Molecular characterization of quail apolipoprotein very-low-density lipoprotein II: disulphide-bond-mediated dimerization is not essential for inhibition of lipoprotein lipase

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As part of the avian reproductive effort, large quantities of triglyceride-rich very-low-density lipoprotein (VLDL) particles are transported by receptor-mediated endocytosis into the female germ cells. Although the oocytes are surrounded by a layer of granulosa cells harbouring high levels of active lipoprotein lipase, non-lipolysed VLDL is transported into the yolk. This is because VLDL particles from laying chickens are protected from lipolysis by apolipoprotein (apo)-VLDL-II, a potent dimeric lipoprotein lipase inhibitor [Schneider, Carroll, Severson and Nimpf (1990) J. Lipid Res. 31, 507–513]. To determine whether this protection depends on dimer formation and constitutes a general mechanism to ensure high levels of yolk triglycerides for embryonic utilization in birds, we have now molecularly characterized apo-VLDL-II in the Japanese quail, a frequently used avian species. Quail apo-

VLDL-II shows 72% amino acid identity with the chicken protein, with most replacements being in the C-terminal region. Importantly, quail apo-VLDL-II lacks the single cysteine residue present eight residues from the C-terminus of chicken apo-VLDL-II, which is responsible for dimerization of the chicken lipoprotein lipase inhibitor. Nevertheless, monomeric quail and dimeric chicken apo-VLDL-II display, on a molar basis, identical inhibitory effects on lipoprotein lipase, underscoring the biological importance of their function. Furthermore secondary structure prediction of the 3'-untranslated region of the quail message supports a role for loop structures in the strictly oestrogen-dependent production of the lipoprotein lipase inhibitors. Our findings shed new light on the essential role of this small, hormonally regulated, protein in avian reproduction.

INTRODUCTION

In egg-laying species lipoprotein metabolism is adapted, on one hand, to facilitate massive transport of nutrients into the growing oocytes, and, on the other, to maintain systemic lipid homeostasis. Very-low-density lipoprotein (VLDL) particles, whose synthesis is massively elevated by oestrogen at the onset of egg-laying [1], function mainly as triglyceride carriers from the liver to the oocytes, where they serve the developing embryo as a major energy source. It is therefore important to ensure that lipolysis of VLDL targeted to oocytes is kept to a minimum. However, just before entering the oocyte proper within the follicle, VLDL passes through the monolayer of ovarian granulosa cells, a major site of lipoprotein lipase (LPL) expression [2]. This paradox has been resolved by the observation that, in chicken, a specific apolipoprotein (apo) on VLDL of egg-laying hens, termed apo-VLDL-II, functions to preserve essentially intact VLDL particles by inhibiting LPL [3]. Endocytosis by the oocyte of the VLDL is mediated by a 95-kDa receptor that is a member of the LDL receptor supergene family and recognizes the apo-B moiety of VLDL [4,5]. Apo-VLDL-II is found exclusively in mature female birds, where its production is under the strict control of oestrogen [6]. The cDNA for chicken apo-VLDL-II encodes a mature 82-residue protein [7] containing a single cysteine residue that forms an intermolecular disulphide bond between two 9.5 kDa subunits. Apo-VLDL-II clearly lacks receptor binding activity [5]. Nevertheless its ability to inhibit LPL on endothelial cells as well as on granulosa cells of the follicle is essential in avian lipoprotein metabolism: it ensures massive triglyceride flow to the growing oocyte.

Interestingly, the protein from chicken seems to be the only apo-VLDL-II so far identified that forms a dimer [8]. This raises the question of whether dimerization of apo-VLDL-II is essential for function. To address this we now have molecularly characterized apo-VLDL-II from the Japanese quail (*Coturnix coturnix japonica*), where it is incorporated into VLDL as a monomer [9]. The cDNA cloning of quail apo-VLDL-II revealed the molecular basis for its structural differences from chicken apo-VLDL-II. Furthermore we investigated in biochemical experiments whether the different structural features are correlated with altered functional properties of this ancient apolipoprotein gene product.

MATERIALS AND METHODS

Animals, diets and lipoprotein isolation

Derco-Brown laying hens and roosters (*Gallus domesticus*) were purchased from Heindl (Vienna) and maintained as described [10]. Roosters (20–30 weeks old) were treated with 17 α -ethinyloestradiol [5]. Japanese quail and quail eggs were purchased from the Institute of Animal Biochemistry and Genetics, Slovak Academy of Science (Ivanka pri Dunaji, Slovak Republic). The birds were reared in an environmentally controlled room (24 °C and 16 h light period) and had free access to both water and diet. Chicken and quail apo-VLDL-II were purified from yolk VLDL [11] as described [5].

