Expression and Conservation of Apolipoprotein AIV in an Avian Species*

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In birds, intestinally derived lipoproteins are thought to be secreted directly into the portal vein rather than to enter the circulation via the lymphatic system as in mammals. Hepatic clearance of these so-called portomicrons must be rapid, but the protein(s) mediating their catabolism, presumably analogues of the 36-kDa mammalian apolipoprotein E, have not been identified. In searching for such a mediator(s), we have isolated a hitherto unknown 38-kDa protein from chicken serum, which we identified by microsequencing and molecular cloning as a counterpart to mammalian apolipoprotein AIV (apoAIV). Mature chicken apoAIV consists of 347 amino acids, lacks cysteine residues, and displays 57% sequence identity with human apoAIV and, to a significantly lesser extent, with apoAIVs of rodents. This first nonmammalian apoAIV characterized is the smallest homologue reported so far, because of the lack of repeated motifs at the carboxyl terminus with the consensus sequence Glu-Gln-Glu/Ala-Gln, a hallmark of mammalian apoAIVs. Chicken apoAIV (isoelectric point, 4.65) is also considerably more acidic than its human counterpart. Agarose gel electrophoresis revealed that unlike human apoAIV, which migrates to a pre-o-position, chicken apoAIV shows fast a migration. Functional characterization demonstrated that the avian protein is able to activate the enzyme lecithin:cholesterol acyltransferase. Roosters and hens express apoAIV predominantly in the gut, one-fifth as much in the liver, and no other sites of expression are identifiable by Northern blot analysis. Although pronounced intestinal synthesis is common to apoAIVs, the features of the avian protein support the notion that it represents a prototype of an apoprotein that evolved to acquire possibly distinct functions in mammals and birds. Key molecules of lipoprotein metabolism in birds and mammals display many common features but also differ in several aspects. For instance, apolipoprotein (apo) B of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) are well conserved among mammals and in the chicken (1–3). However, whereas mammalian plasma apoB exists in two forms, apoB-48 and apoB-100 (4, 5), the chicken produces only apoB-100. Mammalian apoB-48 production arises from apoB mRNA editing (6–8) that generates, via deamination of the C in position 6538 to a U, an in-frame translational stop codon (UAA) corresponding to residue 2153 (glutamine) of the human 512-kDa apoB-100 (9). This process is not observed in the chicken (10). ApoB-100 but not apoB-48 contains the binding domain recognized by the LDL receptor (11) and related receptors, including those of the chicken (12–15).

A further difference concerns the metabolism of intestinally derived lipoproteins in birds; they are not delivered to the lymphatic system but rather are thought to be secreted into the portal vein as so-called, yet to be isolated, portomicrons (16), which are subject to subsequent rapid uptake by the liver. Separation of chicken plasma lipoproteins has been performed by sequential ultracentrifugation (2), by conventional density gradient ultracentrifugation (1), or by a faster vertical spin technique (17); however, a separate portomicron entity has not been identified during the characterization of isolated lipoprotein fractions. This could be due to a lack of circulating portomicrons in peripheral blood or from difficulties in separating portomicrons from hepathically derived VLDL, as both particles are likely to carry apoB-100 in view of the absence of apoB editing in the chicken (10). Likewise, the mediator(s) of hepatic clearance of portomicrons has not been identified, but candidates for receptor binding are apoB-100, lipoprotein lipase, and/or as yet unknown proteins (18). In addition to apoB-100, mammalian apoE was found to be an in vitro ligand for the chicken homologues of the mammalian LDL receptor gene family (13, 19). Importantly, however, synthesis of apoE in chicken has not been demonstrated to date (1, 2, 20), prompting us to investigate further the possibility of expression of an avian apoE or surrogate protein(s).

Recently, a sea lion apolipoprotein with a molecular mass of 36,053 Da has been identified as apoE (21). This protein is 12 residues longer than its human counterpart and thus represents the largest apoE molecule identified so far. In our search for hitherto unidentified chicken apolipoproteins, we discovered and purified an apoprotein with an apparent molecular mass of 38,000 Da, reminiscent of apoE in the sea lion.

