Mutation of conserved cysteines in GPIHBP1 reduces amounts of GPIHBP1 in capillaries and abolishes LPL binding

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Abstract Mutation of conserved cysteines in proteins of the Ly6 family cause human disease—chylomicronemia in the case of glycosylphosphatidylinositol-anchored HDL binding protein 1 (GPIHBP1) and paroxysmal nocturnal hemoglobinuria in the case of CD59. A mutation in a conserved cysteine in CD59 prevented the protein from reaching the surface of blood cells. In contrast, mutation of conserved cysteines in human GPIHBP1 had little effect on GPIHBP1 trafficking to the surface of cultured CHO cells. The latter findings were somewhat surprising and raised questions about whether CHO cell studies accurately model the fate of mutant GPIHBP1 proteins in vivo. To explore this concern, we created mice harboring a GPIHBP1 cysteine mutation (p.C63Y). The p.C63Y mutation abolished the ability of mouse GPIHBP1 to bind LPL, resulting in severe chylomicronemia. The mutant GPIHBP1 was detectable by immunohistochemistry on the surface of endothelial cells, but the level of expression was ~70% lower than in WT mice. The mutant GPIHBP1 protein in mouse tissues was predominantly monomeric. We conclude that mutation of a conserved cysteine in GPIHBP1 abolishes the ability of GPIHBP1 to bind LPL, resulting in mislocalization of LPL and severe chylomicronemia. The mutation reduced but did not eliminate GPIHBP1 on the surface of endothelial cells in vivo. —Allan, C. M., C. J. Jung, M. Larsson, P. J. Heizer, Y. Tu, N. P. Sandoval, T. L. P. Dang, R. S. Jung, A. P. Beigneux, P. J. de Jong, L. G. Fong, and S. G. Young. Mutating a conserved cysteine in GPIHBP1 reduces amounts of GPIHBP1 in capillaries and abolishes LPL binding. J. Lipid Res. 2017. 58: 1453–1461.

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Glycosylphosphatidylinositol-anchored HDL binding protein 1 (GPIHBP1), a GPI-anchored protein of capillary endothelial cells, binds LPL in the interstitial spaces (where it is secreted by myocytes and adipocytes) and transports it across endothelial cells to the capillary lumen (1). When GPIHBP1 production is absent, LPL never reaches the capillary lumen, resulting in defective intravascular triglyceride processing and severe hypertriglyceridemia (chylomicronemia) (1, 2). GPIHBP1 is a member of the Ly6 family of proteins and contains two principal domains—an N-terminal acidic domain followed by a three-fingered Ly6 domain containing 10 cysteines (3). The 10 cysteines form five disulfide bonds that maintain the three-fingered structure of the Ly6 domain (4). An early report suggested that the acidic domain played an important role in LPL binding (5), but recent studies with more refined LPL–GPIHBP1 binding assays showed that the Ly6 domain is primarily responsible for LPL binding, with the acidic domain playing a smaller, accessory role (3).

Since the discovery of GPIHBP1’s role in intravascular triglyceride processing (2), many GPIHBP1 mutations have been identified in patients with familial chylomicronemia (6–11). In some cases, deletions of the entire gene have been documented (12, 13), but most of the mutations have been missense mutations that interfere with GPIHBP1’s ability to bind LPL (6–11, 14). Many missense mutations involve one of the conserved cysteines in the Ly6 domain or an adjacent residue (6, 7, 9, 10). In CHO cell studies, these mutations promote the formation of

Abbreviations: BAT, brown adipose tissue; ER, endoplasmic reticulum; GPIHBP1, glycosylphosphatidylinositol-anchored HDL binding protein 1; LPL, LPL receptor; mAb, monoclonal antibody; PIPLC, phosphatidylinositol-specific phospholipase C; WAT, white adipose tissue.

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inappropriate intermolecular disulfide bonds, leading to protein dimerization/multimerization (11, 15). GPIHBP1 dimers and multimers have no ability to bind LPL. Thus far, no one has identified a clinically significant mutation in GPIHBP1’s acidic domain.

