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Mobility of “HSPG-bound” LPL explains how LPL is able to reach GPIHBP1 on capillaries^S

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Abstract In mice lacking glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1), the LPL secreted by adipocytes and myocytes remains bound to heparan sulfate proteoglycans (HSPGs) on all cells within tissues. That observation raises a perplexing issue: Why isn't the freshly secreted LPL in wild-type mice captured by the same HSPGs, thereby preventing LPL from reaching GPIHBP1 on capillaries? We hypothesized that LPL–HSPG interactions are transient, allowing the LPL to detach and move to GPIHBP1 on capillaries. Indeed, we found that LPL detaches from HSPGs on cultured cells and moves to: 1) soluble GPIHBP1 in the cell culture medium; 2) GPIHBP1-coated agarose beads; and 3) nearby GPIHBP1-expressing cells. Movement of HSPG-bound LPL to GPIHBP1 did not occur when GPIHBP1 contained a Ly6 domain missense mutation (W109S), but was almost normal when GPIHBP1's acidic domain was mutated. To test the mobility of HSPG-bound LPL in vivo, we injected GPIHBP1-coated agarose beads into the brown adipose tissue of GPIHBP1-deficient mice. LPL moved quickly from HSPGs on adipocytes to GPIHBP1-coated beads, thereby depleting LPL stores on the surface of adipocytes. **■** We conclude that HSPG-bound LPL in the interstitial spaces of tissues is mobile, allowing the LPL to move to GPIHBP1 on endothelial cells.—Allan, C. M., M. Larsson, R. S. Jung, M. Ploug, A. Bensadoun, A. P. Beigneux, L. G. Fong, and S. G. Young. **Mobility of “HSPG-bound” LPL explains how LPL is able to reach GPIHBP1 on capillaries.** *J. Lipid Res.* 2017. 58: 216–225.

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LPL, a triglyceride hydrolase that is synthesized and secreted by myocytes and adipocytes, is crucial for the lipolytic processing of triglyceride-rich lipoproteins (TRLs) inside blood vessels (1–3). For decades, dogma held that the LPL produced by myocytes and adipocytes was bound to negatively charged heparan sulfate proteoglycans (HSPGs) in the glycocalyx coating of blood vessels (4). This model was plausible because LPL contains positively charged heparin-binding domains (5–8), and LPL binds avidly to HSPGs on cultured cells and to HSPGs immobilized on 96-well plates (9). Moreover, LPL was known to be released into the plasma with an injection of heparin (1, 2). Over the past 8 years, however, this model for intravascular lipolysis has changed. We now recognize that glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1 (GPIHBP1), a glycosylphosphatidylinositol-anchored protein of capillary endothelial cells, is the binding site for LPL (10). GPIHBP1 captures LPL within the interstitial spaces and shuttles it across endothelial cells to the capillary lumen (10, 11). The GPIHBP1–LPL complex on the surface of capillaries is also crucial for the margination of TRLs along capillaries, making it possible for LPL-mediated TRL processing to proceed (12).

GPIHBP1 expression is required for TRL processing (11). Several mutations in GPIHBP1's cysteine-rich Ly6 domain have been identified in patients with severe hypertriglyceridemia (“familial chylomicronemia syndrome”) (13–22). All of those mutations block the ability of GPIHBP1 to bind and transport LPL. Recent surface plasmon resonance studies have indicated that GPIHBP1's Ly6 domain is primarily responsible for high-affinity LPL binding,

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; GPIHBP1, glycosylphosphatidylinositol-anchored high density lipoprotein binding protein 1; HSPG, heparan sulfate proteoglycan; TRL, triglyceride-rich lipoprotein.

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while the acidic domain at GPIHBP1's N terminus simply promotes the formation of an initial LPL–GPIHBP1 complex (23, 24).

Immunohistochemistry studies on wild-type and GPIHBP1-deficient (*Gpihbp1*^{-/-}) mice have provided key insights into LPL and GPIHBP1 physiology. In wild-type mice, the vast majority of the LPL in sections of heart is bound to GPIHBP1 on capillaries (10), revealing that freshly secreted LPL can move efficiently to endothelial cells. The LPL in wild-type mice can be released into the plasma with an injection of heparin (25). The immunohistochemistry studies on heart sections from *Gpihbp1*^{-/-} mice were arguably more intriguing. In the absence of GPIHBP1, the LPL remained attached to HSPGs on the surface of all cells within the tissue. As in wild-type mice, the LPL in *Gpihbp1*^{-/-} mice could be released into the plasma with an injection of heparin (10, 25). Thus, in the absence of GPIHBP1, the LPL remains bound to HSPGs within the interstitial spaces; that HSPG-bound LPL, which is catalytically active (25), does not simply diffuse away into the lymph and reach the plasma. Indeed, the levels of LPL in the plasma of *Gpihbp1*^{-/-} mice are far lower than in wild-type mice, implying that the binding of LPL to interstitial HSPGs is quite avid and efficient.

The fact that the LPL in *Gpihbp1*^{-/-} mice accumulates in the interstitium, mainly on the surface of cells, and does not enter the plasma compartment poses a conundrum: Why don't the same interstitial HSPG binding sites in wild-type mice capture newly secreted LPL and thereby impede the movement of LPL to endothelial cells? The answer to that question is not known, but we hypothesize that the binding of LPL to interstitial HSPGs is actually dynamic (with high “on” and “off” rates), such that the LPL is constantly bouncing on and off a large pool of HSPG binding sites. Such a scenario would explain how LPL can move to high-affinity GPIHBP1 binding sites on capillaries. In the current study, we tested, using cell culture and mouse models, the concept that the binding of LPL to HSPGs is dynamic and transient, allowing for rapid transfer of LPL to GPIHBP1.

MATERIALS AND METHODS

Immunohistochemistry on the mouse mammary gland

Mammary glands were harvested from lactating wild-type and *Gpihbp1*^{-/-} mice. Frozen sections (8–10 μm) were incubated with primary antibodies [3 μg/ml for monoclonal antibody 11A12; 1:400 for a hamster anti-CD31 antibody (Millipore, Billerica, MA); 1:1,000 for a rabbit anti-collagen type IV antibody (Cosmo Bio USA, Carlsbad, CA); 10 μg/ml for a goat anti-mouse LPL antibody]. Secondary antibodies (Alexa555- or Alexa647-labeled anti-rat IgG, Alexa488- or Alexa647-labeled anti-hamster IgG, Alexa488- or Alexa647-labeled anti-rabbit IgG, and Alexa549- or Alexa488-labeled anti-goat IgG) were used at a dilution of 1:200 for 1 h at room temperature. Images were obtained with an Axiovert 200 MOT microscope equipped with an Apo-tome (both from Zeiss, Germany) or by confocal fluorescence microscopy with a Leica SP2 1P-FCS microscope (Heidelberg, Germany).

