Phospholipid transfer protein (PLTP) causes proteolytic cleavage of apolipoprotein A-I

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Abstract **Plasma phospholipid transfer protein (PLTP) is a factor that plays an important role in HDL metabolism. In this study we present data suggesting that PLTP has an inherent protease activity. After incubation of HDL_3 in the presence of purified plasma PLTP, the d < 1.25 g/ml particles (fusion particles) contained intact 28.2 kDa apoA-I while the d > 1.25 g/ml fraction (apoA-I-PL complexes) contained, in addition to intact apoA-I, a cleaved 23 kDa form of apoA-I. Purified apoA-I was also cleaved by PLTP and produced a similar 23 kDa apoA-I fragment. The cleavage of apoA-I increased as a function of incubation time and the amount of PLTP added. The process displayed typically an 8–10 h lag or induction period, after which the cleavage proceeded in a time-dependent manner. This lag-phase was necessary for the development of the cleavage activity during incubation at 37°C. The specific apoA-I cleavage activity of different PLTP preparations varied between 0.4–0.8 μg apoA-I degraded/h per 1000 nmol per h of PLTP activity. The 23 kDa apoA-I fragment reacted with monoclonal antibodies specific for the N-terminal part of apoA-I, indicating that the apoA-I cleavage occurred in the C-terminal portion. The apoA-I cleavage products were further characterized by mass spectrometry. The 23 kDa fragment yielded a mass of 22.924 kDa, demonstrating that the cleavage occurs in the C-terminal portion of apoA-I between amino acid residues 196 (alanine) and 197 (threonine). The intact apoA-I and the 23 kDa fragment revealed identical N-terminal amino acid sequences. The cleavage of apoA-I could be inhibited with APMSF and chymostatin, suggesting that it is due to a serine esterase-type of proteolytic activity. Recombinant PLTP produced in CHO cells or using the baculovirus-insect cell system caused an apoA-I cleavage pattern identical to that obtained with plasma PLTP. The present results raise the question of whether PLTP-mediated proteolytic cleavage of apoA-I might affect plasma HDL metabolism by generating a novel kinetic compartment of apoA-I with an increased turnover rate.**—Jauhiainen, M., J. Huuskonen, M. Baumann, J. Metso, T. Oka, T. Egashira, H. Hattori, V. M. Olkkonen, and C. Ehnholm. **Phospholipid transfer protein (PLTP) causes proteolytic cleavage of apolipoprotein A-I.** ***J. Lipid Res.*** **1999.** 40: **654–664.**

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A number of epidemiological studies have demonstrated that the plasma concentration of high density lipoprotein (HDL), whether quantified by cholesterol or apolipoprotein A-I (apoA-I) content, is a good predictor of coronary heart disease (CHD). An inverse correlation between CHD risk and serum HDL levels has been established (1). Further evidence for the antiatherogenicity of human apoA-I has been provided by studies in transgenic mice that express human apoA-I (2, 3). The mechanism underlying the antiatherogenicity of HDL is not known in detail but one proposed explanation is the role of HDL in reverse cholesterol transport, i.e., the transport of cholesterol from peripheral tissues to the liver for excretion (4). Although the mechanism of reverse cholesterol transport is far from resolved, several studies demonstrate that certain subpopulations of HDL, denoted as preβ-HDL, are of great importance in the uptake of cholesterol from tissues (5, 6). It is therefore evident that the factors governing the HDL subclass distribution play an important role in cholesterol homeostasis.

The plasma HDL subpopulations differ in their apolipoprotein and lipid composition and arise at least partly through action of plasma factors that remodel the HDL. Of these, cholesteryl ester transfer protein (CETP), lecithin:cholesteryl acyltransferase (LCAT), hepatic lipase (HL), and phospholipid transfer protein (PLTP) have been characterized. A number of studies (7–11) have demonstrated that PLTP can promote conversion of an apparently homogeneous population of HDL_3 into particles with an increased average size. During this conversion process, which occurs in the absence of other lipoproteins

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; kDa, kilodalton; MAb, monoclonal antibody; CHO, Chinese hamster ovary cells; PLTP, phospholipid transfer protein; cDNA, complementary DNA; APMSF, 4-amidinophenylmethanesulfonyl fluoride; PC, phosphatidylcholine; preβ-HDL, preβ-migrating HDL; α-HDL, α-migrating HDL.

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or CETP, lipid-poor apoA-I is released. Studies with reconstituted HDL particles strongly suggest that particle fusion is responsible for the enlargement of HDL observed upon incubation with PLTP (12).

The lipid poor apoA-I that is released during PLTP-mediated HDL interconversion has been suggested to function as a precursor of preβ_1-HDL, the lipoprotein species that serves as the initial acceptor of cellular unesterified cholesterol in the process of reverse cholesterol transport (13). The mechanisms involved in the formation of preβ_1-HDL are therefore of great physiological importance.

The preβ_1-HDL particles have a composition that is distinctly different from that of α-migrating HDL (5, 14, 15). Only about 10% of the total particle mass is lipid. It has been suggested that the conformational state of apoA-I in preβ-HDL exposes protease-sensitive regions, leading to enhanced predisposition to proteolytic cleavage (14, 16, 17). We report in this study that PLTP, in addition to being able to transfer phospholipids and release lipid-poor apoA-I from HDL during the conversion process, also is able to cause proteolytic cleavage of the main apolipoprotein of HDL, apoA-I. The PLTP-associated protease activity as well as the cleavage site in apoA-I are characterized.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine was from Sigma, 1-palmitoyl-2-[1-^{14}C]palmitoyl phosphatidylcholine (DPPC, specific activity, 55 mCi/mmol) and [^{35}S] Sulphur Labelling Reagent (SLR, t-butoxycarbonyl-l-[^{35}S]methionine, N-hydroxy succinimidyl ester, specific activity 800 Ci/mmol) were from Amersham (Bucks, UK). Butyl-Toyopearl 650 (M) and thin-layer chromatography silica plates were from Merck (Darmstadt, Germany). Hydroxylapatite was from Bio-Rad (Richmond, CA). Superose 6 HR gel filtration column, heparin-Sepharose CL-6B, CNBr-activated Sepharose CL-4B, heparin HiTrap columns, and dextran sulfate were obtained from Pharmacia (Uppsala, Sweden). APMSF (4-amidinophenyl methanesulfonylfluoride) was from Boehringer Mannheim (Germany). Thimerosal (ethylmercurithiosalicylate), pepstatin, and chymostatin were from Sigma Chemical Company (St. Louis, MO).

Monoclonal anti-human apoA-I antibodies (MAb A-I) were a kind gift from Dr. Noel Fidge, Baker Medical Research Institute, Prahran, Australia (MAb A-I 4.1) and Dr. Yves Marcel, University of Ottawa Heart Institute, Ottawa, Canada (MAbs 4H1 and 4A12).

