Glycosylation of Asn-76 in mouse GPIHBP1 is critical for its appearance on the cell surface and the binding of chylomicrons and lipoprotein lipase

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Abstract GPIHBP1 is a glycosylphosphatidylinositol-anchored protein in the lymphocyte antigen 6 (Ly-6) family that recently was identified as a platform for the lipolytic processing of triglyceride-rich lipoproteins. GPIHBP1 binds both LPL and chylomicrons and is expressed on the luminal face of microvascular endothelial cells. Here, we show that mouse GPIHBP1 is N-glycosylated at Asn-76 within the Ly-6 domain. Human GPIHBP1 is also glycosylated. The N-linked glycan could be released from mouse GPIHBP1 with N-glycosidase F, endoglycosidase H, or endoglycosidase F1. The glycan was marginally sensitive to endoglycosidase F2 digestion but resistant to endoglycosidase F3 digestion, suggesting that the glycan on GPIHBP1 is of the oligomannose type. Mutating the N-glycosylation site in mouse GPIHBP1 results in an accumulation of GPIHBP1 in the endoplasmic reticulum and a markedly reduced amount of the protein on the cell surface. Consistent with this finding, cells expressing a nonglycosylated GPIHBP1 lack the ability to bind LPL or chylomicrons. Eliminating the N-glycosylation site in a truncated soluble version of GPIHBP1 causes a modest reduction in the secretion of the protein. These studies demonstrate that N-glycosylation of GPIHBP1 is important for the trafficking of GPIHBP1 to the cell surface.—Beigneux, A. P., P. Gin, B. S. J. Davies, M. M. Weinstein, A. Bensadoun, R. O. Ryan, L. G. Fong, and S. G. Young. **Glycosylation of Asn-76 in mouse GPIHBP1 is critical for its appearance on the cell surface and the binding of chylomicrons and lipoprotein lipase.** ***J. Lipid Res.*** **2008.** 49: **1312–1321.**

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Glycosylphosphatidylinositol-anchored HDL binding protein 1 (GPIHBP1) is a cell-surface protein with a key role in the lipolytic processing of triglyceride-rich lipoproteins in the plasma (1). Multiple observations support the role of GPIHBP1 in lipolysis: *1*) a knockout of *Gpihbp1* in mice causes severe chylomicronemia; *2*) GPIHBP1 is located exclusively on the luminal surface of endothelial cells of heart, muscle, and adipose tissue, the sites where lipolysis occurs, but not on endothelial cells from other tissues where lipolytic processing of chylomicrons is negligible (e.g., brain); and *3*) transfection of a *Gpihbp1* expression vector into cultured cells confers the ability to bind both LPL and chylomicrons (1).

GPIHBP1 contains a signal peptide, followed by a highly negatively charged N-terminal domain (with 17 of 25 consecutive residues in the mouse sequence being aspartate or glutamate) (1–3). This acidic domain is followed by a short linker domain (14 amino acids) and then an Ly-6 domain containing 10 cysteines. After the Ly-6 motif, there is a hydrophobic carboxyl-terminal motif that triggers the addition of a glycosylphosphatidylinositol (GPI) anchor (1–3). GPIHBP1 is tethered to the surface of the plasma membrane by the GPI anchor, and the protein can be readily released by cleaving the GPI anchor with phosphatidylinositol-specific phospholipase C (PIPLC) (1–4).

In our initial cell culture studies (1), the GPIHBP1 in transfected CHO or HeLa cells did not migrate as a sharp band on SDS-polyacrylamide gels, raising the possibility that the protein was glycosylated. The proposition that GPIHBP1 could be glycosylated seemed plausible, particularly because other GPI-anchored proteins containing Ly-6 motifs, for example the urokinase-type plasminogen

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Abbreviations: DMPC, dimyristoylphosphatidylcholine; ER, endoplasmic reticulum; GPI, glycosylphosphatidylinositol; GPIHBP1, glycosylphosphatidylinositol-anchored HDL binding protein 1; PIPLC, phosphatidylinositol-specific phospholipase C; PNGase F, N-glycosidase F; sGPIHBP1, truncated soluble GPIHBP1; UPAR, urokinase-type plasminogen activator receptor.

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activator receptor (UPAR), are known to be N-glycosylated (5, 6). In the current study, we investigated whether GPIHBP1 is glycosylated and whether glycosylation affects the trafficking of the molecule to the cell surface.

MATERIALS AND METHODS

Cloning and expression of GPIHBP1

An IMAGE clone (ID #30298145) containing the complete open reading frame for mouse *Gpihbp1* in pDNR-LIB was purchased from Open Biosystems (Huntsville, AL). An *Nco*I site immediately upstream to the translational start site was created by site-directed mutagenesis with the QuikChange kit (Stratagene; La Jolla, CA), and an *Nco*I-*Hind*III fragment containing the complete open reading frame was cloned into the mammalian expression vector pTriEx-4 (Novagen; Madison, WI). A mouse *Gpihbp1* construct with an N-terminal S-protein tag was created by inserting the S-protein coding sequence as described (1). A putative N-glycosylation motif (76NQTQ) in mouse GPIHBP1 was changed to 76IQIS by site-directed mutagenesis with the QuikChange kit and oligonucleotides 5′-GCGGGGAGAGCTG-CATTCAGATCTCG AGCTGCTCCAGCAGC-3′ and 5′-GCTGCTG-GAGCAGCTCGAGATCTGAATGCAGCTC TCCCCGC-3′.

The putative N-linked glycosylation motif in mouse GPIHBP1 was also replaced with the corresponding sequences from other mammalian species. We inserted cow GPIHBP1 sequences