In studies with transfected CHO cells, mutation of any one of the 10 cysteines in the Ly6 domain in human GPIHBP1 abolished the ability of GPIHBP1 to bind LPL (16); however, these mutations have little or no effect on the amount of GPIHBP1 that reaches the cell surface (15–17). In contrast, a GPIHBP1 missense mutation that abolished N-linked glycosylation markedly reduced GPIHBP1 trafficking to the cell surface (17, 18). The fact that Ly6 domain cysteine mutants behaved differently than the glycosylation mutant was surprising—for several reasons. First, cysteine mutations in cysteine-rich repeats within the epidermal growth factor precursor homology domain of the LDL receptor (LDLR) cause protein misfolding, preventing the LDLR from moving from the endoplasmic reticulum (ER) to the Golgi (19). Second, mutation of a cysteine in the Ly6 domain of CD59 abolished CD59 trafficking to the surface of blood cells (resulting in increased complement activation and paroxysmal nocturnal hemoglobinuria) (20). In light of the latter observations, we were concerned that the finding of normal trafficking of GPIHBP1 cysteine mutants to the surface of CHO cells may have represented an artifact of protein overexpression (i.e., that overexpression of GPIHBP1 mutants overwhelmed the ER quality-control surveillance mechanisms that would ordinarily remove misfolded proteins).

In the current studies, we sought to determine whether a mutation in a conserved cysteine in GPIHBP1’s Ly6 domain would prevent GPIHBP1 from reaching the surface of endothelial cells in vivo. To address this issue, we generated mutant mice harboring a p.C63Y mutation in Gpihbp1 [a mutation first identified in a 3-year-old boy with chylomicronemia (9)]. Cys-63, the first of 10 cysteines in the Ly6 domain of GPIHBP1, is disulfide bonded to the fifth cysteine (Cys-88), creating the first finger of GPIHBP1’s Ly6 domain (17).

**MATERIALS AND METHODS**

**Generation of Gpihbp1<sup>C63Y/C63Y</sup> mice**

Zygotes from FVB females were injected with 10 ng Cas9 mRNA transcript, 5 ng sgRNA transcript, and 20 ng single-stranded oligonucleotides at the Transgenic Core Facility of the Gladstone Institutes (San Francisco, CA). To generate a mutant allele containing the p.C63Y, oligonucleotides were obtained from IDTDNA as ultramers of 150 nucleotides (5′-TTAGGTCATGCCCCGGCTCAGGATGGTTAGCATTGTAGAGGTGCTGTGCAC-3′ for oligonucleotide targeting. Cas9 was coinjected with a single stranded oligonucleotides at the Transgenic Core Facility of the Gladstone Institutes (San Francisco, CA). To generate a mutant allele containing the p.C63Y, oligonucleotides were obtained from IDTDNA as ultramers of 150 nucleotides (5′-TTAGGTCATGCCCCGGCTCAGGATGGTTAGCATTGTAGAGGTGCTGTGCAC-3′ for oligonucleotide targeting. Cas9 was coinjected with a single stranded oligonucleotides at the Transgenic Core Facility of the Gladstone Institutes (San Francisco, CA). To generate a mutant allele containing the p.C63Y, oligonucleotides were obtained from IDTDNA as ultramers of 150 nucleotides (5′-TTAGGTCATGCCCCGGCTCAGGATGGTTAGCATTGTAGAGGTGCTGTGCAC-3′ for oligonucleotide targeting. Cas9 was coinjected with a single stranded oligonucleotides at the Transgenic Core Facility of the Gladstone Institutes (San Francisco, CA). To generate a mutant allele containing the p.C63Y, oligonucleotides were obtained from IDTDNA as ultramers of 150 nucleotides (5′-TTAGGTCATGCCCCGGCTCAGGATGGTTAGCATTGTAGAGGTGCTGTGCAC-3′ for oligonucleotide targeting. Cas9 was coinjected with a single stranded oligonucleotides at the Transgenic Core Facility of the Gladstone Institutes (San Francisco, CA). To generate a mutant allele containing the p.C63Y, oligonucleotides were obtained from IDTDNA as ultramers of 150 nucleotides (5′-TTAGGTCATGCCCCGGCTCAGGATGGTTAGCATTGTAGAGGTGCTGTGCAC-3′ for oligonucleotide targeting. Cas9 was coinjected with a single stranded oligonucleotides at the Transgenic Core Facility of the Gladstone Institutes (San Francisco, CA). To generate a mutant allele containing the p.C63Y, oligonucleotides were obtained from IDTDNA as ultramers of 150 nucleotides (5′-TTAGGTCATGCCCCGGCTCAGGATGGTTAGCATTGTAGAGGTGCTGTGCAC-3′ for oligonucleotide targeting. Cas9 was coinjected with a single stranded oligonucleotides at the Transgenic Core Facility of the Gladstone Institutes (San Francisco, CA). To generate a mutant allele containing the p.C63Y, oligonucleotides were obtained from IDTDNA as ultramer...
CHO pgsA-745 cells were electroporated with S-protein–tagged versions of mouse WT GPIHBP1, GPIHBP1-W108S, and GPIHBP1-C63Y. Gel electrophoresis was used to resolve the products, with the WT allele yielding 783 and 217 bp bands, and the mutant allele yielding 462, 321, and 217 bp bands. 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.

