GPIHBP1 stabilizes lipoprotein lipase and prevents its inhibition by angiopoietin-like 3 and angiopoietin-like 4

William K. Sonnenburg,[1,3,]* Daiguan Yu,[1,]* E-Chiang Lee,* Wei Xiong,* Gennady Gololobov,*
Billie Key,* Jason Gay,[†] Nat Wilganowski,[†] Yi Hu,[§] Sharon Zhao,[§] Matthias Schneider,[†]
Zhi-Ming Ding,[†] Brian P. Zambrowicz,*[,†,§] Greg Landes,[2,]* David R. Powell,[†] and Urvi Desai[3,†]

Departments of Biotherapeutics,* Pharmaceutical Biology,[†] and Molecular Biology,[§] Lexicon
Pharmaceuticals, Inc., 8800 Technology Forest Place, The Woodlands, TX 77381

Abstract Glycosylphosphatidylinositol-anchored HDL-binding protein (GPIHBP1) binds both LPL and chylomicrons, suggesting that GPIHBP1 is a platform for LPL-dependent processing of triglyceride (TG)-rich lipoproteins. Here, we investigated whether GPIHBP1 affects LPL activity in the absence and presence of LPL inhibitors angiopoietin-like (ANGPTL)3 and ANGPTL4. Like heparin, GPIHBP1 stabilized but did not activate LPL. ANGPTL4 potently inhibited nonstabilized LPL as well as heparin-stabilized LPL but not GPIHBP1-stabilized LPL. Like ANGPTL4, ANGPTL3 inhibited nonstabilized LPL but not GPIHBP1-stabilized LPL. ANGPTL3 also inhibited heparin-stabilized LPL but with less potency than nonstabilized LPL. Consistent with these in vitro findings, fasting serum TGs of $Angptl4^{-/-}/Gpihbp1^{-/-}$ mice were lower than those of $Gpihbp1^{-/-}$ mice and approached those of wild-type littermates. In contrast, serum TGs of $Angptl3^{-/-}/Gpihbp1^{-/-}$ mice were only slightly lower than those of $Gpihbp1^{-/-}$ mice. Treating $Gpihbp1^{-/-}$ mice with ANGPTL4- or ANGPTL3-neutralizing antibodies recapitulated the double knockout phenotypes.■ These data suggest that GPIHBP1 functions as an LPL stabilizer. Moreover, therapeutic agents that prevent LPL inhibition by ANGPTL4 or, to a lesser extent, ANGPTL3, may benefit individuals with hyperlipidemia caused by gene mutations associated with decreased LPL stability.—Sonnenburg, W. K., D. Yu, E-C. Lee, W. Xiong, G. Gololobov, B. Key, J. Gay, N. Wilganowski, Y. Hu, S. Zhao, M. Schneider, Z-M. Ding, B. P. Zambrowicz, G. Landes, D. R. Powell, and U. Desai. **GPIHBP1 stabilizes lipoprotein lipase and prevents its inhibition by angiopoietin-like 3 and angiopoietin-like 4.** *J. Lipid Res.* 2009. **50:** 2421–2429.

Our understanding of how triglyceride (TG) metabolism is regulated is essential for designing avenues of ther-

apeutic intervention for diseases such as atherosclerosis, pancreatitis, or dyslipidemia associated with metabolic syndrome or type II diabetes (1–3). Central to triglyceride metabolism is lipoprotein lipase (LPL), an extracellular enzyme primarily located in the vascular beds of many tissues (3, 4). LPL catalyzes the hydrolysis of the triglyceride component of chylomicrons (CM) and VLDL, which constitute the major forms of triglycerides in plasma (3, 5). Although LPL is expressed in many different tissues, the enzyme is expressed at high levels in metabolically active tissues, such as adipose, cardiac muscle, and skeletal muscle, where fatty acids released by the action of LPL are stored or used (4).

LPL appears to be regulated by a variety of mechanisms. Several apolipoproteins associated with CM and VLDL, including apolipoprotein CII (APOC2) and apolipoprotein AV (APOA5), stimulate LPL activity (6–9) apparently by increasing its V_{max} (10, 11). In contrast, apolipoproteins CI (APOC1) and CIII (APOC3) can inhibit LPL activity (7, 12). LPL is inherently unstable and proteins or other factors that either stabilize or destabilize LPL are likely to play a role in regulating its in vivo activity (13). The active form of LPL exists as a head-to-tail homodimer, which dissociates into metastable monomers. These monomers can reassociate to form catalytically active LPL or they can undergo conformational changes, forming inactive, stable monomers. The spontaneous in-

activation of LPL is mostly irreversible (14–16). Heparin oligomers greatly increase the in vitro half-life of LPL, suggesting that the association of LPL with vascular heparan sulfate proteoglycans is a factor that regulates in vivo LPL activity (8, 14, 15, 17).

More recently, angiopoietin-like 3 (ANGPTL3) and angiopoietin-like 4 (ANGPTL4), two proteins of the angiopoietin gene family, have been shown to inhibit LPL activity and to regulate triglyceride metabolism (5, 18–23). ANGPTL3 and ANGPTL4 are secreted proteins that contain a signal peptide, a coiled-coil domain, and a fibrinogen-like domain. The coiled-coil domain mediates the formation of higher order oligomers, which appear to be required for the LPL-inhibitory activity of ANGPTL3 and ANGPTL4. The mature protein is proteolytically cleaved between the coiled-coil domain and the fibrinogen-like domain to form an N-terminal fragment that is involved in LPL inhibition. The by which ANGPTL4 inhibits LPL involves the conversion of LPL from the active dimeric form to the inactive monomeric form, a process that appears to be irreversible. This inactivation process requires association of ANGPTL4 with LPL and is not appreciably inhibited by stabilizing concentrations of heparin (24).

ANGPTL4 is expressed primarily in adipose tissue and liver but is also expressed in cardiac muscle, skeletal muscle, and intestine under the control of peroxisome proliferator-activated receptors. ANGPTL3, in contrast, is expressed in the liver under the control of liver X receptors. ANGPTL3 is likely to function as an endocrine regulator that suppresses triglyceride clearance primarily in the fed state. ANGPTL4, in contrast, is likely to function as an autocrine or paracrine regulator as well as an endocrine regulator, preventing uptake of fatty acids from plasma triglyceride sources, particularly in the fasted state (5, 20, 21). Together, these two proteins likely play a role in regulating triglyceride metabolism largely by inhibiting LPL.

