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Contribution of the Carboxy-Terminal Domain of Lipoprotein Lipase to Interaction with Heparin and Lipoproteins

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The C-terminal domain of lipoprotein lipase (LPL) is involved in several important interactions. To assess its contribution to the binding ability of full-length LPL we have determined kinetic constants using biosensor technique. The affinity of the C-terminal domain for heparin was about 500-fold lower than that of full-length LPL (K_d = 1.3 μM compared to 3.1 nM). Replacement of Lys403, Arg405 and Lys407 by Ala abolished the heparin affinity, whereas replacement of Arg420 and Lys422 had little effect. The C-terminal domain increased binding of chylomicrons and VLDL to immobilized heparin relatively well, but was less than 10% efficient in binding of LDL compared to full-length LPL. Deletion of residues 390–393 (WSDW) did not change the affinity to heparin and only slightly decreased the affinity to lipoproteins. We conclude that the C-terminal folding domain contributes only moderately to the heparin affinity of full-length LPL, whereas the domain appears important for tethering triglyceride-rich lipoproteins to heparin-bound LPL.

Key Words: **surface plasmon resonance technique; affinity constants; heparan sulfate; ionic interaction; mutants.**

Heparan sulfate proteoglycans play an essential role for binding of lipoprotein lipase (LPL) to the surface of the vascular endothelium. This is the place where the enzyme hydrolyses circulating lipoproteins (1, 2). Heparan sulfate is also involved in LPL-mediated binding and endocytosis of lipoprotein remnant particles by cells (3). Recent studies suggest that heparan sulfate/LPL complexes contribute to the uptake of atherogenic oxidized lipoproteins by macrophages (4–6).

According to the proposed three-dimensional models of LPL (7, 8), the enzyme can be divided into two distinct folding domains: the larger N-terminal domain containing the active site (residues 1–312) and the smaller C-terminal domain (residues 313–448). The binding of LPL to receptors such as the LDL receptor-related protein (LRP) appears to occur via interaction with segments in the C-terminal domain (9–11). On the other hand, the role of this domain for binding of LPL to heparin and to lipoproteins remains unclear. The purpose of the present study was therefore to elucidate the contribution of the C-terminal domain to the affinity for heparin and, when bound to heparin, its importance in tethering lipoproteins.

It has been proposed that the heparin-binding region of the LPL dimer is formed by four clusters of positively charged amino acid residues localized at different parts of the enzyme (1, 8). The clusters, brought together by folding of the enzyme, form a large positively charged heparin- binding area. Three of these clusters are continuous and located in the N-terminal domain. The fourth, non-continuous cluster is in the C-terminal domain. Site-directed mutagenesis has confirmed the importance of clusters 2 and 3 for heparin binding (12, 13).

A contribution of the C-terminal domain to the heparin affinity of LPL has been shown by studies of chimeric constructs of LPL and hepatic lipase (14, 15), by studies of the expressed recombinant C-terminal domain (11) and also by mutations in the C-terminal domain of avian LPL (16). The heparin affinity of the C-terminal domain, as determined by microtiter binding experiments, was shown to be comparable to the affinity of the LPL monomer (11). Candidate amino acid residues of human LPL involved in heparin binding are Lys319, Lys403, Arg405, and Lys407 (8, 16). It

Abbreviations used: LDL, low density lipoprotein; LPL, lipoprotein lipase; LRP, LDL receptor-related protein; RU, response units; VLDL, very low density lipoprotein.

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was recently proposed that the heparin-binding region in the C-terminal domain is necessary for the specific recognition of heparan sulfate (16).

Both folding domains of LPL appear to be involved in the interaction with lipids (14). It has been proposed that the lipid-binding region is opposite to the heparin binding area and is constituted of the lid region of the N-terminal domain and an exposed loop between the β-strands of the C-terminal domain involving amino acid residues 390–393 (17). The importance of the C-terminal domain for the lipid binding of LPL was demonstrated by limited proteolysis (18) and by studies of chimeras of LPL and hepatic lipase (14, 19). Specifically, it was shown that the cluster of tryptophanes 390, 393, and 394, located in the exposed loop region, play a role in the interaction with lipids (10, 20). Replacement of these tryptophanes by alanines reduced LPL's activity and affinity for lipoproteins (20). On the other hand, recombinant C-terminal domain bound to microtiter plates was shown to bind β-VLDL from cholesterol-fed rabbits with an affinity about 100-fold lower than that of the full-length enzyme (11).

