A Novel Human Apolipoprotein (apoM)*

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A novel human apolipoprotein designated apolipoprotein M (apoM) is described. The unique N-terminal amino acid sequence of apoM was found in an approximately 26-kDa protein present in a protein extract of triglyceride-rich lipoproteins (TGRLP). The isolated apoM cDNA (734 base pairs) encoded a 188-amino acid residue-long protein, distantly related to the lipocalin family. The mRNA of apoM was detected in the liver and kidney. Western blotting demonstrated apoM to be present in high density lipoprotein (HDL) and to a lesser extent in TGRLP and low density lipoproteins (LDL). The first 20 amino acid residues of apoM constituted a novel apolipoprotein, which is mainly associated with HDL, and it has no known function in reverse cholesterol transport. ApoM is distantly related to the lipocalin family (2, 4), and as such, it contains a binding pocket for small hydrophobic ligands. ApoM is linked to the single phospholipid layer of lipoproteins with a hydrophobic signal anchor. In conclusion, a novel human apolipoprotein, the function of which remains to be determined, is described.

In blood, cholesterol and triglycerides (TG)³ are transported in particles, so-called lipoproteins, composed of a single layer of phospholipid surrounding the lipid core, and by a number of surface-associated apolipoproteins (1–3). These specialized proteins play major roles in the structural organization and intravascular metabolism of the lipoproteins. Thus, the apoproteins are involved in specific binding to cellular receptors, the regulation of lipolytic enzymes, and the process of lipid exchange and transfer. The five major lipoprotein classes are defined according to the densities at which they are isolated as high (HDL), low (LDL), intermediate (IDL), and very low density lipoproteins (VLDL) and chylomicrons. HDL and LDL are rich in cholesterol, whereas TG constitute the major lipid in the remaining subclasses.

In man, apoB-100 is the structural apolipoprotein of LDL, IDL, and VLDL, whereas apoB-48 plays this role in chylomicrons (1–3). In HDL, apoA-I and apoA-II are the major protein constituents. HDL and TG-rich lipoproteins (TGRLP) also contain several other apolipoproteins such as apoC-I, apoC-II, apoC-III, and apoE. ApoA-I, apoC-I, and also apoA-IV, which is mainly associated with HDL, activate lecithin-cholesterol acyltransferase and are involved in the HDL-mediated reverse cholesterol transport. ApoA-II activates hepatic lipase, and apoC-II stimulates lipoprotein lipase. ApoE serves as ligand for the lipoprotein receptor, whereas apoC-III inhibits interaction with hepatic receptors. There are several additional apolipoproteins associated with human plasma lipoproteins, e.g. apoD, apoF, apoH, and apoL. The HDL-specific apoA is a member of the lipocalin family (2, 4), and as such, it contains a binding pocket for small hydrophobic ligands. ApoD has greater affinity for heme-related compounds than for cholesterol, and it has no known function in reverse cholesterol transport. ApoF was recently shown to be identical to lipid transfer inhibitor protein, an important regulator of cholesterol ester transfer protein activity (5). ApoL is the most recently identified apolipoprotein (6). The function of this HDL-specific apolipoprotein remains to be elucidated.

In the search for novel apolipoproteins, we have identified, cloned, and partially characterized a novel apolipoprotein, apoM, which is mainly associated with HDL. ApoM is distantly related to the lipocalin family and is found to retain its signal peptide, which may serve to anchor the protein to the surface of the lipoprotein.

EXPERIMENTAL PROCEDURES

Materials—A human liver 5'-stretch plus cDNA library, a human liver Marathon-ready cDNA library, human multiple-tissue Northern blot membranes, Zoo blot membranes, and Express Hyb solution were purchased from CLONTECH. Enzymes were purchased from Roche Molecular Biochemicals, Promega, Appligene, or MBI Fermentas. Oligonucleotides were from DNA Technology A/S (Denmark). The DNA sequencing kit was from PE Applied Biosystems. The rabbit reticulo-lysate system and canine pancreatic microsomal membrane were from Promega. [α-³²P]dCTP, [³⁵S]methionine, and rainbow ¹⁴C-labeled protein markers were from Amersham Pharmacia Biotech. Keyhole limpet hemocyanin was from Pierce.

