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Research paper

A novel estrogen-regulated avian apolipoprotein☆

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ABSTRACT

In search for yet uncharacterized proteins involved in lipid metabolism of the chicken, we have isolated a hitherto unknown protein from the serum lipoprotein fraction with a buoyant density of ≤1.063 g/ml. Data obtained by protein microsequencing and molecular cloning of cDNA defined a 537 bp cDNA encoding a precursor molecule of 178 residues. As determined by SDS-PAGE, the major circulating form of the protein, which we designate apolipoprotein-VLDL-IV (Apo-IV), has an apparent M_r of approximately 17 kDa. Northern Blot analysis of different tissues of laying hens revealed Apo-IV expression mainly in the liver and small intestine, compatible with an involvement of the protein in lipoprotein metabolism. To further investigate the biology of Apo-IV, we raised an antibody against a GST-Apo-IV fusion protein, which allowed the detection of the 17-kDa protein in rooster plasma, whereas in laying hens it was detectable only in the isolated ≤1.063 g/ml density lipoprotein fraction. Interestingly, estrogen treatment of roosters caused a reduction of Apo-IV in the liver and in the circulation to levels similar to those in mature hens. Furthermore, the antibody crossreacted with a 17-kDa protein in quail plasma, indicating conservation of Apo-IV in avian species. In search for mammalian counterparts of Apo-IV, alignment of the sequence of the novel chicken protein with those of different mammalian apolipoproteins revealed stretches with limited similarity to regions of ApoC-IV and possibly with ApoE from various mammalian species. These data suggest that Apo-IV is a newly identified avian apolipoprotein.

1. Introduction

Several avian species, especially the chicken (*Gallus gallus*), are used as model animals to study key molecules and molecular mechanisms governing lipid metabolism in oviparous species. One of the most conspicuous aspects of lipid metabolism in birds is the dramatic difference between mature female and male lipoprotein profiles and apolipoprotein (Apo) expression levels, which are related to the physiological adaptations required for laying lipid-rich eggs. Much has been learned about qualitative and quantitative aspects of avian serum lipoproteins and the structure and function of receptors mediating lipoprotein transport ([1–5]). Thus, many of the proteins, particularly Apos, involved in avian lipoprotein metabolism have been identified and functionally characterized, but yet unknown components with significant roles in avian lipid metabolic processes presumably do exist. It should be noted that ApoE, one of the best studied mammalian Apos ([6–8]) is absent in the chicken ([9–11]), and that the existence of a galline ApoA-II gene remains controversial [12]. We have initiated investigations of chicken apolipoproteins with known homologues in mammals, and have described various molecular aspects of ApoB ([13,14]), ApoA-I [15], ApoA-IV ([4,11],), and ApoA-V ([16,17]).

New insights into the spectrum of apolipoprotein components have been gained from a detailed analysis of serum proteins in male and female chickens. The classical example is ApoVLDL-II, discovered by L. Chan and colleagues ([18–21]). To our knowledge, this Apo served as the first system for the study of mechanisms of mRNA translation and induction by estrogen ([18,22,23]). These studies indicated that, as in man, liver and intestine are the major sources of chicken plasma Apos. ApoVLDL-II is under the strict control of estrogen ([18,24,25]), which induces the hepatic synthesis of ApoVLDL-II upon onset of egg-laying. Functional studies on ApoVLDL-II, a protein not found in mammals, have

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revealed its physiological role as an inhibitor of lipoprotein lipase that assures the transport of energy-rich lipoproteins to the egg yolk ([26,27]). In the current study, we have identified and characterized a second chicken apolipoprotein that appears to be absent from mammals. The 17-kDa apoprotein, which we designate ApoVLDL-IV (in short, Apo-IV), is primarily synthesized in liver and intestine, and its plasma levels are higher in mature roosters than in laying hens. A mammalian counterpart of Apo-IV could not be identified, albeit protein sequence alignment of chicken Apo-IV with different mammalian Apos suggested regions with similarity to rabbit ApoC-IV.

2. Material and methods

2.1. Animals

Mature Derco-Brown (TETRA-SL) hens and roosters (30–40 weeks old) were purchased from Diglas Co. (Feuersbrunn, Austria). Fertilized eggs were incubated under standard conditions for temperature (37.5 °C) and humidity (60–70%). Japanese quail of both sexes and 16 weeks of age were purchased from the Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences (Ivanka pri Dunaji, Slovak Republic). The birds were fed a commercial layer mash diet with free access to water and feed at 20 °C with a daily light period of 16 h. Where indicated, roosters were treated by intramuscular injection with 10 mg/kg body weight of 17α-ethinylestradiol (Sigma) (stock solution, 40 mg/ml 1,2-propanediol) either once, or every 24 h for up to 3 times, and euthanized by decapitation for tissue and organ retrieval. Adult female New Zealand White rabbits (Sommer, Wollmersdorf, Austria) and Balb/c mice (Institute of Biomedical Research, Medical University of Vienna, Austria) were obtained from the indicated sources. All animal procedures were approved by the "Animal Care and Use Committee" of the Medical University of Vienna.

2.2. Protein expression and antibodies

A 351-bp cDNA fragment coding for the central portion of chicken apo-IV was cloned into the pGEX-5X-1 expression vector (Amersham Pharmacia Biotech) for expression of a GST-Apo-IV fusion protein. The primers were as follows: forward, 5′-CAGAATTCGGGGCGTGTGGGGCTGAG-3′ (EcoRI site in bold face); and reverse 5′-GCGGCCGCTTACTGCCCCCTCCCTCTCCA-3′ (NotI site in bold face, and stop codon underlined). The recombinant GST-Apo-IV was expressed in Top10 F′ cells (Invitrogen), and, following induction with 3 mM IPTG, was purified under native conditions using Glutathione Sepharose® 4B (Amersham Pharmacia Biotech). Adult female New Zealand White rabbits and female Balb/c mice were used for raising polyclonal antibodies against the GST-Apo-IV fusion protein. Rabbit antiserum against recombinant Apo-IV was obtained by intradermal injections of 250 μg each of antigen as described previously [28]. Mouse polyclonal antiserum against recombinant apo-IV was obtained by 4 intraperitoneal injections of 50 μg each of antigen on days 0, 28, 56, and 84. Antisera were tested by Western blotting using preimmune serum as control. Rabbit Anti-ApoVLDL-II antibody was obtained as previously described [29].

