Isolation and Characterization of Several Plasma Apolipoproteins of Common Marmoset Monkey

David Crook, Karl H. Weisgraber, Stanley C. Rall, Jr., and Robert W. Mahley

To explore the potential of the common marmoset monkey (Callithrix jacchus) as a model for human plasma lipoprotein metabolism, several marmoset apolipoproteins were isolated and characterized in this study. Based on several properties, including molecular weight, amino acid composition, and sequence, the marmoset apolipoproteins are strikingly similar to human apolipoprotein (apo) A-I, A-II, C-III, E, and A-IV. The first 54 residues of marmoset apo A-I showed 87% sequence identity with the corresponding region of human apo A-I. Amino-terminal sequence analysis of a minor basic apo A-I isoform revealed that it contained an amino-terminal hexapeptide extension (Arg-His-Phe-Trp-Gln-Gln) identical to that found in human proapo A-I. Like apo A-II in most nonhuman primates, marmoset apo A-II differed from human apo A-II in that it did not contain cysteine and therefore existed as a monomer. The complete amino acid sequence of marmoset apo A-II was deduced. The protein contains 77 amino acids, as does human apo A-II, and showed an 82% identity with its human equivalent. In both species, apo C-III and E had similar amino-terminal sequences and amino acid compositions. Like human apo E, marmoset apo E contained minor sialylated isoforms. However, unlike human apo C-III, no sialylated isoforms of marmoset apo C-III were observed. In addition, the marmoset possessed an apolipoprotein whose molecular weight and amino acid composition were similar to those of human apo A-IV. The close structural similarities between corresponding marmoset and human apolipoproteins indicate that the marmoset monkey will be useful as a model for human lipoprotein metabolism.

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Plasma lipids circulate as macromolecular complexes of lipid and protein. These proteins are referred to as apolipoproteins (apo) and are designated according to an alphabetical nomenclature. Plasma apolipoproteins have two general functions. First, they stabilize the micellar lipoprotein structure and impart, in association with phospholipids, a hydrophilic character to the particle surface. Second, apolipoproteins can direct the metabolism of lipoproteins by serving as ligands for lipoprotein receptors (e.g., apo B and apo E) or as cofactors for enzymes involved in lipid metabolism (e.g., apo C-II and apo A-I are activators of lipoprotein lipase and lecithin:cholesterol acyltransferase [LCAT], respectively). The complete structures of several human apolipoproteins have been determined by direct protein sequencing or have been inferred from the sequence of cDNA or genomic DNA clones (for a review, see reference 1).

In this and a companion article, we examine the potential of the common marmoset monkey (Callithrix jacchus) to serve as a model for human lipoprotein metabolism and atherosclerosis. In the companion article, we show that a cholesterol-enriched diet produced changes in marmoset plasma lipoproteins similar to those seen in other animal models and in humans and that the atherosclerosis induced in the arteries of marmosets by cholesterol feeding bears a striking resemblance to early atherosclerotic lesions in humans. In this article, the structure and electrophoretic properties of marmoset plasma apolipoproteins are compared with those of human apolipoproteins, and sequence homologies to human apo A-I, A-II, C-III, E, and A-IV are described. Many similarities were found between human and marmoset apolipoproteins. Together, the results from both studies strongly suggest that the marmoset is a suitable model for human lipoprotein metabolism and atherosclerosis.

Methods

Animals

The source and maintenance, including diets, of both control and cholesterol-fed common marmoset monkeys are described elsewhere.

Lipoprotein Isolation

Techniques for drawing blood and for plasma lipoprotein fractionation and isolation are described elsewhere. Pooled plasma (4.0 to 6.0 ml) from 7 to 10 animals was used in most cases. In some instances, it was necessary to use lipoprotein fractions from several pools. Briefly, sequential preparative ultracentrifugation was used to prepare fractions of d<1.006 g/ml (very low density lipoproteins, VLDL); 1.006 to 1.02 g/ml (intermediate density lipo-
proteins, IDL); 1.02 to 1.09 g/ml (low density lipoproteins; LDL); d<1.09 g/ml (high density lipoproteins, HDL), and d=1.09 to 1.21 g/ml (d>1.09 g/ml HDL). Lipoproteins of d=1.02 to 1.09 g/ml from cholesterol-fed animals were further fractionated by Pevikon block electrophoresis to obtain HDL with apo E. In this case, the HDL with apo E were isolated from four pools of plasma (3.2 to 4.4 ml), and each pool was obtained from three to four animals whose cholesterol levels ranged from 300 to 840 mg/dl.