Volunteers, Collection of Blood Samples, and Separation of Plasma Lipoproteins—Healthy volunteers (20 men and 24 women), 27–55 years of age (38.1 ± 9.4, mean ± S.D.), participated in the study, which was approved by the local ethics committee. Blood was drawn into EDTA (4 mm final concentration) 2–3 h after a breakfast containing approximately 100 g of fat (from butter, cheese, and cream). The plasma was pooled, and TGRLP (d<1.006 kg/liter) were isolated by ultracentrifugation (7). The TGRLP were washed once by ultracentrifugation at a density of 1.006 in the presence of 1 mmol/liter EDTA. LDL and HDL were isolated as described (8). The isolated lipoproteins were delipidated with ethanol:diethyl ether (3:1, volume/volume) at 4 °C for 24 h as described (9). The delipidation procedure was repeated three times, and the protein precipitate was washed twice with cold diethyl ether and
dried under nitrogen. The proteins were solubilized in 50 mMol/liter Tris-HCl buffer (pH 7.4) and passed through a 0.2-μm Millipore filter. Lipoprotein-deficient plasma (LPDP) was obtained from the infranatant after removal of all lipoprotein classes. The isolated lipoprotein subfractions and LPDP were dialyzed extensively against PBS (KCl 2.7 mM, KH2PO4 1.5 mM, NaCl 136.8 mM, and Na2HPO4 8.1 mM, pH 7.4) and then stored at −20 °C. To investigate whether the apoM concentration in TGLRP increased after the fat meal, TGLRP was isolated from 11 individual plasma before and after the fat-rich meal and the relative apoM levels estimated by Western blotting. The protein concentrations of the different lipoproteins and LPDP were determined by the Lowry procedure (10).

Protein Sequencing and cDNA Cloning—De-lipidated human TGLRP (30 μg) was applied to SDS-PAGE (11), and the proteins were then transferred to polyvinylidene difluoride membranes. The membranes were stained using Coomassie Brilliant Blue, and bands ranging from 6 to 45 kDa were subjected to the N-terminal sequencing using an Applied Biosystems Model 494 sequencer equipped with a Model 140C analyzer. In the 26-kDa region, a weakly stained protein band yielded a double sequence, which was followed for 15 cycles. One of the sequences, identified as the N-terminal sequence of apoC-III, after subtrac- tion yielded the novel sequence MHFQIWAFFGVI. BLAST searches of public data bases were performed as described (12). No homologous protein was identified, but several human expressed sequence tags (13) were found to predict the amino acid sequence. Based on their sequences, two oligonucleotides (oligo-1 and oligo-2) corresponding to the first or second strand of the sequence 5′-ATG TTC CAC CAA ATT TGG GCG GCT CTG CTC TAC TTC TAT GGT ATT were designed. The above selected region was 33 nucleotides and the 3′-untranslated region was 9 nucleotides. The oligonucleotides were used to design oligonucleotides, which were then sequenced on both strands using a sequencing kit from PE Applied Biosystems. Rapid amplification of cDNA ends of a Marathon-ready human liver cdNA library (CLONTECH) was performed as described by the manufacturer, to further investigate whether the isolated cdNA was full length.

Western, Southern, and Northern Blotting Analyses—Rabbit antiserum against five synthetic peptides, corresponding to amino acid residues 1–23, 23–42, 74–95, 109–128, and 136–157 of apoM conjugated to keyhole limpet hemocyanin, were raised. SDS-PAGE and Western blotting were performed as described (11, 14). To determine the relative amount of apoM in lipoprotein-deficient plasma, increasing amounts of normal plasma (0.75, 1.25, 2.5, 5, 10, and 20 μg of plasma proteins) were applied on SDS-polyacrylamide gels and then analyzed by Western blotting after the anti-peptide antibody against apoM. Lipoprotein-deficient plasma (LPDP) was applied (10 μg of protein). The apoM bands in normal plasma were quantified by densitometry (Molecular Dynamics) to construct a standard curve from which the amount of apoM in lipoprotein-deficient plasma was estimated. Human Dynamics) to construct a standard curve from which the amount of apoM in lipoprotein-deficient plasma, increasing amounts of normal plasma (0.75, 1.25, 2.5, 5, 10, and 20 μg of plasma proteins) were applied on SDS-PAGE, and the radioactive prestained radiolabeled standard mixture was used as molecular weight marker.