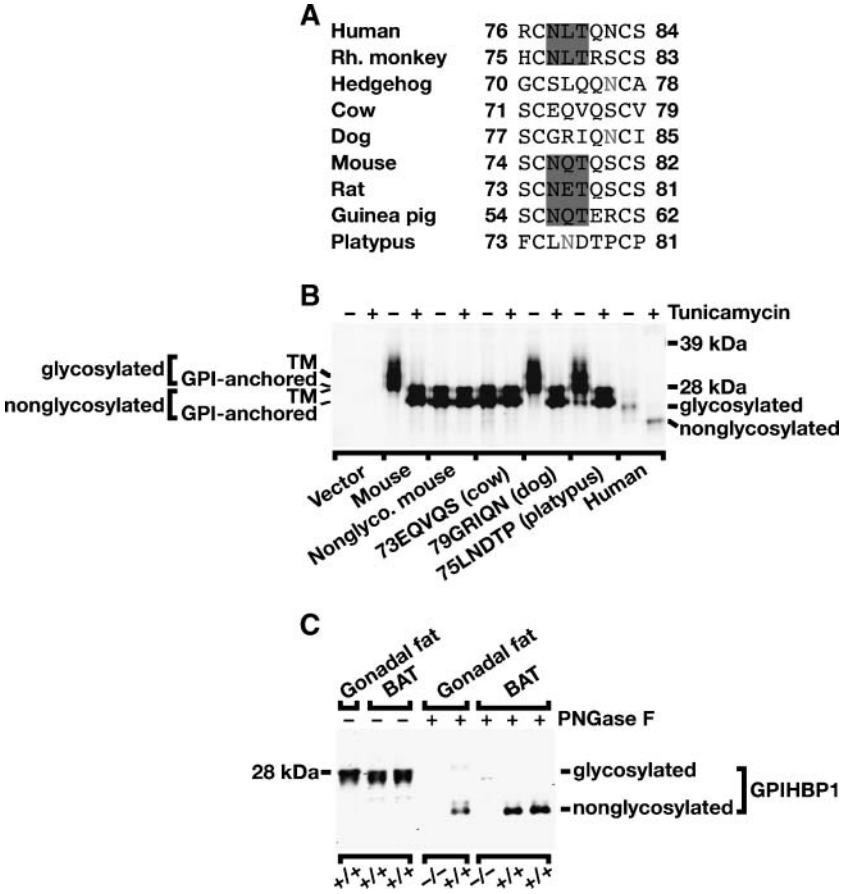

Fig. 1. Identification of an N-linked glycosylation site in glycosylphosphatidylinositol-anchored HDL binding protein 1 (GPIHBP1). A: Multiple amino acid sequence alignment showing a predicted N-linked glycosylation site in GPIHBP1 in nine mammalian species. The NetNglyc server (www.cbs.dtu.dk/services/NetNGlyc) predicts N-glycosylation sites by searching for a signal peptide sequence and an NxS/T consensus motif in the query (motif highlighted in gray in the human, monkey, mouse, rat, and guinea pig sequences). The NetNglyc server did not predict that the asparagines (gray font) in the corresponding region of hedgehog, dog, or platypus would be N-glycosylated. The bovine GPIHBP1 sequence did not contain an asparagine in this region, and the asparagine further downstream (92NTE) was not predicted to be glycosylated. B: Western blot analysis of transfected HeLa cells revealing that human and mouse GPIHBP1 are N-glycosylated. Transfected cells were incubated for 20 h with or without tunicamycin (2.5 μg/ml), and the electrophoretic mobility of GPIHBP1 was assessed by SDS-PAGE. The human and mouse GPIHBP1 cDNAs were inserted into different expression vectors (see Materials and Methods), explaining the lower levels of expression for human GPIHBP1. As expected, there was no N-glycosylation when the N-linked glycosylation motif in the mouse GPIHBP1 sequence was mutated or when the corresponding sequence from the cow was inserted into the mouse *Gpihbp1* expression vector. However, when the N-glycosylation site in the mouse *Gpihbp1* construct was replaced with the corresponding sequences from dog and platypus, the chimeric mutant proteins underwent N-glycosylation. C: Western blot analysis of adipose tissue extracts from wild-type ($^{+/+}$) or *Gpihbp1*-deficient ($^{-/-}$) mice after digestion with N-glycosidase F (PNGase F), revealing that GPIHBP1 is N-glycosylated in vivo. TM, nascent GPIHBP1 with transmembrane domain; Nonglyco., nonglycosylated.

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(73EQVQS) with oligonucleotides 5′-GCGGGGAGAGCTGCGA-ACAGGTACAGAG CTGCTCCAGC-3′ and 5′-GCTGGAGCAGC-TCTGTACCTGTTCGCAGCTCTCCCCGC-3′; dog GPIHBP1 sequences (79GRIQN) with oligonucleotides 5′-GCGGG GAG-AGCTGCGGTCGGATACAGAACTGCTCCAGCAGC-3′ and 5′-GCTGCTGGAG CAGTTCTGTATCCGACCGCAGCTCTCCCC-GC-3′; and platypus GPIHBP1 sequences (75LNDTP) with oligonucleotides 5′-GCGGGGAGAGCTGCCTTAACGACACG CC-GTGCTCCAGCAGCAAACCC-3′ and 5′-GGGTTTGCTGCTGGAG-CACGGCGTGTCGTT AAGGCAGCTCTCCCCGC-3′.

An expression vector for a truncated soluble version of mouse GPIHBP1 (sGPIHBP1) was generated by replacing the GPI anchor attachment recognition site (199SGA) with a stop codon. This mutant was created with the QuikChange kit and oligonucleotides 5′-GGCTAAC CAGCCCCAGTGATCAGGG-GCAGGATACCCTTCAGGC-3′ and 5′-GCCTGAAGGGTAT CCTGCCCCTGATCACTGGGGCTGGTTAGCC-3′.

We obtained a human *GPIHBP1* cDNA IMAGE clone (ID #5754421) from American Type Culture Collection (Manassas, VA) and cloned the open reading frame into pTriEx-4*neo* (Novagen), a mammalian expression vector containing a cytomegalovirus immediate-early promoter. An N-terminal S-protein tag was introduced with PCR-based cloning techniques (7).

Deglycosylation experiments

HeLa cells were transfected with *Gpihbp1* constructs or empty vector with Lipofectamine 2000 (Invitrogen; Carlsbad, CA). Four hours after the transfection, the cell culture medium was replaced with fresh medium containing 0, 0.5, 2.5, or 5 μg/ml of tunicamycin (Sigma; St. Louis, MO), and the cells were allowed to grow for 20 h before collecting the cell extracts in radioimmunoprecipitation assay buffer (RIPA: 1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing complete mini EDTA-free protease inhibitors (Roche; Indianapolis, IN).

Enzymatic deglycosylation by N-glycosidase F (PNGase F) or endoglycosidase H (both from Sigma) was performed on mouse tissue extracts homogenized in RIPA buffer containing complete mini EDTA-free protease inhibitors (Roche), or on HeLa cell extracts collected in the same buffer 24 h posttransfection. Briefly, the tissue or cell extracts were denatured at 100°C for 5 min, chilled on ice, and digested for 3 h with either PNGase F or endoglycosidase H according to the manufacturer's instructions.

Enzymatic deglycosylation by endoglycosidase F1, F2, or F3 (all from Sigma) was performed on HeLa cell extracts collected in 1× PBS containing complete mini EDTA-free protease inhibitors (Roche) 24 h after the transfection. The enzymatic digestions were performed under native conditions (no heat denaturation) according to the manufacturer's instructions.