Recently, Beigneux et al. (25) and Young et al. (26) have shown that LPL and the endogenous substrate CM associate with glycosylphosphatidylinositol-anchored HDL-binding protein (GPIHBP1) (27). This protein attaches to the surface of endothelial cells of adipose tissue, cardiac muscle, and skeletal muscle by a glycosylphosphatidylinositol (GPI) anchor and has been proposed to function as a platform for LPL and its substrates, presumably increasing the efficiency of substrate hydrolysis and uptake of fatty acids by underlying tissues. This proposed function is consistent with the phenotype of $Gpihbp1^{-/-}$ mice, which have elevated plasma triglycerides, largely in the form of CM (25). A mutant form of human GPIHBP1 that contains a Q115P substitution has been shown to be associated with chylomicronemia. This mutation alters the function of GPIHBP1, affecting its ability to bind with LPL and CM (28). Although LPL associates with GPIHBP1, it is not clear whether GPIHBP1 affects LPL activity. In this study, we examined whether GPIHBP1 affects LPL catalytic activity or interacts with LPL to alter its inhibition by ANGPTL3 or ANGPTL4.

EXPERIMENTAL PROCEDURES

Materials and general methods

Bovine LPL and lipase substrate 1,2-O-dilauryl-rac-glycero-3-glutaric acid – (6'-methylresorufin) ester (DGGR) were purchased from Sigma-Aldrich (St. Louis, MO). Primers for PCR amplification and production of polyclonal antisera reactive with mouse GPIHBP1 were purchased from Sigma-Genosys (St. Louis, MO). cDNA for PCR amplification was purchased from CLONTECH Laboratories (Mountain View, CA). Ni-NTA agarose and HiSpeed Plasmid Maxi Kit were purchased from Qiagen (Valencia, CA). Pierce TMB substrate and Pierce Microplate BCA™ Protein Assay Kit–Reducing Agent Compatible were purchased from Thermo Scientific (Rockford, IL). HRP-conjugated goat anti-rabbit IgGs were purchased from Bethyl Laboratories (Montgomery, TX). Mouse anti-human LPL monoclonal IgG1 (clone A00090.01) was purchased from GeneScript (Piscataway, NJ). PCR amplification reactions were performed with Phusion DNA polymerase according to the recommendations supplied by the vendor (New England Biolabs, Ipswich, MA). A549 cells (CCL-185) were purchased from ATCC (Manassas, VA). HyQSFM4-CHO serum-free medium was purchased from Hyclone (Logan, UT). F-12K medium was purchased from MediaTech (Manassas, VA). All other molecular biology techniques, such as restriction endonuclease digests, ligations, and agarose gel electrophoresis, were performed essentially as described in common manuals (29, 30).

Production of recombinant human LPL

Recombinant human LPL was produced in 293F cells and catalytically active LPL was partially purified and concentrated from conditioned medium by heparin Sepharose affinity chromatography as described previously (31). Fractions containing active LPL were pooled, assayed for protein concentration, and stored at −80°C.

Production of recombinant human ANGPTL3 and ANGPTL4

Recombinant human ANGPTL3 (amino acids 17-221 [RefSeq accession NP_055310.1]) and ANGPTL4 (amino acids 26-176 [RefSeq accession NP_647475.1]), both possessing an N-terminal six-histidine tail, were expressed in *Escherichia coli* and subsequently purified by Ni-NTA affinity chromatography as described previously (31). The recombinant proteins were dialyzed against 50 mM sodium phosphate, 500 mM sodium chloride, 10% glycerol, 5 mM β-mercaptoethanol, pH 7.8, assayed for protein concentration, and stored at −80°C.

Production of recombinant soluble mouse GPIHBP1

A cDNA encoding mouse GPIHBP1 amino acids 1–198 (RefSeq accession NP_081006.1) with a C-terminal six-histidine tail was generated by PCR amplification with sense 39mer 5′-GCCACCAAGCTTAGCCACCATGAAGGCTCTCAGGGCTGT-3′ and antisense 54mer 5′-ACTAGTGGATCCTCAATGGTGATGGTGATGGTGTCCCTGGGGCTG GTTAGCCTT-3′. The cDNA was inserted into the Ad5 E1-deleted region of pAd5 under the control of hCMV promoter. Recombinant soluble GPIHBP1 adenovirus was generated, amplified, and purified as described previously

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[4]The *Angptl3*, *Angptl4*, and *Gpihbp1* knockout mice were produced in collaboration between Genentech, Inc. and Lexicon Pharmaceuticals, Inc. to analyze the function of about 500 secreted and transmembrane proteins.

(32). The purified recombinant adenovirus was confirmed by sequencing the cloning region and tested for infectious unit titer by plaque formation in HEK293 cells.

A549 cells were infected with recombinant adenovirus at a multiplicity of infection of 100 in F-12K medium containing 10% fetal bovine serum. After incubating at 37°C overnight, the medium was replaced with HyQSFM4CHO serum-free medium. After incubating at 37°C for about 24 h, the condition medium containing mouse soluble GPIHBP1 protein was harvested, filtered through a 0.22-micron filter unit, and stored at −20°C.

All procedures for purifying recombinant mouse GPIHBP1 were performed at 4°C. Conditioned medium containing GPI-HBP1 was concentrated approximately 2-fold and then diafiltered against 8 vols of Buffer E (50 mM sodium phosphate, 500 mM sodium chloride, pH 7.8) using a GE Kvick Lab System fitted with three 0.11 m^2 PES cassettes (5000 Da MWCO). The retentate was supplemented with imidazole to a concentration of 10 mM and applied to a 1-ml Ni-NTA column at a rate of 1 ml/min. The column was washed with 50 ml of Buffer E containing 20 mM imidazole and then with 12 ml of Buffer E containing 50 mM imidazole. GPIHBP1 was eluted from the column with Buffer E containing 250 mM imidazole. Eluate fractions were analyzed by SDS-PAGE and the fractions containing the peak recombinant protein were pooled, dialyzed against phosphate-buffered saline, and stored at −80°C.

Production of antibodies

Production of affinity-purified rabbit polyclonal IgGs reactive with mouse GPIHBP1 amino acids 27–38 (DADPEPENYNYD) was ordered from Sigma-Genosys. Mouse monoclonal antibodies (mAbs) reactive with keyhole limpet hemocyanin (KLH) (control mAb KLH), ANGPTL4 (mAb 14D12) (18), and ANGPTL3 (mAb 5.50.3) (31) were prepared as described previously.

