Deletion of the propeptide of apolipoprotein A-I reduces protein expression but stimulates effective conversion of preβ-high density lipoprotein to α-high density lipoprotein

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Abstract **The properties of the mature and pro-forms of recombinant apolipoprotein A-I (apoA-I) were compared with those of apoA-I isolated from human plasma. When the synthesis and secretion of pro- and mature forms of apoA-I from a baculovirus/insect cell expression system were compared in parallel experiments, the amount of the pro-form of apoA-I synthesized and secreted was severalfold higher than that of the mature form of apoA-I. A comparison of the properties of the pro- and mature forms of recombinant apoA-I and human plasma apoA-I showed no difference between all three in their secondary structure, their ability to self-associate, lipid-binding capacity, lecithin: cholesterol acyltransferase activation, and binding to the phospholipid transfer protein. The properties of reconstituted high density lipoprotein (HDL) particles formed from the proteins and their ability to promote cholesterol and phospholipid efflux from human skin fibroblasts were also similar. However, their ability to bind to plasma HDL subfractions differed, because twice as much proapoA-I associated with preβ$_1$-HDL and preβ$_2$-HDL subfractions compared with both mature recombinant and plasma apoA-I. Correspondingly, the amount of proapoA-I in α-HDL subfractions, especially in α$_1$-HDL and α$_2$-HDL, was decreased. We conclude that while the propeptide of apoA-I is required for the effective synthesis and secretion of apoA-I, cleavage of this peptide is a requisite for the effective interconversion of HDL subfractions.**—Sviridov, D., L. E. Pyle, M. Jauhiainen, C. Ehnholm, and N. H. Fidge. **Deletion of the propeptide of apolipoprotein A-I reduces protein expression, but stimulates effective conversion of preβ-high density lipoprotein to α-high density lipoprotein.** ***J. Lipid Res.*** **2000.** 41: **1872–1882.**

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Apolipoprotein A-I (apoA-I), the principal apolipoprotein of high density lipoprotein (HDL), is a key element of the reverse cholesterol transport pathway, a process that removes cholesterol from extrahepatic tissues, including the vessel wall, thus protecting against the development of atherosclerosis (1). The role of apoA-I in this pathway includes the organization of HDL structure, promotion of cholesterol efflux, and activation of lecithin:cholesterol acyltransferase (LCAT) (1, 2). New evidence is also emerging, supporting a role for apoA-I in biochemical systems not directly related to the reverse cholesterol transport, but contributing to the protection of the arterial wall against injury. These properties of apoA-I include antioxidant (3), antithrombotic (4), and anti-inflammatory (5, 6) actions. ApoA-I also regulates expression of adhesion molecules (7) and activates intracellular signaling events (4, 8, 9).

Most apoA-I is synthesized in the liver and intestine, although small amounts of apoA-I are produced in avian nerve tissue (10) and kidney (11). ApoA-I is translated as a preproprotein (12) and, after cleavage of the 18-amino acid signal peptide, the proprotein is secreted into the plasma, where the 6-amino acid propeptide is removed by cleavage through an unidentified metalloprotease (13). Maturation of apoA-I in plasma is effective: usually less than 5% of plasma apoA-I is represented by a pro-form (14). The role of the propeptide and requirement for its subsequent cleavage in apoA-I function are unclear. We (15) and others (16) have demonstrated that expression of recombinant apoA-I with the propeptide deleted impairs secretion and decreases the overall efficiency of expression of apoA-I, suggesting that the propeptide functions to facilitate secretion of newly synthesized apoA-I, although its further role and cleavage subsequent to secretion remain unknown. Several in vitro studies that have compared the properties of mature plasma apoA-I with recombinant proapoA-I (17–19) report little difference between them. In vivo, however, it appears that their metabolism differs, because one form of familial HDL deficiency, characterized by

Abbreviations: apoA-I, apolipoprotein A-I; BS^3, bis(sulfosuccinimidyl)suberate; DMPC, dimyristoyl phosphatidylcholine; ELISA, enzyme-linked immunosorbent assay; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; MOI, multiplicity of infection; PLTP, phospholipid transfer protein; POPC, palmitoyloleoyl phosphatidylcholine; rHDL, reconstituted HDL; TLC, thin-layer chromatography.

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hypercatabolism of mature apoA-I but not of proapoA-I, has been described (14, 20). This finding supports the likelihood that cleavage of the propeptide is more than a redundant function of a nonspecific protease, and may be necessary for normal functioning and metabolism of apoA-I.

An understanding of the role of propeptide cleavage also has practical implications: many investigators choose expression systems that produce recombinant proapoA-I, and until a possible confounding effect of the propeptide is eliminated, the findings based on the use of proapoA-I should be treated with caution. Also, the purification procedure for recombinant apoA-I does not involve denaturing agents and thus is different from the established method for purifying plasma apoA-I, which usually involves denaturation-renaturation of apoA-I. Therefore, if differences between recombinant proapoA-I and plasma mature apoA-I are found they may be related to the expression systems and purification procedures rather than to the presence of propeptide. This report presents the first systematic comparison of the properties of the pro-form of apoA-I with the mature recombinant and human plasma apoA-I.

MATERIALS AND METHODS

Expression of recombinant apoA-I

The pro-form and mature form of human apoA-I were expressed in the baculovirus-insect cell expression system as previously described (15, 21). For large-scale production, cells were grown in suspension in a 1-liter spinner flask (working volume, 250 ml) until the cell-doubling time reached 24 h, and then were diluted to a final density of 10^6 cells/ml and infected at a multiplicity of infection (MOI) of 1. All infected cultures were grown in the presence of leupeptin (1 μg/ml) and pepstatin (1 μg/ml) (ICN, Seven Hills, New South Wales, Australia), added daily to prevent degradation of secreted apoA-I (21). Aliquots of culture were removed daily, cells were pelleted by centrifugation, and the apoA-I content in the cells and medium was analyzed by a competitive enzyme-linked immunosorbent assay (ELISA) system using rabbit anti-human apoA-I polyclonal antibody and purified human apoA-I as standard, according to a previously reported procedure (22). ApoA-I was purified from the medium by chromatography on a phenyl-Sepharose column as previously described (21) and delipidated by precipitation with ethanol–diethyl ether 3:1 (v/v).

Human plasma apoA-I was isolated and purified as described previously (23).

