A Carboxyl-terminal Fragment of Lipoprotein Lipase Binds to the Low Density Lipoprotein Receptor-related Protein and Inhibits Lipase-mediated Uptake of Lipoprotein in Cells*

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It has previously been shown that lipoprotein lipase can mediate uptake of remnant lipoprotein particles via binding to the low density lipoprotein-receptor-related protein/α2-macroglobulin receptor (LRP). Binding of lipoprotein lipase, and of triglyceride-rich lipoproteins associated with the lipase, to LRP depends on an intact carboxyl-terminal folding domain of the lipase (Nykjaer, A., Bengtsson-Olivecrona, G., Lookene, A., Moestrup, S. K., Petersen, C. M., Weber, W., Beisiegel, W., and Gliemann, J. (1993) J. Biol. Chem. 268, 15048-15055). Here we show that the site for binding to the receptor is within residues 389-425 of the bovine and residues 378-423 of the human lipoprotein lipase. We demonstrate that a carboxyl-terminal fragment of human lipoprotein lipase (residues 378-448), expressed as fusion protein in Escherichia coli, binds to purified and cellular LRP but not to lipoproteins. Binding of the fragment to purified LRP was blocked by heparin. In addition, the fragment inhibited the binding of lipase and the lipase-mediated binding of lipoproteins to the purified receptor. The fragment exhibited reduced binding to proteoglycan-deficient cells. Moreover, the fragment inhibited the uptake of lipoproteins in cells mediated by the lipase via binding to heparan sulfate proteoglycans and LRP. We conclude that the fragment contains the site for binding to LRP and a candidate site for interaction with heparan sulfate proteoglycans, whereas binding to lipoproteins is inefficient. The fragment can therefore inhibit the lipase-mediated lipoprotein uptake, a process that may promote the development of atherosclerosis when occurring in cells of the arterial wall.

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‡The abbreviations used are: β-VLDL, β-migrating very low density lipoproteins; α, amino acids; apoE, apolipoprotein E; HL, hepatic lipase; LDL, low density lipoprotein; LpL, lipoprotein lipase; LRP, LDL receptor-related protein/α2-macroglobulin receptor; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; h, human; b, bovine; CHO, Chinese hamster ovary; BioTyra, 2-(3-hydroxy-2-hydroxyethyl)-amino)-2-(hydroxyethyl)-propionate-1,3-diol; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Tricine, N-(2-hydroxy-1,1-bis[2-hydroxyethyl]ethyl)glycine; VLDL, very low density lipoprotein; FH, familial hypercholesterolemia.

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residues, and the overall homology is 93% between bLpL and human LpL (hLpL), which is 2 residues shorter at the amino terminus (22). Both bLpL and hLpL can mediate binding and uptake of β-VLDL in cells (4). Earlier studies established that chymotrypsin-truncated bLpL, comprising residues 1–390 (23), does not bind to LRP or β-VLDL (5). Purified chymotrypsin-truncated bLpL1–390 has full catalytic activity against soluble substrates, and the dimeric structure is maintained (23). This suggests that the lack of binding to LRP following the truncation was caused by deletion or disruption of a binding site for the receptor rather than a change in the overall structure of the lipase. We decided to identify the putative LRP binding region in the carboxy-terminal folding domain of bLpL. In addition, we wanted to express the corresponding fragment of hLpL, reasoning that a peptide capable of binding to the receptor, but not to lipoproteins, might inhibit the LRP-mediated uptake of lipoproteins via LRP.