2.3. Lipoprotein isolation

Individual blood samples were collected from the wing veins of laying hens, mature roosters (23G 0.60 × 30 mm needle, 10 ml syringe), and quail (26G 0.45 × 25 mm needle, 2 ml syringe) into tubes containing EDTA (final concentration, 10 mM), and plasma was separated by centrifugation at 3000 × *g* for 15 min at 4 °C. Separation of lipoprotein classes by step gradient ultracentrifugation was performed according to Kelley [30]; 1 ml-fractions were collected from the bottom of the tube, and the density of each fraction was determined. After delipidation, the Apo-IV distribution was analyzed by Western blotting. For the isolation of lipoproteins with densities of ≤1.210 or ≤1.063 g/ml, plasma was adjusted to the respective density by adding solid KBr, and the lipoproteins were floated by ultracentrifugation in a TLA 100.3 rotor at 90.000 rpm for 3 h using a Beckman Optima TLX ultracentrifuge (Beckman Instruments). The VLDL fraction from yolk of freshly layed eggs (yVLDL) was prepared as described [14]. The lipoprotein samples were recovered with a syringe and delipidated in diethylether/ethanol (3:1, v/v) as previously described [29].

2.4. Microsequencing

The lipoprotein fraction of $d \leq 1.063$ of rooster plasma was isolated by ultracentrifugal floatation, delipidated, the residue subjected to SDS-polyacrylamide gel electrophoresis, and blotted onto a polyvinylidene difluoride membrane (Immobilon P, 0.45 mm, Millipore Corp., Bedford, MA). Microsequencing of the 17-kDa protein was performed as previously described [31,32].

2.5. Preparation of triton X-100 protein extracts

Chickens were euthanized as described above and tissues were placed in ice-cold homogenization buffer (4 ml/g wet weight) containing 20 mM HEPES, 300 mM sucrose, 150 mM NaCl, pH7.4, and complete EDTA-free protease inhibitor tablets (Roche), and homogenized with an Ultra–Turrax T25 homogenizer. The homogenates were centrifuged for 10 min at 620 × *g* and 4 °C, and 1/20 volume of 20% Triton X-100 was added to the resulting supernatant. After incubation for 30 min at 4 °C, the mixture was ultracentrifuged using a TLA 100.3 rotor at 50,000 rpm for 1 h using a Beckman Optima TLX ultracentrifuge (Beckman Instruments). Protein concentrations of the extracts were determined by the method of Bradford using the Coomassie Plus assay from Pierce.

2.6. SDS-PAGE and Western Blotting

Plasma, delipidated lipoproteins, and protein extracts were analyzed by one-dimensional 12% SDS-PAGE under reducing (in the presence of 50 mM DTT) or non-reducing conditions, and either stained with Coomassie Blue or electrophoretically transferred to nitrocellulose membranes (Hybond-C Extra, Amersham Pharmacia Biotech) for Western Blotting. Nonspecific binding sites were blocked with TBS (25 mM Tris, 140 mM NaCl, 25 mM KCl, pH7.4) containing 5% nonfat dry milk and 0.1% Tween-20 for 1 h at room temperature. Apo-IV was detected with rabbit anti-GST-Apo-IV antiserum or with mouse anti-GST-Apo-IV antiserum at the indicated concentrations, followed by incubation with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG from Sigma (1:40,000 or 1:1500 dilution), respectively, and developed with the Enhanced Chemiluminescence protocol (Pierce). The sizes of the proteins were estimated using a set of molecular mass standards (10–250 kDa, Bio-Rad).

2.7. cDNA preparation, PCR analysis, and cDNA cloning

Total RNA was isolated using the NucleoSpin® RNAII kit (Marcherey-Nagel), and cDNA was prepared using Superscript™ RNase H- (Invitrogen). PCR amplification was carried out using High Fidelity PCR Enzyme Mix from Fermentas. The sequence of *Gallus gallus* cDNA clone ChEST494i21 (NCBI CR389711) was used for primer design. Primers were: forward, 5′-TATAGGGTCGATGGGGGACT-3′; and

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reverse 5′-CCCCCAAAACAACCCCTC-3′. The reaction conditions were as follows: 1 min 94 °C, 1 min 62 °C, 1 min 72 °C, 3 cycles; 1 min 94 °C, 1 min 58 °C, 1 min 72 °C, 3 cycles; 1 min 94 °C, 1 min 54 °C, 1 min 72 °C, 34 cycles. PCR products were subjected to electrophoresis on a 1% agarose gel and purified using QIAquick® gel extraction kit (Qiagen). Fragments were cloned into the pCR2.1-TOPO vector using the TOPO TA Cloning kit (Invitrogen) and transformed into Top10 cells (Invitrogen). DNA sequencing was carried out by VBC-BIOTECH (Vienna, Austria).

2.8. Northern Blot analysis

Total RNAs (30 μg each), isolated from different laying hen tissues using TRI Reagent® (Molecular Research Center, Inc.), were denatured using glyoxal 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 the membrane was hybridized overnight at 65 °C with a ^{32}P labeled 492-bp PCR-amplified Apo-IV cDNA fragment (Megaprime DNA labeling kit, Amersham Pharmacia Biotech) in 1% BSA, 7% SDS, 0.5 M sodium phosphate buffer pH 6.8, and 1 mM EDTA. The primers used to amplify the probe were as follows: forward, 5′-TCTATAGGGTCGATGGGGGACTTTG-3′; and reverse, 5′-CTGCCCCCTCCCTCTCCAGCGCTCC-3′. Washing was performed at 65 °C in 5% SDS, 40 mM sodium phosphate buffer pH 6.8, 1 mM EDTA, and in 0.5% BSA, 1% SDS, 1 mM EDTA, and 40 mM sodium phosphate buffer pH 6.8. Autoradiography was performed at −80 °C. The relative amounts of RNA loaded were estimated using methylene blue staining of ribosomal RNA.

