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Activation of lipoprotein lipase by native and synthetic fragments of human plasma apolipoprotein C-II

(solid-phase peptide synthesis/plasma triglycerides/lipoproteins)

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ABSTRACT **Apolipoprotein C-II (apoC-II), a protein constituent of human very low density lipoproteins, is the activator for lipoprotein lipase (LPL; triacylglycerol acyl-hydrolase, EC 3.1.1.3). The amino acid sequence of the 78 residues of apoC-II has recently been established in this laboratory. To determine the minimal sequence requirements for activation, we have prepared both native and synthetic fragments of apoC-II and tested them for their ability to activate LPL. Cyanogen bromide fragments of apoC-II coresponding to residues 1–9 and 10–59 had little ability to activate LPL. However, the COOH-terminal cyanogen bromide fragment corresponding to residues 60–78 increased hydrolysis 4-fold compared to an average of 9-fold activation for the same concentration of apoC-II. The synthetic peptide containing residues 60–78 prepared by solid-phase techniques enhanced the lipolysis 3-fold. Addition of five residues produced a synthetic fragment 55–78 that enhanced the release of fatty acid 12-fold compared to 13-fold for intact apoC-II. By contrast, the synthetic peptide containing residues 66–78 did not activate. Removal of the three COOH-terminal residues, Gly-Glu-Glu, from fragment 60–78 decreased the ability to activate LPL by >95%. These studies suggest that the maximal activation of LPL by apoC-II requires a minimal sequence contained within residues 55–78.**

Chylomicrons and very low density lipoproteins (VLDL) are the vehicles for the transport of plasma triglycerides (1, 2). Transfer of fatty acid from the triglyceride-rich lipoproteins to the tissue requires hydrolysis of the triglyceride by lipoprotein lipase at the capillary walls. Apoliprotein C-II from human VLDL (apoC-II) plays an important role in triglyceride metabolism by serving as an activator of lipoprotein lipase (LPL; triacylglycerol acyl-hydrolase, EC 3.1.1.3) (3, 4). The amino acid sequence of apoC-II (Fig. 1) has recently been determined (5). The protein consists of 78 amino acid residues and is lacking cysteine, cystine, and histidine.

We now present results of preliminary studies to define the sequence requirement in apoC-II necessary for the activation of LPL. In addition to testing the three cyanogen bromide (CNBr) fragments corresponding to residues 1–9, 10–59, and 60–78, we have also synthesized and tested the fragments representing residues 66–78, 60–78, and 55–78. On the basis of the results of these studies, we suggest that the sequence determinant in apoC-II that is required for maximal activation of LPL is contained between residues 55 and 78.

MATERIALS AND METHODS

Isolation of apoC-II and CNBr Fragments. apoC-II was isolated as described (6, 7) from VLDL obtained from fasting subjects with type IV or type V hyperlipoproteinemia (8). The isolated apoprotein was homogeneous by polyacrylamide gel electrophoresis in urea and sodium dodecyl sulfate and by amino acid analysis. The three CNBr fragments of apoC-II were prepared by chromatography on Bio-Gel P-30 (5).

The peptide corresponding to residues 60–75 was prepared from a tryptic digest of the COOH-terminal CNBr fragment. To the fragment (800 nmol) was added 200 μg of trypsin (TRT-PCK, Worthington) in 1 ml of 0.1 M ammonium bicarbonate, pH 8.0. After 6 hr at 23°, the reaction mixture was placed directly onto a column (1.6 × 200 cm) of Sephadex G-50 in 0.1 M ammonium bicarbonate. The flow rate was 25 ml/hr, and 4-ml fractions were collected. The peptides were located by absorbance at 226 nm.

Synthesis of Fragments of apoC-II. The tertiary-butyloxycarbonyl (^{t}BOC) amino acids were puchased from Peninsula Laboratories and were pure by thin-layer chromatography in standard solvent systems. The trifluoroacetic acid (Halocarbon Corp.) was redistilled before use. The methylene chloride was Burdick-Jackson "glass distilled." The LS-601 resin (0.32 meq of Cl^- per g) was purchased from Lab Systems and was modified by attaching a spacer chain as previously described by Sparrow (9). The ^{t}BOC-γ-benzylglutamic acid was incorporated into the modified resin according to the procedure of Gisin (10).