Western blots

Proteins were size-fractionated on 12% NuPAGE SDS-PAGE gels with MES buffer, followed by transfer to a nitrocellulose membrane. The nitrocellulose membranes were blocked for 1 h at room temperature with Odyssey blocking buffer (LI-COR) and then incubated with a goat polyclonal antibody against mouse LPL (10 μg/ml) (26) followed by an IRDye800-conjugated donkey anti-goat IgG (LI-COR); a mouse monoclonal antibody against the V5 tag (ThermoFisher Scientific; 1:500) followed by an IRDye800-conjugated donkey anti-mouse IgG (LI-COR); a goat polyclonal antibody against the S-protein tag (Abcam; 1:1,000) followed by an IRDye680-conjugated donkey anti-goat IgG (LI-COR); a rabbit polyclonal antibody against β-actin (Novus Biologicals; 1:1,000) followed by an IRDye800-conjugated donkey anti-rabbit IgG (LI-COR); and antibody 11A12 (3 μg/ml) followed by an IRDye680-conjugated donkey anti-rat IgG. Signals were visualized with an Odyssey scanner (LI-COR).

Release of tissue-associated LPL by heparinase III

Gpihbp1^{-/-} mice were euthanized and perfused with PBS. The interscapular brown adipose tissue and heart were harvested and embedded in OCT medium on dry ice. Tissue sections of the heart (7 μm) and brown adipose tissue (10 μm) were prepared and placed on glass slides. Tissue sections were then incubated for 1 h at room temperature with PBS alone or PBS containing 10 U/ml of heparinase III (Sigma-Aldrich). The solution was collected and analyzed by Western blotting.

Preparation of soluble human GPIHBP1

We produced secreted versions of human wild-type GPIHBP1, GPIHBP1-W109S, and GPIHBP1Δ(25–50) in *Drosophila* S2 cells (27). These GPIHBP1 proteins contained a uPAR tag (27) and the epitope for the GPIHBP1-specific monoclonal antibody 11A12 (28). The GPIHBP1 proteins were purified over agarose beads coated with the uPAR-specific monoclonal antibody R24 (29).

Testing the ability of a GPIHBP1 acidic domain mutant to bind LPL

CHO pgsA-745 cells (2×10^6) were electroporated with 2 μg of a plasmid for S-protein-tagged versions of wild-type human GPIHBP1, GPIHBP1-W109S (27), or GPIHBP1-D,E(25–50)N,Q [a mutant GPIHBP1 in which all acidic amino acids within residues 25–50 were replaced with uncharged amino acids (Asn or Gln)]; the transfected cells were plated on coverslips in 24-well plates. Twenty-four hours later, the transfected cells were incubated with V5-tagged LPL (30) for 1 h at 4°C, washed, and then processed for Western blotting. Cell lysates were collected by incubating cells with mammalian protein extraction reagent (M-PER; Thermo Fisher Scientific) with EDTA-free complete protease inhibitor cocktail (Roche) for 5 min at 4°C. The protein extracts were analyzed by Western blotting.

Preparation of LPL-loaded HepG2 cells

HepG2 cells (8×10^5) were plated in a 24-well plate. The next day, the cells were incubated with V5-tagged human LPL for 1 h at 4°C and then washed. Alternatively, the HepG2 cells were incubated with 1 μg bovine LPL with 0.5% BSA (w/v) for 1 h at 4°C and then washed. HepG2 cells are known to express large amounts of HSPGs on their cell surface (31). To determine whether the LPL on the surface of HepG2 cells was bound to HSPGs, the cells were incubated for 20 min at room temperature with PBS alone, heparin (10–100 U/ml), or heparinase III (15–30 U/ml; Sigma-Aldrich). The supernatant fluid was collected and aliquots were analyzed by Western blotting.

Testing the ability of soluble GPIHBP1 to remove LPL from the surface of HepG2 cells

HepG2 cells (8×10^5) were loaded with V5-tagged human LPL for 1 h at 4°C and then washed. Secreted versions of human wild-type GPIHBP1 or buffer alone were added to the cells in a volume of 200 μ l for 30 min at 4°C. The supernatant fluid was collected and the cells were washed and incubated with 200 μ l heparin (500 U/ml) for 20 min at 4°C. Both the initial supernatant fluid and the heparin-released material were analyzed by Western blotting. Bands were visualized and quantified with an Odyssey scanner (LI-COR). In some experiments, LPL-loaded HepG2 cells were incubated with 10 μ g/ml of wild-type GPIHBP1 in 200 μ l at 4°C for 5, 10, 20, or 30 min, or buffer only for 30 min. Supernatant fluids were analyzed for GPIHBP1 and LPL by Western blotting.

Testing the ability of HSPG-bound LPL on HepG2 cells to detach and move to GPIHBP1

