Evidence for Two Distinct Binding Sites for Lipoprotein Lipase on Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein 1 (GPIHBP1)*

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GPIHBP1 is an endothelial membrane protein that transports lipoprotein lipase (LPL) from the subendothelial space to the luminal side of the capillary endothelium. Here, we provide evidence that two regions of GPIHBP1, the acidic N-terminal domain and the central Ly6 domain, interact with LPL as two distinct binding sites. This conclusion is based on comparative binding studies performed with a peptide corresponding to the N-terminal domain of GPIHBP1, the Ly6 domain of GPIHBP1, wild type GPIHBP1, and the Ly6 domain mutant GPIHBP1 Q114P. Although LPL and the N-terminal domain formed a tight but short lived complex, characterized by fast on- and off-rates, the complex between LPL and the Ly6 domain formed more slowly and persisted for a longer time. Unlike the interaction of LPL with the Ly6 domain, the interaction of LPL with the N-terminal domain was significantly weakened by salt. The Q114P mutant bound LPL similarly to the N-terminal domain of GPIHBP1. Heparin dissociated LPL from the N-terminal domain, and partially from wild type GPIHBP1, but was unable to elute the enzyme from the Ly6 domain. When LPL was in complex with the acidic peptide corresponding to the N-terminal domain of GPIHBP1, the enzyme retained its affinity for the Ly6 domain. Furthermore, LPL that was bound to the N-terminal domain interacted with lipoproteins, whereas LPL bound to the Ly6 domain did not. In summary, our data suggest that the two domains of GPIHBP1 interact independently with LPL and that the functionality of LPL depends on its localization on GPIHBP1.

Significance: The partition of LPL between the binding sites on GPIHBP1 may influence its function in lipoprotein metabolism.
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The binding of LPL to the N-terminal acidic domain was presumable, as LPL associates tightly with negatively charged biopolymers such as heparin, heparan sulfate, dermatan sulfate, and even polynucleotides (19, 20). The fact that heparin reduces the association of LPL with GPIHBP1 suggests that the binding sites for heparin/heparan sulfate and GPIHBP1 on LPL are partially overlapping. The GPIHBP1 binding site does not completely coincide with the heparin/heparan sulfate binding site, as the mutations C418Y and E421K in LPL do not affect heparin affinity but abolish binding to GPIHBP1 (21). A cluster of negatively charged amino acid residues in the N-terminal domain is invariably present in all known GPIHBP1 sequences, but the number of negatively charged residues varies from 17 to 22 between different mammalian species (22). The sequence variation and the high proportion of negatively charged residues of the N-terminal domain of GPIHBP1 raises questions about the role of the N-terminal domain for interaction with LPL. It has been speculated that this domain may function as a magnet to attract LPL at a long distance, whereas the more specific interaction may occur between LPL and the Ly6 domain (5, 18).

Several aspects of the LPL/GPIHBP1 interaction remain unclear. For example, what is the contribution of the two domains of GPIHBP1 to the interaction? Do the domains of GPIHBP1 act as two independent binding sites or as two regions of one binding site? Is the N-terminal domain just needed for tethering LPL to the more specific interaction with the Ly6 domain or does the N-terminal domain play a more specific role on its own? Can kinetic studies performed in vitro explain why LPL preferentially binds to GPIHBP1 and not to other polyanions like heparan sulfate? To address these questions, we have investigated the mechanism of the interaction of LPL with GPIHBP1 using surface plasmon resonance (SPR), fluorescence anisotropy, enzyme activity measurements, and a combination of chemical cross-linking with mass spectrometry. The experiments were performed with mouse GPIHBP1, the Ly6 domain mutant GPIHBP1 Q114P (mouse sequence) (6), the Ly6 domain (GPIHBP1 lacking the N-terminal domain), synthetic peptides corresponding to the N-terminal domain of GPIHBP1 from mouse, human, or bovine sequence (N-terminal peptide), and with bovine LPL.

Experimental Procedures

Reagents—Bovine LPL was purified from milk (23) and stored at −80 °C as a stock solution of 0.5–1 mg/ml in 1.5 M NaCl, 10 mM BisTris buffer, pH 6.5. Synthetic peptides corresponding to the N-terminal sequence of human, mouse, or bovine GPIHBP1 or a composition of human GPIHBP1 sequences were bought from GeneCust (Luxembourg) or Storkbio (Estonia). To allow specific modification by biotinylation or by DyLight 488 Sulphydryl-Reactive dye, an extra cysteine residue was added to the C-terminal end of the peptides. The sequences of the synthetic peptides were as follows: QQEEEEDEDEHPD DYYDEEDEDVEEEETC (human N-terminal peptide, human GPIHBP1 residues 23–51), AQEDGDADPEPENYDDDD DEEEEEETC (mouse N-terminal peptide, mouse GPIHBP1 residues 22–49), AQEDEDDDPAGREGYDDEDEEEEAA (bovine N-terminal peptide, bovine GPIHBP1 residues 22–47), HGPDDYDEDEDEDEVEEEETC (peptide 1, human GPIHBP1 residues 33–51), EEDDEDEVEEETC (peptide 2, human GPIHBP1 residues 40–51), EEEEEETC (peptide 3, human GPIHBP1 residues 45–51) and VDQDYDDHETEDDEEQQDPEEEEGEEEEC (peptide 4, a random sequence of human GPIHBP1 residues 23–51). The sequence of peptide 4 was generated using Microsoft Excel’s functions RAND and RANK. The synthetic peptides were biotinylated at their C-terminal cysteines in 20 mM phosphate buffer, pH 7.5, with 0.8 mM peptide, and 0.8 mM biotin maleimide (Sigma). The reaction mixtures were incubated at room temperature for 30 min. Unreacted biotin was removed by dialysis. Heparan sulfate was biotinylated at amino groups as previously described (24). Labeling of human N-terminal peptide by DyLight 488 Sulphydryl-Reactive dye (Pierce) was performed as follows: a 0.1 mM solution of the peptide in 20 mM phosphate, pH 7.2, was incubated with a 7-fold molar excess of DyLight 488 Sulphydryl-Reactive dye (8.8 mM stock in N,N-dimethylformamide) for 4 h at room temperature in the dark. Unreacted dye was removed with excessive washing on Amicon Ultra 3 MWCO Centrifugal Filter Units (Millipore). Extinction coefficients at 280 nm for determination of peptide/protein concentrations were as follows: LPL, 70440 m−1 cm−1; human N-terminal peptide, bovine N-terminal peptide, peptide 1 and peptide 4, 1480 m−1 cm−1; mouse N-terminal peptide, 2960 m−1 cm−1. The extinction coefficients were calculated according to Gill and von Hippel (25). Concentrations of LPL were calculated using its monomer molecular mass of 55 kDa.

A rat monoclonal antibody, 11A12, against mouse GPIHBP1 (16) was a generous gift from Dr. Stephen G. Young (University of California, Los Angeles). The epitope of this antibody is located at the C-terminal part of GPIHBP1 (16). VLDL and LDL isolated from human plasma by ultracentrifugation (26) was a kind gift from Dr. Elena Makoveichuk (Umea University). Heparin was obtained from LEO Pharma (Denmark). 1,2-O-Dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin) ester (DDGR, 30058 lipase substrate) was purchased from Sigma.

