Mapping the Heparin Binding Domain of Human Hepatic Lipase

by

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ABSTRACT

Hepatic lipase (HL) is an enzyme that plays many roles in lipid metabolism. HL is involved in the catabolism of lipoprotein particles and the hydrolysis of intermediate density lipoprotein (IDL) with the formation of low density lipoprotein (LDL). HL facilitates the remodeling and recycling of anti-atherogenic high density lipoprotein (HDL), an important step in the putative process of reverse cholesterol transport. HL is also involved in the selective hydrolysis and uptake of cholesterol ester (CE) contained within HDL. However, little is known about the mechanisms of action of HL responsible for its functional roles. What is known, is that HL is found bound primarily to hepatocyte cell surface heparan sulfate proteoglycans (HSPG). Previous studies have suggested the heparin-binding domain of HL is located in the terminal 60 amino acids of the HL protein. It was our hypothesis that by the application of multiple strategies we could further define specific amino acid residues responsible for the heparin binding of HL. Regions 294-315 and 453-475 are rich in reported heparin binding consensus sequences but homology modeling implicated only the former region as the latter could not be modeled. A synthetic peptide corresponding to amino acids 304-323, containing R306, R310, K312, K314, R315 and R321, displayed moderate heparin affinity eluting from a heparin-Sepharose column at 0.42M and 0.35M NaCl, in the absence or presence of a secondary structure inducer (TFE), respectively. A synthetic peptide representing residues 455-471, containing K459, K463, K467, K469, displayed no affinity for heparin in the absence of TFE (0.15M), but eluted in two peaks (0.15M and 0.72M NaCl) in the presence of TFE. Molecular identification of the species responsible for the two peaks could not be determined. Peptides of regions 355-377, 419-442, and 452-471 did not display affinity for the heparin-Sepharose column. However, none of the peptide displaced HL bound to the surface of transfected Chinese hamster ovary cells. Additionally, a panel of monoclonal antibodies against the 452-471 failed to associate with HL or inhibit the binding of HL to heparin. These results indicate that regions 304-323 and 455-471 may contribute to HL heparin binding domain.

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AMINO ACID DESIGNATIONS

Amino Acid	Three Letter Code	Single Letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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1. INTRODUCTION

1.1 Atherosclerosis

1.1.1 Etiology

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. In developed countries atherosclerosis is the leading cause of death [1] and it underlies approximately 50% of all deaths in westernized societies [2]. The initial event in the development of atherosclerosis is the formation of the 'fatty streak', an accumulation of cholesterol engorged macrophages, or 'foam cells' in the intimal layer of the artery. The fatty streak is not clinically significant but can lead to advanced lesions characterized by a fibrous cap containing smooth muscle cells (SMC) and extracellular matrix, and a lipid-rich necrotic core. Although occlusion of the artery due to the growing lesion is a serious complication of atherosclerosis, the greatest danger is rupture or erosion of the lesion resulting in thrombosis.

Numerous risk factors ***Table 1*** with strong genetic components have been found to be associated with the development of atherosclerosis and coronary artery disease (CAD). The relative abundance of various lipoproteins appears to be particularly important, as most forms of atherosclerosis are associated with an atherogenic lipoprotein profile. Raised levels of the lipoproteins of the low density (LDL) and very low density (VLDL) classes, particularly the cholesterol fractions (LDL-C and VLDL-C), have been shown through studies of genetic disorders and animal models to contribute to the development of atherosclerosis [3]. High density lipoproteins (HDL) have been shown to have a cardio-protective effect and reduced HDL levels are associated with atherosclerosis [4]. High levels of lipoprotein (a) (Lp(a)) have also been implicated as atherogenic in some studies [5]. Other risk factors with a strong genetic element include: high blood pressure, elevated homocysteine levels [6], a family history of heart disease [7], diabetes and obesity [3], elevated levels of the coagulation factors [3], behavioral traits such as depression [8], gender (male) [9], systemic inflammation [10], and presence of a metabolic syndrome [11].

Studies have also shown associations between atherosclerosis and certain lifestyle factors ***Table 1***, particularly diet. A high fat, high cholesterol diet is strongly associated with the development of atherosclerotic lesions [3]. There is also a strong association between smoking and atherosclerosis while lack of exercise is independently associated with (CAD) [3]. Fat-soluble antioxidants are protective in animal models, however, clinical trials with people at high risk for cardiac events indicates no significant protection is provided [12,13]. There is also preliminary evidence that certain infectious agents, for example *Chlamydia pneumonia*, are associated with CAD [14].

Table 1. Risk Factors for Atherosclerosis.

Non-Modifiable
Age
Family history of heart disease
Gender (male)
Modifiable
High fat diet
Smoking
Lack of exercise
Low antioxidant levels
High blood pressure
Obesity
Behavioral traits
Increased LDL
Increased VLDL
Reduced HDL
High levels of Lp(a)
Elevated homocysteine
Diabetes
Elevated levels of coagulation factors
Systemic Inflammation
Metabolic syndrome
Infectious agents

1.1.2 Lesion Initiation

The fatty streak results from an inflammatory response to endothelial injury [15]. Endothelial injury is more likely to occur in regions of the artery that are subject to a low fluid shear stress, where blood flow is disturbed, such as regions of arterial branching and curvature. Endothelial cells located in tubular regions of the artery are ellipsoid in shape and are aligned in the direction of blood flow whereas endothelial cells in curved or branched regions of artery are polygonal with no specific orientation. The polygonal, unaligned endothelial cells are more permeable to macromolecules including lipoproteins. The influx of lipoproteins across the endothelium into the intima is injurious and these areas of disturbed flow show increased susceptibility to lesion formation [16].

The formation of the fatty streak is initiated by the accumulation of LDL in the subendothelial matrix. LDL passively diffuses through endothelial cell junctions and appears to be retained in the vessel wall due to an interaction between a protein contained in LDL (apo B) and matrix proteoglycans [17]. This accumulated native LDL is not taken up by macrophages at a rate sufficient to result in the formation of foam cells, however, this native LDL is modified as a result of its accumulation on

the subendothelial matrix. Native LDL undergoes lipolysis, proteolysis, aggregation, and most significantly oxidation. The retained LDL is oxidized by exposure to the oxidative waste (reactive oxygen species or ROS) of the vessel wall and possibly by the enzyme 12-15 lipoxygenase [18]. This minimally oxidized LDL (m[O]LDL) may not be sufficiently recognized by macrophage scavenger receptors to elicit foam cell formation, but it is sufficiently modified to spark an inflammatory reaction.

1.1.3 Inflammation

Lesion inflammation is characterized by the recruitment of monocytes and lymphocytes, but not neutrophils, to the arterial endothelial injury. The accumulation of m[O]LDL stimulates the overlying endothelial cells to produce pro-inflammatory molecules including the adhesion molecules ICAM-1, VCAM-1, the P- and E-selectins, and the growth factor monocyte colony stimulating factor (M-CSF). Initially the leukocytes 'roll' along the endothelial surface and adhere to the P- and E-selectins or to ICAM-1 [19,20]. Strong adhesion of the leukocytes is subsequently achieved through binding of the cells to VCAM-1 [21]. Once the leukocytes have entered the lesion, M-CSF stimulates the differentiation and proliferation of macrophages and increases the expression of macrophage scavenger receptors [22].

m[O]LDL also decreases endothelial cell production of nitric oxide (NO), a compound with multiple anti-atherogenic properties. NO activities include vasorelaxation [23-25], inhibition of monocyte and platelet adhesion [26,27] possibly via the inhibition of VCAM-1 [26-30], and the inhibition of SMC migration from the media and extracellular matrix [9].

1.1.4 Foam Cell Formation

The precipitous event to foam cell formation is the conversion of m[O]LDL to a highly oxidized form of LDL (h[O]LDL). Within the subendothelial layer of the artery, m[O]LDL is further oxidized by the enzymes myeloperoxidase [31], sphingomyelinase [32] and secretory phospholipase [33], and presumably the ROS of endothelial cells and macrophages to yield h[O]LDL. This h[O]LDL is recognized by macrophage scavenger receptors and is rapidly taken up by macrophages leading to the formation of foam cells. The foam cell is a dysfunctional cell and the conversion of a macrophage to a foam cell results in apoptosis and necrosis. The death of foam cells leaves behind a growing mass of extracellular lipids and cellular debris.

1.1.5 Fibrous Plaque Formation

There are several hallmarks of the fibrous plaque including the buildup of extracellular lipid, mostly cholesterol and cholesterol ester, the migration and accumulation of smooth muscle cells (SMC) in the subendothelial space, and the proliferation of SMC-derived extracellular matrix. Cholesterol and cholesterol ester are present in the fibrous plaque as a result of earlier lipoprotein infiltration. Macrophages and T-cells in the subendothelial space, stimulated by endothelial cell

injury and m[O]LDL, produce inflammatory cytokines such as IFN-γ, and matrix-degrading proteases. These cytokines and proteases play a key role in the migration and proliferation of SMC and the production of extracellular matrix, and ultimately, the development of advanced lesions [34,35].

1.1.6 Advanced Lesions and Thrombosis

Thrombus mediated acute coronary events appear to be mediated more by the composition and vulnerability of a plaque rather than the severity of occlusion. Vulnerable plaques tend to have thin fibrous caps with large numbers of monocytes and lymphocytes and are inclined to rupture at the edges of the lesion, which are rich in foam cells. Calcification and vascularization, both common features of the advanced lesion, are also associated with plaque vulnerability. The thrombogenicity of the lesion core likely depends on the presence of tissue factor, the production of which is enhanced by oxidized LDL and infection [2].

1.2. Lipoprotein Metabolism

1.2.1. Lipoproteins

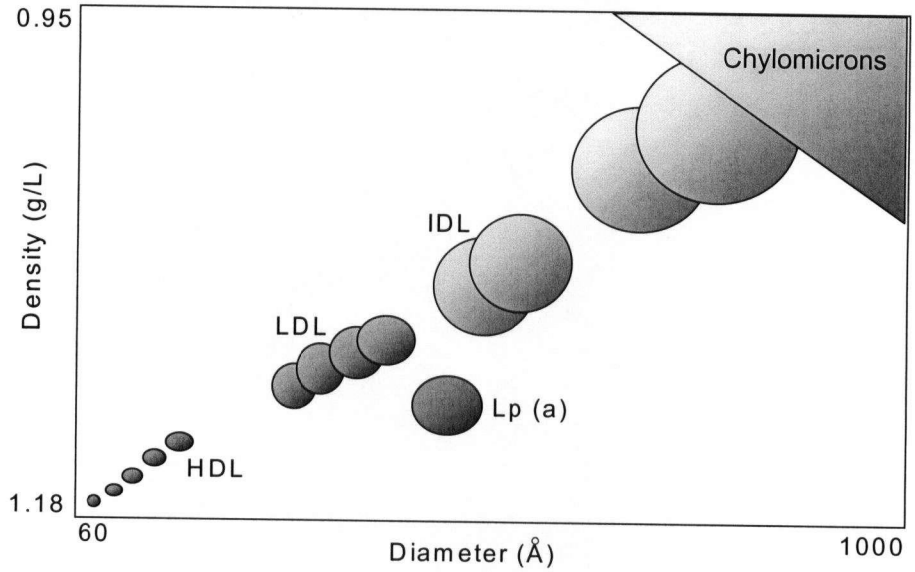


Figure 1. Density versus Diameter of Lipoprotien Subclasses. *Diameter and density increase from the relatively triglyceride poor and cholesterol ester rich HDL particles to the triglyceride rich and cholesterol ester poor VLDL and chylomicrons*

Lipoproteins are globular, sometimes discoid particles that vary in size and composition, but all are composed of a hydrophilic outer surface and hydrophobic core. Lipoproteins can be separated by ultracentrifugation into five main classes: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density lipoproteins (HDL) [36,37] ***Figure 1***. Lipoprotein density increases and size decreases from chylomicrons to HDL. Although this classification assumes a discrete hierarchy of structure, lipoprotein particles in the plasma exist as gradually differentiated particles.

The outer surface of lipoprotein particles is composed of a phospholipid bilayer containing free cholesterol (FC), small amounts of non-polar lipids, and at least one apoprotein. Apoproteins enhance the structural integrity of the lipoprotein and control lipid metabolism through interactions with membrane receptors or by acting as cofactors for enzymes involved in lipoprotein metabolism. Conversely, the lipoprotein core contains mainly cholesterol esters (CE), which can be used for synthetic (bile) or structural purposes, and triglycerides (TG), which are an energy-rich metabolic fuel and the most efficient form of energy utilization and storage.

Lipoproteins exist primarily as a lipid transport system to transport energy-rich TG and cholesterol from the intestine and liver to the adipose tissue. During the life of the lipoprotein, hepatic (HL) and lipoprotein lipases (LPL) successively degrade TG liberating fatty acids (FA) [38] that are subsequently absorbed by peripheral tissues. FA liberated from TG are metabolized mainly in liver and muscle but stored in adipose tissue. Since energy utilization and storage occur in different cells, an efficient inter-organ transport system is necessary. Hydrophobic lipids cannot be transported in free form in the aqueous environment of the plasma and can only be transported bound to albumin or as components of amphipathic lipoprotein particles.

1.2.2. Exogenous Pathway

The Exogenous pathway begins with the consumption of dietary lipid and its movement through the gastro-intestinal tract ***Figure 2***. TG are hydrolyzed in the small intestine by pancreatic lipase (PL) to yield monoglycerides (MG) and FA. The MG and FA are absorbed into the enterocyte cytoplasm where they are repackaged into TG. Similarly, CE is hydrolyzed in the small intestine by cholesterol esterase to produce FC and FA. The FC and FA are likewise absorbed into the cytoplasm of the enterocyte and converted into CE. In this manner, the enterocyte is prepared to synthesize lipoproteins.

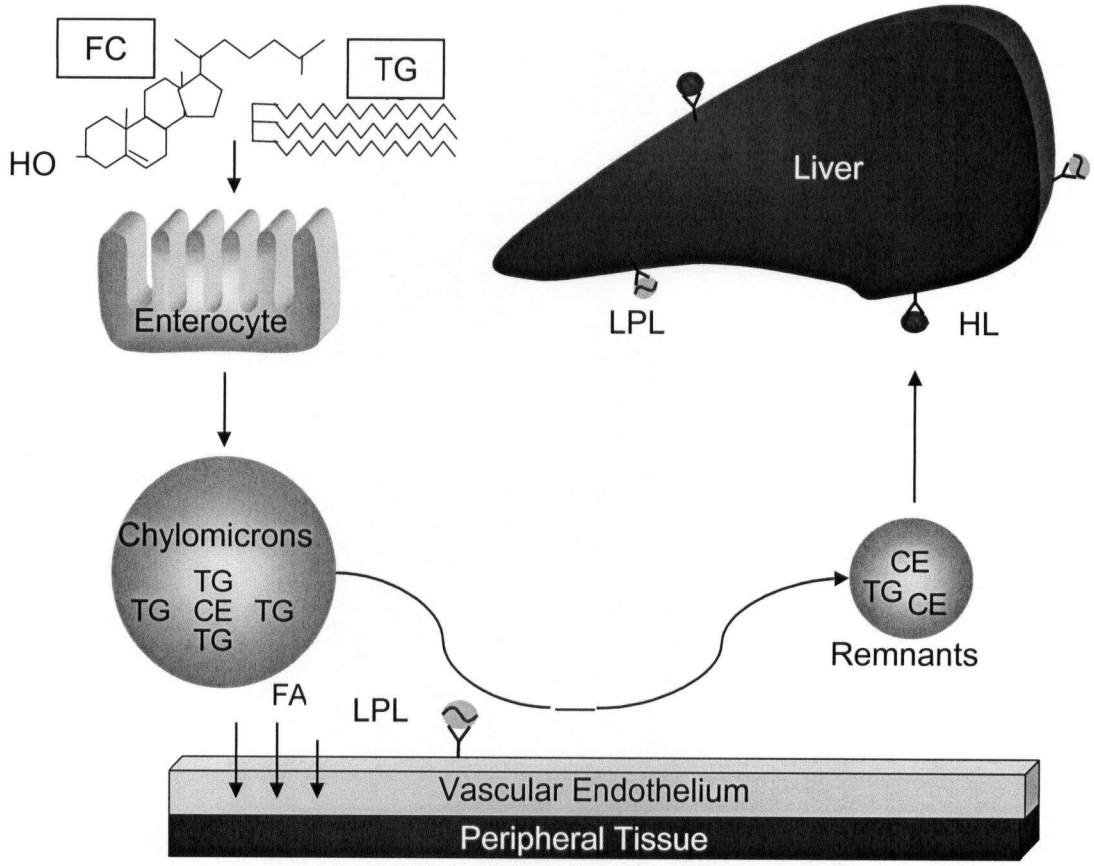


Figure 2. The Exogenous Pathway. *Dietary lipids are absorbed by the enterocyte and packaged with protein into chylomicrons. Chylomicrons are secreted into the plasma where their hydrolysis by LPL yields fatty acids (FA) for peripheral tissue use or storage. Chylomicron remnants are catabolized through the liver. FC=free cholesterol TG= triglyceride CE=cholesterol ester*

The newly re-formed cellular TG and CE are packaged into lipoprotein particles and secreted into the plasma ***Figure 2***. TG and CE are packaged together with enterocyte apoprotein B-48 (apo B-48), and phospholipid bilayer components to produce chylomicrons. Apo B-48 is made up of 2153 amino acids and is essentially the amino terminal 48% of the hepatocyte apoprotein B-100 [39,40]. The enterocyte contains a specific RNA editing enzyme that creates a premature stop codon in apo B-100 resulting in apo B-48. Lipoprotein assembly begins with the secretion of apo B-48 into the rough endoplasmic reticulum (RER) where the protein is minimally lipidated by microsomal TG transfer protein (MTP). This minimally lipidated apo B-48 translocates to the smooth endoplasmic reticulum (SER) where TG rich droplets are formed [41]. The TG rich apo B-48 complex then travels to the RER/SER junction

where expansion of the lipoprotein core occurs and the mature chylomicrons are formed [42]. Chylomicrons are large, buoyant, TG rich and CE poor lipoprotein particle that contain fat-soluble vitamins absorbed from the intestinal lumen. In addition to apo B-48, nascent chylomicrons contains apoproteins A-I, A-II, and A-IV [43]. The enterocyte secretes chylomicrons into the intestinal lymph and they are ultimately emptied into the circulation via the thoracic duct. Chylomicrons, like all lipoproteins, reside in the plasma portion of blood where their metabolism occurs.

In plasma, the chylomicron pool is dynamic and interchanging and acts as a reservoir of cellular energy. While circulating in the plasma, chylomicrons receive apo C-I, C-II, C-III, and apo E from HDL and VLDL [44-46]. Apo C-II and C-III activate and inhibit LPL respectively [38,47], and subsequent to apo C-II acquisition, chylomicrons are acted on by LPL embedded in the vascular endothelium. LPL hydrolyzes chylomicron TG and facilitates the absorption of the consequent FA and glycerol into peripheral tissue. In a fasting state, the hydrolyzed lipids are used in skeletal muscle and the liver to produce energy. In a fed state, the hydrolyzed lipids are transported to adipose for energy storage. Changes in the lipid component of lipoproteins is not the only change that occurs during the metabolism of chylomicrons.

LPL metabolism of chylomicrons and the subsequent compositional changes leads to structural changes. The process of hydrolyzing lipids within chylomicrons by LPL decreases chylomicron mass approximately 20-fold and results in conformational changes of the apoproteins and in the loss of the A apoproteins and phospholipid. The loss of TG also results in the relative concentrating of apo E and B-48 on the surface of the particle. The altered chylomicrons, a class of IDL, are referred to as chylomicron remnants ***Figure 1***. The 'remnant' classification is a functional rather than structural dichotomy as 'remnant particle' is a stage in lipoprotein metabolism that redirects the particle from LPL to catabolic sites at the liver [48].

Although the role of chylomicrons and chylomicron remnants in the development of atherosclerosis is not completely elucidated, it is known that chylomicron concentration may exceed a critical limit above which they can deposit on the artery wall [39,49]. Additionally, TG rich lipoproteins are susceptible to peroxidation and may promote foam cell formation in the absence of oxidative modification [50]. Moreover, smaller remnant particles can penetrate the arterial intima and produce an inflammatory response [51]. Therefore, an efficient means of removing remnants from the circulation is necessary.

