Title

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Journal

Circulation Research, 116(4)

ISSN

0009-7330

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Publication Date

2015-02-13

DOI

10.1161/circresaha.116.305085

Peer reviewed

Published in final edited form as:
Circ Res. 2015 February 13; 116(4): 624–632. doi:10.1161/CIRCRESAHA.116.305085.

GPIHBP1 Missense Mutations Often Cause Multimerization of GPIHBP1 and Thereby Prevent Lipoprotein Lipase Binding

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Abstract

Rationale—GPIHBP1, a GPI-anchored protein of capillary endothelial cells, binds lipoprotein lipase (LPL) in the subendothelial spaces and shuttles it to the capillary lumen. GPIHBP1 missense mutations that interfere with LPL binding cause familial chylomicronemia.

Objective—We sought to understand mechanisms by which *GPIHBP1* mutations prevent LPL binding and lead to chylomicronemia.

Methods and Results—We expressed mutant forms of GPIHBP1 in Chinese hamster ovary cells, rat and human endothelial cells, and *Drosophila* S2 cells. In each expression system, mutation of cysteines in GPIHBP1's Ly6 domain (including mutants identified in chylomicronemia patients) led to the formation of disulfide-linked dimers and multimers. GPIHBP1 dimerization/multimerization was not unique to cysteine mutations; mutations in other amino acid residues, including several associated with chylomicronemia, also led to protein dimerization/multimerization. The loss of GPIHBP1 monomers is quite relevant to the pathogenesis of chylomicronemia because only GPIHBP1 monomers—and not dimers or multimers—are capable of binding LPL. One GPIHBP1 mutant, GPIHBP1-W109S, had distinctive properties. GPIHBP1-W109S lacked the ability to bind LPL but had a *reduced*

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DISCLOSURES
The authors have no conflict of interest to disclose.

propensity for forming dimers or multimers, suggesting that W109 might play a more direct role in binding LPL. In support of that idea, replacing W109 with any of 8 other amino acids abolished LPL binding—and often did so without promoting the formation of dimers and multimers.

Conclusions—Many amino acid substitutions in GPIHBP1's Ly6 domain that abolish LPL binding lead to protein dimerization/multimerization. Dimerization/multimerization is relevant to disease pathogenesis, given that only GPIHBP1 monomers are capable of binding LPL.

Keywords

Lipoprotein lipase; hypertriglyceridemia; multimerization; GPIHBP1; lipids and lipoprotein metabolism; chylomicron; triglycerides; endothelial cell

INTRODUCTION

GPIHBP1, a GPI-anchored protein of the lymphocyte antigen 6 (Ly6) protein family, is expressed exclusively in endothelial cells of capillaries.[1] GPIHBP1 binds lipoprotein lipase (LPL) within the interstitial spaces and shuttles LPL to its site of action in the capillary lumen.[2] In the absence of GPIHBP1, LPL does not reach the capillary lumen and therefore cannot hydrolyze triglycerides in lipoproteins, resulting in both hypertriglyceridemia (chylomicronemia)[1,3–9] and impaired delivery of lipid nutrients to parenchymal cells.[10]

Aside from GPIHBP1, the Ly6 protein family includes the urokinase-type plasminogen activator receptor (uPAR), CD59 (an inhibitor of complement activation), and SLURP1 (a secreted protein of keratinocytes).[11–13] The hallmark of the Ly6 protein family is a 70–80-residue "Ly6 domain" containing 8 or 10 cysteines—all arranged in a characteristic spacing pattern[14] and all disulfide-bonded, creating a three-fingered structural motif. Aside from the conserved cysteine residues, most Ly6 family members display little homology at the amino acid sequence level.

GPIHBP1's Ly6 domain is functionally important. Replacing GPIHBP1's Ly6 domain with that from CD59 eliminates the ability of GPIHBP1 to bind LPL.[15] Mutation of any of the 10 cysteines in GPIHBP1's Ly6 domain abolishes GPIHBP1's ability to bind LPL and to shuttle the LPL across endothelial cells.[16,17] Interestingly, many of the mutations causing chylomicronemia in humans involve a cysteine residue (*e.g.*, C65S, C65Y, C68G, C68Y, C89F).[4–8] Initially, we suspected that the cysteine mutations might prevent trafficking of GPIHBP1 to the cell surface, but this was not the case; they had minimal effects on GPIHBP1 trafficking to the cell surface or on the secretion of soluble versions of GPIHBP1.[16] Mutations in other amino acids, aside from the cysteines, can also cause disease. For example, a Q115P mutation, first identified in a young man with chylomicronemia,[3] introduces a proline adjacent to a cysteine in the Ly6 domain. GPIHBP1-Q115P reaches the cell surface normally but has a markedly reduced capacity to bind LPL.[3] We suspected that the new proline might interfere with disulfide bonding, but we were never able to find evidence for a free thiol with cysteine-modifying reagents. Surendran *et al.*[18] identified a T108R mutation in a patient with chylomicronemia, but the impact of that mutation on LPL binding was not tested.

Beigneux *et al.*[17] performed alanine-scanning mutagenesis on each residue of GPIHBP1's Ly6 domain and identified 12 residues in the Ly6 domain, aside from the cysteines, that reduced the binding and transport of LPL. Nine of the 12 residues were located in the "second finger" of the three-fingered structural motif.[17] Nearly all of these mutants trafficked to the cell surface normally,[17] and the mechanism by which these mutants prevented LPL binding was unclear.

In the current study, we investigated mechanisms by which *GPIHBP1* missense mutations interfere with LPL binding. We show, using mammalian and insect cell expression systems, that many GPIHBP1 mutants (including those identified in chylomicronemia patients) promote the formation of dimers and multimers.

METHODS

GPIHBP1 constructs

Human GPIHBP1 mammalian expression vectors containing an amino-terminal S-protein tag have been described previously.[5,17]

For expression of GPIHBP1 in insect cells, we cloned a cDNA encoding human uPAR domain III (uPAR-DIII) in-frame with human GPIHBP1 amino acids 21–136, followed by mouse GPIHBP1 amino acids 136–198, into pMT/V5-His (Life Technologies).[9]

All mutations were introduced by site-directed mutagenesis with the QuikChange Lightning kit (Stratagene).

Treatment of CHO cells with Phosphatidylinositol-specific Phospholipase C (PIPLC)

Human umbilical vein endothelial cells (HUVECs) or CHO-K1 cells were electroporated with human GPIHBP1 expression vectors with the Nucleofector II apparatus (Lonza). After 24 h, the GPIHBP1 was released into the culture medium by treating the cells with PIPLC (10 U/ml for 20 min at 37° C). In some experiments, we used rat heart microvascular endothelial cells (RHMVECs) that had been transduced with a mouse GPIHBP1 lentivirus.[2] The RHMVECs were treated with PIPLC when they reached 90% confluence. Proteins in the medium and cell extracts were size-fractionated on SDS-polyacrylamide gels under reducing or nonreducing conditions. Western blots were performed with an antibody against the S-protein tag (for human GPIHBP1) and antibody 11A12[16] (for mouse GPIHBP1).

Cell-based assays of LPL binding to GPIHBP1

CHO-K1 cells electroporated with S-protein–tagged human GPIHBP1 constructs were incubated with V5-tagged human LPL ± heparin (250 U/ml) at 4° C.[17] Two hours later, the cells were washed, and cell lysates were prepared. The amounts of GPIHBP1 and LPL in the cell extracts were assessed by western blotting with antibodies against the S-protein tag and the V5 tag, respectively.

