

Apolipoprotein specificity of the chicken oocyte receptor for low and very low density lipoproteins: lack of recognition of apolipoprotein VLDL-II

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Abstract At onset of egg-laying in the chicken, plasma levels of apolipoprotein VLDL-II (apoII) increase dramatically, suggesting a function of apoII in yolk deposition of triglyceride-rich lipoproteins. Thus, the possibility that this female-specific homodimeric protein (M_r of subunit, 9500) is recognized by the oocyte receptor for low and very low density lipoproteins was investigated. ApoII was purified from very low density lipoproteins by a novel, rapid procedure and reconstituted with egg phosphatidylcholine (PC) by detergent-dialysis. The resulting discoidal apoII/PC lipoprotein particles contained 3 mg of apoII per mg of PC and had a buoyant density of 1.062 g/ml. The ability of apoII/PC, as well as of physiological particles containing apoII but devoid of apolipoprotein B (apoB), namely high density lipoproteins (HDL) from laying hens, to interact with the oocyte receptor was tested. Both of these ligands failed to show saturable high affinity binding, in contrast to the apoB-containing ligands, low and very low density lipoproteins. Furthermore, neither laying-hen HDL which contain apoII and apoA-I nor apoII/PC were able to displace receptor-bound apoB-containing lipoproteins, as shown in competitive binding assays as well as by ligand blotting. **■** Thus, we conclude that apoB, but not apoII, participates in binding and uptake of very low density lipoproteins via receptor-mediated endocytosis by growing chicken oocytes. — Nimpf, J., R. George, and W. J. Schneider. Apolipoprotein specificity of the chicken oocyte receptor for low and very low density lipoproteins: lack of recognition of apolipoprotein VLDL-II. *J. Lipid Res.* 1988. **29**: 657-667.

Supplementary key words reconstitution • endocytosis • apoB

We are concerned with the mechanisms underlying the control of oocyte growth. In oviparous (egg-laying) species, the developing embryo is absolutely dependent on the egg components for its nutritional requirements. The major food source is the lipid-rich yolk whose components, at least in avian species, are synthesized in and secreted from the liver under the control of estrogens (1, 2). The quantitatively most important yolk proteins are the lipophosphoglycoprotein, vitellogenin (VTG), and the triglyceride-rich very low density lipoproteins (VLDL). Specific receptors in the plasma membrane of the growing

oocyte are thought to mediate the uptake of these complex macromolecules (3-5), and possibly of other transport proteins, such as transferrin (6, 7), riboflavin-binding protein (8-10), retinol-binding protein (11), thiamin-binding protein (12, 13), certain biotin-binding protein(s) (14), cobalamin-binding protein (15), and cholecalciferol (16).

We have chosen the chicken as our experimental system for the study of receptor-mediated endocytosis and its role in yolk deposition for several reasons. Most importantly, from an experimental viewpoint, because chicken oocytes grow extremely fast: within the last 7 days before oviposition, about 10-15 g of protein are deposited (4, 17). In addition, hepatically synthesized yolk proteins, in particular VLDL and VTG, are highly amenable to isolation from the plasma of laying hens or estrogen-treated roosters. Finally, many of the minor yolk proteins, such as vitamin-binding and iron-transport proteins have been purified and characterized from both egg yolk and plasma. It is reasonable to expect that these giant cells express high levels of the endocytotic machinery required for massive transport, facilitating biochemical studies.

The chicken system also offers the possibility to gain insight into processes of general physiological interest. Of primary significance is the dual role of estrogens; they stimulate hepatic output of the lipid-rich particles, VLDL and VTG, which have detrimental consequences if not cleared from the bloodstream, as indicated by the rapid formation of aortic atherosclerotic lesions in estrogen-treated roosters (18) and in chickens with hereditary hyperlipidemia (19). Conversely, the reproductive effort of the female is completely dependent on the very same lipoproteins. Of foremost interest are the apolipoproteins

Abbreviations: apoII, apolipoprotein VLDL-II; CR, control rooster; ER, estrogen-treated rooster; LH, laying hen; VTG, vitellogenin; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; OG, octylglucoside.

of VLDL and low density lipoproteins (LDL), in that both particle classes of the chicken contain apoB, which is apparently highly homologous to its human counterpart (M_r , 512,000). However, at onset of egg-laying in the hen, and following administration of oestrogen to roosters, the hepatic synthesis of an apolipoprotein apparently uniquely found in birds, termed apolipoprotein-VLDL-II (apoII), is induced dramatically. ApoII, a homodimer of 9.5-kDa subunits (20) becomes associated with secreted VLDL particles, leading to a several hundred-fold elevation of plasma apoII. The synchronization of onset of oviposition with apoII induction suggests that this apoprotein may play a role in directing VLDL to the growing oocyte, i.e., in recognition of VLDL by oocyte receptors.

