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Equivalent binding of wild-type lipoprotein lipase (LPL) and S447X-LPL to GPIHBP1, the endothelial cell LPL transporter

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Abstract

The S447X polymorphism in lipoprotein lipase (LPL), which shortens LPL by two amino acids, is associated with low plasma triglyceride levels and reduced risk for coronary heart disease. S447X carriers have higher LPL levels in the pre- and post-heparin plasma, raising the possibility that the S447X polymorphism leads to higher LPL levels within capillaries. One potential explanation for increased amounts of LPL in capillaries would be more avid binding of S447X-LPL to GPIHBP1 (the protein that binds LPL dimers and shuttles them to the capillary lumen). This explanation seems plausible because sequences within the carboxyl terminus of LPL are known to mediate LPL binding to GPIHBP1. To assess the impact of the S447X polymorphism on LPL binding to GPIHBP1, we compared the ability of internally tagged versions of wild-type LPL (WT-LPL) and S447X-LPL to bind to GPIHBP1 in both cell-based and cell-free binding assays. In the cell-based assay, we compared the binding of WT-LPL and S447X-LPL to GPIHBP1 on the surface of cultured cells. This assay revealed no differences in the binding of WT-LPL and S447X-LPL to GPIHBP1. In the cell-free assay, we compared the binding of internally tagged WT-LPL and S447X-LPL to soluble GPIHBP1 immobilized on agarose beads. Again, no differences in the binding of WT-LPL and S447X-LPL to GPIHBP1 were observed. We conclude that increased binding of S447X-LPL to GPIHBP1 is unlikely to be the explanation for more efficient lipolysis and lower plasma triglyceride levels in S447X carriers.

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Introduction

Lipoprotein lipase is a triglyceride hydrolase that is responsible for hydrolyzing the triglycerides in triglyceride-rich lipoproteins (TRLs; chylomicrons and VLDL) [1, 2]; reviewed in [3]. When LPL is absent, the hydrolysis of plasma triglycerides is severely compromised, leading to markedly elevated plasma triglyceride levels (familial chylomicronemia) [4]. Heterozygosity for LPL deficiency leads to milder forms of hypertriglyceridemia [5, 6]. Subtle missense mutations in LPL can impair catalytic activity and lead to small increases in plasma triglyceride levels [7, 8]. Elevated plasma triglyceride levels can also be caused by increased production of triglyceride-rich lipoproteins [9, 10] or impaired clearance of remnant lipoproteins [11, 12]. Of note, LPL can be released from capillaries onto remnant lipoproteins in the plasma. LPL binds to low density lipoprotein receptor family members; hence, LPL could play a role in the clearance of remnant lipoproteins by the liver [13, 14].

Approximately 10% of the population carries a single-nucleotide polymorphism in *LPL* (the gene for lipoprotein lipase) that converts Ser-447 to a stop codon, shortening LPL by two amino acid residues [15]. The S447X polymorphism has attracted considerable attention because it lowers plasma triglyceride levels by 10–25% [15] and reduces susceptibility to coronary heart disease [15, 16]. However, the mechanism by which this polymorphism affects triglyceride metabolism has remained obscure [15]. One report suggested that the S447X polymorphism alters LPL translation [17], but the mechanism by which this single-nucleotide polymorphism at the end of the coding sequences would alter the efficiency of translation was not clear. Several reports suggested that the S447X polymorphism might increase or decrease LPL activity [18, 19], but others have found no significant effect [20–22]. The stabilities of wild-type LPL (WT-LPL) and S447X-LPL in response to denaturation by heat or guanidine hydrochloride are identical [23].

LPL is produced by myocytes and adipocytes but hydrolyzes triglycerides at the luminal face of capillaries. The transport of LPL to the capillary lumen is mediated by GPIHBP1, a GPI-anchored protein of capillary endothelial cells [24]. In the setting of GPIHBP1 deficiency, LPL is mislocalized to the interstitial spaces surrounding myocytes and adipocytes and never reaches the capillary lumen, resulting in hypertriglyceridemia [24].

When the amount of LPL in capillaries is negligibly low, as in GPIHBP1 deficiency, the levels of LPL in the pre-heparin plasma are quite low [25, 26]. In contrast, S447X carriers have higher-than-normal levels of LPL in the pre-heparin plasma (along with low plasma triglyceride levels) [27], suggesting that the S447X polymorphism leads to higher-than-normal amounts of LPL inside capillaries. GPIHBP1 deficiency also results in lower-than-normal LPL levels in the post-heparin plasma [26, 28, 29]. In contrast, S447X carriers have significantly higher levels of LPL in the post-heparin plasma [15, 30, 31]. These contrasting observations have suggested that there might be increased transport of S447X-LPL to the capillary lumen, perhaps due to more avid binding of S447X-LPL to GPIHBP1. The notion that the S447X polymorphism might affect GPIHBP1 binding seemed plausible because LPL binding to GPIHBP1 is mediated by sequences within the carboxyl terminus of LPL [13]. In the current study, we investigated whether the S447X polymorphism affects LPL

binding to GPIHBP1. To address this issue, we developed two new binding assays that allowed us to directly compare the abilities of freshly synthesized WT-LPL and S447X-LPL to bind GPIHBP1.

MATERIALS AND METHODS

LPL expression vectors

The S447X polymorphism was introduced into a human LPL expression vector [32] with the QuikChange Lightning kit (Agilent) and oligonucleotide primer 5′–CTGAATAAGAAGTGAGGCTGATCCAC–3′. To create human LPL expression vectors with an internal S-protein (SP) tag, we replaced LPL amino acids 220–233 in the lid domain of LPL [33] with an SP tag (KETAAAKFERQHMDS). The SP tag was amplified from pTriEx-4 (Novagen) with primers 5′–CAGCCAGGATGTAACATTAAAGAAACCGCTGCTGCG–3′ and 5′–GCACTTCACTAGCTGGTCCGAGTCCATGTGCTGGCG–3′. To create human LPL expression vectors with an internal V5 tag, we replaced LPL amino acids 220–229 in the lid domain of LPL [33] with a V5 tag (GKPIPNPLLGLDST). The V5 tag was amplified from pcDNA3.1 (Life Technologies) with primers 5′–CAGCCAGGATGTAACATTGGTAAGCCTATCCCTAACCC–3′ and 5′–CACTTCACTAGCTGGTCCACCGTAGAATCGAGACCGAGG–3′. Both tags were introduced into a human LPL expression vector with the InFusion HD cloning kit (Clontech). The constructs encoding the internally tagged LPL proteins were further modified by site-directed mutagenesis to introduce the S447X polymorphism as well as the C418Y mutation into these constructs [13].

