.2). The high-density lipoprotein (HDL), on the other hand, is identified by an absence of

Figure 1.2

Structure and composition of major human lipoproteins. Lipoproteins are shown in their most common classification. The lipid and protein content are expressed in percent by weight. Chylomicrons **(CM)**, very low-density lipoproteins **(VLDL)** and their remnants contain large TG cores. Intermediate-density lipoproteins **(IDL)** contain similar amounts of TG and CE in their core. The low-density lipoprotein **(LDL)** particles and the high-density lipoprotein **(HDL)** particles are relatively rich in core CE. The percent phospholipid **(PL)** is inversely correlated to particle sizes due to surface area to core volume ratios. Likewise, the percent protein **(Prot)** also increases with decreased particle size. (Imaged adapted from Atkinson & Small. *Annu. Rev. Biophys. Biophys. Chem.* 1986., Modified figure was provided as a courtesy of Dr. Chris Harder.)


B48
Lymph
Chylomicron
TG 90 %
CE 2 %
PL 6 %
FC 1 %
Prot < 1%
Size 200
-1200 nm
d <1.006 g/ml
B48
Chylomicron
Remnant
Decreased TG
Size ~100 nm
d <1.006 g/ml
B100
VLDL
TG 65 %
CE 10%
PL 15 %
FC 3 %
Prot < 7%
Size ~60nm
d <1.006 g/ml
B100
IDL
B100
LDL
TG 3 %
CE 50 %
PL 21 %
FC 6%
Prot 20 %
Size ~22 nm
d 1.006 -
1.063 g/ml
Discoidal
HDL
TG 0 %
CE 2 %
PL 50 %
FC 8 %
Prot 40 %
Size ~16 nm
d >1.21 g/ml
HDL3
HDL2
Mean
TG 2 %
CE 20 %
PL 6 %
FC 3 %
Prot 50%
Size 8-11 nm
d 1.063
-1.21 g/ml
Apolipproteins
Non-exchangable
ApoB100
ApoB48
Exchangable
ApoAI
ApoE
ApoAII
ApoC's
ApoAIV
ApoD

apoB, relatively smaller size and higher density. The HDL class is further divided into HDL_2 (large HDL) and HDL_3 (small HDL) depending on its size and density (Fig. 1.2). Lipoproteins also contain various combinations of exchangeable apolipoproteins that help further define their function (Fig. 1.2). The metabolism of lipoproteins with specific regards to its effects on atherosclerosis will be discussed in further detail.

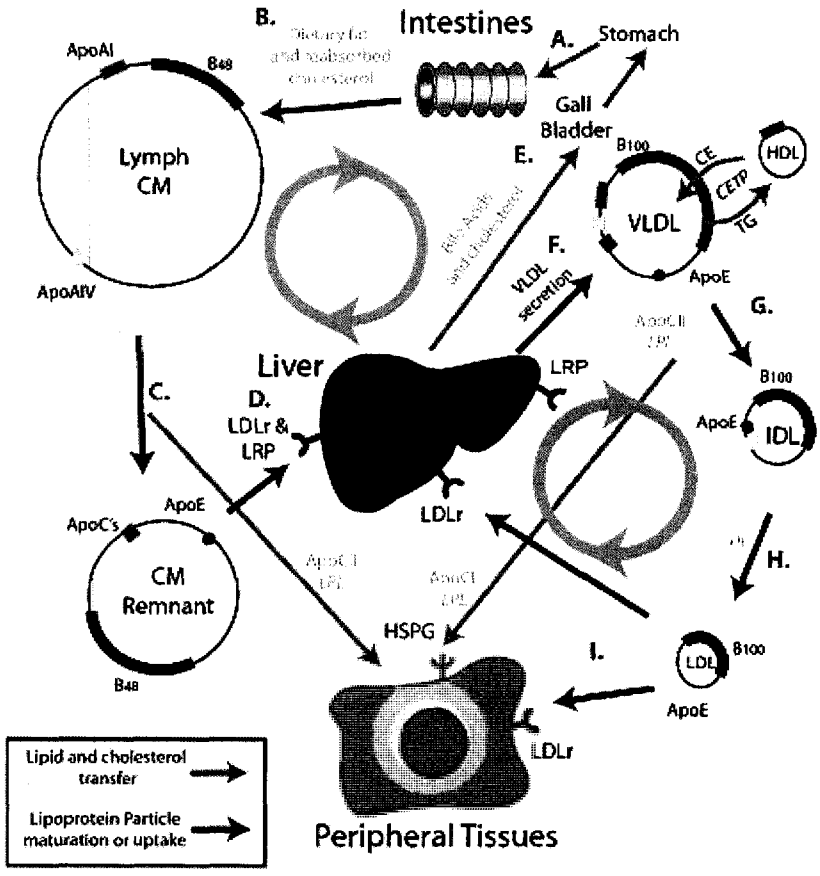
1.2.2 – Metabolism of apoB containing lipoproteins

There are two lipoprotein transport systems that utilize apoB-containing lipoproteins; the intestinal (exogenous origin) and hepatic (endogenous origin) lipoprotein transport systems. In both lipoprotein transport systems, TG is the main energy form that is moved in the body.

Exogenous lipid transport refers to the process of transporting dietary lipid that is carried out by intestinal chylomicrons (Fig. 1.3a). In the digestive tract, dietary lipids are hydrolyzed, emulsified and absorbed by the intestinal epithelium. These dietary lipid molecules are re-esterified into triglyceride (TG), phospholipid (PL), and cholesteryl esters (CE) within the enterocytes and assembled with apoB48 to form chylomicrons (Fig. 1.3b). Chylomicrons that carry these dietary lipids (exogenous lipids) are secreted into the circulation. In the circulation, TG in the core of the chylomicrons will be rapidly hydrolyzed by lipoprotein lipase (LPL) to free fatty acids that are quickly absorbed by adjacent cells and serve as a significant source of energy for peripheral cells in the postprandial state. The particles interact with heparin sulfate proteoglycans (HSPG) on the surface of peripheral cells which facilitates the degradation of the CM core by LPL. The resultant chylomicron remnants are then taken up by the liver through receptor-mediated endocytosis via either the LDL receptor (LDLr) or the LDLr related protein (LRP) (Fig. 1.3d). From synthesis of chylomicrons to the uptake of chylomicron remnants, the non-exchangeable apoB48 is always associated with these lipoprotein particles.

Figure 1.3

ApoB lipoprotein metabolism. Two cyclical pathways ensure the efficient delivery of both dietary (exogenous) and endogenously synthesized lipids. The top cycle consists of the enterohepatic recycling of cholesterol to the liver and the delivery of dietary triglyceride to the periphery in the fasted state. Free fatty acids are released after the hydrolysis of TG by the lipoprotein lipase (LPL), while the CM remnants are cleared by LDLr or LRP. VLDL are secreted by the liver and also deliver free fatty acids to peripheral tissues in the fasted state. This process of LPL-mediated hydrolysis creates IDL and LDL. Hepatic lipase (HL) also contributes to the production of LDL and delivers the freed lipid to the liver. LDL acts as a source of cholesterol for all tissues and is internalized and degraded by the LDLr. Enzymes are italicized. Apolipoproteins are denoted by shapes on the surface of the lipoproteins according to the legend in Fig. 1.2. See text for a detailed description. (Figure provided as a courtesy of Dr. Chris Harder)


B.
Intestines
A.
Stomach
ApoAI
B48
Lymph
CM
ApoAIV
Gall
Bladder
E.
B100
CE
HDL
CETP
TG
VLDL
ApoE
F.
VLDL
secretion
G.
Liver
LRP
C.
D.
LDLr &
LRP
B100
ApoE
IDL
ApoE
ApoC's
CM
Remnant
B48
LDLr
H.
HSPG
I.
LDL
B100
ApoE
LDLr
Lipid and cholesterol transfer
Lipoprotein Particle maturation or uptake
Peripheral Tissues

Endogenous lipid transport refers to the transport of lipids that originate from liver and corresponds to VLDL metabolism (Fig. 1.3f). The substrates utilized for hepatic VLDL assembly, namely cholesterol, phospholipids and fatty acids, are mostly synthesized via the *de novo* pathways. These lipid molecules are assembled with a single molecule of full-length apoB100, and the resulting VLDL particles are secreted into the blood steam by hepatocytes. This VLDL secretion provides a source of TG and cholesterol for peripheral cells in the fasted state. The secreted VLDL contains very little cholesterol and rapidly acquires free cholesterol from other lipoproteins and exchanges TG for CE by the actions of the cholesteryl ester transfer protein (CETP). In circulation, VLDL-TG is hydrolyzed by LPL, and converted into VLDL remnants and smaller intermediate sized IDL (Fig. 1.3g). VLDL remnants (IDL) contain apoE, which is a ligand for a number of hepatic lipoprotein receptors including LDL receptor and LRP. The IDL can be taken up by the liver via LDL receptor or converted to LDL by hepatic lipase that removes excess TG and PL and creates smaller LDL (Fig. 1.3h). Normally, plasma LDL particles which are relatively rich in CE are then taken up by cells in the liver or in the periphery primarily through the LDLr as a source of cholesterol (Fig. 1.3i).

Taken together, apoB lipoprotein metabolism consists of two cyclical processes that ensure sufficient amounts of cholesterol and TG are delivered to the appropriate cells of the body in both the fed and fasted state.

1.2.3 – HDL metabolism and reverse cholesterol transport

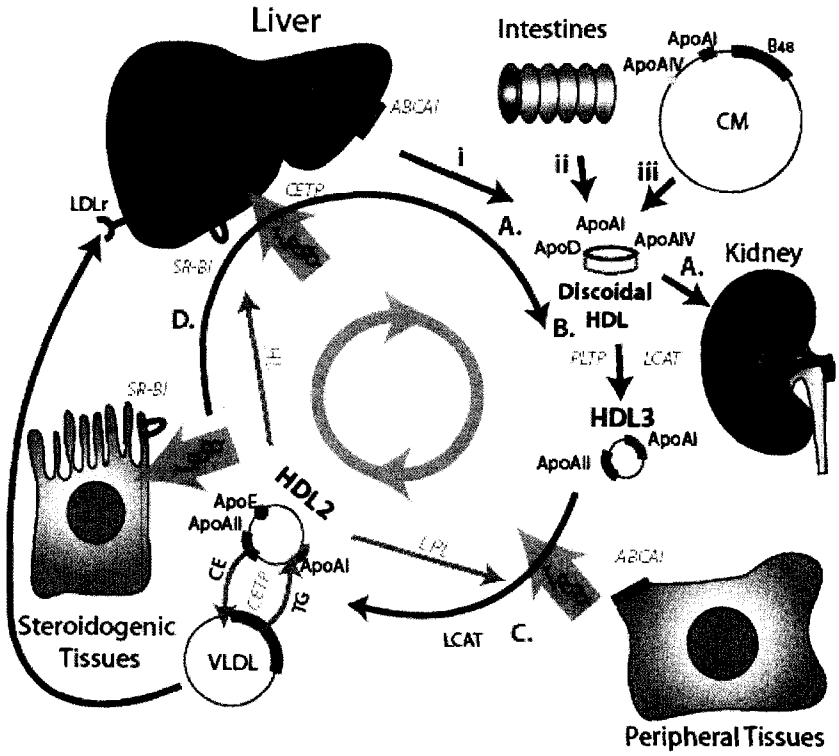
High density lipoproteins (HDL) have been shown to be an important factor in regulating cholesterol storage. Intricately coupled to apoB lipoprotein metabolism, HDL metabolism contributes to the lipid and cholesterol delivery system while also mediating the return of excess cholesterol from the periphery back to the liver. Currently established factors that are known to regulate HDL metabolisms include HDL associated apolipoproteins and

enzymes as well as cholesterol transfer proteins, cellular HDL receptors and a wide variety of transcription factors implicated in lipid metabolism.

ApoA-I, a major apolipoprotein associated with HDL, is secreted as a lipid-protein complex from hepatocytes as well as enterocytes. Each molecule of the newly secreted apoA-I is associated with several molecules of phospholipid. This relatively lipid-poor apoA-I can interact with cell surface ATP binding cassette A-1 (ABCA1) and promote phospholipid and cholesterol efflux. Both the newly synthesized apoA-I as well as the exogenous apoA-I are lipidated with the help of ABCA1 [7]. ABCA1 is also demonstrated to have a role in intracellular lipidation of apo-AI [8]. While the early lipidation of apoA-I in ER is ABCA1 independent, the lipidation in Golgi and at the plasma membrane is shown to be dependent on ABCA1 [8]. Further, possible plasma membrane reorganization by ABCA1 expands the non-raft membrane fractions (membrane fractions with relatively lower cholesterol to protein ratio; further explained in *Section 1.4.1*) and, consequentially, pre-conditions cells for cholesterol efflux [9]. As a result, ABCA1-mediated acquisition of additional phospholipid and cholesterol leads to the formation of poorly-lipidated and unstable discoidal HDL particles (Fig. 1.4a). Lecithin cholesterol acyltransferase (LCAT) catalyzes the esterification of surface free cholesterol to CE in discoidal HDL by fatty acylation of its polar hydroxyl group further stablizing the newly generated HDL [10]. The neutral cholesteryl ester then moves into the hydrophobic core and converts the discoidal particle to a spherical particle (Fig. 1.4b). Continued LCAT activity expands the core leading to an increase in particle volume. Additionally, a phospholipid transfer protein (PLTP) also ensures that the maturing HDL particle has sufficient phospholipid [11;12], while other exchangeable lipoproteins bind and further stabilize the HDL particle. TG hydrolysis catalyzed by lipoprotein lipase in chylomicrons leads to shedding of excess surface material such as phospholipid and exchangeable apolipoproteins that contribute to formation of

Figure 1.4

HDL metabolism and reverse cholesterol transport. The process of HDL maturation begins with the secretion of lipid-poor apoAI by the liver and the intestines or by the transfer of cholesterol, phospholipids, and apolipoproteins from chylomicrons and VLDL during LPL mediated lipolysis **(i,ii and iii respectively)**. The nascent particles acquire cholesterol and phospholipids via ABCAI-mediated efflux from the liver **(A)** and increase in size through the actions of LCAT and PLTP **(B)**. Lipid-poor apoAI and small HDL particles acquire additional cholesterol and phospholipids from cells in the periphery by ABCA1-mediated efflux. This generates progressively more cholesterol enriched HDL **(C)**. The cholesterol is then delivered back to steroidogenic tissues or the liver **(D)** by SR-BI or CETP and the TG is hydrolyzed by HL or LPL. (Figure provided as a courtesy of Dr. Chris Harder)


Liver
Intestines
ApoAI
B48
ApoAIV
CM
ABCA1
i
ii
iii
LDLr
CETP
A.
ApoAI
ApoD
ApoAIV
Kidney
A.
SR-BI
Discoidal
HDL
D.
B.
PLTP
LCAT
HDL3
ApoAI
ApoAII
SR-BI
HDL2
ApoE
ApoAII
ApoAI
CE
TG
LPL
ABCA1
Steroidogenic
Tissues
VLDL
LCAT
C.
Peripheral Tissues

mature HDL in circulation. These activities direct the creation of small dense HDL_3 particles, rich in phospholipid that travels to peripheral tissues. Both ABCA1 (for lipid-poor particles) and ABCG1 (for larger particles) facilitate the peripheral acquisition of free cholesterol [13;14]. The free cholesterol is again converted by the actions of LCAT to CE that moves to the core of the particle (Fig. 1.4c). In addition, CETP increases the TG content of the small HDL_3 particles by exchanging TG for CE with apoB lipoproteins. Together, these processes increase the size of the HDL_3 to form larger CE and TG rich HDL_2 particles. HDL_2 travels through circulation where the TG is hydrolyzed by LPL (for delivery to peripheral tissues) and HL (for delivery to the liver).

HDL metabolism not only results in the efflux of cholesterol from peripheral cells but also involves its delivery to the liver where it can be secreted into bile as bile acid. This pathway is referred to as **reverse cholesterol transport** since cholesterol is normally synthesized in the liver and then delivered to peripheral cells. Reverse cholesterol transport can potentially reduce the cholesterol load in peripheral tissues, including artery walls, and is therefore considered to be an anti-atherogenic pathway. The observation that high HDL cholesterol levels are associated with a decreased risk for coronary artery disease may be, in part, attributed to this pathway.

In the final step of reverse cholesterol transport, HDL derived CE can be returned to the liver by three different routes. First, the CE is selectively removed by SR-BI [15] and by CETP [16] via a process called selective uptake which will be further discussed in *Section 1.2.4*. Secondly, CETP can mediate the transfer of CE from HDL to VLDL in exchange for TG leading to a CE enrichment of VLDL. As mentioned, some VLDL can return the cholesteryl esters that it has acquired to the liver by receptor-mediated endocytosis or VLDL can be converted to IDL and LDL through the actions of lipoprotein lipase and hepatic lipase. IDL and LDL can then be taken by the LDLr on hepatocytes. Lastly, there are other HDL receptors in liver that are yet to

be characterized that can also mediate HDL uptake but this process slightly differs from selective uptake in that it involves HDL holoparticle uptake leading to degradation of the HDL particle. The cholesterol returned to the liver may then be converted into bile acid and secreted into the bile for excretion in the stool. Hence reverse cholesterol transport of HDL-C ensures that peripheral tissues do not accumulate excess cholesterol.

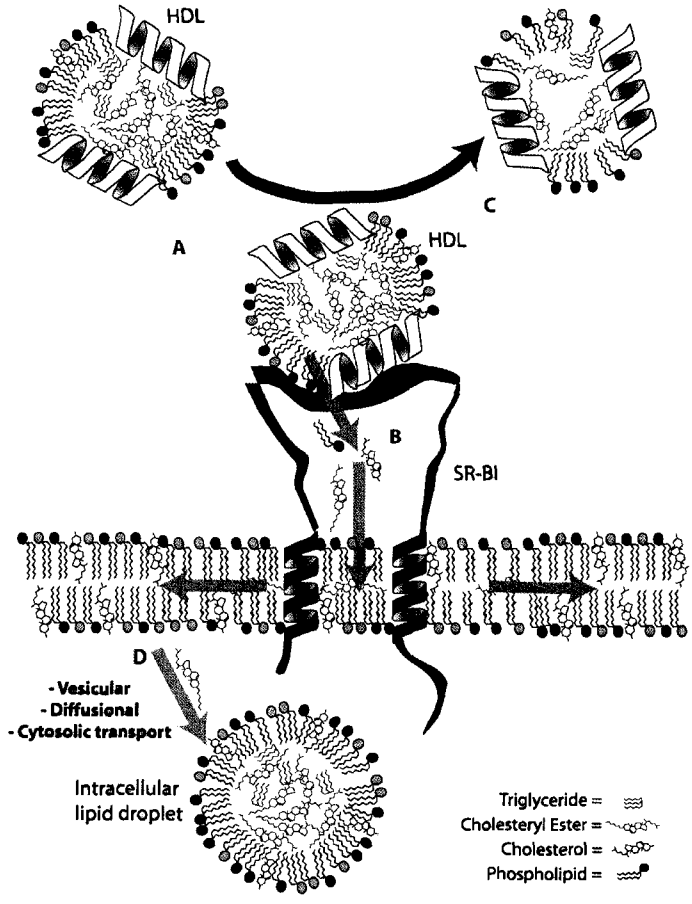
1.2.4 – Selective uptake of HDL derived CE

As mentioned in the previous section, HDL-derived CE can be directly returned to the liver by a process known as selective uptake. Selective uptake by SR-BI has been demonstrated in steroidogenic tissues and hepatocytes [15] and by CETP in adipocytes [17] and hepatocytes [16]. The process of selective uptake has been studied extensively but unresolved issues regarding its exact cellular mechanism still remain. The classical view of selective uptake consists of 3 steps; 1) receptor binding (Fig. 1.5A,B)[18], 2) the reversible incorporation of HDL-derived CE into a plasma membrane pool (Fig. 1.5B)[19] followed by 3) transfer of the lipid to an inaccessible pool by mechanisms not involving coated pit-mediated endocytosis[20] without the uptake or degradation of HDL particle (Fig. 1.5D)[21]. The reversible compartment is proposed to include CE that has entered the plasma membrane and remains accessible to extraction by extracellular, unlabeled HDL (Fig. 1.5D)[22]. The plasma membrane CE is subsequently transferred to an irreversible compartment (i.e. lipid droplets) as it is internalized and becomes inaccessible to extraction by extracellular unlabeled HDL.