The ^{t}BOC-γ-benzylglutamic acid–resin (0.15 meq/g, 5 g) was placed in the shaker vessel of a Schwarz/Mann Bioresearch automated peptide synthesizer and was treated with 1 M acetic anhydride/pyridine to block any unreacted amino groups from the synthesis of the spacer resin as described (9). The ^{t}BOC protecting group was removed with 40% trifluoroacetic acid in methylene chloride, and the resulting salt was neutralized with 5% diisopropylethylamine in methylene chloride. The ^{t}BOC amino acids were coupled as the symmetrical anhydrides generated *in situ* by reaction of 4 mmol of ^{t}BOC amino acid with 2 mmol of dicyclohexylcarbodiimide in a 1:1 mixture of methylene chloride/dimethyl formamide. The program used for the synthesis has been described (11). Samples of resin (1 g) were removed after attachment of residues 60, 66, and 55 and were dried under reduced pressure.

Hydrogen Fluoride Cleavage of Peptide Resins. The peptide resins (1 g) were treated with 20 ml of hydrogen fluoride containing 2 ml of anisole at 0° for 30 min. The HF was removed under reduced pressure at 0° and the resin was transferred to a fritted glass filter funnel for washing with ether. The peptides were then extracted from the resin with anhy-

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Abbreviations: VLDL, very low density lipoproteins; apoC-I, apoC-II, apoC-III, apolipoproteins from human VLDL; LPL, lipoprotein lipase (triacylglycerol acyl-hydrolase, EC 3.1.1.3); CNBr, cyanogen bromide; ^{t}BOC, tertiary-butyloxycarbonyl.

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H_2N-Thr-Glu-Gln-Pro-Gln-Gln-Asp-Glu-Met-Pro-Ser-Pro-Thr-Phe-Leu-Thr-Glu-Val-Lys-Glu-Trp-Leu-Ser-Ser-Tyr-Gln
5 10 15 20 25

Ser-Ala-Lys-Thr-Ala-Ala-Gln-Asn-Leu-Tyr-Glu-Lys-Thr-Tyr-Leu-Pro-Ala-Val-Asp-Glu-Lys-Leu-Arg-Asp-Leu-Tyr
30 35 40 45 50

Ser-Lys-Ser-Thr-Ala-Ala-Met-Ser-Thr-Tyr-Thr-Gly-Ile-Phe-Thr-Asp-Gln-Val-Leu-Ser-Val-Leu-Lys-Gly-Glu-Glu-COOH
55 60 65 70 75

FIG. 1. Amino acid sequence of apoC-II, as determined by Jackson *et al.* (5).

drous trifluoroacetic acid. The trifluoroacetic acid was removed under reduced pressure at 20° and the resulting residue was washed with ether. The precipitated peptide was collected by centrifugation and dissolved in 1 M Tris/6 M guanidine hydrochloride. The pH of this solution was 8.

Purification of Synthetic apoC-II Fragments. The peptides were subjected to chromatography on a column (2.6 × 100 cm) of Bio-Gel P-10 equilibrated with 0.1 M Tris/6 M urea, pH 8.2. The fractions that contained the peptide were pooled, desalted, and lyophilized. The peptides were dissolved in 0.01 M Tris·HCl/6 M urea, pH 8.2, and applied to separate columns (1.6 × 35 cm) of DEAE-cellulose (Whatman DE-52) (Fig. 2). Fragment 66–78 was eluted with a 400-ml linear gradient of

