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Construction and functional characterization of recombinant fusion proteins of human lipoprotein lipase and apolipoprotein CII

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The hydrolysis of triacylglycerols of chylomicrons and very low density lipoproteins by lipoprotein lipase (LPL) requires the presence of apolipoprotein (apo) CII as a cofactor. To obtain further information on the interaction of apo CII and LPL, we generated two fusion proteins consisting of the complete LPL molecule and the mature form of apo CII. The cDNAs of both proteins were either connected directly or by a segment encoding a 16-amino-acid linker peptide. The fused cDNAs were stably expressed in human embryonic kidney (HEK) 293 cells and the enzymic properties of the recombinant proteins were examined. The fusion proteins hydrolysed both emulsified long-chain (lipase) triacylglycerol substrate and a water-soluble short-chain (esterase) fatty acid ester substrate (*p*-nitrophenylbutyrate), regardless of whether or not they contained the linker peptide. In the absence of exogenous apo CII, the fusion proteins had up to 3.5-times higher basal activity than wild-type LPL. Similar to wild-type LPL, the fusion proteins were inhibited by 1 M NaCl, however less than wild-type LPL. A polyclonal antibody specific for apo CII impaired their ability to hydrolyse triacylglycerol emulsions. A similar effect was seen when the tetrapeptide KGEE was used as inhibitor, which corresponds to the carboxy-terminal four amino acids of apo CII.

Keywords: fusion protein; lipoprotein lipase; apolipoprotein CII; cofactor; lipoprotein lipase/apolipoprotein CII complex.

Lipoprotein lipase (LPL) plays an important role in plasma lipoprotein metabolism [1, 2]. LPL is primarily synthesized in adipose tissue, muscle cells and macrophages. LPL is attached to the capillary endothelium by electrostatic interactions with glycosaminoglycans. Its major function is to hydrolyse triacylglycerols transported in circulating lipoproteins, thereby releasing free fatty acids which are used either as a source of energy or are reesterified for storage. Chylomicrons and very low density lipoproteins are the preferred substrate particles. LPL is active as a homodimer; for full enzymic activity, the presence of apolipoprotein (apo) CII is required as a cofactor [3]. Apo CII is an integral component of lipoproteins which serve as the substrate of LPL.

Another role for LPL in lipoprotein metabolism has been suggested recently. *In vitro* studies have shown that LPL may function as a ligand of the low-density-lipoprotein-receptor-related protein, thereby facilitating the receptor-mediated uptake of chylomicron remnants, regardless whether it is enzymically active or not [4, 5]. The binding of other lipoproteins, for instance of very low density lipoproteins or low density lipoproteins to cultured fibroblasts and HepG2 cells is also enhanced by LPL [6, 7]. Whether this *in vitro* function is relevant to the cellular uptake of lipoproteins *in vivo* remains to be studied.

Functional domains can be distinguished in the LPL molecule such as the catalytic domain, an interfacial activation site and specific binding sites for heparin, lipids, and apo CII [8]. Cloning and sequencing of the LPL cDNA [9] and the characterization of naturally occurring mutations, together with site-specific mutagenesis experiments lead to the assignment of particular functions of LPL to structural domains. The catalytic domain of LPL has been shown to consist of Ser132, Asp156 and His241 [10, 11]. Lys147 and Lys148 may be involved in the ionic binding of apo CII [12], whereas the charged residues at positions 279–282 and 292–304 are important for heparin binding [13, 14]. Additional insight came from the comparison of the amino acid sequences of serum LPL and pancreatic lipase (PL), the crystal structure of which has been determined recently [15]. The two lipases have a similarity of 30%. Together with hepatic triacylglycerol lipase (HTGL) they form the triacylglycerol lipase family. A very similar tertiary structure [16] has been predicted on theoretical grounds. Based on this prediction, domains of LPL and HTGL have been exchanged to characterize the roles of the amino-terminal and carboxy-terminal protein domains of the two lipases. The results of these studies were in good agreement with those of the above-mentioned site-specific mutagenesis experiments [17, 18].

Despite the considerable sequence similarity and the putative similarities of their tertiary structures, LPL and PL significantly differ with regard to their cofactors or activators, apo CII and

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Abbreviations. Apo, apolipoprotein; CMV, cytomegalovirus; DMEM, Dulbecco's minimum essential medium; HEK, human embryonic kidney; HTGL, hepatic triacylglycerol lipase; LPL, lipoprotein lipase; PL, pancreatic lipase, SOE, splicing overlap extension. LPL–apo-CII, fusion protein of human LPL and mature human apo CII; LPL–*l*-apo CII, fusion protein of human LPL and mature human apo CII containing a linker polypeptide.

Enzyme. LPL(EC 3.1.1.34).

Table 1. Sequences of oligonucleotide primers.