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1 The abbreviations used are: apo, apolipoprotein; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); PCR, polymerase chain reaction; bp, base pair(s).
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However, as reported here, N-terminal sequencing of the protein revealed high homology to mammalian apoAIV (22–26), an apolipoprotein that shares several characteristics with apoE (27). Molecular cloning of the corresponding cDNA and characterization of the protein indeed demonstrates for the first time that apoAIV expression is not limited to mammals.

**Experimental Procedures**

**Animals**—White leghorn laying hens and roosters were obtained from Heindl (Vienna, Austria) and maintained as described previously (28). Adult female New Zealand White rabbits were used for raising antisera in rabbits.

**Lipoprotein and Apolipoprotein Isolation and Antibody Production**—Blood (10 ml) was drawn from the wing veins of laying hens and mature roosters into tubes containing 1 mg/ml EDTA, placed on ice, and plasma was separated by low speed centrifugation (3000 × g) for 20 min at 4 °C. Plasma was adjusted to a density of 1.063 g/ml by adding solid KBr, and the lipoproteins were floated by centrifugation in a TLA 120.2 rotor at 120,000 rpm for 1 h 40 min (29) using a Beckman Optima TLX ultracentrifuge (Beckman Instruments). The d < 1.063 lipoproteins were recovered with a syringe, dialyzed against 10 mM Tris-Cl, 140 mM NaCl, 1 mM EDTA, pH 7.4, and delipidated in ether/ethanol (1:3, v/v) at −20 °C. The 38-kDa apoprotein was purified from the d < 1.063 lipoprotein fraction by preparative SDS-polyacrylamide gel electrophoresis. Although the isolation from this fraction was highly reproducible, subsequent Western blotting analysis (see below) revealed that more than 80% of the 38-kDa apoprotein resided in the d > 1.063 lipoprotein fraction. Rabbit antibodies were obtained by intracutaneous injection of 200 μg of the protein mixed with complete Freund's adjuvant followed by injections of 200 μg of protein each mixed with Freund's incomplete adjuvant 3, 6, and 8 weeks later. Antisera were tested by Western blotting using preimmune serum as control. Human apoAI and apoAIV were isolated as described (30).

**Microsequencing**—The 38-kDa apolipoprotein was subjected to SDS-polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane (Immobilon P, 0.45 μm, Millipore Corp., Bedford, MA). Microsequencing of the protein was carried out essentially as described (31).

**Lipoprotein and Apoprotein Analysis**—To analyze the apoprotein content of plasma lipoprotein fractions, 200 μl of plasma were separated on a Superose-12 column (1 × 30 cm; Amersham Pharmacia Biotech) operated at a flow rate of 0.3 ml/min in 20 mM Tris-Cl, 140 mM NaCl, 1 mM EDTA, pH 7.4. The positions of lipoproteins in the eluted fractions were determined by cholesterol measurement (Boehringer Mannheim). Aliquots of the fractions were separated by electrophoresis on 4–20% SDS gradient polyacrylamide gels, and apoproteins AI, B, and the 38 kDa-apoprotein were identified by Western blotting with specific antibodies.

Lipoprotein fractions containing apoAI or the 38 kDa apoprotein were immunoprecipitated. To aliquots of the fractions were added equal volumes of immunoprecipitation buffer (50 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 0.1% SDS, pH 7.4), and incubation with the respective antisera at a dilution of 1:60 and protein A-Sepharose beads was carried out for 16 h at 4 °C. The protein A-Sepharose beads were washed three times with phosphate-buffered saline. The beads were resuspended in Laemmli sample buffer containing 2% 2-mercaptoethanol, and the precipitated proteins were eluted by heating to 95 °C for 5 min and subjected to electrophoresis on 4–20% SDS gradient polyacrylamide gels. Chicken and human lipoproteins were separated by agarose gel electrophoresis and crossed immunoelectrophoresis was performed using antibodies to human apoAIV (32) and to the chicken 38-kDa protein, respectively.

