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Apolipoprotein C-II is a novel substrate for matrix metalloproteinases

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Abstract

We previously reported an efficient proteomic approach to identify matrix metalloproteinase (MMP) substrates from complex protein mixture. Using the proteomic approach, apolipoprotein C-II (apoC-II), which is a cofactor of lipoprotein lipase (LPL) and a component of very-low density lipoprotein and chylomicron, has been identified as a putative MMP-14 substrate. Cleavage of apoC-II, with various MMPs, demonstrated that apoC-II is cleaved most efficiently by MMP-14, and also by MMP-7, among the tested MMPs. The 79-amino acid residue apoC-II was cleaved between Asn^{35} and Leu^{36} by MMP-14, and between Phe^{14} and Leu^{15} and between Asn^{35} and Leu^{36} by MMP-7. Cleavage of apoC-II by MMP-14 markedly decreased LPL activity and would thus impair hydrolysis of triglycerides in plasma and transfer of fatty acids to tissues. Our result suggests that cleavage of apoC-II by MMPs would be important for development of pathophysiological situations of apoC-II deficiency such as atherosclerosis.

Keywords: Apolipoprotein C-II; Atherosclerosis; Cleavage site; Lipoprotein lipase; Matrix metalloproteinase; Proteomics

Apolipoprotein C-II (apoC-II) is synthesized in liver and intestine, and is secreted as a surface component of chylomicrons, very low-density lipoproteins (VLDLs), and high-density lipoproteins in blood plasma [1,2]. ApoC-II, the first apolipoprotein for which a specific function was found, activates lipoprotein lipase (LPL) as a cofactor [3]. LPL, which is associated with the luminal side of capillaries and arteries, catalyzes hydrolysis of the triacylglycerol (TG) component of circulating chylomicrons and VLDLs, thereby, providing non-esterified fatty acids and 2-monoacylglycerol for tissue utilization [4,5]. The structure needed for activation of LPL resides in the C-terminal one-third of the apoC-II molecule [6], while lipid binding seemed to be confined to fragments from the N-terminal two-thirds [7]. Lack of functional apoC-II leads to type IB hyperlipoproteinaemia, similar to type I hyperlipoproteinaemia which is caused by LPL deficiency [8].

Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases. Twenty-three different MMPs encoded by 24 genes were identified in humans. MMPs play an important role in many normal processes such as embryonic development, pregnancy, and wound repair, and in pathological situations such as tumor growth, metastasis, atherosclerosis, inflammation, arthritis, periodontal disease, ulceration, and fibrosis [9,10]. While it has been well known that MMPs break down most extracellular matrix components, there is growing evidence that MMPs also act on a variety of non-extracellular matrix proteins [11]. Thus, identification of MMP substrates will provide important understanding of normal and pathological roles of MMPs [9]. In addition, identification of these substrates will improve the efficacy and reduce the side effects of MMP inhibitors used to treat MMP-related diseases [12].

Recently, we described a rapid and efficient method to identify MMP substrates within a complex protein mixture using a degradomic approach [13]. This approach is based on a basic proteomic technique; differential display of a complex protein mixture treated with or without MMP in two-dimensional (2-D) gels, in-gel digestion of the interested spots, and matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) of

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the resultant peptides, followed by peptide mass fingerprinting for protein identification. By applying this method to the action of the catalytic domain of MMP-14 (also known as membrane type-1 MMP) in human plasma, we have found apoC-II as one of novel substrates of MMP-14. We then examined cleavage of the purified apoC-II by major MMPs, determined its cleavage sites by a proteomic approach, and described the change in its LPL cofactor activity upon incubation with MMP-14.

Materials and methods

Preparation of catalytic domains of MMPs and proMMPs. A functional catalytic domain of MMP-14 (cMMP-14) was obtained by refolding of the catalytic and hinge domains (Tyr^{112}-Ile^{318}), containing the N-terminal methionine and the C-terminal hexa-histidines, which were expressed as inclusion body in *Escherichia coli* [14]. The pro, catalytic, and hinge domains of human MMP-1 (pchMMP-1, Phe^{20}-Ala^{277}) containing the N-terminal methionine and the C-terminal hexa-histidines were expressed as inclusion body in *E. coli* and refolded by the same method as cMMP-14. A functional human MMP-7 (Ala^{108}-Lys^{288}) containing the N-terminal methionine and the C-terminal hexa-histidines, which was expressed as inclusion body in *E. coli*, was prepared by refolding [15]. The recombinant human proMMP-2 was expressed in Sf9 cells by infection of the 72Gel baculovirus and purified by gelatin–agarose column chromatography as described previously [16]. The human full-length MMP-9 cDNA (a generous gift from Dr. G.I. Goldberg, Washington University) [17] was cloned into pBlueBac4 (Invitrogen, Carlsbad, CA) and 92Gel baculovirus was produced using the Bac-N-Blue Baculovirus Expression System (Invitrogen). ProMMP-9 was expressed and purified using the same method as proMMP-2. Using pBacPAK9-MMP-3 (a generous gift from Dr. M.-Y. Kim, AngioLab) and the BacPAK Baculovirus Expression System (Clontech, Mountain View, CA), MMP-3 baculovirus was produced. To purify the recombinant proMMP-3 expressed in Sf9 cells, the serum-free medium sample recovered at 4 days after the infection with the recombinant baculovirus was loaded on SP-Sepharose (Pharmacia Biotech, Uppsala, Sweden) column equilibrated with column buffer A (10 mM NaOAc, pH 6.0). The column was washed with the column buffer A and subjected to 0–1.5 M NaCl gradient in the column buffer A. Pooled fractions containing proMMP-3 were dialyzed against column buffer B (5 mM Tris–HCl, pH 8.5, 1 mM $CaCl_2$, and 0.1 mM $ZnCl_2$) and were loaded on Q-Sepharose (Pharmacia Biotech) column. Proteins were eluted by 0–1.5 M NaCl gradient in the column buffer B. Pooled fractions, containing proMMP-3, were dialyzed against MMP reaction buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, and 0.5 mM $ZnCl_2$) and stored at −70 °C in small aliquots.

