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Functional Analysis of the Carboxyl Terminus of Human Hepatic Lipase

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FUNCTIONAL ANALYSIS OF THE CARBOXYL TERMINUS OF HUMAN HEPATIC LIPASE

by

Robert J. Brown

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

Doctor of Philosophy (Ph.D.)

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ABSTRACT

Human hepatic lipase (hHL) plays an important role in the metabolism of several lipoproteins. Unlike hHL that is mainly cell surface anchored via binding to heparan sulfate proteoglycans (HSPG), mouse HL (mHL) has a low affinity to HSPG and thus is largely blood borne. Studies have suggested that the carboxyl terminus of HL mediates cell surface binding. Recombinant hHL, mHL, and chimeric proteins (hHLmt and mHLht, in which the C-terminal 70 amino acids of hHL were exchanged with the corresponding sequence from mHL) were generated in a cell culture model. The hHL, mHL, and hHLmt proteins were catalytically active using triglyceride and phospholipid substrates. In transfected cells, the majority of hHL bound to the cell surface with only 4% of total extracellular hHL released into heparin-free media, whereas under the same conditions, 61% of total extracellular mHL were released. Like mHL, hHLmt showed decreased cell surface binding with 68% of total extracellular hHLmt released. A truncated hHL mutant (hHL_{471}) was also generated by deleting the C-terminal five residues (KRKIR). The hHL_{471} also retained hydrolytic activity, and showed decreased cell surface binding with 40% of total extracellular protein released into the heparin-free media. To determine the functional role of HSPG binding by hHL *in vivo*, hHL and hHLmt were expressed in mice. Low levels of adenoviral expression (up to 1.8×10^{10} virus particles per mouse) of hHL and the chimera hHLmt in C57BL/6J mice resulted in a 4- to 6-fold increase in post-heparin HL activity above uninfected mice. The hHLmt displayed reduced HSPG binding; the activity of hHLmt in pre-heparin plasma was 3-

fold higher than that of hHL. In contrast to mice expressing hHL which had plasma total cholesterol and phospholipid levels comparable control mice, mice expressing hHLmt showed decreased plasma total cholesterol and phospholipids in a time-dependent manner by at least half, and the decrease was mainly attributable to HDL cholesterol and HDL phospholipids, as determined by FPLC. The reduced HDL lipids in the hHLmt-expressing mice were accompanied by markedly decreased plasma and hepatic apoA-I. In primary hepatocytes isolated from hHLmt-expressing mice, the concentration of cell-associated and secreted apoA-I were decreased by 2- to 3-fold as compared to hepatocytes isolated from control mice, whereas the levels of apoB and apoE were unaltered. Surprisingly, primary hepatocytes expressing hHLmt *ex vivo* had reduced secreted apoA-I but exhibited unaltered levels of cell-associated apoA-I. These data show that the C-terminal 70 amino acids of hHL play an important role in HSPG binding, and that the impaired HSPG binding of hHL has a profound HDL-lowering effect (hypoalphalipoproteinemia) *in vivo*.

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ABBREVIATIONS USED

ABCA1: adenosine triphosphate binding cassette transporter A1
apo: apolipoprotein
cAMP: cyclic adenosine monophosphate
cDNA: complementary deoxyribonucleic acid
CE: cholesteryl ester
CETP: cholesteryl ester transfer protein
CHO: Chinese hamster ovary
C-terminal: carboxyl-terminal
DMEM: Dulbecco's modified Eagle medium
DNA: deoxyribonucleic acid
DPPC: 1,2-dipalmitoyl phosphatidylcholine
EL: endothelial lipase
EMEM: Eagle's minimum essential medium
FAF-BSA: fatty acid-free bovine serum albumin
FBS: fetal bovine serum
FPLC: fast performance liquid chromatography
Gal: galactose
GlcA(2S): 2-O-sulfated β-D-glucuronic acid
GlcA: β-D-glucuronic acid
GlcN: glucosamine
GlcNAc(6S): 6-O-sulfated N-acetylated glucosamine
GlcNAc: N-acetylated glucosamine
GlcNS(3,6S): 3,6-O-sulfated N-sulfated glucosamine
GlcNS(3S): 3-O-sulfated N-sulfated glucosamine
GlcNS(6S): 6-O-sulfated N-sulfated glucosamine
GlcNS: N-sulfated glucosamine
HBD: heparin binding domain
HDL: high density lipoprotein
HEK293: human embryonic kidney 293 (cell line)
hHL: human hepatic lipase
HL: hepatic lipase
HRP: horseradish peroxidase
HSPG: heparan sulfate proteoglycan
IDL: intermediate density lipoprotein
IdoA(2S): 2-O-sulfated α-L-iduronic acid
IdoA: α-L-iduronic acid
IP: intraperitoneal
LCAT: lecithin:cholesterol acyltransferase
LDL: low density lipoprotein
LDLR: low density lipoprotein receptor

LpA-I/A-II: apolipoprotein A-I and apolipoprotein A-II-containing high density lipoprotein
LpA-I: apolipoprotein A-I-only-containing high density lipoprotein
LPL: lipoprotein lipase
LRP: low density lipoprotein receptor-related protein
mHL: mouse hepatic lipase
mRNA: messenger ribonucleic acid
NCBI: National Center for Biotechnology Information
N-terminal: amino-terminal
PBS: phosphate buffered saline
PCR: polymerase chain reaction
PL: phospholipid
PLase: pancreatic lipase
PLTP: phospholipid transfer protein
RAP: receptor-associated protein
rHL: rat hepatic lipase
SNP: single nucleotide polymorphism
SR-BI: scavenger receptor class B type I
TC: total cholesterol
TG: triglyceride
VLDL: very low density lipoprotein
VP: virus particle
Xyl: xylose

CHAPTER 1 – INTRODUCTION

The enzyme hepatic lipase (HL), together with other proteins, plays an important role in the metabolism of lipid in the bloodstream. Why and how some of the functions by HL occur remain unclear, however the disruption of specific properties of HL through molecular biology approaches have and can continue to provide clues to the mechanisms behind these functions. The following introduction provides relevant information about the known functions and roles of HL in the metabolism of lipid in the circulation.

1.1 Hepatic lipase and the lipase superfamily

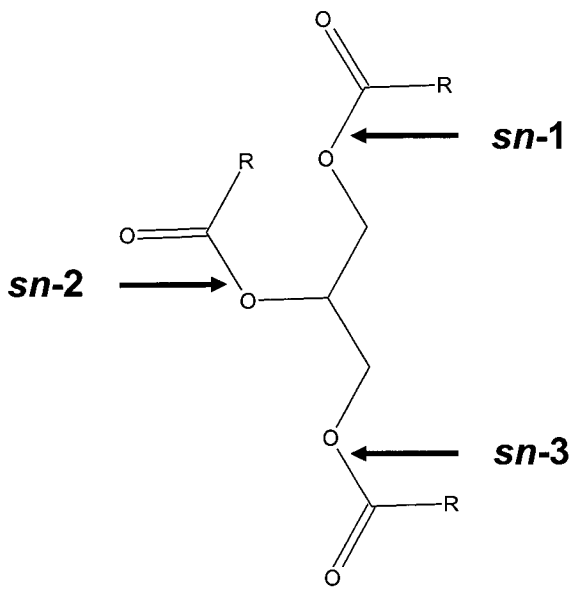
1.1.1 What are the sn-1 lipases?

Lipases are water-soluble enzymes that liberate fatty acids from monoglycerides, diglycerides, triglycerides (TG), phospholipids (PL), and cholesteryl esters (CE) by hydrolyzing the ester bonds. A feature unique to the lipases is the preferential hydrolysis of fatty acids at the *sn*-1 and *sn*-3 positions of fatty-acyl glycerides (**Figure 1.1.1**)(1). The catalytic site of the lipases is similar to that of serine proteases, where a triad of serine, aspartic acid, and histidine provides a charge-relay mechanism that catalyzes the hydrolytic events (2). The serine residue found in the catalytic triad of the lipases is part of an amino acid consensus sequence motif shared by all lipases, which is Gly-Xaa-Ser-Xaa-Gly, where Xaa represents any amino acid (3,4). Lipases have been found in several organisms, ranging from bacteria to plants to mammals (reviewed in (5)). Mammalian

Figure 1.1.1. Positions of esterified fatty-acyl chains in triglyceride and phospholipid

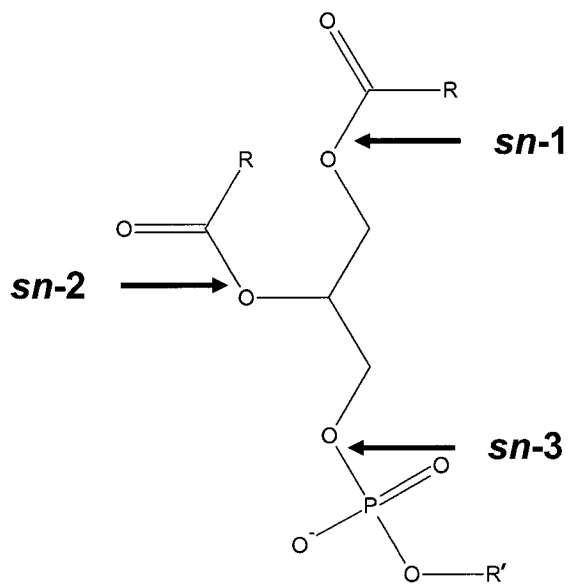
Lipases preferentially liberate free fatty acids from the *sn*-1 and *sn*-3 positions on triglycerides, and from the *sn*-1 position of phospholipids. *Panel A*, the *sn*-1 and *sn*-3 positions of triglycerides are indistinguishable, and the esterified fatty acids at these positions may be hydrolyzed by lipases equally well (assuming the same fatty acyl species is present at both positions). *Panel B*, the *sn*-1 position has a fatty acyl chain, whereas in contrast to triglycerides the *sn*-3 position contains a phosphate head-group.

A **Triglyceride**



R = any fatty acid

B **Phospholipid**



R = any fatty acid

R' = H, ethanolamine, choline, inositol, serine

lipases that affect the levels of circulating lipids have been studied for over a century (6), and three of which that were identified prior to their protein and deoxyribonucleic acid (DNA) sequencing include pancreatic lipase (PLase), lipoprotein lipase (LPL), and HL.

1.1.2 Overview of pancreatic lipase, lipoprotein lipase, hepatic lipase, and endothelial lipase

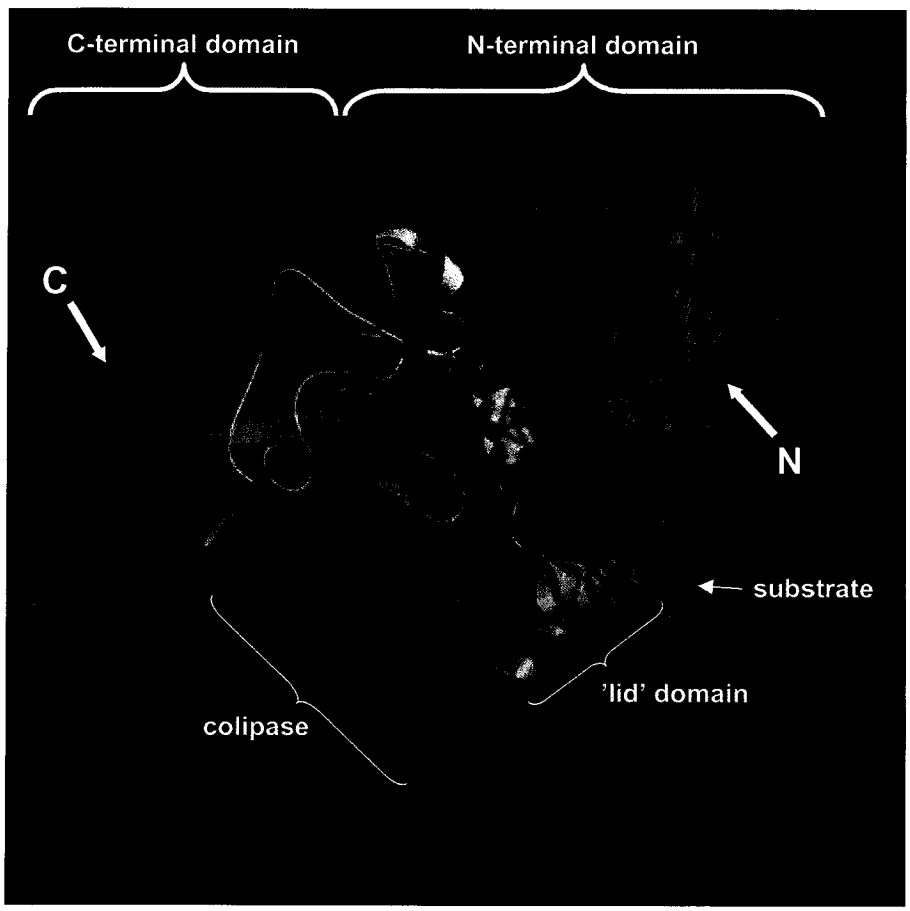
Pancreatic lipase (PLase) is the first reported mammalian lipase and was identified in the nineteenth century (6). The enzyme is synthesized in pancreatic acinar cells and is secreted into the intestinal lumen where it hydrolyzes TG to aid in fatty acid absorption. The hydrolytic activity of PLase is dependent on a cofactor named colipase. These cells also produce other digestive enzymes, including pancreatic amylase, trypsin, chymotrypsin, carboxypeptidase, deoxyribonuclease, and ribonuclease.

Pancreatic lipase was the first mammalian lipase to have its complementary DNA (cDNA) sequenced (7), and the canine and human PLase protein structures were solved in 1989 and 1990, respectively (8,9). **Figure 1.1.2** shows the solved structure of human PLase bound to its cofactor colipase, and to a PL substrate (as adapted from (10)). There are two domains to the structure: a 336 amino acid residue amino-terminal (or N-terminal) globular domain and a 113 amino acid residue carboxyl-terminal (or C-terminal) beta-barrel domain. The N-terminal domain contains the active site. Substrates are trapped by the 'lid domain', which is a structure in the N-terminal domain that folds over the active site and governs substrate specificity. The cofactor colipase interacts with the C-terminal domain, however it does not cause a conformational change to the

Figure 1.1.2. Tertiary structure of pancreatic lipase

Human pancreatic lipase (PLase), complexed with the cofactor colipase (dark blue structure) and diundecyl phosphatidylcholine ("substrate"), has been crystallized as previously described (10) to 3.04 Å resolution. Human PLase has two domains: an N-terminal domain (amino acid residues 1-336) and a C-terminal domain (amino acid residues 337-449). The N-terminal domain is mainly globular in structure and contains the catalytic triad within a pocket that supports the substrate. This pocket is protected by the 'lid' domain that governs substrate specificity. The smaller C-terminal domain forms a β-barrel structure, and is solely responsible for interactions with colipase. The start and end of the protein are indicated by "N" and "C", respectively. The figure was obtained from the structure data set 1LPA, available in the Research Collaboratory for Structural Bioinformatics Protein Data Bank.

Figure 1.1.2


C-terminal domain
N-terminal domain
C
N
substrate
'lid' domain
colipase

structure of PLase (10). Both LPL and HL have a high amino acid sequence homology to PLase (as described below), and thus these lipases are thought to have a similar structure.

The activity of LPL was first reported in 1943, when heparin infusion into dogs was shown to rapidly reduce postprandial lipemia (11). LPL is synthesized in various tissues, most notably in adipose and muscle tissues, and is anchored to the capillary endothelium via heparan sulfate proteoglycans (HSPG) (see Section 1.3). LPL liberates fatty acids from TG-rich lipoproteins for delivery to tissues, and LPL also has non-catalytic functions such as lipoprotein uptake from the circulation. As in the case for PLase, LPL also requires a cofactor, namely apolipoprotein (apo) C-II, for catalytic activity. The cDNA sequences of lipoprotein lipases from a variety of species were reported in 1987 (12-15), and they were found to have a high amino acid sequence homology to PLase. The human pancreatic and lipoprotein lipases share 30% identity and 49% homology.

Hepatic lipase was first identified as a lipase released by the liver, independent of LPL activity, due to its ability to be catalytically active in the presence of high salt (i.e. 1.0 M NaCl) (16). Very shortly after the LPL sequence was characterized, HL was sequenced from various species (14,17-22). Hepatic lipase was found to be highly homologous to LPL; the human lipoprotein and hepatic lipases share 45% identity and 62% homology. Hepatic lipase is synthesized mainly in the liver, and like LPL, it is anchored to the vascular endothelium via HSPGs. Hepatic lipase liberates fatty acids from various lipoproteins, and also has non-catalytic functions that facilitate lipoprotein uptake from the circulation. The catalytic and non-catalytic functions of HL are described in Sections 1.2 & 1.4, respectively.

The high homologies between the pancreatic, lipoprotein, and hepatic lipases suggest that they evolved from a common gene (4). The chromosomal locations of human PLase (chromosome 10q24-q26.1) (23), human LPL (chromosome 8p22) (24), and human HL (hHL – chromosome 15q21-q23) (21,24) are different, however these genes have highly homologous intron/exon sequence boundaries. **Figure 1.1.3** shows the amino acid sequence alignments for these three lipases. These three human lipases have significant sequence identities, (which are highlighted by the red-coloured amino acid residues). Of note are the numbers of conserved cysteine residues (highlighted in yellow) and glycine residues (highlighted in blue) which are key structural residues in PLase.

In 1999, two groups independently cloned endothelial lipase (EL) (25,26). The human lipoprotein lipase and endothelial lipase are highly homologous, having 45% identity and 62% homology with each other. Endothelial lipase is synthesized in various tissues, and like both the lipoprotein and hepatic lipases, it is anchored to the capillary endothelium via HSPGs. Endothelial lipase liberates fatty acids from PL-rich lipoproteins for delivery to tissues. Phylogenetic analyses of EL show that it belongs to the same lipase superfamily (**Figure 1.1.4**).

1.1.3 Hepatic lipase synthesis, secretion, and regulation

The hHL polypeptide matures into a 476 amino acid protein after the trimming of a 23 amino acid signal peptide. Hepatic lipase is synthesized in the endoplasmic reticulum and becomes N-glycosylated. The importance of the glycosylation of HL has been demonstrated in several cell culture studies, where inhibitors of glycosylation impair both the HL catalytic activity and the cellular secretion of HL (28-31). Human HL is

Figure 1.1.3. Alignment of the amino acid sequences of mature human hepatic lipase, lipoprotein lipase, and pancreatic lipase

The mature amino acid sequences (without the signal peptide) of human HL (hHL), human LPL (hLPL), and human PL (hPLase) were aligned using the algorithm of the computer program MultAlin (27). The N- and C-terminal regions of these lipases are not homologous, thus the alignments within these regions should be carefully interpreted. The numbers following each line of sequence corresponds to the numbers of the amino acid residue at the end of each line for the respective lipases. The locations of the serine (S), aspartic acid (D), and histidine (H) that comprise the catalytic triad are marked by "*". The 'lid domain' of the lipases is denoted by "-". Amino acid residues sharing identity are labeled in red. Conserved glycine (G) and cysteine (C) residues are highlighted blue and yellow, respectively. Sites of N-linked glycosylation are boxed.

Figure 1.1.3

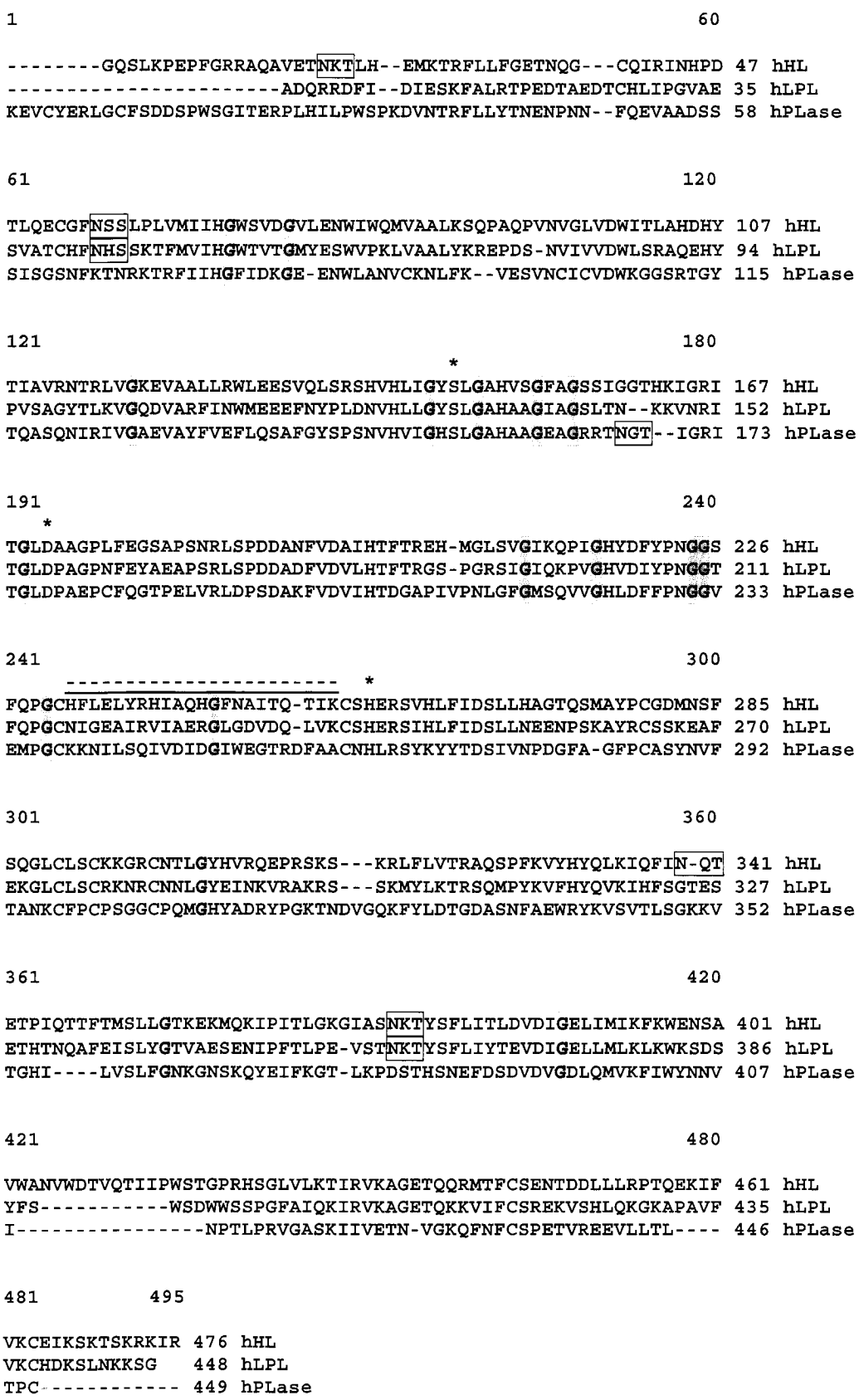
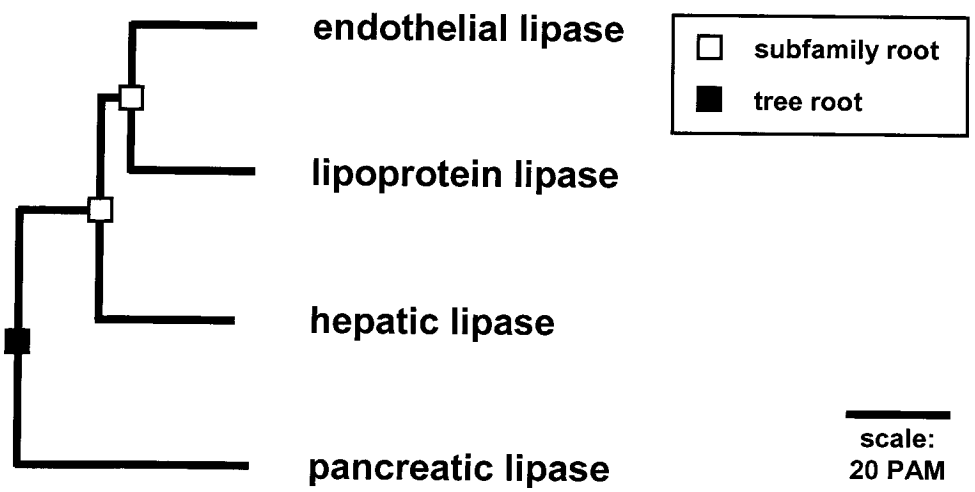

1 60
--------GQSLKPEPFGRRAQAVETNKTLH--EMKTRFLLFGETNQG---CQIRINHPD 47 hHL
----------ADQRRDFI--DIESKFALRTPEDTAEDTCHLIPGVAE 35 hLPL
KEVCYERLGCFSDDSPWSGITERPLHILPWSPKDVNTRFLLYTNENPNN--FQEVAADSS 58 hPLase
61 120
TLQECGFNSSLPLVMIIHGWSVDGVLENWIWQMVAALKSQPAQPVNVGLVDWITLAHDHY 107 hHL
SVATCHFNHSSKTFMVIHGWTVTGMYESWVPKLVAALYKREPDS-NVIVVDWLSRAQEHY 94 hLPL
SISGSNFKTNRKTRFIIHGFIDKGE-ENWLANVCKNLFK--VESVNCICVDWKGGSRTGY 115 hPLase
121 180
TIAVRNTRLVGKEVAALLRWLEESVQLSRSHVHLIGYSLGAHVSGFAGSSIGGTHKIGRI 167 hHL
PVSAGYTLKVGQDVARFINWMEEEFNYPLDNVHLLGYSLGAHAAGIAGSLTN--KKVNRI 152 hLPL
TQASQNIRIVGAEVAYFVEFLQSAFGYSPSNVHVIGHSLGAHAAGEAGRRTNGT--IGRI 173 hPLase
191 240
TGLDAAGPLFEGSAPSNRLSPDDANFVDAIHTFTREH-MGLSVGIKQPIGHYDFYPNGGS 226 hHL
TGLDPAGPNFEYAEAPSRLSPDDADFVDVLHTFTRGS-PGRSIGIQKPVGHVDIYPNGGT 211 hLPL
TGLDPAEPCFQGTPELVRLDPSDAKFVDVIHTDGAPIVPNLGFGMSQVVGHLDFFPNGGV 233 hPLase
241 300
FQPGCHFLELYRHIAQHGFNAITQ-TIKCSHERSVHLFIDSLLHAGTQSMAYPCGDMNSF 285 hHL
FQPGCNIGEAIRVIAERGLGDVDQ-LVKCSHERSIHLFIDSLLNEENPSKAYRCSSKEAF 270 hLPL
EMPGCKKNILSQIVDIDGIWEGTRDFAACNHLRSYKYYTDSIVNPDGFA-GFPCASYNVF 292 hPLase
301 360
SQGLCLSCKKGRCNTLGYHVRQEPRSKS---KRLFLVTRAQSPFKVYHYQLKIQFIN-QT 341 hHL
EKGLCLSCRKNRCNNLGYEINKVRAKRS---SKMYLKTRSQMPYKVFHYQVKIHFSGTES 327 hLPL
TANKCFPCPSGGCPQMGHYADRYPGKTNDVGQKFYLDTGDASNFAEWRYKVSVTLSGKKV 352 hPLase
361 420
ETPIQTTFTMSLLGTKEKMQKIPITLGKGIASNKTYSFLITLDVDIGELIMIKFKWENSA 401 hHL
ETHTNQAFEISLYGTVAESENIPFTLPE-VSTNKTYSFLIYTEVDIGELLMLKLKWKSDS 386 hLPL
TGHI----LVSLFGNKGNSKQYEIFKGT-LKPDSTHSNEFDSDVDVGDLQMVKFIWYNNV 407 hPLase
421 480
VWANVWDTVQTIIPWSTGPRHSGLVLKTIRVKAGETQQRMTFCSENTDDLLLRPTQEKIF 461 hHL
YFS----------WSDWWSSPGFAIQKIRVKAGETQKKVIFCSREKVSHLQKGKAPAVF 435 hLPL
I----------NPTLPRVGASKIIVETN-VGKQFNFCSPETVREEVLLTL---- 446 hPLase
481 495
VKCEIKSKTSKRKIR 476 hHL
VKCHDKSLNKKSG 448 hLPL
TPC---------- 449 hPLase

Figure 1.1.4. Phylogenetic tree of human pancreatic lipase, lipoprotein lipase, hepatic lipase, and endothelial lipase

The mature amino acid sequences (without the signal peptide) of human PLase, LPL, HL, and EL were aligned using the MultAlin algorithm (27), and the alignment data was converted into a phylogenetic tree. The lengths of the branches represent the "percent accepted mutations" (PAM).