Measuring plasma triglycerides

Blood was collected from 3.5- to 4-month-old mice by retroorbital bleeding. Triglycerides were measured in plasma samples with the triglyceride determination kit (Sigma Aldrich) according to the manufacturer’s instructions.

Testing the ability of LPL to bind to GPIHBP1-C63Y by immunocytochemistry

The ability of several GPIHBP1 constructs to bind LPL was tested with a “co-plating assay” (17). CHO pgsA-745 cells were electroporated with either 0.5 μg of a plasmid for S-protein–tagged versions of mouse WT GPIHBP1, GPIHBP1-W108S, or GPIHBP1-C63Y. These cells were then mixed with CHO pgsA-745 cells (5 × 10⁵) that had been independently electroporated with 0.5 μg of a plasmid for V5-tagged mouse LPL and plated on coverslips in 24-well plates. After 24 h, cells were fixed with 3% paraformaldehyde for 15 min and processed for immunocytochemistry. Cells were permeabilized with 0.2% Triton X-100 and blocked with 10% donkey serum in PBS/Ca/Mg. Cells were then incubated overnight at 4°C with a mouse monoclonal antibody (mAb) against the V5 tag (ThermoFisher Scientific; 1:100) and a goat polyclonal antibody against the S-protein tag (Abcam; 1:800), followed by a 30 min incubation with an Alexa656-conjugated donkey anti-goat IgG (ThermoFisher Scientific; 1:800) and an Alexa488-conjugated donkey anti-mouse IgG (ThermoFisher Scientific; 1:800). After washing, the cells were fixed with 3% paraformaldehyde for 15 min and stained with 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.

Testing the ability of LPL to bind to GPIHBP1-C63Y by Western blotting

CHO pgsA-745 cells (2 × 10⁶) were electroporated with 2 μg S-protein–tagged versions of the WT or mutant GPIHBP1 vectors (GPIHBP1-W108S, GPIHBP1-C63Y). After 24 h, cells were incubated with V5-tagged human LPL for 1 h at 4°C (22). After washing cells twice with PBS, cell extracts were prepared using M-PER mammalian protein extraction reagent (ThermoFisher Scientific) with Complete EDTA-free protease inhibitor cocktail (Roche). Extracts were size-fractionated on 12% NuPAGE SDS-PAGE gels with MES buffer, followed by transfer to a nitrocellulose membrane. The membranes were blocked for 1 h at room temperature with Odyssey blocking buffer (LI-COR). The amount of LPL bound to the cells was assessed by Western blotting with an antibody against the V5 tag. The amount of GPIHBP1 in cell extracts was assessed with an antibody against the S-protein tag. Actin was used as a loading control.

indicates extracts of nontransfected cells; the lanes labeled WT, W108S, and C63Y show extracts of cells that had been transfected with expression vectors for WT mouse GPIHBP1, GPIHBP1-W108S, or GPIHBP1-C63Y, respectively. Actin was used as a loading control.
Immunohistochemistry on mouse tissues

