The Signal Peptide Anchors Apolipoprotein M in Plasma Lipoproteins and Prevents Rapid Clearance of Apolipoprotein M from Plasma*

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Lipoproteins consist of lipids solubilized by apolipoproteins. The lipid-binding structural motifs of apolipoproteins include amphipathic α-helices and β-sheets. Plasma apolipoprotein (apo) M lacks an external amphipathic motif but, nevertheless, is exclusively associated with lipoproteins (mainly high density lipoprotein). Uniquely, however, apoM is secreted to plasma without cleavage of its hydrophobic NH₂-terminal signal peptide. To test whether the signal peptide serves as a lipoprotein anchor for apoM in plasma, we generated mice expressing a mutated apoMQ²²A cDNA in the liver (apoMQ²²A-Tg mice (transgenic mice)) and compared them with mice expressing wild-type human apoM (apoM-Tg mice). The substitution of the amino acid glutamine 22 with alanine in apoMQ²²A results in secretion of human apoM without a signal peptide. The human apoM mRNA level in liver and the amount of human apoM protein secretion from hepatocytes were similar in apoM-Tg and apoMQ²²A-Tg mice. Nevertheless, human apoM was not detectable in plasma of apoMQ²²A-Tg mice, whereas it was easily measured in the apoM-Tg mice. To examine the plasma metabolism, recombinant apoM lacking the signal peptide was produced in *Escherichia coli* and injected into wild-type mice. The apoM without signal peptide did not associate with lipoproteins and was rapidly cleared in the kidney. Accordingly, ligation of the kidney arteries in apoMQ²²A-Tg mice resulted in rapid accumulation of human apoM in plasma. The data suggest that hydrophobic signal peptide sequences, if preserved upon secretion, can anchor plasma proteins in lipoproteins. In the case of apoM, this mechanism prevents rapid loss by filtration in the kidney.

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§1 The abbreviations used are: HDL, high density lipoprotein; HRP, haptoglobin-related protein; PON-1, paraoxonase 1; TC, tyramine cellobiose; Tg, transgenic; apo, apolipoprotein; ELISA, enzyme-linked immunosorbent assay.

Lipoproteins consist of lipids (mainly cholesterol, phospholipids, and triglycerides) solubilized by apolipoproteins. The apolipoproteins play essential roles in controlling plasma and tissue lipid homeostasis by interacting with cellular lipoprotein receptors (the low density lipoprotein receptor, the scavenger receptor class B type-I, the ATP-binding cassette transporter AI, etc.) and enzymes (e.g. lecithin-cholesterol acyltransferase, hepatic lipase, and lipoprotein lipase) and lipid transfer protein (cholesteryl ester transfer protein and phospholipid transfer protein). However, several apolipoproteins have roles beyond lipid metabolism. Indeed, a recent shotgun proteomic approach revealed that HDL³ on average contains >40 proteins, of which many affect complement activation or protease inhibition (1). Also, several well known HDL apolipoproteins have roles in inflammation and host defense against microbial infections (1). For instance, apol. is involved in fighting *Trypanosoma* infections (2), and apo (clusterin) has even been proposed to play a significant role in tumorigenesis (3). For apolipoproteins without any (known) effect on plasma lipid metabolism, it is conceivable that the host HDL particle mainly serves as a carrier that prevents rapid removal of its associated apolipoproteins (e.g. by filtration in the kidney) or perhaps aids in their assembly at sites of inflammation.

The structural motifs conferring the lipid-binding capacity of apolipoproteins has been most intensively studied for apoB in low density lipoprotein (4) and apoA-I in HDL (5, 6). Both amphipathic α-helices (7) and β-sheets (4) bind to the lipids. In the case of apoA-I and several other HDL apolipoproteins (e.g. apoE and the apoCs), the non-covalent binding of the amphipathic α-helices are rather weak, allowing exchange of the apolipoproteins between the triglyceride-rich very low density lipoprotein and chylomicron particles and HDL (8). Indeed, some apolipoproteins are so loosely bound to HDL (e.g. apo (9), apoA-IV (10), and lecithin-cholesterol acyltransferase (11)) that they are often stripped from the lipoprotein particles during preparative ultracentrifugation.