Isolation of lipoproteins

Human HDL_3 (1.12–1.21 g/ml) was isolated by sequential ultracentrifugation using solid KBr to adjust the densities (18). Isolation was carried out at 50,000 rpm in a Beckman Ti 50.2 rotor for 40 h. The HDL was dialyzed against 10 mm Tris-HCl, 150 mm NaCl, pH 7.4 (TBS) and stored at 4°C.

Isolation of human apoA-I and apoA-II

ApoA-I and apoA-II were purified from fresh human plasma as described by Scanu (19). The fraction with apoA-II was delipidated overnight with ethanol–diethyl ether 3:1 (v/v) at −20°C in a volume ratio of 1:24 (20). The samples were subjected to ion-exchange chromatography on DEAE-cellulose (21). This protocol afforded homogeneous preparations of apoA-I and apoA-II which appeared as single bands on 12.5% SDS gels stained with Coomassie Brilliant Blue. The purified apoproteins were dialyzed against 50 mm ammonium bicarbonate and lyophilized. The lyophilized proteins were dissolved in TBS and stored at −20°C.

Purification of plasma PLTP

PLTP was purified from human plasma and assayed essentially as described elsewhere (22), the only change being the use of 1–250 mm sodium phosphate buffer gradient in the final hydroxyapatite chromatography step. After this step PLTP activity of the preparation was 6,000 nmol/ml per h, and the protein appeared as a single major band on SDS-PAGE (see Fig. 1). The PLTP preparations were free of cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT) activity.

Expression of PLTP in baculovirus-transfected insect cells

Human PLTP was produced using a baculovirus-insect cell expression system as described recently (23). PLTP was purified from the insect cell medium using two sequential Reactive Red 120 affinity chromatography steps (24). After the second Reactive Red 120 step, the PLTP activity was 1,200 nmol/ml per h and the protein appeared on SDS-PAGE as a single major band.

Expression of PLTP in Chinese hamster ovary cells

Human PLTP was also expressed in Chinese hamster ovary (CHO) cells. A full-length human PLTP cDNA (25) was obtained from human liver cDNA by reverse transcriptase polymerase chain reaction using oligonucleotide primers (5′-primer, 5′-CA GCTCCACCGCTGAGCCCGCTC and 3′-primer, 5′-ACAGCTGC CAGCTTGGGGATTGAGG) and subcloned into pT7 Blue vector (Novagen). The 1.55 kilobase pair PLTP cDNA was sequenced using the dideoxynucleotide method (Amersham, Bucks, UK), and the proper orientation was confirmed. The cloned human mature PLTP cDNA was ligated into mammalian expression vector pEF321-3.1.1, which was made by modifying the vector pEF321 designated by Kim et al. (26). Inhibin (α signal sequence, MVLHLLLFLLLTPQGGHSCQGLELARELVLAK) was added to excrete the mature protein to the culture medium in CHO cells and six histidines were attached at the N-terminus for the purpose of simplifying the purification of recombinant PLTP from the culture medium. Clones containing the mature PLTP cDNA were transfected into CHO cells using liposome-mediated transfection (27). The selectable marker Neo (G418) was used to select for stable colonies by plating the initial transfection at low concentration of cells. After establishing stable colonies expressing PLTP, the cells were cultured in CHO-SFM II medium (GIBCO) and culture medium was collected after every 3-day period. N-terminally His-tagged PLTP expressed in CHO cells was purified from the culture medium following the standard protocol of nickel (Ni-NTA resin) column chromatography provided by the manufacturer (Qiagen, Chatsworth, CA). After the Ni-column step the PLTP activity was 7,500 nmol/ml per h and the protein appeared on SDS-PAGE as a single major band.

Incubation of HDL and apolipoprotein A-I with PLTP

Incubations of HDL in the presence of PLTP were carried out as described (7). The incubation mixtures contained 0.1–0.7 mg of HDL protein and the PLTP activity used ranged from 500 to 1000 nmol/h. Control HDL samples were incubated in the absence of PLTP.

Human apoA-I purified either by the method of Scanu (19) in our laboratory or apoA-I from the Swiss Red Cross Laboratory purified by the method of Lerch et al. (28) were incubated

in the presence of plasma or recombinant PLTP as specified in the figure legends. After incubation the samples were analyzed on 12.5% SDS-polyacrylamide gels, which were stained with Coomassie blue. In addition, proteins were electrotransferred onto nitrocellulose membranes and then visualized with monoclonal antibodies against known epitope regions on human apoA-I.

Effect of proteinase inhibitors on apoA-I cleavage

The effect of proteinase inhibitors on apoA-I cleavage and PLTP-mediated phospholipid transfer was studied by incubating PLTP in the presence of APMSF (final concentration, 0–25 mm), thimerosal (final concentration, 0–50 mm), pepstatin (final concentration, 30 μg/ml) or chymostatin (final concentration, 30 μg/ml). The preincubation of PLTP with APMSF or thimerosal was performed at room temperature for 30 min after which the incubation mixture was dialyzed against TBS. Incubation of PLTP with pepstatin or chymostatin was performed at room temperature for 15 min after which the mixture was immediately used for apoA-I cleavage experiments. The phospholipid transfer and apoA-I cleavage activities after these incubations were analyzed as described elsewhere in the Materials and Methods.

Radiolabelling of proteins

HDL_3, apoA-I, or apoA-II (1–2 mg protein) were dialyzed against 0.1 m sodium borate buffer, pH 8.5, in the volume of 1 ml. The dialyzed samples were mixed with [^{35}S]Sulphur Labelling Reagent (50 μCi/mg protein) and the mixture was kept on ice for 45 min. The reaction was stopped by adding 50 μl of 1 m glycine in 0.1 m Na-borate buffer, pH 8.5, and kept on ice for 5 min. Unbound radioactivity was removed on a PD-10 gel filtration column. The specific radioactivity obtained was 15,000 cpm/μg apoA-I and 9,000 cpm/μg apoA-II.

The radiolabeled apoA-I or apoA-II were incubated in the presence of plasma or recombinant PLTP as described in the figure legends and the labeled apoproteins were analyzed by 12.5% SDS-PAGE. After autoradiography, the radioactive bands were cut out from the gel and minced. Thereafter, 300 μl of 0.4 m NH_4HCO_3, 2% SDS was added followed by 400 μl of 30% H_2O_2, and the mixture was incubated at room temperature for 2 h. Scintillation solvent (HiSafe, Pharmacia) was added and radioactivity was determined in a Wallac β-counter.

Matrix-assisted laser-desorption mass spectrometry

Mass determination of the native- and PLTP-fragmented apoA-I was performed using the MALDI-TOF instrument from Bruker (Bruker, Germany). Native apoA-I (100 μg) was incubated in the presence or absence of PLTP (800 nmol/h) in Tris-buffered saline (TBS), pH 7.4 for 42 h. After incubation each sample was applied onto a Superose 12 HR gel filtration column (Pharmacia, Sweden) equilibrated with 60% formic acid and 0.5-ml fractions were collected. Protein peaks were detected at 280 nm, pooled, and lyophilized. The lyophilized fractions were dissolved in 20% acetonitrile prepared in water and applied to a reversed phase (RP) HPLC purification step (Applied Biosystems Solvent Delivery System 150A; Perkin-Elmer Corp., Norwalk, CT) using a Vydac C-18 column (Vydac Separations Systems, Hesperia, CA) and a 30-min gradient of 0.1% trifluoroacetic acid (TFA) in acetonitrile (0–70%). Protein peaks were detected at 218 nm. Mass analysis was performed with the RP-purified fractions.