ELISA assay for GPIHBP1 binding to LPL

A 96-well Nunc MaxiSorp plate was coated with 1 μg/ml mouse anti-LPL monoclonal antibody in 0.2 M sodium carbonate buffer (pH 9.4) at 4°C overnight. The wells were washed three times with phosphate-buffered saline, 0.05% Tween-20 (PBST) and blocked with 5% human serum albumin in PBST at room temperature for 1 h. Bovine LPL (5.8 nM) was added to the wells and incubated at room temperature for 1 h. After washing the wells three times with PBST, purified mouse soluble GPIHBP1 at concentrations ranging between 0 and 40 nM was added to the wells and incubated at room temperature for 1 h. After washing the wells three times with PBST, rabbit anti-mouse GPIHBP1 peptide antibodies (10 μg/ml) were added and incubated at room temperature for 1 h. After washing three times with PBST, HRP-conjugated goat anti-rabbit antibodies (10 μg/ml) were added and incubated at room temperature for 1 h. The wells were washed three times with PBST and HRP activity was quantitated with TMB substrate according to the manufacturer's protocol. GPI-HBP1 binding with LPL is expressed as absorbance at 450 nm.

LPL activity assay

LPL activity was assayed with the fluorogenic substrate DGGR (33) as described previously (31). All experiments in which LPL activity was measured were performed in LPL assay buffer (50 mM Tris-HCl, 0.12 M sodium chloride, 0.5% Triton X-100, 10 mg/ml BSA, 1.5 mM calcium chloride, pH 7.4). Reactions were performed at room temperature in triplicate and were initiated by adding 90 ul of sample to 10 ul of 0.24 mM DGGR substrate. Hydrolysis of DGGR was measured at 30 s intervals over 10 min with a Cytofluor 4000 Fluorescence Multi-well Plate Reader (Applied Biosystems, Foster City, CA) fitted with a 530/25 nm excita-

tion filter and a 620/40 nm emission filter. The rate of product formation is expressed as the change in relative fluorescence units (RFU) per minute. Under these assay conditions, LPL activity was linear up to at least 120 RFU/min. The initial concentration of active LPL (monomer) in the reaction mixture was approximately 10 nM.

Generation of mutant mice

Generation of the $Angptl4^{-/-}$, $Angptl3^{-/-}$, and $Gpihbp1^{-/-}$ mouse lines[4] has been described previously (18, 25, 31). $Angptl3^{-/-}$ mice were bred with $Gpihbp1^{-/-}$ mice to generate $Angptl3^{-/-}/Gpihbp1^{-/-}$ mice. $Angptl4^{-/-}$ mice were bred with $Gpihbp1^{-/-}$ mice to generate $Angptl4^{-/-}/Gpihbp1^{-/-}$ mice.

Mouse care and study

All procedures involving animals were conducted in conformity with Institutional Animal Care and Use Committee guidelines in compliance with state and federal laws and the standards outlined in the Guide for the Care and Use of Laboratory Animals (34). Mice were housed at 24°C on a fixed 12 h light/12 h dark cycle and had free access to water and diet. All mice were maintained on regular chow (Cat# 5021, Purina, St. Louis, MO).

Analysis of serum lipid levels

Serum samples for lipid analysis were prepared from blood obtained from the retro-orbital plexus. Total TG levels were measured by kit (Serum TG determination kit, Cat# TR0100; Sigma-Aldrich).

Calculations and statistical analyses

Calculations for determining EC$_{50}$ values, IC$_{50}$ values, and confidence intervals were determined by nonlinear regression analysis with sigmoidal dose-response (variable slope) equation. Binding constants (K$_d$) were determined by nonlinear regression analysis with one-site binding (hyperbola) equation. LPL activity decay was determined by nonlinear regression analysis with one-phase exponential decay equation (GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego, CA). Values for GPIHBP1 binding or LPL activity are expressed as the mean (± SEM) of triplicate determinations. Comparisons between two mouse groups were analyzed by unpaired Student's t-test. Comparisons among multiple mouse groups were analyzed by Kruskal-Wallis test followed by a posthoc test if statistical significance was less than 0.05.
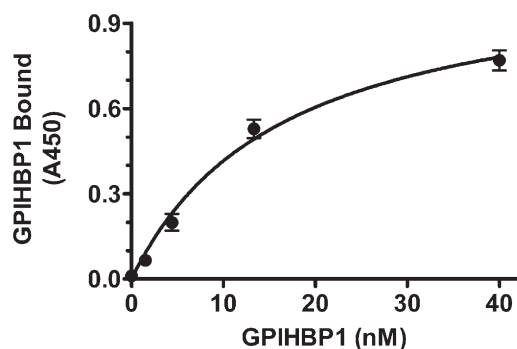
RESULTS

Properties of recombinant soluble mouse GPIHBP1

To investigate the interaction of GPIHBP1 with LPL activity in a chemically defined system, we produced a recombinant GPIHPB1 protein that was freely soluble in aqueous buffers. Mouse GPIHBP1 amino acids 199–228 and human GPIHBP1 amino acids 152–184 contain a C-terminal signal sequence for GPI anchoring (35). By removing the nucleotide sequence encoding this segment and expressing the truncated GPIHBP1 cDNA in our adenoviral expression system, we were able to produce and purify GPIHBP1 from conditioned medium. Although the predicted molecular mass is 20 kDa for recombinant soluble mouse GPIHBP1, the expressed protein displayed slightly higher molecular mass as determined by SDS-PAGE under denaturing, reducing conditions (data not
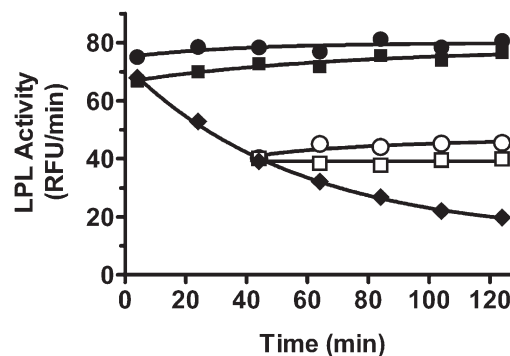
shown). Moreover, the protein migrated as a somewhat broad, unfocused band, suggesting that recombinant soluble mouse GPIHBP1 is glycosylated as expected (36). We tested the ability of soluble mouse GPIHBP1 to bind with LPL in a chemically defined, equilibrium binding ELISA format (**Fig. 1**). The soluble form of GPIHBP1 bound LPL with a K_d of 17 nM (95% confidence interval (CI) = 10–23 nM). This K_d is similar to that reported for the full-length GPIHBP1 expressed on Chinese hamster ovary cells lacking heparan sulfate proteoglycans (36 nM), indicating that the LPL-binding properties of soluble and GPI-anchored GPIHBP1 are comparable (25).