As briefly mentioned, SR-BI is one established HDL receptor that mediates selective CE uptake from HDL in mice. The rate of SR-BI-mediated CE transfer is proportional to the amount of CE in HDL, suggesting that SR-BI may form a hydrophobic channel allowing movement of sterol down the concentration gradient (Fig. 1.5B) [23]. Reconstitution of SR-BI in liposomes also indicated that SR-BI mediates selective CE uptake independent of other cellular

Figure 1.5

SR-BI-mediated selective uptake. A graphic representation of SR-BI-mediated selective uptake from HDL is shown. The process is characterized by HDL binding to SR-BI **(A)** and the selective transfer of lipids down a concentration gradient **(B)**. For continual uptake, the dissociation of the HDL particle from the receptor **(C)** and the irreversible internalization of SR-BI appear to be required **(D)**. Step **(B)** is a reversible process as the transferred lipids in the plasma membrane can be extracted by an excess of poorly lipidated HDL. The intracellular accumulation of non-hydrolyzed CE occurs in the lipid droplet. The irreversible transport of FC and CE to intracellular sites can occur through vesicular transport, diffusion or by cytosolic carrier proteins. (Figure provided as a courtesy of Dr. Chris Harder)


HDL
A
C
HDL
B
SR-BI
D
- Vesicular
- Diffusional
- Cytosolic transport
Intracellular
lipid droplet
Triglyceride =
Cholesteryl Ester =
Cholesterol =
Phospholipid =

53

cofactors [24]. However, although most initial studies suggested that selective uptake occurs strictly at the cell surface, both SR-BI and HDL have been shown to internalize into and recycle from endocytic compartments. Tall and colleagues demonstrated that HDL is internalized by primary murine hepatocytes and recycled in a cholesterol-depleted form[25], a process often referred to as 'retroendocytosis' [26]. Further, Silver and colleagues observed that SR-BI and HDL colocalizes with transferrin in the endocytic recycling compartment (ERC) in CHO cells and in the apical recycling compartment (ARC) in polarized primary hepatocytes, suggesting that HDL undergoes cyclical recycling mediated by SR-BI that involves intracellular deposition of CE in a manner similar to the internalization of iron by transferrin and the transferrin receptor[27]. We have also shown that endocytosis of HDL occurs with early separation of receptor and cargo and recycling of lipid depleted HDL to the cell surface (unpublished data). However, it was later found that endocytosis occurs but is not a requirement for efficient SR-BI-mediated selective uptake[28] suggesting SR-BI may mediate both HDL uptake and recycling as well as selective uptake.

Selective uptake of CE from HDL results in the production of small lipid-depleted apoAI particles that are returned to the circulation, where they may acquire more cholesterol and phospholipids from peripheral cells or be cleared by the kidney.

1.2.5 – Lipoprotein metabolism and atherosclerosis

The cellular regulation of cholesterol is a tightly regulated process as complications arise when the rates of cholesterol ingestion and synthesis exceed the rates of return to the liver and excretion. The metabolism of both apoB lipoproteins and HDL uniquely affects the development of atherosclerosis and it is for this reason that clinicians examine the ratio of the two plasma cholesterol pools when assessing CVD risk.

Pro-atherogenic properties of ApoB lipoproteins

TG-rich lipoprotein remnants and particularly small dense LDL are significant contributors to the progression of atherosclerosis. As a result, LDL-C has remained the primary target for atherosclerosis risk reduction. Besides being an abundant lipoprotein in circulation, it is also small in size which enables it to easily infiltrate the arterial wall and initiate the atherosclerotic process [29]. The high concentration and the slow turnover rate of LDL in circulation also increase its susceptibility to modifications by reactive oxygen species (ROS)-mediated oxidation in the vessel walls [30]. LDL oxidation may explain why smokers are at an increased risk of cardiovascular disease, as LDL is modified by smoke induced oxidation in the lungs [31]. Furthermore, apoB-containing proteins are extremely insoluble and have the tendency to self-aggregate making them prime candidates for macrophage clearance and foam cell formation [32].

Anti-atherogenic properties of HDL

Plasma concentration of high density lipoprotein cholesterol (HDL-C) is shown to be inversely correlated with atherosclerotic cardiovascular disease risk in humans [33]. The Framingham study results further showed that increasing HDL levels is more effective in reducing CVD risk than lowering LDL levels and low plasma concentration of HDL-C is now established as a significant independent risk factor for CVD [34]. The anti-atherogenic claim of HDL is due to its anti-inflammatory [35], antithrombotic [36], anti-oxidative [37] and nitric oxide-inducing properties [38;39]that further attenuate and even regress atherosclerosis. More importantly, HDL stimulates cholesterol excretion via the reverse cholesterol transport from peripheral cells or macrophages to the liver and ultimately biliary excretion and maintains cholesterol homeostasis. Hence, this participation of HDL in reverse cholesterol transport is an important mechanism to explain its anti-atherogenicity [40]. Accordingly, much focus of recent lipid research

has been cast on the metabolism of HDL and RCT and how best to utilize its anti-atherogenic properties.

The reverse cholesterol transport is carried out in all peripheral tissues including cholesterol-loaded macrophages in atherosclerotic lesions where cholesterol efflux relies heavily on acceptor HDL particles. The best-studied hypothesis regarding HDL-mediated protection against atherosclerosis involves the entry of this macrophage-derived cholesterol into the RCT pathway. The process of macrophage cholesterol efflux is mediated by ABCA1 [41] as macrophage-specific ABCA1 knockout mice exhibited increased atherosclerosis [42-44]. ABCG1[45;46], ABCG4 [13] and SR-BI [47;48] also mediate efflux of FC to larger HDL molecules and protect against atherosclerosis. HDL cholesterol is rapidly trafficked to the apical bile canaliculus of polarized hepatocytes and is likely a significant source of cholesterol excretion into bile [49]. Overall, HDL acts as an important mediator in the clearance of excess cholesterol from the body and this property contributes to its strong anti-atherogenicity.

1.3 – Cholesteryl Ester Transfer Protein

1.3.1 – Background on CETP: expression, regulation and function

CETP expression and regulation

Cholesteryl ester transfer protein is a secreted glycoprotein, 476 amino acids in length, that is a member of the lipid transfer protein/lipopolysaccharide binding protein gene family that also includes phospholipid transfer protein (PLTP), lipopolysaccharide binding protein (LBP) and bactericidal/permeability increasing protein (BPI) [50]. The molecular weight of CETP ranges from 66 to 74 kDa depending on the level of glycosylation[51]. The crystal structure of CETP has recently been solved and the resulting structural model predicts that CETP is a 60-Å-long tunnel with two hydrophobic cholesteryl esters buried in the middle and plugged by an

amphiphilic phosphatidylcholine at each end [52]. The two tunnel openings are found to be large enough to allow lipid access, which is aided by a flexible helix and possibly by a mobile flap [52]. Interestingly, point mutations blocking the middle of the tunnel abolish lipid-transfer activities, suggesting that neutral lipids may pass through this tunnel during lipid transfer. Additionally, the curvature of the concave surface of CETP matches the radius of curvature of nascent HDL particles and is suggested to undergo potential conformational changes to accommodate larger lipoprotein particles [52].

In humans, CETP mRNA is predominantly expressed in the liver, spleen and adipose tissue in humans and is secreted to a variable extent from each of these tissues into plasma, where it has an established role in mediating neutral lipid transport between lipoproteins. The human CETP gene contains 16 exons spanning approximately 225 kb of chromosome 16 at locus 16q12-21 near the LCAT locus [53;54]. cDNAs encoding CETP have been cloned from several species including monkey, rabbit, hamster, chicken and tree shrew with variations in homology of between 80-95% compared to human cDNA [55-57]. Interestingly, mice and rats do not have the gene encoding CETP in their genomes [58] making them an ideal model for studying CETP function.

A number of studies have demonstrated that CETP mRNA and protein expression are increased by cholesterol. More specifically, CETP mRNA level is up-regulated by cholesterol by a mechanism that involves both a sterol response element (CRE) which binds YY1 and SREBP-1[59] and by a DR-4 element, which binds LXR/RXR [60]. Indeed, plasma concentrations of CETP are increased in obese states and in response to cholesterol intake [61] resulting from increased CETP mRNA in the liver and adipose tissue [62]. Dietary or pharmacological treatments such as statin therapy, which lower plasma lipids, also reduce circulating CETP levels [63;64]. This is also true in various animal models. In rabbits [65] and hamsters [66], plasma CETP activity was also

shown to increase in response to high fat, high cholesterol diet due to increased production of CETP in the liver [66].

CETP function

As mentioned previoiusly, CETP is secreted from various tissues and the majority of plasma CETP is associated with HDL although CETP is known to bind to all lipoproteins *in vitro* [67]. In plasma, CETP is thought to function as a mediator in transporting lipids between different lipoprotein particles as it is responsible for all CE/TG transfer activity in human plasma and for ~50% of the phospholipid transfer activity [68;69]. More specifically, CETP mediates the exchange of neutral lipid between apoB-containing lipoproteins and HDL causing a net transfer of CE from HDL to apoB-containing lipoproteins. This leads to the CE depletion and TG enrichment of HDL while causing CE enrichment and TG depletion of LDL. Hence, under conditions of efficient hepatic apoB lipoprotein clearance, CETP may promote cholesterol transport from HDL to the liver for subsequent biliary secretion via LDLr mediated pathway (RCT). Cholesterol depletion and TG enrichment of HDL also makes it a better substrate for hepatic lipase, which plays an important role in HDL remodeling and catabolism [70]. Moreover, CETP was also shown to mediate the exchange of LDL CE with VLDL TG [69].

The majority of studies measuring the kinetics of CETP lipid exchange between lipoprotein substrates support a mechanism whereby CETP acts as a shuttling protein [71;72]. Recently, the CETP inhibitor torcetrapib was reported to inhibit CETP transfer activity by creating a non-functional complex between HDL and CETP further supporting the shuttling model for neutral lipid transfer [73;74].

1.3.2 – Role of CETP in selective uptake of HDL derived CE

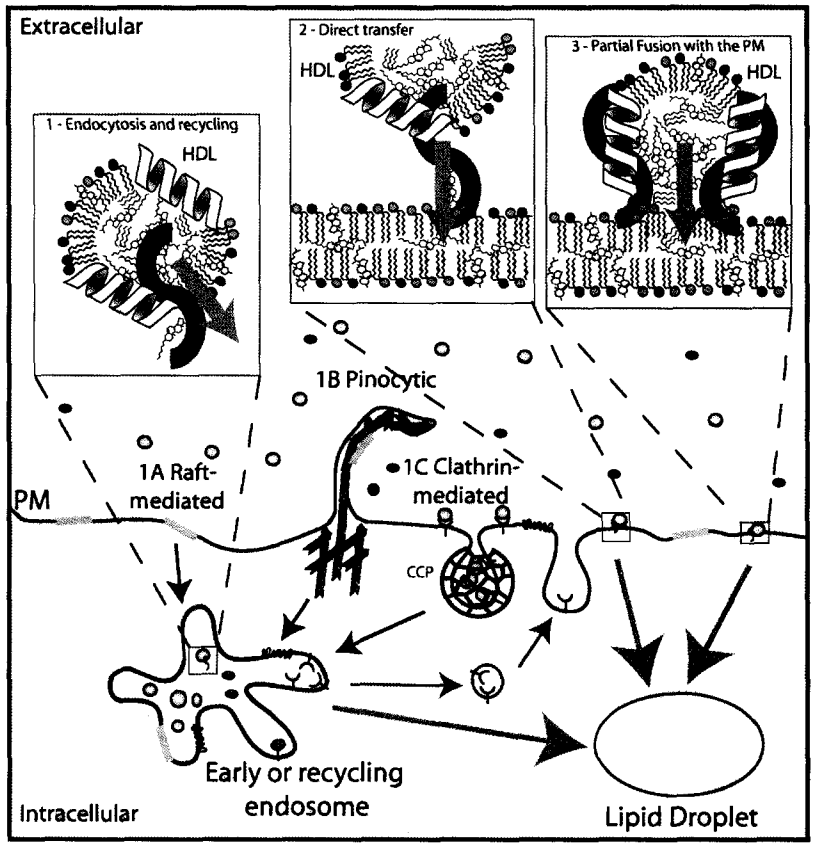
Recently, we have made the novel observation that in adipocytes [17;75;76] and hepatocytes [16], CETP mediates the selective acquisition of CE from HDL, analogous to the role of SR-BI.

Experiments were carried out using primary hepatocytes from either SR-BI-null or LDLr-null mice, and treatments such as receptor-associated protein (RAP) to block the members of the LDLr family through competitive binding were used. We also determined the effects of heparin, which disrupts binding of hepatic lipase to the heparin sulphate proteoglycan (HSPG) matrix. These studies clearly demonstrated that CETP mediates the selective uptake of HDL-derived CE by a mechanism that does not involve SR-BI and which does not require LRP, LDLr or VLDLr nor clathrin-mediated endocytosis. Furthermore, hepatic expression of adenovirus-CETP, by tail vein injection resulted in significant remodeling of plasma HDL, consistent with a major physiological role for hepatocyte CETP as a receptor mediating selective uptake [16]. In further support of our hypothesis, shortly after our findings were published, two separate studies performed in CETP transgenic mice confirmed a similar role of CETP in hepatic HDL-CE uptake [77;78]. In more recent studies, we have also demonstrated that the human CETP transgene protects against diet-induced atherosclerosis in SR-BI deficient mice thereby providing new and important insight into the possible roles of CETP as an alternative route for hepatic HDL-CE clearance [79].

Based on studies in hepatocytes and in adipocytes[80;81], we have originally proposed two pathways to explain CETP-mediated selective uptake of CE; namely shuttling and transient fusion [17]. First, circulating plasma CETP may mediate selective uptake by shuttling CE directly from HDL to the plasma membrane (Fig. 1.6-2). This process may or may not require the participation of another protein on the cell surface but clearly does not require SR-BI, members of the LDL receptor family or an intact HSPG matrix [16]. Secondly, we have shown that cell-associated CETP appears to mediate selective uptake by a mechanism that is insensitive to torcetrapib and thus does not involve CETP-mediated shuttling of CE from HDL to the plasma membrane (hence lipid transfer activity of CETP may not be required in this case) [16]. Rather,

Figure 1.6

Three proposed mechanisms for CETP-mediated CE selective uptake. Three hypothetical underlying mechanisms of CETP mediated selective uptake of CE to hepatocytes and adipocytes exist. The first involves selective depletion of CE during a process of endocytosis and recycling. CETP endocytosis could be raft mediated (1A), via pinocytosis (1B), or through clathrin coated pit (CCP) internalization (1C). The second mechanism may involve CETP tethering HDL to the cell-surface and mediating a direct hydrophobic transfer through its neutral lipid binding pocket (2). The third mechanism, may involve CETP mediating the partial-fusion of HDL with the plasma membrane (3). Importantly, CETP endocytosis and recycling most likely coincides with either direct transfer or partial-fusion mediated selective uptake. Following selective uptake, the CE is mostly likely shuttled to a lipid droplet or to the bile canaliculus in polarized hepatocytes (not shown). (Figure provided as a courtesy of Dr. Chris Harder)


Extracellular
1 - Endocytosis and recycling
HDL
2 - Direct transfer
HDL
3 - Partial Fusion with the PM
HDL
1B Pinocytic
1A Raft-mediated
PM
1C Clathrin-mediated
CCP
Early or recycling endosome
Intracellular
Lipid Droplet

there may be a direct interaction of HDL with CETP on the cell surface. One hypothesis is that plasma membrane associated CETP may mediate the transient fusion of the HDL amphipathic coat with the membrane outer leaflet, allowing CE transfer without HDL particle uptake (Fig. 1.6-3). CETP contains a C-terminal peptide that has a tilted orientation relative to the lipid-water interface, and this peptide has fusogenic properties similar to those described for viral fusion peptides[82;83]. Finally, we cannot disregard the possibility of CETP-mediated endocytosis and recycling of HDL during which selective depletion of CE from HDL occurs (Fig.1.6-1). We suspect that CETP endocytosis could be raft-mediated or occur via pinocytosis or through clathrin-coated pit internalization. As mentioned previously in *Section 1.2.4*, evidence of HDL recycling associated with SR-B1 mediated HDL-CE selective uptake has been presented [27]. This HDL recycling process has also been suggested to be essential in the efficient selective uptake process [25]. It is also possible that HDL internalization and recycling coincides with direct transfer or partial fusion mechanism. Further studies are required to unravel the exact molecular mechanisms of CETP-mediated HDL-CE selective uptake and to establish the possible role of CETP in intracellular cholesterol trafficking.

1.3.3 – CETP and atherosclerosis

The overall role of CETP in development of atherosclerosis remains unclear. Although SR-BI has been shown to be the primary receptor responsible for hepatic HDL-cholesterol clearance in the mouse[84], a species that intrinsically lacks CETP[58], the role of the human homologue of SR-BI, CLA-1, in lipoprotein metabolism is not well established. It is temping to speculate that CETP may play a bigger role in HDL metabolism and atherosclerosis in humans than what is known thus far.

The controversy regarding the potential atherogenicity of CETP stems from its multiple potential functions in lipoprotein metabolism and reverse cholesterol transport as well as the variable effects of polymorphisms in the CETP gene on atherosclerotic risk.

Potential **anti-atherogenicity** of CETP arises from many different reasons. First, as explained previously, under conditions of efficient hepatic apoB lipoprotein clearance, CETP may promote cholesterol transport from HDL to apoB lipoproteins and to the liver for subsequent biliary secretion, hence facilitating reverse cholesterol transport. Additionally, CETP-mediated remodeling of HDL combined with the action of hepatic lipase results in the production of smaller HDL particles which can be a vehicle for both ABCG1-mediated and diffusional cholesterol efflux from peripheral cells. Furthermore, strong evidence has shown that CETP also mediates selective uptake of HDL-CE, adding to its anti-atherogenic claim.

However, CETP has also been proposed to be **pro-atherogenic** by increasing the cholesterol content of the potentially atherogenic apoB lipoproteins. Also, high plasma concentrations of CETP are associated with low levels of HDL-C, which is an important risk factor for atherosclerosis. Further, the observation that genetic deficiency of CETP in humans results in a marked increase in plasma concentrations of HDL-CE [85;86] has led to the development of CETP inhibitors as a potential therapy to reduce atherosclerosis. However torcetrapib, the first of these agents to be tested in a randomized controlled trial, was not found to be clinically effective. This was not unexpected since no conclusive link has been established between CETP-mediated modified HDL levels and its potential atherogenicity. Although plasma concentrations of HDL are elevated in CETP deficiency, it has not been shown to confer protection against CVD. Rather, a study done on a Japanese-American group demonstrated that CETP deficiency is associated with an increased risk of atherosclerosis in spite of high plasma HDL concentrations[87], possibly due to impaired hepatic cholesterol

clearance. Also, in coronary heart disease patients with low plasma CETP concentrations associated with the Taq 1B polymorphism of CETP, further reduction of plasma CETP by pravastatin treatment resulted in progression rather than regression of atherosclerosis [88]. Hence, studies in both animal models and human subjects with atherosclerosis have not provided a clear answer regarding CETP and its link to atherosclerotic risk due to the extensively varied outcomes, apparently contingent upon the level of CETP expression and the genetic background of the animals and individuals studied. The ongoing controversy regarding the role of CETP in atherosclerosis may be a reflection of a complex molecule with multiple functions.

1.4 – Intracellular Trafficking

1.4.1 – Lipid rafts

The term lipid raft refers to a specialized lipid microdomain on the membranes with unique lipid composition that is distinct from the surrounding lipid environment. It is generally believed that lipid rafts are enriched in cholesterol, glycosphingolipids, sphingomyelin, and phospholipids with long unsaturated acyl chains [89-92]. The long hydrocarbon chains of sphingolipids and the small cholesterol molecules incorporated in between the sphingolipids and below their polar head groups allow for a close packing and make these raft domains more rigid than the rest of the relatively fluid plasma membrane forming a liquid-ordered phase (L_o). In contrast, the bent hydrocarbon chains of unsaturated phospholipids which make up the surrounding bulk liquid-disordered bilayer (L_d) cannot pack so closely together and has physicochemical properties that is different from the L_o. These contrasting physical properties contribute to different biochemical properties of raft and non-raft domains.

Lipid rafts are also rich in a variety of other molecules such as glycophosphatidylinositol (GPI)-anchored proteins [93], a number of palmitoylated and mirystoylated transmembrane

proteins (e.g., src family tyrosine kinases)[94], Gα subunits of heterotrimeric G proteins [95;96], and endothelial nitric oxide synthase (eNOS)[97]. This makes lipid rafts the perfect sites for the initiation of cell signaling and signal transduction. In some proteins, their lipid-raft localization is dependent of extracellular signals.