CHO pgsA-745 cells (2×10^6) were electroporated with 2 μ g of a plasmid for S-protein-tagged versions of wild-type human GPIHBP1, GPIHBP1-W109S, or GPIHBP1-D,E(25–50)N,Q; the transfected cells were plated on coverslips in 24-well plates. After 24 h, the coverslips containing the transfected CHO pgsA-745 cells were placed face-down onto the HepG2 cells that had been loaded with V5-tagged human LPL or bovine LPL in PBS/Ca/Mg buffer and incubated at 37°C for 20 min. The pgsA-745 cells were then washed and processed for Western blotting, immunocytochemistry, or LPL activity measurements. In a separate experiment, HepG2 cells were plated on coverslips and placed face-down onto LPL-loaded CHO pgsA-745 cells transfected with an S-protein-tagged version of wild-type human GPIHBP1. For Western blotting, cell lysates were collected as described earlier. For immunocytochemistry studies, the transfected cells were fixed in 3% paraformaldehyde for 15 min and blocked with 10% donkey serum in PBS/Mg/Ca. The cells were then incubated overnight at 4°C with a mouse monoclonal antibody against the V5 tag (Thermo Fisher Scientific; 1:100) and a goat polyclonal antibody against the S-protein tag (Abcam; 1:800), followed by a 30 min incubation with an Alexa568-conjugated donkey anti-goat IgG (Thermo Fisher Scientific; 1:800) and an Alexa647-conjugated donkey anti-mouse IgG (Thermo Fisher Scientific; 1:800). After washing, the cells were fixed with 3% paraformaldehyde for 15 min and stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize DNA. Images were recorded with an Axiovert 200M microscope and processed with Zen 2010 software (all from Zeiss). Within each experiment, the exposure conditions for each construct were identical. LPL activity on the surface of transfected cells was measured with [3 H]triolein-Intralipid (0.5 μ Ci [3 H]triolein per milligram Intralipid triglyceride). Coverslips were placed in 500 μ l incubations containing 50 units of heparin per milliliter, 2 mg of triglyceride per milliliter in PBS/Mg/Ca containing 6% BSA (w/v, pH 7.4), and 5% (v/v) heat-inactivated rat serum (as a source of apo-CII). Samples were incubated at room temperature for 90 min, followed by lipid extraction and scintillation counting (32).

Testing the ability of HSPG-bound LPL on HepG2 cells to move to GPIHBP1-loaded agarose beads

We tested the movement of LPL from V5-LPL-loaded HepG2 cells to agarose beads harboring recombinant human GPIHBP1. To prepare GPIHBP1-agarose beads, we added 8.5 μ g GPIHBP1 proteins [wild-type GPIHBP1, GPIHBP1-W109S, or GPIHBP1 Δ (25–50)] or buffer alone to 100 μ l antibody 11A12-coated agarose beads (33). The agarose beads were then placed adjacent to the coverslips containing V5-LPL-loaded HepG2 cells in 6-well plates and incubated for 2 h at 4°C. The beads (spatially

separated from the coverslips) were then collected and washed, and any LPL that had moved to GPIHBP1 on the surface of the beads was eluted with SDS sample buffer (10 min at 90°C). The amounts of GPIHBP1 and LPL were analyzed by Western blotting.

Testing the ability of GPIHBP1 to capture LPL from HSPGs in tissues of *Gpihbp1*^{-/-} mice

Antibody 11A12-coated agarose beads that had been incubated with wild-type GPIHBP1 (or buffer alone) were injected into the interscapular brown adipose tissue of wild-type or *Gpihbp1*^{-/-} mice (50 μ l of agarose beads containing 6.5 μ g GPIHBP1 injected into each pad). After 45 min, the mice were euthanized and perfused with PBS followed by 3% paraformaldehyde. The brown adipose tissue pad was then harvested and embedded in OCT medium on dry ice. Tissue sections (10 μ m) were fixed with methanol at -20°C for 10 min, permeabilized with 0.2% Triton X-100 for 5 min, and blocked at room temperature with 5% donkey serum, 10% fetal bovine serum, and 0.2% BSA in PBS/Mg/Ca. Tissues were incubated overnight at 4°C with a goat polyclonal antibody against mouse LPL (7 μ g/ml) (26) and a rabbit polyclonal antibody against mouse CD31 (Abcam; 1:50), followed by a 45 min incubation at room temperature with Alexa647-conjugated antibody 11A12 (3 μ g/ml), Alexa568-conjugated donkey anti-goat IgG (Thermo Fisher Scientific; 1:200), and Alexa488-conjugated donkey anti-rabbit IgG (Thermo Fisher Scientific; 1:200). After washing, the cells were fixed with 3% paraformaldehyde for 5 min and stained with DAPI to visualize DNA. Microscopy was performed as described earlier. The mice were fed a chow diet and housed in a barrier facility with a 12 h light-dark cycle. All studies were approved by the University of California Los Angeles Animal Research Committee.

RESULTS

Mislocalization of LPL in the mammary gland of *Gpihbp1*^{-/-} mice

Davies et al. (10) reported that LPL in the hearts of *Gpihbp1*^{-/-} mice is mislocalized, with most of it located on or near the surface of cells (both cardiomyocytes and endothelial cells). We wanted to determine whether the LPL in *Gpihbp1*^{-/-} mice was also mislocalized in the lactating mammary gland, a site with high levels of LPL expression (34). In wild-type mice, most of the LPL in the mammary gland was bound to capillaries, colocalizing with the endothelial cell marker, CD31 (Fig. 1A, B). In *Gpihbp1*^{-/-} mice, LPL did not colocalize with CD31 and, instead, was mislocalized in the interstitial spaces on or near mammary epithelial cells (Fig. 1C).

We have assumed that the LPL within the interstitial spaces of *Gpihbp1*^{-/-} mice was bound to HSPGs because the LPL in those mice was readily released into the plasma with an injection of heparin (25). To test that idea, we treated heart and brown adipose tissue sections from *Gpihbp1*^{-/-} mice with heparinase III, an enzyme that cleaves heparan sulfates. As we had predicted, that treatment released LPL from sections of both heart and brown adipose tissue (supplemental Fig. S1).

Testing the ability of soluble GPIHBP1 to capture LPL from the surface of HepG2 cells

We loaded the surface of HepG2 cells, which are known to be rich in cell-surface HSPGs (31), with V5-tagged