Apolipoprotein Isolation

Marmoset apo A-I, proapo A-I, apo A-II, and apo C-III were isolated from d=1.09 to 1.21 g/ml lipoproteins (15 mg of protein) after delipidation with chloroform/methanol (2:1, vol/vol) and gel filtration on Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ). Fractions within each of the two major peaks (S-300 I and S-300 II) were combined, dialyzed exhaustively against 5 mM NH₄CO₃, lyophilized, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Material contained in S-300 I (10 mg) was further purified by preparative isoelectric focusing (IEF) on a flat-bed Multiphor apparatus (LKB Produkter, Bromma, Sweden) as described, but a mixture of equal volumes of narrow-range ampholines (Serva Fine Biochemicals, Westbury, NY), pH 4.5 to 5.0 and 5.0 to 5.5, was used in the present study. Forty percent of the protein was recovered.

The low molecular weight apolipoproteins of marmoset d=1.09 to 1.21 g/ml lipoproteins (S-300 II) were fractionated by ion-exchange chromatography. The lyophilized apolipoproteins (4.5 mg) were solubilized in 0.01 M Tris-HCl, pH 8.0 (containing 6 M urea), and were applied to 8×0.9 cm columns (Amicon, Lexington, MA) of DEAE Sephacel (Pharmacia Fine Chemicals) maintained at 4°C. The column was eluted with a linear gradient of 0.01 to 0.25 M Tris-HCl, pH 8.0, containing 6 M urea. Column fractions were dialyzed exhaustively against 5 mM NH₄CO₃ and were lyophilized.

Apolipoproteins E and A-IV were isolated from HDL with apo E, which was induced by the atherogenic diet. The HDL with apo E (4.5 mg) obtained by Pevikon block electrophoresis of d=1.02 to 1.09 g/ml lipoproteins of the hypercholesterolemic animals were delipidated and fractionated by preparative SDS-PAGE by using a vertical slab gel apparatus (Bio-Rad, Richmond, CA).

The purity of the isolated apolipoproteins was assessed by SDS-PAGE and by analytical IEF. The effect of cysteamine treatment and neutaminidase digestion on the IEF pattern of marmoset apolipoproteins was determined as described. Two-dimensional electrophoresis of nontreated and neuraminidase-treated apo E was performed as described. Ouchterlony immunodiffusion analysis was performed on 1% agarose gels as described.

For amino acid analysis of purified apolipoproteins, samples (15 to 25 μg) were hydrolyzed in 6 N HCl at 110°C for 20 hours in sealed, evacuated ignition tubes and were analyzed on a Beckman 121MB Analyzer (Beckman Instruments, Fullerton, CA) equipped with a Model 126 integrator. No corrections were made for losses due to hydrolytic destruction.

The amino-terminal amino acid sequences of purified apolipoproteins were determined by the automated Edman technique. Samples (100 to 250 μg) in 0.5 ml of 50% acetic acid were applied along with 2 μg of Polybrene to the cup of a Beckman 890C Sequencer. The first cycle was double-coupled; otherwise, a single-coupling, single-cleavage 0.1 M Quadrol program (No. 122974, Beckman Instruments) was used. After conversion to the phenylthiohydantoin (PTH) derivative, PTH-amino acids were identified and quantified by high-performance liquid chromatography as described.

The amino-terminal pyroglutamic acid residue of marmoset apo A-II (200 μg) was removed by the method of Podell and Abraham by using calf liver pyroglutamylaminopeptidase (Boehringer Mannheim, Indianapolis, IN). For the determination of the complete amino acid sequence of marmoset apo A-II, untreated (blocked amino terminus) marmoset apo A-II (250 μg) was digested with a 30-fold weight excess of CNBr in 70% HCOOH for 24 hours at room temperature. The lyophilized digest was dissolved in 0.5 ml of 50% acetic acid, and the entire digestion mixture was subjected to direct sequence analysis.