PIPLC treatment of transfected cells

CHO pgsA-745 cells (cells with a defect in the sulfation of proteoglycans) (8) were transfected with *Gpihbp1* constructs or empty vector with Lipofectamine 2000 (Invitrogen). Twenty-four hours after the transfection, the culture medium was replaced

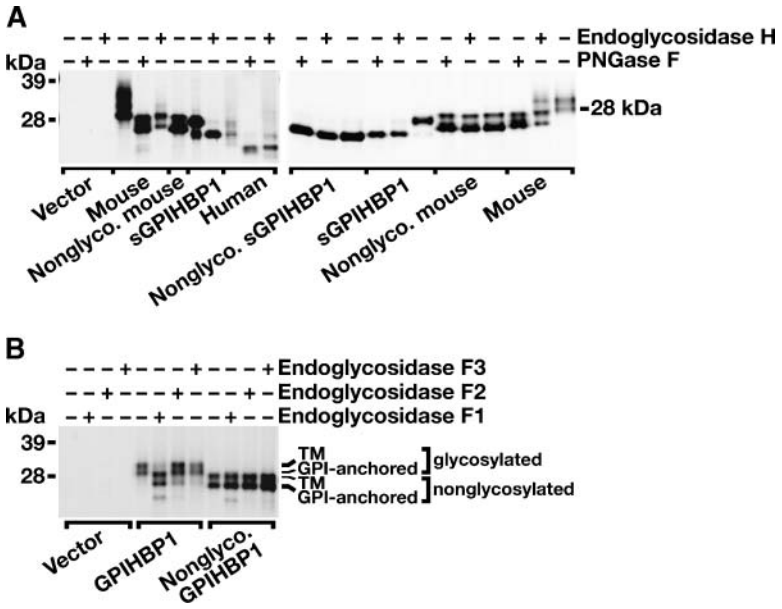

Fig. 2. The N-linked oligosaccharide in GPIHBP1 is sensitive to endoglycosidase H and F1 digestion. A: Western blot analyses showing that the N-linked oligosaccharide of GPIHBP1 is sensitive to endoglycosidase H digestion. HeLa cells were transfected with human or mouse wild-type GPIHBP1, nonglycosylated mouse GPIHBP1, truncated soluble GPIHBP1 (sGPIHBP1), nonglycosylated sGPIHBP1, or empty vector, as described in Materials and Methods. The degree of glycosylation of GPIHBP1 before and after deglycosylation with PNGase F or endoglycosidase H was assessed by SDS-PAGE. The human and mouse GPIHBP1 cDNAs were cloned into two different expression vectors (see Materials and Methods), which explains the lower levels of expression for human GPIHBP1. B: Western blot analysis showing that the N-linked oligosaccharide in GPIHBP1 is sensitive to digestion by endoglycosidase F1, marginally sensitive to digestion with endoglycosidase F2, and resistant to digestion with endoglycosidase F3. HeLa cells were transfected with wild-type mouse GPIHBP1, nonglycosylated mouse GPIHBP1, or empty vector. The degree of glycosylation of GPIHBP1 before and after deglycosylation with endoglycosidase F1, F2, or F3 was assessed by SDS-PAGE. TM, nascent GPIHBP1 with transmembrane domain; Nonglyco., nonglycosylated.

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with fresh medium containing 1 U/ml PIPLC (4). After 1 h at 37°C, the cell culture medium was harvested and cell extracts were collected in RIPA buffer containing complete mini EDTA-free protease inhibitors (Roche).

Binding of human LPL to *Gpihbp1*-transfected CHO cells

CHO pgsA-745 cells were cotransfected with a V5-tagged human LPL cDNA in pcDNA6 (9) (a gift from Dr. Mark Doolittle, University of California, Los Angeles) along with the different mouse *Gpihbp1* constructs or empty vector using Lipofectamine 2000 (Invitrogen). Twenty-four hours after the transfection, the cell culture medium was replaced with fresh Ham F12 medium, with or without 1 U/ml of heparin (American Pharmaceutical Partners; Schaumburg, IL). After 1 h, the medium was harvested and cell extracts were collected in RIPA buffer containing complete mini EDTA-free protease inhibitors (Roche).

Western blot analyses

Proteins were size-fractionated on 4–12% Bis-Tris gradient SDS-polyacrylamide gels (cell culture medium) or 12% Bis-Tris SDS-polyacrylamide gels (mouse tissue extracts or extracts from cultured cells), and then transferred to a sheet of nitrocellulose membrane for Western blotting. The antibody dilutions were 1:250 for a rabbit polyclonal antibody against the S-protein tag

