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

Anne P. Beigneux, Loren G. Fong, André Bensadoun, Brandon S.J. Davies, Monika Oberer, Henrik Gårdsvoll, Michael Ploug, Stephen G. Young

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 patients with chylomicronemia) 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 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 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. (Circ Res. 2015;116:624-632. DOI: 10.1161/CIRCRESAHA.116.305085.)

Key Words: chylomicrons ■ endothelial cells ■ GPIHBP1 protein ■ hypertriglyceridemia ■ lipoprotein lipase ■ protein multimerization ■ triglycerides
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. 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. Interestingly, many of the mutations causing chylomicronemia in humans involve a cysteine residue (eg, C65S, C65Y, C68G, C68Y, and C89F). 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. 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, introduces a proline adjacent to a cysteine in the Ly6 domain.

Homology Modeling of GPIHBP1

The homology model of human GPIHBP1 was created with the program Modeller. The model of human GPIHBP1 amino acids 21 to 136, followed by mouse GPIHBP1 amino acids 136 to 198, was built with the program PyMOL Molecular Graphics System, Version 1.5.0.4.

Methods

GPIHBP1 Constructs

Human GPIHBP1 mammalian expression vectors containing an amino-terminal S-protein tag have been described previously. 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 to 136, followed by mouse GPIHBP1 amino acids 136 to 198, into pcDNA3-His (Life Technologies). All mutations were introduced by site-directed mutagenesis with the QuickChange Lightning kit (Stratagene).

Treatment of Chinese Hamster Ovary Cells With Phosphatidylinositol-Specific Phospholipase C

Human umbilical vein endothelial cells (HUVECs) or Chinese hamster ovary (CHO)-K1 cells were electroporated with human GPIHBP1 expression vectors with the Nucleofector II apparatus (Lonza). After 24 hours, the GPIHBP1 was released into the culture medium by treating the cells with phosphatidylinositol-specific phospholipase C (PIPLC: 10 U/mL for 20 minutes at 37°C). In some experiments, we used rat heart microvascular endothelial cells that had been transfected with a mouse GPIHBP1 lentivirus. The rat heart microvascular endothelial cells were treated with PIPLC when they reached 90% confluency. 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 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. 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 CuSO4 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 and an IRdye800-conjugated monoclonal antibody against the uPAR tag (R24). 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 along with agarose beads coated either with antibody 11A12 or the LPL-specific antibody 5D2. 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-fractionated on 12% Bis-Tris SDS-polyacrylamide gels and subsequently transferred to nitrocellulose. For antibody dilutions, see the Materials in the Online Data Supplement.

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 and uPAR 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. We found that substantial amounts of mutant GPIHBP1 protein can be released from the surface of CHO cells with PIPLC (Figure 1A, middle), 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). In the case of the cysteine mutants, the intensity of the monomer band was reduced, whereas that of dimers and multimers increased (Figure 1A, top). When the intensity of GPIHBP1 monomers was compared with the total GPIHBP1 signal (ie, the signal from all 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 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 rat heart microvascular endothelial cells 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. In this system, the GPIHBP1 protein 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 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% to 90% lower than with wild-type GPIHBP1 (Figure 1B and 1C).

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 and 1C).

The Q115P mutation in GPIHBP1 impairs the ability of GPIHBP1 to bind LPL, but changing Q115 to Lys (the residue found in canine GPIHBP1) has little or no impact on LPL binding. 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...
Table 1. Ratio of GPIHBP1 Monomers to Total GPIHBP1 (Monomers, Dimers, and Multimers) for Different GPIHBP1 Mutants, Expressed as a Percentage of the Ratio With Wild-Type GPIHBP1 (Set at 100%)