Mice were anesthetized with isoflurane and perfused with PBS followed by 3% paraformaldehyde. The brown adipose tissue (BAT), heart, and quadriceps were then harvested and embedded in OCT medium on dry ice. Tissue sections (7 μm for heart and quadriceps; 10 μm for BAT) 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 and 0.2% BSA in PBS/Mg/Ca. Tissues were incubated overnight at 4°C with a goat polyclonal antibody against mouse LPL (12 μg/ml) (23) 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 μg/ml), Alexa568-conjugated donkey anti–goat IgG (ThermoFisher Scientific; 1:200), and Alexa488-conjugated donkey anti–rabbit IgG (ThermoFisher Scientific; 1:200). After washing, the tissues were fixed with 3% paraformaldehyde for 5 min and stained with 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.

Quantifying Lpl and Gpihbp1 transcripts

Mice were anesthetized with isoflurane and perfused with PBS. The BAT and heart were harvested and flash-frozen in Fig. 4. LPL is mislocalized within the interstitial spaces in Gpihbp1<sup>C63Y/C63Y</sup> and Gpihbp1<sup>−/−</sup> mice. Immunohistochemistry studies were performed on sections of BAT (A); heart (B); and quadriceps (C). Sections were stained with antibodies for CD31 (cyan), LPL (green), and GPIHBP1 (red). DNA was stained with DAPI (blue). Scale bar, 50 μm.
**Western blot analysis of mouse tissue homogenates**

Mice were anesthetized with isoflurane and perfused with PBS. The BAT, heart, lung, and gonadal white adipose tissue (WAT) were harvested. Tissues were homogenized on ice for 12–15 s in 50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 2.5 mg/ml deoxycholic acid, 0.1% SDS, and Complete EDTA-free protease inhibitor (Roche). Samples were then centrifuged at 15,000 g for 15 min, and the supernatant fluid was collected. Proteins (40 μg/lane) were size-fractioned by SDS-PAGE, followed by Western blotting with an IRDye680-conjugated mAb against mouse GPIHBP1 (11A12, 3 μg/ml) and a rabbit polyclonal antibody against β-actin (Novus Biologicals; 1:1,000) (followed by an IRDye800-conjugated donkey anti-rabbit IgG from LI-COR). Signals were visualized with an Odyssey infrared scanner (LI-COR).

**Measuring GPIHBP1 on the capillary lumen**

Mice were anesthetized with ketamine/xylazine and given an intravenous injection of 30 μg IRDye680-conjugated antibody 11A12 in 100 μl saline into the inferior vena cava. After 2.5 min, mice were perfused with PBS followed by 3% paraformaldehyde. The BAT, heart, quadriceps, lung, and liver were then harvested and embedded in OCT medium on dry ice. Tissue sections (10 μm for BAT, heart, lung, and liver; 20 μm for quadriceps) were visualized with an Odyssey scanner (LI-COR). The IRDye680-conjugated 11A12 signal was quantified in each tissue sample and normalized to tissue area (using Image J software).

**Assessing GPIHBP1 monomers and multimers in GPIHBP1-transfected CHO cells**

CHO pgsA-745 cells (2 × 10^6) were electroporated with 2 μg of a plasmid for S-protein–tagged versions of mouse WT GPIHBP1, GPIHBP1-W108S, or GPIHBP1-C63Y and plated in 24-well plates. After 24 h, cells were treated with phosphatidylinositol-specific phospholipase C (PIPLC; 10 U/ml) in PBS, or PBS alone, for 20 min at 37°C to release GPI-anchored proteins. The PIPLC-released proteins, along with proteins from cell lysates, were analyzed by Western blotting under reducing and nonreducing conditions with antibody 11A12 (3 μg/ml) and a goat polyclonal antibody against the S-protein tag (Abcam; 1:1,000).