Expression of GPIHBP1, the Mutant Q114P, and the Cleavable Form of GPIHBP1—pTriEx4 plasmid, containing either sequence of mouse GPIHBP1 or the Q114P mutant without the GPI-anchor (residues 1–198) (16), was a kind gift from Dr. Anne Beigneux (University of California, Los Angeles). A thrombin recognition site was generated in the linker region of GPIHBP1 (amino acids 49–55 in the mouse sequence) using the QuickChange Lightning Site-directed Mutagenesis Kit (Agilent Technologies) according to the protocol from the manufacturer using the forward primer, GATGAAGAGGAGGAGGAGCCATAGTGCCCGGTGAGCAGGAGACCTTCTC and reverse primer, AGAGGTGCTCTGTCTCTTCGCCAAGGGCGACTAAGTGTCTCCTTCCTTCCATC. The mutation was verified by DNA sequencing using BigDye 3.1 with TriEx DOWN (TGGTATTGTGCTGCA) and TriEx UP (CAGTGGTATTTGTG) primers. The plasmid was amplified using DH5α cells and purified using the EZ.N.A.® Fastfilter Plasmid Midi Kit according to manufacturer’s protocol. CHO-K1 cells were grown in Ham’s F-12K (Kaighn’s) medium, supplemented with 10% (v/v) FCS and 1:200 penicillin. Cells were transfected using
Amaza® Cell Line Nucleofector® Kit T and a Nucleofector™ 2b Device (Lonza) according to the manufacturer’s protocol optimized for CHO-K1 cells. Proteins were expressed in ProCHO-AT medium in the presence of 5% medium (F-12K, 10% (v/v) FCS, 1:200 penicillin) and 1:200 protease inhibitor mixture (Sigma). Expression media were harvested and concentrated 10 times using Amicon Ultra 10 MWCO Centrifugal Filter Units (Millipore). Protein expression was verified by Western blot using 300 ng/ml of GPIHBP1 antibody 11A12, followed by detection with a 1:20000 dilution of horseradish peroxidase-conjugated goat anti-rat antibody. To obtain the Ly6 domain, the GPHBP1 variant incorporating the thrombin cleavage site was treated with thrombin (0.1 unit of restriction grade thrombin (Novagen) per 50 µl of concentrated expression medium) for 18 h at room temperature under shaking. The obtained protein was analyzed using Western blot under the same conditions as described above (data not shown).

**SPR Measurements**—SPR experiments were performed on a Biacore 3000 instrument using CM5 sensorchips (GE Healthcare). GPIHBP1, the Ly6 domain, or the mutant Q114P were bound to the surface of the sensorchip via antibody 11A12 that was covalently preimmobilized using the amino coupling kit (GE Healthcare). Biotinylated N-terminal peptide or biotinylated heparan sulfate was attached to covalently pre-bound NeutrAvidin (Sigma). The surface densities of non-covalently immobilized ligands were between 0.6 and 1.2 ng/mm², except for the determination of rate constants. Then the surface densities were lower, between 0.06 to 0.12 ng/mm², to avoid mass transport limited association and rebinding effects. Measurements were carried out at 4 °C in running buffer that contained 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, and the indicated concentrations of NaCl unless otherwise stated. Under these conditions LPL was stable and its nonspecific binding to the sensorchip matrix was low. For steady-state measurements sequential injections (120 µl or 60 µl, 20 µl/min) of LPL at increasing concentrations were made over the surface. In parallel, to evaluate nonspecific binding, the same solutions were injected over a surface with only the antibody or NeutrAvidin. For data analysis, nonspecific binding was subtracted. The sensorchip surface was regenerated after each injection using 1.5 M NaCl. When the Ly6 domain was used, the regeneration was not successful and the interaction was therefore studied using single cycle experiments. The amount of proteins and peptides at the sensorchip surface was calculated using the relation: 1 resonance unit corresponds to protein/peptide surface concentration (1 pg/mm²) (Biacore Assay Handbook). Bound LPL per ligand (mol/mol) was calculated based on the monomer molecular masses of the proteins, 55 kDa for LPL, 25 kDa for GPIHBP1, 21 kDa for the Ly6 domain, 3.8 kDa for biotinylated mouse N-terminal peptide, and 25 kDa for the mutant Q114P.

For the interaction between GPIHBP1 and LPL at 0.4 M NaCl, data were fitted to a two-binding site model (Equation 1),

\[
f = \frac{a \cdot L_0}{K_{d1} + L_0} + \frac{b \cdot L_0}{K_{d2} + L_0}
\]  
(Eq. 1)

where \(f\) is bound LPL per GPIHBP1 (mol/mol) at steady-state (equilibrium), \(L_0\) is the concentration of LPL in the injected solution, \(K_{d1}\) is the equilibrium dissociation constant for binding site 1, \(K_{d2}\) is the equilibrium dissociation constant for binding site 2, and \(c\) is the maximal binding capacity of site 2.

For the interactions of GPIHBP1 with LPL at 0.6 M NaCl, the Ly6 domain with LPL at 0.4 and 0.6 M NaCl, respectively, the mutant Q114P with LPL at 0.4 M NaCl and heparan sulfate with LPL at 0.4 M NaCl data were fitted using Equation 2.

\[
f = \frac{a \cdot L_0}{K_{d} + L_0}
\]  
(Eq. 2)

Data for the interaction of LPL with the mouse N-terminal peptide at 0.4 M NaCl were fitted using the Hill’s equation,

\[
f = \frac{a \cdot [L_0]^c}{(K_{d} + [L_0])^c}
\]  
(Eq. 3)

where \(c\) is the Hill coefficient.

**Determination of Kinetic Constants**—Kinetic data were analyzed using standard evaluation software from Biacore (BLAevaluation 4.0.1). For the interaction between binding site 1 of GPIHBP1 and LPL, studied at 0.4 M NaCl, a simple 1:1 Langmuir interaction model was used to calculate kinetic parameters. It was not possible to get reliable fits for the interaction between binding site 2 of GPIHBP1 and LPL studied under the same conditions. For the interactions of the N-terminal peptide with LPL, heparan sulfate with LPL and mutant Q114P with LPL at 0.4 M NaCl, a bivalent analyte interaction model was used that assumes that bivalent analyte A binds to monovalent ligand B (\(A + B \leftrightarrow AB; AB + B \leftrightarrow AB_2\); where A represents LPL and B represents immobilized ligand). In this model, \(k_{on1}\) and \(k_{off1}\) are the forward and reverse rate constants for formation of a 1:1 LPL-ligand complex, and \(k_{on2}\) and \(k_{off2}\) are the forward and reverse rate constants for binding of the LPL-ligand complex to another ligand molecule on the surface. For the interaction between LPL and the Ly6 domain at 0.4 M NaCl only the dissociation rate constant (\(k_{off2}\)) was determined experimentally. Data were analyzed using a monoexponential