We have recently started to investigate the structure and function of oocyte growth-mediating receptors of the chicken and have succeeded in the identification and characterization of the receptor for VLDL (21). This membrane protein has an apparent M_r of 95,000 as determined by SDS-PAGE under non-reducing conditions, and shares many properties with the mammalian apoB_E-(LDL-) receptor. Here, we have analyzed the specificity of the oocyte receptor with respect to the major apolipoproteins found in laying hen plasma. We also report a novel rapid procedure for the purification and reconstitution of apoII.

MATERIALS AND METHODS

Materials

We obtained Protein-A Sepharose and Sephadex G25 columns PD-10 from Pharmacia; egg phosphatidylcholine (Cat. No. P-5763), octyl- β -D-glucoside, PMSF, leupeptin, aprotinin, 17 α -ethinylestradiol, bovine serum albumin (Cat. No. A-7030), Freund's adjuvant (complete and incomplete) were from Sigma; M_r standards were from BRL; acrylamide, bis-acrylamide, and glycine were from Schwarz/Mann Biotech; nitrocellulose paper BA 85 was from Schleicher and Schuell, Keene, NH; Nuflow cellulose acetate membrane filters N25/45 were from Oxoid Ltd., England; goat-anti-rabbit IgG (Cat. No. 0612-3151) was from Cooper; and Na¹²⁵I iodide was from Edmonton Radiopharmaceutical Centre, Edmonton, Canada. Other materials were obtained from previously reported sources (22).

Preparation of chicken lipoproteins

Animals and diets used were previously described (21). Lipoprotein fractions were isolated by differential ultracentrifugation from the plasma of laying hens (LH), untreated roosters (CR), and roosters that had received 17 α -ethinylestradiol dissolved in propyleneglycol (50 mg/kg

body weight, single intramuscular injection) 4 days prior to the experiment (ER). Plasma was prepared and VLDL was purified as described previously (21).

For isolation of high density lipoproteins (HDL) from LH, the bottom fraction obtained after isolation of VLDL was adjusted to a density of 1.04 g/ml by the addition of solid KBr. After centrifugation at 150,000 g_{av} for 12 hr at 4°C, the resulting bottom one-third of the tube contents was adjusted to a density of 1.21 g/ml, and the HDL was floated by centrifugation at 320,000 g_{av} for 24 hr at 4°C.

VLDL and HDL from CR were prepared as described above. CR-LDL was isolated by sequential centrifugations at d 1.006 g/ml (bottom recovered) and d 1.060 g/ml (top recovered), followed by equilibrium density gradient centrifugation. The KBr gradients were prepared as follows, from bottom to top: 3 ml of d 1.15 g/ml solution; 3 ml of d 1.06 g/ml solution; 3 ml of d 1.02 g/ml solution; and 3 ml of the LDL sample that had been dialyzed against buffer containing 0.15 M NaCl, 0.2 mM EDTA, pH 7.4; the same buffer was used to prepare the KBr solutions. Centrifugation was performed in a Beckman SWTi 40 rotor at 39,000 rpm for 12 hr at 4°C. LDL was recovered at a position approximately 4 cm from the top of the tube. Lipoproteins were labeled with ¹²⁵I by the iodine monochloride method as previously described (23). If not indicated otherwise, all lipoproteins were extensively dialyzed against buffer containing 0.15 M NaCl, 0.2 mM EDTA, pH 7.4, and stored at 4°C. Lipoprotein concentrations are expressed in terms of protein content (24).

Isolation and purification of apoII

Freshly prepared VLDL from estrogen-treated roosters (ER) was dialyzed against deionized water and lyophilized, and the dry residue was delipidated by consecutive extractions with ice-cold chloroform-methanol 2:1 (v/v), diethylether-ethanol 3:1 (v/v), and diethylether alone. The residue was dried under a stream of nitrogen, and then lyophilized overnight to remove trace amounts of organic solvents. Typically, 10–25 mg of the resulting white powder was then resuspended in 1–2.5 ml of buffer containing 10 mM Tris-HCl, 60 mM octylglucoside (OG), pH 7.4, and incubated for 16 hr at 4°C. Undissolved material was then removed by centrifugation (5000 g , 5 min, 4°C), and the supernatant containing apoII was dialyzed against distilled water and lyophilized.