Production of LPL

LPL expression vectors or empty vectors were electroporated into CHO pgsA-745 or CHO-K1 cells with the Nucleofector T kit (5 μg/5 × 10^6 cells). After 2 days, the cell culture medium was collected and concentrated 20-fold with an Amicon Ultra 30,000 MWCO filter. Relative amounts of the internally tagged LPLs (*e.g.*, WT-LPL, S447X-LPL, or C418Y-LPL) in the conditioned medium were assessed by western blots with the LPL-specific antibody 5D2 [34]. Band intensities were quantified with an Odyssey infrared scanner (Li-Cor).

Measurements of WT-LPL or S447X-LPL specific activity were performed by transfecting untagged WT-LPL and S447X-LPL expression vectors into HEK-293 cells (which do not produce LPL). LPL activity in fresh medium was assessed in quadruplicate with a [^{3}H]triolein substrate (PerkinElmer), and LPL mass was measured with an ELISA with monoclonal antibodies 5D2 and 5F9 [35]. LPL specific activity (nmol/h/μg LPL) was calculated from the activity and mass measurements. We also created immortalized fibroblasts from *Lpl* knockout mice [36], transfected them with the same vectors, and measured LPL specific activity in the same fashion.

Cell-based LPL–GPIHBP1 binding assay

A GPIHBP1 expression vector was electroporated into CHO-K1 cells (5 μg/5 × 10^6 cells). One day later, GPIHBP1-transfected CHO cells were placed at 4° C for 15 min and washed five times with Dulbecco's phosphate-buffered saline (PBS) containing 100 mg/L $CaCl_2$, 100 mg/L $MgCl_2$, 200 mg/L KCl, 200 mg/ml KH_2PO_4, 8 g/L NaCl, and 2.16 g/L Na_2HPO_4 (Life Technologies). To assess the binding of untagged versions of WT-LPL and S447X-LPL to GPIHBP1, CHO-K1 cells were transfected with either WT-LPL or S447X-LPL plasmids; aliquots of the LPL-containing medium were then added in equivalent and sub-saturating amounts to GPIHBP1-expressing CHO-K1 cells and incubated for 2 h at 4° C [28]. Relative amounts of untagged WT-LPL and S447X-LPL bound to GPIHBP1 were determined by performing western blots of cell extracts [28].

To compare the binding of internally tagged versions of WT-LPL and S447X-LPL to GPIHBP1, equivalent and sub-saturating amounts of the V5- and SP-tagged LPLs (either V5-WT-LPL and SP-S447X-LPL, or SP-WT-LPL and V5-S447X-LPL) were mixed and added to GPIHBP1-expressing cells and incubated for 2 h at 4° C. After washing the cells with PBS, western blots were performed on cell extracts with a mouse V5 monoclonal antibody (Life Technologies) and a goat SP antibody (Abcam) that were labeled with different infrared dyes (IRDye-680 or IRDye-800) (Li-Cor). GPIHBP1 expression was detected with the GPIHBP1-specific antibody 11A12 [28]. Western blot signals were quantified with an Odyssey infrared scanner. The same assay system was used to compare binding of WT-LPL and C418Y-LPL to GPIHBP1. All experiments were performed three times.

Cell-free LPL–GPIHBP1 binding assay

The binding of internally tagged WT-LPL and S447X-LPL to soluble GPIHBP1 (lacking the GPI anchor) was assessed with a cell-free assay [37, 38]. Soluble GPIHBP1 was generated by transfecting CHO pgsA-745 cells with a GPIHBP1 expression vector in which a stop codon was introduced after Gln-197 [28]. After 48 h, the soluble GPIHBP1-containing medium was collected and concentrated 20-fold on an Amicon Ultra 10,000 MWCO filter. The soluble GPIHBP1 was then incubated for 2 h at 4° C with equivalent amounts of internally tagged versions of WT-LPL and S447X-LPL (either V5-WT-LPL and SP-S447X-LPL, or SP-WT-LPL and V5-S447X-LPL) along with agarose beads coated with antibody 11A12. After washing the beads, soluble GPIHBP1 and GPIHBP1-bound LPL were eluted with 0.1 M glycine, pH 2.5. The amounts of GPIHBP1 and LPL in the starting material and in the wash and elution fractions were assessed by western blotting. GPIHBP1 was detected with a GPIHBP1-specific rabbit antibody [32], and the tagged LPLs were detected with V5- and SP-specific antibodies labeled with different IRDyes. Western blot signals were quantified with an Odyssey infrared scanner.

RESULTS

Untagged versions of WT-LPL and S447X-LPL were transfected into HEK-293 cells, and fresh medium was collected for LPL mass and activity measurements. The specific activities of WT-LPL and S447X-LPL (measured in quadruplicate) were similar (50.3 ± 0.35 nmol/h/

μg for WT-LPL *vs.* 45.4 ± 0.32 nmol/h/μg for S447X-LPL). Similar results were obtained with transfected *Lpl*$^{-/-}$ fibroblasts, where the specific activity for WT-LPL was ~8% higher than that for S447X-LPL.

To assess the abilities of WT-LPL and S447X-LPL to bind to GPIHBP1, freshly isolated WT-LPL and S447X-LPL preparations were incubated in sub-saturating amounts with GPIHBP1-expressing CHO cells in the presence or absence of heparin. The binding of WT-LPL and S447X-LPL to GPIHBP1 was similar, as judged by western blotting of cell extracts (Fig. 1).