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decay equation. The corresponding association rate constant was calculated from the equation 
\[k_{a} = k_{d}/K_{d}\]
where \(K_{d}\) is the equilibrium dissociation constant obtained from SPR steady-state analysis. Similarly, dissociation rate constants for the interactions of the Ly6 domain or the N-terminal peptide with 

**Stability Measurements of LPL Activity**—For this analysis 
LPL (10 nm) was preincubated alone or with the bovine N-terminal peptide at 25 °C in 20 mm HEPES, pH 7.4, in the presence of 0.15, 0.23, or 0.3 M NaCl. Remaining LPL activity at the indicated time points was measured using DGGR as a substrate as described previously (27). The curves for inactivation of LPL were analyzed by simple monoeponential decay kinetics. The obtained inactivation rate constants \(k_{i}\) were used for \(K_{d}\) determinations. At 0.15 M NaCl, the concentration of LPL was in the same order of magnitude as the N-terminal peptide. Equation 5 was used for calculation of the \(K_{d}\) values,

\[k_{i} = k_{0}(L_{0} + P_{0} + K_{d} - \sqrt{(L_{0} + P_{0} + K_{d})^{2} - 4L_{0}P_{0}}) + k_{x}\]  
(Eq. 5)

where \(k_{i}\) is the rate constant for inactivation of LPL, \(k_{0}\) is the rate 
constant for inactivation of LPL in the absence of peptide, \(L_{0}\) is 
the concentration of LPL, \(P_{0}\) is the concentration of the peptide, 
\(K_{d}\) is the equilibrium dissociation constant, and \(k_{x}\) is the 
rate constant for inactivation of the LPL-peptide complex.

At 0.23 or 0.30 M NaCl, the concentration of LPL was much lower than the stabilizing N-terminal peptide, and in these cases the \(K_{d}\) values could be calculated by Equation 6.

\[k_{i} = \frac{k_{0} \cdot K_{d}}{K_{d} + P_{0}} + k_{x}\]  
(Eq. 6)

**Fluorescence Anisotropy Measurements**—Fluorescence anisotropy experiments were performed on a Hitachi F-7000 (Hitachi High-Tech, Japan) fluorescence spectrophotometer. The excitation wavelength was 493 nm and the emission wavelength was 518 nm. Measurements were carried out at 4 °C in 20 mM phosphate, pH 7.4, 0.3 M NaCl. DyLight 488 maleimide-labeled N-terminal peptide (100 nm) was mixed with different concentrations of LPL. After binding equilibrium had been reached, within 90 s, fluorescence was measured when excitation and emission polarizer were oriented vertically and when excitation polarizer was oriented vertically and emission polarizer was oriented horizontally. From this data the fluorescence anisotropy was calculated using the Equation 7,

\[r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}\]  
(Eq. 7)

where \(r\) is fluorescence anisotropy, \(I_{\parallel}\) is the observed fluorescence intensity when the emission polarizer was oriented parallel to the direction of the polarized excitation, \(I_{\perp}\) is the observed fluorescence intensity when the emission polarizer was oriented perpendicular to the direction of the polarized excitation.

To calculate the corresponding \(K_{d}\) for this interaction, the dependence between the change of anisotropy and LPL concentration was fitted with Hill’s equation,

\[r = \frac{r_{\text{max}} \cdot [L_{0}]^{b}}{(K_{d})^{b} + [L_{0}]^{b}}\]  
(Eq. 8)

where \(r\) is the change of fluorescence anisotropy, \(r_{\text{max}}\) is the 
value of anisotropy of the complex of LPL with the N-terminal peptide, \(L_{0}\) is the concentration of LPL, \(K_{d}\) is the equilibrium dissociation constant, and \(b\) is the Hill’s coefficient.

**Chemical Cross-linking Combined with Mass Spectrometry**—
The bovine N-terminal peptide was chemically cross-linked to 
LPL using the homobifunctional amino reactive cross-linker 
dissuccinimidyl tartarate (DST) (Pierce). The reaction was 
carried out for 18 h at 4 °C in 20 mM HEPES buffer, pH 7.4, in 
the presence of 0.4 M NaCl. The concentrations of LPL and 
the N-terminal peptide were 7.3 and 73 μM, respectively. A freshly 
prepared stock solution of DST (2.3 mg/ml in dimethyl sulfoxide) 
was added to a final concentration of 300 μM. The reaction was 
stopped by adding 1 M ethanolamine-HCl, pH 8.5, to a final concentration of 50 mM. The mixture was subjected to SDS-PAGE on an 8% gel. Bands with molecular masses of ~59 and ~71 kDa that corresponded to the complexes of 
LPL-N-terminal peptide with stoichiometries 1:1 and 1:4, 
respectively, were excised. After reduction by DTT and alkylation by iodoacetic acid, the complex was in-gel digested by trypsin (28) and the extracted peptides were desalted using C18 StageTips (29). The cross-linked peptide mixture was separated on an Agilent 1200 series nano-LC with in-house packed (3-μm 100-Å ReproSil-Pur C18AQ particles, Dr. Maisch, Entringen, Germany) 15-cm 75-μm inner diameter (tip diameter 8 μm) 
emitter columns (New Objective, Woburn, MA) using 3–50% 
(90 min) separating gradient. Buffer A was 0.5% acetic acid in 
water and buffer B was 0.5% acetic acid in 80% acetonitrile 
(ACN). Separated peptides were eluted at 200 nl/min (nano-ESI 
spray voltage 2.0 kV) to an LTQ Orbitrap mass spectrometer 
(Thermo Electron, Waltham, MA) operating with a top-5 
MS/MS strategy. Briefly, one high-resolution MS scan 350– 
2000 m/z was taken with a resolution setting of \(r = 60,000\) at 
400 m/z, and the 5 most intense precursors were subjected to 
collision-induced dissociation fragmentation combined with 
Orbitrap MS/MS detection (\(r = 15,000\)). Full scan (target value 
1E6 ions) and MS/MS (3E4 ions) maximum injection times 
were 500 and 2,000 ms. Dynamic exclusion (list size: 500) was 
set to 90 s and only charge states \(+2\) were subjected to 
MS/MS. The cross-links were identified using pLink software 
(30). The search parameters were as follows: cross-linker, DST; 
enzyme, trypsin; maximum missed sites, 2; fixed modification, 
carbamidomethylation of cysteines; variable modification, oxida- 
tion of methionines, formation of pyrogallamate of an amino 
terminus glutamine, N-terminal protein acetylation; mass 
accuracy for precursor and fragment ions were 5 and 20 ppm, 
respectively.