Primer	Sequence	Orientation
LPL-1	5′-aggtcgacgcgccccgagatggagagcaaa-3′	sense
LPL-4	5′-ttgctggggctgttgggtgcctgacttcttatt-3′	antisense
LPL-5*	5′-aataagaagtcaggcacccaacagccccagcaa-3′	sense
LPL-7	5′-gggaattcctgttactcctctcccttcagcac-3′	antisense
linker-1	5′-tcccccgggggccgcttccgctgcctgacttcttatt-3′	antisense
linker-2	5′-ggccccgggggaagcggaaccggcggaagcggaagc-3′	sense
apo CII-2	5′-ggcggaggcggaggcggaacccaacagccccagcaa-3′	sense

colipase, respectively. It is, therefore, not possible to derive the structure of the LPL and apo CII complex from the data available for the complex of PL and colipase.

The main binding site for colipase is located in the carboxy terminus of PL around the lid domain of the molecule [19]. In contrast, the apo-CII-binding site appears to reside in the amino-terminal part of LPL and mutagenesis experiments revealed that apo CII does not interact with the lid domain of LPL [20]. Colipase interacts with PL at the water/lipid interphase, but does not influence the catalytic rate [21]. Apo CII, in contrast, increases the catalytic activity of LPL molecules already bound to the interface. The exact mechanism of this activation, however, is still unclear.

Analyses of the interaction of LPL and apo CII have been hampered by poor water solubility of apo CII and the aggregation to multimers, similar to other apolipoproteins [22]. We, therefore, attempted to engineer a water-soluble fusion protein consisting of LPL and its activator apo CII. We obtained an enzymically active LPL apo CII with functional properties closely resembling those of the natural complex of LPL and apo CII.

MATERIALS AND METHODS

Oligonucleotides were synthesized using the phosphoramidite chemistry on an Applied Biosystems 380A DNA synthesizer and purified on SEP-PAK C_{18} cartridges (Millipore). The tetrapeptide KGEE was synthesized on a SP 640 peptide synthesizer (Labortec). Radiolabelled triolein (5 mCi/ml; 10 Ci/mmol) was from Amersham.

A 2.4-kb cDNA fragment encoding wild-type human LPL was kindly provided by M. C. Schotz (Los Angeles, California, USA). The complete apo CII cDNA has been described previously [23], β-tubulin cDNA was a gift of N. J. Cowan [24]. The expression vector pRc/CMV was from Invitrogen. PCR products were purified from agarose gels using the Jetsorb DNA purification kit from Genomed. Human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (Rockville, USA). Polystyrene tissue-culture vessels and microplates for enzyme immunoassays were from Nunc. Dulbecco's minimal essential medium (DMEM), trypsin, additives (glutamine, penicillin G, streptomycin, G418, Ultroser G (for serum replacement)), *Taq* polymerase, restriction endonucleases and fetal bovine serum were from Gibco BRL. A polyclonal antibody against LPL purified from bovine milk according to the method of Socorro et al. [25] was raised in rabbits. Cross-reactivity with human LPL was tested against human LPL purified from human milk according to the method of Zechner [26]. The monoclonal LPL antibody was from the Washington Research Foundation (Seattle, USA). A polyclonal antibody against recombinant apo CII expressed in *Escherichia coli* was raised in rabbits [23]. Nylon and nitrocellulose (type BA85) membranes for nucleic acid and protein blotting, respectively, were purchased from Schleicher und Schüll and all other chemicals from E. Merck or Serva at the highest purity grade available.

Construction of the human LPL–apo-CII fusion vectors. Two fusion constructs were generated using the splicing overlap extension (SOE) method [27] (Fig. 1a). The sequences of the PCR primers used are compiled in Table 1.

To generate the cDNA of the fusion protein in which LPL and apo CII were linked directly (LPL–apo-CII), two PCR reactions were carried out using the primer pairs LPL-1/LPL-4 and LPL-5*/LPL-7 (20 pmol each) and the LPL or the apo CII cDNA (100 ng each), as templates (Fig. 1). In each case, the following parameters of the cycles were used: denaturation at 94°C for 3 min; 80°C, 5 min; 30 cycles of 94°C, 1 min; 45°C, 2 min; 72°C, 2 min (DNA Thermal Cycler, Perkin-Elmer). The two products were purified (Jetsorb), mixed and used to generate the full fusion length LPL–apo-CII cDNA in a second (SOE) PCR with the primers LPL-1 and LPL-7. The cycler program was then denaturation 94°C, 3 min; 80°C, 5 min; 35 cycles of 55°C, 2 min; 72°C, 2 min.