ApoM (188 amino acids) was discovered in 1999 by Xu and Dahlback (12). In plasma, apoM is exclusively found in associ-
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...with lipoproteins (mainly HDL). Recent studies of mice with genetically modified apoM expression showed that apoM affects plasma HDL metabolism and development of atherosclerosis (10, 13), but apoM also has antioxidant effects, and its physiological roles remain to be determined (10). Analysis of the tertiary structure of apoM by in silico homology modeling has revealed that apoM belongs to the lipocalin protein superfamily (14). Like in other lipocalins, the structure of apoM contains eight antiparallel β-sheets forming a small lipid-binding pocket. Recent experimental data confirmed the lipocalin structure of apoM and showed that it can bind retinoic acid and retinol (15). Human apoM can be glycosylated at amino acid Asn-135 surrounded by the glycosylation signal Asn-Glu-Thr (12). Hence, Western blotting of SDS-PAGE gels with reduced human plasma samples typically reveals both glycosylated apoM and a non-glycosylated apoM of ~25 and ~20 kDa (9, 12). The predicted structure of apoM lacks external amphipathic motifs that would explain the lipoprotein association of apoM. NH2-sequencing of plasma apoM has shown that it is secreted to the blood with its NH2-terminal signal peptide (12). This is unusual because intracellular cleavage of the NH2-terminal signal peptide by signal peptidases in the endoplasmic reticulum conventionally is seen as a prerequisite for cellular release of proteins to plasma. We are, however, aware of at least two other plasma proteins with preserved NH2-terminal signal peptides, i.e. paraoxonase-1 (PON-1) (16) and haptoglobin-related protein (HRP) (17). Like apoM, PON-1 and HRP are mainly found in a subfraction of HDL (although not the same HDL fraction as that with apoM (9)), and in vitro studies suggest that the signal peptide in PON-1 can mediate its association with lipoproteins (16). Based on these findings and the highly hydrophobic nature of the signal peptide, we hypothesized that the signal peptide serves as an anchor for apoM in plasma lipoprotein particles.

To test this idea and explore the impact of the signal peptide on the metabolism of plasma apoM, we generated transgenic mice that express a mutated human apoM22A cDNA in the liver. The cDNA encodes a mutated human apoM22A protein where glutamine in position 22 is replaced by alanine. The Gln-22→Ala substitution results in secretion of apoM without its hydrophobic signal peptide (15). The mice expressing apoM22A were compared with transgenic mice expressing wild-type human apoM.

MATERIALS AND METHODS

ApoM-Tg and apoM22A-Tg Mice—Transgenic (Tg) mice expressing a truncated human apoM protein without the signal peptide were generated. Site-directed mutagenesis resulting in substitution of amino acid Gln-22 with Ala in human apoM (apoM22A) was done previously (15). Secretion of apoM22A cDNA from transfected HEK293 cells was assessed as described (15) by Western blotting (9) and ELISA (18). A 774-bp fragment of the apoM22A-encoding cDNA was amplified with Pho DNA polymerase system (Stratagene, AH Diagnostic) and primers (pcDNA3-apoM22A-51:5’-gcgccgcacatgcccgccttttagttggtac-3’ and pcDNA3-apoM22A-31:5’-gcgccgcgcagtaaagtctcccata-3’) and cloned into a PCR-Blunt II-TOPO vector (Invitrogen A/S). The ApoM22A encoding cDNA was subsequently cloned into a pGEMAlbSV40 vector (obtained from Ragnar Matsson, Lund University) between a murine albumin promoter/enhancer sequence and an SV40 intron/poly(A) sequence. The correctness of apoM22A encoding cDNA was verified by DNA sequencing (primers will be given by request). The 4446-bp albumin-apoM22A-SV40 fragment was purified from the plasmid by restriction digestion with Nsi and Apal, agarose gel electrophoresis and the QIAEX II gel extraction kit (Qiagen-Nordic) and used for pronuclear micro-injections into fertilized mouse oocytes (C57/B1 X CBA) at the Transgenic Core Facility, University of Lund, Sweden. Human apoM22A transgenic founders were identified by real-time PCR amplification (LightCycler, Roche Diagnostic) of a 175-bp fragment of human APOM from tail DNA with primers h-apoM-51 and h-apoM-31 (10). The human apoM-transgenic mice expressing wild-type human apoM (apoM-Tg) were described recently elsewhere (as apoM-TgN mice (10)). Both apoM-Tg and apoM22A-Tg mice were backcrossed with C57BL/6 mice for at least five generations before the present studies.

