Guinea pig apolipoprotein C-II: expression in *E. coli*, functional studies of recombinant wild-type and mutated variants, and distribution on plasma lipoproteins

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Abstract Guinea pig apolipoprotein C-II (apoC-II) lacks four amino acid residues in the amino-terminal, lipid-binding part compared to apoC-II from other mammalian species (Andersson et al. 1991. *J. Biol. Chem.* **266:** 4074–4080). To explore whether this structural difference explains the low ability of guinea pig plasma to activate lipoprotein lipase in vitro, we have expressed guinea pig apoC-II in *Escherichia coli* and have constructed an insertion mutant with the four missing amino acid residues compared to human apoC-II. With a synthetic emulsion of long-chain triacylglycerols, both the wild-type guinea pig apoC-II and the insertion mutant stimulated lipoprotein lipase similar to human apoC-II, but with chylomicrons from an apoC-II-deficient patient, 5- to 10-fold more of both wild-type guinea pig apoC-II and the insertion mutant were needed. Studies of tryptophane fluorescence indicated a slight difference in how guinea pig apoC-II interacted with liposomes, and presumably with lipoproteins, as compared to human apoC-II. The level of apoC-II (11.5 ± 5.4 μg/ml) was lower in guinea pig compared to human plasma, and most of guinea pig apoC-II was on HDL-like particles. These had decreased ability to donate apoC-II to lipid emulsions compared to human HDL. Some guinea pig apoC-II was associated with LDL which, as demonstrated by surface plasmon resonance, had higher affinity for lipoprotein lipase than human LDL, and inhibited rather than stimulated the lipase reaction in vitro. We conclude that while guinea pig apoC-II is fully competent to stimulate lipoprotein lipase, the sum of several different factors explains the low ability of guinea pig plasma to accomplish stimulation.—**Andersson, Y., A. Lookene, Y. Shen, S. Nilsson, L. Thelander, and G. Olivecrona.** Guinea pig apolipoprotein C-II: expression in *E. coli*, functional studies of recombinant wild-type and mutated variants, and distribution on plasma lipoproteins. *J. Lipid Res.* 1997. **38:** 2111–2124.

Supplementary key words lipoprotein lipase • liposomes • lipid interaction • surface plasmon resonance

Apolipoprotein C-II (apoC-II) plays an important role for the hydrolysis of lipids in chylomicrons and very low density lipoproteins (VLDL) as an activator for lipoprotein lipase (LPL) (1–4). In humans, apoC-II is mainly synthesized in the liver and there is some synthesis in the intestine (5). ApoC-II is reversibly bound to VLDL and to chylomicrons. On hydrolysis of their lipid constituents, apoC-II leaves the particle and becomes associated with high density lipoproteins (HDL), which serve as reservoir for apoC-II (6).

The complete amino acid (7) and cDNA sequences (5) for human apoC-II were resolved at an early stage. Human apoC-II consists of 79 amino acid residues. Later apoC-II sequences from several different animal species have been determined at the protein level and/or at the cDNA level (8–14). The different sequences show high degree of similarity, especially in the carboxy-terminal third of the molecule. From studies with synthetic fragments of human apoC-II, it was concluded that the functional sites for binding and activation of lipoprotein lipase reside in that part (3, 15, 16). The smallest fragment able to activate lipoprotein lipase is apoC-II$_{61-79}$ (3, 15). The fragment 50–79 of human apoC-II was found to form two rather stable helical structures when studied in solution by ^{1}H-NMR (17, 18). The amino-terminal end of apoC-II binds to lipid–water interfaces probably by formation of amphipathic helices (3, 8).

Plasma from all animal species studied activate lipo-

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; IPTG, isopropylthio-β-D-galactoside; BSA, bovine serum albumin; PBS, 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4; PMSF, phenylmethylsulfonylfluoride; LPL, lipoprotein lipase; apoC-II, apolipoprotein C-II; apoA-I, apolipoprotein A-I; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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protein lipases in vitro (4, 19) with one exception, plasma from guinea pigs (1, 20, 21). Guinea pigs are, however, not hypertriglyceridemic (22) and guinea pig lipoprotein lipase is activated by apoC-II from other species (23). In a previous study, guinea pig cDNA for apoC-II was isolated and sequenced (11). The protein sequence is about 60% homologous to that of human apoC-II. The major difference is a deletion of 4 amino acid residues (residues 16–19, human numbering) in the amino-terminal end. In the carboxy-terminal end most of the substitutions are functionally conserved.

It was not possible to isolate sufficient amounts of apoC-II from guinea pig plasma for functional studies (11). We have, therefore, constructed an expression system for apoC-II in *E. coli* that allows isolation of about 5 mg apoC-II per liter culture medium. The function of the recombinant guinea pig protein was studied both with respect to activation of LPL from three different species and with respect to direct interaction with LPL and with liposomes. To examine the effect of the deletion, we constructed a mutated form of guinea pig apoC-II with the missing amino acids inserted. Furthermore, antibodies were raised to the recombinant protein and an enzyme-linked immunoassay was developed for measurements of plasma levels of apoC-II and of distribution of guinea pig apoC-II on plasma lipoprotein classes.

MATERIALS AND METHODS

Materials

Restriction endonucleases were from International Biotechnologies, Inc., New Haven, CT, and Pharmacia LKB Biotechnology, Uppsala, Sweden. Carbenicillin, IPTG, egg yolk phosphatidylcholine, and horseradish peroxidase were from Sigma, St. Louis, MO. Low melting point agarose (GTG-agarose), FMC, was from Sea Kem, Rockland, ME. The expression vectors pET21a and pET15b, T7-Tag antibody and anti-mouse IgG conjugates were from Novagen Inc. AMS Biotechnology, Täby, Sweden. AmpliTaq DNA polymerase was from Perkin Elmer Cetus, Norwalk, CT. Ni^{2+}-NTA agarose was from Qiagen Inc., Chatsworth, CA. Factor X_a from bovine plasma was from Biolabs, Beverly, MA. Aprotinin was from Bayer, Leverkusen, Germany. Serva Blue G was from Serva, GmbH, Heidelberg, Germany. Low molecular weight markers for SDS-PAGE were from Amersham, Buckinghamshire, England, and high molecular weight markers were from Bio-Rad, Richmond, CA. Intralipid® 10% (KABI-Pharmacia Nutrition, Stockholm, Sweden) is a commercial emulsion of soybean triacylglycerols in egg yolk phosphatidylcholine. BCA Protein Assay was from Pierce, Rockford, IL. Oligonucleotides were synthesized on an Applied Biosystems 392 RNA/DNA synthesizer. CNBr-activated Sepharose, Protein A Sepharose, and the Superose 6 HR 10/30 column were from Pharmacia LKB Biotechnology, Uppsala, Sweden. Lipoprotein lipases were purified from bovine milk and from human post-heparin plasma as described (24) and from guinea pig milk (23). Human apoC-II was purified from plasma by adsorption to Intralipid® as previously described (24). Phosphatidylcholine was measured using an enzymatic kit from Wako Chemicals GmbH, West Germany. Microtiter plates were from Nunc (Nunc-immuno plates). Kits for measurements triacylglycerols, total cholesterol and free cholesterol in plasma were purchased from Boehringer Mannheim, Germany. Immunodiffusion plates for quantification of human apoC-II were obtained from Daiichi Pure Chemicals CO. LTD, Tokyo, Japan. Ready-made polyacrylamide gels (4–20% Tris-Cl) were from Bio-Rad. Dialysis tubes were from Spectrum Medical Industries, Houston, TX.

Animals

Male guinea pigs (Dunkin Hartley), weighing 400–700 g, were from AB Sahlins Försöksdjursfarm, Malmö, Sweden. They were housed in a 12-h light/12-h dark cycle and had free access to a standard pellet diet and water. The animals were anesthetized with Hypnorm (Janseen, Beerse, Belgium) and Dormicum (Hoffmann-La Roche AG, Basel, Switzerland). Blood was taken by aortic puncture from animals that had starved overnight into EDTA-containing tubes. Plasma was collected after centrifugation. For storage of plasma, sodium azide (0.02% w/v), aprotinin (100 unit/ml), and PMSF (0.1 mg/ml) were added. All animal procedures were approved by the Regional Animal Care and Use Committee of Umeå University. Human blood samples were collected from volunteers starved overnight and plasma samples were treated as described above.