To produce the cDNA of the fusion protein in which LPL and apo CII were connected by a linker peptide (LPL–*l*-apo-CII), two PCR reactions were carried out using the primer pairs LPL-1/linker-1 or apo CII-2/LPL-7 (20 pmol each) and LPL–apo-CII fusion cDNA (100 ng) as templates. The cycler program was: denaturation at 94°C, 3 min; 80°C, 5 min; followed by 30 cycles of 94°C, 1 min; 45°C, 2 min; 72°C, 2 min. To elongate the linker segment, the CII-2/LPL-7 product was reamplified using the primer pair linker-2/LPL-7 (20 pmol each). The cycler program for the re-amplification was: denaturation 94°C, 3 min; 80°C, 5 min; 94°C, 1 min; 50°C, 1 min; 72°C, 2 min; 30 cycles. The fragments were purified (Jetsorb) and used in the SOE-PCR with the primers LPL-1 and LPL-7 to generate the full-length fusion cDNA (LPL–*l*-apo-CII). Cycler program: denaturation 94°C, 3 min; 80°C, 5 min; followed by 35 cycles of 55°C, 2 min and 72°C, 2 min.

The 1.7-kb fusion cDNAs (LPL–apo-CII, LPL–*l*-apoC-II) and a 2.4-kb human LPL cDNA fragment were cloned into the expression vector pRc/CMV. The vector was cleaved with *Bst*XI. Ligation was performed blunt ended after filling in the single-stranded overhangs. Prior to transfection into human embryonic kidney (HEK) 293 cells, the expression vectors were characterized by DNA sequencing using the dideoxynucleotide chain termination method.

Expression studies. DNA transfection was performed by electroporation (Gene Pulser, Biorad). Subconfluent HEK 293 cells were harvested by trypsinisation and resuspended in DMEM. 0.5 ml of a suspension containing 5×10^6 cells and 1 μg plasmid DNA were loaded into a 0.4-cm electroporation cuvette. After 10 min on ice, the cuvette was pulsed at 960 μF, 160 V. The cells were then seeded in four 100-mm cell culture dishes

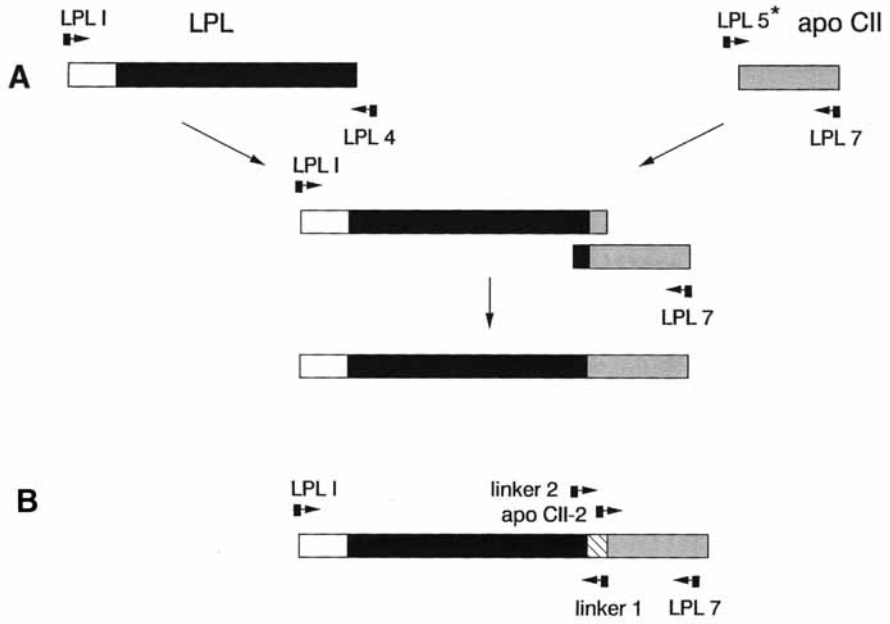

Fig. 1. Construction of fused LPL–apo-CII cDNAs. (a) The cDNA sequence encoding the LPL signal peptide and amino acid residues 1–448 were linked to sequences encoding the mature apo CII using SOE PCR. The LPL and apo CII cDNA fragment of interest were amplified in independent PCR reactions using the primer pairs LPL-1/LPL-4 and LPL-5*/LPL-7 and the respective cDNA clones as templates. The fused LPL–apo-CII cDNA was generated in a second PCR using the primer pairs LPL-1 and LPL-7. (b) The LPL–*l*-apo-CII cDNA was produced in an analogous fashion. Two PCR reactions were carried out using the primer pairs LPL-1/linker-1 and apo CII-2/LPL-7. The CII-2/LPL-7 product was re-amplified using the primer pair linker-2/LPL-7 to elongate the linker segment. The LPL–*l*-apo-CII cDNA was produced by SOE PCR using the LPL-1/linker-1 and the linker-2/LPL-7 fragments as template and oligonucleotides LPL-1 and LPL-7 as primers. Solid bar, LPL signal peptide; open bar, mature LPL; grey bar, mature apo CII; hatched bar, linker segment.

and selected with DMEM, 10% (by vol.) fetal bovine serum, containing 500 μg/ml G418. After two weeks, positive clones were picked and characterized by their total RNA, Northern blotting, enzyme immunoassay, and assays for lipase activity. Cells were adapted to growth in serum-free DMEM supplemented with 2% (by vol.) ultroser G. The medium was harvested and stored at −70 °C until lipase activity and the concentration immunoreactive LPL were determined. For inactivation with phenylmethylsulfonyl fluoride, the cell culture supernatant was incubated with a final concentration of 1 mM phenylmethylsulfonyl fluoride for 2 h at 4 °C. To remove the inhibitor, the inactivated enzymes were purified by heparin-Sepharose chromatography or affinity chromatography on an α-LPL column.