The studies shown in Fig. 1 tested the binding of WT-LPL and S447X-LPL to GPIHBP1 on different tissue culture wells, limiting our confidence in drawing firm conclusions about small differences in the avidity of LPL binding. We reasoned that it would be desirable to develop assays that would make it possible to *directly* compare the abilities of two different LPL preparations to bind to GPIHBP1 in the very same tissue culture well. To pursue this goal, we initially created WT-LPL and S447X-LPL constructs with amino-terminal V5 or S-protein (SP) tags. These constructs proved to be unsatisfactory because the LPL preparations with amino-terminal tags were susceptible to a furin-mediated cleavage event [39] and because our studies suggested that the tags themselves might alter LPL binding to GPIHBP1. To circumvent these issues, we created LPL expression vectors with V5 or SP tags in LPL's lid domain [33] (replacing LPL residues 220–229 or residues 220–233, respectively). We chose this location because the lid domain is a surface loop in LPL's catalytic domain and distant from the GPIHBP1-binding domain [33]. In earlier studies, subtle mutations in the lid domain had small effects on catalytic activity while more substantial mutations abolished LPL's ability to hydrolyze triglycerides [33]. Not surprisingly, we found that the replacement of the lid domain with the epitope tags eliminated LPL catalytic activity (Fig. 2). Again, we found no difference in the catalytic activity of untagged WT-LPL and S447X-LPL. From the perspective of analyzing GPIHBP1–LPL interactions, this absence of catalytic activity with the internally tagged LPLs was not a problem because LPL binding to GPIHBP1 is mediated by the carboxyl terminus of LPL and does not require catalytically active LPL [13].

The internally tagged LPL proteins were secreted efficiently and were not susceptible to the furin cleavage event. Also, the internally tagged LPL preparations bound avidly to GPIHBP1 (unlike LPL monomers which cannot bind to GPIHBP1 [39]) (Fig. 3). To validate the utility of using internally tagged LPLs to investigate LPL binding to GPIHBP1, we compared the binding of internally tagged versions of WT-LPL and C418Y-LPL to GPIHBP1-expressing cells. The C418Y mutation interferes with LPL's capacity to bind to GPIHBP1 [13]. Equal amounts of internally tagged versions of WT-LPL and C418Y-LPL (V5-WT-LPL and SP-C418Y-LPL, or SP-WT-LPL and V5-C418Y-LPL) were mixed and incubated with GPIHBP1-expressing cells for 2 h at 4° C. After washing the cells, western blots of cell extracts were performed with V5- and SP-specific antibodies labeled with different IRDyes. The V5 and SP signals were then quantified with a Li-Cor infrared scanner—both in the starting LPL mixture ("starting") that was incubated with the cells and the LPL that bound to GPIHBP1 on the surface of GPIHBP1-expressing cells ("bound"). We then calculated the "percent bound" by dividing the signal in "bound" LPL by the signal

in the "starting" LPL mixture. Regardless of whether the C418Y-LPL contained an internal V5 or a SP tag, the binding of C418Y-LPL to GPIHBP1-expressing cells was extremely low, whereas the binding of WT-LPL was robust (Fig. 3A–D).

The same cell-based binding assay was used to assess whether the S447X polymorphism affects LPL binding to GPIHBP1. Equivalent and sub-saturating amounts of internally tagged LPLs (V5-WT-LPL and SP-S447X-LPL, or SP-WT-LPL and V5-S447X-LPL) were mixed together and incubated with GPIHBP1-expressing cells. After washing the cells, LPL binding to GPIHBP1 was quantified by western blotting with IRDye-labeled V5 and SP antibodies. The "percent bound" measurement for S447X-LPL was similar to that for WT-LPL, regardless of whether the S447X-LPL contained the V5 or the SP tag (Fig. 3E–H). We repeated the experiment with independent batches of WT-LPL and S447X-LPL—adding either high or low amounts of the LPL mixture to the GPIHBP1-expressing cells (Fig. 4). When the lower amount of the LPL mixture was incubated with the cells, the "percent bound" tended to be higher for both WT-LPL and S447X-LPL (Fig. 4). However, there was no evidence for increased binding of S447X-LPL to GPIHBP1.

We also compared the binding of internally tagged versions of WT-LPL and S447X-LPL to GPIHBP1 in a cell-free assay. Soluble GPIHBP1 was mixed with internally tagged LPLs (SP-WT-LPL and V5-S447X-LPL, or V5-WT-LPL and SP-S447X-LPL) and agarose beads coated with the GPIHBP1-specific antibody 11A12. After 2 h, the beads were washed, and both GPIHBP1 and GPIHBP1-bound LPL were eluted with 0.1 M glycine, pH 2.5. The amounts of WT-LPL and S447X-LPL in the elution fractions (reflecting LPL that was bound to soluble GPIHBP1) were then assessed by western blotting with IRDye-labeled V5 and SP antibodies. Both WT-LPL and S447X-LPL bound to the soluble GPIHBP1 and did so equally (Fig. 5). In the first experiment (Fig. 5A), the SP-WT-LPL band in the elution fraction was 37% as intense as the SP-WT-LPL band in the starting mixture. The V5-S447X-LPL band in the elution fraction was 36% as intense as the V5-S447X-LPL band in the starting mixture. Thus, the efficiency of WT-LPL and S447X-LPL binding to soluble GPIHBP1 was virtually identical. In the second experiment (Fig. 5B), we examined the binding of LPL preparations where the tags were reversed. Again, we found no evidence for preferential binding of S447X-LPL to GPIHBP1. The V5-WT-LPL band in the elution fraction was 21% as intense as the V5-WT-LPL band in the starting mixture, while the SP-S447X-LPL band in the elution fraction was 20% as intense as the SP-S447X-LPL band in the starting mixture.