The mice were housed at the Panum Institute (University of Copenhagen) and fed standard chow (Altromin 1314, Brogaarden). Blood samples were taken from the venous plexus in the orbital cavity into Na2EDTA- or heparin-containing tubes and kept on ice. Plasma was obtained by centrifugation at 3000 rpm for 10 min at 4 °C and stored at ~80 °C or ~20 °C. All procedures were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

mRNA Quantification—RNA was isolated from frozen tissue (~80 °C) with TRIzol (Invitrogen) and examined on an RNA Nano LabChip (Agilent Technologies). First-strand cDNA synthesis and quantitative real-time PCR analysis with a LightCycler was performed as described (19) with primers for human APOM (10), mouse APOM (20), and β-actin (19).

Quantification of Human apoM—Human wild-type apoM and recombinant human apoM22–188 were measured with a sandwich ELISA employing two monoclonal antibodies against human apoM (18). The primary antibodies against human apoM used for 12% SDS-PAGE Western blotting (9) were polyclonal rabbit anti-human apoM IgG (2.5 μg/ml), a monoclonal mouse anti-human apoM antibody (M58; 1:1000 (18)), or a monoclonal mouse anti-human apoM antibody (from BD Biosciences, 1:10,000). Mouse apoM was detected with a polyclonal rabbit anti-mouse apoM antisera (1:500) (10).

Primary Hepatocyte Cell Culture—Primary hepatocytes were isolated from ApoM-TgN, ApoM22A-Tg, and wild-type mice (21). Briefly, mice were perfused with 5 ml of liver perfusion medium (Invitrogen) followed by 5 ml of digestion medium containing 4 mg/ml collagenase type I (C2624, Sigma-Aldrich). The liver was then placed in a sterile tube with 5 ml of digestion medium at 37 °C for 20 min before being placed in ice-cold L-15 cell medium (Invitrogen). Subsequently, the tissue was homogenized with a Pasteur pipette, passed through a 70-μm nylon filter, and washed with hepatocyte washing medium (Invitrogen). Centrifugation between washes was done at 50 × g for 5 min. The cells were resuspended in 5 ml of F-12/Dulbecco’s modified Eagle’s medium (Invitrogen) with 1% penicillin/streptomycin, 10% bovine calf serum, and 2 mM L-glutamine (G6392,
Sigma-Aldrich) and grown on 6-well collagen-coated plates (152034, Nunc). The concentration of human apoM in cell culture medium was measured after 48 h. Lipoproteins (d < 1.21 g/ml) were separated from non-lipoproteins in plasma or concentrated (×4) cell culture medium by adjusting 20 μl of sample to d = 1.21 g/ml with NaBr and ultracentrifugation in a TLA-100 ultracentrifuge (Beckman) using 200-μl tubes at 15 °C for 4 h and 100,000 rpm.

**Plasma Turnover and Tissue Elimination of Human apoM22–188**—To study the plasma turnover of apoM without the signal peptide, recombinant apoM22–188 or wild-type human apoM were injected intravenously into recipient wild-type mice, and the plasma turnover was assessed by measuring human apoM plasma concentrations with ELISA. Recombinant human apoM22–188 was produced as described (18), and human apoM plasma concentrations with ELISA. Recombinant human apoM22–188 was produced as described (18), and protein concentration was measured with the Pierce BCA protein assay kit (Bie and Berntsen A-S) using bovine serum albumin as a standard (9). For each recipient mouse, apoM22–188 (100 μg) in 148 μl of phosphate-buffered saline was mixed with 90 μl of plasma from a wild-type mouse and injected intravenously into a wild-type mouse (n = 3); control mice (n = 3) received 148 μl of phosphate-buffered saline mixed with 90 μl of plasma from human apoMTg mice that overexpress human apoM 10-fold (10). Blood samples were taken after 5, 30, 120, and 1400 min for measurement of human apoM. To assess the lipoprotein association of human apoM, lipoproteins (d < 1.21 g/ml) were separated from proteins in plasma samples taken after 30 min as described, above and the human apoM concentration was determined in the fractions by ELISA.

