Not the mature 56 kDa lipoprotein lipase protein but a 37 kDa protein co-purifying with the lipase mediates the binding of low density lipoproteins to J774 macrophages

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Lipoprotein lipase (LPL) purified from bovine milk showed variable abilities to stimulate the binding of low density lipoprotein (LDL) to J774 macrophages. The presence of a 37 kDa protein in the LPL sample seemed to be of importance for its stimulatory capacity. In order to investigate this, we isolated LPL from bovine milk via heparin Sepharose chromatography using a continuous salt gradient. Fractions containing the 37 kDa protein (as shown by SDS/PAGE under reducing conditions) eluted first from the column, followed by the 56 kDa LPL protein. The LPL enzymatic activity co-eluted with the 56 kDa protein, whereas the amount of 37 kDa protein fully paralleled the stimulatory effect on the binding of LDL to J774 cells. Samples not containing the 37 kDa protein were far less effective in stimulating the binding. Western blotting using a monoclonal antibody 5D2 against amino acids 396–405 in the carboxy-terminal domain of LPL, showed that the 37 kDa protein may be the C-terminal domain of LPL, presumably generated by proteolytic degradation of the mature LPL protein by milk proteases during its isolation. Furthermore, the functional mass of LPL for stimulation of the binding of LDL, as determined by radiation inactivation, was shown to be 30.9 ± 1.8 kDa. We therefore suggest that cleavage of LPL at protease-sensitive sites causes a conformational change, generating an LPL protein which is more effective in mediating the binding and uptake of lipoproteins by cells.

INTRODUCTION

Lipoprotein lipase (LPL) is the major enzyme responsible for the hydrolysis of triglycerides in circulating lipoproteins. It also mediates non-enzymatic interactions between lipoproteins and heparan sulphate proteoglycans [1–3], thereby stimulating the binding and uptake of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) by different cell types [4–7]. LPL is also a ligand for the low density lipoprotein receptor-related protein (LRP) [8–11], the LDL receptor [12,13] and the VLDL receptor [14]. The carboxy-terminal, non-catalytic domain of LPL is thought to be important for LPL binding to either heparan sulphate proteoglycans or receptors [11,15,16], indicating that lipase activity is not required for proteoglycan- or receptor-mediated lipoprotein binding. This is also confirmed by Salinelli et al. [12], who reported that there is no correlation between bridge function and lipolytic activity.

Whether LPL should have the monomeric or dimeric form the LPL-mediated uptake of lipoproteins is still controversial. Krapp et al. [17] and Nykjær et al. [10,15] found that for this function LPL should be in the dimeric state, whereas Williams et al. [11] and Chappell et al. [16] proposed that LPL monomers are also able to mediate uptake of lipoproteins into cells.

The source of the LPL protein varies among different studies. In many reports bovine milk LPL was used [9–11,15–17], but human LPL [12,15–17], and bacterial lipase [10] have also been studied. It was found that the latter does not bind to either α_2MR/LRP or to β-VLDL [10].

In our laboratory, we make use of LPL purified from bovine milk as described previously [7]. The various LPL samples showed different abilities to stimulate the uptake of LDL by J774 macrophages, varying from less than 2-fold to as high as 50-fold at an LPL protein concentration of 5 μg/ml. It seemed that the stimulatory effect of LPL strongly correlated with the presence of a protein band of approx. 37 kDa, which variably co-purifies with LPL (56 kDa).

From our results we suggest that the 37 kDa protein represents a C-terminal part of the LPL molecule, having a conformation more suitable for bridging between lipoproteins and receptors or heparan sulphate proteoglycans than the whole LPL molecule.

EXPERIMENTAL

Cells

Murine macrophage-like J774 cells were cultured in 75 cm² flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 0.85 g/l $NaHCO_3$, 4.76 g/l Hepes, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. The cells were incubated at 37 °C in an atmosphere containing 5% CO_2 in air. For each experiment, cells were plated in 24-well plates. The cells were fed every 3 days, and used for experiments, with DMEM supplemented with 1% (w/v) bovine serum albumin (BSA) and further incubated with DMEM containing 5% (v/v) of lipoprotein deficient serum (LPDS) instead of FCS.