2.9. Cell culture

Chicken liver cells were isolated from newly hatched chicks of mixed sex as described previously [33] with minor modifications. Tissue slices were digested with a solution of 1 mg/ml type II collagenase (Sigma) to generate a single-cell suspension. The liver cells were grown in monolayer culture in DME medium supplemented with 20 mM Glucose, 5% chicken serum, 1% penicillin-streptomycin, 2 mM L-glutamin, at 37 °C in an atmosphere of 7.5% CO_2/92.5% air. Medium was changed every 24 h. Where indicated, cultured cells were incubated with moxestrol, a synthetic analog of 17-β-estradiol, at a final concentration of 50 nM for 24 h [13].

3. Results

3.1. Identification of a new chicken apolipoprotein

Separation by SDS-polyacrylamide gel electrophoresis of the proteins present in the total lipoprotein fraction of chicken plasma resulted in the identification of a protein, which to our knowledge has not been described previously. As shown in Fig. 1, the major proteins in the $d \leq 1.21$ g/ml fraction of laying hen plasma were ApoB, ApoA-I, and the strictly estrogen-dependent Apo-VLDL-II displaying mono- and dimeric forms ([29,26,27]). The lipoprotein-associated proteins in rooster plasma were predominantly ApoB and ApoA-I, with an additional protein that was apparently absent from the fraction isolated from laying hen plasma. The relative mobility of this protein on SDS-polyacrylamide gels indicated an apparent M_r of 17 kDa (Fig. 1). Microsequencing of the 17-kDa band resulted in four peptide sequences: H_2N-Glu-Thr-Pro-Thr-Pro-Glu-Thr-Pro-Leu-Ala-Pro-Leu-Thr, Arg-Leu-Trp-Gly-Ser-Asp-Val-Gly-Gln-Thr-Val-Gln-Ser-Leu-Leu-Thr-Val-Leu-Arg, Arg-Val-Ala-Glu-Tyr-Gly-Ala-Glu-Val-Glu-Gln-Ser-Val-Ala-Ser-Leu-Ser, and Arg-Trp-Gly-Gln-Tyr-Arg. The sequences of all 4 peptides were represented in the cDNA clone *Gallus gallus* ChEST494i21 (NCBI; GenBank CR389711.1). In addition, 3 in-frame methionine residues upstream of the amino-terminal Glu were identified in the translated sequence. The closest upstream Met is located at position −18, and is separated from the Glu by a peptide with predominantly hydrophobic residues, **M**LLVTVVAAAALLGACGA, possibly representing the signal sequence for secretion, while the other two Met residues were not followed by such sequences. The ChEST494i21 sequence was used to produce a 538 bp cDNA fragment by RT-PCR, using RNA from laying hen small intestine with the primer pair (indicated in Fig. 2) 5′-TATAGGGTCGATGGGGGACT-3′ and 5′-CCCCCAAAACAACCCCTC-3′. Northern blot experiments demonstrated that chicken liver and intestine express the highest levels of the specific transcript with a size of approximately 830 nt. Weak signals were also obtained in kidney, abdominal fat, and adrenal gland after prolonged exposure (Fig. 3). This result is in agreement with the tissue expression of clone ChEST494i21 reported in NCBI's EST Profile, Gga.18119.

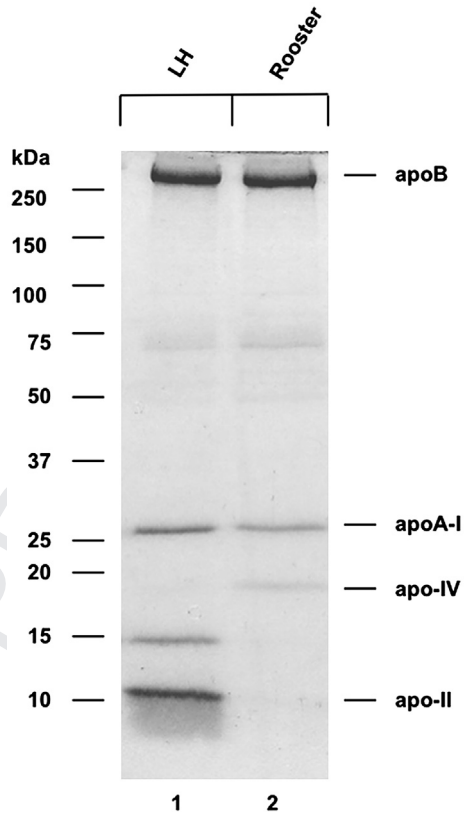

Fig. 1. Analysis of proteins in the lipoprotein fraction of chicken serum. The apolipoproteins present in the serum fraction containing lipoproteins with densities of $d \leq 1.21$ g/ml of laying hen (LH) and rooster serum was analyzed by SDS-PAGE. One μl of the indicated fraction was separated on a 4.5–18% gradient SDS-polyacrylamide gel under non-reducing conditions, followed by Coomassie Blue staining. The positions of the known apolipoproteins, apoB-100, apoA-I, and mono- and dimeric apo-VLDL-II, as well as of the hitherto unknown protein of 17-kDa size (apo-IV) are indicated.

