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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 (Macherey-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 CR389711.1) was used for primer design. Primers were: forward, 5′-TATAGGGTCGATGGGGGACT-3′; and