The existence of lipid rafts has been questioned by many scientists for a long time, and the debate still has not been settled as to whether the rafts are true structures or just experimental artifacts [98]. The controversy arises from the lack of proper techniques required for visualizing lipid rafts in living cells. Lipid rafts are microscopic and dynamic structures (approximately 50 nm in diameter in steady state, few hundred when clustered upon stimulation) and there are obvious technical challenges in studying them. The small size of lipid rafts makes it hard to visualize them with conventional light microscopy as it falls under the resolution power of most light microscopes. The most common method of identifying lipid rafts involves solubilization of cells with detergents such as Triton X-100 at 4°C. Since lipid rafts are relatively insoluble in detergent at low temperature and low in density (due to the relatively higher lipid to protein ratio), one can separate lipid-raft associated proteins and lipids using detergent extraction followed by sucrose gradient centrifugation. Many lipid raft associated proteins have been identified by this method. However, some researchers believe that the membrane composition changes following the detergent treatment and that the separation occurs as a consequence of the membrane alteration instead of projecting the true image of the plasma membrane structure. Over the past decade, however, a significant amount of evidence supporting the lipid raft hypothesis has accumulated due to a number of technical advancements, and although some remain skeptic, rafts are currently accepted as a real biophysical entity. As one example, Sheets and colleagues performed a single particle tracking technique using colloidal

gold conjugated antibodies to prove that the sphingolipid, ganglioside GM1 and Thy-1 (lipid raft-associated protein) were confined to secluded areas on plasma membrane.

Although lipid rafts are now considered a biophysical reality, the functional importance, dynamics and role of lipid rafts in various cellular processes still remain to be resolved. Rafts are considered platforms for the regulation of many distinct types of protein interactions. Indeed, recent studies have implicated rafts in signal transduction, pathogen entry [99], virus budding [100], membrane fusion platform, T cell signalling [101] as well as endocytosis and intracellular trafficking [102;103]. However the exact role of rafts in the context of the metabolism, transport and maintenance of intracellular cholesterol is not well understood.

1.4.2 – Endocytosis

Endocytosis allows cells to internalize macromolecules and particles into transport vesicles derived from the plasma membrane [104]. Multiple endocytic pathways exist to meet the diverse requirements of cargoes destined for specific intracellular sites.

The best-characterized mechanism for internalizing both bulk membrane and specific proteins is the **clathrin-mediated endocytic pathway** which involves the dynamin-dependent pinching of vesicles coated in clathrin (Fig. 1.7A). Before pinching, these vesicles specifically recruit cell-surface receptors with specialized targeting signals and adapter proteins to facilitate the fission of coated pits from the plasma membrane and direct their subsequent intracellular trafficking. LDLr bound to LDL is internalized via this clathrin-mediated pathway [105]. There are also **clathrin-independent endocytic pathways** which include macropinoctyosis, phagocytosis, and caveolar endocytosis as well as raft-mediated (caveolae-independent) endocytosis [106]. They do not recruit known coat proteins to mediate their budding, but instead appear to make extensive use of lateral heterogeneity, anchored receptors and recruited scaffolding to direct the internalization of receptor-associated ligands [107]. These forms of

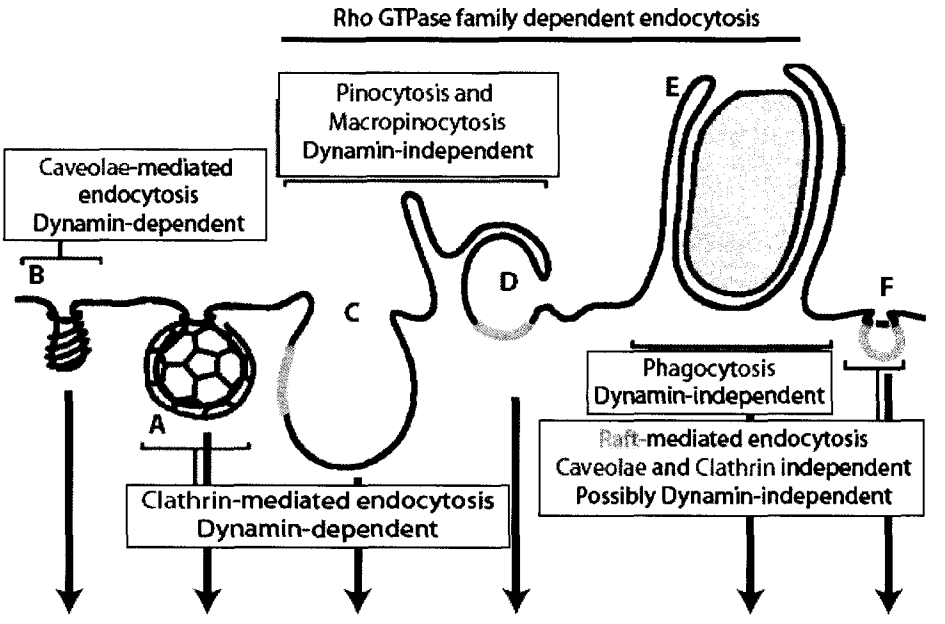
endocytosis involve membranes moving to engulf fluid-phase ligands, often sequestering receptors and their cargo into dynamic membrane microdomains that pinch off and migrate into the cell [107].

The best characterized of the clathrin-independent endocytosis is **caveolae**-mediated internalization (Fig. 1.7B). Caveolae are dynamin-dependent, sphingolipid- and cholesterol-rich "cave-like" invaginations of the plasma membrane induced by the polymerization of the resident protein, caveolin [107]. Binding of certain ligands to the caveolar receptors can induce endocytosis of caveolae, which unlike the clathrin-coated pits, do not fuse with endosomes/lysosomes. Certain pathogens that utilize caveolar transport to the ER and Golgi avoid lysosomal degradation thus ensuring their survival in the host cell.

A number of different forms of dynamin-independent endocytosis make extensive use of actin remodeling to mediate the rearrangement or extension of plasma membranes pseudopods to facilitate cargo entry. **Phagocytosis** is one process whereby large particles are internalized and degraded (Fig. 1.7E). Uptake is usually triggered by binding of the cargo to cell-surface receptors that transduce signals and initiate membrane rearrangement [108]. **Pinocytosis** or "cell-drinking" refers to any mechanism of endocytosis that involves the uptake of fluid phase markers and includes caveolar and clathrin-mediated endocytosis but not phagocytosis (Fig. 1.7C) [104]. Macropinocytosis is the best known form of pinocytosis that is characterized by the formation of large, irregularly-shaped endosomes made by the folding and closure of lamellipodia that are generated primarily at sites of membrane ruffling (Fig. 1.7D) [109]. These plasma membrane ruffles are enriched in specific phosphoinositides and lipid raft markers [110]. After internalization, the membranes of macropinosomes often merge with early endosomes and are recycled back to the plasma membrane by recruiting specific machinery [111].

Figure 1.7

Methods of endocytosis. A graphic representation of the different forms of cellular endocytosis including clathrin-mediated **(A)**, caveolar **(B),** pinocytic and macropinocytic **(C)**, phagocytic **(D)** and raft-mediated **(E)** endosytosis. The clathrin cage is shown in **red**, the cavelin-1 surrounding caveolae is illustrated with **dark green strips**, dynamin is depicted as a **blue ring** around the neck of budding vesicles and an apoptotic cell or bacteria is shown in **pink**. Some forms of raft-mediated endocytosis require dynamin, while others do not **(E)**. (Adapted and modified from – Nichols, B. *et al.*, *JCS*, 2003, Modified figure provided as a courtesy of Dr. Chris Harder)


Rho GTPase family dependent endocytosis
Pinocytosis and
Macropinocytosis
Dynamin-independent
Caveolae-mediated
endocytosis
Dynamin-dependent
B
A
C
D
E
F
Phagocytosis
Dynamin-independent
Raft-mediated endocytosis
Caveolae and Clathrin independent
Possibly Dynamin-independent
Clathrin-mediated endocytosis
Dynamin-dependent

Finally, there appears to be a distinct class of endocytosis that involves membrane rafts [112] (Fig. 1.7F). Recently, it was found that endocytosis of various endogenous plasma membrane molecules occurs in the clathrin independent endocytic pathway [109;113;114]. Most of these molecules are found in non-caveolar lipid rafts suggesting that at least some clathrin- and caveolae-independent endocytosis might be raft-mediated. These rafts can also be endocytosed by other characterized means of endocytosis as well, and are usually classified by common indicators of endocytosis. However, some have been defined that are clathrin, caveolae, and dynamin-independent [115]. Flotillin -1 and decay accelerating factor (DAF) were recently identified as a non-caveolar, non-clathrin raft markers [115;116].

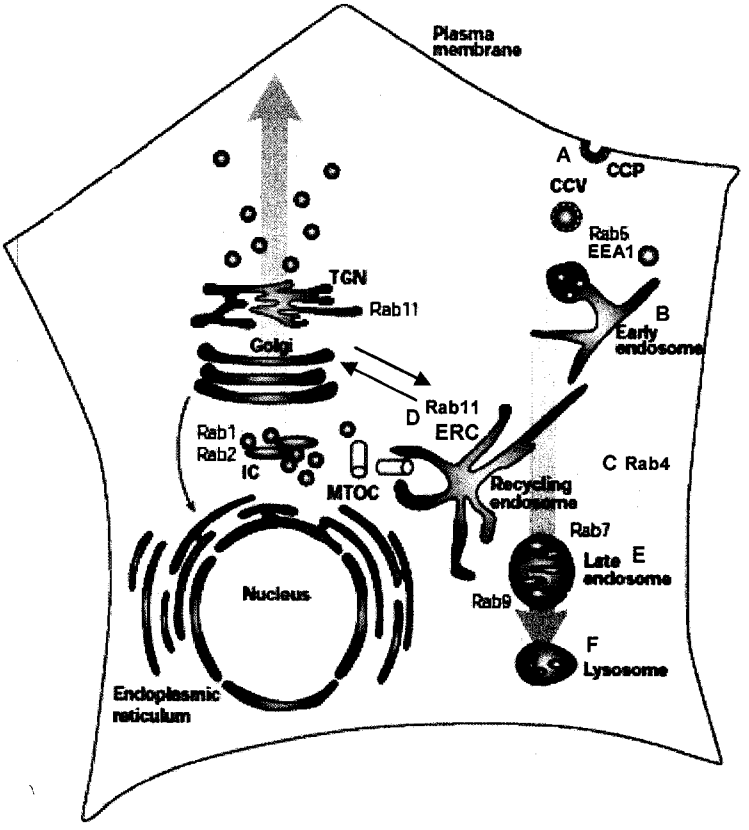
1.4.3 – The classical endocytic pathway

Following the endocytosis of extracellular cargo and its associated plasma membrane, an elaborate network of endosomes ensures the proper processing and delivery of the cargo to its correct intracellular destination.

Although not all endocytosed cargo travels to the same location, the majority ends up in an early sorting compartment termed the early endosome. This compartment, marked by the Rab GTPase (Rab5) or the early endosome antigen-1 (EEA1) sorts the cargo for its appropriate destination (Fig. 1.8b) [117-119]. There is also a substantial amount of heterogeneity in these early compartments, as not all cargo goes into the same early endosomes [120]. Acquiring these proteins (Rab5 and its effectors) is critical for the correct processing of these endocytic entities. Despite the critical sorting function of the early endosome, some cargo bypasses this compartment and either undergoes a rapid recycling back to the plasma membrane or self-matures into a lysosomal-like structure. The former process is often associated with membrane turnover for the purpose of cellular migration and involves membranes being moved from the receding side

Figure 1.8

The classical endocytic pathway. A graphic representation of the classic routes of intracellular trafficking. Most methods of endocytosis **(A)** deliver their contents to an early endosome **(B)**. These include macropinocytosis, raft-mediated and clathrin-mediated endocytosis (a clathrin-coated pit is shown (CCP)). The early endosome has a pH of 6 which helps to separate the ligands from their receptors. Receptors are either rapidly recycled from the early endosome via Rab4-mediated pathway **(C)** or slowly recycled through the ERC **(D)**. As receptors recycle, early endosomes mature into late endosomes **(E)** this process requires the recruitment of hydrolytic enzymes and vacuolar ATPases that degrade proteins and acidify the endosome respectively. Through these recruitments, late endosomes become multivesicular bodies and lysosomes **(F)**. Some cargo can cycle between the ERC and the TGN **(green arrows)** as well as from late endosome or lysosomes to TGN. (Adapted from Zerial and Mcbride, *Nature Reviews Molecular Cell Biology*, 2001)


Plasma membrane
A
CCP
CCV
Rab5
EEA1
B
Early endosome
TGN
Rab11
Golgi
D
Rab11
ERC
Rab1
Rab2
IC
MTOC
C Rab4
Recycling endosome
Rab7
Late endosome
E
Rab9
F
Lysosome
Nucleus
Endoplasmic reticulum

to the advancing side of the cell [121;122]. Cargo sorting in the early endosome is facilitated by changes in pH (achieved by the recruitment of vacuolar ATPases), the creation of local areas of phosphoinositide enrichment or membrane rafts and by tubulation of the early endosome. The low endosomal pH encourages the dissociation of a number of cargos from their receptors while the local concentrations of phosphoinositides in conjunction with the endosome tubulation segregate receptors for their transport to a different cellular locale (often back to the plasma membrane). Different regions of the early endosomes often contain regulatory Rab GTPases that facilitate this sorting [123-125]. For instance, the tubules involved in the direct recycling of receptors back to the plasma membrane are enriched in Rab4 (Fig. 1.8c) [124;126], while other tubules that direct cargo towards a perinuclear ERC are enriched in Rab11 (Fig. 1.8d) [124]. The ERC consists of many cholesterol-rich endosomes [127] and participates in the uptake, sorting, and processing of nutrients, metabolites, hormones, and growth factors. The ERC can also act as an intracellular source of receptors and cholesterol that can be mobilized to the plasma membrane to help maintain the characteristic distribution of the plasma membrane's functional domains [128]. A number of studies have demonstrated that certain molecules can travel back and forth from the ERC and the Golgi (Fig 1.8 – green arrows). As such, the ERC and the trans-Golgi network (TGN) may be involved in general recycling or exocytosis of proteins.

Together, both the rapid (Rab4, (Fig. 1.8c)) and slow (Rab11, (Fig. 1.8d)) recycling pathways continually recycle cargo from the early endosome back to the plasma membrane. At some point, the ligands separated from their receptors by the decreased pH in the early endosomes need to be transported to the late endosomes. Recently, M. Zerial and colleagues demonstrated that early endosomes mature into late endosomes by losing Rab5 and gaining the late endosomal Rab GTPase, Rab7 (Fig. 1.8e) [129]. This maturation of early endosomes to late endosomes requires the recruitment of hydrolytic enzymes and vacuolar ATPases that degrade

proteins and acidify the endosome respectively. Through these recruitments, late endosomes become multivesicular bodies and lysosomes (Fig 1.8f).

Finally, transportation between late endosomes and the TGN is mediated by Rab9. This process is critical for the delivery of hydrolytic enzymes and glycosphingolipids to and from late endosomes [130] and may also represent a recycling route for some cargo [131].

1.4.4 – Intracellular trafficking of HDL derived CE

The route of intracellular trafficking and site of hydrolysis of CE acquired from HDL by the process of selective uptake remains poorly understood. HDL-derived CE takes a distinctly different route of trafficking than that derived from LDL as LDL-CE are targeted to lysosomes for hydrolysis while selective uptake-mediated CE is eventually stored in lipid droplets for storage.

More specifically, in LDL uptake, LDL is recognized by the LDLr, upon which LDLr and CE complex is internalized and transported to sorting endosomes and CE is directed to late endosomes and lysosomes where it is hydrolyzed. Following hydrolysis, free cholesterol is effluxed to cellular compartments including plasma membrane and ER, while LDLr recycles to the plasma membrane via the endocytic recycling compartment. On the other hand, HDL-CE acquired through the selective uptake pathway takes a slightly different route that is thought to involve temperature independent transfer of CE to the plasma membrane, a rapid transfer of lipid followed by lipid accumulation in a perinuclear region (thought to be the Golgi/membrane sorting compartment) and in lipid droplets of the cells [132]. A protein-mediated event may be required for CE internalization from the plasma membrane as N-ethylmaleimide has been shown to block the internalization phase of the selective uptake process [132]. Also it is suggested that hydrophobic CE probably flows through the cell by association with vesicles or intracellular

membrane sheets, rather than a cytosolic pathway [132]. Whether CETP plays a role in internalization and intracellular trafficking of HDL-derived CE is not yet known.

1.5 – Rationale for study

HDL mediates the transport of cholesterol from peripheral tissues to the liver for excretion in bile in reverse cholesterol transport. However, the exact mechanisms mediating the uptake of HDL by hepatocytes and other cells are poorly understood. Increasing evidences suggest CETP as one potential candidate for mediating this hepatic HDL-CE uptake. While the role of CETP on plasma lipoprotein remodeling has been extensively studied, little is known about the function of membrane-bound CETP on HDL uptake or its role on intracellular trafficking of cholesterol. Studies carried out in this report attempted to dissect the molecular mechanisms by which CETP promotes this HDL-CE uptake as well as the effects of CETP on intracellular cholesterol trafficking, which is clearly fundamental to the design of effective HDL-modifying strategies.

CETP is poorly secreted but is shown to be present on the plasma membrane of adipocytes and preadipocytes (unpublished data). Since, CETP does not have a transmembrane domain, it is speculated to be associated with the membrane by hydrophobic or ionic interactions. However, it is not clear whether CETP localizes to specific microdomains on the membrane and whether this localization of CETP has important functional implications. SR-BI, an important HDL receptor, has been shown to exist in lipid raft domains and it has been suggested that this may have specific implications to its function. Furthermore, given that lipid rafts are implicated as having functions of endocytic portals, we speculate that CETP may be associated with lipid rafts in order to mediate HDL endocytosis. Indeed, endocytosis of oxidized LDL through scavenger receptor CD36 was shown to utilize a lipid raft pathway that

does not require caveolin-1 [116]. Additionally, it is possible that CETP itself may create a unique lipid environment since it may alter the cholesterol composition in the plasma membrane by its lipid transfer action.

Strong evidence also suggests an intracellular role of CETP in cellular cholesterol homeostasis. Studies have shown a strong link between CETP and cellular cholesterol homeostasis as CETP biosynthesis in SW872 cells was shown to be directly correlated to cellular lipid status [133]. A recent study carried out by the same group also suggests that CETP may have an intracellular role in adipocyte lipid metabolism and storage as chronic CETP deficiency was shown to disrupt lipid homeostasis and compromises the TG storage function in adipocytes [134].

Based on the previous findings, we hypothesize that CETP localizes to the specialized microdomains or lipid rafts on the plasma membrane of cells. We also hypothesize that these surface-bound or cell-associated CETP can function as a HDL receptor similar to the role of SR-BI and mediate the uptake of HDL by accommodating a direct interaction and docking HDL onto the cell surface. We also propose that CETP plays a role in internalization and intracellular trafficking of HDL-derived CE. We hypothesize that CETP mediates this HDL internalization via non-clathrin, non-caveolar raft-mediated endocytic route and this internalization may be followed by the recycling of the HDL whole particle. The results obtained from this study will hopefully expand upon the previous findings to clarify the cellular function of CETP with specific regards to HDL-CE selective uptake.

2 – Materials and Methods

2.1 – Materials

Cell culture medium and reagents were purchased from Life Technologies and cell culture plasticware was purchased from Falcon. Chemical reagents were acquired from Fisher and Sigma-Aldrich chemicals. Iodobeads, BCA reagents and Super Signal chemiluminescence reagents were purchased from Pierce. Horseradish peroxidase conjugated secondary antibodies, ^{125}I, and [^{3}H] cholesteryl oleate as well as Cy3 and Cy5 protein labeling kits were purchased from Amersham Biosciences. Anti-CETP (TP2) antibody was kindly provided by Dr. Yves Marcel and Dr. Ross Milne (University of Ottawa Heart Institute, Ottawa, Ontario). Monoclonal and polyclonal anti-myc and monoclonal anti-EEA1 antibody were obtained from Abcam. Anti-caveolin1 antibodies were obtained from BD biosciences. Cyclohexamide was obtained from Sigma Aldrich. BODIPY-CE (cholesteryl 4,4-difluoror-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoate), Alexa 488, Alexa 594 and Alexa 647 were obtained from Molecular Probes. Lipofectamine, Lipofectamine 2000 reagents and Taq DNA polymerase were purchased from Invitrogen. Partially purified recombinant CETP (rCETP) was purchased from Cardiovascular Targets, Inc (New York, NY). DakoCytomation fluorescent mounting medium was purchased from DakoCytomation (Glostrup, Denmark). Primers were purchased from Sigma-Genosys. Restriction enzymes were purchased from New England Biolabs. pCMV-3tag2B vector was purchased from Stratagene. Maxiprep and miniprep DNA isolation kits were purchased from Qiagen.