Expression of GPIHBP1 in Drosophila S2 cells

Drosophila S2 cells adapted to suspension culture were transfected with GPIHBP1 expression vectors with the Calcium Phosphate Transfection kit (Life Technologies). The expression of the uPAR–GPIHBP1 fusion protein was induced by adding $CuSO_4$ to the medium. Three days later, the conditioned medium and cell extracts were collected and size-fractionated by SDS-PAGE under reducing or nonreducing conditions. Western blots were performed with IRdye680–antibody 11A12[16] and an IRdye800-conjugated monoclonal antibody against the uPAR tag (R24).[19] Western blots were quantified with an Odyssey infrared scanner (Li-Cor).

To produce soluble GPIHBP1 for cell-free assays of GPIHBP1–LPL binding, the conditioned medium from GPIHBP1-transfected *Drosophila* S2 cells was concentrated 6-fold with an Amicon Ultra 10 MWCO filter (Millipore). The soluble GPIHBP1 was incubated with conditioned medium containing V5-tagged human LPL[17] along with agarose beads coated either with antibody 11A12 or the LPL-specific antibody 5D2.[9,16] After washing the beads, GPIHBP1–LPL complexes captured by the antibody-coated beads were released by heating the samples in SDS-loading buffer. The amounts of GPIHBP1 and LPL in the samples were assessed by western blotting with IRdye680–antibody 11A12 and an IRdye800-labeled V5 monoclonal antibody, respectively.

Western blots

Proteins were size-fractioned on 12% Bis-Tris SDS-polyacrylamide gels and subsequently transferred to nitrocellulose. For antibody dilutions, see the Online Supplemental Material.

Homology modeling of GPIHBP1

The homology model of human GPIHBP1 was created with the protein fold recognition server Phyre 2.[20] The 3D structures of the water-soluble domains of human LYNX1[21] and uPAR[22] were selected as templates. The calculated models were visualized with the PyMOL Molecular Graphics System, Version 1.5.0.4.

RESULTS

The majority of the *GPIHBP1* missense mutations identified in patients with chylomicronemia involve conserved cysteines in the Ly6 domain.[4–8] We found that substantial amounts of mutant GPIHBP1 protein can be released from the surface of CHO cells with phosphatidylinositol-specific phospholipase C (PIPLC) (Figure 1A, middle panel), indicating that these mutations have little effect on GPIHBP1 trafficking to the cell surface. To determine whether the unpaired thiol in the GPIHBP1 cysteine mutants might lead to intermolecular disulfide bonds, PIPLC-released proteins were electrophoresed under nonreducing conditions. With wild-type GPIHBP1, monomers (~28 kDa) were present and easily detected by western blotting; however, dimers (~49 kDa) and multimers were also present (Figure 1A, top panel). In the case of the cysteine mutants, the intensity of the monomer band was reduced while the intensity of dimers and multimers increased (Figure 1A, top panel). When the intensity of GPIHBP1 monomers was compared to the total GPIHBP1 signal (*i.e.*, the signal from all of the GPIHBP1 bands in the lane), the amounts of

monomers with GPIHBP1-C65Y, GPIHBP1-C65S, and GPIHBP1-C68G were 75, 73, and 81% lower, respectively, than with wild-type GPIHBP1. When the GPIHBP1 monomer band was compared only to the GPIHBP1 dimer band, the results were similar; the amounts of GPIHBP1-C65Y, GPIHBP1-C65S, and GPIHBP1-C68G monomers were 72, 71, and 77% lower, respectively, than with wild-type GPIHBP1 (Figure 1A).

To determine whether GPIHBP1 dimerization was a peculiarity of the CHO cell expression system, we examined the electrophoretic migration of wild-type mouse GPIHBP1 and GPIHBP1-C88A in rat heart microvascular endothelial cells (RHMVEC) that had been transduced with mouse GPIHBP1 lentiviral expression vectors. When the proteins were electrophoresed under nonreducing conditions, 82% of the wild-type GPIHBP1 at the surface of RHMVECs was in the form of monomers (Online Figure IA). With GPIHBP1-C88A, the amount of monomers was reduced by 85% (compared with wild-type GPIHBP1), and the amount of dimers was markedly increased (1300-fold as judged by quantification of the western blots with an infrared scanner) (Online Figure IA).

We also expressed soluble versions of human GPIHBP1 in *Drosophila* S2 cells.[19,23,24] In this system, the GPIHBP1 proteins contained an amino-terminal uPAR tag (detectable with antibody R24) and sequences from the carboxyl terminus of mouse GPIHBP1 (detectable with antibody 11A12). Under nonreducing conditions, 48.8 ± 0.02% (n = 20 experiments) of the wild-type GPIHBP1 secreted by *Drosophila* S2 cells was monomeric, as judged by western blots with antibody 11A12 (Figure 1B). With antibody R24, we observed a higher percentage of monomers (72.7 ± 0.03%; n = 20 experiments), reflecting a preference of antibody R24 for a properly folded uPAR tag (Figure 1B). GPIHBP1 cysteine mutants were secreted efficiently, as judged by western blotting under reducing conditions (Figure 1B). However, western blots of nonreduced samples revealed that single cysteine mutants were mainly in the form of dimers and multimers (Figure 1B). The amounts of monomers with the cysteine mutants were 71–90% lower than with wild-type GPIHBP1 (Figure 1B–C). In follow-up studies, we eliminated pairs of cysteines that are connected by a disulfide bridge; the percentage of monomers with these “paired mutants” was greater than with the single-cysteine mutants, but lower than with wild-type GPIHBP1 (Figure 1B–C).

The Q115P mutation in GPIHBP1 impairs the ability of GPIHBP1 to bind LPL,[3] but changing Q115 to Lys (the residue found in canine GPIHBP1) has little or no impact on LPL binding.[17] We suspected that the introduction of a proline immediately adjacent to Cys114 might impair proper disulfide bonding, reduce the secretion of monomers, and promote the formation of dimers and multimers. Indeed, the Q115P mutation reduced the secretion of monomers by 73% (as judged by western blots with antibody 11A12) (Figure 1B, Table 1). The propensity of GPIHBP1-Q115P for dimerization/multimerization likely explains why we could not find evidence for a free thiol with cysteine-modifying reagents. The Q115K mutant had no effect on the amount of GPIHBP1 monomers (Figure 1B).

We predicted that GPIHBP1 dimers and multimers would have little capacity to bind LPL. To test this prediction, we performed a cell-free LPL–GPIHBP1 binding assay. Agarose beads coated with the LPL-specific antibody 5D2[25] were incubated with V5-tagged human LPL[26] and either wild-type GPIHBP1 or GPIHBP1-C68Y. After washing the beads, the

LPL that had been captured by antibody 5D2 (along with any GPIHBP1 bound to the LPL) was eluted from the antibody-coated beads in SDS-sample buffer. The amounts of LPL and GPIHBP1 in the starting material, the flow-through (unbound) fraction, the wash fraction, and the elution fraction were assessed by western blotting with an anti-V5 antibody and antibody 11A12, respectively. Wild-type GPIHBP1 and LPL co-eluted from the beads, indicating that GPIHBP1 had been bound to LPL (Figure 2, top panel). We examined the same samples under nonreducing conditions. Monomers, dimers, and multimers were detected in the starting material, unbound fraction, and wash fraction, but only monomeric GPIHBP1 was present in the elution fraction (Figure 2, bottom panel), indicating that only GPIHBP1 monomers bind LPL. No GPIHBP1-C68Y was released from the agarose beads, consistent with the inability of this mutant to bind LPL (Figure 2, top panel). Under nonreducing conditions, we found that most of the GPIHBP1-C68Y in the starting material was in the form of dimers and multimers (Figure 2, bottom panel).