To determine tissue uptake and degradation, we labeled apoM22–188 or albumin with 125I-TC and injected it into wild-type mice. The 125I-TC moiety is trapped inside cells upon lysosomal degradation of the labeled protein, enabling in vivo studies of degradation sites of plasma proteins (22). First, tyramine cellobiose (TC) (50 nmol) was labeled with 37 MBq 125I in tubes coated with 10 μg of iodogen (Invitrogen) (23). Then, the 125I-labeled TC was activated and coupled to 100–120 μg of apoM22–188 or albumin at pH 9–10. To separate the unbound 125I and 125I-TC from 125I-TC-albumin and 125I-TC-apoM22–188, the mixture was passed over a PD-10 column (Amersham Biosciences). The labeling efficiency was 34% for apoM22–188 and 22% for albumin, and 91 and 86% of the 125I in the doses were precipitated with 15% trichloroacetic acid for apoM22–188 and albumin, respectively. The integrity of the labeled proteins was examined by 12% SDS-PAGE, and visualization of the radioactivity was done with a Fuji BAS 200 bioimaging analyzer (Fuji Photo Film).

The concentration of 125I-TC-labeled proteins was determined by the BCA Pierce kit (Pierce), and 125I-TC-apoM22–188 or 125I-TC-albumin (10 μg, 3.2–5.3 × 106 cpm) was injected into a tail vein of wild-type mice (n = 2 × 3) in a total volume of 330 μl. Blood samples were taken 10 min and 6 h after injection before the mice were anesthetized and perfused with phosphate-buffered saline. Plasma samples and biopsies from liver, kidney, lung, spleen, eyes, heart, brain, gut, and gall bladder were placed in 1 ml of phosphate-buffered saline and counted in a 1470 automatic gamma counter (PerkinElmer Life Sciences) for 30 min.

To examine the cellular distribution of labeled apoM22–188, 1-μm Epon sections were obtained from kidneys fixed by immersion in 4% formalin 6 h after injection of labeled protein. The tissue was postfixed in OsO4, dehydrated, and embedded in Epon 812. The Epon sections were processed for autoradiography using Ilford K2 emulsion, exposed for 1–4 weeks, and developed in Kodak D19. The sections were examined in a Leica DMR microscope equipped with a Leica DFC320 camera. Images were transferred by a Leica TFC Twain 6.1.0 program and processed using Adobe PhotoShop 8.0.

**Exclusion of Kidney Elimination of Plasma Proteins**—To exclude kidney elimination of plasma proteins, apoM22A-Tg (n = 6), apoM-Tg3N (n = 3), and wild-type mice (n = 3), animals were anesthetized, and the kidney was dissected free as described previously (24) to enable bilateral ligation of the kidney artery. The musculoskeletal and skin incisions were then sutured, and the animals were given buprenorphine (0.001 mg/10 g of body, subcutaneously) for analgesia while kept anesthetized for another 4 h. Blood samples were taken before and 1 and 4 h after the kidney artery ligation.

**RESULTS**

**Expression of ApoM22A in Transgenic Mice**—To examine the impact of the signal peptide sequences on the metabolism of apoM, we used a mutated human apoM22A cDNA driven by albumin enhancer-promoter sequences to make Tg mice. The Gln-22 → Ala substitution introduces a signal peptidase cleavage site and results in secretion of a truncated apoM without the signal peptide from HEK293 cells (Fig. 1a). Twelve founders were generated by pronuclear injection of the transgene into fertilized mouse eggs. One transgenic line with an estimated ~32 copies of the transgene was bred for further studies and compared with Tg mice expressing wild-type human apoM (10). The apoM22A-Tg mice thrived well, bred normally, and were indistinguishable from wild-type mice.

