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Molecular Characterization of Heparin Binding Proteins in Triglyceride-rich Lipoprotein Catabolism

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Molecular Characterization of Heparin Binding Proteins in Triglyceride-rich Lipoprotein Catabolism

A dissertation submitted in partial satisfaction of the requirements of the degree Doctor of Philosophy in Biomedical Sciences by

Jon Christopher Gonzales

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Professor Hudson Freeze
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Professor Joseph Witztum

2013
The dissertation of Jon Christopher Gonzales is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2013
DEDICATION

I dedicate this dissertation to my foundation in life: my family.

To my parents, for their love, guidance, and unwavering support. Words cannot express how much you’ve done for me.

To Melis, who has always set the bar impossibly high, but also provides the loving encouragement to strive for that.

To Dave, my brother and best friend, whom I’ve tried to emulate since the day I was born.

To Wayne, Megan, Bella, and Leo, for the love and joy you bring to the family.

And lastly,

To my Stephanie, whose smile and love has brought so much happiness to my life.
EPIGRAPH

“Clear eyes. Full hearts.”

-Coach Eric Taylor

*Friday Night Lights.*
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LIST OF ABBREVIATIONS

AlbCre  Cre transgene driven by rat albumin promoter
APO/Apo  apolipoprotein
APOE/ApoE  apolipoprotein E
APOB/ApoB  apolipoprotein B
APOA/ApoA  apolipoprotein A
APOC/ApoC  apolipoprotein C
APOAV/ApoAV  apolipoprotein AV
AUC  area under the curve
BAEC  bovine aortic endothelial cell
CHO  Chinese hamster ovary
Col18  collagen XVIII
Ext1  exostosin-1
FPLC  fast-phase liquid chromatography
GAG  glycosaminoglycan
GlcA  D-glucuronic acid
GlcNAc  N-acetyl-D-glucosamine
GPIHBP1  glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1
HDL  high density lipoprotein
HL  hepatic lipase
HS  heparan sulfate
Hs2st  uronyl 2-O-sulfotransferase
HSPG  heparan sulfate proteoglycan
IDL  intermediate density lipoprotein
IdoA  L-iduronic acid
LDL  low density lipoprotein
LDLR/Ldlr  LDL receptor
LPL/Lpl  lipoprotein lipase
LRP1/Lrp1  LDL receptor-related protein
NDST/Ndst  N-deacetylase N-sulfotransferase
OST/Ost  O-sulfotransferase
SDC1/Sdc1  syndecan-1
Tie2Cre  Cre transgene driven by the Tie2 promoter
TRL  triglyceride-rich lipoprotein
TC  total cholesterol
TG  triglyceride
VLDL  very low density lipoprotein
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in the liver increases glucose production and is activated during insulin-
ABSTRACT OF THE DISSERTATION

Molecular Characterization of Heparin Binding Proteins in Triglyceride-rich Lipoprotein Catabolism

by

Jon Christopher Gonzales
Doctor of Philosophy in Biomedical Sciences
University of California, San Diego, 2013
Professor Jeffrey D. Esko, Chair

Heparan sulfate proteoglycans mediate multiple aspects of triglyceride-rich lipoprotein metabolism through non-covalent interactions with several lipases and apolipoproteins. In the circulation, lipoprotein lipase bound to the luminal side of the microvascular endothelium catalyzes the lipolysis of triglyceride-rich lipoproteins, resulting in lipoprotein remnants. In the liver, remnant lipoproteins are cleared from the circulation by three major endocytic clearance receptors, including the heparan sulfate proteoglycan syndecan-1. This dissertation describes the identification of the lipoprotein ligands responsible for heparan sulfate mediated clearance and studies suggesting a role for endothelial heparan sulfate proteoglycans in lipolysis. Chapter 1 provides background information on the structure and function of heparan sulfate, its putative role in lipolysis,
and its participation in the clearance of triglyceride-rich lipoprotein remnants. The protein components of triglyceride-rich lipoproteins that bind to heparan sulfate are described. Chapter 2 provides evidence that led to the identification of the physiologic proteins responsible for lipoprotein binding to hepatic heparan sulfate proteoglycans. Chapter 3 describes studies of several mutant cell lines and mice that suggest a role for endothelial heparan sulfate proteoglycans in lipolysis. Chapter 4 presents on-going and future studies and the relevance of these findings to human disease.
Chapter 1

Heparin Binding Proteins and Triglyceride-rich Lipoprotein Catabolism

1.1 Summary

Triglyceride-rich lipoproteins (TRLs) are large buoyant particles that transport hydrophobic triglycerides and cholesterol esters in the blood. TRLs in the circulation undergo lipolytic processing primarily by lipoprotein lipase (LPL) immobilized on the capillary endothelial surface, resulting in triglyceride hydrolysis and the release of free fatty acids for energy production or storage in the surrounding tissue. Lipolysis of TRLs produces remnant lipoproteins that can be cleared from the circulation via one of three major hepatic endocytic receptors, including the heparan sulfate proteoglycan (HSPG) syndecan-1. The goal of this thesis is study how endothelial and hepatic HSPGs mediate the lipolysis and clearance of circulating TRLs through interactions with lipases and apolipoproteins. This introductory chapter will describe the structural components of TRLs that facilitate the transport, interaction, and clearance of the lipoprotein particles and the role of heparan sulfate proteoglycans in this process.

1.2 Hypertriglyceridemia

Recent clinical studies have indicated that hypertriglyceridemia, characterized by the accumulation of plasma TRLs, is an independent risk factor for cardiovascular disease (1, 2). Patients with hypertriglyceridemia have an increased risk of atherosclerosis and coronary artery disease. Hypertriglyceridemia is prevalent among the United States and Western countries, affecting as much as 10-20% of the population. Hypertriglyceridemia can result from genetic deficiencies of receptors, apolipoproteins,
chaperone proteins, and lipases. Poor diet, chronic alcohol consumption, diabetes mellitus, and other environmental factors also contribute to hypertriglyceridemia (3). Thus, the etiology of this disease is complex and much effort has been focused on understanding the synthesis and clearance of TRLs.

1.3 Triglyceride-rich lipoprotein metabolism

Lipoproteins transport hydrophobic lipids through the circulation for distribution and use throughout the body. These particles contain a hydrophobic core consisting of triglycerides and cholesterol esters, surrounded by a monolayer of phospholipid, unesterified cholesterol, and apolipoproteins (4). Lipoproteins are separated into several classes based on their density and compositional differences (Table 1-1). In the plasma, the largest, most buoyant (density less than 1.006 g/ml) particles contain the least amount of protein and the greatest amount of lipid, and are classified as triglyceride-rich lipoproteins.
Table 1-1. Major classes of plasma lipoproteins (adapted from (4, 5)). * TRL particle. †Values given are expressed as the percentage of total mass. (TG: triglyceride; Chol: Cholesterol)

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density (g/mL)</th>
<th>Diameter (nm)</th>
<th>% Protein Content†</th>
<th>% TG‡</th>
<th>% Chol‡</th>
<th>Apolipoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron*</td>
<td>&lt; 0.96</td>
<td>75 - 1200</td>
<td>1 - 2</td>
<td>85</td>
<td>5</td>
<td>B48, AI, AII, AIV, AV, CI, CII, CIII, E</td>
</tr>
<tr>
<td>VLDL*</td>
<td>&lt; 0.96 - 1.006</td>
<td>30 - 80</td>
<td>6 - 10</td>
<td>55</td>
<td>20</td>
<td>B100, AI, AII, AV, CI, CII, CIII, E</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006 - 1.019</td>
<td>25 - 35</td>
<td>10 - 12</td>
<td>25 - 30</td>
<td>35</td>
<td>B100, E</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019 - 1.063</td>
<td>18 - 25</td>
<td>20</td>
<td>5 - 10</td>
<td>60</td>
<td>B100</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063 - 1.210</td>
<td>5 - 12</td>
<td>45 - 55</td>
<td>5</td>
<td>20 - 40</td>
<td>AI, AII, AIV, AV, CI, CIII, E</td>
</tr>
</tbody>
</table>

Triglyceride-rich lipoproteins can be derived from dietary lipids or synthesized de novo by the liver. In enterocytes, dietary triglycerides are packaged into large buoyant chylomicrons that contain the truncated form of apolipoprotein B (apoB48) (4). These nascent chylomicrons are secreted and enter the circulation via the thoracic duct. After secretion, nascent chylomicrons acquire apolipoproteins E (apoE), CII (apoCII) and other apolipoproteins from circulating HDL, and become mature chylomicrons. In hepatocytes, endogenous fats are packaged into lipoprotein particles that have the full-length form of apolipoprotein B (apoB100) in humans, and either apoB48 or apoB100 in rodents, and are classified as very-low-density lipoproteins (VLDL, also of density <1.0063 g/ml). Nascent VLDL also contain small amounts of apoE and apolipoproteins C (CI-CIII), but acquire additional apoCII, apoE, and other apolipoproteins via HDL after secretion into
the circulation, becoming mature VLDL. Collectively, the mature chylomicrons and VLDL are known as triglyceride-rich lipoproteins (TRLs).

In the circulation, TRL particles encounter LPL bound to the surface of capillary endothelial cells and undergo lipolytic processing (6). LPL hydrolyzes the triglycerides in the core of the lipoproteins, releasing free fatty acids that can be used for energy production or storage. Depletion of triglycerides from TRLs results in smaller particles and the loss of apoCII and other apolipoproteins, and these particles are classified as chylomicron remnants and in the case of VLDL, intermediate-density lipoproteins (IDL, density 1.006-1.019 g/ml) (4). Remnant lipoproteins are small enough to enter the fenestrated endothelium of the liver and into the Space of Disse, where the particles can become enriched in apoE, undergo further lipolytic processing by hepatic lipase (HL), and cleared from circulation by several endocytic receptors (7).

The dominant hepatic endocytic receptors for TRL remnants include the LDL receptor (LDLR), the LDLR-related protein 1 (LRP1), and heparan sulfate proteoglycans, most notably syndecan-1 (8, 9). Mice lacking LDLR (ldlr^-/-), or humans with inactivating mutations in the LDLR gene, exhibit modest hypertriglyceridemia, suggesting that LDLR is responsible for a portion of TRL clearance (10-12). Hepatic inactivation of Lrp1 does not cause hypertriglyceridemia, unless compounded with ldlr-deficiency, which results in further accumulation of remnant particles (13). Systemic deletion of the major proteoglycan in the liver, syndecan-1, as well as hepatic inactivation of heparan sulfate biosynthetic enzymes, also result in moderate hypertriglyceridemia due to impaired TRL remnant clearance (14-16). Based on these genetic studies, the current model is that each of these endocytic receptors participates in the clearance of TRL remnants. Each receptor
facilitates clearance through binding of apolipoprotein ligands found on the surface of TRLs. The focus of the majority of this chapter will be on the structural components of TRLs that can mediated interactions with cell surface HSPGs.

Table 1-2. Characteristics of major apolipoproteins (adapted from (4)).

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Molecular Weight (KDa)</th>
<th>Amino Acids</th>
<th>Chromosome</th>
<th>Plasma Concentration (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AI</td>
<td>29</td>
<td>243</td>
<td>11</td>
<td>130</td>
</tr>
<tr>
<td>AII</td>
<td>17</td>
<td>77</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>AIV</td>
<td>45</td>
<td>376</td>
<td>11</td>
<td>40</td>
</tr>
<tr>
<td>AV</td>
<td>39</td>
<td>343</td>
<td>11</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>B100</td>
<td>510</td>
<td>4536</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>B48</td>
<td>240</td>
<td>2152</td>
<td>2</td>
<td>Variable</td>
</tr>
<tr>
<td>CI</td>
<td>6.6</td>
<td>57</td>
<td>19</td>
<td>6</td>
</tr>
<tr>
<td>CII</td>
<td>8.9</td>
<td>79</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>CIII</td>
<td>8.8</td>
<td>79</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>E</td>
<td>34</td>
<td>299</td>
<td>19</td>
<td>5</td>
</tr>
</tbody>
</table>

1.4 Heparan sulfate proteoglycans: structure and synthesis

Heparan sulfate proteoglycans consist of a protein core with one or more covalently attached heparan sulfate, a type of glycosaminoglycan chain (17). Depending on the core protein, the HSPGs can be found at the cell surface, proteolytically shed into the extracellular matrix, secreted, or in secretory vesicles (18). Plasma membrane-associated HSPGs consist of the syndecans 1-4, the glycosylphosphatidylinositol anchored glypicans 1-6, the cell surface adhesion receptor CD44v3, betaglycan, a member of the transforming growth factor beta receptor family (TGFβ Receptor III), and neuropilin-1, a co-receptor for vascular endothelial growth factor. The transmembrane core proteins (syndecans, CD44v3, and betaglycan) can be shed from the cell surface by proteolytic cleavage. The secreted, extracellular matrix associated HSPGs include perlecan, agrin,
and collagen type XVIII. Serglycin is found in secretory granules of hematopoietic cells, most notably in mast cells.

Biosynthesis of heparan sulfate occurs in the Golgi and depends on the coordination of multiple enzymes. Assembly is initiated with the assembly of a tetrasaccharide linker consisting of a glucuronic acid-β1,3-galactose-β1,3-galactose-β1,4-xylose attached to specific serine residues in proteoglycan core proteins (19). After addition of the first N-acetyl-D-glucosamine (GlcNAc) unit, by exostosin-like protein-3 (ExtL3), the glycosyltransferases exostosin 1 and 2 (Ext1/Ext2) polymerize disaccharide units consisting of alternating N-acetyl-D-glucosamine-α1-4 (GlcNAc) and D-glucuronic-β1-4 acid. During polymerization, a group of modifying enzymes act on the nascent sugar chain. The bifunctional GlcNAc N-deacetylase/N-sulfotransferase (Ndst) family of four enzymes removes acetyl groups from a subset of the GlcNAc residues and subsequently adds sulfates to the free amino groups. A C-5 epimerase then converts adjacent D-glucuronic acid units to L-iduronic acid, which can then be sulfated at the C2 position by a 2-O-sulfotransferase (Hs2st). The final modifications come from other O-sulfotransferase families that can add sulfate groups to the C6 (Hs6st1-3) or C3 (Hs3st1-6) of glucosamine units. Additionally, post-synthetic modifications can occur at the cell surface by secreted heparan sulfate processing enzymes, glucosamine-6-O-endosulfatas 1 and 2 (Sulf1, Sulf2) (20, 21).

Sulfation and epimerization occur in a preferred order and in clusters along the heparan sulfate chain, creating variably modified sections. These domains can provide binding sites for protein ligands through electrostatic interactions between the negatively charged sulfates and carboxyl groups on the polysaccharide and positively charged
domains on protein ligands. HS-protein interactions often occur with great specificity and affinity. Factors that bind heparan sulfate include growth factors and morphogens, enzymes, chemokines, and numerous other proteins. Different cell types vary in expression levels of the HSPG core proteins and biosynthetic enzymes, which result in great heterogeneity of HSPG composition across tissue and cell types (18).

Figure 1-1. Heparan sulfate biosynthesis (adapted from (22).

1.5 Heparin binding proteins

Most of the protein ligands that bind to HSPGs in vivo were originally identified by their capacity to bind to heparin, a highly sulfated form of heparan sulfate, and were classified as “heparin-binding proteins” (23). Numerous heparin-binding proteins have been identified and have diverse roles as enzymes, adhesion proteins, chemoattractants and repellents, growth factors, ligands for receptors, and as morphogens (24).

Contrary to other families of glycan binding proteins such as lectins and hyaladherins, heparin-binding proteins do not contain a conserved “carbohydrate recognition domains” (CRDs) that can be identified through protein sequencing or
structural analysis (23). This is mainly due to the variable positioning of sulfate groups along heparan sulfate chains that can interact with various arrangements of amino acids in the binding site of the ligand (25). However, there are some common features among heparin-binding proteins. First, the binding sites within the proteins always contain the basic amino acids lysine and arginine, which presumably interact with the negatively charged sulfate and carboxyl groups through electrostatic interactions. Typically, the three-dimensional structure of the heparin binding protein positions the basic residues into clusters, often creating a cleft that interacts with multiple sugar residues on the heparan sulfate chain (23). Another common feature is that heparin-binding proteins require only small segments of the heparan sulfate chain (~4-10 sugar residues) for binding to occur (25). These binding segments often consist of N-sulfated glucosamines, 2-O-sulfated iduronic acids, and one or more other sulfate modifications. Generally, the interactions between heparin-binding proteins and heparan sulfate are characterized by high-affinity binding and can occur either with great selectivity (e.g. antithrombin) or in an analog fashion, with increasing affinity depending on the degree of sulfation.

1.6 Hepatic heparan sulfate and lipoprotein clearance

Genetic mouse models with hepatocyte specific reduction in N-sulfation and 2-O-sulfation showed that hepatic heparan sulfate contributes to the binding and clearance of triglyceride-rich lipoproteins (14, 16). This was first demonstrated by genetic inactivation of the major enzyme responsible for N-sulfation, GlcNAc N-deacetylase/N-sulfotransferase-1 (Ndst1) in a liver-specific manner through crossbreeding mice bearing a floxed conditional allele (Ndst1) to mice expressing the bacteriophage Cre recombinase
under the hepatocyte-specific albumin promoter \((AlbCre)\) (14, 26). \(Ndst1^{Ef}\)AlbCre\(^+\) mice are viable and fertile in contrast to mice bearing a systemic deletion of \(Ndst1\), which results in perinatal lethality due to defects in lung, brain, and skeletal development (14, 27-29). Disaccharide analysis of hepatic heparan sulfate from \(Ndst1^{Ef}\)AlbCre\(^+\) mice demonstrated reduced N-sulfation (18 sulfates/100 disaccharides vs. 38 sulfates/100 disaccharides in wildtype) and 2-O-sulfation (4 sulfates/100 disaccharides vs. 17 sulfates/100 disaccharides in wildtype) and an overall decrease in sulfation by approximately 50%. However, 6-O-sulfation was unaffected (27 sulfates/100 disaccharides vs. 31 sulfates/100 disaccharides in wildtype). Importantly, careful analysis of \(Ndst1^{Ef}\)AlbCre\(^+\) mice found that mutant mice have a two-fold elevation in fasting plasma triglycerides, as compared to \(Ndst1^{Ef}\)AlbCre\(^-\) littermates, due to impaired clearance of TRL remnants (14). This effect is further exacerbated under postprandial conditions. Crossbreeding \(Ndst1^{Ef}\)AlbCre\(^+\) mice with \(Ldlr^{/-}\) mice resulted in much greater accumulation of TRLs in the compound mutants \((Ldlr^{/-}\) \(Ndst1^{Ef}\)AlbCre\(^+\)) compared to their single mutant counterparts, demonstrating that HSPGs and LDLR are independent, parallel endocytic receptors for TRL remnants.