Soluble mouse GPIHBP1 stabilizes but does not activate LPL

Although GPIHBP1 is capable of binding LPL (25), little is known about whether this association affects activity of LPL. Because $Gpihbp1^{-/-}$ mice display very high plasma triglycerides and chylomicronemia (25), we reasoned that the association of GPIHBP1 with LPL may not only serve as a platform for LPL and its substrates but also might stabilize LPL, like heparan sulfate proteoglycans or heparin (8, 14, 15, 17). To test our hypothesis, we used a simple, rapid kinetic assay that employs the fluorogenic lipase substrate DGGR to measure LPL activity over a period that was short enough to assess LPL stability. We incubated recombinant human LPL (approximately 10 nM) for times ranging between 0 and 124 min in LPL assay buffer that was unsupplemented or supplemented with a stabilizing concentration of either heparin (0.1 U/ml) or soluble GPIHBP1 (100 nM). We then measured LPL activity at 20 min intervals throughout the 124 min preincubation. As shown in **Fig. 2**, unsupplemented LPL activity decreased with preincubation time in a manner that is consistent with a first-order reaction with a half-life of about 40 min. In contrast, when heparin or GPIHBP1 was added to the

Fig. 2. Soluble GPIHBP1 stabilizes LPL. Recombinant human LPL (approximately 10 nM) was preincubated at room temperature in LPL assay buffer for up to 124 min with either no supplement (◆), 0.1 U/ml heparin added at time = 0 min (■), 0.1 U/ml heparin added at time = 40 min (□), 100 nM soluble mouse GPIHPB1 added at time = 0 min (●), or 100 nM soluble mouse GPIHBP1 added at time = 40 min (○). The preincubated samples were then assayed for LPL activity at room temperature with fluorogenic substrate DGGR. LPL activity is expressed as relative fluorescence units (RFUs) per minute. Values are the mean ± SEM of triplicate determinations.
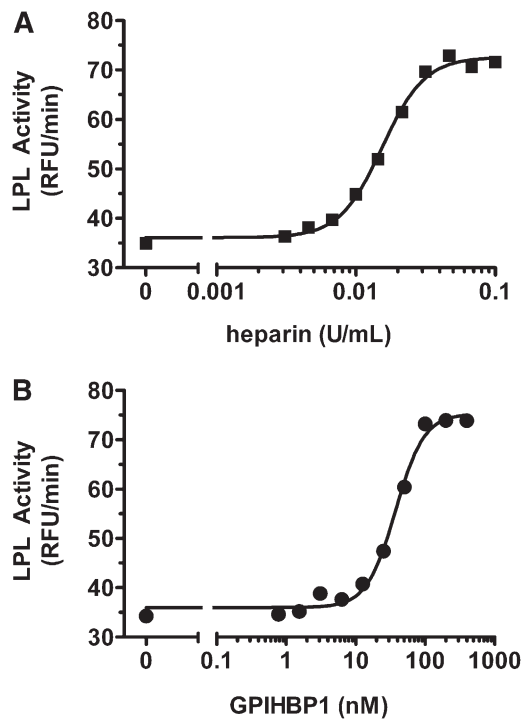
LPL mixture at 0 min, we detected no decay in LPL activity over the 124 min preincubation period. When supplements were added to the LPL mixture at 40 min, a time at which LPL activity had decayed by approximately 50%, both heparin and GPIHBP1 arrested further decay but did not increase LPL activity.

To determine the potency of the stabilizing activity of soluble GPIHBP1 relative to heparin, we incubated LPL in the presence of varying concentrations of either heparin or soluble GPIHBP1 for 60 min at room temperature and then assayed the mixtures for LPL activity (**Fig. 3**). Under these conditions, heparin fully stabilized LPL at approximately 0.03 Units/ml, with an EC_{50} of 0.015 U/ml and GPIHBP1 fully stabilized LPL at 100 nM with an EC_{50} of 38 nM.

Soluble mouse GPIHBP1 antagonizes inactivation of LPL by ANGPTL3 and ANGPTL4

Inactivation of LPL occurs by a mechanism whereby catalytically active dimers dissociate into inactive monomers (15). One physiological regulator of LPL stability is heparan sulfate proteoglycans (8). Our results presented here so far suggest that GPIHBP1 is another regulator that stabilizes LPL. Because ANGPTL4 has been shown to accelerate LPL inactivation (24), we asked whether soluble GPIHBP1 or heparin affect LPL inactivation by ANGPTL4 or LPL inhibition by ANGPTL3, a homolog of ANGPTL4. We incubated varying concentrations of ANGPTL4 (0–20 nM) or ANGPTL3 (62.5–500 nM) with LPL (approximately 10 nM) in a reaction mixture that was either unsupplemented or supplemented with stabilizing concentrations of heparin (0.1 U/ml) or soluble GPIHBP1 (100 nM) for times ranging between 0 and 124 min. The reaction mixtures were then assayed for LPL activity at 20 min intervals.

In **Fig. 4**, we show that ANGPTL4 increased the rate of LPL inactivation in a concentration-dependent manner.

Fig. 1. Soluble GPIHBP1 binds with LPL. Bovine LPL was adsorbed to wells of a mouse anti-LPL monoclonal IgG-coated 96-well plate and then incubated with varying concentrations of soluble mouse GPIHBP1 at concentrations ranging between 0 and 40 nM. The amount of soluble mouse GPIHBP1 bound to LPL was then quantitated by incubating with rabbit anti-GPIHBP1 primary antibodies, then HRP-conjugated goat anti-rabbit secondary antibodies, and finally, TMB substrate (see Experimental Procedures). The amount of soluble mouse GPIHBP1 bound to LPL is expressed as absorbance at 450 nm. Values are the mean ± SEM of triplicate determinations.

Fig. 3. Concentration dependence of heparin or soluble GPIHBP1 on LPL stability. Recombinant human LPL (approximately 10 nM) was preincubated with (A) heparin ranging between 0 and 0.1 U/ml or with (B) soluble mouse GPIHBP1 ranging between 0 and 400 nM in LPL assay buffer at room temperature for 60 min. LPL activity was then assayed at room temperature with DGGR substrate. LPL activity is expressed as RFUs per minute. Values are the mean ± SEM of triplicate determinations.