Northern-blot hybridization analysis of RNA. The total RNA of stably transformed cells was isolated by the acid guanidinium thiocyanate/phenol/chloroform method [28]. 20 μg RNA aliquots were electrophoresed in a 1.5% (mass/vol.) agarose gel containing 6% (mass/vol.) formaldehyde, and transferred to a nylon membrane. The membrane was hybridized using a random primed 850-bp human LPL cDNA (nucleotides 795–1640) fragment or a probe specific for apo CII (nucleotides 67–310 of the cDNA), both of which had been prepared by PCR. The gels were stained with ethidium bromide to confirm that equivalent amounts of RNA were electrophoresed in each lane.

Western-blot analysis of secreted proteins. Western-blot analysis was performed on the total protein in the serum-free conditioned media of cells stably transfected with pRc/CMV control plasmid, pRc/CMV-LPL, pRc/CMV-LPL-apo CII, or pRc/CMV-LPL-*l*-apo CII; the medium was dialysed and lyophilised [29]. Proteins were dissolved in sample buffer, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis as described by Laemmli [30], and electrophoretically transferred to BA85 nitrocellulose. Recombinant proteins were detected using a polyclonal antiserum from rabbits specific for LPL and alkaline-phosphatase-coupled anti-rabbit IgG (Sigma). 5-Bromo-4-chloro-3-indolylphosphate and 4-nitro-blue-tetrazolium-chloride hydrate (Sigma) were used as substrates.

Quantification of lipase activity and mass. To measure lipolytic activities, triolein containing [^{3}H]oleat in all three positions was emulsified in the presence of gum arabic. The assay buffer contained 0.15 M NaCl, 0.5% gum arabic, 2.5% bovine serum albumin, 0.1 M Tris/HCl, pH 8.6, 20 U heparin/ml, 10% heat-inactivated (56 °C, 30 min) human serum as a source of apo CII, 1 mg/ml unlabeled triolein, 1.5 μCi [^{3}H] triolein/ml; the total assay volume was 400 μl. After 30 min incubation at 37 °C, free fatty acids were extracted by a liquid/liquid partition system[31] and assayed for radioactivity in Ultima Gold scintillation counting solution (Packard). The specificity of the apo-CII-mediated activation was examined by incubating the fusion proteins with an apo-CII-specific antibody [23] for 2 h on ice prior to the lipase activity determination.

Esterase activity in the media was quantified using *p*-nitrophenylbutyrate as substrate [32]. Lipase activities were always determined in triplicates.

LPL mass was quantified by a sandwich enzyme-linked immunosorbent assay. This assay uses a polyclonal antibody raised against purified bovine milk LPL as the first antibody (dilution 1:2500). We used a monoclonal antibody specific for LPL (dilution 1:1000) as tracer antibody. Detection was carried out with an alkaline-phosphatase-conjugated anti-mouse serum (Sigma). The assay was standardized against LPL purified from bovine milk.

RESULTS

The cDNAs of human LPL and of its activator, the mature form of human apo CII (Fig. 1), were fused. They were linked

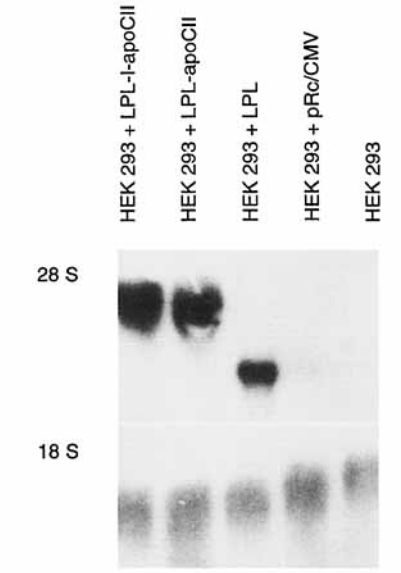

Fig. 2. Northern-blot analysis. 20 µg total RNA isolated from stably transfected HEK 293 clones, expressing LPL and fusion proteins. Lanes from right to left: HEK 293 cells mock-transfected cell with the expression vector pRc/CMV, expression vector pRc/CMV containing a 2.4-kb insert of wild-type LPL cDNA, pRc/CMV constructs containing cDNA inerts of LPL and apo CII fused directly (LPL−apo-CII) or by means of a 48-bp segment encoding a linker peptide (LPL−*l*−apo CII). Hybridization was carried out with a 850-bp cDNA probe specific for human LPL (top panel). Hybridization with an *β*-tubulin probe (bottom panel) demonstrated that the amounts of total RNA electrophoresed per lane were virtually equal.