3.2. Generation of antibodies and immunological analysis of Apo-IV in avian plasma

To obtain an immunological tool for the analysis of Apo-IV, the protein was expressed as a GST fusion protein in Top10 F′ cells, purified, and used to generate antisera in rabbits as well as mice. As shown in Fig. 4A, the rabbit antiserum recognized the recombinant GST-Apo-IV fusion protein at 40 kDa and Apo-IV in rooster plasma at 17 kDa; pre-immune serum showed no reactivity. Next, we

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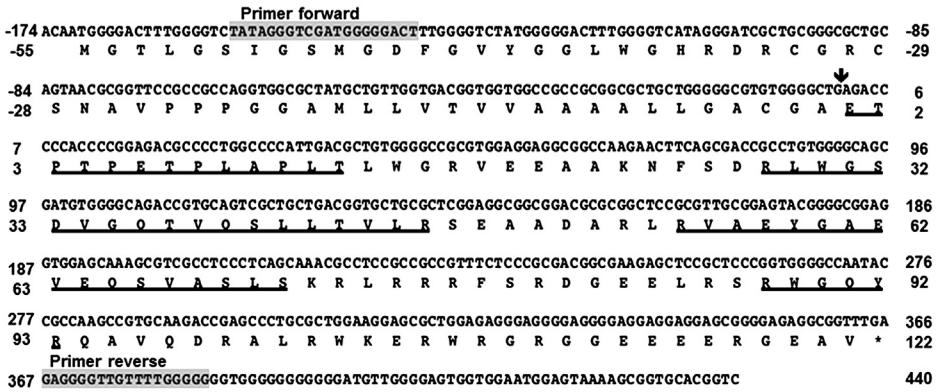

Fig. 2. Nucleotide and amino acid sequences of Apo-IV. The novel protein (see Fig. 1) was isolated from the chicken serum fraction containing lipoproteins with densities of ≤1.063 g/ml. By microsequencing and molecular cloning, we obtained a 537 bp cDNA encoding a 121 residue mature protein. Numbering starts after the predicted signal cleavage site, which is indicated by the arrow. Primers are highlighted in gray, and peptides obtained by microsequencing are underlined.

subjected rooster plasma to gradient ultracentrifugation in the density range from 1.006 to 1.21 g/ml, collected fractions, and tested aliquots by Western blotting for the presence of the protein. Analysis of the plasma fractions with the rabbit anti-GST-Apo-IV antiserum (Fig. 4B) revealed that Apo-IV in plasma is present in 3 fractions at and near the top of the tube, likely representing LDL and VLDL, at densities at and smaller than 1.063 g/ml.

Based on this result, we prepared the $d \leq 1.063$ lipoprotein fractions of the plasma of laying hens and roosters to enrich for Apo-IV. While the protein was indeed clearly present in these lipoprotein fractions of both, hen and rooster (Fig. 5, lanes 1 and 2), the rabbit anti-Apo-IV antiserum detected the 17-kDa protein only in unfractionated plasma of roosters, but not of laying hens (Fig. 5, lanes 3 and 4), in agreement with the results of Fig. 1. In addition, in quail rooster plasma we identified a cross-reactive band, likely representing the quail analog of the galline Apo-IV protein (Fig. 5, lane 5). When the incubation medium contained GST-Apo-IV, no signal was observed (Fig. 5, lanes 6–10), demonstrating the specificity of the immunoreaction; furthermore, preimmune serum showed no reactivity (Fig. 5, lanes 11–15).

3.3. Effect of estrogen

Next, we tested whether hepatic Apo-IV expression is estrogen-sensitive. To directly investigate the effects of estrogen *in vivo*, we treated mature roosters with or without the hormone and analyzed liver extracts by Western blotting. Estrogen treatment of male chickens induces dramatic changes in gene expression levels and hepatic lipid metabolism ([18,34–37]). As shown in Fig. 6A (lanes 1 and 2), hepatic Apo-IV levels in laying hens were much lower than in untreated roosters, as expected; a single dose of estrogen administered to roosters dramatically reduced hepatic Apo-IV within 24 h (compare lanes 2 and 3), after which the level rose and, at 72 h after estrogen administration, reached that observed before treatment (lane 5). In contrast to the single-dose estrogen treatment, multiple estrogen administrations (3 times at 24 h

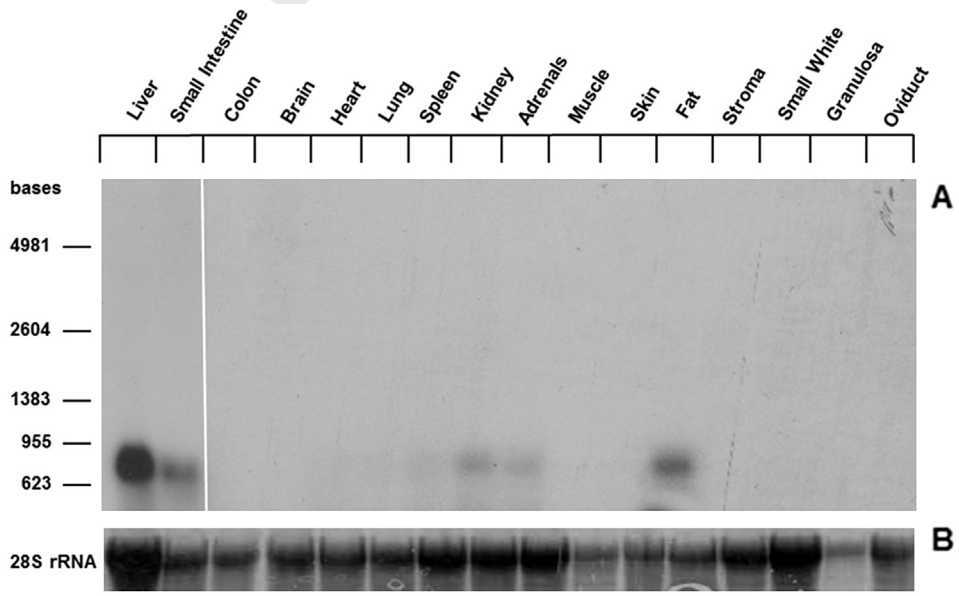

Fig. 3. Northern blot analysis of Apo-IV mRNA tissue distribution. Total RNA (30 μg) isolated from the indicated laying hen tissues was separated on a 1.2% agarose gel and hybridized with a ^{32}P labeled 492-bp PCR fragment. The position of migration of marker RNAs (bases) is indicated. The amount of blotted RNA was visualized by methylene blue staining (lower panel). After 2 d exposure, Apo-IV mRNA was detected in the liver and small intestine (2 lanes on the left). After 5 d exposure, weak signals were obtained in kidney, adrenals, and abdominal fat (lanes to the right). *Stroma*, ovarian follicle tissue; *Granulosa*, granulosa cells isolated from a mature ovarian follicle.