**Assessing GPIHBP1 monomers and multimers in the heart**

To investigate the presence of GPIHBP1 multimers in vivo, mice were anesthetized with isoflurane and perfused with Tyrode’s solution (10 mM HEPES pH 7.4, 136 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 1 mM MgCl₂) containing 10 mM glucose and 1 mM CaCl₂. The heart was then cannulated through the aorta with a blunt 25 gauge needle and perfused with 2 ml of Tyrode’s solution. Hearts were then perfused with 800 μl of antibody 11A12 (50 μg/ml) in Tyrode’s solution (or 800 μl Tyrode’s solution alone) and incubated for 5 min at room temperature. The heart was then perfused with 5 ml of Tyrode’s solution. Next, the heart was perfused with 1 ml of Tyrode’s solution containing 0.2% Triton X-100. The Triton X-100 perfusate was then incubated with 25 μl of Protein G–agarose beads (Thermo-Fisher Scientific) for 1 h at 4°C. The beads were washed with Tyrode’s solution containing 0.2% Triton X-100 and eluted with SDS sample buffer in the absence of reducing agents (10 min at 90°C). Samples were size-fractionated under nonreducing conditions and processed for Western blotting with an IRDye680-conjugated 11A12 (3 μg/ml) and a goat polyclonal antibody against mouse LPL (10 μg/ml) (23), followed by an IRDye800-conjugated donkey anti-goat IgG (LI-COR).

**RESULTS**

Mice harboring a cysteine mutation in *Gpihbp1* were generated with CRISPR/Cas9 techniques. DNA sequencing confirmed the presence of the p.C63Y mutation in exon 3 and an absence of any other coding sequence mutations. Mice heterozygous for the mutation (*Gpihbp1<sup>C63Y/+</sup>*) did not have elevated plasma triglyceride levels on a chow diet, whereas homozygous mice (*Gpihbp1<sup>C63Y/C63Y</sup>*) had plasma triglyceride levels >7,000 mg/dl—higher than those in the *Gpihbp1<sup>+/−</sup>* mice within the same colony (Fig. 1).

**ELISA to measure GPIHBP1 in mouse plasma**

ELISA plates were coated with 0.5 μg mAb 11A12 (a rat mAb against the carboxyl terminus of mouse GPIHBP1) and incubated overnight at 4°C. The next day, plates were blocked for 4 h at room temperature in Starting block (Pierce). Serial dilutions (1:1, 1:2, 1:4) of frozen mouse plasma samples were added to the wells (40 μl/well) and incubated overnight at 4°C. A standard curve with recombinant mouse GPIHBP1 run in parallel (0–800 pg/well). After washing, the amount of GPIHBP1 captured by antibody 11A12 was tested by adding 50 ng HRP-labeled mAb 2A8 (a rat mAb against the N terminus of mouse GPIHBP1) to each well and incubating for 2 h at 4°C. After washing, the plate was incubated on ice for 15 min with TMB substrate (100 μl/well). The reaction was stopped with 2 M sulfuric acid (100 μL/well), and the optical density was read at 450 nm.

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**Fig. 5.** GPIHBP1 protein levels are reduced in tissues of *Gpihbp1<sup>C63Y/C63Y</sup>* mice. Proteins (40 μg) from homogenates of heart (A), BAT (B), lung (C), and gonadal WAT (D) were size-fractionated by SDS-PAGE, and Western blots were performed with the GPIHBP1-specific mAb 11A12 (red) and a polyclonal antibody against β-actin (green).
The Gpihbp1<sup>C63Y</sup> mutation had little or no effect on Gpihbp1 expression; the levels of Gpihbp1 transcripts in BAT and heart were similar in WT, Gpihbp1<sup>C63Y/+</sup>, and Gpihbp1<sup>C63Y/C63Y</sup> mice (Fig. 2A). Lpl transcript levels were also similar (Fig. 2B).

The finding of severe chylomicronemia in Gpihbp1<sup>C63Y/C63Y</sup> mice suggested that the mutant GPIHBP1 would not be able to bind LPL. Indeed, mouse GPIHBP1-C63Y had no ability to bind LPL in cell-based LPL–GPIHBP1 binding assays (Fig. 3), and the LPL in BAT was mislocalized within the interstitial spaces—indistinguishable from findings in Gpihbp1<sup>-/-</sup> mice (Fig. 4A). The mislocalization of LPL in Gpihbp1<sup>C63Y/C63Y</sup> mice was equally apparent in heart and quadriceps (Fig. 4B, C). In these studies, the mutant GPIHBP1 was easily detectable in capillary endothelial cells (Fig. 4A–C).