Plasmid constructions

Guinea pig apoC-II cDNA (11) was cleaved out from pUC18 with NlaIV and Sau3a to give a 237 bp long fragment, the 5′ nucleotide triplett of which encoded the first amino acid residue (Ala) of apoC-II. The pUC18 EcoRI/BamHI was ligated to a linker with a EcoRI site and then ligated to the guinea pig apoC-II cDNA fragment (blunt end and Sau3a). The apoC-II cDNA with the linker was cleaved out from the recombined plasmid by EcoRI/SalI and made blunt-end. The pET21a vector was opened by NotI and made blunt-end. Again the apoC-II insert was cleaved out with NdeI/XhoI from the pET21a-linker-apoC-II plasmid. The final construct, designated pET15Gp, resulted in a vector

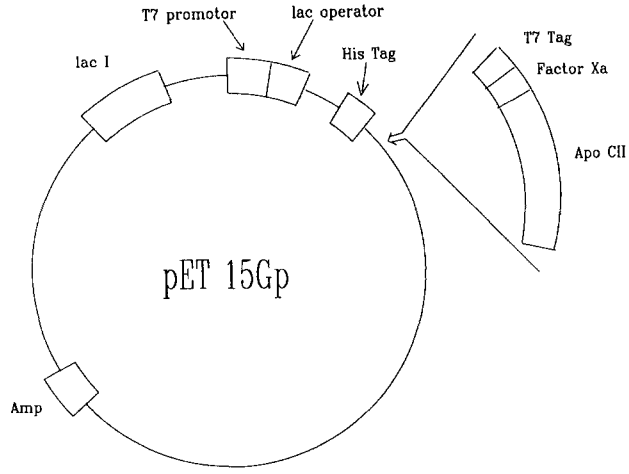

Fig. 1. Construction of the expression vector pET15Gp. Guinea pig apoC-II cDNA (11) was ligated to an oligonucleotide linker encoding the factor X_a cleavage site and the sequence for T7-Tag in vector pET21a. Commercial antibodies against the T7-Tag were used for detection of the fusion protein. For production, the T7-Tag, factor X_a, and apoC-II sequences were cleaved out and ligated to the vector pET15b. This construct (pET15Gp), with His-Tag for simple purification, was the most suitable vector for expression of guinea pig apoC-II.

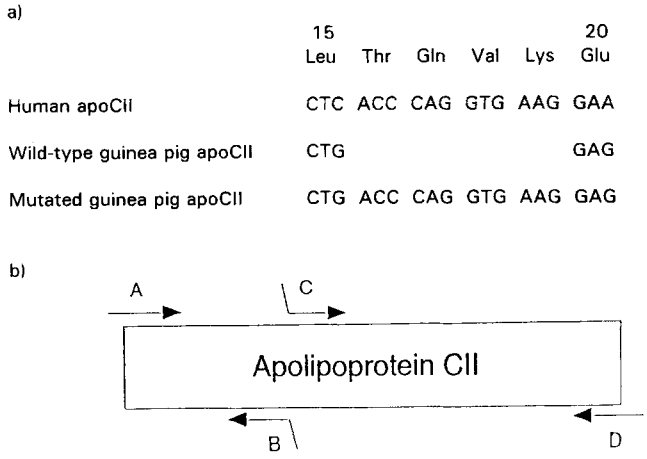

Fig. 2. Sequence differences between human and guinea pig apoC-II and generation of an insertion mutant of guinea pig apoC-II. a) Sequences of segments of apoC-II variants. The human apoC-II nucleotide sequence is shown from amino acid residue 15 to residue 20. The sequence of the wild-type guinea pig apoC-II shows the deletion of four amino acid residues as compared to human apoC-II. A mutated guinea pig apoC-II was constructed by insertion of the four lacking amino acids compared with the human sequence. b) Schematic demonstration of how the nucleotide primers acted to generate the mutated guinea pig apoC-II. The primers used were as follows:
Primer A. 5′TCGGAATTCCGGATCGAGGGTAGGGCCCATCTGA
Primer B. 5′GTCTC**GTTCACCTGGGT**CAGCAGGTCTGGGCTGGTC
Primer C. 5′**ACCCAGGTGAAC**GAGACCCTCTCCACCTACTGGGACA
Primer D. 5′GCAGGTCGACCCATCACTGTTTTCCTTGAAGC
The bold letters show the inserted nucleotides coding for amino acids Thr-Gln-Val-Lys corresponding to the human apoC-II sequence.

with the T7 RNA polymerase promoter followed by the lac operator and by the sequences for His-Tag, T7-Tag, Factor X_a cleavage site and apolipoprotein C-II (**Fig. 1**).

Site-directed mutagenesis of the guinea pig apoC-II

Site-directed mutagenesis by overlap extension PCR was performed using the guinea pig apoC-II (EcoRI/SalI) fragment with factor X_a linker (from the pUC18 construction) and four different primers (**Fig. 2**). In the first run of PCR two sets of reactions were set up. One reaction was with the guinea pig apoC-II DNA fragment, primers A and B, to give a 86 bp long fragment encoding the EcoRI site, the Factor X_a cleavage site, the first 15 amino acids in guinea pig apoC-II, and the four new amino acids (TQVK). The other reaction was with the guinea pig apoC-II DNA fragment and primers C and D, to give a 217 bp long fragment encoding for the four new amino acids (TQVK), the C-terminal 60 amino acids, and the SalI cleavage site (Fig. 2). In the next run, primers A and D were used with a mixture of the 86 bp and the 217 bp fragments as templates. The resulting 286 bp mutagenized apoC-II fragment was cleaved by EcoRI/SalI and ligated to the pET21a (EcoRI/SalI). The new vector was cleaved by NdeI/XhoI and the resulting fragment was ligated to the pET15b (NdeI/XhoI). Both constructs (pET15GpN and pET15GpM for mutated) were verified by dideoxy ribonucleotide sequencing.

Expression and purification of recombinant guinea pig apoC-II

Exponentially growing BL21(DE3) containing the plasmid pET15GpN or pET15GpM was induced with IPTG (0.5 mM) at A_{590} = 1.0 for 2 h. The bacteria were pelleted and lysed by 6 M guaninine-HCl, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl (pH 8.0), overnight at 4°C. After centrifugation at 19,000 rpm for 60 min at 4°C, the supernatant was mixed with Ni^{2+}-NTA agarose and the suspension was gently shaken overnight at 4°C. Then the gel was washed and eluted as recommended by the manufacturer. Briefly, buffers containing 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris base were used to produce a discontinuous gradient from pH 8.0 to pH 4.5. The fusion protein eluted between pH 6.3 and pH 4.5 and was dialyzed overnight against 0.1 M NaCl, 50 mM Tris-Cl (pH 8.0) containing 1 mM $CaCl_2$ and 0.5 M urea. The fusion protein was cleaved by factor X_a (1/250 of the substrate by weight) at room temperature overnight. After inhibition of factor X_a by PMSF (1 mM), guanidinium cloride was added to a concentration of 6 M and the sample was again mixed with Ni^{2+}-NTA-agarose (1 ml agarose/liter culture). After incubation overnight at 4°C the gel was poured into a column. The unbound protein (apoC-II) was dialyzed for 48 h

at 10°C against 10 mM ammonium bicarbonate and was then lyophilized.

Protein expression analyzed by Tricine-SDS-PAGE and Western blotting

The proteins were separated on Tricine-SDS-PAGE (16.5% T, 6% C) (25) and were detected by staining in 0.025% Serva-blue G in 10% acetic acid. For blotting experiments with T7-Tag antibody, the separated proteins were transferred onto a nitrocellulose filter in a Bio-Rad transblotting apparatus. Then the nitrocellulose filter was handled as recommended by the protocol of the manufacturer for the T7-Tag antibody.