MATERIALS AND METHODS

Lipases, LRP, and β-VLDL—The properties of the dimeric bLpL preparation purified from milk, as well as the iodination using lactoperoxidase and glucose oxidase, have been described previously (5). Monomeric bLpL was prepared by incubation with 1 m guanidinium hydrochloride for 3 h at 20°C (24). bLpL was purified from postheparin plasma by chromatography on heparin-Sepharose (4); separate preparations were tested, and one of them was a gift from Dr. J. D. Brunzell, University of Washington. Human hepatic lipase (hHL) was purified from postheparin plasma as described (25). In brief, fresh postheparin plasma was adjusted to 0.4 m NaCl and mixed with heparin-Sepharose for 2 h at 4°C. Following washes of the heparin-Sepharose, elution was performed with 0.7 m NaCl in 10 mM phosphate buffer, pH 7.4. hHL is eluted under this condition whereas hLpL is retained. To remove possible traces of hLpL in the eluate, the preparation was immunoprecipitated with rabbit IgG raised against bLpL (25). Then the eluate was adjusted to 0.2 m NaCl and applied to a Sepharose column (26). After thorough washing with 0.2 m NaCl, hHL was eluted with 0.9 m NaCl. The hHL preparation was enzymatically active (3.5 units/mg). The preparation was not entirely homogeneous on SDS-PAGE but showed two to three additional bands with lower molecular weights than hHL. To exclude the possibility that results obtained with the hHL preparation from plasma might be due to reaction of LRP with contaminating hLpL or fragments thereof, some experiments were performed with hHL isolated from the medium of the human hepatoma cell line HuH-27. The cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1 or 2 units of heparin/ml. The medium was applied to heparin-Sepharose High Trap columns (Pharmacia, Sweden), and hHL was eluted using 10 mM BisTris buffer, pH 7.4, containing 1 m NaCl and 10% glycerol, followed by precipitation by dialysis against 3.4 m ammonium sulfate. The eluted hHL was prepared from the medium of HuH-27 cells to contain hLpL as judged from lack of reactivity with the murine 5D2 monoclonal antibody. Chymotrypsin-truncated bLpL, comprising residues 1–390 (23), does not bind to LRP or β-VLDL (5). Purified chymotrypsin-truncated bLpL1–390 has full catalytic activity against soluble substrates, and the dimeric structure is maintained (23). This suggests that the lack of binding to LRP following the truncation was caused by deletion or disruption of a binding site for the receptor rather than a change in the overall structure of the lipase. We decided to identify the putative LRP binding region in the carboxy-terminal folding domain of bLpL. In addition, we wanted to express the corresponding fragment of hLpL, reasoning that a peptide capable of binding to the receptor, but not to lipoproteins, might inhibit the LRP-mediated uptake of lipoproteins via LRP.

**Expression of Fusion Proteins Containing bLpL Fragments**—Nucleotide sequences encoding residues 378–411, 378–423, or 378–448 of bLpL were amplified by polymerase chain reaction from pUC18 containing full-length bLpL cDNA (courtesy of Dr. R. Lawn, Stanford University Medical Center and Dr. W. K. Hayden, University of British Columbia) as a template using Taq polymerase and the oligonucleotide 5′-CACCAGTCTACGGAAGTGTGAGCCATAAGGAG as the NH2-terminal primer paired either with TTCAAGGTTACCGACTCTTATTTACG-3′, TTCAAGGCTATTTCCTCAGAGGAAG-3′, or TTCAAGGCTACTCCCTGTTTACTCT3′ as the COOH-terminal primer. The fidelity of the polymerase chain reaction-generated products was confirmed by DNA sequencing (34). The fragments were cut with BamHI and HindIII (Boehringer Mannheim) and subcloned into the Escherichia coli T1 expression vectors (35, 36) pT1,HFX (fusion protein designated F-LpL), pT,GFX (fusion protein C1-LpL), or pT,CRLpL (fusion protein C1443-LpL). HFX refers to the hexa-histidine- Factor X substrate sequence MSHG-SEGR. C14 refers to the NH2-terminal 30 as of the lambda C1, phage protein, and MLC refers to the NH2-terminal 116 as of chicken myosin light chain. Expression and purification of the fusion proteins were performed as described (19) except that 2 m glutathione/0.2 m oxidized glutathione was added to allow disulfide reshuffling. The eluted fusion proteins were dialyzed into the aqueous incubation buffer. The fusion proteins containing bLpL278–446 were iodinated as described for bLpL.

**Incubations**—Incubations of PVDF membranes with immobilized proteins and labeled ligands followed by autoradiography were performed as described (5). Most of the labeling was performed using 140 mM NaCl, 10 mM Hepes, 2 mM CaCl2, 1 mM MgCl2, 1% bovine serum albumin, pH 7.8 (buffer A). Immuno- blotting was performed as described (18, 30) using the monoclonal 5D2 antibody. Incubations with bLpL or LRP immobilized in microtiter wells (5) were performed in 100 ml of buffer A. HepB3 hepatoma cells, normal CHO cells, CHO cells (CHO mutant 745 (37)) deficient in proteoglycan content (courtesy of Dr. J. D. Easo, University of Alabama), and LDL receptor-deficient fibroblasts from a patient with familial hypercholesterolemia (French-Canadian mutation, courtesy of Dr. J. Davignon, IRCM, Montreal) were incubated as described previously (4) and details are provided in the legend to Fig. 9. Cross-linking of labeled bLpL and fusion proteins to cells and autoradiography of electrophoretically resolved proteins followed previously published procedures (4). For detection of LRP immunoreactivity, we used specific polyclonal rabbit anti-LRP antibodies (36) directed against a recombinant fragment of the α-chain (aa 2500–2592) (39).