In the present study we have used a quantitative approach for examination of the heparin- and lipoprotein-binding properties of the C-terminal domain using the Biacore biosensor system. We show that although the C-terminal domain interacts with heparin, its affinity is much lower than that for the full-length, dimeric LPL. We conclude that the C-terminal domain may contribute to, but it cannot be solely responsible for, the interaction of LPL with heparin. In addition, the results suggest that the C-terminal domain may play an important role in bridging of triglyceride-rich lipoproteins to heparin/heparan sulfate.

MATERIALS AND METHODS

Materials. Expression and purification of the recombinant proteins are described in a previous study (11). Bovine LPL was purified from milk (21). The amino coupling kit and CM5 sensor chips were from Biacore, Uppsala, Sweden. Streptavidin and avidin were from Sigma. Heparin was purchased from Novo Nordisk A/S. Heparan sulfate purified from umbilical cord endothelial cells was a kind gift from professor Lars-Åke Fransson (University of Lund, Sweden). Heparin and heparan sulfate were biotinylated according to methodology described in a previous study (22). The peptide LPL-(402–423) was synthesized by Sehaeffer-N, Denmark. Chylomicrons were isolated from rat lymph according to the previously described procedure (23). VLDL ($d < 1.006$ g/ml) and LDL ($1.03 < d < 1.04$ g/ml) were isolated from fresh human plasma by sequential ultracentrifugation (24). Biotinylation of VLDL and LDL was performed according to a previous study (25). Liposomes of egg yolk phosphatidylcholine were prepared and analyzed as previously described (26).

Determination of kinetic constants. Experiments were performed on a Biacore 1000 instrument (Biosensor, Uppsala Sweden). Streptavidin and avidin were coupled through amino groups to the activated carboxyl groups on the dextran matrix of CM5 sensor chips according to the procedures recommended by the manufacturer. After immobilization, the change in response, expressed in arbitrary units, RU (Response Units), ranged between 3000 and 5000. Biotinylated heparin was then bound to the covalently immobilized avidin or streptavidin and the proteins were injected over the layer at a constant flow rate (5 μl/min). Studies of protein/heparin interaction were performed in 10 mM Hepes, 0.15 M NaCl, pH 7.4, at 25°C. The protein concentrations varied between 1 nM and 20 μM. The effect of ionic strength on the protein/heparin interaction was studied by varying the concentration of NaCl in the binding buffer. After each binding experiment, the surface of the sensor chip was regenerated by washing with 1 M NaCl or 0.1% SDS.

To determine association and dissociation rate constants, k_{ass} and k_{diss}, respectively, the sensorgrams were analyzed using the BIA evaluation software. The equilibrium constant, K_d, was determined from the equation

$$R_{eq} = \frac{R_{max} \cdot C}{K_d + C},$$

where R_{eq} is the increase of the response values at equilibrium, R_{max} is the capacity of the immobilized heparin to bind a protein (the number of binding sites) expressed in response units and C is the concentration of the injected protein. Nonspecific binding was determined for each protein concentration by flow cells that did not contain immobilized heparin. The nonspecific binding, which ranged between 10 and 30%, was subtracted from the total binding. Competition studies with heparin fragments were performed according to a previous study (22). The data were analyzed by nonlinear regression using the FIG.P version 2.5 program (Biosoft, Cambridge, UK).

Fluorescence measurements. Measurements were performed on a Shimadzu spectrofluorophotometer model RF500. The experiments were done in 20 mM Hepes, 0.15 M NaCl, pH 7.4 at 25°C.