Analysis of HDL particle size

HDL particle size was assessed by non-denaturing polyacrylamide gradient gel electrophoresis (29) using self-made 4–26% polyacrylamide gels (8.0 × 8.0 cm). High molecular weight electrophoresis calibration standards (Pharmacia, Uppsala, Sweden) were used as molecular markers. The stained gels were scanned using the Bioimage System (Millipore Co.).

Other methods

Protein was determined by the method of Lowry et al. (30) using human serum albumin as standard. Human apoA-I and apoA-II were assayed by immunoturbidometry (31). Cholesterol, triglycerides, and phospholipids were determined using enzymatic methods (32). CETP activity was assayed as described by Groener, Pelton, and Kostner (33) and LCAT activity as described by Jauhiainen and Dolphin (34). SDS-PAGE was carried out by the method of Laemmli (35) and Western blotting as described by Towbin, Staehlin, and Gordon (36).

RESULTS

Purified plasma PLTP as analyzed by homogenous SDS-PAGE is shown in **Fig. 1**. The purification procedure and the degree of purification were very repeatable between different PLTP preparations. Human HDL_3 ($1.12 < d < 1.21$ g/ml) was incubated in the presence or absence of purified plasma PLTP at 37°C for 24 h. After the incubation the samples were subjected to ultracentrifugation at density 1.25 g/ml. Products of PLTP-mediated HDL conversion were then isolated by ultracentrifugation. The top fraction ($d < 1.25$ g/ml) contained large particles with a diameter of approximately 11 nm, while the fraction with a density >1.25 g/ml consisted of small (7.5 nm in diameter) lipid-poor particles containing apoA-I as their main protein constituent. Analysis of these two populations of HDL particles by denaturing SDS polyacrylamide gel electrophoresis (**Fig. 2**) revealed that during incubation in the presence of purified PLTP, part of the major apoprotein of HDL, apolipoprotein A-I (apoA-I), was proteolytically cleaved and could be recovered in the lipid-poor fraction

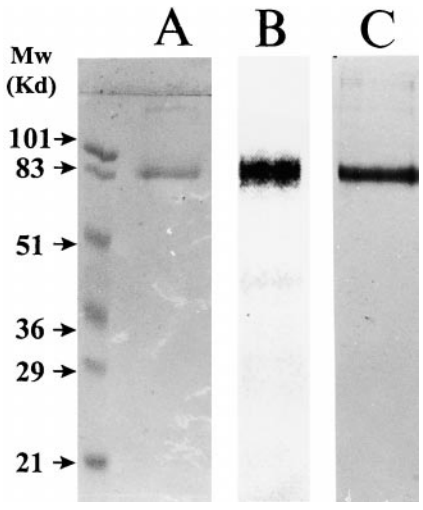

Fig. 1. SDS-PAGE (12.5%) of the purified human plasma PLTP. After the final hydroxylapatite chromatography step, human PLTP was run in a 12.5% SDS-PAGE and detected by Coomassie staining (lane A), silver staining (lane B), and immunoblotting with rabbit anti-human PLTP antibody (lane C). In lanes A and C, 0.4 μg protein and in lane B 0.8 μg protein were loaded. Molecular weight standards are shown on the left.

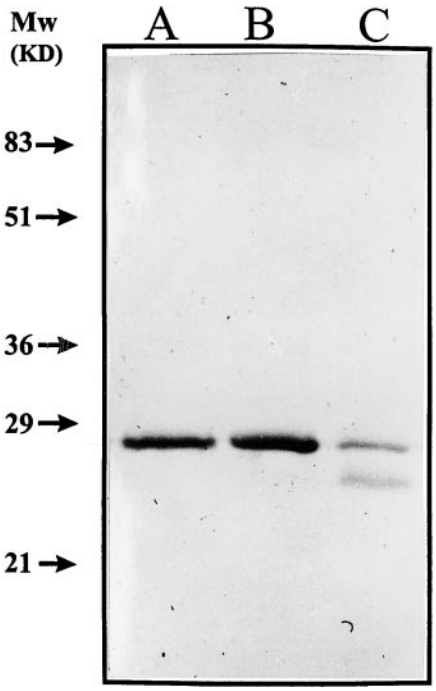

Fig. 2. Cleavage of apoprotein A-I (apoA-I) upon incubation of high density lipoprotein (HDL) in the presence of phospholipid transfer protein. After a 24-h incubation of HDL_3 (500 μg protein) in the presence of PLTP (1,000 nmol/h) the $d < 1.25$ g/ml and $d > 1.25$ g/ml fractions were isolated by ultracentrifugation and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting. The proteins were visualized using monoclonal apoA-I antibody (MAb 4H1); lane A: HDL incubated in the absence of PLTP; lane B: the $d < 1.25$ g/ml fraction isolated after PLTP incubation; lane C: the $d > 1.25$ g/ml fraction isolated after PLTP incubation; 2 μg of protein was applied per lane. The arrows indicate the molecular sizes of standard proteins.

with $d > 1.25$ g/ml. Thus, after incubation, the $d < 1.25$ g/ml fraction (Fig. 2, lane B) contained only intact 28.2 kD apoA-I while the $d > 1.25$ g/ml fraction contained, in addition to intact apoA-I, a cleaved form of the protein with an apparent size of 23 kD (Fig. 2, lane C). Immunoblotting with anti-apoA-II indicated that apoA-II was exclusively located in the large $d < 1.25$ g/ml particle fraction (data not shown). The HDL conversion products were further analyzed by size-exclusion chromatography. In accordance with the ultracentrifugation analysis, immunoblotting of the eluted fractions indicated that the large fusion particles contained intact apoA-I and apoA-II whereas the small HDL particles contained both the intact protein and the 23 kD fragment and no apoA-II (data not shown).

To study whether purified apoA-I is proteolytically cleaved upon incubation with PLTP, we used radioactively labeled apoA-I, which also enabled us to quantitate the cleavage. ^{35}S-labeled apoA-I (50 μg protein) was incubated in the presence of human PLTP (600 nmol/h) at 37°C for 18 h. After incubation, SDS-polyacrylamide gel electrophoresis (**Fig. 3**) revealed two species of apoA-I, one with the size of intact apoA-I, 28.2 kD, and another with an apparent size of 23 kD. In the absence of added PLTP, no significant degradation of apoA-I was observed.