Circular dichroism

Measurements were made with an Aviv (Lakewood, NJ) model 62DS spectrometer at 27°C in a 0.5-mm path length cuvette, at protein concentrations of 0.4–0.5 mg/ml in 20 mM phosphate buffer, pH 7.6. Data were collected from 185 to 250 nm at 0.5-nm intervals. Circular dichroic (CD) spectra were fitted between 190 and 240 nm and the percentage of α helices was calculated as described by Yang, Wu, and Martinez (24).

Preparation of reconstituted high density lipoprotein

The reconstituted high density lipoprotein (rHDL) was prepared by the sodium cholate dialysis method according to Jonas et al. (25, 26), using palmitoyloleoyl phosphatidylcholine (POPC) (Sigma, Castle Hill, New South Wales, Australia), apoA-I, and sodium cholate (Sigma) in a molar ratio of 80:1:80. After the removal of sodium cholate by dialysis, the rHDL preparations were examined by electrophoresis in 3–30% nondenaturing gradient polyacrylamide gels (Gradipore, North Ryde, New South Wales, Australia) run at 2,500 V·h. After staining with Coomassie blue, gels were scanned and analyzed by SigmaGel software (Jandel Scientific, San Rafael, CA). The size of the rHDL particles was calculated against high molecular weight calibration standards (Pharmacia Biotech, Boronia, Victoria, Australia) and their chemical composition was determined according to the Bradford protein assay (27) and enzymatic-fluorometric phospholipid assay (Boehringer Mannheim, Castle Hill, New South Wales, Australia; used according to the manufacturer instructions).

Crosslinking experiments

To crosslink oligomers of lipid-free apoA-I, proteins (final concentration, 1.5 mg/ml) were incubated for 30 min at room temperature with bis(sulfosuccinimidyl)suberate (BS^3) (Pierce, Rockford, IL); (final concentration, 0.5 mM). The reaction was stopped by addition of 50 mM Tris-HCl, pH 7.3, and incubated for a further 15 min at room temperature. Samples were analyzed by gradient (8–16%) sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

To determine the number of apoA-I molecules in rHDL particles, rHDL preparations (final concentration, 15 μM protein) were incubated for 30 min at room temperature with BS^3 (final concentration, 0.25 mM). The reaction was terminated and samples were analyzed as described above.

Interaction of apolipoproteins with phospholipid liposomes

Solubilization of dimyristoyl phosphatidylcholine (DMPC) by apoA-I was studied as described by Ji and Jonas (28). Briefly, dry DMPC (Sigma) was sonicated in Tris buffer, pH 8.0, to form multilamellar liposomes. Apolipoproteins (final concentration, 0.2 mg/ml) were preincubated for 10 min at 24°C and the reaction initiated by adding DMPC liposomes (final DMPC concentration, 0.5 mg/ml). The reduction of absorption at 325 nm (light scattering) was monitored for 2 h at 2-min intervals at 24°C to assess formation of apoA-I/DMPC complexes.

LCAT activation

LCAT was isolated from human plasma by the method of Chen and Albers (29). The substrate particles were prepared by adding apolipoproteins to a solution containing egg phosphatidylcholine, cholesterol (Sigma), and [4-^{14}C]cholesterol (Amersham, Castle Hill, New South Wales, Australia; specific radioactivity, 2 GBq/mmol) in 12 mM sodium cholate in Tris buffer (10 mM Tris, 140 mM NaCl, 1 mM EDTA, pH 7.4). Sodium cholate was removed by dialysis. The final ratio of phosphatidylcholine to cholesterol to apoA-I was 100:10:1 (mol/mol/mol). For LCAT analysis the substrate was diluted in Tris buffer, pH 7.4, to give a final apoA-I concentration ranging from 0.1 to 2 μM. Each reaction mixture contained 63 μl of 2% bovine serum albumin (Sigma; essentially fatty acid free), 12.5 μl of 100 mM 2-mercaptoethanol, and 10 μl of LCAT solution (final volume, 200 μl). After incubation for 30 min at 37°C the reaction was stopped by adding 1 ml of ethanol. Lipids were extracted, and the cholesterol and cholesteryl esters were separated by thin-layer chromatography (TLC) (30); the radioactivity associated with each was quantitated by liquid scintillation counting. The extent of cholesterol esterification was kept below 10% to maintain first-order kinetics. The apparent V_{max} and K_m values were determined from Hanes-Woolf plots of substrate cholesterol concentration (μM) divided by the cholesteryl ester formation rate (nanomoles of cholesteryl ester per hour per milliliter of LCAT) versus substrate cholesterol concentration (μM).

Efflux experiments

Human skin fibroblasts were grown in a CO_2 incubator (5% CO_2, 95% air) in 75-cm^2 flasks or 12-well cell culture clusters (Falcon, Becton Dickinson, Franklin Lakes, NJ). Cultures were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 1% nonessential amino acids, 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), and sodium bicarbonate (3.7 mg/ml) (all reagents from ICN, Costa Mesa, CA).

To label cellular cholesterol and phospholipid, cells were incubated in serum-containing medium with [1α,2α(*n*)-^{3}H]cholesterol (specific radioactivity 1.81 TBq/mmol, final radioactivity 0.2 MBq/ml; Amersham) and [*methyl*-^{14}C]choline (specific activity 2.1 GBq/mmol; final activity 0.4 MBq/ml; Amersham) for 48 h in a CO_2 incubator. After labeling, cells were washed six times with Hanks' balanced salt solution and further incubated for 2 h at 37°C with serum-free medium containing either lipid-free apoA-I at a final concentration of 1 μM, or rHDL at a final POPC concentration of 80 μM. Preliminary experiments showed that the cholesterol efflux was apoA-I concentration dependent within a linear range of 0.1–1 μM of apoA-I. The medium was then collected and centrifuged for 15 min at 4°C at 30,000 *g* to remove cell debris, and the supernatant was used for further analysis. Cells were harvested with a cell scraper and dispensed in 0.5 ml of distilled water, and aliquots were taken for either phospholipid or cholesterol determinations or dissolved overnight by incubation in 0.5 M NaOH and used for the protein assay. Lipids were extracted from aliquots of medium and cells, and cholesterol and phospholipid were isolated by TLC as described previously (30).