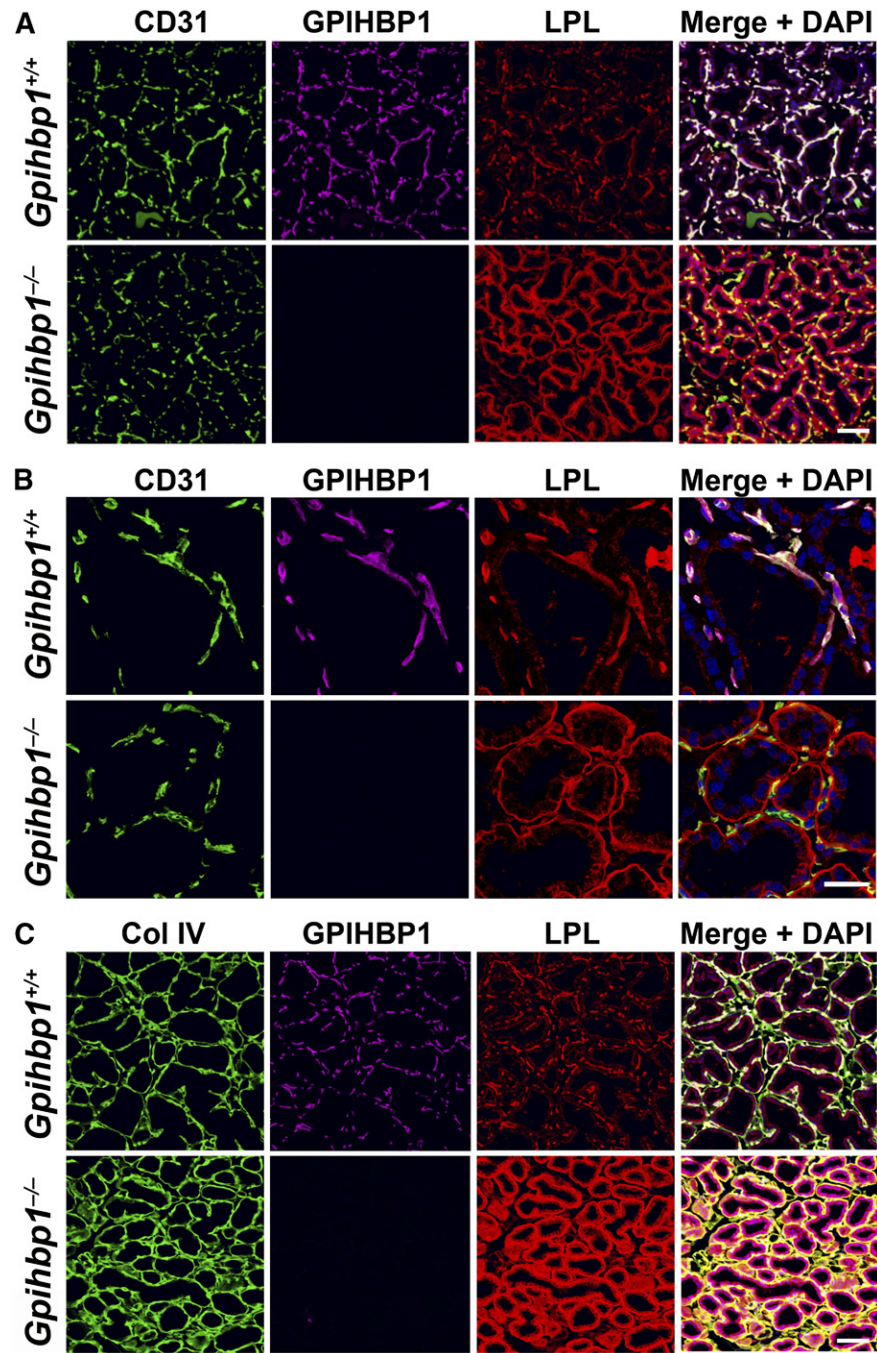


Fig. 1. Localization of LPL in the mammary gland. Mammary glands were harvested from lactating *Gpihbp1*^{+/+} and *Gpihbp1*^{-/-} mice, sectioned, and fixed on slides. Sections were stained with antibodies for CD31 (green), GPIHBP1 (magenta), LPL (red), and collagen IV (green). LPL localization in relation to the endothelial cell marker, CD31, was assessed with 20× (A) and 100× (B) objectives. LPL localization in relation to collagen IV was assessed with a 20× objective (C). DNA was stained with DAPI (blue). Scale bar, 50 μm in (A) and (C); 10 μm in (B).

human LPL. As expected, the LPL on the surface of those cells could be released with heparinase III (supplemental Fig. S2). To determine whether soluble GPIHBP1 (GPIHBP1 lacking the glycosylphosphatidylinositol anchor) would capture LPL from HSPGs on HepG2 cells, we added soluble GPIHBP1 to the medium of HepG2 cells that had been preloaded with V5-LPL. We then assessed the amount of LPL that was released into the medium and the amount of

LPL remaining on the surface of the cells. With increasing amounts of GPIHBP1 in the medium, more LPL was released into the medium and less remained attached to HepG2 cells (Fig. 2A). We also showed that the release of LPL by recombinant GPIHBP1 increased steadily between 5 and 30 min (Fig. 2B). Interestingly, a small amount of LPL was released from cells during a 30 min incubation with buffer alone, but the amount released with buffer was