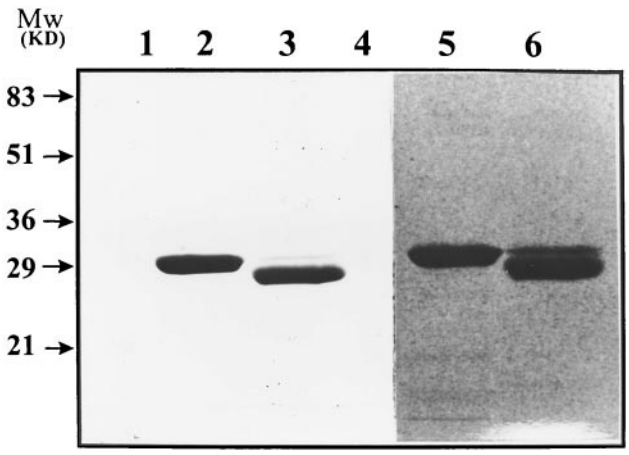

Fig. 3. Cleavage of apoA-I upon incubation in the presence of PLTP. ^{35}S-labeled apoA-I (50 μg protein) was incubated for 18 h at 37°C in the presence of PLTP (600 nmol/h), followed by analysis by 12.5% SDS-polyacrylamide gel electrophoresis. ApoA-I incubated in the absence (lane 2) or the presence (lane 3) of PLTP (4 μg of apoA-I was applied per well). Lanes 1 and 4 show molecular weight standards. Autoradiography of lanes 2 and 3 is shown on the right (lanes 5 and 6).

Labeling of apoA-I with the sulfur labeling reagent did not make apoA-I more susceptible to degradation, as experiments with non-labeled apoA-I gave a similar pattern of degradation.

The cleavage of apoA-I increased as a function of the amount of PLTP added to the incubation (**Fig. 4**). Up to 15% of apoA-I was cleaved when 50 μg of labeled apoA-I was incubated with 900 nmol/h of PLTP activity for 18 h. Although the apoA-I cleavage was apparent with each purified PLTP preparation, we observed differences in the

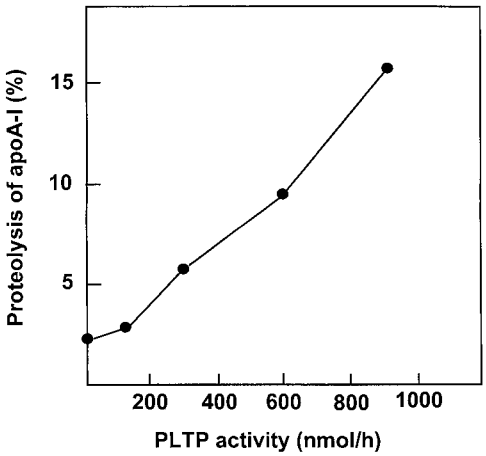

Fig. 4. Cleavage of apoA-I as a function of PLTP activity. ^{35}S-labeled apoA-I (50 μg protein) was incubated at 37°C for 18 h in the presence of increasing amounts of PLTP. After incubation the samples were subjected to 12.5% SDS-polyacrylamide gel electrophoresis to separate the 28 kD apoA-I and the 23 kD apoA-I fragments. The bands were cut out and their radioactivities were determined. Proteolysis of apoA-I is defined as radioactivity of the 23 kDa band as per cent of total radioactivity recovered in the lane (sum of the radioactivity of the 28.2 kDa and 22.9 kDa bands).

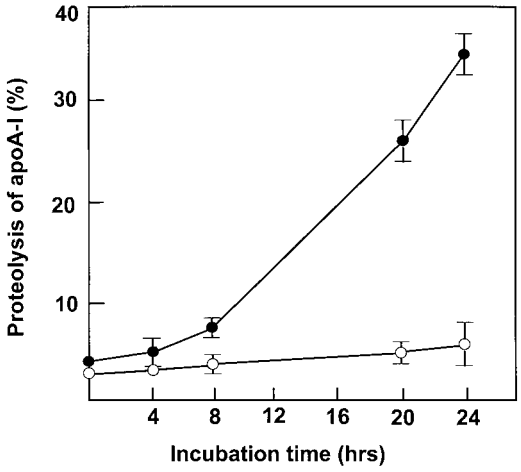

Fig. 5. PLTP-mediated cleavage of apoA-I as a function of incubation time. ^{35}S-labeled apoA-I (50 μg protein) was incubated in the absence (open circles) and the presence (closed circles) of PLTP (1,000 nmol/h) at 37°C for the times indicated. The percentage of apoA-I degraded was calculated as described in the legend to Fig. 4. The results are representative of three separate experiments.

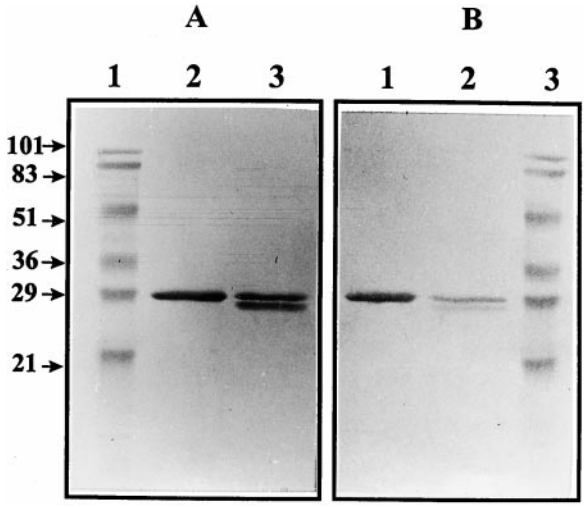

Fig. 6. Characterization of the PLTP-mediated apoA-I cleavage products using monoclonal anti-apoA-I antibodies. ApoA-I (50 μg protein) was incubated in the absence or presence of PLTP (600 nmol/h) for 24 h at 37°C. After incubation, 0.4 μg of protein was applied on an SDS-polyacrylamide gel (12.5%). After electrophoresis and transfer of proteins to the nitrocellulose membrane, the membrane was probed with specific monoclonal anti-apoA-I antibodies. Panel A: immunoblot with anti-apoA-I MAb 4H1; lane 1, molecular weight standards; lane 2, apoA-I incubated in the absence of PLTP; lane 3, apoA-I incubated in the presence of PLTP. Panel B: immunoblot with anti-apoA-I MAb 4A12; lane 1, apoA-I incubated in the absence of PLTP; lane 2, apoA-I incubated in the presence of PLTP; lane 3, molecular weight standards as in panel A. Immunoblotting with the anti-apoA-I MAb A-I 4.1 (epitope between the amino acid residues 211–220) did not recognize the 23 kD fragment.

extent of apoA-I cleavage between batches of PLTP with the same PL-transfer activity (which is also evident in Figs. 3 and 4). The clear established causes for this are not known to us at the moment. In the apoA-I cleavage experiments PLTP was added into the incubation based only on its PL-transfer activity without knowing the exact PLTP mass (because of the lack of specific PLTP mass assay). Therefore, one probable reason for these differences may be that the mass of PLTP added (due to instability of the PL-transfer activity) is slightly different in each incubation. The cleavage of apoA-I was time-dependent (**Fig. 5**). The cleavage rate was slow up to 10 h, whereafter the rate increased significantly. Interestingly, each of the PLTP preparations displayed this lag or induction period (8–10 h) during which the cleavage occurred at very low rates. It is possible that this lag period is necessary for a proper PLTP–apoA-I interaction in order to launch the cleavage process. After a 24 h incubation, approximately 35% of the apoA-I was cleaved. In the absence of added PLTP only marginal time-dependent cleavage of apoA-I (6% over baseline) was observed.

