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Analysis of Protein Structure-Function *in Vivo*

ADENOVIRUS-MEDIATED TRANSFER OF LIPASE LID MUTANTS IN HEPATIC LIPASE-DEFICIENT MICE*

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Hepatic lipase (HL) and lipoprotein lipase (LPL) are key enzymes involved in the hydrolysis of triglycerides and phospholipids present in circulating plasma lipoproteins. Despite their similarities, the role that each of these two lipases play in the metabolism of triglyceride-rich lipoproteins and high density lipoproteins is distinct. In order to identify structural domains that may confer the different substrate specificities between HL and LPL, we have utilized a novel approach for performing structure-function analysis of a protein, *in vivo*, by using recombinant adenovirus vectors to express native and mutant enzymes in an animal model for a human genetic deficiency. HL-deficient mice (n = 19) characterized by increased plasma cholesterol and phospholipid concentrations were injected with adenovirus expressing luciferase (rLucif-AdV), native hepatic (rHL-AdV), and lipoprotein lipase (rLPL-AdV) or lipase mutants in which the lid covering the catalytic site of either enzyme was exchanged (rHL+LPL lid-AdV and rLPL+HL lid-AdV). Mice injected with rLucif-AdV had no changes in post-heparin HL and LPL activities (217 ± 29 and 7 ± 2 nmol/min/ml, respectively) as well as plasma lipids. Despite expression of similar levels of post-heparin plasma lipase activity on day 5 post-adenovirus infusion (9806 ± 915 and 9677 ± 2033 nmol/min/ml, respectively) mice injected with rHL-AdV or rHL+LPL lid-AdV demonstrated marked differences in the reduction of plasma phospholipids (70% and 32%, respectively, p < 0.005). Similarly, despite post-heparin plasma lipolytic activities of 4495 ± 534 and 4844 ± 1336 nmol/min/ml, injection of rLPL-AdV or rLPL+HL lid-AdV resulted in phospholipid reductions of 31% and 81% (p < 0.005). Exchange of the lipase lid did not significantly alter plasma triglyceride concentrations. Thus, preferential *in vivo* hydrolysis of phospholipids was demonstrated in animals expressing lipases containing the HL lid but not the LPL lid. These studies identify the lipase lid as a major structural motif responsible for conferring the different *in vivo* phospholipase activities between HL and LPL, a function which may modulate the distinct physiological roles of these two similar lipolytic enzymes in lipoprotein metabolism. The use of recombinant adenovirus to express mutant proteins in animal models for human genetic deficiencies represents a powerful, new approach for performing structure-function analysis of proteins *in vivo*.

Our understanding of the biological function that a variety of different enzymes, receptors, and transfer proteins play in lipid metabolism has been greatly enhanced by the analysis of functional and mutant proteins synthesized in different *in vitro* expression systems. One of the major limitations of this approach, however, relates to our ability to extrapolate *in vitro* structure-function studies to *in vivo* physiologic processes. In the present report, we describe a method that permits the identification of important functional domains of a lipolytic enzyme, by using recombinant adenovirus to express native and mutant proteins in an animal model for a human genetic deficiency. This approach permits structure-function analysis of proteins *in vivo*, thus circumventing the limitations of as well as complementing the information obtained from *in vitro* expression studies.

As a model system to test this approach, we investigated potential structural elements that could mediate the difference in the phospholipase properties of HL[1] and LPL. HL and LPL are endothelial-bound lipolytic enzymes that hydrolyze triglycerides and/or phospholipids present in circulating plasma lipoproteins (1–3). The lipolytic action of these two enzymes is necessary for the remodeling and maturation, as well as ultimate catabolism, of triglyceride-rich lipoproteins and HDL. Together with pancreatic lipase, these two enzymes belong to a family of lipases that have a common evolutionary origin and share a high degree of primary sequence homology (4–6). Both HL and LPL have similar catalytic sites (7–9) and, like pancreatic lipase (10), appear to be organized into functionally distinct carboxyl- and amino-terminal domains.

Despite their similarities, the physiologic roles that HL and LPL play in the metabolism of triglyceride-rich lipoprotein particles as well as HDL are distinct, a difference that may, in part, be mediated by the different substrate specificities of the two enzymes. Thus, characterization of patients with a genetic deficiency of either HL or LPL indicates that large-triglyceride-rich lipoproteins are the preferred substrate for LPL (2, 11), whereas HL is more active in the hydrolysis of intermediate density lipoprotein as well as HDL (3, 12, 13). Several lines of evidence indicate that unlike LPL, which acts primarily as a triacylglycerol hydrolase (2), HL functions as both a triacylglycerol hydrolase as well as a phospholipase (3, 14–18). This enhanced phospholipase activity may, in fact, play an important role in the ability of HL, as opposed to LPL, to directly modulate HDL metabolism.

One important structural domain present in both HL and

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[1] The abbreviations used are: HL, hepatic lipase; LPL, lipoprotein lipase; HDL, high density lipoprotein; rLucif-Adv, recombinant adenovirus containing the reporter gene luciferase; rHL-AdV, recombinant adenovirus containing native human HL; rLPL-AdV, recombinant adenovirus containing native LPL.