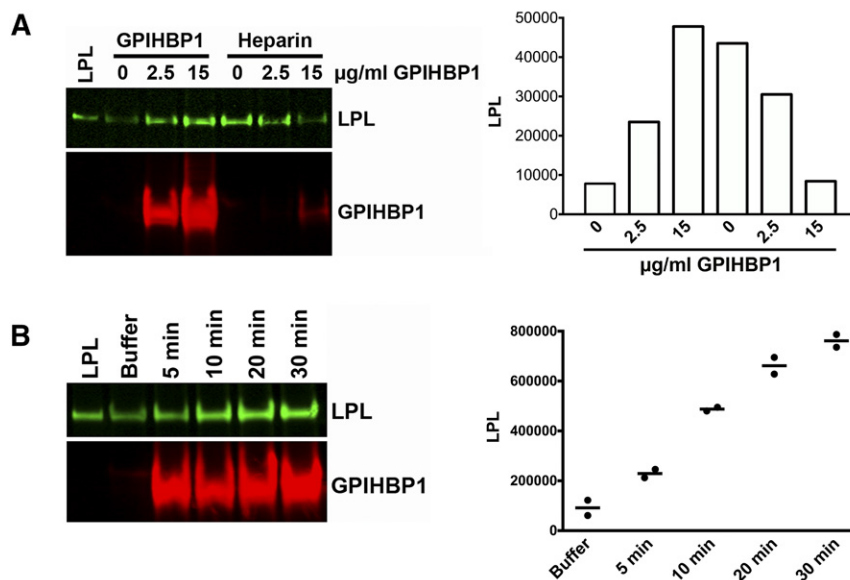


Fig. 2. Removal of LPL from the surface of HepG2 cells with soluble GPIHBP1. **A:** HepG2 cells were incubated with V5-tagged human LPL, allowing the LPL to bind to cell-surface HSPGs. Next, the cells were incubated with buffer alone or buffer containing 2.5 or 15 µg/ml (0.083 µM and 0.5 µM) of wild-type GPIHBP1 for 30 min at 4°C. The V5-LPL remaining on the cells was released with heparin (500 U/ml; 20 min at 4°C). The GPIHBP1 incubation samples and the “heparin-release samples” were analyzed by Western blotting with a monoclonal antibody against the V5 tag (LPL, green) and a monoclonal antibody against GPIHBP1 (11A12, red). The lane labeled “LPL” is the V5-LPL that was loaded onto HepG2 cells. The bar graph shows quantification of the LPL signal on the Western blot, as measured with an Odyssey infrared scanner. **B:** V5-LPL-loaded HepG2 cells were incubated with buffer alone for 30 min or with 10 µg/ml of wild-type GPIHBP1 for 5, 10, 20, or 30 min. Samples were analyzed by Western blotting with a monoclonal antibody against the V5 tag and monoclonal antibody 11A12. The lane labeled “LPL” is the V5-LPL that was loaded onto HepG2 cells. The scatter plot shows quantification of signals from two independent experiments.

less than the amount released during a 5 min incubation with GPIHBP1 (Fig. 2B).

Testing the ability of LPL to move from the HSPGs on HepG2 cells to GPIHBP1-expressing CHO cells

In the next series of studies, we tested whether LPL would move from HSPGs on HepG2 cells to GPIHBP1-expressing cells. We transfected CHO pgsA-745 cells with expression vectors for several human GPIHBP1 proteins [wild-type, a mutant containing an Ly6 missense mutation that prevents LPL binding (W109S), and a mutant in which the glutamates and aspartates in the acidic domain had been replaced with glutamine and asparagine]. [GPIHBP1-W109S lacks the ability to bind LPL in cell-based LPL–GPIHBP1 binding assays, whereas the acidic domain mutant retains the capacity to bind LPL (supplemental Fig. S3).] The GPIHBP1-transfected cells were plated on coverslips; those coverslips were then placed face-down on the V5-LPL-loaded HepG2 cells for 20 min at 37°C. Western blots of cell extracts revealed that the V5-LPL on HepG2 cells had moved to CHO cells expressing wild-type GPIHBP1 or the acidic domain mutant, but not to cells expressing GPIHBP1-W109S (Fig. 3A, B). We performed similar studies in which the HepG2 cells were loaded with bovine LPL. In those studies, we assessed the movement of bovine LPL to transfected CHO pgsA-745 cells by measuring the activity of LPL on the surface of the CHO cells. The cells expressing

wild-type GPIHBP1 and the acidic domain mutant captured catalytically active bovine LPL from the HSPG-bound pool on HepG2 cells (Fig. 3C). The movement of LPL from HepG2 cells to cells expressing wild-type GPIHBP1 and the acidic domain mutant was evident by immunocytochemistry (Fig. 3D). LPL did not move to cells expressing GPIHBP1-W109S. As an additional control, we tested the ability of GPIHBP1-bound LPL to move from GPIHBP1-expressing CHO pgsA-745 cells to HepG2 cells. As expected, movement of LPL from GPIHBP1 to HSPGs on HepG2 cells was not detectable (supplemental Fig. S4).

Assessing the movement of HSPG-bound LPL to GPIHBP1-loaded agarose beads

The experiments in Fig. 2 revealed that soluble GPIHBP1 in the medium captures the LPL that is spontaneously released from HepG2 HSPGs, but the next goal was to determine whether GPIHBP1 was also effective in capturing LPL when GPIHBP1 was spatially separated from the cells. To address this issue, we captured soluble GPIHBP1 on agarose beads coated with the GPIHBP1-specific monoclonal antibody, 11A12, and then placed the agarose beads in 6-well plates adjacent to the coverslips containing LPL-loaded HepG2 cells. We then recovered beads that were spatially separated from the coverslips. Even though the beads were not in contact with the HepG2 cells, the LPL nevertheless diffused away from HSPGs on HepG2