Figure 1.1.4


endothelial lipase
lipoprotein lipase
hepatic lipase
pancreatic lipase
subfamily root
tree root
scale:
20 PAM

glycosylated at four sites (amino acid residues 20, 56, 340, and 375) (32). In contrast, rat HL (rHL) is glycosylated at two sites (amino acid residues 57 and 376) (33), and mouse HL (mHL) is potentially glycosylated at two sites (amino acid residues 57 and 376). Mutagenesis studies of hHL show that amino acid residue 56 must be glycosylated to attain activity and secretion (32). This is analogous to the necessity for the corresponding amino acid residue in LPL (amino acid residue 43) to be glycosylated to attain catalytic activity and cell secretion (34).

Glycosylation alone of HL is essential but insufficient for HL to gain catalytic activity. Human HL has been demonstrated to be catalytically active as a homodimer (35). The dimerization of HL is thought to occur during the intracellular processing of HL in the endoplasmic reticulum. A homogenous oligomeric complex of catalytically inactive HL is found in the endoplasmic reticulum. The glycosylation of HL monomers and the breakdown of the oligomeric HL complex into homodimers raises the specific activity of the newly formed HL homodimers to levels equivalent to that observed with HL secreted from cells (36,37).

The apparent molecular mass of the processed hHL monomer varies from 55- to 69-kDa (20-22) presumably owing to variation in the extent of glycosylation. Hepatic lipase is synthesized and secreted mainly by liver parenchymal cells. Immunocytochemistry studies have revealed that hHL is located in the liver on the sublumenal extracellular matrix component of endothelium, the microvillar surface of hepatocytes in the space of Disse, interhepatocyte space, and lumenal surface of sinusoidal endothelium (38). Infusing heparin *in vivo* increases HL activity in the serum by 1000-fold, suggesting an interaction of hHL with HSPGs (see Section 1.3.3) (39).

Hepatic lipase has also been recently found to be produced in monocytes (40), however the role of HL in these cells is unclear. A truncated version of the HL messenger ribonucleic acid (mRNA) was identified in steroidal tissues to yield a truncated 40 to 45-kDa protein that is not secreted (41-44), however the role of this truncated HL has not been defined.

The promoter of the rHL gene (the HL gene is designated "*LIPC*") was characterized in 1990 and putative regulatory elements were identified for cyclic adenosine monophosphate, estrogen, thyroid hormone, sterols, and glucocorticoids (45). To date, there have been very few studies on the regulation of the *LIPC* promoter function. Prior to the sequencing of the *LIPC* promoter, a correlation between levels of the sex hormone estrogen and HL activity was already established; premenopausal women have lower HL activities relative to men and postmenopausal women (46-48). The data suggesting that estrogen may be a negative repressor of *LIPC* were confirmed in several studies where the treatment of women (49-57) or rats (58-61) with estrogen reduced HL activity, and in studies where the HL mRNA levels were reduced by estrogen (60,61). The sex hormone testosterone was also demonstrated to affect HL, although results are contradictory between models studied. Testosterone replacement therapy in men has been shown to increase HL activities (62-67), however testosterone treatment of rats decreased HL activity and elevated HL mRNA (60). Furthermore, the treatment of a human hepatic cell line (HepG2) with high doses of testosterone resulted in a nearly four-fold elevation of HL mRNA but no change in HL protein (68). Taken together, the testosterone studies suggest that the regulation of HL may occur both at the level at the *LIPC* promoter and at a post-transcriptional level.

Comparing patients with overt hypothyroidism to those with hyperthyroidism, HL activities correlated positively with the circulating levels of the thyroid hormone triiodothyronine (69,70). Furthermore, thyroid hormone replacement therapy of patients with hypothyroidism restores HL activities to those observed in patients with normal thyroid function (69,71-73). Studies with rats show that hypothyroidism results in both decreased HL mRNA and activity, which suggest that thyroxine acts as a positive regulator of HL transcription. With HepG2 cells, studies have shown that HL mRNA and activity are indeed elevated by the stimulation of triiodothyronine at supraphysiological levels (74). However, at physiological levels of triiodothyronine, the HL mRNA levels and rates of protein synthesis were not altered despite an elevation of HL activity (75). These data suggest that like testosterone, thyroid hormones not only appear to regulate HL both at the levels of the *LIPC* promoter and post-transcription, but also possibly at the level of post-translation.

Cell culture studies have shown that cholesterol can regulate the expression of HL. HepG2 cells cultured with an inhibitor of cholesterol biosynthesis increases HL mRNA, mass, and activity (76). The idea that cholesterol acts as a negative regulator of *LIPC* transcription is supported with *in vivo* data showing that cholesterol-fed rats have reduced levels of both HL mRNA and activity (77). Other studies have identified anabolic steroids (78), glucose (79), and leptin (80) as positive regulators of HL mRNA expression. Adrenaline has been shown to be a negative regulator of HL at the post-translational level (81,82).

1.1.4 Polymorphisms of LIPC

Several polymorphisms of *LIPC* have been identified within both coding and non-coding regions. To date, a search of the National Center for Biotechnology Information (NCBI) single nucleotide polymorphisms (SNPs) database for human *LIPC* yields 551 reported SNPs. Four of the 551 polymorphisms are in the coding region, which result in changing the amino acid residues Val^{72} → Met, Asn^{192} → Ser, Leu^{334} → Phe*, and Ser^{417} → Asn. Other polymorphisms in the coding region have been reported that are not in the NCBI database, which include changes to the amino acid residues Asp^{47} → Ser, Ser^{267} → Phe* (83), Val^{319} → Ile (84), Thr^{383} → Met* (85), and Asp^{387} → Ala (84), and an insertion mutation Val^{111} → Val-His-Tyr-Thr-Val-Ala-Val (86). (* These have been reported and numbered on the basis of a 477 amino acid protein and a 22 amino acid signal peptide). Of these polymorphisms in the coding region, the Ser^{267} → Phe, Thr^{383} → Met, and the Val^{111} → Val-His-Tyr-Thr-Val-Ala-Val insertion are associated with familial HL deficiency. The Ser^{267} → Phe and Thr^{383} → Met have been studied in a cell culture model, and despite producing normal protein mass (in comparison with wild-type HL), they both had impaired cell secretion and activity (87).

The polymorphisms within the promoter of human *LIPC* include A^{-763} → G, T^{-710} → C, C^{-586} → T, C^{-514} → T, C^{-480} → T, and C^{-250} → A (88-90). Carriers of the T-allele at -586, T-allele at -514, the T-allele at -480, and the A-allele at -250 are associated with low post-heparin HL activity versus homozygous wild-type allele carriers (88-98). Cell culture studies of the HL promoter show that the T-allele at -514 results in reduced promoter activity, regardless of the allele at -250, which appeared to have no effect on promoter activity (99).

1.1.5 Activity and substrates of hepatic lipase

In vivo and *in vitro* studies show that HL has a broad substrate specificity and is involved in the metabolism of TG-rich lipoproteins and high density lipoproteins (HDL) (100-104) (see Section 1.2). HL liberates fatty acids from the *sn*-1 position of PL, and from mono-, di-, and triglycerides. This is in contrast to LPL which is almost exclusively a TG-lipase, and to EL which is almost exclusively a phospholipase (105). This difference in preference for TG-lipase versus phospholipase activity is attributed to the 'lid domain' of these lipases. *In vivo* and *in vitro* studies with chimeras of HL and LPL having exchanged 'lid domains' show that LPL with the HL 'lid domain' had reduced TG-lipase but enhanced phospholipase activity, whereas HL with the LPL 'lid domain' had enhanced TG-lipase but reduced phospholipase activity (106,107). EL has a shorter 'lid domain' (19 amino acid residues) compared to both LPL and HL (both having 22 amino acid residues) (26), and it is thought that the different length and amino acid residue sequence of the 'lid domain' on EL contributes to its almost-exclusive phospholipase activity.

Hepatic lipase has been shown to have preferences for the type of acylglyceride and type of PL it hydrolyzes. With HDL (see Section 1.2) as a substrate (which contains various mono-, di-, and triglycerides, and PL), HL was shown to preferentially hydrolyze di- over triglycerides (108). Furthermore, HL has preferential hydrolysis for the type of fatty acyl chain on acylglycerols and PL. Short chain acylglycerides have been shown to be better substrates for HL in contrast to long chain acylglycerides (109,110), however the affinity to long chain acylglycerides is greater than short chain acylglycerides (111). Preferential hydrolysis of fatty acyl chains by HL is also exhibited with PL substrates.

HL was shown to preferentially hydrolyze the fatty acyl chains from phosphatidylethanolamine compared with other PL (112-115), thus suggesting that the head group at the *sn*-3 position of PL plays a role in the accessibility of the *sn*-1 fatty acyl chain by HL. Furthermore, the uncleaved *sn*-2 fatty acyl chain can also affect the accessibility of the sn-1 fatty acyl chain by HL. Bulky and long-chain fatty acyl chains in the *sn*-2 position impair the hydrolytic activity of HL to the *sn*-1 position (116).

The activity of LPL requires the cofactor apoC-II and is sensitive to high salt (i.e. 1 M NaCl), whereas the activity of HL is independent of apoC-II and remains active at 1 M NaCl. Studies with a chimeric protein composed of amino acid sequences derived from human LPL and hHL have shown that the catalytic properties and high salt sensitivity of LPL are determined by the N-terminal domain (117), whereas the apoC-II activation of LPL probably involves both the N-terminal (118,119) and C-terminal domains of LPL (118). Although no protein cofactors have been identified that enhance HL catalytic activity, various proteins found in the circulation have been suggested to inhibit HL. These include apoA-I (120,121), apoA-II (121-123), and apoC-I (124). However, different subclasses of the macromolecular complex HDL (see Section 1.2) have been shown *in vitro* to both enhance and inhibit the ability of HL to hydrolyse TG from very low density lipoproteins (VLDL, see Section 1.2). Dense HDL subclasses have been shown to inhibit the HL-mediated hydrolysis of TG from VLDL, whereas buoyant HDL subclasses stimulate the HL-mediated hydrolysis of TG from VLDL (125).

1.2 Lipoproteins

1.2.1 Overview

Lipoproteins are aqueous-soluble microemulsions of lipids and proteins. The structure of a lipoprotein consists of a hydrophobic core surrounded by an amphipathic layer consisting of cholesterol, PL, and proteins (126) (**Figure 1.2.1**). The core typically contains mono-, di-, and triglycerides, CE, and a variety of lipophilic hormones and vitamins (127). Lipoproteins are synthesized and secreted by the liver and intestines, and are also generated in the circulation as a consequence of metabolic events affecting secreted precursor lipoproteins. Lipoprotein lipids can be enzymatically modified, delivered to tissues, and transferred to other lipoproteins. Almost all of the major lipoprotein-associated proteins, apolipoproteins, can be transferred to other lipoproteins (128,129). A list of the major human apolipoproteins and the lipoprotein classes they are associated with can be found in **Table 1.2.1**. The very dynamic nature of circulating lipoproteins yields a variety of distinct classes of lipoproteins, which are typically characterized based on buoyancy and electrophoretic mobility on agarose gels. The lipoprotein classes can also be further divided into subclasses, which are usually based on lipoprotein buoyancy and size.

The major classes of lipoproteins are chylomicrons, VLDL, intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and HDL. **Table 1.2.2** compares the physiochemical properties of these major lipoprotein classes. Note that there is a direct relationship between lipoprotein size and molecular weight, and an inverse relationship between lipoprotein size and lipoprotein buoyancy. The functions of these classes of

Figure 1.2.1. General structure of a lipoprotein

Lipoproteins consist of a hydrophobic core, that contains acylglycerides and CE. The core is surrounded by a PL monolayer, that has free cholesterol and some diglyceride interdispersed throughout the monolayer. Apolipoproteins reside on the lipoprotein surface.

Figure 1.2.1

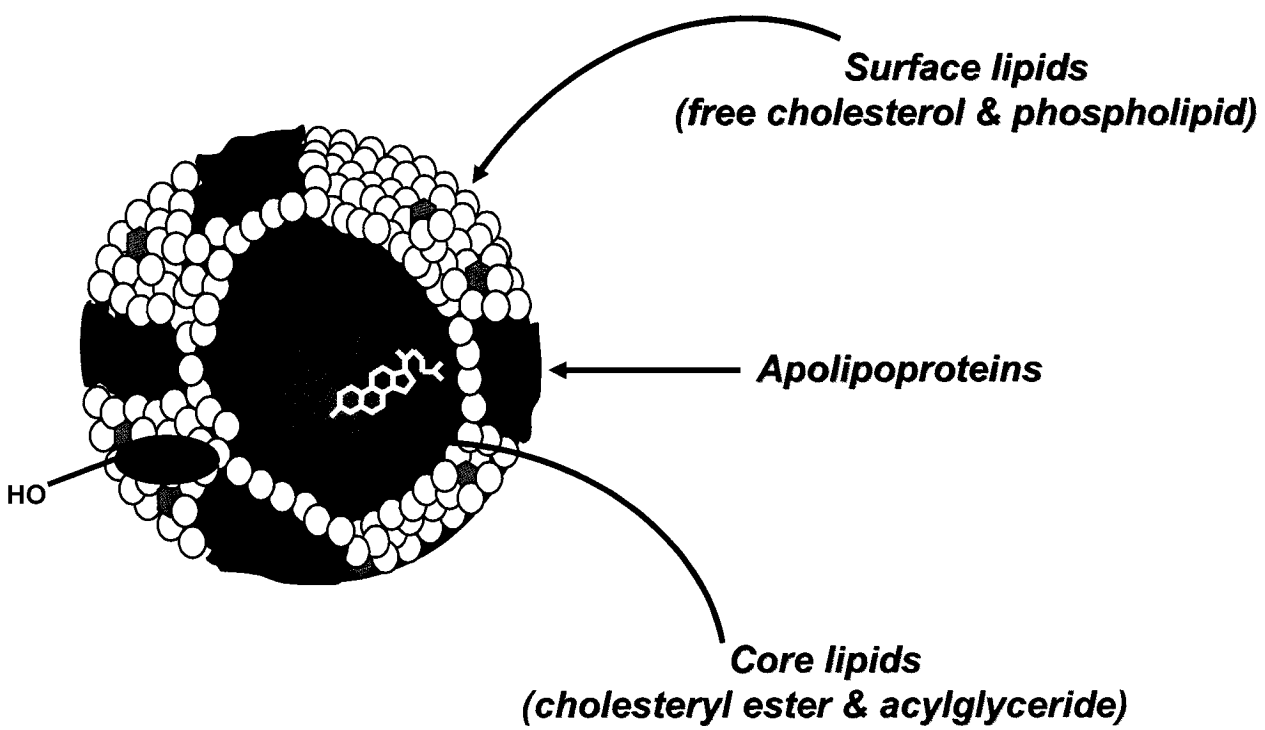

Surface lipids
(free cholesterol & phospholipid)
Apolipoproteins
HO
Core lipids
(cholesteryl ester & acylglyceride)

Table 1.2.1. Major human apolipoproteins associated with lipoproteins*

Apolipoprotein[a]	Molecular weight[b] (*MW* x1000)	Lipoprotein[c]	Plasma conc. (mg/dl)
AI	28.1	CM, HDL	130
AII	8.7†	HDL	40
AIV	44.5	CM	15
(a)	300-800	Lp(a)	0.1-40
B100	512	VLDL, LDL	93
B48	242	CM	
CI	6.6	CM, VLDL, HDL	3
CII	9.0	CM, VLDL, HDL	12
CIII	9.0	CM, VLDL, HDL	12
D	22.0	HDL	12
E	34.2	CM, VLDL, HDL	7

a, Other apolipoproteins are found in plasma; most are in very low concentrations and have undefined functions.
b, The listed molecular weights are of the peptides without taking into consideration any post-translational modifications.
c, CM, chylomicrons
† The molecular weight of the monomeric form. Only human apoA-II is found as a disulfide linked dimer.
* Adapted from (126,130).

Table 1.2.2. Physiochemical properties of various lipoprotein classes*

Class	Diameter (nm)	Density (g/ml)	Chemical composition† (% of dry mass)				
			TG	CE	FC	PL	Protein
Chylomicrons	80-500	<0.94	86	3	2	7	2
VLDL	30-80	0.94-1.006	55	12	7	18	8
IDL	25-35	1.006-1.019	23	29	9	19	19
LDL	18-25	1.019-1.063	6	42	8	22	22
HDL_2	9-12	1.063-1.125	5	17	5	33	40
HDL_3	5-9	1.125-1.210	3	13	4	25	55

† TG, triglyceride; CE, cholesteryl ester; FC, free cholesterol; PL, phospholipid.
* Adapted from (131).

lipoproteins are quite diverse. Chylomicrons are synthesized by the intestine, and transport dietary lipids from the intestine to various tissues (see Section 1.2.3). VLDL, produced by the liver, transport endogenously-produced lipids from the liver to various tissues (see Section 1.2.4). IDL and LDL are generated from the metabolism of VLDL, and deliver cholesteryl esters to various tissues, whereas HDL, produced by various routes (see Section 1.2.5), removes excess cholesterol from tissues for delivery to steroidogenic tissues and the liver. The enzymatic properties of HL play an important role for each class of lipoprotein. Furthermore, HL can combine its enzymatic function with a non-catalytic liganding function, which is described in Section 1.4.

1.2.2 Apolipoprotein B

The common feature associated with lipoprotein classes chylomicrons, VLDL, IDL, and LDL is that they all have the protein component apoB. These lipoproteins contain only one apoB per particle, and this apoB cannot be transferred to other lipoprotein particles (132-134). Human apoB is synthesized as a single polypeptide of 4536 amino acid residues (135-138). This is termed as apoB100, meaning 100% of the full-length polypeptide. ApoB100 is produced in the liver, and is a protein component of VLDL, IDL, and LDL. In the intestines, the apoB mRNA is edited by the enzyme APOBEC-1, which is a cytidine deaminase that converts cytosine to uracil at codon 2153 and generates a stop codon in place of glutamine (139-141). This yields a truncated apoB protein that is 48% of the full-length polypeptide from the N-terminus (designated apoB48). ApoB48 is a protein component of chylomicrons. Unlike that in humans, apoB48 is also generated in the livers in rats and mice (142-144), thus apoB48 can also

be used to generate VLDL, IDL, and LDL. Other apolipoproteins are associated with these apoB-containing lipoproteins, as listed in **Table 1.2.1**.

1.2.3 Chylomicrons and chylomicron remnants

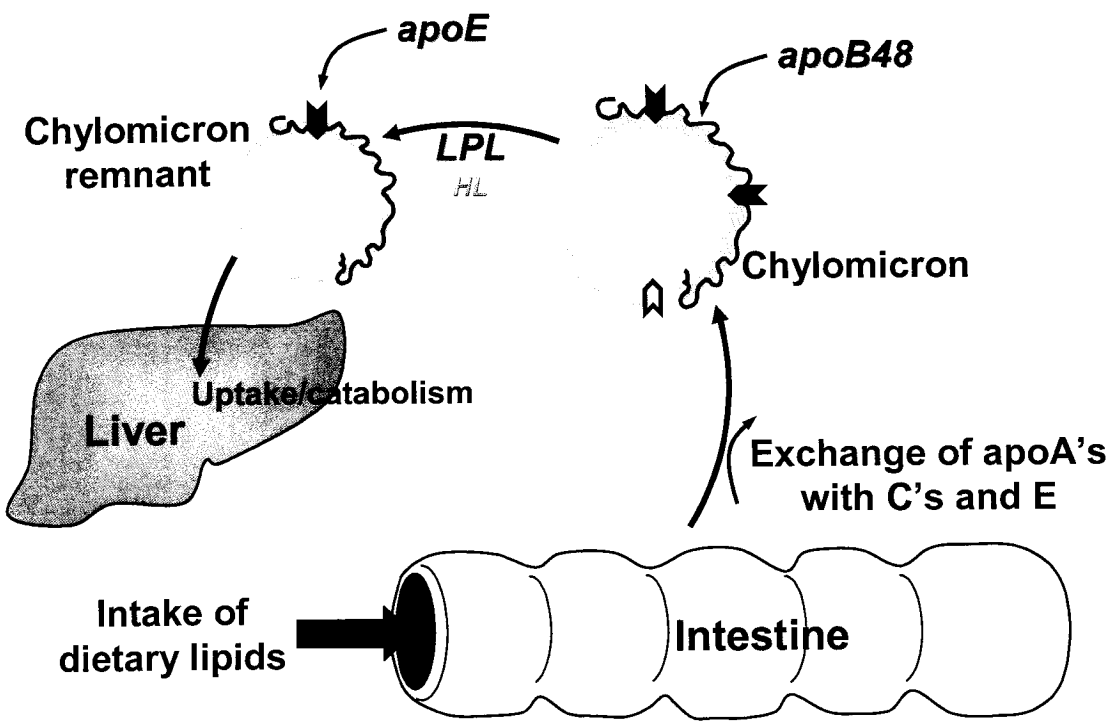
Pancreatic lipase released by the pancreatic acinar cells into the intestine liberates free fatty acids from the hydrolysis of dietary TG and PL. The fatty acids and the byproducts (*sn*-2 monoglycerides and lysophospholipids, respectively) are internalized into intestinal cells (enterocytes), re-esterified to regenerate TG and PL, and are incorporated with apoB48 intracellularly to produce chylomicrons (126). The metabolism of chylomicrons as described below is summarized in **Figure 1.2.2**. Cholesterol and CE are also incorporated into the newly synthesized chylomicrons. These chylomicrons also associate the exchangeable apolipoproteins apoA-I, apoA-II, and apoA-IV (145-147). The chylomicrons are secreted into the circulation through the thoracic duct from the mesenteric lymph (148). The apoA-I and apoA-II are exchanged in the circulation with the exchangeable apolipoproteins apoC-I, apoC-II, apoC-III, and apoE from HDL (146,149). The apoC-II activates endothelium-associated LPL, which rapidly hydrolyses the chylomicron TG to liberate free fatty acids for use in various tissues. HL is also thought to hydrolyse chylomicron TG and degrade some of the chylomicron PL (103). The reduction in TG content in the particles parallels an enrichment in cholesterol and CE concentrations. These modified chylomicrons are termed chylomicron remnants.

Chylomicron remnants are smaller than the chylomicron precursors due to the loss of lipids. These remnants are also depleted of most of their exchangeable

Figure 1.2.2. Metabolism of chylomicrons

As described in Section 1.2.3, chylomicrons are produced in the intestines and are released into the circulation through the thoracic duct from the mesenteric lymph. The chylomicrons contain apoA-I, apoA-II, and apoA-IV, and exchanges these latter apolipoproteins for apoC-I, apoC-II, apoC-III, and apoE once in the circulation. The apoC-II activates endothelium-associated LPL, which rapidly hydrolyses the chylomicron TG to liberate free fatty acids for use in various tissues. HL is also thought to hydrolyze these TG and deplete some of the chylomicron PL. The TG-depleted particles are enriched in cholesterol and CE, and are termed chylomicron remnants. The remnants can be further metabolized by HL to liberate free fatty acids from PL and TG. The remnants are removed from the circulation through various possible routes (see Sections 1.2.3 and 1.4.2).

Figure 1.2.2


apoE
apoB48
Chylomicron
remnant
LPL
HL
Chylomicron
Uptake/catabolism
Liver
Exchange of apoA's
with C's and E
Intake of
dietary lipids
Intestine

apolipoproteins, which have a reduced affinity to the remnants, possibly due to increased cholesterol and CE content (150). Chylomicron remnants are considered atherogenic (151-153), that is they promote atherosclerosis. Atherosclerosis is the accumulation of lipids (especially cholesterol and CE) within the walls of blood vessels (154). This leads to the narrowing of the blood vessels and the subsequent impairment in the deilvery of oxygen to surrounding tissues. The removal of the chylomicron remnants from the circulation is thought to occur under normal physiological conditions through a combination of three mechanisms (as reviewed in (155)). Each mechanism requires the remnants to enter the space of Disse through the sinusoidal endothelium. Within the space of Disse, HL is proposed to further metabolize the remnants through its lipolytic activity. Studies have demonstrated that remnants could be removed via direct binding through the associated apolipoprotein apoE to the LDL receptor (LDLR), and subsequent endocytosis (156,157). A second mechanism is through the binding and uptake of the remnants via direct binding of apoE, HL, or LPL both to the remnants and to HSPG (155). A third mechanism involves the remnants acting as "bridges" binding to both HSPG via the associated apoE, HL, or LPL, and binding to the LDLR-related protein (LRP) (155). The removal of chylomicron remnants by the liganding functions of HL is further discussed in Section 1.4.2.