LPL is the lipase lid. By analogy to pancreatic lipase, the lids of HL and LPL appear to cover the active site and prevent access of the substrate to the catalytic pocket (10, 19–21). Despite the high degree of structural homology, there is very little primary amino acid sequence homology between the lids of HL, LPL, and pancreatic lipase (4, 22), suggesting a potential role of the lipase lid in mediating the different *in vivo* physiologic function of HL and LPL. We have recently demonstrated that the lipase lid (Fig. 1) is essential for the hydrolysis of lipid substrates (22, 23) and may play a major role in conferring the different *in vitro* phospholipase activities of the two lipases (24). In order to extend these *in vitro* findings to an *in vivo* system, we have expressed the native enzymes as well as lipase lid mutants in HL-deficient mice using recombinant adenovirus vectors. HL-deficient mice, like patients with HL deficiency, have increased plasma cholesterol and phospholipid concentrations attributable to increased plasma HDL levels (25) and thus are an excellent animal model to test the *in vivo* phospholipase function of different lipases. Our studies identify the lipase lid as a major structural motif responsible for conferring the different *in vivo* phospholipase activities between LPL and HL and demonstrate the feasibility of performing *in vivo* structure-function analysis of lipolytic enzyme using recombinant adenovirus vectors.

MATERIALS AND METHODS

Recombinant Adenovirus—Recombinant adenovirus utilized in the study were constructed by generating a pXCX2 plasmid (26), which contained the cytomegalovirus promoter and enhancer elements driving the expression of either HL (6, 27), LPL (28), luciferase (29), or mutant lipase cDNA (22, 24), as well as the SV40 splice donor, acceptor, and polyadenylation signals inserted into the E1 region of the human adenovirus (AdV5), and co-transfecting this plasmid with pJM17 (30). Recombinant adenoviruses were identified by the polymerase chain reaction and subjected to two rounds of plaque purification prior to large scale amplification, purification, and titering as described (31).

Study Animals—HL-deficient males (25) between the ages of 2 and 3 months and approximately 25–30 g in weight received 5×10^8 plaque-forming units of recombinant adenovirus via saphenous vein infusion (31). For the procedure, animals were anesthetized by intraperitoneal injection with 0.011 ml/g of animal weight with 2.5% avertin prepared by dissolving 10 g of tribromoethanol in 10 ml of tertiary amyl alcohol.

Determination of in Vivo HL and LPL Expression—HL and LPL enzymic activities were determined in blood samples collected before and 5 min after infusion of 500 units/kg heparin-sodium (Lyphomed, Deerfield, IL) via the tail vein. Post-heparin plasma HL and LPL activities were measured at days 0, 5, 15, and 30 prior to or after adenovirus delivery by using emulsified ^{14}C-labeled triolein as substrate in the presence or absence of 1 M NaCl, for HL and LPL, respectively, as described previously (32, 33).

LPL concentrations in mouse post-heparin plasma were determined in duplicate by an enzyme-linked immunosorbent assay using the 5D2 monoclonal antibody (kindly provided by Dr. J. D. Brunzell, University of Washington, Seattle, WA) for capture and a chicken polyclonal antibody (kindly provided by Dr. I. J. Goldberg, Columbia University, New York, NY) for measurement as described previously (22).

Expression of HL in mouse post-heparin plasma was determined by Western blot hybridization as described (33). Mouse post-heparin plasma (100 μl pooled from 3 mice) was bound to heparin-Sepharose CL-6B (600 μl, Pharmacia Biotech Inc.) equilibrated with 0.01 M sodium phosphate buffer, pH 7.6. The resin and post-heparin plasma were gently rotated for 30 min at 7 °C. Unbound protein was removed by thorough washing the equilibration buffer. Elution of bound proteins was performed stepwise with buffer containing 0.4 M NaCl, 0.8 M NaCl, and 1.5 M NaCl. The eluate was dialyzed into 0.01 M NH_4HCO_3 and dried on a Savant SpeedVac concentrator (Savant Instruments Inc. Farmingdale, NY). The dried samples were resuspended in a minimal volume of sample buffer, separated in a 10% Tris-glycine gel (Novex, San Diego, CA), and transferred to polyvinylidene difluoride microporous membranes (Immobilon PVDF; Millipore, Bedford, MA) as described previously (34). Human HL was identified by blotting with a goat polyclonal antibody (kindly provided by Dr. I. J. Goldberg) and visualized by silver enhanced detection of a gold-labeled rabbit anti-goat antibody (Auroprobe, Amersham Corp.). Protein standards of known molecular weight (SeeBlue, Novex, San Diego, CA) and HL standard were used on each blot. Human HL standard was isolated from medium obtained from 293 cells transfected with HL cDNA under the control of the cytomegalovirus promoter/enhancer (34).