Results

Gel filtration chromatography of control marmoset apo HDL (d>1.09 g/ml) resulted in the separation of two peaks (Figure 1). Peak I contained a single Mr=27 500 apolipoprotein that eluted in a position similar to that of human apo A-I, comigrated with human apo A-I on SDS-PAGE, and gave a positive reaction when tested by immunodiffusion analysis against a rabbit antihuman apo A-I antiserum (data not shown). Analytical IEF of this protein (Figure 2) demonstrated three isoforms: a major band (pI 5.5), together with a minor, more acidic isoform (~1 unit of charge relative to the major band), and a second minor, more basic isoform (~2 units of charge relative to the major band). This pattern is similar to that...
of human apo A-I but is displaced toward the anode relative to human apo A-I. The amino acid composition of peak I was similar to that of human apo A-I (Table 1), confirming that the apolipoprotein was the marmoset equivalent of human apo A-I. Like human apo A-I, marmoset apo A-I was enriched in glutamic acid, leucine, and lysine and lacked isoleucine and cysteine. The amino-terminal sequence of marmoset apo A-I was determined through residue 54 (Figure 3). Residues 1 to 20, 28 to 34, and 38 to 52 were identical in the marmoset and human sequences, representing an 87% identity between the marmoset and human sequences over the 54 residues. The minor basic isoform had an amino acid composition similar to that of the major apo A-I isoform. However, amino-terminal sequencing of this minor isoform gave the single sequence Arg-His-Phe-Trp-Gln-Gln-Asp-Glu-

The marmoset pi 5.1 apolipoprotein migrated on SDS-PAGE as a single protein of $M_c = 8500$ whose electrophoretic mobility was not affected by $\beta$-mercaptoethanol incubation (data not shown). As shown in Table 1, the amino acid composition of this apolipoprotein closely resembled that of human apo A-II: however, the apolipoprotein lacked cysteine and isoleucine, amino acids found in human apo A-II, and contained arginine, an amino acid not present in human apo A-II. In addition, it contained two residues of methionine, whereas human apo A-II contains only one.

Marmoset apo A-II failed to yield an amino-terminal sequence, suggesting that the amino terminus was blocked because of the cyclization of the amino-terminal glutamine, as has been demonstrated in human and rhesus monkey apo A-II. Digestion of marmoset apo A-II with pyroglutamate aminopeptidase enabled us to sequence residues 2 to 35 (Figure 5). One methionine residue was detected at position 26. The second methionine residue of marmoset apo A-II was located by digesting the blocked protein with CNBr and sequencing the unfraccionated digest. The mixture would be expected to contain the blocked amino-terminal CNBr fragment and two nonblocked fragments. Indeed, no sequence corresponding to the amino-terminal sequence of the protein was detected. Instead, the sequences of two peptides were detected, in approximately equivalent amounts. The first sequence corresponded exactly to residues 27 to 35 previously determined from the deblocked intact protein. By homology to human apo A-II, the second sequence could be aligned with human apo A-II. This positioned the second methionine residue of marmoset apo A-II at residue 68 (Figure 5). Because this second peptide washed out of the sequencer cup after seven cycles, continuing the analysis through 40 cycles of the first peptide allowed unequivocal assignment of residues 36 to 66. From this approach and from the amino acid composition of the protein, the entire sequence of marmoset apo A-II could be deduced (Figure 5). Because the last one or two amino acids at the carboxy terminus of a peptide are usually washed out of the sequencer cup, it was necessary to assume that residues 67 and 77 were phenylalanine and glutamine, respectively, based on homology to the human protein. The assignment of alanine at residue 76 was deduced by taking into account the number of threonine and alanine residues determined by amino acid analysis of the intact protein (Table 1). Unlike human apo A-II, but like rhesus apo A-II, the marmoset apo A-II had serine rather than cysteine at residue 8, and consequently it was not able to form disulfide-linked dimers. The overall identity between the marmoset and human apo A-II sequences was 82%.

The marmoset pl 4.8 apolipoprotein had an apparent $M_c = 10000$, and its pl on IEF gels was not affected by treatment with *Clostridium perfringens* neuraminidase (data not shown). The amino acid composition of this apolipoprotein is given in Table 1. Partial amino-terminal amino acid sequencing of the protein gave the sequence Ser-Glu-Ala-Glu-Asp-Thr-Ser-Leu-. This sequence is identical to that reported for human apo C-III except for the substitution of threonine for alanine at residue 6. Thus, by homology, the marmoset pl 4.8 apolipoprotein is the equivalent of human apo C-III, although sialic acid-containing isoforms were not detected.
Table 1. Amino Acid Composition of Human and Marmoset Apolipoprotein A-I, A-II, and C-III

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<th>Human†</th>
<th>Marmoset§ (n=6)</th>
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*From references 9, 10.  †Assuming same total number of residues/mole as human apolipoprotein (apo) A-I.
‡From reference 11.  §Assuming same total number of residues/mole as human apo A-II. Values in parentheses indicate number of residues obtained by sequence analysis of marmoset apo A-II.
‖From reference 12.  ¶Assuming same total number of residues/mole as human apo C-III. **Determined by performic acid oxidation on quadruplicate samples. ††ND=not determined.