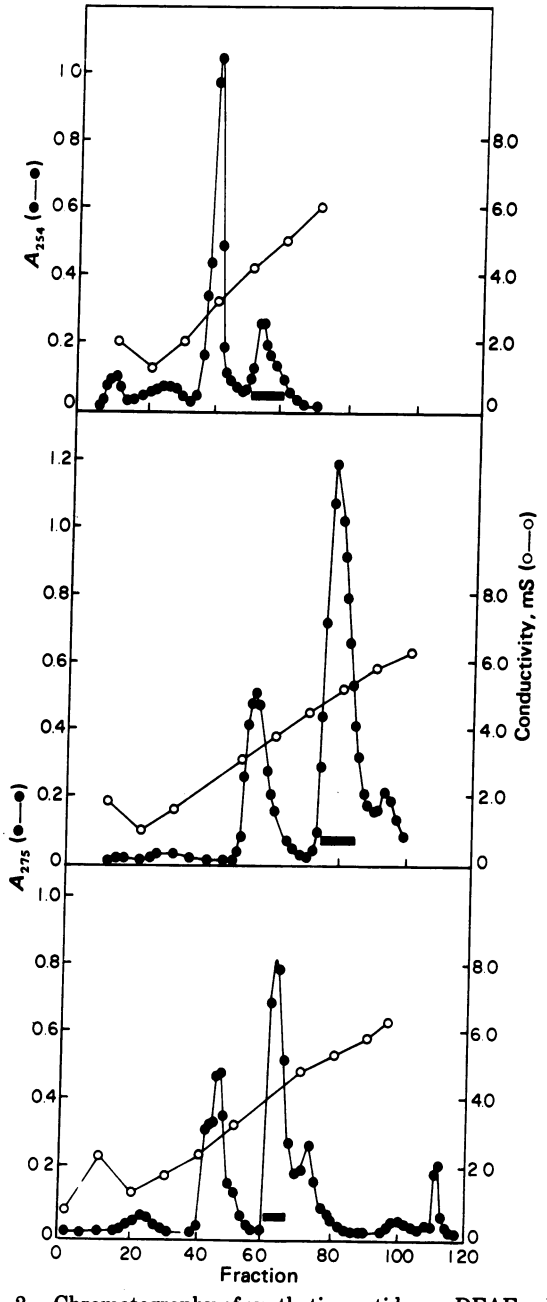

FIG. 2. Chromatography of synthetic peptides on DEAE-cellulose. (*Top*) Fragment 66–78; (*Middle*) fragment 60–78; (*Bottom*) fragment 55–78. The fractions indicated by the solid bar were pooled and desalted. In the *top* panel, the larger UV-absorbing peak contained only a small amount of peptide; the UV spectrum did not correspond to that of phenylalanine.

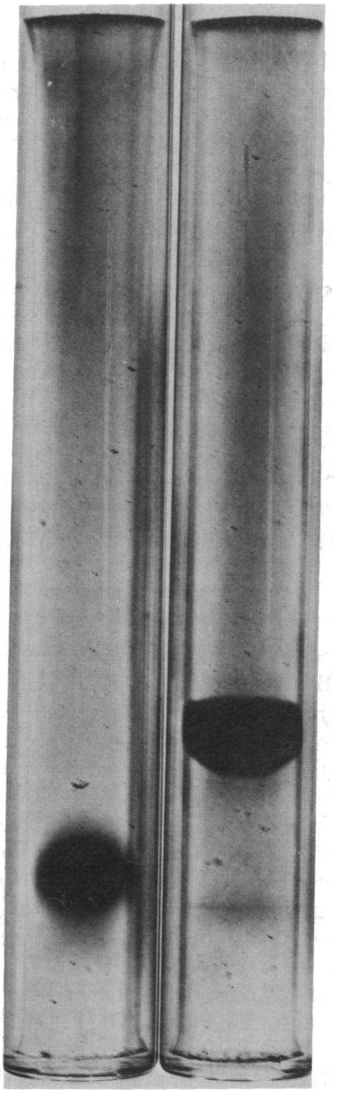

FIG. 3. Polyacrylamide gel electrophoresis of synthetic fragments. (*Left*) Fragment 60–78; (*Right*) fragment 55–78. Electrophoresis was performed on 7.5% acrylamide gels at pH 8.9 in 8 M urea; 100 μg of peptide was applied to each gel. The samples were run for 2 hr at 2 mA per gel. After staining with Coomassie blue, the gels were destained with water/methanol/acetic acid, 87:5:8 (vol/vol).

Table 1. Amino acid composition (residues) of peptides and synthetic fragments of apoC-II*

Amino acid	CNBr fragments of apoC-II			Tryptic fragment†	Synthetic fragments of apoC-II		
	1–9	10–59	60–78	60–75	66–78	60–78	55–78
Aspartic acid	1.1 (1)	3.6 (3)	1.3 (1)	1.2 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Threonine	1.0 (1)	4.5 (5)	2.6 (3)	2.6 (3)	2.7 (3)	3.7 (4)	1.0 (1)
Serine		6.3 (6)	2.3 (2)	1.8 (2)	0.9 (1)	1.7 (2)	2.4 (3)
Glutamic acid	4.9 (5)	6.5 (6)	3.3 (3)	1.3 (1)	3.1 (3)	3.2 (3)	3.1 (3)
Proline	0.9 (1)	2.5 (3)					
Glycine			2.4 (2)	1.2 (1)	1.2 (1)	2.3 (2)	2.0 (2)
Alanine		6.8 (6)					2.0 (2)
Valine		2.4 (2)	1.9 (2)	1.8 (2)	2.2 (2)	2.0 (2)	2.1 (2)
Methionine	0.5 (1)‡	0.5 (1)‡					1.0 (1)
Isoleucine			0.7 (1)	1.0 (1)		0.9 (1)	1.0 (1)
Leucine		5.6 (6)	1.7 (2)	2.0 (2)	2.0 (2)	2.0 (2)	2.0 (2)
Tyrosine		3.9 (4)	0.7 (1)	1.0 (1)		0.9 (1)	1.0 (1)
Phenylalanine	0.2 (0)	0.8 (1)	0.9 (1)	0.9 (1)	1.2 (1)	1.0 (1)	1.0 (1)
Lysine		4.6 (5)	0.8 (1)	0.9 (1)	1.1 (1)	1.0 (1)	1.1 (1)
Arginine		1.0 (1)					

* The numbers represent the values obtained from 22-hr hydrolysates with the expected values in parentheses.
† Obtained from tryptic digestion of the CNBr fragment 60–78.
‡ As homoserine.