Detection of apoC-II activity from gel slices

The lyophilized sample of purified recombinant apoC-II was dissolved in 5 M Urea, 10 mM Tris-Cl (pH 8.2). The protein was then analyzed on a discontinuous alkaline polyacrylamide gel containing urea (26). For detection of apoC-II activity, unstained lanes were cut into 2-mm pieces and put in an incubation mixture containing a ^{3}H-labeled lipid emulsion as previously described (11). Amino acid sequence analyses of proteins transferred to filters were done as previously described (11).

Activation of lipoprotein lipase in vitro

Human and guinea pig apoC-II were dissolved in 5 M Urea, 10 mM Tris-Cl (pH 8.2) to a final concentration of 0.5 mM. The final composition of the assay mixture was 0.15 M Tris-Cl, 0.1 M NaCl, 6% BSA, 1.5 IU heparin and ^{3}H-labeled Intralipid (5 mg triglyceride/ml) in the total volume of 200 µl (27). The pH was 8.5 and the temperature was 25°C. The emulsion was preincubated with apoC-II for 15 min before LPL was added. To each incubation 5 µl of the stock solution of apoC-II, or dilutions thereof in 5 M urea, 10 mM Tris (pH 8.2) was added. The reactions were stopped after 10 min by addition of organic solvents (27). For analyses of activation by isolated lipoprotein fractions, 1-80 µl of the different lipoprotein classes were allowed to preincubate with the emulsion for 15 min. Then LPL was added and the incubation time with lipase was 30 min.

ApoC-II-deficient chylomicrons

Human apoC-II-deficient chylomicrons from a homozygote patient with the apoC-II$_{Hamburg}$ mutation (28, 29) was a kind gift from Ulrike Beisiegel, Hamburg, Germany. Chylomicrons were isolated by centrifugation of plasma (30 min, 30,000 rpm, 4°C) and were obtained as a floating layer. In experiments with the chylomicrons, the fatty acids released were extracted and titrated manually (27). The assay mixture of 1 ml contained 0.15 M Tris-Cl, 0.1 M NaCl, 6% BSA, 1.5 IU heparin/ml, and chylomicrons (2 mg triglycerides/ml). The pH was 8.5 and the temperature was 25°C. The solutions of apoC-II were the same as above and not more than 5 µl of the stock solution or dilutions thereof was added per ml incubation mixture. The reactions were preincubated for 15 min before addition of bovine lipoprotein lipase. To ensure linearity the reactions were stopped after only 5 min by addition of organic solvents (27).

Dansylation of apoC-II and fluorescence measurements

Both human and guinea pig apoC-II were dansylated as previously described (30), but for guinea pig apoC-II 0.2 M sodium bicarbonate (pH 10.0) was used instead of 0.1 M, pH 8.6. Both preparations contained 1.3 mol dansyl groups/mol apoC-II. Fluorescence measurements were done as previously described (30). The relative increase in fluorescence at 490 nm upon excitation at 280 nm was measured after each addition of bovine lipoprotein lipase (in 10 mM Bistris buffer with 1.0 M NaCl) to the solution of dansylated apoC-II in 0.1 M Tris-Cl (pH 8.1). The fluorescence background values of human apoC-II (0.5 mM) and of guinea pig apoC-II (0.5 mM) were subtracted from the experimental values before analysis ($DF = F - F_0$).

Preparation of liposomes and binding experiments with apoC-II

Liposomes of egg yolk phosphatidylcholine were prepared with or without trioleoylglycerol (3 mol %) as described (31). After ultracentrifugation the tubes were divided into three parts and the middle fraction of the tube was collected (31). To study the interaction between apoC-II and liposomes, fluorescence measurements were made in 0.1 M Tris-Cl, pH 8.1, at 25°C on a Shimadzu spectrofluorophotometer model RF500. The tryptophan fluoescence emission spectrum of apoC-II in the absence of lipid was maximal at 345 nm. With addition of liposomes the maximal fluorescence emission wavelength shifted to a minimum at 330 nm and the intrinsic fluorescence of the protein increased. The increase of fluorescence was attributed to the binding of apoC-II to the liposomes. The data were fitted according to the Hill-equation using the computer program Fig.P® for Windows (BIOSOFT, UK).

Preparation of antibodies against guinea pig apoC-II, affinity purification and conjugation

Recombinant guinea pig apoC-II was coupled as a hapten to ovalbumin using glutaraldehyde and was used to raise antibodies by subcutaneous injections to a rabbit. Immunoglobulins were isolated from serum using Protein A Sepharose. For affinity purification of the antibodies, recombinant guinea pig apoC-II was coupled to CNBr-activated Sepharose. Rabbit IgG was incu-

bated with the gel overnight at 4°C. After wash the anti-guinea pig apoC-II antibodies were eluted by 0.2 M glycine-HCl (pH 2.7) followed by 0.2 M glycine with 10% (v/v) dioxan (pH 2.7). The peak fractions were pooled and were then dialyzed against 10 mM Tris, 140 mM NaCl, pH 7.4. For conjugation with horseradish peroxidase, 5 mg of the antibodies was dialyzed against water and then lyophilized. After solubilization in 250 µl PBS, the antibodies were mixed with 10 mg glutaraldehyde-activated horseradish peroxidase.

Enzyme-linked immunosorbent assay (ELISA) for guinea pig apoC-II

Microtiter plates were coated with 100 µl of the affinity purified apoC-II antibodies (15 µg/ml) in PBS for 4 h at 37°C. The plates were washed 4 times with 0.01 M PBS containing 0.05% Tween-20 (pH 7.4). Recombinant guinea pig apoC-II used as standard was diluted in PBS, containing 15% glycerol, 1 mg/ml heparin, 1% Tween-20, and 14 mg/ml BSA, ranging from 1 to 40 ng/ml (100 µl per well). Guinea pig plasma (collected in EDTA-containing tubes) was diluted in the same buffer. Incubations with antigen were performed overnight at 4°C. The plates were washed 4 times with washing buffer as described above and 100 µl of affinity-purified anti-guinea pig apoC-II antibody coupled to horseradish peroxidase (diluted 1/750 in PBS, 0.1% Tween-20 and 4% BSA) was added. After incubation for 3 h at room temperature, the plates were washed 4 times with 0.01 M PBS containing 0.05% Tween. For detection, 1,2-phenylenediamine (0.4 mg/ml) and H_2O_2 (0.012%/ml) in 0.1 M sodium citrate/sodium phosphate (pH 5.0) was used. The reaction was stopped after 30 min by addition of 50 µl of 4 M H_2SO_4. Absorbancy at 490 nm was recorded by a titertek Multiscan spectrophotometer. The assay was linear from 1 to 20 ng apoC-II/ml.

High performance liquid chromatography of plasma lipoproteins

Fresh EDTA plasma was centrifuged at 10,000 rpm for 10 min and was passed through a 0.22-µm filter (Millipore, Molsheim, France). Then 500 µl was applied to a Superose 6 HR 10/30 column. The column was run at 4°C in 50 mM Na-phosphate buffer, pH 7.4, containing 150 mM NaCl. The flow rate was 300 µl/min. Fractions were collected every minute and were analyzed on the same day for triacylglycerol and cholesterol. Samples were frozen for later analysis of apoC-II.

Separation of lipoproteins by density-gradient ultracentrifugation

Discontinuous density gradients were made in 13.2 ml polyallomer centrifuge tubes ($^9/_{16}$-in. diam × $3^1/_2$-in. length) essentially as previously described (32). The plasma samples were adjusted to d 1.21 g/ml by solid potassium bromide (0.325 g/ml plasma) and 4 ml was loaded at the bottom of the gradients. All solutions contained EDTA (0.01%) and were prepared with potassium bromide and sodium chloride. The tubes were ultracentrifuged at 15°C, 40,000 rpm for 24 h in a Beckman SW41 rotor. After centrifugation, fractions were collected from the bottom of tube (Fraction Recovery system, Beckman, Fullerton, CA).