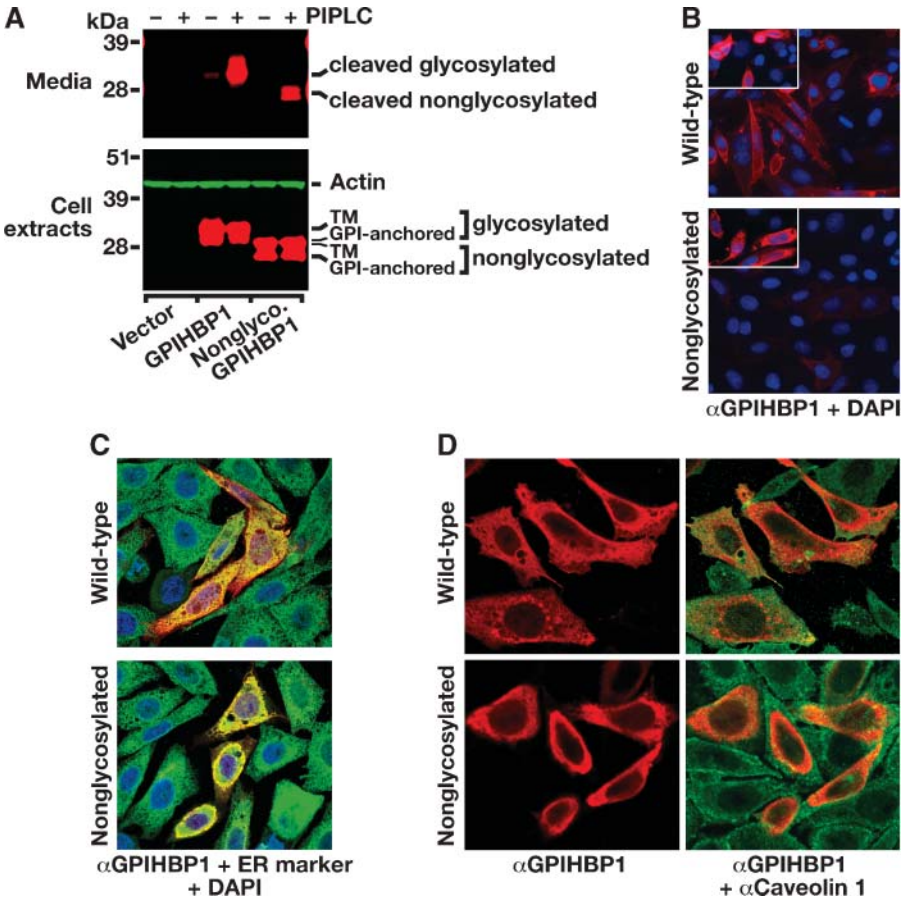

Fig. 3. N-glycosylation is essential for proper targeting of GPIHBP1 to the cell surface. A: Western blot analysis revealing reduced amounts of nonglycosylated GPIHBP1 released into the culture medium by phosphatidylinositol-specific phospholipase C (PIPLC), compared with wild-type GPIHBP1. Total levels of expression for the two constructs were similar, as judged by the amount of GPIHBP1 in cell extracts (lower panel). HeLa cells were transfected with wild-type mouse GPIHBP1, nonglycosylated mouse GPIHBP1, or empty vector. Twenty-four hours after the transfection, cells were treated for 1 h at 37°C with 1 U/ml PIPLC, and the amount of GPIHBP1 protein in the medium and cell extracts was assessed by Western blot analysis. TM, nascent GPIHBP1 with transmembrane domain; Nonglyco., nonglycosylated. B: Immunofluorescence microscopy of nonpermeabilized transfected cells showing decreased cell surface expression of nonglycosylated mouse GPIHBP1, compared with wild-type mouse GPIHBP1. Inserts show GPIHBP1 expression in permeabilized cells from the same experiment. CHO pgsA-745 cells were transfected with either nonglycosylated mouse GPIHBP1 or wild-type mouse GPIHBP1 constructs, and GPIHBP1 expression (in red) was assessed in permeabilized and nonpermeabilized cells by immunofluorescence microscopy. DAPI staining (blue) was used to visualize DNA. C and D: Confocal immunofluorescence microscopy showing that most of the nonglycosylated GPIHBP1 remains trapped in the endoplasmic reticulum (ER). The subcellular localization of wild-type and nonglycosylated GPIHBP1 (in red) was compared with an ER marker (in green, C) and caveolin 1, a cell surface protein (in green, D) in permeabilized CHO pgsA-745 cells. In panel C, DAPI staining (blue) was used to visualize DNA. Wild-type GPIHBP1 can be detected in the ER (C) but also in the periphery of the cell (C and D), whereas most of the nonglycosylated GPIHBP1 is trapped in the ER (C), and little reaches the cell surface (C and D).

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(Abcam; Cambridge, MA); 1:250 for a mouse monoclonal antibody against the V5 tag (Invitrogen); 1:500 for a mouse monoclonal antibody against β-actin (Abcam); 1:2000 for an IRdye680-conjugated goat anti-rabbit IgG (Li-Cor; Lincoln, NE); and 1:2000 for an IRdye800-conjugated goat anti-mouse IgG (Li-Cor). Antibody binding was detected with an Odyssey infrared scanner (Li-Cor).

Immunofluorescence microscopy

For the detection of GPIHBP1 in cultured cells, cells were plated on coverslips at ~25,000 cells per well in 24-well plates, fixed in 3% paraformaldehyde, blocked with 10% sheep serum, and incubated with a rabbit antiserum against GPIHBP1 (Novus Biologicals; Littleton, CO) diluted in blocking buffer (1:2000). In some studies, detergents were omitted, allowing us to visualize GPIHBP1 on the surface of cells, whereas in other experiments, the cells were permeabilized with 0.2% Triton X-100. Bound rabbit IgG was detected with an Alexa 568-conjugated goat anti-rabbit IgG (1:800; Invitrogen). In some experiments, a mouse monoclonal antibody against caveolin 1 (1:250; Abcam) and an Alexa 488-conjugated goat anti-mouse IgG (1:800; Invitrogen) were used. For the endoplasmic reticulum (ER) colocalization experiments, a mouse monoclonal antibody against KDEL (1:200; Stressgen; Ann Arbor, MI) and an Alexa 488-conjugated goat anti-mouse IgG (1:600; Invitrogen) were used. After washing, cells were stained with 4',6 diamino-2-phenylindole dihydrochloride to visualize DNA. Images were obtained with an Axiovert 200 MOT microscope (Zeiss, Germany) with a 63×/1.25 oil-immersion objective and processed with AxioVision 4.2 software (Zeiss). Confocal fluorescence microscopy was performed with a Leica TCS-SP MP confocal inverted microscope (Heidelberg, Germany) equipped with an argon laser (488 nm blue excitation), diode laser (561 nm green excitation), and a two-photon laser tuned at 768 nm for ultraviolet excitation. Individual images were captured sequentially with a 63×/1.32 planapo objective, and merged images were generated with Leica confocal software (version 2.5).

Binding of DiI-labeled chylomicrons to transfected cells

DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) was purchased from Invitrogen. DiI (3 mg) was dissolved in 1.0 ml DMSO; 10 μl of the DiI/DMSO solution was added to 70 μl of serum from a severely hypertriglyceridemic 14-week-old *Gpihbp1*$^{-/-}$ mouse and 250 μl of human lipoprotein-deficient serum and incubated at 37°C overnight. The $d <$ 1.006 g/ml lipoproteins ("chylomicrons") were then isolated by centrifugation at 100,000 rpm in a Beckman TLA-100.3 rotor for 6 h at 4°C. The DiI-labeled chylomicrons were harvested from the top of the tube and dialyzed against PBS containing 5.0 μM EDTA.

To evaluate the binding of the DiI-labeled chylomicrons to GPIHBP1, CHO pgsA-745 cells were transfected with mouse *Gpihbp1* constructs and incubated with 1 μg/ml of DiI-labeled chylomicrons in PBS containing 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.5% BSA for 2 h at 4°C. The cells were then washed five times in the same buffer and fixed in 3% paraformaldehyde, and binding of chylomicrons to the cells was assessed by fluorescence microscopy. GPIHBP1 on the cell surface was detected as described earlier.