**RESULTS**

**Identification of an LRP Binding Region in bLpL**—We first analyzed the binding of 125I-labeled purified human LRP and 125I-labeled rabbit β-VLDL to different mammalian lipases. As shown in Fig. 1A, 125I-LRP and 125I-β-VLDL bound specifically to immobilized (slot blotted) bLpL, i.e. the binding reactions were blocked by excess unlabeled ligand or excess bLpL in solution. Heparin and EDTA inhibited the binding of 125I-LRP, but not of 125I-β-VLDL, to the immobilized lipase. Monomeric bLpL, prepared by treatment of bLpL with guanidinium hydro-
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FIG. 1. Binding of labeled LRP and β-VLDL to lipases. About 1 pg of the indicated lipases (i.e., 10 pmol of the dimeric species: bLpL, monomeric full-length bLpL (m-bLpL), hHL, hLpL, or human pancreatic lipase (hPL)), were slot blotted onto PVDF membranes, incubated in buffer A for 16 h at 4 °C with either human 125I-LRP (50 pm) or rabbit 125I-β-VLDL (1 pg of protein/ml), and followed by wash and autoradiography. The additions were: heparin, 1 unit/ml; EDTA, 10 mM; bLpL, 900 ng; unlabeled LRP, 75 ng; unlabeled β-VLDL, 50 pg of protein/ml.

chloride (24), was capable of binding both ligands individually as described previously (5). bLpL truncated with chymotrypsin and purified to yield bLpL-1-380 (23) did not bind 125I-LRP or 125I-β-VLDL in agreement with previously published results (5). As shown in Fig. 1A, 125I-LRP as well as 125I-β-VLDL bound to immobilized hLpL, and binding of the labeled receptor to hLpL was inhibited by heparin as well as bLpL in solution (not demonstrated). We also analyzed binding to hHL due to its extensive homology with hLpL (40). Fig. 1B shows that 125I-LRP and 125I-β-VLDL bound specifically to immobilized hHL purified from postheparin plasma and that binding of 125I-LRP was inhibited by heparin and EDTA as well as bLpL in solution. Since a slight contamination of plasma-derived hHL with hLpL or hLpL fragments could not be entirely excluded, immobilized hHL purified from the medium of human HUH, hepatoma cells was also incubated with 125I-LRP, and the results were not distinguishable from those shown in Fig. 1B. We used immobilized human pancreatic lipase as a control, and binding of 125I-LRP was not detected (Fig. 1B).

To identify the LRP binding region, bLpL was subjected to fragmentation procedures. Fig. 2A shows the result of partial chymotryptic cleavage followed by electrophoretic separation and electroblootting of the fragments. 125I-LRP bound to a 13-kDa fragment identified as bLpL-1-214 by amino-terminal aa sequencing, but not to bLpL-215-425, which previously has been shown to be the main carboxyl-terminal fragment produced (23). The approximately 45 kDa band containing bLpL-1-380 did not bind 125I-LRP. Fragmentation was next performed using CNBr since residues 379 and 425 of bLpL are methionines. Incomplete cleavage was suspected since methionine 425 is followed by a serine residue. Fig. 2B, lane 2, shows binding of 125I-LRP to 5- and 8-kDa fragments and weak binding to a less abundant 20-kDa fragment. As shown in lane 3, the 8- and 5-kDa fragments also bound a monoclonal antibody, 5D2, reacting within residues 398–407 of bLpL (28). None of the fragments bound 125I-β-VLDL (lane 4).