2.1 – Methods

Generation of adenoviruses

Previously generated and characterized CETP adenovirus (AdCETP) and the luciferase adenovirus (AdLuc) were used in this study [135]. Briefly, human CETP, in the pCMV5 mammalian expression vector, was subcloned into the pShuttle-CMV vector using standard molecular biology techniques; this vector was used for subsequent generation of the adenovirus DNA construct via homologous recombination with the pAd-Easy1 adenovirus DNA vector. For the luciferase control adenovirus, the luciferase cDNA was excised by restriction digest from the pCA13-luciferase construct (kindly provided by Dr. Marcel) and subcloned into the HindIII and XbaI sites of the pShuttle-CMV vector. The adenovirus was then generated and scaled up in 293 cells, purified by cesium-chloride density ultracentrifugation and measured by OD following the manufacturer's instructions (Qbiogene). Virus particles per ml are converted to plaque forming units per ml (PFU/ml). Viruses were added at 25 multiplication of infecticity (MOI) (approximately 6.25 X 10^6 PFU/well) and incubated for 24-48 hrs depending on the experiment.

Cloning of myc-tagged CETP

First, CETP signal sequence (Met1 to Gly10 of mature CETP peptide) was generated by dimerizing complementary oligonucleotides (CETPss Forward: 5' – ATGCTGGCTGCCACAGTCCTGACCCTGGCCCTGCTGGGCAATGCCCATGCCTGAT CCAAAGGCACCTCGCACGAGGCAGGC – 3' & CETPss Reverse: 5' – GGCCGCCTGCCTCGTGCGAGGTGCCTTTGGAGCAGGCATGGGCATTGCCCAGCA GGGCCAGGGTCAGGACTGTGGCAGCCAGCATGC – 3'). NotI and SacII restriction sites were designed into the 5' and 3' ends of the signal sequences, respectively. Annealed signal sequence was then sub-cloned into the multiple cloning site of a myc vector (pCMV-3tag2B) using restriction digest followed by ligation (NotI and SacII). Meanwhile, pCMV5-CETP was used as a template to generate the mature CETP sequence by PCR using the following primers:

Forward – 5' GCCAAGGATCCCCTGCTCCAAAGGC 3' & Reverse – 5' GCCAAAAGCTTCTAGCTCAAGCTCTGG 3'. These primers were designed to contain BamHI and HindIII restriction sites so that once the PCR product was generated, it could be cut with these restriction enzymes and sub-cloned into the multiple cloning site of the myc-vector. CETP PCR product was then cloned into the vector which now contains the sequence for myc-tag as well as the signal sequence. Resulting ligation product was used to transform Top10 cells via heat-shock method and grown in bacterial culture overnight to be purified by miniprep DNA isolation kit (Qiagen). The resulting plasmids were screened for positives using standard molecular biology methods and the selected positive clone containing all three construct (Myc tag, signal sequence and CETP) was further prepared and purified using Maxiprep DNA isolation kit (Qiagen) and sequenced to make sure no point mutations were introduced during the cloning.

Lipoprotein purification and labelling

All HDL was purified by density gradient ultracentrifugation using plasma from healthy normolipemic donors [136] and dialyzed against 4L of nitrogen-sparged PBS, pH7.4 with 2g of Chelex (Biorad). The purified HDL was labeled with ^{3}H-cholesteryl oleate using a modified protocol as described by Reaven [132]. Human apo-AI was kindly provided by Dr. D. Sparks (Univ. of Ottawa Heart Institute, Ottawa, Canada) and was labeled with ^{125}I as described [137]. The ^{125}I apoAI HDL was exchanged onto HDL for 36 h, followed by a 16 h 1.21 g/ml density spin at 60 000 g. The HDL was then dialyzed again 3 times in 4L of nitrogen-sparged PBS. HDL containing BODIPY-CE was made as described previously [132;138;139]. HDL was also labeled using Cy3 and Cy5 mono-reactive protein labeling kits. 1 mg of protein were labeled using the manufacturer's protocol and separated from free label on a 30 cm P10 column. Concentration was determined by BCA (bicinchoninic acid) protein assay using a BSA standard.

Cell culture

HeLa and COS-7 cell lines were cultured in basic medium (Dulbecco's modified Eagle's medium (DMEM); 10% FBS, 2 mM L-glutamine, penicillin (50 units/ml) and streptomycin (50 units/ml)) and grown at 37°C with 5% CO_2. COS-7 cells used for selective uptake were cultured in media containing 5 % FBS instead of 10% since serum may hinder selective uptake. Transient and stable transfections were performed using Lipofectamine and Lipofectamine 2000 according to manufacturer's protocol (Invitrogen, Canada)

Animals and primary murine hepatocyte isolation and culture

C57Bl6J (wild-type) and SR-BI deficient mice (strain B6.129S2-Srb1^{tm1Krh}) mice were purchased from Jackson laboratories and experiments were carried out on 4-6 months old mice. Mice do not express CETP making them an ideal model for these studies. The mice were maintained on a 12 h light/12 h dark schedule on a normal chow diet. Primary hepatocytes were prepared from mice according to established protocols [140;141]. Briefly, mice were sedated and livers perfused with collagenase solution. The cells were seeded on fibronectin coated (4ug/well) 6 well plates (for immunofluorescence studies) or 10 cm dishes (for plasma membrane isolation) to be confluent in William's medium containing penicillin (50 units/ml), streptomycin (50 units/ml), 1% antibiotic/antimycotic and 10% fetal bovine serum (Sigma). 4 hours following the initial plating, the cells were washed in William's medium without fetal bovine serum. For experiments using adenovirus-mediated expression of CETP, Ad-CETP or control Ad-Luc was added to the wells at a concentration of 6.25 X 10^6 PFU/well (25 MOI). The infected cells were left to express the transgene for 36 to 48 hrs prior to the experiments.

Selective uptake assay

COS-7 cells were plated in 24-well plates to be confluent on the day of the experiment. Cells were infected with 6.25x10^6 PFU/ml (25 MOI) of adenovirus construct containing CETP

or luciferase 2 days prior to the experiment. On the day of experiment, cells were washed twice at 37°C with 2 ml of ligand buffer (HBSS, 20mM HEPES, 5mg/ml BSA, pH 7.4) and then pretreated in ligand buffer for 30 min. Cells were then incubated at 37°C with 300 ul of ligand buffer containing either ^{3}H-CE-HDL or ^{125}I-ApoAI-HDL (50ug/ml) for 8 hrs. At the end of the incubation, the ligand buffer was removed and cells were washed 6 times on ice with 4°C HBSS. After the final wash, the remaining buffer was removed and the cells were solubilized with 500 μl of 0.2 N NaOH at room temperature with gentle shaking overnight. The protein content of 40 μl from each well was measured using a BSA standard and the BCA protein assay reagent according to the manufacturer's instructions (Pierce). The cell-associated [^{3}H] or [^{125}I] radioactivity in 400 μl of each cell lysate was measured by liquid scintillation counting using Ecolite (ICN, Costa Mesa, CA) or by gamma counting, respectively. The cell-association of radioactivity was measured in units of the amount of label in 1 ng (protein content) of HDL and corrected for levels of total cell protein (ng HDL/mg cell prt). Measured this way, an equivalent amount of 3H-CE and 125I-apoA-1 labels represents HDL holoparticle uptake and any additional 3H-CE cell association is attributed to selective uptake. Six replicates were done for each condition.

Plasma membrane isolation and sucrose gradient fractionation

Plasma membrane isolation and subcellular fractionation of COS-7 and primary mouse hepatocytes was adapted from previously described protocols [142;143]. Briefly, 10 X 10cm dishes were grown to sub-confluence (90% confluent) and either transfected with pCMV5CETP (COS-7) or infected with AdCETP (hepatocyte) to over-express CETP. Cells were left to express the transgene for 24-36 hours. Cells were washed with PBS containing protease inhibitor cocktail and collected in 3 ml ice cold HES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, protease inhibitor cocktail (1X), pH7.4) and were homogenized by 12 strokes

through a chilled ball bearing homogenizer. The homogenate was centrifuged in an SS34 rotor (16 000g, 20 min, 4 C). At this point, the supernatant contains the high and low density microsomes and the pellet contains the nuclei, mitochondra and the plasma membrane. Resulting pellet was suspended in 1 mM of HEP buffer (20 mM HEPES, 1 mM EDTA, 1X protease inhibitor cocktail, pH 7.4) and further homogenized by 20 strokes in the ball bearing homogenizer, diluted in 9 ml of HEP buffer, overlaid atop a 1 ml of 1.12 M sucrose (in HEP) cushion. The sample was centrifuged in SW41 rotor (100 000g, 1h, 4°C) in order to separate the plasma membrane from the nuclei and mitochondria. Plasma membrane band was collected and adjusted to 2 ml HEP buffer and centrifuged in TLA100.4 rotor (30 000g, 30 min, 4°C) to obtain pellet. Plasma membrane pellet was then incubated in 0.5 ml of TNE buffer (25 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, pH 7.5) containing 1% Triton-X 100 for 30 minutes on ice to allow for detergent extraction. The cell lysates were then mixed with 0.5 ml 80% sucrose in TNE buffer and corrected to 40% sucrose. A step sucrose gradient was formed by layering of 2.5 ml of 38% (wt/vol) sucrose in TNE and 1 ml of 5% (wt/vol) sucrose in TNE. After centrifugation of the samples (TLA100.4 rotor, 38 000 rpm, 3h, 4°C), 12 fractions of equal volumes were collected from top to bottom. Following the spin, the interface between the 5% and 38% sucrose layer should contain most of DRMs. The fractions were precipitated using trichloroacetic acid (TCA) according to an established protocol. Briefly, the fractions were diluted to 1 ml with PBS containing 10% TCA, vortexed and precipitated for several hours on ice. The precipitate was then pelleted in a cold microfuge (14000 rpm, 30 min, 4°C), supernatant was discarded and 500 ul of cold acetone was added subsequently. Samples were again pelleted and briefly air-dried before adding SDS PAGE loading buffer. The distribution patterns of CETP and cav-1 were analyzed by immunoblotting. Cav-1 was used as a marker for raft membranes.

Western blot analysis

TCA precipitated fractions were resuspended in 25 ul of denaturing/reducing loading buffer (10% SDS and 0.1% β-mercaptoethanol) and heated for 5 minutes at 95°C before being subjected to SDS-polyacrylamide gel electrophoresis (10% with 4% stacking gel) at 100V for 1.5 hours and transferred to a nitrocellulose membrane at 100V for 1 hour at 4°C. Blocking of the blot was done with 5% skim milk in PBS solution for 1 hour at room temperature and was followed by incubation with primary antibodies (monoclonal anti-CETP antibody (TP2, 1ug/ml) or polyclonal anti-caveolin-1 antibody (1ug/ml)) overnight at 4°C. The blot was washed in PBS (3 X 10 minutes) and incubated with 1:5000 dilution of secondary antibody (anti-mouse or anti-rabbit) conjugated to horseradish peroxidase. CETP bands were detected by chemiluminescent detection using the Super Signal solutions (Pierce).

HDL binding and internalization

For binding study, HeLa cells were plated on 12 well plates, transfected with pCMV5CETP (kindly provided by Dr. Ross Milne, University of Ottawa Heart Institute) one day prior to the experiment. Cells were treated with cyclohexamide (100 ug/ml) for 1 hour and cooled at 4°C for 1 hour. Media was then changed and cells were incubated with Cy3HDL (50ug/ml) for 1 hour at 4°C. Cells were carefully washed in PBS and prepared for immunofluorescence microscope imaging analysis. For internalization study, Cy3HDL as well as HDL labeled with Bodipy-CE in the core was used. Both HeLa cells and SR-BI null primary mouse hepatocytes were allowed to express CETP via transfection or adenoviral infection. Labeled HDL was then added to the cell in warm media and incubated for 30 minutes. Where indicated, exogenous rCETP (0.48 ug/ml) was added at the same time as HDL. For colocalization studies with different endocytic markers, HeLa cells were either co-transfected with CETP and an endocytic marker (plasmid containing Rab9 or Rab11 construct tagged with

green fluorescent protein (GFP)), or stained for the marker post fixing using specific antibodies (EEA1).

Pulse chase experiment with HDL-Bodipy-CE or Cy5 labelled HDL

HeLa cells were transfected with CETP and allowed to bind labeled HDL as described above. Following binding, media was changed to fresh media such that only HDL bound to the cell membrane remained. Cells were slowly warmed up to 37°C to allow for internalization. At indicated time points, cells were quickly washed 3 times in PBS and prepared for immunofluorescence imaging analysis.

Immunofluorescence

After treatments, cells were washed in PBS and fixed in 3.3% paraformaldehyde (PFA) for 15 min. The paraformaldehyde was removed and the cells were rinsed 3 times again with PBS and then quenched for 20 min with 50 mM NH_4Cl. The NH_4Cl was removed and the cells were permeabilized with 0.2% Triton-X 100 for 3 min. Triton-X 100 was removed; the cells were rinsed, and blocked for 20 min in 0.2 % gelatin/PBS blocking solution. Cells were incubated with the primary antibody (1ug/ml for both CETP and EEA1) in 0.2 % gelatin/PBS for 30 min. Following primary incubation, cells were washed 3 times with 0.2 % gelatin/PBS to remove non-specific binding. Cells were then incubated with the secondary fluorescent antibodies in 0.2 % gelatin/PBS for 30 min (Alexa488, Alexa594 and Alexa 647 were used). Following secondary incubation, cells were washed again with PBS, rinsed with distilled water and mounted with DakoCytomation fluorescent mounting medium (DakoCytomation).

Confocal microscopy

For confocal analyses, cells were seeded on 10 mm glass coverslips placed in 12 or 24 well plates. After the specified treatment, cells were visualized with an Olympus 100X oil immersion objective (numerical aperture 1.4) on an Olympus IX80 Laser scanning confocal

microscope operated by FV1000 software version 1.6a. The Bodipy, GFP and Alexa488 were excited with the 488 nm line of a multiple line argon ion laser, the Cy3 and Alexa594 were excited with the 543 nm line of helium/neon green laser and the Cy5 and Alexa 647 were excited with the 633 nm line of helium/neon red laser. For colocalization studies, sequential line scanning was done to prevent bleed through. Images were exported from the FV1000 software and processed in Adobe photoshop software.

Statistical analysis

Results are expressed as the mean ± SEM. Where indicated, the statistical significance of the differences between groups was determined using Student's *t* test using GraphPad InStat v.3.06 statistical analysis software (GraphPad Software Inc.).

3 – Results

3.1 – Localization of CETP on the plasma membrane

3.1.1 – Cell-associated CETP mediates selective uptake in COS-7 cells

In order to establish that CETP mediates selective uptake in other mammalian cell lines as well as primary hepatocytes, we performed selective uptake assay on COS-7 cells (African green monkey kidney cell line). The cell-association of radioactivity was measured in units proportional to the amount of these labels contained in 1 ng (protein content) of HDL and corrected for levels of total cell protein (ng HDL/ug cell protein). Measured this way, an equivalent amount of ^{3}H-CE and ^{125}I-apoAI labels represents HDL holoparticle uptake and any additional ^{3}H-CE cell association is attributed to selective uptake. In Fig. 2.1, we show that COS-7 cells over-expressing CETP exhibit approximately two-fold greater selective uptake than the control cells infected with Ad-luciferase (two-tailed p-value < 0.05, unpaired t-test).

3.1.2 – CETP localizes to the detergent resistant membrane (DRM) in the plasma membrane of COS-7 and primary murine hepatocytes

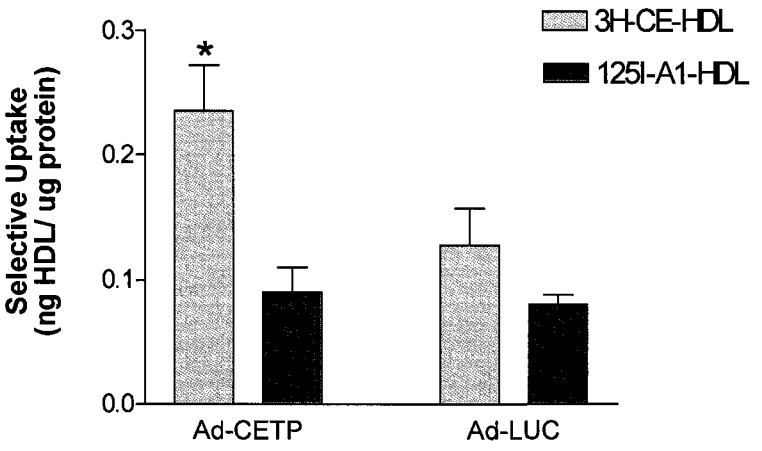
Using biochemical plasma membrane floatation followed by detergent extraction and sucrose density fractionation, we determined that CETP localizes in part to low density, detergent-resistant membrane fractions in both COS-7 cells (Fig. 2.2A) and primary murine hepatocytes (Fig. 2.2B). These data also confirm that there is a significant amount of CETP associated with the plasma membrane. Control cells infected with Ad-luc did not demonstrate CETP expression. In order to determine that the spin resulted in successful separation, the samples were also immunoblotted for caveolin-1, a marker protein for caveolae, which is a type of raft. The result shows that caveolin-1 is also found in the low density fractions, confirming

Figure 2.1 CETP mediates selective uptake in COS-7 cells.

A) **Selective uptake assay** was performed using COS-7 cells infected 2 days prior to experiment with 6.25 X 10^6 PFU/ml (25 MOI) of adenovirus construct containing CETP or luciferase (control). Cells were incubated with HDL labeled with either ^{3}H-CE or ^{125}I-apoAI (50 ug/ml). The cell-association of ^{3}H-CE or ^{125}I-apoAI is measured in units proportional to the amount of these labels contained in 1 ng (protein content) of HDL. Measured this way, an equivalent amount of ^{3}H-CE and ^{125}I-apoA-1 labels represents HDL holoparticle uptake and any additional ^{3}H-CE cell association is attributable to selective uptake. CETP infected cells exhibit approximately two-fold greater selective uptake than the control cells infected with luciferase. * two-tailed p-value < 0.05, unpaired t-test.

B) **Illustration of radioactively labeled HDL** – a) HDL with core labeled with ^{3}H-cholesteryl oleate (CE) and b) HDL with surface apolipoprotein A1 labeled with ^{125}I.