**RESULTS**

Identification and Cloning of a Novel Apolipoprotein—A previously unrecognized amino acid sequence (MHFQIWAFFGVI) was identified when a 26-kDa protein in TGLRP was analyzed by N-terminal sequence. BLAST searches of public data bases failed to identify any identical or similar protein sequence, but several expressed sequence tags were found that encode the identified protein sequence. These sequences were used to design oligonucleotides, which were then used to isolate the cdNA encoding the protein. Two different human cdNA libraries were used and yielded identical cdNA sequences. An open reading frame encoding a putative protein of 188 amino acid residues was identical with the first 15 amino acid residues of the sequenced protein (Fig. 1). The 5′-untranslated region was 33 nucleotides and the 3′-untranslated region...
Novel Apolipoprotein Mainly Associated with HDL—

ApoM in different lipoprotein subclasses and in plasma. A, apolipoproteins from TGRLP, LDL, and HDL (5 μg in each lane) and LPDP (5 μg of plasma proteins) were applied to 4–15% gradient SDS-PAGE under reducing and nonreducing conditions and detected by Western blotting with pooled anti-peptide apoM antisera. B, Increasing amounts of normal plasma proteins were applied to 8–18% gradient SDS-PAGE and analyzed by Western blotting using the pooled anti-peptide antisera. Lanes 2-7 contain 0.75, 1.25, 2.5, 5, 10, and 20 μg of plasma proteins, respectively. In parallel, LPDP (10 μg of protein) was analyzed.
ApoM is a minor component of HDL. To elucidate the relative amount of apoM in HDL as compared with the other lipoproteins, 10 μg of delipidated HDL was applied in duplicate to 10%-PAGE run in the presence of SDS under reducing conditions. One lane was silver-stained and the other subjected to immunoblotting as described in the legend to Fig. 3. The silver-stained gel was scanned with a personal densitometer (Molecular Dynamics); the OD profile is shown to the right. The apoM is not seen as an individual band on the silver-stained gel, suggesting that its concentration is lower than those of the other major apolipoproteins.

The identification, characterization, and cloning of a novel human apolipoprotein, designated apoM, is described. ApoM fulfills the criteria of an apolipoprotein because the majority of apoM in plasma is associated with lipoproteins. A small amount of apoM could be detected in lipoprotein-depleted plasma, but it is possible that this apoM is associated with small lipoprotein particles remaining in the plasma after ultracentrifugation rather than existing in free form. Among the various lipoprotein subclasses, HDL appeared to contain somewhat more apoM than TGRLP and LDL. In all of these lipoproteins, apoM appeared to be a minor component as compared with the major apolipoproteins. On Northern blotting, apoM mRNA was prominent in both liver and kidney but not in other organs. The synthesis of apoM in liver may be associated with the production of HDL, whereas the physiological importance of the apoM mRNA in the kidney is less obvious.

ApoM is unusual in having its hydrophobic signal peptide retained in the mature protein, where it presumably serves as a phospholipid anchor. The phospholipid in lipoproteins form a single layer in contrast to the double-layer phospholipid that constitutes the cell membranes of eukaryotic cells (1). The attachment of a protein to single-layer phospholipid via a hydrophobic uncleaved signal peptide is unique (21). In contrast, many transmembrane proteins of type I (N terminus located outside the cell) or type II (N terminus located in the membrane) are surrounded by hydrophilic sequences localized inside or outside the membrane. The single-layer phospholipid found in lipoproteins cannot accommodate a protein with such transmembrane regions because the inner core of the lipoprotein is hydrophobic. Many of the apolipoproteins bind to the phospholipid via hydrophobic surfaces formed by amphipathic helices (1). These helices have a characteristic spatial arrangement of hydrophobic and hydrophilic amino acid residues, yielding a hydrophobic face of the helix, which intercalates between the fatty acids of the phospholipid. The uncleaved signal peptide of apoM is predicted to form a hydrophobic α-helix similar to the transmembrane regions in trans-membrane proteins.

It is tempting to speculate that the synthesis of apoM is closely linked to the HDL assembly and secretion from the liver. Several results are consistent with such a hypothesis. Thus, the hydrophobic N-terminal part of apoM targets apoM for translocation through the endoplasmic reticulum. Moreover, the hydrophobic signal sequence remains attached to the protein and may be instrumental as a phospholipid anchor. ApoM has the characteristics of a membrane integral protein, and it is possible that apoM is incorporated into the lipoprotein.

**DISCUSSION**

The identification, characterization, and cloning of a novel human apolipoprotein, designated apoM, is described. ApoM fulfills the criteria of an apolipoprotein because the majority of human DNA, two bands (6.6 and 1.9 kb) were observed, which agreed with the presence of an EcoRI site in the cDNA sequence. A strong signal at the bottom of the gel in the lane containing rabbit DNA suggested that the rabbit gene is less than 2 kb in length.