The mixture following cleavage with CNBr was then chromatographed on heparin-Sepharose using a NaCl gradient (Fig. 3A), and the fragments in the consecutively eluted fractions 1–5 were resolved by electrophoresis, electroblootting, and incubated with 125I-LRP. As shown in Fig. 3B, the 8- and 5-kDa receptor binding fragments, which were eluted at 0.8–1.2 M NaCl, were identified by aa sequencing as bLpL-1-214 and bLpL-215-425, respectively. This suggested that an LRP binding site is within residues 380–425. The 18–20-kDa band identified as bLpL-118-288 (or bLpL-118-311) did not bind 125I-LRP in agreement with the result obtained with bLpL-1-380. We also sequenced the minor 30-kDa fragment capable of binding 125I-LRP since we were unable to detect reactivity with the 5D2 antibody, and LRP binding therefore might occur to a site not present within
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residues 380–425. However, the 30-kDa fragment was identified as bLpL118–450 (Fig. 3B). We ascribe the apparent lack of reactivity (cf. Fig. 2B) to the low abundance of the 30 kDa band as compared with the 8- and 5-kDa bands. We found that the 5D2 antibody not only inhibits lipase activity of LpL, perhaps by preventing its access to emulsified lipid substrates (41), but also inhibits the binding of 125I-LRP to immobilized bLpL. Prior binding of 125I-LRP to the weak 30 kDa band may therefore block binding of the 5D2 antibody. As shown in Fig. 3B, fragments containing peptides NH2-terminal to residue 311, as well as the fragment comprising residues 426–450 of bLpL, did not bind 125I-LRP.

The results show that the region comprising residues 380–450 of bLpL contains a binding site for LRP. Since bLpL1–390 is unable to bind to LRP even though its overall structure is maintained (23), the presence of additional LRP binding sites is highly unlikely. In addition, the results suggested that the LRP binding site is within residues 380–425 of bLpL. However, due to the qualitative nature of the blotting procedures, it was not possible to assess the affinities of bLpL380–450 and bLpL380–425. To address this question and in order to obtain material for functional studies, we expressed fusion proteins containing carboxyl-terminal fragments of hLpL.

**Binding of Fusion Proteins Containing hLpL Fragments to Purified LRP**—Fusion proteins containing hLpL378–448 (homologous with bLpL380–450), hLpL378–423 or hLpL378–411 were expressed in E. coli. Fig. 4 shows the purity of the preparations as evaluated by Tricine-SDS-PAGE and their reactivity with the 5D2 antibody. The fusion proteins were slot blotted onto PVDF membranes and, as shown in Fig. 5, 125I-LRP bound to the fusion proteins containing hLpL378–448 and hLpL378–423. Little or no binding was observed to hLpL378–411, and no binding could be detected to expression products not containing hLpL sequences. Heparin (1 unit/ml) abolished the binding to hLpL378–423 and hLpL378–448 in agreement with the previous observation that heparin affinity is partially linked with the carboxyl-terminal folding domain (14, 42). The 40-kDa α1-macroglobulin receptor-associated protein, previously reported to inhibit binding of bLpL to LRP (5), also inhibited the binding of 125I-LRP to hLpL378–448 (not shown).

The results obtained with the fusion proteins demonstrate the presence of a LRP binding site within residues 378–423 of hLpL in agreement with the results on the fragments of wild-type bLpL. Moreover, residues within aa 412–423 and, as deduced from the lack of 125I-LRP binding to bLpL392–450 (cf. Fig. 2A), residues within aa 378–390 of hLpL are necessary for the binding to LRP. Binding of 125I-β-VLDL was not observed to the fusion proteins (Fig. 5) in agreement with the results obtained with the fragments of bLpL.

**Inhibition of 125I-LRP Binding, but Not 125I-β-VLDL Binding, to bLpL**—In order to obtain estimates of the affinities for binding to LRP, we measured the abilities of the fusion proteins in solution to inhibit binding of 125I-LRP to bLpL immobilized in microtiter wells. Fig. 6A shows that the fusion proteins containing hLpL378–448 and hLpL378–423 had the same inhibitory potencies whereas the potency of hLpL378–411 was at least 25-fold lower. The concentration of monomeric bLpL causing half-maximal inhibition of 125I-LRP to the immobilized bLpL was similar to that of the fusion proteins containing hLpL378–448 or hLpL378–423. However, the inhibitory potency of dimeric bLpL was several-fold higher, possibly due to the interaction with two sites in LRP (5) or to a conformation of each site dependent on the dimeric structure. Monomeric bLpL was soluble in the aqueous buffer only to the concentration that could inhibit binding of 125I-LRP by about 50% (Fig. 6A), and experiments were performed in the presence of taurodeoxycholate (1 mM) to increase the solubility (Fig. 6B). Surprisingly, the detergent caused a 10–15-fold increase in the inhibitory potencies of dimeric as well as monomeric bLpL whereas the potency of hLpL378–448 remained unchanged. LpL has been shown to bind to deoxycholate (43), and the present results suggest that interaction of the detergent in regions within residues 1–378 of hLpL may cause an increase in its affinity for binding to the receptor. These experiments show that residues 423–448 are not important for the affinity of the fragment. The higher affinity of LpL, as compared with the fragment, appears dependent on its dimeric structure, and the affinity of LpL may be further increased by detergents such as bile acids.