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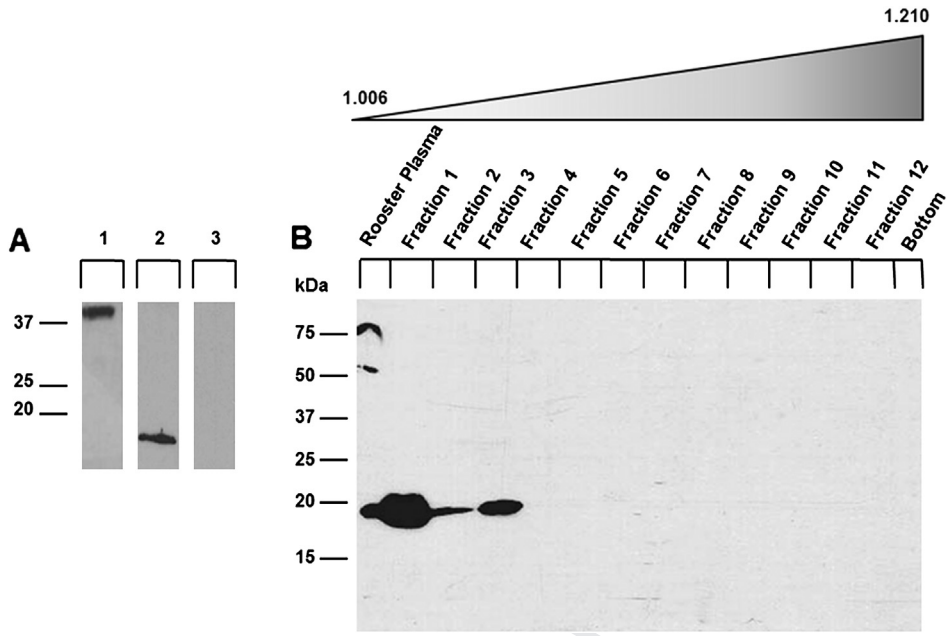

Fig. 4. Immunological analysis of Apo-IV in chicken plasma. (A): Reactivity of GST-Apo-IV antiserum. Lane 1, GST-Apo-IV fusion protein in Top10 cells; lanes 2 and 3, 1 μl rooster plasma. Lanes 1 and 2 were incubated with rabbit anti-GST-Apo-IV antiserum (dilution, 1:250), and lane 3 with rabbit preimmune serum (1:250). For all 3 lanes, peroxidase-conjugated goat anti-rabbit IgG was used as detection antibody. (B): Rooster plasma was subjected to density gradient ultracentrifugation as described in Experimental procedures. Fractions of 1 ml volume were collected from the top of the tube, and 10 ml of each fraction were analyzed by 15% SDS-PAGE under non-reducing conditions. One ml rooster plasma served as control (left lane); fractions 1–12 had densities from 1.006 to 1.210 g/ml; *Bottom*, proteins sedimented in a soft pellet at the bottom of the tube after ultracentrifugation. In all samples, Apo-IV was analyzed by Western blotting with rabbit anti-GST-Apo-IV antiserum (dilution 1:250) and peroxidase-conjugated goat anti-rabbit IgG.