For reduction and alkylation of apoII, 0.75 mg of the freeze-dried protein was dissolved in 1 ml of buffer (0.5 M Tris-HCl, 7 M guanidine, 2 mM EDTA, 30 mM 2-mercaptoethanol, pH 8.2) and incubated at 37°C for 2 hr. Solid iodoacetamide was then added to give a final concentration of 50 mM and the mixture was incubated for a further 2 hr at room temperature in the dark, followed by exhaustive dialysis against 0.15 M NaCl, 0.2 mM EDTA, pH 7.4, to stop the reaction.

Reconstitution of apoII with egg phosphatidylcholine

At 23°C, the freeze-dried apoII was dissolved in buffer containing 75 mM NaCl, 50 mM Tris-HCl, and 60 mM OG, pH 7.4, to give a concentration of 2 mg/ml. The indicated amounts of egg phosphatidylcholine were combined with 4 times the weight of apoII of solid OG in chloroform-methanol 9:1 (v/v), the solvent was removed under a stream of nitrogen, and the residue was dissolved in chloroform-methanol 2:1 (v/v). The solution was dried under a stream of nitrogen during vortexing so as to produce a thin lipid film on the glass wall. After removing traces of solvent by a 2-hr lyophilization step, the apoII solution was added under vigorous mixing. The resulting clear solution was dialyzed extensively against buffer containing 50 mM Tris-HCl, 100 mM NaCl, and 0.1 mM EDTA, pH 7.4, at 23°C. The dialyzed material, designated apoII/PC, was then subjected to ultracentrifugation on a KBr density gradient consisting of (from the bottom) 2 ml each of d 1.21 g/ml, d 1.15 g/ml, d 1.06 g/ml, and d 1.02 g/ml solutions, the dialyzed apoII/PC, and water. Centrifugation was performed in a Beckman SWTi 40 rotor at 39,000 rpm for 24 hr at 4°C, and fractions of 1 ml each were collected from the bottom using a tube-fractionator (Hoefer Scientific, San Francisco). Densitometry of the fractions was performed with a digital densitometer DM 40 (A. Paar, Graz, Austria). Phospholipid concentrations were measured by a colorimetric method (absorbance at 710 nm) according to Raheja et al. (25). Protein concentrations were determined by a modification of the method of Lowry et al. (24) as described previously (26). ¹²⁵I-Labeled apoII/PC was prepared by the Iodogen method (27) with 200 μg of iodogen and 1 mCi of ¹²⁵I per mg of protein.

Electron microscopy

ApoII/PC samples were negatively stained with 2% sodium phosphotungstate (pH 7) and photographed in a Philips EM420 operated at 100 kV. The instrumental magnification was 60,000×, and the low-dose unit was employed in order to minimize specimen damage.

Filtration assay for the binding of radiolabeled lipoproteins and apoII/PC to oocyte extracts

A solid-phase filtration assay, originally developed for the binding of lipoproteins to the bovine and human LDL receptor (26) and previously adopted to chicken oocyte extracts (21), was used. In brief, oocyte membranes were prepared from small oocytes (3–15 mm in diameter) by differential ultracentrifugation. The membranes were solubilized in a buffer containing 40 mM OG and, after removing undissolved material by centrifugation, the OG concentration was decreased to below its critical micellar concentration by dilution with 7 volumes of detergent-free buffer (28). The resulting precipitate containing the oocyte

VLDL-receptor was recovered by sedimentation, resuspended by aspiration with a 22-gauge needle in buffer (20 mM Tris-HCl, 40 mM NaCl, 1 mM CaCl₂, pH 8), and used for binding assays. Assay mixtures contained in a volume of 100 μl: 12.5 mM Tris-HCl, 25 mM NaCl, 2 mM CaCl₂, 16 mg/ml BSA, the indicated amount of precipitated oocyte membrane OG-extract, and the indicated concentrations of labeled lipoproteins in the absence or presence of unlabeled lipoproteins as indicated in the figure legends. Incubations were carried out for 2 hr at room temperature and unbound ligand was separated from receptor-bound by filtration as described (28).