Sequential ultracentrifugation of lipoproteins

Lipoproteins were isolated by sequential ultracentrifugation (33). For guinea pig plasma the following density ranges were used: VLDL, d < 1.006 g/ml; LDL, d 1.006–1.10 g/ml, and HDL, d 1.10–1.21 g/ml. After aspiration of the top 1.0 ml in each tube, the following 2.0 ml was recovered and centrifuged again under the same conditions. The material that floated at the top was recovered and pooled with the material from the first centrifugation. Each lipoprotein fraction was dialyzed overnight against 150 mM NaCl, 20 mM Tris-Cl (pH 7.4) in dialysis tubes with a cut off at 3500 kDa.

Delipidation of lipoprotein

Lipoproteins (50–200 µl) were delipidated as previously described (34). The protein pellets were dissolved in sample buffer for SDS-PAGE (1 µg/µl). After 30 min at room temperature the samples were heated for 4 min at 95°C.

Interaction of guinea pig and human LDL with LPL as studied by surface plasmon resonance technique

Binding experiments were made using a BIAcore (Pharmacia Biosensor, Uppsala, Sweden) and using conditions that were described previously (35, 36). For the present experiments biotinylated LPL was coupled to streptavidin which was, in turn, covalently attached to the dextran matrix of the sensor chips. Solutions of LDL in 10 mM HEPES, pH 7.4, containing 0.15 M NaCl and 3.4 mM EDTA were passed over the surface layer. Binding was studied as the change in refractive index (relative response units, RU). Dissociation was studied when buffer without LDL was passed over the surface layer. Calculations of rate constants were made as previously described (35).

RESULTS

Expression of guinea pig apoC-II in *E. coli*

Several different expression vectors were tried (pET 8c, pKK 332-2, pRIT 5, and pET 21a). Finally we selected a modified system using the pET 15b with a His-

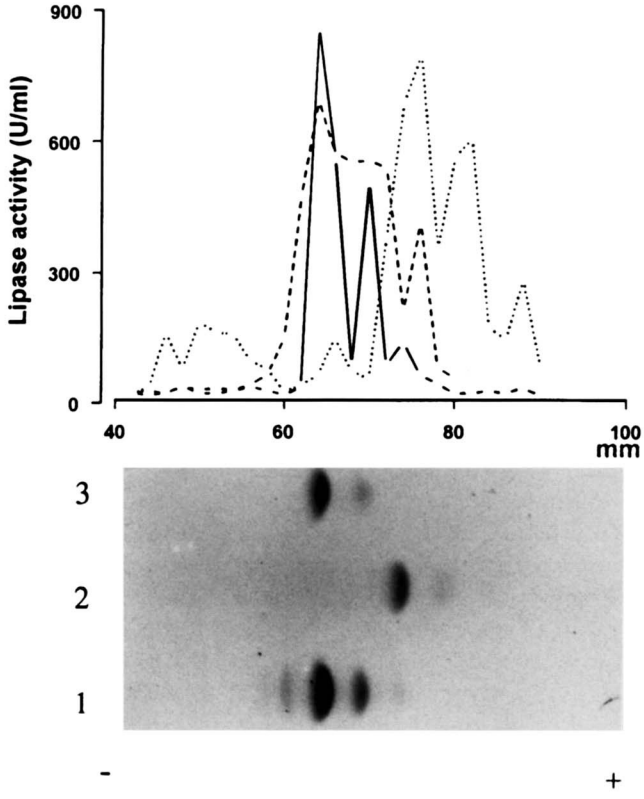

Fig. 3. Electrophoresis of wild-type and mutated guinea pig apoC-II and recovery of lipoprotein lipase-stimulating activity from gel slices. Lower panel: lane 1, human apoC-II purified from plasma (20 μg); lane 2, wild-type guinea pig apoC-II from *E. coli* (15 μg); lane 3, mutated guinea pig apoC-II (15 μg). The proteins were separated on a PAGE in urea (26). Minus and plus signs indicate the positions of cathode and anode, respectively. Migration was from left to right. Upper panel: the graph shows stimulation of activity of bovine LPL by extracts of gel slices, (–––) human apoC-II; (. . . . .) wild-type guinea pig apoC-II; (——) mutated guinea pig apoC-II. The lipase activity is calculated per ml of enzyme added (1.1 mg LPL/ml). One U corresponds to 1 μmol fatty acids released per min at 25°C.

Tag, a T7-Tag, and sequences for a factor X_a recognition site. The pET 21a itself did not work. The expression of the fusion protein was analyzed by Tricine-SDS-PAGE and Western blotting using anti T7 antibody. After induction with IPTG, a protein with the expected molecular mass was seen (13 kD). The highest yield of the fusion protein was obtained after a 2 · h induction period. The same conditions were used for expression of the mutated variant of apoC-II in which 4 amino acids (residues 16–19, TQVK) were inserted. Protein determinations revealed that the yield of apoC-II after purification was approximately 5 mg/l culture medium.

The purity of the recombinant variants of apoC-II was analyzed by electrophoresis in an alkaline polyacrylamide gel system containing urea (**Fig. 3**). Both the wild-type and the mutated forms of guinea pig apoC-II gave two protein bands (Fig. 3, lower panel). Amino acid sequence analyses showed that the proteins in the major band had the expected amino-terminal sequences, while the minor bands contained a mixture of apoC-II variants. One sequence in these minor fractions was 5 amino acids shorter in the amino-terminal end than full-length guinea pig apoC-II. Traces of the intact amino-terminal sequences were also found. The reason for additional cleavages both at the amino-terminus, and probably also at the carboxy-terminus, is unknown, but could be due to unspecific cleavage by Factor X_a during the long incubation time needed for optimal recovery of apoC-II. Scanning of the gels showed that the minor bands constituted less than 10% of the total material. Both the major and the minor bands of native and mutated apoC-II extracted from gel slices activated lipoprotein lipase (Fig. 3, upper panel). It should be emphasized that this analysis is only qualitative and no detailed comparisons regarding activation ability can be made. For comparison, a preparation of human apoC-II purified from plasma was run. The two strongest bands corresponded to pro- and mature apoC-II (mature lacking the amino-terminal hexapeptide according to the nomenclature of Fojo et al. (37)). The mutated form of guinea pig apoC-II with the four extra amino acids (TQVK) inserted had the same net charge as human apoC-II and therefore the same electrophoretic mobility. Wild-type guinea pig apoC-II had a faster mobility than the others which is consistent with the lack of one positively charged lysine residue (Fig. 2). Due to the relatively low proportion of cleaved or modified versions of the recombinant proteins and also due to the fact that at least the main part of the modified form was able to activate lipoprotein lipase, we did not attempt to further purify the recombinant proteins. On SDS-PAGE we were not able to discriminate between the major and the minor bands by size, demonstrating that no major truncations had occurred (data not shown).