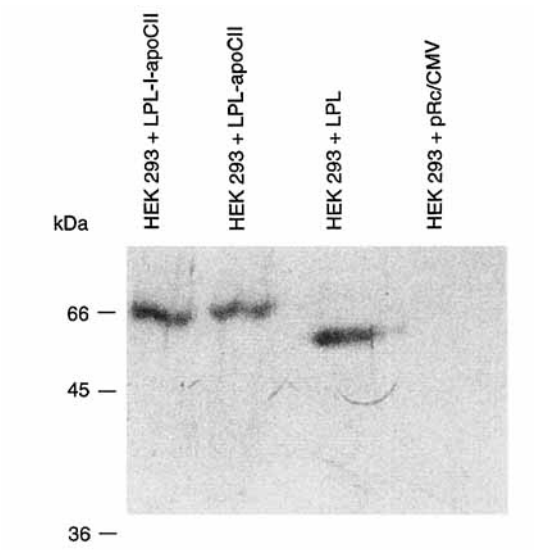

Fig. 3. Western-blot analysis of proteins secreted in the medium of stably transfected HEK 293 cells. Proteins of the conditioned medium of stably transfected cells were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and blotted to nitrocellulose. The recombinant proteins were visualized using a polyclonal rabbit anti-(bovine LPL) serum.

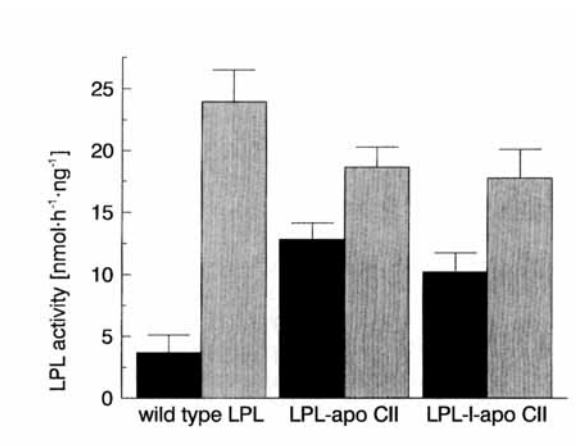

Fig. 4. Hydrolysis of a long-chain fatty acid triacylglycerol emulsion by recombinant LPL and LPL–apo-CII fusion proteins. HEK 293 cells were stably transfected with expression vectors encoding either wild-type LPL or chimeric proteins in which LPL and apo CII were linked directly (LPL−apo-CII) or by means of a linker peptide (LPL−*l*-apo-CII). LPL activity was determined in the conditioned medium of the transfected cells as described in Materials and Methods, either in the absence (solid bars) or in the presence (grey bars) of human serum as a source of apo CII during the incubation. Values are mean standard deviations of triplicate assays. Lipase activities are normalized to the concentration of immunoreactive LPL in the conditioned medium determined by means of a sandwich enzyme immunoassay.

either directly or by a 16-amino-acid linker polypeptide. The linker polypeptide consisted of threonine, serine and glycine, which have small, polar residues. The linker sequence (*SGSG***PGGSGT***GGSGSG*) was derived from naturally occurring linker peptides such as the **GGSGT** motif in phospholipase A2 and *GSGSG* motif in the immunoglobulin A light-chain Fab fragment [33]. Sequence analysis confirmed that the fused genes contained the desired in-frame linkage of LPL and apo CII cDNAs. Clones of the wild-type LPL and the fusion constructs were stably expressed in HEK 293 cells. (Fig. 2). There were no major differences in mRNA abundance between the wild-type LPL and the fusion constructs (Fig. 2). As expected, the two fusion mRNAs were 700-nucleotides smaller than the wild-type LPL mRNA and hybridized with probes specific for both LPL and apo CII (data not shown).

Western blotting of protein secreted into the conditioned serum-free media by the stably transfected cell lines, expressing wild type LPL or the fusion proteins (Fig. 3) was performed with an antibody prepared against purified bovine LPL. Cells mock-transfected with the pRc/CMV vector were used as a control. The molecular masses of the fusion proteins differed by 9 kDa from the wild-type LPL, which corresponds to the molecular mass of the attached non-glycosylated apo CII domain.

The hydrolase activity of wild-type LPL and the fusion proteins with long-chain fatty acid triacylglycerols (emulsion of gum-arabic-stabilized trioleine) as substrate was determined, (Fig. 4). The results are expressed as specific activities. The lipolytic activities were normalized to the concentrations of immunoreactive LPL in the conditioned media of the transfected HEK 293 cells.