Electrophoresis and blotting procedures

One-dimensional SDS gel electrophoresis was performed according to Laemmli (29) using a minigel system from Bio-Rad (Mini Protean™ II Slab Cell). Electrophoresis was conducted on gradient gels (4.5–18% polyacrylamide) at 200 V at 10°C for 40–50 min. Lipoprotein samples, delipidated with 20 volumes of chloroform-methanol 2:1 (v/v), contained 10 mM dithiothreitol and were heated to 90°C for 5 min. Gels were calibrated with a mixture of the following *M_r* standards: myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), and lysozyme (14 kDa). Protein bands on gels were stained with Coomassie Blue. Electrophoretic transfer of the proteins to nitrocellulose was performed with the Mini Trans-blot system from Bio-Rad for 1 hr at 200 mA at 4°C using 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol as transfer buffer. Proteins transferred to nitrocellulose were stained with 0.2% Ponceau S in 3% TCA and destained with 10% acetic acid. Western blotting was performed as described (30) with 5% (w/v) skim milk powder as blocking agent. ¹²⁵I-Labeled goat-anti-rabbit IgG, used as second antibody for Western blotting, was prepared by the Iodogen method (27), to specific activities of 300 to 500 cpm/ng. For ligand blotting experiments, OG-extracted proteins from oocyte membranes were electrophoretically separated on SDS-polyacrylamide gels (16 × 12 × 0.15 cm) and transferred to nitrocellulose as described previously (30). The ligand blotting procedure was identical to that described for the B/E receptor and human lipoproteins (31). Autoradiography was performed by exposing the dried nitrocellulose to Kodak XAR-5 film at room temperature for the indicated times.

Preparation of antibodies

Polyclonal antibodies were raised in adult female New Zealand White rabbits by subcutaneous injection of purified and reconstituted apoII, prepared as described above, and apoA-I, respectively. ApoA-I was isolated by electroelution from an SDS-polyacrylamide gel as follows. CR-

HDL apoproteins were separated on a 4.5–18% gradient gel, the gel was stained, and the gel piece containing the apoA-I band ($M_r = 28,000$) was cut out. Electroelution from the gel was performed according to the method of Hunkapiller et al. (32). For the first injection (day 0), the antigens were mixed with Freund's complete adjuvant, and for successive booster injections, with Freund's incomplete adjuvant (days 14 and 28). The antisera were tested by Western blotting and IgG fractions were purified by affinity chromatography on Protein-A Sepharose (33).

RESULTS

Characterization of lipoproteins and purification of apoII

Fig. 1 (lane B) shows the results of SDS-polyacrylamide gel electrophoresis of a typical preparation of VLDL prepared from the plasma of an estrogen-treated rooster (ER) as described in Materials and Methods. Of the three protein bands detected, the high M_r band corresponds to apoB; the other two (apparent M_r s of 9,500 and 16,000, respectively) represent the monomeric and dimeric forms of apoII. The disulfide bridge between the identical

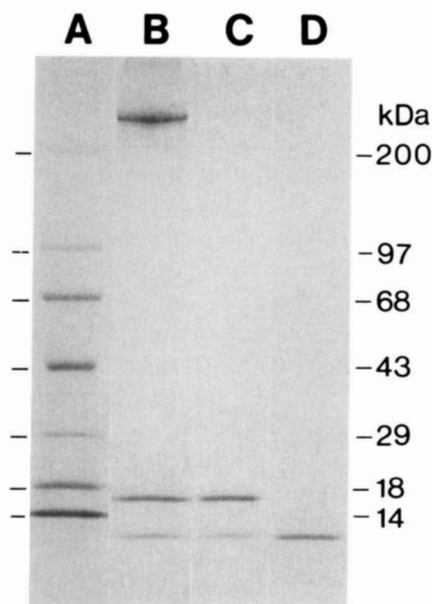


Fig. 1. SDS-polyacrylamide gel electrophoresis of chicken apoVLDL-II. ER-VLDL and apoII were purified as described in Materials and Methods. VLDL was delipidated by extraction with 20 volumes of ice-cold chloroform-methanol 2:1 (v/v) prior to electrophoresis on a 4.5–18% SDS polyacrylamide gradient minigel. All samples contained 10 mM dithiothreitol and were heated to 90°C for 5 min. Lane A: M_r standards, as described in Materials and Methods; lane B: 15 μ g of protein of VLDL from estrogen-treated rooster; lane C: 5 μ g of purified apoII; lane D: 5 μ g of apoII that had been reduced and alkylated as described in Materials and Methods. The proteins were stained with Coomassie Blue.