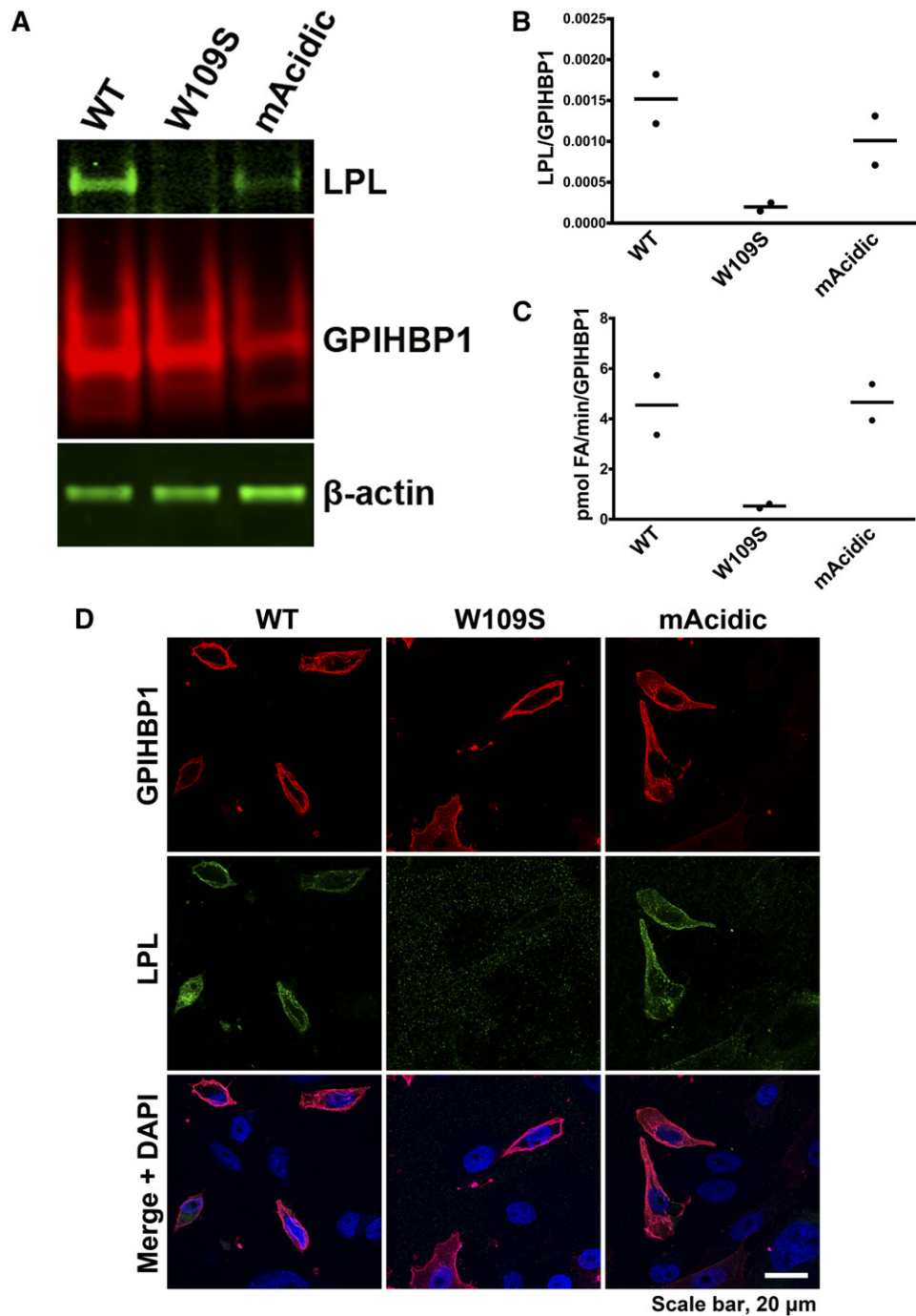


Fig. 3. Movement of HSPG-bound LPL on the surface of HepG2 cells to GPIHBP1 on GPIHBP1-transfected CHO pgsA-745 cells. A, B: HepG2 cells were incubated with V5-tagged human LPL, allowing the LPL to bind to cell-surface HSPGs. The CHO pgsA-745 cells were transfected with expression vectors for S-protein-tagged versions of wild-type GPIHBP1 (WT), GPIHBP1-W109S (W109S), or GPIHBP1-D,E(25–50)N,Q (mAcidic) and plated on coverslips; those coverslips were then placed face-down on the LPL-loaded HepG2 cells and incubated for 20 min at 37°C. Western blots were performed on cell extracts from the transfected CHO pgsA-745 cells with an antibody against the V5 tag (LPL); an antibody to the S-protein tag (GPIHBP1); and an antibody against β -actin. Signals from two independent experiments were quantified, and the amount of V5-LPL transferred to the CHO pgsA-745 cells was normalized to levels of GPIHBP1. C: Movement of bovine LPL loaded on HSPGs at the surface of HepG2 cells to the surface of CHO pgsA-745 cells that had been transfected with wild-type GPIHBP1 (WT), GPIHBP1-W109S (W109S), or GPIHBP1-D,E(25–50)N,Q (mAcidic). The scatter plot shows measurements of LPL activity on the surface of GPIHBP1-transfected CHO pgsA-745 cells after an incubation with bovine LPL-loaded HepG2 cells. The bar graph shows results from two independent experiments. D: Immunocytochemical detection of LPL movement from V5-LPL-loaded HepG2 cells to the surface of GPIHBP1-transfected CHO pgsA-745 cells. The GPIHBP1-expressing cells were fixed and stained with antibodies against the S-protein (red) and V5 (green) tags to visualize GPIHBP1 and LPL, respectively. DNA was stained with DAPI (blue).

cells and was captured/concentrated on agarose beads coated with wild-type GPIHBP1 or the acidic domain GPIHBP1 mutant (where the release was 87.9% of the amount released with wild-type GPIHBP1). We observed far lower migration of LPL to agarose beads coated with GPIHBP1-W109S (Fig. 4).

Testing the mobility of HSPG-bound LPL in *Gpihbp1*^{-/-} mouse tissue to GPIHBP1 on agarose beads

We next tested to determine whether LPL would move from HSPGs in tissues of *Gpihbp1*^{-/-} mice to GPIHBP1-loaded agarose beads *in vivo*. For this purpose, we injected GPIHBP1-loaded agarose beads (or simply 11A12-coated beads) into the interscapular brown adipose tissue of wild-type or *Gpihbp1*^{-/-} mice. Thousands of beads were injected. After 45 min, the brown adipose tissue was harvested and processed for immunohistochemistry. In the absence of any bead injection, the LPL in brown adipose of *Gpihbp1*^{-/-} mice was mislocalized to the interstitial spaces, whereas the LPL in the brown adipose tissue of wild-type mice was associated with capillaries (colocalizing with CD31) (Fig. 5, supplemental Fig. S5). In *Gpihbp1*^{-/-} brown adipose tissue that was injected with the GPIHBP1-loaded beads, the interstitial LPL moved to the beads, and that movement was accompanied (in three independent experiments) by reduced LPL staining within the interstitium of the brown adipose tissue (Fig. 5, supplemental Fig. S5). In *Gpihbp1*^{-/-} brown adipose tissue injected with 11A12-coated beads (i.e., no GPIHBP1), the LPL did not move to the beads and there was no detectable depletion of LPL in the interstitium (Fig. 5). In wild-type mice, we expected

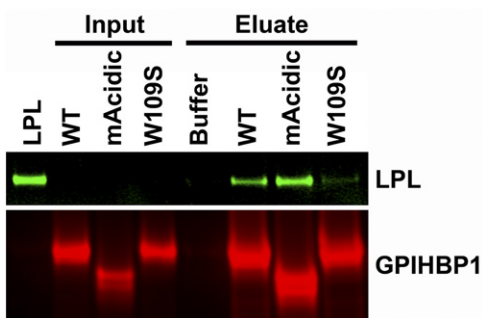


Fig. 4. Movement of LPL on the surface of HepG2 cells to GPIHBP1-loaded agarose beads. V5-LPL-loaded HepG2 cells were grown on coverslips and transferred into a 6-well plate. Antibody 11A12-coated agarose beads were then loaded with wild-type GPIHBP1 (WT), GPIHBP1 Δ (25–50) (mAcidic), or GPIHBP1-W109S (W109S). Next, the agarose beads were placed adjacent to the coverslips in the 6-well plates and incubated for 2 h at 4°C. The beads that were physically separated from the coverslips were then collected; the bound proteins were eluted with SDS sample buffer and analyzed by Western blotting with an antibody against the V5 tag and with a GPIHBP1-specific antibody. The lane on the left labeled “LPL” is the V5-LPL that was loaded onto the HepG2 cells. The lane labeled “Buffer” shows an experiment in which monoclonal antibody 11A12-coated beads (without any GPIHBP1) were incubated with HepG2 cells. The amounts of LPL that moved to beads coated with GPIHBP1 Δ (25–50) and GPIHBP1-W109S (normalized to the amount of GPIHBP1 on the beads) averaged 87.9% and 23.3%, respectively, in three separate experiments.

that some freshly secreted LPL would be captured by GPIHBP1-coated agarose beads. Indeed, movement of LPL to GPIHBP1-loaded agarose beads was also observed in brown adipose tissue of wild-type mice (Fig. 5, supplemental Fig. S5).