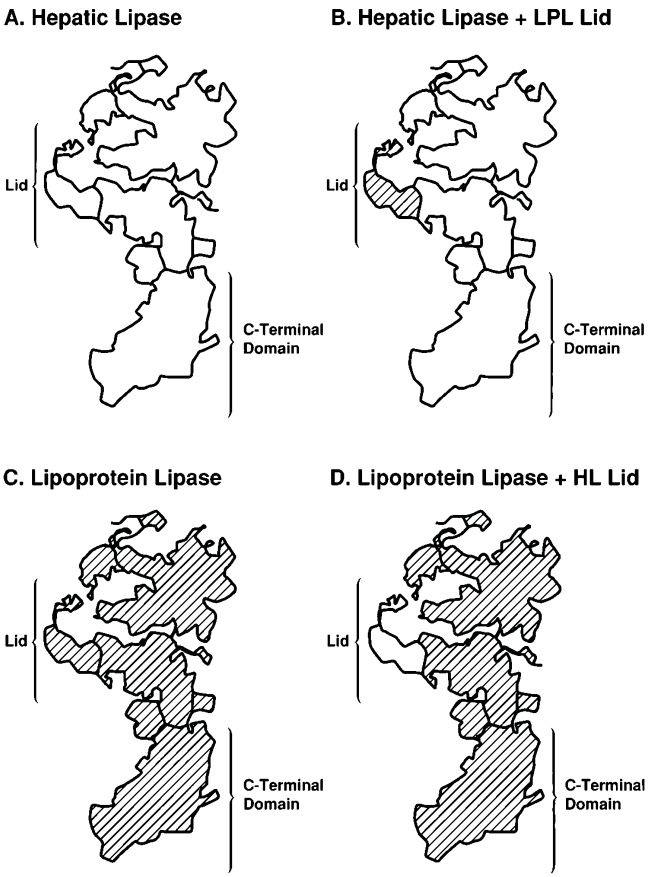

FIG. 1. **Schematic representation of the native and mutant lipases expressed *in vivo* using recombinant adenovirus.** The structure of pancreatic lipase (44) was utilized to represent the exchange of the lid domain between HL and LPL in *panels A–D*. Native HL is shown in by *white* areas (*panel A*), and native LPL is illustrated by *striping* (*panel C*). The structure of the lipase mutant containing the HL backbone (residues 1–476) with amino acids 217–238 of the LPL lid replacing the HL lid is shown in *panel B*, whereas *panel D* illustrates the structure of the lipase mutant containing the LPL backbone (residues 1–448) with amino acids 232–253 of HL replacing the LPL lid.

Quantitation of Plasma Lipids—Plasma cholesterol, triglyceride and phospholipid levels were determined enzymatically in 10 μl of fasting plasma diluted 1:50 with phosphate-buffered saline using commercially available kits (cholesterol, Sigma Diagnostics; phospholipid, Wako Chemical USA, Inc., Richmond, VA) and the Cobas Miras Plus automated chemistry analyzer (Roche Diagnostic Systems, Inc., Branchburg, NJ). HDL cholesterol was determined as the cholesterol remaining in plasma after precipitation of the apolipoprotein B-containing lipoproteins with heparin and calcium as described (35).

Analysis of Plasma Lipoproteins—Plasma lipoprotein analysis was performed by separating plasma lipoproteins by permeation chromatography using two Superose 6 HR 10/30 columns (Pharmacia) connected in series. Lipoproteins from 50 μl of mouse plasma were eluted at 0.3 ml/min with phosphate-buffered saline buffer containing 1 mM EDTA and 0.02% sodium azide. Lipids in the recovered fractions were quantitated as described above. Lipoprotein elution volumes were: very low density lipoprotein, 15–16 ml; intermediate density lipoprotein/low density lipoprotein, 20–24 ml; and HDL, 30–31 ml.

RESULTS

Generation of Native and Mutant Recombinant Adenovirus—To perform these studies, we generated recombinant adenovirus vectors expressing the reporter gene luciferase, human HL, and LPL, as well as lipase mutants in which the lids of HL and LPL were exchanged as described previously (24). Fig. 1 illustrates the structure of native HL (*panel A*) and LPL

(*panel C*) as well as the mutant, chimeric lipases containing either the human HL backbone with the LPL lid (*panel B*) or the human LPL backbone with the HL lid (*panel D*), which were expressed in HL-deficient mice using these recombinant adenovirus vectors.

Quantitation of in Vivo Lipase Expression—Systemic infusion of recombinant adenovirus via saphenous vein injection resulted in significant expression of both native and chimeric lipases by day 5 in HL-deficient mice (Fig. 2 and Table I). Thus, Western blot analysis of post-heparin plasma after heparin-Sepharose affinity chromatography using antibodies monospecific for human HL established the presence of a major 60-kDa immunoreactive band in the plasma of treated animals demonstrating expression of native HL or the HL + LPL lid lipase mutant *in vivo* (Fig. 2, *panel B*). Analysis of post-heparin plasma HL activity on day 5 demonstrated similar HL expression in mice injected with either the rHL-AdV or the rHL+LPL lid-AdV (Fig. 2, *panel A*). Likewise, comparable levels of expression as determined by LPL activity and plasma concentrations were present in the post-heparin plasma of mice injected with either rLPL-AdV or rLPL+HL lid-AdV (Table I). Determination of day 5 plasma lipase activities before and after heparin infusion indicated that greater than 95% of all four expressed lipases were heparin-bound (data not shown).

Mice injected with adenovirus expressing the reporter gene, luciferase, had no increase in post-heparin lipolytic activity.