monomers of apoII is highly resistant to reduction (34). ApoII could be extracted from delipidated and freeze-dried VLDL with 60 mM octyl- β -D-glucoside as described in Materials and Methods. We found that a single extraction resulted in pure apoII, but that extracts obtained by repeated OG treatment contained significant amounts of high- M_r contaminants, presumably proteolytic fragments of apoB. This could not be avoided by the addition of a mixture of protease inhibitors including PMSF, aprotinin, leupeptin, 2-mercaptoethanol, and EDTA. From 25 mg of delipidated and freeze-dried ER-VLDL, we obtained 4–6 mg of pure apoII. As shown in Fig. 1, lane C, the isolated apoII showed the same resistance to dissociation by reduction as the lipoprotein-bound protein. Following reduction and alkylation of apoII, however, a single band with an apparent M_r of 9,500 was observed both in the presence (Fig. 1, lane D) and absence (not shown) of dithiothreitol.

For further analysis of apolipoprotein contents of plasma and lipoproteins, we prepared polyclonal antibodies directed against apoII and apoA-I, respectively. Fig. 2 demonstrates the specificity of these antibodies. When equal aliquots of delipidated plasma from either CR or LH were analyzed by SDS-polyacrylamide gel electrophoresis (lanes B and C) and Western blotting (lanes D–G), the expected differences between the two sources were revealed. Namely, Coomassie staining demonstrated the presence of a protein with $M_r \sim 220,000$ in LH plasma (lane C) which was totally absent from CR plasma (lane B), representing the vitellogenins (35). Western blotting confirmed the absence of apoII from CR plasma (lane D), and its induction in LH (lane E); the anti-apoII antibody did not cross-react with any other protein in chicken plasma (Fig. 2, lanes D and E). Immunoblotting with the anti-apoA-I antibody demonstrated a reduction of apoA-I levels in LH plasma compared to CR plasma. This finding is consistent with the marked decrease in HDL levels in the estrogenized state as previously described for estrogen-treated versus untreated immature hens (34). Our rabbit anti-apoA-I antibody also was monospecific (Fig. 2, lanes F and G).

While VLDL from estrogen-treated roosters consistently contained large amounts of apoII, the presence of this protein was highly variable in VLDL of laying hens. An example of LH-VLDL relatively poor in apoII is shown in Fig. 3a, lane B. For this reason, we routinely used ER-VLDL as source for apoII. LDL was prepared from CR, which do not produce apoII (36); these particles had a mean buoyant density of 1.04 g/ml as determined by equilibrium density gradient centrifugation as described in Materials and Methods.

HDL prepared from LH had an apoprotein composition very different from that of CR-HDL: the particles from LH contained, in addition to apoA-I, approximately equal amounts of apoII (Fig. 3a, lane C). In contrast, CR-HDL