Analogous experiments (not demonstrated) were performed using 125I-β-VLDL. The results showed that whereas 10 nM bLpL in solution inhibited binding of the labeled lipoprotein to immobilized bLpL half-maximally, no inhibition was obtained when using 2 μM fusion protein containing hLpL378–448. This result shows, together with the lack of binding of 125I-β-VLDL to fragment immobilized onto PVDF membranes, that hLpL378–448 binds poorly to β-VLDL.

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2 A. Nykjaer, unpublished observation.
Inhibition of LpL-mediated Binding of β-VLDL to Purified LRP—Since the aim was to use the fragment for inhibition of LpL-mediated binding of lipoprotein, we first analyzed whether hLpL 378-448 and hLpL 378-423 might mediate binding of 125I-β-VLDL to LRP immobilized in microwell plates. As shown in Fig. 7A, the presence of up to 4 µM fragment in the incubation medium did not cause significant binding of the labeled lipoprotein to the immobilized receptor whereas bLpL mediated the binding of 125I-β-VLDL to the receptor as reported previously (5, 6). The binding of 125I-β-VLDL was maximal at about 10 nM bLpL and half-maximal at about 3 nM bLpL, in close agreement with previously reported results using 125I-labeled normal human VLDL (6). As shown in Fig. 7B, the binding of 125I-β-VLDL mediated by 4 nM bLpL was essentially blocked by the fragment at concentrations of 1–2 µM, which will cause occupation of a large fraction of the immobilized LRP molecules, whereas the expression product not containing hLpL sequences had no effect. Thus, the fragment can bind to LRP and inhibit the LpL-mediated binding to the receptor, apparently because it binds poorly to lipoproteins.

Inhibition of LpL-mediated Binding and Uptake in Cells—We used the following cell types: Hep3b hepatoma cells since previous experiments with hepatic cells have shown cross-linking of 125I-bLpL to a large membrane protein compatible with LRP as well as bLpL-mediated β-VLDL binding and uptake (4); CHO cells since a mutated form not expressing proteoglycans is available (CHO mutant 745 (37)); and fibroblasts from a patient with homozygous familial hypercholesterolemia (FH) since they lack functional LDL receptors. We first used chemical cross-linking, followed by SDS-PAGE and electrophoretic transfer, to elucidate the nature of binding of bLpL and fragment to Hep3b cells. As shown in Fig. 8A, autoradiography (lane 1) and immunoblotting of the same filter using specific anti-LRP antibodies (lane 2) indicated cross-linking of 125I-LpL to LRP. Hep3b cells were then incubated with 125I-bLpL or 125I-labeled fusion protein containing hLpL 378-448 in the absence or presence of unlabeled bLpL followed by cross-linking. Fig. 8B shows that labeled bLpL and labeled fragment were cross-linked to a high molecular weight component of Hep3b cells, compatible with LRP, and that the binding of labeled fragment was inhibited by unlabeled bLpL. The results strongly suggest that hLpL 378-448, like bLpL, can be cross-linked to LRP following binding to cells, although some cross-linking to high molecular weight proteoglycans cannot be excluded.

The following experiments were designed to analyze the effect of unlabeled fusion proteins containing hLpL 378-448 on the LpL-mediated binding and uptake of β-VLDL in cells. In initial experiments (not shown) we measured the heparin-releasable binding at 4 °C of the 125I-labeled fragment to Hep3b cells and normal CHO cells and found a 75–85% inhibition by 1 µM unlabeled fragment as well as by 200 nM bLpL. This result confirmed the similar nature of the binding of bLpL and the fragment to the cell surfaces. Other experiments showed that the fusion proteins containing hLpL 378-448 and hLpL 378-423 (2 µM) did not mediate binding of 125I-β-VLDL in Hep3b cells and normal CHO cells, in agreement with the results obtained with purified LRP. By contrast, as demonstrated in Fig. 9, upper panel, hLpL 378-448 inhibited the LpL-mediated heparin-releasable binding of 125I-β-VLDL in normal CHO cells, and the specificity of the binding is shown by the inhibition in the presence of unlabeled β-VLDL. Since heparin-releasable binding may occur both to proteoglycans and to LRP, we performed parallel experiments (not shown) in proteoglycan-deficient CHO cells, and the results showed a 56–77% reduction in binding of labeled fusion proteins containing hLpL 378-448 in agreement with previous experiments using bLpL (44). These results are compatible with binding of the fragment to proteoglycans and partial competition with bLpL at this level in addition to the competition for binding to LRP. The LpL-mediated binding of 125I-β-VLDL was in different experiments reduced by 30–80% in the proteoglycan-deficient mutant CHO cells as compared with the normal CHO cells. The inhibition of LpL-mediated 125I-β-VLDL binding by the fragment was difficult to assess in the mutant CHO cells.
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VLDL binding by the fragment (1 ficient cell-associated radioactivity in the mutant cells, we found incubation at Moreover, the fragment inhibited the incorporation of in agreement with the inhibition of the LpL-mediated binding.