A

CONSTRUCT	HL ACTIVITY (nmoles/min/ml)	
	day 0	day 5
rHL-Adv (n = 4)	4 ± 3	9806 ± 915**
rHL+LPL Lid-Adv (n = 4)	13 ± 5	9677 ± 2033*
rLucif-Adv (n = 3)	9 ± 2	7 ± 2

*p < 0.05, **p < 0.01 paired t – analyses (compared to day 0)

B

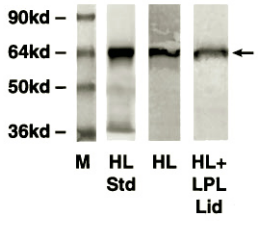

FIG. 2. **Expression of native HL and HL + LPL lid mutant after recombinant adenovirus infusion in HL-deficient mice is demonstrated.** *Panel A* summarizes the post-heparin plasma HL activity in HL-deficient mice before (day 0) and after (day 5) infusion of recombinant adenovirus. Post-heparin plasma HL activity in control, untreated C57BL/6 mice = 387 ± 73 nmol/min/ml (n = 4). Analysis of day 5 post-heparin plasma by Western blotting using a monospecific anti-human HL antibody after heparin-Sepharose affinity chromatography is shown in *panel B*.

The distribution of adenoviral-mediated gene delivery, as assessed by quantitation of luciferase activity per 100 mg of homogenized tissue (data not shown) in liver (>90%), spleen (4%), heart and skeletal muscle (4%), lung (2%), as well as kidney, testes, and fat (<1% combined), indicated as previously reported (31, 36–38) that the major organ of transgene expression after systemic adenovirus delivery is the liver.

Analysis of Plasma Lipid Changes—Adenovirus-mediated expression of all four lipases led to decreases in plasma cholesterol and phospholipid concentrations on day 4 compared to base-line (day 0) values (paired t test, $p < 0.05$, Table II). Plasma HDL cholesterol values were also significantly reduced in animals expressing HL ($p < 0.005$), HL+ LPL lid ($p < 0.05$), and LPL + HL lid ($p < 0.005$). Interestingly, base-line plasma triglyceride concentrations were only significantly reduced in animals expressing native LPL ($0 < 0.05$).

However, the percent change in plasma cholesterol and phospholipid was significantly different when animals injected with rHL-AdV or rHL+LPL lid-AdV and animals receiving rLPL-AdV or rLPL+HL lid-AdV were compared. This difference was most evident when the phospholipid levels were analyzed. Thus, despite expression of similar post-heparin lipolytic activities on day 5 (9806 ± 915 and 9677 ± 2033 nmol/min/ml, respectively, Fig. 2), injection of the recombinant virus expressing native HL (rHL-AdV) resulted in a much more significant reduction in plasma phospholipid concentrations (70% decrease from base-line value, $p < 0.001$) than infusion of the adenovirus expressing the mutant lipase containing the HL backbone and the rHL + LPL lid-AdV (base-line phospholipids reduced by only 32%, $p < 0.05$). Similarly, injection of adenovirus expressing either rLPL-AdV or a mutant lipase containing the LPL backbone and the rLPL + HL lid-AdV resulted in post-heparin lipolytic activities of 4495 ± 534 and 4844 ± 1336 nmol/min/ml (Table I) and phospholipid reductions of 31% ($p < 0.05$) *versus* 81% ($p < 0.005$), respectively (Table II). Thus, the simple exchange of the lids between LPL and HL, without other structural alterations, markedly altered the ability of the lipases to hydrolyze phospholipids *in vivo*.

Analysis of Plasma Lipoproteins—Fig. 3 illustrates the effect of adenovirus-mediated expression of native enzymes and lipase lid mutants on plasma lipoproteins in HL-deficient mice as analyzed by FPLC. HL-deficient mice injected with rHL-AdV had a more significant reduction in HDL-phospholipid and cholesterol than mice expressing the HL+LPL lid mutant (*panels A* and *B*). Similarly, in contrast to animals expressing native LPL, mice injected with rLPL+HL lid-AdV demonstrated a marked reduction in HDL-phospholipid and cholesterol (*panels C* and *D*). Thus, regardless of the lipase backbone, the presence of the HL lid led to a more dramatic reduction in HDL-associated cholesterol and phospholipid, thereby resulting in a marked decrease in HDL particle concentrations.

Time Course of Plasma Lipid Changes—Fig. 4 illustrates the time course of changes in the cholesterol and phospholipid concentrations of HL-deficient mice after infusion of different recombinant adenovirus. In contrast to mice injected with rLucif-AdV, animals expressing the four lipases demonstrated a

TABLE I

Postheparin plasma LPL activity and concentration in hepatic lipase-deficient mice after infusion of recombinant adenovirus

LPL activity is expressed in nmol/min/ml, and LPL concentration is expressed in ng/ml. Post-heparin plasma LPL activity in control, untreated C5BL/6 mice = 255 ± 13 nmol/min/ml (n = 6). Data are presented as mean ± S.E. *, $p < 0.05$; **, $p < 0.01$ (paired t analyses, compared to day 0).

Construct	Day 0	Day 5	
	LPL activity	LPL activity	LPL concentration
rLPL − AdV (n = 4)	549 ± 209	4495 ± 534**	4931 ± 1292
rLPL + HL lid AdV (n = 4)	575 ± 120	4844 ± 1336*	5050 ± 1992
rLucif-AdV (n = 3)	203 ± 41	217 ± 29	

TABLE II

Plasma lipids and lipoproteins in HL-deficient mice before and after adenovirus infusion

*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$; ****, $p < 0.001$. (paired t-analyses compared to day 0). TC, total cholesterol; TG, triglyceride; PL, phospholipids; HDL-C, thigh density lipoprotein-cholesterol; ND, not done.