Studies with \(Ndst1^{Ef}\)AlbCre\(^+\) mice demonstrated that TRL uptake in the liver is dependent on the degree of sulfation of the heparan sulfate chains. However, to determine the contribution of specific sulfate residues to TRL binding, such as 2-O-sulfated iduronic acid and 6-O-sulfated glucosamine, mouse models were generated with liver specific inactivation of uronyl-2-O-sulfotransferase \((Hs2st^{Ef}\)AlbCre\(^+\)) or glucosaminyl 6-O-sulfotransferase-1 \((Hs6st1^{Ef}\)AlbCre\(^+\)) (16). Interestingly, \(Hs6st1^{Ef}\)AlbCre\(^+\) mice did not exhibit any changes in plasma triglycerides, whereas
Hs2st^{Eff} AlbCre^{+} mice accumulated plasma triglycerides to a similar extent as Ndst1^{Eff} AlbCre^{+} mice and also display similarly impaired postprandial TRL clearance. Analysis of hepatic heparan sulfate in Hs2st^{Eff} AlbCre^{+} mice demonstrated that 2-O-sulfation was dramatically reduced (4 sulfates/100 disaccharides vs. 21 sulfates/100 disaccharides in wildtype), while surprisingly, N-sulfation and 6-O-sulfation were both increased (69 N-sulfates /100 disaccharides and 48 6-O-sulfates /100 disaccharides vs. 48 N-sulfates/100 disaccharides and 19 6-O-sulfates/100 disaccharides in wildtype mice).

Table 1-3. Distribution of sulfate groups on hepatocytes from wildtype and heparan sulfate deficient mice (from (16)).

<table>
<thead>
<tr>
<th>Sulfate groups per 100 disaccharides</th>
<th>Ndst1^{Eff} Albcre^{-}</th>
<th>Ndst1^{Eff} Albcre^{+}</th>
<th>Hs2st^{Eff} Albcre^{+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Sulfate</td>
<td>38</td>
<td>18</td>
<td>69</td>
</tr>
<tr>
<td>2-O-Sulfate</td>
<td>17</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>6-O-Sulfate</td>
<td>31</td>
<td>27</td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>49</td>
<td>121</td>
</tr>
</tbody>
</table>

Additionally, studies by Williams and colleagues demonstrate that impaired TRL clearance in a mouse model of type 2 diabetes was due to overexpression of hepatic Sulf2, and that reduction of Sulf2 synthesis restores TRL clearance (30, 31). Collectively, these studies have demonstrated that TRL clearance by HSPGs depends on the fine structure of the chains, and suggest ligand specificity in terms of TRL binding to heparan sulfate.

Recently, syndecan-1 was identified as the major proteoglycan that mediates the clearance of triglyceride-rich lipoproteins in vivo (15). Stanford et al. demonstrated that syndecan-1-deficient mice (Sdc1^{-/-}) accumulate plasma triglycerides to a similar extent as Ndst1^{Eff} AlbCre^{+} mice and have similarly impaired clearance of postprandial TRL remnants. The compound mutant Sdc1^{-/-}; Ndst1^{Eff} AlbCre^{+} did not have accentuated
accumulation of plasma triglycerides compared to the each single mutant. In vitro binding studies confirmed that hepatocytes isolated from Sdc1−/− mice were not able to bind human VLDL in a heparan-sulfate dependent manner. Further studies demonstrated that HSPG-mediated TRL binding and uptake in primary human hepatocytes also depends on syndecan-1 (32). Taken together, these results provide strong evidence that syndecan-1 is the major proteoglycan mediating TRL clearance by hepatocytes in mice and humans, and henceforth, will be used synonymously with HSPG-mediated clearance in the rest of this dissertation.

1.7 Apolipoprotein and lipase interactions with hepatic heparan sulfate

The liver-specific inactivation of heparan sulfate biosynthetic enzymes Ndst1 and Hs2st demonstrated that the arrangement of sulfate groups in heparan sulfate is important for mediating TRL clearance and suggested specific interaction between heparan sulfate chains and some component of the TRL particles. We could exclude lipids as potential binding moieties based on their neutral, acidic, or zwitterionic character and that the mutations in HSPGs only affected TRLs, not other classes of lipoproteins. Thus, it was reasonable to assume that Sdc-1 bound to one or more of the proteins found on the surface of TRLs. Many of the apolipoproteins associated with and lipases that act upon triglyceride-rich lipoproteins have been previously demonstrated to bind to heparin and/or HSPGs (Table 1-4). Each of these potential ligands will be discussed in further detail below.
Table 1-4. Apolipoproteins and lipases that bind heparin/heparan sulfate.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Binding Domain</th>
<th>Amino Acid sequence</th>
<th>HS Specificity</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE</td>
<td>N-terminal (136-147)</td>
<td>Arg-142, Lys-143, Arg-145, Lys-146, Arg-147</td>
<td>Sequences rich in GlcNS6S-IdoA2s</td>
<td>(33-36)</td>
</tr>
<tr>
<td>ApoB100</td>
<td>C-terminal (3359-3369)</td>
<td>Arg-3359, Arg-3362, Lys-3363, Arg-3364, Lys-3367</td>
<td>Unknown, but binds to heparin</td>
<td>(37)</td>
</tr>
<tr>
<td>ApoB48</td>
<td>N-terminal (84-94)</td>
<td>Lys-88, Lys-89, Lys-91</td>
<td></td>
<td>(38, 39)</td>
</tr>
<tr>
<td>ApoAV</td>
<td>C-terminal (186-227)</td>
<td>Arg-210, Lys-211, Lys-215, Lys-217</td>
<td>Unknown, but binds to heparin</td>
<td>(40, 41)</td>
</tr>
<tr>
<td></td>
<td>C-terminal (465-476)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPL</td>
<td>C-terminal (400-430)</td>
<td>Lys-321, Arg-405, Arg-407, Lys-409, Lys-416</td>
<td>Affinity for modestly sulfated sequences (6-10mers) and sequences rich in GlcNS6S-IdoA2S</td>
<td>(45, 46)</td>
</tr>
</tbody>
</table>

**Apolipoprotein E**

ApoE is one of the most abundant apolipoproteins in circulation (plasma concentrations ~ 4-7 mg/dl) and is a major determinant for receptor-mediated clearance of lipoproteins via LDLR and LRP1. ApoE can be found on most lipoproteins, including TRLs, IDL, and HDL. Expression of apoE occurs in many tissues, but the majority of apoE production occurs in the liver (>75%) and brain. ApoE is a 299 amino acid (34 kDa protein) consisting of two structural domains: the amino-terminal, receptor binding domain, and a carboxy-terminal lipid-binding domain that facilitates association of apoE into the lipid layer of lipoproteins.
Early on, apoE was shown to have a crucial role in lipid clearance and that its variants can contribute to cardiovascular disease in animals and humans (47). Intravenous infusion of apoE significantly reduced plasma lipoprotein clearance in cholesterol-fed rabbits (48, 49). Furthermore, \( \text{ApoE}^{-/-} \) mice exhibit impaired lipoprotein clearance, elevated plasma triglycerides and cholesterol, and extreme susceptibility to atherosclerosis (50). Innerarity et al. demonstrated that lipoproteins enriched for apoE have a very high affinity for LDLR, suggesting that apoE mediates TRL clearance through the LDL receptor (51). However, interbreeding of \( \text{ApoE}^{-/-} \) mice with \( \text{Ldlr}^{-/-} \) mice showed that plasma triglyceride levels were similar in \( \text{Ldlr}^{-/-},\text{ApoE}^{-/-} \) and \( \text{ApoE}^{-/-} \) mice, but higher than in \( \text{Ldlr}^{-/-} \) mice, suggesting a second TRL clearance receptor that is dependent on apoE (52). Subsequently, Beisiegel et al. identified LRP1 as another hepatic endocytic receptor that mediates clearance of TRL remnants strictly via apoE (53). While LRP1-mediated clearance is quite important when either LDLR or HSPG-mediated clearance is inactivated, the contribution of LRP1 to TRL clearance in a normal physiologic setting seems to be minimal (13).

Other data have suggested the importance of apoE for TRL clearance through syndecan-1. First, apoE was identified as a heparin binding protein and the critical basic residues important for heparin binding have been mapped (35, 54-56). Further, Ji et al. showed that addition of apoE to rabbit \( \beta \)-VLDLs resulted in enhanced binding and uptake via cell surface heparan sulfate proteoglycans, and Wilsie et al. showed that apoE-enrichment of human VLDL enhanced binding to syndecan-1 transfected cells (57, 58). ApoE is found at high concentration on the sinusoidal side of the basal membrane of hepatocytes, which led to the idea that apoE produced by hepatocytes might enrich
lipoproteins in the space of Disse and enhance remnant clearance through proteoglycans, an idea put forth by Mahley and coworkers known as the “secretion-capture” hypothesis (59-61). Despite the substantial indirect evidence suggesting a role for apoE in HSPG-mediated clearance, direct evidence is lacking.

**Apolipoprotein B**

ApoB is the critical structural component of TRLs and LDL. There are two forms of apoB, apoB48 and apoB100, which are derived from a single gene. ApoB48 is a truncated form of apoB100, and is a result of processing by RNA editing machinery in the intestine (mice and humans) and liver (mice only)(62). Full-length apoB100 is 4536 amino acids and a mass of ~550 kDa, whereas apoB48 consists of the N-terminal 2153 amino acids (48% of apoB100 residues) and is ~260 kDa. ApoB-deficiency in mice is embryonic lethal due to severe defects in development, but mice expressing a single isoform of apoB (apoB<sup>100/100</sup> “apoB100-only” and apoB<sup>48/48</sup> “apoB48-only” mice) are viable and fertile (63, 64).

ApoB plays a structural role in lipoprotein assembly and mediates interactions with endocytic receptors. ApoB100, but not apoB48, interacts with LDLR via basic residues found in the C-terminus. The interaction facilitates binding and clearance of LDL (and IDL) through LDLR (12). ApoB does not bind to LRP1, but apoB48- bearing particles bearing apoE bind and are cleared through LRP1 (65, 66).

Prior to the studies reported here, it was unknown if apoB mediates clearance through syndecan-1, but studies using radiolabeled heparin identified apoB48 and apoB100 as heparin-binding proteins (67). Other studies demonstrated that mouse apoB48-LDL and apoB100-LDL can bind equally well to heparin-Sepharose. The
heparin binding sites for apoB100 and apoB48 were characterized through affinity chromatography of peptide fragments on heparin-Sepharose and by transgenic expression of site-directed mutants of in mice (37-39, 68). Intriguingly, when apoB\textsuperscript{100/100} and apoB\textsuperscript{48/48} mice were bred to LDLR-deficient mice, fasting plasma triglyceride accumulated to a greater extent when apoB48 was lacking (i.e., in the ApoB100-only mice), suggesting that apoB48 particles are cleared more efficiently than apoB100 particles by endocytic receptors other than LDLR (65). Thus, it was hypothesized that apoB48 might mediate HSPG-dependent clearance of TRLs, although it was just as likely that the elevated triglycerides results from decreased clearance through LRP1.

**Apolipoprotein AV**

Rubin and colleagues identified apoAV in 2001 by comparative genome sequencing and described a significant association of plasma triglyceride levels and single nucleotide polymorphisms (SNPs) in the APOA5 locus in humans (69). They further demonstrated that ApoAV\textsuperscript{−/−} mice accumulate plasma triglycerides 4-fold, and transgenic mice overexpressing apoAV have significantly reduced plasma triglycerides. Subsequent studies in humans and mice found that deficiency in apoAV results in very large TRLs due to impaired lipolysis (70, 71). This accumulation of large TG-rich particles is thought to be due to diminished interactions with LPL and/or endothelial LPL receptors, such as GPIHBP1 and endothelial HSPGs (72, 73).

ApoAV is mainly produced and secreted by the liver (74). Plasma apoAV is found on TRLs and HDL, but the plasma concentration of apoAV is extremely low (20-500 ng/ml in humans) (75). Based on plasma concentrations, Merkel and colleagues have estimated that apoAV is found on 1 out of every 24 VLDL particles (76), thus in much
less abundance than the other major lipoproteins such as apoE (5-7 per particle) and apoB (1 per particle). Regardless, apoAV is a potent modulator of plasma triglycerides, suggesting it may act as an effector rather than a structural component.

ApoAV also has been suggested to promote clearance of TRLs by acting as a ligand for receptor-mediated endocytosis (71, 77). This hypothesis derives from the observation that the apoAV is made hepatocytes and apoAV can bind to one or more endocytic receptors. Nilsson et al. demonstrated through cell culture studies and surface plasmon resonance (SPR) that apoAV can bind to LRP1 and Sortilin, another member of the LDLR family (78). Subsequent studies found that apoAV readily binds to heparin and that the heparin-binding sites on apoAV correspond to sites responsible for binding to GPIHBP1 and LRP1 (40, 41). However, due to confounding lipolysis defects, in vivo studies have yet to demonstrate a role for apoAV in mediating TRL remnant clearance.

**Hepatic Lipase**

HL is a member of the extracellular lipase family that includes endothelial lipase (EL) and LPL (79). As its name suggests HL is produced by hepatocytes, and has triglyceride hydrolytic and phospholipase activity (80). HL is secreted by hepatocytes into the Space of Disse where it binds to the cell surface and to the sinusoidal endothelium of the liver (81). Humans lacking HL have hypertriglyceridemia and accumulate TRL remnants, but also have several other lipoprotein abnormalities (82). However, inactivation of HL in mice does not affect plasma triglycerides or clearance of exogenously introduced chylomicrons, but rather results in an increase in HDL cholesterol (83).
Nevertheless, a number of in vitro studies with isolated cells and in vivo studies in which HL was overexpressed suggest a role for HL in clearance. *In vitro* studies with hepatoma cells overexpressing HL found enhanced binding and uptake of TRLs (84). Diard et al. found that heat-inactivated HL accelerates the uptake of TRL remnants in rat hepatocytes and Dichek et al. found that transgenic expression of catalytically inactive HL increased TRL remnant clearance in a LDLR-independent manner (85, 86). Studies by Mahley and colleagues demonstrated that HSPGs are the important endocytic receptors mediating the clearance of HL-enriched lipoproteins (84, 87). Subsequent studies found that HL avidly binds to heparin and contains two distinct sites important for heparin binding, and is thus hypothesized to be tethered to HSPGs in the Space of Disse (43, 44). The liver heparan sulfate-deficient mice (*Ndst1<sup>AlbCre</sup>*<sup>+</sup>) have elevated plasma HL compared to wildtype mice, further supporting the role of HSPGs in binding and sequestering of HL in the Space of Disse (14). Importantly, much of the evidence supporting HL as a ligand for HSPG-mediated clearance is from transgenic overexpression or HL-enriched models; the *in vivo* relevance of these observations has not been demonstrated.

*Lipoprotein Lipase*

LPL is the critical enzyme responsible for the lipolysis of triglyceride-rich lipoproteins in the peripheral circulation (6). Genetic ablation of LPL in mice results in severe hypertriglyceridemia (~15,000 mg/dl), reduced HDL, and neonatal death (88). Patients with homozygous LPL deficiency also have severe hypertriglyceridemia, with pronounced chylomicronemia, reduced plasma HDL, and episodes of pancreatitis (89).
The predominant site of action for LPL is at the endothelial surface of the microvasculature, where LPL will encounter circulating TRLs. Additionally, intravenous injection of artificial triglyceride emulsions and immunohistochemical analyses suggest that LPL is displaced from the surface of the endothelium during lipolysis and travels with the remnant lipoproteins into the space of Disse (90, 91). LPL readily interacts with heparin through a cluster of basic amino acids near the carboxy-terminus of the enzyme and thus was speculated to participate in TRL interactions with heparan sulfate (92, 93). In support of this hypothesis, *in vitro* studies demonstrated that LPL enrichment of chylomicrons enhanced heparan sulfate dependent binding to a human hepatoma cell line (94, 95). Merkel et al. found that transgenic expression of catalytically inactive LPL increases uptake of TRLs and Heeren et al. observed that clearance of TRLs isolated from patients with catalytically inactive LPL was increased by ~35% after enrichment with inactive LPL (96). Enrichment of TRLs and other lipoproteins with LPL clearly can provide additional capacity to interact with HSPGs, but the physiologic relevance of these observations with respect to hepatic clearance remains questionable and difficult to assess because of the impact of altering LPL on lipolysis.

Collectively, the various heparin-binding apolipoproteins and lipases have significant evidence suggesting their involvement in mediating TRL clearance through syndecan-1. To determine the relevant ligands, a thorough study requiring genetic crossbreeding of heparan sulfate-deficient mice with apolipoprotein deficient mice, as well as *in vitro* analyses to replicate a physiologic setting will be required to determine which ligand(s) mediate clearance. Chapter 2 provides the first genetic evidence in mice that both apoE and apoAV mediates binding to TRLs to syndecan-1 in mice and that
apoB and the lipases do not appear to participate in proteoglycan mediated clearance in the liver.