In control experiments ^{35}S-labeled apolipoprotein A-II (apoA-II) was used. After incubation in the presence of PLTP, no degradation of apoA-II could be detected even after a prolonged 44-h incubation. Neither were albumin nor IgG, which were used as controls, cleaved when incubated in the presence of PLTP (data not shown).

To determine whether the cleavage of apoA-I occurred in the N-terminal or the C-terminal portion of the molecule, we used monoclonal site-specific apoA-I antibodies. Radiolabeled apoA-I (50 μg of protein) was incubated in the presence of PLTP (600 nmol/h) for 24 h. After incubation, a sample (0.4 μg of apoA-I) was subjected to polyacrylamide gel electrophoresis and immunoblotting (**Fig. 6**). It is evident that the 23 kD apoA-I fragment formed reacts with the monoclonal antibody 4HI specific for the N-terminal part of apoA-I (epitope between amino acid residues 2–8), indicating that the apoA-I cleavage occurs in the C-terminal portion (Fig. 6A). The location of the cleavage region in apoA-I was verified using two mono-

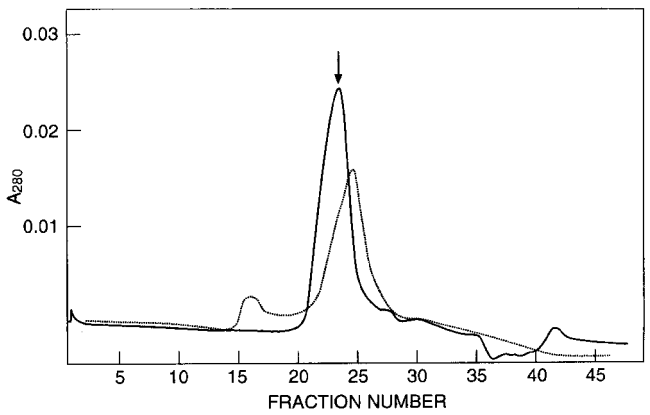

Fig. 7. Separation of apoA-I and the 23 kD fragment by size-exclusion chromatography. Native apoA-I (100 μg) was incubated in the absence or presence (800 nmol/h) of PLTP in Tris-buffered saline, pH 7.4. After incubation, the samples were separately applied onto a Superose 12 HR column as described in detail in Materials and Methods. Fractions (0.5 ml) were detected by absorbance measurements at 280 nm. The solid line represents the elution pattern of native apoA-I (mol mass 28.2 kD) and the dotted line that of the 23 kD apoA-I fragment.

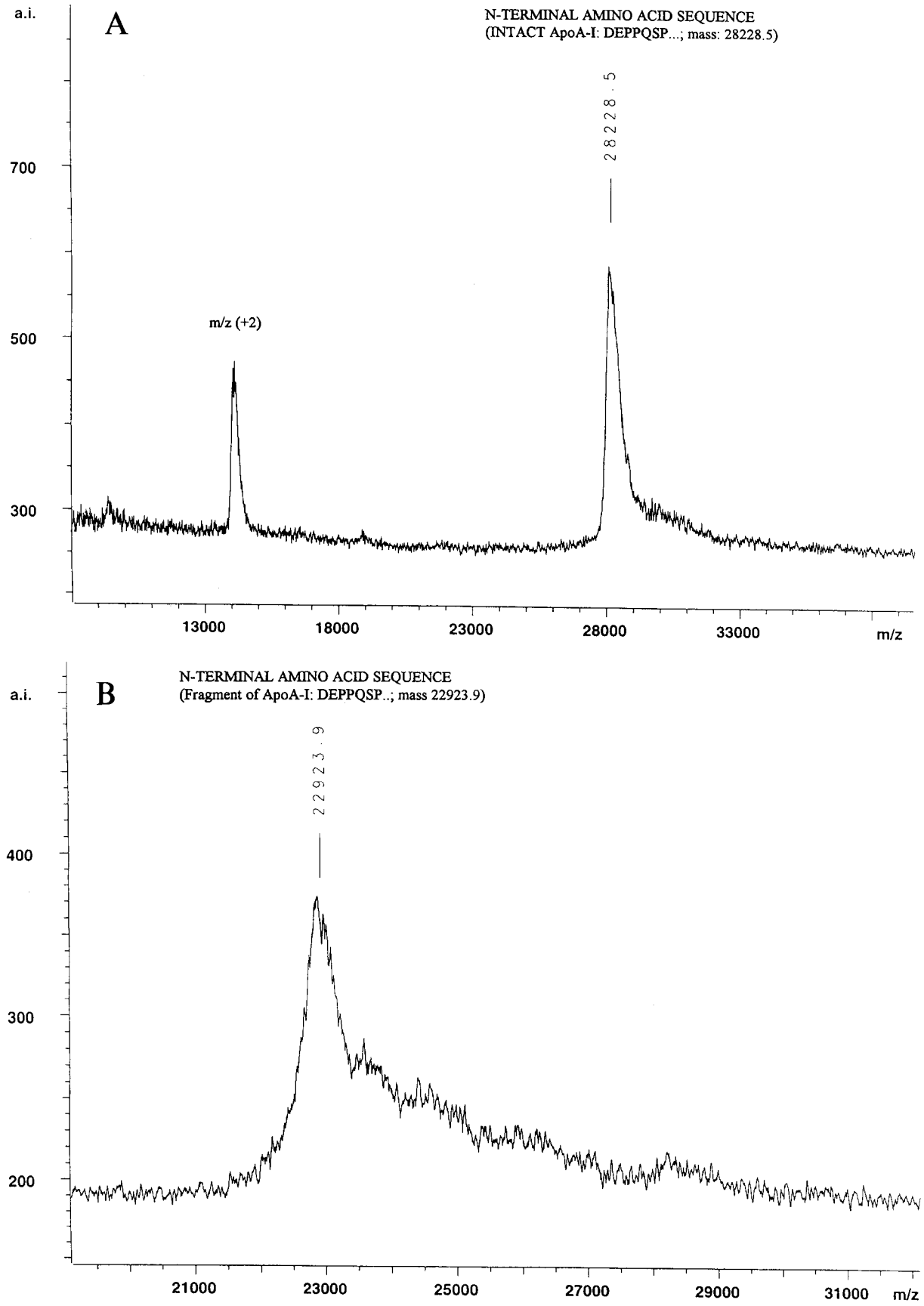

Fig. 8. Matrix-assisted laser-desorption mass spectrometric analysis of apoA-I and the 23 kD fragment. Mass determination of the native and PLTP-fragmented apoA-I was performed using MALDI-TOF mass spectrometry. Samples for mass analysis were prepared as described in the Materials and Methods. Panel A: mass spectrum of native apoA-I; panel B: mass spectrum of the apoA-I 23 kD fragment. N-terminal amino acid sequences of intact apoA-I and the 23 kD proteolytic fragment are shown above the corresponding peaks.

clonal antibodies with epitopes in the carboxy terminal portion of the protein. Monoclonal antibody Mab A-I 4.1, which recognizes an epitope between amino acid residues 211–220, did not recognize the 23 kD cleavage product, whereas Mab 4A12, the epitope of which is between residues 173–205, did recognize this fragment (Fig. 6B). These results suggest that the cleavage site is in the C-terminal half of the apoA-I molecule, located in the region around amino acid residue 200.