Construct	TC		TG		PL		HDL-C	
	Day 0	Day 4	Day 0	Day 4	Day 0	Day 4	Day 0	Day 4
HL								
rHL − AdV ($n = 4$)	193 ± 22	56 ± 15***	58 ± 6	67 ± 21	343 ± 30	106 ± 27****	181 ± 19	51 ± 11***
rHL + LPL lid ($n = 4$)	176 ± 11	105 ± 8*	64 ± 19	30 ± 5	323 ± 24	218 ± 11*	156 ± 20	92 ± 3*
LPL								
rLPL − AdV ($n = 4$)	166 ± 14	110 ± 18*	69 ± 17	28 ± 7*	304 ± 17	206 ± 15*	152 ± 12	101 ± 7[a]
rLPL + HL lid AdV ($n = 4$)	177 ± 9	44 ± 19***	47 ± 11	21 ± 1[a]	312 ± 6	57 ± 18***	160 ± 7	18 ± 11***
rLucif − AdV ($n = 4$)	160 ± 15	147 ± 6	70 ± 17	65 ± 15	279 ± 23	265 ± 8	ND	ND
rHL − AdV[b] ($n = 4$)	76 ± 3	24 ± 8***	55 ± 13	55 ± 24	176 ± 14	65 ± 18***	ND	ND

[a] $p = 0.079$.

[b] Lipid values in control, C57BL/6 mice before (day 0) and after (day 4) infusion of rHL-AdV. Values are expressed as mean ± S.E.

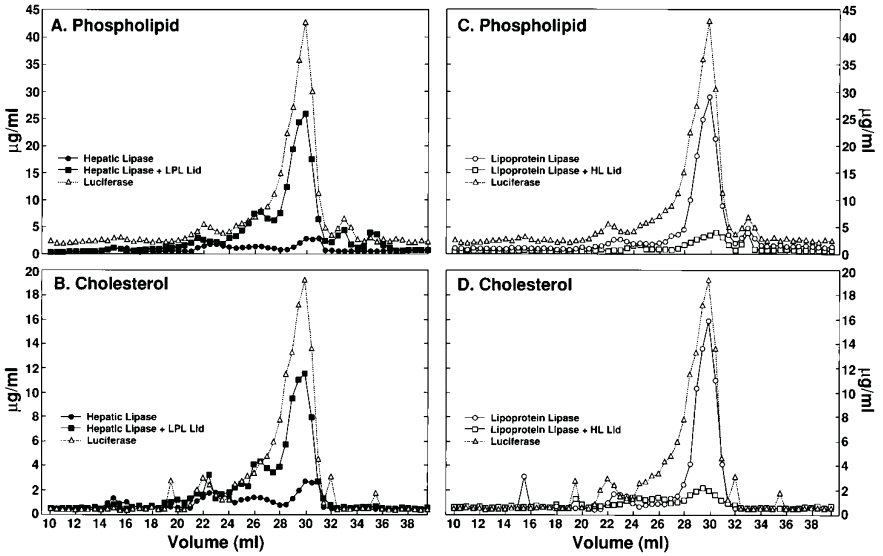

FIG. 3. **FPLC separation of plasma lipoproteins 5 days after infusion of recombinant adenovirus in HL-deficient mice.** The distribution of lipoprotein-associated cholesterol and phospholipids in FPLC fractions is illustrated for HL-deficient mice injected with recombinant adenovirus expressing either hepatic lipase (●), hepatic lipase + LPL lid (■), lipoprotein lipase (○), lipoprotein lipase + HL lid (□), and luciferase (△). Each profile represents the analysis of one representative animal out of several analyzed for each study group.

change in the plasma cholesterol and phospholipids that was evident by day 2 and greatest by day 4, when the post-heparin plasma lipolytic activities were at their maximum. Significant differences between the cholesterol and phospholipid reduction achieved in animals expressing HL and HL+LPL lid were evident at days 2 and 4 post-adenovirus injection (Fig. 4, *panels A* and *B*). Similarly, the decrease in plasma phospholipids and cholesterol detected in mice injected with rLPL-AdV was significantly less than that present in mice injected with rLPL+HL lid-AdV at days 4, 8, and 12 post-adenovirus injection (Fig. 4, *panels C* and *D*), demonstrating that the differences in phospholipid hydrolysis mediated by the lipase lid were sustained for several days after initial virus infusion and were especially evident during maximal *in vivo* lipase expression.