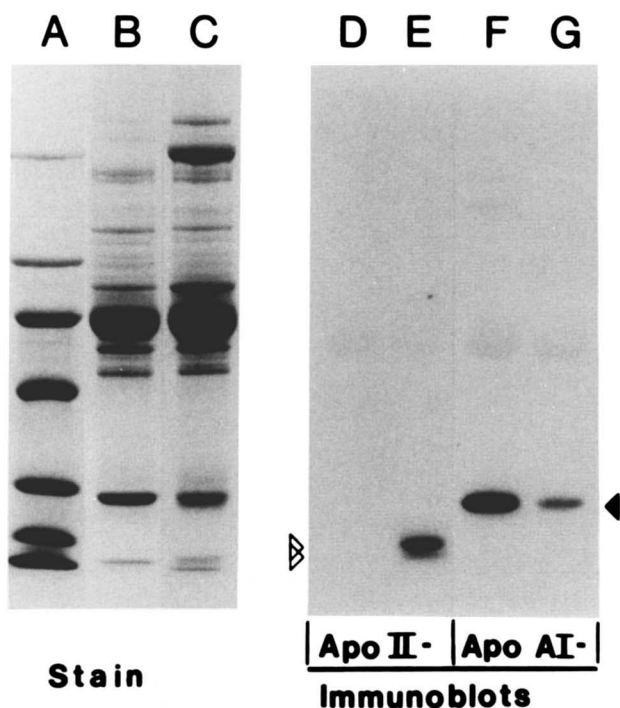


Fig. 2. SDS-polyacrylamide gel electrophoresis and Western blotting of chicken plasma. Chicken plasma was obtained as described in Materials and Methods. Plasma samples were delipidated with 20 volumes of ice-cold chloroform-methanol 2:1 (v/v), and aliquots corresponding to 1 μ l of plasma were electrophoresed on 4.5–18% SDS polyacrylamide gradient minigels. Samples contained 10 mM dithiothreitol and were heated to 90°C for 5 min. Lane A: M_r standards, same as in Fig. 1; lane B; plasma from control rooster; lane C: plasma from laying hens. Lanes A–C were stained with Coomassie Blue. For lanes D–G, proteins from gels run in parallel with the stained one were electrophoretically transferred to nitrocellulose as described in Materials and Methods. Lanes D and F were identical to lane B; lanes E and G were identical to lane C. The nitrocellulose replicas were incubated with the indicated antibodies at a concentration of 1 μ g/ml. The second antibody, 125 I-labeled goat-anti-rabbit IgG was used at a concentration of 5 μ g/ml (sp act, 320 cpm/ng). Exposure of the dried nitrocellulose to Kodak XAR-5 film was for 12 hr at room temperature.

was devoid of apoII as expected, and contained small amounts of a protein of approximately 12 kDa as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions; the prominent protein component of CR-HDL was the 28-kDa apoA-I (Fig. 3a, lane D). In Fig. 3b, the apoprotein content of LH-VLDL, LH-HDL, and CR-HDL was analyzed by Western blotting with the above-described (Fig. 2) antibodies to apoII and apoA-I, respectively. The results demonstrate that LH-VLDL indeed does not contain detectable amounts of apoA-I, and that CR-HDL is devoid of apoII. Furthermore, our anti-apoII antibody reacted equally well with the monomeric and dimeric forms of the antigen, respectively, and anti-apoA-I bound to a diffuse band of M_r ~80,000 present in CR-HDL which is also seen by Coomassie Blue staining (Fig. 3a, lane D and Fig. 3b, lane 9). The 12-kDa protein of CR-HDL was recognized by neither anti-apoII nor anti-apoA-I antibodies.

The ability of apoII to associate with lipid was tested in attempts to recombine egg phosphatidylcholine (PC) and the protein into soluble complexes of defined composition. To this end, we prepared mixtures containing apoII and PC in various ratios in the presence of solubilizing concentrations of OG. Mixtures with apoII/PC ratios (by weight) of 3:1, 2:1, and 1:1, respectively, were dialyzed extensively, and the resulting material was analyzed by equilibrium centrifugation on KBr density gradients. At all apoII/PC ratios tested, the major portion of the dialyzed material sedimented at d 1.062 g/ml; at apoII/PC 3:1, this was the only peak observed (Fig. 4). When the ratio was decreased to 2:1, a second peak at d 1.028 g/ml emerged, containing 12% of the total protein, and 18% of the total phospholipid applied to the gradient, respectively; at apoII/PC 1:1, excess free egg PC vesicles floated to a position with d 1.01 g/ml, whereas at ratios greater than 3:1, excess apoII sedimented to the bottom of the tube (data not shown).

The material with d 1.062 g/ml consisted of apoII/PC complexes with 2.97 ± 0.07 mg of apoII/mg of egg PC, as determined by direct measurement of protein and PC in the pooled peak fractions of six different reconstitution experiments. With the assumption of an average M_r for egg PC of 800, in the reconstituted particles each mole of apoII monomer (M_r , 9500) bound approximately 4 mol of egg PC.

Electron microscopy of the d 1.062 g/ml complexes demonstrated that their morphology was that of discoidal lipoproteins with a diameter of 20–25 nm and a thickness of approximately 4 nm (Fig. 5).