In order to characterize in detail the cleavage products of apoA-I, we isolated native apoA-I and the 23 kD fragment by size-exclusion chromatography (**Fig. 7**) and by reversed phase HPLC using a C-18 column. The native apoA-I and the 23 kDa proteolytic fragment were subjected to mass spectrometric analysis. The mass determined for native apoA-I was 28.2 kDa (detailed mass 28,229 Da) (**Fig. 8A**), while analysis of the N-terminal fragment yielded a mass of 22.9 kDa (detailed mass 22,924 Da) (Fig. 8B), demonstrating that the cleavage of apoA-I had occurred between amino acid residues 196 (alanine) and 197 (threonine) in the C-terminal portion of apoA-I. Both the intact apoA-I and the 23 kDa fragment revealed identical N-terminal amino acid sequences (DEPPQSP), verifying that no cleavage had occurred at the N-terminus (Fig. 8). We were not able to isolate and characterize the C-terminal peptide released from apoA-I (residues 197–243).

The cleavage pattern of apoA-I resulting in a 22.9 kDa fragment was reproducibly obtained with 35 different PLTP preparations from the plasma of 13 individuals. Next we calculated the apoA-I cleavage activity for the different PLTP preparations. The cleavage activity varied between 0.4–0.8 μg apoA-I degraded/h per 1000 nmol per h of PLTP activity. These calculations are based on the total incubation time, including the lag period. To further strengthen the conclusion that the protease activity is present in intact PLTP, we performed immunoprecipitation experiments using a monospecific anti-human PLTP polyclonal antibody that only detects PLTP band. This antibody was raised in rabbits by immunizing them with purified plasma PLTP as shown in Fig. 1. In these experiments we incubated purified PLTP (500 nmol/h) with this antibody (50 μg) for 30 min at room temperature. After this protein A-Sepharose (100 μl, 1:1 v/v dilution in PBS) was added and incubated for a further 15 min at room temperature. Finally the immunocomplex–protein A-Sepharose was precipitated by centrifugation in an Eppendorf centrifuge for 10 min (15,000 rpm). The supernatant was used for apoA-I cleavage experiments where 50 μg of apoA-I was incubated with the supernatant for 24 h. After immunoprecipitation with anti-PLTP, there was no apoA-I cleavage activity in the supernatant or immunostainable PLTP. A non-relevant control rabbit antibody did not affect the cleavage activity (data not shown). These experiments support the concept that PLTP contains inher-

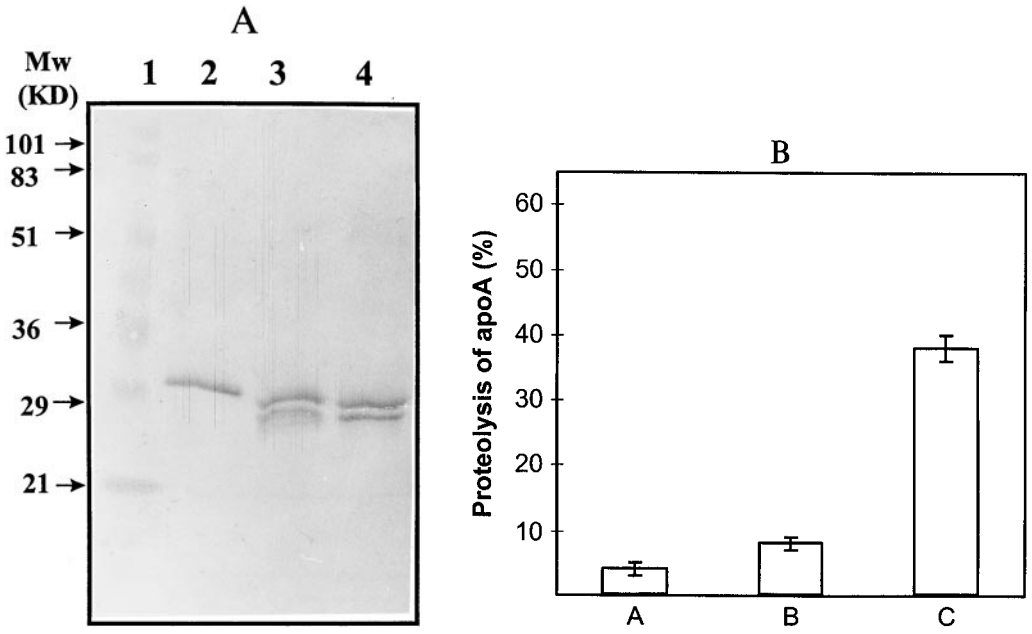

Fig. 9. Cleavage of apoA-I by recombinant human PLTP produced in Chinese hamster ovary (CHO) cells. Panel A: apoA-I was incubated for 24 h at 37°C in the absence or presence (500 nmol/h) of purified plasma PLTP or PLTP expressed in CHO cells as described in Materials and Methods. After incubation, 0.4 μg of apoA-I was applied on an SDS-polyacrylamide gel (12.5%) and immunoblotting was performed with the anti-apoA-I MAb 4H1. Lane 1, molecular weight standards; lane 2, apoA-I incubated in the absence of PLTP; lane 3, apoA-I incubated in the presence of purified plasma PLTP; lane 4, apoA-I incubated in the presence of human PLTP expressed in CHO cells. Panel B: Radiolabeled [^{35}S]apoA-I was incubated in Tris-buffer, pH 7.4, in the absence (A) or presence (C) of recombinant PLTP (500 nmol/h) purified from the CHO cell growth medium as described in Materials and Methods. Incubation time was 22 h at 37°C. After incubation, the apoA-I cleavage was quantitated as described in the legend to Fig. 4. Column B: [^{35}S]apoA-I incubated in the presence of CHO cell control medium (collected from cells transfected with the expression vector containing only the selectable marker NEO) treated analogously to the recombinant PLTP-containing medium. The results (mean ± SD) shown are from three independent experiments.

ent protease activity although it does not absolutely exclude the possibility of a separate protease associated with PLTP.