1.8 Endothelial heparan sulfate and lipolysis

LPL is found in most tissues in the body but is most abundant in the heart and skeletal muscle and adipose tissue (97). LPL is synthesized and secreted in its active dimeric conformation by parenchymal cells, such as myocytes and adipocytes. The enzyme then traverses the basement membrane and is transported across the endothelial cells to its site of action on the luminal side of the endothelium.

The majority of LPL’s physiologic actions occur at the luminal surface of endothelial cells (4, 98), where lipolysis of circulating lipoproteins occurs, releasing free fatty acids that can be used for energy or storage. Early studies of LPL lipolysis demonstrated that LPL readily associates with heparin and intravenous injection of heparin would displace LPL from the surface of endothelial cells (99, 100). Due to the rapid and strong association of LPL with heparin, it was logically assumed that LPL resided on the surface of endothelial cells via heparan sulfate proteoglycans, and intravenous heparin competed with the heparan sulfate chains. Multiple in vitro studies found that the enzyme binds to endothelial cells in a heparan sulfate-dependent manner (101, 102). It was further shown that heparin and heparan sulfate stabilizes and preserves enzymatic activity of LPL (93, 103). Additional studies with affinity columns and purified oligosaccharides demonstrated specificity in the interactions between LPL and heparan sulfate (45, 104, 105). Thus, it was widely believed that LPL resides on the surface of endothelial cells bound to heparan sulfate proteoglycans.
Recent genetic studies from Young and colleagues demonstrated that the endothelial protein glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) is responsible for the transport of LPL across the endothelium and immobilization of LPL at the luminal surface (106, 107). GPIHBP1 deficient mice have severe defects in lipolysis, resulting in large TRL particles and hypertriglyceridemia, with plasma triglyceride levels greater than 3000 mg/dl. Importantly, GPIHBP1 expression is lost when primary cells are passaged in culture, thus most of the previous in vitro studies were done in the absence of GPIHBP1, possibly overemphasizing the role of HSPGs which are stably expressed on cultured endothelial cells. These findings have prompted a reexamination of the proposed role of HSPGs in the binding of LPL on the surface of endothelial cells (Chapter 3).

1.9 Conclusion

Hypertriglyceridemia, characterized by the accumulation of plasma TRLs, increases the risk of atherosclerosis and cardiovascular disease. HSPG interactions with lipases apolipoproteins are thought to be important mediators of TRL lipolysis and clearance. Recent genetic studies have highlighted hepatic HSPGs, most notably syndecan-1, as major endocytic receptors mediating clearance of TRLs from circulation. Evidence suggests that apolipoproteins and lipases on the surface of TRLs mediate binding to heparan sulfate, but the physiologic ligand(s) have not yet been conclusively identified. In the endothelium, HSPGs were long considered the primary receptors that immobilized LPL at the luminal surface of blood vessels. However, the recent discovery of GPIHBP1 demonstrates that LPL binding to endothelial heparan sulfate may not play
the role in peripheral lipolysis as originally suggested, but instead might facilitate coordinated action with apoAV and GPIHBP1 in docking and lipolysis of TRLs. It is the goal of this thesis to resolve the importance of the different apolipoproteins and lipases in TRL binding, lipolysis and clearance. An important outcome of these studies is the demonstration that HSPG receptors in the liver have an atheroprotective role in vivo.
1.10 Literature cited


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Chapter 2

Apolipoproteins E and AV Mediate Lipoprotein Clearance by Hepatic Proteoglycans

2.1 Summary

The heparan sulfate proteoglycan, syndecan-1, acts as a major receptor for triglyceride-rich lipoprotein (TRL) clearance in the liver. Here, we sought to identify the relevant apolipoproteins on TRLs that mediate binding to syndecan-1 and determine their clinical relevance. Evidence supporting apoE as a major determinant of binding arose from enrichment of apoE in TRLs from mice defective in hepatic heparan sulfate \((Ndst1^{f/f} AlbCre^+\)), decreased binding of \(ApoE^{-/-}\) TRLs to heparan sulfate proteoglycans on human hepatoma cells, and decreased clearance of \(ApoE^{-/-}\) TRLs in vivo. Evidence for a second ligand was suggested by the faster clearance of \(ApoE^{-/-}\) TRLs after injection into wild-type mice versus \(Ndst1^{f/f} AlbCre^+\) mice and elevated fasting and post-prandial plasma triglycerides in \(Ndst1^{f/f} AlbCre^+;ApoE^{-/-}\) mice compared to either single mutant. ApoAV emerged as a candidate based on a 6-fold enrichment of apoAV in TRLs accumulating in \(Ndst1^{f/f} AlbCre^+\) mice, decreased heparan sulfate-dependent binding of apoAV-deficient particles to hepatocytes, and decreased binding of TRLs to proteoglycans after depletion of apoAV or addition of anti-apoAV monoclonal antibody. Importantly, disruption of hepatic heparan sulfate-mediated clearance increased atherosclerosis by 2.25-fold. We conclude that clearance of TRLs by hepatic heparan sulfate proteoglycans is atheroprotective and mediated by multivalent binding to apoE and apoAV.
2.2 Introduction

Accumulation of plasma triglyceride-rich lipoproteins (TRLs) is now considered an independent risk factor for cardiovascular disease (1-4). TRLs consist of chylomicrons derived from dietary fats in the intestine, very low-density lipoproteins (VLDL) derived from de novo synthesized lipids in the liver, and remnant particles that arise from lipolysis of these lipoproteins in the peripheral circulation. TRLs vary in size dependent on their origin, but have similar low buoyant density ($\delta<1.006 \text{ g/ml}$) due to their high content of triglycerides and cholesteryl esters relative to protein. TRL-associated proteins include apoB-100 (VLDL) or apoB-48 (chylomicrons in humans, chylomicrons and VLDL in rodents), apoE, apoCI-III, and multiple less abundant apolipoproteins and lipases, including apolipoproteins AI, AII, AIV, AV, lipoprotein lipase (LPL), hepatic lipase (HL), and endothelial lipase. Many of the apolipoproteins have profound effects on lipoprotein metabolism by acting as structural proteins, as cofactors for activation of enzymes involved in lipolysis, or as ligands for receptor-mediated clearance of TRLs in the liver and peripheral tissues (5).

TRLs in the circulation undergo lipolytic processing primarily by LPL immobilized on the capillary endothelial surface by way of its receptor GPIHBP1, resulting in triglyceride hydrolysis and the release of free fatty acids for energy production or storage in the surrounding tissue (6-8). The remnant TRLs then undergo rapid clearance in the liver by receptors located on the basal membrane of hepatocytes facing the space of Disse. The dominant endocytic receptors in the liver for TRL remnants include the LDL receptor (LDLR), the LDLR-related protein 1 (LRP1), and heparan sulfate proteoglycans (HSPGs), most notably syndecan-1 (9, 10). LDLR
preferentially clears apoB-containing lipoproteins, thus mediating the removal of LDL and subclasses of TRLs containing apoB-100 or combinations of apoB-48 and apoE (11, 12). Consistent with these observations, mice lacking LDLR exhibit mild hypertriglyceridemia under fasting conditions and in the post-prandial state (13, 14). LRP1 shows overlapping specificity with LDLR, preferentially interacting with apoE-bearing lipoproteins. However, inactivation of LRP1 in hepatocytes does not cause hypertriglyceridemia presumably due to compensation by LDLR (15). Genetic evidence in mice demonstrates the importance of HSPGs in TRL clearance, in particular syndecan-1 (16-18). Syndecan-1 works in parallel to, but independently of, LDLR based on the compound effect of altering hepatic heparan sulfate and LDLR deficiency on plasma triglycerides (16).

Studies of mice bearing mutations in enzymes involved in heparan sulfate biosynthesis showed that TRL binding to syndecan-1 depends on specific subsets of sulfate groups on the heparan sulfate chains, which presumably facilitate interaction of syndecan-1 with protein ligands involved in lipoprotein clearance (16-20). Proteins associated with TRLs that can interact with heparan sulfate or heparin (a highly sulfated form of heparan sulfate) include apoB-48 and apoB-100 (21-24), apoE (25-30), apoAV (31), LPL and HL (32-35). Cell culture studies of model lipoproteins enriched with apoE, LPL or HL have demonstrated enhanced binding and uptake by HSPGs (36-45). Mutated lipases lacking enzymatic activity can also act as bridging molecules between lipoproteins and proteoglycans (46-49). Furthermore, all of these factors are manufactured in the liver and secreted into the space of Disse where they can potentially bind to heparan sulfate proteoglycans. These observations led Mahley and coworkers to
suggest that TRL clearance might occur through a “secretion-capture” hypothesis in which TRLs entering the space of Disse become enriched in apoE or lipases thus facilitating their binding to HSPGs (for recent reviews see (9, 10, 50, 51). In spite of all this information, the physiological protein ligand(s) responsible for TRL binding and uptake via HSPGs in vivo remain undefined.

In the current study, we sought to identify the relevant protein ligands on TRLs for HSPG-mediated clearance. We show that mutants containing undersulfated hepatic heparan sulfate accumulate TRLs enriched in apoE and apoAV. Direct evidence for a dominant role of these apolipoproteins was obtained by analysis of apolipoprotein mutants, depletion/reconstitution studies and competition with monoclonal antibodies using a novel lipoprotein-proteoglycan flotation assay. Our findings suggest that HSPGs clear a unique subset of particles enriched in both apoE and apoAV and support a model in which the heparan sulfate chains on syndecan-1 form multivalent contacts with both apoE and apoAV on the surface of TRLs. Furthermore, disruption of this clearance mechanism results in increased plasma triglycerides and a significant increase in the development of atherosclerosis in ApoE−/− mice, demonstrating for the first time that proteoglycan-mediated clearance of TRLs is atheroprotective.

2.3 Results

2.3.1 ApoE mediates TRL binding and clearance by heparan sulfate

To identify the proteins on TRLs responsible for HSPG-mediated clearance, we analyzed TRLs from mice deficient for the liver heparan sulfate biosynthetic enzyme N-deacetylase-N-sulfotransferase-1 (Ndst1<sup>fl/fl AlbCre<sup>+</sup></sup>). Ndst1-deficiency induced in this way
results in undersulfation specifically of hepatocyte heparan sulfate and accumulation of TRLs in both the fasted and post-prandial state due to altered hepatic clearance (16). We reasoned that if HSPGs preferentially clear subspecies of TRLs, then lipoproteins of unique composition should accumulate in the mutant. Thus, plasma TRLs from fasted \( Ndst1^{\text{cfl}} AlbCre^+ \) (wild-type) and \( Ndst1^{\text{cfl}} AlbCre^- \) (mutant) mice were isolated by density ultracentrifugation (\( \delta < 1.006 \) g/ml) and equal amounts of protein were analyzed by SDS-PAGE and silver staining (Figure 2-1, A). ApoE and apoC’s were enriched in TRLs from \( Ndst1^{\text{cfl}} AlbCre^+ \) as compared to \( Ndst1^{\text{cfl}} AlbCre^- \) mice, whereas no consistent change in apoB-48 or apoB-100 was observed. Similar results were obtained when post-prandial TRLs were analyzed. Of the apolipoproteins detected in this way, only apoE and apoB bind to heparan sulfate or to heparin, thus focusing our initial experiments on these apolipoproteins.

To examine the participation of apoE in binding to hepatic HSPGs, we developed a cell surface receptor-binding assay using metabolically labeled post-prandial \(^3\text{H}\)TRLs derived from mice orally gavaged with \(^3\text{H}\)retinol and corn oil. Binding to cell surface HSPGs was assessed by incubation of the radiolabeled TRLs with Hep3B human hepatocarcinoma cells, a cell line that expresses a similar profile of HSPGs as primary human and murine hepatocytes (17, 52). Incubation of cells at \( 4^\circ \)C with \(^3\text{H}\)TRLs derived from wild-type mice demonstrated saturable binding (\( B_{\text{max}} = 3.8 \pm 1.9 \) µg TRL protein/mg cell protein) with an apparent \( K_D \) of 38 µg/mL (\( R^2 = 0.83 \)), which compares well with values obtained previously for human TRL binding to primary murine hepatocytes (\( B_{\text{max}} = 8.2 \pm 1.1 \) µg VLDL protein/mg cell protein; \( K_D = 43 \pm 10 \) µg/mL) (17). Subsequent measurements were made with 50 µg/mL of purified TRLs in order to
econonize the use of radioactive TRLs. Under these conditions, $3.3 \pm 0.4\, \mu g$ TRL protein/mg cell protein bound to the cells. Treatment of Hep3B cells with heparin lyases, which degrade the heparan sulfate chains on syndecan-1, significantly reduced cell surface binding to $1.3 \pm 0.1\, \mu g$ TRL protein/mg cell protein ($P < 0.001$; Figure 2-1, B). The residual binding presumably reflects other receptors (e.g. LDLR) or incomplete removal of heparan sulfate.

In contrast to the behavior of wild-type TRLs, $[^3\text{H}]$TRLs isolated from $ApoE^{-/-}$ mice bound to Hep3B cells poorly ($0.7 \pm 0.1\, \mu g$ TRL/mg cell protein) and the residual binding did not depend on heparan sulfate (Figure 2-1, B). Reconstitution of $[^3\text{H}]$TRLs from $ApoE^{-/-}$ mice with recombinant human apoE3 restored binding to Hep3B cells (2.5-fold compared to apoE-deficient particles; $P = 0.002$), and the enhanced binding was heparan sulfate dependent ($P = 0.009$) (Figure 2-1, C). Analysis of apoE-deficient TRLs by SDS-PAGE showed that in addition to the loss of apoE, the particles were enriched with apoAI and apoAIV (Supplemental Figure S2-1, A). Interestingly, reconstitution of apoE-deficient TRLs with recombinant human apoE3 resulted in displacement of apoAIV, but not apoAI or other apolipoproteins, as determined by gel electrophoresis and silver staining or Western blotting (Supplemental Figures 2-1, B and C) (53, 54).
Figure 2-1. Murine TRLs require apoE for binding to cell surface heparan sulfate. (A) TRLs (δ < 1.006 g/ml) were isolated from fasted Ndtt<sup>fl/fl</sup>AlbCre<sup>−</sup> (wildtype, n = 4) and Ndtt<sup>fl/fl</sup>AlbCre<sup>+</sup> (mutant, n = 4) mice and analyzed by gradient SDS-PAGE. Individual proteins were visualized by silver staining. A representative gel is shown with n = 2 per genotype. (B) Binding of [³H]TRLs (50 µg/ml) from wild-type and ApoE<sup>−/−</sup> mice to Hep3B cells was measured before (filled bars) and after (open bars) treatment with heparin lyases (n = 5). (C) ApoE-deficient [³H]TRLs were reconstituted with rhApoE3 and purified by ultracentrifugation (δ < 1.006 g/ml). Cell surface binding of the reconstituted particles was measured (n = 8).

Additional evidence demonstrating the importance of apoE for HSPG-mediated TRL clearance was obtained by intravenously injecting [³H]TRLs into wild-type Ndtt<sup>fl/fl</sup>AlbCre<sup>−</sup> and mutant Ndtt<sup>fl/fl</sup>AlbCre<sup>+</sup> mice and measuring their clearance from the plasma. Injected [³H]TRLs cleared rapidly in wild-type mice with a t<sub>1/2</sub> of ~4 min (Figure 2-2, A). In contrast, clearance was much delayed in Ndtt<sup>fl/fl</sup>AlbCre<sup>+</sup> mice (t<sub>1/2</sub> >20 min), confirming the importance of the HSPG-mediated pathway in vivo. ApoE-deficient [³H]TRLs injected into Ndtt<sup>fl/fl</sup>AlbCre<sup>+</sup> mice showed similarly delayed clearance kinetics. The lack of an additive effect of compounding these two alterations supports the idea that apoE on TRLs interacts with hepatic HSPGs.
Figure 2-2. ApoE-independent clearance of TRLs by heparan sulfate proteoglycans. (A) Isolated $[^3]$HTRLs from wild-type (filled symbols) and ApoE$^{-/-}$ (open symbols) mice were injected intravenously into Ndst1$^{fl/fl}$AlbCre$^-$ (circles) or Ndst1$^{fl/fl}$AlbCre$^+$ mutant (squares) mice ($n = 3$). Clearance of $[^3]$HTRLs was assessed by measuring the counts remaining in the plasma relative to counts recovered one min post injection. (B) Plasma triglycerides were measured in plasma samples collected from fasted Ndst1$^{fl/fl}$AlbCre$^-$ (n = 9, filled squares), Ndst1$^{fl/fl}$AlbCre$^+$ (n = 10, open squares), Ndst1$^{fl/fl}$AlbCre$^+$;ApoE$^{-/-}$ (n = 19, filled circles), and Ndst1$^{fl/fl}$AlbCre$^+$;ApoE$^{-/-}$ (n = 15, open circles) mice (8-weeks old). (C) $[^3]$Hretinol ester excursion in 10-week old Ndst1$^{fl/fl}$AlbCre$^+$;ApoE$^{-/-}$ (n = 11, filled circles) and Ndst1$^{fl/fl}$AlbCre$^+$;ApoE$^{-/-}$ (n = 8, open circles) was measured after mice were gavaged with corn oil and $[^3]$Hretinol. (D) Plasma triglycerides four hours post gavage was measured.