Interaction of apoA-I with HDL subfractions

Different forms of apoA-I were labeled with ^{125}I, using IodoGen (Pierce) according to the manufacturer instructions. Final specific activities were similar for each apoA-I, ranging between 1×10^3 and 1.5×10^3 dpm/ng protein; labeled proteins were used in the experiments immediately after iodination. Plasma was obtained by centrifuging heparinized blood from healthy volunteers at 4°C (1,000 *g*, 15 min) and frozen at −80°C. It was shown previously that freezing and thawing of plasma does not affect the distribution of apoA-I-containing lipoprotein subclasses or interconversion of HDL subfractions (31).

^{125}I-labeled apoA-I was added to the plasma while on ice, warmed to 37°C for 1 min, and then added to a monolayer of human skin fibroblasts in 24-well cell culture clusters (Falcon). Cells were incubated at 37°C for either 5 min or 1 h, after which plasma was removed and snap-frozen.

HDL subfractions were separated by a modification of nondenaturing two-dimensional electrophoresis described by Miccoli et al. (32). In brief, lipoproteins from 20 μl of plasma were mixed with Sudan black stain and separated by electrophoresis on a 0.75% agarose gel run in 50 mM merbital buffer (Serva, Heidelberg, Germany), pH 8.7, for 20 h at 50 mA at 4°C. The gel strips containing α-lipoproteins and preβ-lipoproteins were dissected perpendicular to the direction of electrophoresis and laid on top of vertical slabs of 3–15% polyacrylamide gels (Gradipore). Strips were fixed in position with agarose and the second-dimension electrophoresis was performed in 25 mM Tris, 200 mM glycine buffer, pH 8.3, for 1.5–2 h at 300 V. Lipoproteins were then transferred onto nitrocellulose filters and Western blotting was performed with monoclonal anti-apoA-I antibody (AI-4.1) and goat anti-mouse second antibody (Bio-Rad, Regents Park, New South Wales, Australia). The areas corresponding to the individual HDL subfractions were excised and counted in a γ counter. The identity of HDL subfractions was based on the nomenclature suggested by Asztalos et al. (33).

Other methods

Phospholipid transfer protein (PLTP) binding to immobilized apoA-I was assessed by the ELISA method as described previously (34). Binding of ^{125}I-labeled apoA-I to human skin fibroblasts (final concentration of apoA-I added to cells, 2.5 μg/ml) was assessed as described previously (35). ApoA-I concentrations and the protein content of cells were determined by the Bradford protein assay (27). Cell cholesterol and phospholipid content was determined by enzymatic fluorometric assays (Boehringer Mannheim) according to the manufacturer instructions.

RESULTS

Synthesis and secretion of apoA-I

To compare the synthesis and secretion of recombinant pro- and mature forms of apoA-I expressed in insect cells, the same batch of Sf21 cells was infected with the recombinant baculovirus at an MOI of 1 and the cells were cultivated in suspension, in parallel, as described in Materials and Methods. While the shape of time-dependent production curves was similar for the two proteins, the total amount of proapoA-I (cells plus medium) synthesized at maximum expression levels after 5 days of incubation was 2.4 times higher than that of mature apoA-I (**Fig. 1A**). Moreover, while about half of the proapoA-I was secreted into the medium, on average, 80% of mature apoA-I was retained in the cells (Fig. 1A). The ratio of the amount of apoA-I secreted into the medium versus that retained in the cells was about 0.3 for mature apoA-I and about 1 for proapoA-I (Fig. 1B); the increase in the ratio in the last 2 days of the incubation is most likely due to release of apoA-I from cells undergoing lysis. Thus, when expressed in the baculovirus/insect cells expression system, the deletion of the propeptide from apoA-I results in a reduced efficiency of expression and retarded secretion of the newly synthesized apoA-I.

Secondary structure

The α-helix content of lipid-free apoA-I was approximately 50%, the rest of the molecule comprising random conformation and β-turn regions. No β-sheet regions were detected in apoA-I. This conformation of apoA-I is consistent with our previous finding (36), as well as with the data of Ji and Jonas (28). No differences in secondary structure were found among plasma apoA-I and the mature and pro-forms of recombinant apoA-I.

Self-association properties

To study the self-association properties of pro- and mature forms of apoA-I, we performed crosslinking experiments using the crosslinking reagent BS^3. Plasma apoA-I, the mature form and the pro-form of recombinant apoA-I all formed dimers, trimers, tetramers, and pentamers to similar degrees (in addition to monomers), when they were present in solution at a concentration of 1.5 mg/ml (not shown).

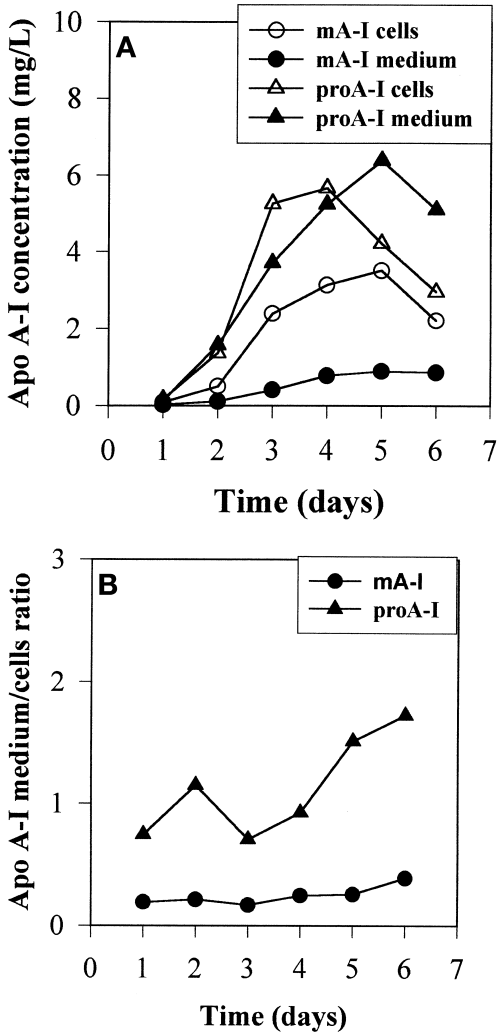

Fig. 1. Synthesis and secretion of apoA-I in a baculovirus/insect cell expression system. (A) Time course of the appearance of the pro-form (proA-I; triangles) and mature form (mA-I; circles) of apoA-I in the cells (open symbols) and the medium (closed symbols). (B) Ratio of the amount of protein found in the medium versus that retained in the cells for proapoA-I (triangles) and mature apoA-I (circles).