**Isoelectric Focusing and Determination of Isoelectric Point**—Apolipoproteins were analyzed by isoelectric focusing in 6 × urea-containing gels with the PhastSystem essentially as described (33). Plasma was diluted 50-fold and 1 μl was applied close to the cathode. Isoelectric focusing was performed with a gel containing only one sample lane at one side to determine the position of the protein. Immediately after isoelectric focusing, the lane containing the sample was cut off and subjected to Western blotting. The remaining gel was cut into 2-mm strips and each about 25 μl of sample was applied overnight at 20 °C in plastic tubes containing 1.4 ml of distilled water and the resulting pH was measured (34). The position of the protein was then localized within the established pH gradient and assigned an isoelectric point.

**Western Blotting**—Delipidated apolipoproteins or aliquots of column fractions were separated on 4–20% SDS gradient polyacrylamide gels (35) and transferred electrophoretically to nitrocellulose (36). The nitrocellulose membranes were blocked with 5% powdered milk in phosphate-buffered saline containing 0.1% Tween 20, incubated with antiserum (diluted 1:1000), developed with goat anti-rabbit IgG conjugated to horseradish peroxidase, and detected with the enhanced chemiluminescence method (37).

**Isolation of Human and Chicken Lecithin:Cholesterol Acyltransferase**—LCAT was isolated from human plasma by a combination of ultracentrifugation, affinity chromatography on blue Sepharose, ion exchange chromatography, and hydroxypatite chromatography as described (38). Chicken LCAT was isolated from 200 ml of pooled plasma essentially as described (39) with the following modifications. After ultracentrifugation at a buoyant density of 1.21 g/ml, the clear middle fractions were recovered from the tubes, dialyzed against 0.5 mM NaCl, applied to a phenyl-Sepharose column (2.6 × 12 cm) equilibrated with the same buffer, and eluted with deionized water. LCAT activity was determined by incubating aliquots of the eluted fractions with artificial substrate complexes containing human apoAI-egg yolk lecithin (28–30). Cholesterol fractions containing LCAT activity were pooled, and purifying of the enzyme was continued by DEAE-Sepharose and hydroxyapatite chromatography as described for human LCAT (38).

**Apolipoprotein Cofactor Function for Human and Chicken LCAT**—Apolipoprotein-phospholipid (egg yolk lecithin or 1,2-lysophatidylcholine-β-palmitoyl-γ-oleoyl)-3H-cholesterol complexes as substrates for LCAT were prepared by the cholate dialysis procedure (40) at a molar ratio of 1:2 phopholipid/apoprotein of 150:1. The assays were performed by incubating the substrate complexes with purified enzyme as described (38). At various time points the amount of cholesteryl ester formed was determined in aliquots of the incubation mixture, and the formation of cholesteryl esters was found to be linear for at least 2 h. As human apolipoprotein A1 serves as a cofactor for chicken LCAT as well (39), this apoprotein, complexed to egg yolk lecithin, was used as an internal standard. The reactivity of each set of substrates with a given LCAT preparation was expressed relative to the reactivity with human apoAI-egg yolk lecithin complexes (100%). All determinations were performed in duplicate, and zero time blanks were subtracted.

**cDNA Preparation and PCR Analysis and cDNA Cloning**—Total RNA was prepared by the Trisolve method (Biotex), and poly(A) + RNA was prepared by passing 1 mg of total RNA twice over an oligo(dT) column. First-strand cDNA was prepared by mixing 3 μg of poly(A) + RNA with 0.2 nmol of a random hexamer (Boehringer Mannheim) in a total volume of 10 μl and heating for 10 min at 75 °C. After quick chilling, 0.5 nmol each of dNTPs and 200 units of Superscript II (Moloney murine leukemia virus RNase H-reverse transcriptase from Life Technologies, Inc.) were added, and DNA synthesis was allowed to proceed at 37 °C for 5 min. PCR amplification was carried out in 100 μl final volume of the following reaction mixture: 2 μl of the cDNA solution, 500 pmol of each degenerated primer (see below), 200 μmol each of the deoxyribonucleotides (Amersham Pharmacia Biotech), 10 mM Tris-Cl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 0.01% Triton X-100, 100 μg/ml of bovine serum albumin, 0.01 μg/ml of Taq polymerase (Promega). The reaction conditions were as follows: 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C, 30 cycles in a Perkin-Elmer 480 DNA Thermal Cycler. PCR products were subjected to electrophoresis on a 1.5% agarose gel (SeaKem GTGTM, FMC Bioproducts) and purified using a Qiagen gel extraction kit before cloning into the vector pBluescript II (Stratagene). Phage DNA was isolated by the alkaline lysis method (37). cDNA libraries were generated using T7 and SP6 primers and a Sequenase Version 2.0 kit (US Biochemical Corp.).