0.01 M Tris·HCl/6 M urea, pH 8.2, and 400 ml of the same buffer containing 0.2 M sodium chloride. Fragments 60–78 and 55–78 were eluted with the gradient as above except that the sodium chloride concentration was 0.125 M. The fractions indicated by the bars were pooled, desalted, and lyophilized. Each peptide showed the predicted amino acid composition.

Measurement of LPL Activation. LPL, purified from bovine skim milk as described (12), was homogeneous by polyacrylamide gel electrophoresis in sodium dodecyl sulfate and had a molecular weight of 55,000. The assay system was similar to that described (13, 14) and contained in a total volume of 0.5 ml: 2.4 nmol of glycerol tri[*1*-^{14}C]oleate (Amersham/Searle); 1.5 μmol of nonradioactive triolein (Sigma); 5 mg of gum arabic; 10 mg of fatty acid-poor bovine serum albumin; 0.1 mmol of Tris·HCl buffer, pH 8.4; and 5 units of heparin (Upjohn). The amount of apoC-II or fragment added to each assay is indicated in the figures. The value for each experimental point is the mean of duplicate determinations; the SEM was ±3.5%.

RESULTS

Preparation of Native and Synthetic Fragments of apoC-II. The three CNBr fragments of apoC-II were isolated as described (6) by chromatography on Bio-Gel P-30 in 25% formic acid. The amino acid compositions of the fragments were consistent with the expected sequences (Table 1). The synthetic fragments corresponding to residues 66–78, 60–78, and 55–78 were prepared by solid-phase peptide methods (9, 11). Amino acid analysis of each peptide, chromatographed separately, gave the expected composition (Table 1). Polyacrylamide gel electrophoresis gave single bands for synthetic fragments 60–78 and 55–78 (Fig. 3); fragment 66–78 diffused from the gel during destaining.

Activation of LPL. apoC-II enhanced the activity of LPL 6-fold at a concentration of 0.5 μM† (Fig. 4). CNBr fragment 1–9 was totally inactive at concentrations up to 4.8 μM. The middle CNBr fragment containing residues 10–59 gave slight stimulation, which can be accounted for as a 2–5% contamination by intact apoC-II. The COOH-terminal CNBr fragment (residues 60–78) gave a 3.9-fold stimulation at 4.8 μM.

Because the COOH-terminal CNBr fragment contains only one residue of lysine, at position 75, it was of interest to cleave this peptide with trypsin and to determine its activation properties. The peptide was prepared as described in *Materials and Methods* and had an amino acid composition consistent with the sequence. At concentrations as high as 4.8 μM, the 60–75 fragment was inactive.

The above study with the CNBr fragments suggested that the COOH-terminal portion of apoC-II was involved in the activation of LPL. Because the activation was less than that with intact apoC-II, it was of interest to prepare synthetic fragments to determine the minimal requirements for activation. Fragment 66–78 did not stimulate LPL (Fig. 5). Synthetic fragment 60–78 enhanced LPL activity 2.5-fold at 5.0 μM compared to 3.9-fold at 4.8 μM for the CNBr fragment. Addition of five residues to the 60–78 fragment—i.e., the 55–78 peptide—re-

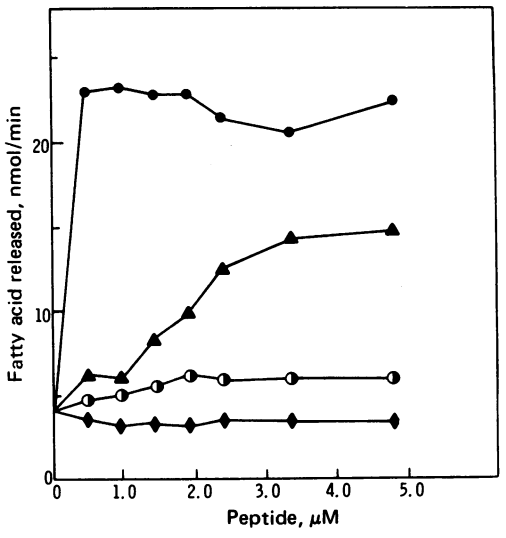

FIG. 4. Activation of LPL by CNBr fragments of apoC-II. The reaction mixture contained 0.04 μg of LPL and the indicated amounts of peptide: apoC-II (●-●); CNBr 60–78 (▲-▲); CNBr 10–59 (◑-◑); CNBr 1–9 (♦-♦).