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The present results show that residues 378–423 in the carboxyl-terminal folding domain of hLpL contain a site for binding to LRP. This conclusion is based on the identification of the homologous LRP binding fragments of bLpL and on the binding of bLpL fragments expressed as fusion proteins as summarized in Fig. 10A. We propose that this region contains the only site for binding to the receptor for the following reasons. First, bLpL1–390 did not bind to LRP, indicating that regions NH2-terminal to residue 380 of bLpL (residue 378 of hLpL) do not interact with the receptor. Second, the apparent affinity of hLpL378–423 was identical with that of hLpL378–446, indicating the absence of accessory sites within residues 424–448 of hLpL. Third, the concentration required to inhibit binding of labeled LRP to immobilized blpL half-maximally was similar for the fragments and for monomeric bLpL in aqueous buffer. Finally, the binding of 125I-LRP to immobilized blpL was completely inhibited by the fusion proteins containing hLpL378–446 or hLpL378–423, demonstrating that only sites inhibitable by the fragment could cause binding of blpL to the receptor. However, the inhibitory potency of dimeric blpL was higher than that of monomeric blpL and the fragments. In addition, we observed that the addition of taurodeoxycholate caused an increase in the affinity of the full-length blpL (monomeric or dimeric) but not of the fragment. The mechanism of this effect remains to be elucidated. When taken together, the results indicate that full length dimeric blpL is required for optimal LRP binding affinity, even though the region comprising residues 378–423 of the hLpL monomer is likely to contain the only site interacting directly with the receptor.

After completion of this study, Williams et al. (46) reported that the carboxyl-terminal folding domain of hLpL (residues 313–448) binds to LRP and mediates binding of normal human VLDL particles to the purified receptor immobilized in microtiter wells. The result concerning location of the LRP binding site is in agreement with the present results as well as the previous observation that binding of blpL to LRP is dependent on an intact carboxyl-terminal folding domain (5). In contrast to the present results on hLpL378–446, blpL313–448 bound to li-

Fig. 6. Inhibition by the fusion proteins of 125I-LRP binding to immobilized blpL. A, blpL (approximately 250 fmol) was immobilized in microtiter wells, and the incubations were performed in buffer A for 16 h at 4 °C with dimeric blpL ( ), monomeric blpL (○), C1MLC-LpL378–446 (□), C1MLC-LpL378–446 ( ), C1MLC-LpL378–423 ( ■), F-LpL378–448 ( ), F-LpL378–448 ( ), F-LpL378–411 ( ), or C1MLC-LpL378–423 ( × ) at the concentrations indicated. B, the experiment (C1MLC-LpL378–423 not included) was performed the same as that shown in panel A, except that the incubation buffer contained 1 μg taurodeoxycholate. The binding of 125I-LRP in the absence of inhibitors (maximum binding) ranged between 25 and 30% of the added tracer. The points are the mean values of triplicates.