The liver expression of human apoM mRNA in the apoM22A-Tg mice was ~40% of that in apoM-Tg mice (Fig. 1b). However, on Western blotting of mouse plasma with a human apoM-specific polyclonal antibody, apoM was only detectable in the apoM-Tg and not in apoM22A-Tg mice (Fig. 1c). Of note, the polyclonal anti-human apoM antibody recognized recombinant apoM22–188 as well as wild-type human apoM (Fig. 1c). This implies that the lack of apoM in apoM22A-Tg mouse plasma does not reflect the lack of reactivity of the antibodies toward truncated apoM. In agreement with the Western blotting results, human apoM could not be detected in the plasma from apoM22A-Tg mice with a human apoM-specific sandwich ELISA when the plasma was diluted as little as 50 times. The ELISA uses two different monoclonal antibodies to human apoM and reacts well with both wild-type human apoM and recombinant apoM without the signal peptide sequences (Fig. 1e). The detection limit is ~0.3 nM. Hence, the concentration of human apoM in apoM22A-Tg mice is <15 nM. In contrast, the concentration of human apoM is ~2 μM in the human apoM-Tg mice. The absence of detectable apoM22A in plasma was also seen in analyses of eight additional apoM22A-Tg founders and thus was not dependent on the site of transgene integration in the mouse genome. Overex-
pression of human apoM had no discernable effect on the plasma levels of endogenous mouse apoM (Fig. 1).

Since apoM^{Q22A} is secreted from transfected HEK293 cells, we reasoned that the absence of apoM^{Q22A} in the apoM^{Q22A}-Tg mouse plasma most likely did not reflect impaired secretion from the mouse liver. To test this assumption, primary hepatocytes from apoM^{Q22A}-Tg, apoM-Tg, and wild-type mice were grown in tissue culture plates. Analysis of the medium by ELISA (Fig. 2) and Western blotting (not shown) showed that human apoM was secreted into the medium from both apoM^{Q22A}-Tg and apoM-Tg hepatocytes. The concentration of apoM^{Q22A} in the medium was ~40% of that of wild-type human apoM. Hence, the relative rate of secretion of apoM^{Q22A} and wild-type human apoM from the cultured hepatocytes was similar to the relative amount of apoM^{Q22A} and wild-type human apoM mRNA expression in the liver. The virtual absence of apoM^{Q22A} in plasma despite ample secretion from the liver suggested that the lack of the signal peptide might cause rapid clearance of apoM^{Q22A} from plasma because apoM^{Q22A} cannot associate with plasma lipoproteins.

**FIGURE 1. Expression of human apoM in apoM^{Q22A}-Tg and apoM-Tg mice.**

- **a**, Western blot showing secretion of a truncated human apoM from HEK293 cells expressing the apoM^{Q22A} cDNA (15). Human plasma (0.5 µl) and concentrated (~ × 40) cell culture medium from transfected HEK293 cells (3 µl) were analyzed by Western blotting after SDS-PAGE using an anti-human apoM antibody from BD Biosciences. **b**, ethidium bromide-stained 2% agarose gel showing human apoM mRNA expression in apoM^{Q22A}-Tg and apoM-Tg mouse livers; wild-type mouse liver was included as negative control. **c**, Western blot of plasma from apoM^{Q22A}-Tg, apoM-Tg, and wild-type mice with a polyclonal human apoM antibody. Human plasma (1, 0.5, or 0.25 µl of a plasma pool with ~ 23 µg/ml apoM) and recombinant apoM apoM^{22–188} (50, 25, or 5 ng/lane) were included as controls to document that the antibody reacts with both wild-type human apoM (h-apoM) and apoM without the signal peptide. **d**, Western blot of mouse apoM (m-apoM) in plasma (0.5 µl) from apoM-Tg, apoM^{Q22A}-Tg, and wild-type mice using a polyclonal mouse apoM antibody. Human plasma was included as negative control. **e**, the relationship between the concentration of human apoM (in the well) versus resulting absorbance in the human apoM ELISA. The results were obtained with a dilution series of human plasma (open circles) or recombinant apoM^{22–188} (filled circles).
Impact of the Signal Peptide on Plasma Metabolism of apoM—To explore the impact of the signal peptide on the plasma metabolism of apoM, we produced a recombinant truncated human apoM22–188 without the signal peptide (corresponding to amino acids 22–188 of wild-type human apoM) in Escherichia coli and injected it (100 μg) into wild-type mice. ELISA measurements showed that apoM22–188 was rapidly cleared from plasma with only ~5% of the injected dose remaining in plasma 2 h after the injection (Fig. 3a). We also examined the plasma metabolism of wild-type human apoM by injecting plasma from apoM-Tg mice into wild-type mice; the clearance of wild-type human apoM was much slower than that of apoM22–188 with ~50 and ~5% of the injected wild-type human apoM remaining in plasma after 2 and 24 h, respectively (Fig. 3a). To analyze the lipoprotein association of apoM22–188, lipoproteins were separated from the remaining plasma proteins by ultracentrifugation at d = 1.21 g/ml using plasma collected 30 min after injection of apoM22–188 or wild-type human apoM particles. ApoM22–188 was recovered in the protein fraction, whereas wild-type human apoM predominantly was in the lipoprotein fraction (Fig. 3b).