A)


3H-CE-HDL
125I-A1-HDL
Selective Uptake
(ng HDL/ ug protein)
0.3
0.2
0.1
0.0
*
Ad-CETP
Ad-LUC

B)

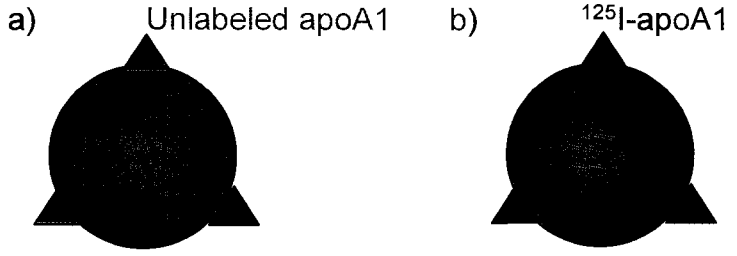
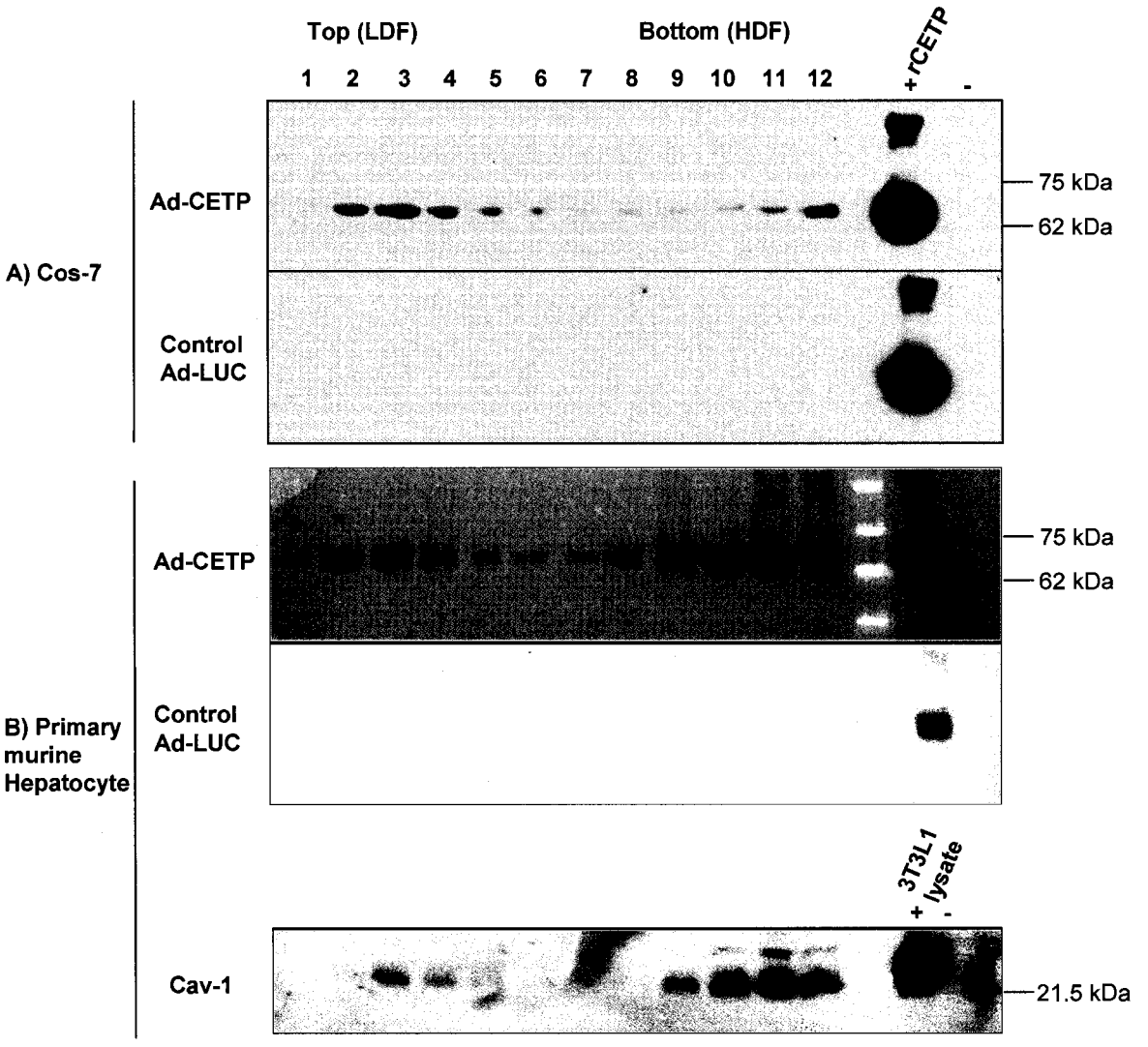

a)
Unlabeled apoA1
b)
125I-apoA1

Figure 2.2 CETP localization in the plasma membrane of COS-7 and primary murine hepatocytes.

Plasma membrane was isolated from ten confluent 10 cm dishes for each treatment (Ad-CETP and Ad-luciferase) by flotation gradient centrifugation. Isolated plasma membrane was solublized in TNE buffer containing 1% TritonX-100 and step sucrose gradients were centrifuged at 38,000 rpm for 3 h. Twelve fractions were collected from top to bottom and numbered accordingly as noted in the figure. Distributions of CETP and caveolin-1 were analyzed by immunoblotting. CETP is, in part, found in low density, detergent resistant membranes in both COS-7 (A) and primary murine hepatocytes (B). In primary hepatocytes, caveolin-1 was also present in low-density fractions indicating successful separation.


Top (LDF)
Bottom (HDF)
1 2 3 4 5 6 7 8 9 10 11 12
+ rCETP
-
A) Cos-7
Ad-CETP
Control Ad-LUC
75 kDa
62 kDa
B) Primary murine Hepatocyte
Ad-CETP
Control Ad-LUC
75 kDa
62 kDa
+ 3T3L1 lysate
-
Cav-1
21.5 kDa

that the spin had resulted in successful separation of membrane lipids and proteins according to their buoyant density.

3.1.3 – Serum availability affects CETP localization to lipid rafts

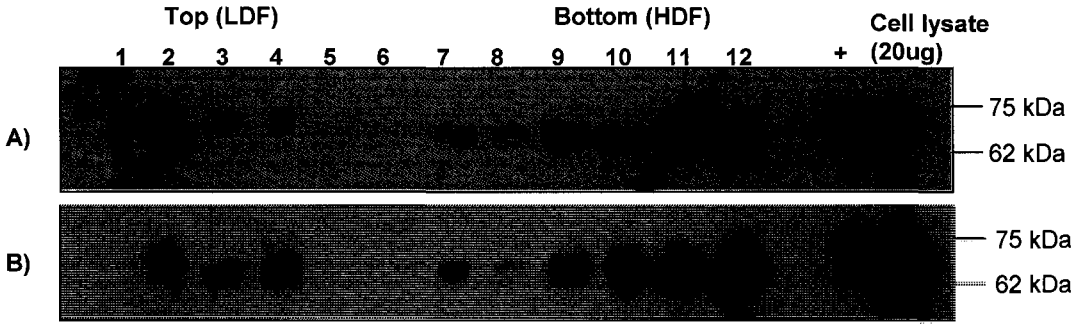
CETP is a lipid transfer protein and it is possible that CETP activity may alter the neighbouring lipid environment of the membrane. In order to determine whether serum availability alters CETP localization to lipid raft, membrane fractionation was carried out under two different conditions; cells were either kept in serum-deprived media for 24 hours prior to lysis or kept in normal media (10% serum) and were further treated with HDL for 1 hour prior to lysis. All experimental procedures were otherwise identical, including the exposure time for the blot. We found that less CETP is found in DRM when the cells are serum-deprived as compared to the HDL treated cells (Fig. 2.3). Densitometry results in (C) show the integrated density value (IDV) of CETP expression in each fraction as a percentage value of the whole plasma membrane CETP expression (sum of the IDV of all 12 fractions) in each blot. It is worth noting that in serum and HDL treated cells, a higher percentage of CETP expression is present in low density fractions (fractions 2, 3 and 4) as compared to the serum-starved cells.

Figure 2.3 Serum availability affects CETP localization to lipid rafts in the plasma membrane of COS-7 cells.

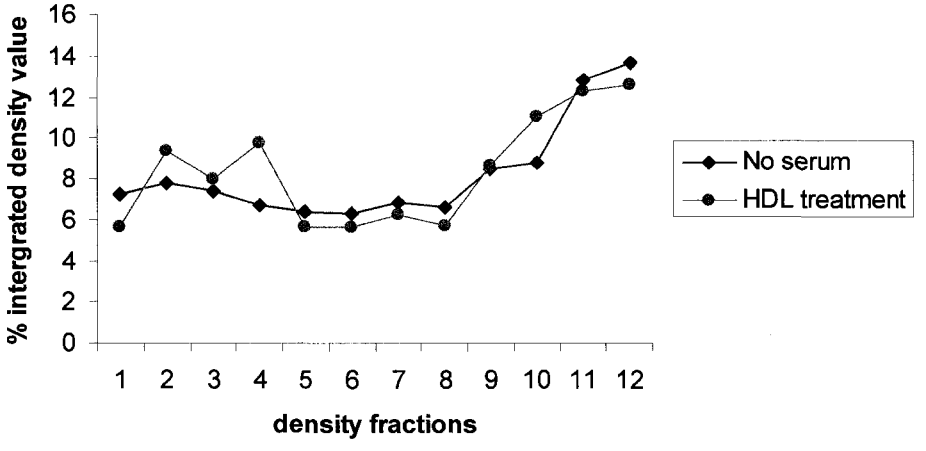
Plasma membrane was isolated from ten confluent 10 cm dishes for each treatment by flotation gradient centrifugation. Isolated plasma membrane was solublized in TNE buffer containing 1% TritonX-100 and step sucrose gradients were centrifuged at 38,000 rpm for 3 h. Twelve fractions were collected from top to bottom and numbered accordingly as noted in the figure. Distributions of CETP was analyzed by immunoblotting. CETP is found in low density, detergent resistant membranes in both (A) serum-starved and (B) HDL treated cells (50 ug/ml). Qualitative observation indicates that HDL-treated cells contain more CETP in both low density membrane fractions and whole plasma membrane as compared to serum-starved cells. (C) Densitometry of the CETP expression. Each fractions is expressed as a percentage value of the sum of the integrated density value of all 12 fractions. Low density fractions (fractions 2-4) in HDL treated cells contain a greater percentage of CETP (corrected to the total CETP expressed in the plasma membrane of each conditions) as compared to serum-starved cells.

A) Serum starved COS-7 cells

B) HDL treated COS-7 cells



C)



3.2 – Intracellular events following CETP mediated selective uptake of HDL-CE

3.2.1 – CETP mediates HDL binding to HeLa cell plasma membrane

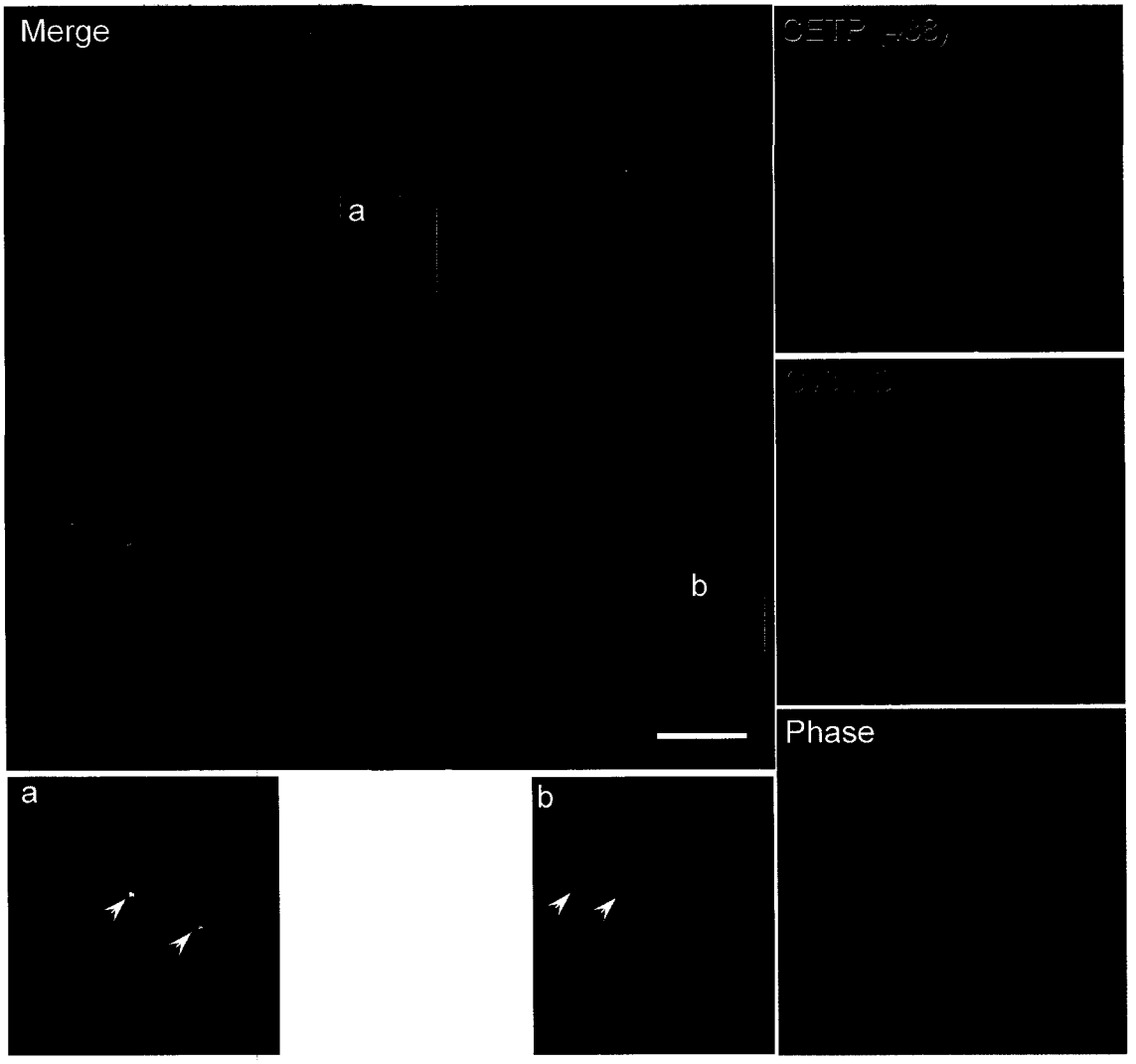
In the HDL binding study, we found that cells expressing CETP exhibit significantly more HDL binding to the cell surface as compared to the cells without CETP expression. This was evident by qualitative observation (Fig. 2.4 A and B). HDL binding is evident on the plasma membrane of cells with high CETP expression (Fig. 2.4 A) as well as ones with low CETP expression (Fig. 2.4 B). Importantly, some of the membrane bound HDL appears to colocalize perfectly with CETP (Fig. 2.4 A, B (a, b), arrowheads). In cells lacking CETP expression, there is noticeably less HDL bound to the plasma membrane (Fig. 2.4 C).

This qualitative observation is further confirmed by quantitation of the fluorescence intensity of the bound Cy3HDL in each condition. The average total fluorescence intensity of Cy3HDL was analyzed by the FV1000 software. Average total fluorescence intensity (FI) was measured in each image field and was divided by the number of cells in the field to obtain average FI/cell. 5 fields (containing 5 - 7 cells for transfected, 7-9 cells for non-transfected) were taken for each condition under identical laser power. Data obtained clearly shows that there was approximately three-fold more HDL bound to the cell surface of the CETP expressing cells (average FI/cell = 22.7) as compared to control cells that did not express CETP (average FI/cell = 7.85) (Fig. 2.4 D) ($p < 0.01$, student's t test).

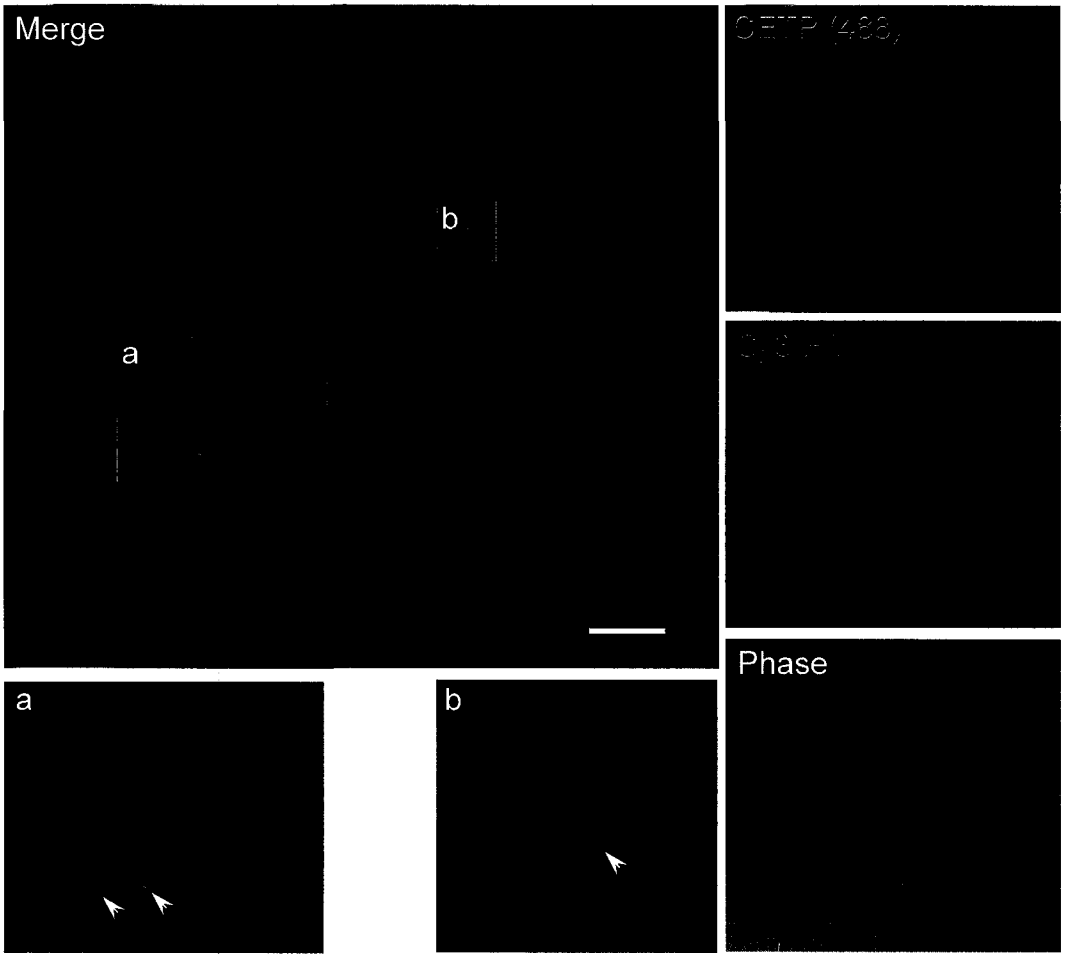
Figure 2.4 CETP mediates HDL binding on cell surface of HeLa cells.

HeLa cells were transiently transfected with hCETP and left to express the transgene for 24 hours prior to the experiment. Cells were pre-incubated with 100ug/ml cyclohexamide for 1 hour, changed to cold media and incubated at 4°C for 1 hour. Cells were then loaded with 50 ug/ml of Cy3HDL for 1 hour at 4°C to allow for HDL binding. Following PBS washes, cells were than fixed in 3.3% paraformaldehyde, quenched in 50 mM NH_4Cl and stained for CETP (TP2, Alexa488) prior to mounting. Images were obtained by confocal microscopy. (A) Cells overexpressing CETP (green) show evidence of HDL (red) binding on the plasma membrane. (a,b) Cy3HDL and CETP colocalization (yellow) is indicated by arrowheads. Some but not all HDL colocalizes with CETP on the cell surface. (B) Cells with lower CETP expression also mediate HDL binding. Colocalization between CETP and HDL is again evident (a,b) (C) Non-transfected cells without CETP expression exhibit an absence of HDL binding to cell surface. (D) When CETP was expressed in HeLa cells there was a three fold increase in the amount of HDL binding compared to control cells not expressing CETP. *two-tailed p-value < 0.01 (unpaired t test). Scale Bars = 10 μm

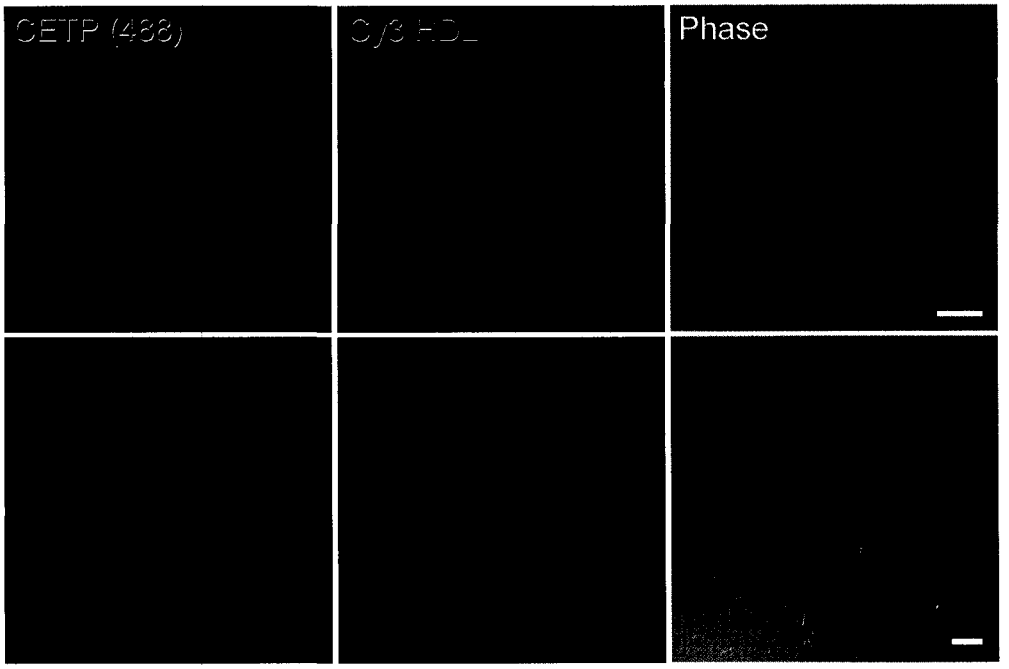
A) HeLa cells overexpressing CETP



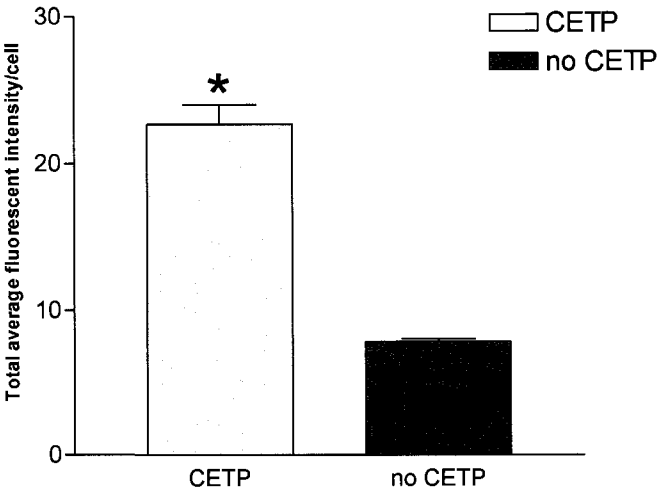
B) HeLa cells with low expression of CETP



C) Non-transfected HeLa cells



D) HDL binding is increased in HeLa cells expressing CETP



3.2.2 – CETP colocalizes with HDL derived CE on the cell surface and intracellularly

Using HDL labeled with Bodipy-CE in the core, we found that CETP colocalizes with Bodipy-CE on the cell surface as well as in intracellular compartments after a 30-minute incubation at 37°C.