Discussion

GPIHBP1 deficiency markedly reduces LPL levels within capillaries [24] and results in high plasma triglyceride levels and low levels of LPL in the pre- and post-heparin plasma [25, 26, 28, 29]. S447X carriers have low plasma triglyceride levels and increased levels of LPL in the pre- and post-heparin plasma [15, 30, 31]. These contrasting observations prompted us to hypothesize that the S447X polymorphism might lead to more avid binding of LPL to GPIHBP1. This hypothesis seemed plausible, given that LPL's GPIHBP1-binding domain is located within the last 30–40 amino acids of LPL [13]. However, we found little support for the notion that S447X-LPL binds more avidly to GPIHBP1. In our cell experiments, we

compared the binding of untagged versions of WT-LPL and S447X-LPL to GPIHBP1-expressing cells and found no obvious differences. However, we worried that this approach might fail to detect small differences in WT-LPL and S447X-LPL binding to GPIHBP1. For this reason, we developed a new cell-based LPL–GPIHBP1 binding assay with internally tagged versions of LPL. The new assay made it possible to *directly compare* the GPIHBP1-binding properties of two different LPL preparations in the same tissue culture well. With this assay, we uncovered no differences in the binding of WT-LPL and S447X-LPL to GPIHBP1. We are confident that this cell-based assay was suitable for detecting any differences in GPIHBP1 binding because the very same assay system uncovered a striking inability of C418Y-LPL to bind to GPIHBP1. Moreover, we were confident in the validity of the cell-based LPL–GPIHBP1 binding assay because our results were consistent in independent experiments—and in experiments in which the assignment of epitope tags were switched. Finally, we found consistent results with a completely different assay system—a cell-free LPL–GPIHBP1 binding assay. In the cell-free assay, we *directly compared* the binding of internally tagged versions of WT-LPL and S447X-LPL to soluble GPIHBP1 and found no differences. An attractive feature of both the cell-based and cell-free assays is that they used freshly synthesized LPL rather than LPL that had been subjected to multiple biochemical purification steps.

The fact that the S447X polymorphism had no significant effect on LPL binding to GPIHBP1 suggests that low plasma triglyceride levels in S447X carriers are unlikely to be due to changes in the binding of LPL by GPIHBP1. However, one can imagine possible caveats. For example, it is conceivable that differences in WT-LPL and S447X-LPL binding to GPIHBP1 would emerge under certain circumstances, for example in the setting of active lipolysis and high local concentrations of free fatty acids. It is also conceivable that differences in the avidity of WT-LPL and S447X-LPL binding to GPIHBP1 could emerge when LPL is in the presence of a different spectrum of regulatory factors (*e.g.*, apolipoprotein CII, apolipoprotein CIII, apolipoprotein AV, ANGPTL4, and ANGPTL3). Addressing whether one or more of those factors has an enhanced effect on S447X-LPL will almost certainly require a much better understanding of the mechanisms by which the various regulatory factors influence LPL activity. Finally, it is conceivable that there are subtle differences between WT-LPL and S447X-LPL in terms of their ability to bind to remnant lipoproteins and/or promote their uptake in the liver.

In keeping with results from earlier studies [20–22], we detected little difference in the specific activities of WT-LPL and S447X-LPL. However, given that plasma triglyceride levels are lower in S447X carriers [15], it seems likely that S447X-LPL is more active *in vivo*. Also, an S447X-LPL adenovirus, when injected intramuscularly into mice, is more effective than a WT-LPL adenovirus in lowering plasma triglyceride levels and in preventing perinatal lethality in *Lpl* knockout mice [23]. The S447X-LPL adenovirus also led to higher levels of LPL catalytic activity in the post-heparin plasma [23]. Our current studies imply that the higher LPL activities are unlikely to be a consequence of more avid binding of S447X-LPL to GPIHBP1. Further efforts to define mechanisms surrounding the S447X polymorphism are warranted but will likely require an improved understanding of the mechanisms by which LPL activity is regulated by various regulatory factors. These

efforts are worthwhile because they could lead to fresh insights into strategies for lowering plasma triglyceride levels and coronary risk.

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Highlights

- We hypothesized that the S447X polymorphism in LPL increases binding to GPIHBP1.
- We found no difference in the binding of wild-type LPL and S447X-LPL to GPIHBP1.
- We found no difference in the catalytic activity of wild-type LPL and S447X-LPL.
- The S447X polymorphism does not lead to more avid binding of LPL to GPIHBP1.