For cDNA cloning, we screened a chicken liver Agt11 cDNA library (CLONTECH) in duplicate with a specific 32P-labeled 190-bp PCR fragment (see under “Results”) under the following conditions: 2% SSC, 10−4 Denhardt's solution, 0.1% SDS, 100 μg/ml salmon sperm DNA. Filters were washed in 2 × SSC, 0.1% SDS at 65 °C. Inserts from three strongly hybridizing phases were subcloned into pBluescript II SK + vectors and sequenced.

**Northern Blot Analysis**—Total RNAs (15 μg each) from homogenized organs (intestinal epithelium was scraped off the submucosa with scalpel layers with a scalpel before isolation of RNA) were denatured in 1 ml of denatured glyoxal, 48% dimethyl sulfoxide, and 10 mM sodium phosphate buffer, pH 6.8, for 1 h at 50 °C and separated on a 1.2% agarose gel in 10 mM sodium phosphate buffer, pH 6.8. After transfer to Hybond N membrane (Amersham Pharmacia Biotech), RNA was immobilized by UV cross-linking and filters were hybridized with a specific 32P-labeled 190-bp PCR fragment (see under “Results”) in 1× BSA, 7% SDS,
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RESULTS

Purification and Characterization of a Novel Chicken Serum Protein—Preparation by SDS-polyacrylamide gel electrophoresis of the proteins present in the d < 1.063 g/ml lipoprotein fraction resulted in the isolation of a hitherto unknown apolipoprotein. The relative mobility of this protein on SDS-polyacrylamide gels, corresponding to an apparent molecular mass of 38,000, did not change whether or not the reducing agent dithiothreitol was used (Fig. 1). By microsequencing of the 38-kDa protein, we determined an amino-terminal sequence of Asp-Val-Ser-Tyr-Leu-Ala-Thr-Val-Leu-Trp-Arg-Phe and partial sequences of two tryptic peptides, Leu-Val-Pro-Phe-Ala-Thr-Leu-Pro-Asp-Gln-Val-Ala-Thr-Val-Leu-Trp-Arg-Phe and parallel sequences from the T3 and T7 promoters of the vector. All three of these sequences were highly homologous to apoAIV of human (22) and monkey (25, 26) and to a lesser degree to apoAIV of mouse and rat. Based on this information, we designed a pair of degenerated primers, 5'-CCYGYACARGTS-GCHACHTG-3' (A) and 5'-YTCVTRGRCRAADGGVCAC-3' (B) (IUPAC ambiguity code) for PCR amplification of cDNA. With this primer pair, a 190-bp PCR product was obtained by amplification of chicken liver cDNA. Sequencing of the PCR fragment again revealed high nucleotide sequence homology to that of mammalian apoAIVs. Furthermore, the 190-bp fragment encoded an open reading frame which included both peptide coding sequences used to design the primer pair and overall was highly homologous to known apoAIV sequences. Thus, the PCR fragment most likely represented a newly found avian homologue of apoAIV, prompting us to clone the corresponding full-length cDNA.

In preliminary Northern blot experiments, we had identified liver and intestine as organs expressing the highest levels of the specific transcript. Therefore, a chicken liver Agt11 cDNA library was screened with the PCR-amplified and labeled 190-bp cDNA fragment. The inserts of three positive clones were isolated and subcloned into Bluescript II SK+ vector. We sequenced the largest (1236 bp) insert on both strands, a 1204-bp insert on one strand, and of the third insert, over 420 nucleotides each from both the T3 and T7 promoters of the vector. All of the sequences matched and confirmed that obtained from the 1236-bp insert, which also contained the sequence of the above described 190-bp PCR fragment. The nucleotide and derived amino acid sequences are shown in Fig. 2.

