Binding Preferences for GPIHBP1, a Glycosylphosphatidylinositol-Anchored Protein of Capillary Endothelial Cells

Peter Gin, Anne P. Beigneux, Constance Voss, Brandon S.J. Davies, Jennifer A. Beckstead, Robert O. Ryan, André Bensadoun, Loren G. Fong, Stephen G. Young

Objective—To define the ability of GPIHBP1 to bind other lipase family members and other apolipoproteins (apos) and lipoproteins.

Methods and Results—GPIHBP1, a GPI-anchored lymphocyte antigen (Ly)6 protein of capillary endothelial cells, binds lipoprotein lipase (LPL) avidly, but its ability to bind related lipase family members has never been evaluated. As judged by cell-based and cell-free binding assays, LPL binds to GPIHBP1, but other members of the lipase family do not. We also examined the binding of apoAV-phospholipid disks to GPIHBP1. ApoAV binds avidly to GPIHBP1-transfected cells; this binding requires GPIHBP1’s amino-terminal acidic domain and is independent of its cysteine-rich Ly6 domain (the latter domain is essential for LPL binding). GPIHBP1-transfected cells did not bind high-density lipoprotein. Chylomicrons bind avidly to GPIHBP1-transfected Chinese hamster ovary cells, but this binding is dependent on GPIHBP1’s ability to bind LPL within the cell culture medium.

Conclusion—GPIHBP1 binds LPL but does not bind other lipase family members. GPIHBP1 binds apoAV but does not bind apoAI or high-density lipoprotein. The ability of GPIHBP1-transfected Chinese hamster ovary cells to bind chylomicrons is mediated by LPL; chylomicron binding does not occur unless GPIHBP1 first captures LPL from the cell culture medium. (Arterioscler Thromb Vasc Biol. 2011;31:176-182.)

Key Words: apolipoproteins ■ lipids ■ lipoproteins ■ metabolism

GPIHBP1, a glycosylphosphatidylinositol (GPI)-anchored glycoprotein of capillary endothelial cells, plays a crucial role in the lipolytic processing of triglyceride-rich lipoproteins. The importance of GPIHBP1 in plasma triglyceride metabolism was uncovered by examining phenotypes of Gpihbp1-knockout mice (Gpihbp1−/−). Gpihbp1−/− mice display severe hypertriglyceridemia, even on a low-fat diet, with plasma triglyceride levels of 3000 to 6000 mg/dL. Subsequent studies proved that GPIHBP1 is responsible for transporting lipoprotein lipase (LPL) into the lumen of capillaries. In the absence of GPIHBP1, LPL is mislocalized to the interstitial spaces and, therefore, cannot hydrolyze triglycerides in plasma lipoproteins.

GPIHBP1 contains a single lymphocyte antigen 6 (Ly6) domain containing 10 cysteines, which are arranged in a characteristic spacing pattern. Mutation of any of these cysteines abolishes GPIHBP1’s capacity to bind LPL. A second noteworthy feature of GPIHBP1—and the one that distinguishes it from other Ly6 protein family members—is a striking acidic domain at its amino terminus. This domain is highly enriched in negatively charged amino acids (21 of 26 consecutive residues in human GPIHBP1 are aspartate or glutamate). Mutating the acidic domain abolishes GPIHBP1’s ability to bind LPL.

The binding of LPL to GPIHBP1 appears to depend on a positively charged heparin-binding domain in LPL. Mutating the positively charged amino acids in LPL’s principal heparin-binding domain eliminates its ability to bind to GPIHBP1. Two other lipase family members, hepatic lipase (HL) and endothelial lipase (EL), also contain heparin-binding domains. Whether these other heparin-binding lipases also bind to GPIHBP1 has never been assessed.

Transfection of Chinese hamster ovary (CHO) pgsA-745 cells with GPIHBP1 confers the ability to bind apolipoprotein (apo) AV–phospholipid disks. ApoAV contains a strong heparin-binding domain, but the structural features of GPIHBP1 required for apoAV binding are not understood. It is not known whether the binding of apoAV, like the binding of LPL, depends on both the Ly6 domain and the acidic domain. Also, Beigneux et al reported that GPIHBP1-transfected CHO
cells bind chylomicrons \( (d<1.006 \text{ g/mL}) \) for lipoproteins from \( \text{Gpihbp1}^{-/-} \) mice, but the mechanism for chylomicron binding has been obscure.