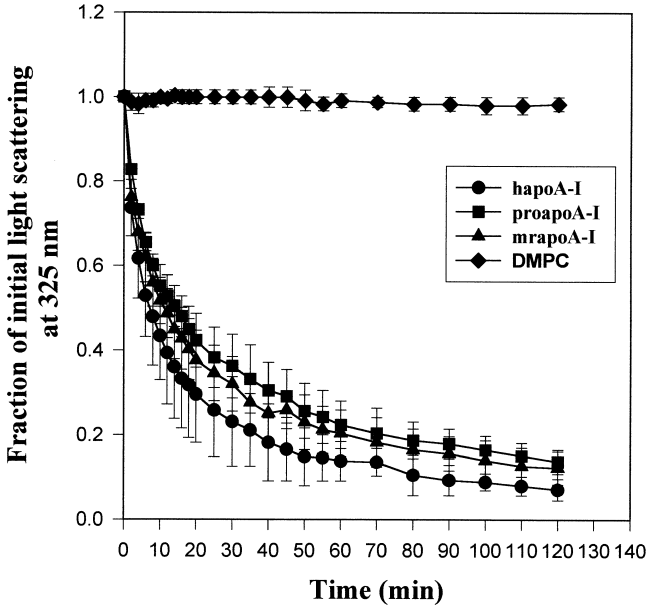

Fig. 2. Time course of the interaction of apoA-I with DMPC liposomes. Multilamellar DMPC liposomes were added to the apoA-I preparations at a final phospholipid-to-protein ratio of 2.5:1 (w/w) and the formation of particles was assessed by monitoring reduction in absorbance at 325 nm, measured at 24°C. The data represent means ± standard errors of three independent experiments. hapoA-I, human plasma apoA-I; proapoA-I, proform apoA-I; mrapoA-I, mature recombinant apoA-I; DMPC, dimyristoyl phosphatidylcholine.

Interaction with DMPC

To determine the effect of the presence of the propeptide on the ability of apoA-I to bind and reorganize phospholipid, we studied the ability of the three forms of apoA-I, human plasma apoA-I and the mature form and proform of recombinant apoA-I, to clear multilamellar liposomes. All forms effectively cleared the liposomes when combined in a 2.5:1 w/w ratio (DMPC to apoA-I) (**Fig. 2**), the $t_{1/2}$ values being 7, 8, and 11 min for human plasma apoA-I, the mature form and pro-form of recombinant apoA-I, respectively; there was no statistically significant difference between the three curves.

In a similar assay the delipidated recombinant apoA-Is were compared with the nondelipidated forms (see Materials and Methods). Nondelipidated proapoA-I was 50% less effective in forming apoA-I/DMPC complexes than delipidated protein, while nondelipidated mature recombinant apoA-I did not bind DMPC at all (not shown). This suggests that recombinant apoA-I may acquire cellular lipids during or shortly after secretion and requires delipidation prior to further experiments.

LCAT activation

The ability of different forms of apoA-I to activate LCAT was tested under conditions chosen so that the initial reaction velocities could be measured and analyzed by standard Michaelis-Menten kinetics. Dose-dependence curves of cholesterol esterification are illustrated in **Fig. 3**. When the kinetic parameters were analyzed, the only statistically significant difference observed was a marginally lower K_m for the mature recombinant apoA-I (1.1 ± 0.2 μM vs. 1.5 ± 0.6 μM, $P < 0.03$). However, the overall ability to activate LCAT was similar for all three apoA-I species: V_{max}/K_m (nmol/h/ml/μM) was 20.4, 24.1, and 21.4 for plasma apoA-I, mature recombinant apoA-I, and proapoA-I, respectively. This demonstrates that the propeptide has little effect on activation of LCAT by apoA-I.

Properties of reconstituted HDL particles

To determine the properties of rHDL particles formed with the different forms of apoA-I, reconstitution was carried out with POPC at an initial ratio of POPC to apoA-I of 80:1 (mol/mol). The particles obtained with human plasma apoA-I, and the mature forms and pro-forms of recombinant apoA-I were of similar size (**Fig. 4**), the major peak of each product ranging from 10.4 to 10.6 nm, and composition (**Table 1**), and all contained two molecules

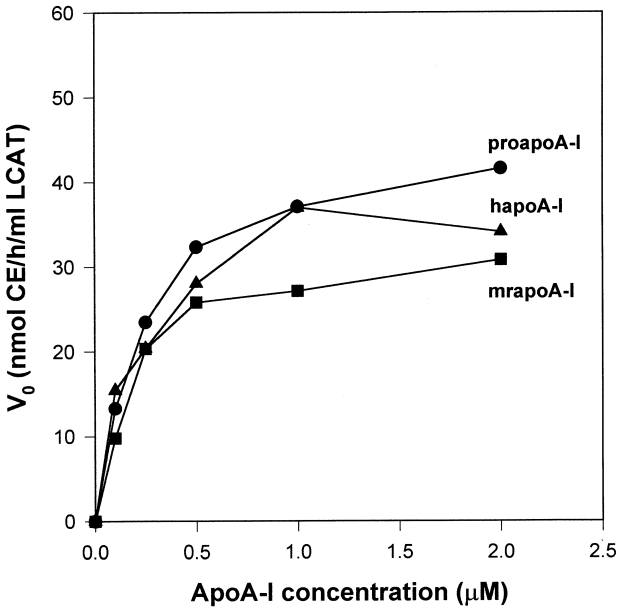

Fig. 3. LCAT activation using apoA-I. Small unilamellar vesicles containing [^{14}C]cholesterol and human plasma apoA-I (hapoA-I; triangles), recombinant mature apoA-I (mrapoA-I; squares), or proapoA-I (proapoA-I; circles) [final ratio of cholesterol to apoA-I, 10:1 (mol/mol)] were incubated with LCAT for 30 min at 37°C and the amount of cholesteryl esters formed was calculated as described in Materials and Methods.

of apoA-I per particle. Particles formed with recombinant proapoA-I and particularly with mature recombinant apoA-I were somewhat more heterogeneous compared with human plasma apoA-I (Fig. 4). This may be attributed to the presence of a small proportion of partially degraded apoA-I (most likely truncated at the carboxyl-