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Ratio of Monomer to Total GPIHBP1 (% of Wild-Type GPIHBP1)</th>
<th>Mab 11A12</th>
<th>Mab R24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y66A</td>
<td>44.3±4.3 (n=3)</td>
<td>52.7±7.5</td>
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</tr>
<tr>
<td>L71A</td>
<td>43.2±6.1 (n=3)</td>
<td>60.5±16.2</td>
<td>(n=3)</td>
</tr>
<tr>
<td>T91A</td>
<td>68.4±9.7 (n=3)</td>
<td>62.4±15.8</td>
<td>(n=3)</td>
</tr>
<tr>
<td>L92A</td>
<td>114±4.9 (n=3)</td>
<td>96.4±12.5</td>
<td>(n=3)</td>
</tr>
<tr>
<td>I93A</td>
<td>60.6±3.9 (n=3)</td>
<td>58.9±12.0</td>
<td>(n=3)</td>
</tr>
<tr>
<td>G101S</td>
<td>72.2±2.1 (n=3)</td>
<td>73.1±7.0</td>
<td>(n=3)</td>
</tr>
<tr>
<td>T104A</td>
<td>68.4±1.9 (n=3)</td>
<td>81.2±7.9</td>
<td>(n=3)</td>
</tr>
<tr>
<td>T105A</td>
<td>45.0±2.7 (n=3)</td>
<td>57.7±12.3</td>
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<tr>
<td>H106L</td>
<td>38.6±3.0 (n=3)</td>
<td>58.6±11.0</td>
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<tr>
<td>S107C</td>
<td>4.84 ± 0.82 (n=3)</td>
<td>14.9±9.6</td>
<td>(n=3)</td>
</tr>
<tr>
<td>T108A</td>
<td>144±4.9 (n=3)</td>
<td>111±7.7</td>
<td>(n=3)</td>
</tr>
<tr>
<td>T108R</td>
<td>70.4±1.9 (n=4)</td>
<td>80.6±5.4</td>
<td>(n=3)</td>
</tr>
<tr>
<td>W109A</td>
<td>138±7.1 (n=5)</td>
<td>139±3.4</td>
<td>(n=5)</td>
</tr>
<tr>
<td>W109S</td>
<td>155±3.7 (n=7)</td>
<td>139±7.3</td>
<td>(n=7)</td>
</tr>
<tr>
<td>Q115K</td>
<td>99.6±6.2 (n=3)</td>
<td>102±2.5</td>
<td>(n=2)</td>
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<tr>
<td>Q115P</td>
<td>27.5±4.1 (n=4)</td>
<td>42.3±10.6</td>
<td>(n=3)</td>
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<tr>
<td>V126A</td>
<td>34.9±2.0 (n=3)</td>
<td>46.3±7.7</td>
<td>(n=3)</td>
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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 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, 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 hour 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 was 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).

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 were incubated with V5-tagged human LPL and either wild-type GPIHBP1 or GPIHBP1-C68Y. After washing the beads, the LPL that had been captured by antibody 5D2 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 anti-GPIHBP1 (ie, the GPIHBP1 and LPL), unbound fraction, wash fraction, and elution fraction were assessed by Western blotting. LPL was 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 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 whether 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, and 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 versus 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 whether 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 non-reducing 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). 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 >50% when compared with 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 hour, 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–5C). Small amounts of LPL binding were observed with G101S, T104A, and T108A mutants, in agreement with previous findings17 (Figure 5A–5C).

Discussion

Previous 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.1–5 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 3-fingered motif—or whether the mutations interfere with LPL binding in a more direct fashion. In the present study, we investigated that topic and uncovered 3 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 patients with chylomicronemia,3,18 led to nearly as many dimers/multimers as the cysteine mutants. Mutations in other residues found to be important for LPL binding (eg, Tyr66, Leu71, Thr91, Ile93, Gly101, Thr104, Thr105, His106, and Val112)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 noncysteine 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 (eg, 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

Table 2. Ratio of Monomers to Total GPIHBP1 (Monomers, Dimers, and Multimers) With GPIHBP1-W109 Mutants,Expressed as Percentage of the Ratio With GPIHBP1-W109S (Set at 100%)

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

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).
interfaces often disrupt protein–protein interactions.\textsuperscript{27} Also, when structures of protein–protein complexes are examined, tryptophans are the most overrepresented residue in the core of binding interfaces.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 present 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 et al. 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 have been identified in freshly isolated venom from the Naja kaouthia cobra. α-Cobratoxin is a cysteine-rich neurotoxin in the same protein family as mammalian Ly6 proteins; α-cobratoxin has 10 cysteines, all arranged in the same pattern as in GPIHBP1 and CD59 and all are disulfide-bonded. Interestingly, the α-cobratoxin–α-cobratoxin 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 patients with chylomicronemia) increase the propensity of GPIHBP1 to form disulfide-linked dimers and multimers. Mutations in other residues in the Ly6 domain, including several identified in patients with chylomicronemia (eg, Q115P and T108R), also promote the formation of dimers-multimers. This discovery is relevant to pathogenesis because LPL binds preferentially to GPIHBP1 monomers. We identified 1 mutant, W109S, which 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.