**Propeptide**

Marmoset: Arg-His-Phe-Trp-Gln-Gln-Asp-Glu-Pro-Pro-Gln-Ser-Pro-Pro-Gl

Hunan:

Thr-Val-Tyr-Val-Arg-Asp-Asp-Glu-Val-Leu-Asp-Arg-Glu-

Figure 3. Amino-terminal amino acid sequence of marmoset apolipoprotein A-I. The amino acid differences at specific positions in the human sequence\textsuperscript{11,13} are indicated beneath the lines of the marmoset sequence.

Marmoset apo E, isolated by preparative SDS-PAGE of HDL- with apo E (d<1.09 g/ml) obtained from hypercholesterolemic animals, had an apparent $M_r=37500$ on SDS-PAGE, significantly higher than that obtained for human apo E (34000 to 35000). Mixing experiments (not shown) confirmed the difference in apparent molecular weight. Two-dimensional electrophoresis (Figure 6A) of marmoset apo E demonstrated a single major band with minor acidic isoforms of higher apparent molecular weight, typical of polysialylated forms of apo E.\textsuperscript{18} Digestion of marmoset apo E with neuraminidase shifted these minor isoforms to the major isoform position (Figure 6B), confirming their identity as sialylated isoforms. The pl of the major isoform of marmoset apo E was 5.7; cysteamine treatment (data not shown) did not affect the position of marmoset apo E on IEF gels, indicating that it did not contain cysteine. The amino acid composition of marmoset apo E resembled that of human apo E\textsuperscript{3,10} in that it was rich in glutamic acid, leucine, alanine, and arginine (9.7 mol%) (Table 2). Although cysteine was detected, it amounted to less than 1 residue/mole. It is likely that the presence of cysteine in this preparation of
 apo E represents a contaminant, since cysteamine treatment failed to shift the position of the apo E isoforms on IEF gels, and disulfide dimers were not apparent in preparations of marmoset apo E electrophoresed on SDS-PAGE in the absence of β-mercaptoethanol (data not shown). The homology between the partial amino-terminal sequence of this protein (Lys-Val-Glu-Gln-Val-Leu-Glu-Pro-Glu-Leu-Glu-Pro-Glu-) and that of human, baboon, and cynomolgus monkey apo E confirmed that this protein was marmoset apo E. The marmoset sequence differs from the human sequence at residues 5, 6, 8, and 10, where the human protein has Ala, Val, Thr, and Pro, respectively. It differs from the baboon sequence at residues 5, 6, 10, and 13, and from the cynomolgus monkey sequence at residues 5, 6, and 10. In addition, the marmoset apo E cross-reacted with rabbit antisera raised against human apo E (not shown).

As indicated in the companion study, apo A-IV often appeared as a doublet (M, ~48 000), in which both bands stained with similar intensities. The amino acid composition of a preparation containing both bands was similar to the composition reported for human apo A-IV (Table 2), suggesting that both bands represented marmoset apo A-IV. Both bands also reacted against antisera to human apo A-IV (not shown). In one isolation, we were able to obtain limited amounts of both bands. On IEF gels, both bands gave similar patterns: four to five bands with isoelectric points between 5.5 and 5.8 (data not shown). However, the relative intensity of the isoforms was different with each band. The basis for the molecular weight heterogeneity is not clear.

**Discussion**

Apo A-I, the major apolipoprotein of marmoset HDL, resembled the human protein in molecular weight, amino
acid composition, amino-terminal amino acid sequence, and appearance on IEF. The pattern of apo A-I isoforms demonstrated by the latter technique represented a shift toward the anode that is characteristic of apo A-I of New World primates, in contrast to the cathodal shifts seen in Old World primates.\(^{23}\) Determination of the amino-terminal amino acid sequence of marmoset apo A-I revealed a striking homology to human apo A-I, including the prolines at positions 3 and 4. Humans and several nonhuman primates have two proline residues at these positions, whereas apo A-I from subprimate species has only one, at residue 3; therefore, from position 4 onward the subprimate sequences are one amino acid out of phase with the homologous human protein.\(^{6,10}\) The additional proline at position 4 in human and primate apo A-I may be an insertion mutation. The marmoset was also shown to possess a circulating proapo A-I whose amino-terminal amino acid hexapeptide sequence was identical to that seen in human proapo A-I.\(^{14,16}\)

Apo A-II, the second most abundant protein of marmoset HDL, resembles monomeric human apo A-II in size, pl, and amino acid sequence and in having a blocked amino terminus. However, human apo A-II contains cysteine at residue 6 and consequently can exist as a disulfide homodimer or as a heterodimer complexed with apo E.\(^{24}\) In contrast, marmoset apo A-II, like that of the rhesus monkey,\(^{16}\) contains serine at this site and consequently exists only as a monomer. Among nonhuman primates, only the chimpanzee (Pan troglodytes) has been shown to have cysteine-containing apo A-II.\(^{26}\) The metabolic significance of the presence of cysteine in apo A-II is unknown. Recently, the structure of rat apo A-II has been deduced from a cDNA clone;\(^{26}\) it does not contain cysteine but has aspartic acid at residue 6.