RESULTS

Interaction with heparin. To identify residues in LPL important for binding to heparin, we first tested the recombinant C-terminal folding domain of human LPL (LPL-(313–448)), the triple mutant (K403A/R405A/K407A) and the double mutant R420A/K422A of the same domain. Figure 1 shows that LPL-(313–448) bound to immobilized heparin. The triple mutant was essentially unable to bind, indicating that the basic residues K403, R405 and K407 were necessary. The double mutant R420A/K422A bound similarly to LPL-(313–448) at low protein concentrations, suggesting that R420 and K422 were not critical. The relationship of ΔR_{eq} versus concentration of LPL-(313–448) was, however, not a Langmuir-type hyperbola (Fig. 1). An apparent plateau was reached at 2–3 μM, followed by a further increase at higher concentrations of the protein in the flow phase. The R420A/K422A mutant bound in a similar way at low concentrations, but the binding increased even more at high concentrations. We believe that binding at high protein concentrations led to aggregation of the proteins on the surface of the sensor chip. In support of this hypothesis we found that at high protein concentrations SDS was needed to elute the bound proteins from the sensor chip. For protein concentrations lower than 3 μM, 1 M NaCl was sufficient for regeneration. Also, the association kinetics

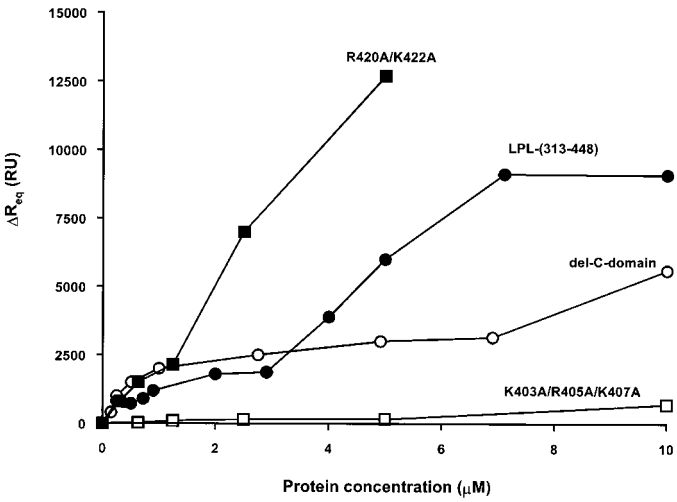

FIG. 1. Binding to heparin of the recombinant C-terminal domain of LPL (313–448) (●), the triple mutant K403A/R405A/K407A (□), the double mutant R420A/K422A (■) and the del-C-domain lacking amino acids 390–393 (○). Biotinylated heparin was immobilized to the surface of a sensor chip. The proteins were injected over the sensor chip, and relative responses (ΔR_{eq}) corresponding to the bound fraction at the equilibrium were determined for the protein concentrations indicated on the abscissa. The experiments were performed in 10 mM Hepes, 0.15 M NaCl, pH 7.4.

followed the simple bimolecular model A + B ⇄ AB only at low protein concentrations. In order to circumvent the problem of surface-induced aggregation, we constructed the deletion mutant, designated del-C-domain, lacking amino acids 390-393 (WSDW). We assumed that deletion of this hydrophobic, and presumably exposed segment (8) should decrease the aggregation on the sensor chip. Indeed, binding of the del-C-domain followed the simple bimolecular binding model up to 7 μM (Fig. 1). When only low protein concentrations (up to 3 μM) were used for affinity determination, the equilibrium dissociation constants were quite close for LPL-(313–448) and for the del-C-domain: 0.80 and 0.58 μM, respectively. We interpret the data at low protein concentrations to show similar heparin binding for LPL-(313–448), the R420A/K422A double mutant and the del-C-domain (Fig. 1). In the following experiments the del-C-domain was therefore chosen as a model to assess heparin-binding affinity of the C-terminal domain.