2-D polyacrylamide gel electrophoresis (PAGE), in-gel digestion, MALDI-TOF MS, and peptide mass fingerprinting analysis. Plasma proteins, which were incubated without or with cMMP-14, were differentially displayed on 2-D gels, and protein spots of interest were subjected to in-gel digestion with trypsin, MALDI-TOF MS of the resultant peptides, and peptide mass fingerprinting, as described previously [13].

Cloning of the human apoC-II cDNA and its expression and purification. The human apoC-II cDNA [18] was amplified by polymerase chain reaction (PCR) using *Pfu* polymerase (Stratagene, La Jolla, CA) and the human liver cDNA as a template. A pair of primers were 5′-GAATCTCCAT*ATG*ACCCAACAGCCCCAGCAAG-3′ (*Nde*I site is underlined and the N-terminal methionine codon is italicized) and 5′-GCGGATCC*TTA*CTCCTCTCCCTTCAGCAC-3′ (*Bam*HI site is underlined and the termination codon is italicized). The PCR product that was digested with *Nde*I and *Bam*HI was ligated into pET-21a(+) vector (Novagen, Cambridge, UK) cleaved with the same enzymes. The ligated DNA was transformed into *E. coli* BL21 (DE3). The resultant plasmid (pET-21a(+)-apoC-II) was confirmed by restriction enzyme digestion and DNA sequencing.

A human recombinant apoC-II was expressed and purified as described [19] with some modifications. The *E. coli* BL21 (DE3) cells harboring pET-21a(+)-apoC-II were cultured in 100 ml LB broth containing 100 μg/ml ampicillin until absorbance at 600 nm reached 0.6. Isopropyl-1-β-D-galactopyranoside was added to a concentration of 1 mM and the cells were cultured for 3 h at 37 °C. The cell pellet was resuspended in 10 ml TN buffer (50 mM Tris–HCl, pH 8.0, and 100 mM NaCl) and sonicated for 10 cycles on an ice bath. The solution was diluted by the addition of 10 ml TN buffer and centrifuged at 11,000*g* for 20 min. The pellet containing inclusion body was washed twice with TN buffer containing 0.1% Triton X-100 and once with TN buffer alone. The inclusion body recovered by centrifugation was resuspended in 0.5 ml of 8 M urea. The soluble fraction was applied to Superdex 75 column (Pharmacia-Biotech), equilibrated with 6 M urea in 20 mM Tris–HCl, pH 8.0, and eluted with the same solution. Pooled fractions containing denatured apoC-II were diluted to 3 M urea by adding the same volume of 20 mM Tris–HCl, pH 8.0. ApoC-II was refolded at 4 °C by gradient dialysis from 3 M urea in 20 mM Tris–HCl, pH 8.0 to 20 mM Tris–HCl, pH 7.4. The concentration of the apoC-II was determined using the Bradford assay (Bio-Rad, Hercules, CA).

Cleavage of apoC-II by various MMPs in vitro. Pro-forms of MMPs were activated immediately prior to use by incubating them with 1 mM 4-aminophenyl mercuric acetate (APMA) at 37 °C; for 30 min at 100 nM pchMMP-1 and proMMP-2, for 3 h at 100 nM proMMP-9, and for 3 h at 500 nM of proMMP-3. ApoC-II (1.5 μg) was incubated with the activated forms of MMPs or catalytic forms of MMPs (MMP-7 and cMMP-14) in a 1:160 enzyme/substrate molar ratio for 1 h at 37 °C in 15 μl MMP reaction buffer. The reactions were terminated by adding EDTA to a final concentration of 10 mM. The reaction products were subjected to 15% Tris–Tricine sodium dodecyl sulfate (SDS)–PAGE or mass analysis.

Mass determination of apoC-II cleaved with MMPs. The reaction mixtures of apoC-II digested with MMPs were desalted with Poros resin (Applied Biosystems, Foster City, CA) and dispensed onto a MALDI-TOF sample plate along with 0.5–1 μl of the matrix (α-cyano-4-hydroxycinnamic acid dissolved in 0.1% trifluoroacetic acid, 70% acetonitrile). Mass analysis was performed on a Voyager-DE STR MALDI-TOF MS (Applied Biosystems). MS spectra were acquired in the linear mode. Data were collected from at least three independent experiments. Expected positions of the digested fragments were assigned from the mean ± SD masses of the peaks. Theoretical molecular masses of polypeptides were calculated using the Web-based programs, such as ExPASy PeptideMass (http://kr.expasy.org/tools/peptide-mass.html).