Due to the small size (~20 kDa) and the lack of lipoprotein association of apoM22–188, we suspected that it would be rapidly removed by filtration in the kidney. We could, however, not detect any human apoM in the urine of apoM22A-Tg mice by ELISA or Western blotting, suggesting that if the kidney was a primary site for removal of apoM without the signal peptide, then the filtered apoM might be taken up in the proximal tubule rather than being excreted into the urine. Indeed, we previously showed that the endocytic receptor megalin, which is highly expressed on the luminal side of the proximal tubule epithelium, can bind and internalize apoM (26). To explore the tissue sites for uptake and degradation of apoM22–188, we labeled apoM22–188 with 125I-TC and injected it into wild-type mice. Parallel studies were done with human albumin. 125I-TC label-
did not affect the integrity of apoM<sub>22–188</sub> or albumin as both proteins migrated as one band with the expected size in SDS-PAGE gels (Fig. 4a). Similar to non-labeled apoM<sub>22–188</sub>, 125I-TC-apoM<sub>22–188</sub> was rapidly removed from plasma (Fig. 4b). Six hours after injection, the main portion of the 125I-TC-apoM<sub>22–188</sub> was recovered in the kidney, whereas 125I-TC-albumin was recovered in the plasma, liver, and kidney (Fig. 4c). To examine which cells had taken up 125I-TC-apoM<sub>22–188</sub>, sections of the recipient mouse kidneys were subjected to autoradiography. The vast proportion of 125I in the kidney was seen in proximal tubule epithelial cells, whereas only a few grains were seen in glomerular cells or distal tubules (Fig. 4d). The rapid plasma elimination and uptake by kidney proximal tubule cells of human apoM<sub>22–188</sub> were reiterated when using 125I-TC-labeled recombinant mouse apoM without its signal peptide sequences (data not shown).

The turnover studies using recombinant apoM<sub>22–188</sub> suggested that the absence of human apoM in the plasma of apoM<sub>Q22A</sub>Tg mice reflects rapid removal of apoM<sub>Q22A</sub> in the kidney and that human apoM would accumulate in the plasma of the apoM<sub>Q22A</sub>Tg mice if kidney elimination were prevented. To test this idea, we excluded kidney degradation of plasma
proteins by bilateral ligation of the renal arteries in anesthetized apoM^{Q22A}-Tg, apoM-Tg, and wild-type mice and measured human apoM in plasma with ELISA. Indeed, human apoM became detectable in plasma of the apoM^{Q22A}-Tg mice already 1 h after eliminating kidney excretion (Fig. 5), which strongly supports the conclusion that apoM can be secreted from the liver without its signal peptide but that the lack of signal peptide results in rapid elimination of the truncated plasma protein by the kidney. The concentration of human apoM in apoM-Tg mice did not increase after bilateral ligation of the kidney arteries (Fig. 5).

DISCUSSION

The NH₂-terminal signal peptide plays important roles in the intracellular processing of proteins. It can determine protein folding (e.g. EspP (27)) and direct proteins into specific secretory pathways (e.g. pro-opiomelanocortin (28)) or cellular compartments (e.g. PB2 of the influenza virus is directed to the mitochondria (29)). Also, the hydrophobic nature of the signal peptide can anchor proteins in the cell membrane. Failure of cleavage of the signal peptide of proteins that are normally secreted can result in intracellular degradation (30). In the present study, however, human wild-type apoM and human apoM^{Q22A} appeared to be equally effectively secreted from primary mouse hepatocytes. The results suggest that the signal peptide is responsible for apoM’s lipoprotein association and is crucial to prevent rapid clearance of apoM from plasma. The present in vivo findings are in accord with a recent in vitro observation by Axler et al. (25), suggesting that apoM^{Q22A} fails to associate with HDL in the culture medium when expressed in HEK293 cells.