Figure 2A represents saturation curves for binding of the del-C-domain at different NaCl concentrations used for determination of the kinetic constants. The K_d values increased and the maximal number of binding sites on the immobilised heparin chains decreased with increasing NaCl concentration. The rate constants were determined from the association and dissociation curves obtained at the various concentrations of the del-C-domain for each NaCl concentration and compared with results obtained when using full-length, dimeric LPL. Table 1 shows the kinetic parameters for the del-C-domain, for full-length LPL dimer and also for the synthetic peptide LPL-(402–423) at 0.3 M NaCl. This NaCl concentration was chosen because the affinity of the LPL dimer is too high at lower concentrations of NaCl to be correctly determined by the Biacore system (22). Binding of the del-C-domain to heparin was characterized by a markedly lower association rate constant (k_{ass}) as compared with full-length, dimeric LPL. However, the absolute value for k_{ass} (3.0×10^4 M^{-1} s^{-1}) is still fairly high compared with most interactions between macromolecules. The synthetic peptide LPL-(402–423) had a much lower k_{ass}, 800 M^{-1} s^{-1}. The k_{diss} values were high (0.1–0.6 s^{-1}) and in the same order of magnitude for all of the studied proteins, in-

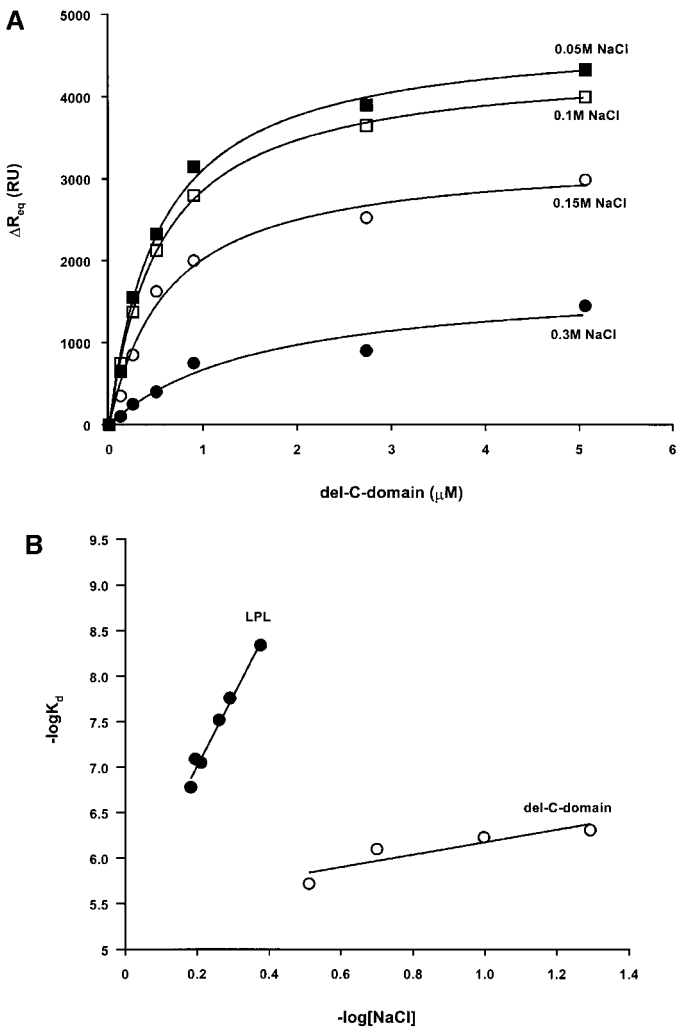

FIG. 2. Effect of NaCl on the interaction with heparin. (A) Different concentrations of del-C-domain were passed over the sensor chip and the binding at equilibrium was determined. Saturation curves for binding of the del-C-domain were obtained for different concentrations of NaCl, as indicated. (B) Comparison of the effect of [NaCl] on the heparin affinity of full-length dimeric LPL (●) and of the del-C-domain (○). The determination of K_d values is described in the text. The numbers of efficient ionic interactions were calculated from the slopes of the regression lines.