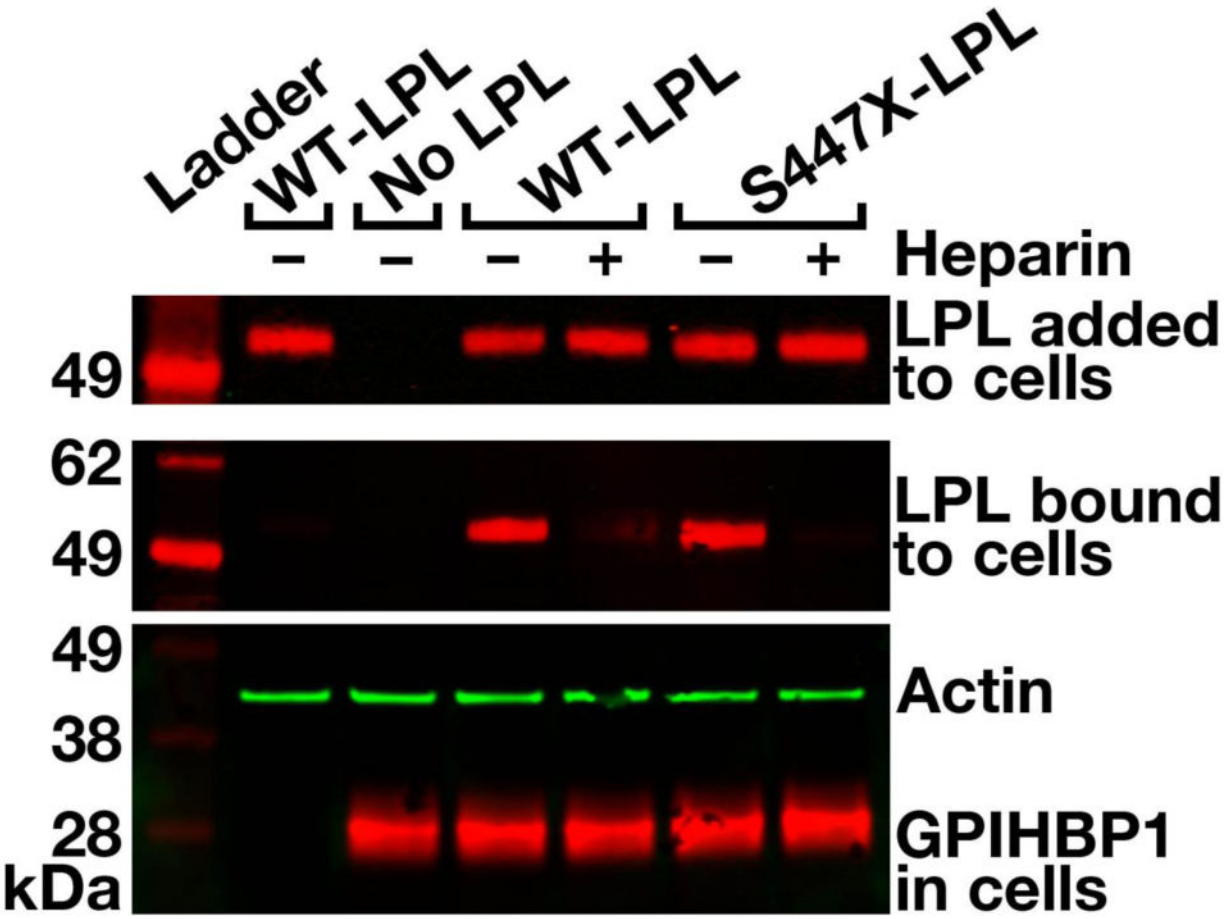

Fig. 1.

Western blot assessing the binding of untagged versions of wild-type (WT) human LPL and S447X-LPL to GPIHBP1-expressing CHO-K1 cells in the presence or absence of heparin (250 U/ml). After incubating the cells with the different LPL preparations, the cells were washed with PBS, and western blots of cell extracts were performed with GPIHBP1- and LPL-specific antibodies [28]. A rabbit antibody against ß-actin (Abcam) was used as a loading control. “Ladder” indicates the lane containing molecular weight markers.

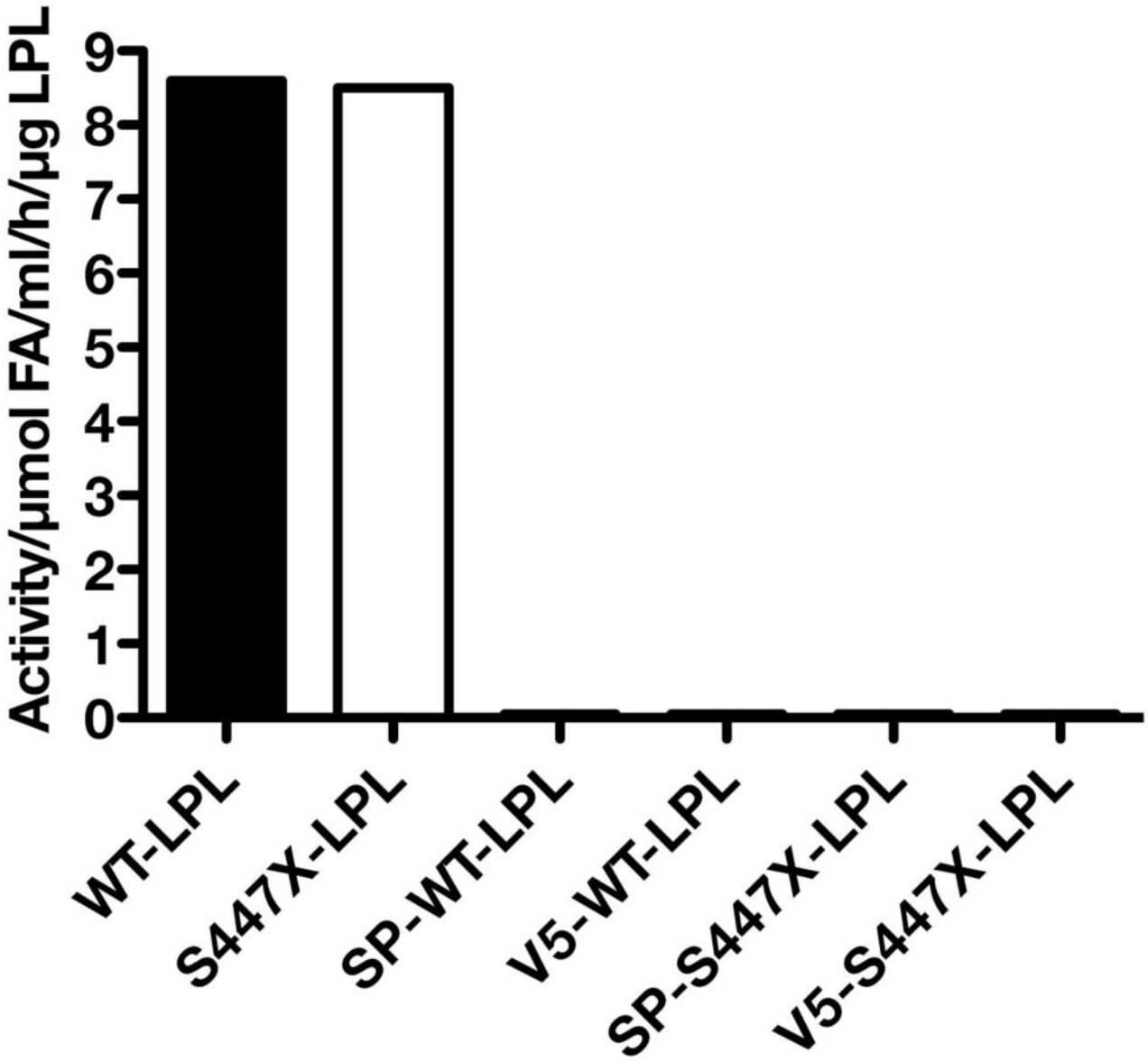

Fig. 2.