Electrophoresis and transfer to nitrocellulose

One-dimensional SDS/PAGE under reducing (10 mM dithiothreitol) or non-reducing conditions was performed by the

Abbreviations used: apo, apolipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; VLDL, very-low-density lipoprotein.

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method of Laemmli [12]. Relative molecular masses of proteins were estimated with the low-range molecular mass standard (3–43 kDa) from Bio-Rad. Protein staining, electrophoretic transfer to nitrocellulose membranes (Hybond-C, Amersham), and Western blotting were performed as described [4].

cDNA preparation, PCRs and sequencing

Total RNA was isolated by the guanidinium isothiocyanate method [13] from the livers of laying hens. Poly(A)⁺ RNA was purified from total RNA by oligo(dT)-cellulose chromatography [14].

cDNA was prepared by using poly(A)⁺ RNA primed with an excess of (dT)₁₅. The reaction was driven with Superscript[®] Moloney Murine Leukaemia Virus reverse transcriptase (BRL) in accordance with the manufacturer's instructions. The cDNA was used as template for PCR reactions (DynaZyme[®]) amplifying the 3' end of the Apo-VLDL-II cDNA with a degenerate oligonucleotide primer, GC(GAT) GC(GAT) TAC AT(TCA) TAC GAA GC(GAT) GT, coding for the C-terminal portion of both quail and chicken apo-VLDL-II, and (dT)₁₅. The resulting product was cloned into the pGEM-T vector (Promega), propagated in *Escherichia coli* JM109 and sequenced.

cDNA coding for the N-terminal portion of apo-VLDL-II was prepared by the use of the degenerate primer AC(ATC) GCT TCG TA. The resulting cDNA was tailed with terminal deoxynucleotidyl transferase and dATP, and the resulting tailed single-stranded cDNAs were used as templates for PRC amplifications with (dT)₁₅ and the degenerate primer CC(TG) TGT TCA C(AC)G CTT CGT AGA TGT A. The resulting products were cloned into the Promega pGEM-T vector; sequencing analysis revealed perfect agreement between the multiple overlapping clones.

The sequences of the extreme 5' and 3' ends of the cDNAs cloned were used to design a third set of primers used to amplify the contiguous quail apo-VLDL-II cDNA. This set of primers had the sequences 5'-CCT CAG CTT CAG CCT GGG AGA GAG and 3'-TTT ATT AGA GGG TCA ATG GCC AAG. The amplifications were performed in triplicate, and each of the resulting products was cloned into pGEM-T and sequenced. The resulting sequences were identical and again in perfect agreement with those of the overlapping partial clones described above obtained by PCR with 5'- and 3'-rapid amplification of cDNA ends.

Sequencing of the cDNA clones was performed in both directions by the dideoxy-chain termination method with Sequenase[®] (USB). A structural model of the 3' untranslated region of the quail apo-VLDL-II mRNA was generated with the RNA Secondary Structure Prediction algorithm of the Intelligenetics PC/Gene[®] program and reconciled with the previously published structure of the 3' untranslated region of chicken apo-VLDL-II mRNA [15].

Lipase assay

For the continuous monitoring of lipoprotein lipase activity we used the fluorogenic alkyl diacylglycerol, 1-trinitrophenylamino-dodecanoyl-2-pyrenedecanoyl-3-O-hexadecyl-*sn*-glycerol, prepared from the corresponding 1-O-trityl-3-O-hexadecyl-*sn*-glycerol as described [16]. Enzyme solutions (5–20 µl of lipoprotein lipase from bovine milk resulting in a final concentration of 120–350 ng/ml [17]) in 0.1 M Tris/HCl, pH 6.5, containing 1 M NaCl were added to 2 ml of the substrate solution (final concentration 2 µM) as described [18]. Rates of lipolysis were

determined from the progressive increase in fluorescence intensity at 378 nm (excitation wavelength 342 nm, slit widths 10 nm each) on a Shimadzu RF-540 spectrofluorophotometer for 5 min at 37 °C. Aqueous solutions of apo-VLDL-II from chicken and quail were added to the substrate equilibrated at 37 °C before lipolysis was started by addition of the enzyme.