TABLE 1

Kinetic Constants Which Characterise the Interaction of Full-Length LPL of del-C-Domain and of Peptide 402–422 with Heparin

	K_d (M)	k_{ass} (M^{-1} s^{-1})	k_{diss} (s^{-1})	
			Determined	Calculated
Full-length LPL	$(3.3 \pm 0.5) \times 10^{-9}$	$(3.0 \pm 0.2) \times 10^{7}$	0.10 ± 0.03	0.10
Del-C-domain	$(1.6 \pm 0.4) \times 10^{-6}$	$(3.0 \pm 0.2) \times 10^{4}$	0.20 ± 0.05	0.05
Peptide (402–422)	$(7.5 \pm 0.9) \times 10^{-4}$	$(0.8 \pm 0.1) \times 10^{3}$	0.40 ± 0.15	0.60

Note. Conditions for the experiments: 10 mM Hepes, 0.3 M NaCl, pH 7.4, 25°C. The calculated k_{diss} represents $K_d \cdot k_{ass}$. Experimental data are expressed ± SD.

dicating that the interactions were controlled mainly by association.

The effect of NaCl concentration on affinity was used to estimate the number of ionic interactions involved in the association of the del-C-domain to heparin. This approach is based on the theory of macromolecule/polyelectrolyte interactions (27). According to this theory, Na^+ and other cations displace proteins that are bound to heparin via ionic interactions. A graph of log K_d versus log[NaCl] is linear with a slope indicating the number of ionic contacts. Figure 2B represents such a graph showing that the binding of del-C-domain was less sensitive to salt than binding of full-length LPL. The estimated number of efficient ionic interactions for the del-C-domain/heparin complex was 1–2 as compared to 9–10 interactions for the LPL/heparin complex.

Binding of the del-C-domain was also analyzed on a chip with immobilized heparan sulfate derived from endothelial cells and previously shown to bind full-length LPL with high affinity (22). It was not, however, possible to distinguish any specific binding of the del-C-domain from non-specific interaction. Thus, the affinity of the isolated C-terminal domain to these proteoglycans was too poor to be determined by the Biacore system.

Interaction with lipoproteins. Similar to full-length, dimeric LPL, both LPL-(313–448) and the del-C-domain increased binding of lipoproteins to immobilized heparin. Figure 3A demonstrates enhancement of chylomicron binding in the presence of LPL-(313–448). Other heparin binding proteins, like extracellular superoxide dismutase, antithrombin and protamin, did not increase the binding of lipoproteins (data not shown). The presence of these proteins at the surface even decreased the amount of bound lipoproteins. This demonstrated that the binding of lipoproteins to the heparin-bound C-terminal domain of LPL was specific. Figure 3B presents sensorgrams for binding of chylomicrons to immobilized heparin via the del-C-domain in an experiment performed at different chylomicron concentrations. The interaction of chylomicrons with del-C-domain/heparin was characterized by a high association rate constant ($k_{ass} = 3 \times 10^4$ M^{-1} s^{-1}) and by a low dissociation rate ($K_d = 10^{-4}$ s^{-1}). At comparable surface density (mol/mm^2), LPL-(313–448) and the del-C-domain were 2- and 3-fold less efficient, respectively, than full-length LPL in enhancing binding of chylomicrons (Fig. 4A). In the case of VLDL, the difference between LPL and the del-C-domain was almost the same as for chylomicrons (data not shown). However, the del-C-domain was less efficient in mediation of LDL binding (Fig. 4B). Compared with LPL, an approximately 10-fold higher surface density of the del-C-domain was required to obtain the same binding of LDL (Fig. 4B). We also compared binding of full-length LPL and the del-C-domain to VLDL and LDL, which were first biotinylated and immobilized to the sensor chip surface via streptavidin. The association rate constants were 1.8×10^6 M^{-1} s^{-1} and 2.1×10^6 M^{-1} s^{-1} with full-length LPL and VLDL and LDL, respectively. The del-C-domain associated with VLDL about 50 times more slowly than full-length LPL did. Furthermore, binding of the del-C-domain to LDL was so poor that it was not possible to distinguish from the non-specific interaction (not shown). We conclude from these results that the C-terminal domain, particularly when bound to heparin, is important for tethering lipoproteins, and that the domain appears to contribute more to the interaction with the large, triglyceride-rich lipoproteins (chylomicrons and VLDL) than to interaction with the smaller, cholesterol-rich LDL.