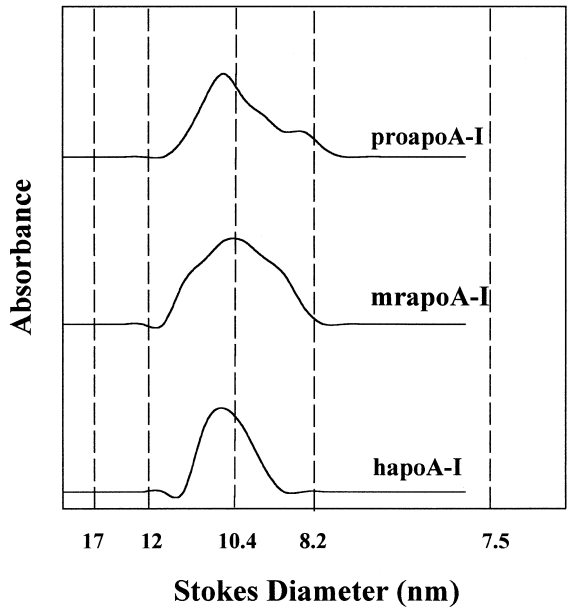

Fig. 4. Characterization of rHDL particles by native gradient gel electrophoresis. proapoA-I, proform apoA-I; mrapoA-I, mature recombinant apoA-I; hapoA-I, human plasma apoA-I.

TABLE 1. Properties of rHDL particles

	Initial POPC/ApoA-I Ratio	Final POPC/ApoA-I Ratio	Diameter	Number of ApoA-I per Particle
	mol/mol	*mol/mol*	*nm*	
Plasma apoA-I	80:1	99:1	10.6	2
Mature apoA-I	80:1	94:1	10.4	2
Pro-form apoA-I	80:1	81:1	10.6	2

terminal end) that accompanied most preparations of recombinant protein (21). Overall, we did not observe a significant difference among the ability of mature plasma apoA-I, and the mature and pro-forms of recombinant apoA-I to form rHDL particles.

Cholesterol and phospholipid efflux

To study cholesterol and phospholipid efflux to different species of apoA-I and rHDL particles formed with these proteins, human skin fibroblasts were incubated with [^{3}H]cholesterol and [^{14}C]choline to label cholesterol and phospholipid (see Materials and Methods). Labeled and washed cells were incubated for 2 h with lipid-free human plasma apoA-I or with the mature form and pro-form of recombinant apoA-I (final concentration, 1 μM) or with rHDL particles containing the same proteins (final concentration of POPC, 80 μM). Lipids released to the medium were separated by TLC and the radioactivity in cholesterol and phospholipid fractions was determined. There was no statistically significant difference between the different forms of apoA-I, either in the lipid-free form or as a part of rHDL, in promoting efflux of either cholesterol (**Fig. 5A**) or phospholipid (Fig. 5B).

Binding to cells

Binding of apoA-I was assessed by incubating different forms of apoA-I labeled with ^{125}I (final protein concentration, 2.5 μg/ml) with human skin fibroblasts for 2 h at 4°C. Specific binding of ^{125}I-labeled apoA-I to the cells (defined as the difference between total and nonspecific binding, determined in the presence of a 50-fold excess of unlabeled human plasma apoA-I) was 2.8 ± 0.5, 2.8 ± 0.3, and 3.0 ± 0.04 ng/mg cell protein/2 h for human plasma apoA-I, recombinant mature apoA-I, and recombinant proapoA-I, respectively. There was no statistically significant difference between the abilities of the proteins to bind to the cells.

Interaction with HDL subfractions

To investigate the ability of native apoA-I and mature and pro-forms of recombinant apoA-I to bind to various subfractions of human plasma HDL and participate in their interconversion, apoA-I preparations were labeled with ^{125}I and incubated for 5 and 60 min with human plasma at 37°C. Because conversion of preβ-HDL subfractions at this temperature occurs rapidly [within a few minutes (37)], and results in depletion of the plasma preβ-HDL fraction (38), incubation was carried out in the presence of a monolayer of human skin fibro-

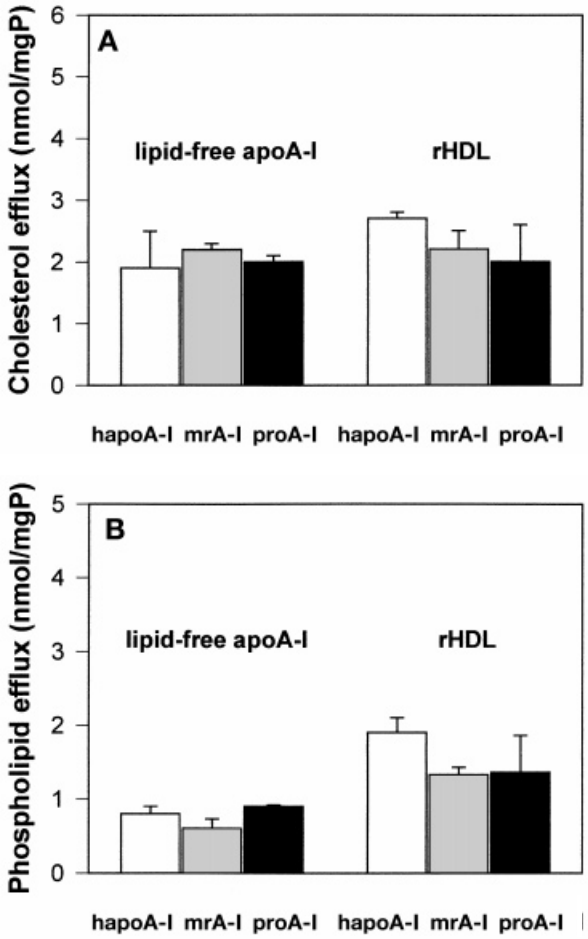

Fig. 5. Cholesterol (A) and phospholipid (B) efflux to apoA-I and rHDL. Human skin fibroblasts were labeled with [^{3}H]cholesterol and [^{14}C]cholate as described in Materials and Methods. Cells were then incubated with the lipid-free apoA-I (final concentration, 1 μM) or rHDL (final concentration of POPC, 80 μM) for 2 h at 37°C and the amount of [^{3}H]cholesterol and [^{14}C]phospholipid released to the medium was analyzed by TLC after separation of lipids. rHDL, reconstituted HDL; hapoA-I, human plasma apoA-I; mrA-I, mature recombinant apoA-I; proA-I, proform apoA-I.

blasts, which prevents preβ-HDL depletion (38). HDL subfractions were then separated by a new modification of nondenaturing two-dimensional electrophoresis, based on the system described by Miccoli et al. (32). This modification allows separation of up to nine samples in one run, which greatly reduces interexperimental variations.