Inspection of the data presented in Figure 2-2A indicated slower clearance of apoE-deficient $[^3]$HTRLs in Ndst1$^{fl/fl}$AlbCre$^+$ mice compared to their clearance in wild-type mice ($t_{1/2} \sim 20$ min vs. $\sim 10$ min, respectively), suggesting that there might be another ligand on TRLs that can interact with hepatic HSPGs. We reached a similar conclusion
by analysis of plasma triglycerides in \( Ndst1^{\text{f/f}} AlbCre^+ \), \( ApoE^{-/-} \) and compound \( Ndst1^{\text{f/f}} AlbCre^+ ; ApoE^{-/-} \) mice. As shown previously, Ndst1 and apoE deficiency resulted in modest hypertriglyceridemia compared to wild-type mice (105 ± 16 mg/dl in wildtype mice, \( n = 9 \); 228 ± 25 mg/dl in \( Ndst1^{\text{f/f}} AlbCre^+ \), \( n = 10 \); and 197 ± 12 mg/dl in \( ApoE^{-/-} \) mice; \( n = 19 \)) (Figure 2-2, B) (16-18, 55). The absence of apoE led to increased plasma cholesterol as well, but Ndst1-deficiency did not (Supplemental Figure S2-2). Importantly, plasma triglycerides accumulated to a greater extent in \( Ndst1^{\text{f/f}} AlbCre^+ ; ApoE^{-/-} \) mice (334 ± 23 mg/dl; \( n = 15 \)) compared to each single mutant. Clearance of intestinally derived TRLs was also significantly delayed in \( Ndst1^{\text{f/f}} AlbCre^+ ; ApoE^{-/-} \) compared to \( ApoE^{-/-} \) mice (area under curve = 9300 vs. 5800, respectively; Figure 2-2, C). Postprandial plasma triglycerides were also elevated in \( Ndst1^{\text{f/f}} AlbCre^+ ; ApoE^{-/-} \) mice compared to their \( Ndst1^{\text{f/f}} AlbCre^+ ; ApoE^{-/-} \) littermates (538 ± 63 mg/dl; \( n = 9 \) vs. 251 ± 21 mg/dl; \( n = 11 \); Figure 2D). The increase in fasting plasma triglycerides and impairment in clearance of dietary lipids in the compound mutant compared to the single mutants, along with the slower clearance of injected apoE-deficient \([3H]\)TRLs in \( Ndst1^{\text{f/f}} AlbCre^+ \) mice compared to wild-type mice strongly suggested that HSPG-mediated TRL clearance does not exclusively depend on apoE.

2.3.2 The apoE-independent pathway of TRL clearance by heparan sulfate does not involve apoB

In light of these findings, we examined the contribution of other TRL associated proteins known to bind to heparin. We excluded any contribution from apoB based on several criteria. Firstly, binding of \([3H]\)TRLs derived from \( ApoE^{-/-} \) mice to hepatocytes
did not depend on heparan sulfate (Figure 2-1, B), yet these particles contain apoB, mostly apoB-48 (Supplemental Figure S2-1, A). Secondly, binding of [3H]TRLs derived from ApoB^{100/100} and ApoB^{48/48} mice (Supplemental Figure S2-3) was similar to wild-type TRLs (2.9 ± 0.2 and 2.9 ± 0.5 µg for TRLs from the mutants, respectively vs. 3.3 ± 0.4 µg of TRL/mg cell protein for TRLs from wild-type mice; Figure 3A). Thirdly, binding of both apoB-restricted mutant particles was significantly reduced after treatment of Hep3B cells with heparin lyases, but not to any greater extent than wild-type TRLs (Figure 2-3, A). Fourthly, triglyceride accumulation in Ndst1^F^ AlbCre^+;ApoB^{100/100} and Ndst1^F^ AlbCre^+;ApoB^{48/48} was not elevated compared to Ndst1^F^ AlbCre^+ mice (Figure 2-3, B). Finally, binding of TRLs to HSPGs was not affected in vitro by monoclonal antibodies raised against different domains of apoB. In these latter experiments, we took advantage of the observation that shed syndecan-1 ectodomains will form complexes with purified TRLs from human plasma (δ<1.006 g/ml) based on an assay in which ^35S-labeled ectodomains are mixed with hTRLs and subjected ultracentrifugation (52). Under these conditions complexes of hTRLs with ^35S-ectodomains floated in saline solution (150 mM NaCl) containing non-ionic iodixanol (δ = 1.019 g/ml), while unbound ^35S-ectodomains sedimented to the bottom of the centrifuge tube. Binding was concentration-dependent and saturable (Figure 2-3, C), and under these conditions about 25-35% of ^35S-ectodomains bound to hTRLs. Heparin, a highly sulfated derivative of heparan sulfate, abolished binding of the ectodomains. Using this assay, we showed that particles bearing only apoB-48 or apoB-100, which were separated by immunoprecipitation with an apoB-100 specific antibody, did not differ in ectodomain binding (Figure 2-3, D). Furthermore, apoB mAbs MB19 (which binds to the N-terminal domain of apoB and reduces LDL
binding to heparin), MB43 (apoB-100-specific), MB47 (apoB-100-specific) or a combination of MB19 and MB47 had no impact on TRL binding to ectodomains (Figure 3E and 3F) (56-58). Other monoclonal antibodies that recognize epitopes on both apoB isoforms (MB2, MB3, and MB11) also had no effect (58, 59). Thus, although particles that bind to heparan sulfate contain apoB, this apolipoprotein appears to be dispensable for the interaction.
Figure 2-3. TRL binding to heparan sulfate is independent of ApoB. (A) Binding of $[^3H]$TRLs from $ApoB^{100/100}$ and $ApoB^{48/48}$ mice to Hep3B cells was measured before (filled bars) or after (open bars) treatment with heparin lyases ($n = 4$). Binding of wildtype $[^3H]$TRLs done at the same time and shown in Figure 1B is repeated here for comparison. (B) Fasting plasma triglycerides from $Ndst1^{fl/fl} AlbCre^{-};ApoB^{100/100}$ ($n = 12$, filled triangles), $Ndst1^{fl/fl} AlbCre^{+};ApoB^{100/100}$ ($n = 13$, open triangles), $Ndst1^{fl/fl} AlbCre^{-};ApoB^{48/48}$ ($n = 8$, filled circles) and $Ndst1^{fl/fl} AlbCre^{+};ApoB^{48/48}$ ($n = 13$, open circles) mice were measured. Plasma triglycerides from $Ndst1^{fl/fl} AlbCre$ animals shown in Figure 2A are included for comparison. (C) Binding of purified $[^{35}S]$HSPG ectodomains to human TRLs (hTRLs) was measured by ultracentrifugation (Experimental Procedures). Binding of $[^{35}S]$HSPGs to hTRLs (filled circles) occurred in a saturable manner and binding was inhibited by heparin (open circles). (D) Binding of $[^{35}S]$HSPGs to apoB48-only (open circles) or mixed hTRLs (filled circles) was measured. Inset: SDS-PAGE and silver staining for apoB of purified hTRLs. (E) Diagram of epitope map for apoB mAbs. Specific residues recognized by antibodies are shown in parentheses and heparin binding sites are shaded gray (58). (F) $[^{35}S]$HSPG binding to hTRLs was measured by ultracentrifugation in the absence (black) or presence of mouse IgG (dark gray) or mAbs specific for domains spanning apoB (MB19, MB43, MB47; light gray). A control experiment done without hTRLs is shown for comparison (white).
2.3.3 The apoE-independent pathway of TRL clearance by heparan sulfate involves apoAV

To investigate other candidate heparin-binding proteins, fasting TRLs from \( Ndst1^{Ef}\) AlbCre\(^-\) and \( Ndst1^{Ef}\) AlbCre\(^+\) mice were subjected to SDS-PAGE and analyzed by Western blotting. Previous work has suggested that LPL and HL can facilitate remnant clearance by acting as bridge between TRLs and hepatic HSPGs (46, 60, 61). However, we did not detect either lipase in TRL preparations derived from wild-type or heparan-sulfate deficient mice (\( n = 4 \) per genotype, Figure 2-4, A). In contrast, apoAV was detected and enriched nearly 6-fold on TRLs derived from \( Ndst1^{Ef}\) AlbCre\(^+\) (\( n = 13 \)) compared to the wildtype (3.1 ± 0.6 units [\( n = 13 \)] vs. 0.5 ± 0.2 units [\( n = 8 \)], \( P = 0.0029; \) Figure 2-4, B). We previously did not observe apoAV in TRLs that accumulate in \( Ndst1^{Ef}\) AlbCre\(^+\) mice presumably because of a lack of sensitivity of the staining techniques used (16). ApoAV also was enriched in TRLs in \( ApoE^{-/-}\) mice (5.1 ± 1.2 units in the mutant vs. 0.5 ± 0.2 units in the wildtype), consistent with the role of ApoE in clearance mediated by HSPGs. Importantly, mice deficient in Ldlr and hepatic Lrp1 (\( Ldlr^{-/-};Lrp1^{Ef}\) AlbCre\(^+\)) also have elevated plasma triglycerides (361 ± 44 mg/dl; \( n = 7 \)) but did not accumulate TRLs bearing apoAV (0.4 ± 0.1 units, \( n = 7 \); Figure 2-4, B). These findings demonstrate that apoAV accumulation in \( Ndst1^{Ef}\) AlbCre\(^+\) mice strictly depends on loss of hepatic heparan sulfate and does not simply reflect impaired TRL clearance.
Figure 2-4. TRLs from mice with impaired HSPG-mediated TRL clearance accumulate apoAV. (A) Western blot of mouse TRLs from \textit{Ndst1}^{f/f} \textit{AlbCre}^- and mutant \textit{Ndst1}^{f/f} \textit{AlbCre}^+ mice using antibodies against LPL, HL, and apoAV. Homogenates of heart and liver were used as positive controls, respectively. hTRLs were also probed for the presence of apoAV with a human anti AV mAb, as shown on the right. (B) Fasting TRLs from \textit{Ndst1}^{f/f} \textit{AlbCre}^-, \textit{Ndst1}^{f/f} \textit{AlbCre}^+, and \textit{Ldlr}^-/-;\textit{Lrp1}^{f/f} \textit{AlbCre}^+ mice were analyzed by SDS-PAGE and probed for the presence of apoAV by Western blotting. The blots were scanned by densitometry and the amount of apoAV was normalized to apoB for each sample. (\textit{Ndst1}^{f/f} \textit{AlbCre}^-, n = 8; \textit{Ndst1}^{f/f} \textit{AlbCre}^+, n = 13; \textit{Ldlr}^-/-;\textit{Lrp1}^{f/f} \textit{AlbCre}^+ n = 7).

Although it was tempting to test for genetic interactions by crossbreeding apoAV mice with \textit{Ndst1}^{f/f} \textit{AlbCre}^+ animals, apoAV deficiency alters peripheral lipolysis, resulting in the accumulation of very large TRLs that might confound the interpretation of the results (62). However, we could circumvent this problem and compare the behavior of comparably sized particles by depleting TRLs of apoAV through repeated challenge with antibodies directed against apoAV. Treatment of hTRLs with a mAb to human apoAV depleted the particles of the apoAV by 85% (Figure 2-5, A inset), without affecting the content of apoB and apoE. Binding of apoAV-depleted hTRLs to $[^{35}\text{S}]$ectodomains was significantly reduced as compared to hTRLs treated with non-specific murine IgG.
Furthermore, anti-apoAV mAb inhibited binding of unmodified hTRLs in a dose-dependent manner (IC\textsubscript{50} \sim 0.5 \mu g/ml) similar to anti-apoE mAb (IC\textsubscript{50} \sim 0.25 \mu g/ml), whereas nonspecific mouse IgG had no effect (Figure 2-5, B).

Treatment of mTRLs derived from \textit{Ndst1}\textsuperscript{EF}\textit{AlbCre}\textsuperscript{+} mice with mAb to murine apoAV also resulted in apoAV displacement (> 95%) allowing us to examine if clearance of apoAV-depleted [\textsuperscript{3}H]mTRLs might be impaired after injection into wild-type mice. Only a modest difference was observed when depleted [\textsuperscript{3}H]mTRLs were injected compared to [\textsuperscript{3}H]mTRLs treated with nonspecific IgG (t\textsubscript{1/2} \sim 3.5 min vs. 3.2 min, respectively; n = 4). The difference was accentuated by depleting apoAV from TRLs derived from \textit{ApoE}\textsuperscript{-/-} mice (7.6 min vs. 5.6 min, respectively; n = 4), but in neither case did the difference reach significance (P = 0.4145 and P = 0.1320, respectively). To circumvent potential confounding effects of apolipoprotein reconstitution that might occur in vivo, we examined binding of depleted particles to hepatocytes in culture. Binding of apoAV-depleted [\textsuperscript{3}H]mTRLs to Hep3B cells was significantly reduced as compared to [\textsuperscript{3}H]mTRLs treated with non-specific IgG (Figure 2-5, C, P = 0.0048).
**Figure 2-5.** TRL binding to HSPGs depends on apoE and apoAV. (A) hTRLs were subjected to three rounds of immunoprecipitation using nonspecific mouse IgG or apoAV-specific mAb, and the cleared, antibody-free solution was collected. $[^{35}\text{S}]$HSPG binding to the preparations was measured by ultracentrifugation. Inset: SDS-PAGE and Western blot for apoAV content of hTRLs after treatment with nonspecific IgG and apoAV-specific IgG. (B) $[^{35}\text{S}]$HSPG binding to hTRLs in the presence of increasing amounts of mouse IgG (filled circles) or mouse mAbs specific for apoE (open squares) or apoAV (open circles). (C) $[^{3}\text{H}]$mTRLs from Nd$st^{1/2}\text{AlbCre}^+$ were subjected to three rounds of immunoprecipitation using nonspecific mouse IgG or apoAV-specific mAb, and the antibody-free solution was collected. $[^{3}\text{H}]$mTRL binding to Hep3B cells at 4°C was measured before and after treatment with heparin lyases (n = 3) and expressed as $\mu$g TRL bound/mg cell protein.

Syndecan-1, like other HSPGs, contain up to three attachment sites for heparan sulfate clustered near the N-terminus of the protein. Because binding of TRLs to ectodomains appeared to depend on both apoE and apoAV, we examined if the interaction depended on the presentation of clustered heparan sulfate chains on the ectodomains. Because of their large hydrodynamic volume, $[^{35}\text{S}]$ectodomains elute towards the void volume during gel filtration chromatography on CL-4B resins (Figure 6A (63). Alkaline treatment of the ectodomains results in beta-elimination and liberation
of the individual chains, as demonstrated by the more retarded elution of individual chains compared to the native ectodomains (Figure 2-6, A). Binding of individual chains to TRLs also occurred in the floatation assay and, like binding to intact ectodomains, binding to individual chains was sensitive to added heparin (Figure 2-6, B). Typically, 20-35% of the $[^{35}\text{S}]$ectodomains or $[^{35}\text{S}]$heparan sulfate chains would associate with the hTRLs in this assay, suggesting that only a subpopulation of the ectodomains and chains have the capacity to bind under these conditions. Monoclonal antibodies directed against apoE or apoAV prevented the formation of complexes to the same extent (Figure 2-6, C). These findings suggest that individual chains contain binding sites for both of apoE and apoAV and that simultaneous interaction with these apolipoproteins provides for optimal binding.

Figure 2-6. ApoE and apoAV mediate binding of TRLs to heparan sulfate chains. (A) Gel filtration chromatography of $[^{35}\text{S}]$HSPG ectodomains (black circles) and $[^{35}\text{S}]$heparan sulfate chains (open circles). (B) Binding of $[^{35}\text{S}]$heparan sulfate (HS) or $[^{35}\text{S}]$HSPGs to hTRLs was measured by ultracentrifugation. (C) Inhibition of $[^{35}\text{S}]$HS binding to hTRLs by mAbs against apoE or apoAV.

2.3.4 Clearance of TRLs by HSPGs is atheroprotective

To determine the relevance of hepatic TRL clearance mediated by heparan sulfate proteoglycans, we examined 6-month old $Ndst1^{EF}AlbCre^{+};ApoE^{+/+}$ and $Ndst1^{EF}AlbCre^{+}$
ApoE−/− mice for spontaneous atherosclerosis. Like the younger animals described in Figure 2B, the older Ndst1−/− AlbCre⁺;ApoE−/− mice showed elevated plasma triglycerides compared to Ndst1−/− AlbCre⁺;ApoE−/+ (250 ± 82 mg/dl vs. 169 ± 33 mg/dl) and plasma cholesterol remained unchanged (356 ± 57 mg/dl vs. 378 ± 66 mg/dl). Analysis of the plasma lipoproteins by gel filtration FPLC showed accumulation of triglycerides in large TRLs and no change in cholesterol-rich lipoproteins (Figures 2-7, A and B). En face analysis of aortas from chow-fed Ndst1−/− AlbCre⁺;ApoE−/− mice demonstrated a striking 2.25-fold increase in plaque formation in the aortic arch compared to their Ndst1−/− AlbCre−;ApoE−/+ littermates (1.24 ± 0.27 mm² vs. 2.81 ± 0.40 mm², respectively; Figure 2-7, C). Both females and males were affected to the same extent. These findings provide the first evidence that HSPG-mediated clearance of TRLs is atheroprotective.
Figure 2-7. Disruption of HSPG-mediated TRL clearance enhances the development of atherosclerosis. Lipoproteins from pooled plasma samples (n = 6) from Ndst1^{ftf}AlbCre^{*};ApoE^{*} (filled circles) and Ndst1^{ftf}AlbCre^{*};ApoE^{*} (open circles) mice were analyzed by FPLC gel filtration and the amount of (A) triglyceride and (B) cholesterol was determined. Ndst1^{ftf}AlbCre^{*};ApoE^{*} (n = 8; 6 month old) and Ndst1^{ftf}AlbCre^{*};ApoE^{*} (n = 12; 6-month old) mice on standard chow diets were analyzed for spontaneous atherosclerosis development. (C) Plaque formation in the aortic arch from males (squares) and females (circles) were quantified. (D) Three representative images of stained aortas from females for each genotype are displayed.