LPL assay. To analyze the activity of apoC-II as an activator of LPL, LPL assay was performed by the method previously described [19] with some modifications. The 2-fold concentrated solution of lipid substrate was prepared by mixing 1.4 nmol of glycerol tri[9,10-^{3}H]oleate (specific activity 14 Ci/mmol, Amersham Bioscience) in toluene, 14 μmol of trioleoylglycerol (Sigma–Aldrich, St. Louis, MO), and 1.4 μmol of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Sigma–Aldrich) in chloroform. The solvents were evaporated under a stream of nitrogen gas. The dried lipids were emulsified in 5 ml of 50 mM NH_4OH-HCl (pH 8.5) four times for 1 min each in an ice bath using ultrasonic cleaner (Bransonic, Danbury, CT). The assay mixture, with a final volume of 100 μl, contained 50 mM NH_4OH-HCl (pH 8.5), 1.4 mM trioleoylglycerol, 0.14 mM 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, various amounts of apoC-II (from 0.125 to 2 μg/ml), and 20 μl of the 5.25 μg/ml bovine milk LPL (Sigma–Aldrich). Reaction was performed for 1 h at 37 °C and terminated by the addition of 3.2 ml of chloroform/heptane/methanol (5:4:5.6, v/v/v) and 1.2 ml of 0.2 M NaOH. Samples were centrifuged and 1.2 ml of the aqueous phase was mixed with 10 ml of Ecolite (MP Biomedical, Montreal, Canada), and the radioactivity was determined in a Beckman scintillation counter. One milliunit of enzymatic activity was defined as the release of 1 nmol of oleic acid per minute at 37 °C.

To analyze effect of the MMP-14-cleaved apoC-II on LPL activity, apoC-II (1.5 μg) was digested by cMMP-14 in various enzyme/substrate molar ratios for 3 h at 37 °C. The reactions were terminated by the addition of GM6001 (Chemicon, Temecula, CA) to a final concentration of 1 μM. LPL assay was done in the presence of 0.5 μg/ml of the cleaved apoC-II as described above.

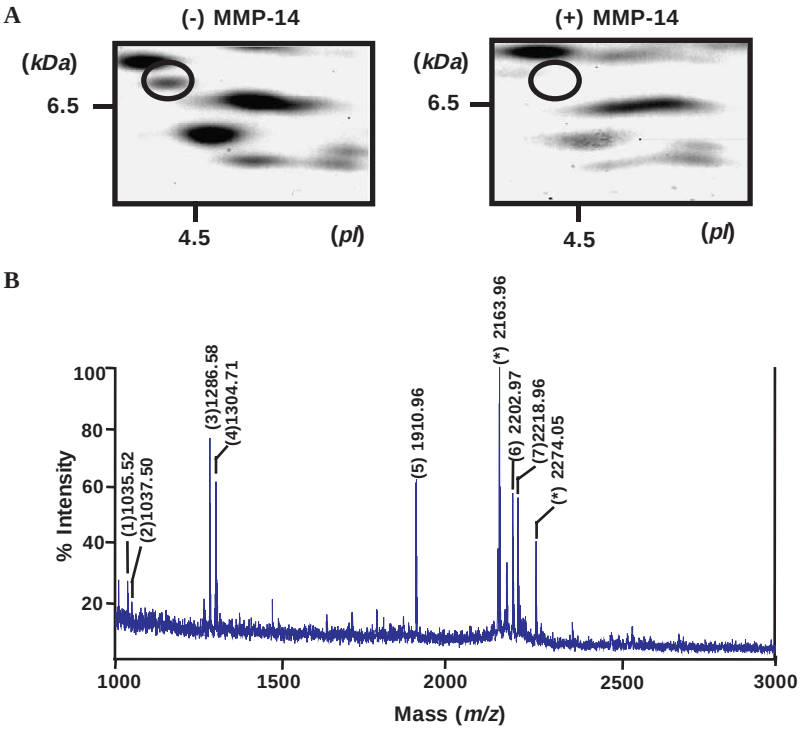
Results

ApoC-II as a novel substrate of MMP-14

Plasma proteins that were incubated in the presence and the absence of cMMP-14 were displayed in two-dimensional gels. By comparison of the gels, a protein spot which was present in the gel of the non-treated plasma proteins disappeared in the gel of the MMP-14-treated plasma proteins (Fig. 1A). MALDI-TOF MS spectrum of the trypsin-digested peptides derived from the spot matched with apoC-II. The matched trypsin-digested peptides are located between T^{1} and K^{55} of apoC-II (Figs. 1B and C). From this result we assume that apoC-II would be a novel substrate of MMP-14.