† The maximum activation by apoC-II varied with different preparations and appears to be related to the extent of aggregation and/or carbamylation that occurred during chromatography in 6 M urea.

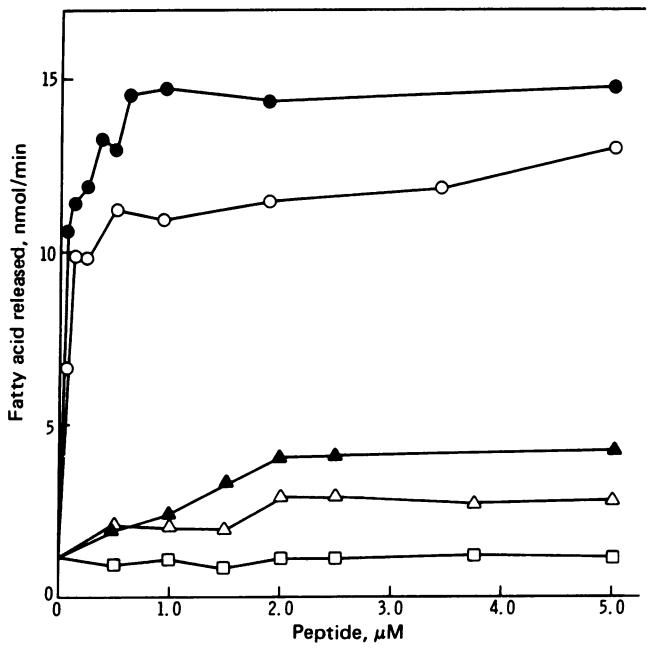

FIG. 5. Activation of LPL by synthetic peptide fragments of apoC-II. The reaction mixture contained 0.01 μg of LPL and the indicated amounts of peptide: apoC-II (●-●); synthetic peptide 55–78 (○-○); CNBr fragment 60–78 (▲-▲); synthetic peptide 60–78 (△-△); synthetic peptide 66–78 (□-□).

sulted in a marked increase in ability to activate LPL. The 55–78 fragment enhanced LPL activity 11.7-fold at 1.0 μM compared to 13.3-fold for intact apoC-II.

DISCUSSION

Relatively little is known about the specific interactions between apoC-II and LPL. Studies with surface monolayer techniques (15) have demonstrated that apoC-II forms a stable surface film and that apoC-II and LPL associate as a stable complex at the interface, even in the absence of lipid. These results suggest that the activation of LPL by apoC-II involves a direct protein–protein interaction. With knowledge of the sequence of apoC-II, we undertook experiments to determine the minimal sequence of apoC-II required for activation of LPL, purified to homogeneity from bovine milk. There was only a slight difference in the activation curves of the synthetic and native 60–78 fragments; each gave a 3- to 4-fold increase in activity (Fig. 4). However, when the five residues (Ser_{55}-Thr-Ala-Ala-Met_{59}) were added to the 60–78 fragment, there was a marked increase in activation, approaching 90% of that obtained with intact apoC-II. These data imply that the COOH-terminal 24 residues of apoC-II contain the active site for stimulation of LPL. Preliminary studies with the 55–78 fragment indicate that the peptide has a disordered structure by circular dichroism and does not form a soluble complex with phospholipid. Because the intact apoC-II interacts with phospholipids, we propose that the NH_2-terminal portion of the apoprotein contains the lipid-binding region. Experiments are currently in progress to prepare and test the 1–49 fragment for its lipid-binding properties.

Miller and Smith (15) have shown that the specific LPL–apoC-II interaction could be reversed by high concentrations of inorganic salts. This salt-mediated inhibition of LPL activation is anion-specific (16). Because the removal of Gly-Glu-Glu from the 60–78 fragment greatly decreases its activation ability, it may be speculated that these two COOH-terminal glutamic acid residues are sites for ionic interaction between LPL and apoC-II and that residues 55–65 are involved in the enhancement of enzymatic catalysis. To extend this line of argument further, the NH_2-terminal 49 residues would interact with phospholipids and, thus, bind apoC-II to the chylomicron and VLDL surface film, leaving the COOH-terminal residues available for interaction with LPL on the capillary wall.

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