**Homology Modeling of Bovine LPL**—A homology model of 
bovine LPL was obtained from the protein structure predic- 
tion service Phyre (31) using horse pancreatic lipase (32) as 
a template. The resulting structure was visualized using 
Chimera from the University of California San Francisco 
(33).
Results

**SPR Experiments for Studies of the Interaction between GPI-HBP1 and LPL**—Solutions containing different concentrations of LPL were injected to flow cells with immobilized GPI-HBP1, the mouse N-terminal peptide, the Ly6 domain, or the mutant GPI-HBP1 Q114P. For comparison, binding of LPL to heparan sulfate was performed under the same conditions. LPL associated with all the ligands, but the observed binding characteristics varied largely and were dependent on the concentration of NaCl. Binding curves for these interactions at equilibrium conditions and corresponding sensorgrams are presented in Figs. 1 and 2, respectively. We started the analysis with the equilibrium data at 0.4 and 0.6 M NaCl. In the case of the interaction of LPL with GPI-HBP1 at 0.4 M NaCl, a nanomolar high affinity binding and a micromolar low affinity binding was evident (Fig. 1A). The data were fitted to a two-binding site model (Equation 1), which assumed two independent LPL binding sites on GPI-HBP1. Calculated $K_d$ values for the sites differed more than 600-fold: 6.4 ± 1.3 nM for the high affinity binding site (site 1) and 4.2 ± 0.9 µM for the low affinity binding site (site 2). In contrast, data collected at 0.6 M NaCl could be fitted to a simple 1:1 binding model (Equation 2) with a $K_d = 16.0 ± 0.8$ nM. At this concentration of NaCl, the capacity of the immobilized GPI-HBP1 to bind LPL was reduced to the level comparable with the capacity of binding site 1 recorded at 0.4 M NaCl. This suggests that the binding of LPL to binding site 2, observed at 0.4 M NaCl, was not detectable at 0.6 M NaCl.

The high affinity of LPL for binding site 1 of GPI-HBP1 was not observed for binding of LPL to the N-terminal peptide when studied at 0.4 M NaCl. This binding became detectable when the concentration of LPL in the flow phase was in the micromolar range (Fig. 1B). The binding curve was slightly upward, suggesting cooperativity. Therefore, the data were fitted to the Hill’s equation (Equation 3). The obtained $K_d$ of 3.9 ± 0.1 µM was comparable with the binding of LPL to site 2 of GPI-HBP1 at 0.4 M NaCl ($K_d = 4.2 ± 0.9$ µM). No measurable binding of LPL to the N-terminal peptide was detected at 0.6 M NaCl, indicating a crucial role of ionic forces for the interaction.

Binding of LPL to the Ly6 domain was observed already at nanomolar concentrations of LPL and the corresponding binding curve was well described by a simple 1:1 model (Equation 2) at both 0.4 and 0.6 M NaCl (Fig. 1C). The $K_d$ values at these salt concentrations (93 ± 5 and 270 ± 30 nM, respectively) were 15-fold higher than that for the interaction of LPL with site 1 of GPI-HBP1. It should be noted that this difference in the $K_d$ values is not very significant. Addition of the thrombin recognition site into the sequence of GPI-HBP1 already lowered the apparent site 1 affinity for LPL by 5-fold ($K_d = 35$ nM, data not shown). Similar to the characteristics of binding site 1, the interaction of LPL with the Ly6 domain was only slightly influ-
enced by salt. The data therefore suggest that site 1 of GPIHBP1 corresponds to the Ly6 domain and site 2 to the N-terminal domain.

To further explore the contributions of different parts of GPIHBP1 to the interaction with LPL we used the Ly6 domain mutant GPIHBP1 Q114P (6). LPL bound to this mutant with much lower affinity than to site 1 of GPIHBP1 and more similar to the affinity of site 2 (Fig. 1 D). The affinity of the Q114P mutant for LPL at 0.4 M NaCl \( (K_d = 1.6 \pm 0.2 \mu M) \) was similar to that of LPL for the N-terminal peptide \( (K_d = 3.9 \pm 0.1 \mu M) \). In accordance, the Q114P mutant did not bind LPL at 0.6 M NaCl. These observations suggest that the Q114P mutant is unable to interact with LPL via the Ly6 domain.

For comparison we studied binding of LPL to immobilized heparan sulfate and found an affinity of 2.2 ± 0.4 \( \mu M \) at 0.4 M NaCl (Fig. 1 E). This is comparable with the affinity of LPL for the N-terminal domain of GPIHBP1.

**Effects of Heparin and the N-terminal Peptide on the Binding of LPL to GPIHBP1**—Based on the results in Fig. 1 we hypothesized that the interaction of LPL with GPIHBP1 includes two separate binding events: one that occurs to the N-terminal domain of GPIHBP1 and another that engages the Ly6 domain. To test this model, SPR displacement experiments were performed by injecting heparin (10 IU/ml) or the N-terminal peptide (100 \( \mu M \)) to the flow cells in which LPL was bound to immobilized GPIHBP1, the N-terminal peptide, or the Ly6 domain (Fig. 3). Heparin was able to partly dissociate LPL from GPIHBP1 (Fig. 3 A) and almost completely from the N-terminal peptide (Fig. 3 B). In contrast, heparin did not dissociate LPL from the immobilized Ly6 domain (Fig. 3 C). The N-terminal peptide behaved similar to heparin with regard to dissociation of LPL, but the effect was less pronounced (data not shown). These observations suggest that heparin and the N-terminal peptide were only able to elute LPL from the N-terminal domain of GPIHBP1.

The proposed two-binding site model was tested using competition experiments in which LPL was mixed with the competing N-terminal peptide before injection to the flow cells with immobilized GPIHBP1, N-terminal peptide, or the Ly6 domain. At a concentration of 100 \( \mu M \) the N-terminal peptide blocked about 75% of the association of LPL to immobilized N-terminal peptide (Fig. 4 B) but only 25% of the association of

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**FIGURE 2.** SPR sensorgrams that show binding of LPL to GPIHBP1 variants and heparan sulfate. Binding of LPL to GPIHBP1 (A), mouse N-terminal peptide (B), the Ly6 domain (C), the mutant GPIHBP1 Q114P (D), and heparan sulfate (E) at 0.4 M (left) or 0.6 M NaCl (right) is shown. In the case of the Ly6 domain, single cycle analysis was performed. Concentrations of LPL were as followed: 10 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 \( \mu M \), 1.5 \( \mu M \), 3 \( \mu M \), and 6 \( \mu M \) in A (left panel), all at 0.4 M NaCl; 1 nM, 2 nM, 4 nM, 8 nM, 20 nM, 40 nM, 80 nM, 200 nM, 500 nM, 1 \( \mu M \), and 4 \( \mu M \) in A (right panel), all at 0.6 M NaCl; 100 nM, 200 nM, 400 nM, 600 nM, 800 nM, 1 \( \mu M \), 1.2 \( \mu M \), 1.6 \( \mu M \), 2 \( \mu M \), 3 \( \mu M \), 4 \( \mu M \), 5 \( \mu M \), and 6 \( \mu M \) in B (left panel), all at 0.4 M NaCl; 100 nM, 200 nM, 400 nM, 800 nM, 1 \( \mu M \), 3 \( \mu M \), and 6 \( \mu M \) in B (right panel), all at 0.6 M NaCl; 1 nM, 4 nM, 10 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 \( \mu M \), 3 \( \mu M \), and 5.2 \( \mu M \) in C (left panel), all at 0.4 M NaCl; 10 nM, 20 nM, 40 nM, 80 nM, 180 nM, 360 nM, 720 nM, 1.44 \( \mu M \), and 4 \( \mu M \) in C (right panel), all at 0.6 M NaCl; 260 nM, 550 nM, 1.1 \( \mu M \), 2.1 \( \mu M \), 3.6 \( \mu M \), and 6.3 \( \mu M \) in D (left panel), all at 0.4 M NaCl; 550 nM, 1.1 \( \mu M \), 3 \( \mu M \), and 6.3 \( \mu M \) in D (right panel), all at 0.6 M NaCl; 29 nM, 146 nM, 760 nM, 1.1 \( \mu M \), 1.46 \( \mu M \), and 2.5 \( \mu M \) in E, all at 0.4 M NaCl.
LPL to GPIHBP1 (Fig. 4A) or the Ly6 domain (Fig. 4C). Interestingly, in the experiments with immobilized GPIHBP1 or the Ly6 domain, the kinetic constants were not affected by the presence of the peptide. More than 80% of LPL was calculated to be bound to the competing N-terminal peptide in the injection mixture when using the $K_d$ value of 22 $\mu$M that was obtained for the interaction of LPL with the N-terminal peptide by SPR in competition experiments at 0.4 M NaCl (Table 3). The results in Fig. 4 indicate that the soluble complex of LPL with the N-terminal peptide is able to associate with the immobilized Ly6 domain or GPIHBP1 via the Ly6 domain.