Preparation of the functional recombinant apoC-II

As reported [19], human recombinant apoC-II expressed in *E. coli* was detected mostly in the insoluble fraction. After the inclusion bodies were solubilized with urea, the apoC-II was purified by gel permeation chromatography in denatured condition and refolded by dialysis. Typical yield of the refolded apoC-II was approximately 210 μg/100 ml culture. It was an electrophoretically homogeneous protein, purer than plasma apoC-II commercially avail-



C

No	Mass $[M+H]^+$ (Da)		Delta (Da)	Missed cleavage	Modification	Residues	Peptide sequence
	Measured	Calculated					
1	1035.52	1035.54	0.02	0		40-48	TYLPAVDEK
2	1037.50	1037.53	0.03	0		31-39	TAAQNLYEK
3	1286.58	1286.59	0.01	0		20-30	ESLSSYWESAK
4	1304.71	1304.72	0.01	1		40-50	TYLPAVDEKLR
5	1910.96	1911.02	0.06	2		40-55	TYLPAVDEKLRDLYSK
6	2202.97	2203.07	0.1	0		1-19	TQQPQQDEMPSPTFLTQVK
7	2218.96	2219.06	0.1	0	MSO	1-19	TQQPQQDEMPSPTFLTQVK

Fig. 1. A portion of 2-D gel pattern of plasma proteins that was incubated with or without MMP-14 and identification of apoC-II from MALDI-TOF MS. (A) Isoelectric focusing was carried out on pH 3–10 non-linear immobilized pH gradient strip (18-cm) which was loaded with 1.5 mg of plasma proteins incubated with or without 2 μg cMMP-14. SDS–PAGE was done on 9–16% gradient and the gel was stained with Coomassie brilliant blue G-250. A circle in the non-treated sample indicates the spot that disappeared in the cMMP-14-treated sample. The spot was subjected to in-gel trypsin digestion and MALDI-TOF MS. (B) Mass peaks corresponding to tryptic peptides derived from apoC-II are numbered. Asterisks indicate mass peaks from autoproteolyzed trypsin. (C) Tryptic peptides derived from ApoC-II identified by trypsin in-gel digestion and mass fingerprinting are shown. MSO refers to mono-methionine sulfoxide modification.

able, and approximately 9 kDa in size, similar to the calculated molecular weight of 8.9 kDa (Fig. 2A).

To examine functionality of the recombinant apoC-II, its ability in activating LPL was examined by LPL assay using glycerol tri[9,10-^{3}H]oleate as substrate. As shown in Fig. 2B, the presence of apoC-II increased the LPL activity in a dose-dependent manner. Approximately 1.5 μg/ml apoC-II saturated the LPL cofactor activity, at which the LPL activity was about 4-fold of that without apoC-II. In addition, the recombinant apoC-II has the LPL cofactor activity as much as the plasma apoC-II.

Cleavage of apoC-II with MMP-14 and other MMPs

To confirm that apoC-II is a substrate of MMP-14 and other major MMPs, susceptibility of the recombinant apoC-II to various MMPs was examined. As shown in Fig. 3, apoC-II was cleaved the most efficiently by MMP-14 and also by MMP-7. In addition, it was cleaved by high concentration of MMP-3 (data not shown). When the recombinant apoC-II was incubated for 0–6 h with MMPs, 9-kDa apoC-II was processed into a fragment of approximately 5-kDa and then smaller fragments by MMP-14 (Fig. 4A) and slowly into fragments of 7-, 5-kDa, and smaller fragments by MMP-7 (Fig. 4B). However, it was not degraded in the absence of the MMPs for 6-h incubation. These results demonstrate that apoC-II is a novel substrate for MMP-14 and MMP-7.

Determination of cleavage sites of apoC-II by MMP-14 and MMP-7

To determine the precise cleavage site of apoC-II, the mass of apoC-II cleaved by MMP-14 or MMP-7 was measured by MALDI-TOF spectrometry in linear mode and the relative position of each fragment within the apoC-II

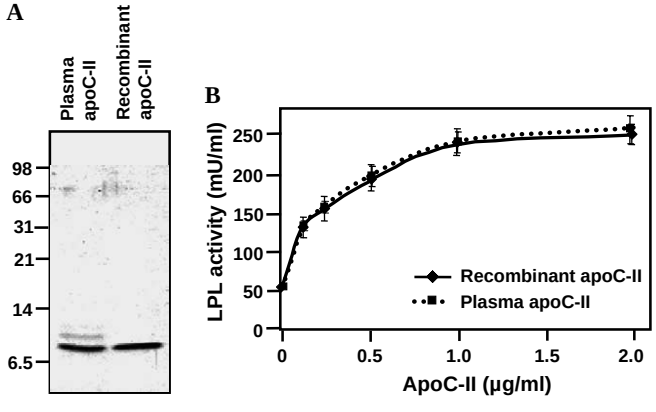


Fig. 2. Expression and purification of a recombinant human apoC-II. (A) A recombinant human apoC-II was expressed in *E. coli*, purified using Superdex 75 gel filtration chromatography, and refolded. The recombinant apoC-II and a commercial plasma apoC-II were subjected to 15% Tris–Tricine SDS–PAGE and stained with Coomassie brilliant blue R-250. (B) LPL assay was performed in the presence of recombinant apoC-II and plasma apoC-II as described under Materials and methods.