In addition to purified plasma PLTP, we used recombinant human PLTP produced in two different cell systems (CHO cells and the baculovirus-insect cell system) and purified by different chromatographic procedures. **Figure 9** and **Fig. 10** illustrate the results of incubations of apoA-I in the presence of these two recombinant PLTP proteins. The recombinant PLTP produced in the mammalian CHO cells yielded an apoA-I cleavage pattern similar to that produced by purified plasma PLTP (Fig. 9A). Incubation of [^{35}S]apoA-I (50 μg) in the presence of PLTP produced in the CHO cells (activity of 500 nmol/h) caused almost 40% degradation of apoA-I in 24 h (Fig. 9B). Control medium from CHO cells lacking the human PLTP cDNA chromatographed on a Ni-NTA column caused only marginal 8% apoA-I cleavage (Fig. 9B). The recombinant protein produced in baculovirus-infected insect cells caused an apoA-I cleavage pattern similar to that generated by plasma PLTP or PLTP produced in CHO cells (Fig. 10). ApoA-I incubated in the presence of mock-medium treated analogously to the recombinant PLTP-containing insect cell medium caused no degradation of apoA-I (Fig. 10, lane 4).

To probe the nature of the proteolytic cleavage of apoA-I, we used inhibitors directed against different classes of proteases. The effects of these inhibitors on the PLTP-induced fragmentation of apoA-I as well as on the PLTP phospholipid transfer activity were studied. To follow the effects of APMSF, an inhibitor of serine-esterases, and thimerosal, an inhibitor of thiol-proteases, on the PLTP-mediated apoA-I degradation, we used radioactively labeled apoA-I, while the effects of these inhibitors on PLTP-mediated phospholipid transfer were recorded using the liposome–HDL PC transfer assay.

As shown in **Fig. 11**, panel A, APMSF inhibited the protease activity of the PLTP preparation without significantly affecting the phospholipid transfer activity. The radioactivity recovered in the 22.9 kD fragment was reduced from 34% (no inhibitor added) to 3.3% (25 mm APMSF). Furthermore, another serine–esterase inhibitor, chymostatin (final concentration, 22 μg/ml), which is specific for chymotrypsin-like proteases, totally inhibited the apoA-I cleavage without affecting PLTP activity. Thimerosal, an inhibitor of thiol-proteases did not influence the apoA-I cleavage but caused about 90% inhibition of the phospholipid transfer activity of PLTP (Fig. 11, panel B). Thus, the two activities, protease and phospholipid transfer, are affected differently by the two types of inhibitors. The other protease inhibitors studied (leupeptin, 0.01 mm; trasylol, 50 U/ml; E-64, 1 mm, N-ethylmaleimide, 40 mm) had no significant effect either on the PLTP-mediated phospholipid transfer activity or the apoA-I cleavage. These data suggest that the cleavage of apoA-I is due to a serine esterase-type of activity. In separate experiments we performed PLTP purification and apoA-I cleavage assays in the presence of sodium azide (0.02%) and gentamycin sulfate (0.1 mg/ml). These antimicrobial agents had no effect on the PLTP-mediated apoA-I cleavage, indicating that protease function does not originate from bacterial sources during purification (data not shown).

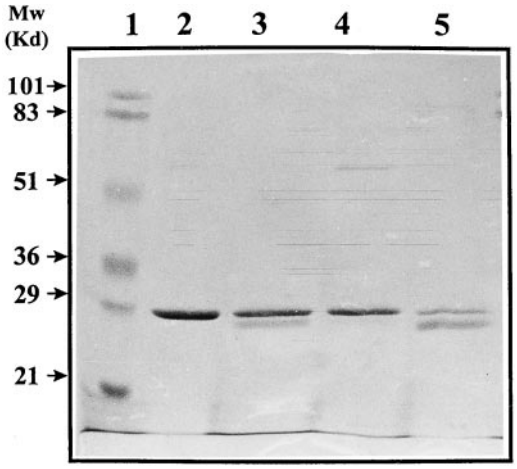

Fig. 10. Cleavage of apoA-I by recombinant human PLTP produced in the baculovirus-insect cell system. ApoA-I was incubated for 21 h at 37°C in the absence or presence (400 nmol/h) of plasma PLTP or a recombinant PLTP produced in insect cells, which were purified as described in Materials and Methods. After incubation, 0.4 μg of apoA-I was applied on an SDS-polyacrylamide gel, electrophoresed, and stained with Coomassie blue. Lane 1, molecular weight standards; lane 2, apoA-I incubated in the absence of PLTP; lane 3, apoA-I incubated in the presence of recombinant PLTP; lane 4, apoA-I incubated in the presence of insect cell mock-medium (collected from cells infected with wild-type baculovirus) treated analogously to the recombinant PLTP-containing medium; lane 5, apoA-I incubated in the presence of purified plasma PLTP.

DISCUSSION

We have previously reported on the interconversion of HDL subclasses mediated by plasma PLTP (7, 10, 37). Based on results obtained using reconstituted HDL particles, we proposed a two-stage mode for the PLTP-mediated interconversion (12). In this model, PLTP first causes transfer of phospholipid molecules between particles. This event results in the release of apoA-I with a small amount of bound phospholipids, which leads to the destabilization of HDL particles which fuse to form an HDL population with a larger particle diameter. The released surface components, apoA-I and phospholipids, form small particles that can be isolated by either gel filtration or ultracentrifugation (37), and display pre-β mobility in agarose gel electrophoresis (22, 37). We have demonstrated here that part of the apoA-I in the small particles is proteolytically cleaved to a 23 kDa fragment that is not present in the larger fusion particles. The cleavage was observed using PLTP purified from human plasma or recombinant PLTP purified by different chromatographic protocols from two different cell systems. Thus, although we cannot absolutely exclude the possibility that the proteolytic activity could be due to a copurifying protease tightly bound to PLTP, we consider this unlikely. We therefore suggest that PLTP, in addition to