**Combined Analysis of Binding Kinetics Using SPR, Fluorescence Anisotropy, and Stabilization Experiments**—Due to high nonspecific binding of LPL to sensorchip matrix, it was not possible to use SPR at NaCl concentrations lower than 0.3 M. Therefore we studied effects of the N-terminal peptide on the stability of LPL by measurements of enzymatic activity for estimations of $K_d$ values for the interaction between LPL and the peptide. The N-terminal peptide stabilized LPL from thermal inactivation in a concentration-dependent manner (Fig. 5). The inactivation curves were fitted to the monoexponential decay equation. To derive the $K_d$ value, the dependence between inactivation rate constants for LPL and the concentrations of the N-terminal peptide were fitted to Equation 5. At 0.15 M NaCl, the $K_d$ value for the interaction between LPL and the N-terminal peptide was 2.0 ± 0.6 nM. Analogous experiments were done in a buffer containing 0.23 or 0.30 M NaCl (inactivation curves not shown). In these cases, higher concentrations of the N-terminal peptide were needed to stabilize LPL and $K_d$ calculations were performed using Equation 6. The corresponding $K_d$ values were 180 ± 14 nM and 2.4 ± 0.7 $\mu$M, respectively.
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The $K_a$ values for the interactions between LPL and the Ly6 domain and between LPL and the N-terminal peptide determined at different concentrations of NaCl were obtained by a combination of results from fluorescence anisotropy (Fig. 6), SPR (Fig. 1, Table 3), and measurements of LPL stabilization (Fig. 5). Results of this analysis are presented in Fig. 7. As indicated, binding of LPL to the N-terminal peptide was highly influenced by salt, whereas the interaction between LPL and the Ly6 domain was only slightly influenced by salt. To estimate the $K_a$ value for the interaction of LPL with the Ly6 domain at 0.15 M NaCl, the line of the relationship $-\log K_a$ versus $-\log[NaCl]$ was extrapolated to this salt concentration. The $K_a$ value was $\sim 45$ nM using this technique. The combined data analysis revealed that LPL binds to both the Ly6 domain and the N-terminal peptide with nanomolar affinity at 0.15 M NaCl. This affinity is comparable with the affinity of LPL for heparan sulfate (20).

To further characterize the interaction of LPL with GPIHBP1 and the GPIHBP1 domains, binding dynamics was examined by determination of rate constants, $k_{on}$ and $k_{off}$. For comparison, kinetic parameters were determined for binding of LPL to heparan sulfate and to the mutant GPIHBP1 Q114P. At 0.15 M NaCl, only $k_{off}$ values were determined by SPR. For these experiments, LPL was first associated with the immobilized ligands in the presence of 0.4 M NaCl, whereas the dissociation was monitored at 0.15 M NaCl. In the case of the N-terminal peptide, 10 IU/ml of heparan was injected during the dissociation phase to avoid rebinding of dissociated LPL to the immobilized N-terminal peptide (20). SPR sensograms and their analysis are presented in Fig. 8. Calculated parameters obtained in the presence of 0.4 or 0.15 M NaCl are summarized in Tables 1 and 2, respectively. As can be seen in Table 1, the N-terminal peptide/LPL and the heparan sulfate/LPL interactions had similar binding dynamics characterized by moderately fast association ($k_{on} \sim 10^4$ M$^{-1}$ s$^{-1}$) and fast dissociation ($k_{off} \sim 0.1$ s$^{-1}$) at 0.4 M NaCl. The interaction of the mutant Q114P with LPL was characterized by similar rate constants supporting our previous observations that the Q114P mutant interacts with LPL via the N-terminal domain. When SPR data were combined with measurements of stabilization of LPL activity at 0.15 M NaCl, very fast association rate ($k_{on} = 7.7 \times 10^7$ M$^{-1}$ s$^{-1}$) was estimated for the binding of LPL to the N-terminal peptide (Table 2). Because of a fast dissociation rate ($k_{off} = 0.15$ s$^{-1}$), resulting in a calculated average lifetime of 7 s for the N-terminal peptide-LPL complex, the affinity of this interaction was not significantly stronger than the interaction between LPL and the Ly6 domain. Thus, the high affinity for the binding of LPL to the N-terminal peptide is determined by the very high $k_{on}$ value. The complex between LPL and the Ly6 domain was formed more slowly and the proteins stayed together for a longer time. The determined off-rate values were similar at both concentrations of NaCl (0.4 and 0.15 M), giving an average lifetime of 9 min for the complex between LPL and the Ly6 domain (Table 2). However, the $k_{on}$ value over $10^4$ M$^{-1}$ s$^{-1}$ is still high, suggesting a diffusion-controlled association mechanism (34). Interestingly, the $k_{off}$ values for the interaction of LPL with binding site 1 of GPIHBP1 or the Ly6 domain at 0.4 M NaCl were similar ($\sim 10^{-3}$ s$^{-1}$), whereas $k_{on}$ values for association of LPL to site 1 of GPIHBP1 was measured to be 62-fold faster than that to the Ly6 domain ($k_{on} = 1.8 \times 10^6$ M$^{-1}$ s$^{-1}$ and $k_{on} = 2.9 \times 10^6$ M$^{-1}$ s$^{-1}$, respectively). This indicated that to some extent, the N-terminal domain influenced the interaction between LPL and the Ly6 domain.