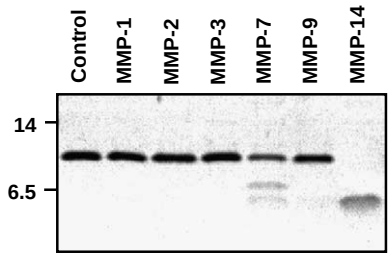


Fig. 3. Cleavage of apoC-II by various MMPs in vitro. Pro-forms of MMPs (pchMMP-1, proMMP-2, proMMP-3, and proMMP-9) were activated by APMA. The activated MMPs (MMP-1, MMP-2, MMP-3, and MMP-9) and catalytically active MMPs (MMP-7 and cMMP-14) were used for cleavage of apoC-II. The purified apoC-II (1.5 μg) was digested by MMPs in a 1:160 enzyme/substrate molar ratio for 1 h at 37 °C. Digestion was stopped by addition of SDS sample buffer. Samples were subjected to 15% Tris–Tricine SDS–PAGE and stained with Coomassie brilliant blue R-250.

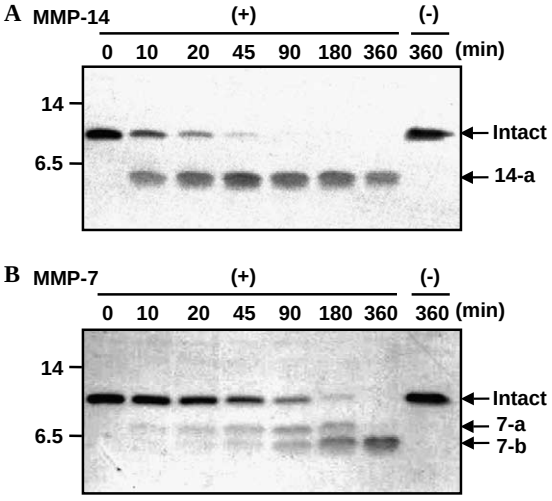
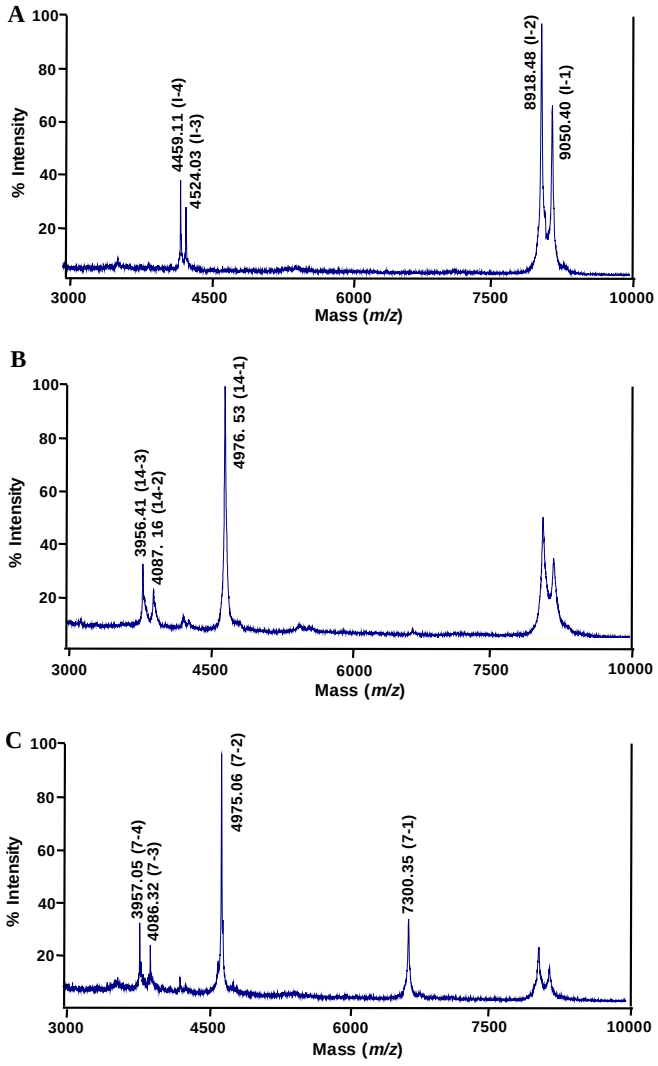


Fig. 4. Digestion of apoC-II by MMP-14 and MMP-7 as a function of incubation time. ApoC-II (1.5 μg) was digested by cMMP-14 (A) or MMP-7 (B) in a 1:160 enzyme/substrate molar ratio at 37 °C. At the indicated time intervals, aliquots were taken and digestion was stopped by addition of SDS sample buffer. Samples were analyzed by 15% Tris–Tricine SDS–PAGE and stained with Coomassie brilliant blue R-250. The "14-a" band indicates an apoC-II fragment generated by MMP-14 cleavage and "7-a," and "7-b" bands indicate those by MMP-7 cleavage.

polypeptide was assigned. Mass analysis of the uncleaved apoC-II showed four peaks, I-1 to I-4 (Figs. 5A and D). Masses of peak I-1 (9049.34 ± 3.50 $[M+H]^{+}$) and peak I-2 (8915.44 ± 2.05 $[M+H]^{+}$) were matched with the calculated masses of apoC-II with N-terminal methionine (9047.12 $[M+H]^{+}$) and without methionine (8915.93 $[M+H]^{+}$), respectively. Masses of peaks I-3 and I-4 were doubly charged masses ($[M+2H]^{2+}/2$) of the peaks I-1 and I-2, respectively. It was thus confirmed that the recombinant apoC-II was a mixture of two forms, with and without the N-terminal methionine, as previously reported [19]. Mass analysis of the MMP-14-digested apoC-II revealed a singlet peak (14-1) and doublet peaks (14-2 and 14-3) (Fig. 5B and D). The doublet peaks were derived from