1.2.3. Endogenous Pathway

As opposed to the exogenous path that concludes in the liver, the endogenous path begins in the liver with the synthesis of VLDL particles ***Figure 3***. The hepatocyte contains FA, CE, and other lipids as a result of lipogenesis, absorption of plasma FA, or acquired from chylomicron remnants. In contrast to enterocytes that build lipoprotein particles on an apo B-48 backbone, hepatocytes incorporate TG on the backbone of apo B-100 [52]. Apo B-100 is a 4536 amino acid

apoprotein synthesized in the hepatocyte. Apo B-100 is secreted into the cytoplasm, losing 27 NH_2 terminal amino acid residues, and locates to the cytosolic side of the RER. At the surface of the RER apo B-100 is lipidated by microsomal TG transfer protein (MTP) acquiring TG, phospholipid, and CE [53]. This lipidation results in the translocation of the apo B-100-lipid complex to the luminal side of the RER [52,54] and its eventual secretion through the fenestrae of the hepatic sinusoidal endothelium to enter the blood as a VLDL. As well as containing one apo B-100 molecule, VLDL contain apo A-I and A-IV. As with chylomicrons, VLDL function for intracellular TG transport and as vehicles for transport of fat-soluble vitamins [42].

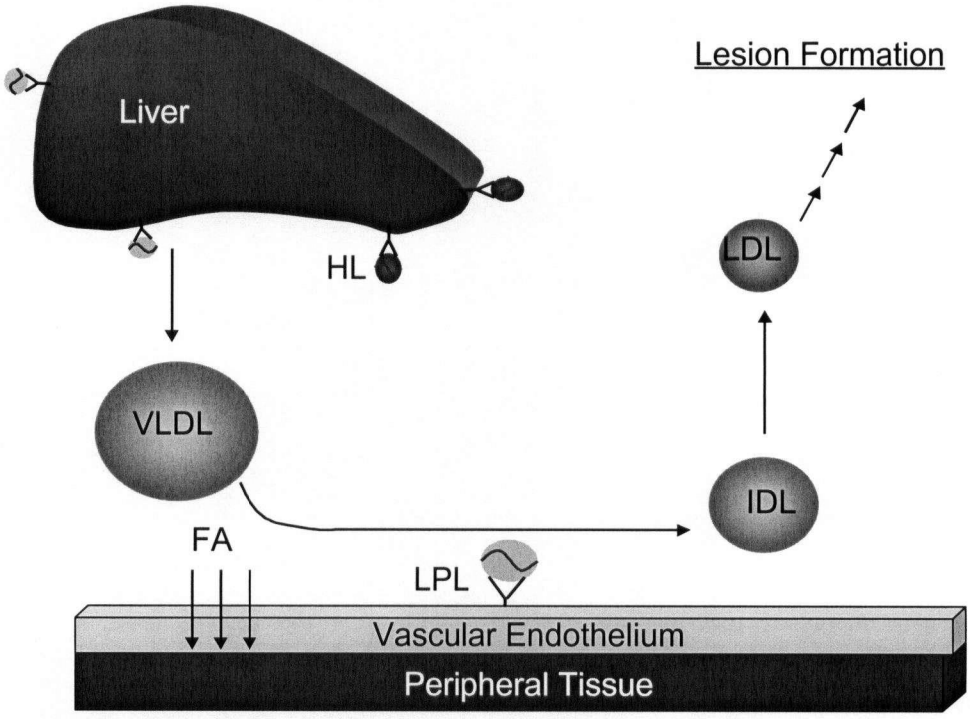


Figure 3. The Endogenous Pathway. Hepatocytes secrete lipoproteins termed VLDL. VLDL are acted on by LPL liberating fatty acis (FA) and resulting in the formation of IDL. IDL is catablolized through the liver or acted on at the surface of the hepatocyte yielding the atherogenic lipoprotein species LDL.

The metabolic fate as well as the production of VLDL in the circulation mimics that of the chylomicrons. VLDL circulating in the plasma interacts with HDL acquiring apo C-I, C-II, and C-III, and apo E in exchange for TG [45]. However, VLDL also receives TG from HDL in exchange for CE through the enzymatic action of cholesterol ester transfer protein (CETP). The apo C-II of VLDL likewise activates LPL resulting in hydrolysis of the lipid core and the release of FA and glycerol into the peripheral tissues. Similar to chylomicrons, VLDL decreases in size after LPL

hydrolysis resulting in a change of conformation of its apoproteins. The VLDL remnant, also a class of IDL, transfers apo A-I and A-IV to HDL thus concentrating the amount of apo E and B-100 contained in the lipoprotein. The majority of VLDL remnants are targeted to the liver for catabolism in a similar manner as chylomicron remnants.

However, a fraction of VLDL remnants are not internalized via hepatocyte receptors. Instead, approximately 10-20% [55] of VLDL remnants are acted on by HL at the hepatocyte surface, losing TG, phospholipid, apo C and apo E [48,56], creating a low density lipoprotein (LDL) ***Figure 3***. LDL contains only a single apo B-100 molecule and no other protein. LDL is directly atherogenic and is the lipoprotein most often implicated in the formation of atherosclerotic lesions in humans. LDL is less efficiently removed from the circulation than other remnants and is capable of entering the vascular endothelium and initiating an inflammatory atherogenic response. Efficient removal of all classes of remnant particles is essential for normal health.

1.2.4. Remnant Particle Catabolism

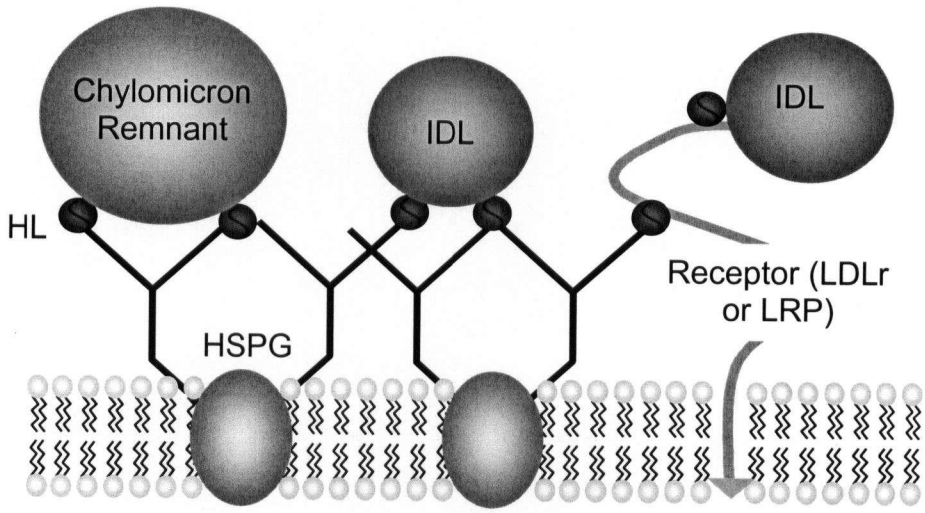


Figure 4. Remnant Particle Catabolism. Chylomicron remnants and IDL are believed to be catabolized by the liver through two concurrent processes. One is receptor mediated endocytosis via the LRP or LDLr, while the other is believed to involve HL tethering the lipoprotein particles to hepatocyte heparan sulfate proteoglycans (HSPG) with concomitant lipid hydrolysis.

Chylomicron remnants are catabolised at the surface of liver through at least two receptors ***Figure 4***. The first is the LDL receptor (LDLr) which recognizes both apo B and apo E - although it recognizes apo E with a higher affinity. Chylomicron remnant removal via the LDLr has been shown by *Choi et al.* [57], *Choi and Cooper* [58], *Ishibashi et al.* [59], *Rohlmann et al.* [60], and *Mortimer et al.* [61]. LDLr removal of chylomicron remnants follows a classical receptor-mediated pathway of endocytosis [62] that is mediated through apo E. The second receptor, termed the LDL receptor-related protein (LRP), is structurally related to LDLr and also recognizes apo E. Although numbers relating the removal of chylomicron remnants through LDLr or LRP are not certain, one study with lipid monolayers suggests that only 35% of chylomicron remnants are catabolised through LDLr [63] with the remaining chylomicron remnants removed via the LRP [64]. This seems logical considering the truncation of apo B-48 could reduce the affinity of chylomicron remnants for the apoB/E receptor LDLr. On the other hand, *Havel 1998* [62], reported that LRP in rat liver bound weakly to chylomicron remnant apo E and did not play a significant role in the initial uptake of chylomicron remnants. However, *Havel* found the affinity of chylomicron remnants for the LRP was increased in the presence of proteoglycan enriched in apo E. As well, HL appeared to mediate binding of chylomicron remnants to the LRP. *Havel 1998* [62] further suggested that lipolysis of chylomicron remnant lipid by HL promoted but was not required for their interaction with LRP and LDLr, and that HSPG bound HL may also mediate remnant particle catabolism through and apo E independent mechanism. The clearance of VLDL remnants is comparable to that of chylomicron remnants.

As a result of the concentrating of apo E and B-100, VLDL remnants are catabolised via the hepatocyte receptors LDLr and LRP. However, the HSPG-LRP path does not appear to play a significant role [65], and the majority of VLDL remnants are removed via the apo B/E receptor LDLr. The removal of lipoprotein remnants from plasma is obviously complex.

1.2.5. Role of HSPG in Remnant Particle Catabolism

A role for HSPG in the catabolism of chylomicron remnants was supported by *Zeng et al. 1998* [66]. Binding of remnant particles to HepG2 cells *in vitro* decreased 60-65% in the presence of monoclonal antibodies to heparan-sulfate or LDLr, while remnant binding decreased by 25% in the presence of the glycosylation inhibitor beta-nitrophenylxylopyranoside. These results infer an important role for both the heparan-sulfate and proteoglycan components of HSPG.

In vitro and *in vivo* studies have established a role for HSPG in the binding and uptake of apo E enriched lipoprotein remnants [61,67,68]. HSPG binding of apo E at the sinusoidal microvilli of the hepatocyte, more specifically the space of Disse, may cause the accumulation of apo E, the major ligand facilitating remnant catabolism, and create a reservoir of apo E [69]. HSPG may in this manner

participate in the sequestration and capture of lipoproteins. However, studies with LDLr-deficient mice suggest that the clustering of remnant particles is not dependent on HSPG but that HSPG may modify the binding of remnants, possibly by stabilizing the association with LRP [70]. *Ji et al. 1993* [69], showed that heparinase treatment greatly decreased the binding and uptake of apo E enriched remnants, and studies on Chinese hamster ovary cell (CHO) mutants lacking HSPG indicate that LRP expression alone is insufficient to mediate the binding and uptake of apo E enriched remnants. Importantly, heparinase treatment had no effect on LRP or LDLr structure or function. These results indicate that HSPG-LRP mediated endocytosis plays a significant part in the catabolism of apo B-48 remnants, and that HSPG are required for LRP mediated remnant catabolism.

Mahley & Ji 1999 [71], suggest four pathways responsible for the catabolism of lipoprotein remnant particles. The first path is the receptor-mediated endocytosis of remnant lipoproteins through LDLr. Next are two pathways involving the cooperative action of HSPG and LRP. Both of the supposed HSPG-LRP mechanisms involve the initial interaction of HSPG with the remnant particle through an ionic association with apo E. Subsequent to the HSPG-remnant interaction, the lipoprotein remnant can be transferred to LRP for internalization, or the HSPG-remnant can form a complex with LRP and the entire LRP-HSPG-remnant complex is internalized.

In addition to facilitating LRP mediated endocytosis, HSPG may directly mediate the uptake of remnants into the liver. The fourth pathway of remnant clearance suggested by *Mahley & Ji 1999* [71] is mediated solely through HSPG. *Ji et al. 1993* [69] demonstrated the binding and uptake of remnant particles in $LRP^{-/-}$ fibroblasts by HSPG alone. These findings complement studies suggesting the existence of a non-LDLr, non-LRP pathway of remnant catabolism [60,72]. HSPG appear to make a major independent contribution to the metabolism of apo E enriched lipoproteins.

1.2.6. HDL Metabolism and Reverse Cholesterol Transport

HDL is synthesized in the liver and intestine as small, dense, discoid particles termed pre-β HDL. Pre-β HDL is a cholesterol-poor, phospholipid complex that contains 5 apoproteins: apo A-I, the most numerous, apo A-II, A-IV, C, and apo E. In addition to exchanging apo's B and C with remnants to facilitate their hydrolysis and catabolism by hepatocyte membrane receptors, HDL also contains paraoxanose, an enzyme that prevents the accumulation of lipoperoxides in LDL, thus inhibiting LDL oxidation. HDL is also implicated as an important lipoprotein in the putative anti-atherogenic process of reverse cholesterol transport.

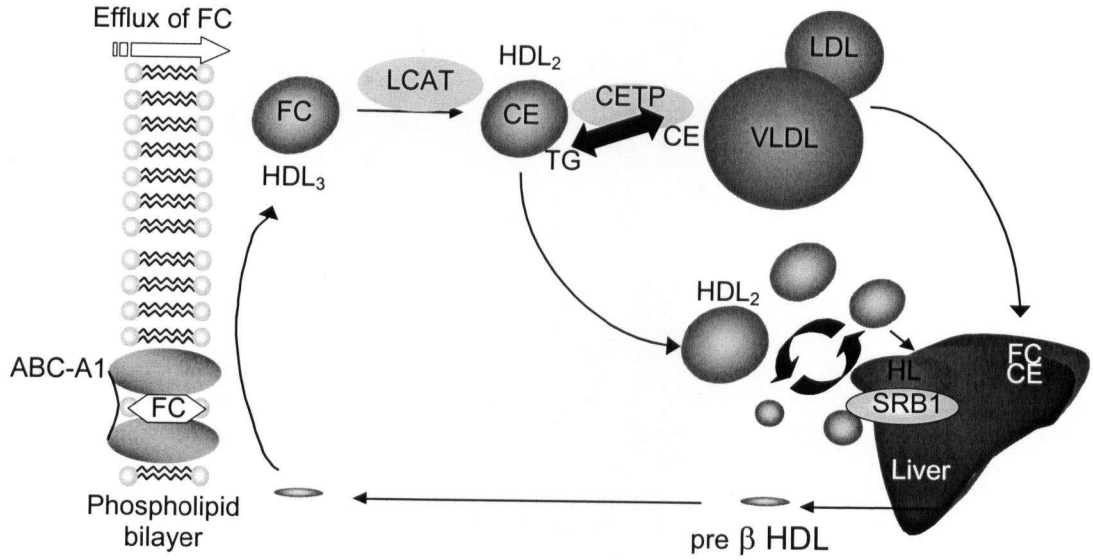


Figure 5. Reverse Cholesterol Transport. *Preβ HDL particles absorb free cholesterol (FC), through ABC-A1 transporter, from the peripheral tissues becoming larger HDL_2 particles whereupon the FC is acted on by LCAT to yield cholesterol ester (CE). Via the action of CETP, the HDL_2 particles exchange CE for triglyceride (TG) form TG-rich lipoproteins, which are removed from the circulation by the liver. HDL_2 proceed to the liver where selective uptake of HDL-CE occurs.*

Reverse cholesterol transport is a putative mechanism by which excess peripheral cholesterol is shuttled by HDL to the liver for catabolism. HDL is rich in phospholipid that attracts cholesterol from the peripheral tissues to the surface endothelium ***Figure 5***. FC is transported across the vascular endothelium by the ATP binding cassette protein A1 (ABC-A1), and associates with the small, discoid, lipid poor pre-β HDL particles that, as a result, become relatively lipid rich HDL_3 particles. Lecithin cholesterol acyl transferase (LCAT), which requires apo A-I as a cofactor, transforms the FC of HDL_3 to CE [73] creating what is termed an HDL_2 particle. The CE ester translocates to the core of the HDL particle increasing lipoprotein buoyancy and size, and decreasing lipoprotein density. Cholesterol ester transfer protein (CETP) acts on the resulting spherical HDL_2 particles, gaining TG, and transporting CE from HDL to VLDL and LDL [74]. The remnants continue on to peripheral tissue for the transport of energy and ultimately to the liver where FC and CE can be cleared from the blood. As a result of the TG acquisition, HDL particles become more buoyant ***Figure 5***. High CE content of HDL stabilizes the particle and protects it from degradation [75]. The exchange of CE for TG by CETP thus generates an HDL particle more susceptible to degradation. The majority of HDL

clearance occurs via the liver and most reverse cholesterol transport occurs without the internalization of HDL. Much HDL instead binds to the hepatocyte surface where HL facilitates the hepatic internalization of CE.

1.3 Hepatic Lipase

1.3.1 Ligand for Remnant Particle Catabolism

HL is produced by hepatocytes and found on the surface of luminal sinusoidal endothelial cells and on the surface of hepatocyte microvilli in the space of Disse [76]. Recent evidence also suggests HL is synthesized de novo by macrophages and may have a direct role in the pathogenesis of atherosclerosis [77]. Additionally, HL is found extra-hepatically associated with the endothelium of the steroidogenic adrenal glands and ovaries [78-81]. There are several lines of evidence that indicate a role for HL in remnant catabolism. HL deficiency often results in the accumulation of remnant particles in the plasma [82-85], and anti-HL antibodies inhibit remnant clearance in animals *in vivo* [86-88]. HL has also been shown to be activated by apo E [63] and HL hydrolysis of remnants increases the exposure of apo E on remnants [89]. Hence, HL clearly plays a role in remnant catabolism.

Additionally, *Ji et al. 1994* [90] demonstrated that rat hepatoma cells transfected with human HL exhibited 3-fold binding and uptake of rabbit βVLDL (a term referring collectively to chylomicron and VLDL remnants) and canine chylomicron remnants compared with non-transfected controls. Addition of apo E to the remnant particles did not affect the degree of enhanced binding. Furthermore, heparinase treatment of the hepatoma cells abrogated the HL-mediated enhanced binding and uptake. Pretreatment of the non-transfected cells with heparinase prevented the enhanced binding and uptake of βVLDL incubated with conditioned medium from HL-secreting cells. These findings suggest that HL contributes to the enhanced cell association of some remnants, independent of apo E, by initiating their binding to HSPG. This hypothesis was supported by findings that HL activity in HepG2 cells significantly enhances the uptake of VLDL remnants and the addition of heparin, heparinase, or heparitinase blocks the enhanced uptake [91]. HL appears to enhance remnant particle catabolism independent of apo E but require attachment of HL to HSPG. This HL-HSPG interaction may be an initial step in receptor mediated hepatic remnant clearance.

Several *in vitro* studies have shown HL binds to LRP suggesting a possible mechanism of HL enhancement of lipoprotein uptake. Results from *Kounnas et al. 1995* [92], report that LRP binds and internalizes HL in a process involving an initial and obligatory delivery by HSPG. *Krapp et al. 1996* [93] demonstrated that LDLr is not essential for HL-mediated uptake of remnant particles, as $LDLr^{-/-}$ human fibroblasts displayed HL induced remnant uptake. The same study confirmed the binding of HL to LRP via cross-linking experiments in which HL was incubated with hepatoma cells and bound ligand was chemically linked to LRP. Samples subsequently underwent electrophoresis and showed one significant band at 600

kDa corresponding to the mobility of cross-linked LRP. *Krapp et al 1996* concluded that HL mediated remnant uptake is initiated by HSPG binding and requires LRP.

Conversely, *Choi et al 1994* [94], tested the hypotheses that HL can act as a cofactor in chylomicron and chylomicron remnant uptake and found no connection. The uptake of chylomicron and chylomicron remnants by either LDLr or LRP was not altered in CHO cells transfected with rat HL, human apo E, or both. However, the aforementioned study did not consider the possibility of an LRP independent mechanism of HL-mediated remnant particle uptake which would also be consistent with evidence that HL-mediated remnant uptake is apo E independent.

In vivo studies with transgenic mice confer a role of HL in the uptake of apo B containing remnants and strongly suggest this role is independent of HL catalytic activity. *Dichek et al 1998* [95] developed and characterized human HL transgenic mice. Mice fed a high fat, high cholesterol diet to increase endogenous apo B containing remnants had decreased plasma cholesterol levels of 33% and 75% in heterozygotes and homozygotes, respectively. Lipoprotein remnant levels were likewise reduced. Human HL was subsequently expressed in human apo B transgenic and apo E deficient mice, both of which have elevated levels of apo B containing remnant particles. The cholesterol and remnant lipoprotein levels decreased in both murine populations. To determine if HL catalytic activity was required for the reduction in cholesterol and apo B containing remnant lipoprotein particles, *Dichek et al.* expressed catalytically inactive HL in apo E deficient mice. Plasma cholesterol and apo B containing remnant lipoprotein levels were approximately 60% lower in apo $E^{-/-}$ mice with catalytically inactive HL than in their apo $E^{-/-}$, catalytically active HL counterparts. That is, mice expressing catalytically inactive HL displayed greater cholesterol and remnant clearance than mice expressing catalytically active HL. Furthermore, the remaining clearance of remnants by apo $E^{-/-}$ mice with catalytically active HL was similar to that of non-transfected mice expressing only endogenous rat HL. Thus, the catalytic activity of HL does not appear critical for its mediation of remnant particle binding and uptake, which suggests that HL may act as a ligand in this process. However, not all studies reproduce this evidence.