In the absence of human serum as a source of apo CII, the fusion proteins showed up to 3.5-times higher specific activities (12.8 nmol · h^{-1} · ng^{-1}) than wild-type LPL (3.7 nmol · h^{-1} · ng^{-1}). With apo CII (5%, by vol., human heat-inactivated serum) added to the incubation mixtures, wild-type LPL was activated 6.45-fold and reached a specific activity of 23.9 nmol · h^{-1} · ng^{-1}, which is approximately twice the activity of the fusion proteins in the absence of exogenous apo CII. Interestingly, the fusion proteins LPL−apo-CII or LPL−*l*-apo-CII could be activated further by addition of excess apo CII supplied as heat-inactivated human serum. We observed an average increases in activity of 45% and 74%, respectively. The fusion proteins thus exhibited almost the same specific lipolytic activity as wild-type

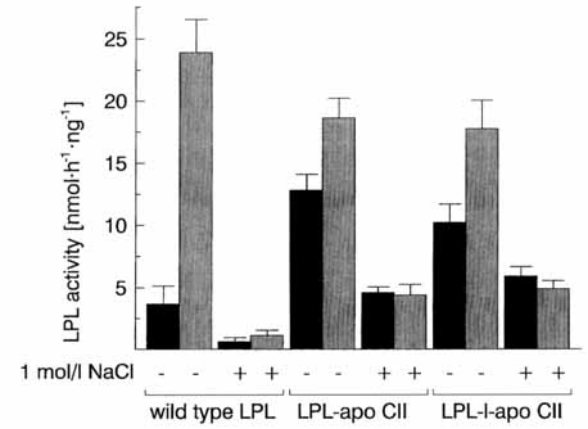

Fig. 5. Effect of 1 M NaCl on the hydrolysis of a long-chain fatty acid triacylglycerol emulsion by recombinant LPL and LPL–apo-CII fusion proteins. HEK 293 cells were stably transfected with expression vectors encoding either wild-type LPL or fusion proteins (LPL−apo-CII or LPL−*l*-apo-CII). LPL activity was determined in the conditioned medium of the transfected cells as described in Materials and Methods in the presence (+) or in the absence (--) of 1 M NaCl and in the presence (grey bars) or in the absence (solid bars) of apo CII in the incubation mixtures. Values are means ± standard deviations of triplicate assays. Lipase activities are normalized to the concentration of immunoreactive LPL (in the conditioned medium) as determined by means of a sandwich enzyme immunoassay.

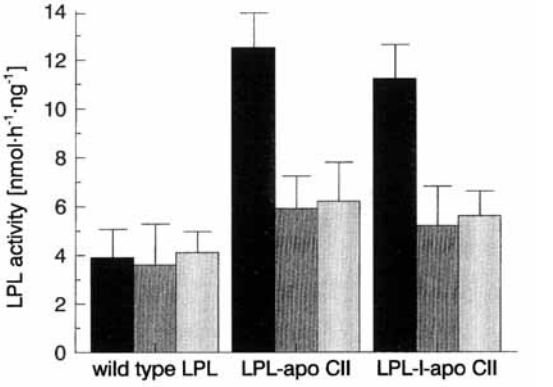

Fig. 6. Effect of a polyclonal monospecific anti-(apo CII) on the hydrolysis of a long-chain fatty acid triacylglycerol emulsion by recombinant LPL and LPL–apo-CII fusion proteins. HEK 293 cells were stably transfected with expression vectors encoding either wild-type LPL or the fusion proteins, LPL−apo-CII or LPL−*l*-apo-CII. LPL activity was determined in the conditioned medium as described in Materials and Methods section. Solid bar, control; dark grey bar, polyclonal rabbit anti-(apo CII) only; light grey bars, rabbit anti-(apo CII) *plus* apo CII (60 mg/l) supplied as human serum. Values are mean standard deviations of triplicate assays. Lipase activities are normalized to the concentration of immunoreactive LPL in the conditioned medium determined by means of a sandwich enzyme immunoassay.

LPL together with apo CII when they were supplemented with additional apo CII.

The active form of LPL is a homodimer. To examine whether the higher basal activity of the fusion proteins was produced by intramolecular activation or by interdimer interactions between the apo-CII-domains of the fusion proteins and the apo-CII-binding sites of free LPL, the lipase activity of the fusion proteins was inactivated by phenylmethylsulfonyl fluoride, final concentration 1 mM. After removing the lipase inhibitor phenylmethylsulfonyl fluoride the inactivated fusion proteins were used as a source of apo CII in lipase assays with bovine LPL. The bovine LPL was not activated, regardless whether LPL−apo-CII or LPL−*l*-apo-CII was used as the source of apo CII (data not shown). This suggests that the apo CII domains within the LPL−apo-CII hybrid proteins was not able to activate LPL molecules other than those they were fused to. As expected, apo CII heat-inactivated human serum (5% by vol.) in the control experiment increased the activity of the bovine LPL five-fold.