D

Peak	Measured Mass	Expected region	Calculated Mass
I-1	9049.34±3.50	M + T^1-G^{79}	9047.12
I-2	8915.44±2.05	T^1-G^{79}	8915.93
I-3	4523.87±2.80*	M + T^1-G^{79}	4524.06*
I-4	4457.43±2.37*	T^1-G^{79}	4458.47*
14-1	4976.22±1.10	L^{36}-G^{79}	4976.55
14-2	4090.60±3.10	M + T^1-N^{35}	4089.50
14-3	3958.82±2.15	T^1-N^{35}	3958.30
7-1	7297.95±1.77	L^{15}-G^{79}	7300.19
7-2	4973.42±1.20	L^{36}-G^{79}	4976.55
7-3	4085.42±1.00	M + T^1-N^{35}	4089.50
7-4	3955.26±1.23	T^1-N^{35}	3958.30

Fig. 5. Determination of a cleavage site of apoC-II by MMP-14. ApoC-II uncleaved or cleaved with cMMP-14 or MMP-7 was analyzed by MALDI-TOF MS in linear mode. (A) Mass spectra of the uncleaved apoC-II. (B) Mass spectra of apoC-II cleaved by cMMP-14 in a 1:160 enzyme/substrate molar ratio at 37 °C for 5 min. (C) Mass spectra of apoC-II cleaved by MMP-7 in a 1:160 enzyme/substrate molar ratio at 37 °C for 60 min. Mass spectra shown in A–C are representative of at least three independent experiments. (D) Assignment of mass peaks of apoC-II cleaved by cMMP-14 and MMP-7. Masses are averaged from the respective spectra measured at least three times (mean ± SD). All masses were shown as $[M+H]^+$ (Da), except doubly charged masses, $[M+2H]^{2+}/2$, marked by asterisks. M in the expected region indicates the translation-initiating methionine.

the N-terminal fragments, which correspond to the amino acid residues of T^1 to N^{35} with and without the N-terminal methionine, respectively. The singlet peak was the C-terminal fragment corresponding to the L^{36} to G^{79} residues. This result indicates that MMP-14 catalyzed the initial cleavage of apoC-II at N^{35}–L^{36} (Fig. 6). Mass analysis of the MMP-7-digested apoC-II showed two singlet peaks (7-1 and 7-2) and doublet peaks (7-3 and 7-4) (Figs. 5C and D). The two singlet peaks were the C-terminal fragments corresponding to the L^{15} to G^{79} residues and L^{36} to G^{79} residues, respectively. The doublet peaks were derived from the N-terminal fragment, which correspond to the amino acid residues T^1 to N^{35} with and without the methionine, respectively. This result demonstrates that MMP-7 catalyzed the initial cleavage of apoC-II at F^{14}–L^{15} and N^{35}–L^{36} bonds (Fig. 6).

To identify which fragments assigned from mass peaks correspond to the bands (14-a, 7-a, and 7-b) detected in the gels of the apoC-II cleaved by MMP-14 and MMP-7 (Fig. 4), tryptic digests of the bands were analyzed by MALDI-TOF mass spectroscopy and peptide fingerprinting. Peptides derived from the 14-a band encompassed the L^{36} to G^{79} residues and those from the 7-a and 7-b bands covered the L^{15} to G^{79} residues and the L^{36} to G^{79} residues, respectively (Table 1). We thus found that 14-a, 7-a, and 7-b bands (Fig. 4) correspond to the 14-1, 7-1, and 7-2 fragments (Fig. 5), respectively.

ApoC-II cleaved by MMP-14 loses the LPL cofactor activity

In order to assess whether the LPL cofactor activity of apoC-II is altered by its cleavage with MMP-14, the recombinant apoC-II was incubated with various concentrations of MMP-14 (Fig. 7A) and LPL activity in the presence of LPL and the MMP-14-cleaved apoC-II was assayed (Fig. 7B). The cofactor activity of apoC-II decreased in an MMP-14 dose-dependent manner. However, its remaining cofactor activity was not correlated with the residual number of the intact apoC-II molecules but with the sum of the intact and 5-kDa molecules. Thus, not until the 5-kDa fragment disappeared was the cofactor activity lost.

Discussion

Among apolipoproteins, apoA-I [20], apoA-IV, apoE, and apoJ [13], were reported as substrates of MMPs. Here we have shown that apoC-II is a novel substrate for MMP-14 using an in vitro degradomic approach that we had developed [13]. Cleavage of the recombinant apoC-II by six different MMPs (MMP-1, -2, -3, -7, -9, and -14) showed that apoC-II is cleaved the most preferentially by MMP-14 and then by MMP-7.