DISCUSSION

When one considers GPIHBP1 and plasma triglyceride metabolism, there is a tendency to focus on GPIHBP1's function in the capillary lumen. GPIHBP1 transports LPL to the capillary lumen; GPIHBP1 is the binding site for LPL in the capillary lumen; and GPIHBP1 is required for TRL margination in the capillary lumen (12). These findings are key elements in any outline for intravascular lipolysis, but LPL and GPIHBP1 physiology in the interstitial spaces is equally important and intriguing. When GPIHBP1 is absent, the LPL secreted by myocytes, adipocytes, and mammary epithelial cells remains attached to HSPGs on or near the surface of these cells. The ability of interstitial HSPGs to capture LPL is obviously robust, given that levels of LPL in the plasma are lower in *Gpihbp1*^{-/-} mice than in wild-type mice (25). Given the robust binding of LPL to the interstitial HSPGs in *Gpihbp1*^{-/-} mice, we did not understand why the LPL in wild-type mice would not be captured by the same interstitial HSPGs, thereby limiting the ability of LPL to move to capillaries. The most straightforward explanation for this conundrum is that the HSPG-bound LPL in the interstitium is highly mobile. In the past, others had shown that the binding of fibroblast growth factor to HSPGs is avid, but that the “off-rate” of fibroblast growth factor from HSPGs is high (35). Biacore experiments have suggested that the off-rate of LPL from HSPGs is also high (36, 37), raising the possibility that the LPL in the interstitial spaces of wild-type mice would be capable of “moving past” the HSPG binding sites. Our current studies support this concept. We found that LPL, when bound to HSPGs on the surface of HepG2 cells, moved within minutes to: 1) soluble GPIHBP1 in the medium; 2) GPIHBP1 on the surface of GPIHBP1-transfected cells; and 3) GPIHBP1 on the surface of nearby agarose beads. Thus, it is clear that LPL readily detaches from HSPG binding sites, allowing it to move to higher affinity binding sites, namely, GPIHBP1 on endothelial cells. An obvious limitation of these cell culture studies, however, is that we had no way of knowing whether the behavior of HSPG-bound LPL on HepG2 cells is relevant to the binding of LPL to the interstitial HSPGs *in vivo*.

To be confident that our cell culture findings were physiologically relevant, we captured GPIHBP1 on antibody 11A12-coated beads and then injected those beads into the interscapular brown adipose tissue of *Gpihbp1*^{-/-} mice. Our goal was to determine, by immunohistochemistry, whether any of the LPL that is bound to interstitial HSPGs in *Gpihbp1*^{-/-} mice would be capable of moving to the GPIHBP1-coated beads. The results from these studies were clear and consistent: within 45 min, the LPL in brown adipose tissue moved to GPIHBP1-coated beads and the LPL