ANGPTL4 was able to inactivate both nonstabilized (Fig. 4A) and heparin-stabilized (Fig. 4B) LPL. The rate of inactivation of LPL by ANGPTL4 appeared to be greater in the presence of heparin (Fig. 4B) than in the absence of heparin (Fig. 4A). In contrast, soluble GPIHBP1 greatly diminished the rate of inactivation of LPL by ANGPTL4 (Fig. 4C). After 80 min with 10 nM ANGPTL4, GPIHBP1-stabilized LPL showed only a 15% decrease in activity whereas nonstabilized and heparin-stabilized LPL showed nearly undetectable activity.

Like ANGPTL4, ANGPTL3 inhibited LPL activity in a time- and concentration-dependent manner (**Fig. 5**). Although less potent than ANGPTL4, ANGPTL3 inhibited nonstabilized (Fig. 5A) and heparin-stabilized (Fig. 5B) LPL. Unlike ANGPTL4, ANGPTL3 was less effective at inhibiting heparin-stabilized LPL than nonstabilized LPL (Fig. 5A, B). When incubated with 500 nM ANGPTL3 for 45 min, nonstabilized LPL showed nearly undetectable activity whereas heparin-stabilized LPL showed only a 20% decrease in activity. Moreover, LPL stabilized by soluble GPIHBP1 resisted inhibition by ANGPTL3 (Fig. 5C). When incubated with 500 nM ANGPTL3, soluble GPIHBP1-stabilized LPL showed only a 5% decrease after 45 min.

To more directly compare the potency with which heparin or soluble GPIHBP1 prevents inhibition of LPL by ANGPTL4 or ANGPTL3, we preincubated nonstabilized LPL, heparin-stabilized LPL, or soluble GPIHBP1-stabilized
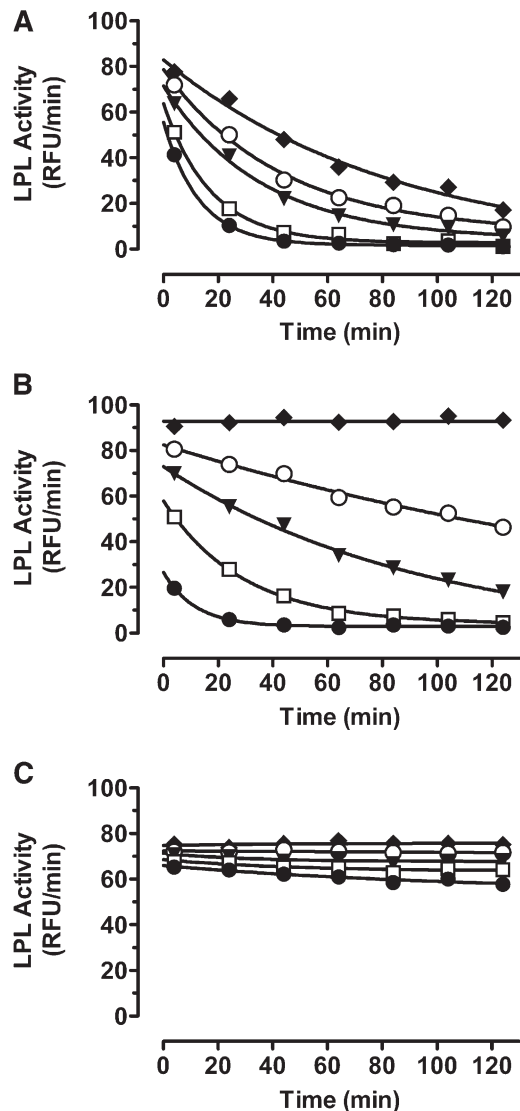
Fig. 4. Soluble GPIHBP1 but not heparin prevents inactivation of LPL by ANGPTL4. Recombinant human LPL (approximately 10 nM) with (A) no supplement, (B) 0.1 U/ml heparin, or (C) 100 nM soluble mouse GPIHBP1 was preincubated at room temperature in LPL assay buffer containing human ANGPTL4 at 0 nM (♦), 2.5 nM (○), 5 nM (■), 10 nM (□), or 20 nM (●). LPL activity was then assayed at room temperature with DGGR substrate. LPL activity is expressed as RFUs per minute. Values are the mean ± SEM of triplicate determinations.

LPL with varying concentrations of ANGPTL4 (**Fig. 6A**) or ANGPTL3 (Fig. 6B) and then assayed the reaction mixtures for LPL activity. ANGPTL4 (Fig. 6A) inactivated nonstabilized LPL (IC_{50} = 6.1 nM; 95% CI = 5.8–6.5 nM) and heparin-stabilized LPL (IC_{50} = 4.3 nM; 95% CI = 3.8–5.0 nM) with nearly equal potency. ANGPTL4, however, was 20-fold less potent at inactivating LPL stabilized by 100 nM soluble GPIHBP1 (IC_{50} = 100 nM; 95% CI = 94–110 nM) and 50-fold less potent at inactivating LPL stabilized by 400 nM soluble GPIHBP1 (IC_{50} = 250 nM; 95% CI = 240–270 nM) than at inactivating nonstabilized or heparin-stabilized LPL.