In the current study, we sought to define binding preferences for GPHBP1. We tested the ability of other lipase family members to bind to GPHBP1 and assessed the structural features of GPHBP1 required for apoAV binding. In addition, we investigated the mechanism underlying the ability of chylomicrons to bind to GPHBP1-transfected CHO cells.

**Methods**

**Expression of Lipases in Cultured Cells**
We obtained an expression vector for a V5-tagged human LPL (h-LPL). We used a commercially available kit (In-Fusion Advantage PCR Cloning kit) to insert open reading frames for human HL (h-HL), mouse HL (m-HL), mouse EL (m-EL), and human pancreatic lipase (h-PL) into plasmid vector used to clone DNA (pCMV) 6/5-V5-His (in frame with the carboxyl-terminal V5 tag). The expression plasmids (5 \( \mu \text{g} \)) were electroporated into CHO-K1 or Chinese hamster lung (CHL)-11 cells, and the cells were then grown for 24 hours in serum-free medium containing protease inhibitors. The lipase-containing cell medium was concentrated 10-fold with a filter (Amicon Ultra 10,000 normal molecular weight limit [NMWL] filter).

**Assessing the Binding of Lipases to GPHBP1-Expressing CHO Cells**
A total of \( 5 \times 10^6 \) CHO-K1 cells were electroporated with 5 \( \mu \text{g} \) of an S-protein–tagged GPHBP1 expression vector and then seeded into triplicate wells of 24-well plates. After 24 hours, the cells were incubated for 2 hours at 4°C with 400 \( \mu \text{L} \) of concentrated conditioned medium from cells expressing h-LPL, h-HL, m-HL, m-EL, or h-PL. Cells were then washed, and the amounts of LPL and GPHBP1 in the cell extracts were assessed with Western blots, as previously described.7

**Assessing the Binding of Lipases to Soluble GPHBP1 With a Cell-Free Assay**
CHO pgsA-745 cells were electroporated with a mouse GPHBP1 expression vector truncated at residue Q197 (immediately before the GPI-anchoring sequence).7 After 48 hours, the medium was collected and concentrated 20-fold with a filter (Amicon Ultra 10,000 filter). Binding of lipases to soluble GPHBP1 was assessed with a cell-free binding assay, as previously described.7 Briefly, soluble mouse GPHBP1 was mixed with the following: (1) V5-tagged h-LPL, h-HL, m-HL, m-EL, or h-PL; and (2) agarose beads coated with a mouse GPHBP1-specific monoclonal antibody (11A12)2 in PBS containing 0.2% NP-40 for 2 hours. The beads were washed, the soluble GPHBP1 was eluted with 0.1-mol/L glycine (pH 2.5), and the relative amounts of GPHBP1 and lipase in the starting material and in each wash and elution fraction were assessed with Western blots.7 We also used this assay to test the ability of nonadenatured and denatured h-LPL to bind to GPHBP1. h-LPL was denatured by heating to 50°C for 16 hours or by 3-mol/L guanidine hydrochloride for 16 hours (the LPL was then dialyzed against PBS).16

**Western Blots**
Proteins were size fractionated on 12% acrylamide SDS-BIS-TRIS gels and then transferred to nitrocellulose. The antibody dilutions for Western blots were: 1:1000 for a goat polyclonal antibody against the S-protein tag; 1:200 for a mouse monoclonal antibody against the V5 tag; 1:500 for a rabbit polyclonal antibody against \( \beta \)-actin; 1:1000 for a rabbit polyclonal antibody against GPHBP1; 1:1000 for a mouse monoclonal antibody against apoA-1; 1:200 for 11A12, a rat monoclonal antibody against mouse GPHBP1; 1:1000 for SD2, a monoclonal antibody against bovine LPL; 1:5000 for a goat polyclonal antibody against mouse LPL; 1:5000 for an IRdye800-conjugated donkey anti–goat IgG; 1:500 for an IRdye800-conjugated donkey anti–mouse IgG; 1:2000 for an IRdye680-conjugated donkey anti–rabbit IgG; 1:1000 for an IRdye680-conjugated donkey anti–mouse IgG; 1:2000 for an IRdye800-conjugated donkey anti–rat IgG; and 1:5000 for an IRdye680-conjugated donkey anti–goat IgG. Antibody binding was detected with an infrared scanner (Odyssey).