HL may facilitate uptake of remnants in the space of Disse through its lipolytic function. *Ji et al. 1997* [96] demonstrated that HL catalytic activity in addition to ligand properties may be important in hepatic uptake of βVLDL. Hepatoma cells were transfected with wild type HL, catalytically inactive HL, or LRP binding defective HL. Although all cell lines displayed an increased clearance of βVLDL, the wild type enhancement was 20-35% greater than the cell line with catalytically inactive or LRP binding defective HL. An *in vivo* study by *Crawford and Borensztajn 1999* [97] supports a lipolytic role for HL in hepatic remnant catabolism as well. In this study, both normal mice fed a high-fat diet and apo E deficient mice displayed inhibited removal of injected non-HL-lipolyzed remnants while the clearance of HL-lipolyzed chylomicrons was not suppressed. Additionally, the removal of HL from the surface of the lipolyzed particles by proteolytic digestion did not affect their uptake, indicating that the hepatic recognition of lipoproteins is not mediated by HL. These findings

suggest that HL mediated enhancement of remnant catabolism is a result of its catalytic activity and is independent of apo E.

Qui et al 1998 [98], found that although the clearance of chylomicron remnants in HL knockout mice was reduced, the clearance of cholesteryl ester-labeled chylomicrons was unimpaired. As well, the novel approach of using transfected hepatoma cells expressing HL [99] suggest that factors in addition to HL play a major role in hepatic lipoprotein removal. *Donner et al. 1998* [99] demonstrated that HL-transfected tumor cells have increased remnant particle removal, however, normal liver cells more efficiently removed chylomicron remnants. Interestingly, transfected tumor cells had approximately twice as much LRP or LDLr per milligram of cell membrane as normal hepatocytes suggesting an inverse correlation between the level of LDL or LRP receptor and lipoprotein uptake. This seems somewhat at odds with evidence showing HL affinity to LRP. Nevertheless, the findings of *Amar et al. 1998* [100], corroborate *Dichek et al. 1998* [95]. *Amar et al.* over-expressed native or catalytically inactive HL in apo E knockout mice. Plasma clearance and hepatic uptake of VLDL cholesterol was increased in mice expressing the catalytically active or inactive HL indicating that catalytic activity is not required for HL mediated remnant clearance.

More recently, *Zambon et al. 2000* [101], discovered a distinct increase in cholesterol and apo B levels in the IDL and VLDL of two patients lacking both HL activity and mass compared to normal controls and one patient lacking only HL activity. All three patients – the two lacking both HL mass and activity and the one lacking only HL activity – displayed an increase in lipoprotein TG content in all lipoprotein classes and also showed more buoyant LDL particles compared to normal controls. *In vivo* studies by *Dichek et al.* [102], also indicate a role for HL in the removal of remnant lipoproteins through a non-catalytic ligand function independent of LDLr. Whatever role HL plays in lipoprotein remnant particle catabolism, evidence describing the interactions HL plays with other components of the hepatic remnant removal machinery, if any, remains vague.

HL and apo E have been postulated to participate as ligands in an initial sequestration step prior to receptor-mediated uptake of βVLDL in the liver; a process occurring within the space of Disse ***Figure 4***. Transgenic mice expressing rat apo E display apo E localization to the hepatic sinusoids [103], presumably reflecting distribution in the space of Disse, and rat apo E has been shown to accumulate in the space of Disse [104]. It has been hypothesized that apo E plays a secretion-capture role interacting with remnants in the space of Disse enhancing their binding to cell surface HSPG [69,105]. Substantial amounts of HL are also present in the space of Disse and HL has also been postulated to participate with apo E in the sequestration of remnant lipoproteins in the space of Disse [71]. However, much evidence previously described purports an apo E independent mechanism of HL mediated remnant uptake.

Yu et al 2000 [106], in studies employing wild-type, apo E knockout and apo E/LDLr knockout mice, also found that apo E was not critical for the rapid initial removal of chylomicron remnants, the process that HL is purportedly involved in. *Yu*

et al 2000, found that hepatically localized apo E did not accelerate the process of HL mediated remnant removal. *Medh et al. 2000* [107], on the other hand, determined that HL promoted VLDL degradation via an apolipoprotein E-dependent mechanism. The study suggests in the absence of apo E, lipase stimulated surface binding of VLDL to HSPG is greatly increased. Thus apo E appears to inhibit VLDL binding to HSPG. HSPG is also found in large quantities in the space of Disse, and *Mahley and Ji 1999*, speculate that the inhibitory effect of heparinase on lipoprotein remnant catabolism may be caused by the release of heparan-sulfate oligosaccharides from HSPG in the liver causing the depletion of apo E in the space of Disse, or interfering with HL interactions in the liver.

Interestingly, *Ramsamy et al. 2000* [108] found that lipid hydrolysis by HL was inhibited when the enzyme was associated with HSPG, a result that is contrary to much of the evidence reported. This study also demonstrated an ability of apolipoprotein A-I to displace HL from HSPG and to inhibit the hydrolysis of VLDL lipids by HL. *Ramsamy et al.* suggest that the hydrolysis of TG enriched lipoproteins may require the displacement of HL from cell surface HSPG. Furthermore, since apo A-I directly affects the association of HL with HSPG, the amount of loosely bound apo A-I in plasma may control the ligand functions of HL and affect the uptake and clearance of remnant lipoprotein particles.

In summary, evidence of the mechanisms of HL action are equivocal. It appears most likely from current evidence that HL plays both a lipolytic and non-lipolytic role in the process of hepatic lipoprotein remnant catabolism ***Figure 4***. HL mediated remnant clearance appears to be independent of apo E but requires interaction with HSPG in the space of Disse although interaction on the sinusoidal endothelium has not been disproven. Whether HL functions in association with hepatic lipoprotein receptors is uncertain.

1.3.2 Conversion of IDL to LDL

Human and animal studies have revealed the involvement of HL in the formation of LDL from IDL. *Nicoll and Lewis 1980* [109] assessed the kinetics of VLDL-apo B removal in patients with non-functional LPL and also evaluated the relative capacities of HL and LPL to hydrolyze TG of different lipoprotein species. Results of the study revealed that HL activity increased as lipoprotein size decreased. Goldberg *et al. 1982* [88] found that administration of HL antiserum resulted in defective conversion of IDL to LDL in the cynomolgus monkey, and *Demant et al. 1988* found that HL deficiency in humans results in an almost complete lack of IDL conversion [110]. More recently, *Qiu et al. 1998* [98] found that IDL to LDL conversion was reduced 6-fold in HL-deficient mice compared with controls. These studies establish a critical role for HL in the formation of LDL from IDL.

HL not only significantly contributes to the formation of LDL but also to the functional characteristics of LDL. Clinical studies have revealed a correlation between HL activity and LDL size and metabolism. A study by *Auwerx et al. 1989* [111] comparing normal and HL deficient subjects provides evidence that HL

determines normal LDL characteristics. *Auwerx et al. 1989* compared three subjects with decreased or absent HL levels with 18 normal subjects. HL deficient persons displayed larger more buoyant LDL than controls, and peak density and average diameter of the LDL particles correlated with HL activity. Additionally, LDL isolated from HL deficient subjects contained more TG compared to LDL isolated from control subjects.

Decreased plasma LDL size has been shown to be associated with premature CAD. *Campos et al. 1992* [112] found that small LDL particles are more prevalent in CAD patients versus controls while the prevalence of large LDL particles was lower in CAD patients than controls. Furthermore, small LDL particle size was highly associated with elevated TG and decreased HDL cholesterol (HDL-C), both risk factors in the onset and progression of CAD. Similar results have been obtained in other studies [112-115].

As HL appears to be necessary for the conversion of IDL to LDL, it is reasonable to believe HL activity can modulate LDL atherogenicity. *Zambon et al. 1993* [116] suggested that HL modulates the physical and compositional properties of LDL. This study compared HL activity and LDL of 21 patients with CAD and 23 normolipidemic subjects and found that LDL size and buoyancy were inversely associated with HL activity in both CAD and normolipidemic subjects. The free cholesterol (FC) content of LDL and the FC to phospholipid ratio in LDL particles also correlated with HL in CAD and normal subjects suggesting that HL influences LDL lipid composition by affecting surface lipid components regardless of the presence of CAD. Similarly, *Jansen et al. 1994* [117] found that HL activity was lower in a CAD population with predominantly large LDL versus members of the same population with predominantly small LDL. The association of low HL activity with large, buoyant LDL particles is well documented [118,119].

In summary, HL is a vital enzyme in the formation of small, dense LDL and modulates their phospholipid and TG content. The higher the HL activity, the smaller, denser, and more atherogenic the resulting LDL particles.

1.3.3 HDL Remodeling

In vitro studies clearly indicate the ability of HL to hydrolyze HDL lipid components. HL catalyzes the hydrolysis of HDL phospholipid and TG [120,121], and experiments on isolated HDL particles suggest that HL hydrolyzes phospholipids and TG at similar rates [122-124]. This is in contrast to LPL, which hydrolyzes TG at a 10-fold higher rate than phospholipid. *Deckelbaum et al. 1992* [125] hypothesized that HL acts to remodel HDL by increasing the shedding of HDL surface material that is necessary for the conversion of larger HDL particles to smaller HDL particles after the depletion of the HDL core TG and CE. *Braschi et al. 1998* created transgenic mice expressing human HL. A 23-fold increase in HL activity significantly decreased HDL cholesterol (HDL-C), phospholipid and TG 64%, 60%, and 24%, respectively. Obviously, HL promotes the metabolism of HDL *in vivo* but the precise mechanism in HL mediated HDL remodeling is not understood.

Catalytic activity of HL, however, may not be required for its HDL modification effects. Dugi *et al. 2000* [126] injected adenovirus expressing either native or catalytically inactive HL into HL deficient mice. Both the active and inactive forms of HL significantly reduced plasma cholesterol and phospholipid; however, the reduction was greater in the mice with catalytically active HL. This study indicates an *in vivo* role for HL in HDL metabolism independent of lipolysis, though HL activity does appear to increase HDL remodeling. HL therefore, may not only act in an enzymatic function promoting HDL remodeling, but may in a similar manner to remnant particle catabolism, serve a ligand function.

Apoproteins contained in HDL may regulate its modification by HL. Although lipolysis of HDL occurred with active or inactive HL, *Dugi et al. 2000* found that catalytically active HL reduced plasma levels of apo A-I while inactive HL had no effect on apo A-I levels. Both forms of HL significantly reduced apo A-II. HL lipolysis therefore may involve in the catabolism of apo A-I leading to enhanced HDL removal. *Hime et al 2001* [127] reconstituted HDL containing only apo A-I, only apo A-II, or both apo A-I and A-II. HL mediated phospholipid hydrolysis was assessed in mixtures of the different HDL particles. Apo A-II only-HDL inhibited phospholipid hydrolysis of apo A-I only-HDL but phospholipid hydrolysis of apo A-II only-HDL was increased in the presence of apo A-I only-HDL. Additionally, the rate of phospholipid hydrolysis was greater in reconstituted HDL containing both apo A-I and A-II than in reconstituted HDL containing only apo A-II. Apo A-I appears to enhance HL mediated phospholipid hydrolysis in apo A-II containing HDL regardless of whether apo A-I is present in the same or different particles. Perhaps apo A-I in addition to, or instead of, being altered by HL activity, enhances HL lipolytic function. Supporting this notion is evidence from *Ramsamy et al. 2000* [108] who reported that apo A-I activated HL *in vitro* by binding and liberating HL from cell surface proteoglycans. It is possible that HL acts as a ligand by binding apo A-I consequently facilitating HDL transformation.

HL-apo A-I interaction may require TG enrichment of HDL. *Rashid et al. 2002* [128] conducted experiments with *ex vivo* TG-enriched, apo A-I containing rabbit HDL *in vivo*. Similar to results from *Dugi et al. 2000* [126] already mentioned, active HL modified apo A-I containing TG enriched HDL more rapidly than inactive HL. Additionally, TG enriched apo A-I HDL was modified more rapidly than TG poor apo A-I containing HDL by both active and inactive HL. TG enrichment of HDL with concomitant HL hydrolysis, possibly through interaction with apo A-I, may lead to enhance HDL remodeling.

In summary, HL plays a role in the alteration of HDL particles leading to their clearance from the plasma ***Figure 6***. HL hydrolyzes HDL surface components, phospholipid and TG, and although this lipolytic activity enhances HDL clearance, HL can facilitate HDL clearance independent of its lipolytic activity possibly by acting as a ligand for hepatic HDL removal. HL lipolytic and ligand functions may act in concert to facilitate HDL catabolism in a process requiring, or enhanced by apo A-I contained within HDL particles.

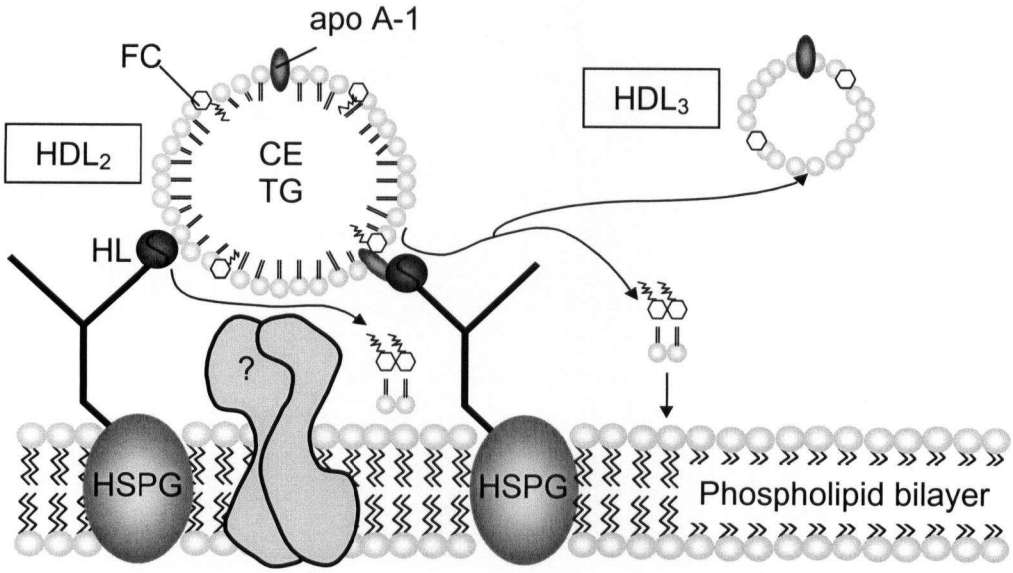


Figure 6. HDL Remodeling. *HL assists in the shedding of surface materials after depletion of the HDL_2 lipid core. HL may serve as a ligand for another as yet unknown protein (?) or may directly catalyze the hydrolysis of phospholipid and FC possibly through an interaction with apo A-I. HL-mediated hydrolysis results in remodeling of HDL2 into the smaller, denser, relatively lipid poor HDL_3. CE=cholesterol ester.*

1.3.4 Selective Uptake of Cholesterol Ester

HL also plays a significant role in the hydrolysis of HDL core lipid components and is a player in reverse cholesterol transport ***Figure 5***. *Knecht et al. 1989* demonstrated HL converts HDL_2 to HDL_3 *in vitro* [129]. In patients with hyperalphalipoproteinemia, a disorder characterized by increased apo A-I, low HL activity is associated with an increased HDL_2/HDL_3 ratio. Low HL activity is also associated with increased HDL_3 in normotriglyceridemic men [130], and in patients with HL deficiency. As previously noted, HDL participates in the removal of cholesterol from peripheral tissues in a process known as reverse cholesterol transport. The extracted cholesterol is subsequently delivered to the liver for excretion as bile acids [73,131,132] or to steroidogenic tissues for hormone synthesis [80,117,133]. Three simultaneous processes may be involved in the removal of plasma HDL.

First, the action of cholesterol ester transfer protein (CETP) may enhance HL-mediated selective uptake of CE within the core of the HDL particle ***Figure 5***. CETP catalyzes the transfer of TG from large, TG-rich apo B-containing remnant particles to HDL in exchange for CE from HDL. The remnant-TG is then carried to the liver for removal. *Rye et al. 1999* [134] suggested that both HL and CETP are required to

deplete HDL core TG and CE and remove excess surface lipids to form smaller HDL particles as neither protein alone was able to convert large HDL to small HDL. The same study also found that when HDL cholesterol ester (HDL-CE) was exchanged for TG, HL action produced smaller, possibly discoid HDL. Additionally, *Rye et al. 1999* determined spherical HDL particles are better substrates for HL phospholipid hydrolysis (HDL remodeling) than discoid HDL particles. This is complemented by the findings of *Rashid et al 2002* [128] who found that TG enrichment of HDL – as occurs through the action of CETP in the plasma – combined with HL lipolysis enhances HDL clearance. These studies suggest larger, TG-rich HDL particles are targets of HL and the combined action of HL and CETP are involved in the conversion of larger HDL to smaller HDL.

The second mechanism of HDL uptake involves the receptor mediated endocytosis of whole HDL particles [135-138], while the third proposed method of HDL clearance concerns the hepatic uptake of HDL-CE without concomitant uptake of the HDL particle. Experiments with HL-transfected hepatocytes indicate that HL may be involved in both the whole particle uptake of HDL as well as the selective uptake of HDL-CE [96]. *Ji et al 1997* [96] demonstrated enhanced binding and uptake of HDL and HDL-CE by HL-transfected hepatocytes. Enhanced binding and uptake were slightly diminished by an inhibitor of LRP and were almost completely blocked by heparinase. This suggests two pathways of HL-mediated HDL uptake: an HSPG-dependent, LRP-dependent route, and an HSPG dependent, LRP-independent route. The LRP-dependent route is presumably involved in endocytosis of HDL particles while the absence of a receptor requirement in the LRP-independent path may indicate uptake of the cholesterol ester component of HDL particles.

However, in the same study, catalytically inactive HL only minimally reduced HL mediated HDL clearance, suggesting that much of the HL mediated HDL catabolism requires neither HL binding the LRP nor lipolytic processing. Again this may indicate a possible ligand role for HL in HDL catabolism. Moreover, in the same study, HL increased the selective uptake of radio-labeled HDL-CE by HL-transfected hepatocytes in a process that was nearly completely abrogated by heparinase but was unaffected by the LRP inhibitor, again suggesting the LRP independent route of HL mediated HDL clearance involves the selective uptake of HDL-CE. So it appears that HL is involved the uptake of HDL via both an LRP-dependent, seemingly receptor-mediated endocytosis, and an LRP-independent pathway, likely selective uptake of HDL-CE. Additionally, both the HL mediated selective uptake of HDL-CE and LRP mediated whole particle removal appear to require HSPG.

Alternatively, another cell surface receptor, the scavenger receptor B1 (SR-B1) appears to be involved in HDL-CE selective uptake ***Figure 7***. *Stangl et al. 1998* [139] demonstrated that SR-B1 can rapidly exchange cholesterol between HDL and cell membranes, and SR-B1 was shown in one study to be of major importance in the clearance of HDL-CE in mice [140]. Additionally, selective uptake of HDL-CE by SR-B1 may require SR-B1 interaction with apo A-1 [141,142].

HL mediated HDL-CE selective uptake has also been shown *in vivo*. *Lambert et al. 2000* [143] investigated the relevance of HL in HDL-CE metabolism in HL deficient mice. The HL deficient mice had decreased plasma clearance and

decreased hepatic uptake of HDL-CE. Thus, HL appears to play an important role in facilitating the selective uptake of HDL-CE *in vivo.*

Conflicting results have been obtained regarding the importance of HL activity and HDL catabolism. *Rinninger et al. 1998* [144], supported *Ji et al. 1997*, when they demonstrated an HL mediated increase in HDL_3-CE selective uptake by hepatoma cells that was independent of lipolysis and independent of LRP and LDLr. However, *Dichek et al. 1998* [95] determined that catalytic activity of HL was necessary for HDL-CE selective uptake in HL transgenic mice.