A T108R missense mutation was recently encountered in a patient with chylomicronemia,[18] but the ability of GPIHBP1-T108R to bind LPL was not tested. To address that issue, we transfected CHO-K1 cells with wild-type GPIHBP1 or GPIHBP1-T108R and then incubated the cells with V5-tagged human LPL. After 2 h, the cells were washed, and the amount of LPL bound to the cells was determined by western blotting. LPL bound avidly to cells expressing wild-type GPIHBP1, and this binding was inhibited by heparin (Figure 3A). Cells expressing GPIHBP1-T108R lacked the capacity to bind LPL (Figure 3A). We also used a cell-free assay to assess the binding of LPL to wild-type GPIHBP1 and GPIHBP1-T108R. The GPIHBP1 proteins were incubated for 1 h with V5-tagged human LPL and antibody 11A12–coated agarose beads. After washing the beads, GPIHBP1 (and any GPIHBP1-bound LPL) were eluted from the beads with SDS-sample buffer. The amounts of GPIHBP1 and LPL in the starting material, the flow-through fraction (unbound), wash fraction, and elution fraction were assessed by western blotting. LPL bound avidly to wild-type GPIHBP1 and therefore was found in the elution fraction (Figure 3B). LPL did not bind to GPIHBP1-Q115P, GPIHBP1-T108R, or GPIHBP1-C68Y, and therefore was not present in the elution fraction (Figure 3B).

To test whether the T108R substitution renders GPIHBP1 more prone to dimerization/multimerization, we compared the migration of wild-type GPIHBP1 and GPIHBP1-T108R by SDS-PAGE under nonreducing conditions. Most of the wild-type human GPIHBP1 was secreted as a monomer, although dimers and multimers were also present (Figure 3C). In contrast, most of the GPIHBP1-T108R was in the form of dimers and multimers (Figure 3C).

We previously identified 12 residues in GPIHBP1's Ly6 domain that are important for LPL binding, which were predominantly located in β-strands C and D (forming the "second finger" of the Ly6 domain).[17] To determine if the impaired ability of these mutants to bind LPL was associated with an increased propensity to dimerize/multimerize, we characterized these mutants with the *Drosophila* S2 cell expression system. Most of the GPIHBP1 mutants (Y66A, L71A, I93A, T104A, T105A, H106L, S107A, T108R, V126A) had an increased propensity to form dimers and multimers (Figure 4A, Table 1). The L92A mutant was similar to wild-type GPIHBP1 with respect to the relative amounts of monomers *vs*. dimers/

multimers (Figure 4A, Table 1). The W109S mutant displayed a *reduced* propensity to form dimers and multimers (Figure 4A, Table 1); this finding was consistent in 7 independent experiments. The propensities of W109Y, W109H, W109A, and W109F mutants to form dimers/multimers were similar to GPIHBP1-W109S (Figure 4A, Table 2). The W109C, W109P, and W109T mutants had a greater propensity to form dimers/multimers than GPIHBP1-W109S (Figure 4A, Table 2).

To determine if the lower-than-normal propensity of "W109 mutants" to form dimers/multimers was a peculiarity of the *Drosophila* cell expression system, we expressed GPIHBP1-W109S and several other GPIHBP1 mutants in CHO cells, incubated the cells with PIPLC, and then examined PIPLC-released proteins under reducing and nonreducing conditions (Figure 4B). PIPLC released similar amounts of wild-type GPIHBP1 and the GPIHBP1 mutants from the surface of CHO cells, as judged by western blots of the reduced samples (Figure 4B, middle panel). When we examined nonreduced samples, we observed *larger* amounts of GPIHBP1 monomers with GPIHBP1-W109S than with wild-type GPIHBP1 (2.5 ± 0.23-fold increase, $n = 4$ experiments) (Figure 4B). GPIHBP1-C65S and GPIHBP1-T108R monomer levels were only 14 ± 2 and 19 ± 3%, respectively, of wild-type GPIHBP1 (Figure 4B). With the L92A mutant, the monomer levels were 51 ± 3% as much as wild-type GPIHBP1 (Figure 4B). We obtained similar findings with HUVECs. The amount of monomers in GPIHBP1-W109S-expressing HUVECs was 2.4-fold greater than in HUVECs expressing wild-type GPIHBP1. In HUVECs expressing GPIHBP1-C65S or GPIHBP1-T108R, the amounts of GPIHBP1 monomers were reduced by more than 50% when compared to wild-type GPIHBP1 (Online Figure IB).

We next tested the ability of wild-type and mutant GPIHBP1 proteins to bind LPL. GPIHBP1 was mixed with V5-tagged human LPL and antibody 11A12–coated agarose beads. After 1 h, the beads were washed, and the GPIHBP1 (along with GPIHBP1-bound LPL) was eluted from the beads with SDS-loading buffer. Wild-type GPIHBP1 bound LPL avidly, but there was no binding of LPL to the L92A or W109S mutants (Figure 5A–C). Small amounts of LPL binding were observed with G101S, T104A, and T108A mutants, in agreement with earlier findings[17] (Figure 5A–C).

Tryptophans are overrepresented in the binding interfaces of protein complexes.[27–29] For this reason, we suspected that Trp-109 in GPIHBP1 might be important for LPL binding. Indeed, replacing Trp-109 with Ser, Tyr, His, Ala, Phe, Cys, Pro, or Thr abolished the ability of GPIHBP1 to bind LPL (Figure 5D–F).

DISCUSSION

Earlier studies revealed that some cases of familial chylomicronemia in humans are caused by amino acid substitutions in GPIHBP1's Ly6 domain that interfere with LPL binding, but the molecular mechanisms were not explored.[3–5] It has remained unclear whether the amino acid substitutions block LPL binding by interfering with the ability of the protein to form disulfide bonds and fold into the hallmark three-fingered motif—or whether the mutations interfere with LPL binding in a more direct fashion. In the current study, we investigated that topic and uncovered three important findings. The first is that mutations involving the

conserved cysteines in GPIHBP1's Ly6 motif promote the formation of GPIHBP1 dimers/multimers and reduce the formation of monomers. This was the case in both CHO and *Drosophila* S2 expression systems. The elimination of any single cysteine in the Ly6 domain results in a reactive thiol, which can promote intermolecular disulfide bridges with a neighboring GPIHBP1 molecule, resulting in covalent dimers (resistant to dissociation when heated in SDS). The presence of the unpaired thiol may also interfere with additional disulfide bonds, leading to further illicit intermolecular disulfide bonds and the formation of multimers. The introduction of an extra cysteine into GPIHBP1's Ly6 domain also leads to the formation of dimers and multimers.[9]

A second important finding is that mutations in other amino acid residues—aside from the conserved cysteines—promote the formation of GPIHBP1 dimers and multimers. For example, the Q115P and T108R mutants, identified in chylomicronemia patients,[3,18] led to nearly as many dimers/multimers as the cysteine mutants. Mutations in other residues found to be important for LPL binding (*e.g.*, Tyr66, Leu71, Thr91, Ile93, Gly101, Thr104, Thr105, His106, Val126)[17] also led to an increase in the formation of dimers/multimers. The increased propensity of GPIHBP1 mutants to form dimers/multimers is important. Only GPIHBP1 monomers—and not dimers or multimers—are capable of binding LPL. The fact that both cysteine and "non-cysteine" mutations reduce the formation of functionally active monomers represents a new lesson in the human genetics of chylomicronemia.