2.4 Discussion
Our studies demonstrate that apoE and apoAV are the dominant particle-associated proteins involved in TRL binding to HSPGs in mice. This conclusion is based on (i) accumulation of TRLs bearing apoE and apoAV in Ndst1<sup>wt</sup>AlbCre<sup>+</sup> mice (Figures 2-1 and 2-4); (ii) failure of TRLs isolated from ApoE<sup>-/-</sup> mice to bind to hepatocytes in a heparan sulfate-dependent manner, with restoration of binding by reconstitution of apoE-deficient TRLs with recombinant human apoE3 (Figure 2-1); (iii) decreased clearance of apoE-deficient TRLs in wild-type mice and lack of any further reduction in Ndst1-deficient mice (Figure 2-2); (iv) inhibition of binding of human TRLs to <sup>35</sup>S-labeled HSPG ectodomains by monoclonal antibodies specific to apoE and apoAV, but not to apoB (Figures 2-3 - 2-5); (v) reduction of binding after depletion of apoAV from hTRLs and mTRLs (Figure 2-5); and (vi) decreased clearance of apoAV-depleted particles in vivo. Although the in vivo data was derived from genetic studies in mice, we infer from the in vitro studies of human TRLs that these same factors may be relevant to remnant clearance in humans.

ApoE is found at high concentration on the sinusoidal side of the basal membrane of hepatocytes (64), which led to the idea that apoE produced by hepatocytes might enrich lipoproteins in the space of Disse and enhance clearance through proteoglycans (“secretion-capture” hypothesis) (65, 66). The binding site in apoE for heparin has been characterized (25, 29, 67, 68). Furthermore, several naturally occurring apoE variants that exhibit reduced binding to heparin have been found in patients with hyperlipoproteinemia (28, 39, 69, 70). These findings coupled with the experimental evidence presented here firmly establish the importance of apoE in TRL clearance by HSPGs.
ApoAV also has been suggested to promote clearance of TRLs by acting as a ligand for receptor-mediated endocytosis (71-73). The accumulation of TRLs bearing apoAV in Ndst1<sup>εf</sup> AlbCre<sup>+</sup> mice and the inhibition of binding to HSPG by depletion of apoAV or by the addition of neutralizing antibodies provides experimental evidence for a role in apoAV in proteoglycan-mediated clearance. Although apoAV has been suggested to act as a ligand for LDL receptor family members (71), we favor the idea that apoAV facilitates TRL binding to hepatic proteoglycan receptors, which act independently of members of the LDL receptor family (16, 17). The lack of accumulation of TRLs bearing apoAV in Ldlr<sup>-/-</sup>; Lrp1<sup>εf</sup> AlbCre<sup>+</sup> mice, in which HSPG-mediated clearance is intact, provides further support for this conclusion.

Remarkably, the concentration of apoAV in plasma is extremely low compared to other apolipoproteins and its accumulation in Ndst1<sup>εf</sup> AlbCre<sup>+</sup> mice required Western blotting for detection. It has been estimated that as few as one in twenty four VLDL particles carries apoAV in plasma (74). Superficially, the low abundance of apoAV bearing particles would appear to be inconsistent with the observation that HSPG receptors, notably syndecan-1, account for a large proportion of TRL clearance (~50% under fasting conditions and as much as 70% under post-prandial conditions) (17). However, the six-fold enrichment in apoAV in TRLs from Ndst1<sup>εf</sup> AlbCre<sup>+</sup> mice suggests that ~25% of TRLs contain apoAV in the absence of HSPG-mediated clearance. Thus, the low concentration of apoAV in wild-type mice most likely reflects efficient clearance of apoAV-bearing lipoproteins from the circulation. One should also keep in mind that plasma apoAV concentration might not reflect events occurring in the liver, the site of apoAV production (75). Although the concentration of apoAV in the space of Disse is
unknown, it is intriguing to speculate that a dual “enrichment-capture” mechanism might exist, in which remnant lipoproteins could become enriched for apoAV as well as apoE when they enter the liver (73, 76, 77).

ApoB, which also binds to heparin, does not appear to participate directly in HSPG-mediated clearance. Clearance of apoB\(^{48/48}\) and apoB\(^{100/100}\) particles occurs normally in \(Ndst1^{+/+} AlbCre^{+/+}\) mice and no additive effects were noted in compound mutants. Moreover, \(apoE^{-/-}\) TRLs contain apoB-48 but no longer bind to hepatocytes in a heparan sulfate dependent manner. Monoclonal antibodies directed against apoB had no effect on binding of human TRLs to proteoglycan ectodomains as well. Thus, the heparin-binding capacity of apoB does not appear to facilitate binding of TRLs to HSPGs. Conceivably, domains involved in heparin binding might not be exposed in TRLs and only appear as the particles are reduced in size or converted to LDL (56, 78). The observation that LDL can form complexes with arterial heparan sulfate appears to be consistent with this idea (79). In regards to hepatic clearance, we propose that apoB is primarily responsible for interaction with members of the LDL receptor family, which also function in remnant particle clearance.

Multiple studies have demonstrated that triglyceride lipases, HL and LPL, could act as ligands for TRL binding to HSPGs, independent of their catalytic capacity (46, 60, 61, 80-82). Both lipases bind to heparan sulfate and enrichment of TRLs with either lipase led to enhanced binding to cell surface HSPGs (36, 40, 43, 83, 84). However, the importance of these lipases in HSPG-mediated clearance in vivo has been difficult to assess, because knockout models of the lipases are confounded by lipolysis defects and other effects on lipoprotein metabolism. We were unable to detect LPL or HL on freshly
isolated TRLs from wild-type or \( \text{Ndst1}^{\text{Ef}} \text{AlbCre}^{+} \) mice and human TRLs. Furthermore, anti-human LPL mAb has no effect on binding of hTRLs to HSPG in vitro (Gonzales, J. and Esko, J.D., unpublished results). Enrichment of TRLs and other lipoproteins with LPL or HL clearly can provide additional capacity to interact with HSPGs, but the physiologic relevance of these observations with respect to hepatic clearance remains questionable based on the findings presented here.

Based on the ability of free heparan sulfate chains to bind to human VLDL particles, we believe that binding of TRLs to syndecan-1 most likely depends on multivalent interactions. Interestingly, gel filtration of the heparan sulfate chains from hepatoma cells and human liver indicate an average molecular mass of \( \sim 16 \) KDa, corresponding to \( \sim 60 \) disaccharides per chain (85). Most heparin-binding proteins require only 4-6 disaccharides for binding (86), thus the chains can easily bind multiple protein ligands if they possess the appropriate arrangement of sulfated sugars and epimers of uronic acids. Thus, we propose a model in which a TRL particle binds to one or more heparan sulfate chains on syndecan-1 via multiple contacts with apoE or combinations of apoE and apoAV.

Hypertriglyceridemia is an independent risk factor for cardiovascular disease and there is increasing evidence that remnant TRLs contribute to the development of atherosclerosis (1-4). Our findings support this conclusion and provide the first evidence that TRL clearance mediated by apoE and apoAV binding to HSPGs is atheroprotective. Intriguingly, \( \text{Ndst1}^{\text{Ef}} \text{AlbCre}^{+};\text{ApoE}^{-/-} \) mice have elevated plasma triglycerides, but plasma cholesterol is not affected, suggesting that the impaired clearance of triglyceride-rich lipoproteins drives the more extensive development of atherosclerosis in this model. This
conclusion is in agreement with studies showing that transgenic over-expression of human apoAV, which reduces plasma triglycerides, provides atheroprotection in mouse models of dyslipidemias (87, 88).

In conclusion, our studies provide new, significant insights into the mechanism of HSPG-mediated clearance of TRLs and provide the first genetic evidence of atheroprotection by hepatic HSPGs. We have identified apoE and apoAV as the dominant ligands on TRLs that bind to hepatocyte HSPGs and demonstrate that disruption of this multivalent interaction results in increased atherosclerosis in mice.

2.5 Experimental procedures

2.5.1 Mice and animal husbandry

Ndst1\textsuperscript{ff}\textsubscript{AlbCre}\textsuperscript{+} mice were described previously (16). Mice deficient for apoE (ApoE\textsuperscript{-/-}) were from Jackson Laboratory (89), and apoB-48 and apoB-100 deficient mice (ApoB\textsuperscript{100/100} and ApoB\textsuperscript{48/48}, respectively) were provided by Steve Young, University of California, Los Angeles, CA) (90). Ldlr\textsuperscript{-/-};Lrp1\textsuperscript{ff}AlbCre\textsuperscript{+} mice were generated by crossbreeding Lrp1\textsuperscript{ff}, Ldlr\textsuperscript{-/-}, and AlbCre\textsuperscript{+} mice purchased from Jackson Laboratory. All animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved vivaria in the School of Medicine, University of California San Diego following the standards and procedures approved by the local Institutional Animal Care and Use Committee. Mice were weaned at 3 weeks, maintained on a 12-hr light-dark cycle, and fed \textit{ad libitum} water and standard rodent chow (Harlan Tekland). Genotyping was performed as described in the original publications (16, 89-91).
2.5.2 Cell culture

The human hepatocarcinoma cell line Hep3B was obtained from the American Type Culture Collection (HB-8064, Manassas, VA) and cultured in Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum (Gemini Bio-Products), non-essential amino acids, sodium pyruvate, penicillin, and streptomycin.

2.5.3 TRL Isolation

Mouse TRLs were isolated from blood samples drawn by cardiac puncture into BD Microtainer tubes with EDTA. Samples (200 µl per tube) were centrifuged for 4 hr at 175,000 x g in a Beckman 42.2Ti rotor. The top fraction (50 µl, δ<1.006 g/ml) was collected and used for subsequent studies. Human TRLs (hTRLs, δ<1.006 g/ml) were isolated from plasma by centrifugation for 16 hr at 135,000 x g in a Beckman 50.3Ti rotor. Donors were healthy, normally fed volunteers (3-4 hr post-meal) with informed consent. Isolated TRLs were quantified by BCA protein assay (Pierce).

2.5.4 Lipoprotein analysis

Plasma samples were prepared from blood drawn via submandibular puncture from overnight fasted mice. Total cholesterol and triglyceride levels were determined enzymatically (Triglyceride-SL and Cholesterol-SL, Sekisui Diagnostics) using an automated plate reader (Cobas Mira; Roche Diagnostics).

TRLs were analyzed by SDS-PAGE on 4-12% Bis-Tris gradient gels (NuPage, Invitrogen). Proteins were visualized by silver staining (Pierce) or after transfer to Immobilon-FL PVDF membrane (Millipore). Membranes were blocked with Odyssey
Blocking Buffer (LI-COR Biosciences) for 1 hr and incubated overnight at 4°C with specific antibodies. Rabbit and mouse antibodies were incubated with secondary Odyssey IR dye antibodies (1:15,000 dilution) and visualized with Odyssey IR Imaging system (LI-COR Biosciences). Goat and guinea pig antibodies were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, 1:10,000 dilution) and visualized by SuperSignal chemiluminescent substrate for HRP detection (Pierce). Blots were analyzed by densitometry with ImageJ software (NIH) and the amount of each apolipoprotein was normalized to apoB. The values therefore are ratiometric and provided in arbitrary units.

Western blot antibodies included: rabbit anti-mouse apoB (Abcam ab20737; 1:1,000 dilution), rabbit anti-mouse apoE (Meridian Life Sciences, K23100R, 1:1,000 dilution), rabbit anti-human (and mouse) hepatic lipase (Santa Cruz Biotechnology, sc-21007, 1:200 dilution), guinea pig anti-mouse LPL (antisera prepared from animals immunized with recombinant mouse LPL; 1:300 dilution; Gonzales and Esko, unpublished), rabbit anti-mouse apoAV (Santa Cruz Biotechnology, sc-66810, 1:200 dilution), mouse anti-human ApoE (Abcam, ab1906, 1:1,000 dilution), and mouse anti-human apoAV (Santa Cruz Biotechnology sc-32810, 1:200 dilution). Positive controls for Western blotting of LPL and HL consisted of clarified tissue homogenates prepared from heart and liver of a wildtype mouse, respectively. Freshly isolated tissue was homogenized in RIPA buffer and centrifuged at low-speed (2,000 x g). Protein concentration was measured by BCA assay.

2.5.5 Immunoprecipitation of TRLs
To separate apoB-48 bearing TRLs from apoB-100 bearing TRLs, purified apoB-100-specific mAb MB47 (10 µg) was incubated with 100 µl Protein G coated magnetic beads (Dynabeads, Invitrogen) for 15 min and washed twice with PBS using a magnet. Beads with captured IgG were resuspended in PBS with 10 µg of human TRLs and rotated gently overnight at 4°C. The beads were collected with a magnet, washed twice with PBS, and eluted with SDS-PAGE reducing buffer (Invitrogen). hTRLs, hTRLs cleared of apoB-100 containing lipoproteins, and apoB-100 bearing lipoproteins bound to the beads were analyzed by SDS-PAGE and silver staining.

To deplete apoAV from hTRLs, 10 µg of anti-human apoAV antibody (Santa Cruz Biotechnology, sc-32810) was incubated with 100 µL Protein G coated magnetic beads (Dynabeads, Invitrogen) for 15 min and washed twice with PBS using a magnet. Beads with captured IgG (10 µg) were resuspended in PBS with 10 µg of hTRLs and rotated for 2 hr at room temperature. The beads were collected with a magnet and the supernatant was challenged with fresh beads two more times. The final solution was collected and used for [35S]HSPG binding experiments and Western blot analysis. Immunodepletion with nonspecific mouse IgG (Jackson Immunoresearch, 015-000-003) was performed in parallel as a control. Depletion of apoAV from mTRLs was accomplished under the same conditions with an anti-mouse apoAV antibody (Santa Cruz Biotechnology, sc-66810) using a nonspecific rabbit IgG as the control (Jackson Immunoresearch 011-000-003). The final solution was collected and used in hepatocyte binding assay and TRL clearance assays. Material bound to the beads was washed twice in PBS and eluted with SDS-PAGE reducing buffer (Invitrogen).
2.5.6 Murine TRL binding to hepatocytes

Overnight fasted mice (n = 5 per preparation) were gavaged with 300 µl of corn oil (Sigma Aldrich) and 10 µCi of [11,12-³H] retinol (Perkin-Elmer, 43.0 Ci/mmol). Three hours post-gavage, mice were sacrificed, blood was withdrawn by cardiac puncture, and postprandial [³H]TRLs were isolated from plasma by ultracentrifugation. The samples were pooled from multiple tubes and the radiospecific activity of the particles was calculated by liquid scintillation counting and BCA protein assay.

In some experiments, Hep3B cells were incubated for 15 min at 37°C in serum free media in the absence or presence of 2 mU/ml each of recombinant heparin lyases I, II, and III. Treated and untreated cells were washed twice with serum-free growth medium, chilled on ice for 20 min and then incubated with 50 µg/ml [³H]TRLs in serum-free medium at 4°C for 1 hr. Cells were rinsed with ice-cold PBS three times and solubilized by adding 0.1 M sodium hydroxide containing 0.1% sodium dodecyl sulfate for 45 min at room temperature. Bound TRLs was quantitated by liquid scintillation counting of the solubilized material, and the values were expressed as µg of TRL protein bound per mg of cell protein based on the radiospecific activity of the particles.

2.5.7 Reconstitution of apoE-deficient TRLs

Purified ApoE⁻/⁻ [³H]TRLs (50 µg) were incubated in the absence or presence of 50 µg recombinant human apoE3 (rhApoE3, R&D Systems) at 37°C for 1 hr in PBS (38). ApoE reconstituted [³H]TRLs were reisolated by ultracentrifugation, and both the top (δ<1.006 g/ml) and bottom fractions (δ>1.006 g/ml) were collected. Incorporation of rhApoE3 was verified by SDS-PAGE with silver staining and Western blotting of top and
bottom fractions using antibodies against human apoE (Abcam, ab1906, 1:1000), mouse apoAI (BioDesign K23500R; 1:200) and mouse apoAIV (Santa Cruz Biotechnology, sc-19036, 1:200). Reconstituted particles were used in binding studies described above.

2.5.8 Clearance of $[^3\text{H}]$TRLs

Overnight fasted $Ndst1^{AlbCre^-}$ and $Ndst1^{AlbCre^+}$ mice ($n = 3$) were injected via the tail vein with purified $[^3\text{H}]$TRLs from wildtype or apoE-deficient mice. In some experiments purified $[^3\text{H}]$TRLs from $Ndst1^{AlbCre^+}$ and $apoE^-\pm$ mice were immunodepleted of apoAV as described above and injected into wildtype mice ($n = 4$ per group). Serial blood samples were taken by submandibular venous puncture at the indicated times. TRL clearance was measured by liquid scintillation counting of an aliquot of plasma and expressing the values at different time points relative to the number of counts in the circulation 1 min after injection.

2.5.9 Retinyl ester excursion

$[11,12-^3\text{H}]$ retinol (25 µCi) (Perkin-Elmer, 43.0 Ci/mmol) in ethanol was mixed with 1 ml of corn oil (Sigma-Aldrich). Each mouse received 200 µl by oral gavage and blood was sampled at indicated times by submandibular puncture. Radioactivity in plasma was measured in duplicate by scintillation counting (55).

2.5.10 HSPG-TRL flotation assay

Hep3B cells were incubated for 24 hr in growth medium supplemented with 10% dialyzed fetal bovine serum (Gibco) containing 100 µCi/ml Na$[^35\text{S}]$O$_4$ (PerkinElmer Life
Science). Cells were treated for 5 hr in serum-free medium with 250 nM phorbol myristic acid (Sigma-Aldrich) to induce shedding of $[^{35}\text{S}]$syndecan-1 ectodomains (52). Shed $[^{35}\text{S}]$ectodomains were isolated from conditioned medium and purified by anion-exchange chromatography as previously described (92). $[^{35}\text{S}]$ectodomains (~5,000 counts in 15 µL) were incubated with 0 - 1 µg hTRLs in 200 µL of a solution of iodixanol (OptiPrep Density Gradient Medium, Sigma-Aldrich) prepared in 150 mM NaCl ($\delta=1.019$ g/ml) in the absence or presence of 1 µg of heparin (Celsus). For competition studies with monoclonal antibodies, hTRLs (1 µg) were mixed with the indicated concentration of purified antibody. The mixtures were incubated for 1 hr and centrifuged at 38,000 rpm (175,000 x g) for 3 hr at 18°C in a Beckman 42.2Ti rotor. The top 75 µL of each tube was removed and designated the top fraction. The remaining 125 µL was designated the bottom fraction. Both fractions were assayed for radioactivity by liquid scintillation counting, and the top fraction was expressed as a percentage of the total. In some experiments, $[^{35}\text{S}]$heparan sulfate was liberated from purified $[^{35}\text{S}]$HSPG ectodomains by beta-elimination (0.4 N sodium hydroxide for 24 hr at 4°C) and purified by anion-exchange chromatography.