In contrast to ANGPTL4, ANGPTL3 was a 5-fold less potent inhibitor of heparin-stabilized LPL (IC_{50} = 690
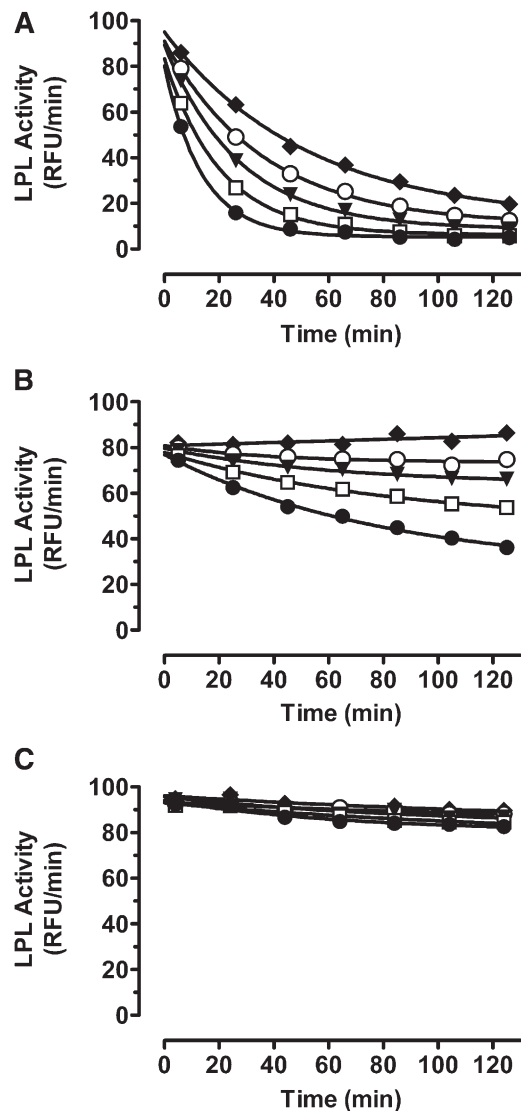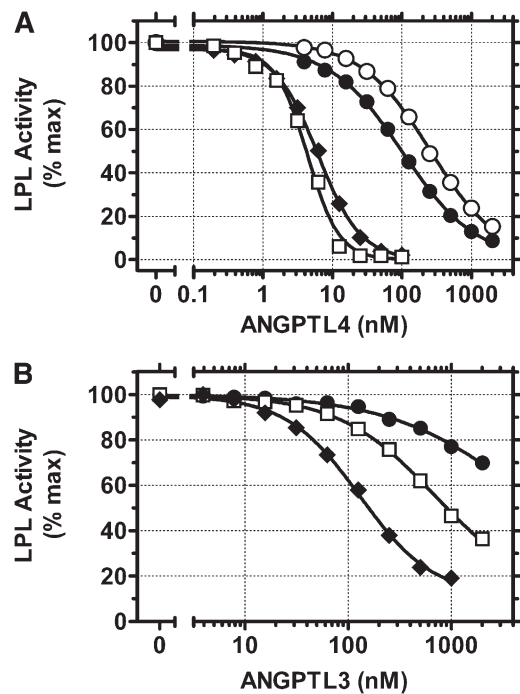
Fig. 5. Soluble GPIHBP1 and heparin prevents inhibition of LPL by ANGPTL3. Recombinant human LPL (approximately 10 nM) with (A) no supplement, (B) 0.1 U/ml heparin, or (C) 100 nM soluble mouse GPIHBP1 was preincubated at room temperature in LPL assay buffer containing human ANGPTL3 at 0 nM ($\blacklozenge$), 62.5 nM ($\bigcirc$), 125 nM ($\blacksquare$), 250 nM ($\square$), or 500 nM ($\bullet$). LPL activity was then assayed at room temperature with DGGR substrate. LPL activity is expressed as RFUs per minute. Values are the mean ± SEM of triplicate determinations.


Fig. 6. Concentration dependence of inhibition of LPL by ANGPTL3 or ANGPTL4 in the presence of stabilizing concentrations of heparin or soluble GPIHBP1. Recombinant human LPL with no supplement ($\blacklozenge$), 0.1 U/ml heparin ($\square$), 100 nM soluble mouse GPIHBP1 ($\bullet$), or 400 nM soluble mouse GPIHBP1 ($\bigcirc$) was preincubated at room temperature in LPL assay buffer containing either (A) human ANGPTL4 for 20 min or (B) human ANGPTL3 for 60 min at concentrations ranging between 0 and 2000 nM. LPL activity was then assayed at room temperature with DGGR substrate. LPL activity is expressed as RFUs per minute. Values are the mean ± SEM of triplicate determinations.

nM; 95% CI = 650–740 nM) than of nonstabilized LPL (IC_{50} = 130 nM; 95% CI = 120–140 nM). Moreover, the inhibitory potency of ANGPTL3 for LPL stabilized with 100 nM soluble GPIHBP1 (IC_{50} = 5200 nM; 95% CI = 4300–6200 nM) was 40-fold less than that for nonstabilized LPL (Fig. 6B).

Inactivation of *Angptl4* markedly lowers TG levels in *Gpihbp1*$^{-/-}$ mice

Gpihbp1$^{-/-}$ mice display chylomicronemia with serum TG levels 50- to 100-fold higher than those of wild-type mice (25). Our in vitro findings described above suggest that the chylomicronemia observed in *Gpihbp1*$^{-/-}$ mice might be caused, at least in part, by inactivation of LPL by

ANGPTL4 or, to a lesser extent, by ANGPTL3. We hypothesized that inactivating the *Angptl4* or *Angptl3* genes in *Gpihbp1*$^{-/-}$ mice might lower their serum TG levels.

To test this hypothesis, we first intercrossed *Angptl4*$^{+/-}$ / *Gpihbp1*$^{+/-}$ mice to generate *Angptl4*$^{+/-}$ / *Gpihbp1*$^{-/-}$ mice and *Angptl4*$^{-/-}$ / *Gpihbp1*$^{-/-}$ mice. All genotypes were viable and appeared healthy. Because the effect of ANGPTL4 deficiency on serum TG levels is most pronounced in the fasted state (18, 31, 37), we then compared fasted serum TG levels of *Angptl4*$^{+/-}$ / *Gpihbp1*$^{-/-}$ mice and *Angptl4*$^{-/-}$ / *Gpihbp1*$^{-/-}$ mice to those of *Gpihbp1*$^{-/-}$ mice. As shown in **Fig. 7A**, serum TG levels averaged 3900 mg/dl in *Gpihbp1*$^{-/-}$ mice, were modestly reduced to 2700 mg/dl in *Angptl4*$^{+/-}$ / *Gpihbp1*$^{-/-}$ mice, and were significantly reduced ($P < 0.05$) to 280 mg/dl (93%) in *Angptl4*$^{-/-}$ / *Gpihbp1*$^{-/-}$ mice. In contrast, *Gpihbp1*$^{+/+}$ / *Angptl4*$^{-/-}$ mice had serum TG levels of 50 mg/dl, which rose only slightly to 130 mg/dl in the presence of two functional *Angptl4* alleles.