DISCUSSION

Despite the similarity in the structure and hydrolytic function of HL and LPL, the role that these two enzymes play in lipoprotein metabolism is distinct. One of the major functional differences between HL and LPL resides in the ability of HL to hydrolyze HDL phospholipids (3, 14, 15, 17). Thus, together with lecithin cholesterol acyltransferase and cholesterol ester transfer protein, HL is involved in the remodeling and metabolism of plasma HDL (14, 17, 18, 39, 40), processes that are essential for reverse cholesterol transport (41) and, perhaps, ultimately modulate the development of atherosclerosis. LPL, on the other hand, catalyzes the hydrolysis of triglycerides present in larger, triglyceride-rich lipoproteins (2). A deficiency of this enzyme predisposes to the development of pancreatitis, but not cardiovascular disease (2, 11). The enhanced ability of HL compared to LPL to function as a phospholipase may, in fact, ultimately determine the different roles that these two enzymes play in the metabolism of HDL, as well as other plasma lipoproteins. Previous studies have demonstrated that the lid in human pancreatic lipase covers the active site of the enzyme and must be repositioned to permit access of the lipid substrates for hydrolysis (10). During this process the lid comes into intimate contact with both substrate and cofactor (19). Based on the pancreatic lipase model, the HL and LPL lids have also been proposed to modulate access of the substrate to the catalytic site and, thus, play a central role in mediating lipase interaction with lipids and lipoproteins. This latter concept has been tested using site-directed mutagenesis and expression of lipase lid mutants in mammalian cells (22). Utilizing this *in vitro* approach, we have demonstrated the importance of the HL and LPL lids and their amphipathic helices in mediating the interaction of the lipase with the lipid substrates (22).

In the present study, we investigate the lipase lid as a potential structural domain that may confer the different phospholipase activities between HL and LPL. Despite the high degree of structural homology between HL and LPL, there is little conservation of the amino acid residues present in the lids of HL and LPL (22). This difference in amino acid sequence identifies the lipase lid as a potential structural motif that could mediate differences in lipase function. In the current

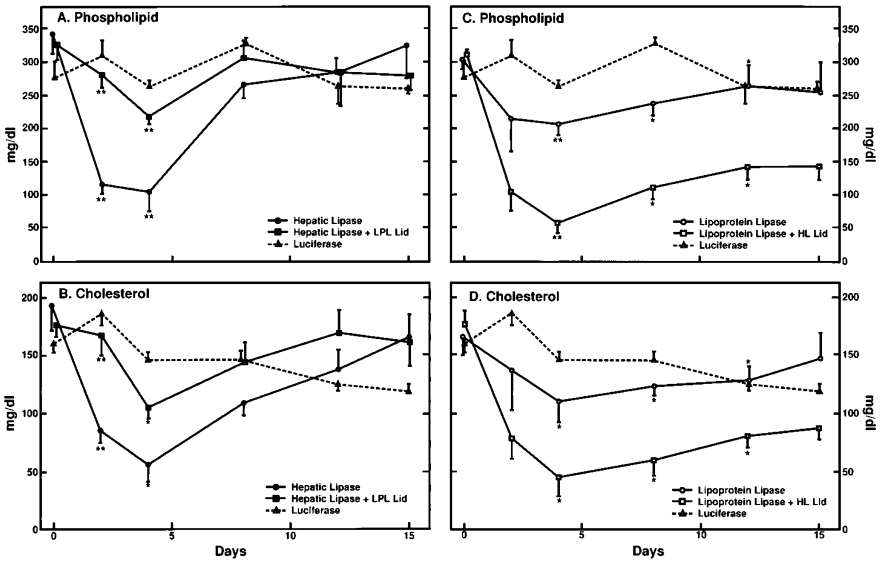

FIG. 4. **Time course of plasma lipid changes in HL-deficient mice after infusion of recombinant adenovirus.** Alterations in plasma cholesterol and phospholipids of HL-deficient mice injected with recombinant adenovirus expressing either hepatic lipase (●), hepatic lipase + LPL lid (■), lipoprotein lipase (○), lipoprotein lipase + HL lid (□), and luciferase (△) are illustrated for days 0–15 post-adenovirus infusion. *, $p < 0.05$; **, $p < 0.01$ (unpaired t test, lipid reduction from base-line value measured in animals expressing the native lipase was compared to mice expressing the lipase lid mutants).

study we utilize a novel approach for performing structure-function analysis of a protein, *in vivo*, by using recombinant adenovirus to express the native enzymes as well as lipase lid mutants in HL-deficient mice. This approach permits direct assessment of the physiologic consequences of altering specific structural elements of a protein *in vivo*, thus circumventing some of the limitations of an *in vitro* expression system. HL-deficient mice represent an excellent animal model to investigate structural domains that may modulate differences in phospholipase activity between the two enzymes since, compared to normal mice, HL-deficient mice have increased plasma phospholipid concentrations (25, 33).

Our findings demonstrate successful adenovirus-mediated expression of functional native and mutant lipases in HL-deficient mice, which resulted in significant reductions in plasma concentrations of total and HDL cholesterol as well as phospholipids. However, despite similar levels of expression, significant differences in the ability of lipase mutants to hydrolyze phospholipids were evident. Thus, *in vivo* expression of lipases containing the HL lid (native HL and LPL+HL lid mutant) demonstrated a 70–81% decrease in circulating plasma phospholipids. In contrast, *in vivo* expression of lipases containing the LPL lid (native LPL and HL+LPL lid mutant) resulted in only 30–31% reduction in plasma phospholipids. Similarly, FPLC analysis of plasma lipoproteins demonstrated a more significant decrease in the cholesterol and phospholipid content of HDL in animals expressing lipases containing the HL lid. Changes in plasma total cholesterol and HDL cholesterol concentrations reflected alterations in phospholipids. Thus, the simple exchange of the lids present in HL and LPL, without other alterations in the lipase backbone, markedly changed the ability of the expressed lipases to hydrolyze phospholipids *in vivo*. These studies suggest that the enhanced phospholipase activity present in mice injected with lipases containing the HL lid as compared to the LPL lid accelerated *in vivo* HDL catabolism, emphasizing the importance of HL-mediated phospholipid hydrolysis for the initiation of HDL metabolism.