Stimulation of lipoprotein lipases from three different species by guinea pig apoC-II variants and by human apoC-II

Lipoprotein lipases from three different species (guinea pig, cow, and human) were used in an in vitro system with Intralipid® as the substrate (**Fig. 4**). Human apoC-II stimulated all three lipases. The amounts of the different lipase preparations were chosen so that they would reach approximately the same maximal level of activity. The lipases differed in their activity without apoC-II; guinea pig LPL had the lowest basal activity, while bovine LPL had the highest. Hence, the stimulation was highest for guinea pig LPL, about 13-fold, compared to about 3-fold for bovine LPL. High amounts of apoC-II (>1 μg/ml) caused inhibition. This was most

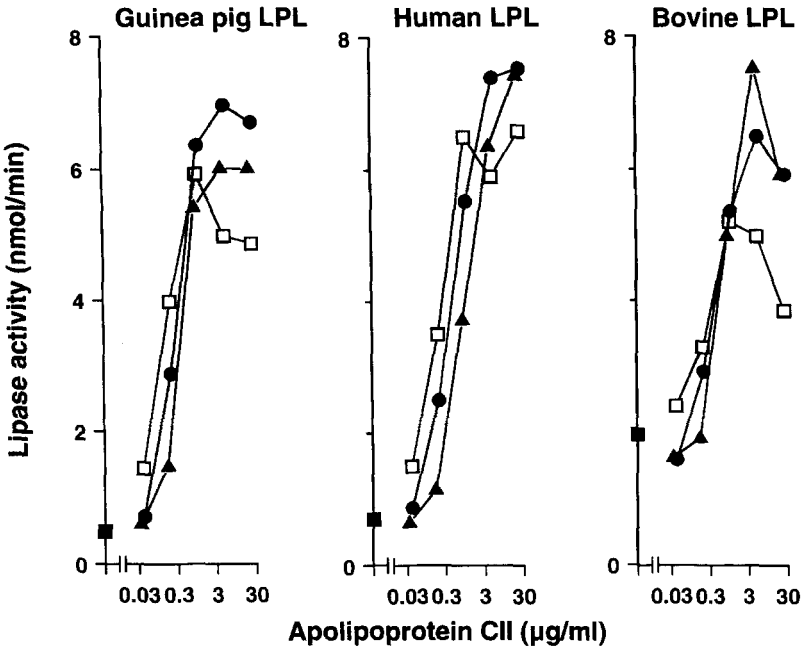

Fig. 4. Activation of lipoprotein lipases from three different animal species by guinea pig apoC-II variants and by human apoC-II. ApoC-II (□, human; ●, guinea pig, wild-type; ▲, guinea pig, insertion mutant) was incubated with LPL from three different species (~5 ng of each) in a total volume of 200 μl containing ^{3}H-labeled Intralipid®. The incubations were carried out for 10 min. Each data point is the mean of duplicate determinations. The experiment was repeated three times with similar results. Due to a comparatively large between-assay variation, the data were not combined.

clearly seen with bovine LPL. We found, quite unexpectedly, that wild-type guinea pig apoC-II stimulated LPL from all three species at least to the same level as human apoC-II. In fact, the tendency for inhibition with higher amounts of the activator was less pronounced with guinea pig apoC-II than with human apoC-II. The insertion mutant of guinea pig apoC-II was similarly effective. Evaluation of several similar experiments indicated, however, that when small amounts of the activators were used (0.03–0.2 μg/ml), human apoC-II was more effective than wild-type guinea pig apoC-II which, in turn, was slightly more effective than the insertion mutant (Fig. 4 and data not shown). This was the case with LPL from all three species.

Stimulation of lipoprotein lipase activity against apoC-II-deficient human chylomicrons

We used chylomicrons from an apoC-II-deficient patient to study the ability of guinea pig apoC-II to stimulate LPL against a natural lipoprotein (**Fig. 5**). In this case the basal activity was very low, even with bovine

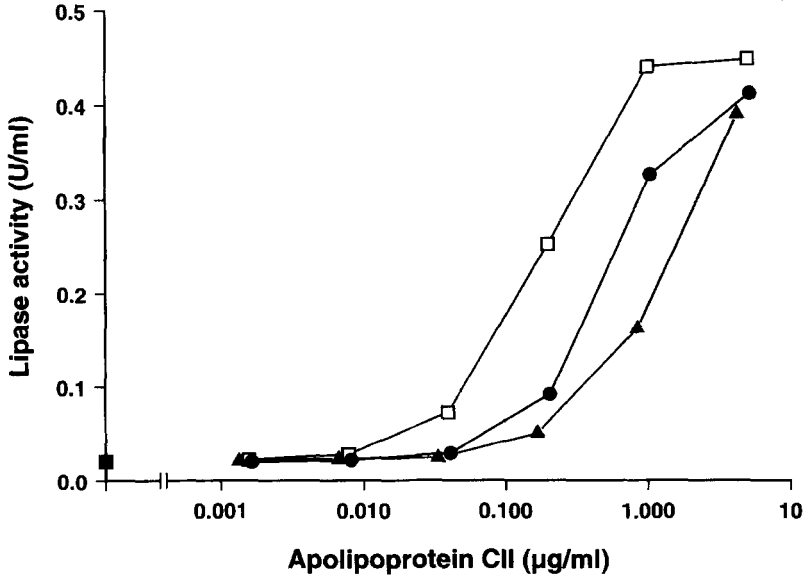

Fig. 5. Stimulation of lipoprotein lipase by the apoC-II variants with chylomicrons from an apoC-II-deficient patient as substrate. Lipoprotein lipase (bovine, 1.2 ng) was incubated for 5 min at 25°C in a total volume of 1 ml with chylomicrons from an apoC-II-deficient patient (2 mg triglycerides/ml) and with either human apoC-II (□), with guinea pig wild-type apoC-II (●), or with guinea pig apoC-II, insertion mutant (▲). The apoC-II variants were dissolved to a final concentration of 4.9 mg/ml in 5 M urea, 10 mM Tris-Cl (pH 8.2) Five μl of this stock solution, or dilutions thereof, in the same buffer was added. Each data point is the mean of duplicate samples. The fatty acids released were extracted and titrated manually. Lipase activity is expressed in U/ml incubation mixture.

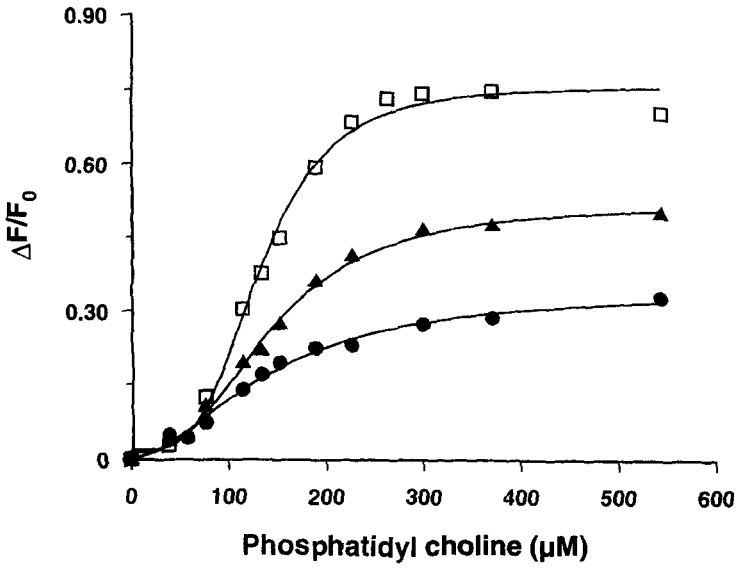

Fig. 6. Binding of apoC-II variants to liposomes as measured by fluorescence. Liposomes were successively added to apoC-II (2.3 μM) either human or guinea pig (wild-type or mutated) in 2 ml 0.1 M Tris-Cl (pH 8.1) to the indicated final phospholipid concentration. The liposomes were prepared with trioleoylglycerol. After each addition the fluorescence intensity was measured. The excitation wavelength was 280 nm and the measurements were made at maximal emission (490 nm). The fluorescence value given was adjusted for the change in concentration due to the additions of liposomes and ΔF was determined after subtraction of the fluorescence background from apoC-II ($\Delta F = F - F_0$). The ΔF values were normalized to background fluorescence ($\Delta F/F_0$). Human apoC-II (□), guinea pig, wild-type (●) and guinea pig, insertion mutant (▲).

LPL, which was used for the experiment. Both forms of guinea pig apoC-II were able to stimulate the activity to about the same level as human apoC-II. However, this substrate emphasized the difference in relative efficiency between the apoC-II variants which was seen already with the synthetic lipid emulsion in Fig. 4. Human apoC-II was clearly more efficient. About 5- to 10-fold more of the guinea pig apoC-II variants was needed for half maximal stimulation. As the amount of this rare substrate was limited we could only repeat the experiment once. In both cases slightly more of the mutated form of guinea pig apoC-II appeared to be required compared to the wild-type form.