Next, we intercrossed *Angptl3*$^{+/-}$ mice to obtain *Angptl3*$^{-/-}$ mice; these mice were present in normal Mendelian ratios at weaning and appeared healthy throughout their normal lifespan. We then intercrossed *Angptl3*$^{+/-}$ / *Gpihbp1*$^{+/-}$ mice to generate *Angptl3*$^{+/-}$ / *Gpihbp1*$^{-/-}$ mice and *Angptl3*$^{-/-}$ / *Gpihbp1*$^{-/-}$ mice. All genotypes were again viable and appeared healthy. Because the effect of
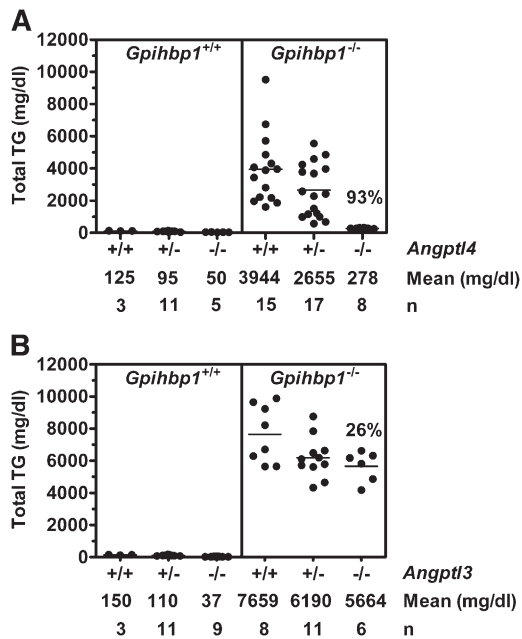
Fig. 7. Disrupting *Angptl4* gene expression normalizes TG levels in hyperlipidemic $Gpihbp1^{-/-}$ mice. A: Fasting serum TG levels were determined in 8-week-old male mice lacking zero, one, or two copies of *Angptl4* in $Gpihbp1^{+/+}$ or $Gpihbp1^{-/-}$ backgrounds. TG levels of $Angptl4^{-/-}/Gpihbp1^{-/-}$ mice were 93% lower than those of $Angptl4^{+/+}/Gpihbp1^{-/-}$ mice. B: Fed serum TG levels were determined in 18 to 20-week-old male mice lacking zero, one, or two copies of *Angptl3* in the $Gpihbp1^{+/+}$ or $Gpihbp1^{-/-}$ background. TG levels of $Angptl3^{-/-}/Gpihbp1^{-/-}$ mice were 26% lower than those of $Angptl3^{+/+}/Gpihbp1^{-/-}$ mice. The bars represent the mean total serum TG level, which is also given below the genotype labels for each group. The number of mice in each group (n) is also shown.

ANGPTL3 deficiency on serum TG levels is most pronounced in the fed state (18, 31, 37), we then compared fed serum TG levels of $Angptl3^{+/-}/Gpihbp1^{-/-}$ mice and $Angptl3^{-/-}/Gpihbp1^{-/-}$ mice to those of $Gpihbp1^{-/-}$ mice. As shown in Fig. 7B, serum TG levels averaged 7700 mg/dl in $Gpihbp1^{-/-}$ mice and were reduced to 6200 mg/dl in $Angptl3^{+/-}/Gpihbp1^{-/-}$ mice and 5700 mg/dl in $Angptl3^{-/-}/Gpihbp1^{-/-}$ mice. In contrast, in a $Gpihbp1^{+/+}$ background, $Angptl3^{-/-}$ mice had serum TG levels of 40 mg/dl whereas $Angptl3^{+/+}$ mice had serum TG levels of 150 mg/dl. Thus, inactivation of both *Angptl3* alleles resulted in a slight reduction in serum TG levels.

Neutralizing mAbs to ANGPTL3 and ANGPTL4 lower TG levels in $Gpihbp1^{-/-}$ mice

The above data suggest that mAbs that neutralize the activity of ANGPTL4 and, to a lesser extent, ANGPTL3 should lower serum TG levels in $Gpihbp1^{-/-}$ mice. The serum TG levels of fasted $Gpihbp1^{-/-}$ mice treated with anti-ANGPTL4 mAb 14D12 were significantly ($P = 0.0009$) lower than those of $Gpihbp1^{-/-}$ mice treated with anti-KLH mAb, resulting in a 75% serum TG reduction (**Fig. 8A**). The serum TG levels of fed $Gpihbp1^{-/-}$ mice treated with anti-ANGPTL3 mAb 5.50.3 were slightly lower than those of $Gpihbp1^{-/-}$ mice treated with anti-KLH mAb, resulting in a 24% serum TG reduction (Fig. 8B).
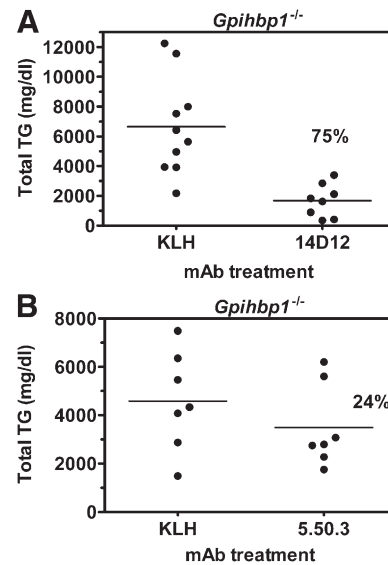
Fig. 8. Neutralizing ANGPTL4 with mAb 14D12 reduces serum TGs in hyperlipidemic $Gpihbp1^{-/-}$ mice. A: Serum TG levels were determined in $Gpihbp1^{-/-}$ mice treated with either ANGPTL4-neutralizing mAb 14D12 or control mAb KLH. Mice were injected intraperitoneally with 30 mg/kg mAb 14D12 (n = 8) or mAb KLH (n = 10) on day 0. Blood was drawn from fasted mice for serum TG measurements on day 4. B: Serum TG levels were determined in $Gpihbp1^{-/-}$ mice treated with either ANGPTL3-neutralizing mAb 5.50.3 or control mAb KLH. Mice were injected intraperitoneally with 30 mg/kg mAb 5.50.3 (n = 7) or control mAb KLH (n = 7) on day 0 and day 4. Blood was drawn from fed mice for serum TG measurements on day 8. The bars represent the mean total serum TG level for each group. The percent reduction in TG levels is also shown.

DISCUSSION

GPIHBP1 is an important regulator of triglyceride metabolism in vivo (25, 26). In $Gpihbp1^{-/-}$ mice, plasma triglycerides, mainly in the form of CM, are increased to levels as high as 5000 mg/dl (25), levels that are 50-fold higher than those of wild-type mice. The increase in plasma triglyceride levels appears to be caused by a substantial decrease in intravascular LPL activity, which is required for processing CM and VLDL (25, 38). Young et al. (26) proposed that GPIHBP1 functions as a platform for LPL and its substrate CM, placing this substrate in close proximity with LPL and presumably increasing its catalytic efficiency. Moreover, Weinstein et al. (38) showed that the release of LPL into plasma after injection with heparin is slower in $Gpihbp1^{-/-}$ mice than in wild-type mice, suggesting that LPL is largely absent from the intravascular pools where it is normally bound with GPIHBP1 and is instead released from extravascular sites. Thus, GPIHBP1 appears to play a major role in anchoring LPL to the lumen of capillary beds.