ApoM shares its preservation of the NH₂-terminal signal peptide sequences with PON-1 and PAI-2 (15, 16). In the case of HRP, however, the signalP software predicts that the signal peptide can be cleaved, and in vitro expression in 293 cells of an HRP encoding cDNA showed that the major fraction of HRP indeed is secreted from the cultured cells without the signal peptide (35). Nevertheless, plasma HRP does contain the signal peptide. The present data provide a possible explanation for the apparent discrepancy; even if only a subfraction of HRP is secreted with the signal peptide sequences, the anchoring of HRP with the signal peptide in HDL may prevent clearance from plasma, whereas HRP without the signal peptide (~35 kDa) predictably will be rapidly removed from plasma.

The physiological role of apoM remains to be elucidated. Genetic elimination or overexpression of apoM in mice affects plasma lipid metabolism (10, 13), and in healthy humans, the plasma apoM concentration is positively associated with total cholesterol concentration (18). Nevertheless, apoM may have roles beyond lipid metabolism; HDL carries multiple proteins with roles in inflammation and innate immune responses, and HDL has pronounced anti-inflammatory effects in animal models (1, 36). Thus, it is possible that apoM, only being present in ~5% of the plasma HDL particles (9), may also have effects unrelated to lipid metabolism. For instance, apoM protects against Cu²⁺-induced lipid oxidation (9, 10) and binds retinol and retinoic acid in vitro (15). The latter observation is explained by the lipocalin structure of apoM where a small hydrophobic binding pocket enables binding of small lipophilic molecules (14). It is interesting to note that apoM is also found in a highly conserved major histocompatibility complex class III cluster in primitive organisms such as fish, indicating conservation of the cluster for more than 450 million years (37), further suggesting that apoM may have essential biological functions. The present data document that the signal peptide in apoM serves an important function by anchoring the apolipoprotein to lipoproteins, thus inhibiting the elimination of apoM in the kidney and maintaining stable apoM concentrations in plasma.