The third important finding is that the W109S mutation in GPIHBP1 abolishes GPIHBP1's ability to bind LPL yet *reduces* dimers/multimers. These findings raise the possibility that the W109S mutation may impair LPL binding by a more direct mechanism (*e.g.*, adversely affecting the binding interface between GPIHBP1 and LPL). This scenario is plausible. Tryptophans are the most overrepresented residue in protein–protein interfaces, and mutation of tryptophans in binding interfaces often disrupts protein–protein interactions.[27] Also, when structures of protein–protein complexes are examined, tryptophans are the most overrepresented residue in the core of binding interfaces.[28,29] Two additional observations lend support to the notion that W109 might play a direct role in LPL binding. The first is the finding that replacing W109 with any of 8 other amino acids (including other aromatic amino acids) abolished LPL binding. The second is that W109 is one of only a handful of Ly6 domain residues (aside from the cysteines) that are perfectly conserved in mammalian evolution.[30]

GPIHBP1-L92A also lacked the ability to bind LPL despite the ability to form half-normal amounts of monomers, raising the possibility that L92 might also play a direct role in binding LPL. Interestingly, homology modeling of the GPIHBP1 structure predicts that W109 and L92 are located on β-strands D and C, respectively, and that their side chains are adjacent on a solvent-exposed region of the molecule (Figure 6).

The reduced propensity of GPIHBP1-W109S to form dimers/multimers was intriguing. W109 is located adjacent to C110 (which forms a disulfide bond with C83). Interestingly, tryptophans are underrepresented in primary sequences adjacent to cysteines that are engaged in disulfide bonds and are more frequent in the sequences close to free cysteines, prompting the conclusion that tryptophans are a hindrance to disulfide bond formation.[31]

This finding may help to explain the better-than-normal capacity of GPIHBP1 to form monomers when W109 is replaced with a serine.

In the current study, we used CHO cell, endothelial cell, and *Drosophila* cell expression systems to investigate GPIHBP1 dimerization/multimerization. In CHO and endothelial cells, we examined the *GPI-anchored* form of GPIHBP1 on the surface of cells, whereas in the insect cells we examined *secreted* versions of GPIHBP1. In the case of the W109S mutant, both expression systems revealed a reduced propensity of GPIHBP1-W109S to dimerize/multimerize. In the case of the L92A mutant, the *Drosophila* cell expression system did not uncover an increased propensity to form dimers/multimers, but the CHO cell system revealed reduced monomers and increased dimers/multimers at the cell surface. These observations suggest that the mammalian GPI-anchored expression system may be more sensitive for uncovering a predilection for GPIHBP1 misfolding and the formation of intermolecular disulfide bonds.

In the *Drosophila* and CHO systems, and to a somewhat lower extent in the HUVEC expression system, dimers and multimers were detectable with wild-type GPIHBP1. It is conceivable that dimerization of wild-type GPIHBP1 is an artifact of protein overexpression, but dimers and multimers have been observed with other Ly6 proteins in the absence of protein overexpression. First, Fletcher and coworkers[32] released CD59 (a GPI-anchored Ly6 protein) from the surface of erythrocyte membranes with PIPLC and then size-fractionated the proteins by SDS-PAGE under nonreducing conditions. Most of the CD59 on erythrocytes was in the form of monomers, but there were substantial amounts of dimers and multimers. Second, homodimers of α-cobratoxin (αCT) have been identified in freshly isolated venom from the *Naja kaouthia* cobra.[33] αCT is a cysteine-rich neurotoxin in the same protein family as mammalian Ly6 proteins; αCT has 10 cysteines, all arranged in the same pattern as in GPIHBP1 and CD59 and all disulfide-bonded. Interestingly, the αCT–αCT homodimers had altered function (a reduced capacity to compete with α-bungarotoxin for binding to the α7-nicotinic acetylcholine receptor). Finally, in recent studies, we examined proteins released by PIPLC from capillaries of mouse hearts. When the PIPLC-released proteins were size-fractionated under nonreducing conditions, we observed GPIHBP1 dimers in addition to monomers (A. Beigneux, L. Fong, unpublished observations).

In summary, we demonstrated that the GPIHBP1 cysteine mutants (identified in chylomicronemia patients) increase the propensity of GPIHBP1 to form disulfide-linked dimers and multimers. Mutations in other residues in the Ly6 domain, including several identified in chylomicronemia patients (*e.g.*, Q115P, T108R), also promote the formation of dimers/multimers. This discovery is relevant to pathogenesis because LPL binds preferentially to GPIHBP1 monomers. We identified one mutant, W109S, that abolished LPL binding but reduced GPIHBP1's propensity to form dimers and multimers. We propose that W109 may play a more direct role in LPL binding. L92, predicted to be located adjacent to W109, might also play a more direct role in LPL binding because it abolished LPL binding but had little effect on protein dimerization in the *Drosophila* system and reduced GPIHBP1 monomers by only ~50% in the mammalian cell system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the work of a graduate student, Mr. Calvin Leung, who worked on dimerization/multimerization of mutant Ly6 proteins.

SOURCES OF FUNDING

This work was supported by grants from the NIH (HL094732, HL090553 and HL087228), and a Leducq Transatlantic Network grant (12CVD04).

Nonstandard Abbreviations and Acronyms

GPIHBP1	glycosylphosphatidylinositol-anchored high density lipoprotein–binding protein 1
GPI	glycosylphosphatidylinositol
LP	lipoprotein lipase
CHO	Chinese hamster ovary
uPAR	urokinase-type plasminogen activator receptor
Ly6	Lymphocyte antigen 6

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Novelty and Significance

What Is Known?

- GPIHBP1, a cell-surface protein of capillary endothelial cells, binds lipoprotein lipase (LPL) in the subendothelial spaces and shuttles the enzyme to the capillary lumen, where LPL hydrolyzes triglycerides within triglyceride-rich lipoproteins (VLDL and chylomicrons).
- *GPIHBP1* and *LPL* missense mutations that disrupt GPIHBP1–LPL interactions abolish LPL transport to the capillary lumen and cause familial chylomicronemia.
- Most GPIHBP1-related cases of chylomicronemia involve amino acid substitutions in GPIHBP1's three-fingered domain that abolish LPL binding.

What New Information Does This Article Contribute?

- Most of the *GPIHBP1* missense mutations causing chylomicronemia interfere with the folding of GPIHBP1's three-finger motif and result in the production of disulfide-linked GPIHBP1 dimers and multimers.
- LPL binds to monomeric GPIHBP1 but not to GPIHBP1 dimers/multimers.
- Enhanced formation of dimers/multimers by mutant forms of GPIHBP1 represents an important mechanism for defective LPL binding and chylomicronemia.

GPIHBP1, a cell-surface protein of capillary endothelial cells, binds LPL in the subendothelial spaces and shuttles it to the capillary lumen. We sought to define mechanisms by which *GPIHBP1* missense mutations abolish LPL binding and result in chylomicronemia.

Most *GPIHBP1* missense mutations involve residues in GPIHBP1's three-fingered motif. Most of these mutations interfere with the folding of GPIHBP1 and result in the appearance of GPIHBP1 dimers and multimers at the cell surface. Reduced amounts of GPIHBP1 monomers at the cell surface is highly relevant to the pathogenesis of chylomicronemia because only GPIHBP1 monomers—and not dimers or multimers—bind LPL. We identified one mutant, GPIHBP1-W109S, with distinctive properties. GPIHBP1-W109S did not bind LPL but had a *reduced* propensity for forming dimers or multimers, raising the possibility that W109 might play a direct role in forming the GPIHBP1–LPL interface.