The effect of a high salt concentration on the activities of wild-type LPL and of the fusion proteins is shown (Fig. 5). Wild-type LPL was highly salt sensitive; 1 M NaCl decreased its specific activity by 84% and 95% in the absence and in the presence, respectively of apo CII. LPL−apo-CII and LPL-*l*-apo-CII were also salt sensitive, but to a lower degree, in the absence of apo CII, LPL−apo-CII and LPL-*l*-apo-CII were inhibited by 64% and 42%, respectively; in its presence LPL−apo-CII and LPL-*l*-apo-CII were inhibited by 76% and 72.3%, respectively. Interestingly, the residual specific activities of the fusion proteins in the presence of 1 M NaCl (4.6 nmol · h^{-1} · ng^{-1} and 5.9 nmol · h^{-1} · ng^{-1}, respectively) were greater than the basal activity of the wild-type LPL (3.7 nmol · h^{-1} · ng^{-1}).

As expected, a polyclonal antibody specific for apo CII did not affect the basal activity of wild-type LPL, but completely inhibited its activation by apo CII (Fig. 6). The basal activity of the fusion proteins was nearly halved by the addition of the polyclonal anti-(apo CII), but again remained above that of the wild-type LPL level.

We investigated the influence of the peptide KGEE, which corresponds to the carboxy-terminus of apo CII, known to inhibit LPL activity [34]. The activity of the wild-type LPL and of the fusion proteins was diminished in a concentration-dependent manner, both in the absence and in the presence of exogenous apo CII (Fig. 7). The specific activity of the wild-type LPL was affected to a higher extent than those of the fusion proteins.

Apo CII inhibits the hydrolysis of short-chain fatty acid esters by LPL [35]. In our experiments, hydrolase activity towards the short-chain fatty acid ester *p*-nitrophenylbutyrate was not affected by the apo CII moiety of the fusion proteins; wild-type LPL, LPL−apo-CII and LPL−*l*-apo-CII exhibited almost identical specific hydrolase activities (149.3, 157.8 and 148.4 nmol · h^{-1} · mg^{-1}, respectively).

DISCUSSION

In this study, we report on the structure and functional analysis of a fusion protein consisting of an enzyme and its activator. Numerous fused genes have been constructed for the expression of heterologous proteins in *E. coli* by connecting cDNAs with expressed host sequences (e.g. lacZ). Another group of fused genes (in which domains of closely related genes have been exchanged) code for chimeric proteins e. g. LPL-HTGL chimeras [17, 18]. The studies of the LPL-HTGL chimeras and recent research on the tertiary structure of hybrid proteins [36] have shown that distinct domains of chimeric proteins mostly retain their original structure and function.

In the design of the fused LPL−apo-CII constructs, we considered both the orientation of the enzyme and activator domains and the lengths and sequences of the connecting peptides between the domains. We made the following considerations: the LPL binding and activation sites of apo CII resides within the carboxy-terminal half of the protein [34], therefore the amino-terminus of apo CII was connected with the carboxy-terminus of LPL. A reverse orientation would presumably immobilize the

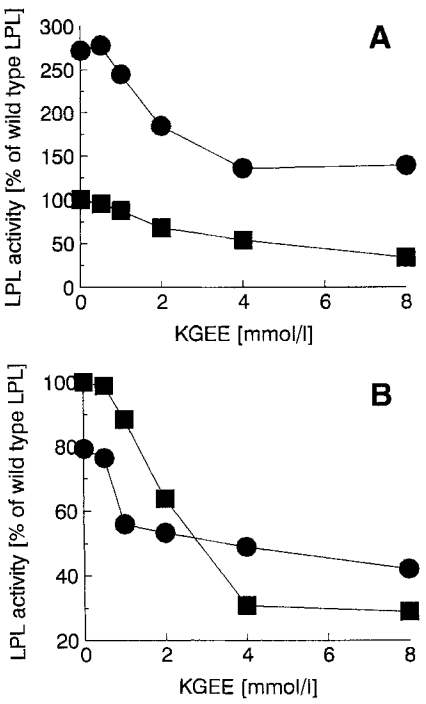

Fig. 7. Effect of the apo CII carboxy-terminal tetrapeptide KGGE on the hydrolysis of a long-chain fatty acid triacylglycerol emulsion by recombinant wild-type LPL and the LPL–apo-CII fusion protein. HEK 293 cells were stably transfected with expression vectors encoding wild-type LPL or the fusion protein LPL–apo-CII. Recombinant LPL activity was determined in the conditioned medium as described in Materials and Methods section. Squares, recombinant wild-type LPL; circles, LPL activities are expressed relative to the wild-type LPL. (a) Apo CII absent; (b) apo CII (heat-inactivated human serum).