Electron microscopy and analytical methods

VLDL preparations were transferred to activated carbon-coated copper grids, washed twice by placing the grids on top of a drop of deionized water, and stained with 1% (w/v) sodium phosphotungstate. Samples were examined in a Philips EM 300 electron microscope with an instrumental magnification of × 35000. Total and free cholesterol levels were determined by using the enzymic CHOD-iodide-method test kit (Merck, Darmstadt, Germany). Enzymic determination of phospholipids and triglycerides was performed with test kits (Bio-Merieux, Marcy l'Etoile, France) as recommended by the manufacturer. Protein concentrations were determined as described by Lowry et al. [19]. The protein concentration of samples containing lipoproteins or detergent was determined with a modified Lowry procedure [20].

RESULTS

Quail apo-VLDL-II, by analogy with the apoprotein from all other fowl except the chicken, has been reported to exist only in the monomeric form on isolated VLDL [9]. To confirm that this result, obtained by analysis of plasma VLDL purified by sequential ultracentrifugation, was not due to a procedural artifact, we characterized apo-VLDL-II in total plasma and yolk by Western blotting and compared the results with those obtained with isolated VLDL. We used a rabbit antiserum [5] that recognizes dimeric and monomeric chicken apo-VLDL-II and cross-reacts with the quail protein. As shown in Figure 1, total plasma and

Figure 1 Western blot analysis of apo-VLDL-II in serum and egg yolk from laying quail and chicken

Serum and yolk proteins were separated by one-dimensional SDS/PAGE on a 15% (w/v) slab gel under non-reducing conditions and transferred to nitrocellulose. Apo-VLDL-II was visualized with a polyclonal rabbit IgG (0.18 µg/ml) directed against purified chicken apo-VLDL-II. Bound antibodies were detected with Protein A-horseradish peroxidase and the chemiluminescence system as described in the Materials and methods section. Lane 1, laying-quail serum (50 µg of protein); lane 2, quail egg yolk (50 µg of protein); lane 3, laying-chicken serum (25 µg of protein); lane 4, chicken egg yolk (10 µg of protein); lane 5, oestrogen-treated rooster serum (10 µg of protein); lane 6, control rooster serum (25 µg of protein). The positions of marker proteins are indicated.

Figure 2 Electrophoretic analysis of purified apo-VLDL-II from quail and chicken

Apo-VLDL-II samples were purified from quail and chicken serum and egg yolk as described in the Materials and methods section. Proteins were subjected to one-dimensional SDS/PAGE on a 15% (w/v) slab gel under reducing and non-reducing conditions as indicated. Protein bands were revealed by Coomassie Blue staining. Purified protein (10 μ g per lane) from the following sources were applied: lane 1, quail serum; lane 2, chicken serum; lane 3, quail egg yolk; lane 4, chicken egg yolk. Migration positions of the molecular mass markers are indicated.

total yolk from laying quail hens contain an immunoreactive band with a relative molecular mass of approx. 6 kDa under non-reducing conditions. Abnormal electrophoretic mobility has been reported previously for the 9.5 kDa apo-VLDL-II monomer [21]. In contrast, when laying-chicken hen serum (Figure 1, lane 3) and yolk (Figure 1, lane 4) were analysed under non-reducing conditions, only the apo-VLDL-II dimer (migrating at approx. 15.5 kDa) was present. As further controls we used sera from oestrogen-treated (lane 5) and untreated (lane 6) roosters. Because the synthesis of apo-VLDL-II is under the strict control of oestrogen, immunoreactive protein was completely absent from untreated roosters.

Quail apo-VLDL-II in purified VLDL from yolk and serum has the same electrophoretic mobility as apo-VLDL-II in unfractionated yolk and serum (compare Figure 2, lanes 1 and 3, with Figure 1). Significantly, it co-migrates under reducing as well as non-reducing conditions with the monomer of chicken apo-VLDL-II, which can be generated only by reduction (Figure 2, lanes 2 and 4); reduction of the quail protein did not alter its electrophoretic mobility.

To gain an understanding at the molecular level of the reason for this different property of quail apo-VLDL-II, we cloned its cDNA as described in the Materials and methods section (Figure 3). Quail apo-VLDL-II cDNA codes for a 105-residue pre-

Figure 3 Nucleotide and deduced amino acid sequence of the cDNA encoding quail apo-VLDL-II

The nucleotide sequence of quail apo-VLDL-II is shown, together with the deduced amino acid sequence. Numbering of the nucleotides begins with the first residue of the initiation codon, with negative numbers referring to the 5'-untranslated region. Numbering of the amino acid sequence starts at the methionine residue corresponding to the initiation codon. Differences in the chicken nucleotide sequence are shown above the quail sequence and differences in the deduced amino acid sequence of chicken apo-VLDL-II are shown below the quail sequence. The open arrowhead indicates the cysteine residue responsible for homodimerization of chicken apo-VLDL-II. Sequences shown in bold are found in the 3'-untranslated regions of both apo-VLDL-II and vitellogenin cDNAs. The underlined sequence is present in several copies in the 3'-untranslated region of the transferrin receptor cDNA.