There are only two tryptophan residues in the del-C-domain, namely at positions 382 and 394. We tested whether these residues were involved in lipid binding of the del-C-domain by measurements of fluorescence using liposomes of phosphatidylcholine as a model system. The fluorescence intensity of the del-C-domain increased about 50% in the presence of liposomes. This increase accompanied by a blue shift of the emission maximum from 342 nm to 339 nm upon excitation at 280 nm (data not shown), indicated that when the del-C-domain bound to liposomes, at least one of the tryptophane residues was located in a more hydrophobic environment. In contrast to liposomes, heparin (100

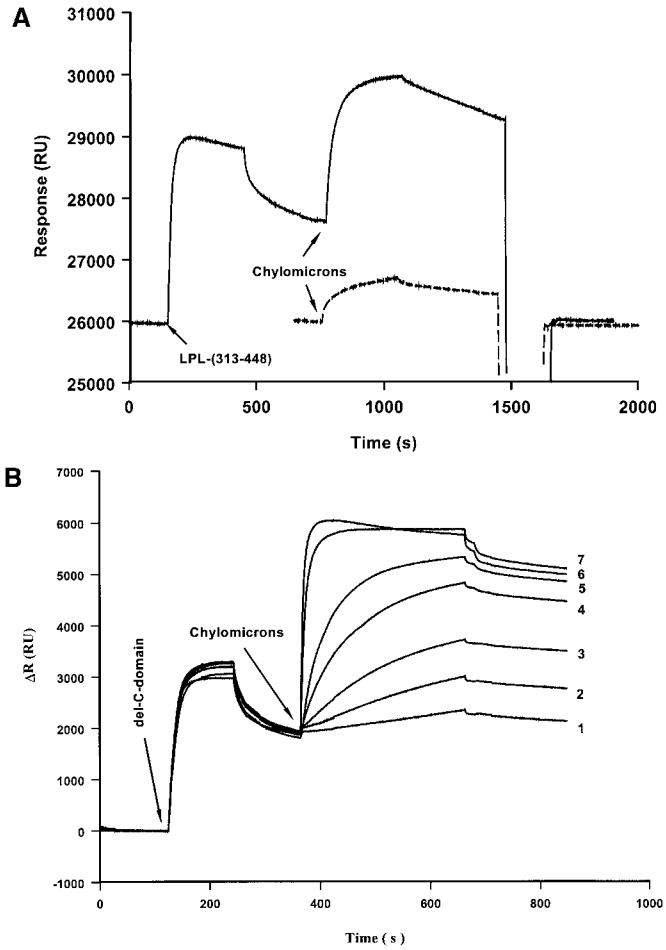

FIG. 3. Binding of chylomicrons to heparin complexes with the C-terminal domain (313–448) and del-C-domain. (A) Sensorgrams demonstrating that LPL-(313–448) increased the binding of lipoproteins to heparin. Binding of chylomicrons (0.3 mg triglycerides per ml) to heparin is presented in the presence (—) and in the absence of LPL-(313-449) (- - -). The arrows show starting points of injections. (B) Sensorgrams demonstrating binding of chylomicrons to the del-C-domain (injected concentration was 3.1 μM). At 390 s, chylomicrons were injected. Concentrations expressed in mg triglycerides/ml were: 1, 0.015; 2, 0.03; 3, 0.06; 4, 0.15; 5, 0.3; 6, 0.75; and 7, 1.5.

μM) had no effect on the fluorescence spectrum of the del-C-domain.