Lipoproteins

Blood was obtained from healthy volunteers. Serum was separated from the cells by centrifugation at 1200 ***g*** for 10 min. LDL was isolated from the serum by density gradient ultracentrifugation by the method of Redgrave et al. [18]. The protein

Abbreviations used: LPL, lipoprotein lipase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; LRP, low density lipoprotein receptor-related protein; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; BSA, bovine serum albumin; LPDS, lipoprotein deficient serum; PBS, phosphate buffered saline.

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content of the LDL fraction was determined by the method of Lowry et al. [19], after extensive dialysis against phosphate buffered saline (PBS) containing 10 μM EDTA. LDL samples were iodinated by using the method of Bilheimer et al. [20]. The specific activity ranged from 200 to 300 c.p.m./ng of protein. After iodination the LDL samples were dialysed extensively against PBS, pH 7.4, stored at 4 °C and used within 2 weeks. Whenever unlabelled LDL was used, dialysis against DMEM was performed at 4 °C.

Lipoprotein lipase

LPL was isolated from fresh bovine milk as described previously [7], with some minor modifications. In short, Heparin-Sepharose (CL-6B, Pharmacia) was equilibrated with 0.4 M NaCl and added to the skimmed milk. The mixture was shaken gently for 3 h at 4 °C, washed, and transferred to a column. After washing, 1.5 M NaCl, 10 mM KH_2PO_4, pH 6.8 was used as an eluent buffer at a flow rate of 1.5 ml/min. Protein-containing fractions were pooled and diluted with 10 mM KH_2PO_4, pH 6.8 to a final salt concentration of 1 M NaCl, 10 mM KH_2PO_4, pH 6.8. The pooled fractions were applied to a HiTrap Heparin affinity column (Pharmacia) equilibrated with 1 M NaCl, and eluted with a linear gradient of 1–1.7 M NaCl, 10 mM KH_2PO_4, pH 6.8; 1 ml fractions were collected at a flow rate of 1 ml/min. To determine the protein constituents, every fraction was submitted to SDS polyacrylamide gel electrophoresis (SDS/PAGE) using 4–20 % gels. Thereafter, as judged by the Coomassie Brilliant Blue stained gels, approximately every third fraction of the elution profile was pooled, and the protein was precipitated and collected as described [7]. After resuspension of the protein in 20 mM NaH_2PO_4, 50 % glycerol, the samples were again applied to SDS/PAGE. Proteins were either stained using Coomassie Brilliant Blue or transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) followed by incubation with either a polyclonal antibody against bovine LPL (dilution 1:500) or a monoclonal antibody 5D2 (prepared by Dr. J. Brunzell et al., University of Washington, Seattle [21] at a dilution of 1:250) to detect the protein bands corresponding to lipoprotein lipase. The 5D2 antibody is reported to react within residues 396–405 of the C-terminal folding domain of lipoprotein lipase [21]. As a secondary antibody either swine anti-rabbit or rabbit anti-mouse IgG conjugated to peroxidase (DAKO, Denmark) was used at a dilution of 1:1000. The bands were visualized by using 4-chloro-1-naphthol staining (Merck, Darmstadt, Germany).

Samples were reduced before being applied to SDS/PAGE, using a solution containing a final concentration of 1 % SDS, 2.5 % β-mercaptoethanol, 5 % glycerol and 0.0025 % bromophenol blue.

Lipoprotein lipase enzymatic activity in the different fractions was measured using a glycerol-stabilized glycerol [9,10 (*n*-^{3}H)]trioleate suspension as described by Nilsson-Ehle and Schotz [22].

Radiation inactivation of LPL

The functional molecular mass of LPL with respect to its bridging function was determined by radiation inactivation, a method for determining the functional size of a protein without the need for prior purification. LPL samples were irradiated at different doses as described by Schoonderwoerd et al. [23] and subsequently, the effect of the irradiation dose on the stimulatory effect of the LPL sample on the binding of LDL was tested in a binding experiment as described below. The fractional activity ($A/A0$) is the stimulatory capacity of the irradiated LPL samples (A), divided by the stimulatory capacity of the non-irradiated LPL samples ($A0$). For calculating the functional molecular mass (M), the ln ($A/A0$) is plotted against the irradiation dose. The relationship between the molecular mass (M) and the dose is given by the equation $M = c \cdot K \cdot St$, where $c = 6400$ kGy/kDa, $St = 2.88$ (correction factor for the irradiation temperature of -135 °C) and K = slope of the ln (fractional activity $A/A0$) versus dose plot [23].