The novel cDNA contains an open reading frame of 1098 bp following a 37-bp 5'-untranslated region. A single ATG (for methionine) codon is followed by a stretch of hydrophobic amino acid residues with a consensus signal sequence cleavage site (41) 19 residues downstream. The mature protein is predicted to contain 347 amino acids with a molecular mass of 38,975 Da. Sequence similarity is highest to human apoAIV at both the nucleotide (66% identity) and protein levels (57% identity) and lower to monkey, rat, and mouse apoAIVs. Over the entire length of human apoE (317 residues), the chicken apoprotein displayed 45% similarity and 23% amino acid identity, respectively. However, as discussed below, the carboxy-terminal glutaminyl- and glutamyl-rich stretch found in mammalian apoAIVs appears diminished or lacking; thus, chicken apoAIV represents the smallest homologue identified to date (Fig. 3). Nevertheless, the protein ends with the same two residues, Glu-Ser, as the mammalian counterparts. The 3'-untranslated region of the cDNA contained a polyadenylation

FIG. 1. Analysis of chicken serum proteins. After isolation by ultracentrifugation of the lipoprotein fraction floating at a density of 1.063 g/ml and dialysis, aliquots were delipidated in ethanol/ether (3:1, v/v), and 50 µg/lane were applied to a 4-20% gradient SDS-polyacrylamide gel in the presence (lane A) or absence (lane B) of 10 mM dithiothreitol. Proteins were visualized by staining with Coomassie Blue. The positions of broad range molecular mass markers (in kilodaltons) (Bio-Rad), apoAI (28 kDa), apoAII, and the 38-kDa protein (apoAIV) are indicated.

FIG. 2. Nucleotide and deduced amino acid sequence of the cDNA for chicken apoAIV. The amino acids are presented in the single-letter code beneath the second base of the codon. The amino acids are presented in the single-letter code beneath the second base of the codon. The amino acids are presented in the single-letter code beneath the second base of the codon.
signal (AATAAA); a poly(A) tail was not present in our clone. The calculated molecular mass of mature chicken AIV is in good agreement with the apparent molecular mass of the plasma protein of 38 kDa upon SDS-polyacrylamide gel electrophoresis (Fig. 1). Thus, the protein does not seem to be significantly glycosylated. Indeed, no potential N-glycosylation sites (NXS/T) could be identified. We noticed a four-amino-acid insert, Glu-Arg-Leu-Ala, at positions 297–300 of the chicken protein, as compared with the human sequence (Fig. 3) and to the sequences of monkey, rat, and mouse apoAIV, respectively (data not shown).

A hallmark of exchangeable apolipoproteins is the presence of 11-residue-long amino acid repeats that have evolved into multiple 22-mer tandem repeat units exhibiting the periodicity of an amphipathic α-helix (42). Many of the repeats have proline residues as the first amino acid, and indeed, most proline residues (10 of 11) in the newly discovered chicken protein are in positions corresponding to multiples of 11-mers, i.e. in the following positions (numbering in the mature protein), with spacing in parentheses: 62, (33), 95, (22), 117, (22), 139, (22), 161, (22), 183, (22), 205, (22), 227, (22), and 249, (33) (Fig. 2). This characteristic feature of the primary structure is in strong agreement with the prediction that the newly identified chicken protein is indeed a functional exchangeable apolipoprotein.