1.2.4 Very low density lipoproteins, intermediate density lipoproteins, and low density lipoproteins

In humans, VLDL are generated in liver cells (hepatocytes) by the incorporation of PL, cholesterol, CE, and endogenously-produced TG with apoB100 (126). VLDL is

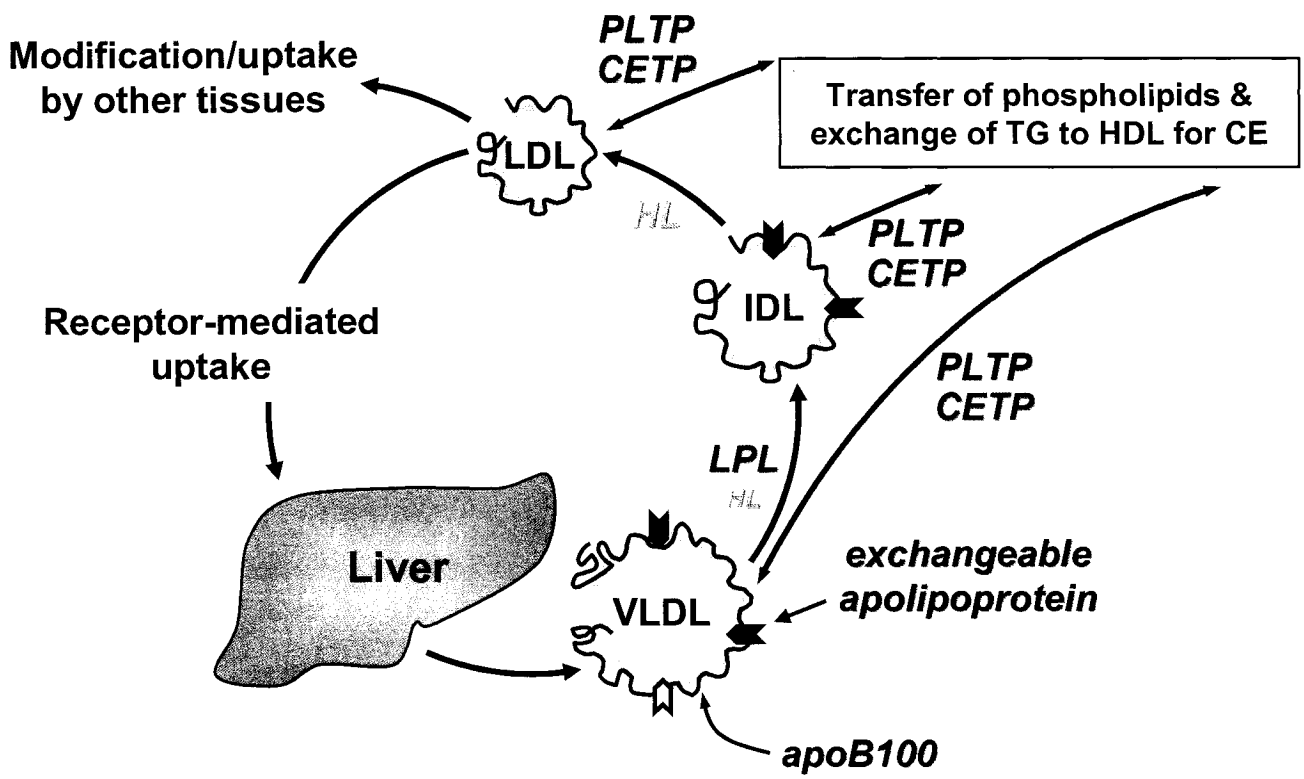
the precursor to the IDL and LDL classes of lipoproteins, and the metabolic processes of these lipoproteins described below are summarized in **Figure 1.2.3**. VLDL are secreted from the hepatocytes into the circulation, and they contain the exchangeable apolipoproteins apoA-I, apoA-II, apoC-II, apoC-III, and apoE (see also **Table 1.2.1**). VLDL provides apoC-II to activate endothelium-associated LPL, which hydrolyses TG to liberate free fatty acids for delivery to various tissues. HL is also thought to hydrolyze VLDL TG and PL (158,159). The hydrolysis of VLDL causes some apolipoproteins to dissociate from the particles and yields the smaller, denser class of lipoprotein IDL. IDL can be removed from the circulation through the association of apoE with the LDLR and LRP (156,160,161), or the IDL TG and PL can be further hydrolyzed exclusively by HL (162). This latter process generates the LDL class of lipoproteins, which are depleted of exchangeable apolipoproteins (see also **Table 1.2.1**). VLDL, IDL, and LDL are not only modified from the hydrolysis of lipids by LPL and HL, but are also modified by the exchange of lipids via the transfer proteins phospholipid transfer protein (PLTP) and CE transfer protein (CETP) with the HDL class of lipoproteins (see Section 1.2.5).

The hydrolysis of VLDL into the smaller and denser LDL class of lipoproteins changes the conformation of apoB. This change exposes a positively charged region on apoB (amino acid residues 3359-3369) that under normal physiological conditions interacts with negatively charged amino acid residues on the LDLR to remove LDL from the circulation (163). LDL that is not efficiently removed from the circulation may be modified by processes such as oxidation to become highly atherogenic (164).

Figure 1.2.3. Metabolism of very low density lipoproteins, intermediate density lipoproteins, and low density lipoproteins

As described in Section 1.2.4, VLDL are produced and secreted by the liver and are the precursor to both IDL and LDL. VLDL provides the apolipoprotein apoC-II, that activates endothelium-associated LPL, which hydrolyses TG to liberate free fatty acids for delivery to various tissues. HL is also thought to hydrolyse these TG and deplete some of the VLDL PL. The hydrolysis of VLDL causes some apolipoproteins to dissociate from the particles and yields the smaller, denser class of lipoprotein IDL. The IDL TG and PL can be further hydrolyzed exclusively by HL. This latter process generates the LDL class of lipoproteins, which are depleted of exchangeable apolipoproteins. Triglycerides from VLDL, IDL, and LDL can be delivered to HDL in exchange for CE from HDL, via the transfer protein CETP. Furthermore, PL can be transferred from VLDL, IDL, and LDL to HDL by the transfer protein PLTP. LDL can be removed from the circulation by different processes (see Sections 1.2.4 and 1.4.3).

Figure 1.2.3


Modification/uptake by other tissues
PLTP
CETP
Transfer of phospholipids & exchange of TG to HDL for CE
LDL
HL
PLTP
CETP
IDL
Receptor-mediated uptake
PLTP
CETP
LPL
HL
Liver
VLDL
exchangeable apolipoprotein
apoB100

1.2.5 High density lipoproteins

High density lipoproteins are produced in the liver, intestines, and circulation, and are metabolized in the circulation. The HDL class of lipoproteins are the most complex class of lipoproteins, that consist solely of exchangeable apolipoproteins and contain at least one molecule of apoA-I (see also **Table 1.2.1**). There are several subclasses of HDL that can be defined on the basis of density, size, charge, and protein composition. Two main classes of HDL have been identified based on density, namely HDL_2 (ρ = 1.063-1.125 g/ml) and HDL_3 (ρ = 1.125-1.21 g/ml) (165). Other density classes of HDL have been isolated, including the very buoyant HDL_1, the very dense HDL_4, and very high density lipoproteins, however their circulating levels are very low (165). Separating HDL by size using gradient gel electrophoresis shows that the diameter of HDL_2 are larger (9-12 nm) than the diameter of HDL_3 (5-9 nm) (166). Furthermore, using the same technique, HDL_2 and HDL_3 can be further separated into additional subclasses based on size (HDL_{2a} and HDL_{2b}; HDL_{3a}, HDL_{3b}, and HDL_{3c}, respectively) (166).

With agarose gel electrophoresis, HDL can be separated on the basis of surface charge (in comparison to the migrations of α- and β-macroglobulins) into three classes: α-migrating HDL, pre-α-migrating HDL, and pre-β-migrating HDL (167). The α-migrating HDL particles are the most electronegative, have a lipid-rich core, and account for at least 85% of circulating HDL-associated apoA-I mass, whereas the pre-β-migrating HDL particles are less electronegative, poorly lipidated, and account for up to 15% of circulating HDL-associated apoA-I mass (167,168). Pre-β-HDL can be further separated by non-denaturing electrophoresis to reveal three subclasses: pre-β_1-HDL (the smallest HDL subclass consisting of one or two apoA-I molecules associated with PL), pre-β_2-

and pre-β_3-HDL (which are generated from pre-β_1- and pre-β_2-HDL, respectively, following the accumulation of PL and free cholesterol) (169).

High density lipoproteins can also be identified based on associated apolipoproteins using immunoaffinity chromatography: HDL containing only apoA-I (LpA-I), and HDL containing both apoA-I and apoA-II (LpA-I/A-II) (170). LpA-I particles account for approximately 30% of the circulating HDL-associated apoA-I mass, and the remainder is associated with LpA-I/A-II (171).

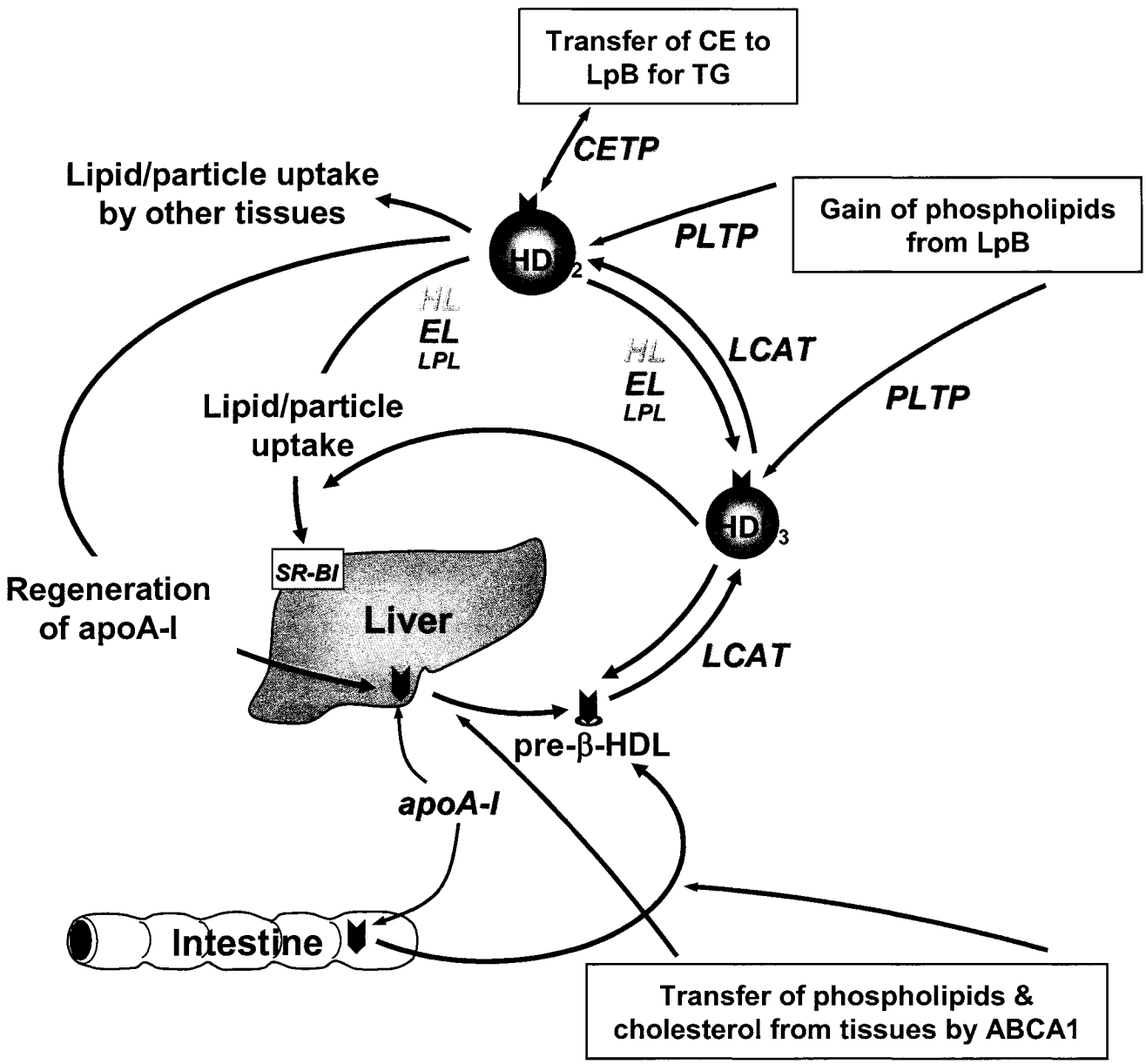
Although there are different methods of classification for HDL species, the classifications share some commonality. The most obvious overlap is that the buoyancy of HDL correlates with the size of HDL: the more buoyant HDL, the larger in size. Both HDL_2 and HDL_3 make up the α-migrating HDL (166). Also, the majority of LpA-I particles are found as HDL_2, whereas the majority of LpA-I/A-II particles are found as HDL_3 (166).

The metabolism of HDL is regulated by a variety of proteins, including the membrane-bound proteins adenosine triphosphate binding cassette transporter A1 (ABCA1) (172) and scavenger receptor class B type I (SR-BI) (173), plasma proteins CETP (174), PLTP (175), and lecithin:cholesterol acyltransferase (LCAT) (176), and the cell-associated lipases EL, LPL, and HL (177). The processes described below in HDL metabolism are summarized in **Figure 1.2.4**. The major protein component of HDL is the exchangeable apolipoprotein apoA-I, which is primarily synthesized by the liver and intestines. ApoA-I acquires a small amount of lipid (possibly during the synthesis and secretion of apoA-I from cells) to form the very small and dense lipoprotein subclass pre-β_1-HDL (169). Pre-β_1-HDL can also be generated in the circulation during the

Figure 1.2.4. Metabolism of high density lipoproteins

As described in Section 1.2.5, the metabolism of HDL is very complex. The major protein component of HDL, apoA-I, is primarily synthesized by the liver and intestines. ApoA-I acquires a small amount of lipid to form the very small and dense lipoprotein subclass pre-β_1-HDL. The membrane-bound protein ABCA1, which is found in a variety of tissues, transfers membrane PL and free cholesterol to the pre-β-HDL particles to make larger pre-β-HDL particles. The pre-β-HDL gains additional lipid in the circulation by a combination of the transfer of PL from apoB-containing lipoproteins to HDL by PLTP, and the esterification of free cholesterol by LCAT using a fatty acyl chain from phosphatidylcholine. The actions of PLTP and LCAT generate the more buoyant subclass HDL_3. The HDL_3 gains additional PL and CE through the same PLTP/LCAT pathway to generate the more buoyant HDL_2 subclass. Particles of HDL_2 can become TG-enriched in the circulation through the action of the plasma protein CETP, which exchanges CE for TG from HDL to apoB-containing lipoproteins. The lipases LPL, HL, and EL can hydrolyze the TG and PL of the HDL particles to liberate free fatty acids which can be transferred to various tissues and generate smaller, denser HDL. The membrane protein SR-BI, found in various tissues, promotes the uptake of CE, free cholesterol, and PL from HDL into tissues. Together, these processes recycle the apoA-I particles to generate pre-β-HDL. HDL can also be removed from the circulation (see Section 1.4.4).

Figure 1.2.4


Transfer of CE to LpB for TG
CETP
Lipid/particle uptake by other tissues
HDL2
PLTP
Gain of phospholipids from LpB
HL
EL
LPL
LCAT
HL
EL
LPL
PLTP
Lipid/particle uptake
HDL3
SR-BI
Liver
Regeneration of apoA-I
LCAT
pre-β-HDL
apoA-I
Intestine
Transfer of phospholipids & cholesterol from tissues by ABCA1

metabolism of VLDL, whereby apoA-I can acquire a small amount of lipid from the apoB-containing lipoprotein as it dissociates (178). The membrane-bound protein ABCA1, which is found in a variety of tissues, has been demonstrated through an unknown mechanism to transfer membrane PL and free cholesterol to the pre-β-HDL particles to generate discoidal HDL (179), so named because of its shape as identified by electron microscopy (180). The discoidal HDL gains additional lipid to become the more buoyant HDL subclass HDL_3. The lipid is gained in the circulation by a combination of the transfer of PL from apoB-containing lipoproteins to discoidal HDL by PLTP (175), and the esterification of free cholesterol by LCAT using a fatty acyl chain from phosphatidylcholine to generate CE and lysophosphatidylcholine (176). The excess PL aids in supporting an enlarged neutral lipid core that holds the increased CE content. The HDL_3 gains additional PL and CE through the same PLTP/LCAT pathway to become the more buoyant HDL subclass HDL_2. Particles of HDL_2 can become TG-enriched in the circulation through the action of the plasma protein CETP, which exchanges CE for TG from HDL to apoB-containing lipoproteins (174).

The fate of the buoyant HDL_2 is complex and results in the delivery of various lipids to several tissues, especially to the liver and steroidogenic tissues (181,182). The delivery of lipids from peripheral tissues back to the liver for excretion is termed "reverse cholesterol transport", as opposed to "forward cholesterol transport" whereby cholesterol is transported from the liver to peripheral tissues (183). The lipases LPL, HL, and EL can hydrolyze the TG and PL of the HDL particles to liberate free fatty acids, monoglycerides, and lyso-PL, which can be transferred to various tissues (177). The

membrane protein SR-BI promotes the uptake of CE, free cholesterol, and PL into tissues (173,181). Together, these processes recycle the apoA-I particles to generate pre-β-HDL.

Unlike chylomicron remnants (generated from chylomicrons) or LDL (generated from VLDL) that are atherogenic, apoA-I-containing HDL are thought to be anti-atherogenic lipoproteins. Low circulating levels of HDL in patients with cardiovascular disease is the most common lipoprotein abnormality. Proposed protective roles by HDL include reverse cholesterol transport from periphery tissues to the liver (184), antioxidant effects against LDL oxidation (185), inhibition of inflammation through actions of the associated protein paraoxonase (186), and enhancement of the production of nitric oxide (187).

1.3 Heparin binding by lipases

1.3.1 Heparin and heparan sulfate proteoglycans

Heparin is a heterogeneously-sized and complex polysaccharide that is produced and stored in cytoplasmic secretory granules of mast cells from connective tissues (188). Purified heparin has been used clinically since 1935 as a potent anticoagulant (189). Endogenously-produced heparin plays important roles in mast cells. In mice deficient in N-deacetylase/N-sulfotransferase 2 (which is necessary for heparin production), the levels of various proteases normally found in the cytoplasmic secretory granules were markedly reduced (190). The latter study suggested that the negatively-charged heparin (described below) carries positively-charged proteases into the granules. Histamine, which is stored in the cytoplasmic secretory granules of the mast cells, is also greatly reduced in N-deacetylase/N-sulfotransferase 2 deficient mice (190,191). Histamine has been shown by nuclear magnetic resonance studies to bind heparin (192), thus heparin is believed to be involved in the storage of histamine in the secretory granules.

The complex polysaccharide of heparin consists of subunits of disaccharides containing uronic acid and glucosamine (GlcN), which can be sulfated and acetylated resulting in a negatively charged chain (193). The uronic acid component can be either α-L-iduronic acid (IdoA) or β-D-glucuronic acid (GlcA), and they can both be 2-O-sulfated (abbreviated IdoA(2S) and GlcA(2S), respectively). The 1-hydroxyl group of the uronic acid is linked with the 4-hydroxyl group of the GlcN component of the disaccharide. The GlcN component can either be N-acetylated (abbreviated GlcNAc) or N-sulfated (abbreviated GlcNS), and both GlcNAc and GlcNS can be 6-O-sulfated

(abbreviated GlcNAc(6S) and GlcNS(6S), respectively). Furthermore, both GlcNS and GlcNS(6S) can be 3-O-sulfated (abbreviated GlcNS(3S) and GlcNS(3,6S), respectively). Altogether, these combinations yield 24 different disaccharide units that can make up the heparin polysaccharide (193). These combinations are summarized in **Figure 1.3.1**.

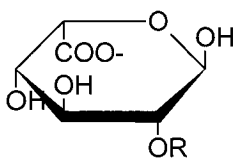
Heparin is linked with a protein called serglycin; the complex is termed as a heparin proteoglycan (194). Serine residues within various 'linkage motifs' of the serglycin and the heparin chains are linked through a tetrasaccharide of xylose (Xyl), which is covalently bound to the serine, two galactose (Gal), and a GlcA (yielding the chain β-GlcA-(1,3)-β-Gal-(1,3)-β-Gal-(1,4)-β-Xyl-Ser) (195,196). The monosaccharides GlcNAc and GlcA are alternately added and linked in 1→4 linkages to generate a polysaccharide chain of $[GlcA\text{-}(1,4)\text{-}GlcNAc]_n$ subunits (193). N-acetyl groups of GlcNAc are randomly removed and sulfated in the same reaction (to produce GlcNS), and this N-deacetylation/N-sulfation continues along the polysaccharide until a single GlcNAc-containing subunit is surrounded by two GlcNS-containing subunits (193). Following this sulfation step, the GlcA units can be randomly converted into IdoA by epimerization of the 5-hydroxyl group (197-201). This epimerization permits the 2-O-sulfation of IdoA by 2-O-sulfotransferase (yielding IdoA(2S)), and the 6-O-sulfation of GlcNS by 6-O-sulfotranferase (yielding GlcNS(6S)) (193,202). However, IdoA cannot be sulfated if the adjoining GlcNS is already 6-O-sulfated. Though to a lesser extent than the 2-O-sulfation of IdoA and the 6-O-sulfation of GlcNS, additional sulfation reactions also occur: 2-O-sulfation of GlcA, 6-O-sulfation of GlcNAc, and 3-O-sulfation of both GlcNS and GlcNS(6S) (203-206). The resulting and very complex heparin polysaccharide chain can then be randomly cleaved at some of the GlcA locations by the

Figure 1.3.1. Building blocks of heparin and heparan sulfate

Heparin and heparan sulfate chains comprise of disaccharides of a uronic acid and glucosamine (GlcN). The two uronic acids utilized are α-L-iduronic acid (IdoA) and β-D-glucuronic acid (GlcA), both of which can be 2-O-sulfated (yielding IdoA(2S) and GlcA(2S), respectively. The GlcN may be sulfated (GlcNS) or acetylated (GlcNAc), and these may be further sulfated as shown. Altogether, these building blocks provide 24 different disaccharide subunits.

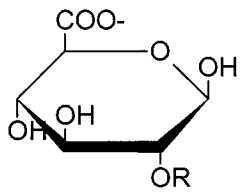
Figure 1.3.1

α-L-iduronic acid



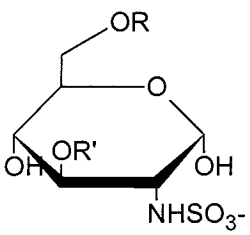
IdoA	R = H
IdoA(2S)	R = SO_3^-

β-D-glucuronic acid



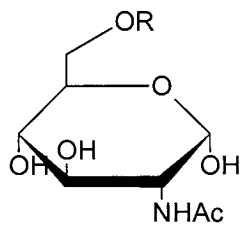
GlcA	R = H
GlcA(2S)	R = SO_3^-

N-sulfo-α-D-glucosamine



GlcNS	R = R' = H
GlcNS(6S)	R = SO_3^- , R' = H
GlcNS(3S)	R = H, R' = SO_3^-
GlcNS(3,6S)	R = R' = SO_3^-

N-acetyl-α-D-glucosamine



GlcNAc	R = H
GlcNAc(6S)	R = SO_3^-

enzyme endo-β-D-glucuronidase to liberate free heparin chains of various lengths (193).

Heparan sulfate (which was originally named heparitin sulfate) was identified as an impurity in the preparation of heparin (207). Heparan sulfate, like heparin, consists of subunits of disaccharides containing uronic acid and GlcN and bound to a core protein associated with the cell membrane (193); the complex is termed as a heparan sulfate proteoglycan, or HSPG. However, unlike heparin, heparan sulfate is very rarely cleaved from its protein. HSPGs have been found in almost all mammalian cell types, and there are various families of HSPGs characterized by the core protein (208). The difference between heparin and heparan sulfate lies within the modifications of the polysaccharide chain. There are an approximately equal number of GlcNS and GlcNAc units in the heparan sulfate chain, unlike heparin which has approximately four-fold more GlcNS units than GlcNAc units (208). Heparan sulfate also has at least 3-fold less N- and O-sulfation versus heparin (209), thus the highly-sulfated, more negatively-charged heparin can readily displace proteins bound to heparan sulfate. There is also a wide variation in heparan sulfate produced between tissues, likely due to the wide variety of isoforms of enzymes responsible for the modifications to the $[GlcA\text{-}(1,4)\text{-}GlcNAc]_n$ subunits (210-213).

1.3.2 Heparin binding motifs

Various functions are associated with HSPG, and involve the interactions of proteins at the cell surface with the heparan sulfate chains of HSPG. These functions include the regulation of cell growth and proliferation, angiogenesis, blood coagulation, viral infection, and some processes in lipoprotein metabolism (208). Although proteins

that bind to HSPG may use various non-covalent interactions such as van der Waals interactions, the binding of proteins to HSPG primarily involve electrostatic interactions by basic amino acid residues interacting with the negatively-charged sulfate groups on the heparan sulfate chains (214) that can be interrupted by increasing salt concentrations. Attempts to identify consensus sequences or motifs for the binding of proteins to HSPG and heparin have yielded sequences on the basis of both linear and tertiary protein structures (215-217). Common structural motifs were identified when sequences of a number of heparin binding proteins were compared (214); they are xBBxBx, xBxxBBBx, and xBBxxBBBxxBBx, respectively, where "B" represents a basic amino acid and "x" represents any amino acid. In the case of antithrombin III and apoE, a distinct distribution of two basic amino acids located about 20 Å apart (xBxxxxxxxxxxxxBx in an α-helix structure and xBxxxxxxBx in a β-strand structure) has been suggested to be critical in HSPG binding (218).

1.3.3 Heparin binding by lipoprotein lipase, hepatic lipase, and endothelial lipase

Lipoprotein lipase, HL, and EL are heparin binding proteins (219). Lipoprotein lipase and HL have been compared and contrasted for their heparin binding affinities by using heparin-affinity column chromatography. Studies with chimeric proteins of LPL and HL have suggested that the C-terminal domains of HL and LPL play a role in determining their heparin affinity. The affinity of HL and LPL towards heparin can be estimated from the concentration of NaCl required to elute the lipases from immobilized heparin: 1.1 M NaCl to elute LPL and 0.75 M NaCl to elute HL (119). The heparin affinity of LPL is thus higher than that of HL. For chimeric proteins in which the C-

terminal sequences of human LPL were substituted with either rat (119) or human HL (117) sequences, reduced heparin-binding affinity of the chimeric proteins was observed. From these studies, it has been suggested that the major determinant of heparin binding resides within the C-terminal domain of hHL.

Through various site-directed mutagenesis studies and studies with peptide sequences, specific basic amino acid residues have been identified in LPL (220-226) to be involved in heparin binding. Peptides containing sequences of human LPL encompassing amino acid residues Lys^{403}, Arg^{405}, and Lys^{407} have been shown to be involved in heparin binding (220). However in another study, peptides containing Arg^{405} and Lys^{407} were shown to not be involved in heparin binding (221). The three analogous amino acid residues in avian LPL (Arg^{405}, Arg^{407}, and Lys^{409}) have been shown by site-directed mutagenesis to indeed be involved in heparin binding. Thus, the three amino acid residues together may constitute a single heparin binding domain (HBD) that does not align with previously proposed HBD sequences (214,218). In mutagenesis studies with human LPL, amino acid residues Arg^{279}, Lys^{280}, Arg^{282} (which fit the xBBxBx HBD sequence (214)), and amino acid residues 291-304 (which has homology to the xBBBxBx HBD sequence (214)), were shown to be involved in heparin binding (223,224). However, basic amino acid residues alone may not account for all of the heparin binding properties of LPL; peptides containing the amino acid residues 388-395 of human LPL (Phe-Ser-Trp-Ser-Asp-Trp-Trp-Ser) also show strong binding to heparin (221). Very little work has been done to elucidate the HBDs of HL. One study using site-directed mutagenesis on rHL shows that six basic amino acid residues located in two clusters (Cluster 1: Lys^{297}, Lys^{298}, and Arg^{300}; Cluster 4: Lys^{436} and Arg^{443}) were involved in

heparin binding (227). These two clusters of basic amino acid residues have the xBBxBx and xBxxxxxxBx HBD sequences, respectively.