Interaction with Lipoproteins—Previous studies have shown that LPL can interact with lipoproteins while being bound to GPIHBP1 (13, 35). Here we examined whether this important ability was affected by the type of complex LPL had formed with GPIHBP1. To minimize effects of triglyceride lipolysis by LPL, these experiments were first performed with triglyceride-poor, human LDL. GPIHBP1, the Ly6 domain, or the N-terminal pep-
**FIGURE 8.** Kinetic analysis of SPR sensorgrams. Presented are interactions between LPL and GPIHBP1 (A), LPL and mouse N-terminal peptide (B), LPL and the mutant GPIHBP1 Q114P (C), LPL and heparan sulfate (D), LPL and the Ly6 domain (E). In the case of the Ly6 domain, only the dissociation phase was analyzed. Black lines represent raw data, red traces are the fits to the experimental data curves. Kinetic rate constants obtained from these fits are given in Table 1. Concentrations of LPL were as follows: 50, 100, and 500 nM in A; 600 nM, 800 nM, and 1 μM in B; 550 nM, 1.1 μM, and 2.1 μM in C; 146 nM, 760 nM, and 1.1 μM in D; and 500 nM, 1 μM, and 3 μM in E. Measurements were performed at 4 °C in 20 mM phosphate, pH 7.4, 2 mg/ml of BSA, 0.4 M NaCl.

**TABLE 1**

| Ligand                  | \( k_{on} \) \( s^{-1} \) | \( k_{off} \) \( s^{-1} \) | \( K_d \) \( \text{M} \) | \( K_d \) \( \text{eq} \) |
|-------------------------|----------------------------|----------------------------|----------------|-----------------|
| GPIHBP1                 |                           |                           |                 |                 |
| Binding site 1          | (1.8 ± 0.8) × 10^6        | (7.1 ± 0.8) × 10^{-3}      | 4.0 nM          | 6.4 nM          |
| Binding site 2          |                           |                           |                 |                 |
| Ly6 domain              | (2.9 ± 0.7) × 10^4        | (2.7 ± 0.7) × 10^{-3}      | 93 nM           |                 |
| N-terminal peptide      | (4.8 ± 1.1) × 10^4        | 0.07 ± 0.02               | 1.5 μM          | 3.9 μM          |
| Heparan sulfate         | (2.9 ± 1.5) × 10^4        | 0.10 ± 0.07               | 3.4 μM          | 2.2 μM          |
| GPIHBP1 Q114P           | (4.9 ± 2.6) × 10^4        | 0.18 ± 0.03               | 3.7 μM          | 1.6 μM          |

**TABLE 2**

| Ligand                  | \( k_{on} \) \( s^{-1} \) | \( k_{off} \) \( s^{-1} \) | Average lifetime |
|-------------------------|----------------------------|----------------------------|-----------------|
| N-terminal peptide      | (7.7 ± 1.5) × 10^7        | 0.15 ± 0.03               | 7 s             |
| Ly6 domain              | (1.9 ± 1.1) × 10^{-3}     |                           | 9 min           |

**Addition of LDL to running buffer containing 0.15 M NaCl.** As can be seen in Fig. 9A, binding of LDL to the sensorchip containing the N-terminal peptide was markedly increased when addition of LDL to running buffer containing 0.15 M NaCl. As can be seen in Fig. 9A, binding of LDL to the sensorchip containing the N-terminal peptide was markedly increased when...
there was LPL present on the chip. In contrast, LPL did not stimulate binding of LDL to the Ly6 domain, but rather decreased the interaction (Fig. 9B). When complexed with GPIHBP1, LPL is distributed between binding sites 1 and 2. The occupancy of the sites depends on the concentration of LPL in the flow phase (Fig. 1A). Therefore it was possible to examine whether localization of LPL on GPIHBP1 affects binding of LDL. Calculations of binding site occupancy from the data in Fig. 1A and using Equation 1 indicated that the occupancy of binding site 2 on GPIHBP1 was increased from 13 to 42% when the concentration of LPL in flow phase was increased from 0.6 to 3 μM, whereas the occupancy of binding site 1 was near complete at both LPL concentrations. As can be seen in Fig. 9C, the increase of the occupancy of binding site 2 resulted in a more than 10-fold increased LDL binding. These observations indicate that when LPL is bound to site 2 of GPIHBP1, or to the acidic N-terminal peptide, the enzyme is able to simultaneously associate with LDL. Analogous binding studies were performed with VLDL. Unfortunately the data obtained with the Ly6 domain or GPIHBP1 were not completely reliable, although a similar tendency to the results of LDL was observed. The high nonspecific binding of VLDL to the immobilized GPIHBP1 antibody made interpretation of the data difficult. However, when LPL was bound to the N-terminal peptide, which was in turn immobilized via NeutrAvidin, the LPL/VLDL interaction was reliably detectable (Fig. 10).

Specificity of the Interaction between LPL and the N-terminal Peptide from GPIHBP1—The observations that LPL binds at two distinct binding sites on GPIHBP1, and that binding to site 2 in the N-terminal domain may be compatible with binding of LPL also to lipoproteins, whereas binding to the Ly6 domain is not, prompted us to focus on the interaction of LPL with the acidic N-terminal domain. To investigate the specificity of this interaction, experiments were performed with synthetic peptides that corresponded to various regions of the human N-terminal domain of GPIHBP1 and with a peptide that was composed of the same amino acids as the whole N-terminal domain but whose sequence was randomly generated. The affinity of these peptides for LPL at 0.4 M NaCl was studied in the solution by SPR competition experiments. Corresponding $K_d$ values are shown in Table 3. No significant differences in the $K_d$ values were observed for the human, bovine, or mouse N-terminal peptide, despite variations in their length and number of negatively charged residues. Deletion of 10 residues from the N-terminal end (peptide 1) slightly decreased the affinity in comparison with that of the intact peptide. Subsequent deletion of the next 7 residues (peptide 2) resulted in a 13-fold increased $K_d$ value. The shortest peptide tested contained 8 amino acid residues (peptide 3) and was unable to compete with the immobilized human N-terminal peptide, demonstrating that a longer stretch is required for formation of the binding site for LPL. The $K_d$ value for the interaction of LPL with the random-sequence peptide (peptide 4) was only 3-fold higher than that of the corresponding native peptide. The high affinity peptides (human, mouse, bovine, peptide 1, and peptide 4) with $K_d$ values lower than 70 μM shared similarities at their C-terminal parts. In contrast, the N-terminal parts of the peptides were different with regard to the number of negatively charged residues and dis-
played low sequence similarities. The observation that peptide 2 showed only 7–13 times lower affinity than the full-length N-terminal domain peptides suggests that the affinity for LPL is largely determined by the sequence stretch that corresponded to this peptide in the intact proteins (residues 40–51 in the human GPIHBP1 sequence). This assumption is supported by the reasonable sequence similarities in the respective regions in all high affinity peptides used in the current study. Because the affinity of peptide 1 almost matched that of the intact N-terminal peptides, we propose that the last 19 residues at the C-terminal part of the N-terminal domain (residues 33–51 in the human GPIHBP1 sequence) are crucial in the interaction with LPL.