Binding of ^{125}I-LDL to J774 macrophages

J774 macrophages were cultured in 24-well plates as described above. DMEM supplemented with 5 % (v/v) of LPDS instead of FCS was added to the cells 24 h before the start of the experiment. J774 cells were incubated for 3 h at 4 °C with 10 μg/ml of ^{125}I-labelled LDL, either in the absence or in the presence of 5 μg/ml of the different LPL fractions and either in the presence or in the absence of a 20-fold excess of unlabelled LDL. The receptor-mediated (specific) cell-binding was calculated by subtracting the amount of labelled lipoproteins that was cell-bound after incubation in the presence of a 20-fold excess of unlabelled lipoprotein (aspecific) from the amount of labelled lipoprotein that was cell-bound after incubation in the absence of unlabelled lipoprotein (total cell binding). After removing the medium, the cells were washed four times with ice-cold PBS containing 0.1 % (w/v) of BSA, and subsequently with PBS without BSA. Cells were then dissolved in 1 ml of 0.2 M NaOH. Protein content was measured according to Lowry et al. [19]. In an aliquot, the radioactivity represented the amount of cell-bound lipoprotein.

RESULTS

In Figure 1, we compared different LPL samples with regard to their pattern on SDS/PAGE and their stimulatory effect on the binding of LDL to J774 macrophages. As shown in Figure 1A, on SDS/PAGE gel some LPL samples contain almost exclusively the 56 kDa band (samples 4 and 5), whereas others display a major protein band of approx. 37 kDa in addition (samples 1–3 and 6). LPL samples containing the 56 kDa band only, stimulated the binding of ^{125}I-LDL to J774 cells to a very small extent (compare lanes 4 and 5 in Figures 1A and 1B). The ability of LPL to stimulate the binding of LDL to J774 cells seemed to depend on the presence of a protein of approx. 37 kDa co-purifying with the lipase (lanes 1–3 and 6).

In order to investigate the effect of the presence or the absence of this 37 kDa protein in LPL samples on the binding of LDL to J774 cells, we isolated LPL from bovine milk via heparin Sepharose chromatography. The heparin binding fraction was subsequently applied to a HiTrap Heparin affinity column and eluted using a continuous salt gradient. A representative LPL isolation is shown in Figure 2A. SDS/PAGE of fractions across the elution profile indicated several protein constituents (Figure 2B). In the fractions eluting first from the HiTrap column, a protein with an apparent molecular mass of about 37 kDa was present. Fractions eluting from the column at higher salt concentrations contained a major protein band of approx. 56 kDa. A minor 46 kDa protein and some low molecular mass proteins of about 15–20 kDa were also found. In Figure 2C it is shown that fractions containing the 56 kDa band displayed the highest LPL activity. The effect of the different LPL fractions on the binding of ^{125}I-LDL to J774 cells is shown in Figure 2D. Fractions containing only the 37 kDa protein (fractions 1–4) stimulated the binding of LDL to the cells up to about 25-fold. Furthermore, the stimulatory effect of the LPL sample corresponded roughly to the amount of 37 kDa protein in the sample, rather than to the 56 kDa protein.