Several mouse monoclonal antibodies against hTRL-associated proteins were used in competition experiments: unspecific mouse IgG (Jackson Immunoresearch, 015-000-003), anti-apoE (Abcam, ab1906), anti-apoAV (Santa Cruz Biotechnology, sc-32810), and anti-LPL (Abcam, ab21356). Ascites fluids with antibodies specific for apoB (MB2, MB3, MB11, MB19, MB43, and MB47) were a generous gift from Dr. Linda Curtiss (The Scripps Research Institute, La Jolla, CA) (57, 59, 93). IgG was purified from ascites fluid on a 1 ml Protein G chromatography cartridge (Pierce) following directions
provided by manufacturer. Inhibition curves were generated with each antibody up to a maximum of 1 µg/assay.

2.5.11 Plaque analysis

Fasted 6-month old Ndst1<sup>ff</sup> AlbCre<sup>+</sup>;ApoE<sup>−/−</sup> (n = 8) and Ndst1<sup>ff</sup> AlbCre<sup>+</sup>;ApoE<sup>−/−</sup> (n = 12) mice were perfused with 10 ml of PBS following cardiac puncture. The heart and ascending aorta down to the iliac bifurcation were removed and incubated in formalin. The heart and adventitial tissue were removed, the aortas were incised longitudinally, pinned flat and stained for neutral lipids using Sudan IV. Images were acquired using a Sony DXC-960MD CCD color video camera mounted on 4x objective/Nikon 80i microscope. Lesion area of blinded samples was measured using ImageJ analysis software (NIH). The data are expressed as the Sudan IV positive lesion areas within the aortic arch.

2.5.12 Statistical methods

Statistical analyses were performed using Prism software (version 5, GraphPad Software). The data was analyzed by two-tailed Student t-test or one-way ANOVA and is presented as mean ± SEM. P values less than 0.05 were considered significant.

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2.8 Supplementary Figures

Supplemental Figure S2-1. Analysis of apoE-deficient and reconstituted TRL particles. (A) Purified TRLs (1 µg) from wildtype and ApoE<sup>−/−</sup> mice were analyzed by SDS-PAGE and silver staining (n = 3 per genotype). The identification of the individual bands was determined by Western blotting with mAbs to apoE, apoB, and apoAIV, by molecular mass (apoC’s), and by loss of the bands in the mutants. (B) ApoE<sup>−/−</sup> TRLs (lanes 1 and 2) were reconstituted with rhApoE3 (lanes 3 and 4) and purified by ultracentrifugation (δ < 1.006 g/ml). The top (lanes 1 and 3) and bottom (lanes 2 and 4) fractions of each flotation were analyzed by SDS-PAGE and silver staining or (C) by Western blotting for apoE, apoAIV, and apoAI.
Supplemental Figure S2-2. Fasting plasma cholesterol from compound mutant mice. Plasma cholesterol was measured in fasting plasma samples collected from Ndst1^f/f AlbCre^- (n = 9, filled squares), Ndst1^f/f AlbCre^+ (n = 10, open squares), Ndst1^f/f AlbCre^-;ApoE^-^- (n = 19, filled circles), and Ndst1^f/f AlbCre^-;ApoE^-^- (n = 15, open circles) mice (8-weeks old).

Supplemental Figure S2-3. Analysis of apoB-restricted TRLs. Purified TRLs from ApoB^{100/100} and ApoB^{48/48} (1 µg, n = 3 per genotype) were analyzed by SDS-PAGE and silver staining. The identification of the individual bands was determined by molecular mass and by loss of the bands in the mutant.
Chapter 3

Endothelial Heparan Sulfate Proteoglycans Modulate Lipolysis of Triglyceride-rich Lipoproteins

3.1 Summary

Lipoprotein lipase (LPL) degrades the triglycerides in triglyceride-rich lipoproteins (TRLs) in the peripheral circulation, liberating free fatty acids for energy production and storage. Hydrolysis occurs when enzyme bound to the luminal surface of endothelial cells, encounters circulating TRLs. Recent genetic studies showed that the endothelial protein glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 (GPIHBP1) is the dominant receptor for LPL. Here, we used biochemical binding assays in combination with mutants in heparan sulfate assembly to reexamine the association of LPL with endothelial cells and the role of endothelial HSPGs in lipolysis. Flow cytometry assays performed with endothelial cells overexpressing GPIHBP1 demonstrated that a significant portion of LPL binding remains heparan sulfate dependent. Studies of CHO cells demonstrated that binding depends on the degree of sulfation of the chains. However, binding of LPL to endothelial cells derived from mice bearing defects in heparan sulfate sulfotransferases was unaltered and in vivo studies did not exhibit any alteration in TRL metabolism. Nevertheless, inactivation of the glycosyltransferase exostosin-1 resulted in elevated fasting and postprandial plasma triglycerides. These findings suggest that endothelial HSPGs act as a receptor for LPL and modulate triglyceride-rich lipoprotein metabolism.
3.2 Introduction

LPL is synthesized and secreted by parenchymal cells in the heart, skeletal muscle, and adipose tissue, and is translocated to the lumen of capillaries. At the luminal surface, circulating TRLs encounter LPL and undergo lipolytic processing in which LPL hydrolyzes triglycerides within the lipoproteins, releasing free fatty acids that can be used for energy or storage (1-3). LPL-deficiency in humans causes familial chylomicronemia, characterized by severe hypertriglyceridemia (>2000 mg triglycerides/dL), reduced plasma HDL, and episodes of pancreatitis (4). In mice, genetic ablation of LPL results in neonatal death due to complications arising from severe hypertriglyceridemia and chylomicronemia (>15,000 mg triglycerides/dL) (5). Impairments in the transport, function, or metabolism of LPL result in hypertriglyceridemia due to insufficient lipolysis of TRLs and increase the risk for cardiovascular disease (3, 6, 7).

LPL readily interacts with heparin through a cluster of basic amino acids near the carboxyl-terminus of the enzyme and thus was speculated to interact with the anionic heparan sulfate chains on proteoglycans expressed on endothelial cells (8, 9). Heparan sulfate proteoglycans (HSPGs) consist of a protein core with one or more covalently attached heparan sulfate chains that resemble heparin in structure (10, 11). Heparin and heparan sulfate assemble through sequential, coordinated reactions catalyzed by multiple biosynthetic enzymes. Among these, the glycosyltransferase complex consisting of exostosin 1 and 2 (Ext1/Ext2) catalyze chain elongation, the bifunctional GlcNAc N-deacetylase-N-sulfotransferases (Ndst1-4) generate N-sulfated glucosamines, and multiple O-sulfotransferases add sulfate to C2 of uronic acids (Hs2st) and to C6 (Hs6st1-3) and C3 (Hs3st1-6) of glucosamine units (12). These biosynthetic enzymes create
variably sized sulfated and non-sulfated regions that provide binding sites for protein ligands, such as LPL.

Early studies demonstrated that LPL could be released from the luminal surface of endothelial cells by intravenous injection of heparin and that LPL readily interacts with heparin, a highly sulfated form of heparan sulfate (HS) (13, 14). In vitro studies demonstrated that LPL also binds to HSPGs on the surface of aortic endothelial cells and that binding stabilizes and preserves enzyme activity (15, 16). The cluster of cationic amino acids on LPL and the high affinity of LPL for heparin suggested that the degree of sulfation on heparan sulfate chains would be important for mediating LPL binding to endothelial cells.

Recent genetic studies identified the extracellular endothelial protein GPIHBP1 as the dominant receptor responsible for immobilization of LPL at the luminal surface and for its transendothelial transport from its site of synthesis in the parenchyma (17, 18). GPIHBP1-deficient mice have severe defects in lipolysis, resulting in large TRL particles and hypertriglyceridemia (>3000 mg/dL). GPIHBP1 contains a domain rich in acidic amino acids that mimics the sulfated domains in heparin and heparan sulfate, providing an alternate explanation for the displacement of LPL from GPIHBP1 by exogenous heparin.

For unknown reasons, GPIHBP1 expression is lost when primary endothelial cells are passaged in culture (19). Thus, many of the biochemical binding studies of LPL to endothelial cells were done in the absence of GPIHBP1. Here, we have reevaluated the contribution of HSPGs in the immobilization of LPL on the luminal endothelial surface using a combination of biochemical studies and analysis of mutants altered in HSPG
assembly. We show that endothelial HSPGs are responsible for a significant portion of LPL binding in cells expressing both HSPGs and GPIHBP1, and that LPL binding to HSPGs is dependent upon interactions with the heparan sulfate chains. We further demonstrate that endothelial-specific ablation of heparan sulfate synthesis results in hypertriglyceridemia that is further exacerbated under postprandial conditions, and may be due to impaired lipolysis of TRLs. Our findings suggest endothelial HSPGs contribute to LPL-mediated lipolysis and provide further insight into this complex process.

3.3 Results

3.3.1 Heparan sulfate-dependent binding of LPL at the cell surface

To examine cell surface binding of LPL, we developed a flow cytometry-based assay in which cells were incubated with purified (bovine) biotinylated LPL (bLPL) and bound bLPL was detected via a streptavidin-conjugated fluorophore. Biotinylation of purified LPL did not alter enzymatic activity or affinity for heparin-Sepharose, indicating the enzyme retained its active-dimeric conformation (Supplemental Figure 1). To restore expression of GPIHBPI, bovine aortic endothelial cells (BAECs) were transduced with Gpihbp1 or empty vector and were analyzed for their capacity to bind bLPL. The transduced cells express GPHBP1 at very high expression levels as compared to freshly isolated cardiac endothelial cells (Figure 3-1 A, Right Panel). After incubation of the cells with bLPL, samples were solubilized and analyzed by SDS-PAGE and Western blotting, which showed that a single 56 KDa band, the molecular weight of bovine LPL. Under saturating conditions, Gpihbp1-transduced BAECs bound approximately twice as much LPL as compared to vector-transduced BAECs (13.6 ± 0.3 vs. 7.3 ± 1 fold change
in fluorescence; \( n = 3, \ P < 0.001 \) (Figure 3-1A, C). Heparin lyase treatment of \( Gpihbp1 \) transduced BAECs, which removes cell surface heparan sulfate, significantly reduced cell surface binding of LPL to that observed in vector-transduced cells (13.6 ± 0.3 before heparin lyase treatment vs. 7.4 ± 0.4 after treatment; \( n = 3, \ P < 0.001 \) (Figure 3-1, B and C). These results suggested that HSPGs make up a significant portion of binding sites for LPL in the presence or absence of GPIHBP1.

**Figure 3-1.** Heparan sulfate-dependent binding of LPL in the presence of GPIHBP1. (A) Left Panel: Binding of biotinylated LPL (bLPL) to transduced bovine aortic endothelial cells (BAECs) expressing \( Gpihbp1 \) (black line) or empty vector (gray line). Cells were incubated in the presence of 50 ng/mL of bLPL or in its absence (gray shaded). Right Panel: Western blot analysis for GPIHBP1 expression in cell lysate of transduced BAECs (\( n = 2 \) per cell line) and freshly isolated wildtype mouse cardiac endothelial cells (MCEC, \( n = 2 \)). (B) Binding of bLPL to \( Gpihbp1 \)-transduced cells before (black solid line) and after (black dashed line) treatment with heparin lyases. (C) Quantitation of flow cytometry analyses (\( n = 3 \)) for bLPL binding to control (gray) and \( Gpihbp1 \)-transduced cells (black) ± heparin lyase treatment. Data is presented as fold increase over the background measured in the absence of bLPL.
LPL preferably binds to both high and low sulfated oligosaccharide sequences in HS (20, 21). To examine how sulfation of the chains affects LPL binding, mutant Chinese Hamster Ovary (CHO) cell lines with mutations in HS biosynthetic enzymes were analyzed by flow cytometry. Incubation of wildtype CHO cells with bLPL demonstrated a robust fluorescent signal that was almost entirely HSPG-dependent based on heparin lyase treatment, consistent with the absence of GPIHBP1 expression in CHO cells (Figure 2A) (17, 22). CHO cells lacking the heparan sulfate copolymerase exostosin-1 (Ext1) also did not bind bLPL (Figure 2B). CHO cells with reduced N- and 2-O-sulfation also exhibited significantly reduced binding (~72% and 92% reduction, respectively) (Figure 2B). These data demonstrate that LPL binds to HSPGs dependent on the degree of sulfation of the heparan sulfate chains.

Figure 3-2. LPL binding to CHO cell heparan sulfate. (A) Binding of bLPL to wildtype (left) and (B) heparan sulfate-deficient CHO cells was measured before (black solid line) and after (black dashed line) treatment with heparin lyases. The filled shaded curve represents background signal obtained in the absence of added bLPL (C) Fold increase in geometric means (n = 3) for bLPL binding to wildtype CHO and CHO cells lacking heparan sulfate (pgsD677; Ext1<sup>−/−</sup>) or with reduced sulfation of heparan sulfate chains (pgsE606; Ndst1<sup>−/−</sup>, or pgsF17; Hs2st<sup>−/−</sup>).
3.3.2 Modifications to the fine structure of endothelial heparan sulfate does not impact LPL binding

The primary site of action for LPL-mediated lipolysis of TRLs is at the endothelial cell surface of the microvasculature, and impairments in the transport or function of LPL result in elevated plasma triglycerides (6). In light of the in vitro binding studies using CHO and BAEC cells, we examined if plasma triglycerides might be elevated in mice bearing defects in heparan sulfate biosynthesis specifically in endothelial cells (23, 24). Fasting plasma samples were taken from mice with endothelial targeted-deletion of N-deacetylase-N-sulfotransferase-1 (Ndst1\textsuperscript{Eff}Tie2Cre) or uronyl-2-O-sulfotransferase (Hs2st\textsuperscript{Eff}Tie2Cre). Interestingly, neither Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{+} or Hs2st\textsuperscript{Eff}Tie2Cre\textsuperscript{+} mice had elevated plasma triglycerides compared to their wildtype littermates (69 ± 7 mg/dL in Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{+} [n = 8] vs. 66 ± 4 mg/dL in Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{−} [n = 10]; P > 0.05; 59 ± 4 mg/dL in Hs2st\textsuperscript{Eff}Tie2Cre\textsuperscript{+} [n= 9] vs. 68 ± 5 mg/dL in Hs2st\textsuperscript{Eff}Tie2Cre\textsuperscript{−} [n = 9]; P > 0.05) (Figure 3A). Wildtype and mutant mice were also orally gavaged with corn oil, but plasma triglycerides were not elevated in either Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{+} or Hs2st\textsuperscript{Eff}Tie2Cre\textsuperscript{+} mice two hours post-gavage (168 ± 30 mg/dL in Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{+} [n = 4], 111 ± 13 mg/dL in Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{−} [n = 6]; P > 0.05; 142 ± 13 mg/dL in Hs2st\textsuperscript{Eff}Tie2Cre\textsuperscript{+} [n = 4] and 122 ± 16 mg/dL in Hs2st\textsuperscript{Eff}Tie2Cre\textsuperscript{−} [n =5]; P > 0.05) (Figure 3B). Retinyl ester excursion studies were also performed to measure if hepatic clearance rates of intestinally derived TRLs might be affected. No difference in hepatic clearance was detected between Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{+} and Ndst1\textsuperscript{Eff}Tie2Cre\textsuperscript{−} mice and wildtype mice as well (Supplemental Figure 2).
Primary microvascular endothelial cells were isolated from wildtype and mutant mice and examined by flow-cytometry using bLPL to determine if the alteration in heparan sulfate in the mutants affected LPL binding. Prior studies showed that inactivation of Ndst1 reduced overall sulfation of the chains by ~2-fold (23), whereas inactivation of Hs2st results in loss of 2-O-sulfation and increases in N- and 6-O-sulfation (24). Surprisingly, wildtype, Ndst1- and Hs2st-deficient primary microvascular endothelial cells displayed similar binding of bLPL (Figure 3C). Parallel experiments in which primary cells were incubated with biotinylated FGF2, a well-characterized HSPG ligand whose binding depends on the sulfation of HS (25), demonstrated significantly reduced binding to mutant endothelial cells as compared to the wildtype cells. Thus, altering the sulfation of endothelial HSPGs does not impair LPL-mediated binding in vitro or lipolysis in vivo.
3.3.3 Hypertriglyceridemia in mice with targeted inactivation of endothelial heparan sulfate biosynthesis

To more severely deplete endothelial cells of heparan sulfate, we decided to inactivate Ext1, which encodes one of the subunits of the heparan sulfate copolymerase (26). Germline inactivation of Ext1 in endothelial cells (Ext1<sup>Fl</sup>Tie2Cre<sup>-</sup>) causes

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**Figure 3-3.** Modifying endothelial heparan sulfate sulfation does not affect plasma triglycerides or LPL immobilization. (A) Fasting plasma triglycerides were measured from Ndst1<sup>Fl</sup>Tie2Cre<sup>-</sup> (n = 10), Ndst1<sup>Fl</sup>Tie2Cre<sup>+</sup> (n = 8), Hs2st<sup>Fl</sup>Tie2Cre<sup>-</sup> (n = 9), and Hs2st<sup>Fl</sup>Tie2Cre<sup>+</sup> (n = 9) mice. (B) Ndst1<sup>Fl</sup>Tie2Cre<sup>-</sup> (n = 6), Ndst1<sup>Fl</sup>Tie2Cre<sup>+</sup> (n = 4), Hs2st<sup>Fl</sup>Tie2Cre<sup>-</sup> (n = 5), and Hs2st<sup>Fl</sup>Tie2Cre<sup>+</sup> (n=4) mice were orally-gavaged with corn oil and plasma triglycerides were measured two-hours later. (C) bLPL binding to primary microvascular endothelial cells isolated from Ndst1<sup>Fl</sup>Tie2Cre<sup>-</sup> (gray line) and Ndst1<sup>Fl</sup>Tie2Cre<sup>+</sup> (black line) and (D) Hs2st<sup>Fl</sup>Tie2Cre<sup>-</sup> (gray line) and Hs2st<sup>Fl</sup>Tie2Cre<sup>+</sup> (black line).
embryonic lethality due to defects in vascularization (27, 28). To circumvent this problem, we inactivated Ext1 in adult mice using an endothelial-specific, tetracycline-inducible promoter ($rTA\text{-}Tie2\text{Cre}^+$) (29). $Ext1^{+/+}\text{-}rTA\text{-}Tie2\text{Cre}^+$ (control) and $Ext1^{f/f}\text{-}rTA\text{-}Tie2\text{Cre}^+$ (knockout) mice were administered doxycycline (~2.5 mg/kg/day) in 5% sucrose-water for 21 days, which resulted in >90% ablation of $Ext1$ expression (28). At this time, fasting plasma triglycerides from $Ext1^{f/f}\text{-}rTA\text{-}Tie2\text{Cre}^+$ were significantly elevated compared to doxycycline treated $Ext1^{+/+}\text{-}rTA\text{-}Tie2\text{Cre}^+$ mice (287 ± 29 mg/dL vs. 146 ± 13 mg/dL, $P = 0.0011$) (Figure 4A). This elevation in plasma triglycerides was further exaggerated in plasma samples two hours post-gavage of corn oil (569 ± 83 mg/dL vs. 214 ± 35 mg/dL, $P = 0.0046$) (Figure 4B).