**Chemical Cross-linking of LPL and the N-terminal Peptide**—To localize the binding region of the N-terminal peptide on LPL, chemical cross-linking was combined with mass spectrometry. The experiment was performed using the homobifunctional amino group-specific chemical cross-linker DST. Because the N-terminal amino group is the only reactive amine in this peptide, it was possible to localize its position at the surface of LPL. DST bridged covalent complexes between LPL and the N-terminal peptide resulting in molecular masses ∼59 or ∼71 kDa, corresponding to LPL monomers with one (1:1) or four (1:4) bound peptides (Fig. 11A). In the 1:1 complex several cross-linked lysines (residues 410, 417, 440, and 425 in bovine LPL) were identified in the C-terminal domain of LPL. Only two cross-linked lysines, at positions 275 and 303, were identified in the N-terminal domain of LPL. In the case of the 1:4 complex, also lysines 151, 263, 270, 295, and 444 were cross-linked with the peptide, in addition to the cross-linked residues of the 1:1 complex. The abundance of the cross-links with lysine residues in the C-terminal domain of LPL was higher than that of the N-terminal domain in all performed experiments, indicating a higher probability for formation of the complex in which the N-terminal amino group of the peptide is localized at the C-terminal domain of LPL. For localization of the cross-linked lysine residues on the three-dimensional structure of bovine LPL, we first modeled the structure of this lipase using the protein structure prediction service Phyre (31). The final LPL structure was very similar to that obtained in previous studies (36, 37). The cross-linked lysines on the three-dimensional model of bovine LPL are shown in Fig. 11B. As can be seen, all of the identified cross-linked lysines are located on the same side of the enzyme subunit. Based on the distances between the cross-linked residues on the model of LPL, and taken in account that the length of the spacer arm of the cross-linker is 6.4 Å, it was obvious that the peptide could be bound to LPL in various orientations. In addition to the cross-links between LPL and the peptide, we were able to detect several intramolecular LPL cross-links. All of them were consistent with the model of bovine LPL, if the distances between α-carbon atoms in the model were compared with the maximum cross-linkable distance (6.4 Å DST spacer arm + 2 × 6 Å lysine side chain = 18.4 Å). Identified intramolecular cross-linked lysine pairs and their α-carbon distances were Lys70–Lys77 (Ca–Ca 10.1 Å), Lys776–Lys303 (Ca–Ca 9.7 Å), Lys270–Lys307 (Ca–Ca 9.8 Å), Lys775–Lys307 (Ca–Ca 13.0 Å), Lys410–Lys417 (Ca–Ca 4.6 Å), and Lys431–Lys433 (Ca–Ca 7.0 Å).

**Discussion**

In the present study we demonstrate that LPL binds to two distinct binding sites on GPIHBP1: one within the acidic N-terminal domain and one within the Ly6 domain. The contribution of both GPIHBP1 domains for the interaction with LPL have been demonstrated in several previous studies (5, 16, 17), but we have here shown that the two domains act as two distinct and functionally different binding sites. Our measurements suggest that LPL can be independently bound to the N-terminal domain or to the Ly6 domain, whereas occupancy of either site does not prevent binding to the other site. This conclusion is based on several observations. The first was that the binding of LPL to wild type GPIHBP1 could only be fitted to a two-binding site model. The second observation was that a salt-dependent and salt-insensitive binding component could be clearly distinguished. Binding of LPL to the Ly6 domain appeared similar to binding to the salt-insensitive site of GPIHBP1 (binding site 1), whereas binding to the acidic N-terminal domain resembled binding to the salt-dependent site of GPIHBP1 (binding site 2). The third observation was that the capacity of wild type GPIHBP1 to bind LPL was about two times higher than that of the N-terminal or Ly6 domain. The fourth observation was that heparin could nearly completely dissociate LPL from the N-terminal peptide, but was totally ineffective with regard to release LPL from the Ly6 domain. In the case of the interaction with GPIHBP1, heparin eluted only a fraction of the bound LPL, indicating that the binding was not homogenous. The fifth observation was that while associated with the N-terminal peptide, LPL could still bind to the Ly6 domain and also to GPIHBP1. Moreover, the kinetic parameters of the interactions were not influenced by the presence of the peptide. We conclude that the previously observed lowered or abolished binding of LPL to modified variants of GPIHBP1, with one of the two domains mutated or deleted (5, 16, 17), was likely caused by a reduced number of binding sites leading to a reduced overall binding capacity, rather than a complete prevention of the interaction.

The observation that heparin efficiently eluted LPL only from the N-terminal domain of GPIHBP1 is intriguing. It is assumed, but seldom demonstrated, that heparin removes essentially all LPL from the capillary endothelium in tissues (1, 38). Determination of LPL activity in post-heparin plasma has been used for many years as a surrogate measure of the functional pool of LPL on the capillary bed in the body. Intravenous

### Table 3

**Equilibrium dissociation constants (K_d) for the interactions between LPL and different peptides as determined by competition experiments using SPR.**

| Peptide | Sequence | \( K_d \) (µM) |
|---------|----------|----------------|
| Human N-terminal peptide | QCQEEEEEEDDEHPDDYDEEEDECVEEEEETC | 18±3 |
| Peptide 1 | HPVDDYDEEEDECVEEEEETC | 32±5 |
| Peptide 2 | EDEEEDECVEEEEETC | 20±41 |
| Peptide 3 | EVEEETC | no binding |
| Peptide 4 | VQQQYEDHEDEEEDECVEEEEETC | 63±4 |
| Mouse N-terminal peptide | AQEQDAQPIPI PENNYDDDDDDDEEEETC | 22±2 |
| Bovine N-terminal peptide | AQEDEEDPPPDAAGFYDEEDEEEDETC | 23±5 |
administration of heparin into humans and animals releases LPL from the endothelial binding sites, and most likely also from the subendothelial tissue but at a slower rate, leading to more than a 100-fold increase of LPL activity in plasma (39). According to our study, heparin dissociates LPL only from the N-terminal domain of GPIHBP1 (binding site 2). This observation suggests that LPL is bound mainly to the N-terminal domain of GPIHBP1 at the luminal side of the endothelium. Interestingly, our data also indicate that only the fraction of LPL that is bound to the N-terminal domain of GPIHBP1 is able to interact with lipoproteins, as concluded from the binding studies with LDL. Analogous binding experiments with VLDL were possible to conduct only with the N-terminal domain. In the case of the Ly6 domain or wild type GPIHBP1, the data were difficult to interpret due to nonspecific effects. However, previous studies indicate that the nature of the interaction of LPL with LDL is similar to that with VLDL (40–42). It is therefore reasonable to assume that LDL can be used as a surrogate model for analysis of the interaction of LPL with apoB100-containing lipoproteins, but details in composition of lipids and apolipoproteins in the surface layer of the particles are likely to affect the binding parameters in each case.