Binding of apolipoprotein A-V-phospholipid disks to *Gpihbp1*-transfected CHO cells

Apolipoprotein A-V (apoA-V) was expressed and purified as previously described (10). To generate apoA-V-phospholipid complexes, dimyristoylphosphatidylcholine (DMPC) was dissolved in chloroform-methanol (3:1; v/v), and the solvent was evaporated under a stream of nitrogen. The sample was then incubated in vacuo for 16 h to remove residual solvent. DMPC was resuspended in a solution containing 50 mM sodium citrate and 150 mM NaCl (pH 3.0), followed by the addition of apoA-V (DMPC-apoA-V; 6:1; w/w). The mixture was sonicated until clear, dialyzed against 150 mM NaCl, 50 mM phosphate, pH 7.4, and filter-sterilized (0.22 μm). Protein concentration was determined with the BCA Protein Assay Kit (Pierce; Rockford, IL), and the phospholipid content was determined with an enzyme-based colorimetric assay (Wako; Richmond, VA). To evaluate the binding of the apoA-V-DMPC disks to GPIHBP1, CHO pgsA-745 cells were transiently transfected with the S-protein-tagged *Gpihbp1* cDNA and incubated with 5 μg/ml of apoA-V-DMPC disks in PBS containing 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.5% BSA for 2 h at 4°C. The binding of apoA-V-DMPC disks was detected with a mouse anti-apoA-V antibody (1:1000; Invitrogen) and an Alexa 568-conjugated goat anti-mouse IgG antibody (1:800; Invitrogen); the S-protein-tagged GPIHBP1 was detected with an FITC-conjugated goat antibody against the S-protein tag (1:400; Abcam).

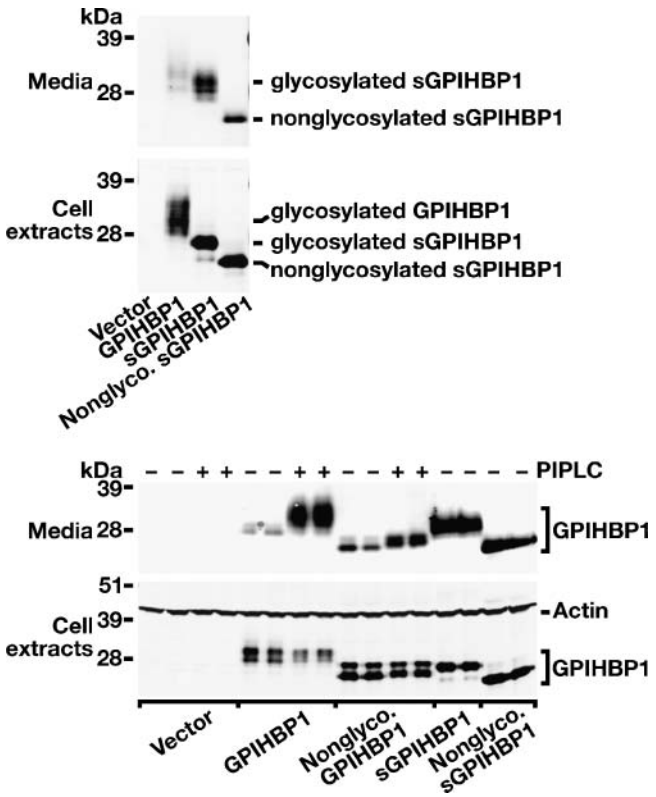

Fig. 4. N-glycosylation is less crucial for the secretion of an sGPIHBP1. Western blot analyses from two independent transfection experiments showing a 48% reduction in the amount of nonglycosylated truncated GPIHBP1 secreted into the culture medium compared with a normally glycosylated truncated GPIHBP1 (as judged by quantification of band intensities with a Li-Cor scanner). In contrast, with the glycosylphosphatidylinositol (GPI)-anchored proteins, the absence of glycosylation resulted in a 79% reduction in GPIHBP1 expression on the cell surface, as judged by PIPLC release. HeLa cells were transfected with mouse wild-type or nonglycosylated GPIHBP1, an sGPIHBP1, truncated before the GPI anchor attachment motif, nonglycosylated (Nonglyco.) sGPIHBP1, or empty vector. Twenty-four hours after the transfection, the relative amounts of GPIHBP1 in cell culture medium and cell extracts were assessed by Western blot analysis.

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RESULTS

Sequence analysis of GPIHBP1 proteins from different mammalian species

Amino acid sequences for GPIHBP1 of multiple mammalian species were obtained from Ensembl (http://www.ensembl.org) (**Fig. 1A**). The NetNglyc program (www.cbs.dtu.dk/services/NetNGlyc) identified a putative N-linked glycosylation site within the Ly-6 domain of GPIHBP1 from human (78NLT), rhesus monkey (77NLT), mouse (76NQT), rat (75NET), and guinea pig (56NQT) (Fig. 1A). In the human GPIHBP1 sequence, there is a second asparagine at residue 82 that also could be N-glycosylated. The NetNglyc program did not identify any N-glycosylation sites in the Ly-6 domain of GPIHBP1 from hedgehog, dog, platypus, or cow (Fig. 1A).

Identification of an N-glycosylation site in GPIHBP1

To determine whether human or mouse GPIHBP1 was N-glycosylated, HeLa cells were transfected with wild-type mouse or human GPIHBP1, a mutant mouse GPIHBP1 in which the putative N-glycosylation motif was eliminated, and mutant mouse GPIHBP1 proteins in which the putative N-glycosylation site was replaced with corresponding sequences from cow, dog, or platypus. Transfected HeLa cells were incubated in the presence or absence of tunicamycin, which inhibits the addition of N-linked oligosaccharides to nascent polypeptides (11), and the electrophoretic migration of GPIHBP1 was assessed by SDS-polyacrylamide gel electrophoresis and Western blotting (Fig. 1B). In tunicamycin-treated cells, the electrophoretic migration of mouse GPIHBP1 was more rapid and two bands could be detected (with and without the GPI anchor) (Fig. 1B). Human GPIHBP1 from tunicamycin-treated cells also migrated more rapidly (Fig. 1B). When the putative N-glycosylation site in mouse GPIHBP1 was mutated, tunicamycin did not alter the electrophoretic mobility of the protein, consistent with the existence of a single N-glycosylation site. When the putative N-glycosylation site in mouse GPIHBP1 was replaced with corresponding sequences from dog and platypus, the proteins were clearly glycosylated, because their electrophoretic migration was changed by tunicamycin. When the corresponding sequences from cow were inserted into mouse GPIHBP1, there was no evidence for glycosylation, consistent with the absence of an asparagine in the cow sequence.