**Immunofluorescence Microscopy**
A total of \( 1 \times 10^6 \) CHO-K1 cells were electroporated with 2 \( \mu \text{g} \) of plasmid DNA for wild-type GPHBP1 or a lipase, and the 2 groups of transfected cells were mixed and plated on coverslips in 24-well plates. The next day, the cells were permeabilized with 0.2% Triton X-100; fixed in 3% paraformaldehyde; blocked with blocking buffer (PBS containing 1-mmol/L magnesium chloride, 1-mmol/L calcium chloride, and 10% donkey serum); and incubated with a goat antiserum against the S-protein tag (1:500), followed by an Alexa Fluor 568–conjugated donkey anti–goat IgG (1:800), and a mouse monoclonal antibody against the V5 tag (1:1200), followed by an Alexa Fluor 488–conjugated donkey anti–mouse IgG (1:800). After washing, the cells were stained with 4’,6-diamidino-2-phenylindole to visualize DNA. The images were recorded with a 200 M microscope (Axiovert) equipped with a 63X objective, an AxioCam MRm, and an ApoTome; the images were processed with computer software (AxioVision 4.2).

**Assessing the Binding of ApoAV to GPHBP1-Expressing Cells With Immunofluorescence Microscopy**
CHO pgsA-745 cells were plated on cover slips in 24-well plates and then transfected with 0.8 \( \mu \text{g} \) of a mouse GPHBP1 expression vector (or an empty vector). After 24 hours, the cells were washed 3 times and incubated with 0.5 \( \mu \text{L} \) of apoAV-1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) disks (1 \( \mu \text{mol/L} \) in binding buffer (PBS containing 1-mmol/L magnesium chloride, 1-mmol/L calcium chloride, and 0.5% BSA). Cells were washed, fixed in 3% paraformaldehyde, blocked with blocking buffer, and then incubated with a rabbit polyclonal antibody against the S-protein tag (1:1000) and a mouse monoclonal antibody against apoA-1 (1:1000). After washing, the cells were incubated with an Alexa Fluor 488–conjugated donkey anti–rabbit IgG (1:500) and an Alexa Fluor 568–conjugated donkey anti–mouse IgG (1:500). After washing, the cells were stained with 4’,6-diamidino-2-phenylindole to visualize DNA, and images were captured with a 200 M microscope (Axiovert). We also tested binding of apoAV disks to soluble GPHBP1 immobilized on agarose beads (supplemental data; available online at http://atvb.ahajournals.org).

**Assessing Binding of Lipoproteins to GPHBP1-Transfected Cells With Immunofluorescence Microscopy**
CHO pgsA-745 and CHL-11 cells were plated on cover slips in 24-well plates and then transfected with 0.8 \( \mu \text{g} \) of a S-protein–tagged GPHBP1 expression vector or an empty vector using Lipofectamine 2000. After 24 hours, the cells were washed with binding buffer and incubated for 1 hour at 4°C with 400 \( \mu \text{L} \) of concentrated conditioned medium from cells expressing a V5-tagged version of h-LPL (or other lipase family members). Some CHO pgsA-745 cells were incubated with heparin (500 \( \text{U/mL} \) for 30 minutes and washed 3 times with binding buffer before incubation with LPL. Cells were then washed 3 times with binding buffer and incubated with 0.5-\( \mu \text{L} \) 1,1’-dioctadecyl-3,3,3,3’-tetramethylindocarbocyanine perchlorate (DM)–labeled chylomicrons (1 \( \mu \text{g/mL} \) in binding buffer, washed, fixed in 3% paraformaldehyde, blocked with blocking buffer, and

**Gin et al Binding Preferences for GPHBP1 177**
incubated with a rabbit polyclonal antibody against the S-protein tag (1:1000) and a mouse monoclonal antibody against LPL (5D2) (1:1000). After washing, cells were incubated with an Alexa Fluor 488–conjugated donkey anti–rabbit IgG (1:500) and an Alexa Fluor 647–conjugated donkey anti–mouse IgG (1:500). Images were captured with a 200 M microscope (Axiovert).

In separate studies, CHO pgsA-745 cells were plated on cover slips in 24-well plates and transfected with 0.8-H9262 g cDNA of a GPIHBP1 expression vector, an expression vector for green fluorescent protein–low-density lipoprotein (LDL) receptor fusion,21 an expression vector for scavenger receptor class B, member 1 (SR-B1)–green fluorescent protein fusion (supplemental data), or an empty vector. After 24 hours, the cells were washed with binding buffer and incubated with 1-H9262 g/mL DiI-labeled LDL or DiI-labeled high-density lipoprotein (HDL) for 2 hours at 4°C. The cells were then washed with binding buffer, fixed in 3% paraformaldehyde, blocked with blocking buffer, and incubated with the rabbit polyclonal antibody against the S-protein tag (1:1000), followed by an Alexa Fluor 488–conjugated donkey anti–rabbit IgG (1:500). After washing, images were captured with a 200 M microscope (Axiovert).