carboxy-terminal residues Glu78 and Glu79 of apo CII. These residues have been proposed to be responsible for the initial ionic interaction with the Lys147 and Lys148 of the LPL. Whether other positively charged side chains of LPL are involved remains to be studied. The first ten amino acids of mature apo CII are supposed to have random secondary structure [2]. They can be regarded as a preformed linker peptide in the hybrid. Experiments with truncated LPL produced by chymotrypsin cleavage [37] and with the chimeric LPL-HTGL molecules have shown that the carboxy-terminus of LPL is not involved in the interaction with apo CII. The truncated LPL showed reduced activity towards some substrates, but resembled wild-type LPL with regard to most of the functional properties studied. Similar results were obtained with naturally occurring truncated LPL mutants [38]. Hence, we reasoned that the attachment of the apo CII domain to the carboxy-terminus rather than to the amino-terminus would cause the least steric interference within the chimeric enzyme-activator protein.

In addition to the directly fused LPL and apo CII, we generated a construct in which a linker peptide was inserted between the two domains. Domains of genuine proteins are on average linked by six or seven amino acids [33]. They usually couple domains of the proteins which do not interact with each other within the same molecule. Given the estimated Stoke's radius of 4.4 nm for bovine LPL [39] and the head-to-tail model of the LPL dimer [17], we reasoned that a linker of 16 amino acids would provide the degree of freedom required for interaction of the apo CII activator domain with the activation site of LPL within the chimeric molecule. The additional linker peptide did not influence the specific activity of the fusion protein.

The fusion proteins had up to 3.5-times higher basal activity than wild-type LPL. By using heat and phenylmethylsulfonyl-fluoride-inactivated fusion proteins as a source of apo CII in lipase assays, we showed that the apo CII domain activated the lipase intramolecularly. Several lines of evidence indicate that the higher basal activities of the fusion proteins were caused by direct activation of LPL by apo CII via the apo CII domain of a homodimeric form rather than by a conformational change induced by the fusion. First, an inhibitory antibody specific for apo CII reduced the basal activity of the fusion proteins to one half. Second, the carboxy-terminal tetrapeptide of apo CII, KGEE, represents an inhibitor of LPL [34]. In agreement with this finding, we observed that the tetrapeptide inhibited wild-type LPL in a concentration-dependent fashion, both in the absence as well as in the presence of apo CII. Likewise, the fusion proteins were inhibited, but again to a lower extent.

These results demonstrate that the apo CII domains of the fusion proteins are in fact responsible for the activation of the basal activity of the LPL domain. The antibody and the tetrapeptide, however, did not inhibit the enzymic activity of the fusion proteins to the same degree as the wild-type LPL activity. Therefore, a minor additional activating effect, possibly brought about by the fusion itself, must be considered.

LPL and HTGL activities in post-heparin plasma are generally separated by their susceptibility to inhibition by high salt concentrations. The amino terminus of LPL is responsible for the inhibition of LPL by high salt concentrations [17, 18]. Although the precise mechanism of inhibition is still a matter of controversy, changes in the salt concentration may induce reversible, conformational changes of the LPL dimer [40]. The recombinant wild-type LPL is salt sensitive, regardless of the presence or absence of apo CII. The fusion proteins were also inhibited by high salt concentrations. However, they retained an activity exceeding that of the wild-type LPL basal level but similar to the residual activity of the fusion proteins after blocking the anti-(apo CII). This suggests that the apo CII domain within the fusion protein stabilizes the active conformation of the enzyme.

The basal activity of the fusion proteins amounted to 54% of the fully activated wild-type LPL; supplementation of the fusion proteins with additional apo CII increased their enzymic activity by as much as 74%. These findings demonstrate that apo CII was less effective as an activator of LPL when covalently linked in the fusion proteins rather than being freely stable. Also, the possibility that the fusion protein may form a less stable dimer than the wild-type LPL must be considered. A conclusive explanation for this phenomenon cannot be given. A fusion protein in which the distance between the LPL and apo CII domain is expanded by a longer linker exhibited essentially the same specific activity as the two fusion proteins described here. Another reason for the lower enzymic activity of the fusion protein in comparison to fully activated wild-type LPL may be that more than one apo-CII-binding site exists/LPL monomer and that the additional binding site(s) required exogenous apo CII to become fully saturated. This is, however, not very likely in view of published data strongly supporting the 1:1 stoichiometry of LPL and apo CII in enzymically active complexes [41, 42].

In conclusion, we have expressed and characterized two fusion proteins consisting of human LPL and mature human apo CII. Both fusion proteins hydrolyse emulsified long-chain fatty acid triacylglycerols as well as short-chain fatty acid esters. Inhibition studies using the competitive apo CII tetrapeptide KGEE and monospecific anti-(apo CII) indicate that the activity of the fusion proteins mainly depends on the endogenous apo CII domain. This first paradigm of engineered recombinant enzyme activator chimeric protein might provide a strategy for further

studies of the molecular mechanism of enzyme activation by protein (peptide) cofactors.

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