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Disclosures

None.

References


GPIHP1, 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 GPIHP1 missense mutations abolish LPL binding and result in chylomicronemia. Most GPIHP1 missense mutations involve residues in GPIHP1′s 3-fingered motif. Most of these mutations interfere with the folding of GPIHP1 and result in the appearance of GPIHP1 dimers and multimers at the cell surface. Reduced amounts of GPIHP1 monomers at the cell surface are highly relevant to the pathogenesis of chylomicronemia because only GPIHP1 monomers—and not dimers or multimers—bind LPL. We identified 1 mutant, GPIHP1-W109S, with distinctive properties. GPIHP1-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 GPIHP1′-LPL interface. In summary, our studies show that GPIHP1 dimerization/multimerization represents an important mechanism by which many amino acid substitutions in GPIHP1 abolish LPL binding and cause chylomicronemia.

**Novelty and Significance**

**What Is Known?**

- GPIHP1, 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 (very low-density lipoprotein and chylomicrons).
- GPIHP1 and LPL missense mutations that disrupt GPIHP1′-LPL interactions abolish LPL transport to the capillary lumen and cause familial chylomicronemia.
- Most GPIHP1-related cases of chylomicronemia involve amino acid substitutions in GPIHP1′s 3-fingered domain that abolish LPL binding.

**What New Information Does This Article Contribute?**

- Most of the GPIHP1 missense mutations causing chylomicronemia interfere with the folding of GPIHP1′s 3-finger motif and result in the production of disulfide-linked GPIHP1 dimers and multimers.
- LPL binds to monomeric GPIHP1 but not to GPIHP1 dimers/multimers.
- Enhanced formation of dimers/multimers by mutant forms of GPIHP1 represents an important mechanism for defective LPL binding and chylomicronemia.
GPIHBP1 Missense Mutations Often Cause Multimerization of GPIHBP1 and Thereby Prevent Lipoprotein Lipase Binding
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http://circres.ahajournals.org/content/suppl/2014/11/11/CIRCRESAHA.116.305085.DC1
Detailed Online Methods

GPIHBP1 Constructs

Human GPIHBP1 expression vectors containing an amino-terminal S-protein tag have been described previously. Additional vectors containing GPIHBP1 mutations were created by site-directed mutagenesis with the QuikChange Lightning kit (Stratagene).

For transient expression of GPIHBP1 in insect cells, we used an established system for expressing Ly6 fusion proteins. We cloned a cDNA encoding human uPAR domain III (uPAR-DIII), an enterokinase cleavage site, human GPIHBP1 amino acids 21–136, and mouse GPIHBP1 amino acids 136–198 into pMT/V5-His (Life Technologies) with the In-Fusion HD cloning kit (Clontech). The human GPIHBP1 sequences contained the entire Ly6 domain; the mouse GPIHBP1 sequences (immediately upstream from the GPI anchoring signal) encoded sequences for the GPIHBP1-specific rat monoclonal antibody 11A12. Mutant vectors were created with the QuikChange Lightning kit.

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

Human umbilical vein endothelial cells (HUVEC) or CHO-K1 cells (5 × 10^6) were electroporated with 5 µg of a human GPIHBP1 expression vector 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 (RHMVEC) that had been transduced with a mouse GPIHBP1 lentivirus. The RHMVEC 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 a goat polyclonal antibody against the S-protein tag (Abcam) (for human GPIHBP1) and rat monoclonal antibody 11A12 (for mouse
GPIHBP1).