Results

When V5-tagged h-LPL was added to CHO-K1 cells that had been transiently transfected with GPIHBP1, the LPL bound avidly to the GPIHBP1-expressing cells (Figure 1A and 1B). Because HL and EL contain heparin-binding domains, we suspected that they might also bind to GPIHBP1. However, this was not the case. V5-tagged versions of h-HL, m-HL, and m-EL did not bind to GPIHBP1-expressing cells (Figure 1A and 1B), even though ample amounts of those lipases were added to the cells (Figure 1C and 1D).

We also examined the binding of V5-tagged lipases to GPIHBP1-transfected cells by immunofluorescence microscopy (Figure 2). For these studies, we simply mixed CHO-K1 cells that had been transiently transfected with 1 of the lipases with other CHO-K1 cells that had been transiently transfected with wild-type GPIHBP1. The results were unequivocal. The h-LPL secreted by the h-LPL–transfected cells bound to the surface of the GPIHBP1-transfected cells; however, there was no detectable binding of the other lipases (h-HL, m-HL, m-EL, and h-PL) to GPIHBP1-transfected cells (Figure 2).
The ability of other lipase family members to bind to GPIHBP1 was also assessed with a cell-free binding assay (Figure 3). Soluble mouse GPIHBP1 that had been captured on agarose beads coated with a GPIHBP1-specific monoclonal antibody (11A12)7 bound LPL avidly but did not bind h-HL, m-HL, m-EL, or h-PL (Figure 3). In parallel, we found that LPL that had been denatured by heating or guanidine hydrochloride was unable to bind to GPIHBP1 (supplemental Figure I).

As judged by the Western blot binding assay, LPL binding to GPIHBP1 can be abolished by certain missense mutations in GPIHBP1’s Ly6 domain (eg, changing any of the 10 cysteines to alanine or changing glutamine-115 in the human sequence [glutamine-114 in the mouse sequence] to a proline).7,22 In this study, we used the immunofluorescence microscopy assay to confirm those findings (supplemental Figure I and II) and then used this approach to test the impact of Ly6 missense mutations on GPIHBP1’s ability to bind to apoAV (Figure 4). As expected, DMPC-apoAV disks bound avidly to cells transfected with wild-type GPIHBP1 (Figure 4). However, the apoAV disks bound equally well to cells transfected with mutant GPIHBP1 proteins (GPIHBP1-C88A and GPIHBP1-Q114P) harboring Ly6 mutations that abolish GPIHBP1’s ability to bind to LPL (Figure 4). These findings led us to suspect that apoAV binding to GPIHBP1 depends primarily on GPIHBP1’s acidic domain. Consistent with that idea, mutation of GPIHBP1’s acidic domain abolished apoAV binding (Figure 4). By using the monoclonal antibody–based cell-free assay, apoAV-DMPC disks bound to wild-type GPIHBP1. A mutation in GPIHBP1’s acidic domain abolished apoAV binding, whereas mutations in the Ly6 domain did not (supplemental Figure III). Soluble GPIHBP1 did not bind DMPC disks containing the 22-kDa fragment of apoE (another heparin-binding apo)23,24 or apoCIII (supplemental Figure IVA and IVB). Also, we found no evidence that soluble GPIHBP1 binds HDL (supplemental Figure IVC and IVD).

Although LPL and apoAV both bound to soluble GPIHBP1 in the cell–free monoclonal antibody–based assay, we were not able to readily compete away LPL binding to GPIHBP1 with excess apoAV (supplemental Figure V). One interpr-
tation of these studies is that GPIHBP1 binds LPL with high affinity.