The initial distribution of apoA-I among HDL subfractions in plasma is shown in **Table 2** and remained unchanged during the experiment. After a 5-min incubation with native plasma ^{125}I-labeled apoA-I, approximately 10% of the label was incorporated into HDL. However, the distribution of ^{125}I-labeled apoA-I varied considerably from unlabeled apoA-I, being significantly higher in preβ$_1$-HDL and α$_3$-HDL particles and significantly lower in preβ$_2$-HDL and α$_2$-HDL subfractions (Table 2). These data suggest that lipid-free ^{125}I-labeled apoA-I when added to plasma does not distribute proportionally to pre-existing HDL particles, but rather participates in the cyclical interconversion of HDL subfractions, with preβ$_1$-HDL and α$_3$-HDL being the points of entry.

The distribution of the mature form of recombinant ^{125}I-labeled apoA-I among HDL subfractions was similar to that of plasma ^{125}I-labeled apoA-I (**Fig. 6A**). However, the proportion of ^{125}I-labeled proapoA-I bound to the preβ-HDL subfractions was significantly higher than that of mature apoA-I. The proportion of ^{125}I-labeled proapoA-I found in preβ$_1$-HDL was 2-fold higher, and in preβ$_2$-HDL was 1.6-fold higher, than that of the mature forms of apoA-I (Fig. 6A). Correspondingly, the proportion of ^{125}I-labeled proapoA-I in all α-HDL subfractions was significantly lower when compared with mature ^{125}I-labeled apoA-I, with an approximately 1.5-fold decrease in the amount of labeled protein in α$_1$-HDL and α$_2$-HDL, and an approximately 17% decrease in the α$_3$-HDL subfraction (Fig. 6A).

After 1 h of incubation, 17% of added ^{125}I-labeled apoA-I was recovered in the HDL subfractions and the specific activity of plasma ^{125}I-labeled apoA-I in different HDL subfractions had almost equilibrated, with the exception of the higher specific activity of the preβ$_1$-HDL fraction (Table 2). The difference in the distribution of the mature and pro-forms of ^{125}I-labeled apoA-I among HDL subfractions was also diminished (Fig. 6B). However, there was still significantly more ^{125}I-labeled proapoA-I in preβ$_1$-HDL compared with the mature apoA-I preparations (Fig. 6B). It is possible that equilibration of ^{125}I-labeled apoA-I among HDL subfraction is related to the cleavage of the propeptide from ^{125}I-labeled proapoA-I, although only a small proportion of proapoA-I is processed during a 1-h incubation (L. E. Pyle, D. Sviridov, and N. H. Fidge, unpublished observation).

Binding of PLTP to immobilized apoA-I

One of the explanations for the differences between pro- and mature apoA-I in the conversion of preβ-HDL is that the propeptide may impair binding of phospholipid

TABLE 2. Initial distribution of human plasma apoA-I and binding of human plasma ^{125}I-labeled apoA-I to HDL subfractions

	Preβ$_1$-HDL	Preβ$_2$-HDL	α$_1$-HDL	α$_2$-HDL	α$_3$-HDL
			% of total		
Initial distribution of apoA-I	7.3	6.9	13.1	59.9	12.8
^{125}I-labeled apoA-I distribution					
5-min incubation	13.5 ± 0.7	4.3 ± 0.05	15.2 ± 1.6	34.9 ± 5.2	32.1 ± 3.8
1-h incubation	12.6 ± 0.6	6.9 ± 0.6	19.8 ± 2.8	46.3 ± 5.4	14.4 ± 0.5

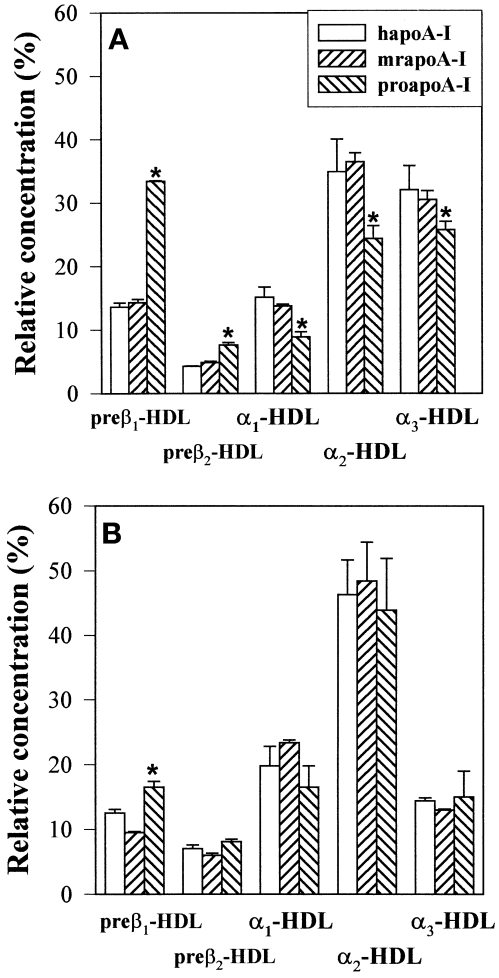

Fig. 6. Interaction of apoA-I with HDL subfractions. Different forms of ^{125}I-labeled apoA-I (final concentration, 0.1 μM) were incubated with human plasma for 5 min (A) or 1 h (B) in the presence of human skin fibroblasts. Plasma HDL subfractions were then separated by nondenaturing two-dimensional electrophoresis as described in Materials and Methods; the areas corresponding to the individual HDL subfractions were excised and counted in a γ counter. Relative distribution of ^{125}I-labeled apoA-I among HDL subfraction is shown. $*P < 0.01$. HDL, high density lipoprotein; hapoA-I, human plasma apoA-I; mrapoA-I, mature recombinant apoA-I; proapoA-I, proform apoA-I.