From the data obtained by SPR, fluorescence anisotropy, and stabilization measurements, it can be concluded that LPL binds to both of the sites on GPIHBP1 with nanomolar affinity, but the nature of these interactions are quite different. Although LPL and the N-terminal domain formed a complex with a relatively short lifetime, the complex between LPL and the Ly6 domain formed more slowly, but stayed stable for a longer time. The average lifetimes were 7 s and 9 min, respectively. The high affinity of the binding of LPL to the N-terminal domain was explained by a very high $k_{\text{on}}$ value, which stems from the strong ionic interaction between the highly negatively charged peptide and the well known positively charged clusters on LPL. Accord-
ing to the concept of electrostatic steering, polyelectrolytic biopolymers with opposite charge attract each other from a long distance, resulting in a very high association rate (34). In contrast, the slower association of LPL with the Ly6 domain was relatively insensitive to the concentration of NaCl, and therefore not primarily electrostatic. This is in accordance with that hydrophobic residues in the Ly6 domain are important for the interaction with LPL, as previously demonstrated by site-directed mutagenesis (17). The observation that the affinity of LPL for the Ly6 domain was somewhat lower than its affinity for binding site 1 of the wild type GPIHBP1 (Ly6 domain in the intact protein) indicates that the two binding sites do not act completely independently and suggests cooperativity in their interaction with LPL. If cooperativity exists, binding of LPL to the N-terminal domain should increase the affinity of LPL for the Ly6 domain in the intact GPIHBP1. However, the LPL-N-terminal peptide complex and LPL alone bound to the Ly6 domain or GPIHBP1 with similar kinetic parameters. We can therefore exclude the possibility of cooperativity between the two domains. A more likely explanation is that the N-terminal domain influences the conformation of the Ly6 domain. An effect of the two domains of GPIHBP1 on each other was supported by our observation that insertion of the thrombin cleavage site between the domains slightly increased the $K_d$ value for binding of LPL to GPIHBP1. However, under non-equilibrium conditions, as in vivo, it is possible that long range electrostatic steering effects of the N-terminal domain increase the association rate of LPL for binding to the Ly6 domain.

Analysis of our SPR data suggested that the Q114P mutant (representing the mouse sequence) was unable to bind to LPL via the Ly6 domain, but that binding to LPL via the N-terminal domain was still possible. Previous studies in cell cultures have demonstrated that neither mouse GPIHBP1 Q114P nor the corresponding human GPIHBP1 Q115P showed detectable binding to LPL (6, 17). In contrast, the human mutants Q115A, Q115K, and Q115E bound to LPL as wild type GPIHBP1 (17). These observations, together with our results, suggest that the reduced affinity of the Q114P mutant for LPL is due to some conformational change that leads to a non-functional Ly6 domain. Also, a comparison by homology modeling of wild type GPIHBP1 and GPIHBP1 Q115P, using the protein structure prediction service Phyre (31), revealed that the structure of the Q115P mutant was different (data not shown). In the case of patients carrying the Q115P mutation, previous studies have shown that the amount of LPL in their post-heparin plasma is very low, demonstrating a crucial role of the Ly6 domain of GPIHBP1 in the transport of LPL to the luminal side of vascular endothelium (6). Together with our data this suggests that binding of LPL only to the N-terminal domain of GPIHBP1 is not sufficient to trigger the transport of LPL.

One question addressed in this study was what mechanism ensures the transfer of LPL from other extracellular binding sites, such as heparan sulfate proteoglycans, to GPIHBP1. It is possible that GPIHBP1 provides binding sites for LPL with considerably higher affinity than those of heparan sulfate or other extracellular ligands, but it is also possible that the distribution of LPL on the endothelium is controlled by kinetics. As discussed in a number of excellent recent reviews, association and dissociation rate constants are often more relevant parameters than equilibrium constants to describe interactions in vivo (34, 43). Our study demonstrates, however, that the interaction of LPL with GPIHBP1 is not stronger than the interaction with heparan sulfate. Even the association rates of these interactions were comparable. Furthermore, heparan sulfate proteoglycans can be assumed to be much more abundant on cell surfaces than GPIHBP1 and they should therefore be the dominating binding partner. To some extent, the interaction of LPL with heparan sulfate proteoglycans is similar to the interaction with the N-terminal domain of GPIHBP1. Both interactions are characterized by very fast association and dissociation rates. We had previously described that LPL dissociates very slowly from sensorchips with heparin or heparan sulfate (20) and the same appears to be the case with sensorchips with immobilized N-terminal peptide. In both cases, injection of free heparin in the flow phase caused a tremendous increase in the observed dissociation rate, demonstrating that, in the absence of free heparin, avid rebinding of LPL to the sensorchip surface is the reason of the slow dissociation.

Because the N-terminal domain of GPIHBP1 is highly negatively charged, and its sequence varies largely among species (22), it is reasonable to assume that the interaction between LPL and the N-terminal domain is not perfectly specific, even though the affinity is in the nanomolar range. However, based on comparison of the affinities of the N-terminal domain peptides, we propose that the last 19 residues at the C-terminal part of the N-terminal domain (residues 33–51 in the human GPIHBP1 sequence) are crucial in the interaction with LPL. The cross-linking data, demonstrating that the amino group of the N-terminal peptide could be attached at different positions on the LPL subunit, indicated various possibilities for formation of the complex. This is likely to be an additional reason for the fast association rate. However, the binding of the peptide to LPL was not random, because the peptide preferentially bound to LPL in the orientation where the N-terminal group of the peptide was identified in the C-terminal domain of LPL. Interestingly, LPL and the N-terminal peptide formed complexes with two stoichiometries 1:1 and 1:4. It is possible that formation of the cross-linked 1:4 LPL-N-terminal peptide complex is due to nonspecific interactions. However, we cannot rule out the possibility that the 1:4 complex has physiological significance. First, GPI-anchored proteins are usually clustered in lipid rafts of plasma membranes (44, 45). The N-terminal domains of closely located GPIHBP1 proteins may be able to simultaneously interact with LPL, leading to formation of up to 1:4 complexes. Second, it is noteworthy that we were able to detect formation of 1:1 and 1:4 complexes but not 1:2 and 1:3 complexes. Third, based on the results of binding studies (Table 3), the cross-linking experiments were performed under conditions where not all LPL molecules were associated with the peptide (about 80%) to prevent nonspecific effects of oversaturation. Usually nonspecific binding is clearly weaker than specific binding. According to LPL models, the enzyme has an asymmetrical charge distribution with four clusters of positively charged amino acid residues that form a large positively charged area opposite to the active site (36). It has been shown that this area is involved in the interaction with heparin (46–
All cross-linked lysines in the 1:1 and 1:4 complexes were located on this side of the LPL molecule. Thus, it is likely that these clusters are also involved in the interaction with the acidic N-terminal domain of GPIHBP1. More detailed cross-linking studies would reveal more precisely how the 1:1 and 1:4 complexes are formed.

All of our experiments were performed with bovine LPL purified from milk. This was because production of recombinant mammalian LPL in amounts sufficient for biophysical studies has not been successful so far. However, we are quite convinced that the results would not be different if LPL from other sources had been used. First, because the degree of amino acid sequence identity between LPL from different mammalian species is more than 90% (51), and second, because bovine LPL has successfully been used in numerous previous model studies. Regardless of their origin all tested LPL ligands, such as apolipoprotein CII, lipoproteins, lipoprotein receptor-related protein (LRP), angiopoietin-like proteins 3 and 4, and GPIHBP1 interact similarly with bovine LPL.

The main observations of this study are summarized schematically in Fig. 12 where the interaction modes under different circumstances are proposed. We have demonstrated that two domains of GPIHBP1 act as two largely independent units. Our data also suggest that the role of LPL in lipoprotein metabolism may depend on how the enzyme is located on GPIHBP1.

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