In Fig. 2.5, panel (A), although some CETP/CE complexes are seen intracellularly, most of the CE and CETP colocalization is seen on the cell surface (arrowheads). As this cell exhibits less CETP expression as compared to other cells, we speculate that HDL internalization is delayed. It is also possible that the CETP bound to this cell may have originated from the secreted CETP into media.

In Fig. 2.5, panel (B), CETP and CE are shown to colocalize intracellularly indicating internalization of the CETP/CE complex. Bodipy-CE is seen to enter intracellular compartments that are punctate in appearance and scattered throughout the cell. These CETP/CE complexes seem to come together forming a larger irregularly shaped aggregate (arrowheads).

Additionally, it is worth noting that extensive cell surface presentation of CETP is observed (in box (i)). Accumulation of Bodipy-CE is also found in the perinuclear region. This pool of CE does not contain CETP and may represent lipid droplets.

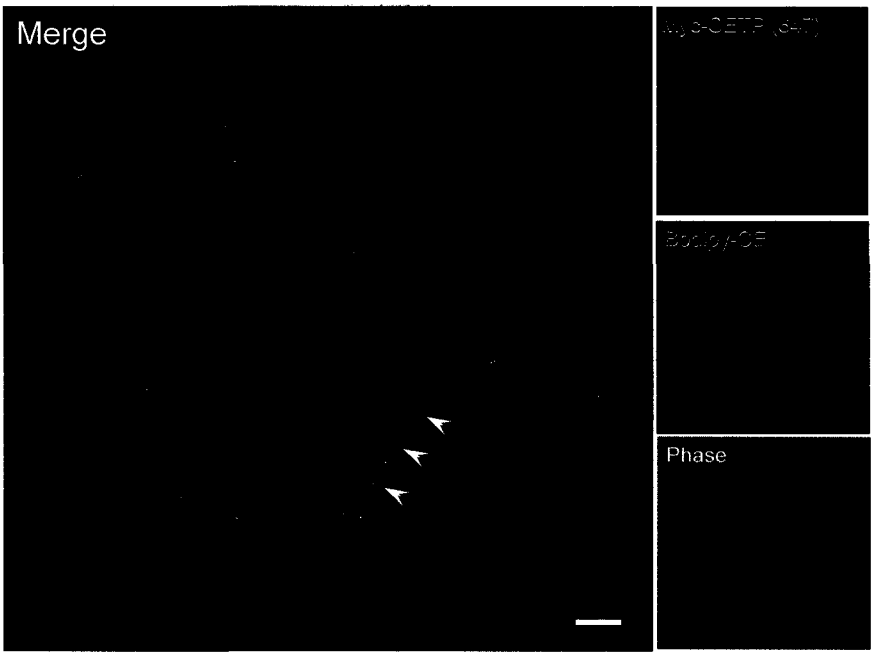
3.2.3 – CETP colocalizes with HDL and HDL derived CE in SR-BI null primary mouse hepatocytes

In primary mouse hepatocytes lacking SR-BI, a cell line more physiologically relevant in the context of this study, CETP is also shown to colocalize with HDL (Fig. 2.6) as well as CE derived from HDL (Fig. 2.7) after a 30 minute incubation. Both exogenous rCETP and adenovirally expressed CETP colocalize with HDL and HDL-CE in intracellular compartments. Some of this colocalization is perinuclear (shown by arrowheads). Most but not all internalized

Figure 2.5 CETP colocalizes with HDL derived CE in HeLa cells both on cell surface and in intracellular compartments.

HeLa cells were transiently transfected with myc-CETP (red) 24 hours prior to the experiment. Cells were loaded with HDL containing Bodipy-CE (50 ug/ml) for 30 min in 37°C, washed in PBS, fixed in 3.3 % PFA and quenched in 50 mM NH_4Cl. Cells were then stained for CETP (TP2, Alexa 647) prior to mounting. (A) Representative picture of a cell demonstrating binding and internalization of CE. CETP (red) colocalizes with CE (green) mostly on the cell surface. This cell has low transgene expression; CETP bound to the cell may have originated from CETP secreted into the media. (B) Representative picture of a cell demonstrating later stages of CETP/HDL-Bodipy-CE internalization. CETP and Bodipy-CE colocalize intracellularly indicating internalization of the CETP/HDL-CE complex. (i) Abundant CETP is found anchored to the cell surface. In both (A) and (B), CE accumulation in the perinuclear region is apparent. This pool of CE has apparently separated from CETP. Scale Bars = 10 μm

A) Cell surface binding and early endocytosis of HDL in CETP expressing HeLa cells



B) Internalization of CETP & Bodipy-CE

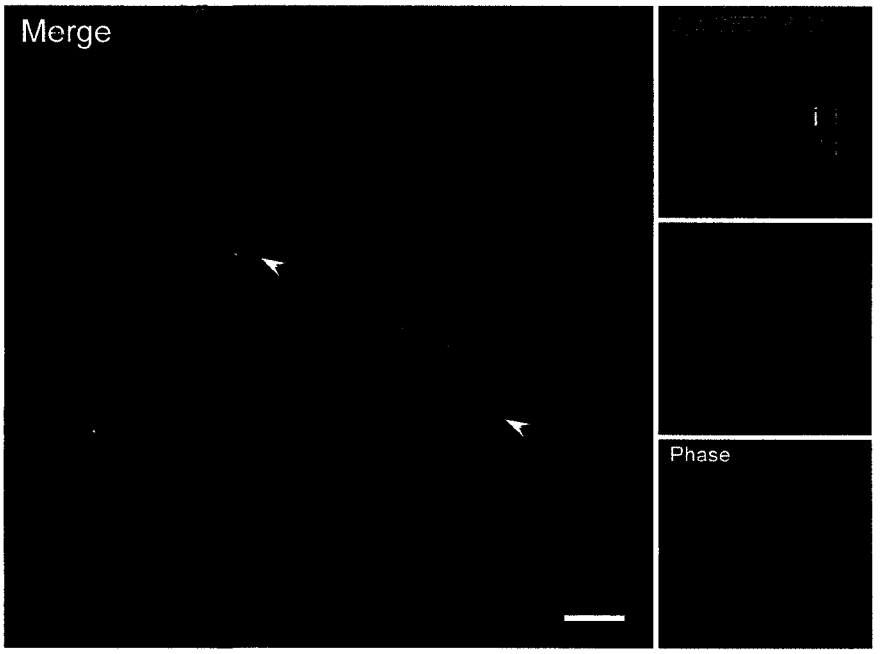


Figure 2.6 CETP colocalizes with HDL whole particle in SR-BI knockout mouse hepatocytes. Primary hepatocytes were isolated from a SR-BI KO mouse and seeded on glass coverslips. Cells were either (A) incubated with exogenous CETP (rCETP, 0.48 ug/ml) and Cy3HDL (50 ug/ml) for 30 min in 37°C or (B) infected with AdCETP (25MOI, 6.25 $X10^6$ PFU/ml) a day before experiment and then incubated with Cy3HDL for 30 min in 37°C. Cells were stained for CETP (TP2, Alexa488). With both exogenously added CETP and adenovirally expressed CETP, colocalization of CETP and HDL particles were observed in intracellular compartments (arrows). The identity of these compartments is unknown. Most but not all HDL colocalizes with CETP. Scale bars = 10 μm

CETP mediates HDL internalization in SR-BI deficient hepatocytes

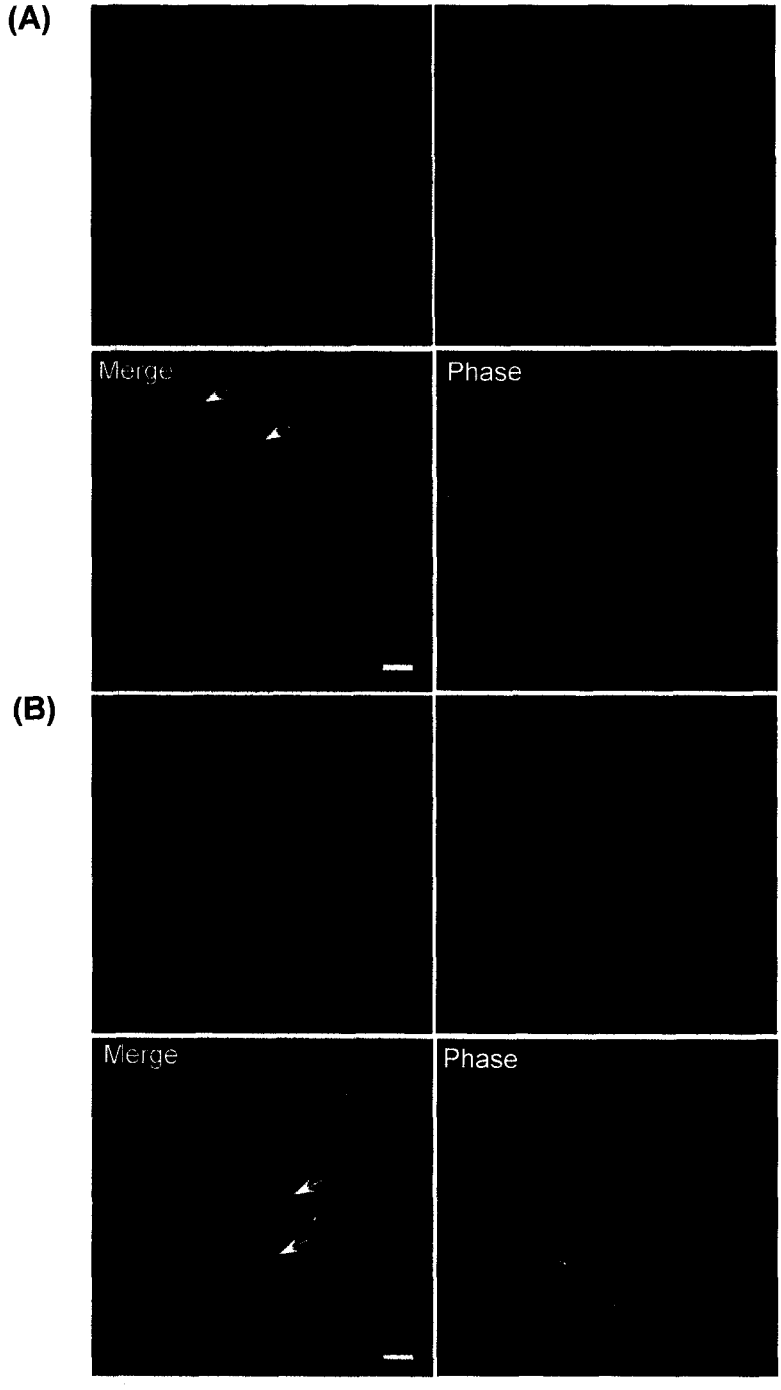
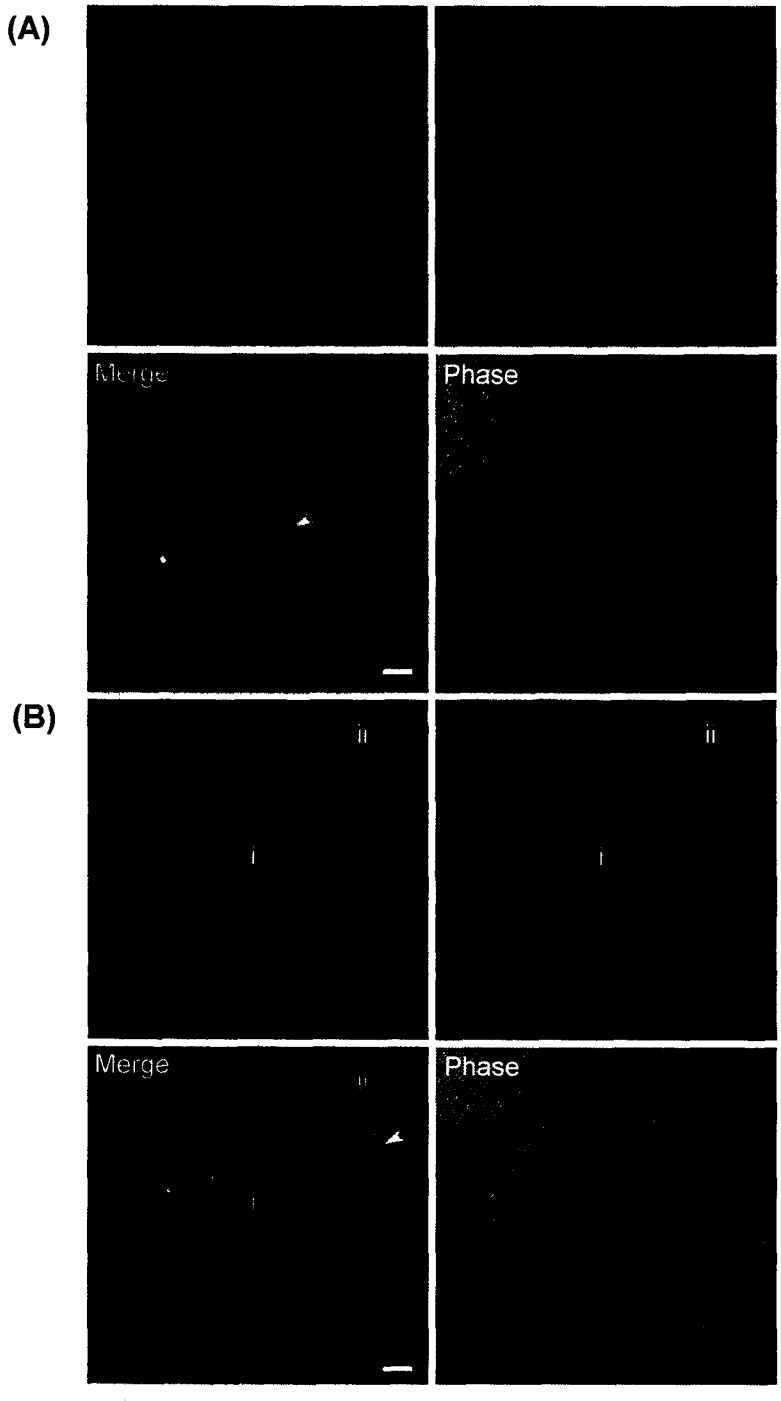


Figure 2.7 CETP colocalizes with HDL derived CE in SR-BI knockout mouse hepatocytes. Primary hepatocytes were isolated from a SR-BI deficient mouse and seeded on glass coverslips. Cells were incubated with either (A) exogenous CETP (recombinant CETP, 0.48 ug/ml) and HDL-Bodipy-CE (50 ug/ml) for 30 min in 37°C or (B) infected with AdCETP (25MOI, 6.25 X10^6 PFU/ml) 24 h prior to the experiment and then incubated with HDL-Bodipy-CE for 30 min in 37°C. Cells were stained for CETP (TP2, Alexa647) prior to mounting and visualized by confocal microscopy. With both exogenously added CETP and adenovirally expressed CETP, colocalization of CETP and HDL derived CE is evident (arrows). In (B), cells with extensive CE internalization also exhibit marked CETP and CE colocalization (cells with nucleus marked with i, ii). These cells demonstrate lower amounts of CETP in the cytosolic compartment as compared to neighbouring cells without intracellular CETP/Bodipy-CE association. Thus HDL-CE may recruit cytosolic CETP as part of an endocytic itinerary. Scale bars = 10 μm

CETP co-localizes with HDL-Bodipy-CE in SR-BI KO hepatocytes



HDL-CE is associated with CETP. However, cells that have extensive CE internalization also exhibit marked CETP and CE colocalization (Fig. 2.7). Interestingly, these cells have lower abundance of CETP expression in the cytosol (Fig. 2.7, cells with nucleus marked with i & ii) as compared to the neighbouring cells without noticeable CE internalization. It is possible that cellular CETP distribution is altered by HDL exposure.

3.2.4 – CETP and HDL are found in a subset of early endosomes at t = 10 min

Specific endocytic markers were used in order to identify the route of CETP-mediated HDL endocytosis. EEA1 is a known Rab5 effector and is an early endosomal marker. Here, we demonstrate that CETP and HDL complex is found in a subpopulation of early endosomes marked by EEA1 (Fig. 2.8). Although there is definite HDL and CETP association (Panel A, cyan, arrowheads), not all of the internalized HDL associates with CETP (Panel A, blue). CETP without HDL is also found in EEA1-positive compartments (Panel B, yellow, arrowheads). Overlapping of the three labels reveal that a portion of CETP and HDL indeed colocalize in EEA1-positive early endosomes (Panel C, white, arrowheads). Additionally, CETP/HDL complexes are found in a specific subset of EEA1 positive endosomes in close proximity to the nucleus.