In this paper, we describe new functional properties of GPIHBP1: stabilization of LPL and prevention of its inhibition by ANGPTL3 and ANGPTL4. LPL is an unstable enzyme that exists as a head-to-tail homodimer in its active form and monomers in its inactive form and its conversion from active to inactive forms is a process that is essentially

irreversible (14–16). GPIHBP1 is a multidomain protein, consisting of a short acidic N-terminal domain with very high linear charge density, a lymphocyte antigen 6 (Ly6) superfamily domain (39, 40), and a C-terminal GPI anchor, which mediates attachment of GPIHBP1 to the luminal surface of endothelial cells. The acidic domain as well as the Ly6 domain of GPIHBP1 have been shown to play an important role in binding with the heparin binding domain of LPL (25, 26, 41). Here, we show that soluble GPIHBP1, which consists of only the acidic and Ly6 domains, is secreted into media by virally transduced cell lines and retains the ability to bind with LPL. Using our in vitro assay we showed that, like heparin, GPIHBP1 is capable of stabilizing LPL for at least 2 h. Moreover, when we added GPIHBP1 to LPL that had decayed, GPIHBP1 prevented any further decrease in LPL activity but did not return LPL to its initial level; thus, GPIHBP1 stabilizes but does not activate LPL.

ANGPTL4 has been shown to inhibit LPL in the absence and presence of stabilizing concentrations of heparin and this process appears to involve interaction of ANGPTL4 with dimeric, catalytically active LPL and acceleration of its irreversible inactivation (24). Our experiments confirm this earlier finding and further show that ANGPTL4 apparently inactivates both nonstabilized and heparin-stabilized LPL with nearly equal potency under our assay conditions. The time course of inhibition of LPL by ANGPTL3 is similar to that of ANGPTL4, suggesting that ANGPTL3 also inhibits LPL by inactivation. ANGPTL3, like ANGPTL4, was capable of inhibiting heparin-stabilized LPL. However, the potency of ANGPTL3 to inhibit heparin-stabilized LPL was 5-fold less than that to inhibit nonstabilized LPL. The mechanism by which heparin appears to protect LPL from inhibition by ANGPTL3 and not by ANGPTL4 is not clear and will require further investigation.

In our in vitro assay system, we demonstrated that GPIHPB1 reduces the LPL-inhibitory potency of ANGPTL4 by 20-fold and the LPL-inhibitory potency of ANGPTL3 by 40-fold. Moreover, we showed that inhibition of LPL by ANGPTL4 or ANGPTL3 was antagonized more strongly by GPIHBP1 than by heparin. These in vitro observations suggest that, in vivo, GPIHBP1 may buffer LPL from the inhibitory effects of circulating ANGPTL4 and ANGPTL3. To test this hypothesis in vivo, we generated $Gpihpb1^{-/-}$ mice with 0, 1, or 2 functional $Angptl4$ alleles. Whereas $Gpihpb1^{-/-}$ mice with two $Angptl4$ alleles had TG levels of 3900 mg/dl, those with one $Angptl4$ allele had serum TG levels of 2700 mg/dl (33% reduction) and those with no functional $Angptl4$ allele had serum TG levels of 280 mg/dl (93% reduction). These results suggest that, in vivo, GPIHBP1 plays an important role in buffering the inactivation of LPL by ANGPTL4. Moreover, consistent with our in vitro results, heparan sulfate proteoglycans are relatively unable to protect LPL from ANGPTL4 inactivation in the absence of GPIHBP1. These results also suggest that ANGPTL3 is not a very effective replacement for ANGPTL4 in the absence of GPIHBP1. This interpretation is consistent with our in vitro results showing that ANGPTL3 is a much less potent inhibitor of LPL than is ANGPTL4, par-

ticularly in the presence of heparin. Moreover, our results suggest that heparan sulfate proteoglycans may to some extent protect LPL from inhibition by ANGPTL3 in vivo. Although our findings suggest that GPIHBP1 shields LPL from the effects of ANGPTL4 in vivo, it is also likely that GPIHBP1 prevents inhibition of LPL by other factors, probably including ANGPTL3, because the serum TG levels of $Angptl4^{-/-}$ mice with both functional $Gpihbp1$ alleles are 50 mg/dl whereas those of $Angptl4^{-/-}$ mice with no functional $Gpihbp1$ allele are 280 mg/dl.

The results presented here support our contention that GPIHBP1 not only functions as a platform for LPL and its substrates (25, 26) but also stabilizes LPL in certain metabolic contexts (42), essentially participating with circulating ANGPTL4 in the regulation of LPL activity. GPIHBP1 is highly expressed on the luminal surface of endothelial cells of the vasculature of adipose tissue, cardiac muscle, and skeletal muscle (25, 43). In adipose tissue, LPL plays an important role in providing free fatty acids from lipoproteins for fat storage. In contrast, LPL in cardiac tissue likely plays an important role in supplying free fatty acids from lipoproteins for β-oxidation. Thus, the distribution, storage, and utilization of lipids in skeletal muscle, cardiac muscle, and adipose tissue is likely to be orchestrated, at least in part, by the level of ANGPTL4, ANGPTL3, and GPIHBP1 in these tissues and their interaction with LPL.

Our study also suggests that mutations in GPIHBP1 or LPL that compromise the ability of GPIHBP1 to stabilize LPL could result in hyperlipidemia associated with fasting chylomicronemia. In such cases, agents that directly or indirectly stabilize LPL may be useful for treating the pancreatitis that often accompanies severe hypertriglyceridemia. We have shown here that monoclonal antibodies that neutralize ANGPTL4 or, to a lesser extent, ANGPTL3, can decrease the elevated TG levels associated with absence of functional GPIHBP1; this occurs by a mechanism that most likely involves indirect LPL stabilization resulting in increased LPL activity. Thus, neutralizing anti-ANGPTL4 or anti-ANGPTL3 antibodies should be considered as a therapeutic option for treating individuals with recurrent pancreatitis associated with mutations in LPL or in GPIHPB1 that lead to inappropriate inactivation or destabilization of LPL.█

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