In some of our immunohistochemistry studies, GPIHBP1 staining was less intense in Gpihbp1<sup>C63Y/C63Y</sup> mice than in WT mice, but this finding was inconsistent (Fig. 4). However, Western blots of protein extracts from mouse tissue homogenates unequivocally demonstrated that the amounts of GPIHBP1 protein were lower in Gpihbp1<sup>C63Y/C63Y</sup> mice than in WT mice (Fig. 5). Quantification of the Western blot signals revealed that the amounts of GPIHBP1 in the heart, BAT, lung, and gonadal WAT of Gpihbp1<sup>C63Y/C63Y</sup> mice were reduced by 72.4, 77.0, 73.8, and 65.8%, respectively.

The Western blot studies on tissue homogenates obviously could not provide insights into the location of GPIHBP1–C63Y expression (e.g., whether it was on the plasma membrane of endothelial cells or trapped within secretory organelles). For that reason, we used recently validated immunohistochemical methods (21) to determine whether the GPIHBP1 in Gpihbp1<sup>C63Y/C63Y</sup> mice was actually present on the luminal surface of capillaries. We injected mice intravenously with an IRDye-labeled mAb against the carboxyl terminus of GPIHBP1 (11A12) and then quantified antibody 11A12 binding to capillaries in tissue sections. The binding of antibody 11A12 to tissues was easily detectable in Gpihbp1<sup>C63Y/C63Y</sup> mice—demonstrating that the GPIHBP1-C63Y is present on the luminal surface of endothelial cells; however, the amount of antibody binding was lower in Gpihbp1<sup>C63Y/C63Y</sup> mice than in WT mice. Compared with littermate WT mice, amounts of mAb 11A12 binding to heart, BAT, lung, quadriceps, and liver of Gpihbp1<sup>C63Y/C63Y</sup> mice were reduced by 79.7, 61.2, 83.3, 70.1, and 39.6% (Fig. 6). Immunohistochemistry studies confirmed that GPIHBP1-C63Y was found on the luminal surface of capillaries (supplemental Fig. S1A, B).

![Fig. 6.](https://example.com/fig6.png) Levels of GPIHBP1 in the capillary lumen are substantially reduced in tissues of Gpihbp1<sup>C63Y/C63Y</sup> mice. Gpihbp1<sup>C63Y/C63Y</sup> (n = 4), Gpihbp1<sup>C63Y/-</sup> (n = 5), and WT (n = 4) mice, along with littermate Gpihbp1<sup>-/-</sup> (n = 3) and Gpihbp1<sup>-/-</sup> mice (n = 4) (3.5–4 months old), were injected intravenously with IRDye680-labeled 11A12 (30 µg). After 2.5 min, the mice were perfused, and tissues were harvested. Tissue sections from heart (A), BAT (B), lung (C), quadriceps (D), and liver (E) were prepared, and the intensity of the IRDye680 signal was quantified with an Odyssey infrared scanner. The IRDye680 signal (i.e., from labeled mAb 11A12 in the lumen of capillaries) was normalized to tissue area, and the signal in WT mice was set at 100%. GPIHBP1 levels in Gpihbp1<sup>C63Y/C63Y</sup> mice were substantially lower than in WT mice (*P < 0.0001 for heart, BAT, lung, and quadriceps; *P = 0.0055 for liver).
We recently used a pair of human GPIHBP1–specific mAbs to create a sandwich ELISA to measure levels of GPIHBP1 in human plasma (27). Why a GPI-anchored protein such as GPIHBP1 is found in plasma is not known, but we suspect that it is secreted from endothelial cells without a GPI anchor. Here, we took advantage of a pair of mouse-specific Gpihbp1 mAbs (11A12 and 2A8) to create a sandwich ELISA for measuring levels of GPIHBP1 in mouse plasma. GPIHBP1 was easily detectable in the plasma of WT and Gpihbp1<sup>C63Y/C63Y</sup> mice, but the levels were somewhat lower in Gpihbp1<sup>+/−</sup> mice. As expected, GPIHBP1 was undetectable in plasma samples from Gpihbp1<sup>−/−</sup> mice (Fig. 7).