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REFERENCES

1. Vaisar, T., Pennathur, S., Green, P. S., Gharib, S. A., Hoofnagle, A. N., Cheung, M. C., Byun, J., Vuletic, S., Kassim, S., Singh, P., Chea, H., Knopp, R. H., Brunzell, J., Geary, R., Chait, A., Zhao, X. Q., Elk, K., Marcovina,
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S., Ridker, P., Oram, J. F., and Heinecke, J. W. (2007) J. Clin. Investig. 117, 746–756
2. Vanhamme, L., Paturiaux-Hancoq, F., Poelvoorde, P., Nolan, D. P., Lins, L., Van Den, A. J., Pays, A., Tebabi, P., Van, X. H., Jacquet, A., Moguilevsy, N., Dieu, M., Kane, J. P., De, B. P., Brasseur, R., and Pays, E. (2003) Nature 422, 83–87
3. Trougakos, I. P., and Gonos, E. S. (2002) Int. J. Biochem. Cell Biol. 34, 1430–1448
4. Segrest, J. P., Jones, M. K., De, L. H., and Dashti, N. (2001) J. Lipid Res. 42, 1346–1367
5. Davidson, W. S., and Silva, R. A. (2005) Curr. Opin. Lipidol. 16, 295–300
6. Davidson, W. S., Hazlett, T., Mantulin, W. W., and Jonas, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 13605–13610
7. Segrest, J. P., Jackson, R. L., Morrisett, J. D., and Gotto, A. M., Jr. (1974) FEBS Lett. 38, 247–258
8. Atkinson, D., and Small, D. M. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 403–456
9. Christoffersen, C., Nielsen, L. B., Axler, O., Andersson, A., Johnsen, A. H., and Dahlback, B. (2006) J. Lipid Res. 47, 1833–1843
10. Christoffersen, C., Jauhiainen, M., Moser, M., Porse, B., Ehnholm, C., Boesl, M., Dahlback, B., and Nielsen, L. B. (2007) J. Biol. Chem. 282, 1839–1847
11. Cheung, M. C., Wolf, A. C., Lum, K. D., Tollefson, J. H., and Albers, J. J. (1986) J. Lipid Res. 27, 1135–1144
12. Xu, N., and Dahlback, B. (1999) J. Biol. Chem. 274, 31286–31290
13. Wolfrum, C., Poy, M. N., and Stoffel, M. (2005) Nat. Med. 11, 418–422
14. Duan, J., Dahlback, B., and Villoutreix, B. O. (2001) FEBS Lett. 499, 127–132
15. Ahnstrom, J., Faber, K., Axler, O., and Dahlback, B. (2007) J. Lipid Res. 48, 1754–1762
16. Sorenson, R. C., Bisgaier, C. L., Aviram, M., Hsu, C., Billecke, S., and La Du, B. N. (1999) Arterioscler. Thromb. Vasc. Biol. 19, 2214–2225
17. Raper, J., Fung, R., Ghiso, J., Nussenzweig, V., and Tomlinson, S. (1999) Infect. Immun. 67, 1910–1916
18. Axler, O., Ahnstrom, J., and Dahlback, B. (2007) J. Lipid Res. 48, 1772–1780
19. Bartels, E. D., Lauritsen, M., and Nielsen, L. B. (2002) Diabetes 51, 1233–1239
20. Faber, K., Axler, O., Dahlback, B., and Nielsen, L. B. (2004) J. Lipid Res. 45, 1272–1278
21. Sabol, S. L., Brewer, H. B., Jr., and Santamarina-Fojo, S. (2005) J. Lipid Res. 46, 2151–2167
22. Pittman, R. C., Carew, T. E., Glass, C. K., Green, S. R., Taylor, C. A., Jr., and Attie, A. D. (1983) Biochem. J. 212, 791–800
23. Juul, K., Nielsen, L. B., Munkholm, K., Stender, S., and Nordestgaard, B. G. (1996) Circulation 94, 1698–1704
24. Bro, S., Moeller, F., Andersen, C. B., Olgaard, K., and Nielsen, L. B. (2004) J. Am. Soc. Nephrol. 15, 1495–1503
25. Axler, O., Ahnstrom, J., and Dahlback, B. (2008) FEBS Lett. 582, 826–828
26. Faber, K., Hvidberg, V., Moestrup, S. K., Dahlback, B., and Nielsen, L. B. (2006) Mol. Endocrinol. 20, 212–218
27. Szabady, R. L., Peterson, J. H., Skillman, K. M., and Bernstein, H. D. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 221–226
28. Cool, D. R., Fenger, M., Snell, C. R., and Loh, Y. P. (1995) J. Biol. Chem. 270, 8723–8729
29. Carr, S. M., Carnero, E., Garcia-Sastre, A., Brownlee, G. G., and Fodor, E. (2006) Virology 344, 492–508
30. Kotsova, Z., and Wolf, D. H. (2003) EMBO J. 22, 2309–2317
31. Vanhollebeke, B., Nielsen, M. J., Watanabe, Y., Truc, P., Vanhamme, L., Nakajima, K., Moestrup, S. K., and Pays, E. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 4118–4123
32. Palmiter, R. D., Gagnon, J., and Walsh, K. A. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 94–98
33. Ye, R. D., Wun, T. C., and Sadler, J. E. (1988) J. Biol. Chem. 263, 4869–4875
34. von, H. G., Liljestrom, P., Mikus, P., Andersson, H., and Ny, T. (1991) J. Biol. Chem. 266, 15240–15243
35. Nielsen, M. J., Petersen, S. V., Jacobsen, C., Oxvig, C., Rees, D., Moller, H. I., and Moestrup, S. K. (2006) Blood 108, 2846–2849
36. Barter, P. J., Nicholls, S., Rye, K. A., Anantharamaiah, G. M., Navab, M., and Fogelman, A. M. (2004) Circ. Res. 95, 764–772
37. Deakin, J. E., Papenfuss, A. T., Belov, K., Cross, J. G., Coggill, P., Palmer, S., Sims, S., Speed, T. P., Beck, S., and Graves, J. A. (2006) BMC. Genomics 7, 281

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