Tris–Tricine SDS gel analysis showed that the 9-kDa apoC-II was initially cleaved into the 5-kDa fragment by MMP-14 and the 7-kDa and 5-kDa fragments by MMP-7. Since the fragments disappeared upon further incubation, we expected that there are other cleavage sites by

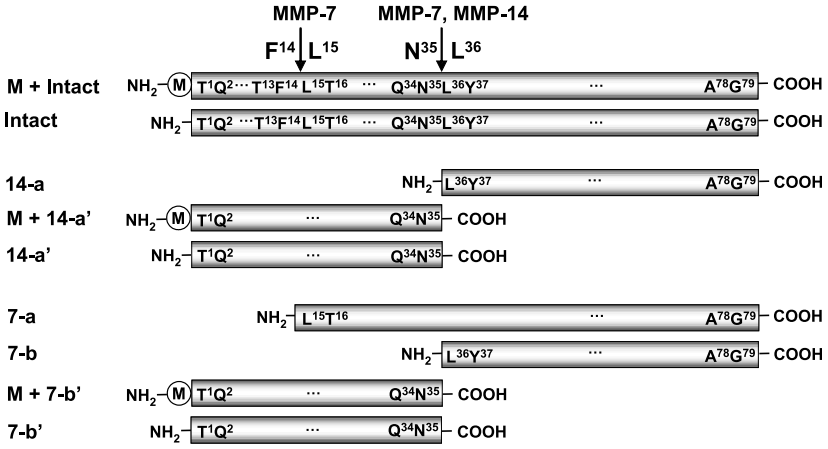


Fig. 6. Schematic diagram of the apoC-II cleavage pathway for MMP-14 and MMP-7. The cleavage sites by cMMP-14 and MMP-7 are marked with arrows. Cleavage of apoC-II was between F^{14}–L^{15} bond by cMMP-14, and between F^{14}–L^{15} bond and between N^{35}–L^{36} bond by MMP-7. The a′ and b′ fragments mean counterparts of a and b fragments, respectively.

Table 1
MALDI-TOF mass analysis of tryptic peptides of the MMP-cleaved apoC-II fragments

Fragment	Peak No.	Mass $[M+H]^+$ (Da)		Modification	Residues	Peptide sequence
		Measured	Calculated			
14-a	1	1035.43	1035.54		40–48	TYLPAVDEK
	2	1838.97	1838.01		36–50	LYEKTYLPAVDEKLR
	3	1911.07	1911.02		40–55	TYLPAVDEKLRDLYSK
	4	2548.26	2548.25		56–79	STAAMSTYTGIFTDQVLSVLKGEE
	5	3125.81	3124.62	MSO[a]	49-76	LRDLYSKSTAAMSTYTGIFTDQVLSVLK
	6	3153.46	3154.55		51–79	DLYSKSTAAMSTYTGIFTDQVLSVLKGEE
	7	3170.51	3170.54	MSO	51–79	DLYSKSTAAMSTYTGIFTDQVLSVLKGEE
7-a	1	1304.72	1304.72		40–50	TYLPAVDEKLR
	2	1855.93	1855.94		15–30	LTQVKESLSSYWESAK
	3	1911.02	1911.02		40–55	TYLPAVDEKLRDLYSK
	4	2054.03	2054.04		31–48	TAAQNLYEKTYLPAVDEK
	5	2233.14	2233.14		56–76	STAAMSTYTGIFTDQVLSVLK
	6	2249.13	2249.14	MSO	56–76	STAAMSTYTGIFTDQVLSVLK
	7	2305.10	2305.10		20–39	ESLSSYWESAKTAAQNLYEK
	8	2323.23	2323.23		31–50	TAAQNLYEKTYLPAVDEKLR
	9	2548.25	2548.25		56–79	STAAMSTYTGIFTDQVLSVLKGEE
	10	2564.25	2564.24	MSO	56–79	STAAMSTYTGIFTDQVLSVLKGEE
	11	2874.45	2874.45		15–39	LTQVKESLSSYWESAKTAAQNLYEK
	12	3154.55	3154.55		51–79	DLYSKSTAAMSTYTGIFTDQVLSVLKGEE
	13	3171.56	3170.54	MSO	51–79	DLYSKSTAAMSTYTGIFTDQVLSVLKGEE
7-b	1	1568.81	1568.82		36–48	LYEKTYLPAVDEK
	2	1838.97	1838.01		36–50	LYEKTYLPAVDEKLR
	3	1911.03	1911.02		40–55	TYLPAVDEKLRDLYSK
	4	2548.23	2548.25		56–79	STAAMSTYTGIFTDQVLSVLKGEE
	5	2564.24	2564.24	MSO	56–79	STAAMSTYTGIFTDQVLSVLKGEE
	6	3154.41	3154.55		51–79	DLYSKSTAAMSTYTGIFTQVLSVLKGEE

ApoC-II which was cleaved with cMMP-14 or MMP-7 was displayed on SDS gels. Protein bands were subjected to in-gel digestion with trypsin, MALDI-TOF MS of the resultant peptides, and peptide mass fingerprinting, as described previously [13].