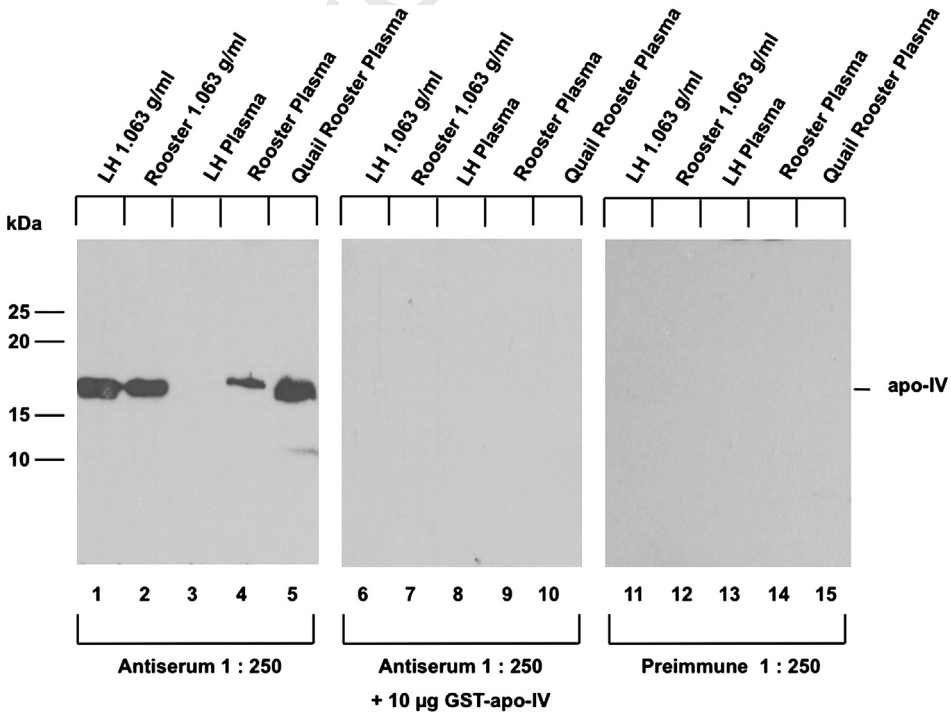

Fig. 5. Detection of Apo-IV in chicken and quail plasma. Plasma lipoproteins with densities of ≤1.063 g/ml were delipidated, and 50 μg apolipoproteins from laying hen (lanes 1, 6, 11) or rooster (lanes 2, 7, 12), or 1 μl of whole plasma from laying hen (lanes 3, 8, 13), rooster (lanes 4, 9, 14), and quail rooster (lanes 5, 10, 15) were separated under reducing conditions on 12% SDS-polyacrylamide gels. After transfer to membranes, the blots in lanes 1–10 were incubated with rabbit anti-GST-Apo-IV antiserum (dilution 1:250) in the absence (lanes 1–5) or presence (lanes 6–10) of 10 μg/ml GST-Apo-IV, followed by peroxidase-conjugated goat anti-rabbit IgG. In lanes 11–15, preimmune rabbit serum was used instead of the anti-GST-Apo-IV rabbit antiserum.

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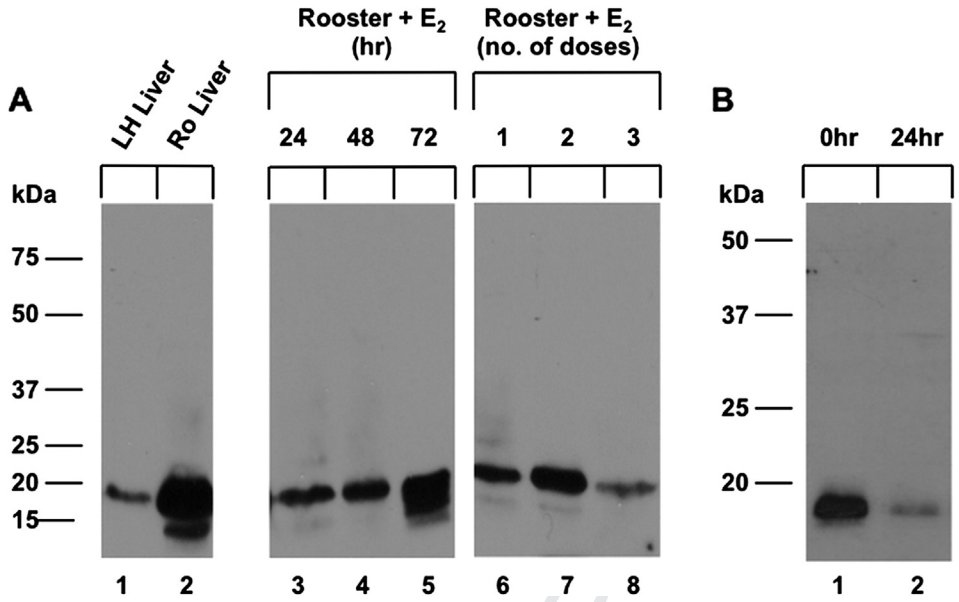

Fig. 6. Effect of estrogen administration on Apo-IV levels in the liver of roosters (A) and cultured hepatocytes (B). (A): Liver extracts (70 μg protein/lane) from laying hen (LH, lane 1), rooster (Ro, lane 2), and roosters treated with estrogen (Ro + E_2; lanes 3–8) were separated by 12% SDS-PAGE under non-reducing conditions. The roosters had been treated as follows: a single dose (10 mg/kg body weight) at 0 h, euthanized at 24 h (lane 3); a single dose at 0 h, euthanized at 48 h (lane 4); a single dose at 0 h, euthanized at 72 h (lane 5); 1 dose at 0 h, euthanized at 24 h (lane 6); 1 dose each at 0 and 24 h, euthanized at 48 h (lane 7); and 1 dose each at 0, 24, and 48 h, euthanized at 72 h (lane 8). Tissue extracts were prepared as described in Experimental Procedures. (B): Cultured primary chicken hepatocytes were harvested before (lane 1) or after incubation for 24 h with 50 nM moxestrol (lane 2). Cell lysates (40 μg protein/lane) were separated by 12% SDS-PAGE in the presence of 50 mM DTT. In (A) and (B), Apo-IV was visualized by Western blot analysis using our mouse anti-GST-Apo-IV antiserum (dilution 1:1500), followed by peroxidase-conjugated rabbit anti-mouse IgG.

intervals) led to a persistently decreased Apo-IV protein level over the entire treatment period (Fig. 6A, lanes 6–8). Furthermore, to gain insight into the effect of estrogen at the cellular level, we isolated primary liver cells from 1- to 3-day old chicks of mixed sex and treated them with the synthetic estrogen, moxestrol. As shown in Fig. 6B, cellular Apo-IV protein clearly decreased after incubation with estrogen for 24 h. These data, together with our observations in roosters in comparison to hens, establish that Apo-IV expression is directly suppressed by estrogen both *in vitro* and *in vivo*.

Finally, we tested whether Apo-IV-containing VLDL particles accumulate in the yolk of oocytes. One important property of yolk-targeted VLDL particles is that they harbor the unique protein ApoVLDL-II [26]. As shown in Fig. 7 for control purposes, laying hen plasma and VLDL isolated from yolk indeed contains ApoVLDL-II, whereas Apo-IV is present only in plasma. Even though in this experiment we used our mouse anti-GST-Apo-IV antiserum, which has greater sensitivity towards the antigen than our rabbit anti-GST-Apo-IV antiserum (see Fig. 5, lanes 3 and 4, where the rabbit antiserum did not detect Apo-IV in hen plasma), Apo-IV in yolk VLDL was not detected by Western blotting. Thus, it appears that Apo-IV-containing VLDL particles are, at least to a significant extent, excluded from uptake into yolk.