Interaction of guinea pig apoC-II with lipoprotein lipase in solution

The activator proteins were dansylated to allow direct comparison of the interaction between human and guinea pig apoC-II and LPL. Energy transfer from tryptophans in LPL to the dansyl group was measured as fluorescence at 490 nm upon excitation at 280 nm as previously described (30, 38). The K_d for the interaction was about 0.2 μM for both guinea pig apoC-II and for human apoC-II (data not shown).

Interaction of guinea pig apoC-II with lipids

The relative affinities of wild-type guinea pig apoC-II, the insertion mutant, and human apoC-II for lipid/water interfaces were studied using liposomes of phosphatidylcholine containing a small amount of trioleoylglycerol (31). With increasing amounts of liposomes the tryptophan fluorescence of both human apoC-II and of the guinea pig apoC-II variants increased, indicating that their single tryptophan was exposed to a more hydrophobic environment (**Fig. 6**). Binding of apoC-II to liposomes was rapid; the fluorescence did not change from 30 sec to 60 min (data not shown). A notable difference was that the maximal fluorescence increase was around 2-fold higher with human apoC-II compared to that for wild-type guinea pig apoC-II. The curve for the insertion mutant fell in between those for the other two. The binding curves were in all cases S-shaped, indicating that cooperativity was involved. When the curves were analyzed by the Hill equation, the estimated half maximal values were achieved at concentrations of phosphatidylcholine similar for all three apoC-II variants. The apparent dissociation constants were 134 ± 4 μM, 143 ± 12 μM, and 146 ± 5 μM for human, for wild-type guinea pig, and for mutated guinea pig apoC-II, respectively. Similar results were obtained with pure liposomes without any incorporated trioleoylglycerol, except that the mutated form of guinea pig apoC-II then behaved more like the wild-type guinea pig apoC-II (data not shown).

Concentration of apoC-II in guinea pig plasma

The antiserum raised against the recombinant wild-type guinea pig apoC-II reacted well with purified apoC-II or with guinea pig plasma on Western blots, but poorly or not at all with human apoC-II or with plasma from other species (rat, cow, and chicken, data not shown). The antibodies were used in an enzyme-linked immunoassay (ELISA). Standard curves with different amounts of purified recombinant guinea pig apoC-II were parallel to dilution curves for guinea pig plasma (data not shown). Plasma samples from nine different (male) guinea pigs were analyzed. Their mean apoC-II concentration was 11.5 ± 5.4 μg/ml plasma. This is lower than those reported for human plasma: 36 ± 10 μg/ml (39) or 39.8 ± 7.1 μg/ml (40).

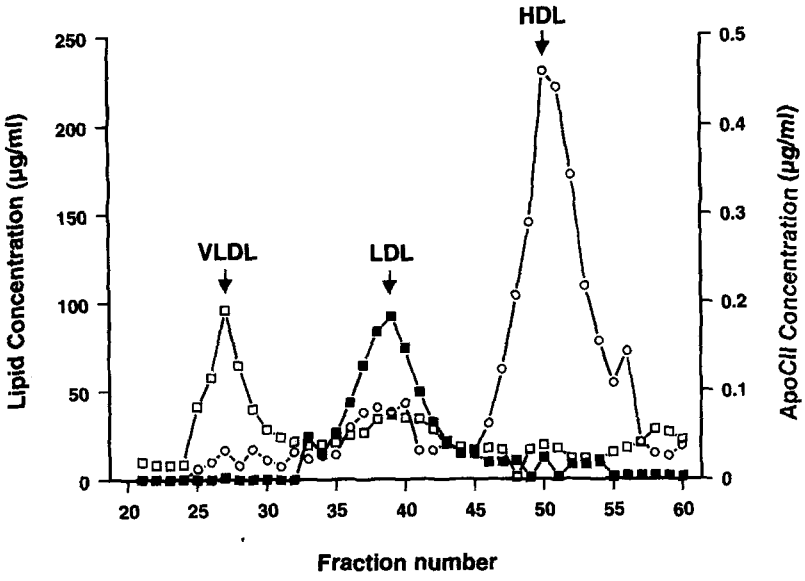

Fig. 7. Separation of plasma lipoproteins by gel permeation chromatography. Plasma from a guinea pig fasted overnight (triacylglycerol = 475.8 µg/ml and cholesterol = 322.9 µg/ml) was run on the column; (□) triacylglycerol, (■) cholesterol, (○) apoC-II. The arrows indicate the elution positions of human VLDL, LDL, and HDL.

Distribution of apoC-II on plasma lipoproteins isolated by HPLC or by gradient ultracentrifugation

Lipoprotein analysis by gel permeation chromatography confirmed the previously known fact that LDL is the dominating cholesterol-containing lipoprotein in guinea pigs (**Fig. 7**), while the level of HDL is extremely low. Most of the apoC-II in guinea pig plasma was associated with particles of HDL size. Additional information was obtained by separation of plasma lipoproteins by density gradient ultracentrifugation (**Fig. 8**). For comparison, we used the same density gradient as previously described for human lipoproteins. Fractions 8–9 represented the boundary between densities 1.21 g/ml and 1.063 g/ml. With guinea pig plasma the lipid concentration in fractions 6–9 was extremely low, but still 40% of the total apoC-II was found in these four fractions (Fig. 8a). Some apoC-II was also found associated with lipoproteins of LDL density in agreement with the pattern from the Superose column (Fig. 7). SDS-PAGE on material from fractions 6–9 showed that it contained apoB-100. Thus, in contrast to the corresponding human fraction, the guinea pig fraction contained a mixture of HDL and LDL with apoB-100 representing at least 80% of the total apolipoprotein (data not shown). In human plasma, apoC-II was distributed between the HDL and VLDL fractions (Fig. 8b). In guinea pig VLDL, only small amounts of apoC-II were found (Fig. 7 and Fig. 8a).

Separation of lipoproteins by sequential ultracentrifugation

Based on experiments such as those shown in Fig. 8, we chose the density range 1.006–1.100 g/ml for separation of LDL from guinea pig plasma and HDL was therefore isolated in the density range 1.10–1.21 g/ml

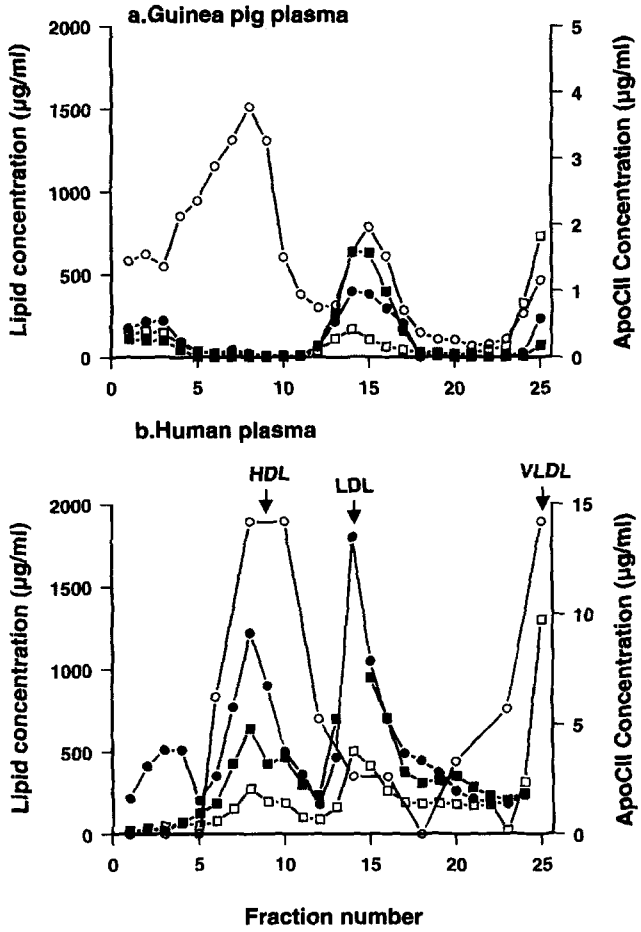

Fig. 8. Separation of lipoproteins by density gradient ultracentrifugation. Fractions were collected from the bottom of the tubes. Panel a shows the profile of guinea pig plasma; panel b for human plasma; (□) triacylglycerol, (■) cholesterol, (●) phospholipid, (○) apoC-II. The y-axis shows the concentration of each of the measured lipids.