1.3.4 Comparison of human and mouse hepatic lipase heparin binding

The heparin binding properties between human and rat HL are similar, such that they have a similar affinity to heparin-Sepharose (39). Infusing heparin *in vivo* increases hHL activity in the serum by 1000-fold, suggesting a strong interaction of hHL with HSPG (39). In contrast to that of hHL, the major proportion of mHL mass and activity (60–70% of total releasable by intravenous heparin administration) circulates in the plasma (39). It should be noted that in animal studies where heparin is administered intraperitoneally (IP), plasma mHL activities are indifferent from plasma mHL activities in non-heparinized animals (228), due to an insufficient delivery of heparin to the liver. The low affinity of mHL to the cell surface could be attributable to the lack of glycosaminoglycans specific for HL binding that are present in humans but not in mice. Alternatively, the low affinity of mHL could be due to the lack of amino acid sequence elements that are present in human but not in mouse HL. Two pieces of evidence suggest that the determinants of HSPG binding lie within the amino acid sequence of HL. First, infusion of hHL into mice resulted in nearly 100% association of hHL mass and activity with HSPGs (39). Second, mHL has a lower affinity for heparin versus hHL as demonstrated by its earlier elution from heparin-Sepharose; 0.7–0.8 M NaCl was required to elute hHL (39,115,229-231), and 0.48 M NaCl for mHL (39). The difference in heparin binding between human and mouse HL may not be the only difference between these two HL species (as demonstrated within this thesis).

1.4 Liganding functions of hepatic lipase in lipoprotein metabolism

1.4.1 Overview

As well as being an enzyme, HL is shown to have a ligand activity that can enhance the binding and uptake of all classes of lipoproteins independent of its catalytic activity. However many of these binding and uptake processes may be enhanced by the catalytic activity of HL. The binding and uptake of the various lipoproteins with HL generally involve a receptor and/or an association of HL with HSPGs.

1.4.2 Liganding functions of hepatic lipase with remnant lipoproteins

In rats injected with anti-rHL antibodies, the removal of chylomicron remnants from the circulation was two-fold slower compared with control rats (232). This suggested an involvement of HL in the removal of these lipoproteins from the circulation. The involvement of HL in the removal of remnant lipoproteins was also demonstrated with isolated rat livers preperfused with either heparin or anti-HL antibodies, which displayed reduced hepatic uptake of remnants and reduced hydrolysis of remnant TG (103). In rat hepatoma cells overexpressing hHL, the majority of the hHL was cell-associated, and it accounted for a three-fold increase in remnant lipoprotein binding and uptake compared with control cells (233). The enhanced uptake was abolished by treatment of the cells with heparinase (to digest and degrade HSPGs) and chlorate (which inhibits HSPG synthesis). Chinese hamster ovary (CHO) cells with impaired proteoglycan synthesis, and wild-type CHO cells incubated with hHL in the presence of heparin, showed reduced remnant lipoprotein uptake compared with wild-type CHO cells

incubated with hHL in the absence of heparin (234). Furthermore, studies with primary rat hepatocytes incubated with purified active or heat-inactivated HL show that both species of HL could enhance the uptake of remnant lipoproteins (235). The ability for inactive hHL to efficiently enhance the clearance of remnant lipoproteins was also demonstrated in mice. Overexpression of either hHL or a catalytically inactive hHL (by mutagenesis of the catalytic active site $Ser^{145} \rightarrow Gly$) in mice deficient in apoE (236) or the LDLR (237) reduced the levels of apoB-containing lipoproteins, including remnant lipoproteins.

The removal of remnant lipoproteins from the circulation is thought to be mediated primarily through the interactions of apoE (bound to the lipoproteins) with both HSPGs, and the LDLR and the 'remnant lipoprotein receptor' LRP (as reviewed in (155)). Remnant-associated apoE is a key ligand for both the LDLR and LRP, for the binding and uptake of remnant lipoproteins. Humans with naturally-occuring mutations of apoE exhibit dramatically elevated levels of remnant lipoproteins in the circulation (see reviews (238-242)). Such a phenotype has also been demonstrated in mice deficient in apoE (243), and in mice with inducible knockout of LRP (244). (A complete knockout of LRP is embryonic-lethal). Since HL has been shown to play a role in the removal of remnant lipoproteins from the circulation, it was thought that HL may also interact with LRP. Crosslinking studies showed that purified hHL interacts with human LRP from human hepatoma cells (234). Binding studies also show that purified hHL can interact with LRP, and this interaction can be blocked by the LRP chaperone termed receptor-associated protein (RAP, which very tightly binds LRP) (245). However, HL-deficient mice do not have elevated levels of remnant lipoprotein in the circulation and exhibit a

normal rate of chylomicron clearance (104). The normal rate of clearance was shown to be due to an upregulation of apoE in these mice, which may compensate for the loss of any liganding roles by HL (246). Whether the removal of remnant lipoproteins from the circulation is by an interaction with LRP and/or HSPGs remains unclear.

1.4.3 Liganding functions of hepatic lipase with very low density lipoproteins and low density lipoproteins

In apoE knockout mice, the adenovirus-mediated overexpression of wild-type hHL or a catalytically inactive hHL resulted in enhanced selective uptake of CE from VLDL, without affecting the levels of apoB-associated VLDL as compared with control mice (247). Selective uptake (which is different from particle uptake) is a process whereby one or more types of lipoprotein lipid can be removed from the circulation without removing the protein component of the lipoprotein. These data suggested a liganding function by HL with VLDL, however the mechanism for the selective uptake is not defined.

The overexpression of rHL (248), or the overexpression of a membrane-anchored rHL (anchored by glycosylphosphatidylinositol) (249) in CHO cells lead to enhanced binding and degradation of LDL compared to control cells. The binding and degradation of LDL by HL could be inhibited by adding anti-LDLR antibodies to the cells. However, the overexpression of wild-type hHL or catalytically inactive hHL in LDLR knockout mice reduces the levels of LDL (236,237), suggesting that HL may remove LDL from the circulation through a mechanism independent of the LDLR. Cell culture studies show that the binding and clearance of LDL by CHO cells overexpressing rHL was not through

LRP (248). Thus, the clearance of LDL may be mediated through the binding of HL to both LDL (likely through a protein-protein interaction with apoB (250)) and HSPGs, or perhaps to other unidentified receptors.

1.4.4 Liganding functions of hepatic lipase with high density lipoproteins

In contrast to wild-type control mice, *LIPC* knockout mice have an impaired ability to remove cholesteryl ester from HDL in the plasma, even though the fractional catabolic rates of the HDL proteins apoA-I and apoA-II were comparable (251). This shows that HL is involved in the selective uptake process of HDL lipids, which was suggested in early cell culture studies prior to the identification of the HL cDNA. The early studies in which purified HL was incubated with HDL resulted in the generation of smaller, denser HDL particles that were able to selectively deliver cholesterol to the cells better than HDL that was not incubated with HL (252). In *LIPC* knockout mice, adrenal levels of SR-B1 mRNA were increased even though female mice had decreased cholesterol levels (253). This suggested that HL and SR-B1 played an integral role in the selective uptake of HDL lipids. This was confirmed in cell culture studies that showed the co-overexpression of both hHL and SR-B1 in the human embryonic kidney 293 (HEK293) cell line had a synergystic increase in the uptake of CE from HDL above the levels of uptake by hHL or SR-B1 alone (254). Furthermore, the co-overexpression of a catalytic-inactive hHL and SR-B1 also resulted in increased uptake of CE from HDL. The enhanced selective uptake in the presence of active or inactive hHL was impaired by heparin. Active and inactive HL have also been shown to be involved in the selective uptake of CE from HDL using human hepatoma cells (255). In addition to the combined

role of HL and SR-B1 in the selective uptake of HDL lipid, the overexpression of hHL in rat hepatoma cells has been shown to enhance the binding and uptake of HDL through a mechanism involving HSPGs (heparinase impairs binding and uptake) and partially through LRP (RAP reduced binding and uptake) (256). LRP alone has been shown in adipose cells to be involved in the selective uptake of CE from HDL (257).

1.5 Rationale and hypotheses

The function of hHL binding to HSPG is unclear. Studies addressing the liganding functions of HL suggest that an association of HL with HSPG is necessary (see Section 1.4), however these studies utilized heparin or heparinase, both of which also indiscriminantly remove other HSPG binding proteins from cell surfaces that may also potentially play a liganding role with lipoproteins. Therefore, to address the HSPG binding properties of hHL alone with respect to liganding functions, it was necessary to identify and disrupt the HSPG binding properties of hHL. **The first tested hypothesis of the current study is that the C-terminal region of hHL confers high-affinity cell surface binding activity through interaction with HSPG.** This was tested using chimeric HL proteins. The data shown in the Results (Chapter 3) support the notion that the difference in cell surface association between human and mouse HL is caused by the divergence in the C-terminal amino acids between the two proteins. Portions of these data have been published recently (258). One of the chimeric HL proteins (designated as hHLmt) in these studies shares the catalytic properties of hHL and the *in vitro* HSPG binding properties of mHL. The chimeric hHLmt was subsequently used to address the second hypothesis (see below).

The displacement of lipases from cell surfaces by heparin *in vivo* rapidly reduces lipemia (11). This raises the notion that the lipolytic functions of HL (in the absence of heparin) may not actually require cell surface binding to HSPG. A previous study demonstrated that hHL could be displaced from purified HSPG by exogenous HDL and by apoA-I, but not by other lipoproteins (120). This displacement of hHL by HDL and

apoA-I was also recently demonstrated using cell culture models, and the displaced hHL could efficiently hydrolyze the TG from VLDL (125). From these studies, it may be inferred that the displaced hHL entering the circulation may rapidly enhance lipoprotein metabolism. The idea that HL (and LPL) needs to be displaced to hydrolyse TG and PL has been postulated for apoB containing lipoproteins. Pre-heparin activities for both HL and LPL directly correlate with LDL cholesterol levels, suggesting that a greater displacement of HSPG-associated HL and LPL results in increased metabolism of apoB containing lipoproteins to generate LDL (259). **The second tested hypothesis of this study is that increases in circulating hHL activity *in vivo* in the absence of heparin are responsible for rapid metabolism of lipoproteins in the circulation**. Portions of this work have also been recently published (260), showing that at low levels of expression the HSPG binding-impaired chimeric hHLmt activity in pre-heparin plasma rapidly reduced HDL lipid and protein *in vivo*.

CHAPTER 2 – EXPERIMENTAL PROCEDURES

2.1 Materials

C57BL/6J mice were purchased from Charles River Laboratories (Wilmington, MA, United States). Transgenic C57BL/6J mice expressing hHL were generated in-house as previously described (261), and have been deposited at The Jackson Laboratory as strain TgN(LIPC)6784His. The bacterial cell line DH5α and the mammalian cell lines COS-7 and HEK293 were purchased from the American Type Culture Collection (Manassas, VA, United States). CHO cells deficient in LRP expression (designated as CHO 13-5-1) (262) were a generous gift of Dr. David FitzGerald (University of Texas Southwestern Medical Center, Dallas, TX, United States). Triolein, tributyrin, 1,2-dipalmitoyl phosphatidylcholine (DPPC), cholesterol, fatty acid-free bovine serum albumin (FAF-BSA), heparin, fibronectin, fumed silica, and fetal bovine serum (FBS) were purchased from Sigma (Oakville, ON, Canada). Dulbecco's modified Eagle medium (DMEM), Ham's F12 medium, Eagle's minimum essential medium (EMEM), William's medium, Hepatozyme, penicillin, streptomycin, Fungizone, and G418 were purchased from Gibco BRL/Invitrogen (Burlington, ON, Canada). The Hi-Trap heparin-Sepharose columns, Superose-6 columns, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody, HRP-conjugated goat anti-rabbit IgG antibody, and [^{35}S]methionine/cysteine were purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada). [^{3}H]triolein was purchased from Dupont. [^{14}C]tributyrin and

[^{14}C]DPPC were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, United States). Enzymes used for subcloning (KpnI, HindIII, BamHI, DpnI, PflmI, XbaI, PacI, PmeI, and T4 DNA ligase) were purchased from New England Biolabs (Mississauga, ON, Canada). Pfu DNA polymerase was purchased from Stratagene (La Jolla, CA, United States). A polyclonal anti-hHL antibody (263) was a generous gift of Dr. Ann White (University of Texas Southwestern Medical Center, Dallas, TX, United States). The monoclonal anti-hHL antibody XHL3-6 (231) was a gift of Dr. André Bensadoun (Cornell University, Ithaca, NY, United States). A polyclonal anti-rat HL antibody was a gift of Dr. Howard Wong (University of California, Los Angeles, CA, United States). Rabbit polyclonal antibodies against mouse apoA-I, apoB, and apoE were purchased from Biodesign International (Saco, ME, United States). Purified hHL was isolated from post-heparin human plasma as described previously (120). The AdEasy adenoviral system was purchased from Q-Biogene (Carlsbad, CA, United States). The LIPO lipoprotein agarose gel electrophoresis system was purchased from Beckman Coulter (Fullerton, CA, United States). Total cholesterol (TC) and PL assay kits were purchased from Roche (Laval, QC, Canada).

2.2 Methods

2.2.1 Preparation of hepatic lipase expression plasmids

The hHL cDNA was excised from the pLiv10.hHL vector (provided by Dr. John Taylor at The Gladstone Institute, San Francisco, CA, United States) (264) by digestion with KpnI and HindIII, and the KpnI-HindIII fragment was inserted into the polylinker region of the pCMV5 expression vector (265) to create pCMV5.hHL. Similarly, the mHL cDNA was excised from the pSP64.mHL vector (provided by Dr. Hans Will at Hamburg University, Hamburg, Germany) (17) by digestion with HindIII and XbaI, and the HindIII-XbaI fragment was inserted into pCMV5 to create pCMV5.mHL. To create the chimeric construct hHLmt encoding amino acids 1–406 of hHL and amino acids 409–488 of mHL, the respective hHL and mHL cDNAs were excised from pCMV5.hHL and pCMV5.mHL by digestion with KpnI and XbaI, and subcloned into pBluescript (pBlue.hHL and pBlue.mHL). A PflmI-XbaI fragment was excised from pBlue.hHL and replaced with a PflmI-XbaI fragment from pBlue.mHL, generating an in-frame chimeric HL construct (pBlue.hHLmt). The KpnI-XbaI fragment was excised from pBlue.hHLmt and inserted into pCMV5 to create pCMV5.hHLmt. A chimeric construct encoding the amino acids 1–408 of mHL and the amino acids 407–476 of hHL, designated pCVM5.mHLht, was similarly constructed. A second pCMV5.mHLht (designated pCMV5.mHLht$_{DN}$) construct was generated to convert amino acid residue Asp^{410} (corresponding to amino acid residue 408 from hHL) to Asn. Forward (CAGTGTGGGCCAATGTCTGGAACACGGTCCAGACCATCATC) and reverse (GATGATGGTCTGGACCGTTGTCCAGACATTGGCCCACACTG) primers were

used to introduce the mutation (underlined). The product was generated using Pfu DNA polymerase, and the reaction was digested with DpnI to remove trace pCMV5.mHLht template. The completed polymerase chain reaction (PCR) mixture was transformed into DH5α bacteria and positive clones were screened by sequence analyses. To generate the plasmid encoding hHL_{471}, forward (ACGTAAGCTTGCCACCATGGACACAAGTCCCCTGTGT) and reverse (ACGTGGATCCTCATCTGATCTTTCGCTATGATGT) PCR primers were designed such that the C-terminal five residues of wild type hHL were eliminated. The hHL_{471} PCR product was generated in collaboration with Dr. John Hill (University of British Columbia, Vancouver, BC, Canada). The PCR product was inserted into pCMV5 using the HindIII and BamHI restriction sites that were encoded within the primers (underlined). All wild-type and mutant HL constructs were sequence verified to ensure no errors were generated, and were transiently transfected (266) into COS-7 cells (cultured in DMEM containing 10% FBS) to ensure the HL proteins could be expressed prior to stable cell line generation (described below).

2.2.2 Generation of stable Chinese hamster ovary cell lines expressing recombinant hepatic lipase

CHO 13-5-1 cells were cultured in Ham's F-12 medium containing 10% FBS. Cell lines were generated by co-transfecting 10 μg of the corresponding pCMV5 plasmid with 0.10 μg pSV2*neo* using the calcium precipitation method (266). Stable cell lines were selected with 500 μg/ml G418 and screened for HL expression by immunoblot analysis (details are described in figure legends). Antibodies were diluted 1:5,000.

2.2.3 Semi-purification of recombinant hepatic lipase by heparin-Sepharose chromatography

Stably transfected cells were grown to confluency (T175 flasks), washed three times with phosphate buffered saline (PBS), and incubated overnight at 37 °C with Ham's F-12 medium containing 1% FBS, 500 μg/ml G418, and 10 U/ml heparin. Media (up to 400 ml) were collected, centrifuged at 1200 rpm for 10 min at 4 °C to remove any cell debris, and the supernatant was adjusted with glycerol to a final concentration of 20%. The media was cooled to 4 °C, then applied to a Hi-Trap heparin-Sepharose column at 4 °C pre-equilibrated with 90% Buffer A (10 mM sodium phosphate; 20% glycerol; pH 7.2) and 10% Buffer B (10 mM sodium phosphate; 1.5 M sodium chloride; 20% glycerol; pH 7.2). Following re-equilibration of the column, 33% Buffer A and 67% Buffer B (final NaCl concentration of 1.0 M) was used to elute bound proteins. The fractions (1 ml) were collected and those containing protein (as detected using the absorbance at 280 nm) were stored at -80 °C. In other experiments, confluent cells (T175 flasks) were washed three times with PBS and incubated with 20 ml serum-free media containing 100 U/ml heparin. Following 4 h incubation at 37 °C, the media were treated as above and applied to a Hi-Trap heparin-Sepharose column at 4 °C pre-equilibrated with 90% Buffer A and 10% Buffer B. Following re-equilibration of the column, a gradient of Buffer A and Buffer B (NaCl concentration from 0.15–1.5 M) was used to elute the bound proteins. The fractions (1 ml each) were collected and stored at -80 °C.

2.2.4 Measurement of hepatic lipase activity

The activity of HL obtained by chromatography was determined using a [^{3}H]triolein emulsion (267), and using [^{14}C]tributyrin (268) and [^{14}C]DPPC (105), as previously described. Briefly, varying amounts of triolein emulsion (final reaction concentrations 0.35-17.3 mM, with 3.2 μCi [^{3}H]triolein) were incubated for 1 h with up to 2.2 μg semi-purified HL (together with final concentrations 0.1 mM Tris-HCl, 1 M NaCl, 1.5 mM $CaCl_2$, 1% FAF-BSA, pH 8.3). [^{3}H]oleate was extracted and quantified. Varying amounts of tributyrin (final reaction concentrations 0.01-0.50 mM with 0.025 μCi [^{14}C]tributyrin) were incubated for 30 min with semi-purified HL (together with final concentrations 50 mM Tris-HCl, 1 M NaCl, 2% acetonitrile, 2% FAF-BSA, pH 8.3). [^{14}C]butyrate was extracted and quantified. Varying amounts of DPPC (final reaction concentrations 0.016-0.33 mM with 0.07 μCi [^{14}C]DPPC) were incubated for 1 h with semi-purified HL (together with final concentrations 50 mM Tris-HCl, 1 M NaCl, 0.75% FAF-BSA, 4.6 mM cholesteryl oleate, pH 8.0). [^{14}C]palmitate was extracted and quantified. Plasma HL activities (using 10 μl plasma) were measured using the [^{3}H]triolein emulsion. Protein content of HL samples was determined using a modified Lowry assay (269).

2.2.5 Heparin treatment of cells

Stably transfected cells (100-mm dishes) were grown to confluency, washed three times with PBS, and incubated with either 4 ml serum-free Ham's F-12 medium supplemented with G418 or serum-free media containing 100 U/ml heparin. After 4 h incubation at 37 °C, the media were collected, centrifuged at 1200 rpm for 10 min to

remove any cell debris, and the HL proteins in the supernatant were concentrated with fumed silica (50 mg) by overnight incubation at 4 °C (256). The absorbed HL proteins were eluted from fumed silica with 200 µl of lysis/gel loading buffer (38.5 mM Tris-HCL, 0.1% EDTA, 2% SDS, 6 M urea, 0.1% dithiothreitol, 0.05% reduced glutathione, 0.001% bromophenol blue) by occasional mixing at 90 °C for 20 min, then stored at -80 °C until needed for immunoblot analysis.

2.2.6 Pulse-chase analyses

Confluent cells (60-mm dishes) were washed three times with PBS and pulse-labeled with [^{35}S]methionine/cysteine (200 µCi/ml) for 2 h in 2 ml methionine/cysteine- and serum-free DMEM. Media were replaced and cells were chased for 2 or 4 h in 2 ml serum-free media ±100 U/ml heparin. The media were collected at the end of chase, and an aliquot (1 ml) was mixed with 5 µl of the anti-hHL polyclonal antibody overnight at 4 °C. The cells were lysed with RIPA buffer (50 mM Tris-HCL, pH 8.0, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1 mM dithiothreitol, 150 mM NaCl, 0.015% phenylmethylsulfonyl fluoride, 0.1% SDS), and cell-associated HL was likewise immunoprecipitated from the cell lysates. The antibody-HL complexes were adsorbed to Protein A and washed six times with PBS (for medium samples) or RIPA buffer (for cell samples). The HL was eluted from Protein A with 200 µl of gel loading buffer at 100 °C for 10 min, separated by electrophoresis on 10% polyacrylamide gel containing 0.1% SDS (SDS–PAGE), and analyzed by fluorography.

2.2.7 Generation of hepatic lipase adenoviruses

Adenoviruses were generated using the AdEasy adenoviral system. Briefly, a luciferase cDNA, and the hHL and hHLmt cDNAs (in the pCMV5 constructs as described in Section 2.2.1) were subcloned into an *E1/E3*-deleted recombinant adenovirus vector backbone containing the cytomegalovirus promoter to generate pAd.luc, pAd.hHL, and pAd.hHLmt, respectively. The pAd plasmids were transfected into the HEK293 cell line and positive plaques containing adenoviruses expressing luciferase (Ad.luc), hHL (Ad.hHL), and hHLmt (Ad.hHLmt) were propagated using the HEK293 cell line in confluent 150-mm dishes. The adenoviruses were purified by CsCl density gradient ultracentrifugation and dialyzed in PBS. The desalted virus stock was adjusted to 4% sucrose, quantified by measuring the absorbance at 260 nm, and stored at -80 °C until required.

2.2.8 Animals and primary hepatocyte cultures

Female C57BL/6J mice (6-8 week old) and female TgN(LIPC)6784His mice (6-8 week old) were maintained on a normal chow diet and 12 h light/12 h dark cycle. Pre- and post-heparin plasma were collected from TgN(LIPC)6784His and control mice via the saphenous vein. Plasma were stored at -80 °C until needed. Heparin (500 U/kg) was administered to these mice IP and were bled 20 min post-heparin.

For adenoviral studies, female C57BL/6J mice were injected with 200 µl saline, or with up to 1.8 x 10^{10} virus particles (VP) of Ad.luc, Ad.hHL, or Ad.hHLmt by injection of virus in 200 µl total volume via the tail vein. Blood collections via the saphenous vein were performed on 7 h fasted mice at various time points. Following 1 h

recovery, mice were also injected with 500 U/kg heparin via the tail vein and were bled 5 min post-heparin. Primary hepatocytes from adenovirus-infected or uninfected mice were prepared according to previously described methods (270,271). The primary hepatocytes were plated at a density of 1-2 x 10^6 cells per well in fibronectin-coated (25 μg/well) 6-well plates containing William's medium with 100 U/ml penicillin, 100 U/ml streptomycin, 250 ng/ml Fungizone, and 10% FBS. Six hours after initial plating, cells were washed twice in William's medium without FBS, then incubated in Hepatozyme medium. After 4 h, media were collected and spun at 2000 rpm for 10 min to remove cell debris. Fumed silica (12.5 mg) was added into 1 ml media to bind lipoproteins and incubated 16 h at 4 °C while rotating. The media was removed after centrifugation at 3000 rpm for 5 min and the fumed silica was washed twice with ice-cold PBS. Following washes, a cell lysis/SDS-PAGE loading buffer (200 μl of 10 mM Tris; 8 M urea; 2% SDS; 10% glycerol; 5% β-mercaptoethanol; 0.001% bromophenol blue; pH 8.3) was added and samples were heated at 75 °C for 20 min to elute bound proteins. The samples were centrifuged at 3000 rpm for 5 min and the supernatant was stored at -80 °C until needed. The cell lysis/SDS-PAGE loading buffer (200 μl) was added to cells and the mixture was stored at -80 °C until required. *Ex vivo* adenoviral infections of primary hepatocytes were achieved by adding adenovirus at a concentration of 20 VP/cell to the Hepatozyme medium for 24h. Following infection, cells were washed twice with Hepatozyme medium, then incubated in Hepatozyme medium for 4 h. Cells and media were collected and treated as described above.

2.2.9 Plasma lipid analyses

Fresh plasma (3 µl) were separated on 0.6% agarose gels for 30 min at 100 volts direct current in 1% sodium barbital pH 8.6. Agarose gels were fixed for 30 min (with 54% ethanol; 19% acetic acid in ddH_2O), then were dried for 1 h at 65 °C. The dried gel was stained with 7% Sudan black B in methanol to detect migrated lipid. To separate lipoproteins by fast performance liquid chromatography (FPLC), pooled plasma (500 µl) from saline, Ad.luc, Ad.hHL, or Ad.hHLmt injected mice were loaded onto two in-series Superose-6 columns, equilibrated with PBS. One ml fractions were collected and lipids were measured. TC and PL from plasma (2 µl per measurement) or FPLC (30 µl per measurement) were measured using commercially available kits according to manufacturer's specifications.

2.2.10 Apolipoprotein analyses

Plasma and primary hepatocyte samples were separated using 3-15% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes, and the membranes were immunoblotted (antibodies 1:5,000) for different apoproteins. To detect plasma apoB, 2 µl of plasma was used. Plasma was diluted 1:10000 for the detection of apoA-I.

2.2.11 Statistical analyses

Data where statistical values are provided were analyzed using either the paired t-test ("[t-test]") or one-way analysis of variance ("[ANOVA]"). Statistical values with multiple comparisons were corrected with the Bonferroni Adjustment. Error bars on the data are ± SD.