In summary, HL seems to facilitate HDL catabolism on three different levels. HL and CETP function together to remove HDL-CE via lipid exchange with apo B containing remnant particles and their subsequent catabolism ***Figure 5***. HL contributes to HDL whole particle removal by LRP in a process that does not appear to require HL catalytic activity and may represent another ligand function of HL ***Figure 7***. Lastly, HL mediates HDL-CE selective uptake by the liver in a process that appears to require HL activity and may be enhanced by the HDL TG-enrichment of CETP ***Figure 7***. In all cases, HL seems to require the presence of cell surface HSPG. This requirement infers the importance of the HL-HSPG interaction in modifying the progression of atherosclerosis.

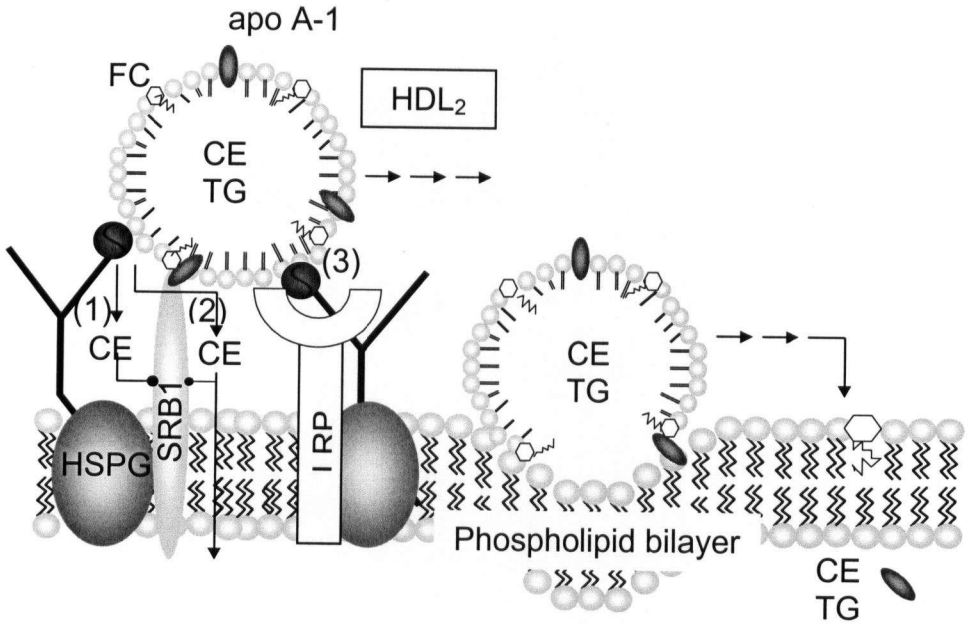


Figure 7. Selective uptake of HDL-CE*. HL may facilitate the hepatic uptake of HDL cholesterol ester (HDL-CE) through (1) its lipolytic function, or by acting as a ligand for both (2) SRB1 mediated HDL-CE uptake and (3) LRP-mediated whole particle uptake.*

1.3.5 HL Deficiency

HL deficiency is extremely rare and has been reported in only 6 families worldwide [82,145,146]. Patients determined to have HL deficiency were initially tested on the basis of hyperlipidemia, therefore, inferences based on these individuals is limited by selection bias. As such, it is not known what proportion of individuals with HL deficiency have hyperlipidemia or whether HL deficiency is sufficient to cause hyperlipidemia.

At any rate, all patients shown to have complete hepatic lipase deficiency exhibit elevated plasma cholesterol and TG [147] and have lipoprotein profiles characterized by the presence βVLDL and IDL. The presence of βVLDL in these patients indicates a defect in the catabolism of VLDL remnants reflecting the lipolytic and ligand role HL plays in remnant clearance.

Patients with complete HL deficiency display normal conversion of large VLDL to small VLDL, impaired conversion of small VLDL to IDL, and an almost complete lack of conversion of IDL to LDL [110]. Also, LDL are almost exclusively large and enriched with TG [84,111]. These findings reiterate the role of HL in the conversion of IDL to LDL and HL regulation of LDL size and atherogenicity.

Additionally, plasma HDL-C levels above the 90th percentile have been observed in several patients despite concomitant hypertriglyceridemia [84,148,149]. The elevated HDL-C levels and TG enrichment echoes the role of HL in normal HDL remodeling and selective uptake of CE, as well as the combined efforts of HL and CETP in HDL metabolism.

The largest and most studied population with HL deficiency is the Ontario HL-Deficient Kindred or OHLD. Two mutations found in the OHLD are unique to this group: S267F and T383M. The three compound heterozygotes in the group all developed CAD [150]. One had a fatal MI at 51 years of age, while another displayed angina at 50 years with severe multiple vessel coronary atherosclerosis and subsequently suffered a severe MI at 58 despite treatment. The third compound heterozygote had a history of angina commencing in mid 50's and required 3 vessel coronary bypass surgery at 57 years of age. Consequently, a complete HL deficiency appears to be associated with an accelerated from of atherosclerosis.

1.3.6 HL Biochemistry

1.3.6.1 Physical Properties

The mature HL protein consists of 476 amino acids corresponding to a molecular weight of 65 kDa [151,152]. The HL gene is located on chromosome 15q21 and is 35kb in size with 2 exons that encode 1.5 kb of mRNA [153]. Human HL is only active as a noncovalent homodimer in solution [154-156] and is glycosylated at four points: Asn 20, 56, 340, and 375 [156], however only glycosylation at Asn-56 is required for activity and secretion [156]. HL has significant sequence homology with LPL and pancreatic lipase (PL) and these lipases make up

a gene family [153,157]. Recent studies have also included endothelial lipase and phosphatidylserine phospholipase A1 as members of the same gene family [75,158,159]. HL is distinguished from LPL by its resistance to inactivation by 1M NaCl and by its independence from activation by apo CII. HL is found primarily bound to cell surface HSPG of the hepatocyte microvilli and sinusoidal endothelium [76]. HL is also found extra-hepatically associated with the endothelium of the steroidogenic adrenal glands and ovaries [78-80,133], and may also be synthesized *de novo* by macrophages [77].

1.3.6.2 Catalytic Properties

HL catalyzes the hydrolysis of fatty acyl ester bonds of phospholipid, and triglycerides in all major classes of lipoproteins [160-162]. HL does however, display a preference for phosphatidylethanolamine and phosphatidylcholine containing the long chain unsaturated fatty acids linoleate and arachidonate [163]. HL is a phospholipase A_1 and hydrolyzes fatty acids from the 1 and 3 positions of TG and produces 2-acyl lysophosphatidylcholine and 2-acyl lysophosphatidylethanolamine upon hydrolysis of phosphatidylcholine and ethanolamine respectively. HL chiefly acts on TG and phospholipids in small VLDL and IDL but is also involved in the hydrolysis of HDL particles.

1.3.6.3 Functional Domains

Crystal structures of members of the mammalian lipase gene family have not been forthcoming and PL is the only human lipase that has been successfully crystallized [164]. The pancreatic lipase enzyme can be divided into an NH_2 terminal domain joined by a short linking region to a smaller COOH-terminal domain ***Figure 8***. The PL NH_2 terminal domain is composed of a series of 9 β sheets existing in a fan-like pattern. The NH_2 domain contains the Ser-His-Asp catalytic triad with the active site Ser centrally located in a pentapeptide consensus sequence (GXSXG) between one end of a β strand and the start of the adjacent α helix. A surface loop covers the catalytic triad in the inactive state and this loop or 'lid' must move in order to accommodate the lipid substrate. In the closed conformation, the catalytic site is inaccessible to substrate. The surface loop converts to the open form upon interaction with lipid.

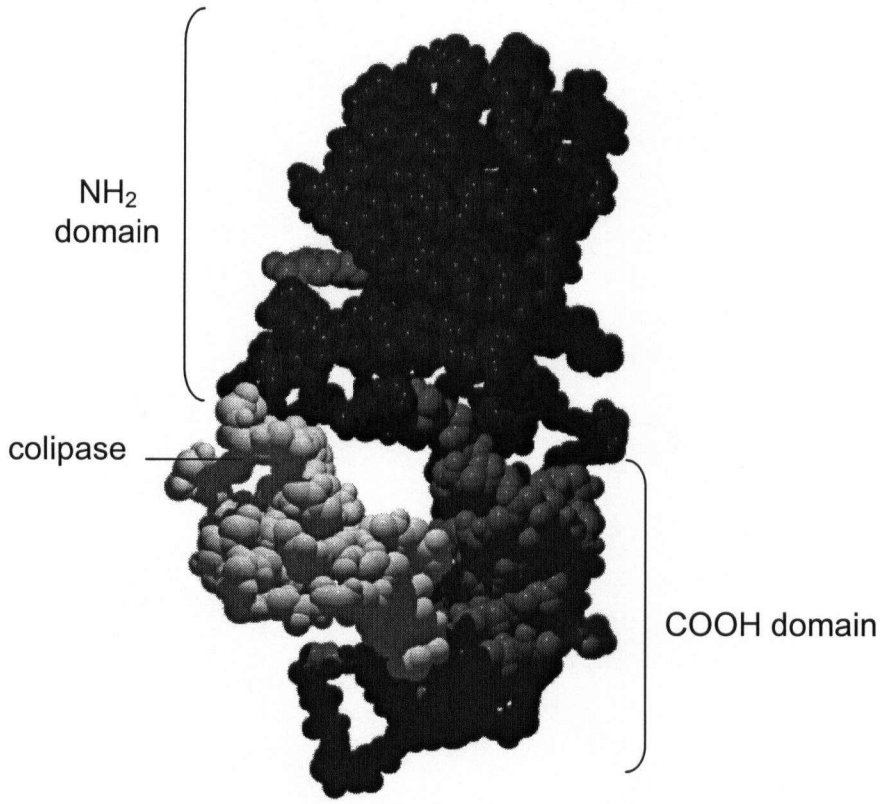


Figure 8. Crystal Structure of Pancreatic Lipase (PL). *PL is composed of two domains. The larger NH_2 terminal domain contains the catalytic triad with active site serine and thus possesses catalytic activity. The smaller COOH terminal domain does not have any catalytic properties, but does bind to colipase a molecule that allows PL binding to micelles in the small intestine.*

In contrast to the NH_2 domain of PL, the COOH domain of PL does not possess any known catalytic functions. The smaller COOH domain ***Figure 8*** consists of a series of β sheets layered one on the other in a sandwich configuration. Although the COOH domain does not have catalytic function, it does bind the surface loop in order to cover the catalytic site in its inactive, closed conformation. The COOH domain is also attached to a colipase molecule via the PL terminus. Colipase allows PL to bind to micelles in the small intestine by alleviating bile salt inhibition of PL.

Although only pancreatic lipase has an available crystal structure, computer modeling studies based on a PL backbone have been used to predict the structures of other lipases and suggest that HL and LPL are also 2 domain enzymes [165,166]. This is not surprising as HL and LPL share a high degree of amino acid homology, approximately 50% homology within NH_2 domain and 30% with COOH domain, and

conservation in number and location of disulfide bonds. However, PL functions as a monomer while HL and LPL function as homodimers, therefore homology-modeling studies are limited.

The need for a better understanding of structure and function relationships in HL led to domain exchange strategies [165,166]. *Wong et al. 1991* [167] constructed a chimeric lipase composed of the NH_2 terminal 329 residues of rat HL (rHL) linked to the COOH terminal 135 residues of human LPL (hLPL). The chimera displayed both esterase and lipase function closely resembling native HL, but monoclonal antibody against LPL inhibited the lipase activity while the esterase activity was unaffected. Thus the NH_2 domain of HL appears to direct catalytic activity, the COOH domain seems to be essential for the hydrolysis of long chain substrates.

Davis et al. 1992 [168] conducted similar experiments to *Wong et al.* and furthered their findings. *Davis et al. 1992* constructed a similar rat HL-human LPL chimera (rHL/hLPL) as well as the reverse chimera: human LPL NH_2 terminus and rat HL COOH terminus (hLPL/rHL). hLPL/rHL had catalytic properties, such as requirement for apo C-II and 1M NaCl inhibition, characteristic of hLPL and kinetic parameters almost identical to hLPL. The rHL/hLPL had catalytic properties like that of native rHL that did not require apo C-II and were not inhibited by salt. These results indicate that the NH_2 domain of HL and LPL is responsible for catalytic efficiency and kinetic parameters. Additionally, hLPL/rHL eluted from a heparin-Sepharose column at 0.75M NaCl while the rHL/hLPL eluted at 1.1 M NaCl. Native rat HL and human LPL eluted at 0.75M and 1.1M respectively. These elution points established that the COOH domain controls heparin binding. Interestingly, hLPL/rHL phospholipid hydrolysis was greater than hLPL and rHL/hLPL phospholipd hydrolysis was higher than rHL and hLPL/rHL. So, although the COOH terminal domain does not possess any catalytic function, it may serve to modulate substrate specificity.

Dicheck et al. 1993 [169] proposed a role for the lipase COOH terminus in substrate interaction. *Dicheck et al.* also constructed a LPL/HL chimera, but both domains were of human origin. Treatment of the chimera with an anti-HL COOH terminus monoclonal antibody abolished the chimera's ability to hydrolyed TG emulsion but not short chain substrates (esterase function). This implies a major role for the lipase COOH terminal in mediating interaction with long chain TG substrates. Unlike *Davis et al. 1992*, the chimera of *Dichek et al. 1993* eluted at 0.9 M NaCl, displaying a heparin-Sepharose elution pattern intermediate of that of LPL and HL. *Dichek et al.* suggest both the NH_2 domain and the COOH domain contribute to heparin binding.

Experiments with chimeric lipases have also been employed to determine the contribution of the NH_2 domain lid to the function of HL and LPL. *Dugi et al. 1995* [170] generated four chimeric lipases: 1) LPL with the 22 amino acid lid of HL, 2) HL with the 22 amino acid lid of LPL, 3) LPL/HL chimera, and 4) LPL/HL with the 22 amino acid lid of HL. The LPL with the HL lid had reduced trioleinase activity and increased phospolipase activity than normal LPL while the HL with the LPL lid had increased tioleinase activity and reduced phospholipase activity than normal HL. Also, LPL/HL with the HL lid had lower trioleinase activity and increased phospholipase activity than the LPL/HL chimera. The exchange of lids resulted in a

reversal of phospholipase and neutral lipase ratios establishing a crucial role for the lid in lipase substate specificity. Although these *in vitro* studies provide consistent evidence regarding the roles of the NH_2 and COOH terminus in HL function, studies showing structure-function relationships *in vivo* are preferred.

Kobayashi et al. 1996 [171] conducted research to identify structural domains that may confer the different substrate specificities *in vivo*. *Kobayashi et al.* used recombinant adenovirus vectors to express enzymes in HL deficient mice. rHL and rHL with LPL lid displayed a striking difference in phospholipid levels in the HL deficient mice. Expression of rHL resulted in 70% reduction while the expression of HL with the LPL lid resulted in only a 32% reduction of phospholipids. Likewise, rLPL and rLPL with the HL lid showed differences in phospolipid reduction with rLPL reducing phospholipids by only 31% and rLPL with the HL lid reduced phospholipids by 81%. The exchange of lipase lids did not alter TG levels. Thus preferential phospholipid hydrolysis *in vivo* was demonstrated with the lid of HL but not LPL. Therefore, the lid may be a major structural element responsible for conferring the different *in vivo* phospholipase activities of HL and LPL.

Studies determining the orientation of the HL and LPL homodimers have also provided important functional information. *Wong et al. 1997* [172] engineered an 8 amino acid linker between the NH_2 domain of one LPL monomer and the COOH domain of another LPL monomer. As the linker region was very short, the dimer molecule was restricted to a 'head-to-tail' conformation. The LPL molecule diplayed kinetic parmeters, salt inhibition, cofactor dependence, and heparin binding identical to that of native LPL. This provides evidence that LPL monomers, and likely HL monomers, are arranged head to tail within the active normal dimer.

The use of chimeras has also shed light on the structure of HL as it relates to the essential cell surface heparan-sulfate proteoglycan binding capacity of the enzyme. *Hill et al. 1998* [173] constructed two human chimeric lipases: one consisted of HL with the proximal half (approximately 60 amino acids) of the COOH domain substitutied with the corresponding LPL portion (HL-LPLc1) and the other was HL with the distal half (approximately 60 amino acids) of the COOH domain substituted with the corresponding LPL portion (HL-LPLc2). HL-LPLc1 eluted from a heparin-Sepharose column at 0.8M, equivalent to the native HL control. HL-LPLc2 eluted at 1.3M NaCl, equivalent to the native LPL control. This study strongly suggests that the heparin binding of HL and LPL associated with the COOH terminal is deterimined by the distal half of the enzymes.

In summary, the NH_2 domain of HL, containing the catalytic triad of Ser 145-His171-Asp256, appears to be primarily responsible for catalytic activity. The NH_2 domain also contains the surface loop or lid which covers the catalytic site in the closed, inactive conformation which may also play a significant role in modulating substrate preferences. The COOH domain appears to be primarily responsible for binding of HL to cell surfaces, and heparin binding has been attributed to the distal half of the COOH domain. Although the COOH domain does not possess catalytic activity, it may participate in determining substrate specificity.

1.3.7 Polymorphisms

Many polymorphisms of the gene encoding HL (*LIPC*) have been identified. Polymorphisms not causing amino acid substitutions and thus not affecting the structure or function of HL include V133V, T202T [174], T457T [175], G175G [176], T344T[145], T19T, L31L, and F47F [177]. Polymorphisms effecting amino acid substitutions in HL include V73M, N193S, S267F, T383M [147,174], L334F [145], R186H [178], A1075C [179], and N37H [177]. Some of these polymorphisms have been very well studied while others are so rare comprehensive studies of their physiological effects have not been feasible.

Reports have connected the variant alleles resulting in amino acid substitutions to altered HL activity including clinical HL deficiency. Reports have linked the S267F, L334F, T383M and R186 polymorphisms to decreased plasma HL activity [145,147,178]. Studies of the OHLD with controls identified S267F and T383M as being unique to HL Deficiency and were co-inherited in the three affected patients [180]. In parallel transformations of primate renal cells [181] the T383M polymorphism resulted in HL activity 10% of wild type HL while the S267F polymorphism resulted in no detectable HL activity. HL activity is completely abrogated in compound heterozygotes [150]. Considerable variation of HL activity exists between individuals with T383M suggesting that T383M related HL deficiency is moderated by other factors. S267F has been reported to be associated with TG enrichment of LDL and HDL lipid fractions in an older female heterozygote of the OHLD while T383M has been associated with TG enrichment of LDL and HDL fractions in multiple older members of the OHLD [150]. As expected, multiple factors may affect the presence and severity of different phenotypes associated with variant alleles of the HL gene.

Fang et al. 2002 [182] identified and examined the effects of an A to C substitution at position 1075 (1075C) of the HL gene in the Chinese population. In males, HDL-C and apo A-I levels were positively related with the 1075C allele with HDL-C levels highest in homozygotes for the polymorphism, intermediate in heterozygotes, and lowest in A/A homozygotes. Although there was no difference in the frequency of the allele in men and women, there was no connection between the variant allele and HDL-C or apo A-I levels in females. The OHLD studies and the results of *Fang et al.* strongly indicate that age and gender may modulate the affects of allelic variation in the HL gene. In addition to moderating lipid profiles, allele variation may influence the development of atherosclerosis.

Moenning et al. 2000 [177] studied whether HL gene polymorphisms contribute to the formation of CAD. *Moenning et al.* sequenced the entire coding and boundary regions of LIPC and analyzed the results in 20 patients with low HDL, high TG, and diagnosed CAD. In addition to finding the previously unidentified H37H, the V73M and S267F polymorphisms were found, but only the V73M was sequenced in both CAD subjects and controls. The study identified these uncommon alleles in 10% of CAD patients and suggests that these alleles may contribute to the formation of dyslipidemic phenotypes and the development of premature CAD. However, as the

study selected CAD patients with existing low HDL and high TG, profiles associated with HL deficiency, they may have pre-selected for HL insufficiency thus artificially inflating the allele frequencies in the CAD group. That said, results from *Hoffer et al. 2000* [183] lend support to the *Moenning et al.* proposal. *Hoffer et al.* reported the V73M variant is associated with significantly higher levels of total cholesterol and apo B in persons with familial combined hyperlipidemia (overproduction of hepatic VLDL apo B), and suggest the variant may explain part of the variability in phenotype of these subjects. Polymorphisms in non-coding sections of the LIPC gene also alter HL function.