Previous *in vitro* expression studies evaluating potential structural domains that may confer the phospholipase properties of HL and LPL have suggested that the carboxyl terminus (42) rather than the lipase amino-terminal domain (24) may be most important in modulating the phospholipase function of the two enzymes. Interestingly, in these studies the chimeric lipase demonstrating the greatest phospholipase activity contained the amino terminus of HL, which included the HL lid (42). These latter findings are in fact consistent with the present *in vivo* studies as well as *in vitro* transfection experiments (24), which indicate that the lipase lid is a major structural motif contributing to the enzyme's phospholipase function. Thus, (24), regardless of the lipase backbone, the relative phospholipase/triacylglycerol hydrolase activities of enzymes containing the HL lid was significantly greater than that of the lipases containing the LPL lid. Like the animal studies, which demonstrated a phospholipid reduction of 70% and 81% in animals expressing lipases with the HL lid, the ability of native and mutant lipases expressed after transfection of human embryonal kidney 293 cells (24) to hydrolyze phospholipids *in vitro* was markedly enhanced by the presence of the HL lid.

The mechanism by which the 22-amino acid lid mediates the different phospholipase properties of HL and LPL is not clearly understood. By analogy to fungal lipases (21, 43) and pancreatic lipase (19, 20), the HL and LPL lids may require repositioning to permit access of the lipid substrates to the catalytic site. As a result of this movement, a large hydrophobic area that may function as a binding site for lipid and phospholipid substrates (19) is exposed. There is little homology between the amino acid residues present in the HL and LPL lids (15 out of 22 residues are different). In addition, there is a significant difference in the content of basic *versus* acidic residues between the two lids (24). Thus, the overall structure and organization of the lipase lid may determine the ability of the lid to interact with as well as accommodate different lipids within the hydrophobic catalytic cavity, modulating the preference of HL and LPL for different lipid substrates.

In the present report, we describe a novel approach for performing structure-function analysis of a protein, *in vivo*, by using recombinant adenovirus to express native and mutant enzymes in an animal model for a human genetic deficiency. Our studies demonstrate that the presence of the HL lid markedly enhances the ability of native or mutant lipases to hydrolyze phospholipid substrates. Thus the lipase lid is an important structural motif, which determines the different *in vivo* phospholipase activities of HL and LPL, a property that may modulate the distinct physiological roles that these two similar lipolytic enzymes play in lipoprotein, and especially HDL metabolism. The use of recombinant adenovirus vectors to express mutant proteins in animal models for human genetic deficiencies represents a powerful new approach for performing protein structure-function analysis *in vivo*.