The C-terminal regions of apoAIVs are of particular interest. Namely, the mammalian proteins possess hydrophilic extensions with a high density of glutamyl and glutaminyl residues, typically present as repetitive incomplete -E-Q-E(A)-Q- motifs. The lengths of these motifs account for the size differences of apoAIVs in various species. The contribution of the missing -E-Q-E(A)-Q- sequence in chicken apoAIV (Fig. 3) to the overall hydrophobicity was further evaluated. Calculations of mean residue hydrophobicity and helical hydrophobic moment using a hydrophobicity scale according to Eisenberg (43) revealed that chicken apoAIV is the most hydrophobic apoAIV reported so far, and that it also has the highest hydrophobic moment (data not shown). The higher hydrophobicity is, however, not due to a lack of the C-terminal repeated sequence but rather to the structure of the protein itself. Fig. 4 depicts the hydrophobicities of human and chicken apoAIV versus their sequence, created by using a median sieve filter (44) that reveals the borders of helical domains. Chicken apoAIV has more hydrophobic domains, particularly between residues 150–200, and its hydrophilic domains are smaller. Furthermore, truncation of mammalian apoAIVs at residue 347, thus omitting the repetitive incomplete -E-Q-E(A)-Q- sequences, does not significantly alter their overall hydrophobic properties.

Reactivity of Chicken apoAIV with Chicken and Human LCAT—Incubation of purified LCAT with substrate complexes containing either apoAI-egg yolk lecithin (100% control; Table I) or containing apoAIVs, with egg yolk lecithin or L-a-phosphatidylcholine-L-palmitoyl-g-oleoyl, was used to determine the cofactor function of chicken apoAIV. As shown in Table 1, chicken apoAIV activated both chicken and human LCAT; however, with any combination of substrate and source of LCAT, chicken apoAIV was less potent than human apoAIV. Chicken LCAT was somewhat more active with chicken apoAIV than...
with the human protein regardless of substrate phospholipid. Finally, 1-α-phosphatidylcholine-β-palmitoyl-γ-oleoyl was the better substrate for both enzymes, but only it and not egg yolk lecithin enhanced the activation by human apoAIV of human LCAT beyond that of the avian enzyme.

**Sites of Chicken apoAIV Expression**—Northern blot analysis of RNA from tissues of laying hens and roosters revealed a single 1.2-kilobase transcript in liver and intestine (Fig. 5). apoAIV mRNA could not be detected in brain, heart, lung, spleen, adrenal, kidney, ovary, or testis (data not shown). Control hybridization was performed with a rat glyceroldehyde-3-phosphate dehydrogenase probe hybridizing to a 1284-nucleotide chicken mRNA (45). The chicken apoAIV mRNA is significantly smaller than the 1.7–1.8-kilobase apoAIV transcripts reported for rats (46) and humans (22, 47).

To further characterize the expression of apoAIV in avian liver and intestine, Northern blots were performed on RNA from liver and intestinal mucosa from duodenum, upper and lower jejunum, ileum, and rectum (Fig. 5). In both genders, the highest levels of expression were found in the cranial parts of the gut, whereas only minor expression was found in ileum and rectum. Fig. 5 (upper panel) shows the distribution of apoAIV mRNA from two different regions of the liver as well as from duodenum and upper and lower jejunum of a mature rooster. Standardization based on glyceroldehyde-3-phosphate dehydrogenase hybridization (Fig. 5, lower panel) revealed that apoAIV mRNA levels in liver of both genders are approximately one-fifth of that in the gut (duodenum, upper and lower jejunum). This expression pattern is similar to that in rats and mice (48, 49) but different from humans, where apoAIV expression occurs almost exclusively in the intestine (47).

**Plasma Chicken apoAIV Protein**—The antibody raised against the purified 38-kDa protein reacted with a single protein in unfractionated chicken plasma, allowing us to determine the isoelectric point (pI) by immunoblotting following isoelectric focusing of total plasma. The migration pattern of chicken apoAIV isoforms resembled that of human apoAIV in that there were one major band (pI, 4.65) and two minor more acidic bands (Fig. 6). The measured pl agrees well with that calculated for the mature protein predicted from the cDNA (pI, 4.54), indicating that at 6 M urea the protein exposes all critical charged residues. The results shown in Fig. 6 also confirm that chicken apoAIV is considerably more acidic than its human counterpart (pI, 4.97) (50). Because potential N-glycosylation sites are absent, the two minor bands may arise either by O-glycosylation or by carboxymylation, known to occur in human apoAI (51).