Guerra et al. 1997 [184] sequenced the HL gene in male relatives of patients with documented premature coronary atherosclerosis and discovered 4 polymorphisms in the 5' flanking region. The polymorphisms included a G to A substitution at -250 (-250A), a C to T substitution at –514 (-514T), a T to C substitution at -710 (-710C), and an A to G substitution at -763 (-763G). These polymorphisms are in complete linkage disequilibrium and together define a common lipase allele. A C to T substitution causing splice site polymorphism in intron 1 of the HL gene has also been discovered [146].

The aim of the study by *Guerra et al. 1997* was to examine the relationship between HL gene polymorphisms and plasma HDL-C concentrations. In addition to HL gene sequencing, *Guerra et al. 1997* also conducted linkage analysis and association studies. Linkage analysis in 1456 Caucasian Americans indicate that allelic variation in or close to the HL gene accounts for 25% of plasma HDL-C variation in this CAD population. Association studies on the –514T allele revealed that the presence of this allele is associated with a modest increase in plasma HDL-C and apo AI in men but not women, and that homozygosity for this allele in men is connected to markedly higher HDL-C and apo AI levels. The –514T allele appears to lead to decreased HL activity resulting in increased HDL-C, at least in men.

Vega et al. 1998 [185] showed –514T to be associated with lower HL activity and higher HDL-C levels in both Caucasian and African American males, however the allele appears 3 times more common among African Americans than Caucasian Americans. *Tahvanainen et al. 1998* [186] evaluated the association between –514T and post heparin plasma HL activity and fasting lipoproteins in Finnish men with CAD and HDL-C below 1.1 mmol/L. Similarly to *Guerra et al.* and *Vega et al, Tahvanainen et al.* found a significant association between –514T and decreased HL activity. The TG content of LDL, IDL, and HDL were also elevated as was IDL-C. Although –514T appears to decrease HL activity and to be significantly associated with an increasein HDL-C, there was no association between the –514T allele and CAD. The –514T linked decrease in HL activity and increase in HDL-C in men is well documented [130,187-189].

Murtomaki et al. 1997 [190] investigated the distribution of several HL gene polymorphisms and their association with lipid values in male and female subjects with a paternal history of MI at a young age and matched controls. The –514T substitution showed an association with elevated HDL, apo A1 and LpA-1 levels in both experimental and control groups. However, *Ji et al. 2002* found no connection between –514T mediated decrease HL activity and HDL-C variation in women [189].

Hubacek et al. 2001 [191] studied the association between the –514T polymorphism and HDL-C in the Czech population. The -514T allele was associated with higher HDL-C values in women but not men. Males with the –514T allele did however display higher total cholesterol than non-carriers. Despite these reported differences in the effects of lipoproteins related to gender, *Hokanson et al. 2002* [192] found that the –514T genotype was associated with sub-clinical CAD independent of gender. These occasionally differing results in men and women indicate that estrogen and testosterone levels may modify the affect of the –514T polymorphism on HL activity [193,194].

In accordance with HL function, the –514T mediated increase of HDL-C in men materializes in larger HDL particles. The reported increase in HDL-C as a result of decreased HL activity induced by the –514T variant of HL [184,185] appears to occur entirely in the HDL_2 fraction. *Grundy et al. 1999* [130], using NMR and gradient gel electrophoresis, determined that the –514T variant mediated increase in HDL-C in normolipidemic men was located in the HDL_2 fraction while the cholesterol content of the HDL_3 fraction was actually lower with the –514T variant. This specific affect on the HDL_2 subclass is consistent with the role of HL in remodeling of HDL_3 occurring in the reverse cholesterol transport pathway and with the selective uptake of HDL cholesterol ester. The –514T allele is not the only promoter allele to alter HL activity. More recently, *Fang et al. 2002* [182] found no association between the –480T allele and HDL-C in Chinese men or women. Again, a secondary factor such as ethnicity may modulate allelic variations in lipid profiles.

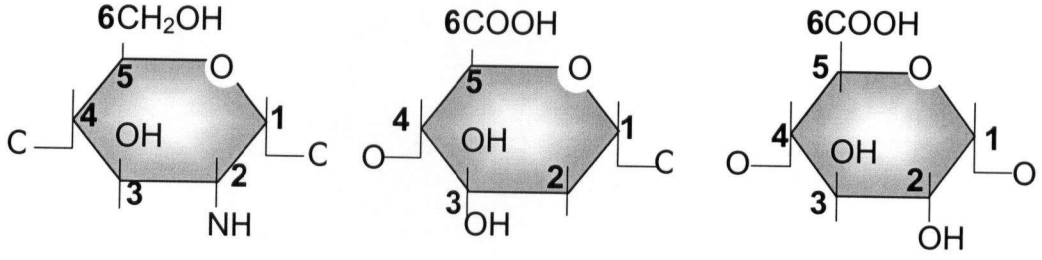
In summary, allelic variations in the HL gene and promoter regions appear to result in reduced activity of HL with a concomitant increase in HDL_2-C. The resulting depression of HL activity may also increase the TG and cholesterol content of other lipoproteins. Although allelic variations are associated with reduced HL activity and increased HDL-C, the effect of a polymorphism on gene expression can vary among individuals, and the resulting phenotypes are likely modified by interactions with other factors such as gender, age, and ethnicity.

1.4. Heparin Versus Heparan-Sulfate

Heparin and heparan-sulfate are compositionally similar polysaccharides but important structural differences do exist. Heparin is composed of polydisperse, repeating disaccharide units containing an uronic acid residue, either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA), and D-glucosamine (GlcN) ***Figure 9***. The GlcN residue is either N-sulfated or N- acetylated, and the dissaccharides may be O-sulfated at the C6 and/or C3 position of GlcN and at the C2 position of the uronic acid. Thus, each disaccharide monomer can exist with 4 possible structures in the uronic acid position and 8 possible structures at the GlcN position resulting in 24 possible different disaccharides. Heparin is a highly sulfated polysaccharide ***Figure 10*** that is found exclusively in the intracellular compartment as a component of the secretory granules of mast cells [195], and is released externally when mast cells degranulate in response to an external signal. Since heparin occurs only in the

intracellular compartment, its physiological significance in protein binding is less apparent.

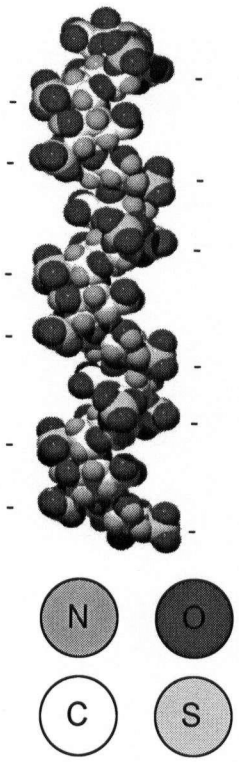
Figure 9. The Monosaccharides of Heparinoids. *All heparinoids consist of repeating units of D-glucosamine with either β-D-glucuronic acid or α-L-iduronic acid. Carbon backbone line drawings are provided with carbon atoms numbered.*



D-glucosamine β-D-glucuronic acid α-L-iduronic acid

On the other hand, heparan-sulfate is involved in a variety of physiological processes. In contrast to heparin, which is located only in mast cell granules, heparan-sulfate occurs in most animal cells. Heparan-sulfate is normally secreted from animal cells and is a main element of the extracellular matrix. "Heparan-sulfate" is generally considered the total pool of heparinoids extracted from a tissue and is therefore not fractionated and contains a range of structures. As heparan-sulfate occurs in large quantities in the extracellular matrix, it is well positioned to participate significantly in many different aspects of animal physiology. Heparan-sulfate is structurally similar to heparin in that it contains the same repeating disaccharide units ***Figure 9*** but contains larger numbers of non and mono-sulfated disaccharides and comparatively smaller numbers of sulfated Ido A containing disaccharides leading to a lower Ido A/GlcN ratio. Although heparin and heparan sulfate do have differences in degree of sulfation, and presumably differences in biological activity exist, both are collectively referred to as heparinoids.

Figure 10. 3D Space Filled Structure of Heparin (PDB code 1HPN). Heparin contains many contiguous highly sulfated disaccharides and thus is a very negative species.



Heparinoids appear to be essential for health. As mentioned, heparinoids, or heparan-sulfate, are found in almost every animal species that has been studied [196], and this ubiquitous existence suggests an important role for heparinoids in animal physiology [197-199]. In fact, three baby boys with congenital enterocyte heparan-sulfate deficiency exhibited severe clinical problems, including massive enteric protein loss, secretory diarrhea, and intolerance of enteral feeds, that resulted in death [200]. The presence of enterocyte heparan-sulfate appears to be of primary importance for small intestine function. Heparinoid function may require their *in vivo* attachment to protein.

Heparinoids occur *in vivo* attached to protein cores in complexes known as heparan-sulfate proteoglycans (HSPG). The heparinoid structures attach via a terminal xylose to a Ser located in the core protein [201-203]. The protein core of HSPG is variable and regulates HSPG metabolism. Many cells secrete HSPG and any one type of cell can express many different heparinoid core proteins. The heparan-sulfate chains expressed by different cells may be different, but the

heparan-sulfate chains on different core proteins of the same cell are identical. These core proteins regulate the cellular trafficking of HSPG and control the amount of HSPG produced by a cell. Even though all heparan-sulfate chains synthesized by one cell are identical, the cell can change the structure of its heparan-sulfate chains in response to environmental changes. The heparan-sulfate chains in turn control the responses of each cell to extracellular proteins.

The list of proteins that bind heparin if not cell surface HSPG is extensive and continues to grow. Heparin-binding proteins include proteins of the circulatory system, growth factors, extracellular matrix proteins, and proteins involved in lipid metabolism including HL. Areas of these proteins that have been shown to bind heparinoids generally have multiple basic amino acids, mostly Lys and Arg, in a fairly short amino acid sequence. There are many proteins that contain short stretches containing multiple basic amino acids that bind heparinoids but would never come into contact with them *in vivo*. However, HSPG are ubiquitous and any protein that occurs extracellularly will have the opportunity to interact with HSPG, so if a protein does occur extracellularly and does interact with heparan-sulfate there is a possibility the interaction is functionally significant.

1.5. Heparinoid-Protein Interactions

Heparinoids exhibit a tertiary structure associated with poor protein binding but possess an important structural feature that greatly increases heparinoids protein binding capacity. The linear, helical tertiary structure that heparinoids exhibit has been shown in other glycosaminoglycans to bind poorly to proteins [204,205]. The difference is the incorporation by heparinoids of IdoA ***Figure 9***. In contrast to other glycosaminoglycans the inclusion of IdoA leads to highly sulfated configurations that impart numerous, closely displayed sulfate and carboxyl groups available for ionic association with basic amino acids of proteins. The presence of IdoA in heparinoids is thought to bestow great flexibility of structure [206]. Although slight rotations occur about glycosidic linkages conferring some flexibility, IdoA undergoes rapid conformational changes [207] that can offer proteins for interaction many different arrangements of negatively charged groups.

Several aspects of heparinoid tertiary structure must be considered. Heparin contains long stretches of highly sulfated IdoA containing disaccharides while heparan-sulfate contains blocks of highly sulfated disaccharides with variably and usually small numbers of adjacent IdoA residues. Additionally, it appears that the ability of heparan-sulfate to undergo an induced conformational change occurs primarily over these relatively short IdoA containing blocks with little ability for induced fit over the other portions of the polysaccharide [207,208]. The minimal size for binding a monomeric protein ranges from hexa- to octasaccharide, and for dimeric proteins, deca-, dodecasaccharides or longer [206].

1.6. The Protein Binding Partner

Although it is now clear that there is no single consensus sequence that facilitates heparin binding by all heparin-binding proteins, several consensus sequences exist that may assist heparin binding by multiple proteins. *Cardin and Weintraub 1989* [209] used 12 known heparin binding sequences from vitronectin, apo E, apo B-100, and platelet factor 4 to formulate two search strings for a computer database and found 49 potential heparin binding domains in 21 proteins ***Table 2***. Many of the sequences conforming to the X-B-B-X-B-X or X-B-B-B-X-X-B-X consensus sequence of *Cardin and Weintraub* were calculated by predictive algorithms and circular dichroism to show amphipathic structures of either α-helical or β-strand conformations. Additionally computer modeling of vitronectin indicated that its heparin-binding domain formed a hydrophilic pocket that enveloped a heparin octasaccharide. In light of these results, *Cardin and Weintraub* suggest that multiple regions representing a discontinuous heparin-binding domain may be brought together by secondary structure folding to form a cleft for heparinoid binding.

Although the database used by *Cardin and Weintraub* was relatively small, several of their predictions have been established as true. Some of the predicted sequences have been established to be heparin binding domains, for example with thrombospondin, antithrombin, heparin cofactor II, protein C inhibitor, while others, namely fibroblast growth factors, are now known to not participate in heparin binding.

Sobel et al. 1991 [210] identified another consensus heparin binding sequence ***Table 2***. *Sobel et al.* examined 7 proteins with established heparin binding domains by aligning their sequences based on their distribution of basic and acidic residues, and found a 13 residue consensus sequence, X-B-B-X-X-B-B-B-X-X-B-B-X. A peptide representing the motif was constructed and displayed high heparin affinity. A 23 amino acid sequence in von Willebrand Factor (vWF) that matched the consensus motif was located (Tyr^{567}-Ala^{587}) and a peptide corresponding to this region was assessed for heparin affinity and ability to compete with vWF. The Tyr^{565}-Ala^{587} peptide exhibited heparin affinity equal to that of the consensus peptide and native vWF. Additionally, the Tyr^{565}-Ala^{587} peptide readily suppressed vWF heparin binding. Furthermore, circular dichroism performed in this study suggested that the Tyr^{565}-Ala^{587} peptide undergoes a conformational change upon interaction with heparin.

Table 2. Heparin Binding Consensus Sequences. *X represents any non-basic amino acid and B represents any basic amino acid.*

Sequence	Inverse	HL Sequence Correspondents
XBBXBX[a]	XBXBBX	293-CKKGKC-298 311-SKSKRL-316
XBBBXXBX[a]	XBXXBBBX	468-SKTSKRKI-475
XBBXXBBBXXBBX[b]	N/A	None
$XBX_{12}BX$[c] *α helix homology* *X_{12} must contain at least 1B*	N/A	296-GRCNTLGYHVRQEPRS-311 320-TRAQSPFKVYHYQLKI-335 361-QKIPITLGKGIASNKT-376 313-SKRLFLVTRAQSPFKV-328 453-LRPTQEKIFVKCEIKS-468
XBX_6B[c] *β strand homology* *X_6 must contain at least 1B*	N/A	5-KPQPFGRRA-13 112-RNTRLVGKE-120 314-KRLFLVTRA-322 467-KSKTSKRKI-475

[a] Cardin & Weintraub 1989
[b] Sobel et al. 1991
[c] Margalit et al. 1993

Similarly to *Sobel et al.*, *Margalit et al. 1993* [211] examined heparin-binding domains and discovered a heparin-binding motif ***Table 2***. *Margalit et al.* studied sequences that had been shown experimentally to be directly involved in heparin binding and compared the spatial distribution of cationic residues among the sequences with defined three dimensional structures using computer graphics

techniques. Common among all of the binding sequences was the placement of 2 basic amino acids approximately 20 Å apart from each other ***Table 2***. The two residues point in opposite directions in an α-helix. The motif appears to result in an amphipathic structure with the intervening basic residues projecting from one side of the helix while intervening non-polar residues project from the opposite side, X-B-X_{12}-B-X. The computer graphics techniques also found that other heparin binding sequences that maintained the same spatial distribution were compatible with a β-strand structure, X-B-X_6-B-X. *Margalit et al.* also determined that the 20 Å gap accommodates a pentasaccharide and that the spatial distribution suggests an intertwining of the heparin-protein complex. The inducing fit of heparinoid and protein as intimated by *Cardin and Weintraub, Sobel et al.,* and *Margalit et al.* is supported by findings that peptides binding to heparin can also undergo changes in secondary structure [212,213].

In summary, the protein interaction with heparinoid structures is realized through ionic forces between the basic residues of the protein and the anionic carboxyls and sulfates of the heparinoid. No one sequence exists that describes the heparin binding regions of peptides, but the domains responsible appear to be rich in Arg and Lys residues in specific spatial or sequence distributions. It also appears that the interaction between protein and polysaccharide results in the induction of a favorable structure in both partners.

1.7. Triflouroethanol (TFE)

The changes effected by TFE on polypeptide chain confirmation are diverse and appear to depend on the particular amino acid sequences involved, the cosolvent concentration, other solution conditions, and the structures involved [214-219]. Though it is now established that there is a preference for the stabilization of helical conformations when α-helix is the conformational bias of the primary sequence [220,221], structures containing turns, β-hairpins, β-sheets, hydrophobic clusters and associated surfaces are also possible in the presence of TFE [222]. Three possible mechanisms have been proposed for the action of TFE on proteins and peptides.

TFE may enhance secondary structure through enhancing internal hydrogen bonding. Direct interactions of TFE molecules with peptides have been inferred [222] and a study by *Mizuno et al. 1984* [223], suggests that non-halogenated alcohols have a weak hydrogen bonding capacity for donors and acceptors. TFE itself is a better hydrogen bond donor than water but a poorer hydrogen bond acceptor. As such, TFE has been suggested to preferentially bind the main chain carbonyl oxygen group, which is capable of accepting two hydrogen bonds [222]. Additionally, evidence exists that this preferential interaction minimized solvent exposure to the main chain amide, thus enhancing its hydrogen bonding [224] and hence intra-polypeptide hydrogen bonding [225].

TFE may also disrupt water structure lessening the hydrophobic effect. Molecules of TFE have been shown to associate with protein surfaces as well as

their interiors [226] and appear to preferentially associate with hydrophobic sites on protein surface [227]. There is evidence that TFE weakens hydrophobic-hydrophobic interactions [228,229], which may account for the ability of TFE to lessen hydrophobic interactions between amino acids distant in sequence. However, structures that rely on local interactions, such as short peptides, are preferentially stabilized [222].

Lastly, the penetration of TFE molecules into the protein core and their preferential solvation of certain groups of the polypeptide chain is proposed to stabilize compact structures [214]. In fact, this may be the main mechanism at low cosolvent concentration [230].

1.8. Homology Modeling

Although X-ray crystallography and NMR spectroscopy are the only methods to acquire detailed structural information of a protein, these techniques are laborious and technically difficult and many proteins, including HL, fail to crystallize or be obtained in quantities necessary for NMR spectroscopy. Homology modeling on the other hand does not require isolation or crystallization of protein and is relatively easy to carry out.

Homology modeling predicts the three dimensional structure of a protein of unknown structure by comparing the sequence of the protein with the template of a known protein. Homology modeling is useful only because it provides structural information about a protein, and in the absence of X-ray crystallography or NMR spectroscopy data, is the only reliable way to obtain structural information [231-233]. Interestingly, comparisons of homologous proteins have determined that tertiary structure is better conserved throughout evolution than primary structures [234-236]. The feasibility of creating structures through homology modeling is well established [237-242]. Several free and easy to use homology-modeling programs are available for download from the Internet: 3DJIGSAW, FAMS, CPH MODEL, SWISS MODEL, and Esy3D.

Although homology modeling of unknown proteins is becoming common, several important limitations exist. Errors in modeling algorithms can be divided into five categories [231,243,244]: (1) side chain packaging errors, (2) distortions of regions that are aligned correctly with the template, (3) distortions of regions that do not have a corresponding segment in the template, (4) distortions of regions not correctly aligned with the template, and (5) template misalignment errors.

As template and protein of interest sequence diverge, the packaging of side chains differ. Errors of side chain packing can be deleterious to the model if they occur in regions involved in protein function; such is the case with catalytic and binding sites. Even if main chain conformations don't change, template-target protein sequence divergence can also result in the template being locally different from the sequence of interest. This can result in errors within correctly aligned sequences. Additionally, modeling regions of a protein that do not have a corresponding sequence in the template is obviously difficult. Although insertions of less than 9 residues can be successfully modeled [245] accurate modeling of these

sequences depends primarily on the accuracy of the modeling of the surrounding sequences. However, distortions of regions that are not correctly aligned with the template are the major source of error with modeling programs especially when sequence homology falls below 30%. Furthermore, when a distant protein is used as a template, the resulting models are unreliable [243], and distinguishing between sequences modeled on a correct or incorrect portion of the template becomes difficult. Though, if key functional or structural residues are conserved between the template and target proteins, the confidence of the corresponding tertiary structure increases.