3.2.5 – Perinuclear pool of CETP and HDL is identified as Rab11 positive, endocytic recycling compartment

In a pulse-chase experiment with Cy5- labeled HDL, we find that CETP mediates both binding and intracellular trafficking of HDL (Fig. 2.9). At 5 min, Cy5HDL is found mostly on the cell surface. Again, some of these HDL colocalize with CETP (box i) confirming the previous data. At 30 min, CETP and HDL are colocalized and scattered throughout the cell (box ii). The majority of this CETP/HDL complex is trafficked to the perinuclear region in HeLa cells at 1 hour (box iii). We also find that this perinuclear pool of CETP and HDL

Figure 2.8 CETP/HDL complex is found in EEA1 positive early endosomes in HeLa cells. HeLa cells were transiently transfected with myc-CETP and left to express the transgene for 24 hours. Cells were pre-incubated with 100 ug/ml cyclohexamide for 1 hour, changed to fresh media and incubated with Cy3HDL for 10 minutes. Cells were prepared for immunostaining by quick PBS washes, 15 min fixation in 3.3% paraformaldehyde followed by 20 min quenching in 50 mM NH_4Cl. Cells were then permeablized in 0.2% TritonX 100 and stained for CETP (polyclonal anti-myc, Alexa488), and EEA1 (monoclonal anti-EEA1, Alexa647). (A) Colocalization of HDL and CETP (cyan, arrowheads). (B) Colocalization of CETP and EEA1 (yellow, arrowheads). (C) HDL (blue), CETP (green) colocalize in EEA1 (red) positive structures, demonstrating that CETP mediates endocytosis of HDL into an early endosomal compartment. Colocalization of the three labels is indicated in white (arrowheads). Scale bars = 10 μm

Co-localization of CETP and HDL in EEA1 positive early endosomes (HeLa cells)

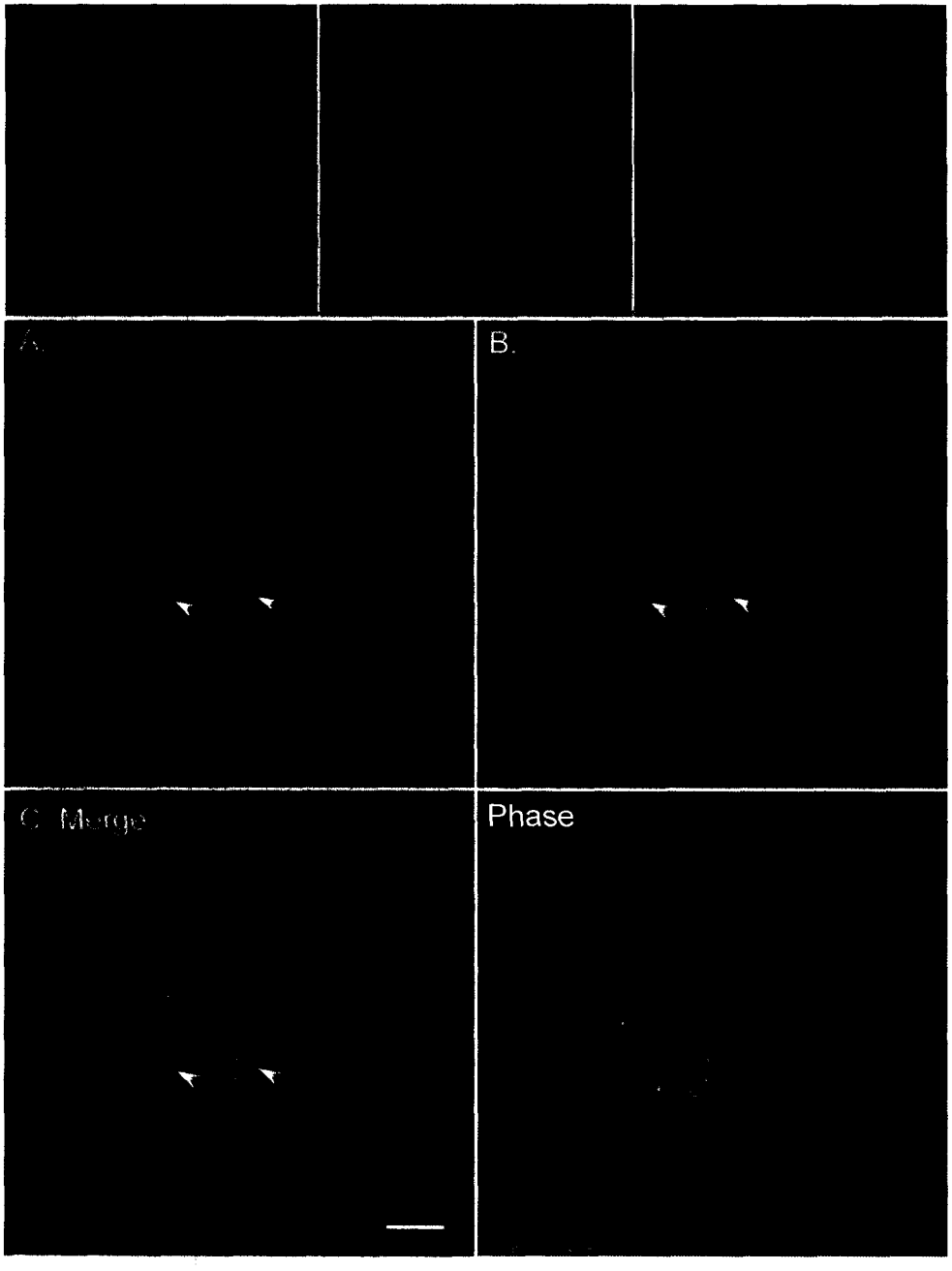


Figure 2.9 CETP and HDL complex are trafficked to perinuclear region in HeLa cells at 1 hour. HeLa cells were transiently transfected with CETP 24 hours prior. Cells were pre-treated with cyclohexamide (100 ug/ml) for 1 hour. Binding of Cy5HDL was carried out at 4°C for 1h prior to endocytic uptake at 37°C. Labeled HDL was then removed and cells were changed to fresh media. Cells were then washed, such that only lipoproteins bound to the plasma membrane remained. Cells were then warmed to 37°C to allow for internalization and were subsequently fixed with 3.3% paraformaldehyde at times 1, 30, or 60 min following internalization. Cells were then permeablized in 0.2% Triton-X 100, stained for CETP (TP2, Alexa488), and visualized by confocal microscopy. (A) At t = 1 min, HDL binding on the plasma membrane is evident. Some of the HDL (red) bound to the cell surface colocalizes with CETP (green) (arrowhead, box (i)). (B) At t = 30 min, HDL and CETP colocalization is evident both on cell surface as well as in intracellular compartments (arrowhead, box (ii)). (C) At t = 60 min, HDL and CETP colocalization is apparent in a perinuclear region, reminiscent of endocytic recycling compartment or trans-Golgi network (arrowhead, box (iii)). Scale bars = 10 μm

CETP and HDL traffic to a perinuclear compartment

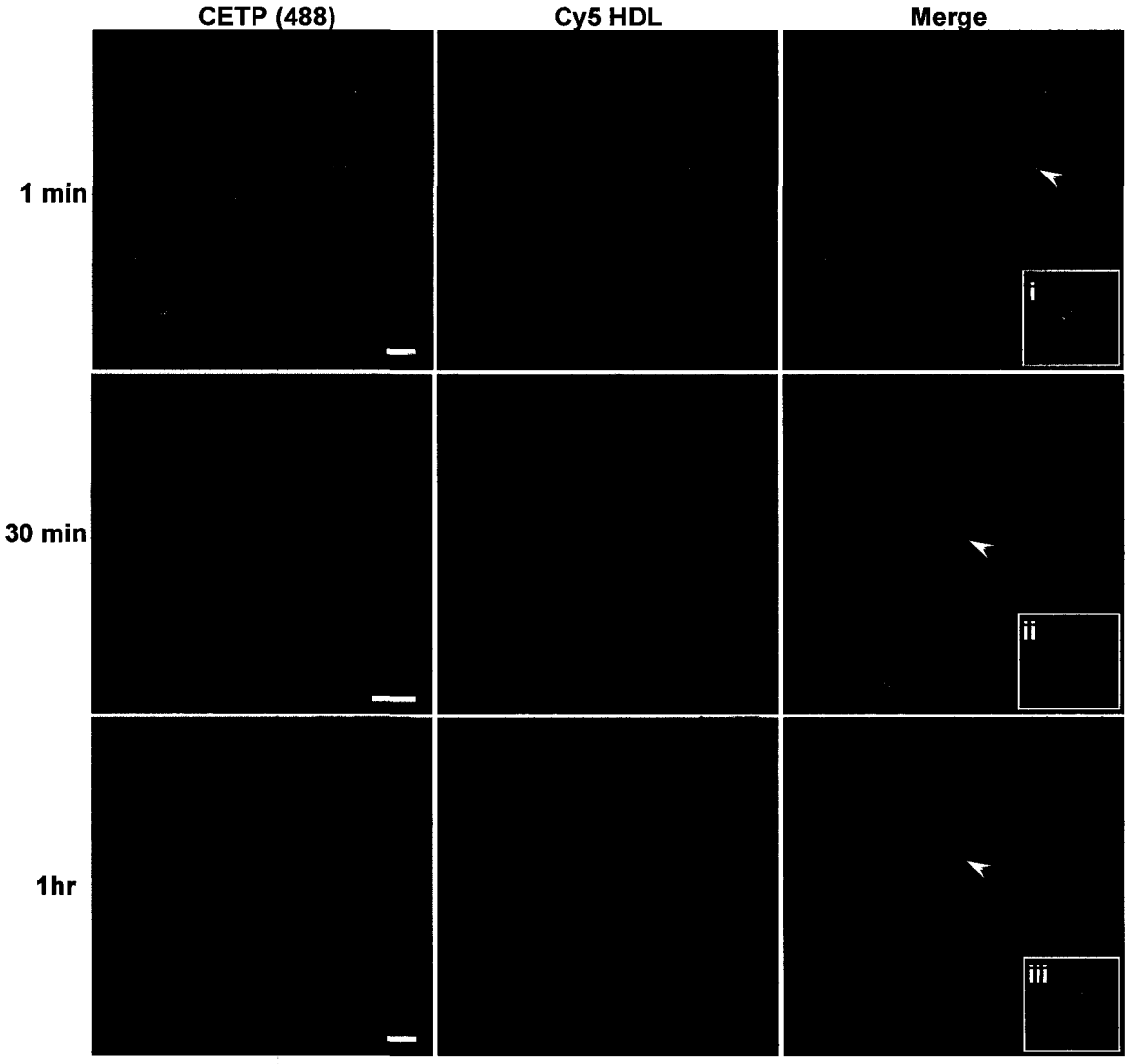
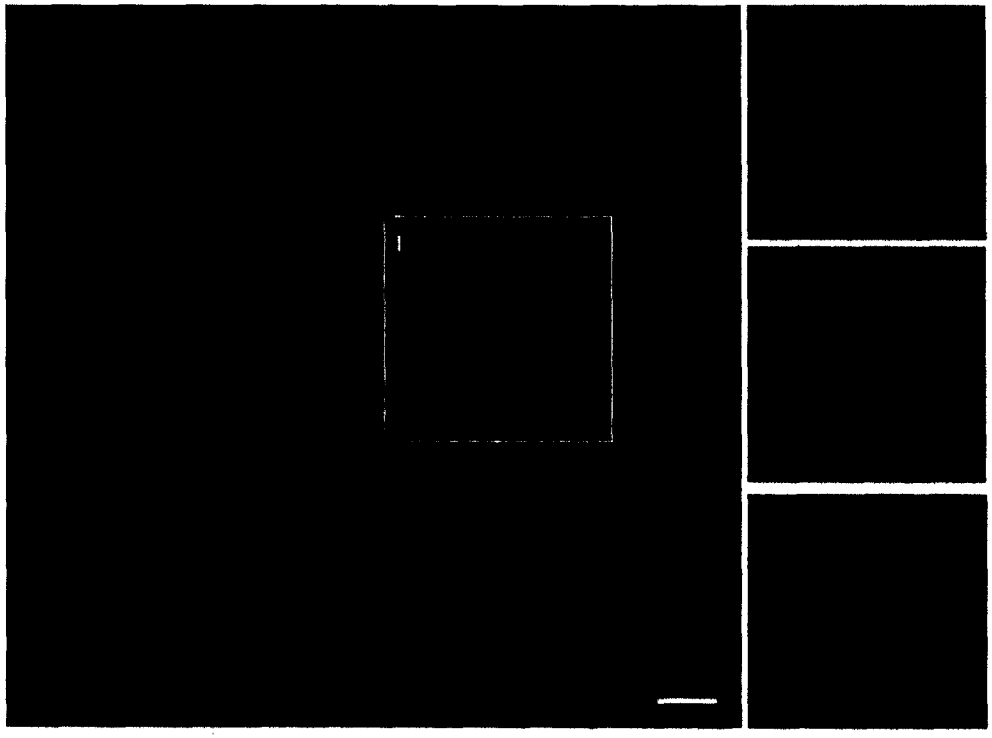


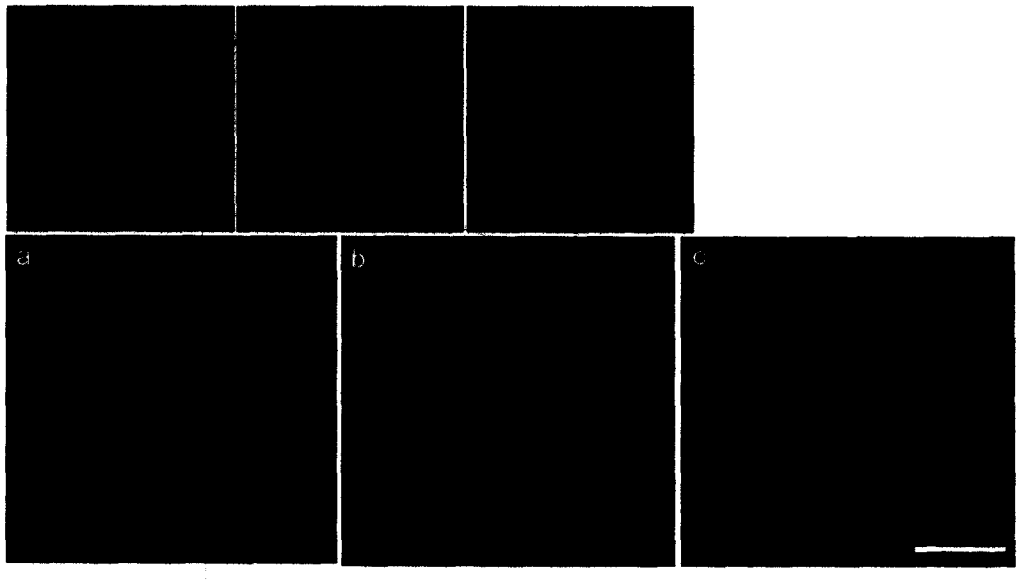
Figure 2.10 Perinuclear pool of CETP and Cy3HDL is identified as a Rab 11 positive endocytic recycling compartment. Hela cells were co-transfected with CETP and Rab11GFP constructs. Cells were pre-treated with 100 ug/ml cyclohexamide for 1 hour in 37˚C and changed to cold media for to allow for cooling for 1 hour. Cy3HDL was allowed to bind to cells in 4˚C for 1 hr. Labeled HDL was removed and cells were incubated with fresh media at 37˚C for 1 hr to allow for HDL internalization. Cells were prepared for immunostaining by quick PBS washes, 15 min fixation in 3.3 % PFA and quenched for 20 min in 50 mM NH_4Cl. Cells were then permeablized in 0.2 % TritonX 100 and stained for CETP (TP2, Alexa647). (A) HDL (blue) and CETP (red) colocalize in Rab11 (green) positive compartments. (B) A close up view of area (i) is shown. (a) CETP and Rab11 colocalizes in yellow. (b) Rab11 and HDL colocalizes in cyan. (c) All three labels colocalize in white. Scale bars = 10 μm

Co-localization of CETP and Cy3HDL in a Rab11 endocytic recycling compartment

(A)



(B)



complex colocalizes with Rab11, an established marker for the endocytic recycling compartment (ERC) (Fig. 2.10).

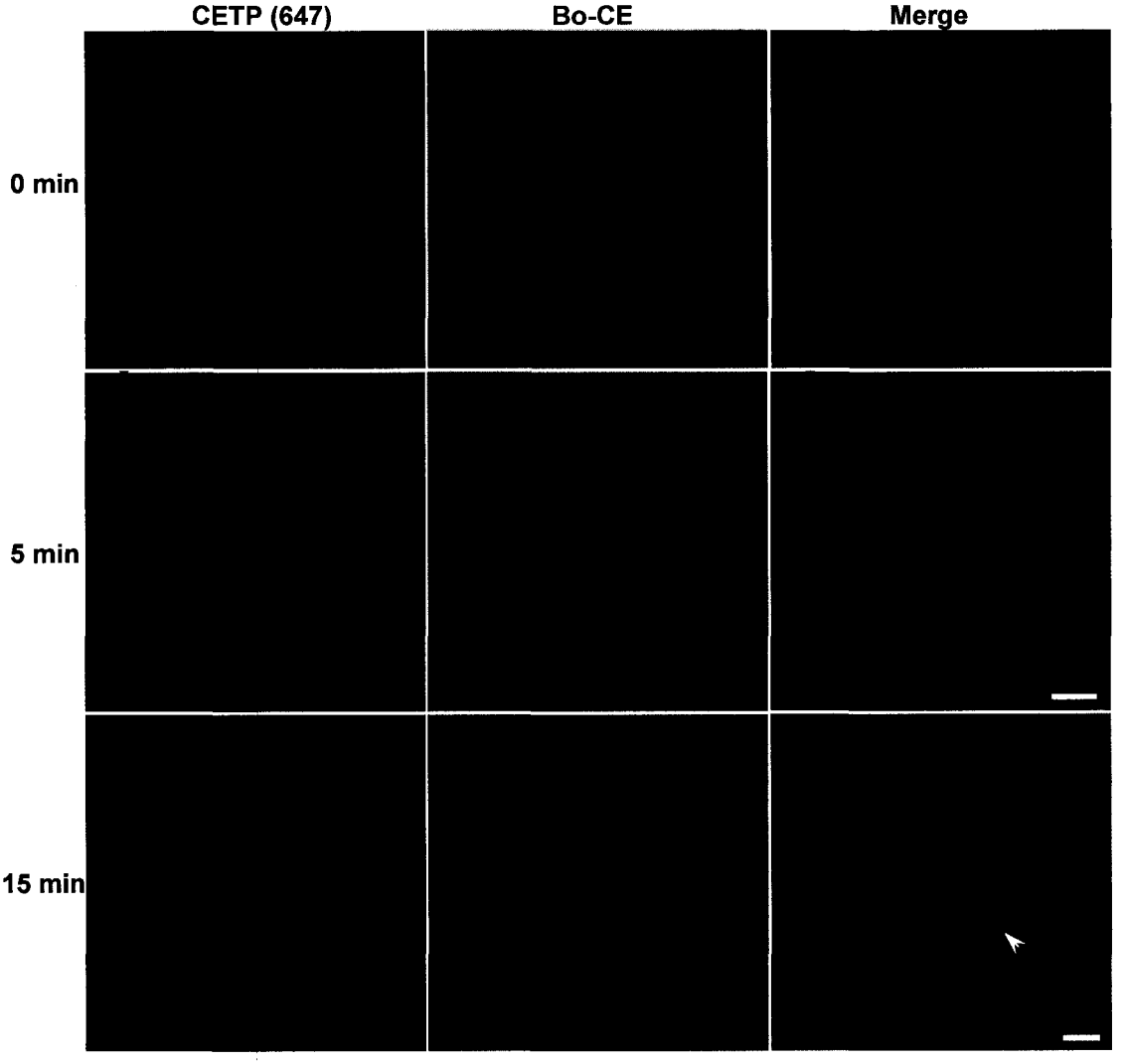
3.2.6 – Longer pulse-chase study reveals separation of CETP from HDL-derived CE

A longer pulse-chase experiment was carried out using HDL-core labeled with Bodipy-CE (Fig. 2.11) to determine the itinerary of CETP and CE following CE accumulation in lipid droplets. At t = 0, CE binding on the plasma membrane is again apparent and CETP and CE are shown to colocalize on the cell surface. At early time points (t = 5, 15 min), CETP and CE begin to show colocalization intracellularly as well as on cell surface. Note that not all internalized CE is colocalized with CETP (t = 15 min, arrowheads). At t = 30, 60 min, CETP and CE complex is mostly found colocalized in the perinuclear region. At t = 120 min, CETP is clearly separated from CE as colocalization is no longer evident (Green, arrowheads).

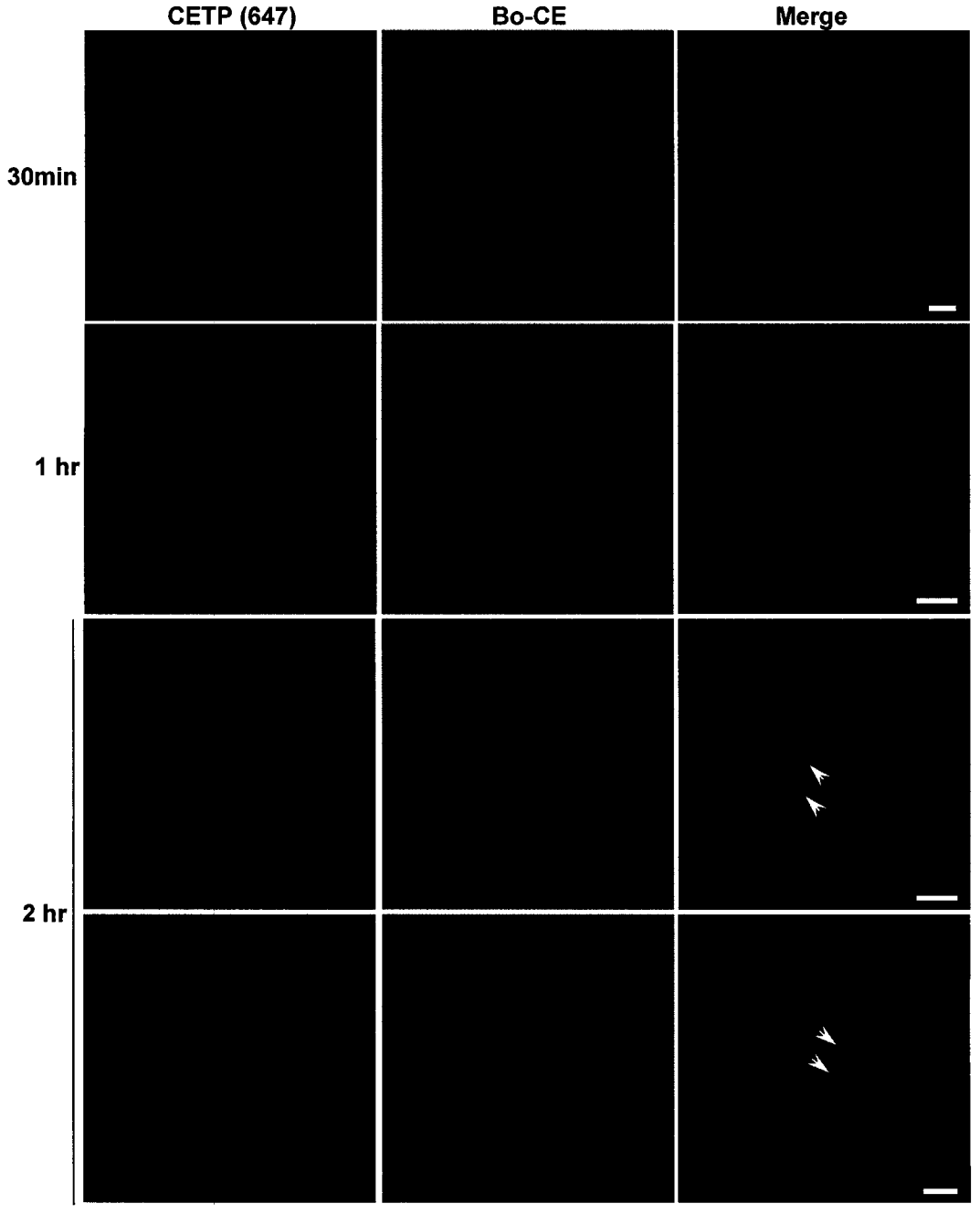
Figure 2.11 CETP dissociates from HDL derived CE at t = 120 min in longer pulse chase.