In summary, our studies show that GPIHBP1 dimerization/multimerization represents an important mechanism by which many amino acid substitutions in GPIHBP1 abolish LPL binding and cause chylomicronemia.

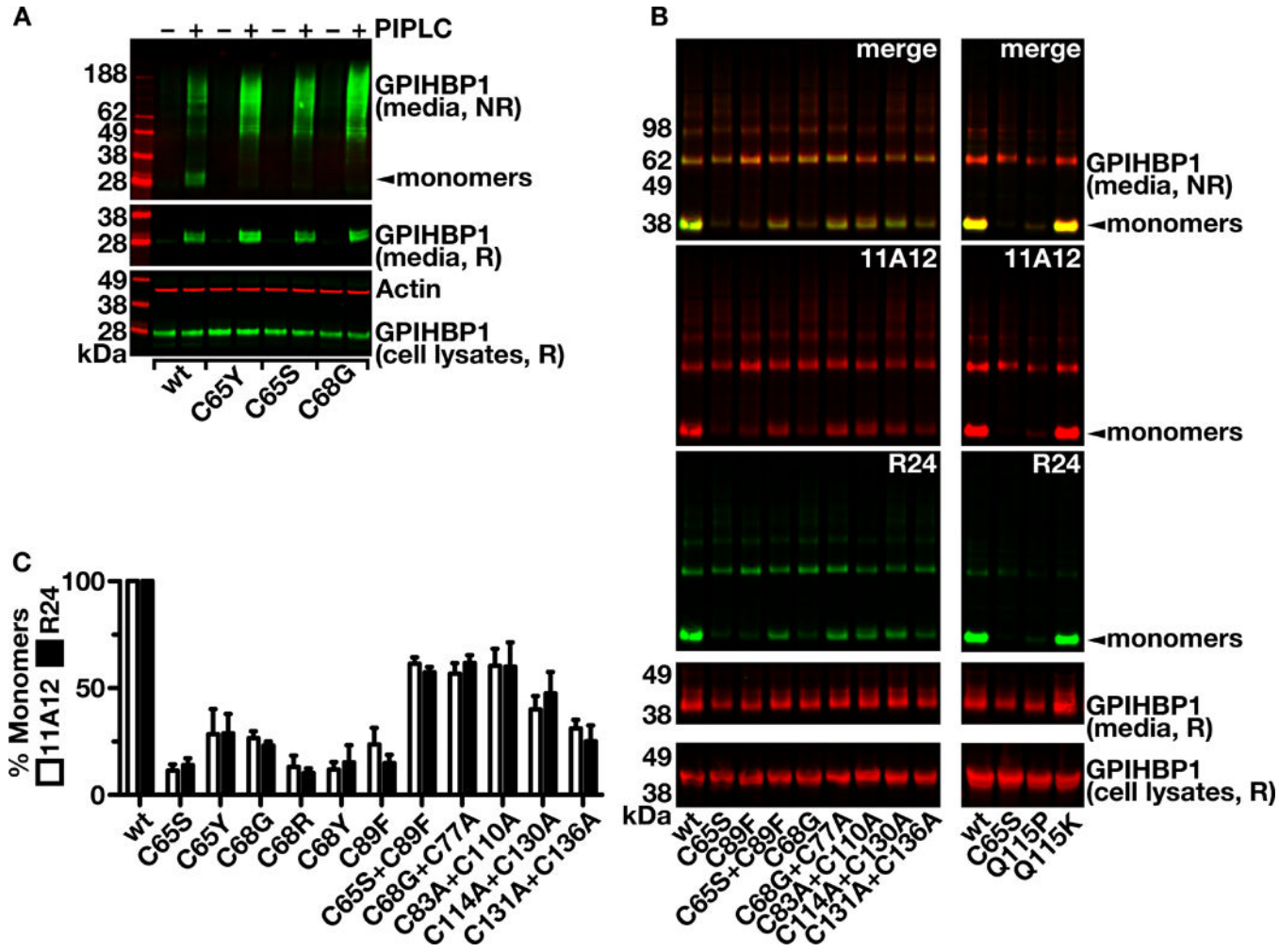

Figure 1. Defining the properties of GPIHBP1 cysteine mutants

A, Western blot analysis of S-protein–tagged GPIHBP1 proteins released from the surface of GPIHBP1-transfected CHO-K1 cells with phosphatidylinositol-specific phospholipase C (PIPLC). 24 h after the transfection, the cells were washed and then incubated for 20 min at 37 °C with PIPLC (10 U/ml). PIPLC-released proteins were size-fractionated by SDS-PAGE under nonreducing (NR) and reducing (R) conditions; cell lysates were examined under reducing conditions. GPIHBP1 was detected with an S-protein antibody; actin was used as a loading control. GPIHBP1 monomers (~28 kDa) are indicated with an arrowhead. **B**, Western blots of secreted versions of GPIHBP1 (from *Drosophila* S2 cells) with IRdye680–antibody 11A12 and IRdye800–antibody R24. The top three panels show GPIHBP1 proteins under nonreducing conditions; the bottom two show GPIHBP1 under reducing conditions. GPIHBP1 monomers (~38 kDa) are noted with an arrowhead. **C**, Bar graph showing the ratio of GPIHBP1 monomers to total GPIHBP1, expressed as a percentage of the ratio with wild-type (wt) GPIHBP1 (set at 100%). Mean ± SEM from at least three independent experiments. Band intensities were quantified with a Li-Cor scanner.

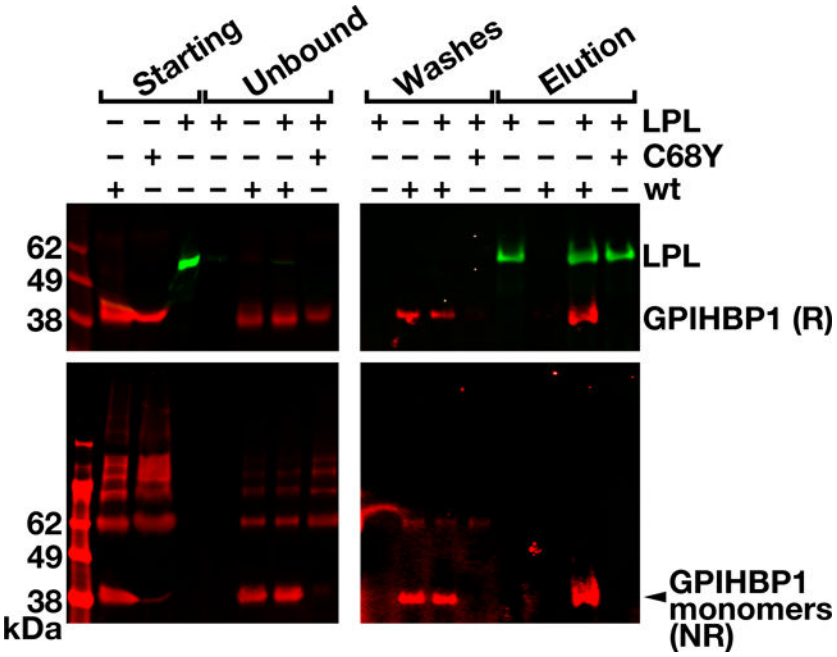

Figure 2. Assessing the ability of GPIHBP1 monomers, dimers, and multimers to bind LPL

Secreted versions of wild-type (wt) GPIHBP1 and GPIHBP1-C68Y were expressed in *Drosophila* S2 cells. The GPIHBP1 in the medium was incubated with V5-tagged human LPL and agarose beads coated with the LPL-specific monoclonal antibody 5D2. After incubating the mixture for 1 h at 4 °C, the beads were washed, and the LPL (and any bound GPIHBP1) was released by heating the beads in SDS-sample buffer. Western blots were performed on the starting material (*i.e.*, the GPIHBP1 and LPL), unbound fraction, wash fraction, and eluted proteins with an IRdye800-V5 antibody (green) and an IRdye680–antibody 11A12 (red) under reducing (R) and nonreducing (NR) conditions. This experiment was repeated twice with virtually identical results.