Errors in regions correctly aligned and errors in region without a corresponding template are infrequent with sequences that share 40% homology with the template and models are approximately 90% accurate [246]. When homology between modeled sequence and template is 30-40% more disparity exists between the sequence to be modeled and the template and hence errors (2)-(4) increase. As a result, the accuracy of the model falls to approximately 80% [246]. At below 30% sequence homology the alignment errors affect roughly 20% of the target residues and the model is of less quality [246]. As many of the sequence and template proteins compared by homology modeling programs are less than 40% homologous [247], alignment errors limit their usefulness. Nevertheless, when alignment errors occur, the model is generally closer to the actual protein structure than the template structure [244]. Therefore, using a model is superior than using the template when actual protein structures are not available.

1.9. Rational

HL is significantly involved in many facets of human lipid metabolism including remnant particle catabolism, HDL remodeling, and the selective uptake of cholesterol ester. However, the mechanisms of the various roles of HL are not currently known and only beginning to be elucidated. The role of HL is complex and its overall anti-atherogenic or pro-atherogenic nature is unclear. The truth undoubtedly lies somewhere in between these two extremes. Taken separately, some HL functions are pro-atherogenic while others are anti-atherogenic. In reality, it is likely the balance between these multiple effects that is required for normal lipid metabolism and good health.

What is known about HL is that it is only active as a homodimer and is bound predominantly to cell surface HSPG and the presence of HSPG appears to be required for many HL functions. Obviously the interaction between HSPG and HL is of primary physiological significance. The heparin-binding domain of human hepatic lipase of HL has previously been suggested by chimera studies to be located in the terminal 60 amino acids of the HL carboxyl terminus. Nonetheless, consensus sequence information and site-directed mutagenesis implicate additional regions of the HL enzyme in heparin binding. It was our intention, guided by past chimera studies, consensus sequence predictions, and homology modeling to pancreatic lipase, to further define regions or specific amino acids within the HL enzyme responsible for its heparin binding.

1.10. Specific Aims

1) To create a three-dimensional model of HL based on the known structure of PL and assess each basic residues' potential heparin binding according to visual characteristics.
2) To apply heparin-Sepharose chromatography on synthetic peptides representing regions of HL rich in basic amino acids to assess their affinity to heparin.
3) To perform competitive binding experiments with said peptides to assess their ability to compete with cell surface HSPG in an environment that more closely mimics the *in vivo* environment of HL action.
4) To determine the ability of a panel of monoclonal antibodies raised against a synthetic peptide to inhibit the binding of HL to heparin-Sepharose.

1.10.1. Homology modeling

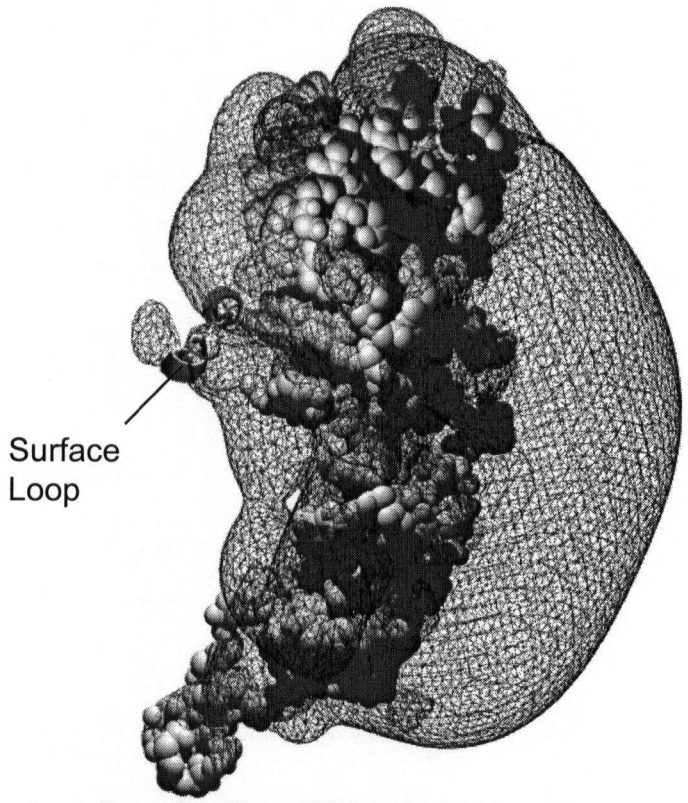


Figure 11. Electron Density Map (EDM) of FAMS Modeled HL. *The EDM of modeled HL was calculated using Swiss Viewer Software. HL is grey while the electron densities are represented by the blue (positive charge density) and red (negative charge density) scaffolding. The surface loop is shown in green and as its predicted secondary structure (α helix)*

Several heparin binding consensus sequences occur within HL. In particular, the regions corresponding to amino acids 293-316 and 453-475 are rich in reported consensus heparin binding sequences. Molecular models of HL were generated using web based homology programs and results were used in conjunction with consensus sequence identification to guide our examination of specific candidate residues in HL. Our models were based on homology with PL, an enzyme with substantially fewer COOH domain residues than HL, and as such distal COOH terminal residues 453-475 could not be modeled. An electron density map (EDM) ***Figure 11*** was generated for each HL model, and all indicated a uniformly large area of high positive charge density along the long axis of the HL monomer opposite the NH_2 domain surface loop. Each candidate basic amino acid was then assessed in 5 different models for its potential to contribute to a linear heparin binding domain corresponding to the area of positive charge density.

1.10.2. Heparin-Sepharose Affinity Chromatography

Based on consensus sequence information, our homology modeling results, and the chimera study of *Hill et al.* 1998 [173] implicating the terminal 60 amino acids of the HS COOH domain, synthetic peptides were created and assessed for heparin affinity ***Figure 12***. Peptide 304-323 being rich in consensus sequences and having scored positive for the regions potential to contribute to a heparin binding domain via homology modeling was expected to display heparin-Sepharose column affinity. Peptides 452-471 and 455-471 also being rich in consensus sequences and having been implicated by *Hill et al. 1998*, were also expected to display affinity for the heparin-Sepharose column. Previous studies in our lab which utilized a truncated recombinant form of HL (HL-471) in which the final five residues were deleted indicated only a modest effect on heparin affinity. Consequently, these residues were not included in the peptide sequences. Although rich in basic amino acids, regions represented by peptides 355-377 and 419-442 were not indicated by previous studies to contain consensus heparin binding sequences, or to be part of the HL hparin-binding domain.

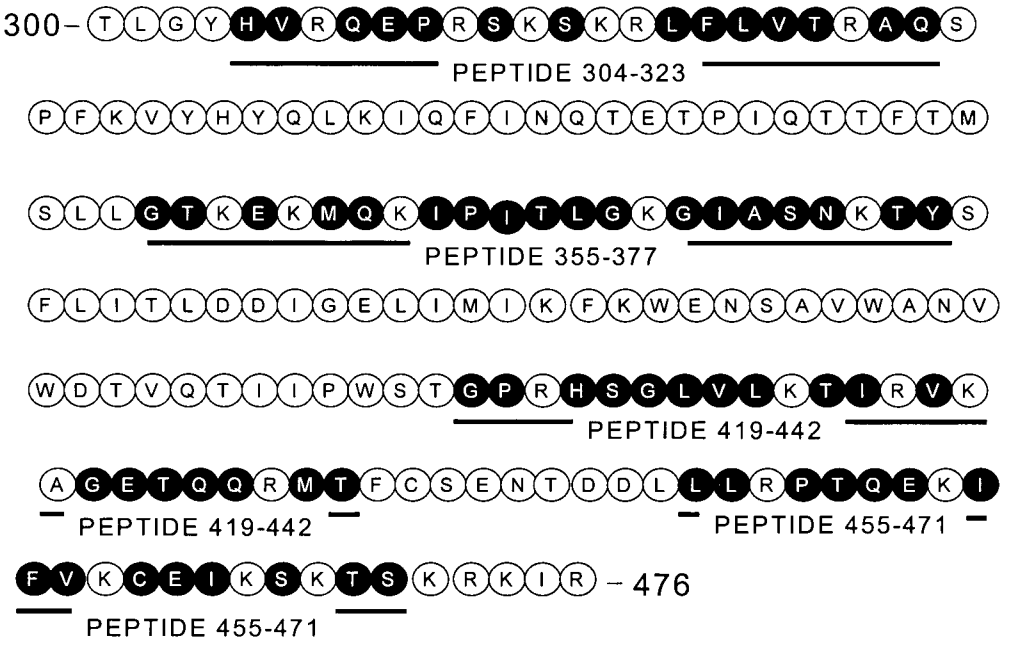


Figure 12. Amino Acid Sequence of the Distal NH_2 (amino acids 300-329) and COOH domains (amino acids 340-376) of human HL. *Amino acids are represented by their single letter abbreviations. Charged residues are grey while segments represented by synthetic peptides are black.*

A possible limitation associated with the use of peptides is that their secondary structure may not mimic the same region within the intact enzyme. To this effect, heparin-Sepharose affinity analyses was conducted in the presence and absence of trifluoroethanol (TFE), a cosolvent which has been shown to induce secondary structure in experimental peptides [230,248-250]. Results of chromatography with TFE were expected to compensate for secondary structure interactions and to enhance our initial findings. Although the epitope(s) respon sible for the heparin binding of HL could be discontinuous, the use of synthetic peptides may still permit the identification of region(s) that contribute to such an epitope.

1.10.3. Competitive Binding

Competitive binding experiments were executed in which individual peptides were added to previously established HL secreting Chinese hamster ovary (CHO) cells to evaluate the ability of each peptide to compete for the same HL surface binding sites. By measuring the amount of enzyme activity released we assessed the relative affinity of each peptide for cell surface heparan sulfate proteoglycans. The use of peptides in a cell culturing system is intended to more closely mimic *in*

vivo binding of human HL. The same secondary structure limitations exist as with the chromatography.

1.10.4. Antibody Inhibition

Based on the presence of multiple consensus sequences in the 453-475 region of the HL sequence and from chimera studies indicating the distal 60 amino acids of the COOH terminal domain play a key role in the heparin binding of HL [173], a peptide-specific monoclonal antibody was raised against 452-471 of human HL. The monoclonal antibody was tested for the ability to inhibit the heparin binding of human HL. Due to the high costs of antibody production, only one of the candidate regions was selected as a peptide source for the creation of a monoclonal antibody.

2 MATERIALS AND METHODS

2.1 Homology Modeling

Models of HL were generated using the 2.46 angstrom resolution structure of human pancreatic lipase-colipase complex inhibited by a C11 alkyl phosphonate (PDB access code: 1LPB) ***Figure 8***. The pancreatic lipase-colipase-C11 alkyl phosphonate complex shares 30% amino acid homology with human HL [251]. Models were created using five different algorithms (1) 3D-JIGSAW [252], (2) SWISS-MODEL [253], (3) CPHmodels [254], (4) Esy3D and (5) FAMS, and analysed using Swiss-Pdb Viewer [255].

Each basic residue was assessed for accessibility to solvent and visualized in all three axes to evaluate its potential to contribute to a linear binding domain. Since arginine and lysine, and not histidine, are protonated at the pH employed, only these residues were classified as basic. Residues that were at least 30% accessible to solvent, as calculated by Swiss Viewer, and appeared to fit a linear binding domain on all three axes on the majority of algorithms tested were assigned a positive score. However, it is important to note that all models lacked distal COOH terminal residues ranging from 407 to 459 to the terminal end. This region is rich in basic amino acids and reported heparin binding consensus sequences.

2.2 Heparin-Sepharose Affinity Chromatography

Synthetic peptides were synthesized by the Nucleic Acids Protein Services Unit at the University of British Columbia ***Figure 12***. Peptides were dissolved in the column equilibration buffer (50mM Tris-HCl, pH 8.0, 0.15M NaCl), herein referred to as Buffer A, to a concentration of 1mg/ml. After equilibration of the column with 10 column volumes of Buffer A, five hundred μg of each peptide were loaded separately onto a 2-mL heparin-Sepharose column connected to a BioRad Econo Chromatography System operating at a flow rate 0.5 ml/min. A NaCl gradient from 0.15 to 1.5M NaCl was applied over a 40-minute time period and 2mL fractions collected. The column was then washed for 10 column volumes with Buffer B (50 mM Tris-HCl, pH 8.0, 1.5M NaCl). A conductivity meter was be used to measure the conductivity, and consequently NaCl concentration, for each collected fraction. Elutions were conducted in the absence and presence of trifluoroethanol (TFE), a cosolvent that has been shown to induce secondary structure in peptides and proteins [222,230,248-250], to assess the role of secondary structure. TFE was purchased from Sigma. One peptide was modified by p-hydroxyphenylglyoxal (HPG) which selectively modifies arginine residues [256,257] to evaluate the relative contribution of arginine residues to heparin binding. HPG was purchased from Pierce.

2.3 Competitive Binding

HL expressing Chinese hamster ovary cells (CHO-HL) were grown to confluency in 10cm dishes at 37°C and 5% CO_2. Cells were grown in DMEM, purchased from Sigma, containing 20% FBS, 5% antibiotic (penicillin, streptomycin sulfate and amphotericin B) and 0.25μg/ml Fungizone (amphotericin B), all purchased from GibcoBRL. At confluency, baseline samples of media were taken and synthetic peptide added to the CHO-HL cells at 0.375μg/mL (a synthetic peptide:HL molar ratio of 100:1) and incubated for 24 hours. HL activity of the samples was measured using a trioleinase activity assay [173] using a triolein emulsion containing radiolabeled triolein: Triolein (7.5 mg), phosphatidylcholine (1mg) and 50μCi of [^{3}H]triolein were dried under nitrogen. A volume of 2.1 mL of 1.2M Tris-HCl, pH 8.0 and 0.4 mL of 1% bovine serum albumin in 1.2 M Tris-HCl, pH 8.0 were added to the lipid mixture before it was sonicated on ice for 8 min at 40% pulse. After sonication, 0.5 ML of 4% bovine serum albumin in 0.2 Tris-HCl, pH 8.0, was added to the substrate mixture.

2.4 Antibody Inhibition

Peptide-specific monoclonal antibodies were produced by Immuno-Precise Antibodies Ltd., Victoria B.C., by their rapid-prime inoculation method. Flip in™ CHO-HL cells were grown to confluency in 10cm dishes at 37°C and 5% CO_2. Cells were grown in F-12 Nutrient Mixture containing 10% FBS, 5% antibiotic (penicillin, streptomycin sulfate and amphotericin B), 0.25μg/ml Fungizone (amphotericin B), and 1x10^{-3}M L-Glutamine, all purchased from GibcoBRL. At confluency, heparin sodium salt, purchased from Sigma, was added to the Flip in™ CHO-HL cells to a final concentration of 10 units/mL in OPTI-MEM. The CHO-HL tissue/heparin salt mixture was incubated at 37°C and 5% CO_2 overnight (16-20hr) to allow the heparin displacement of endogenous HL to reach equilibrium. 500 μL of CHO-HL conditioned OPTI-MEM media (assuming [HL] of 1μg/mL) was incubated on ice for 1 hr with 75μg of monoclonal antibody (50 μg/mL) and subject to heparin-Sepharose chromatography as described above. The resulting fractions were then assessed for HL activity using the trioleinase activity assay [173]. However, the affinity and/or specificity of monoclonal antibodies produced by this method cannot be guaranteed.

3 RESULTS

3.1 Homology Modeling of Human Hepatic Lipase

Results of the homology modelling are listed in ***Table 3*** and ***Table 4***. Basic residues K163, K294, K295, R297, R306, K314, R315, and K375 satisfied all criteria with the 3D-JIGSAW algorithm while residues K294, K295, R297, R306, K314, R315, and R440 satisfied analysis criteria as modeled on the CPH algorithm. The Esy3D algorithm indicated residues K163, K294, K295, R297, R306, R310, K312, K314, R315, R431, and R440 may be part of a linear binding domain, while the FAMS algorithm implicated K294, K295, R297, R306, R310, K312, K314, R315, K375, and R440. Lastly, the SWISS-MODEL implicated residues K294, K295, R297, R306, R310, K312, K314, R315, and K334.

***Table 3. Homology Modelling Results**. Residues are listed according to their position in the HL primary structure. Each basic residue was assessed for its potential to contribute to a linear binding domain. A (✓) indicates the residue met the restrictions while a (-) indicates the residue did not. Blackened squares represent omitted residues in the modelling algorithm.*

Candidate Amino Acid	Modelling Algorithm					
	3DJIGSAW	HL CPH	Esy3D	FAMS	SWISSGPL	Score
$K5_{(non\ coding)}$	-	-				-
$R11_{(non\ coding)}$	-			-		-
$R12_{(non\ coding)}$	-	-		-		-
$K20_{(non\ coding)}$	-	-		-	-	-
K26	-	-	-	-	-	-
R28	-	-	-	-	-	-
R42	-		-	-	-	-
K85	-	-	-	-	-	-
R112	-	-	-	-	-	-
R115	-	-	-	-	-	-
K119	-	-	-	-	-	-
R126	-	-	-	-	-	-
R136	-	-	-	-	-	-
K163	✓		✓	-	-	-
R166	-	-	-	-	-	-
R185	-	-	-	-	-	-
R202	-	-	-	-	-	-
K212	-	-	-	-	-	-
R238	-	-	-	-	-	-
K253	-	-	-	-	-	-
R258	-	-	-	-	-	-
K294	✓	✓	✓	✓	✓	✓
K295	✓	✓	✓	✓	✓	✓
R297	✓	✓	✓	✓	✓	✓
R306	✓	✓	✓	✓	✓	✓
R310			✓	✓	✓	✓
K312			✓	✓	✓	✓
K314	✓	✓	✓	✓	✓	✓
R315	✓	✓	✓	✓	✓	✓
R321	-	-	-	-	-	-
K327	-	-	-	-	-	-
K334	-	-	-	-	✓	-
K357	-	-	-	-	-	-
K359	-	-	-	-	-	-
K362	-	-	-	-	-	-

Table 4. Homology Modelling Results continued.

Candidate Amino Acid	Modelling Algorithm					
	3DJIGSAW	HL CPH	Esy3D	FAMS	SWISSGPL	Score
K375	✓	-	-	✓	-	-
K394	-	-	-	-	-	-
K396	-	-	-	-	-	-
R421	-		-	-		-
K428	-	-	✓	-		-
R431	-	-	-	-		-
K433	-	-	-	-		-
R440	-	✓	✓	✓		✓
R454	-	-		-		-
K459						
K463						
K467						
K469						
K472						
R473						
K474						
R476						

All 5 modeling programs included residues K294, K295, R297, R306, R310, K312, K314, R315, except when the residues were not included in the modeling program ***Table 3*** and ***Table 4***. These residues appear to have high potential to be part of a linear heparin-binding domain.

3.2 Heparin-Sepharose Affinity Chromatography

The synthetic peptides spanning residues 455-471 and 452-471 ***Figure 12*** and ***Table 5*** represent distal COOH terminal portions of HL and are rich in reported consensus heparin binding sequences. However, in the absence of TFE, neither peptide displayed heparin binding both, eluting at 0.15M NaCl ***Figure 13, 14*** and ***Table 5***.