CHAPTER 3 – RESULTS

3.1 Identification of a heparin binding region in human hepatic lipase

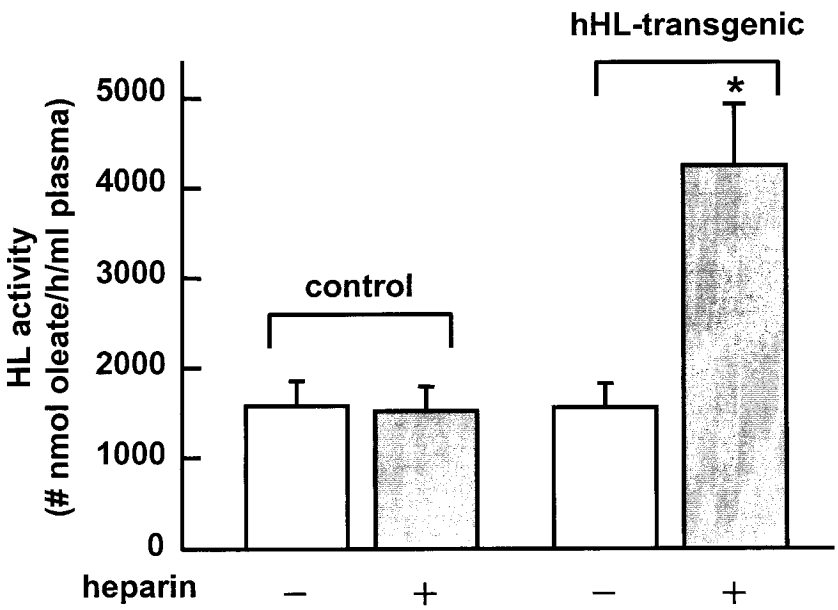
3.1.1 Comparison of human and mouse hepatic lipase in vivo

Previous biochemical studies have demonstrated that hHL had a stronger affinity to heparin-Sepharose than mHL (39). In the same studies, radiolabeled hHL injected into mice became HSPG associated and could be released by injection of heparin. This was also demonstrated with mice transgenically expressing hHL in this current study. Hepatic lipase activities of female C57BL/6J (control) and female TgN(LIPC)6784His (hHL-transgenic) mice were contrasted in **Figure 3.1.1**. Plasma HL activities of control mice were 1603 ± 247 nmol oleate/h/ml. With IP administration of heparin, no significant change was observed with plasma activities (1541 ± 247 nmol oleate/h/ml), thus demonstrating that mHL is mainly blood-borne. A similar result on plasma HL activity following IP administration of heparin has been observed in other unrelated studies (228). In contrast, hHL-transgenic mice injected with heparin IP had about a four-fold elevation of plasma HL activity compared with non-heparinized hHL-transgenic mice (4262 ± 658 *versus* 1578 ± 243 nmol oleate/h/ml, respectively). This increase is attributed to the release of proteoglycan-bound hHL into the mHL background. These data show that hHL is sequestered *in vivo* and that this binding can be disrupted by heparin infusion. The data also suggest that the human and mouse HSPGs are similar, and that the different affinities between hHL and mHL are likely due

Figure 3.1.1. Wild-type and human hepatic lipase-transgenic mouse plasma hepatic lipase activities

Control (C57BL/6J, n=3) and hHL-transgenic (TgN(LIPC)6784His, n=3) female mice were injected without or with 500 U/kg heparin in PBS IP. Twenty minutes post-injection, mice were bled through the saphenous vein and plasma was obtained. HL activity was determined using [^{3}H] triolein as the substrate, as described previously (267). Data are expressed as the mean ± S.D. * p<0.001 post-heparin hHL-transgenic *versus* pre-heparin transgenic plasma or control plasma [t-test].

Figure 3.1.1


hHL-transgenic
*
control
HL activity
(# nmol oleate/h/ml plasma)
5000
4000
3000
2000
1000
0
heparin
–
+
–
+

to differences in primary amino acid sequence.

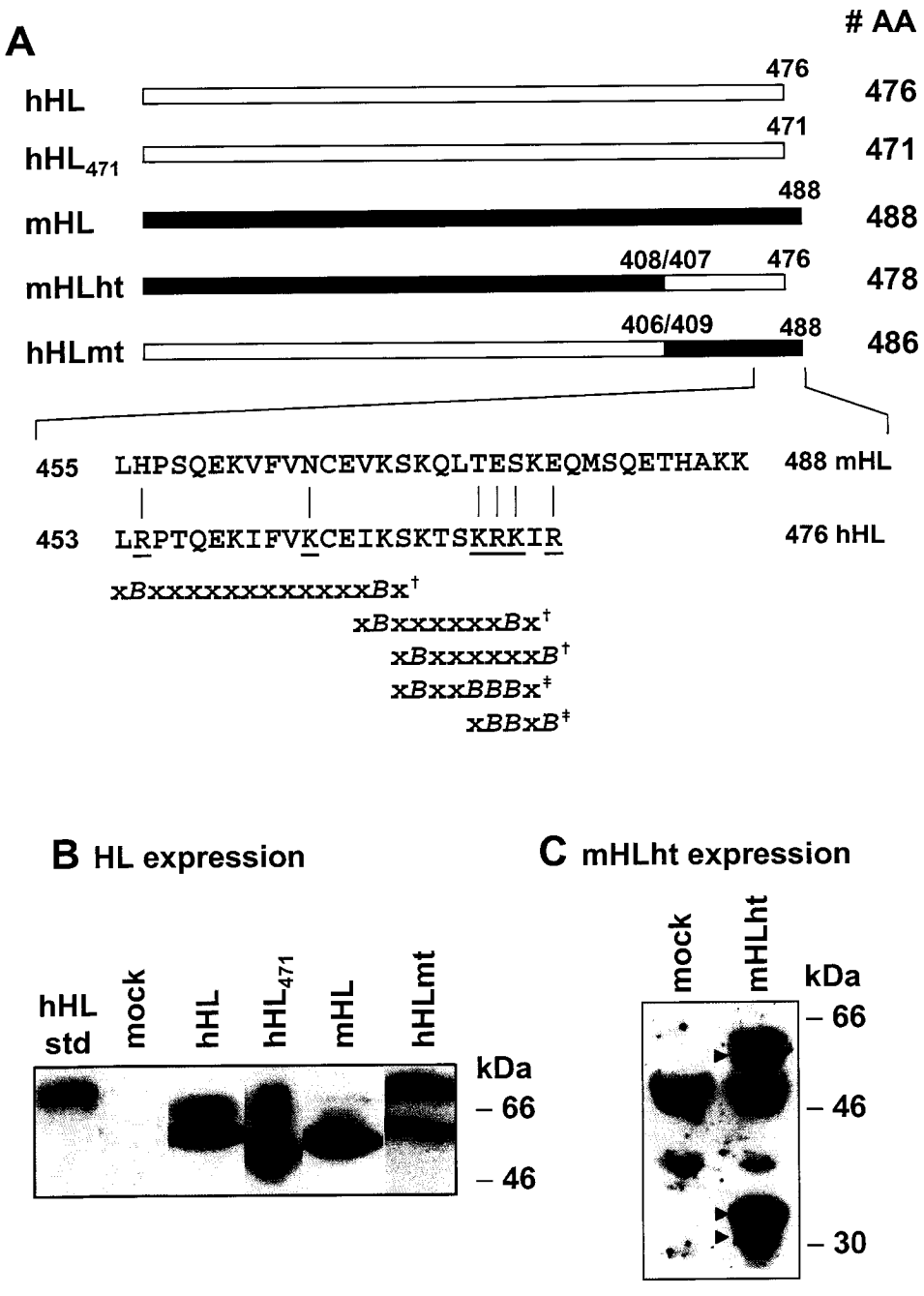
3.1.2 Expression of chimeric hepatic lipase proteins

The amino acid sequence of the C-terminus of hHL differs significantly from that of mHL. Not only does hHL have ten fewer C-terminal amino acid residues than mHL, but also the putative HBDs (214,218) found in hHL are absent in mHL (**Figure 3.1.2, *panel A***). Furthermore, the amino acids in mHL corresponding to the six basic amino acid residues (R or K) located within the putative HBD of hHL (underlined in **Figure 3.1.2, *panel A***) were found to be either acidic or neutral. Notably, four of the basic amino acid residues are present in the C-terminus of hHL. The sequence divergence between hHL and mHL led to the postulate that the C-terminal amino acids govern cell surface association.

To test this hypothesis, the chimeric proteins hHLmt and mHLht were prepared by exchanging the C-terminal regions of hHL and mHL, respectively. A convenient in-frame PflmI restriction endonuclease site within the human and mouse HL cDNAs was utilized, that allowed the swapping of the C-terminal 70 amino acid residues of hHL with the corresponding 80 amino acids derived from the C-terminus of mHL. A truncated hHL mutant (designated hHL_{471}), in which the C-terminal five residues (KRKIR) were deleted, was also generated to more closely examine the amino acid residues involved in heparin binding. Expression of hHL, mHL, hHLmt, and hHL_{471} was achieved in CHO 13-5-1 cells that are deficient in LRP expression (262). The use of LRP-null cells circumvented complications of potential interaction between HL and LRP (234,256). Immunoblots of whole cell extracts from representative hHL, mHL, hHLmt, and hHL_{471}

Figure 3.1.2. Structure and transient expression of recombinant hepatic lipase

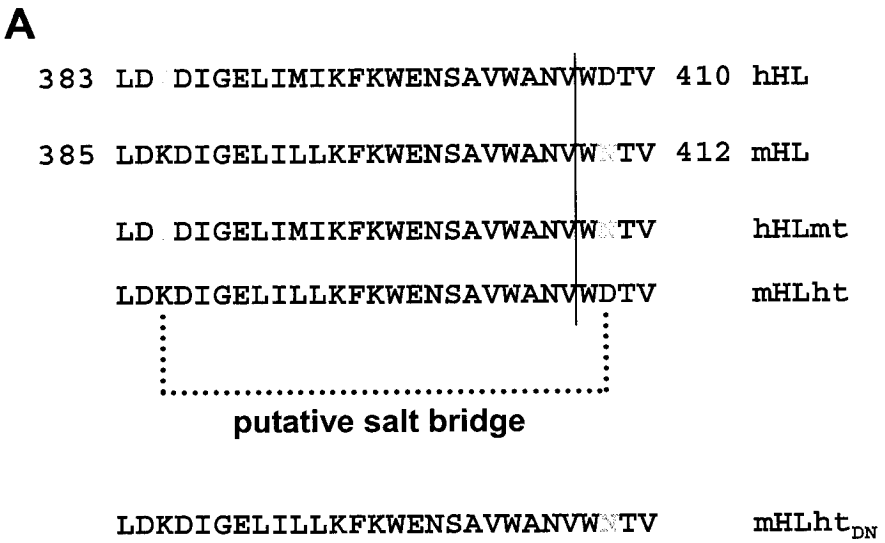
Panel A, schematic diagrams of hHL, hHL_{471}, mHL, mHLht, and hHLmt. The numbers indicate the amino acid positions of the corresponding proteins. The amino acid length of each protein is indicated under "# AA". The C-terminal 33 amino acids of mHL are aligned with the C-terminal 23 amino acids of hHL. Six basic residues (underlined) of hHL are found to be either neutral or acidic in mHL. Shown at *bottom* are the basic amino acids of hHL that align with previously described HBD consensus sequences (†, see ref. (218); ‡, see ref. (214)). *Panel B*, immunoblots of HL proteins from whole cell extracts of stably transfected CHO 13-5-1 cells. The cells were lysed and an aliquot of the sample (10 μg of cell protein) was resolved by SDS–PAGE. Following transfer to nitrocellulose membranes, the HL proteins were probed using either XHL3-6 (for hHL, hHL_{471}, and hHLmt) or anti-rHL (for mHL) and visualized by chemiluminescence. Immunoblots are representative of ten or more stable cell lines for each HL. *Panel C*, expression of mHLht in COS-7 cells. Cells were transfected with mHLht plasmid (10 μg of DNA). Forty-eight h after transfection, cells were lysed and an aliquot of cell lysate was resolved by SDS–PAGE and mHLht was visualized by immunoblotting using the anti-rat HL antibody. Degradation products of mHLht are indicated by *arrowheads*. A 50-kDa protein was detected with the anti-rHL antibody in both mock and mHLht transfected COS-7 cells; the identity of this protein is unknown. The immunoblot is representative of two independent experiments.

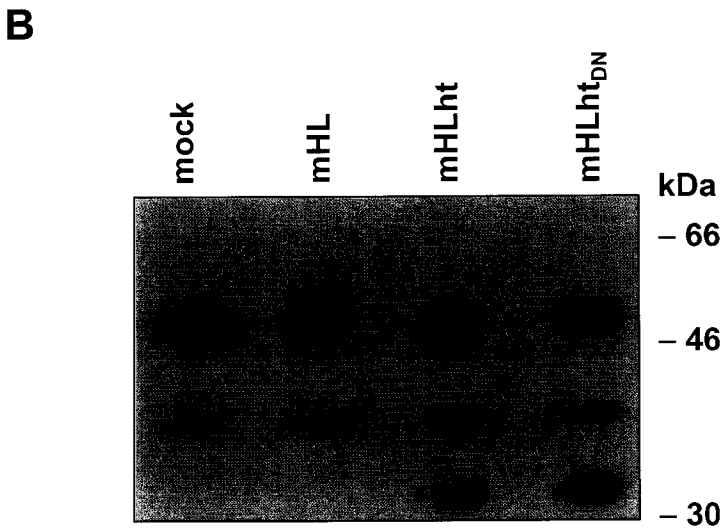

A
AA
hHL
476
476
hHL471
471
471
mHL
488
488
mHLht
408/407
476
478
hHLmt
406/409
488
486
455 LHPSQEKVFVNCEVKSKQLTESKEQMSQETHAKK 488 mHL
453 LRPTQEKIFVKCEIKSKTSKRKIR 476 hHL
xBxxxxxxxxxxxxxBx†
xBxxxxxxxBx†
xBxxxxxxxB†
xBxxBBBx‡
xBBxB‡
B HL expression
hHL std
mock
hHL
hHL471
mHL
hHLmt
kDa
– 66
– 46
C mHLht expression
mock
mHLht
kDa
– 66
– 46
– 30

stable cells are shown in **Figure 3.1.2, *panel B***. The doublet of hHL and hHL_{471} (53 kDa and 66 kDa), similar to what was seen for rat HL in primary hepatocytes (31), represented differently glycosylated species. Likewise, the doublet of hHLmt (57 kDa and 72 kDa) was seen in the whole cell extracts. The observed molecular mass of mHL was 55 kDa. Expression of hHL, mHL, hHLmt, and hHL_{471} was also achieved in COS-7 cells, HEK293 cells, and CHO-K1 cells (data not shown). The chimeric protein mHLht transiently expressed in COS-7 cells was unstable and showed multiple degradation products in addition to the full-length protein after a prolonged exposure of the immunoblot (**Figure 3.1.2, *panel C***). Analysis of the amino acid residues surrounding the junction of the mHLht protein shows that the basic amino acid residue Lys^{387} from mHL and the Asp^{408} from hHL were retained (**Figure 3.1.3, *panel A***). At least one of the corresponding amino acid residues in hHL, mHL, and hHLmt were neutrally charged, thus a salt bridge might have been formed between the Lys and Asp in mHLht and that this putative bridge led to the instability of this chimeric protein. In an attempt to disrupt the potential salt bridge and gain a stable mHLht chimeric protein, the Asp (corresponding to Asp^{408} from hHL) was mutagenized to the neutral amino acid residue Asn, (which corresponds to Asn^{410} from mHL). The new chimeric protein (designated as $mHLht_{DN}$) was transiently expressed in COS-7 cells and, like mHLht, it was also unstable and had one major degradation band (based on the short-term exposure of the immunoblot) (**Figure 3.1.3, *panel B***). The degradation of mHLht could not be prevented by the disruption of the putative salt bridge, thus mHLht was not further studied in the following experiments.

Figure 3.1.3. Correction of a putative salt bridge in mHLht

Panel A, amino acid sequences of hHL, mHL, mHLht, and hHLmt near the fusion site. The numbers indicate the amino acid positions of the corresponding proteins. Amino acid residues 383-410 of hHL are aligned with the corresponding sequences of mHL, hHLmt, and mHLht. The fusion site between the hHL and mHL sequences for the hHLmt and mHLht proteins is indicated with the red line. The Lys^{387} of mHL (in red) and the Asp^{408} of hHL (in blue) were conserved in mHLht and were proposed to form a potential salt bridge (indicated by dashed bridge). The retained Asp in mHLht was mutagenized into Asn to disrupt the potential salt bridge, creating the new mHLht (designated $mHLht_{DN}$). *Panel B*, expression of mHL, mHLht and $mHLht_{DN}$ in COS-7 cells. Cells were transfected with respective HL plasmid (10 μg of DNA). Forty-eight h after transfection, cells were lysed and an aliquot of cell lysate was resolved by SDS–PAGE. Proteins were visualized by immunoblotting using the anti-rHL antibody. Mouse HL was used as a control transfection. Degradation products of mHLht and $mHLht_{DN}$ are indicated by *arrowheads*. The immunoblot is representative of two independent experiments.


A
383 LD DIGELIMIKFKWENSAVWANVWDTV 410 hHL
385 LDKDIGELILLKFKWENSAVWANVWDTV 412 mHL
LD DIGELIMIKFKWENSAVWANVWDTV hHLmt
LDKDIGELILLKFKWENSAVWANVWDTV mHLht
putative salt bridge
LDKDIGELILLKFKWENSAVWANVWDTV mHLht$_{DN}$


B
mock
mHL
mHLht
mHLht$_{DN}$
kDa
– 66
– 46
– 30

3.1.3 Enzymatic activity of recombinant hepatic lipase

To ascertain whether or not the recombinant HL proteins were catalytically active, the hHL, mHL, hHLmt, and hHL_{471} proteins were semi-purified from conditioned media (in the presence of heparin) by heparin-Sepharose affinity chromatography. All four of the recombinant HL proteins showed catalytic activity towards the short-chain triglyceride tributyrin (**Figure 3.1.4, *panel A***), and the long-chain triglyceride triolein (**Figure 3.1.4, *panel C***). No salt-insensitive hydrolytic activity was detected from control mock-transfected cells. The rates of butyrate production and oleate production per μg of semi-purified hHL, mHL, hHLmt, and hHL_{471} were assessed at different concentrations of tributyrin (**Figure 3.1.4, *panel A***) and triolein (**Figure 3.1.4, *panel C***). The apparent K_m and V_{max} values for the semi-purified HLs (**Table 3.1.1**) were obtained from Lineweaver-Burk plots (**Figure 3.1.4, *panels B & D***) using the data shown in **Figure 3.1.4, *panels A & C***, respectively. The apparent K_m values for hHL, hHLmt, and hHL_{471} were comparable, being 0.63 ± 0.17 mM, 0.59 ± 0.13 mM, and 0.57 ± 0.11 mM tributyrin, respectively. These data suggest that the mutant HL proteins had catalytic properties similar to those of hHL when using tributyrin as a substrate. In contrast, mHL has an apparent K_m of 2.83 ± 0.66 mM tributyrin ($p<0.002$ *versus* other HL proteins) and an apparent V_{max} that was at least six-fold greater than the hHL-derived proteins. Compared to tributyrin, the apparent K_m data using triolein for hHL, hHLmt, and mHL were similar, being 0.57 ± 0.16 mM, 0.74 ± 0.14 mM, and 0.63 ± 0.12 mM triolein, respectively. The apparent K_m value for hHL_{471} was 0.78 ± 0.07 mM, respectively. Although the apparent K_m for hHL_{471} was statistically significantly different compared to

Figure 3.1.4. Enzyme kinetics of semi-purified recombinant hepatic lipase proteins

Semi-purified hHL (5 µl of 0.346 mg/ml – diamonds), mHL (5 µl of 0.369 mg/ml – squares), hHLmt (5 µl of 0.427 mg/ml – triangles), and hHL_{471} (5 µl of 0.167 mg/ml – circles) were assayed for tributyrin hydrolysis (*panel A*) and triolein hydrolysis (*panel C*), as previously described (267,268). Data were obtained using a range of tributyrin concentrations from 0.01 to 0.50 mM, and triolein concentrations from 0.35 to 17.3 mM. Each point represents measurement from three independent experiments. Lineweaver-Burk double-reciprocal plots of data obtained from *panels A* and *C* were generated and are shown in *panels B* and *D*, respectively. Apparent K_m and V_{max} values determined from these plots, and their statistical analyses, are shown in **Table 3.1.1**.

Figure 3.1.4

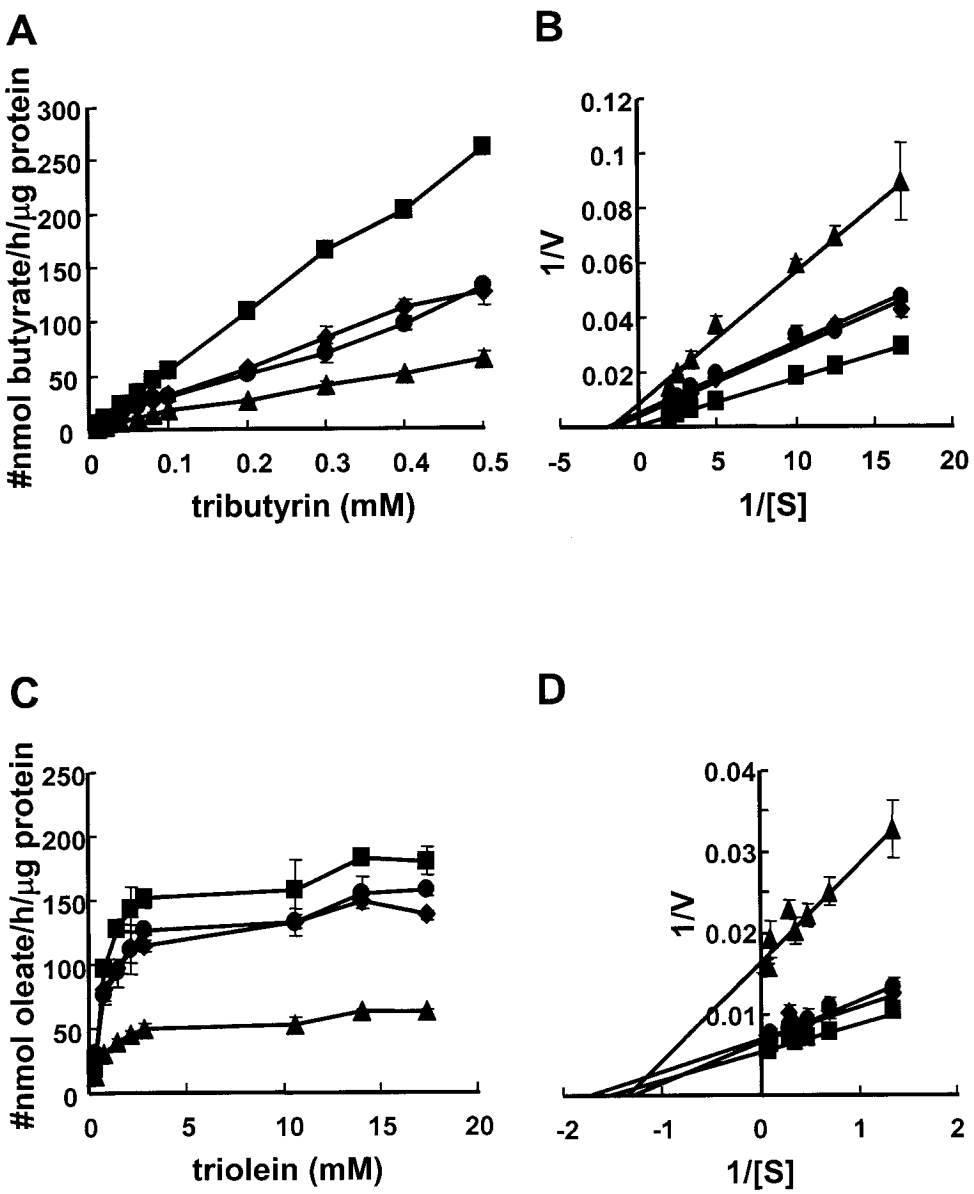

A
#nmol butyrate/h/µg protein
300
250
200
150
100
50
0
0
0.1
0.2
0.3
0.4
0.5
tributyrin (mM)
B
1/V
0.12
0.1
0.08
0.06
0.04
0.02
-5
0
5
10
15
20
1/[S]
C
#nmol oleate/h/µg protein
250
200
150
100
50
0
0
5
10
15
20
triolein (mM)
D
1/V
0.04
0.03
0.02
0.01
-2
-1
0
1
2
1/[S]

Table 3.1.1. Apparent K_m and V_{max} values of the recombinant hepatic lipases

HL Species	Tributyrin (n=3)		Triolein (n=3)	
	K_m	V_{max}	K_m	V_{max}
	mM tributyrin	*nmol butyrate/h/μg*	*mM triolein*	*nmol oleate/h/μg*
hHL	0.63 ± 0.17	260 ± 46[b]	0.57 ± 0.16	137 ± 4[b]
mHL	2.83 ± 0.66[a]	1663 ± 391[a]	0.63 ± 0.12	179 ± 13[b]
hHLmt	0.59 ± 0.13	118 ± 16[b]	0.74 ± 0.14	60 ± 2[b]
hHL_{471}	0.57 ± 0.11	218 ± 36[b]	0.78 ± 0.07[c]	152 ± 5[b]

a: $p<0.002$ *vs.* other HLs [ANOVA]. *b*: $p<0.01$ *vs.* other HLs [ANOVA]. *c*: $p=0.02$ *vs.* only hHL [ANOVA].

hHL (p=0.02), it was not significantly different to the other HL proteins. Thus, these data suggest that the four HL proteins had a similar catalytic activity when using triolein as a substrate, and that the catalytic site of the chimeric lipase was not grossly affected by the C-terminal amino acid swap. At this time, the observed differences in apparent K_m and V_{max} values for mHL *versus* hHL with the two different triglyceride substrates cannot be explained, and it is a unique feature of mHL that may warrant further investigation.