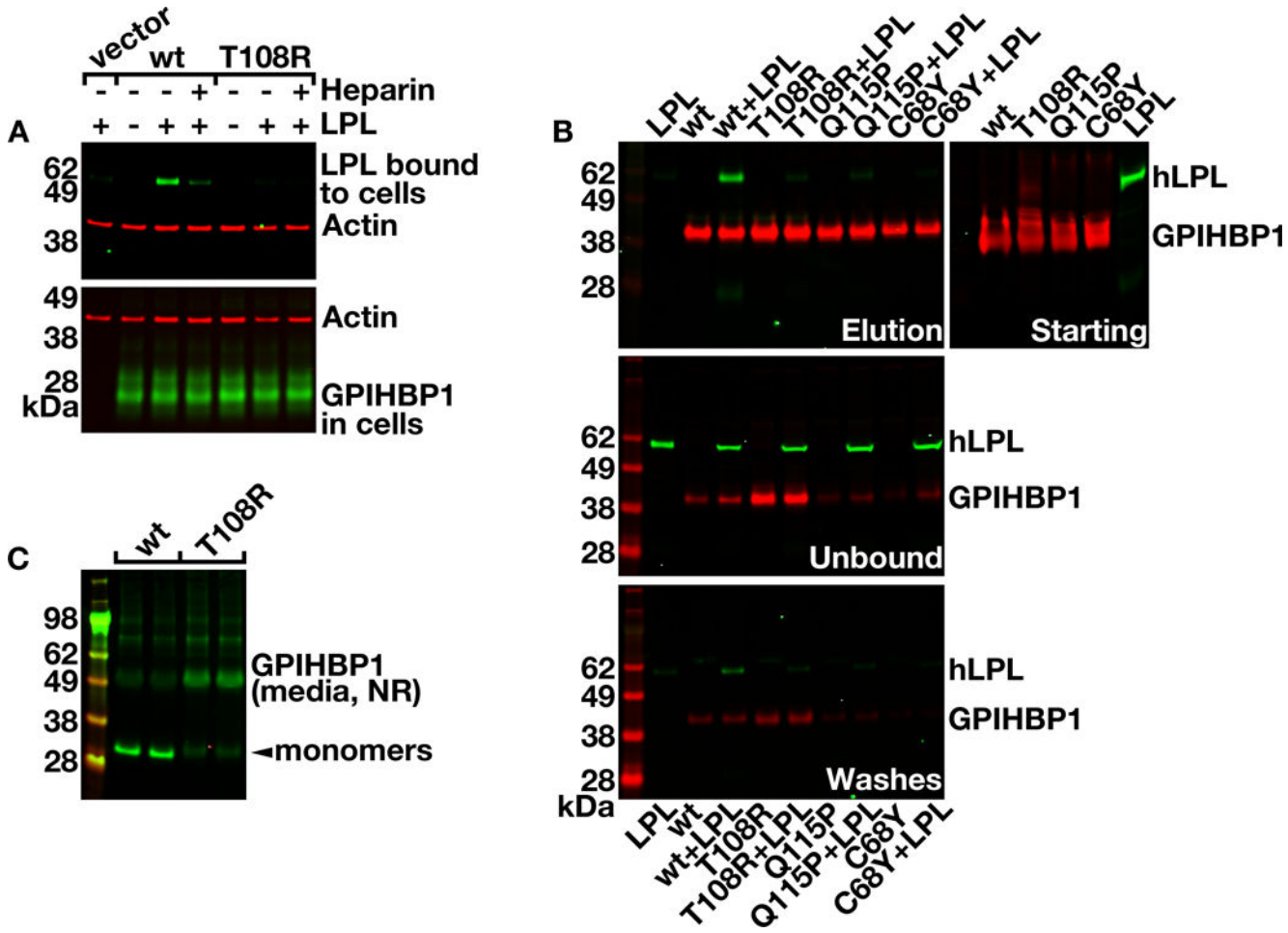

Figure 3. Characterization of GPIHBP1-T108R

A, Cell-based LPL–GPIHBP1 binding assay. CHO-K1 cells were transfected with S-protein–tagged wild-type GPIHBP1 (wt) or GPIHBP1-T108R. 24 h later, the cells were incubated for 2 h at 4°C with V5-tagged human LPL in the presence or absence of heparin (250 U/ml). After washing the cells, western blots of cell lysates were performed with a goat antibody against the S-protein tag (to detect GPIHBP1) and a V5-specific monoclonal antibody (to detect GPIHBP1-bound LPL). Actin was used as a loading control. This experiment was repeated three times with similar results. **B**, Cell-free LPL–GPIHBP1 binding assay. Secreted versions of GPIHBP1 were expressed in *Drosophila* S2 cells. GPIHBP1 was incubated with V5-tagged human LPL and 11A12-coated agarose beads for 1 h at 4 °C. After washing the beads, GPIHBP1 (and any GPIHBP1-bound LPL) was released by heating the beads in SDS-sample buffer. Western blots were performed on the starting material (*i.e.*, the GPIHBP1 and LPL), unbound fraction, wash fraction, and eluted material with an IRdye800-V5 antibody (green) and IRdye680–antibody 11A12 (red). This experiment was repeated twice with virtually identical results. **C**, Western blot of wild-type GPIHBP1 (wt) and GPIHBP1-T108R expressed in *Drosophila* S2 cells with antibody R24; the samples were electrophoresed under nonreducing conditions. The GPIHBP1 monomer band (~28 kDa rather than 38 kDa because this human GPIHBP1 construct did not have the carboxyl-terminal mouse GPIHBP1 sequences) is indicated with an arrowhead. The

monomer/dimer ratio was 6.4 for wild-type GPIHBP1 and 0.55 for GPIHBP1-T108R. This experiment is representative of three independent binding assays.