***Figure 13. Heparin Affinity Chromatography Elutions**. Peptides were eluted from a heparin-Sepharose column through a salt gradient of 0.15M to 1.50M NaCl. (■) indicate points on the NaCl gradient and (▲) represent peptide as measured by absorbance at 220nm. NaCl concentration of fraction containing the peak absorbance is given on graph. Dashed lines represent the standard error of the mean (SEM).*

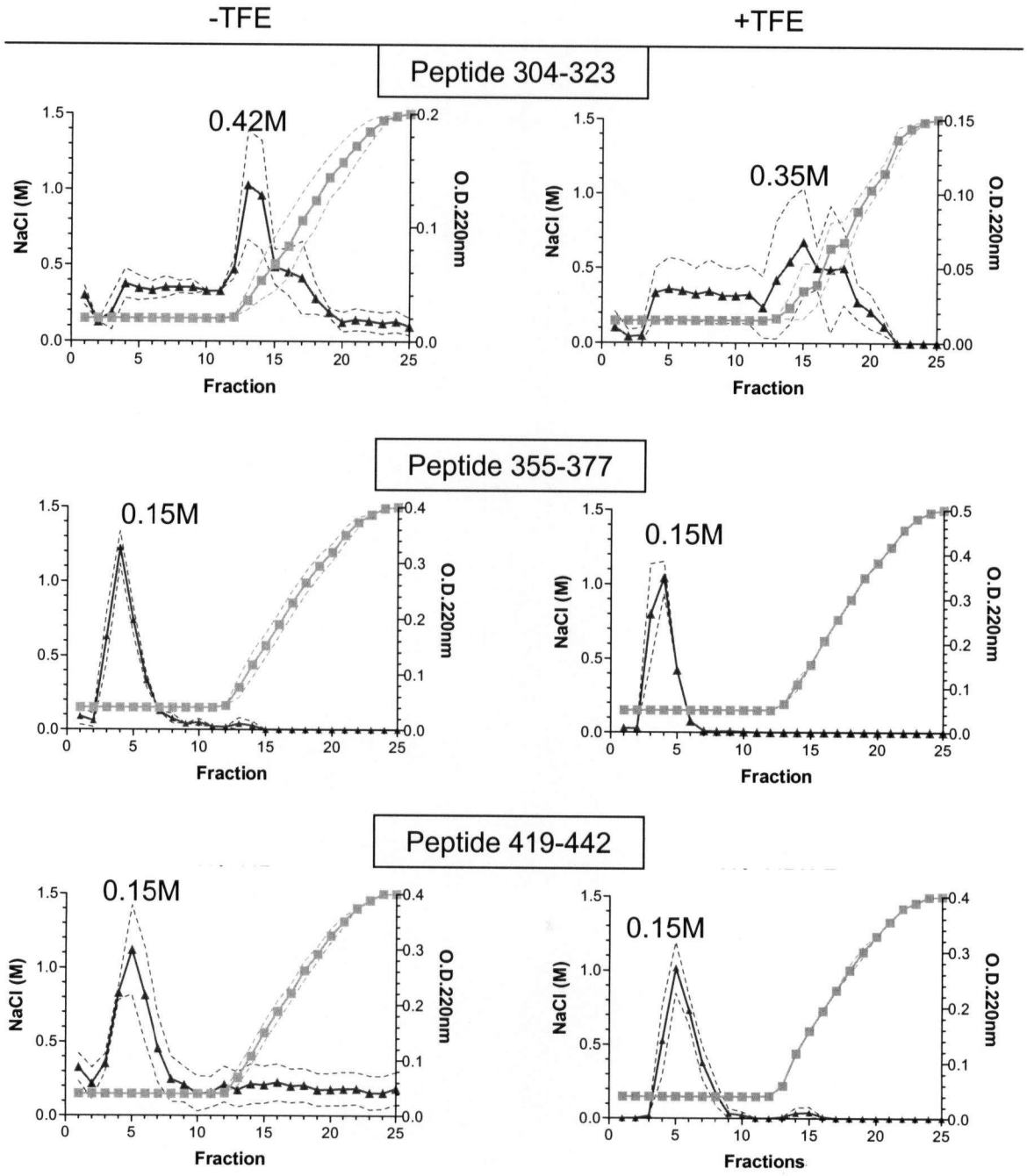
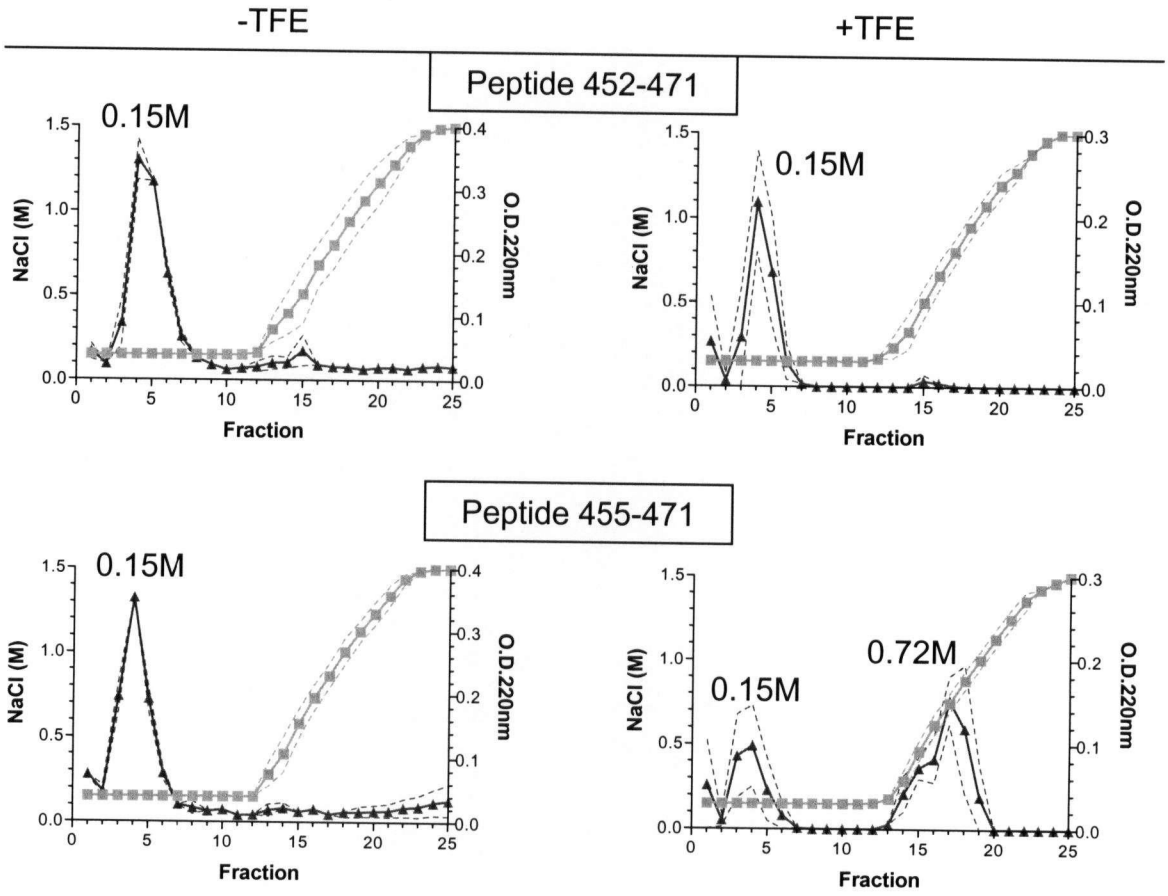


***Figure 14. Heparin Affinity Chromatography Elutions continued**. Peptides were eluted from a heparin-Sepharose column through a salt gradient of 0.15M to 1.50M NaCl. (■) indicate points on the NaCl gradient and (▲) represent peptide as measured by absorbance at 220nm. NaCl concentration of fraction containing the peak absorbance is given on graph. Dashed lines represent the standard error of the mean (SEM).*



Similarly, peptide 419-442, which represents residues located in the proximal portion of the HL COOH terminus, eluted at the bottom of the NaCl gradient indicting the absence of heparin binding ***Figure 13, 14*** and ***Table 5***. Peptide 304-323, which was expected to show heparin affinity based on computer modeling and consensus sequence information, did so eluting from the heparin-Sepharose column at 0.42 M NaCl in the absence of TFE ***Figure 13, 14*** and ***Table 5***.

Table 5. Heparin-Sepharose Chromatography of Synthetic Peptides. *Chromatography was conducted with synthetic peptides of candidate regions in the presence and absence of the secondary structure inducer trifluoroethanol (TFE). The heparin affinity of wild type HL is 0.8M NaCl. The number of ✓indicate the displayed affinity for heparin.*

PEPTIDE	NACL ELUTION (M)		HEPARIN AFFINITY
	-TFE	+TFE	
304-323	0.42	0.35	✓✓
355-377	0.15	0.15	-
419-442	0.15	0.15	-
452-471	0.15	0.15	-
455-471	0.15	0.15 & 0.72	✓

The presence of the secondary structure inducer TFE (10% v/v), did not affect the heparin affinity of peptide 452-471 or 419-442 which continued to display no column binding, or peptide 304-323 which continued to show column binding eluting at 0.34M NaCl ***Figure 13, 14*** and ***Table 5***. However, intriguing results were obtained when peptide 455-471 was eluted in the presence of TFE. Peptide 455-471 eluted in two fractions: one demonstrating no heparin binding, eluting at 0.15M NaCl, and the other exhibiting heparin affinity nearly equivalent to that of wild type human HL, eluting at 0.72M NaCl ***Figure 13, 14*** and ***Table 5***.

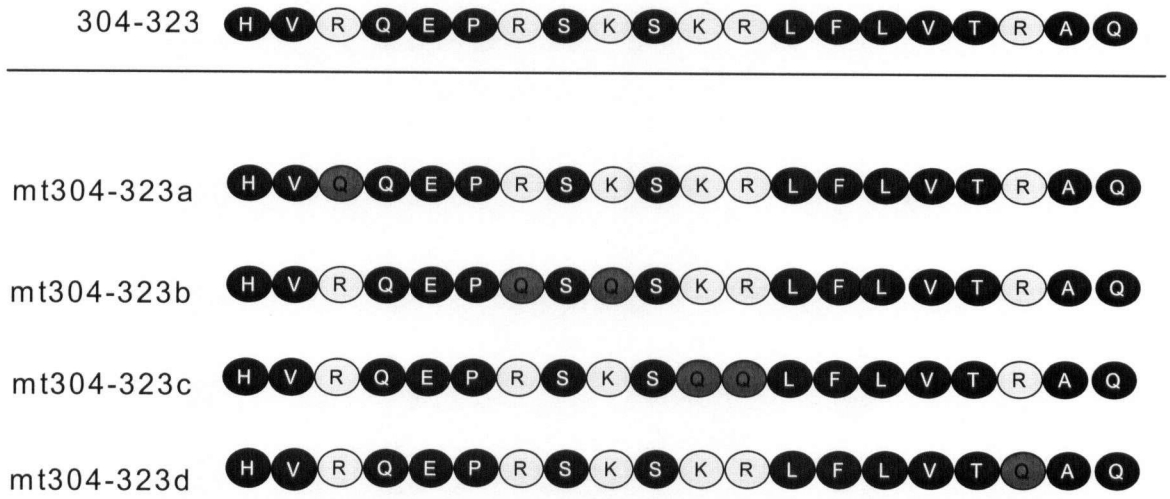


Figure 15. Mutant Synthetic Peptides of Region 304-323*. Candidate basic amino acids were mutated to glutamate (Q). The resulting mutant peptides were assessed for heparin affinity by heparin-Sepharose chromatography. Charged residues are light grey while mutated residues are dark grey.*

Based on the above results, mutant peptides of the 304-323 regions were generated ***Figure 15*** by the Nucleic Acids Protein Services Unit at the University of British Columbia and similarly tested for heparin affinity. The double mutations of mt304-323b (R310Q & K312Q) and mt304-323c (K314Q & R315Q) resulted in the abolition of heparin-Sepharose binding in both the presence and absence of TFE ***Figure 16, 17*** and ***Table 6***. Conversely, the single mutations of mt304-323a (R306Q) and mt304-323d (R321Q) were less damaging. Both peptides retained some heparin affinity as they eluted from the column in two fractions: one that did not bind to heparin-Sepharose and one that bound with roughly equivalent strength as the original 304-323 peptide ***Figure 16, 17*** and ***Table 6***. However, the presence of TFE abrogated the heparin affinity of both mutated peptides.

***Figure 16. Heparin Affinity Chromatography of Mutated Peptides**. Peptides were eluted through a salt gradient of 0.15M to 1.50M NaCl. (■) indicate points on the NaCl gradient curve and (▲) represent peptide absorbance at 220nm. NaCl concentrations of fractions containing the peak absorbance are given on graph. Dashed lines represent the standard error of the mean (SEM).*

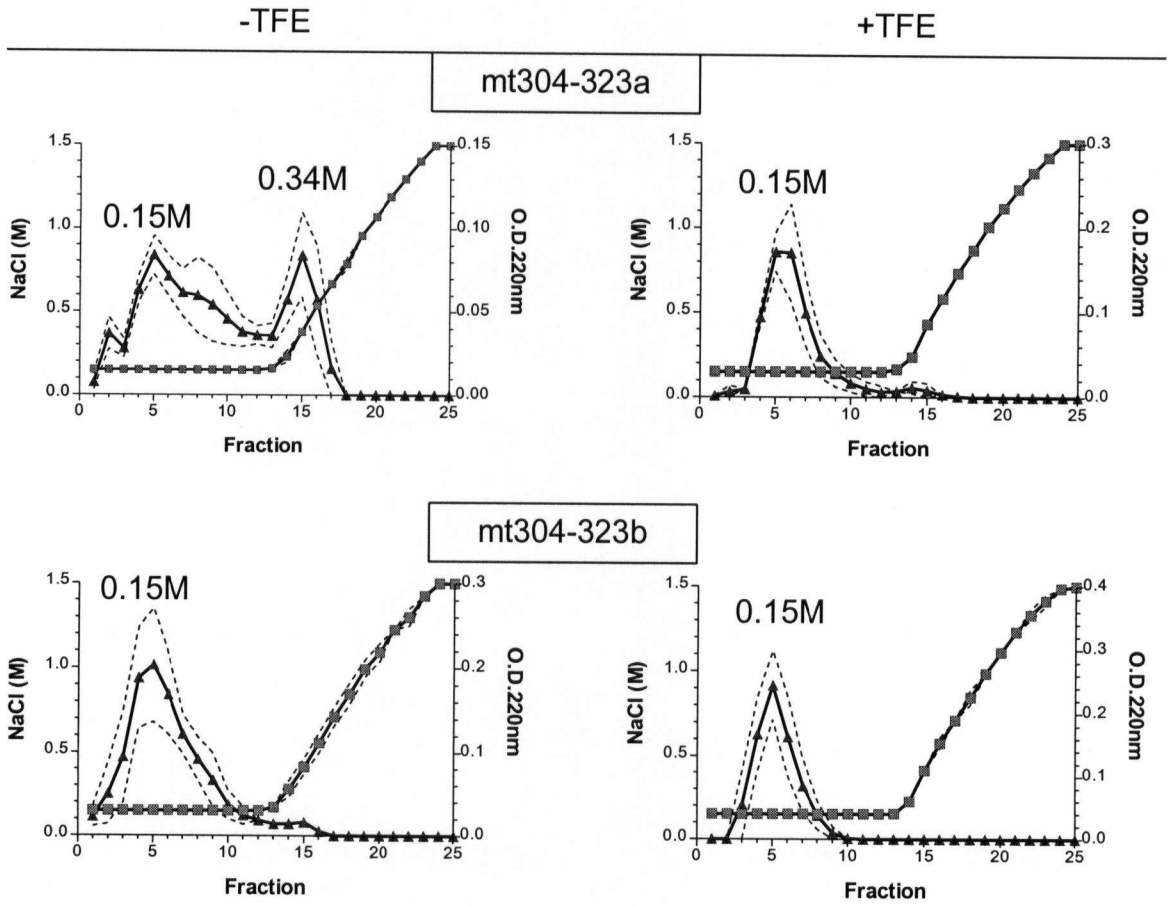


Figure 17. Heparin Affinity Chromatography of Mutated Peptides continued. *Peptides were eluted through a salt gradient of 0.15M to 1.50M NaCl. (■) indicate points on the NaCl gradient curve and (▲) represent peptide absorbance at 220nm. NaCl concentrations of fractions containing the peak absorbance are given on graph. Dashed lines represent the standard error of the mean (SEM).*

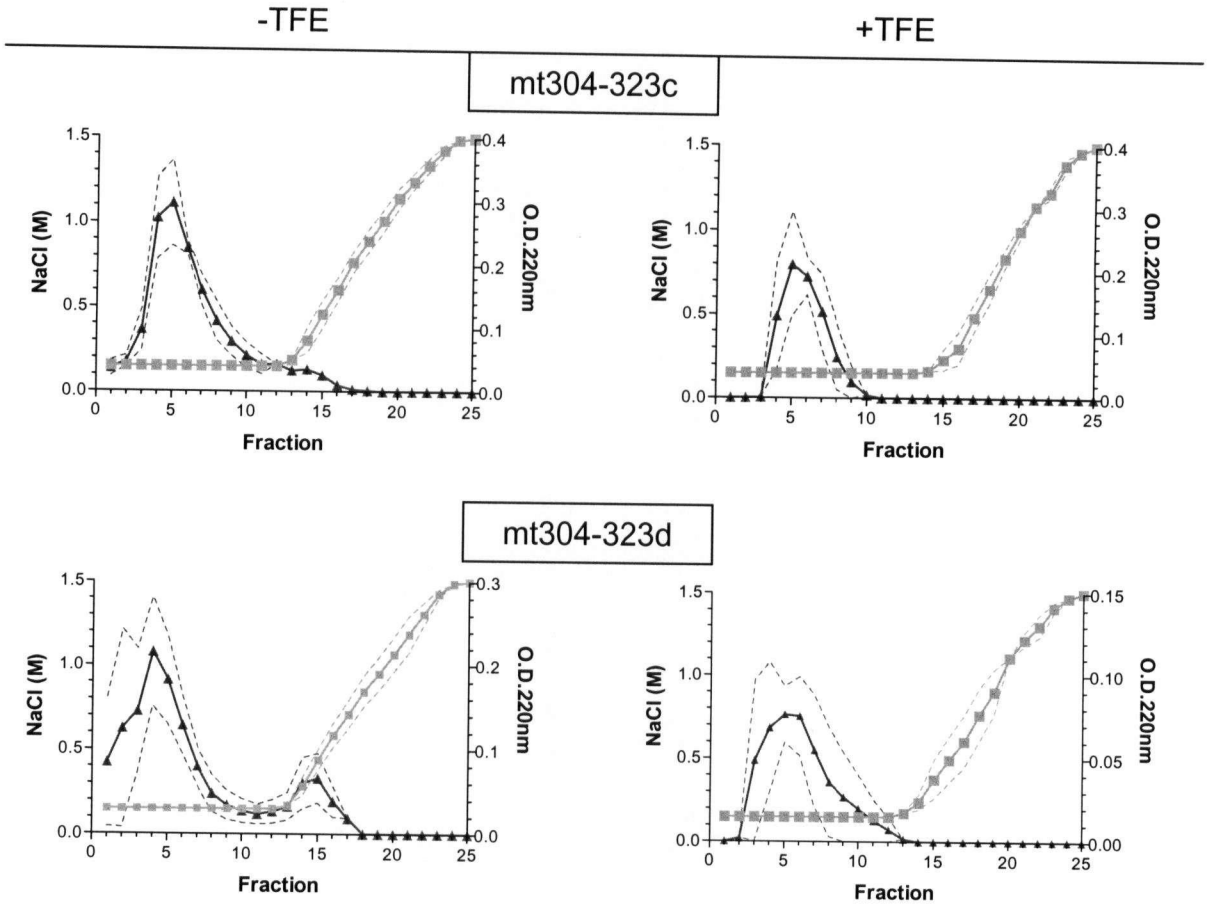


Table 6. Heparin-Sepharose Chromatography Results of Mutant Peptides. *Candidate amino acids were mutated to glutamine and assessed for heparin affinity by heparin-Sepharose affinity Chromatography. The ✓indicate the displayed affinity for heparin.*

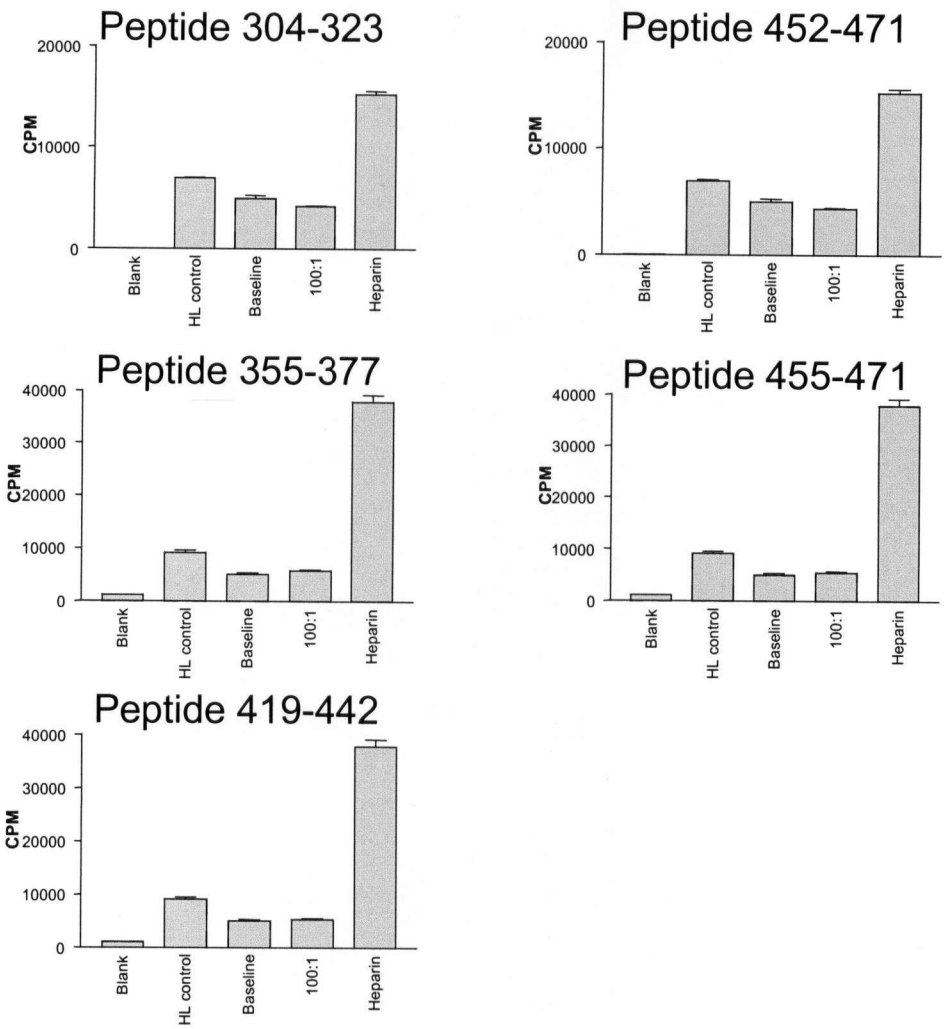
PEPTIDE	NACL ELUTION (M)		HEPARIN AFFINITY DISPLAYED
	-TFE	+TFE	
mt304-323a	0.15 & 0.34	0.15	✓
mt304-323b	0.15	0.15	-
mt304-323c	0.15	0.15	-
mt304-323d	0.15 & 0.46	0.15	✓
HPG modified 304-323	0.15	0.15	-

To confirm the results obtained from the mutant peptides we modified the original 304-323 peptide with p-hydroxyphenylglyoxal (HPG). HPG selectively [257] and covalently [256] modifies arginine residues thus eliminating the possibility of their contribution to an ionic interaction between the synthetic peptides and the heparin-Sepharose column. The treatment of peptide 304-323 with HPG resulted in the loss of binding to the column ***Figure 16, 17*** and ***Table 6***.