Ion-exchange chromatography of low molecular weight apolipoproteins associated with marmoset HDL resulted in the isolation of a minor peptide whose molecular weight, pl, and amino-terminal sequence were similar to those of human apo C-III. Analogues of human apo C-III have been isolated from the plasma HDL of the vervet monkey (Cercopithecus aethiops)\(^{27}\) and rat.\(^{28}\) Inter-species differences in the degree of sialylation of this protein have been noted: apo C-III has been reported to contain 0, 1, or 2 mol of sialic acid/mole of protein (human),\(^{29}\) 0 or 3 mol of sialic acid/mole of protein (rat),\(^{28}\) or 2 mol of sialic acid/mole of protein (vervet monkey).\(^{27}\) In contrast, marmoset apo C-III did not contain sialic acid. This fact may prove useful in elucidating the significance of apo C-III sialylation. The significance of such sialylation, and indeed the function of apo C-III, is not understood.

### Table 2. Amino Acid Composition of Human and Marmoset Apolipoprotein E and A-IV

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<th>Amino Acid</th>
<th>Marmoset Apolipoprotein E (n=4)</th>
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*Calculated from the sequence in reference 19.
\(^{1}\) Determined by performic acid oxidation of a single sample.
\(^{2}\) Calculated from the sequence in reference 20.
Along with marmoset apo A-II and apo C-III, marmoset HDL contained low molecular weight apolipoproteins of pI 5.8 and 6.2. These apolipoproteins eluted from the DEAE column at ionic strengths between those required to elute marmoset apo A-II and apo C-III, but they were incompletely separated by this technique. Proteins with similar isoelectric points, shown to be analogues of human serum amyloid antigen (SAA), have been detected in vervet monkey HDL, particularly when the animals are subjected to chair restraint procedures. However, preliminary amino acid compositions determined for the pI 5.8 and 6.2 apolipoproteins of marmoset HDL (isolated by preparative IEF) do not show homology to those of human or vervet SAA (data not shown).

The distribution of marmoset apo E within the lipoprotein classes resembles that of human apo E: it is prominent in VLDL and IDL and is present in HDL with apo E isolated by Pevikon block electrophoresis of d=1.02 to 1.09 g/ml lipoproteins. To obtain sufficient marmoset apo E for characterization, we induced HDL-apo E by feeding marmosets a diet rich in cholesterol and saturated fat. The apo E from these hypercholesterolemic animals comigrated on SDS-PAGE with apo E from control marmosets (data not shown), and both had a higher apparent molecular weight than did human apo E (M,=37 500 vs. 35 000, respectively). This higher apparent molecular weight of marmoset apo E is characteristic of New World primates. Electrophoresis of marmoset apo E on two-dimensional gels revealed a single major isoform and a number of minor acidic isoforms of higher apparent molecular weight than the major isoform. These acidic isoforms were susceptible to neuraminidase digestion and thus represent polysialylated isoforms, like those described for human apo E. Although marmoset apo E was polysialylated, we found no evidence of charge heterogeneity due to amino acid interchanges, as is common in human apo E. Determination of the extent of homozymosity of apo E within the marmoset population will require screening of large numbers of animals from different colonies and, perhaps, from the wild. Marmoset apo E had an amino-terminal sequence similar to that of humans, baboons, and cynomolgus monkeys and thus differs from apo E of the dog, swine, and rat.

In addition, the marmoset possessed a M,=46 000 apolipoprotein whose amino acid composition strongly resembled that of human apo A-IV.

In summary, plasma apolipoproteins in the marmoset monkey show a striking similarity to human apo A-I, A-II, C-III, E, and A-IV. This should enable investigators to use existing cDNA probes for these human apolipoproteins to study the expression and regulation of marmoset apolipoproteins in response to a variety of metabolic perturbations. The results from this study and the companion study demonstrate that the marmoset will be a valuable model for human lipoprotein metabolism and atherosclerosis.

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