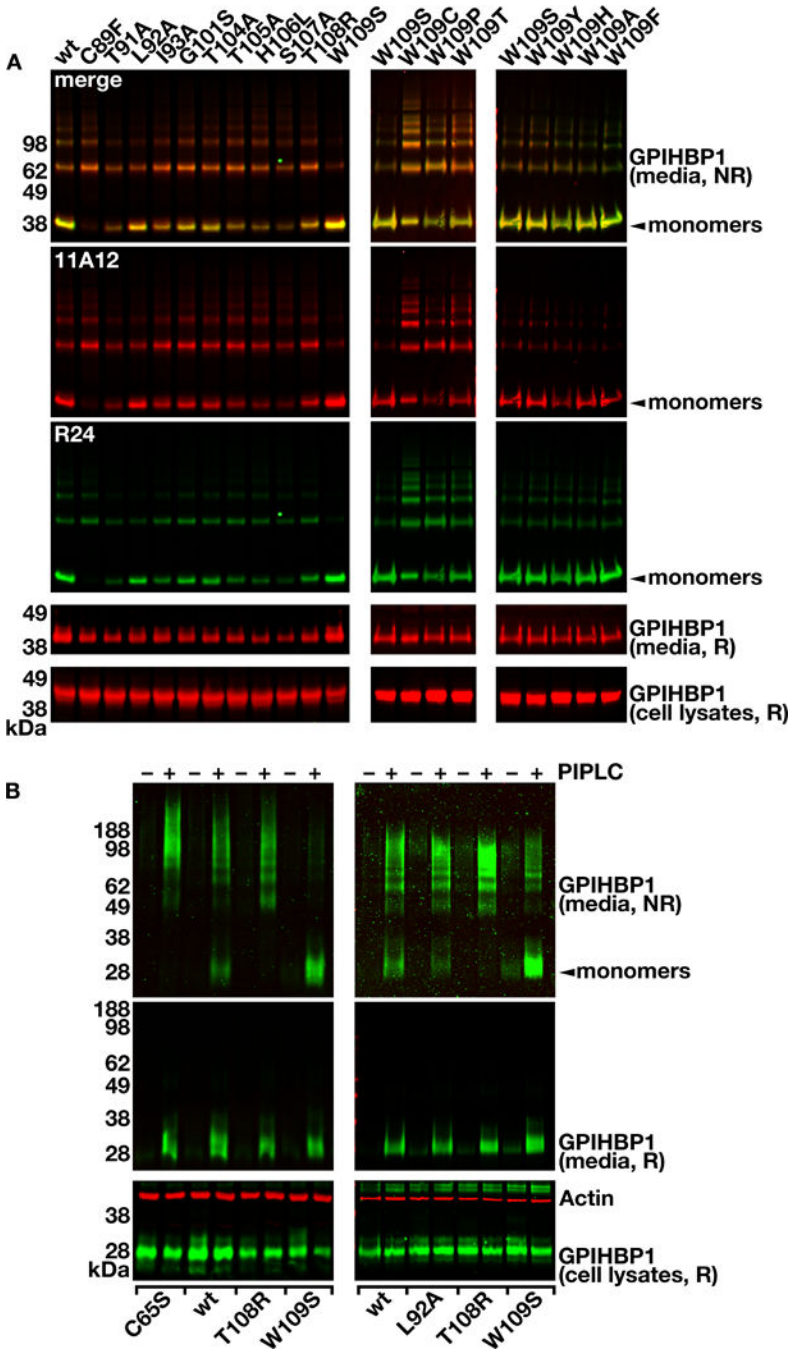

Figure 4. Migration pattern of GPIHBP1 mutants that are defective in LPL binding

A, Western blot of GPIHBP1 proteins (from *Drosophila* S2 cells) with IRdye680–antibody 11A12 and IRdye800–antibody R24. The top three panels show samples under nonreducing conditions (NR); the bottom two panels show samples under reducing conditions (R). GPIHBP1 monomers (~38 kDa) are indicated with an arrowhead. Shown here are representative western blots (see Table 1 and 2 for numbers of replicates). **B**, Western blots, from two independent experiments, of GPIHBP1 that had been released from the surface of GPIHBP1-transfected CHO-K1 cells with phosphatidylinositol-specific phospholipase C

(PIPLC). The CHO-K1 cells had been transfected with S-protein–tagged GPIHBP1 constructs. 24 h later, the cells were washed and incubated for 20 min at 37 °C with PIPLC (10 U/ml). Western blots, using an S-protein antibody (green) were performed on PIPLC-released protein samples under nonreducing (NR) and reducing (R) conditions, and on cell lysates under reducing conditions (R). Actin (red) was used as a loading control. GPIHBP1 monomers (~28 kDa) are indicated with an arrowhead.

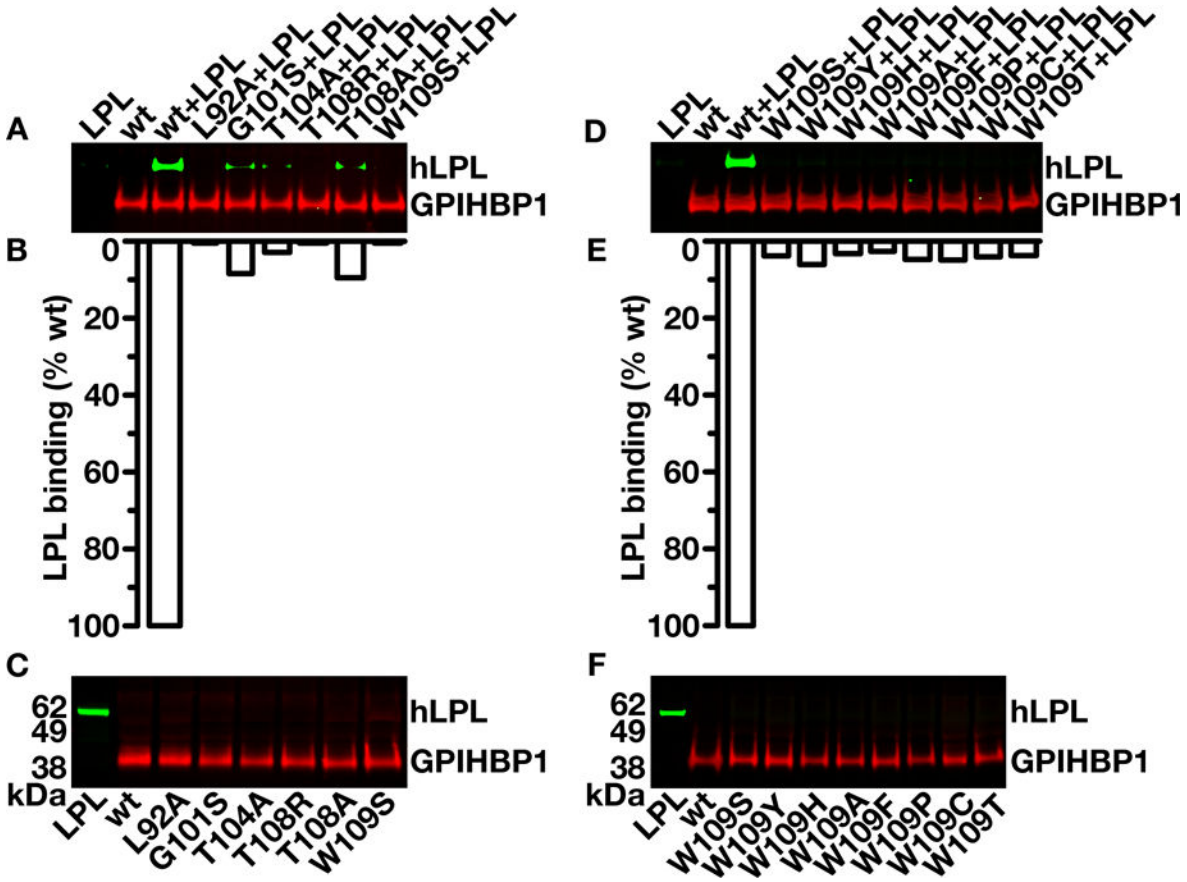

Figure 5. Cell-free LPL–GPIHBP1 binding assays

Secreted versions of wild-type (wt) and mutant GPIHBP1 were expressed in *Drosophila* S2 cells. The GPIHBP1 proteins were incubated with V5-tagged human LPL and agarose beads that had been coated with the GPIHBP1-specific antibody 11A12. After a 1-h incubation at 4 °C, the beads were washed, and the GPIHBP1 (and any GPIHBP1-bound LPL) was released from the beads with SDS-sample buffer. Western blots were performed with an IRdye800–V5 antibody (green) and IRdye680–antibody 11A12 (red). Shown here are western blots on the elution fractions (**A** and **D**) and the starting material (*i.e.*, the GPIHBP1 and LPL added to the assay) (**C** and **F**). The presence of LPL in the elution fraction reflects binding of LPL to the antibody 11A12–immobilized GPIHBP1. **B and E**, Quantification of LPL binding to GPIHBP1, expressed as a percentage of LPL binding with wild-type GPIHBP1 (set at 100%). These experiments were repeated twice with virtually identical results.