4. Discussion

This study describes a hitherto unknown chicken apolipoprotein present in the plasma fraction containing lipoproteins with densities of ≤1.063 g/ml. The levels of the novel 17-kDa protein, which we designate *Gallus gallus* apolipoprotein-VLDL-IV (Apo-IV), differ between hens and roosters, and are negatively regulated by estrogen *in vitro* and *in vivo*. In the course of our extensive literature search for other apolipoproteins that may show negative estrogen responsiveness, we became aware of a study [38], which describes a protein in VLDL of untreated, but not estrogen-treated roosters with a reported apparent size (on SDS-polyacrylamide gels) of

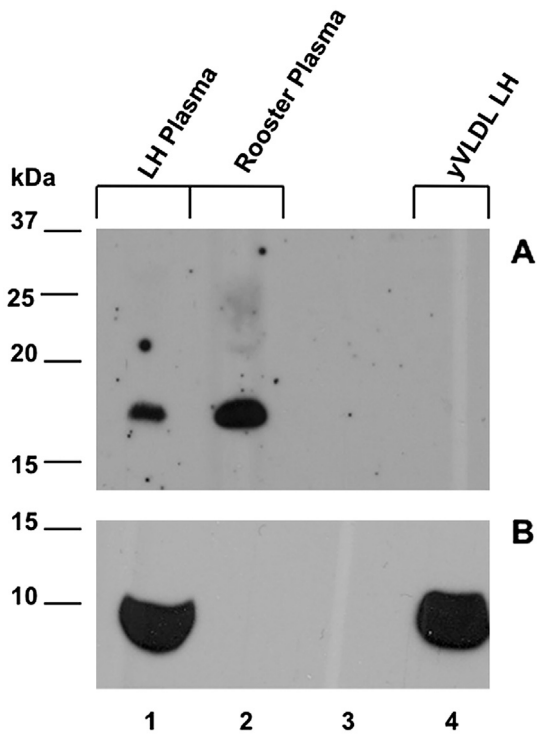

Fig. 7. Yolk VLDL does not harbor detectable amounts of Apo-IV. One μl of plasma from laying hen (LH) or rooster, or 50 μg delipidated VLDL isolated from egg yolk (yVLDL) were separated by 12% (A) or 15% (B) SDS-PAGE in the presence of 50 mM DTT. (A) Western blotting analysis of Apo-IV using mouse anti-GST-Apo-IV antiserum (dilution 1:1500), followed by peroxidase-conjugated rabbit anti-mouse IgG. (B) ApoVLDL-II was visualized by Western blot analysis using rabbit anti-apoVLDL-II antiserum (dilution 1:1500) and peroxidase-conjugated goat anti-rabbit IgG.

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19 kDa (Fig. 2, lanes 4 and 5 in Ref. [38]). In addition, Miller and Lane ([24]) compared the apolipoprotein composition of VLDL secreted by cultured chick hepatocytes under control and estradiol-induced conditions. One particular difference between the two conditions was observed in the levels of a protein whose molecular mass was estimated at 17 kDa. The authors found that the relative secretion rate of this apoprotein decreased from 10 to 4% upon exposure of the hepatocytes to estradiol ([24]). However, we are not aware of any subsequent reports about properties of this/these candidate chicken apolipoprotein(s).

The molecular characterization of the protein identified in the current work revealed several interesting properties. Extensive analyses of DNA- and protein sequence data bases failed to identify a mammalian protein with significant similarity to Apo-IV. There are two stretches with resemblance to ApoC-IV proteins in mammals, particularly in rabbit [39], one of which is a proline-rich motif located at the aminoterminus (identified by protein microsequencing, see Fig. 2) of both proteins. The proline-rich sequences are ETPTPETPLAPL and EPEGTPTPLPAP in Apo-IV and rabbit Apo-C-IV, respectively. The other region of similarity is specified by LWGSDVGQTVQSLLTVLR (Apo-IV) and LVPSSVKELVGPLLTRTR (rabbit Apo-C-IV). However, these two stretches are separated by different numbers of residues in the two proteins (16 in the chicken protein, 6 in rabbit Apo-C-IV). Furthermore, the remaining sequences cannot be aligned in any meaningful way. In man, the gene encoding ApoC-IV is part of a cluster encoding ApoE, ApoC-I, ApoC-IV, and ApoC-II, located on chromosome 19 ([40–42]). Likewise, the genes for human Apo-AI, ApoA-IV, ApoA-V, and ApoC-III form a cluster on chromosome 11q23 [43].