To further investigate this phenotype, TRLs ($\partial < 1.006 \text{ g/ml}$) were isolated from postprandial plasma samples and the ratio of triglyceride to protein was determined. TRLs from $Ext1$-deficient mice had a triglyceride to protein ratio that was twice as large as TRLs from control mice (16 vs. 8 µg triglyceride/µg protein; $n = 4$ samples pooled), suggesting impaired lipolysis of circulating TRLs (Figure 4C). Taken together, these findings demonstrate that inactivation of $Ext1$ results in elevated plasma triglycerides possibly due to impaired lipolysis.
3.4 Discussion

The identification of GPIHBP1 as the dominant endothelial receptor mediating the endothelial transcytosis of LPL altered our perspective on LPL-mediated lipolysis. Prior to this discovery, it was long assumed that HSPGs were the endothelial receptors that immobilized and presented LPL at the luminal surface of the endothelium (6). This assumption was based on the observation that intravenous injection of heparin releases LPL and causes rapid clearance of postprandial lipemia (14, 30). Furthermore, immunocytochemical studies showed that LPL was localized at the luminal surface of the microvasculature (2, 31). LPL also has high affinity for heparin and heparan sulfate, and the association with heparin increases enzyme stability (15, 16, 32). Thus, the identification of GPIHBP1 as the primary endothelial receptor for LPL was surprising. Inspection of the primary sequence of GPIHBP1 demonstrated a negatively charged domain rich in aspartate and glutamate residues that was necessary for docking to LPL,
which contains a complementary domain rich in lysine and arginine residues. Consequently, the ability of heparin to displace LPL from its binding site on endothelial cells can be explained by its ability to bind to the positively charged domain in LPL and compete with acidic domain in GPIHBP1.

Several studies of CHO cells and CHO cell mutants altered in heparan sulfate have documented significant binding of LPL dependent on heparan sulfate (33-35) As shown here, binding of LPL to HSPGs on CHO cells depends on the degree of sulfation of the heparan sulfate chains based on studies of pgs606 cells, which lack Ndst1, and pgsF17 cells, which lack Hs2st (Figure 3). Analysis of endothelial cells also demonstrated saturable binding of LPL to HSPGs. Although binding increased after transfection with GPIHBP1, HSPGs accounted for nearly half of the total amount of LPL associated with the cells even when GPIHBP1 was overexpressed at very high levels. Based on these findings, we were surprised by the lack of hyperlipidemia in Ndst1^{Tie2Cre} and Hs2st^{Tie2Cre} mice, which bear mutations in the same genes that are altered in the CHO mutants. Subsequent analysis of endothelial cells derived from these mutants showed that LPL binding was not affected as in the CHO mutants, but nevertheless binding remained dependent on heparan sulfate based on loss of binding after treatment with heparin lyases. An explanation for the difference in LPL binding to CHO cells vs. endothelial cells is not yet available. Disaccharide analysis of heparan sulfate from endothelial cells and CHO cells show differences in composition (Table 3-1), and the arrangement of the disaccharides might differ, possibly resulting in differences in affinity of the binding sites. Prior studies suggest LPL has the capacity to bind to poorly sulfated heparan sulfate sequences, but how binding depends on the specific arrangement
of sulfated disaccharides is unknown (21). The types of proteoglycans expressed by the
two cells types may differ as well.

**Table 3-1.** Distribution of sulfate groups from wildtype and heparan sulfate deficient
models.

<table>
<thead>
<tr>
<th></th>
<th>Ndst1&lt;sup&gt;Δf&lt;/sup&gt;Tie2Cre&lt;sup&gt;+ &lt;/sup&gt;(23)</th>
<th>Ndst1&lt;sup&gt;Δf&lt;/sup&gt;Tie2Cre&lt;sup&gt;+&lt;/sup&gt; (23)</th>
<th>Hs2st&lt;sup&gt;Δf&lt;/sup&gt;Tie2Cre&lt;sup&gt;+&lt;/sup&gt; (24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Sulfate</td>
<td>42</td>
<td>28</td>
<td>60</td>
</tr>
<tr>
<td>2-O-Sulfate</td>
<td>16</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>6-O-Sulfate</td>
<td>29</td>
<td>14</td>
<td>38</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Wildtype (CHO-K1)(36)</th>
<th>Ndst1&lt;sup&gt;Δf&lt;/sup&gt;(pgsE-606)(37, 38)</th>
<th>Hs2st&lt;sup&gt;Δf&lt;/sup&gt;(pgsF-17)(36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Sulfate</td>
<td>45</td>
<td>21</td>
<td>65</td>
</tr>
<tr>
<td>2-O-Sulfate</td>
<td>30</td>
<td>12</td>
<td>&lt;1</td>
</tr>
<tr>
<td>6-O-Sulfate</td>
<td>25</td>
<td>10</td>
<td>34</td>
</tr>
</tbody>
</table>

Although changes in sulfation did not cause elevation in plasma triglycerides,
targeted inactivation of Ext1 in Ext1<sup>Δf</sup>rTA-Tie2Cre<sup>+</sup> mice resulted in fasting and post-
prandial hypertriglyceridemia, uncovering a role for endothelial heparan sulfate in
lipolysis. Induction of Cre under these condition results in ~90% loss of endothelial
heparan sulfate (28), suggesting that the alterations in sulfation induced in
Ndst1<sup>Δf</sup>Tie2Cre<sup>+</sup> and Hs2st<sup>Δf</sup>Tie2Cre<sup>+</sup> mice were simply insufficient to uncover the
contribution of heparan sulfate to LPL binding. Interestingly, the large reduction of
heparan sulfate expression in Ext1<sup>Δf</sup>rTA-Tie2Cre<sup>+</sup> mice does not result in gross
abnormalities to the vasculature or altered expression of other endothelial proteins such
as CD31, although differences in lymphocyte homing and chemokine presentation have
been noted (28). The observation of hypertriglyceridemia and accumulation of particles that resemble chylomicrons suggests that removal of heparan sulfate in this way alters LPL presentation or activity. Additional studies are needed to confirm this conclusion.

Intravenous injection of heparin into wildtype mice results in dramatic release of LPL into the plasma (14, 30). Heparin also induces rapid release of ~30% of LPL in Gpihbp1<sup>−/−</sup> mice, consistent with the idea that a portion of LPL might be bound to other sites, such as heparan sulfate (17). Interestingly, there is also a difference in severity of hypertriglyceridemia in Lpl<sup>−/−</sup> mice, which die hours after birth with plasma triglycerides >15,000 mg/dL, and Gpihbp1<sup>−/−</sup> mice, which develop normally and have plasma triglycerides >3000 mg/dL (5, 17). This difference in degree of triglyceride accumulation could be explained by a second pool of LPL bound to HSPGs (17, 39). Whether this pool exists simply because of the absence of GPIHBP1 in Gpihbp1<sup>−/−</sup> mice is difficult to assess.

Another plausible explanation for hypertriglyceridemia in Ext1<sup>fl/fl</sup>rTA-Tie2Cre<sup>+</sup> mice concerns collagen XVIII (Col18), a ubiquitous component of basement membranes (40). Defects in the HSPG collagen XVIII (Col18) results in delayed lipolysis and mild chylomicronemia due to decreased presentation of LPL on the lumenal side of the endothelium and in the plasma, suggesting that some tissue LPL may be normally bound to HSPGs (41). Col18 contains 1-3 heparan sulfate chains and mutations affecting the splice variant expressed in vascular tissues results in thickening of basement membranes and alterations in LPL transfer from the parenchyma. Thus, alterations in Ext1 expression in the endothelium could affect the assembly of HS chains on Col18, which then affects interstitial transport of LPL, a step upstream from GPIHBP1 mediated
transendothelial transport. We are currently examining if the hyperlipidemia observed in $Ext^{fr}rTA-Tie2Cre^+$ mice might be due to alterations in Col18 assembly or expression.

Based on the impact of altering GPIHBP1 on plasma triglycerides, GPIHBP1 is clearly the dominant endothelial protein for LPL-mediated lipolysis, but our findings suggest that endothelial HSPGs also play a significant role. Work is underway that focuses on elucidating the mechanism of endothelial HSPGs in the catabolism of triglyceride-rich lipoproteins.

3.5 Methods

3.5.1 Cell culture

Bovine aortic endothelial cells transduced with a Lentiviral vector containing $Gpihbp1$ (or empty vector control) were obtained from Dr. Stephen Young at UCLA (18). Transduced BAECs were grown in MCDB-131 complete media (VEC Technologies) and kept under puromycin selection (0.5µg/ml). Wild-type CHO cells (CHO-K1) were obtained from the American Type Culture Collection, Manassas VA (CCL61). Culture conditions and CHO mutants pgsA745 ($XylT2^−$, deficient in all sulfated glycosaminoglycans), pgsD677 ($Ext^−$, deficient in heparan sulfate), pgsE606 ($Ndst1^−$, deficient in N-sulfation of heparan sulfate), pgsF17 ($Hs2st^−$, deficient in 2-O-sulfation of heparan sulfate) were described previously (36, 42-44). Isolation and culture of mouse lung microvascular endothelial cells from adult mice was performed as described (23, 45).

3.5.2 Isolation of primary endothelial cells
Lungs were excised from adult wildtype and mutant mice, and the tissue was minced and digested with collagenase type II (Worthington Biochemical, LS004176). The cell suspension was centrifuged in Percoll (Fisher Scientific, GE17089102), a low osmotic, non-toxic silica colloid density medium that forms density gradients during centrifugation. The cells that banded at the Percoll-PBS interface were enriched for the endothelial cells by microbead cell selection with anti-CD31 (platelet endothelial cell adhesion molecule, PECAM-1) antibodies (Invitrogen, RM52043). The anti-CD31 antibodies were bound by an anti-IgG antibody fused to a magnetic microbead (MACS, Miltenyi Biotec, 130-048-501). Application of a magnetic field retained the labeled cells along the sides of the tube. Analysis of the final cell preparation by flow cytometry with anti-CD31 mAb (Invitrogen, RM52043) showed that the cells were greater than 97% pure.

3.5.3 Lipoprotein lipase

Bovine LPL generously provided by Gunilla Olivecrona (Department of Biomedical Sciences, Umeå University, Umeå Sweden)(46). Enzyme activity was determined using $^3$H radiolabeled substrate as previously described (47). Molar ratio of biotin to LPL was determined in a HABA displacement assay (Pierce Biotin Quantitation Kit).

LPL binding assays were performed similar to as previously described (45, 48). Cells were harvested using Accutase cell detachment solution (Millipore) and washed twice with PBS. Cells were incubated for 15 min at 37°C in serum free media in the absence or presence of 5 mU/ml each of recombinant heparin lyases I, II, and III. Treated
and untreated cells were washed twice with PBS, chilled on ice for 20 min and incubated with 50 nM biotinylated LPL in 1% BSA supplemented PBS at 4°C for 1 hour. Following the incubation, cells were washed twice in ice-cold PBS and incubated with 0.4 µg/mL Phycoerythrin-Cy5 conjugated Streptavidin in 1% BSA supplemented PBS at 4°C. A set of cells was exposed only to Phycoerythrin-Cy5 conjugated Streptavidin and was used as background control. Cells were washed twice with PBS and analyzed by flow cytometry. As a control for heparan sulfate-dependent binding, binding studies were performed in parallel with 5 nM biotinylated FGF2 ± heparin lyase treatment.

3.5.4 Heparin-Sepharose chromatography

Affinity chromatography was performed using an FPLC-System (Biologic DuoFlow Chromatography Systems, BioRad) with a 1mL HiTrap™ Heparin HP column (GE Healthcare) at 4°C as described (34).

3.5.5 Mice and animal husbandry

Generation and characterization of Ndst1^{fl/fl}Tie2Cre+ and Hs2st^{fl/fl}Tie2Cre+ mice has been described previously (23, 24, 45). Animals were housed in Association for Assessment and Accreditation of Laboratory Animal Care-approved vivaria in the School of Medicine, University of California San Diego following the standards and procedures approved by the local Institutional Animal Care and Use Committee. Mice were weaned at 3 weeks, maintained on a 12-hr light-dark cycle, and fed water and standard rodent chow ad libitum (Harland Tekland). Ext1^{fr} rTA-Tie2Cre+ mice were provided by Dr. Minoru Fukuda and housed at Sanford-Burnham Medical Research Institute (28). Mice
were treated according to guidelines of the National Institutes of Health, and experiments were approved by the Animal Research Committee of the Sanford-Burnham Medical Research Institute. Genotyping was performed as described in the original publications.

### 3.5.6 Plasma triglyceride analysis

Plasma was prepared from submandibular bleeds after fasting the animals for 6 hours in the morning. (10 µL/gram body weight) of corn oil (Sigma) was administered to mice by oral gavage and blood was sampled at various time points. Plasma samples were prepared from blood drawn via submandibular puncture from overnight fasted mice. Total cholesterol and triglyceride levels were determined enzymatically (Triglyceride-SL and Cholesterol-SL, Sekisui Diagnostics) using an automated plate reader (Cobas Mira; Roche Diagnostics).

### 3.5.7 Retinyl ester excursion

[11,12-³H] retinol (25 µCi) (Perkin-Elmer, 43.0 Ci/mmol) in ethanol was mixed with 1 ml of corn oil (Sigma-Aldrich). Each mouse received 200 µl by oral gavage and blood was sampled at indicated times by submandibular puncture. Radioactivity in plasma was measured in duplicate by scintillation counting (49).

### 3.5.8 Statistical methods

Statistical analyses were performed using Prism software (version 5, GraphPad Software). The data was analyzed by unpaired two-tailed Student t-test or one-way
ANOVA and is presented as mean ± SEM. $P$ values less than 0.05 were considered significant.

3.6 Acknowledgments

This work will be completed and submitted for publication to *Arteriosclerosis, Thrombosis, and Vascular Biology* with coauthors Ding Xu, Motohiro Nonaka, Philip L.S.M. Gordts, Minoru Fukuda, and Jeffrey D. Esko. The dissertation author was the primary author of the work contained in this chapter. Together with his coauthors he thanks Steve Young and Brandon Davies for providing the transduced BAECs and Gunilla Olivecrona for providing the bLPL. This work was supported by a Ruth L. Kirschstein NRSA award F31HL977212 (to J.C.G.), and NIH grant GM33063 (to J.D.E). Work was conducted within the department of Cellular and Molecular Medicine and the Glycobiology Research and Training Center, University of California, San Diego, La Jolla, California.
3.7 Literature cited


3.8 Supplemental Figures

**Supplemental Figure 3S-1.** Biotinylated LPL retains its active dimeric conformation. FPLC chromatograph of 100µg of biotinylated LPL on a HiTrap™ Heparin HP column. UV absorbance at 280nm (solid) and NaCl concentration (dashed) were continuously measured.

**Supplemental Figure 3S-2.** Clearance of dietary lipids is not different when *Ndst1* is inactivated in the endothelium. Retinyl ester excursions were measured at the times indicated in *Ndst1<sup>EF</sup>Tie2Cre<sup>−</sup>* (open circles, n = 4) and *Ndst1<sup>EF</sup>Tie2Cre<sup>+</sup>* mice (filled circles, n = 6). Mice were given 200µL of corn oil containing [³H]retinol by oral gavage. Blood samples were taken at the indicated times and radioactivity in 10µL serum was measured by liquid scintillation counting.
4.1 Syndecan-1

In 2007, our laboratory genetically demonstrated that hepatic heparan sulfate proteoglycans (HSPGs) mediate the clearance of remnant triglyceride-rich lipoproteins (TRLs) independently of the other major hepatic endocytic lipoprotein receptors LDLR and LRP1 (1). When I began this thesis work in 2008, the laboratory was in the midst of studies focused on the identification of the relevant HSPG, which led to the identification of syndecan-1 (Sdc1) as the primary hepatic proteoglycan that mediates remnant TRL clearance (2). Based on the structural features of Sdc1, we developed a model for Sdc1 interaction with TRLs (Figure 4-1). We then set out to test this model through two approaches: characterizing the structural-components of Sdc1 and identifying the ligand(s) on TRLs that mediate binding to Sdc1 heparan sulfate chains.