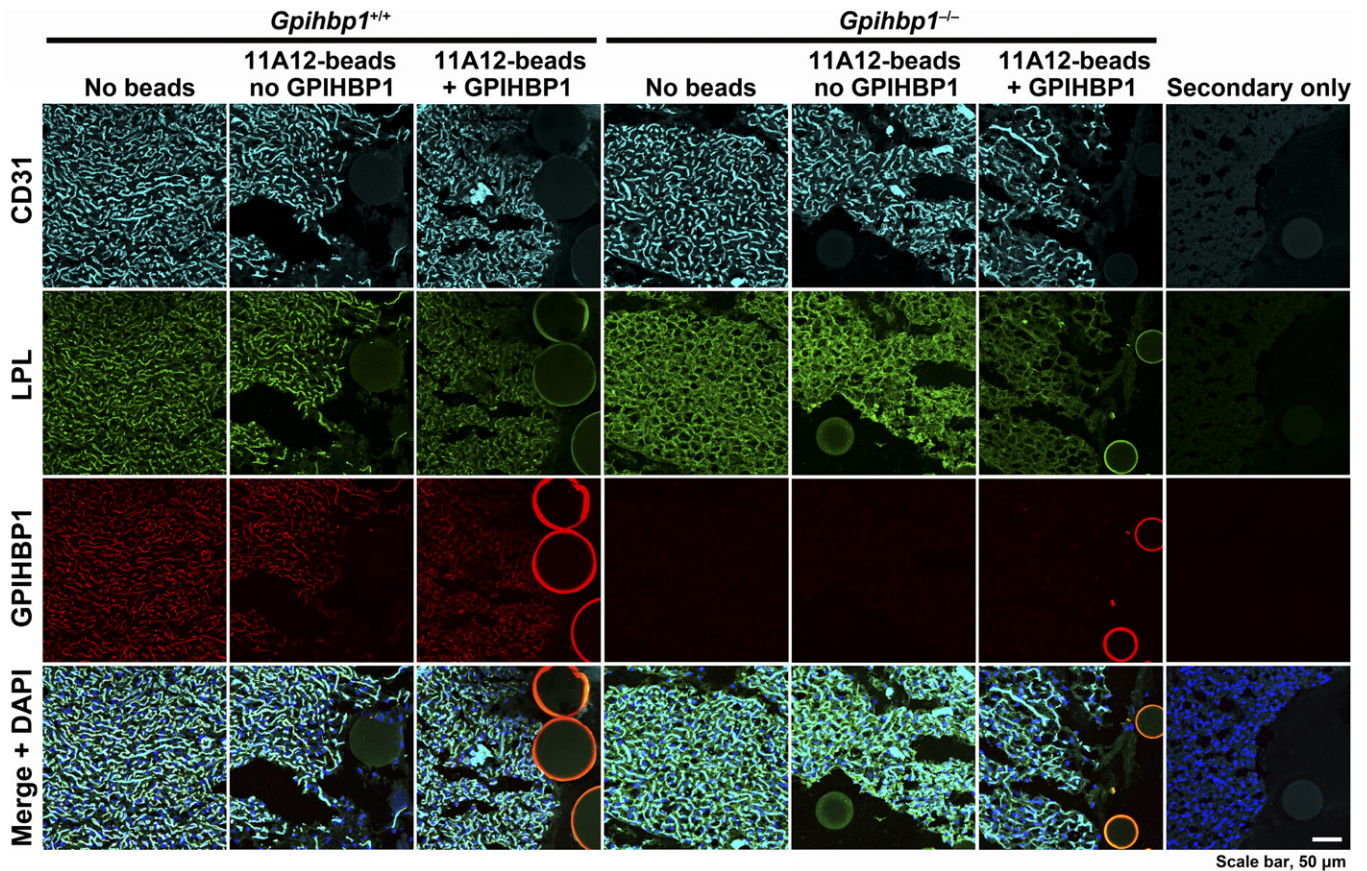



Fig. 5. Movement of LPL in the interstitial spaces of brown adipose tissue in a *Gp1hb1*^{-/-} mouse to agarose beads coated with GPIHBP1. Antibody 11A12-coated agarose beads were incubated with wild-type GPIHBP1 (11A12-beads + GPIHBP1) or buffer only (11A12-beads, no GPIHBP1) and injected into the brown adipose tissue of anesthetized *Gp1hb1*^{+/+} and *Gp1hb1*^{-/-} mice. After 45 min, the brown adipose tissue was harvested, sectioned, and stained with an antibody against the endothelial marker CD31 (cyan); a goat polyclonal antibody against mouse LPL (green); and the GPIHBP1-specific monoclonal antibody 11A12 (red). DNA was stained with DAPI (blue). The different sizes of beads are due to the fact that the beads themselves are sectioned in different planes. Shown are representative images from three independent experiments. In each experiment involving GPIHBP1-loaded beads, we observed reduced LPL staining in the interstitium of *Gp1hb1*^{-/-} brown adipose tissue; reduced LPL staining was never observed when 11A12-coated beads were injected. The agarose beads displayed low levels of endogenous fluorescence (41); hence, it was sometimes possible to visualize the beads in the absence of specific antibody staining. In those instances, the fluorescence was not as sharply localized to the edge of the beads.

stores in the interstitial spaces fell. In contrast, LPL did not move to agarose beads coated with monoclonal antibody 11A12 alone. Thus, “bound LPL” within the interstitial spaces can move to GPIHBP1. LPL also moved to GPIHBP1-agarose beads in the brown adipose tissue of wild-type mice. We did not consider the latter result surprising, simply because the amount of GPIHBP1 on the surface of the beads was very substantial (>5 μg per injection) and there was no reason to believe that it would lack the capacity to bind freshly secreted LPL.

In our studies, we observed little or no movement of HSPG-bound LPL to a mutant GPIHBP1 harboring an amino acid substitution in GPIHBP1’s Ly6 domain (W109S), whereas there was substantial movement of HSPG-bound LPL to “acidic domain GPIHBP1 mutants” (i.e., GPIHBP1 that lacked the acidic domain or GPIHBP1 in which the Asp and Glu residues in the acidic domain had been replaced with Asn or Gln). We chose to test GPIHBP1-W109S because earlier studies had revealed that that mutation virtually eliminates the binding of LPL to GPIHBP1 and does

so without causing inappropriate intermolecular disulfide bond formation and protein multimerization (27). The discovery that GPIHBP1 lacking the acidic domain is capable of capturing LPL from HSPGs is consistent with recent findings by Mysling et al. (24). Using purified proteins and surface plasmon resonance experiments, they showed that GPIHBP1’s Ly6 domain is primarily responsible for high-affinity LPL–GPIHBP1 interactions. Early and less refined LPL–GPIHBP1 binding assays had suggested that GPIHBP1’s acidic domain was important for LPL binding (38), but the surface plasmon resonance studies by Mysling et al. (24) revealed that the acidic domain contributes minimally to the kinetics of LPL–GPIHBP1 binding and only by increasing the on-rate. With the time resolution of our cell culture experiments, it was not possible to detect the small contribution of the acidic domain to LPL binding. Mysling et al. (24) went on to show that GPIHBP1’s acidic domain has another crucial role in lipolysis—preventing the unfolding of LPL’s serine hydrolase domain and thereby stabilizing LPL catalytic activity (24).

Another study using surface plasmon resonance (37) reported that the affinity of LPL binding was greater for GPIHBP1's Ly6 domain than for heparan sulfate. In these studies, the on-rates for LPL binding to GPIHBP1 and heparan sulfate were similar, but the off-rate was far greater with heparan sulfate. These measurements support our experimental results showing that LPL readily moves from HSPGs to GPIHBP1.

Our studies suggest a framework for understanding LPL physiology within tissues. Immunohistochemistry studies on sections of *Gpihbp1*^{-/-} tissues appear to show tight binding of LPL within the interstitial spaces, but our current studies indicate that the seemingly tight binding of LPL to HSPGs is actually dynamic—explaining how LPL in wild-type mice can “move past” HSPGs to GPIHBP1 on capillary endothelial cells. We believe that the duality of LPL binding sites in tissues (HSPG binding sites in the interstitial spaces and high-affinity GPIHBP1 binding sites on capillary endothelial cells) is crucial for triglyceride metabolism. LPL is secreted by parenchymal cells and its activity is regulated by locally produced factors such as ANGPTL4 (39, 40). We suggest that abundant HSPG binding sites in the interstitial spaces are essential for keeping the catalytically active LPL in the local environment and preventing it from disappearing into the lymph. The dynamic nature of LPL–HSPG interactions is also important, allowing LPL to detach and move to GPIHBP1 on the basolateral surface of capillary endothelial cells. The high-affinity GPIHBP1 binding sites on endothelial cells are also vital; GPIHBP1 binds LPL and shuttles it to the capillary lumen, limits its escape into the plasma, and allows TRLs to marginate along capillaries. Thus, the combination of interstitial HSPG binding sites and high-affinity GPIHBP1 binding sites serves to keep LPL in the local environment and thereby focuses intravascular triglyceride hydrolysis according to the requirements of local cells and tissues. 

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