In earlier studies, Beigneux et al. (15) showed, using CHO cell studies, that cysteine mutations in human GPIHBP1 interfere with proper disulfide bond formation and promote the formation of intermolecular disulfide bonds, such that a substantial amount of GPIHBP1 on the surface of cells was in the form of dimers and multimers (15). They also found that substituting Ser for a conserved Trp at residue 109 (Trp-108 in mouse GPIHBP1) resulted in lower-than-normal amounts of GPIHBP1 dimers and multimers. We observed similar findings in mouse GPIHBP1 (Fig. 8A). To determine whether GPIHBP1 dimers were present in capillaries of Gpihbp1<sup>C63Y/C63Y</sup> mice, we perfused mouse hearts with mAb 11A12, followed by Tyrode’s solution. We then released GPIHBP1 from endothelial cells by perfusing the heart with 0.2% Triton X-100. Antibody 11A12 in the perfusate was then immunoprecipitated with Protein G–agarose beads. The immunoprecipitates were size-fractionated by SDS-PAGE under nonreducing conditions, and Western blots were performed with IRDye680-labeled mAb 11A12. Consistent with our findings of reduced amounts of GPIHBP1 in Gpihbp1<sup>C63Y/C63Y</sup> tissues (Figs. 5, 6), the amount of GPIHBP1 in the immunoprecipitates from Gpihbp1<sup>C63Y/C63Y</sup> hearts was lower than in WT mouse hearts (Fig. 8B). In WT hearts, virtually all of the GPIHBP1 was in the form of monomers (Fig. 8B). In Gpihbp1<sup>C63Y/C63Y</sup> mice, the vast majority of the GPIHBP1 was monomeric, but trace amounts of dimers were detected (Fig. 8B). We repeated these studies twice (supplemental Fig. S2). In each experiment, the amount of GPIHBP1 in the immunoprecipitate was reduced in Gpihbp1<sup>C63Y/C63Y</sup> hearts. In immunoprecipitates from WT hearts, LPL was detectable along with the GPIHBP1 (reflecting GPIHBP1-bound LPL). Only trace amounts of LPL could be detected in immunoprecipitates from Gpihbp1<sup>C63Y/C63Y</sup> hearts and LPL was present regardless of whether the heart had been perfused with mAb 11A12 (supplemental Fig. S2).

**DISCUSSION**

Many GPIHBP1 missense mutations have been identified in patients with chylomicronemia, and most involve conserved cysteines in the Ly6 domain or adjacent residues (6–11, 14). Patients who are homozygous for these missense mutations have reduced amounts of LPL in the plasma (9–11), likely reflecting reduced amounts of LPL in capillaries, but our understanding of how GPIHBP1 missense mutations cause disease is incomplete. In human subjects, it is not possible to determine whether missense mutations prevent GPIHBP1 from reaching the surface of capillary endothelial cells. Also, no one has determined whether these mutations, which leave GPIHBP1’s acidic domain intact, might retain partial function and therefore be associated with less severe disease. To address these issues, we created mice harboring a cysteine-to-tyrosine substitution (p.C63Y) in GPIHBP1. Gpihbp1<sup>C63Y/C63Y</sup> mice manifested chylomicronemia, and the LPL in tissues was mislocalized to the interstitial spaces, where it is useless for hydrolyzing triglycerides in the bloodstream. The p.C63Y mutation did not reduce levels of Gpihbp1 transcripts but nonetheless reduced, by ∼70%, amounts of GPIHBP1 protein on capillary endothelial cells. Finding reduced levels of GPIHBP1-C63Y on the plasma membrane of endothelial cells contrasted with virtually normal amounts of mutant GPIHBP1 proteins on the surface of CHO cells (16), but these findings were not altogether surprising, given that mutations of cysteines in CD59 and the LDLR virtually abolished trafficking of those proteins to the cell surface (19, 20, 28, 29). In the case of a cysteine mutation...
in LDLR, pulse-chase studies in fibroblasts revealed defective trafficking of the mutant LDLR from the ER to the Golgi (29). We suspect that impaired protein trafficking accounts for reduced amounts of GPIHBP1-C63Y on endothelial cells of Gpihbp1 C63Y/C63Y mice. It would obviously be desirable to explore this suspicion with pulse-chase studies in capillary endothelial cells from mice, but these experiments would be next to impossible because Gpihbp1 expression disappears very rapidly after isolating primary microvascular endothelial cells (30).