Electrophoresis of total plasma under non-denaturing conditions revealed that the bulk of chicken apoAIV migrates to a position defined as “fast α” for human lipoproteins, i.e. ahead of HDLs (Fig. 7). All detectable apoAIV-containing lipoproteins in chicken and human plasma are present in single peaks; however, human apoAIV migrates entirely to a pre-α-position, i.e. slower than the α-lipoproteins (Fig. 7). The plasma distribution of apoAIV was further investigated by gel filtration on Superose-12 (Fig. 8). apoAIV was detected in two peaks, a minor one migrating between the presumed LDL and HDL fractions, and the major one on the descending part of the HDL (apoAI-containing) peak as shown for the plasma of a rooster. In hyperlipidemic plasma of laying hens the distribution was essentially identical, and the predominant VLDL fraction contained only trace amounts of apoAIV (data not shown). When rooster plasma fractions containing both apoAI and apoAIV were immunoprecipitated with antibodies to apoAI or to apoAIV, only trace amounts of apoAIV or apoAI, respectively, were recovered in the precipitates. This indicates that although parts of the apoAI-containing and apoAIV-containing peaks overlap, there is not a significant amount of particles harboring both apoproteins.
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**DISCUSSION**

Here we describe the identification and molecular cloning of a hitherto unknown chicken protein. We believe that the purified 38-kDa serum protein and the product specified by the isolated cDNA for the first avian homologue of mammalian apoAIV are identical for several reasons: (i) the amino acid sequences at the amino terminus as well as at the carboxyl terminus of the cDNA; (ii) the sequence of a PCR fragment obtained with primers designed according to the protein sequence determined by microsequencing; and (iii) the calculated size of the protein predicted from the apoAIV cDNA (38,975 Da) and the apparent molecular mass determined by SDS-polyacrylamide gel electrophoresis are in excellent agreement. This further supports the notion that the protein does not seem to be significantly glycosylated.

Although human apoAIV was reported to contain 6% carbohydrate by weight (52), no potential N-glycosylation sites are identifiable in the sequence. Furthermore, according to our previous results (53) the human isoform apoAIV-1 is also likely to be a glycoprotein. Chicken apoAIV is the smallest homologue described to date; the mature plasma form consists of 347 amino acids as compared with 371 for rats (23), 478, 375 for mice (24), 376 for humans (22), 397 for baboons (25), and 409 for cynomolgus monkeys (26). On the other hand, chicken apoAIV possesses a 19-residue signal peptide and, like mammalian apoAIV, lacks a propeptide (46). With the exception of one strain of mice (SWR/J) (24, 54), which also has a 19-residue apoAIV prepeptide, 20-residue signal peptides are found in the mammalian proteins. The mature chicken apoAIV has the highest degree of identity with human (57%) and nonhuman primate sequences (51%), whereas the identities with apoAIV of mice and rats are only 39 and 43%, respectively. These differences in sequence identities between avian and primate apoAIV on one hand and the rodent homologue on the other hand might argue for chicken apoAIV being a partially evolved prototype of a new apoprotein, i.e. mammalian apoAIV. Further analyses of homologous genes in a wide variety of species will be necessary to strengthen this view.

Although less potent than human apoAIV, chicken apoAIV is a significant activator of LCAT isolated from chicken and human plasma. However, based on the more pronounced hydrophobicity and relative higher affinity for lipids in comparison to the human protein, more potent LCAT activation might be expected from chicken apoAIV. In this respect, Finer-Moore plots revealed that chicken apoAIV displays much better defined repeated α-helical structure than human apoAIV (data not shown). However, according to De Loof et al. (55), amphiphilic lipid binding helices in apolipoproteins can be separated into two types, helices whose hydrophobicity profile has a maximum at 100° and those whose hydrophobicity periodicity shows a second maximum at about 160°. Chicken apoAIV has a lower mean moment at 165° than human apoAIV or apoAI (data not shown). Because these latter helices play an important role in LCAT activation (55), less effective LCAT activation by chicken apoAIV would be predicted, which is in agreement with our finding (Table I).