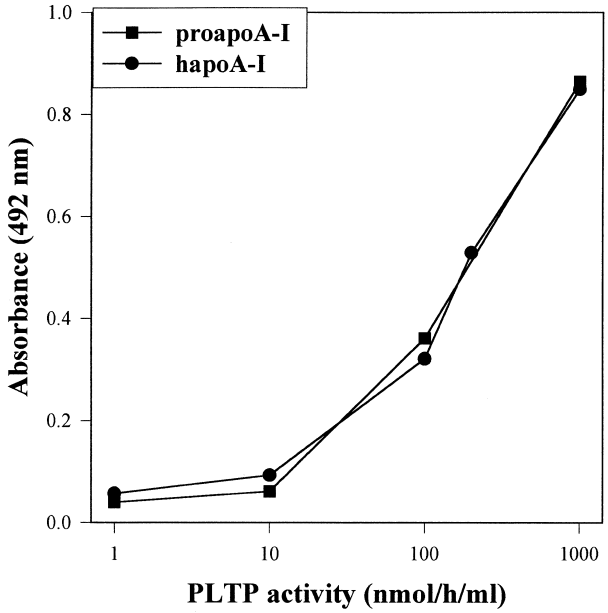

Fig. 7. Binding of PLTP to apoA-I. ELISA plates were coated with proform apoA-I (proapoA-I) or human plasma apoA-I (hapoA-I) at 5 μg/ml, and incubated with serial dilutions of PLTP. The binding of PLTP was monitored with peroxidase-labeled monoclonal anti-PLTP (G11).

transfer protein to apoA-I, a process that involves the amino-terminal end of apoA-I (34). We therefore tested the binding of PLTP to the immobilized pro- and mature apoA-I by ELISA (34). No statistically significant difference was found between proapoA-I and human plasma apoA-I in their ability to bind PLTP (**Fig. 7**).

DISCUSSION

ApoA-I is synthesized as a preproprotein, with the pre- and propeptides cleaved intracellularly and extracellularly, respectively (12). There are several possible structural and functional roles for the apoA-I propeptide and several explanations for the significance of its cleavage. First, the propeptide may be required to form a functional cleavage site to enable intracellular cleavage of the prepeptide. However, we have eliminated this mechanism as a prerequisite for correct cleavage of the prepeptide, which was shown not to be affected by deletion of the propeptide (15).

Second, the propeptide may play a role in the expression and secretion of apoA-I. In a previous article (15) we demonstrated that expression and secretion of mature apoA-I (ΔproapoA-I) in a baculovirus/insect cell expression system was less effective than would be expected on the basis of the results obtained with full-length apoA-I (21). However, because of variations in the efficiency of expression and secretion of proteins in this expression system, depending on the batch, passage number of cells and virus, MOI, and formulation of the medium, a comparison of the efficiency of expression of full-length apoA-I and ΔproapoA-I required experiments to be performed in parallel to eliminate as many variables as possible. Although quantitatively different, the results of such experiments confirmed our previous finding: the deletion of propeptide causes a reduction in the efficiency of apoA-I expression and secretion. This finding outlines one possible function of the propeptide: facilitating the effective production and secretion of apoA-I, for example, by promoting an optimal structure for interaction with cell components, such as chaperone or carrier proteins that may be involved in the secretion, or by being an intramolecular chaperone (39). This is consistent with a report by Folz and Gordon (40), who found that deletion of the propeptide reduces the efficiency of cotranslational translocation/processing of apoA-I in reticulocyte and wheat germ lysates without affecting the fidelity

of signal peptide cleavage. It should be noted, however, that the process of secretion of proteins from insect cells infected with baculovirus might differ from that in differentiated mammalian cells. Cell lysis and variations in the recombination of apoA-I cDNA into the baculovirus genome may affect synthesis and secretion of apoA-I in insect cells; thus, despite the observed high reproducibility in the experiments, quantitative data should be interpreted with caution.

The third possibility is that the propeptide affects the structural and/or functional properties of apoA-I. Several of these properties have been studied in vitro, mainly to validate the use of recombinant proapoA-I to study the functions of this protein, and little difference was found between human plasma mature apoA-I and recombinant proapoA-I (17, 18, 41, 42). However, Batal et al. (14) have identified a familial HDL deficiency that is characterized by hypercatabolism of the mature form, but not the pro-form, of apoA-I. This is the first evidence that the pro- and mature forms can be distinguished during HDL metabolism in vivo; moreover, this phenomenon may be associated with an increased risk of coronary heart disease (20).

In this article we present data derived from a systematic comparison of the structural and functional properties of recombinant proapoA-I with two forms of mature apoA-I, recombinant or native apoA-I. Comparing recombinant proapoA-I with both recombinant and human plasma mature apoA-I accounted for the differences, which may have been caused not by the presence of propeptide but rather by differences in preparation of human plasma and recombinant proteins and/or by the presence of small amounts of degraded protein or other impurities in the preparations of recombinant protein. As reported by our (36) and other laboratories, (17, 18, 41, 42), we found no difference between the three forms of apoA-I in their physicochemical properties, self-association, ability to bind phospholipid liposomes, LCAT activation, or in the properties of reconstituted HDL particles formed with these proteins. We also found that the apoA-I forms were similar regarding binding of PLTP, binding to cells, and in their ability, either in the lipid-free form or when reconstituted into rHDL, to promote efflux of cholesterol and phospholipid from human fibroblasts. The latter finding is inconsistent with a previous report from our laboratory (35), which described that lipid-free proapoA-I (but not rHDL) had only half the activity of human plasma apoA-I in promoting efflux of cholesterol and phospholipid from HepG2 cells. One possible reason for this discrepancy might be that the recombinant proapoA-I used in the earlier experiments was expressed in a different expression system (pGEX/*Escherichia coli*) and may have contained bacterial lipids that were not completely removed by the delipidation procedure used, or that apoA-I was incorrectly folded in bacteria.