[a] MSO refers to mono-methionine sulfoxide modification.

the MMPs. The initial cleavage sites of apoC-II by MMP-14 and MMP-7 were determined by MALDI-TOF mass analysis of the cleaved products and mapping of polypeptide mass. Because of the size of the uncleaved apoC-II and its cleaved products, we used the linear mode in the acquisition of MALDI-TOF MS data, which can detect a wider range of mass and thus is more informative than the reflector mode. Since the recombinant apoC-II was a mixture with and without the N-terminal methionine, we easily differentiated the N-terminal and C-terminal fragments. In this way, it was found that apoC-II is cleaved at N^{35}–L^{36} peptide bond by MMP-14 and at F^{14}–L^{15} bond and at N^{35}–L^{36} bond by MMP-7. Mass analysis of tryptic peptides derived from the bands in the SDS gels showed that the 5-kDa fragment detected in the cleavage with MMP-14 and MMP-7 and the 7-kDa fragment in the

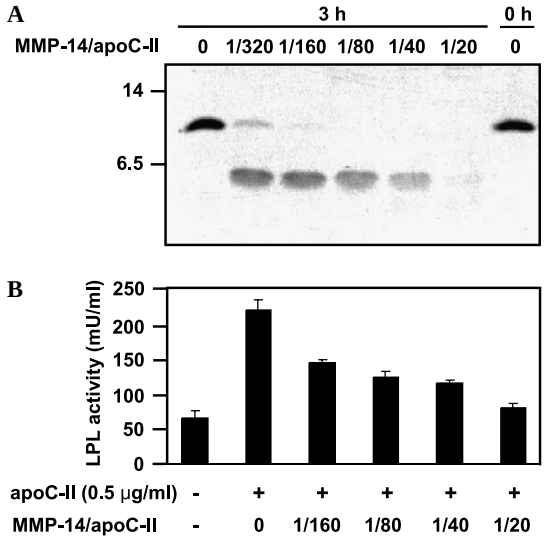


Fig. 7. ApoC-II that was cleaved with MMP-14 loses its LPL cofactor activity. (A) The purified recombinant human apoC-II (1.5 μg) was digested by cMMP-14 in various enzyme/substrate molar ratios (0, 1:320, 1:160, 1:80, 1:40, and 1:20) for 3 h at 37 °C. Samples were analyzed by 15% Tris–Tricine SDS–PAGE. (B) LPL assay was performed in the presence of apoC-II that was incubated with various molar ratios of cMMP-14.

cleavage with MMP-7 correspond to the C-terminal fragments, N^{35}–G^{79} and L^{15}–G^{79}, respectively, and supported that the cleavage sites determined above were correct.

When initial cleavage sites of apoC-II by MMP-14 and MMP-7 were compared with the MMP cleavage site motifs (P5–P3′) determined using peptide libraries [21], two out of three cleavage sites were poor matches; only two amino acid residues matched the MMP-14 cleavage site (TAAQN^{35}↓L^{36}YE; matched amino acid residues are underlined) and one MMP-7 cleavage site (TAAQN^{35}↓L^{36}YE) although five residues matched the other MMP-7 cleavage site (PSPTF^{14}↓L^{15}TQ). Based on studies by NMR [22], the human apoC-II polypeptide in complex with SDS micelles contains three regions with helical conformation, residues 16–36, 50–56, and 63–77, with the intervening regions showing more loosely defined helical conformation. Thus, MMP-14 cleaves in the vicinity of the C-terminus of its first helical region and MMP-7 does near the N- and C-termini of its first helical region. In these regards, we assumed that the position in the tertiary structure, as well as a cleavage site motif in the primary sequence, would be important for its recognition by MMPs.

It has been known that the lipase-activating region of apoC-II is localized to the C-terminal one-third of the sequence, from residue D^{56}, and that its lipid-binding region is in the N-terminal two-thirds [7,23,24]. Studies using synthetic peptides of apoC-II have shown that the maximal activation of LPL by apoC-II requires a minimal sequence contained within residues 56–79 [6]. Consistent with these findings, the L^{36} to G^{79} fragment of apoC-II, which derived from MMP-14 cleavage of apoC-II, showed LPL cofactor activity, and the cofactor activity was lost upon further cleavage of the fragment into smaller fragments. However, it was also shown that the lipid-binding part of apoC-II is needed for full activity of LPL against chylomicron, as a physiologically most relevant substrate [25]. Therefore, we cannot exclude a possibility that even initial cleavage of apoC-II by MMPs may affect LPL activity in a physiological situation.

To our knowledge, our study is the first demonstration for degradation of apoC-II by MMPs including MMP-14 and MMP-7. In addition, we have shown that cleavage of apoC-II by MMPs decreases the LPL cofactor activity of apoC-II. LPL is an acylglycerol hydrolase whose physiological function is to catalyze the hydrolysis of long chain TG for the transport of TG-fatty acids into peripheral tissues [3]. Clinically and biochemically, apoC-II deficiency closely simulates LPL deficiency, or hyperlipoproteinaemia type I, which is associated with several pathophysiological conditions including atherosclerosis, chylomicronaemia, obesity, Alzheimer's disease, and dyslipidaemia [5], and is thus referred to as hyperlipoproteinaemia type IB [8]. Moreover, it is interesting to note that MMP-14 and MMP-7 are expressed at high levels in atherosclerotic plaques [26,27], as well as in a variety of cancer cells [28]. Thus, degradation of apoC-II by MMPs may play an important role in development of pathophysiological conditions of apoC-II deficiency such as atherosclerosis.

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