DISCUSSION

In most previous studies on heparin-binding proteins, the relative binding affinity was evaluated comparing the concentrations of NaCl required for elution of the proteins from heparin-Sepharose columns (1). According to the polyelectrolyte theory, which has been proven to describe properly the salt effects in protein/polyelectrolyte interactions, the sensitivity of an interaction to salt is determined by the number of ionic contacts formed between two interacting macromolecules (27). However, the concentration of NaCl required to displace a protein is not a thermodynamic characteristic. Therefore, data obtained in this way can only be used for a qualitative characterization of the interaction. Deviations between data from heparin-Sepharose chromatography and direct affinity measurements have been demonstrated in several previous studies (28–30).

In the present study we have compared binding affinities of LPL and fragments of LPL for heparin in terms of K_d, k_{ass} and k_{diss} values as measured by the Biacore system. We found that the C-terminal domain of LPL bound to heparin with an affinity comparable to those of several other heparin-binding proteins (1). The

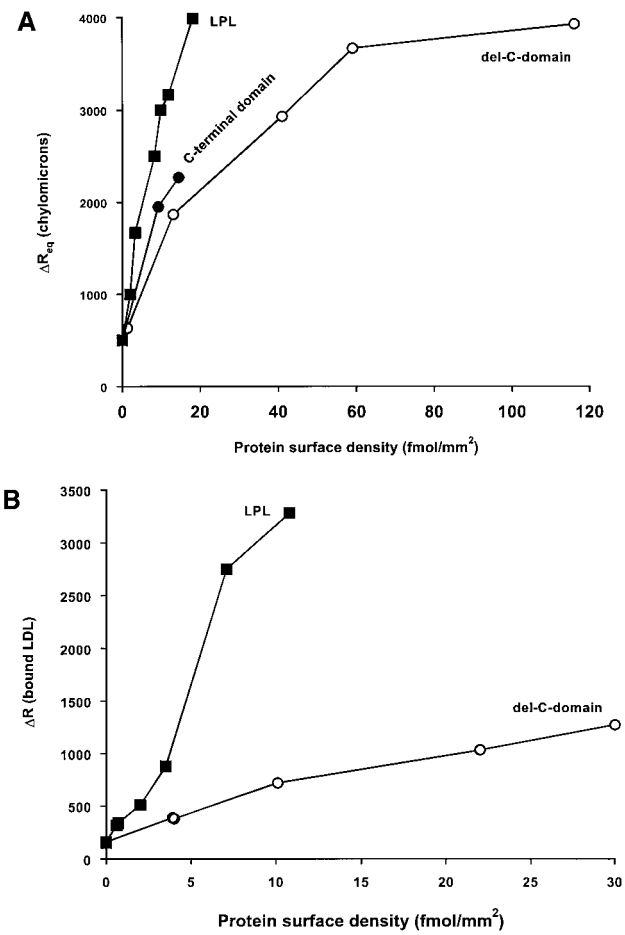

FIG. 4. Effect of surface density of full-length LPL, the C-terminal domain and the del-C-domain on the binding to chylomicrons and LDL. Different amounts of full-length LPL (■), the C-terminal domain (LPL-(313–448)) (●) and the del-C-domain (○) were first bound to heparin on the sensor chips. Note that the fragment LPL-(313–448) was tested only at moderate surface concentrations to avoid precipitation on the chip. (A) Binding of chylomicrons (0.5 mg triglycerides/ml in the flow phase). (B) Binding of LDL (corresponding to 0.1 mg protein/ml in the flow phase). The relative responses shown in the figure were determined close to equilibrium. Protein surface densities were calculated using MW = 110 kDa for dimeric LPL; and MW = 15 kDa for LPL-(313–448) and the del-C-domain.

interaction was, however, much weaker than that previously found for full-length LPL dimer (22). The lower affinity of the C-terminal domain was due to a much slower association rate than that of full-length LPL. Since, the association rate constant, k_{ass}, is proportional to the probability for two molecules to encounter in a proper orientation, this probability is higher when the interacting areas are larger. Therefore we interpret the data to show that the heparin-binding area of the C-terminal domain is significantly smaller than that of full-length LPL dimer. This hypothesis was supported by the observation that the C-terminal domain formed only 1–2 efficient ionic contacts with heparin, compared to 9–10 ionic contacts for the interaction between full-length, dimeric LPL and heparin (22). Assuming that both C-terminal domains in the LPL dimer contributed equally to the interaction with heparin, their contribution to the heparin-binding of the full-length LPL dimer could at the most be between 20 and 40%.