While the amount of GPIHBP1 on capillary endothelial cells was reduced in Gpihbp1 C63Y/C63Y mice, we doubt that this finding was responsible for the mislocalization of LPL and the chylomicronemia. Instead, the inability of GPIHBP1-C63Y to bind LPL was almost certainly the primary cause of disease. In CHO cells, substantial amounts of GPIHBP1-C63Y monomers reached the cell surface, but there was little or no LPL binding. Moreover, large amounts of GPIHBP1-C63Y monomers, but little LPL, were released from hearts of Gpihbp1 C63Y/C63Y mice during a short perfusion with 0.2% Triton X-100.

GPIHBP1’s Ly6 domain is largely responsible for LPL binding, but the acidic domain contributes to the avidity of LPL–GPIHBP1 interactions (3). GPIHBP1’s acidic domain was unaltered by the p.C63Y mutation, but the retention of the acidic domain was not sufficient to permit LPL binding in the CHO cell experiments—or to lessen the severity of the chylomicronemia in mice. The plasma triglyceride levels were clearly not lower in Gpihbp1 C63Y/C63Y mice than in Gpihbp1−/− mice, where all GPIHBP1 coding sequences were absent (2).

While developing Gpihbp1 C63Y/C63Y mice, we simultaneously created mice in which all of the acidic residues in GPIHBP1’s acidic domain (encoded by exon 2) were replaced with alanine. Our goal was to use these “acidic domain mutant mice” to examine the in vivo functional relevance of the acidic domain. We were delighted that the numerous nucleotide substitutions in exon 2 did not perturb mRNA splicing, but we were disappointed to find that the Gpihbp1 transcript levels in the mutant mice were reduced by >98%. Not surprisingly, the acidic domain mutant mice manifested severe chylomicronemia. We do not understand why the acidic domain mutations extinguished Gpihbp1 expression, but it seems possible that exon 2 sequences could serve as a transcriptional enhancer. Others have found evidence for enhancers within protein-coding sequences (31). Alternatively, Gpihbp1 transcripts lacking exon 2 sequences could be unstable and quickly degraded.

In our studies, we were able to detect GPIHBP1 in mouse plasma with a mAb–based sandwich ELISA. In earlier studies (27), an ELISA detected GPIHBP1 in human plasma. The explanation for the presence of GPIHBP1 in plasma is unknown, but we suspect that small amounts of “soluble GPIHBP1” (GPIHBP1 lacking a GPI anchor) are secreted by capillary endothelial cells. In GPIHBP1-expressing CHO cells, where there is an imbalance between the production of GPIHBP1 and GPI anchors, large amounts of soluble GPIHBP1 are secreted from cells. We suspect that this
same phenomenon occurs, to a lesser degree, in capillary endothelial cells of mice. The amount of GPIHBP1 in tissues of Gpihbp1<sup>C63Y/C63Y</sup> mice was only slightly lower than in WT mice, whereas the amount of GPIHBP1 in tissues of Gpihbp1<sup>C63Y/G65Y</sup> mice was reduced by ~70%. We do not fully understand this discrepancy, but it is possible that soluble GPIHBP1-C63Y is more capable of avoiding the quality-control surveillance mechanisms in the ER. In support of this idea, eliminating the N-linked glycosylation site in a full-length GPIHBP1 markedly reduced GPIHBP1 trafficking to the surface of CHO cells (18), whereas the glycosylation site mutation had minimal effects on the secretion of soluble GPIHBP1 (a GPIHBP1 that was truncated before the GPI-anchoring site) (18).

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