Testing the catalytic activity of untagged wild-type (WT) human LPL, untagged S447X-LPL, and internally tagged versions of WT-LPL and S447X-LPL. Plasmids for the different LPL constructs were transfected into cultured cells, and the catalytic activity of the LPL in the conditioned medium was measured [13, 32]. In this bar graph, the LPL catalytic activity is normalized to LPL mass. The absence of catalytic activity with the internally tagged LPL constructs was confirmed in an independent transfection experiment.

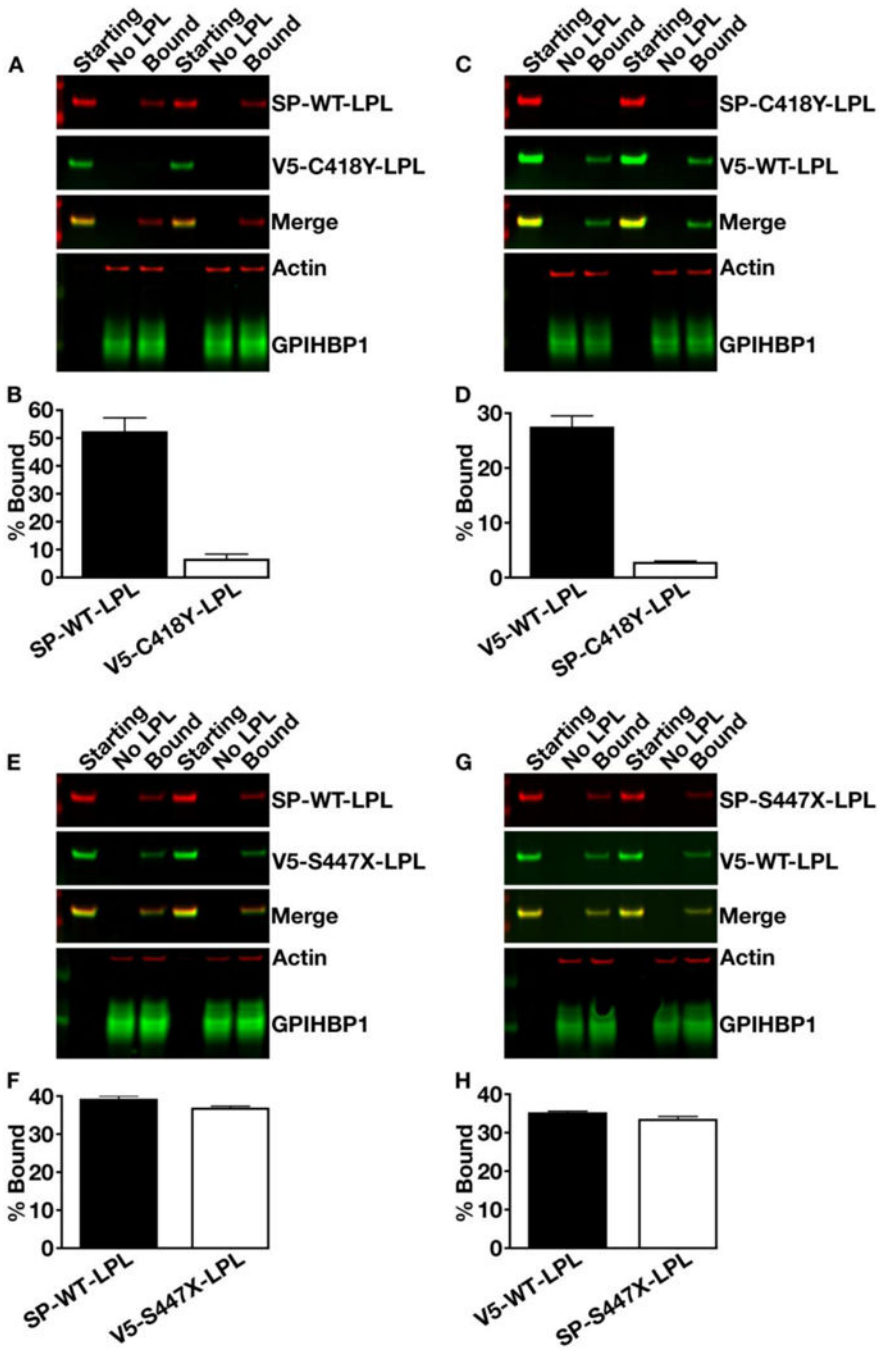

Fig. 3.

A cell-based LPL–GPIHBP1 binding assays using internally tagged versions of human LPL. (A–D) Comparing the binding of wild-type (WT) human LPL and C418Y-LPL to GPIHBP1-expressing CHO cells. Identical amounts of S-protein (SP)-tagged WT-LPL and V5-tagged C418Y-LPL preparations were mixed and incubated with GPIHBP1-expressing CHO cells for 2 h. After washing the cells, the relative amounts of SP-WT-LPL and V5-C418Y-LPL bound to the cells were assessed by western blotting. ß-actin served as a loading control. (A) WT-LPL was detected with an IRDye-680–labeled SP antibody (red),