The only significant difference between the mature and pro-form of apoA-I found in the present study was in their interaction with human HDL. Compared with the mature forms of apoA-I, up to twice as much proapoA-I was found in preβ-HDL subfractions with a corresponding decline in the proportion of proapoA-I in α-HDL. The major pathway for the formation of preβ-HDL species, and particularly preβ_1-HDL, involves a transfer of lipid from the cell plasma membrane (43, 44) and/or other lipoproteins (45, 46) to lipid-free apoA-I; within minutes these particles undergo further conversion with the formation of α-HDL (1). Differences in the specific activities of ^{125}I-labeled apoA-I in HDL subfractions after 5 min of incubation, but almost complete equilibration of ^{125}I-labeled apoA-I after 1 h of incubation, suggest that lipid-free apoA-I enters this pathway rather than binds to pre-existing HDL particles, and particularly in the case of proapoA-I further interconversion is delayed. The mechanism responsible for this delay is unclear. It appears not to be related to the ability of apoA-I to bind lipids, bind to cells, promote lipid efflux, activate LCAT, bind PLTP, or form complexes with phospholipid, as indicated above. Batal et al. (14) demonstrated that in patients with familial HDL deficiency an increased proportion of plasma proapoA-I coincides with an increased proportion of preβ-HDL, as well as with a decrease in the average α-HDL particle size. This was apparently due to faster catabolism of mature apoA-I and slower catabolism of the newly secreted proapoA-I in preβ-HDL particles. However, the differences in the catabolic rates may be a result of delayed conversion of proapoA-I containing preβ-HDL into α-HDL particles. A similar phenomenon, that is, an almost complete block in the conversion of preβ_1-HDL into α-HDL, both in vivo and in vitro, has been observed for apoA-I_{pisa} [apoA-I(L141R)] (32), although this mutation affects the LCAT activation site of apoA-I. These findings demonstrate that apoA-I structural differences may severely affect interconversion between HDL subfractions, although the mechanism of these effects can be different. Patients with Tangier disease are also unable to convert preβ-HDL into α-HDL, which is believed to be related to the absence of a lipid transfer factor in the cells and plasma of the Tangier disease patients (47, 48). The cellular factor has been identified as the ATP-binding cassette transporter 1 (49–51) and it seems to play an important role in the phospholipid and cholesterol efflux from cells (52, 53), which in turn is essential for the effective interconversion of preβ-HDL (1). The plasma factor of Tangier disease is still unidentified. It is possible to speculate that the presence of the propeptide may interfere with the interaction of apoA-I with these factors. The decrease in the average size of α-HDL particles observed by Batal et al. (14) is also consistent with our finding that the proportion of apoA-I found in α_3-HDL, the smallest α-HDL particles, was not as greatly reduced as that in α_1-HDL and α_2-HDL, the two larger α-HDL subfractions. Combined, these observations suggest that interconversion between HDL subfractions is affected by the presence of the apoA-I propeptide, a segment that requires removal to allow effective HDL metabolism.

The possible role of the propeptide in apoA-I metabolism is schematically illustrated in **Fig. 8**. ApoA-I is synthesized and secreted by the liver and intestine as a pro-form,

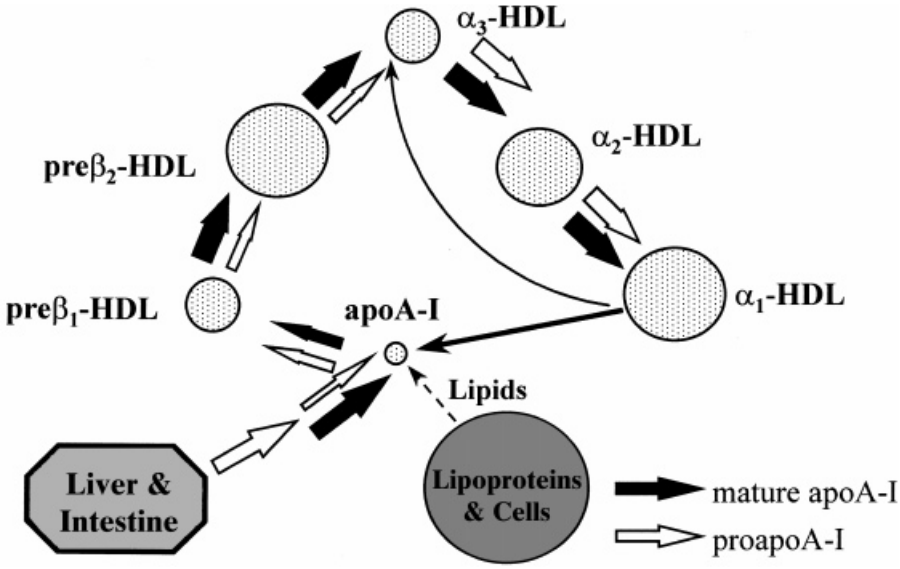

Fig. 8. Schematic representation of the apoA-I interconversion cycle and the role of propeptide. ApoA-I is synthesized and secreted by the liver and intestine as a pro-form (proapoA-I), and the propeptide facilitates effective synthesis and secretion of apoA-I. Newly synthesized apoA-I undergoes processing with cleavage of propeptide and mixes with apoA-I derived from remodeling of α_1-HDL. Lipid-free or lipid-poor apoA-I acquires lipids from the plasma membrane of the cells and other lipoproteins (dashed arrow). Immediately after acquiring lipids apoA-I forms preβ_1-HDL particles, which rapidly transforms into preβ_2-HDL and then into α_3-HDL particles. The presence of propeptide slows this conversion and leads to the accumulation of preβ-HDL particles. Further conversion and remodeling of α-HDL particles apparently is not affected by the presence of propeptide. α_1-HDL undergoes remodeling, releasing α_3-HDL and lipid-free apoA-I, which enters the cycle. Greater width of arrows indicates predicted faster rate of conversion.

and the propeptide facilitates effective synthesis and secretion of apoA-I. Newly synthesized apoA-I undergoes processing with cleavage of the propeptide and combines with apoA-I derived from the remodeling of α_1-HDL (54, 55). Lipid-free or lipid-poor apoA-I acquire lipids from the plasma membrane of the cells (43, 44) and other lipoproteins, a process that requires a number of cofactors, including nonesterified fatty acids (45). It is unclear what proportion of proapoA-I undergoes processing before acquiring lipids, but it is likely that most proapoA-I is cleaved before or shortly after entering the cycle of interconversion. Immediately after acquiring lipids apoA-I forms preβ_1-HDL particles, which rapidly transform into preβ_2-HDL and then into α_3-HDL particles. The presence of propeptide slows this conversion and leads to the accumulation of preβ-HDL particles. Further conversion and remodeling of α-HDL particles apparently is not affected by the presence of propeptide. Alternatively, prolonged exposure of proapoA-I in the preβ-HDL particles facilitates cleavage of propeptide and only mature apoA-I participates in further conversions.

The data presented in this report suggest that while the propeptide of apoA-I is required to facilitate effective production and secretion of newly synthesized apoA-I, cleavage of the propeptide may be necessary to achieve effective conversion of preβ-HDL to α-HDL.

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