Fig. 7. Lack of mediation by the fusion proteins of 125I-b-VLDL binding to immobilized LRP and inhibition of the blpL-mediated binding. LRP (approximately 150 fmol) was immobilized in microtiter wells and incubated in buffer A with 125I-b-VLDL (0.5 μg of protein/ml) and fusion protein or blpL. A, incubations were performed with the indicated concentrations of C1MLC-LpL378–446 (crosses) or blpL either in the absence (closed circles) or presence (open circles) of heparin (10 units/ml). Experimental points obtained with C1MLC-LpL378–446 or C1MLC alone were superimposable on those obtained with C1MLC-LpL378–446. The displayed points are mean values of triplicates. B, incubations were performed in the presence of 4 μg blpL without further addition (hatched bar) or with C1MLC-LpL378–446 (filled bars), C1MLC-LpL378–423 (cross-hatched bars), or C1MLC alone (open bars). The LpL-mediated binding of 125I-b-VLDL without further addition is set at 100%. The bars represent the mean values of triplicates ± 1 S.D.
Figure 8: Cross-linking of labeled bLP L and labeled fusion protein to LRP in Hep3B cells. A, confluent Hep3B cells in 10 cm Petri dishes were incubated with 100 pm [125]bLP L (4 x 10^6 cpm/ml) for 60 min at 4°C. Following washes in phosphate-buffered saline, the cross-linking reagents (EDC-Sulfo NHS, Pierce) were added for 30 min at 22°C. The cells were scraped off, pelleted, and solubilized in the detergent Nonidet P-40 (1%) containing 50 mM dithiothreitol. The solubilized proteins were resolved by SDS-PAGE (5%), electrophoretically transferred to a PVDF membrane, and autoradiographed. The membrane was then incubated with the polyclonal rabbit anti-LRP antibody, followed by incubation with peroxidase-labeled goat anti-rabbit antibodies. Lane 1, autoradiogram demonstrating cross-linked [125]bLP L. Lane 2, peroxidase reaction indicating the location of LRP immunoreactivity. B, confluent Hep3B cells in 6-cm Petri dishes were incubated with labeled ligand for 60 min at 4°C, and cross-linking was performed as in A. The cells were solubilized in 20 mM ethylmorpholin containing 50 mM dithiothreitol and 5% SDS, followed by SDS-PAGE, electrophoretic blotting and autoradiography. Lane 1, 5 nm [125]bLP L (8 x 10^4 cpm/ml); lane 2, 62 nm [125]C-MMC-LP L (1.2 x 10^4 cpm/ml); lane 3, 190 nm [125]C,-LP L (1.2 x 10^4 cpm/ml); lane 4, as in lane 3 plus 1.3 pm bLP L.

poproteins and was capable of mediating their binding to LRP (46). However, whereas binding of [125]labeled human VLDL (6) and rabbit b-VLDL (present results) to purified LRP was mediated by bLP L in the concentration range of 1–10 nm bLP L, approximately 1000-fold higher concentrations of hLP L (5–448) (1–5 pm) were required to cause efficient binding of the lipoproteins to the receptor (46). The low potency of hLP L (5–448) in terms of mediading binding of lipoprotein to LRP may be due to a low affinity of this binding to the lipoproteins (46). Since we have previously reported poor binding of [125]labeled rabbit b-VLDL to bLP L (1–480) (5) and of [125]labeled rat chylomicrons to rat chylomicrons, it seems likely that regions both in the NH2-terminal folding domain (14, 46) and in the carboxyl-terminal folding domain are required for efficient binding of lipoprotein to LpL. In the present work, the absence of binding of hLP L (5–448) to lipoproteins, at concentrations of the fragment that essentially blocked the binding of bLP L to LRP in the solid phase assay, provided the basis for its inhibition of LpL-mediated binding of b-VLDL.

In addition to the LRP binding site, it is likely that hLP L (5–448) has a heparin binding site since heparin blocked the binding to LRP and since LRP does not bind heparin (30). This conclusion is supported by the observation that binding of the fragment was reduced in proteoglycan-deficient cells, and it is in agreement with previous observations on chimeras of hLP L and rat HL (42) as well as predictions from three-dimensional models of hLP L (47). It seems likely that part of the inhibitory effect of the fragment on the LpL-mediated binding of b-VLDL in cells may be due to competition for binding of LpL to proteoglycans. This would decrease the amount of LpL at the cell surface available for binding to lipoproteins as well as LRP and act in concert with the direct inhibition of binding of LpL to LRP. Since previous results have shown that the 40-kDa a-macroglobulin receptor-associated protein can inhibit LpL-mediated degradation of [125]labeled lipoproteins (5, 6), it is likely that uptake in fact largely occurs via endocytosis of LRP. However, it cannot be excluded that some LpL-mediated uptake of b-VLDL may occur via endocytosis of high molecular weight proteoglycans. Such a mechanism may depend on the cell type as was recently reported for LpL-mediated uptake of LDL. Thus, whereas LpL-mediated uptake of LDL appears to proceed via endocytosis of LpL in fibroblasts, uptake via endocytosis of proteoglycans may account for part of this uptake in THP-1 macrophages (48).