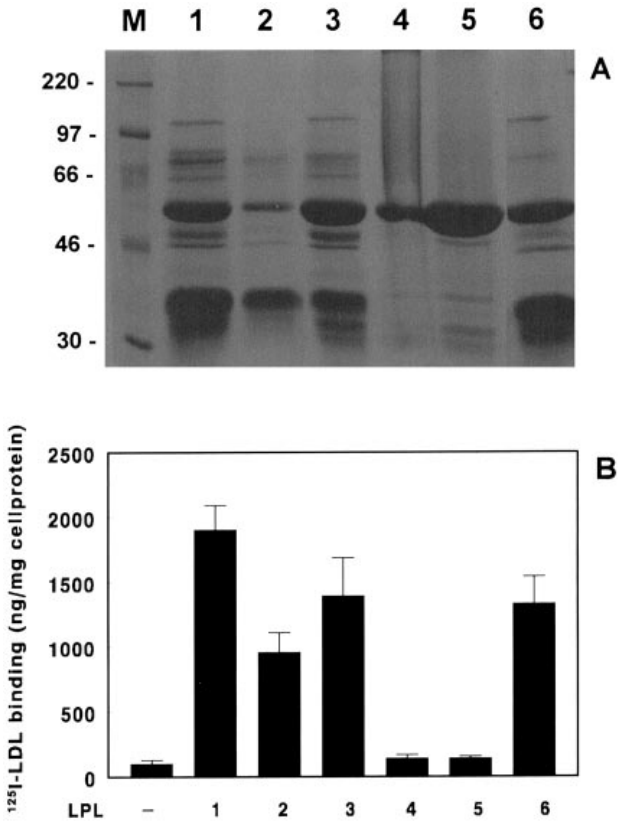

Figure 1 Effect of LPL samples on binding of ^{125}I-LDL to J774 macrophages

About 35 μg of protein was applied to 4–20% SDS-polyacrylamide gradient gels. After electrophoresis the gels were stained using Coomassie Brilliant Blue (**A**). M, Molecular mass marker; lanes 1–6, different LPL samples. The effect of the respective LPL samples on the binding of ^{125}I-LDL was measured after a 3-h incubation of the cells with 10 μg/ml of ^{125}I-labelled LDL at 4 °C in the absence or in the presence of 5 μg/ml of LPL (**B**). Binding is expressed as ng of ^{125}I-LDL per mg cell protein and was determined as described in the Materials and methods section. Values represent the mean ± S.D. of three measurements.

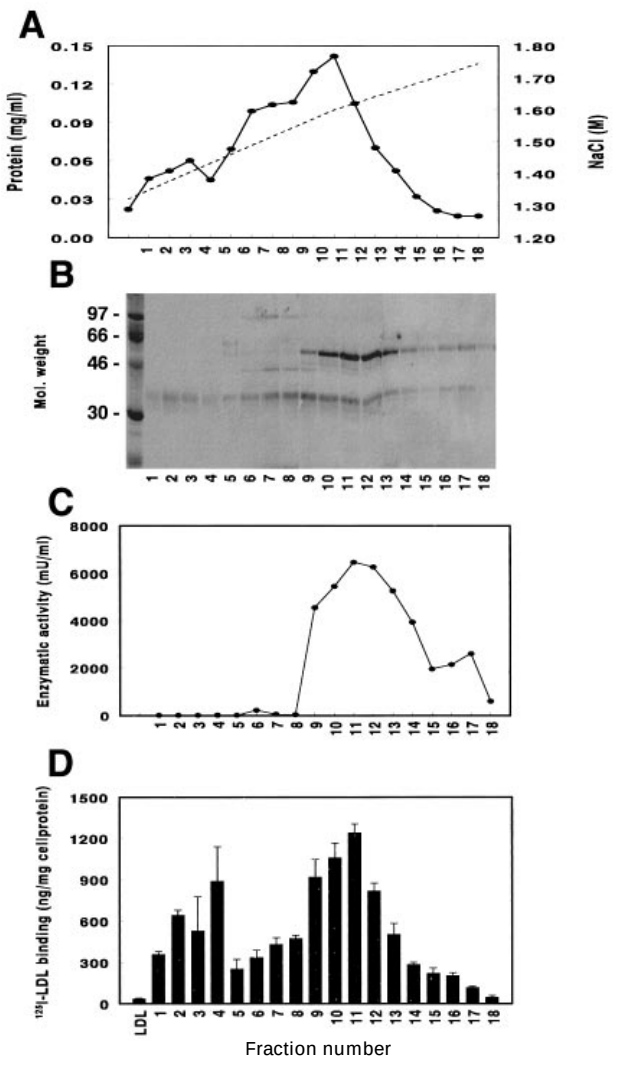

Figure 2 Chromatography on heparin-Sepharose, SDS/PAGE, enzymatic activity and effect on cellular ^{125}I-LDL binding of bovine lipoprotein lipase