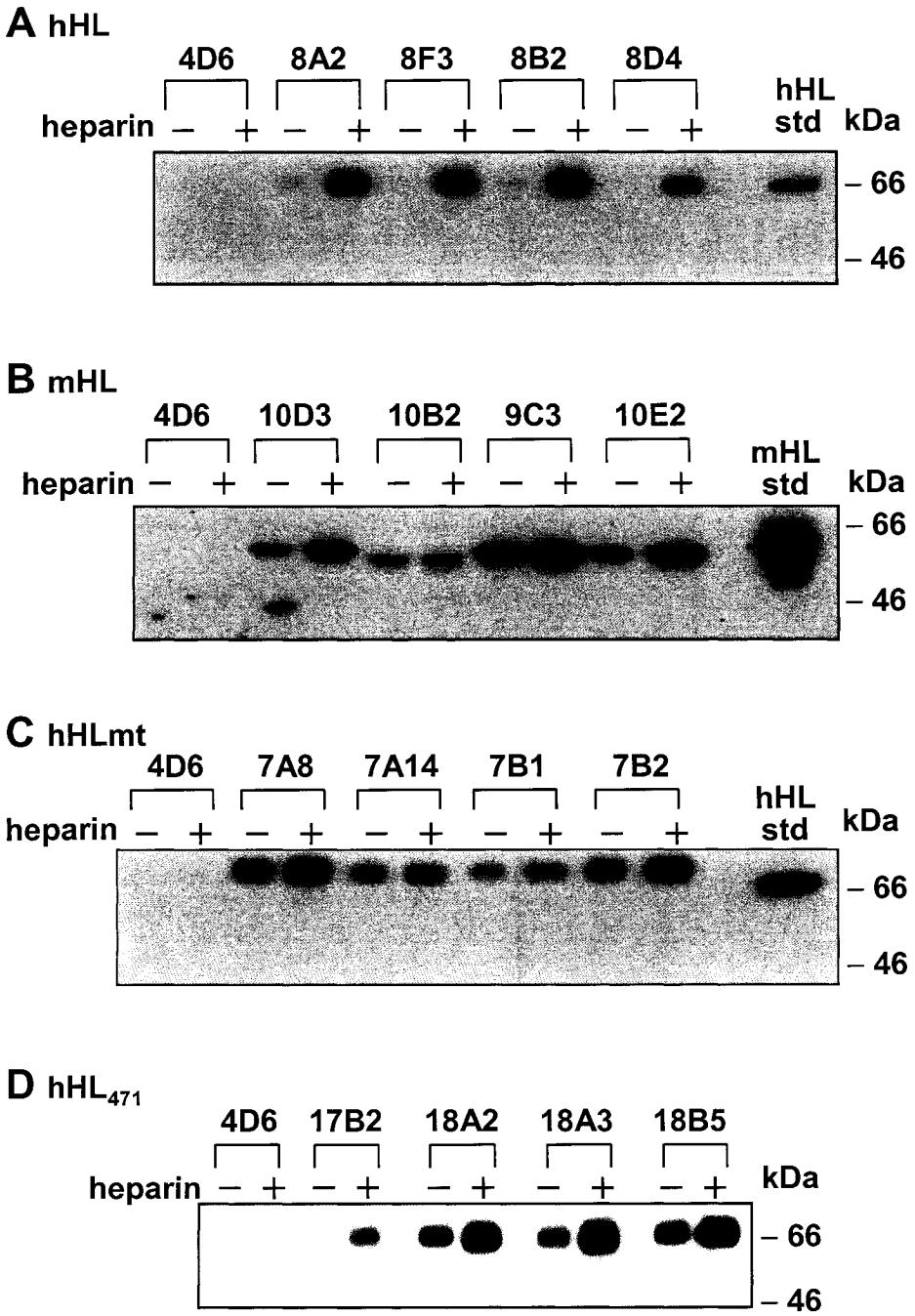
3.1.4 Cell surface association of recombinant hepatic lipase

Wild-type hHL was demonstrated to have a strong cell surface affinity in the following cell culture experiments. In the absence of heparin during a 4 h incubation period, very little hHL (66 kDa form) was detected in media by immunoblot analysis, regardless of the level of hHL expression among different clones (**Figure 3.1.5, *panel A***). In contrast, a spontaneous release of mHL into heparin-free media was observed in all stable transfectants which expressed different levels of the mHL protein (**Figure 3.1.5, *panel B***). Thus, there is a striking difference in cell surface binding between hHL and mHL. Similar to the observations with mHL, a spontaneous release of hHLmt (72 kDa form – **Figure 3.1.5, *panel C***) and hHL_{471} (66 kDa form – **Figure 3.1.5, *panel D***) into heparin-free media was also observed in all stable transfectants regardless of the level of expression.

These data suggest that one or more elements within the C-terminal 70 amino acid residues of hHL are necessary for cell surface binding. On average (calculated from densitometry data obtained from at least four individual stable cell lines), release of hHL

Figure 3.1.5. Analysis of hepatic lipase secretion in the absence or presence of heparin

Four different clones of hHL (*panel A*), mHL (*panel B*), hHLmt (*panel C*), and hHL_{471} (*panel D*) expressing cells were incubated with or without heparin for 4 h. Media HL were analyzed by immunoblot analysis using XHL3-6 (hHL, hHLmt, and hHL_{471}) or anti-rat HL (mHL). Clone 4D6 is the neomycin-resistant mock-transfected control cell line.


A hHL
4D6
8A2
8F3
8B2
8D4
heparin – + – + – + – + – +
hHL std
kDa
– 66
– 46
B mHL
4D6
10D3
10B2
9C3
10E2
heparin – + – + – + – + – +
mHL std
kDa
– 66
– 46
C hHLmt
4D6
7A8
7A14
7B1
7B2
heparin – + – + – + – + – +
hHL std
kDa
– 66
– 46
D hHL471
4D6
17B2
18A2
18A3
18B5
heparin – + – + – + – + – +
kDa
– 66
– 46

into heparin-free media was only 4% of the total hHL released by heparin over 4 h (**Figure 3.1.6**). In contrast, release of mHL and hHLmt in the absence of heparin was 61% and 68%, respectively, of the corresponding total heparin-releasable proteins. Thus, mHL and hHLmt exhibited >20-fold reduced affinity towards the cell surface as compared with hHL in transfected CHO 13-5-1 cells. Intermediate between hHL and mHL, 40% of total extracellular hHL_{471} was released into heparin-free media. The percentages of mHL and the mutant HL proteins into heparin-free media were statistically significantly different relative to hHL ($p<0.001$).

To determine cell surface association of the recombinant HLs, we metabolically labeled the cell lines and monitored the release of HL into media in the absence or presence of heparin. A polyclonal antibody that reacted with native hHL (263) was able to immunoprecipitate mHL and hHLmt from media in the presence of heparin (**Figure 3.1.7, *panel A***). Preliminary pulse-chase experiments with hHL-transfected cells showed that the recombinant hHL was recovered almost completely from the cells and media during chase. Semi-quantification of immunoblots by scanning densitometry showed that the release of newly synthesized hHL (66 kDa form) into media was linear for up to 2 h, and ~40% of the initially labeled hHL (53 kDa form) from cells was recovered from the medium as the 66 kDa form at the end of chase (**Figure 3.1.7, *panel B***).

With the polyclonal antibody (characterized above), quantitative pulse-chase experiments were performed to determine the effect of heparin treatment on the release of hHL (cell line 8A2), mHL (9C3), hHLmt (7A15), and hHL_{471} (17C3) into the chase medium over a 4-h period. The rates of HL release in the absence or presence of heparin

Figure 3.1.6. Semi-quantitative analysis of hepatic lipase secretion

The intensities of medium HL protein secreted in the absence or presence of heparin (from **Figure 3.1.5**) were quantified by scanning densitometry of the immunoblots. Data are presented as HL released into heparin-free medium as a percentage of that released into medium containing heparin (*i.e.* total extracellular HL). * $p<0.001$ for mHL, hHLmt, or hHL_{471} *versus* hHL [ANOVA].

Figure 3.1.6

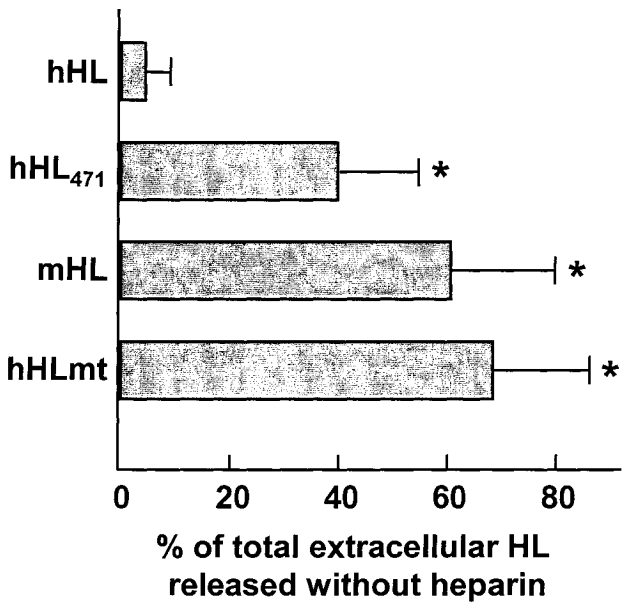
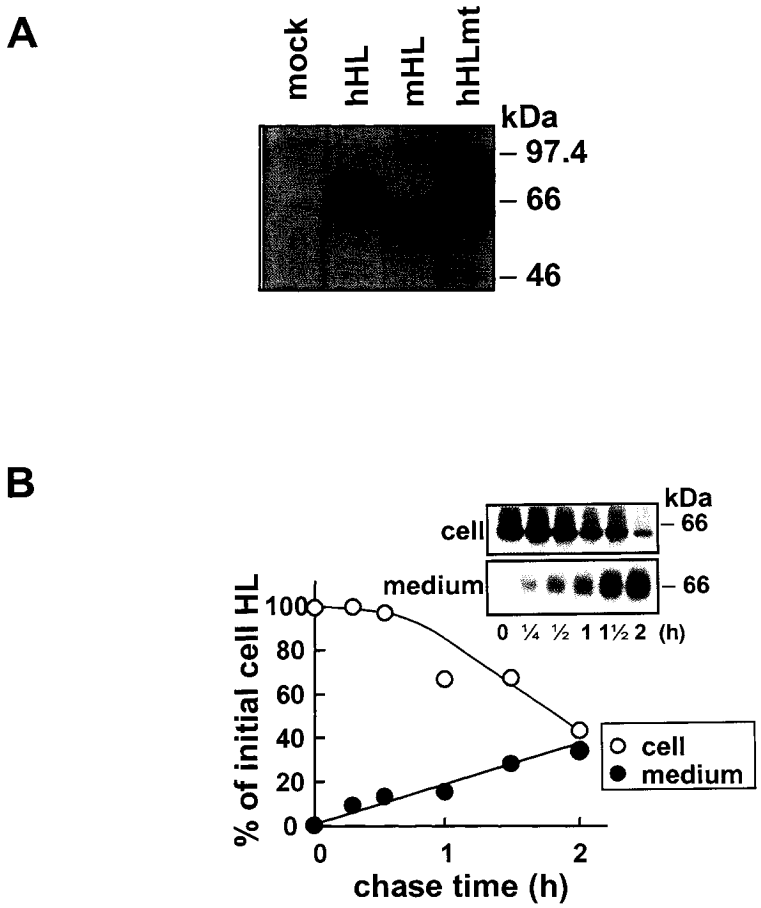

hHL
hHL471
mHL
hHLmt
*
*
*
0
20
40
60
80
% of total extracellular HL
released without heparin

Figure 3.1.7. Pulse-chase analysis of hepatic lipase secretion

Panel A, human HL, mHL, and hHLmt expressing cells were pulse-labeled with [^{35}S]methionine/cysteine for 2 h, and chased for 2 h in the presence of 100 U/ml heparin. The HL proteins from media were immunoprecipitated using the polyclonal anti-hHL antibody, resolved by SDS-PAGE, and analyzed by fluorography. *Panel B*, human HL expressing cells were pulse-labeled as above and chased for 2 h in the presence of heparin. Human HL was immunoprecipitated from the cells and media, respectively, resolved by SDS-PAGE, and analyzed by fluorography (insets). The radioactivity associated with cell and medium hHL was semi-quantified by scanning densitometry, and data are presented as "% of initial cell HL" (*i.e.* cell HL at the zero time of chase). *Panel C*, the hHL expressing cells (cell line 8A2) were pulse-labeled for 2 h and chased for 4 h with or without heparin. Proteins were resolved by SDS-PAGE and the radioactivity associated with the medium hHL was excised from the gel and quantified. The rates of hHL release with or without heparin were determined for each time point and the mean rates are shown in **Table 3.1.2**. Similar pulse-chase data (not shown) for mHL, hHLmt and hHL_{471} were assessed and their rates are also shown in **Table 3.1.2**.


A
mock
hHL
mHL
hHLmt
kDa
97.4
66
46
B
kDa
cell
66
medium
66
0 ¼ ½ 1 1½ 2 (h)
% of initial cell HL
100
80
60
40
20
0
0
1
2
chase time (h)
cell
medium
C
release of 35S-hHL (cpm × 10³/dish)
12
10
8
6
4
2
0
+ heparin
– heparin
0
1
2
3
4
chase time (h)

into media were determined at three different time points (0.5, 2.5, and 4 h), and the data are summarized in **Table 3.1.2**. The release of HL proteins into media is linear, as demonstrated with the hHL pulse-chase data (**Figure 3.1.7, *panel C***). The ratio of the rates for HL release in the absence *versus* presence of heparin provide the percentage of total extracellular HL released without heparin. The values for hHL, mHL, hHLmt, and hHL_{471} are 12%, 74%, 63%, and 44%, respectively. These data obtained by pulse-chase agree with the densitometry data obtained from multiple cell lines (**Figure 3.1.6**).

3.1.5 Heparin-Sepharose affinity of recombinant hepatic lipase proteins

Conditioned media (in the presence of heparin) collected from hHL, mHL, hHLmt, and hHL_{471} cells were subjected to heparin-Sepharose affinity chromatography. Proteins were eluted into 1 ml fractions using a 0.15 M to 1.50 M NaCl gradient, and the eluted proteins were detected by immunoblotting. The hHL was eluted as one peak at 0.76 M NaCl (fraction #34 – **Figure 3.1.8, *panel A***), whereas the mHL was eluted at lower concentrations of NaCl (**Figure 3.1.8, *panel B***). The eluted mHL from the heparin-Sepharose column exhibited a molecular mass above 220 kDa by SDS-PAGE, suggesting the formation of tetramers during chromatography. The hHLmt chimeric protein had a weaker affinity to heparin-Sepharose compared to hHL, with the peak immunoreactivity eluting at 0.62 M NaCl (fraction #26 – **Figure 3.1.8, *panel C***). Thus, substituting the C-terminal 70 amino acids of hHL with mouse sequences decreased the heparin binding to a level comparable to that of mHL. The hHL_{471} mutant was eluted from the heparin-Sepharose column at 0.69 M NaCl (fraction #30 – **Figure 3.1.8, *panel D***). Thus, deletion of the C-terminal five residues of hHL partially reduced the

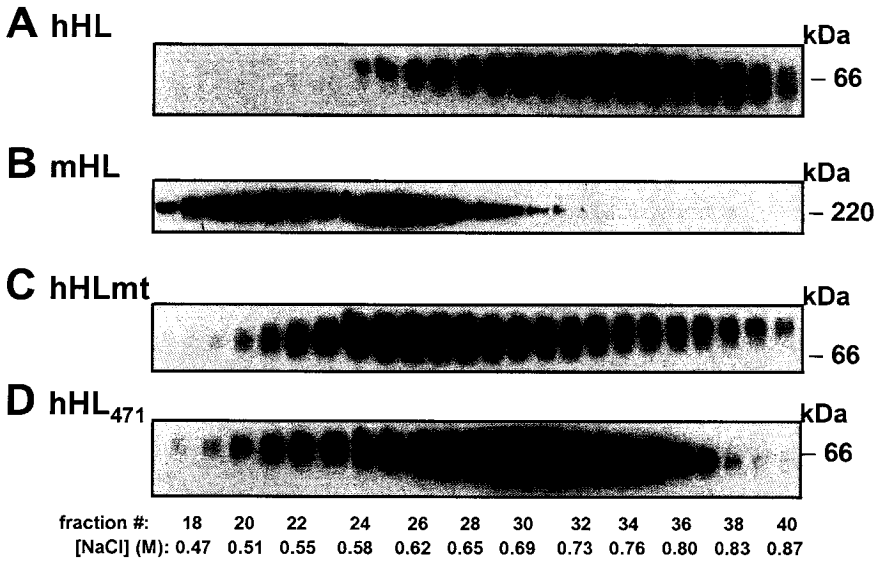
Table 3.1.2. Rates of ^{35}S-hepatic lipase release from cells into media

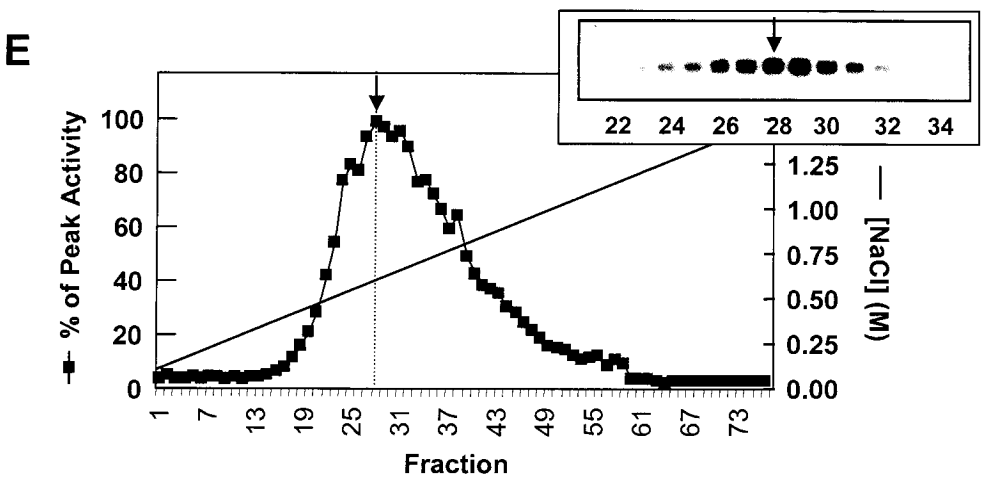
HL Species	no heparin	100 U/ml heparin
	cpm ^{35}S/h	*cpm ^{35}S/h*
hHL[a]	177 ± 155	1428 ± 367
mHL[a]	4947 ± 2366	6623 ± 2995
hHLmt[a]	3865 ± 918	6169 ± 333
hHL_{471}[a]	1984 ± 844	4555 ± 1229

a: $p<0.03$ between heparin *vs.* no heparin for each HL [t-test]. $n=3$ for each condition.

Figure 3.1.8. Heparin-Sepharose chromatography of hepatic lipase

Conditioned media (40 ml) from hHL (*panel A*), mHL (*panel B*), hHLmt (*panel C*), and hHL_{471} (*panel D*) stable cells were collected and subjected to heparin-Sepharose chromatography. Proteins were eluted using a 0.15 M to 1.5 M NaCl gradient, and fractions were resolved by SDS–PAGE and detected by immunoblotting using the antibody XHL3-6 (hHL, hHLmt, and hHL_{471}) or the anti-rHL antibody (mHL). Profiles are representative of two experiments. *Panel E*, conditioned media (400 ml) from hHL stable cells were collected and subjected to heparin-Sepharose chromatography in a separate experiment from *panels A-D* above. Proteins were eluted using a 0.15 M to 1.5 M NaCl gradient, and fractions were assayed for triolein hydrolysis and assessed by immunoblot analysis (insert) using the XHL3-6 antibody. The peak activity and immunoreactivity are indicated by an arrow.


A hHL
kDa
66
B mHL
kDa
220
C hHLmt
kDa
66
D hHL471
kDa
66
fraction #: 18 20 22 24 26 28 30 32 34 36 38 40
[NaCl] (M): 0.47 0.51 0.55 0.58 0.62 0.65 0.69 0.73 0.76 0.80 0.83 0.87


E
% of Peak Activity
100
80
60
40
20
0
1 7 13 19 25 31 37 43 49 55 61 67 73
Fraction
22 24 26 28 30 32 34
1.25
1.00
0.75
0.50
0.25
0.00
[NaCl] (M)

concentration of NaCl required to elute hHL from heparin-Sepharose. In a separate experiment, HL immunoreactivity was shown to correlate with HL activity (**Figure 3.1.8, *panel E***). Conditioned media (400 ml in the presence of heparin) from hHL expressing cells were subjected to heparin-Sepharose chromatography and eluted proteins using a NaCl gradient as above. In this experiment, the peak activity using triolein was found at fraction #28 (0.68 M NaCl, 3.3 μmol oleate/h/fraction), and this peak activity also corresponded with peak immunoreactivity (shown in the inset).

3.2 Effects of heparan sulfate proteoglycan binding-impaired human hepatic lipase *in vivo*

3.2.1 Expression of recombinant hepatic lipase proteins in vivo

The objective of this study was to determine the functional differences between the HSPG-anchored hHL and an HSPG binding-impaired HL *in vivo*. The approach used involved the generation and expression of adenoviruses expressing hHL, hHLmt, and a luciferase control into female C57BL/6J mice. Mice were injected with saline, or 1.8 x 10^{10} VP of adenoviruses expressing either HL or luciferase. Plasma HL activities were measured in both pre- and post-heparin plasma seven days after infection, using ^{3}H-triolein as a substrate (**Figure 3.2.1**). The pre-heparin plasma HL activities in hHLmt-expressing mice were three-fold higher than that in hHL-expressing mice (9.8 ± 1.7 *versus* 3.1 ± 0.5 μmol oleate/h/ml plasma), indicating a reduced HSPG binding affinity of hHLmt. The pre-heparin plasma HL activity in hHL-expressing mice exhibited background levels similar to that in control mice injected with saline or infected with luciferase. The post-heparin plasma HL activities between hHL- and hHLmt-expressing mice were comparable (17.6 ± 6.6 *versus* 25.4 ± 7.6 μmol oleate/h/ml plasma), suggesting that similar amounts of the HL were expressed.

3.2.2 Evaluation of plasma lipids and lipoprotein profiles

Pre-heparin plasma from mice infected for seven days with adenovirus encoding hHL or hHLmt (up to 1.8 x 10^{10} VP) were analyzed by agarose gel electrophoresis (**Figure 3.2.2, *panel A***). A marked reduction of lipid staining for α-migrating

Figure 3.2.1. Hepatic lipase activity in mice expressing hHL or hHLmt

Female C57BL/6J mice (6-8 week old) were infected with virus (1.8×10^{10} VP) encoding luciferase, hHL, or hHLmt or injected with saline. Seven days post-infection, mice were fasted for 7 h followed by bleeding. Sixty min after bleeding, mice were injected with 500 U/kg heparin via the tail vein and bled again 5 min post-heparin. HL activity was determined in duplicate from five or more mice using ^{3}H-triolein as a substrate. * $p <$ 0.02 post-heparin hHL or hHLmt *versus* post-heparin saline or luciferase controls [ANOVA]. ** $p < 0.02$ pre-heparin hHLmt *versus* pre-heparin saline or luciferase controls [ANOVA].

Figure 3.2.1

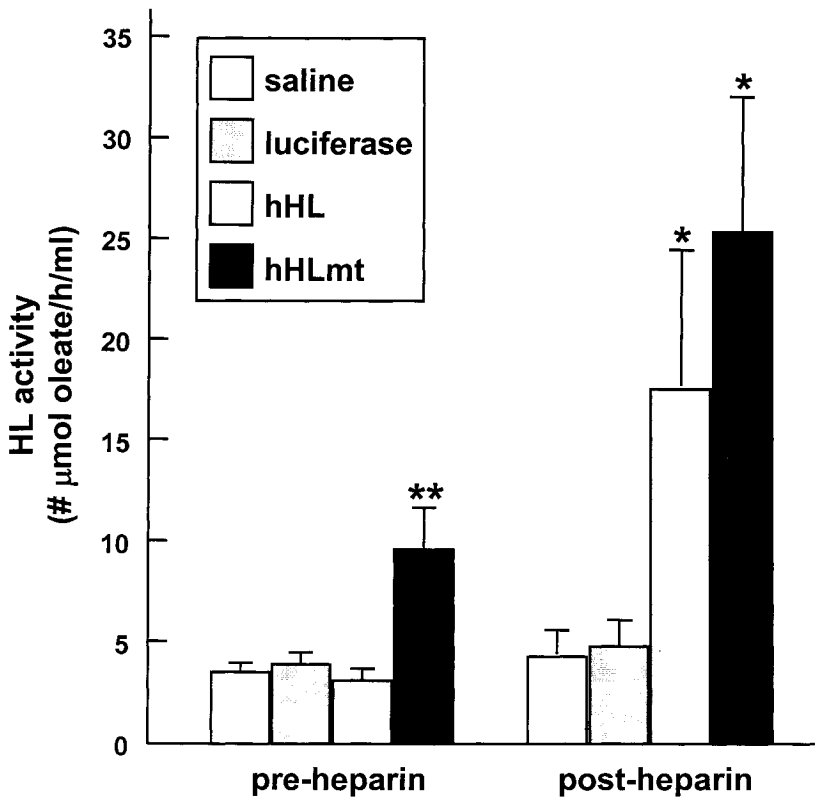
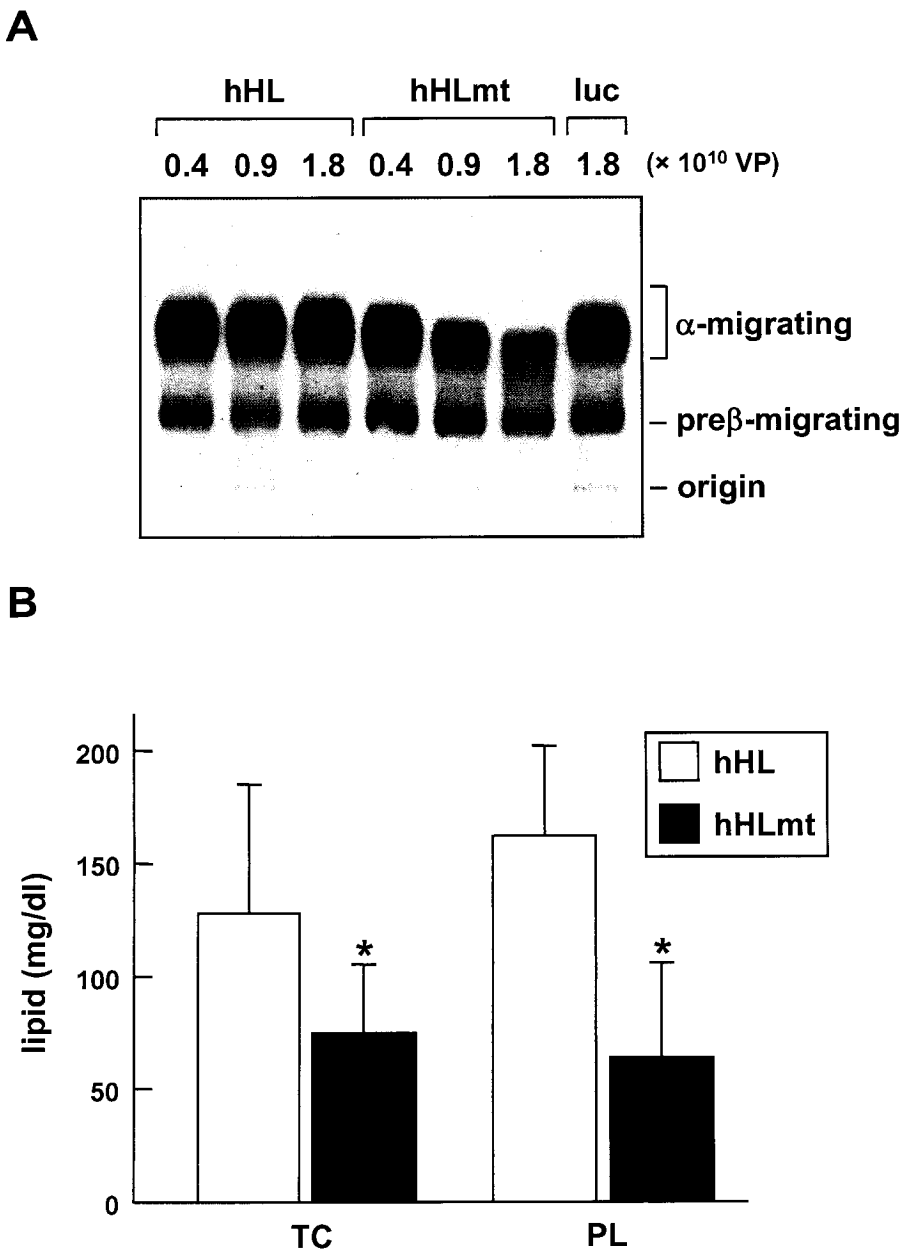

35
30
25
20
15
10
5
0
HL activity
(# μmol oleate/h/ml)
saline
luciferase
hHL
hHLmt
**
*
*
pre-heparin
post-heparin

Figure 3.2.2. Analysis of plasma HDL lipid, total cholesterol, and phospholipid

Panel A, female C57BL/6J mice (6-8 week old) were infected with different amounts of virus (ranging from 0.4 to 1.8 x 10^{10} VP) encoding hHL or hHLmt, or virus encoding luciferase (1.8 x 10^{10} VP). Seven days post-infection, mice were fasted for 7 h followed by bleeding. Three µl of plasma were separated on 0.6% agarose gels. Gels were stained for neutral lipid. *luc*, luciferase. *Panel B,* total cholesterol (*TC*) and phospholipid (*PL*) were measured using fasted blood from mice at 7 days post-infection with virus (1.8 x 10^{10} VP) encoding hHL or hHLmt. Data are measurements in duplicate from five or more mice. * $p < 0.05$ hHLmt *versus* hHL [t-test].