Hela cells were transfected with CETP 24 hours prior to the experiment. Binding of HDL-Bodipy-CE (green) was carried out at 4°C for 1h prior to endocytic uptake at 37°C. HDL was then removed and cells were washed and changed to fresh media and warmed to 37°C to allow for internalization. Cells were fixed with 3.3% paraformaldehyde at times 0, 5, 15, 30, 60 and 120 min following internalization. Cells were stained for CETP (TP2, Alexa647) and visualized by confocal microscopy. At t = 5, 15 min, most but not all Bodipy-CE (green) is associated with CETP (red). At t = 30, 60 min, CETP and Bodipy-CE colocalize in the perinuclear region. By t = 120 min, Bodipy-CE is present in distinct structures (possibly lipid droplets) which do not contain CETP (arrowheads). Scale bars = 10 μm

A) CETP and Bodipy-CE colocalize during early endocytosis



B) Dissociation of Bodipy-CE from CETP at 2 h



4 – Discussion

4.1 – Localization of CETP on the plasma membrane

As briefly mentioned in the Introduction, CETP has been shown to be associated with the plasma membrane of adipocytes and preadipocytes (unpublished data). Although it is plausible that CETP may associate with the plasma membrane by hydrophobic or ionic interactions, the mechanism of CETP localization on plasma membrane has not yet been characterized and it is not clear whether CETP localizes to a specific domain on the membrane and whether this has important functional implications.

We hypothesized that CETP may localize to raft domains since rafts are known to be platforms for various cellular processes including endocytosis. We have shown that CETP functions in a somewhat analogous fashion to the established HDL receptor, SR-BI. There has been much debate on the localization of SR-BI on the plasma membrane and evidence supporting its raft and non-raft localization has been presented. Currently, little is known about the method of SR-BI endocytosis. In several studies, SR-BI has been shown to be associated with raft domains and it has been suggested that this may have specific implications regarding its function specifically in HDL-CE selective uptake. In HepG2 cells, SR-BI and carboxyl ester lipase (an enzyme involved in hydrolysis of HDL-CE in liver) were found together in lipid rafts and this localization was thought to be important for efficient selective uptake. SR-BI was also noted by some investigators, but not others, to be associated with caveolae, one type of lipid raft [144]. However, this caveolar localization was not found to be a requirement for SR-BI-mediated selective uptake [145]. More recent work has determined that SR-BI is present in microdomains that lack caveolin-1 [146]. Using a mutant form of dynamin1 (dynK44A), Silver and colleagues concluded that SR-BI endocytosis is dynamin-independent [27] which further ruled out caveolar-

and clathrin-mediated endocytosis as a means of SR-BI entry as both are dynamin-dependent. Conflicting data from David Williams' group indicated that SR-BI does not associate with lipid rafts but rather associates with patches on microvillar extensions which they speculate to be a docking station for cholesterol trafficking from HDL [142]. They suspect that SR-BI is present in a more fluid membrane domain (L_d) that has a lower cholesterol content which, in theory, will favor rapid cholesterol flux [142]. However, it is possible that SR-BI may actually be associated with a different type of lipid raft and perhaps this alternate lipid raft functions as the vehicle for SR-BI endocytosis.

Here, we show that CETP is indeed found in low density, detergent resistant membranes (DRM) or lipid rafts in both COS-7 (Fig. 1.1) and primary murine hepatocytes[16]. CETP has been shown to mediate selective uptake in both cell lines. Since hepatocytes do not have abundant amounts of caveolae, it is possible that CETP is associated with non-caveolar raft. Additionally, we show that when cells are maintained in a serum-starved condition, CETP association with lipid rafts is still present but to a lesser extent as compared to cells provided with media supplemented with serum and HDL. We also observed that the addition of serum and HDL to the media increased CETP recruitment to lipid rafts as the percent integrated density value in the low density fractions was higher in the serum and HDL treated cells as compared to the serum-starved cells. A possible explanation for this could be that CETP binds to *de novo* rafts under serum-deprived conditions but also creates more rafts when provided with serum and HDL. Indeed, CETP is a lipid transfer protein and may transfer CE directly to the plasma membrane. This CE can then be hydrolyzed to free cholesterol and inserted into the membrane thereby altering the local lipid composition of the membrane and creating more raft domains. It is also possible that HDL binds to cells via known or unknown process and recruits CETP to the site of contact.

This localization of CETP in lipid rafts raises the possibility of CETP/raft-mediated involvement in HDL endocytosis as rafts are implicated in various endocytic processes. It is possible that CETP mediates HDL internalization through its association with lipid rafts. Indeed, we found that the action of cell-associated CETP may differ from that of plasma CETP as it does not seem to require lipid transfer activity. While circulating CETP functions as a shuttling molecule, cell-associated CETP may act as a docking protein and mediate the engulfment of the whole HDL particle. It is tempting to speculate that possible membrane remodeling by CETP may also promote its function in HDL uptake.

Several improvements in experimental design can be made to complement the data presented in this section. First of all, a non-raft marker such as transferrin receptor (Tfr) should also be tested on the membrane fractions in order to further confirm the validity of the density centrifugation. Theoretically, non-raft markers should only be found in the high density fractions. Secondly, in order to further investigate the effect of serum or HDL deprivation/addition on CETP-mediated raft formation, we will repeat the experiment with additional conditions involving varying amounts of serum and/or HDL in cells with or without CETP expression. We will also examine the effect of HDL addition on CETP-mediated raft formation in serum-deprived cells. We will then probe the membrane for caveolin-1 or other raft markers to determine whether serum and HDL deprivation prevented CETP-mediated raft formation or if it caused a generalized decrease in rafts.

4.2 – Intracellular events following CETP mediated selective uptake of HDL-CE

In an attempt to observe CETP and HDL interaction on the cell-surface as well as to dissect the intracellular events following the selective uptake of HDL-CE mediated by CETP, we incubated HeLa cells and SR-BI deficient primary mouse hepatocytes overexpressing CETP

with fluorescently labeled HDL and HDL containing labeled CE. HeLa cells were chosen for their ease of transfection and since they are an established cell line for intracellular trafficking studies. HeLa cells also do not express SR-BI making it an ideal cell model for this type of study.

First, in a HDL binding study, we demonstrate that CETP expressing cells effectively bind HDL on the cell surface while control cells that do not express CETP lack HDL binding (Fig. 2.4). We also note that some of the HDL bound to cell surface colocalizes with CETP, again supporting a role for CETP as a possible HDL ligand/receptor (Fig. 2.4 A, arrowheads). We have previously speculated that CETP may act as another HDL receptor on the membrane similar to the function of SR-BI but this is a first study to clearly demonstrate HDL anchoring on the plasma membrane mediated by CETP. We have previously shown evidence for CETP-mediated HDL-CE selective uptake by both exogenous and cell-associated CETP in hepatocytes [16]. Torcetrapib binds CETP to HDL and inhibits CETP mediated shuttling of CE between lipoproteins and cell membranes. Our previous findings demonstrated torcetrapib sensitive and insensitive portions of selective uptake and concluded that this torcetrapib insensitive portion of CETP mediated selective uptake may be due to the CETP that is associated with the plasma membrane [16]. We speculate that cell-associated CETP may mediate selective uptake via a different mechanism than circulating plasma CETP and hence this mechanism is not affected by torcetrapib which hinders its shuttling/lipid transfer ability. The data presented here clearly shows interaction between CETP and HDL on the plasma membrane and further strengthens this argument that a distinct plasma membrane-associated pool of CETP exists and is able to interact directly with HDL. As previously suggested, this HDL docking may be followed by a direct transfer of CE to the plasma membrane or partial fusion of the HDL bilayer with the plasma membrane. Additionally, the fact that CETP is found in raft domains also raises the possibility that a raft-mediated endocytic process may be involved in CETP-mediated selective

uptake. It will be important to determine whether cell-surface anchored CETP mediates internalization of HDL via one or more of these mechanisms. Various endocytic inhibitors will help to further unravel this process.

Other data also indicate an intracellular role of CETP in cellular cholesterol homeostasis. Other studies by Morton and colleagues have provided a strong link between CETP and cellular cholesterol homeostasis as CETP biosynthesis in SW872 cells was shown to be directly correlated to cellular lipid status [133] and CETP was suggested to have an intracellular role in adipocyte lipid metabolism and storage [134]. CETP has neutral lipid binding pockets that allow for CE binding as well as a C-terminal peptide with potential fusogenic properties which make it an ideal candidate for mediating cellular CE trafficking between vesicles. We suspect that vesicular transport may be involved in CETP-mediated lipid transfer.

Using HDL labeled with Bodipy-CE in the core, we demonstrate that CETP colocalizes with CE on the cell surface as well as in intracellular compartments (Fig. 2.5). Bodipy-CE is a useful tool in HDL selective uptake study since it is not hydrolyzed by neutral cholesterol esterases and can specifically identify CE that is internalized via a selective uptake pathway. The small amount of apo E containing HDL endocytosed via LDLr mediated endocytic pathways is routed through lysosomal compartments where the acidic pH actively hydrolyzes the probe [147]. CE derived from HDL selective uptake however, bypasses this by taking an extralysosomal route and is targeted straight to the lipid droplets without hydrolysis and is stored as CE [147]. We have illustrated two cells that appeared to be in different phases of HDL internalization. The first cell (Fig. 2.5 Panel A) seems to be in the early cycle of endocytosis as most of the CETP/HDL-CE complex is found on the cell surface. The second cell (Fig. 2.5, Panel B) seems to have progressed further in the HDL-CE internalization as we observe the CETP/HDL-CE complex mostly intracellularly in aggregates. It is also notable that HDL

treatment redistributes cellular CETP. Subsequent to HDL loading, CETP seems to be mobilized to HDL both on the cell surface (Panel A, arrowheads) and also intracellularly (Panel B, arrowheads). Furthermore, extensive cell-surface presentation of CETP was also evident (Fig. 2.5, Panel B, box i). It is possible that in response to increased HDL concentration in the media, CETP is recruited to the cell surface in order to facilitate the internalization. Significant CE accumulation in the perinuclear region is also observed which is seen typically in intracellular trafficking of HDL-CE derived from selective uptake [132]. This perinuclear pool of CE does not appear to associate heavily with CETP.

Cy3HDL and HDL-Bodipy-CE also colocalized with CETP in SR-BI-deficient primary mouse hepatocytes, a more physiologically relevant cell model (Fig. 2.6 and 2.7). After a 30-minute incubation (37°C) of CETP-expressing cells and cells provided with exogenous CETP and either Cy3HDL or HDL-Bodipy-CE, we found that the internalized Cy3HDL and Bodipy-CE colocalize with both exogenous and cell-associated CETP. We also observed that some of this colocalization was perinuclear and punctate in appearance. This staining pattern was similar for CETP/Cy3-HDL and CETP/HDL-Bodipy-CE indicating that the HDL whole particle and the core CE may initially be trafficked together in the presence of CETP. Additionally, CETP - expressing cells which had extensive CE internalization also exhibited marked CETP and CE colocalization (Fig. 2.7). These cells had less cytosolic CETP (Fig. 2.7, cells with nucleus marked with i, ii) as compared to the neighbouring cells without noticeable CE internalization. One explanation for this could be that CETP is mobilized to the cell surface in the presence of HDL and CETP and HDL then traffic together through distinct endocytic compartments.

In order to identify the compartments through which CETP/HDL/HDL-CE complexes traffic, we carried out a series of colocalization studies using a number of specific endosomal markers at different time points. First, we observed that CETP/HDL complexes

were found in EEA1 positive early endosomes at an early time point (t = 10 min) suggesting a possible raft-mediated endocytic route (Fig. 2.8). We also noted that the CETP/HDL complex colocalized with a subpopulation of early endosomes (Fig. 2.8 C). It has been suggested that the early endosomes may have heterogeneous subpopulations that direct the cargo in different paths. Thus, identification of this particular subset of early endosome may be crucial in mapping out the intracellular pathway that CETP/HDL/HDL-CE complex takes.

In a short pulse-chase experiment, we again observed marked colocalization between CETP and HDL. At 1h post treatment, labeled HDL and CETP were seen to separate from the early endosomes and segregate in perinuclear structures that are reminiscent of the endocytic recycling compartment or trans-Golgi network (Fig. 2.9, bottom panel). Both of these compartments have been identified to be rich in cholesterol[148] and involved in recycling of membrane lipids and proteins[149]. Indeed, colocalization studies identify these structures as Rab11 positive endocytic recycling compartments (Fig. 2.10). We suspect that CETP plays a role in directing HDL-CE to the ERC after which CE may be directed to lipid droplets for storage and HDL may be recycled back to the plasma membrane.

In a longer pulse-chase experiment with CETP and HDL-Bodipy-CE, we again demonstrated that CETP association with CE is evident from early time points (t = 5, 15 minutes). At t = 30 min and 1 hour, we observed the colocalization of CETP and CE in mainly perinuclear compartments which confirms our previous result. However, at 2 hrs post treatment, CETP was shown to separate from HDL derived CE. It is tempting to speculate that HDL-derived CE may have been directed to lipid droplets for storage. The identity of these compartments needs to be further explored using Nile Red staining to identify lipid droplets.

A number of novel findings reported here provide valuable insight into the current understanding of CETP function in a simple non-polarized cell model as well as primary mouse

hepatocytes. We present evidence that CETP indeed acts as a HDL receptor and also may have a role in intracellular trafficking of CE derived from HDL. First of all, these recent data support the argument that CETP expressed by hepatocytes may play a local role in reverse cholesterol transport, in addition to providing a source of plasma CETP. These results also suggest that the role of CETP as an HDL docking protein may be one mechanism for CE selective uptake and that this process may not require CETP-mediated neutral lipid transfer activity. Secondly, studies carried out as part of this thesis also highlight and strengthen an argument for an intracellular role for CETP in CE trafficking. CETP has hydrophobic pockets that are suitable for carrying lipid, and CETP may associate with HDL-derived CE intracellularly and transfer it to lipid droplets following HDL-CE selective uptake. Additionally, since adipocytes depend on lipoproteins to meet cholesterol requirements, cell-associated CETP may provide an important pathway for cholesterol accumulation during adipocyte development. Indeed, CETP mRNA is abundant in adipose tissue and is highly expressed in adipocytes during development but is down-regulated in response to adipocyte hyperplasia [150]. The recent findings on the involvement of CETP in adipocyte lipid metabolism and storage further support this hypothesis. Lastly, this study also supports the involvement of HDL endocytosis and recycling in CETP-mediated selective uptake. It has been suggested that SR-BI-mediated selective uptake process may, in part, involve HDL internalization and recycling. The finding that CETP is internalized with HDL and CE and co-localizes in both early endosomes and the ERC is evidence for the holoparticle uptake of HDL and recycling mediated by CETP. The subsequent trafficking itinerary of CETP and cholesterol-depleted HDL requires further exploration.

There are a number of limitations to the experimental design used in the above studies that needs to be addressed with additional experiments. Although many precautions such as choosing fluorophores that are at least 100 nm apart as well as sequential scanning were taken,

there is still a possibility of bleed-through, especially for very strong fluorophores such as Bodipy and GFP which can weaken the arguments made by the colocalization studies. A transferrin control experiment would help to confirm the colocalization of the CETP and HDL complex with each of EEA1 (early endosome) and Rab11 (recycling endosome) positive compartments. Transferrin is known to be internalized and recycled through the slow recycling endocytic pathway and can act as a positive control for early endosomes as well as the late recycling endosomes. However, the fact that not all strongly labeled Bodipy-positive CE colocalize with CETP (Fig. 2.5) rules out the possibility of bleed-through accounting for these observations.

5 – Conclusions and future directions

Cardiovascular disease is one of the leading causes of global deaths. This work has attempted to address the cellular mechanism of CETP mediated HDL-CE uptake in hopes to gain insight which may be found valuable in designing therapies to raise plasma HDL levels.

The first aspect investigated was to determine the specific locale of cell-surface anchored CETP as plasma membrane is known to be heterogeneous and is composed of different type of microdomains that may have different functions. The characteristics of its associated microdomain or whether it is even associated with a microdomain may have critical implications on CETP's function. Here, we find that CETP is localized to the low-density, detergent resistant membrane also known as rafts in both COS-7 and primary mouse hepatocyte. We also noted that even with serum deprivation, CETP is still found in lipid rafts suggesting that CETP associates with *de novo* rafts. However, with the addition of serum and HDL to the media, significantly more CETP was found in the lipid raft domains. It is possible that CETP, as a lipid transfer protein, may transfer CE to the membrane thereby altering the local lipid composition hence creating more lipid raft. Also, rafts are implicated in various endocytic processes and it is possible that CETP mediates HDL internalization through this association with lipid rafts. Further experiments are required to determine the exact type of raft with which CETP associates and whether this localization is relevant to CETP-mediated selective uptake. We speculate that CETP associates with a subtype of raft that is of non-caveolar and non-clathrin nature. By better understanding the specific localization of CETP on plasma membrane, insights could be gained in determining the exact mechanism of CETP function in selective uptake.

The second aspect investigated was the internalization and trafficking of CETP-mediated HDL-CE selective uptake. Here, we clearly show that CETP plays a role in mediating HDL binding and also show CETP interacting with HDL on the plasma membrane suggesting CETP-mediated docking of HDL. Further, in a series of trafficking studies, we find that the CETP/HDL complex is internalized. The complex is found in early endosomes at early timepoints and at later time points in a perinuclear compartment identified as a Rab 11 positive endocytic recycling compartment (t = 60 min) from where HDL may be recycled back to the plasma membrane and CE may be targeted to lipid droplets. It appears that CETP-mediated HDL recycling involves a slow recycling pathway via the ERC. We also observed that CETP then separated from CE at t = 120 min. The subsequent trafficking of CE may be to lipid droplets but this needs to be further established. Overall, the findings presented in this thesis support the involvement of CETP-mediated HDL whole particle uptake and potentially recycling in selective uptake.

These findings highlight a new and exciting feature of CETP relevant to its role as a HDL receptor. However, additional studies are required to further dissect the cellular mechanisms of CETP-mediated selective uptake. First, the physiological significance of raft localization in CETP-mediated HDL endocytosis should be explored in more detail. We will first determine whether raft localization of CETP is linked to endocytosis by disrupting rafts with filipin or methyl β cyclodextrin (MβCD) treatment and examining the effect on CETP-mediated HDL-CE internalization. We will also investigate the effects of different endocytic inhibitors on CETP/HDL/CE internalization and trafficking. First, we will test if endocytosis is, in fact, a requirement for effective CETP-mediated selective uptake. Second, we will determine which type of endocytic mechanism is used in CETP-mediated selective uptake by using different mutants such as caveolin-1 dominant negative as well as a dynamin mutant.

Additionally, double-labeled HDL (Cy5HDL-Bodipy-CE) will be used to specifically identify the cellular location where HDL releases its CE content. CETP association with the HDL and/or CE will also be observed at the same time. Lastly, we will further assess the significance of CETP in intracellular cholesterol trafficking and selective uptake in more physiologically relevant models such as polarized hepatocytes. Although trafficking studies done in HeLa cells offer a general insight into CETP mediated HDL-CE trafficking, it is a less physiologically relevant cell type for reverse cholesterol transport as compared to hepatocytes. Repeating these trafficking studies in a polarized hepatocyte cell line such as Wif-B should provide additional insights into CETP-mediated HDL-CE selective uptake. However, such experiments would require creation of a number of new reagents since transfection of Wif-B cells requires viral vectors.

As mentioned in introduction, SR-B1 is the main protein in mice that mediate HDL-CE selective uptake and the role of its human analog (Cla-1) is less clear. We speculate that in humans, CETP may play a more significant role as a murine SR-B1 counterpart.

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Contributions of collaborators

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