CHO cells expressing wild-type GPIHBP1 bound chylomicrons avidly, whereas cells expressing GPIHBP1-Q115P did not. The fact that GPIHBP1’s chylomicron-binding properties mirrored its LPL-binding properties led us to suspect that chylomicron binding might be mediated by LPL. Our suspicions were heightened when we discovered that our CHO cell lines produced LPL; the hamster LPL was readily detectable by Western blots with a goat antibody against mouse LPL or a monoclonal antibody against bovine LPL (5D2) (supplemental Figure VI). The CHO cell–derived LPL can also be detected by immunocytochemistry (the LPL binds to GPIHBP1-transfected CHO cells) (Figure 5). When the hamster LPL was released from the surface of GPIHBP1-transfected cells with heparin, the cells lost their ability to bind chylomicrons (Figure 5). Adding h-LPL back to the heparin-treated cells restored their ability to bind chylomicrons (Figure 5).

To further explore this issue, we tested the ability of Dil-labeled chylomicrons to bind to GPIHBP1-transfected CHL-11 cells (a hamster cell line that expresses only trace amounts of LPL). We were unable to detect binding of Dil-labeled chylomicrons to CHL-11 cells that had been transfected with wild-type GPIHPB1 (Figure 6A). However, we observed avid binding of chylomicrons after h-LPL (supplemental Figure VII) was added to the GPIHPB1-transfected cells (Figure 6A). Other lipases (ie, h-HL, m-HL, or m-EL) (supplemental Figure VII) were incapable of promoting chylomicron binding (Figure 6A). Also, cells expressing GPIHPB1-C88A, a mutant that cannot bind LPL, were unable to bind chylomicrons (Figure 6A). Thus, chylomicron binding to GPIHPB1-expressing cells depends on GPIHPB1-bound LPL. LPL’s ability to promote lipoprotein binding appeared to be confined to the d<1.006 g/mL lipoproteins. Dil-labeled human HDL and LDL were unable to bind to GPIHPB1-transfected cells, even when the experiments were performed in CHO cells, which produce LPL (Figure 6B and 6C). As expected, Dil-HDL bound avidly to cells expressing SR-B1, and Dil-LDL bound avidly to cells expressing the LDL receptor (Figure 6B and 6C).

**Discussion**

The most striking structural feature of GPIHBP1 is its amino-terminal acidic domain. The existence of this negatively charged domain, along with the severe hyperlipidemia in Gpihbp1−/− mice, prompted Beigneux et al to predict that GPIHBP1 would bind LPL (a protein containing positively charged heparin-binding domains). This prediction was quickly confirmed, but the early studies begged the question of whether GPIHBP1 would bind other lipases with heparin-binding domains. In the current study, 3 independent assays showed that neither HL nor EL binds to GPIHBP1. The first, developed by Beigneux et al, uses Western blots to detect the binding of lipases to GPIHBP1-transfected CHO cells. The second, also developed by Beigneux et al, assesses the ability of different lipases to bind to soluble mouse GPIHPB1 captured on antibody-coated agarose beads. The third, new with this article, uses immunofluorescence microscopy to detect binding of freshly secreted lipases to GPIHPB1-transfected cells. The microscopy assay is attractive because it avoids manipulation of lipases and obviates the need for Western blot analyses. Also, because each high-powered field contains multiple GPIHBP1-transfected and nontransfected cells, the extent of this nonspecific binding can quickly be judged. More important, the 3 binding assays yielded concordant results: among the lipases that we tested, only LPL was capable of binding to GPIHBP1.

ApoAV contains a strong heparin-binding domain and binds to heparin and heparan sulfate proteoglycans. The initial report by Beigneux et al demonstrated that apoAV-DMPC disks bind to GPIHPB1-transfected cells. Since then, other experiments have revealed that the binding of LPL to GPIHBP1 depends on GPIHPB1’s acidic domain and its Ly6
In the current study, we showed that this is not the case for apoAV. As judged by both cell-based and cell-free assays, mutating GPIHBP1’s acidic domain abolishes the binding of apoAV-DMPC disks to GPIHBP1, but mutating the Ly6 domain does not. Other apos (eg, apoE) contain a strong heparin-binding domain (located in the 22-kDa amino-terminal domain of the molecule). However, we found no binding of the 22-kDa apoE fragment to GPIHBP1. Similarly, human LDL did not bind to GPIHBP1, even though LDL’s principal protein component, apoB100, contains multiple heparin-binding domains.26