TABLE 1. Average composition of guinea pig LDL-like (d 1.006–1.10 g/ml) and HDL-like (d 1.10–1.21 g/ml) lipoproteins and human LDL and HDL after isolation by sequential ultracentrifugation

	Guinea Pig		Human	
Component	LDL	HDL	LDL	HDL
	weight %			
Triacylglycerol	8.4 ± 2.3	6.5 ± 2.0	4.9	2.6
Free cholesterol	7.2 ± 1.7	[a]	11.5	3.1
Cholesterol esters	28.8 ± 2.0	15.2 ± 7.1	29.8	14.3
Phospholipids	21.5 ± 2.0	16.7 ± 1.8	25.9	27.5
Protein	34.2 ± 3.5	61.3 ± 7.8	27.9	52.4
	weight % of total protein			
ApoC-II	1.1 ± 0.4	2.7 ± 0.6	0.3	1.2

Data for guinea pig lipoproteins are mean values ± SD of 4 different preparations; data for human lipoproteins are from one preparation.

[a] In one preparation there was 1.2% while in the other three preparations free cholesterol was undetectable.

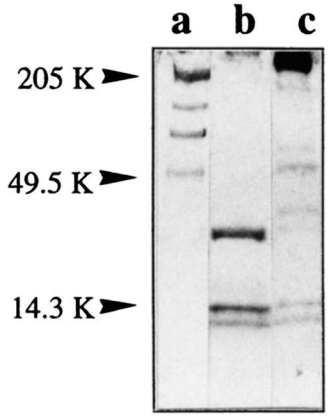

Fig. 9. Analyses of apolipoproteins in guinea pig LDL and HDL by SDS-PAGE. Lane a: molecular weight markers, the arrows indicate the migration of standard proteins; lane b: HDL, density 1.100–1.21 g/ml (15 μg protein); lane c: LDL, density 1.006–1.100 g/ml (10 μg protein).

(**Table 1**). The LDL fraction contained 34% protein (by weight) of which apoC-II constituted about 1.1%. HDL contained 61% protein of which apoC-II constituted 2.7%. More than 45% of total apoC-II was found in the density range 1.006–1.100 g/ml and less than 30% was recovered with the HDL-like particles in the density range 1.100–1.21 g/ml. In human plasma more than 50% of total apoC-II was found in the HDL fraction (d 1.063–1.21 g/ml) and less than 20% was found in the LDL fraction (d 1.006–1.063 g/ml) (**Table 2**).

Analysis of apolipoproteins in guinea pig LDL and HDL by SDS-PAGE

The major apolipoprotein in guinea pig LDL (isolated by sequential centrifugation in the density range between 1.006–1.100 g/ml) was apoB (>205 kDa, **Fig. 9,** lane c). Minor apolipoprotein components were also present confirming earlier data reported by other laboratories (41, 42). The apparent molecular weight of the major apolipoprotein in guinea pig HDL (d 1.10–1.21 g/ml) was about 28 kDa. N-terminal amino acid sequencing revealed the sequence DDPKQQDDVVKEF AN, which probably corresponds to apoA-I (43). The second major apolipoprotein migrated to a position resembling that of human apoA-II. Several protein sequences were found in this band. The smallest apolipoprotein was apoC, about 7 kDa. The major N-terminal sequence in this band was EEIQESSLLGVMKDY, corresponding to guinea pig apoC-III (11).

Effects of lipoproteins from human and guinea pig plasma on LPL activity against Intralipid®

Compared to human lipoproteins, lipoproteins from guinea pig plasma were less effective in stimulating the activity of lipoprotein lipase (**Fig. 10**). Based on the content of apoC-II, about 3-fold more of guinea pig VLDL and of guinea pig HDL had to be added to obtain similar stimulation levels as with the corresponding human fractions. Guinea pig LDL inhibited the lipase, while the human LDL fraction instead stimulated the activity.

Relative affinities of LPL for LDL isolated from guinea pig or human plasma

The interactions of LDL with bovine LPL were directly studied using the surface plasmon resonance

TABLE 2. Distribution of apoC-II in lipoproteins of guinea pig and human plasma

	Guinea Pig Plasma		Human Plasma	
Fraction	Density	ApoC-II	Density	ApoC-II
	g/ml	*% of total*	*g/ml*	*% of total*
VLDL	<1.006	17.3	<1.006	17.7
LDL	1.006–1.100	45.3	1.006–1.063	17.8
HDL	1.100–1.121	27.9	1.063–1.21	52.5
Remaining plasma	>1.21	9.5	>1.21	12.1

Lipoproteins were separated by sequential ultracentrifugation. Data for both guinea pig and human plasma are from one preparation each.

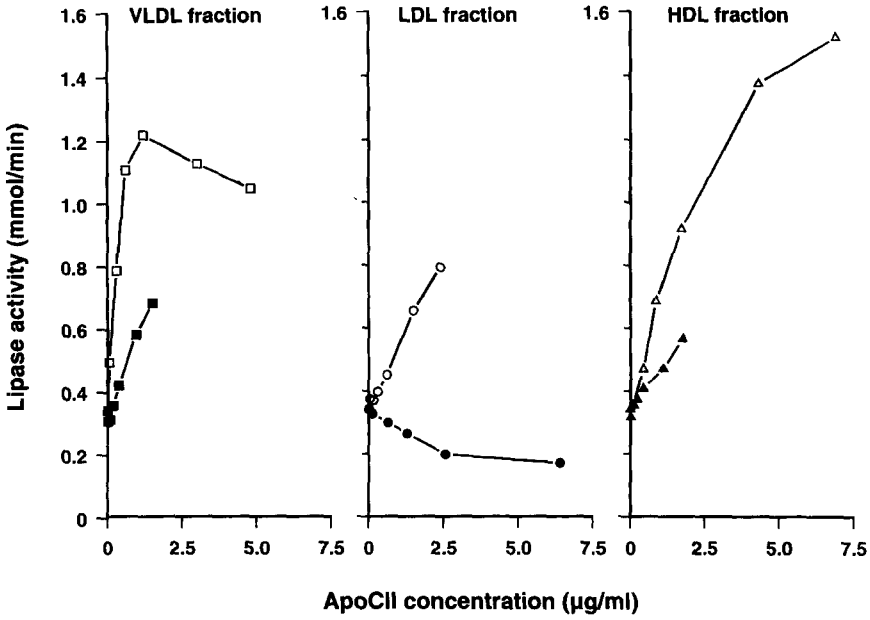

Fig. 10. Activation of lipoprotein lipase by lipoproteins isolated by sequential ultracentrifugation of guinea pig and human plasma. Bovine lipoprotein lipase (26 ng) was incubated with radiolabeled Intralipid® as substrate and with guinea pig lipoproteins (solid symbols) or human lipoproteins (open symbols) as source of apoC-II. Lipoproteins were isolated from guinea pig plasma (triacylglycerol = 280.9 µg/ml; cholesterol = 430.6 µg/ml) and human plasma (triacylglycerol = 500 µg/ml; cholesterol = 1588 µg/ml) by sequential ultracentrifugation. The content of apoC-II in the lipoprotein fractions was determined as described in Materials and Methods. To allow direct comparison, the lipase activity (expressed in mmol free fatty acid released per min) is plotted against the concentrations of apoC-II in the incubation mixtures.

based technology on a BIAcore (**Fig. 11**). In this system the kinetics for both association and dissociation can be directly followed. Compared to human LDL, the association rate for guinea pig LDL was faster and the dissociation rate was slower. The calculated values for the association rate constants were $8.9 \times 10^4\ M^{-1}s^{-1}$ and $2.7 \times 10^4\ M^{-1}s^{-1}$ for guinea pig and for human LDL, respectively. The values for the dissociation rate constants were $2.1 \times 10^4\ M^{-1}s^{-1}$ and $4.5 \times 10^4\ M^{-1}s^{-1}$, respectively. The equilibrium dissociation constants (K_d) calculated as k_{ass}/k_{diss} differed about 7-fold (2.4 nM for guinea pig LDL and 17 nM for human LDL). A plausible explanation for this difference could be that guinea pig LDL contain apoC-II in a form that cannot leave the particle but can increase the affinity of these particles for LPL. This could explain the inhibition seen by guinea pig LDL on LPL activity against the synthetic lipid emulsion (Fig. 10).