**Cell-based Assays of LPL Binding to GPIHBP1**

GPIHBP1 expression vectors were electroporated into CHO-K1 cells. 24 h later, the cells were washed and incubated with V5-tagged human LPL ± heparin (250 U/ml) for 2 h at 4°C. At the end of the incubation period, cells were washed 6 times with ice-cold PBS containing 1 mmol/L MgCl₂ and 1 mmol/L CaCl₂ to remove unbound LPL, and cell lysates were prepared. To assess LPL binding to GPIHBP1, the amounts of GPIHBP1 and LPL in the cell extracts were assessed by western blotting with a goat polyclonal antibody against the S-protein tag (Abcam) and a mouse monoclonal against the V5 tag (Life Technologies), respectively.

**Expression of GPIHBP1 in Drosophila S2 cells**

12 × 10⁶ Drosophila S2 cells (Life Technologies) that had been adapted to suspension culture were transfected with 19 µg of plasmid DNA with the Calcium Phosphate Transfection kit (Life Technologies). 24 h later, the expression of the GPIHBP1 fusion protein was induced with Schneider’s medium (Sigma) containing 1% heat-inactivated fetal bovine serum (Life Technologies), 0.1% Pluronic F-68 (Sigma), and 500 µM CuSO₄. Protein production was induced for 3 days before harvesting the medium.

To characterize the GPIHBP1 produced by Drosophila S2 cells, the conditioned medium and cell extracts were size-fractionated by SDS-PAGE under reducing or nonreducing conditions. Western blots were performed with an IRdye680-conjugated GPIHBP1-specific monoclonal antibody (11A12) and an IRdye800-conjugated monoclonal antibody against the uPAR tag (R24). The intensity of the band corresponding to monomeric GPIHBP1 (~38 kDa) and the signal intensity for total GPIHBP1 (including monomers, dimers, and multimers) were quantified on an Odyssey infrared scanner (Li-Cor).
To produce soluble GPIHBP1 for cell-free GPIHBP1–LPL binding assays, the conditioned medium from GPIHBP1-transfected Drosophila S2 cells was concentrated 6-fold with an Amicon Ultra 10 MWCO filter (Millipore). Soluble GPIHBP1 was incubated for 1 h at 4°C with conditioned medium containing V5-tagged human LPL along with agarose beads coated either with antibody 11A12 or the LPL-specific antibody 5D2. After washing the beads, GPIHBP1–LPL complexes captured by the antibody-coated beads were released by heating the samples in SDS-loading buffer (10 min, 70°C). The amounts of GPIHBP1 and LPL in the starting material, the unbound (flow-through) fraction, the wash fraction, and the eluted proteins 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 then transferred to nitrocellulose. The antibody dilutions were: 1:1,000 for a goat polyclonal against the S-protein tag (Abcam); 1:200 for the V5 monoclonal antibody (Life Technologies); 1:500 for a rabbit polyclonal against β-actin (Abcam); 1:1,000 for the IRdye680–antibody 11A12; 1:1,000 for the IRdye800–antibody R24; 1:500 for an IRdye800–V5 antibody; 1:2,000 for an IRdye800–donkey anti-goat IgG (Li-Cor); 1:2,000 for an IRdye680–donkey anti-rabbit IgG (Li-Cor).

**Homology Modeling of GPIHBP1**

The homology model of human GPIHBP1 was created with the protein fold recognition server Phyre 2. The 3D structures of the water-soluble domain of human LYNX1 and uPAR were selected as templates. The calculated models were visualized using the PyMOL Molecular Graphics System, Version 1.5.0.4.
Online Figure I. Defining the properties of GPIHBP1 mutants in endothelial cells. 
A, Western blot analysis of mouse GPIHBP1 proteins released by phosphatidylinositol-specific phospholipase C (PIPLC) from the surface of RHMVECs that had been transduced with a mouse GPIHBP1 lentiviral expression vector. After reaching ~90% confluence, 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 a rat monoclonal antibody against mouse GPIHBP1 (11A12). GPIHBP1 monomers (~28 kDa) are indicated with an arrowhead.

B, Western blot analysis of S-protein–tagged GPIHBP1 proteins released from the surface of GPIHBP1-transfected HUVECs with 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. GPIHBP1 monomers (~28 kDa) are indicated with an arrowhead.