The initial study by Beigneux et al1 found that GPIHBP1-transfected CHO cells bound Dil-labeled chylomicrons. However, the explanation for this finding was enigmatic. One possibility was that chylomicron binding was mediated by apoAV (given that apoAV-DMPC disks bind to GPIHBP1). However, against that possibility was the observation that GPIHBP1’s chylomicron-binding properties mirrored those of LPL (and not apoAV), in that chylomicron binding was abolished by Ly6 mutations. These observations led us to suspect that chylomicron binding to GPIHBP1 may depend on LPL. In the current study, we demonstrated that CHO cells secrete LPL and showed that chylomicron binding to GPIHBP1-transfected CHO cells is dependent on GPIHBP1-bound LPL. Also, GPIHBP1-transfected CHL-11 cells, which express only trace amounts of LPL, could not bind chylomicrons unless they were first incubated with LPL. The involvement of LPL in binding chylomicrons makes sense, given that LPL contains hydrophobic domains that are known to be important for the binding and hydrolysis of triglyceride substrates.20

Ioka and coworkers6 initially identified GPIHBP1 in expression cloning studies as a novel protein that conferred on CHO cells the ability to bind Dil-labeled HDL. In our study, we found no binding of Dil-HDL to Gpihbp1-transfected CHO cells nor did we find any evidence that HDL binds GPIHBP1 captured on agarose beads. We do not understand the reasons for the conflicting results. One possibility is that the HDL preparation used by Ioka and coworkers contained more apoAV. Alternatively, perhaps the HDL used by Ioka and coworkers was prepared from postheparin plasma and contained LPL. Finally, the expression cloning studies by Ioka and coworkers were performed in CHO cells. It is conceivable that, under their experimental conditions, some CHO cell–derived LPL became associated with Dil-HDL, facilitating its binding to GPIHBP1.

In summary, our current studies contain 4 major findings, each addressing a key issue in GPIHBP1 physiology. First, GPIHBP1 binds LPL but not HL or EL, even though both EL and HL bind avidly to heparin. Second, the binding of apoAV to GPIHBP1 depends on interactions with GPIHBP1’s acidic domain. Ly6 mutations that abolish LPL binding do not interfere with apoAV binding. Third, the binding of chylomicrons to GPIHBP1-transfected cells is mediated by LPL. When LPL is absent, chylomicrons do not bind to GPIHBP1-expressing cells. Fourth, we found little or no binding of LDL, HDL, or apoE-DMPC disks to GPIHBP1-transfected cells.

Figure 6. Immunofluorescence microscopy assessing the binding of Dil-labeled chylomicrons, LDL, and HDL to GPIHBP1-expressing cells. A, CHL-11 cells, which produce only trace levels of LPL, were transfected with empty vector, S-protein–tagged wild-type GPIHBP1, or GPIHBP1-C88A. The cells were incubated with V5-tagged h-LPL, h-HL, m-HL, m-EL, or cell culture medium from nontransfected CHL-11 cells. B and C, CHO pgSA-745 cells were transfected with empty vector, S-protein–tagged GPIHBP1, an LDL receptor–green fluorescent protein (GFP) fusion protein21 (B), or an SR-B1–GFP fusion protein (C). GPIHBP1 was stained with an antibody against the S-protein tag (green), and lipases were detected with an antibody against the V5 tag (purple).
Acknowledgments
We thank Peter Tontonoz, MD, PhD, for the LDL receptor–green fluorescent protein construct; Mark Doolittle, PhD, University of California, Los Angeles, for providing an expression vector for a V5-tagged h-LPL; and John Brunzell, MD, University of Washington, Seattle, for providing 5D2, a mouse monoclonal antibody against bovine LPL.

Sources of Funding
This study was supported by the Western States Affiliate of the American Heart Association (Dr Beigneux); and grants R01 HL094732 (Dr Beigneux), R01 HL086683 and R01 HL073061 (Dr Ryan), and 1RC1 HL100008, P01 HL090553, and R01 HL087228 (Dr Young) from the National Institutes of Health.

Disclosures
None.

References
Binding Preferences for GPIHBP1, a Glycosylphosphatidylinositol-Anchored Protein of Capillary Endothelial Cells

Peter Gin, Anne P. Beigneux, Constance Voss, Brandon S.J. Davies, Jennifer A. Beckstead, Robert O. Ryan, André Bensadoun, Loren G. Fong and Stephen G. Young

Arterioscler Thromb Vasc Biol. 2011;31:176-182; originally published online October 21, 2010; doi: 10.1161/ATVBAHA.110.214718

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/31/1/176

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2010/10/21/ATVBAHA.110.214718.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Material

Binding Preferences for GPIHBP1, a GPI-Anchored Protein of Capillary Endothelial Cells