A Possible Basis for the Binding Properties of the COOH-terminal Fragment—Fig. 10A provides a comparison with the primary structures of the aligned (40) homologous regions in hLP L and hHL, which bind to LRP, and human pancreatic lipase, which does not bind. It should be noted that rat HL, which lacks heparin binding affinity in the COOH-terminal region (42), does not possess the sequence corresponding to hLP L residues 402–423 (identical in bLP L: XBBXXBXXBBXXBXX, where B

Figure 9: Inhibition by the fusion proteins of LpL-mediated binding and uptake of b-VLDL in cells. All incubations were performed in the presence of 2 nm bLP L. Binding or uptake without additions are set at 100% (n = 5). Upper panel, after incubation of normal CHO cells with [125]b-VLDL (1 pm protein/ml) for 30 min at 4°C and washes, the bound ligand was released with heparin (770 units/ml). The binding of [125]b-VLDL, without LpL estimated in parallel experiments (about 25% of the total binding) was subtracted. Depending on the b-VLDL and cell preparation, this contribution was 5, 16, and 363 ng/mg cell protein. Additions were: C,-MMC-LP L (7–448), 1 pm; C,-MMC, 2 pm; b-VLDL, 50 pm of protein/ml. Middle panel, for measuring uptake of [125]b-VLDL (2 pm of protein/ml), Hep3B cells were incubated for 90 min at 37°C and washed, and surface-bound ligand was removed by heparin followed by lysis of the cells. The uptake without additions ranged from 40 to 70 ng/mg cell protein. Uptake without LpL, measured in parallel experiments (29–50% of the total uptake), was subtracted. Additions were: C,-MMC-LP L (7–448), 2 pm; C,-MMC-LP L (7–448), 1 pm; C,-MMC, 2 pm. Lower panel, [14]C-oleate without LpL (11–36% of the total incorporation) was subtracted. This control esterification ranged between 0.09 and 0.9 nmol of cholesterol esterifying of cell protein. Error bars represent 1 S.D. (n = 3) or range (asterisk, * n = 2).
represents basic residues and X any residue. Such a sequence may constitute a binding site for heparan (47, 49). The LRP binding site and the putative heparin binding site may be overlapping since the motif of hLpL residues 404–412 (Fig. 10A, box), identical in human pancreatic lipase, but absent in hPL, is likely to participate in binding to LRP. Since basic residues are important for binding of several ligands to LRP (20, 39), it is of note that an 403–412 of hLpL (402–412 of hHL) comprise three lysine (K) or arginine (R) residues. In fact, Williams et al. (46) showed that mutation of lysine 407 to alanine reduced the affinity of hLpL by approximately 10-fold. Since no binding of bLpL392–450 could be detected, residues within the tryptophan-rich region hLpL378–396 (and a corresponding region in hHL) may participate in binding to LRP. Since hLpL378–411 showed greatly reduced affinity, the region comprising residues 412–423 of hLpL may help maintain the conformation of the residues binding directly to LRP. It should be noted that a disulfide bridge between cysteine residues 418 and 438 is not necessary for binding activity since hLpL378–411 showed the same apparent binding affinity as hLpL378–448 and since blocking of the cysteine residues of hLpL378–448 with iodoacetamide did not change the affinity.3

Inhibition of β-VLDL Uptake in Cells—Fig. 10B shows a possible model for the inhibition of lipoprotein uptake by the COOH-terminal fragment. The LpL dimer is drawn according to the proposed head-to-tail arrangement of monomers (41, 47). The upper panel shows association of lipoprotein, e.g. VLDL, to LpL bound to heparan sulfate proteoglycans at the cell surface. Some of the LpL molecules are thought to bind via the carboxyl-terminal domain since the fragment showed reduced binding to proteoglycan-deficient CHO cells and since it partially inhibited binding of LpL to normal cells. Other LpL molecules associated with VLDL bind to LRP, and multiple LpL molecules are thought to strengthen the binding (5). The lower panel illustrates that the fragment competes with LpL for binding primarily to LRP and in part to proteoglycans. Since the fragment does not bind to lipoprotein, it is unable to mediate uptake on its own but functions as an inhibitor of the LpL- and LRP-mediated lipoprotein uptake. The mechanism of inhibition of lipoprotein uptake described in this report may provide the basis for a new principle in treatment of atherosclerosis. This proposal is based on the results suggesting that the LpL-mediated pathway is important in foam cell precursors of the arterial wall (9, 10, 16, 17) whereas the hepatic clearance may depend more on uptake via LDL receptor as well as uptake via the LDL receptor (52). Future studies should include in vivo models to test whether such inhibitors may help prevent atherosclerotic lesions.

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