Bovine skimmed milk was adjusted to 0.4 M NaCl and added to heparin-Sepharose. The mixture was shaken gently for 3 h at 4 °C, washed, and transferred to a column. After washing, protein was eluted, pooled and applied to a Hi-Trap Heparin affinity column as described in the Materials and methods section. Fractions were eluted with a linear gradient of 1.0–1.7 M NaCl, 10 mM KH_2PO_4, pH 6.8. Approximately every third fraction of the elution profile was pooled, and protein was precipitated and subsequently resuspended in 20 mM NaH_2PO_4, 50% glycerol. The protein content of the pooled fractions was measured according to Lowry as described in the Materials and methods section (**A**). Thirty microlitres of each LPL fraction was applied to 4–20% SDS-polyacrylamide gradient gels. After electrophoresis the gels were stained using Coomassie Brilliant Blue (**B**). Lipoprotein lipase activity was measured with a glycerol-stabilized glycerol [9,10] [*n*–^{3}H]trioleate suspension as described in the Materials and methods section (**C**). The effect of the respective LPL samples (30 μl/well) on the binding of [^{125}I]LDL (**D**) was measured as described in the legends for Figure 1. Binding is expressed as ng of ^{125}I-LDL per mg cell protein and was determined as described in the Materials and methods section. Values represent the mean ± S.D. of three measurements.

To find out whether the 37 kDa protein is (a part of) LPL, Western blotting of fractions from another LPL-isolation was performed, using a polyclonal antibody against bovine milk LPL (Figure 3A). The 37 kDa protein bound to this antibody and it is shown that the stimulatory effect of an LPL fraction on the binding of ^{125}I-LDL to J774 cells, corresponded dose-dependently to the 37 kDa protein and not to the 56 kDa protein (Figure 3B).

Since it is known that binding sites for lipoproteins and receptors are within the carboxy-terminal domain of LPL [10,11,13,16], we hypothesized that the 37 kDa protein may be the C-terminal part of LPL. To test this hypothesis, we performed Western blotting using the monoclonal antibody 5D2, which is reported to react within residues 396–405 of the C-terminal domain of LPL [21]. We found that the 5D2 antibody binds to both the 56 kDa protein and the 37 kDa protein, in a similar pattern as shown in lanes 10–12 of Figure 3A.

To prove that mainly the 37 kDa protein rather than the 56 kDa LPL protein mediates the binding of lipoproteins to cells, we subjected an LPL fraction which contained both the 37 and the 56 kDa protein, to radiation inactivation. With this technique, the biological activity of a sample is decreased by exposure to increasing doses of radiation, whereby relatively large molecules are inactivated at relatively low radiation doses as compared to small molecules. Thus, by using an LPL sample that has been irradiated with different doses, the size of the smallest unit required to stimulate the binding of LDL to cells (functional molecular mass) can be determined without the need for prior purification [24]. The effect of various doses of irradiation on the stimulatory effect of an LPL sample on the binding of ^{125}I-LDL

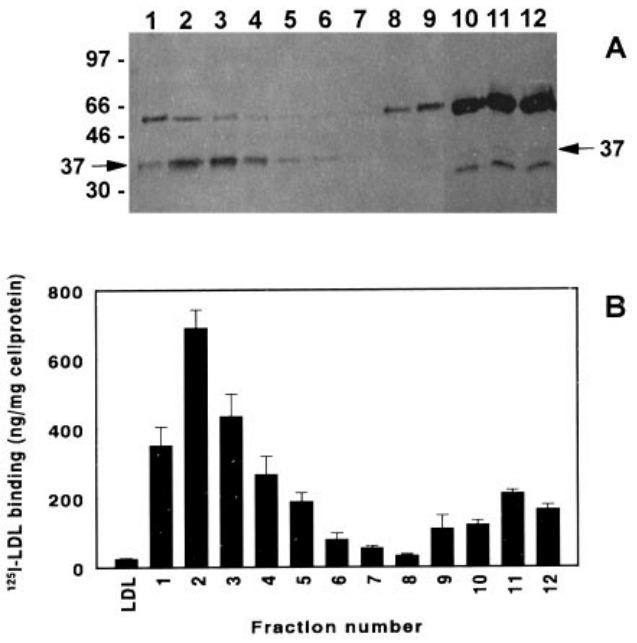

Figure 3 Western blot analysis using a polyclonal antibody and effect on cellular [^{125}I]LDL binding of bovine lipoprotein lipase

About 1 μg of protein was subjected to 4–20% SDS-polyacrylamide gradient gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antibody against bovine LPL at a dilution of 1:500. As a secondary antibody swine anti-rabbit IgG conjugated to peroxidase was used at a dilution of 1:1000. The bands were visualized by using the 4-chloro-1-naphthol staining (**A**). The effect of the respective LPL samples (at concentration of 5 μg/ml) on the binding of ^{125}I-LDL (**B**) was measured as described in the legends for Figure 1. Binding is expressed as ng of ^{125}I-LDL per mg cell protein and was determined as described in the Materials and methods section. Values represent the mean $\pm$ S.D. of three measurements.