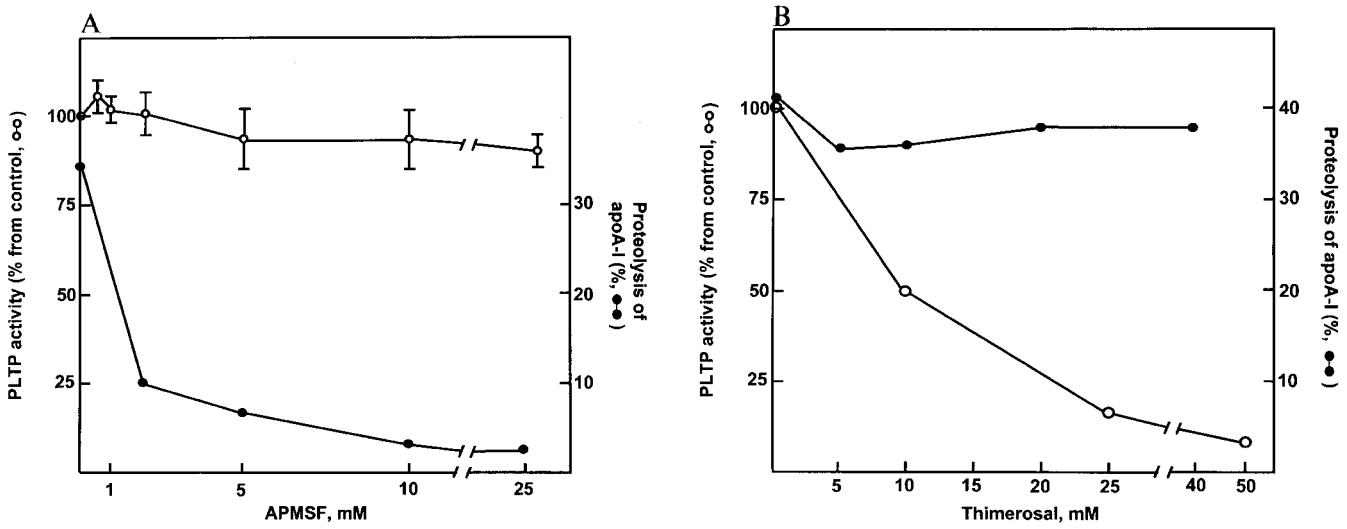

Fig. 11. Effects of aminophenylmethane sulfonyl fluoride (APMSF) and thimerosal on PLTP-mediated apoA-I cleavage and phospholipid transfer activity. Panel A: PLTP (500 nmol/h) was incubated in the presence of APMSF (2, 5, 10, or 25 mm, final concentration) for 30 min at room temperature and then dialyzed against TBS, pH 7.4. PLTP activity was measured as described in Materials and Methods. Cleavage of radiolabeled apoA-I was assayed by incubating [^{35}S]apoA-I in the presence of the APMSF-treated PLTP samples for 20 h at 37°C. Quantitation of the cleavage was carried out as described in the legend to Fig. 4. Panel B: PLTP (500 nmol/h) was incubated in the presence of thimerosal (10, 25, or 50 mm, final concentration) for 30 min at room temperature and then dialyzed against TBS, pH 7.4. PLTP activity and apoA-I cleavage were determined as described in panel A. The data in panels A and B represent average values from three independent experiments.

facilitating the transfer of lipid compounds, displays protease activity. At the moment, it is not known whether PLTP cleaves apoA-I on the surface of HDL particles, or whether apoA-I is first displaced from the lipoprotein surface and forms small apoA-I–PL complexes which act as substrate for the PLTP protease function. Kunitake and his colleagues (16) demonstrated that apoA-I of preβ-HDL displays much higher sensitivity to proteolytic cleavage by plasmin and several other proteases than does the apoA-I of α-HDL. This suggests that the conformational state of apoA-I in preβ-migrating HDL or its spatial relationship to lipids is significantly different from that of apoA-I in α-migrating HDL, and furthermore, that this conformation of apoA-I appears to expose a protease-sensitive region. Preβ-HDL contains 80–90% protein, predominantly apoA-I, which apparently yields a particle that has a structure quite different from that of α-HDL (38, 39).

The cleavage of apoA-I at amino acid 196 results in the formation of two fragments, the N-terminal peptide consisting of amino acids 1–196 and a C-terminal peptide consisting of residues 197–243. The cleavage takes place in the middle of the eighth amphipathic helix of apoA-I (40). Ji and Jonas (41) demonstrated that the fragment consisting of the N-terminal 192 amino acid residues of apoA-I causes a dramatic decrease in the ability of the protein to bind lipid and to spontaneously form reconstituted HDL. Consistent with this observation, deletion of residues 185–243 or 209–243 from apoA-I severely impaired the ability of the truncated proteins to bind to HDL (42). These data were recently corroborated by Laccotripe et al. (43) who suggested that specific hydrophobic residues in the putative loop region (223–231) and within helix 8 (187–223) of apoA-I are important for binding to HDL and for the initial association of apoA-I with multilamellar phospholipid vesicles. In contrast, studies with an amino-terminal deletion mutant (residues 1–43) of apoA-I indicated that the N-terminal region is not necessary for lipid and HDL binding (44). It is therefore plausible that the PLTP-mediated cleavage between amino acids 196 and 197 strongly influences the apoA-I–lipid interactions in the HDL.

The PLTP-mediated apoA-I cleavage described here is important from a physiological point of view. Analyses of patients with reduced plasma concentration of HDL have suggested that accelerated catabolism of apoA-I is the most common cause leading to low HDL levels (45, 46). Although the tissues responsible for HDL clearance from the circulation have not been fully characterized, a major part of apoA-I seems to be cleared by the kidneys. It has been demonstrated that free or lipid-poor apoA-I and small molecular size HDL are first filtered through the glomerulus, then reabsorbed, and finally degraded by proximal tubule cells (47, 48). Proteolysis is known to regulate the clearance of several plasma proteins (49–51). Although the N-terminal domain of apoA-I can undergo limited proteolysis (52), the carboxy-terminal domain seems to be particularly sensitive to proteolysis as substantiated by previous reports where limited enzymatic proteolysis produced 22–26 kDa N-terminal fragments (16, 17, 41). These in vitro proteolytic studies suggest that enzymatic cleavage of apoA-I may also occur in vivo. To address the role of the structural motifs of apoA-I in its metabolic clearance, Schmidt et al. (53) showed that deletion of the carboxy-terminal domain of human apoA-I resulted in a 14-fold increase of the clearance rate after bolus injection in rabbits. The role of the carboxy-terminus in apoA-I clearance was further supported by a recent study in

which human mutant apoA-I with deletion of residues Ala190–GLn243 was overexpressed in mice (54). The fractional catabolic rate of the mutant was 4.9-fold higher than that of native apoA-I. Our data suggest that PLTP-mediated C-terminal cleavage of apoA-I reduces its lipoprotein association, and may result in the production of a novel kinetic pool of apoA-I which has an increased turnover rate. Recent in vivo studies have demonstrated that adenovirus-mediated overexpression of human PLTP in mice dramatically reduces plasma HDL levels (55, 56). The reduced HDL levels in mice expressing high levels of PLTP may partially be explained by the PLTP protease function that may lead to increased catabolism of the cleaved apoA-I via glomerular function.

The cleavage of apoA-I by PLTP can be inhibited by APMSF and chymostatin, specific inhibitors of serine-esterase proteases, suggesting that a catalytic triad-type of pocket should exist in PLTP. An important aspect here was the regular observation that the apoA-I cleavage needs a relatively long incubation time; in the beginning there always seems to be a lag or induction period (8–10 h) during which the cleavage occurs at very low rates. Generally, many serine-esterase-type of enzymes have much faster catalytic rate. It is possible that this lag period is needed for PLTP and apoA-I to form a complex the organization of which launches the fast rate cleavage of apoA-I.

The origin of PLTP apoA-I cleavage activity is presently not known. Recently, the crystal structure of human bactericidal/permeability-increasing protein (BPI) was reported (57). BPI shares functional and sequence similarity with human PLTP. Thus, the crystal structure of BPI is now used in our laboratory for molecular modelling of PLTP structure in order to locate a catalytic center responsible for the protease function of PLTP.

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