Figure 4 Effect of chicken and quail apo-VLDL-II on lipoprotein lipase activity

Activity of bovine milk lipoprotein lipase was determined at 37 °C with fluorogenic alkyldiacyl glycerol (2 µM) as substrate in 0.1 M Tris/HCl, pH 7.4, containing 2 mM octylglucoside. Purified apo-VLDL-II from chicken (▲) and quail (■) was added to the indicated final concentrations as described in the Materials and methods section. Results are expressed as percentages of control activity (in the absence of apo-VLDL-II). Data points are mean values from three independent experiments; standard deviations are shown as error bars.

protein; the mature protein is 81 residues long. The nucleotide sequence of the coding region of quail apo-VLDL-II cDNA shows 92% identity with that of chicken; the two proteins are identical in 72% of the positions, with most of the differences in the C-terminal one-quarter. The most noticeable difference is the exchange of the cysteine residue at position 75 of the chicken protein for a tryptophan residue in the quail homologue. This cysteine residue is responsible for the dimerization of chicken apo-VLDL-II, and its absence renders the quail protein monomeric.

The 3'-untranslated region of the quail cDNA, 65 nt longer than that in chicken, is highly homologous with the chicken cDNA. Importantly, it shares sequences also present in the 3'-untranslated region of the chicken vitellogenin cDNA (Figure 3, sequences in bold) [22], which have been implicated in the control of the oestrogen-mediated stability of chicken apo-VLDL-II mRNA [23]. Analysis based on a secondary-structure prediction model (results not shown) revealed that looped structures in chicken apo-VLDL-II mRNA are very similar to those predicted for the quail mRNA. Sites in the 3'-untranslated regions believed to be cleaved in response to oestrogen withdrawal [23] are conserved; in fact, the quail mRNA contains an extra copy of these sequences (nt 501–512).

Because these biochemical and genetic data demonstrated that quail apo-VLDL-II cannot form disulphide-linked dimers, we next wished to determine whether such dimerization is essential for its function as lipoprotein lipase inhibitor. We purified apo-VLDL-II from chicken and quail yolk (Figure 2) and used them in a newly developed lipoprotein lipase assay. In accordance with our previously obtained results with a different assay procedure [3], increasing concentrations of purified chicken apo-VLDL-II markedly decreased substrate lipolysis by bovine milk lipoprotein lipase (Figure 4). Half-maximal inhibition was observed at approx. 2 µg/ml protein, and concentrations of 5 µg/ml almost completely abolished the action of the lipase. With purified quail apo-VLDL-II, inhibition of enzyme activity was found at even lower protein concentrations (Figure 4); 1 µg/ml was sufficient to inhibit lipolysis by 50%. Because, on the basis of protein

Table 1 Chemical composition of VLDL from laying chicken and laying quail

VLDL was purified by sequential ultracentrifugation from plasma of laying chickens (LH) and laying quail (Q), respectively. The chemical composition of the particles was measured as described in the Materials and methods section. Results are mean values from triplicate determinations and are given in percentages by weight. Abbreviations: TG, triglycerides; PL, phospholipids; FC, free cholesterol; CE, cholesteryl esters; n.d., not detectable.

Source	Protein	TG	PL	FC	CE
LH-VLDL	10.0	69.2	16.6	4.2	n.d.
Q-VLDL	9.3	69.2	15.4	4.9	1.2

Figure 5 Ultrastructural analysis of VLDL from quail and chicken

VLDL particles were purified by sequential ultracentrifugation from laying-quail (A) and laying-chicken (B) sera as described in the Materials and methods section. Particles were analysed by negative-stain electron microscopy at a magnification of $\times 110\,200$.

concentrations, quail apo-VLDL-II was twice as effective as chicken apo-VLDL-II, on a molar basis the quail monomer's inhibitory action is indistinguishable from that of chicken apo-VLDL-II.

Having demonstrated that lack of dimerization of apo-VLDL-II did not abolish its ability to inhibit lipoprotein lipase, we tested whether quail VLDL particle size or composition would be altered in comparison with those of chicken VLDL. Thus we analysed VLDLs purified by sequential ultracentrifugation from mature female chicken and quail plasma. SDS/PAGE of these preparations revealed that apo-B and apo-VLDL-II (dimer in chicken; monomer in quail) were the sole protein components

(results not shown). Importantly, the chemical compositions of laying-chicken and quail VLDLs showed essentially no differences (Table 1). Interestingly, the proportion of cholesteryl esters, which constitute a significant component of human VLDL [24], is very small in chicken VLDL (1.2%) and undetectable in quail VLDL.