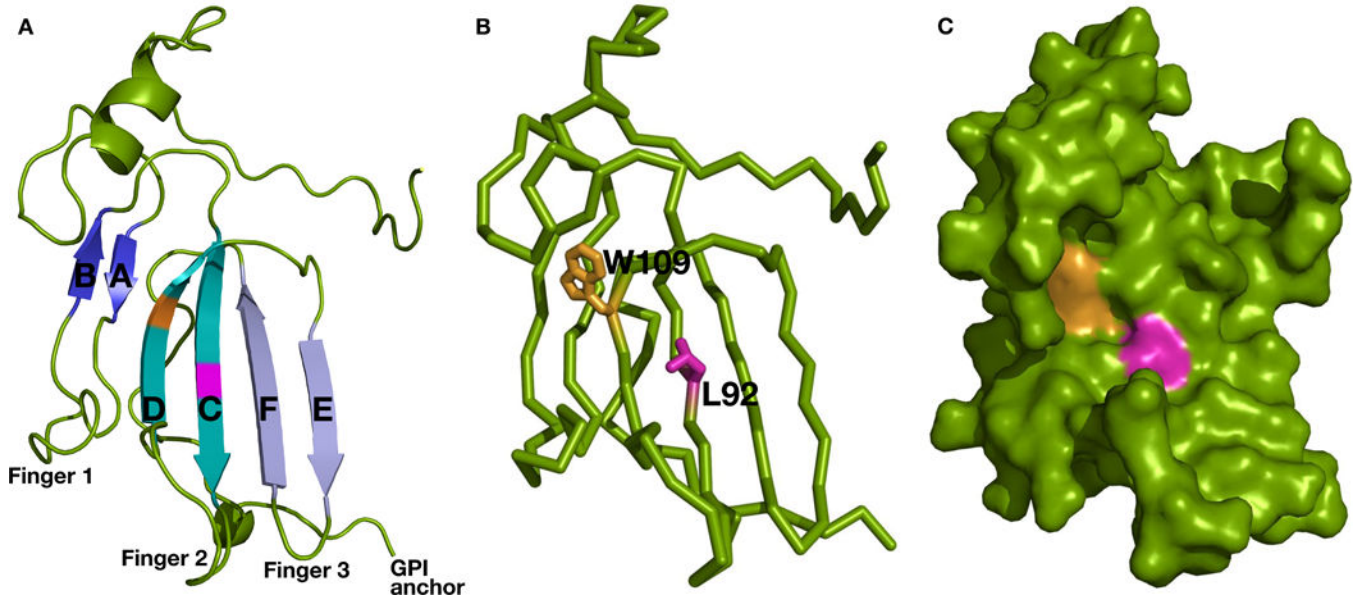

Figure 6. Homology models of human GPIHBP1 (residues 21–151)

The amino terminus is at the top right; the carboxyl terminus (GPI anchor) is at the bottom right. Residues W109 and L92 are highlighted in orange and pink, respectively. **A**, Ribbon representation highlighting the paired β-strands (A–F) and the three fingers of the Ly6 domain. **B and C**, Stick (**B**) and space-filling (**C**) representations depicting the positions of W109 and L92.

TABLE 1

Ratio of GPIHBP1 monomers to total GPIHBP1 (monomers, dimers, multimers) for different GPIHBP1 mutants, expressed as a percentage of the ratio with wild-type GPIHBP1 (set at 100%)

GPIHBP1 from *Drosophila* S2 cells was size-fractioned by SDS-PAGE under nonreducing conditions. Western blots were performed with IRdye680–antibody 11A12 and IRdye800–antibody R24. Band intensities for GPIHBP1 monomers and total GPIHBP1 were quantified on a Li-Cor scanner. Shown here are mean ratios ± SEM and the number of independent experiments. For wild-type GPIHBP1, the absolute ratio of monomers to total GPIHBP1 was 48.8 ± 0.02% with antibody 11A12 and 72.7 ± 0.03% with antibody R24 (n = 20 independent experiments).

	Ratio of Monomeric to Total GPIHBP1 (% of wild-type GPIHBP1)	
Mutation	**Mab 11A12**	**Mab R24**
Y66A	44.3 ± 4.3 (n = 3)	52.2 ± 7.5 (n = 3)
L71A	43.2 ± 6.1 (n = 3)	60.5 ± 16.2 (n = 3)
T91A	68.4 ± 9.7 (n = 3)	62.4 ± 15.8 (n = 3)
L92A	114 ± 4.9 (n = 3)	96.4 ± 12.5 (n = 3)
I93A	60.6 ± 3.9 (n = 3)	58.9 ± 12.0 (n = 3)
G101S	72.2 ± 2.1 (n = 3)	73.1 ± 7.0 (n = 3)
T104A	68.4 ± 1.9 (n = 3)	81.2 ± 7.9 (n = 3)
T105A	45.0 ± 2.7 (n = 3)	57.7 ± 12.3 (n = 3)
H106L	38.6 ± 3.0 (n = 3)	58.6 ± 11.0 (n = 3)
S107C	4.84 ± .82 (n = 3)	14.9 ± 9.6 (n = 3)
T108A	144 ± 4.9 (n = 3)	111 ± 7.7 (n = 3)
T108R	70.4 ± 1.9 (n = 4)	80.6 ± 5.4 (n = 3)
W109A	138 ± 7.1 (n = 5)	139 ± 3.4 (n = 5)
W109S	155 ± 3.7 (n = 7)	139 ± 7.3 (n = 7)
Q115K	99.6 ± 6.2 (n = 3)	102 ± 2.5 (n = 2)
Q115P	27.5 ± 4.1 (n = 4)	42.3 ± 10.6 (n = 3)
V126A	34.9 ± 2.0 (n = 3)	46.3 ± 7.7 (n = 3)

TABLE 2

Ratio of monomers to total GPIHBP1 (monomers, dimers, multimers) with GPIHBP1-W109 mutants, expressed as percentage of the ratio with GPIHBP1-W109S (set at 100%)

GPIHBP1 from *Drosophila* S2 cells was size-fractioned by SDS-PAGE under nonreducing conditions. Western blots were performed with IRdye680–antibody 11A12 and IRdye800–antibody R24. Band intensities for GPIHBP1 monomers and total GPIHBP1 were quantified on a Li-Cor scanner. Shown here are mean ratios ± SEM and the number of independent experiments. For GPIHBP1-W109S, the absolute ratio of monomers to total GPIHBP1 was 75.4 ± 1.8% with antibody 11A12 and 92.8 ± 3.8% with antibody R24 (n = 7 experiments).

	Ratio of Monomeric to Total GPIHBP1 (% of GPIHBP1-W109S)	
Mutation	**Mab 11A12**	**Mab R24**
W109C	20 ± 0.2 (n = 2)	36 ± 2.3 (n = 2)
W109P	33 ± 2.5 (n = 2)	48 ± 4.0 (n = 2)
W109T	31 ± 2.0 (n = 2)	55 ± 1.1 (n = 2)
W109Y	87 ± 16 (n = 2)	91 ± 2.9 (n = 2)
W109H	72 ± 17 (n = 2)	81 ± 5.6 (n = 2)
W109A	86 ± 7.1 (n = 2)	99 ± 3.5 (n = 2)
W109F	85 ± 9.6 (n = 2)	93 ± 0.2 (n = 2)