3.3 Competitive Binding

The addition of synthetic peptides to HL-secreting CHO cells in culture did not result in the significant displacement of HL from cell surfaces to the culture medium ***Figure 18***. None of the synthetic peptides, including peptides 304-323 and 455-471, which displayed heparin affinity upon heparin-Sepharose chromatography, competed with HL for cell surface HSPG.

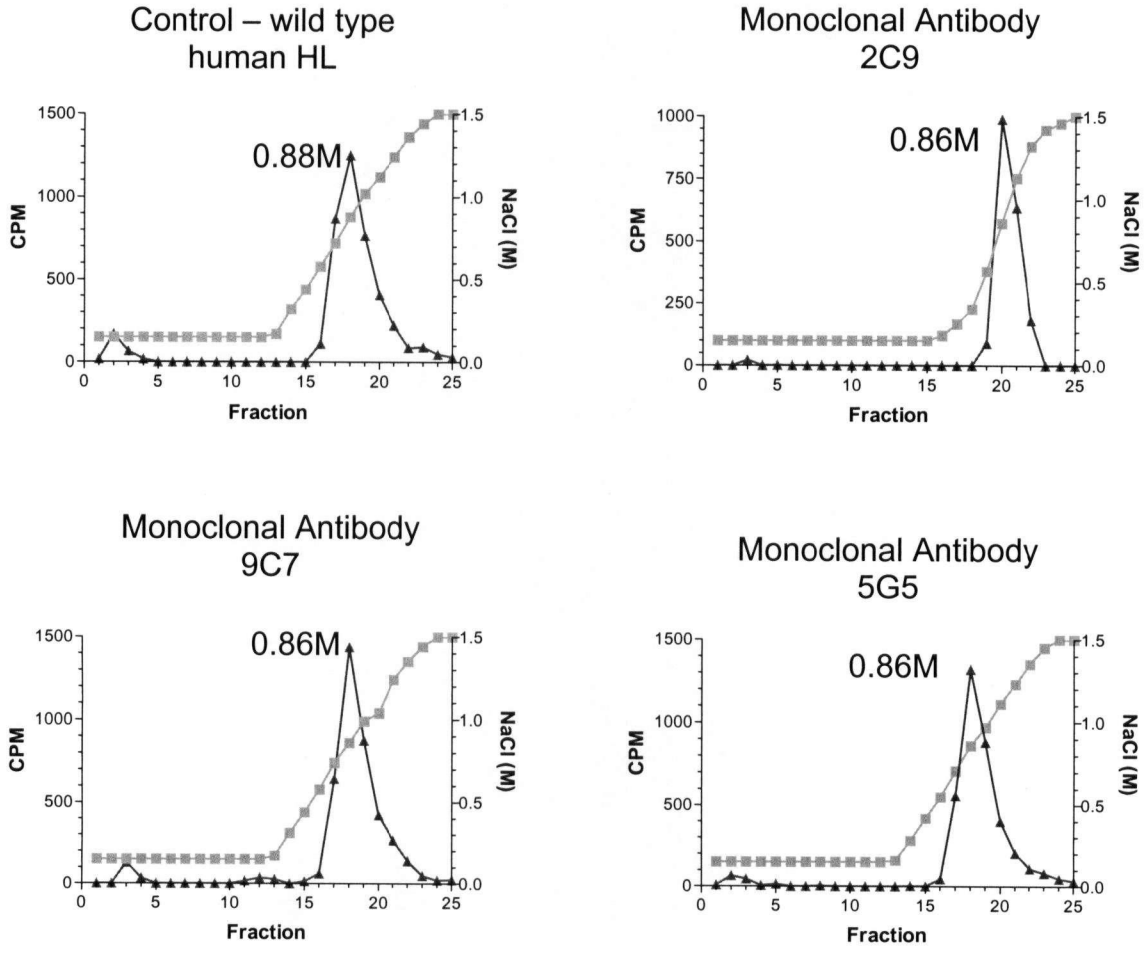
Figure 18. Competitive Binding Results. *Peptides were incubated wit HL-secreting Chinese Hamster ovary cells at a 100:1 molar ratio overnight and HL displaced into the culture media was measured by enzyme activity using a triolein substrate. The amount of HL measured in the media is given as CPM for each sample.*



3.4 Antibody Inhibition Studies

None of the monoclonal antibodies raised against the synthetic peptide corresponding to the 452-471 region of human HL were able to inhibit the heparin binding of wild type HL ***Figure 19***. Positive control human HL from heparin stimulated CHO-HL cell media eluted from the heparin-Sepharose column at a salt concentration of 0.88M NaCl. This is consistent with the heparin affinity of monomeric wild type human HL determined in other studies [168,169,173].

Figure 19. Antibody Inhibition Results*. A panel of monoclonal antibodies raised against peptide 452-471 were assessed for their ability to inhibit the heparin binding of wild type human HL. (■) indicate points on the NaCl gradient curve and (▲) represent HL activity of each fraction as measured using a triolein substrate. The elution point of HL is given on each graph.*



4 DISCUSSION

All 5 modeling programs identified residues K294, K295, R297, R306, R310, K312, K314, R315, and K334 as part of a possible linear heparin binding domain of HL ***Table 3*** and ***Table 4***. Not only are these residues gathered in an area displaying the largest positive charge density as calculate by Swiss Viewer software ***Figure 11***, but this area is also rich in consensus heparin binding sequences ***Table 2***. These data together seem to implicate that these residues may in fact play a role in heparin binding *in vivo.* However, since all models were based on a template of a similar but different protein, the accuracy of the models cannot be certain. Additionally, all models lacked distal COOH terminal residues and sometimes other interspersed omissions increasing the possibility of omission and alignment errors greatly affecting the tertiary structure of the model. Additionally, should the missing residues alter the tertiary structure of HL significantly, the appearance of identified residues within a possible linear heparin binding could be quite different. The missing residues may also change the EDM. Furthermore, visualization of amino acids was done by eye and thus the scoring of candidate residues was subject to human error.

Heparin-Sepharose affinity chromatography of synthetic peptides was conducted in the absence and presence of TFE. Tertiary structure is particularly important in determining epitope interactions and the use of peptides may be limited for this reason, however, the use of synthetic peptides may help determine regions within such an epitope. Based on our results however, the heparin affinity of peptides 355-377 and 419-442 were not affected by the presence of TFE with both displaying no heparin affinity with or without the secondary structure inducer ***Figure 13, 14*** and ***Table 5***. The secondary and tertiary structure of HL are unknown, and the synthetic peptides used are likely too short to form β sheets, therefore the relevance of the secondary structure induction is uncertain.

The heparin-Sepharose affinity chromatography results suggest that regions 355-377 and 419-442 of the HL domain are not sections of a discontinuous HL heparin-binding domain. However, both of these peptides contain an internal proline, a bulky amino acid and a known α helix breaker. As such, these peptides may be unable to form a secondary structure in the presence of TFE and the inability of these peptides to bind to the heparin-Sepharose column in the presence of TFE may not be due to a physiological lack of affinity. However, the results in the absence of TFE, where the internal proline may not be relevant, are negative for both peptides and it seems likely that these regions do not contribute to a discontinuous heparin-binding domain.

Conversely, peptide 304-323 has moderate heparin affinity for the heparin-Sepharose column that was not altered by the presence of TFE ***Figure 13, 14*** and ***Table 5***. This may be because the peptide assumes a secondary structure in the absence of the inducer or conversely because secondary structure is irrelevant. Interestingly, the 304-323 peptide also contains an internal proline that does not seem to affect the peptide's heparin affinity either in the presence or absence of TFE. This strengthens the assertion that peptides 355-377 and 419-442 lack

heparin binding properties and that these regions do not contribute to a discontinuous heparin-binding domain.

The significance of the distal portion of the carboxyl terminus appears low if the focus is placed on the longer peptide 452-471 as it appears to have no heparin binding with our without TFE ***Figure 13, 14*** and ***Table 5***. However, this peptide contains an NH_2 terminal cysteine and an internal proline residue. The cysteine was included in the peptide sequence to facilitate a coupling reaction to a carrier protein and the subsequent complex was used to generate the monoclonal antibodies. Cysteine is known to stabilize α helices and it seems unlikely to interfere with the induction of peptide 452-471 into a secondary structure. However, the exact affect of cysteine on the heparin binding ability of peptide 452-472 is unclear. Although the proline in peptide 304-323 did not appear to interfere with its heparin binding, proline 455 may have hindered the ability of peptide 452-471 to form a secondary structure in the presence of TFE. The 452-471 region in the natural enzyme may be stabilized by surrounding regions and form a secondary and/or tertiary structure capable of binding heparan sulfate proteoglycans *in vivo*. However, given that peptide 304-323 contained an internal proline and displayed heparin affinity, it seems unlikely that peptide 452-471 did not show heparin affinity solely due to an internal proline.

The results of peptide 455-471 are enigmatic ***Figure 13, 14*** and ***Table 5***. The two elution fractions that occur with TFE may be the result of a chemical reaction between the peptide and TFE that results in the presence of two different peptide species, or the incomplete conversion of the peptide to a secondary structure. Notably, peptide 455-471 contains an internal cysteine residue. An internal cysteine residue of a fibroblast growth factor-1 synthetic peptide was found to result in the dimerization of synthetic peptide doubling the species heparin affinity [258]. This may have occurred with peptide 455-471. Affinity elutions carried out later to the original elutions were completed in order to isolate the two fractions of peptide for subsequent mass spectrometry (MS). MS can determine the exact identity of the molecular species and was to be used to determine if covalent interaction of cysteine residues resulted in dimerization of peptide. However, the attainment of two peaks with peptide 455-471 could not be reproduced. Peptide 455-471 is also missing the three NH_2 terminal amino acids of 452-471 and contains an NH_2 terminal proline residue. Although proline is a helix breaker, it can be accommodated as the terminal residue in an α helix. *In vivo* however, proline 455 would not be an NH_2 terminal residue and the functional significance of the heparin affinity of peptide 455-471 is not certain. Further studies are required to elucidate the role of this region in heparin affinity.

The results of heparin-Sepharose affinity chromatography of the mutant 304-323 peptide may indicate R306 and R321 are not as vital as R310, K312, K314, and R315 to the heparin binding of peptide 304-323 ***Figure 16, 17*** and ***Table 6***. On the other hand, the differing results may be because mt304-323a and mt304-323d had only one mutation each whereas mt304-323b and mt304-323c had two mutations each ***Figure 15***. The results of the mutant peptides may indicate that basic amino acids contribute in an additive fashion to heparin affinity, and that this affinity is only partially negated by the mutation of one of the basic residues but is wholly negated

by the mutation of two or more basic residues. The affinity elutions of the HPG modified peptide may indicate that some or all of R306, R310, R315, and R321 are essential in the heparin binding of this peptide or again that basic amino acids contribute in an additive fashion to heparin binding. It is also interesting to note that while R321 was not implicated as an important residue by homology modeling a linear binding domain, its mutation resulted in a greater reduction of heparin binding than the R306 mutation as determined by the area under the peak displaying heparin affinity.

On the other hand, the failure of the synthetic peptides to displace HL from CHO-HL cells ***Figure 18*** may be the result of physiological irrelevance of the regions represented or because the peptides were too short or lacked the appropriate tertiary structure to effectively compete with native HL. The differences between heparin and heparan sulfate may also be responsible for the lack of peptide binding. Heparin, as is present in the column, is highly sulfated and contains numerous adjacent IdoA containing disaccharides. Thus, heparin is expected to react maximally with cationic peptide species. Heparan sulfate on the other hand, contains relatively few neighboring IdoA containing disaccharides, and the sulfated disaccharides in heparan sulfate tend to occur in blocks separated by poorly sulfated sections. As such, heparan sulfate is expected to have an intermediate ability to interact with cationic peptide species, and may require longer segments and/or more positive charge density to bind a peptide species.

Although none of the peptides competed with native HL for HSPG binding the 304-323 and possible the 452-471 regions may be physiologically significant. Given that all 5 peptides contained 4-6 basic amino acids but only two displayed affinity for the heparin-Sepharose column, indicates that there is a specific affinity existing between the reacting species and that the affinity is not due to non-specific charge density interactions. It is the opinion of this investigator that the affinity of peptide 304-323 and possibly 455-471 to a heparin-Sepharose matrix holds some relevance.

Monoclonal antibodies were also studied for their ability to inhibit wild type human HL. Peptide 452-471 corresponded to a region with HL containing many consensus sequences and lies within a region previously described as containing the HL heparin binding domain [173]. Since this region could not be modeled with any of the homology algorithms, it was decided to raise a panel of monoclonal antibodies raised against this region and evaluate their ability to interfere with wild type human HL to bind a heparin-Sepharose column. However, none of the generated antibodies were able to bind to wild type HL via a western blot (data not shown). Likewise, none of the antibodies inhibited wild type HL-heparin binding ***Figure 19***. Since the antibodies generated were unable to bind wild type human HL, it is likely that their failure to inhibit the heparin binding of HL is the result of an inability to bind HL and not insignificance of the corresponding sequence of HL.

Overall, our study indicates that residues K294, K295, R297 – located in the distal NH2 domain - and R306, R310, K312, K314, R315 – located in the proximal COOH domain - are important in the heparin binding of human HL. *Sendak et al. 2000* [259] similarly determined that basic residues in rat HL corresponding to K294, K295, R297 in human HL were essential for the heparin binding of rat HL. It is intriguing to note that residues 294-315 come together to form a trough or cleft

Figure 20 in the HL tertiary structure on all 5 modeling algorithms used. Computer modeling of vitronectin by *Cardin and Weintraub 1989* [209] suggested the vitronectin heparin binding domain formed a hydrophilic pocket that enveloped a heparin octasaccharide. In light of these results, *Cardin and Weintraub* suggest that multiple regions representing a discontinuous heparin binding domain may be brought together by secondary structure folding to form a cleft for heparinoid binding. Similarly, *Busby et al. 1995* [260] demonstrated that 6 positively charge amino acids that are remote in the sequence of fibronectin module III-13 are brought together on one side of the protein to form a 'cationic cradle' into which the heparin molecule is proposed to fit. It is interesting to speculate that residues 294-315 may form the same sort of cationic cradle for heparin binding ***Figure 20***.

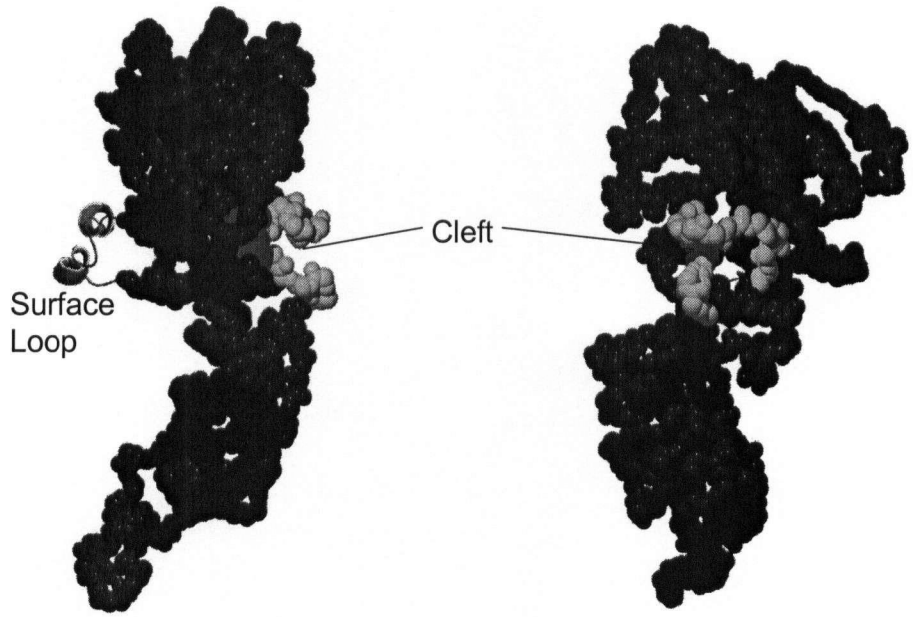


Figure 20. Model of Proposed Heparin Binding Cleft*. Space filled structure of HL with residues 294-315 displayed orange. The surface loop is displayed in secondary structure (α helix) and green. The model on the right is identical to that on the left but is rotated 90° to left.*

Though the relative contribution of histidine residues was not evaluated in this study, some studies have inferred a role of histidine residues in the heparin binding of other peptide species [261,262]. Even though peptide 304-323 contained only one histidine and peptide 455-471 contained none, experiments designed to assess the relative contribution of histidine may shed light on the heparin binding of human HL.

5 FUTURE DIRECTIONS

Certainly site directed mutagenesis of HL in the region of 304-323 and possibly 452-471 could yield important insights into the importance of individual residues with respect to determining a HL heparin binding domain. Creating a panel of CHO cell lines expressing HL with point mutations of: R306, R310, K312, K314, R315, R321, R454, K459, K463, K467, K469; and testing endogenous HL activity in culture media would give an idea of the importance of these residues to the ability of HL to bind the heparan sulfate proteoglycans of CHO cells. Additionally, an *in vitro* solid phase binding assay might yield insights into the involvement of the other peptides in heparin binding. Heparin-Sepharose affinity chromatography of synthetic peptides representing histidine rich regions of HL, carried out at a pH sufficient to ensure protonation of Histidine residues, would allow an initial evaluation of their relative contribution to HL heparin binding.

Importantly, little research has been conducted to determine the contribution of different heparinoid species to any particular protein interaction. HSPG of different cells can vary significantly, and may result in differential protein binding and interaction. More research in this area is needed.

HL has various functions in normal lipid physiology, from participating in the recyling of HDL in reverse cholesterol transport, to transforming IDL into atherogenic LDL particles, to facilitating remnant particle catabolism. HL is found in active form primarily bound to cell surfaces through an association with HSPG and evidence points to a requirment for HSPG for many of these processes. It is likely that the interaction between HL and HSPG is a critical step facilitating HL-mediated lipoprotein metabolism. As such, the delineation of regions within HL responsible for heparin binding could begin to clarify HL mechanisms of action in lipid metabolism.

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