To determine whether mouse GPIHBP1 is N-glycosylated in vivo, adipose tissue extracts from a wild-type mouse and a *Gpihbp1*$^{-/-}$ mouse were digested with PNGase F, an enzyme that removes virtually all N-linked oligosaccharides from glycoproteins. Digestion of wild-type mouse tissue extracts with PNGase F converted a broad GPIHBP1 band into a

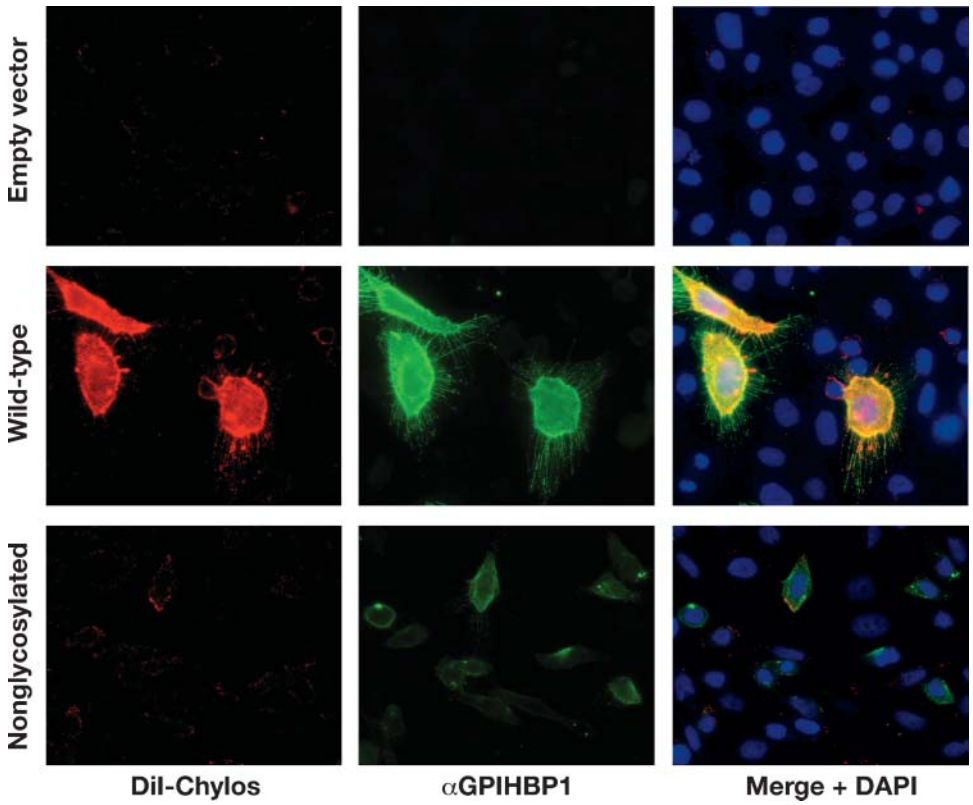

Fig. 5. Decreased expression of nonglycosylated GPIHBP1 at the cell surface is associated with decreased chylomicron binding. CHO pgsA-745 cells were transfected with nonglycosylated mouse GPIHBP1, wild-type mouse GPIHBP1, or empty vector. The binding of DiI-labeled chylomicrons (in red) to wild-type or mutant GPIHBP1 (in green) was assessed by immunofluorescence microscopy as described in Materials and Methods. DAPI staining (blue) was used to visualize DNA.

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sharper and more rapidly migrating band, indicating that GPIHBP1 is N-glycosylated in vivo (Fig. 1C).

Characterization of the N-linked glycan on GPIHBP1

To characterize the N-linked glycan on GPIHBP1, extracts from HeLa cells transfected with mouse or human wild-type GPIHBP1, a nonglycosylated mutant mouse GPIHBP1, an sGPIHBP1, or a nonglycosylated sGPIHBP1 were digested with either PNGase F or endoglycosidase H (an enzyme that cleaves oligomannose and most hybrid glycans). The vast majority of mouse GPIHBP1 was sensitive to endoglycosidase H digestion (**Fig. 2A**, right and left panels). Similar results were obtained with sGPIHBP1 (Fig. 2A, right and left panels) and human GPIHBP1 (Fig. 2A, left panel). The electrophoretic mobility of endoglycosidase H-digested GPIHBP1 was similar to that of PNGase F-digested GPIHBP1 and that of the nonglycosylated mutant GPIHBP1, suggesting that the N-linked glycan is probably an oligomannose or a hybrid glycan. As expected, endoglycosidase H or PNGase F treatment of extracts from cells transfected with a nonglycosylated mouse GPIHBP1 mutant did not change the electrophoretic migration of GPIHBP1 (Fig. 2A, right panel).

To further explore this finding, extracts of HeLa cells transfected with wild-type mouse GPIHBP1 or the nonglycosylated mouse GPIHBP1 mutant were digested with endoglycosidase F1, F2, or F3. Endoglycosidase F1 cleaves oligomannose and hybrid structures but not complex N-linked oligosaccharides. Endoglycosidase F2 cleaves between the two N-acetylglucosamine residues in the diacetylchitobiose core of the oligosaccharide, generating a truncated glycan. Endoglycosidase F2 cleaves biantennary complex oligosaccharides and, at a low rate, high mannose oligosaccharides but not hybrid structures. Endoglycosidase F3 cleaves biantennary and triantennary structures but not oligomannose or hybrid molecules. In our studies, digestion with endoglycosidase F1 removed the oligosaccharide from the vast majority of mouse GPIHBP1, causing it to migrate with nonglycosylated GPIHBP1 on SDS-polyacrylamide gels (Fig. 2B). Approximately 10% of mouse GPIHBP1 was sensitive to endoglycosidase F2, whereas it was resistant to endoglycosidase F3 digestion, suggesting that the N-linked glycan on GPIHBP1 is an oligomannose rather than a hybrid or a complex glycan.

N-glycosylation of GPIHBP1 is important for its trafficking to the cell surface

To assess the importance of glycosylation for the trafficking of GPIHBP1 to the cell surface, we first transfected HeLa cells with wild-type mouse GPIHBP1 or nonglycosylated mouse GPIHBP1 constructs and then released the GPIHBP1 from the surface of cells with PIPLC. The wild-type and the nonglycosylated mutant construct yielded similar levels of expression in cells, as judged by Western