and C418Y-LPL was detected with an IRDye-800–labeled V5 antibody (green). “Starting,” the starting SP-WT-LPL/V5-C418Y-LPL mixture that was added to GPIHBP1-expressing cells; “No LPL,” cell lysates from GPIHBP1-expressing cells that had been incubated with the medium from empty vector–transfected cells; “Bound,” lysates from GPIHBP1-expressing cells that had been incubated with the SP-WT-LPL/V5-C418Y-LPL mixture. (B) Quantification of LPL signals in the western blot shown in panel A. The amount of bound LPL, relative to the LPL in the starting material, was lower for V5-C418Y-LPL than for SP-WT-LPL ($n = 3$; $P = 0.0013$). (C) A similar LPL–GPIHBP1 binding assay except that the V5 and SP epitope tags were reversed. (D) Quantification of LPL signals in the western blot in panel C. The amount of bound LPL, relative to the LPL in the starting material, was lower for SP-C418Y-LPL than for V5-WT-LPL ($n = 3$; $P = 0.0004$). (E–H) Comparing the binding of WT-LPL and S447X-LPL to GPIHBP1-expressing CHO cells. (E) Western blot assay of SP-WT-LPL and V5-S447X-LPL binding to GPIHBP1-expressing cells. (F) Quantification of LPL signals in the western blot in panel E. No differences were observed ($n = 3$; $P = 0.124$) (G) Western blot assay of V5-WT-LPL and SP-S447X-LPL binding to GPIHBP1-expressing CHO cells. (H) Quantification of LPL signals in the western blot in panel G. No differences were observed ($n = 3$; $P = 0.189$)

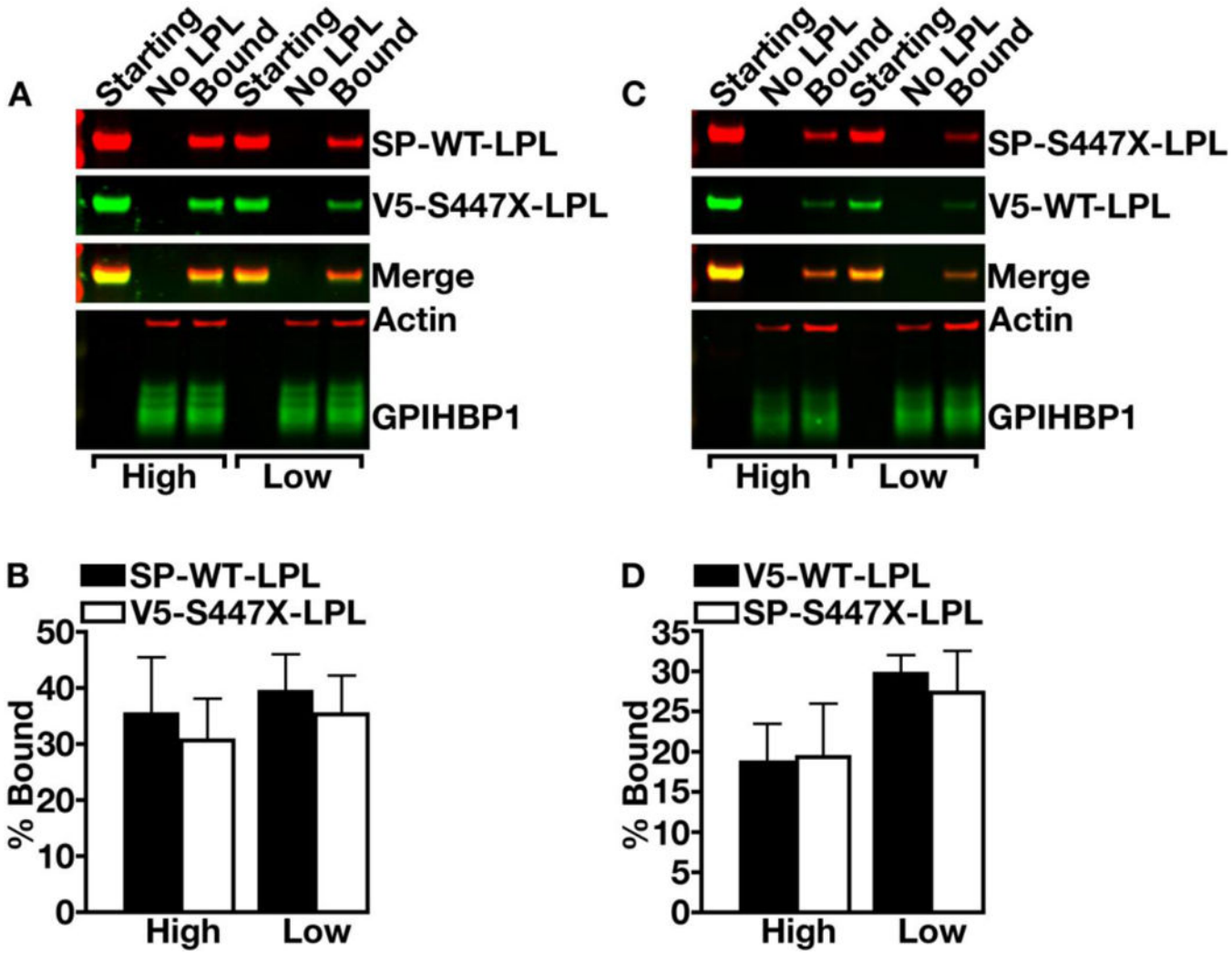

Fig. 4.

Cell-based LPL–GPIHBP1 binding assays comparing the ability of internally tagged versions of WT-LPL and S447X-LPL to bind to GPI-anchored GPIHBP1. (A) Assessing the binding of SP-WT-LPL (red) and V5-S447X-LPL (green) to GPIHBP1-expressing CHO cells. Equivalent amounts of SP-WT-LPL and V5-S447X-LPL were mixed; two different amounts of this mixture (high and low, differing by a factor of two) were added to GPIHBP1-expressing cells. The LPL binding assays were performed as described in Figure 3. (B) Quantification of LPL signals in the western blot shown in panel A. The amount of bound LPL, relative to the LPL in the starting material, was similar for SP-WT-LPL and V5-S447X-LPL, regardless of whether the cells were incubated with high or low amounts of the mixture. Also, the IRDye-680/IRDye-800 ratio, reflecting the ratio of SP-WT-LPL and V5-S447X-LPL, was nearly identical in the starting material and the bound fraction. No statistically significant differences were observed in the ratio ($n = 3$; $P = 0.792$ for the high concentration and 0.9391 for the low concentration). (C) A binding assay identical to that shown in panel A, except that the assignment of epitope tags for WT-LPL and S447X-LPL was reversed. (D) Quantification of LPL signals of the western blot in panel C. No

statistically significant differences were observed in the ratio ($n = 3$; $P = 0.698$ for the high concentration and 0.704 for the low concentration).