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REFERENCES

1. Brewer, H. B., Jr., Santamarina-Fojo, S., and Hoeg, J. M. (1995) in *Endocrinology* (DeGroot, L. J., Besser, M., Jameson, J. L., Loriaux, D., Marshall, J. C., Odell, W. D., Potts, J. T., Jr., Rubenstein, A. H., Cahill, G. F., Jr., Martini, L., and Nelson, D. H., eds) pp. 2731–2753, W. B. Saunders Co., Philadelphia
2. Brunzell, J. D. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 1913–1932, McGraw-Hill, Inc., New York
3. Olivecrona, T., and Bengtsson-Olivecrona, G. (1993) *Curr. Opin. Lipidol.* **4,** 187–196
4. Hide, W. A., Chan, L., and Li, W. H. (1992) *J. Lipid Res.* **33,** 167–178
5. Kirchgessner, T. G., Svenson, K. L., Lusis, A. J., and Schotz, M. C. (1987) *J. Biol. Chem.* **262,** 8463–8466
6. Datta, S., Luo, C.-C., Li, W.-H., VanTuinen, P., Ledbetter, D. H., Brown, M. A., Chen, S.-H., Liu, S., and Chan, L. (1988) *J. Biol. Chem.* **263,** 1107–1110
7. Davis, R. C., Stahnke, G., Wong, H., Doolittle, M. H., Ameis, D., Will, H., and Schotz, M. C. (1990) *J. Biol. Chem.* **265,** 6291–6295
8. Faustinella, F., Smith, L. C., Semenkovich, C. F., and Chan, L. (1991) *J. Biol. Chem.* **266,** 9481–9485
9. Emmerich, J., Beg, O. U., Peterson, J., Previato, L., Brunzell, J. D., Brewer, H. B., Jr., and Santamarina-Fojo, S. (1992) *J. Biol. Chem.* **267,** 4161–4165
10. Winkler, F. K., D'Arcy, A., and Hunziker, W. (1990) *Nature* **343,** 771–774
11. Santamarina-Fojo, S. (1992) *Curr. Opin. Lipidol.* **3,** 186–195
12. Demant, T., Carlson, L. A., Holmquist, L., Karpe, F., Nilsson-Ehle, P., Packard, C. J., and Shepherd, J. (1988) *J. Lipid Res.* **29,** 1603–1611
13. Hayden, M. R., Ma, Y., Brunzell, J., and Henderson, H. E. (1991) *Curr. Opin. Lipidol.* **2,** 104–109
14. Deckelbaum, R. J., Ramakrishnan, R., Eisenberg, S., Olivecrona, T., and Bengtsson-Olivecrona, G. (1992) *Biochemistry* **31,** 8544–8551
15. Ehnholm, C., Shaw, W., Greten, H., and Brown, W. V. (1975) *J. Biol. Chem.* **250,** 6756–6761
16. van Tol, A., van Gent, T., and Jansen, H. (1980) *Biochem. Biophys. Res. Commun.* **94,** 101–108
17. Shirai, K., Barnhart, R. L., and Jackson, R. L. (1981) *Biochem. Biophys. Res. Commun.* **100,** 591–599
18. Nicoll, A., and Lewis, B. (1980) *Eur. J. Clin. Invest.* **10,** 487–95
19. Van Tilbeurgh, H., Egloff, M. P., Martinez, C., Rugani, N., Verger, R., and Cambillau, C. (1993) *Nature* **362,** 814–820
20. Van Tilbeurgh, H., Roussel, A., Lalouel, J.-M., and Cambillau, C. (1994) *J. Biol. Chem.* **269,** 4626–4633
21. Brzozowski, A. M., Derewenda, U., Derewenda, Z. S., Dodson, G. G., Lawson, D. M., Turkenburg, J. P., Bjorkling, F., Huge-Jensen, B., Patkar, S. A., and Thim, L. (1991) *Nature* **351,** 491–494
22. Dugi, K. A., Dichek, H. L., Talley, G. D., Brewer, H. B., Jr., and Santamarina-Fojo, S. (1992) *J. Biol. Chem.* **267,** 25086–25091
23. Santamarina-Fojo, S., and Dugi, K. (1994) *Curr. Opin. Lipidol.* **5,** 117–125
24. Dugi, K. A., Dichek, H. L., and Santamarina-Fojo, S. (1995) *J. Biol. Chem.* **270,** 25396–25401
25. Homanics, G. E., de Silva, H. V., Osada, J., Zhang, S. H., Wong, H., Borensztajn, J., and Maeda, N. (1995) *J. Biol. Chem.* **270,** 2974–2980
26. McKinnon, R. D., Bacchetti, S., and Graham, F. L. (1982) *Gene* (*Amst.*) **19,** 33–42
27. Stahnke, G., Sprengel, R., Augustin, J., and Will, H. (1987) *Differentiation* **35,** 45–52
28. Wion, K. L., Kirchgessner, T. G., Lusis, A. J., Schotz, M. C., and Lawn, R. M. (1987) *Science* **235,** 1638–1641
29. de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987) *Mol. Cell. Biol.* **7,** 725–737
30. McGrory, W. J., Bautista, D. S., and Graham, F. L. (1988) *Virology* **163,** 614–617
31. Kashyap, V. S., Santamarina-Fojo, S., Brown, D. R., Parrott, C. L., Applebaum-Bowden, D., Meyn, S., Talley, G., Paigen, B., Maeda, N., and Brewer, H. B., Jr. (1995) *J. Clin. Invest.* **69,** 1612–1620
32. Iverius, P.-H., and Brunzell, J. D. (1985) *Am. J. Physiol.* **249,** E107–E114
33. Applebaum-Bowden, D., Kobayashi, J., Kashyap, V. S., Brown, D. R., Berard, A. M., Meyn, S., Parrott, C., Maeda, N., Shamburek, R. D., Brewer, H. B., Jr., and Santamarina-Fojo, S. (1996) *J. Clin. Invest.* **97,** 799–805
34. Dichek, H. L., Parrott, C., Ronan, R., Brunzell, J. D., Brewer, H. B., Jr., and Santamarina-Fojo, S. (1993) *J. Lipid Res.* **34,** 1393–1401
35. Burstein, M., Scholnick, H. R., and Morfin, R. (1970) *J. Lipid Res.* **11,** 583–95
36. Smith, T. A. G., Mehaffey, M. G., Kayda, D. B., Saunders, J. M., Yei, S., Trapnell, B. C., McClelland, A., and Kaleko, M. (1993) *Nat. Genet.* **5,** 397–402
37. Ishibashi, S., Brown, M. S., Goldstein, J. L., Gerard, R. D., Hammer, R. E., and Hertz, J. (1993) *J. Clin. Invest.* **92,** 883–893
38. Kopfler, W. P., Willard, M., Betz, T., Willard, J. E., Gerard, R. D., and Meidell, R. S. (1994) *Circulation* **90,** 1319–1327
39. Tall, A. R. (1993) *J. Lipid Res.* **34,** 1255–1274
40. Glomset, J. A., Assmann, G., Gjone, E., and Norum, K. R. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., Valle, D., Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., eds) pp. 1933–1951, McGraw-Hill, Inc., New York
41. Glomset, J. A. (1968) *J. Lipid Res.* **9,** 155–167
42. Davis, R. C., Wong, H., Nikazy, J., Wang, K., Han, Q., and Schotz, M. C. (1992) *J. Biol. Chem.* **267,** 21499–21504
43. Derewenda, U., Brzozowski, A. M., Lawson, D. M., and Derewenda, Z. S. (1992) *Biochemistry* **31,** 1532–1541
44. Bourne, Y., Martinez, C., Kerfelec, B., Lombardo, D., Chapus, C., and Cambillau, C. (1994) *J. Mol. Biol.* **238,** 709–732