**Fig. 4.** ApoM is a minor component of HDL. To elucidate the relative amount of apoM in HDL as compared with the other lipoproteins, 10 μg of delipidated HDL was applied in duplicate to 10%-PAGE run in the presence of SDS under reducing conditions. One lane was silver-stained and the other subjected to immunoblotting as described in the legend to Fig. 3. The silver-stained gel was scanned with a personal densitometer (Molecular Dynamics); the OD profile is shown to the right. The apoM is not seen as an individual band on the silver-stained gel, suggesting that its concentration is lower than those of the other major apolipoproteins.

**Fig. 5.** Slight increase in apoM levels in TGRLP after fat feeding. Blood was drawn from 11 volunteers before and after fat feeding. The TGRLP were isolated by ultracentrifugation. The protein mass was determined by the Lowry method and adjusted to 1 mg/ml. The TGRLP (5 μl, corresponding to 5 μg of protein) from each individual sample were applied to 8–18% PAGE run in the presence of SDS under reducing conditions and subjected to Western blotting. Lanes marked P represent fasting samples, and those marked F were derived from postprandial samples. B, the blots were scanned and the intensity of the bands quantified. The fasting samples were considered as 100%. After a fat meal, the apoM signal increased about 32%, which was significant (<0.01) according to a two-tailed Wilcoxon matched pairs test.

**Fig. 6.** Northern blot analysis of apoM. The human multiple tissue Northern blots were probed at high stringency with a radiolabeled full-length cDNA of apoM. The positions and sizes of apoM mRNA are indicated at the right.
particles together with phospholipid from the endoplasmic reticulum and then secreted to plasma.

After the completion of the cDNA sequencing, a BLAST search of public data bases identified a hypothetical mouse gene (NG20) (GenBank accession number AF109719), which encodes the mouse homologue of the human apoM. The NG20 gene is located in the major histocompatibility complex class III region on chromosome 17, between the HLA-B-associated transcripts BAT3 and BAT4 and close to the TNF gene. In the human genome, this chromosome region corresponds to position p21.3 on chromosome 6. Recently, the genomic sequence of this region was determined and the human apoM gene identified (GenBank accession number AF129756). In the human gene, the apoM gene is surrounded by BAT4 and NG34 on one side and BAT3 on the other. Both the mouse apoM gene and its human counterpart are predicted to contain 6 exons enclosed in a 1.6-kb genomic region, which is consistent with the results of Southern blotting; it can therefore be concluded that there is only one copy of the gene. The amino acid sequences of human and mouse apoM are 79% identical, and just like human apoM, the mouse sequence predicts the presence of a signal anchor, as there is no predicted signal peptidase cleavage site (Fig. 1A). Even though the regular BLASTP searches failed to identify any similar protein sequence, a Washington University BLASTP search of the Swiss Prot Database revealed distinct similarity to a short protein sequence (NSMNQXPEXSQTLTG) of rat protein px, which was isolated from rat HDL (22). The rat protein px sequence is similar to residues 18-33 in human apoM, suggesting that rat apoM is cleaved after its signal peptide, which is in contrast to our findings on human apoM. It remains to be determined whether the N-terminal part of rat apoM is different in principle from those of mouse and human apoM.

The function of apoM in relation to lipoproteins remains to be determined. Apart from the hypothetical involvement in the synthesis of HDL, it is possible that apoM serves a specific function in the metabolism and transfer of cholesterol. Alternatively, apoM could have the ability to transport hydrophobic low molecular weight compounds, the nature of which remains to be determined. In search for protein functions, sequence comparisons are sometimes very useful. However, in the case of apoM, sequence similarity searches of public data bases using the BLAST program failed to identify any certain similar protein sequence. However, when the apoM sequence was analyzed by a recently described fold recognition comparison method using sequence-derived predictions (23), a possible similarity between apoM and rat major urinary protein (Protein Data Bank accession code 1mup) was identified. The Z-score of the prediction was found to be 6.29, which was above the confidence threshold of the method (4.8 ± 1.0). Even though the amino acid sequence identity between 1mup and apoM was only 19%, it appears likely that the apoM is related to the large lipocalin family, of which 1mup is a member (24). The fold of the lipocalins is well conserved, and it is characterized by a hydrophobic binding pocket, which in 1mup binds pheromones that affects the behavior and sexual response of female rats. Using molecular modeling methods, we have found preliminary data suggesting that apoM is indeed a member of the lipocalin family. ApoM is only distantly related to the other HDL-associated lipocalin, apoD, but both proteins demonstrate specific features of this protein family (Fig. 1B). The identification of apoM as a member of the lipocalin family will hopefully be helpful in elucidating the physiological function of apoM.

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