While a gene cluster homologous to that at human 11q23 is located on chicken chromosome 24 (see, e.g. [16]), the question of whether there is an ApoE/C-I/C-IV/C-II cluster in chicken or any other avian species remains unanswered. To address the complexity of this question, we need to consider that while lipid metabolic pathways in birds and mammals share several important properties, they also show significantly different features, particularly in lipoproteins of intestinal and peripheral origin ([4,44],). Accordingly, certain mammalian apolipoproteins are apparently absent from or divergent in aves, and vice versa. *First*, specific differences to mammals include features of avian apolipoprotein B (apoB), which serves an essential role in the assembly and secretion of triglyceride-rich lipoproteins and in lipid transport. In mammals, plasma apoB exists in two forms, i.e. apoB-48, found in intestinally synthesized chylomicrons, and apoB-100 ([45,46]), derived from hepatic synthesis. In the chicken only apoB-100 [47] is produced in both the liver and intestine. ApoB-100, but not apoB-48, contains the binding domain recognized by the LDL receptor, VLDL receptor, and LDL receptor-related protein 1 (LRP1) in mammals and chicken [2]. *Second*, in mammals, apolipoprotein E (ApoE) is a constituent of chylomicrons, chylomicron remnants, VLDL, and specific HDL subclasses (HDL1, HDLc) with high binding affinity for the LDL receptor and other apoB/E receptors ([48–50]). An ApoE or ApoE-like protein in chicken has not been demonstrated to date ([4,9,10]). *Third*, in humans, apolipoprotein A-I (ApoA-I), a major protein constituent of plasma high-density lipoproteins (HDLs), is synthesized only in the liver and intestine [51]. On the other hand, chicken ApoA-I is the major apolipoprotein component of HDL, but it is also found on VLDL, IDL, and LDL, and unique yolk-sac derived lipoprotein particles ([9,52,53]). In adult birds, ApoA-I is synthesized in the liver and intestine [54], but in contrast to mammals, chicken apoA-I mRNA and protein synthesis were also detected in several peripheral tissues such as skin, kidney, ovarian granulosa, and yolk sac endodermal epithelial cells ([10,53,55–57]). *Fourth*, mammalian HDL contains a second major component, apolipoprotein A-II (apoA-II). In chicken, to date an ApoA-II homologue has not been characterized beyond doubt ([12,58]). For instance, the DFCI *Gallus gallus* Gene Index (GgGI) lists TC424643 as the most relevant EST, which is, however, only 38% identical with human ApoA-II in two stretches comprising 71 residues, whereas bona-fide galline apolipoproteins in general show at least 73% identity with mammalian homologues. *Fifth*, human ApoA-IV is synthesized by liver and intestine, and as a prominent component of newly secreted chylomicrons is delivered into the lymph and reaches the plasma via the thoracic duct. Chicken ApoA-IV is synthesized primarily in the intestine ([4,11]); however, in contrast to mammals, in birds intestinally synthesized lipoproteins are not delivered to the lymphatic system. Instead, they are secreted directly into the portal vein as so-called portomicrons [44], which are rapidly taken up by the liver mediated by a yet unidentified receptor. *Sixth*, human ApoA-V, expressed in the liver, plays a key role in the regulation of triglyceride metabolism ([43,59]). In chicken, ApoA-V is expressed in liver and small intestine and also in brain, kidney, and ovarian follicles, and binds to the major chicken LDL receptor family member, LR8 [16]. *Seventh*, the ApoCs comprise four low molecular weight apolipoproteins, designated ApoC-I, -C-II, -C-III, and -C-IV. In mammals, during postprandial lipemia, ApoCs relocate, at least in part, from HDL to nascent chylomicrons and are returned to HDL upon lipoprotein lipase (LPL)-mediated metabolism of chylomicrons [60]. ApoC-I, the smallest apo in mammals, has several clearly defined functions ([61,62]), but a chicken ApoC-I homolog has not been identified to date. ApoC-II plays a crucial role as cofactor of LPL in mammalian as well as galline lipoprotein metabolism ([63–65]). In contrast, whereas human apoC-III is a recognized regulator of lipoprotein metabolism via inhibition of LPL as well as binding of lipoproteins to cell surface heparan sulfate proteoglycans and receptors ([66,67]), the chicken homologue has not been characterized to date. The most recently identified member of the apoC-family is mammalian apoC-IV ([39,68]), which is predominantly found in the VLDL plasma fraction, but at concentrations much lower than that of other apoCs. Again, in the chicken, apoC-IV has not been identified to date. *Finally*, thus far only one apolipoprotein that is absent from mammals has been identified and characterized in birds, i.e., ApoVLDL-II, a unique inhibitor of LPL in laying hens [26]. As mentioned in Introduction, expression of ApoVLDL-II is strictly estrogen-dependent ([18,24,25]), assuring the induction of hepatic synthesis of apoVLDL-II exactly upon onset of egg-laying.

Thus, the above described metabolic features and the available data at the molecular level indicate strongly that chickens lack a gene cluster that could be considered homologous to the mammalian ApoE/C-I/C-IV/C-II region on chromosome 19. Consequently, the similarity between 2 short regions in chicken Apo-IV and rabbit ApoC-IV, albeit intriguing, may merely indicate a distant evolutionary relationship between Apo-IV and mammalian ApoCs, as expected for small lipophilic proteins with modulatory roles in lipoprotein metabolism. In contrast to chicken ApoB and ApoVLDL-II, two key apolipoproteins in the hormone-induced process of egg laying, Apo-IV expression responds negatively to estrogen, a property that likely explains the difference in serum and hepatic levels of the protein in mature roosters and hens (Figs. 5 and 6). The only other known chicken apolipoprotein that shows reduced expression under the influence of estrogen is ApoA-I ([15,69,70]). ApoA-I does not appear to play a direct role in the egg-laying process, but may have an indirect effect on the production of oocyte-directed VLDL particles, which require large amounts of ApoVLDL-II and ApoB for rapid assembly. In this context, the metabolic characteristics of Apo-IV and ApoA-I in the chicken show a further analogy, i.e., their lack of detectable uptake into oocytic yolk (Fig. 7) and [71]. While the majority of VLDL particles are taken up into oocytes via an oocyte-specific chicken homologue of the human VLDL receptor, termed LR8 ([3,72],),

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Walzem et al. [37] have suggested that there is a population of VLDL particles different from this major fraction. The results of Fig. 7 are in agreement with this possibility, as they indicate that VLDL particles containing Apo-IV are excluded from oocytic uptake, possibly by interfering with binding to LR8. The Apo-IV-containing subfraction, however, may well satisfy nutrient requirements of somatic cells, which do not rely on LR8 for lipoprotein uptake ([1]). Such a metabolic role for the ApoIV-containing VLDL particles would be more important in roosters than in hens, where massive estrogen-induced VLDL production provides large amounts of components to oocytes as well as somatic cells. In fact, estrogen-induced ApoVLDL-II is an important regulator of differential lipoprotein flow between somatic and germ cells in laying hens; by analogy, this may be the function of the estrogen-sensitive Apo-IV in roosters. Further investigations along these lines are now underway.

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