On comparative electron microscopical inspection of laying-quail and laying-chicken VLDLs (Figure 5), both preparations appeared as spherical particles of uniform size. The mean diameter [determined on 50 particles from each source (results $\pm$ S.E.M.)] of quail VLDL was somewhat larger (427 ± 4.7 nm) than that found for laying-chicken VLDL (361 ± 3.0 nm), in good agreement with earlier reports on particle size of laying-chicken VLDL isolated from plasma and yolk respectively [11,25]. Importantly, from a physiological point of view, both lipoproteins are sufficiently small to allow their passage through the granulosa cell basement membrane [3].

DISCUSSION

We have previously begun to extend our investigations on genes of lipoprotein metabolism in egg-laying species from the chicken to quail [26] and *Xenopus* [27]. These studies are important (1) to test whether metabolic and genetic principles defined in a particular species are unique or commonly applicable, and (2) to gain insight into evolutionary strategies for diversification and/or conservation of functional units. So far we have characterized the oocyte receptors for yolk precursor uptake in chicken [4], *Xenopus* [27] and quail [26]. Here we present the molecular basis for a significant structural difference between chicken and quail in an apolipoprotein involved in egg laying, and show that this difference does not influence the function of the protein.

Apo-VLDL-II is a major component of hepatically derived VLDL in mature female birds, where its expression has been shown to be strictly dependent on oestrogen [6]. This strict dependence is due to efficient control at the levels of both transcription and mRNA stability [23]. Degradation of chicken apo-VLDL-II mRNA is regulated by oestrogen-dependent binding of a protein to the 3' end of the transcript containing several predicted loop structures in the AU-rich regions. On oestrogen withdrawal these sequences become the target of exonucleases, affording further message degradation. Interestingly, analysis of the 3'-untranslated region of the quail apo-VLDL-II transcript predicts conservation of such loop structures, further suggesting that the regulatory principles underlying oestrogen-mediated induction of apo-VLDL-II production in chicken and quail are similar, if not identical.

As originally demonstrated in chicken [3], the main recognized function of apo-VLDL-II in laying hens is to inhibit lipoprotein lipase, thereby preventing the loss of triglycerides from VLDL on their way from the liver to growing oocytes. The import of VLDL, and not LDL, into the oocytes ensures the accumulation of triglycerides in the female germ cell for subsequent energy utilization by the developing embryo [28]. In chicken, apo-VLDL-II is a homodimer formed by a disulphide bond between the single cysteine residues at position 75 of the mature protein. We now know that in quail apo-VLDL-II, Cys-75 is changed to Trp, and the protein does not contain any other cysteine residue. This property may not be unique for quail because apo-VLDL-II from other birds apparently also lack cysteine [8]; however, confirmation by molecular cloning of these proteins has not been reported.

We tested whether this structural difference would have implications for the function of the apolipoprotein in quail, i.e.

would change its ability to inhibit lipolysis. The effects on lipolysis of purified apo-VLDL-II from chicken (a dimer) and quail (a monomer) were determined with a newly developed, highly sensitive lipase assay [18], which facilitates the rapid and reliable measurement of reaction rates in the absence of activating cofactors. Under the conditions used, both avian apolipoproteins markedly inhibited the activity of lipoprotein lipase. Importantly, when the inhibitory effects are plotted as functions of the molar concentrations of the homologous apo-VLDL-IIs, their effects are indistinguishable. This suggests that apo-VLDL-II exerts its inhibitory effect in a strictly stoichiometric manner and does not require dimerization of the inhibitor. However, we cannot exclude that cysteine-less apo-VLDL-II from quail or other birds form functional dimers on association with lipid. Chemical analysis of VLDLs from chicken and quail clearly shows that the total protein content in both particles is indistinguishable. Because VLDL particles contain only one copy of apolipoprotein-B100, we can assume that both VLDLs also carry similar numbers of apo-VLDL-II monomers, and thus, as the case may be, of functional dimeric structures.

The dimerization via a disulphide bridge of this apolipoprotein in chicken could be an evolutionary variation that results in a functional product by a different mechanism. Alternatively it could be the manifestation of evolutionary pressure in that a protein that normally requires lipid for formation of a functional dimer became dimeric by acquisition of a cysteine residue; at the DNA level, the relevant difference is a single exchange of G for T. The results of further studies on evolutionary aspects of this interesting and important apolipoprotein, in combination with biochemical and physicochemical investigations, are expected to distinguish between these possibilities.

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