A
hHL
hHLmt
luc
0.4 0.9 1.8 0.4 0.9 1.8 1.8 (× 10^10 VP)
α-migrating
– preβ-migrating
– origin
B
lipid (mg/dl)
200
150
100
50
0
hHL
hHLmt
*
*
TC
PL

lipoproteins was observed in mice expressing hHLmt in a dose-dependent manner. At the same doses of infection, mice expressing hHL displayed no changes in lipid staining of lipoproteins. Quantification of total cholesterol and phospholipids, the major lipid constituents of HDL, in the plasma of mice seven days post-infection (with 1.8 x 10^{10} VP) showed decreased total cholesterol (hHL, 127 ± 58 mg/dl; hHLmt, 76 ± 30 mg/dl) and phospholipids (hHL, 162 ± 40 mg/dl; hHLmt, 63 ± 43 mg/dl) as a result of the expression of the HSPG binding-deficient hHLmt (**Figure 3.2.2, *panel B***).

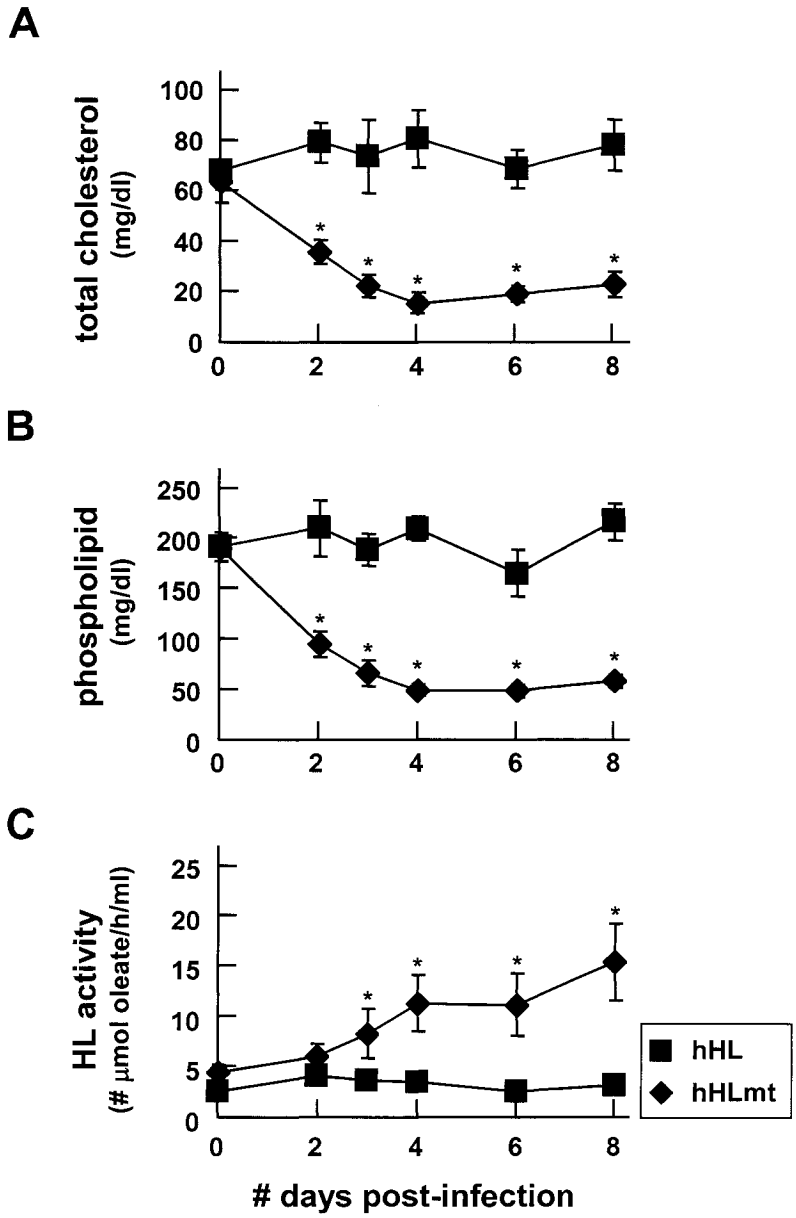
The concentrations of total cholesterol and phospholipid between hHL- and hHLmt-expressing mice, infected with respective viruses, were compared for up to eight days. Both total cholesterol (**Figure 3.2.3, *panel A***) and phospholipid (**Figure 3.2.3, *panel B***) concentrations decreased to minimum at four days post-infection, whereas the pre-heparin plasma hHLmt activity gradually increased to maximum (**Figure 3.2.3, *panel C***). In contrast, there were no significant changes in plasma total cholesterol, phospholipids, or pre-heparin HL activity in mice expressing the wild-type HL throughout the entire post-infection period examined. The average plasma total cholesterol (74.4 ± 11.6 mg/dl), phospholipid levels (196.9 ± 27.1 mg/dl), and pre-heparin HL activities (3.3 ± 0.9 μmol oleate/h/ml) in hHL-expressing mice during the post-infection period were comparable to control mice injected with saline (TC: 74.3 ± 9.3 mg/dl; PL: 199.2 ± 20.3 mg/dl; HL activity: 5.0 ± 1.2 μmol oleate/h/ml) or mice infected with luciferase (TC: 70.6 ± 9.7 mg/dl; PL: 182.2 ± 35.9 mg/dl; HL activity: 4.2 ± 1.0 μmol oleate/h/ml).

Fractionation of lipoproteins in the fasted pre-heparin plasma by FPLC showed that reductions in total cholesterol and phospholipid were mainly attributable to

Figure 3.2.3. Time course of pre-heparin plasma total cholesterol, phospholipid, and hepatic lipase activity

Female C57BL/6J mice (6-8 week old) were infected with virus (1.8×10^{10} VP) encoding hHL or hHLmt. On days 0, 2, 3, 4, 6, and 8 post-infection, mice were fasted for 7 h followed by bleeding. Each point represents measurement in duplicate with four or more mice. *Panel A*, plasma total cholesterol. *Panel B*, plasma phospholipid. *Panel C*, pre-heparin plasma HL activity. * $p < 0.01$ hHLmt *versus* hHL [t-test].

Figure 3.2.3


A
total cholesterol (mg/dl)
100
80
60
40
20
0
0
2
4
6
8
B
phospholipid (mg/dl)
250
200
150
100
50
0
C
HL activity (# µmol oleate/h/ml)
25
20
15
10
5
0
hHL
hHLmt
days post-infection

decreased HDL. Data presented in **Figure 3.2.4** show lipid profiles of plasma samples pooled from three mice seven days post-infection with 1.8 x 10^{10} VP. The most prominent peak of total cholesterol in the plasma of hHL-expressing mice was for HDL (7.4 mg/dl), which was 5-fold lower (1.4 mg/dl) in the plasma of hHLmt-expressing mice (**Figure 3.2.4, *panel A***). Likewise, the most prominent peak of phospholipid in the plasma of hHL-expressing mice was also for HDL (12.0 mg/dl), and the level of which in hHLmt-expressing mice was 5-fold lower (2.3 mg/dl) (**Figure 3.2.4, *panel B***). These results suggest strongly that expression of the HSPG binding-deficient hHLmt has profoundly decreased plasma concentrations of HDL lipids.

3.2.3 Evaluation of plasma apoA-I

The loss of HDL lipids may be associated with changes in apoA-I, the major apolipoprotein constituent of HDL, thus plasma apoA-I levels were analyzed. Immunoblot analysis of pre-heparin plasma for up to eight days, obtained from mice infected with virus (1.8 x 10^{10} VP) encoding hHL or hHLmt, showed markedly diminished apoA-I in hHLmt-expressing mouse plasma two days post-infection (**Figure 3.2.5, *panel A***). There was no change in plasma apoA-I in mice infected with hHL or luciferase, or in mice injected with saline. Semi-quantitative analysis by scanning densitometry of the immunoblots showed comparable levels of apoA-I for hHL-expressing mice, luciferase-expressing mice, and saline-injected mice throughout the post-infection period (**Figure 3.2.5, *panel B***).

Figure 3.2.4. Fast performance liquid chromatography of pre-heparin plasma lipoproteins

Blood was collected from 7 h fasted mice (n=3) seven days post-infection with virus (1.8 x 10^{10} VP) encoding hHL or hHLmt. Plasma samples were pooled (total of 500 µl), and fractionated (1 ml each fraction) by FPLC. Total cholesterol (*panel A*) and PL (*panel B*) in each fraction were quantified. *IDL/LDL*, intermediate- and low-density lipoproteins.

Figure 3.2.4

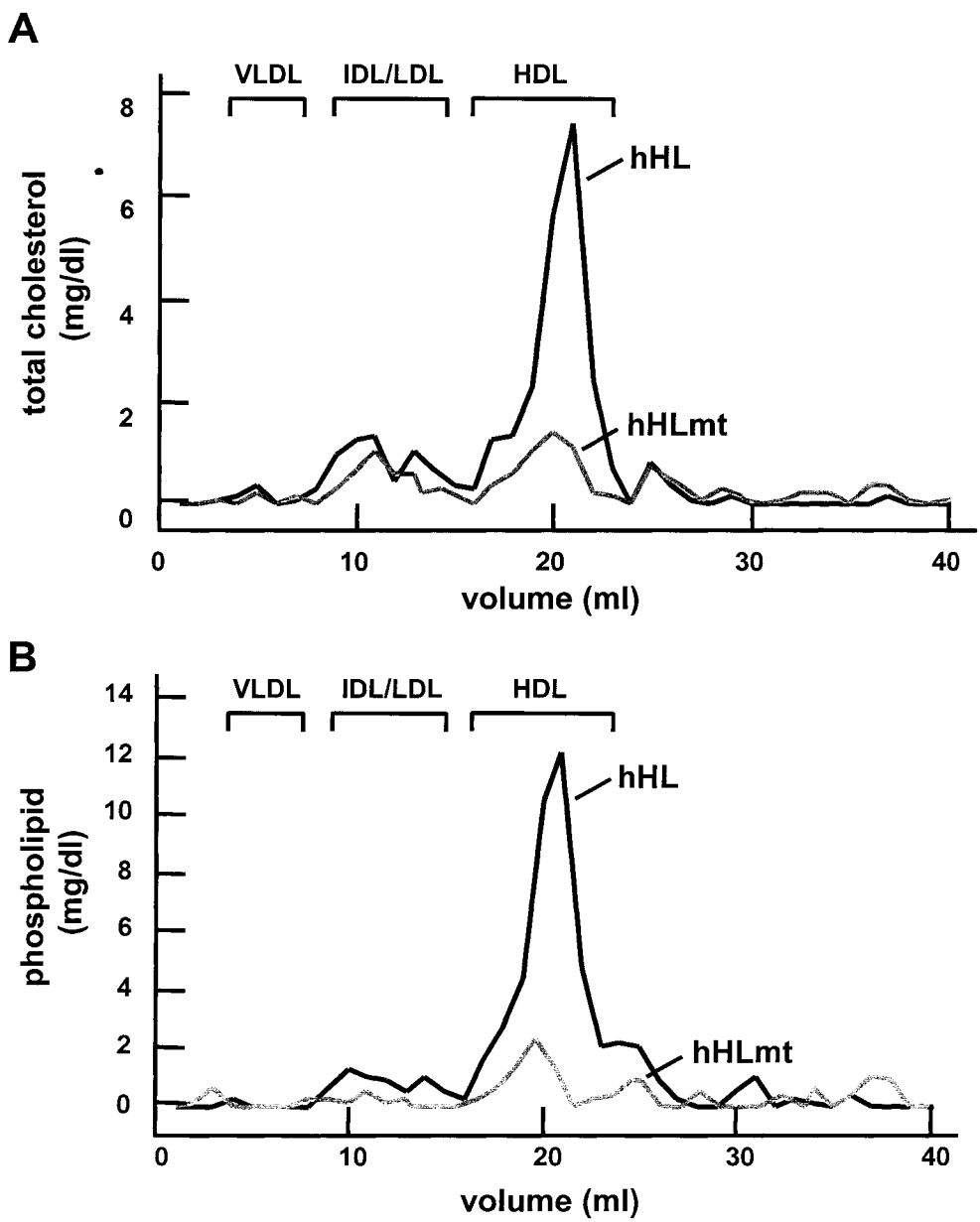
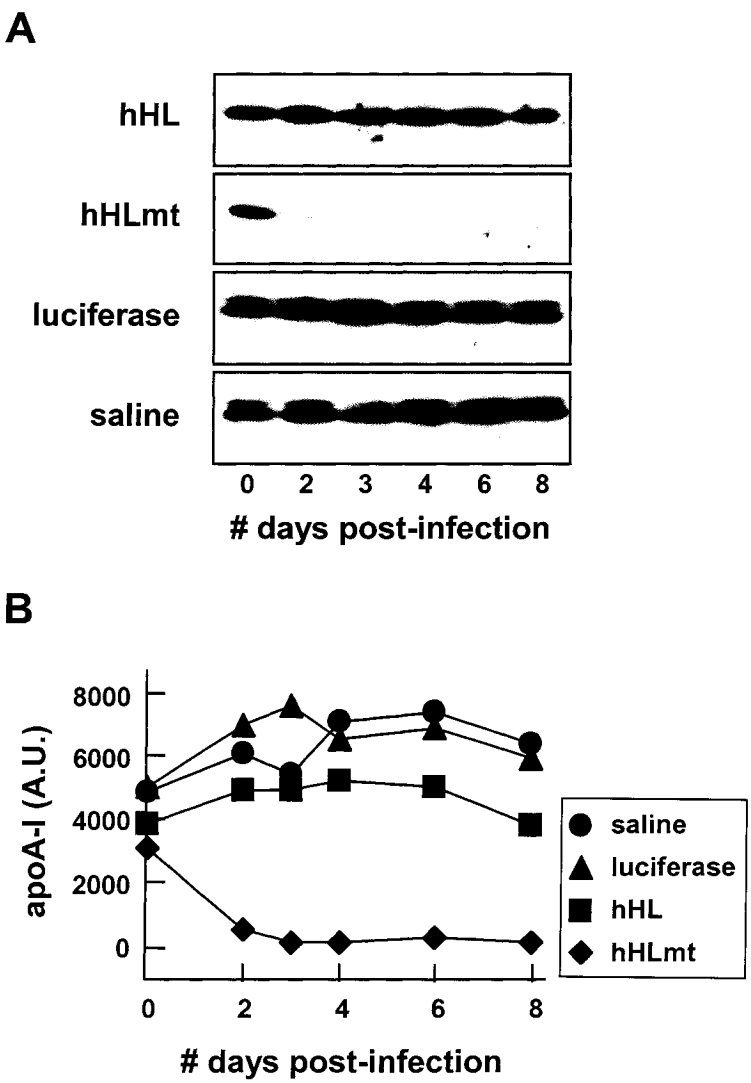

A
VLDL
IDL/LDL
HDL
hHL
hHLmt
total cholesterol (mg/dl)
0
2
4
6
8
0
10
20
30
40
volume (ml)
B
VLDL
IDL/LDL
HDL
hHL
hHLmt
phospholipid (mg/dl)
0
2
4
6
8
10
12
14
0
10
20
30
40
volume (ml)

Figure 3.2.5. Time course of pre-heparin plasma apoA-I

Three female C57BL/6J mice (6-8 week old) were infected with virus (1.8 x 10^{10} VP) encoding hHL, hHLmt, or luciferase or injected with saline. On days 0, 2, 3, 4, 6, and 8 post-infection, mice were fasted for 7 h followed by bleeding. *Panel A,* plasma were diluted 1:10,000 and separated by SDS-PAGE (3-15% gel). Proteins were transferred to nitrocellulose and immunoblotted for apoA-I using a polyclonal antibody against mouse apoA-I. Immunoblots are representative of two experiments. *Panel B,* semi-quantitative analysis of plasma apoA-I by scanning densitometry of immunoblots shown in *Panel A.*

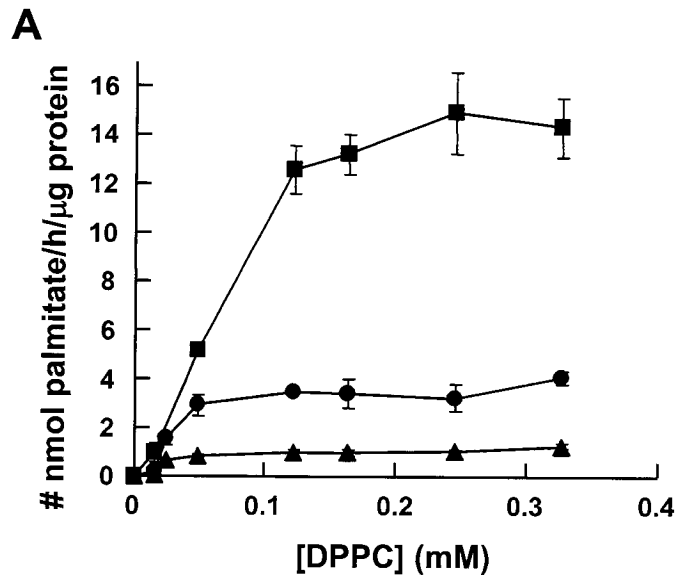

A
hHL
hHLmt
luciferase
saline
0 2 3 4 6 8
days post-infection
B
8000
6000
4000
2000
0
apoA-I (A.U.)
0 2 4 6 8
days post-infection
saline
luciferase
hHL
hHLmt

3.2.4 Phospholipase activity of recombinant hepatic lipase

The reduced levels of HDL phospholipid in the circulation suggest that the phospholipase activities of hHL and hHLmt may differ, such that the chimeric hHLmt may have increased phospholipase activity compared to hHL. To test this hypothesis, the phospholipase activities of semi-purified recombinant hHL, hHLmt, and mHL (from conditioned media of stable CHO 13-5-1 cells as in Section 3.1.2) toward DPPC were compared. The three recombinant HL proteins showed catalytic activity toward DPPC (**Figure 3.2.6, *panel A***). No salt-insensitive hydrolytic activity was detected from control mock-transfected cells. The rates of palmitate production per μg of semi-purified hHL, hHLmt, and mHL were assessed at different concentrations of DPPC (**Figure 3.2.6, *panel A***). The apparent K_m and V_{max} values for the semi-purified HLs (**Table 3.2.1**) were obtained from the Lineweaver-Burk plot (**Figure 3.2.6, *panel B***) using the data shown in **Figure 3.2.6, *panel A***. The apparent K_m values for hHL and mHL were comparable, being 0.044 ± 0.013 mM and 0.063 ± 0.007 mM DPPC, respectively. In contrast, hHLmt had a significantly lower apparent K_m (0.018 ± 0.005 mM DPPC). Furthermore, the apparent V_{max} data show that mHL has at least a four-fold greater phospholipase activity compared with hHL, a feature similar to that seen with tributyrin (see Section 3.1.2). In contrast, hHLmt showed a reduced V_{max} and appeared to be less active than hHL. Thus, these data show that reduced plasma PL levels by hHLmt were not due to a "hyperphospholipase" activity. These data also show that mHL is uniquely different versus hHL with respect to DPPC hydrolysis.

3.2.6 Enzyme kinetics of semi-purified hepatic lipases toward dipalmitoyl phosphatidylcholine

Panel A, semi-purified hHL (circles), mHL (squares), and hHLmt (triangles) (as in **Figure 3.1.4**) were assayed for DPPC hydrolysis as previously described (105). Data were obtained using a range of DPPC concentrations from 0.016 to 0.33 mM. Each point represents measurement from six independent experiments. Lineweaver-Burk double-reciprocal plots of data obtained from *panel A* were generated and are shown in *panel B*. Apparent K_m and V_{max} values determined from these plots, and their statistical analyses, are shown in **Table 3.2.1**.


A
nmol palmitate/h/μg protein
16
14
12
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
[DPPC] (mM)

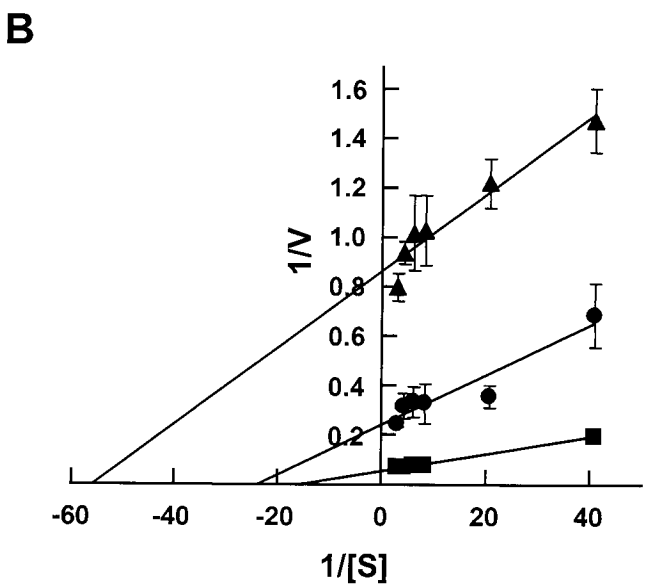

B
1/V
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
-60
-40
-20
0
20
40
1/[S]

Table 3.2.1. Apparent K_m and V_{max} values of recombinant hepatic lipases toward dipalmitoyl phosphatidylcholine

HL Species	K_m	V_{max}
	mM DPPC	*nmol palmitate/h/μg*
hHL	0.044 ± 0.013	4.49 ± 0.58[a]
mHL	0.063 ± 0.007	16.43 ± 1.21[a]
hHLmt	0.018 ± 0.005[a]	1.16 ± 0.08[a]

a: $p<0.001$ *vs.* other HLs [ANOVA]. $n=6$ for each condition.

3.2.5 Evaluation of hepatic apolipoproteins

The majority of the plasma apoA-I originates from the intestines and liver. Since adenoviruses injected via the tail vein primarily infects the liver, the effect of HL expression on the hepatic apoA-I levels in the infected mice was examined. Immunoblot analysis of apoA-I present in total liver homogenates of three mice infected with 1.8 x 10^{10} VP revealed marked reduction of hepatic apoA-I in mice expressing hHLmt compared to those expressing hHL (**Figure 3.2.7, *panel A***). Scanning densitometry analysis of the hepatic apoA-I immunoblots showed a 10-fold decrease in mice expressing hHLmt (**Figure 3.2.7, *panel B***). Thus, expression of HSPG binding-deficient hHLmt results in decreased apoA-I in the liver, which may partly underlie the reduced plasma apoA-I concentrations.

To ascertain that hHLmt expression indeed results in attenuated hepatic apoA-I expression and secretion, we prepared primary hepatocytes from mice that had been infected with virus encoding hHLmt or luciferase for ten days. Cell-associated apoA-I and apoA-I in the conditioned media of cells cultured with a serum-free medium for 4 h was determined by immunoblot analysis. As shown in **Figure 3.2.8, *panel A***, apoA-I levels in media from hHLmt-infected hepatocytes were reduced by three-fold (determined by scanning densitometry) as compared to media from luciferase control hepatocytes. The decrease was specific to apoA-I, as no appreciable changes were observed for apoE or apoB48. [The level of apoB100 was below detection.] The level of cell-associated apoA-I in hHLmt-expressing hepatocytes was reduced by two-fold (determined by scanning densitometry) as compared to that in luciferase control cells

Figure 3.2.7. Analysis of apoA-I from mouse liver homogenates

Three 6-8 week old female C57BL/6J mice were infected with virus (1.8×10^{10} VP) encoding hHL or hHLmt. Seven days post-infection, mice were fasted for 7 h. Fasted mice were sacrificed and whole livers were removed and homogenized. *Panel A*, homogenates were separated by SDS-PAGE (3-15% gel). Proteins were transferred to nitrocellulose and immunoblotted for apoA-I using a polyclonal antibody against mouse apoA-I. *Panel B*, semi-quantitative analysis of mouse liver homogenate apoA-I by scanning densitometry of immunoblots shown in *A*. * $p < 0.001$ hHLmt *versus* hHL [t-test].