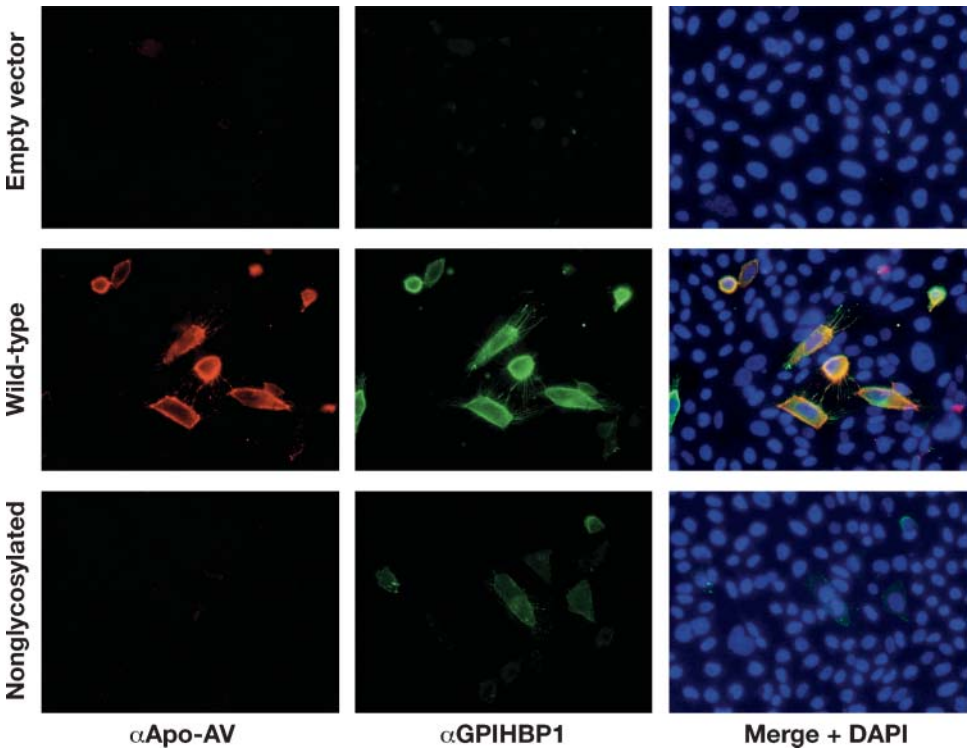

Fig. 6. Decreased expression of nonglycosylated GPIHBP1 at the cell surface is associated with decreased binding of apoA-V-dimyristoylphosphatidylcholine (DMPC) disks. CHO pgsA-745 cells were transfected with empty vector, wild-type mouse GPIHBP1, or the nonglycosylated mouse GPIHBP1. The binding of apoA-V-DMPC disks (in red) to wild-type or mutant GPIHBP1 (in green) was assessed by immunofluorescence microscopy as described in Materials and Methods. DAPI staining (blue) was used to visualize DNA.

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blotting of cell extracts (**Fig. 3A**). However, the amount of GPIHBP1 released into the medium by PIPLC was approximately five-fold lower with nonglycosylated GPIHBP1 than with wild-type GPIHBP1 (see Figs. 3A and 4, lower panel), as judged by quantitative analysis of the Western blots with a Li-Cor scanner.

We also examined the expression of wild-type and nonglycosylated GPIHBP1 on the surface of cells by immunofluorescence microscopy. Only minimal amounts of nonglycosylated GPIHBP1 were observed on the surface of nonpermeabilized cells, whereas the cell surface expression of wild-type GPIHBP1 was robust (Fig. 3B). However, the overall expression levels of the wild-type and nonglycosylated mutant GPIHBP1 were similar, as judged by staining of cells that had been permeabilized with 0.2% Triton X-100 (see inserts in Fig. 3B).

The subcellular localization of wild-type and nonglycosylated GPIHBP1 was assessed by confocal fluorescence microscopy (Fig. 3C, D). Nonglycosylated mouse GPIHBP1 was almost exclusively perinuclear, colocalizing with an ER marker, and there was little expression of the mutant GPIHBP1 at the edges of cells (Fig. 3C). In contrast, wild-type GPIHBP1 expression extended beyond the perinuclear region, with less colocalization with the ER marker (Fig. 3C). Similar findings were apparent in immunocytochemistry experiments with GPIHBP1 and caveolin-1 (Fig. 3D). Again, most of the nonglycosylated GPIHBP1 mutant was perinuclear, failing to reach the edges of the cell, whereas expression of the wild-type GPIHBP1 extended to the edges of the cell (Fig. 3D).

sGPIHBP1 mutants

To create an sGPIHBP1, the GPI anchor attachment motif in GPIHBP1 was replaced with a stop codon. Substantial amounts of sGPIHBP1 were secreted by transfected cells into the medium (**Fig. 4**). Interestingly, significant amounts of a nonglycosylated version of sGPIHBP1 also were secreted into the culture medium (Fig. 4). Relative to the amount of GPIHBP1 in the cell extract, the amount of secretion of the nonglycosylated sGPIHBP1 was reduced by 48%, compared with the fully glycosylated sGPIHBP1, as judged by band intensity quantification with a Li-Cor scanner. Thus, the absence of glycosylation only modestly reduced the secretion of sGPIHBP1 from cells.

Reduced expression of nonglycosylated GPIHBP1 at the cell surface is associated with reduced binding of GPIHBP1 ligands

Because little nonglycosylated GPIHBP1 reaches the cell surface, we predicted that cells transfected with the nonglycosylated mouse GPIHBP1 construct would exhibit a reduced capacity to bind chylomicrons and apoA-V-DMPC disks. This prediction was upheld. CHO pgsA-745 cells expressing the nonglycosylated mouse GPIHBP1 had little if any capacity to bind chylomicrons (**Fig. 5**) or apoA-V-DMPC disks (**Fig. 6**), compared with cells expressing wild-type mouse GPIHBP1.

We also predicted that cells transfected with the nonglycosylated GPIHBP1 would manifest a reduced number of binding sites for LPL, simply because little nonglycosylated GPIHBP1 reaches the cell surface. To test this idea, we cotransfected plasmids encoding a tagged version of LPL and GPIHBP1 into CHO pgsA-745 cells. Normally, the LPL secreted by these cells is bound by GPIHBP1, and little LPL appears in the medium. However, significant amounts of LPL appear in the medium after the GPIHBP1-bound LPL is released with heparin (**Fig. 7**). In cells expressing nonglycosylated GPIHBP1, the amount of LPL in the cell culture medium was no different in the presence or absence of heparin (i.e., little nonglycosylated GPIHBP1 reached the cell surface, so there were fewer LPL binding sites and no additional heparin-releasable LPL) (Fig. 7).