Peter Gin, Anne P. Beigneux, Constance Voss, Brandon S. J. Davies, Jennifer A. Beckstead, Robert O. Ryan, André Bensadoun, Loren G. Fong, and Stephen G. Young

Supplementary Figure Legends

Figure I. Native human LPL, but not human LPL that had been denatured by heat or guanidine hydrochloride, binds to soluble GPIHBP1 captured by antibody 11A12–coated agarose beads. V5-tagged human LPL (h-LPL) and soluble GPIHBP1 were added to antibody 11A12–coated beads. After washing the beads, the mouse GPIHBP1 (along with any bound LPL) was eluted from the agarose beads with 0.1 M glycine, pH 2.5. The starting materials, the unbound fraction, the three wash fractions, and the three elution fractions were assessed by western blotting with a rabbit polyclonal antibody against GPIHBP1 (red) and a mouse monoclonal antibody against the V5 tag (green). (A) Binding of nondenatured V5-tagged h-LPL to GPIHBP1. (B) Absence of h-LPL binding to GPIHBP1 after denaturing the LPL by heating to 50°C for 16 h. (C) Absence of LPL binding to GPIHBP1 after denaturing the LPL with 3 M guanidine hydrochloride for 16 h.
Figure II. Immunofluorescence microscopy showing that CHO cells expressing wild-type GPIHBP1 bind V5-tagged LPL avidly, while cells transfected with GPIHBP1 proteins carrying critical Ly6 domain mutations do not. CHO-K1 cells that had been transiently transfected with wild-type or mutant human GPIHBP1 proteins with key amino acid substitutions within the Ly6 domain (Q115P, C114A) were mixed with other cells that had been transiently transfected with V5-tagged human LPL. The next day, the cells were permeabilized and stained for GPIHBP1 with a goat antibody against the S-protein tag (red) and for LPL with a mouse monoclonal antibody against the V5 tag (green). LPL bound to cells expressing wild-type GPIHBP1 (note the “yellow” hue of the two GPIHBP1-expressing cells). The mutant GPIHBP1 proteins harboring Q115P and C114A substitutions did not bind LPL.

Figure III. Binding of apoAV–DMPC disks to wild-type and mutant GPIHBP1 proteins immobilized on antibody 11A12–coated agarose beads. ApoAV–DMPC disks and the soluble GPIHBP1 were added to antibody 11A12-coated beads. After washing the beads, the GPIHBP1, along with any bound apoAV, was eluted from the beads with 0.1 M glycine, pH 2.5. The starting materials, the unbound fraction, the three wash fractions, and the three elution fractions were assessed for GPIHBP1 (red) by western blotting with a rabbit polyclonal antibody against GPIHBP1 (A–D) or a rabbit antibody against the S-protein tag (E), and were assessed for apoAV (green) with a mouse monoclonal antibody against apoAV. (A) Binding of apoAV to wild-type soluble GPIHBP1. (B) Binding of apoAV to antibody 11A12–coated beads in the absence of GPIHBP1. (C) Binding of apoAV to soluble GPIHBP1-Q114P. (D) Binding of apoAV to soluble GPIHBP1-C81A. (E) Binding of apoAV to soluble GPIHBP1-D,E(38–48)A. Each of these mutations abolishes GPIHBP1’s ability to bind LPL.1–4
Figure VI. Assessing the ability of DMPC disks containing apoCIII or apoE3, and human HDL, to bind to soluble GPIHBP1 that had been immobilized on antibody 11A12–coated agarose beads. (A–C) Western blot studies of experiments in which soluble mouse GPIHBP1 (concentrated conditioned medium from cells that had been transfected with soluble mouse GPIHBP1) was mixed with DMPC–apoCIII or apoE disks, or human HDL and then incubated with agarose beads coated with antibody 11A12. After washing the beads, the mouse GPIHBP1, along with any bound apoCIII, apoE3, or apoAI, was eluted from the beads with 0.1 M glycine, pH 2.5. The starting materials, the unbound fraction, the wash fractions, and the elution fractions were analyzed by western blotting. (A) Assessing apoE binding to GPIHBP1. The western blot was probed with a rabbit antibody against GPIHBP1 (red) and a mouse monoclonal antibody against apoE (green). (B) Assessing apoCIII binding to GPIHBP1. The western blot was probed with a rabbit antibody against GPIHBP1 (red) and a mouse monoclonal antibody against apoCIII (green). (C) Assessing binding of human HDL to GPIHBP1. Western blots were probed with a rat monoclonal antibody against GPIHBP1 (red) and a rabbit polyclonal antibody against apoAI (green). (D) A similar experiment assessing binding of human HDL to GPIHBP1. Instead of performing a western blot, the polyacrylamide gel was stained with silver. The first lane shows multiple proteins in the concentrated conditioned medium of cells that had been transfected with soluble GPIHBP1, while the second lane shows strong apoAI staining in HDL. The third lane shows that most of the apoAI, along with most of the proteins in the conditioned medium, was found in the unbound fraction. Some apoAI was visible in the first wash fraction. Only soluble GPIHBP1, and no apoAI, was detectable in the first elution fraction.