Figure 3.2.7

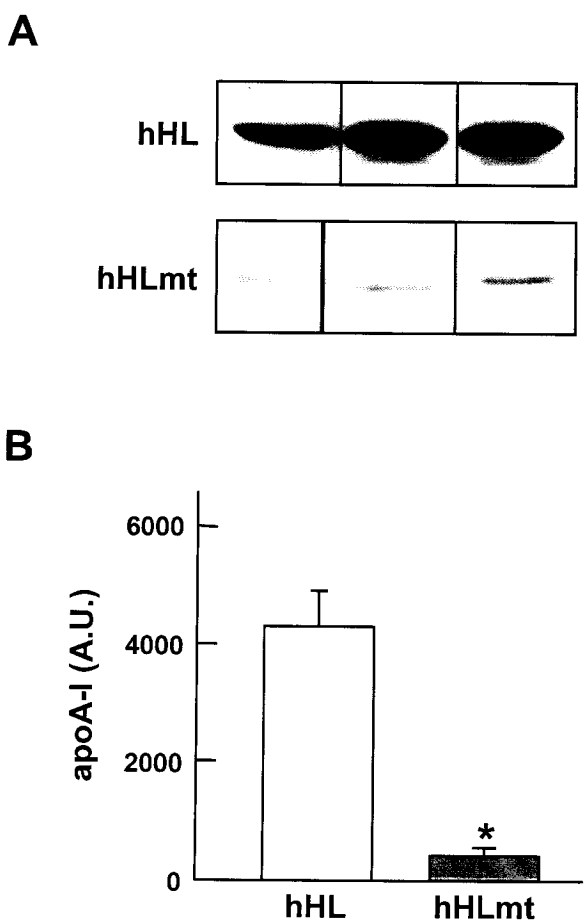
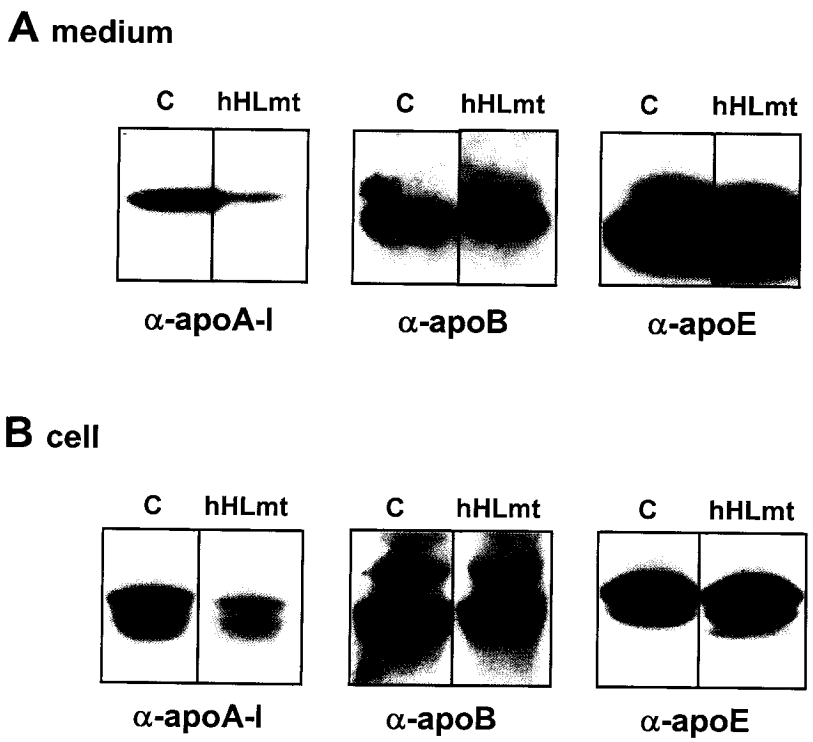


Figure 3.2.8. Analysis of apoproteins from *in vivo*-infected primary hepatocytes

Female C57BL/6J mice (6-8 week old) were infected with virus (1.8×10^{10} VP) encoding luciferase (*C*) or hHLmt. Ten days post-infection, primary hepatocytes were isolated from the infected mice and plated. Six h after initial plating, cells were washed and incubated with serum-free media for 4 h. After incubation, media were collected and apolipoproteins were adsorbed to fumed silica and eluted, and cells were collected and lysed. The experiment was performed in duplicate and presented data are representative. *Panel A*, the fumed silica-treated media samples were separated by SDS-PAGE (3-15% gel). Proteins were transferred to nitrocellulose and immunoblotted for mouse apoA-I, apoB, and apoE, respectively. *Panel B*, cell samples were similarly separated by SDS-PAGE and analyzed by immunoblotting.

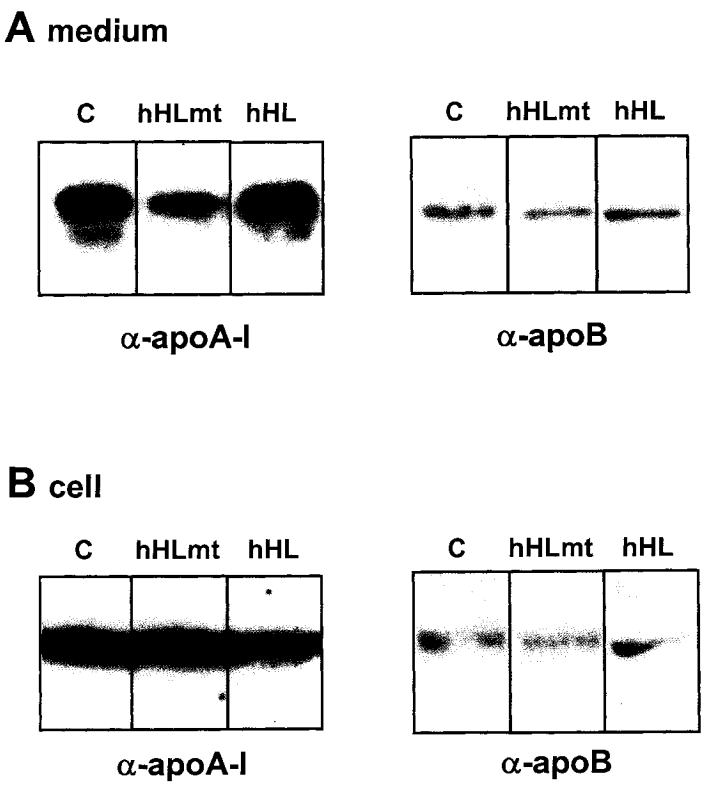

A medium
C
hHLmt
C
hHLmt
C
hHLmt
α-apoA-I
α-apoB
α-apoE
B cell
C
hHLmt
C
hHLmt
C
hHLmt
α-apoA-I
α-apoB
α-apoE

(**Figure 3.2.8, *panel B***). The hepatic apoE or apoB48 concentrations were not changed between hHLmt-expressing and luciferase control cells.

Reduced hepatic secretion of apoA-I was also observed with primary hepatocytes isolated from normal mice that were infected with virus encoding hHLmt *ex vivo*. One day post-infection, the cells were incubated with serum-free media for 4 h and the accumulation of apoA-I at the end of incubation was determined by immunoblot analysis. As shown in **Figure 3.2.9, *panel A***, accumulation of apoA-I in the conditioned media of cells infected with hHLmt virus was decreased as compared to cells infected with either hHL or luciferase. There was no difference in accumulation of apoB48 in the media between hHLmt-expressing cells and hHL- or luciferase-expressing cells. Unlike the primary hepatocytes isolated from mice infected with hHLmt where hepatic apoA-I was reduced (**Figure 3.2.8, *panel B***), the cell-associated apoA-I in cells transiently infected with hHLmt *ex vivo* did not show alterations as compared to cells infected with luciferase control (**Figure 3.2.9, *panel B***). The *in vivo* and *ex vivo* data together suggest that the impaired hepatic apoA-I production, combined with the elevated plasma pre-heparin HL activity, may underlie the hypoalphalipoproteinemia effect from expression of the HSPG binding-defective hHLmt.

Figure 3.2.9. Analysis of apoproteins from *ex vivo*-infected primary hepatocytes

Primary hepatocytes were isolated from 6-8 week old female C57BL/6J mice. Cells were infected with 20 VP/cell of luciferase (*C*), hHLmt, or hHL virus, and 24 h post-infection the cells were incubated in a serum-free medium for another 4 h. Cell (*panel A*) and medium (*panel B*) samples were prepared after incubation and analyzed by immunoblotting as in **Figure 3.2.8**. The experiment was performed in duplicate and presented data are representative.


A medium
C
hHLmt
hHL
α-apoA-I
C
hHLmt
hHL
α-apoB
B cell
C
hHLmt
hHL
α-apoA-I
C
hHLmt
hHL
α-apoB

CHAPTER 4 – DISCUSSION

4.1 Overview

The present work attempts to examine the functional and physiological significance of the cell surface anchorage of hHL. To address the importance of cell surface binding, the normal HSPG-binding of hHL was disrupted using recombinant DNA techniques. The effects of mild (up to 5-fold) overexpression of wild-type hHL and an HSPG binding-impaired hHL (hHLmt) on lipoprotein metabolism in mice were compared and contrasted. The data have shown that the expression of the heparin binding-deficient hHLmt results in a severe hypoalphalipoproteinemia phenotype in mice, as exemplified by markedly decreased HDL lipids and proteins.

4.2 Cell surface association of hepatic lipase

A "loss of function" approach was used in cell culture studies to identify a high affinity region of cell surface binding function by hHL by generating the chimeric mutant hHLmt and the deletion mutant hHL_{471}. The results suggest that at least one determinant of binding to the cell surface exists within the C-terminal 70 amino acids of hHL, of which the last five residues contribute significantly to HSPG binding. Alignment analysis of the primary amino acid sequences shows divergence at the C-termini between hHL and mHL, particularly among the last 23 amino acids of hHL that contain several basic amino acids that are not found in mHL (**Figure 3.1.2, *panel A***). Substituting the C-

terminal sequence of hHL with that of mHL decreased the cell surface association of hHL by more than 20-fold (**Figure 3.1.6**). The C-terminal five amino acid residues of hHL (KRKIR) account for the majority of the cell surface association by the C-terminal 70 amino acid residues, as evidenced by the 15-fold reduction of association by the deletion of these five residues in hHL_{471} (**Figure 3.1.6**). Thus, our results provide the first *in vitro* evidence that the difference in cell surface association between hHL and mHL is attributable to divergence in the C-terminal sequence, most likely in the basic amino acid residues.

While the present studies derived from the "loss-of-function" approach are suggestive, they do not allow one to draw a definitive conclusion that the C-terminus of hHL possesses the HBD. A "gain-of-function" approach was conducted by creating the mutant mHLht, in which the C-terminal sequences of the mHL were substituted with hHL sequences. Unfortunately, this chimeric protein was extremely unstable and was degraded in transfected cells with no detectable secretion (**Figure 3.1.2, *panel C***). The instability was proposed to have been caused by the formation of a potential salt bridge, thus an attempt to remove this potential bridge was made (through the generation of $mHLht_{DN}$). Unfortunately, the attempt to create a stable mHLht protein was unsuccessful (**Figure 3.1.3, *panel B***). An explanation for the instability of the mHLht protein cannot be made at this time without the availability of structural information on both the hHL and mHL proteins.

Although our study suggests that the C-terminal basic amino acid residues are the major factors for HL cell surface association, it by no means implies that these are the sole determinants. In fact, the *in vitro* studies show that mHL exhibits weak HSPG

association (**Figures 3.1.6 and 3.1.8**). Approximately one-third of total releasable mHL and hHLmt remained associated to the cell surfaces in the absence of heparin (**Figure 3.1.6**). These results suggest that sequences other than the C-terminal region must contribute to the remaining overall affinity of mHL. A recent study reported that six basic amino acid residues located in two clusters (Cluster 1: Lys^{297}, Lys^{298}, and Arg^{300}; Cluster 4: Lys^{436} and Arg^{443}) of rHL were involved in heparin binding (227). These two clusters of basic amino acid residues have the xBBxBx and xBxxxxxxBx arrangement, respectively, that are characteristic of the putative HBD (214,218). Alignment of these residues can show that these two clusters of the rat sequences were conserved in both hHL and mHL. Hence, it is likely that these residues may constitute the weak binding affinity components of hHL and mHL.

It is generally considered that an electrostatic interaction between the positively charged amino acid residues and the negatively charged sulfate groups of HSPG is important for protein binding to heparin (208). However, specific positioning of the positively charged residues within a structural motif is perhaps also important. One of the proposed HBD sequences, xBxxxxxxBx, has been modeled to exist as β-strands in some proteins and as α-helices in others. In either case, the two basic residues are spaced 20 Å apart (218). Recent studies with human vitronectin show that residues 343–356, which are involved in heparin binding, are arranged in the xBBxBx sequence (272), which is thought to assume a β-sheet conformation. It should be noted that the C-terminal ten amino acids of hHL have both the xBBxBx and xBxxxxxxBx sequence (**Figure 3.1.2, *panel A***), both of which are absent in the hHLmt and hHL_{471} mutants. Thus, the cell surface-binding motif of hHL may reside at this region in a β-strand

conformation. What remains to be determined is the critical residue(s) within the KRKIR motif of hHL (amino acid residues 472-476) that confer to HSPG binding. In addition to electrostatic contacts, protein-heparin interactions involve other forces. Studies with fibroblast growth factor receptor, for example, have indicated that its interaction with heparin requires substantial van der Waals contact (273). An understanding of HSPG-binding motifs within hHL awaits detailed structural analysis of the protein.

Unlike the *in vitro* studies where two-thirds of the total heparin-releasable hHLmt can be released from cell surfaces in the absence of heparin (**Figure 3.1.6**), only half of the total heparin-releasable HL (both mHL and hHLmt) activity was detected in the plasma of mice expressing hHLmt in the absence of heparin (**Figure 3.2.1**). The possibility cannot be excluded that cell surface binding sites may exist outside of the liver that could be capable of capturing circulating hHLmt, thus reducing the circulating hHLmt activity in the absence of heparin. These sites may include extrahepatic HSPGs, receptors such as LRP (234,256), and even an identified hepatic cell surface HL binding protein, that is heparin-releasable from cell surfaces but with an unidentified function (274). It also may be possible that factors in mouse plasma may exist that have some inhibitory effect on hHL and hHLmt in the absence of heparin, thus potentially masking the amount of measurable pre-heparin activity in hHLmt-expressing mice. For example, human apoC-I has been shown to inhibit VLDL hydrolysis by rHL *in vitro*, in a dose-dependent manner (124). It may be possible for mouse apoC-I to have a greater inhibitory effect on hHL and hHLmt versus any potential inhibitory effects on mHL in the absence of heparin. Detailed studies on the enzyme kinetics of different HLs in the

presence of inhibitory apolipoproteins have yet to be performed, and the potential apolipoprotein interaction sites on these HLs have yet to be identified.

4.3 Enzyme kinetics of recombinant hepatic lipases

The activities of the recombinant HL proteins toward different substrates were assessed *in vitro* to ensure that the wild-type (hHL and mHL) and mutant (hHLmt and hHL_{471}) HL proteins were indeed catalytically active enzymes. All HL proteins were active enzymes (**Figures 3.1.4 and 3.2.6**), and provided interesting results.

Using the triglyceride substrates triolein and tributyrin, the chimeric hHLmt exhibited a mild reduction in activity (apparent V_{max}) relative to hHL (**Table 3.1.1**), despite sharing the same catalytic sites. This could suggest that substituting the C-terminal sequences of hHL with those from mHL grossly disrupted the protein structure to reduce the enzymatic activity of hHLmt, however the affinities (apparent K_m) by hHLmt to these triglyceride substrates were comparable to the affinities by hHL (**Table 3.1.1**). Thus, sequences within the C-terminal 70 amino acid residues of hHL may play a role in the enzymatic activity; such a function has been proposed for C-terminal sequences of LPL (275). Using the phospholipid substrate DPPC, the chimeric hHLmt had a reduced activity (due to both tighter particle binding and a lower apparent maximal rate of hydrolysis), relative to hHL (**Table 3.2.1**). These data not only suggest that hHLmt may actually have a reduced activity toward HDL lipid, but also suggest that the disappearance of the HDL lipid in hHLmt-expressing mice is not due to a gain of a "hyperphospholipase" activity.

The most notable enzymatic results come from the comparisons between hHL and mHL. This is the first study to compare the enzymatic properties between these two lipases, and the data suggest that mHL has a much greater hydrolytic activity, compared with hHL, toward the water-soluble tributyrin and polar DPPC, despite comparable catalytic properties toward the long-chained TG triolein (**Tables 3.1.1 & 3.2.1**). These enzymatic data suggest that there may be a structural difference in the substrate binding pocket between these two lipases. In fact, the 'lid' domains between these two lipases (amino acid residues 231-254 for hHL, 234-257 for mHL) have four amino acid substitutions between each other, that may result in mHL to be more catalytically active toward tributyrin and DPPC. However, the difference between these two lipases may be more complex. The eluted mHL from the heparin-Sepharose chromatography exhibited a molecular mass above 220 kDa by SDS-PAGE, suggesting the tetramerization of the protein (**Figure 3.1.8, *panel B***). Such tetramerization may give mHL its different enzymatic properties, in contrast to those of hHL that has been previously reported to be active as a homodimer (35).

4.4 Hypoalphalipoproteinemia by hHLmt *in vivo*

The advantage of comparing the HSPG binding-impaired hHLmt *in vivo* to the wild-type hHL is that an elevation of circulating HL levels *in vivo* is achieved (using hHLmt) without heparin administration that can indiscriminately remove all HSPG-binding proteins from the endothelium surface. The hHL and mHL proteins could not simply be compared and contrasted, because the enzyme kinetics data toward the short-chained TG tributyrin and the phospholipid DPPC were strikingly different between these

two HL species (**Tables 3.1.1 & 3.2.1**). Although some differences exist for the catalytic properties between hHL and hHLmt, they do share the same N-terminal sequences for catalytic activity, thus these lipases were more suitable for comparison *in vivo*.

In humans, high plasma HL activity (measured using post-heparin samples) is associated with reduced HDL cholesterol and small HDL particles (276). The levels of HDL cholesterol levels and post-heparin hHL activities have also been shown to be directly related to polymorphisms within the *LIPC* promoter. For example, in comparison to humans carrying the T-allele at the -514 position of the *LIPC* promoter, humans carrying the C-allele have up to a two-fold elevation of plasma post-heparin hHL activity and down to a four-fold reduction of HDL cholesterol (94,98). Conversely, genetic HL deficiency is associated with modestly elevated HDL cholesterol and large HDL particles (19,277,278). Reduced HDL lipid and HDL proteins observed in mice expressing the HSPG binding-deficient hHLmt suggest that the level of circulating HL may be catalytically more active than the cell surface-anchored HL in the catabolism of HDL. Three-fold more HL activity is present in the pre-heparin plasma of hHLmt-expressing mice compared to hHL-expressing mice (**Figure 3.2.1**). However, the hypoalphalipoproteinemia phenotype in hHLmt-expressing mice may involve a combination of both the HL enzymatic function and also a non-enzymatic function. These enzymatic versus non-enzymatic roles by the heparin binding-impaired hHLmt *in vivo* have not been addressed, but could be addressed in future studies by generating and expressing a catalytic-inactive version of hHLmt *in vivo*.

Factors that regulate the dissociation of HL from cell surfaces *in vivo* are unclear. Recent studies demonstrated that apoA-I and HDL can displace hHL from purified

proteoglycans *in vitro* and from cultured cell surfaces (12,120,125), and that hHL is active only when it is free in solution. The present study with the HSPG binding-deficient hHLmt provides *in vivo* evidence that the metabolism of HDL may occur in the circulation by the blood-borne HL, whereas the cell surface bound hHL is less involved. To date, no mutations within hHL have been reported that affect HSPG binding or affect HDL lipid and protein levels. A positive correlation has been suggested between the post-heparin hHL activity and the pre-heparin hHL activity (259). Hence, it is possible that the aforementioned inverse relationship between post-heparin hHL activity and plasma HDL may also reflect a relationship between pre-heparin hHL activity and HDL.

It is noteworthy that a major difference in the experimental design between the present experiments and previous ones [including (31,121,236,264,279)] is the low dose of adenoviruses (1.8×10^{10} VP) utilized, which resulted in an elevation of post-heparin hHL or hHLmt activity at maximum only 5-fold above control levels. The HDL lowering effect of hHLmt in mice is unlikely the artifact of an overly high level expression of the recombinant enzyme proteins, because expression of the wild-type hHL at similar levels exerts no effect. The expression of recombinant HL activity at this low level would be of more physiological relevance than the massively increased HL activity (by 50- to 100-fold) achieved in mice (121,236,279) or rabbits (264) by adenovirus infection or transgeneic technologies. These previous works showed that hHL overexpression was invariably associated with reduced total cholesterol and phospholipid in plasma and HDL, indicating that HL plays a major role in HDL metabolism and enhancing the catabolism of HDL. However, the possibility that excessive HL expression in the transgenic animals might render elevated free HL in circulation that in

turn affects HDL metabolism was not examined previously. Thus, the current studies have revealed an important functional significance for HSPG binding of hHL in the metabolism of HDL that originate from the liver.

Although no mutations in hHL have been reported to date that are associated with hypoalphalipoproteinemia, other proteins in HDL metabolism with naturally occuring mutations have been reported. The observed phenotype in the hHLmt-expressing mice closely resembles the phenotype observed in Tangier disease, which is associated with mutations in ABCA1 (172,280). Subjects with Tangier disease exhibit very little to no α-migrating HDL and HDL cholesterol, and have only 1-3% of normal plasma apoA-I levels that are almost exclusively associated with pre-β_1-HDL (281) due to impaired HDL maturation and enhanced apoA-I catabolism. The circulating hHLmt in mice may be rapidly metabolizing the HDL lipids and promoting the uptake and catabolism of the HDL particles by various tissues. Although not addressed in this study, the circulating hHLmt may also be effectively hydrolyzing lipids in the circulation (including lipids provided by ABCA1) that could be used for HDL maturation, thus likely enhancing apoA-I catabolism. To date, no investigations have been carried out to determine if any interaction exists between ABCA1 and HL.

Naturally occuring mutations of LCAT are also associated with low levels of circulating HDL lipid and protein (282). Targeted deletion of LCAT in mice also reduces HDL lipid and protein levels to 10% of normal, however a five-fold increase of VLDL cholesterol and phospholipid levels was also observed in these mice when fed a normal chow diet (283). Such an increase in both the VLDL cholesterol and phospholipid was not observed in the hHLmt-expressing mice (**Figure 3.2.4**). However, the possibility of

hHLmt potentially disrupting LCAT function to yield the observed hypoalphalipoproteinemia phenotype cannot be excluded, due to the depletion of phospholipid substrate for LCAT. Mouse models for the targeted deletions of PLTP (284) and LPL (285) also exhibit hypoalphalipoproteinemia, however, major differences in lipoprotein profiles exist between the hHLmt-expressing mice and these knockout mice. In PLTP-deficient mice that exhibit hypoalphalipoproteinemia as a result of hypercatabolism of HDL proteins, the secretion of lipoproteins containing apoB is also diminished (286). The lack of an effect of hHLmt expression on apoB (**Figures 3.2.8, *panel A* & 3.2.9, *panel A***) excludes the possibility of altered PLTP activity in hHLmt-expressing mice. In LPL-deficient mice, low HDL cholesterol are accompanied with severe hypertriglyceridemia, likely as a consequence of impaired lipolysis of TG-rich lipoproteins (285). However in hHLmt-expressing mice, low HDL was associated with no apparent change in apoB-containing lipoproteins (**Figures 3.2.2, *panel A* & Figure 3.2.4**), ruling out the possibility that hHLmt expression suppresses LPL-mediated lipolysis.

It should be noted that unlike hHL whose post-heparin activity is negatively correlated with plasma HDL concentrations, post-heparin plasma LPL activity is positively correlated with HDL cholesterol levels (276). Expression of a heparin binding-deficient human LPL in mice results in elevated TG and cholesterol associated with very low density lipoproteins (287). Thus, the mode of action is distinct between LPL and HL in regulating plasma lipoproteins including HDL. The studies comparing wild-type hHL and the heparin binding-impaired hHLmt have not addressed the fates of the apoB-containing lipoproteins. Although the expression of hHLmt did not affect apoB

levels, the possibility exists that the hHLmt expression lead to the enhanced production of smaller and denser LDL, which is thought to occur by elevated levels of pre-heparin hHL activity (259). This speculation can be studied using animal models that have high levels of apoB-containing lipoproteins, including *LIPC*-null mice (104), apoE-deficient mice (243), and human apoB transgenic mice (288).

Together with the severe hypoalphalipoproteinemia phenotype in mice expressing hHLmt, an additional unexpected observation made in the present study is that decreased extracellular apoA-I from the hepatocytes of mice infected with virus encoding hHLmt (for ten days) is accompanied with reduced cell-associated hepatic apoA-I (**Figure 3.2.8**). This apoA-I lowering effect of the HSPG binding-deficient hHLmt appears to be specific, as apoB and apoE levels were unaffected (**Figure 3.2.8**). The mechanism by which expression of the HSPG binding-deficient hHLmt decreases hepatic apoA-I production and its contribution to the observed hypoalphalipoproteinemia phenotype is unclear and remains to be defined. Reduction in cell-associated hepatic apoA-I, however, was not observed with hepatocytes acutely infected with hHLmt *ex vivo*. Thus, the decreased hepatic apoA-I production appears to be a slow response to hHLmt expression. Whether or not there are extrahepatic factors that are altered due to hHLmt expression that inhibit hepatic apoA-I synthesis remains to be determined. The factor(s) may include a protein or perhaps even a specific species of lipid that is affected by hHLmt. Nevertheless, decreased extrahepatic of apoA-I from primary hepatocytes acutely infected with virus *ex vivo*, in the face of normal cellular apoA-I (**Figure 3.2.9**), suggest that the HDL-lowering effect of hHLmt observed *in vivo* is at least partly attributable to impaired hepatic secretion of apoA-I.

4.5 Summary and conclusions

In summary, the present work shows that the C-terminal 70 amino acid residues of hHL contain a high-affinity HSPG binding region, and the C-terminal 5 amino acid residues contribute significantly to this high-affinity region. Although the molecular biology approach for identifying regions or amino acid residues involved in HSPG binding are powerful, the risk exists of generating proteins that are not functional perhaps due to an altered structure, as evidenced with the mHLht data (**Figures 3.1.2, *panel C* & Figure 3.1.3, *panel B***). Detailed structural data is necessary in understanding the exact amino acid residues responsible for the HSPG binding by hHL.

The short-term expression of the HSPG binding-deficient hHLmt at low levels results in severe hypoalphalipoproteinemia, which suggests that the lipolytic degradation of HDL occur not at cell surfaces but rather away from cell surfaces. The extracellular and intracellular mechanisms of how hHLmt lead to the hypoalphalipoproteinemia need to be elucidated. The heparin binding-impaired hHLmt may enhance the metabolism of HDL lipids, and the subsequent clearance and catabolism of HDL and apoA-I perhaps through both catalytic and non-catalytic roles. These possible events may be assessed in cell culture studies, where the pool size of apoA-I can be controlled. The reduction of hepatic apoA-I by the heparin binding-impaired hHLmt is novel and requires extensive study to identify extrahepatic or intracellular mechanisms leading to this reduction.

Although genetic variation of the HL gene *LIPC* is an important source of variation in HDL levels in general population (177), the relationship between HL and atherosclerosis is complex. Data support HL as being both pro-atherogenic and anti-

atherogenic (28,289,290). Since hypoalphalipoproteinemia is frequently associated with atherosclerosis, it will be of interest to determine whether or not a long-term expression of both a catalytically active and catalytically inactive hHLmt will pose a risk for atherosclerotic vascular diseases.

CONTRIBUTION OF COLLABORATORS

Section 3.1, Identification of a heparin binding region in hHL

Dr. Kerry Ko (generation of some hHL expressing cell lines);

Dr. John Hill (generation of the hHL_{471} for insertion into pCMV5);

Dr. Tanya Ramsamy (isolation of hHL from human plasma for standards under supervision of Dr. Dan Sparks);

Dr. Ann White (generation of the anti-hHL polyclonal antibody).

Section 3.2, Effects of HSPG binding-impaired hHL *in vivo*

Vivian Chow (HL kinetics data using DPPC);

André Gauthier (injection of viruses and isolation of primary hepatocytes under supervision of Dr. Ruth McPherson);

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** Techniques used include: yeast culture, yeast transfection, homologous recombination of yeast genome, mutagenesis, PCR, DNA isolation.

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