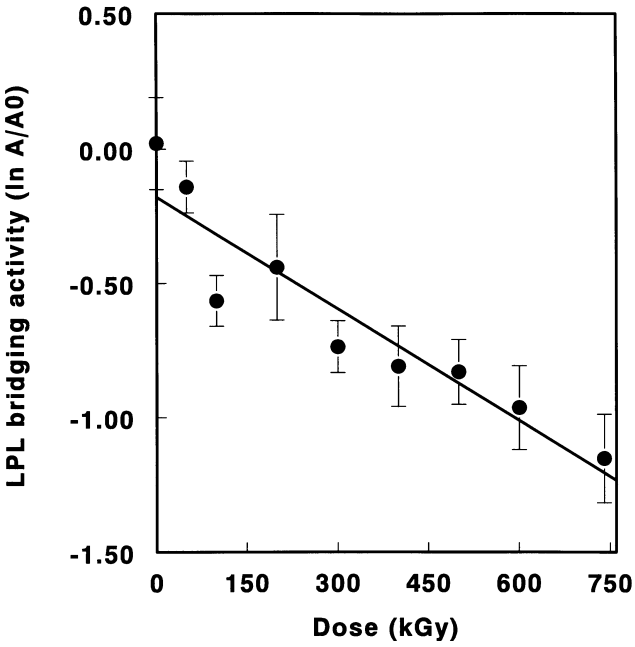

Figure 4 Radiation inactivation of LPL

The effect of irradiated LPL samples on the binding of ^{125}I-LDL to J774 macrophages was determined as described in the Materials and methods section. Data are expressed as ln [stimulation factor of irradiated samples (*A*) divided by the stimulation factor of non-irradiated samples (*A*0)]. The fitted line is from least-square analysis and the functional molecular mass (*M*) is calculated according to the formula $M = c \cdot K \cdot St$ as described in the Materials and methods section.

to J774 cells is shown in Figure 4. The fractional activity $A/A0$ decreased with increasing irradiation doses. The functional molecular mass was calculated to be 30.9 ± 1.8 kDa, which is in the same order of magnitude as 37 kDa. We therefore conclude that in an LPL sample, the 37 kDa protein presumably representing the C-terminal 300 amino acids of lipoprotein lipase, is the major component that stimulates the binding of ^{125}I-LDL to cells.

DISCUSSION

The present study was conducted to elucidate the role of a 37 kDa protein, co-purifying with bovine milk lipoprotein lipase, in the binding of LDL by J774 macrophages. LPL samples containing this protein (as shown in Figure 1A by SDS/PAGE under reducing conditions) were much more potent in stimulating the binding and uptake of ^{125}I-labelled LDL by J774 macrophages than LPL samples without the 37 kDa protein band. In this figure, there is no correlation between the amount of 37 kDa protein present in the sample and the extent of stimulation of LDL-binding. This can be explained by the fact that the LPL samples shown in Figure 1A are obtained from different, independent LPL isolations over a 2-year time period. Since the activity of a freshly isolated LPL sample, with respect to its bridging function, varies between the different isolations, the LPL samples shown in Figure 1 cannot be compared with regard to the amount of stimulatory effect on the binding of LDL.

We also found that the presence of the 37 kDa protein in LPL samples resulted in an increased stimulatory effect on the association and degradation of LDL to J774 cells, in a similar way as for the binding (data not shown). This indicates that the 37 kDa protein can affect the intracellular lipid accumulation in macrophages and may indeed influence the atherogenic process.