DISCUSSION

To investigate the question of why guinea pig plasma appears to be devoid of an activator for LPL it was necessary to develop an efficient expression system for guinea pig apoC-II. We found that expression of apoC-II as a fusion protein made it more stable against proteolytic degradation than we had experienced in other systems. Furthermore, the inducible expression of apoC-II made it less toxic to the bacteria. Because, in our case, the His-Tag made purification of the fusion protein rather simple, the overall yield of reasonably pure apoC-II was about 5 mg/l culture medium.

We studied activation of LPL isolated from three different sources (guinea pig, cow, and human), as tendencies for a species specificity might strengthen differences between the apoC-II variants. Using a synthetic phospholipid-stabilized emulsion of triacylglycerols (Intralipid®) we got from 3- to 13-fold stimulation with human apoC-II of the three different lipases due to differences in their basal activities. Wild-type guinea pig apoC-II stimulated all three lipases to an even higher level than human apoC-II. Detailed comparisons of the maximal stimulation are difficult, however, due to inhibition of the reaction by higher amounts of apoC-II protein. This has been seen also in other studies in vitro (44) and also on overexpression of apoC-II in transgenic mice (45). With Intralipid® similar amounts of all three apoC-II variants were needed for half maximal stimulation, but at low concentrations of activator there was a tendency for human apoC-II to be more efficient. Direct binding studies between bovine LPL and dansyl-labeled guinea pig apoC-II showed that the

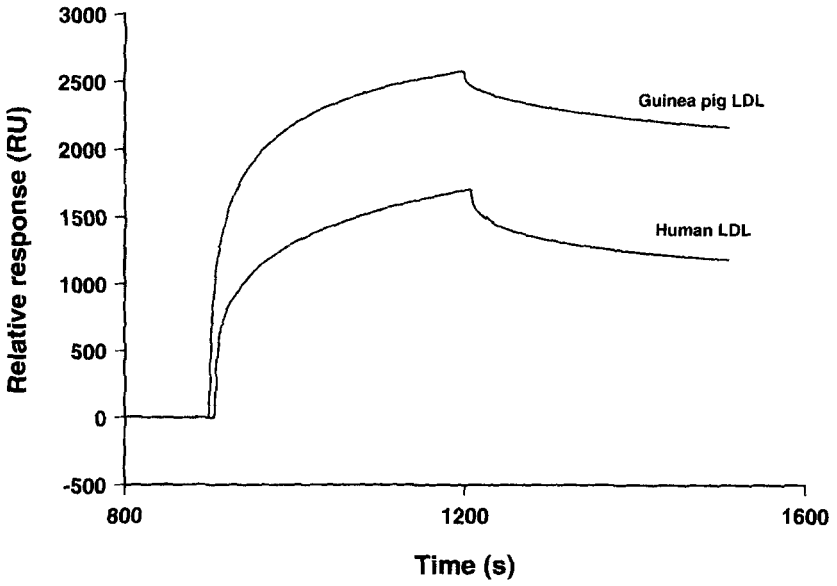

Fig. 11. Kinetics for binding of guinea pig LDL and human LDL to immobilized lipoprotein lipase. The experiments were performed on a BIAcore as described in the Methods section. Biotinylated bovine lipoprotein lipase was bound to sensor chips that were covered by covalently immobilized streptavidin. Solutions of LDL (3.9 nM) were passed over the sensor chips. The increase in response units (RU) is proportional to the amount of bound LDL. At time 1200 s, the flow was changed to buffer without LDL for study of dissociation.

apparent binding constants were similar to those previously obtained for the interaction between bovine LPL and human apoC-II (30) and also carboxy-terminal fragments of human apoC-II (46). The sequence similarities are strong in this part of the molecule between human and guinea pig apoC-II and most of the substitutions are functionally conservative (11). Therefore the mode of interaction between the apoC-II variants and LPL is probably similar or identical.

With human chylomicrons from an apoC-II-deficient patient, the small differences in efficiency of the apoC-II variants seen with Intralipid® were increased. Five- to 10-fold more of guinea pig apoC-II was required than of human apoC-II to achieve the same level of stimulation. This indicated that there might be differences in how well the guinea pig activators can bind to chylomicrons as compared to binding of human apoC-II. The difference was not due to the four residue deletion in the N-terminal domain, as the insertion mutant was, if anything, less efficient than the wild-type guinea pig apoC-II. To investigate the lipid interaction of guinea pig apoC-II, we studied the binding of the activators to liposomes of phosphatidylcholine with and without trioleoylglycerol. The binding did not follow a simple hyperbolic function, but showed sigmoidal curves. The apparent affinities for the lipid were, however, similar. The increase in intrinsic tryptophan fluorescence was largest for human apoC-II, about 1.7-fold, while the effect on the wild-type guinea pig apoC-II was the smallest (1.3-fold). The difference in the increase of the fluorescence between the two species of apoC-II suggested more changes in the conformation of human apoC-II compared to guinea pig apoC-II, or a slightly different mode of lipid interaction. Addition of four amino acid residues into the amino-terminal part of guinea pig apoC-II made the tryptophan fluorescence more sensitive to binding to liposomes, but the change was still smaller than that for human apoC-II. Thus, the whole difference seen in the lipid interaction between human and guinea pig apoC-II variants is not explained by the absence of the four amino acid residues in the putative lipid-binding domain.

Guinea pigs represent an animal species with low levels of HDL (47, 48). In humans HDL is a rich source of apoC-II, harboring around 60% of the total apoC-II pool for transfer to nascent triacylglycerol-rich lipoproteins (1, 6, 49). We found that guinea pigs had somewhat lower concentrations of apoC-II in plasma than has been reported for humans, 11.5 ± 5.4 μg/ml as compared to 36 ± 10 μg/ml. Even though the HDL levels are very low in guinea pig plasma, most apoC-II was associated with HDL and only small amounts were associated with VLDL. It has been suggested that the total amount of apoC-II in human plasma does not necessarily reflect the amount available for efficient transfer to newly synthesized lipoproteins or to lipid emulsions (49, 50). About 20–30% may not be transferable. In guinea pigs, the LDL-like lipoprotein fraction, isolated by sequential ultracentrifugation, contained about 45% of the total apoC-II, but instead of stimulat-

ing it rather inhibited the action of LPL on Intralipid®. Compared to human LDL, guinea pig LDL had higher binding affinity for LPL when it was directly studied by the surface plasmon resonance technique. Thus, guinea pig LDL may not be a good reservoir for transferable apoC-II, but may rather act as a competitive ligand for lipoprotein lipase at least under in vitro conditions.

We conclude that several factors contribute to the low stimulation of lipoprotein lipase by guinea pig plasma: requirement of more guinea pig apoC-II for stimulation of physiologically relevant substrates, lower absolute level of apoC-II in guinea pig plasma, small differences in the mode of lipid interaction of guinea pig apoC-II compared to human apoC-II, a different distribution between lipoprotein classes than seen in other species (humans), a decreased ability of guinea pig HDL to donate apoC-II to synthetic lipid emulsions, and the presence in guinea pigs of apoC-II-containing LDL with high affinity for lipoprotein lipase and with inhibitory effect on the activity of LPL. Furthermore, we have demonstrated that the difference in behavior between human apoC-II and guinea pig apoC-II is not explained by the missing 4 amino acid residues in the amino-terminal domain of guinea pig apoC-II.

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