Based on studies of chicken LPL it was previously proposed that the C-terminal domain is responsible for the specific binding to heparan sulfate (16). Our present study does not support this hypothesis. In contrast, we found that the affinity of the C-terminal domain for endothelial heparan sulfate was very low and practically not measurable by the Biacore system. The cluster of positive charges on the C-terminal domain is probably not large enough to be properly attached to the relatively low-sulfated heparan sulfate chains, while this sulfation density is sufficient to bind to the full-length LPL dimer. It is possible that the C-terminal domain plays a more important role in binding of LPL to highly sulfated, more heparin-like, heparan sulfate proteoglycans found for example in the liver (1). The high affinity of full-length LPL for heparin and heparan sulfate is probably the result of a synergetic action of several negatively charged regions. The binding pattern may be different for different proteoglycans. It may therefore be impossible to define the most crucial cluster or amino acid residue in LPL for the interaction.

The heparin affinity of the C-terminal domain was completely wiped out when the positive charges on residues 403, 405, and 407 were neutralized by alanine exchange. According to the LPL model these amino acid residues may, together K319, K413, and K414, form the fourth heparin-binding cluster of the enzyme (8). A synthetic peptide corresponding to residues 402–423 of human LPL had about 100-fold lower affinity for heparin than the C-terminal domain, suggesting contribution of other residues of this region to the interaction, or emphasizing the importance of proper protein conformation.

Like full-length LPL, the C-terminal domain was able to simultaneously bind to heparin and to lipoproteins and thereby function as a bridge supporting binding of lipoproteins to heparin-covered surfaces. Thus, it is likely that the heparin bound C-terminal domain had an orientation/conformation similar to that of the heparin-bound, full-length LPL. This is in accordance with the LPL model, according to which the heparin- and lipoprotein-binding regions are on opposite sides of the C-terminal domain of LPL (8). The finding that the exposed loop, containing the cluster of tryptophanes, contributed little to the lipoprotein binding is in accordance with a previous study on LPL in which these tryptophanes were replaced by alanine residues (20). Very important for the study was, however, the finding that deletion of residues 390–393 (WSDW) apparently decreased the stickiness of the C-terminal domain, thus enabling binding studies on the Biacore. It was previously demonstrated that LPL truncated in the C-terminal domain, at residues F388 and W390, by chymotrypsin did not bind to lipoproteins or to the receptor LRP, while the heparin-binding ability was retained (18, 31). In this case, the cleavage in the exposed loop region may have induced a major conformational change in the C-terminal domain. This modification resulted in loss of the bridging function (31).

It was an intriguing observation that the C-terminal domain bound much stronger to chylomicrons and VLDL than to LDL. This is different from full-length LPL, which binds to these lipoproteins with similar affinity (25). In a previous study it was found that β-VLDL from cholesterol-fed rabbits bind to the C-terminal domain with an affinity several magnitudes lower than that of LPL (11). Taking these observations together, it seems that lipoprotein interaction with the C-terminal domain depends on the lipid composition of the lipoproteins. The lipid-binding site of the C-terminal domain may prefer triglyceride-rich lipoproteins, suggesting that the C-terminal domain can specifically interact with triglyceride monomers. This hypothesis is supported by the observation that residues in the C-terminal domain are involved in regulation of the substrate specificity of LPL (20). An alternative explanation is that the surface properties, such as lipid packing or surface pressure, of the triglyceride-rich lipoproteins may favor interaction with C-terminal domain. The important role of these physical properties for binding of proteins to lipid–water interfaces are previously well documented and discussed (32).

In summary, we have demonstrated that the C-terminal domain contributes moderately to the heparin affinity of LPL. Our data suggest that the C-terminal domain may be specially important for binding of triglyceride-rich lipoproteins to LPL, but the structures involved in this binding are not yet defined.

ACKNOWLEDGMENTS

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