Several lines of evidence indicated that the 37 kDa protein may be the C-terminal domain of lipoprotein lipase. First, Western blotting using a monoclonal antibody 5D2 against amino acid residues 396–405 of LPL (originally prepared by Brunell et al., University of Washington, Seattle [21]), showed that the 37 kDa protein contained the carboxy-terminal region of LPL. Furthermore, by using the method of radiation inactivation, we showed that the functional molecular mass of lipoprotein lipase with respect to its bridging function was about 31 kDa, which is of the same order of magnitude as 37 kDa. However, it may be argued that the graph shown in Figure 4 can also be drawn in a bi-phasic manner, indicating that there may be two sizes of the LPL molecule that can mediate the binding of lipoproteins to cells; one being approx. 100 kDa, presumably representing the LPL dimer. The second, in accordance with our previous findings, is approx. 30 kDa. This is in accordance with our finding that the LPL protein itself can stimulate the binding of LDL to cells (Figure 3), however to a much lesser extent than the 37 kDa protein.

If the 37 kDa protein indeed represents the C-terminal part of bovine LPL, it would approximately comprise residues 150–450 of the LPL molecule. Although an attempt was made to establish definite proof, sequencing the N-terminal part of the 37 kDa protein was not entirely successful. However, the first two residues were analysed as lysines, and in bovine LPL, a Lys-Lys sequence only occurs at residues 149–150 and 414–415. Thus, the finding

that the N-terminal sequence starts with two lysine residues, sustains our hypothesis that the 37 kDa protein may represent the C-terminal part of the LPL molecule. However, we cannot definitely rule out the possibility that the 37 kDa band represents (an)other protein(s), which are able to stimulate the binding of LDL to cells.

It has been shown by several groups that the carboxy-terminal domain of LPL binds to several receptors and stimulates the receptor-mediated binding and catabolism of lipoproteins by different cell types, in a process facilitated by cell surface proteoglycans [10,11,13,16]. Furthermore, Socorro and Jackson have shown that milk proteases, co-purifying with the LPL, cause degradation of the lipase [25,26]. They reported LPL samples containing additional proteins with molecular masses of 36 kDa and 18–22 kDa, which represented proteolytic degradation products of LPL. Bengtsson-Olivecrona et al. [27] also reported on the presence of protease-sensitive regions within the LPL molecule. Cleavage of the LPL protein at these sites by trypsin or plasmin resulted in the production of three fragments of apparent sizes of about 32–38 kDa; one of these (the T1 fragment) appeared to be the carboxy-terminal domain of LPL. The fragments are held together by disulphide bonds, and they are only shown on SDS/PAGE gels under reducing conditions. Furthermore, it is known that plasmin is present in milk [28].

From these previous reports and the results presented in this study, we hypothesize that LPL can be degraded by milk proteases during its isolation, resulting in the appearance of a 37 kDa protein band on SDS/PAGE gels under reducing conditions. Furthermore, we hypothesize that the proteolytic cleavage of LPL results in such a conformational change of the LPL molecule, that its ability to bridge between lipoproteins and receptors and/or proteoglycans is dramatically increased. This hypothesis is sustained by the current thought that binding sites for lipoproteins and lipoprotein receptors are in close proximity within the carboxy-terminal domain of LPL [10]. Cleavage of the LPL subunit may facilitate binding of both the lipoprotein and the receptor to the LPL monomer at the same time.

In Figure 3 we showed that the 56 kDa protein itself can also stimulate the binding of LDL to cells, although this stimulation is much less effective as compared to the stimulatory effect of the 37 kDa protein. The finding that the 56 kDa LPL protein can stimulate the binding of lipoproteins, is in accordance with the results of Salinelli et al. [12], who performed binding experiments by using transfected COS cells producing the wild type human LPL and several mutant LPLs. They found that the binding and catabolism of VLDL was stimulated in the cells producing the wild type LPL, whereas the non-transfected cells did not bind or degrade VLDL. However, from their experiments the specific activity of LPL in this respect cannot be deduced, since the amount of LPL produced by the transfected COS cells was not reported. Furthermore, in their system the LPL molecule may also have been cleaved.

It has been reported that macrophages in atherosclerotic plaques synthesize LPL and that the concentration of LPL in the vessel wall is related to the concentration of cholesterol in the vessel wall [29]. Thus, in the intima, the LPL-mediated increase of lipoprotein uptake by macrophages most probably occurs and may be sufficient to enhance the formation of foam cells and, eventually, atherosclerotic plaques. However, if LPL cleavage does indeed occur *in vivo* in the intima, the LPL-mediated stimulation of the uptake of lipoproteins by macrophages would be even more relevant with respect to the development of atherosclerosis.

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