Figure V. Apolipoprotein AV does not appear to block the binding of LPL to soluble
**GPIHBP1 captured by antibody 11A12–coated agarose beads.** V5-tagged human LPL (h-LPL, estimated to be ~10 ng), soluble GPIHBP1, and the indicated amounts of apoAV–DMPC disks were added to antibody 11A12–coated beads. After washing the beads, mouse GPIHBP1 (along with any bound LPL) was eluted from the agarose beads with 0.1 M glycine, pH 2.5. The starting materials, the unbound fraction, three wash fractions, and three elution fractions were assessed by western blotting with a rabbit polyclonal antibody against GPIHBP1 (red) and a mouse monoclonal antibody against the V5 tag (green). (A) Binding of V5-tagged h-LPL to GPIHBP1 in the absence apo-AV. (B) Binding of h-LPL binding to GPIHBP1 in the presence of 50 ng apo-AV. (C) Binding of h-LPL binding to GPIHBP1 in the presence of 5 mg apo-AV.

**Figure VI. Western blots of conditioned medium from CHO cells demonstrating that those cells synthesize and secrete LPL.** We examined conditioned medium from CHO-K1 cells transfected with a V5-tagged human LPL, nontransfected CHO-K1 cells, and nontransfected CHO pgsA-745 cells. Western blots were performed with a polyclonal goat antibody against mouse LPL,5 5D2, a mouse monoclonal antibody against bovine LPL,6, 7 and a mouse monoclonal antibody against the V5 tag.

**Figure VII. Western blot showing the presence of lipases in the cell culture media that were used in the immunofluorescence microscopy experiments in Figure 6A.** CHL-11 cells were transfected with V5-tagged human LPL (h-LPL), human HL (h-HL), mouse HL (m-HL), or mouse EL (m-EL). Conditioned medium was collected and lipase levels were assessed by western blotting with a mouse monoclonal antibody against the V5 tag.
Supplemental Methods

Binding of Apolipoproteins to GPIHBP1 with a Cell-free Binding Assay

Conditioned medium containing soluble mouse GPIHBP1 was mixed with 50 ng of apoAV–dymristoylphosphatidylcholine (DMPC) disks\(^8,9\) and antibody 11A12–coated agarose beads. After 2 h, the beads were washed and eluted as described in the Methods. The relative amounts of GPIHBP1 and apolipoproteins in the starting material, wash, and elution fractions were assessed by western blotting.\(^3\) We used the same western blot assays to assess the ability of soluble GPIHBP1 to bind to human HDL (10 µg) and DMPC disks containing the amino-terminal 22-kDa fragment of apoE (50 ng) or apoCIII (50 ng). Additionally, HDL binding to soluble GPIHBP1 was assessed by staining SDS-polyacrylamide gels with silver (SilverXpress, Invitrogen).

Constructing a Scavenger Receptor Class B Type I (SR-B1)–Green Fluorescent Protein (GFP) Fusion Vector

A plasmid vector encoding an SR-B1–GFP fusion protein was created by amplifying the SR-B1 open reading frame with forward primer 5′–GGACTCAGATCTCGAGATGGGCGGCAGCTCCAGGGC–3′ and reverse primer 5′–CCGCCTTACGGTGACCGACGCTTGTGCTTTGCAGCAGCACC–3′. To generate the SR-B1–GFP fusion construct, the amplicon was cloned into pEFGP-N1 with the In-Fusion Cloning Kit (Clontech).
References


IV

A

B

C

D

GPIHBP1  ApoE3-22 kDa  Unbound  Washes  Elutions

GPIHBP1  ApoCIII  Unbound  Washes  Elutions

GPIHBP1  HDL  Unbound  Washes  Elutions

GPIHBP1  HDL  Unbound  Washes  Elutions

ApoE3-22 kDa

ApoCIII

HDL

HDL
VII