DISCUSSION

Several GPI-anchored proteins containing Ly-6 domains, such as UPAR and CD59, are known to be N-glycosylated (5, 6, 12, 13). In the current study, we examined the idea

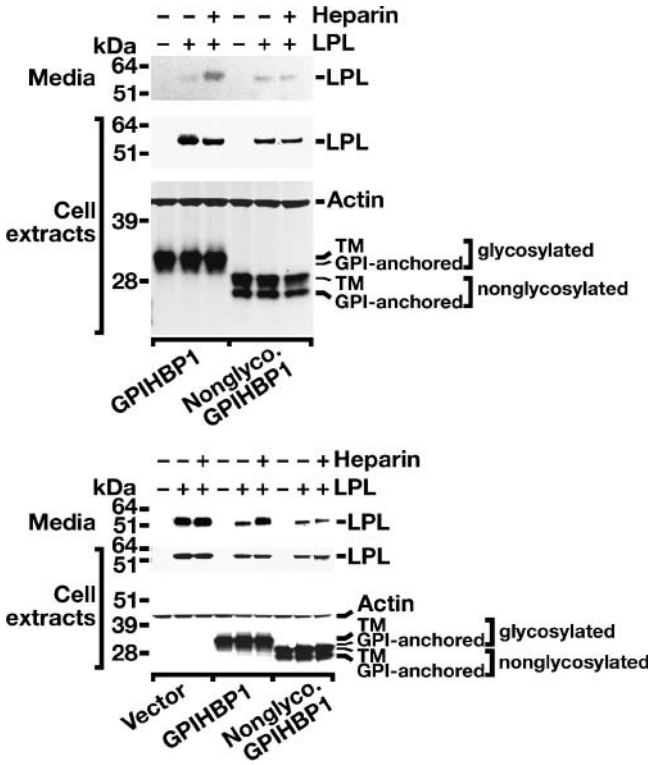

Fig. 7. Decreased expression of nonglycosylated GPIHBP1 at the cell surface is associated with decreased binding of human LPL. Western blot analyses from two independent transfection experiments showing reduced binding of a V5-tagged human LPL to CHO pgsA-745 cells expressing nonglycosylated GPIHBP1. CHO pgsA-745 cells were cotransfected with a V5-tagged human LPL and either wild-type mouse GPIHBP1 or nonglycosylated mouse GPIHBP1, as described in Materials and Methods. Twenty-four hours after transfection, cells were incubated with or without 1 U/ml of heparin for 1 h at 37°C. The amount of bound LPL released into the culture medium by heparin was assessed by Western blotting.

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that GPIHBP1, a structurally related protein with a key role in chylomicron processing (1), might also be glycosylated. Computer-assisted analysis of GPIHBP1 sequences revealed a putative N-glycosylation site in both mouse and human GPIHBP1. A combination of tunicamycin treatment of cells, site-directed mutagenesis, and enzymatic digestion studies revealed that mouse GPIHBP1 was N-glycosylated (at Asn-76). Human GPIHBP1 was also glycosylated, and the corresponding amino acid sequences from several other mammalian species also directed N-glycosylation. Enzymatic digestion studies revealed that the N-linked oligosaccharide in mouse GPIHBP1 was of the oligomannose type rather than a hybrid or a complex glycan. The glycosylation of GPIHBP1 was functionally important. Eliminating the N-glycosylation site in mouse GPIHBP1 caused the protein to be retained in the ER, and little reached the cell surface. Consequently, cells expressing a nonglycosylated GPIHBP1 had fewer binding sites on their surface for LPL, chylomicrons, and apoA-V-DMPC disks. Interestingly, the bovine GPIHBP1 sequence in the databases does not contain an N-linked glycosylation site, raising the possibility that glycosylation may not be as important in that species.

The fact that N-glycosylation of mouse GPIHBP1 was functionally important was not altogether surprising, but neither was it predictable. In the case of UPAR, a related GPI-anchored Ly-6 protein, N-glycosylation is clearly important (5). UPAR contains three Ly-6 domains and four N-glycosylation sites (6, 14). A UPAR mutant lacking all of the N-glycosylation sites remained trapped in the ER and did not reach the cell surface (5). In the case of CD59, however, N-glycosylation did not appear to be crucial, inasmuch as eliminating the N-glycosylation site did not reduce the cell-surface expression of the molecule or adversely affect its function (12).

Interfering with the glycosylation of GPIHBP1 reduced the ability of GPIHBP1 to reach the cell surface. Interestingly, however, eliminating glycosylation had a less dramatic effect on the secretion of an sGPIHBP1. Once again, this finding was not altogether surprising, but neither was it predictable. In the case of UPAR, eliminating all of the glycosylation sites in a truncated soluble version of the protein largely abolished the secretion of the protein (15). Very different results were obtained with Thy-1, a GPI-anchored Ly-6 protein with three N-glycosylation sites, all of which are utilized (16). Eliminating all three N-glycosylation sites in Thy-1 prevented the appearance of the protein at the cell surface (16). However, eliminating the same glycosylation sites in a truncated soluble version of Thy-1 had little if any effect on the secretion of the protein (16).

The reason that glycosylation would be so critical for the GPI-anchored form of GPIHBP1, but less important for the secretion of an sGPIHBP1, is mysterious. The mystery is compounded when one considers the fact that N-glycosylation of proteins occurs cotranslationally, whereas the addition of the GPI anchor occurs after translation is complete (17). It is conceivable that some wild-type GPIHBP1 fails to undergo glycosylation in cells, but that this nonglycosylated protein is detected and degraded when the protein is tethered to the membrane by a GPI anchor. This removal of nonglycosylated GPIHBP1 may be less efficient for soluble proteins lacking the GPI anchor. A similar proposal was advanced by Devasahayam et al. (16) to explain why the trafficking of GPI-anchored Thy-1 was utterly dependent on glycosylation, whereas the secretion of a truncated soluble Thy-1 was not.

In any case, the observation that an sGPIHBP1 is secreted efficiently is likely to be helpful for future investigations of the molecule. GPIHBP1 is critically important for the lipolytic processing of triglyceride-rich lipoproteins (1). At this point, however, it is fair to state that almost nothing is known about GPIHBP1 structure, and which amino acid residues might be important in the binding of ligands. The fact that nonglycosylated sGPIHBP1 is efficiently secreted from eukaryotic cells will be a boon for those interested in crystallizing the protein and defining its structure.

Finally, it is interesting to note that LPL, GPIHBP1's partner in the lipolytic processing of chylomicrons, is utterly dependent on N-glycosylation for secretion (18–21). LPL contains two N-glycosylation sites (22). When one of these, Asn-43, is eliminated by site-directed mutagenesis, LPL is degraded in the ER and the secretion of LPL from cells is abolished (22). Long after the importance of Asn-43 for LPL secretion was documented, Kobayashi et al. (23) identified a homozygous Asn43Ser mutation in a human subject with chylomicronemia; this subject had a complete absence of LPL in postheparin plasma. Our current studies show that glycosylation of GPIHBP1 is critical for its trafficking to the cell surface. Sooner or later, we suspect, a clinical geneticist will uncover a hypertriglyceridemic patient with a mutation eliminating N-linked glycosylation in human GPIHBP1.

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