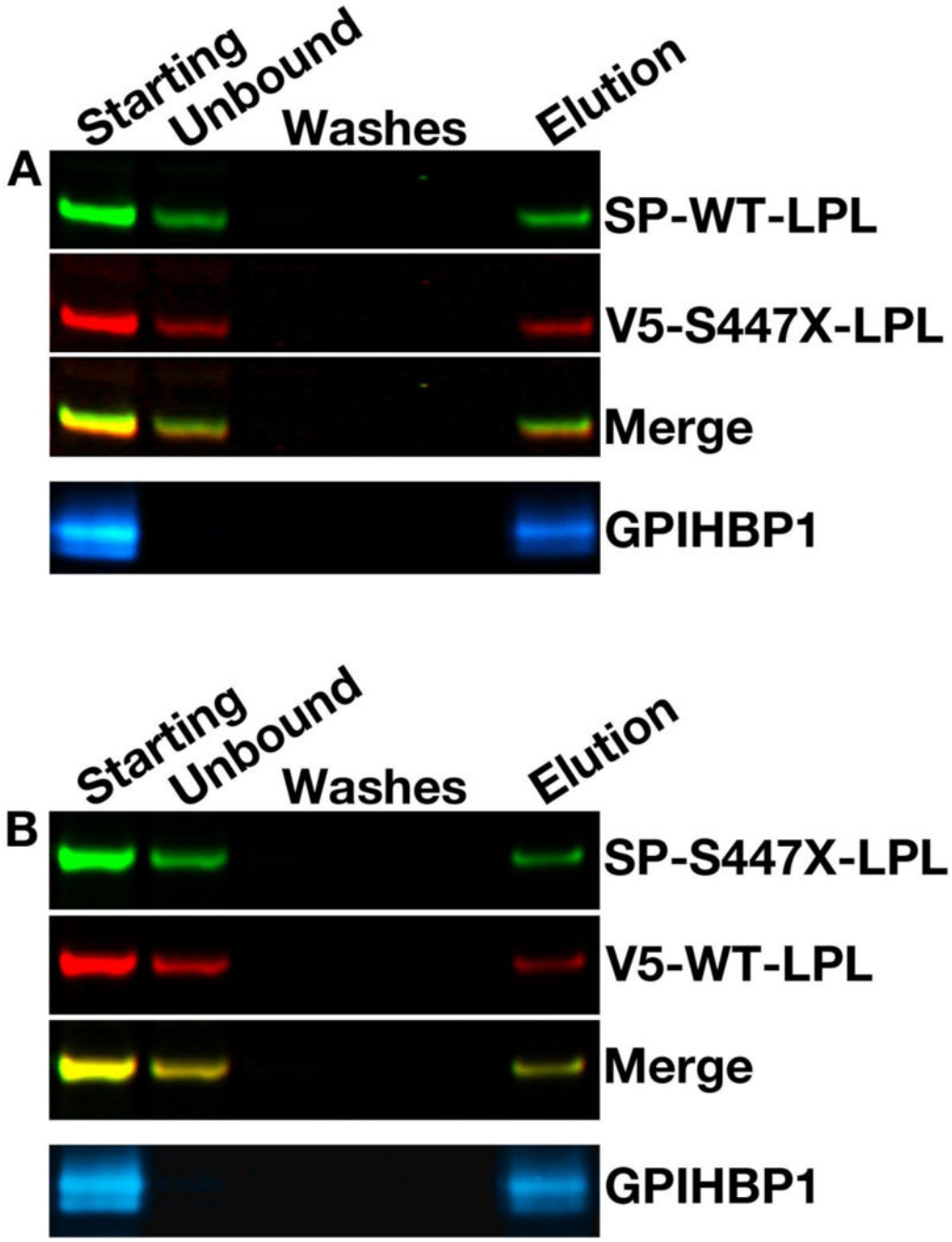

Fig. 5.

Cell-free assays comparing the ability of internally tagged versions of WT-LPL and S447X-LPL to bind to soluble GPIHBP1. (A) Comparing the binding of SP-WT-LPL and V5-S447X-LPL to soluble GPIHBP1. A mixture containing equivalent amounts of SP-WT-LPL and V5-S447X-LPL was added to soluble GPIHBP1 and agarose beads coated with the GPIHBP1-specific antibody 11A12. After 2h, the agarose beads were washed, and both GPIHBP1 and GPIHBP1-bound LPL were eluted with 0.1 M glycine, pH 2.5. “Starting,” the LPL and soluble GPIHBP1 added to the antibody-coated beads; “Unbound,” the flow-

through from the antibody-coated beads; “Washes,” the material that eluted during 3 consecutive washes of the beads; “Elution,” material eluted from the beads with 0.1 M glycine, pH 2.5. SP-WT-LPL was detected with an IRDye-800–labeled SP antibody (green); V5-S447X-LPL was detected with an IRDye-680–labeled V5 antibody (red); GPIHBP1 was detected with a GPIHBP1-specific rabbit antibody [32], followed by an IRDye-800–labeled donkey anti–rabbit IgG. The SP-WT-LPL band in the elution fraction was 37% as intense as the SP-WT-LPL band in the starting mixture, while the V5-S447X-LPL band in the elution fraction was 36% as intense as the V5-S447X-LPL band in the starting mixture. (B) A comparison of the binding of V5-WT-LPL and SP-S447X-LPL to soluble GPIHBP1. Again, the binding of V5-WT-LPL and SP-S447X-LPL to soluble GPIHBP1 was nearly equivalent; the V5-WT-LPL band in the elution fraction was 21% as intense as the V5-WT-LPL band in the starting mixture, while the SP-S447X-LPL band in the elution fraction was 20% as intense as the SP-S447X-LPL band in the starting mixture.