Another interesting feature of apoAIVs relates to their highly variable extreme C-terminal regions which contain imperfect repeats specified by the consensus sequence, E-Q-A/V-Q. For instance, in nonhuman primates this region resembles a polyglutamine tail (25, 26). In the chicken, the protein terminates with a single such peptide motif, E-Q-A-E-S. In various strains of mice, insertions or deletions of 12 nucleotides coding for these repeats are responsible for polymorphic variation unrelated to their phylogenetic descent (54). Also, a human insertion/deletion polymorphism has been identified at this site (56, 57). The polymorphisms at the apoAIV locus in baboons and cynomolgus monkeys result in carboxyl-terminal extensions of up to 9 and 12 imperfect E-Q-X-Q repeats, respectively (25, 26). Similar extensions, consisting of -CAG- (Glu) repeats resembling polyglutamine domains, might have additional pathophysiologival significance. For instance, expansions of CAG repeats located within the coding regions of genes have been implicated in the pathogenesis of neurological disorders such as Huntington’s disease (58, 59); however, the exact mechanism underlying pathogenicity is not known.

Similarly, the physiological significance, if any, caused by the different length of E-Q-X-Q extensions has not yet become obvious. Modeling of truncated versions of mammalian apoAIVs lacking the C-terminal repeated sequences does not reveal significantly altered overall hydrophobic properties. The pronounced hydrophobicity of chicken apoAIV, resulting from more hydrophobic and smaller hydrophilic domains along the entire protein, predicts that chicken apoAIV should have a higher lipid affinity. This may be the reason for the presence of

![FIG. 7. Crossed immunoelectrophoresis of plasma from chicken (A) and humans (B). Ten μl of each plasma were applied, subjected to electrophoresis in the first dimension, and developed in the second dimension against rabbit antibodies to chicken apoAIV (A) and human apoAIV (B), respectively, as described under “Experimental Procedures.”](image)

![FIG. 8. Gel permeation chromatography of rooster plasma.](image)
apoA-containing particles floating in the d < 1.063 g/ml density fraction after ultracentrifugation (Fig. 1); however, in the chicken, apoAIV does not seem to be associated with classical VLDL or LDL particles, as crossed immunoelectrophoresis revealed all detectable apoAIV in fractions other than those corresponding to LDL or VLDL (Fig. 7). Furthermore, upon gel filtration of chicken plasma, apoAIV elutes prior to and in the descending part of the HDL fraction, respectively. When fractions containing both apoAI and apoAIV (Fig. 8) were precipitated with antibodies to apoAI or to apoAIV, only trace amounts of apoAIV or apoAI, respectively, were recovered. Taken together, these data indicate that the majority of lipid-associated apoAIV, unlike the human counterpart, is not associated with apoAI-containing lipoproteins. Finally, the electrophoretic migration of chicken apoAIV-containing lipoprotein particles suggests that they are distinct from pre-β-migrating apoAIV-containing particles observed in humans (60). This difference may in part be due to variation in surface charge arising from the presence of acidic lipids in the surface of the particle and from the conformational status of the apoprotein (61). Further experiments to biochemically characterize these lipoprotein fraction(s) in the plasma of roosters and hens which contain apoAIV are now underway.

The expression pattern of chicken apoAIV closely resembles that of lipoproteins which also produce significant amounts of apoAIV in liver (48, 49). Wu and Windmueller (62), using differential labeling of liver and intestinal proteins with [3H]leucine and [14C]leucine, calculated a 41% contribution of the liver to the overall production of apoAIV in rats. The finding of pronounced expression in the gut of the chicken strengthens the notion that intestinal synthesis is a common feature of apoAIV. In this context it is of interest that unlike mammals, birds are thought to secrete intestinally derived lipoproteins directly into the portal vein (16). With the development of apoB editing in mammals, the structure and function of apoAIV directly into the portal vein (16). With the development of apoB editing in mammals, the structure and function of apoAIV might have undergone distinct evolutionary change. A hypothesis that can be tested is that chicken apoAIV could be involved in the formation and secretion of as yet unidentified triglyceride-rich lipoproteins by enterocytes.

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