Figure 4-1. Structural features of syndecan-1 (reprinted with permission from (3)).
Sdc1 is a 311-amino acid type-I transmembrane proteoglycan, containing three attachment sites for heparan sulfate distal from the membrane (amino acids 37, 45, and 47), and two sites for chondroitin sulfate attachment more proximal to the membrane (amino acids 217 and 227) (4, 5) (Figure 4-1). Occupation of these sites by glycosaminoglycan chains varies in different tissues and has not been characterized in hepatocytes, but the most active form of Sdc1 in other systems contains heparan sulfate at all three attachment sites (6, 7). In the proposed model we depict the interaction of TRLs with syndecan by way of multiple interactions with the heparan sulfate chains on syndecan-1 (3). In this model, termed the “proteoglycan hug,” the lipoprotein particle docks to each of the three-heparan sulfate chains by way of protein ligands on the surface of the lipoprotein particle (2). This model seemed reasonable for several reasons. First, remnant triglyceride-rich lipoprotein particles have diameters ranging from 40-80 nm and a molecular mass of ~5 x 10^7 Da (8). The average heparan sulfate chain in the liver consists of ~50 sugar residues and is about 50 nm in length, which is approximately the diameter of a TRL (9, 10). Most protein ligands that bind to heparin or heparan sulfate interact by way of a small segment of the chain (~4-10 sugar residues) (11). Given the relatively large size of the TRL remnants, it seemed unlikely that the interaction of a single protein with a small segment of a chain would be sufficient to tether the lipoprotein particle to Sdc1. Rather, a more likely explanation was that TRLs required multiple interactions with heparan sulfate to stabilize the lipoprotein-proteoglycan complex prior to and after endocytosis.

This model could also explain why syndecan-1, which has three heparan sulfate attachment sites, is better suited to facilitate hepatic clearance of TRL remnants as
compared to the other HSPGs expressed by hepatocytes. Additional support of the “hug” model comes from preliminary studies in which recombinant forms of syndecan-1 were expressed by way of adenoviral vector in the livers of Sdc1−/− mice (E.M. Foley and J.D. Esko, unpublished observations). Intriguingly, syndecan-1 lacking a single heparan sulfate attachment site at residue 45 could not rescue HSPG-mediated TRL clearance in mice, whereas wildtype syndecan-1 did. Future structure-function studies of syndecan-1 using mutants lacking individual chains at different sites or multiple chains should provide further insight into this interesting problem.

The proposed model also depicts the heparan sulfate chains of Sdc1 binding to a protein ligand on the surface of the TRLs. Multiple heparin binding proteins had previously been suggested to mediate TRL binding to hepatic HSPGs, including apoB-48 and apoB-100 (12-15), apoE (16-21), apoAV (22), LPL and HL (23-26). Chapter 2 contains data showing that apoE and apoAV are the dominant ligands on TRLs that mediate binding to Sdc1. The requirement for two ligands is consistent with the “proteoglycan hug” model in that the individual chains can dock with multiple ligands. Using the flotation assay, we found that both apoE and apoAV can interact with a single heparan sulfate chain. Thus, we need to modify the model shown in Figure 4-1 somewhat to include multivalent interactions with individual chains. Precedence for this concept has recently been provided by a study showing that two HDL apolipoproteins, serum amyloid A (SAA) and apoAI, simultaneously bind to a single heparan sulfate chain (27). Future studies should focus on mapping the relevant heparin binding sites on the apolipoproteins and the sequence of sulfated sugars in heparan sulfate that mediate binding.
The proposed model cannot properly depict the unique microenvironment of the hepatic Space of Disse where Sdc1 is concentrated on hepatocyte microvilli and poised to capture TRLs (2, 28). Hepatocytes are the major site of synthesis for apoE and apoAV, and apoE is found at high concentration on the sinusoidal side of the basal membrane of hepatocytes (29). While apoAV localization in the Space of Disse has not been analyzed, it is known that plasma apoAV is synthesized and secreted by hepatocytes (30). Thus one can speculate that within the space of Disse the local concentrations of these apolipoproteins might favor a dual “enrichment-capture” mechanism in which remnant lipoproteins could become enriched for apoE and apoAV; thus facilitating binding to Sdc1 poised on the microvilli of the hepatocytes (30-32).

Sdc1 accounts for a large proportion of TRL clearance, with estimates as high as 50% of fasting TRL clearance and as much as 70% post-prandial TRL clearance (2). Interestingly, apoE is enriched on post-prandial TRL particles (9-15 apoE per particle) compared to fasting TRLs (5-7 per particle) (33, 34). Other studies have suggested that plasma apoAV concentrations are elevated in post-prandial conditions (32, 35), and Western blot analysis of fasting vs. postprandial TRLs from wildtype mice found significant enrichment of both apoE and apoAV on postprandial TRLs (Gonzales, J.C. and Esko, J.D. Unpublished observations). These findings support the idea that post-prandial TRLs become enriched for the ligands that facilitate their clearance through Sdc1.

4.2 Hepatic HSPG clearance of lipoproteins provides atheroprotection
Atherosclerosis is characterized by the accumulation of lipids, inflammatory cells and fibrous elements in the large and medium-sized arteries in fibrofatty plaques and is the primary cause of cardiovascular disease (36-38). Hypertriglyceridemia is an independent risk factor for cardiovascular disease and there is increasing evidence that remnant TRLs contribute to the development of atherosclerosis (39-42). The studies of atherosclerosis described in chapter 2 support this conclusion and provide the first evidence that TRL clearance mediated by apoE and apoAV binding to HSPGs is atheroprotective. This conclusion is in agreement with other studies showing that transgenic over-expression of human apoAV, which reduces plasma triglycerides, provides atheroprotection in mouse models of dyslipidemias (43, 44).

ApoE<sup>−/−</sup> mice exhibit impaired lipoprotein clearance, elevated plasma triglycerides and cholesterol, and is a well-established mouse model for spontaneous development of atherosclerosis (45, 46). These characteristics have been attributed to the essential role of apoE as a requisite ligand for the clearance of lipoproteins through hepatic endocytic receptors. Intriguingly, the observation that Ndst1<sup>fl/fl</sup>AlbCre<sup>+</sup>;ApoE<sup>−/−</sup> mice have elevated plasma triglycerides, but not plasma cholesterol, and significantly increased atherosclerosis suggests that impaired clearance of triglyceride-rich lipoproteins drives the more extensive development of plaques in this model. Our findings are based on a single time point (6-month old Ndst1<sup>fl/fl</sup>AlbCre<sup>+</sup>;ApoE<sup>−/−</sup> mice). To further understand the progression of disease in this model, plaque development should be measured in 1, 2, 4 and 6-month old mice and correlated with fasting and postprandial plasma triglyceride and cholesterol levels. Studies of Ndst1<sup>fl/fl</sup>AlbCre<sup>+</sup>Ldlr<sup>−/−</sup> mice on normal chow and high fat diet should be undertaken to confirm these results and to exclude complicating effects
of apoE deficiency. Localization studies using labeled-TRLs to detect TRL-remnants within lesion areas would also be informative.

4.3 Can variants of APOE, APOA5, GLCE and other heparan sulfate biosynthetic enzymes explain cases of hypertriglyceridemia?

The identification of apolipoproteins E and AV as the dominant ligands that mediate TRL clearance through HSPGs provides insight into an important mechanism that mediates triglyceride metabolism. Impairments in this mechanism could provide explanation for hypertriglyceridemia of unknown etiology in patients and warrants further evaluation of the genetic variants in APOE, APOA5, and genes involved in heparan sulfate biosynthesis.

There are three major isoforms of apoE (apoE2, E3, and E4) which differ at two amino acids sites on the protein (112 and 158). ApoE3, the most common isoform, has a cysteine and arginine at positions 112 and 158, respectively, whereas apoE4 has arginine residues at both sites and apoE2 has cysteine residues (16, 47). ApoE3 and apoE4 are equally capable of interacting with the hepatic endocytic receptors LDLR and LRP1, whereas apoE2 has impaired binding to LDL receptors. All three isoforms bind to HSPGs (20). For reasons not fully understood, only ~10% of individuals homozygous for apoE2 develop hyperlipidemia and cardiovascular disease, whereas most individuals are normo- and even hypocholesterolemic with no increase in cardiovascular risk (48). The development of overt hyperlipidemia therefore might require a second genetic or environmental factor in addition to apoE2 expression. Our findings suggest that genetic
variation in apoAV or in hepatic heparan sulfate composition could be factors that
determine whether apoE2/E2 individuals develop dysbetalipoproteinemia.

Another rare variant of apoE, apoE-Leiden is associated with hyperlipidemia and
increased cardiovascular risk as well (49). ApoE-Leiden is a variant of apoE3, containing
an in-frame insertion of seven amino acids (50). Interestingly, apoE-Leiden significantly
reduces the capacity of apoE to bind to HSPGs, but not to LDLR and LRP1. Further
studies of allelic variants of APOE (and APOA5) that affect heparin-binding are clearly
warranted to determine if altered TRL-HSPG interactions contribute to
hypertriglyceridemia and atherosclerosis in humans. In mice, Ndst1<sup>Le</sup>AlbCre<sup>+</sup> mice could
be bred to transgenic apoE-Leiden mice, which like humans exhibit hyperlipidemia and
increased atherosclerosis (51). One would expect no accentuation of atherosclerosis and
hyperlipidemia in the compound mutant if the impairment in ApoE-Leiden was due to
altered HSPG-TRL interactions.

ApoAV was originally identified by comparative genome sequencing and was
found to have a strong association with plasma triglycerides and single nucleotide
polymorphisms (SNPs) across the APOA5 locus in humans (52). Human population
studies have shown a positive correlation between single nucleotide polymorphisms in
APOA5 and plasma triglycerides, particularly in individuals with hypertriglyceridemia
(53-55). Our findings suggest that impairments in HSPG-mediated TRL clearance could
account for this positive correlation and suggest that elevated plasma apoAV could be a
marker for impaired hepatic clearance of remnant lipoproteins mediate by HSPGs.

Another factor that might contribute to variation in plasma triglycerides is the
composition of heparan sulfate, which varies amongst different individuals (56). Recent
studies of a Turkish population showed that SNPs in the heparan sulfate enzyme glucuronic acid epimerase (GLCE) were associated with elevated triglyceride and HDL cholesterol levels, a finding supported by studies of Glce+/− mice fed a high fat diet (57). These findings suggest further analyses of hepatic heparan sulfate and allelic variants of genes involved in heparan sulfate metabolism to determine if other alterations of HSPGs could also contribute to hypertriglyceridemia and atherosclerosis.

Another way to study the contribution of HSPGs to hypertriglyceridemia and atherosclerosis would be to employ the mouse hybrid diversity panel, which consists of 114 inbred mouse strains derived from the classical inbred strains of mice (58). Plasma lipid traits have been analyzed and correlated with genetic variation and gene expression levels in the strains (59). With access to these databases, one could examine if SNPs in the genes of interest (Apoa5, Apoe, Sdc1, Glce, other heparan sulfate biosynthetic enzymes) correlate with plasma triglyceride levels. If correlations exist, then the effect of particular SNP variants on syndecan-1 expression, heparan sulfate composition, binding characteristics to apolipoproteins, and capacity of the apolipoprotein variants to bind to heparan sulfate would need to be determined. Collectively, this approach could identify important genetic variants that impact plasma triglyceride levels.

### 4.4 Endothelial heparan sulfate proteoglycans and lipolysis

A second major question addressed by this thesis concerned the role of endothelial heparan sulfate in affecting lipolysis of circulating lipoproteins. Lipolysis of circulating TRLs occurs by the action of LPL located at the lumen of the endothelium. This process requires the coordination of multiple proteins to facilitate the transport and
luminal presentation of LPL. GPIHBPI is the dominant endothelial protein responsible for the transport of LPL across the endothelium (60). Genetic evidence supports this conclusion as Gpihbp1−/− mice have severe chylomicronemia and mislocalization of LPL to the basement membrane and interstitial spaces surrounding myocytes and adipocytes.

The findings described in Chapter 3 demonstrate that targeted inactivation of endothelial Ext1 results in fasting and postprandial hypertriglyceridemia, and suggests that endothelial HSPGs also play a significant role in the catabolism of circulating TRLs. This novel finding opens questions regarding the etiology of the hypertriglyceridemia in Ext1-deficient mice and the role of endothelial HSPGs in LPL-mediated lipolysis of TRLs.

**Figure 4-2.** Potential mechanisms of the role of endothelial HSPGs in LPL-mediated lipolysis of triglyceride-rich lipoproteins. (A) Endothelial HSPGs immobilize LPL at the cell surface. (B) Endothelial HSPGs are important for TRL margination.
One hypothesis is that endothelial HSPGs are an important receptor (alongside GPIHBP1) that immobilize LPL at the endothelial surface (Figure 4-2, A). The in vitro findings described in Chapter 3 (Figure 3-1) demonstrate that a significant proportion of LPL binding to endothelial cells is heparan sulfate-dependent in the presence or absence of GPIHBP1 expression. These findings are in line with other in vitro studies performed with Chinese Hamster Ovary cells that found a significant increase in LPL binding in cells expressing GPIHBP1 (and heparan sulfate) as compared to glycosaminoglycan-deficient cells expressing GPIHBP1 (61). Gpihbp1+/− mice have a significant (~30% as compared to wildtype mice) heparin-releasable pool of LPL, which is consistent with the idea that some LPL might be immobilized by way of HSPGs. Thus, the hypertriglyceridemia we observed in Ext1-deficient mice might be due to the impaired immobilization of LPL at the luminal surface of the endothelium.

To test this hypothesis, it is critical to determine the distribution of LPL in Ext1-deficient (Ext1fl/fl rTA-Tie2Cre+) mice. An increase in plasma LPL in Ext1-deficient mice would support the hypothesis that some of the enzyme is immobilized by HSPGs. If correct, then a portion of the enzyme should be releasable by intravenous injection of Intralipid (a fat emulsion), which displaces luminally-bound enzyme (62). How this value relates to the pool of enzyme released by heparin could also be addressed. Immunohistochemical analysis of LPL in tissue sections from the heart, skeletal muscle, and adipose tissues of wildtype and Ext1-deficient mice would also be informative.

The second hypothesis is that endothelial HSPGs might play a role in TRL docking to the capillary surface, where it can be lipolytically processed by LPL residing on GPIHBP1 (Figure 4-2, B). In support of this hypothesis, studies have suggested that
arterial proteoglycans can mediate the binding of circulating lipoproteins at least in the context of atherosclerotic lesions (63-65). The observation that both apoAV and LPL bind to heparan sulfate (22) and our recent demonstration of the importance of apoAV in remnant TRL clearance in the liver (Chapter 2) also supports the possibility that endothelial HSPGs might dock to TRLs.

To study TRL margination, analysis of fluorescently-labeled TRL binding to wild-type and Ext1-deficient hearts could be performed. In these experiments, mice would be perfused with fluorescently-labeled TRLs and then quickly sacrificed. Heart, liver, and other relevant tissue would be excised and paraffin embedded. Visualization of fluorescent-TRLs bound within the heart tissue can be achieved on tissue sections using fluorescent imaging systems and quantitated to give an overall amount of TRL bound to each section of tissue. One would predict co-localization of TRLs with HSPG and GPIHBP1.

A third possible explanation for hypertriglyceridemia in Ext-1 deficient mice arises from a recent study by our lab showing that the HSPG collagen XVIII (Col18) is important for mediating the subendothelial transport of LPL from the enzyme’s site of synthesis (parenchymal cells) to basoluminal GPIHBP1 (66). Importantly, the study demonstrated that humans lacking a vascular splice variant of Col18, or mice with genetic deletion of this splice form, exhibit elevated fasting plasma triglycerides and reduced luminal presentation of LPL. A corresponding decrease in pre-heparin plasma LPL was also detected in Col18ΔΔ mice, further highlighting the impaired subendothelial transport of the enzyme. Assuming that endothelial cells are the primary source of
subendothelial Col18, inactivation of endothelial Ext1 might affect the heparan sulfate chains on Col18, thus phenocopying Col18-/- mice.

Understanding the role of endothelial HSPGs in mediating the lipolysis of circulating TRLs in the microvasculature is a complex issue. The findings described in Chapter 3 represent an important first step that identified a role for HSPGs in this process. The experiments described above will provide further insight into this system.

4.5 Concluding remarks

Hypertriglyceridemia is characterized by the accumulation of triglyceride-rich lipoproteins and is now recognized as an important risk factor for cardiovascular disease. (67-72). Hypertriglyceridemia (>150mg/dl) is prevalent in the United States, affecting an estimated 10-20% of the population (and 25-35% of adults over the age of 20 according to the Third National Health and Nutrition Examination Survey) (39-42). Most cases of hypertriglyceridemia are complex, resulting from a mixture of predisposing genetic and environmental factors, and only in rare instances is hypertriglyceridemia the result of homozygosity for recessive alleles in genes related to triglyceride metabolism, including GPIHBP1, LPL, LMF1, and APOC2 (33, 73). Thus, a thorough understanding of the multiple factors involved in triglyceride-rich lipoprotein metabolism are critically important for understanding the cause of hypertriglyceridemia. Prior genetic studies have demonstrated the important contribution of hepatic heparan sulfate proteoglycans in mediating hepatic clearance of triglyceride-rich lipoproteins (1-3). The goal of this thesis was to characterize the interactions that take place between heparin-binding proteins involved in lipolysis and clearance and heparan sulfate proteoglycans. The experiments
described in chapter 2 led to the identification of apolipoproteins E and AV as the dominant ligands that mediate lipoprotein clearance through hepatic HSPGs, an important finding that will inspire future studies into the structural basis of these interactions. Furthermore, the demonstration that heparan sulfate-mediated clearance of triglyceride-rich lipoproteins provides atheroprotection in mice will undoubtedly encourage future studies of the proteoglycans in the development of atherosclerosis in humans.
4.6 Literature cited