Receptor binding studies

Previously, we have established conditions for binding of 125 I-labeled chicken lipoproteins to the chicken oocyte receptor for low and very low density lipoproteins (21). When we applied these conditions in order to test whether radioiodinated apoII/PC, LH-HDL (containing apoII and apoA-I as shown in Fig. 3), LH-VLDL, and CR-LDL would bind to oocyte membrane detergent extracts, high affinity, saturable binding of LH-VLDL and CR-LDL, but not of particles lacking apoB was observed (Table 1). All apoB-containing ligands, but neither LH-HDL nor apoII/PC, showed a saturable binding component; their nonspecific linear binding was also unchanged in the presence of 20 mM EDTA or a 100-fold excess of unlabeled ligand (not shown).

To further investigate the binding characteristics of the chicken oocyte receptor, we performed competitive binding experiments. 125 I-labeled VLDL from LH was effectively displaced from the receptor site by both unlabeled LH-VLDL and CR-LDL (Fig. 6A). As suggested by the direct binding experiments (Table 1), CR-LDL appeared to display a somewhat higher affinity for the receptor than

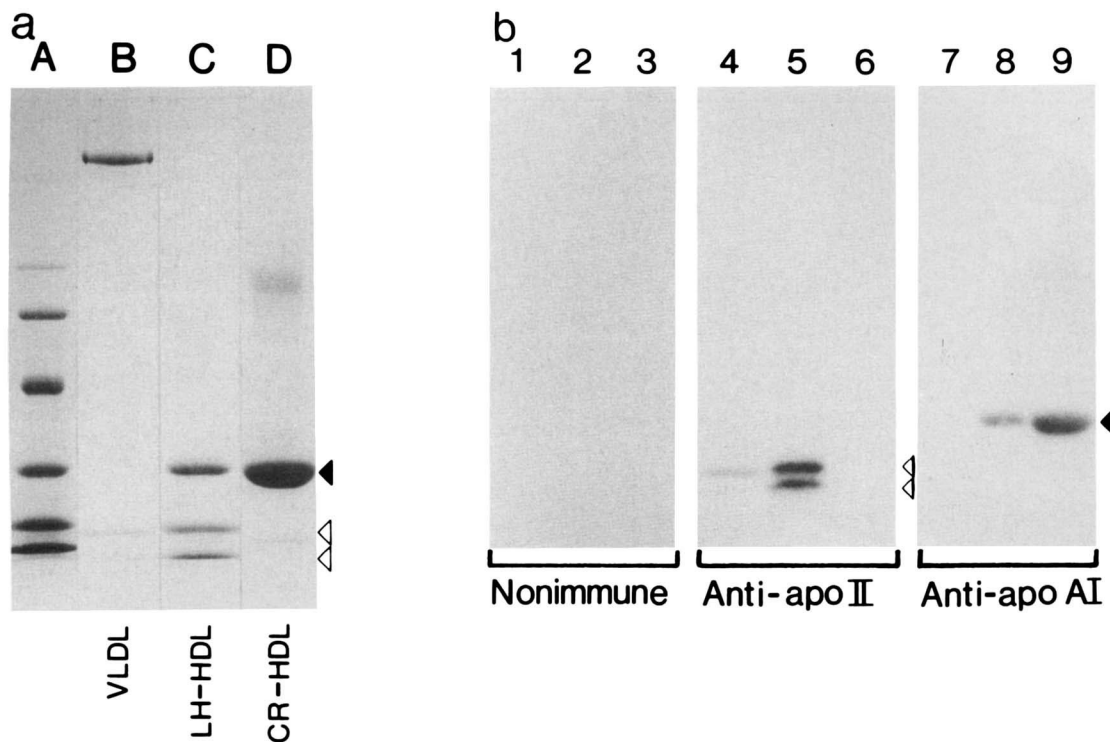


Fig. 3. SDS-polyacrylamide gel electrophoresis and Western blotting of chicken lipoproteins. Chicken lipoprotein fractions were isolated as described in Materials and Methods. Aliquots of the samples were delipidated by extraction with 20 volumes of ice-cold chloroform-methanol 2:1 (v/v) and were subjected to electrophoresis on 4.5–18% SDS polyacrylamide gradient minigels. Samples contained 10 mM dithiothreitol and were heated to 90°C for 5 min. Lane A: M_r standards; same as in Fig. 1; lane B: 10 μ g of protein of LH-VLDL; lane C: 15 μ g of protein of LH-HDL; lane D: 15 μ g of protein of CR-HDL. In (a) the protein bands were stained with Coomassie Blue. In (b) the proteins from gels run in parallel with the one in (a) were electrophoretically transferred to nitrocellulose. Lanes 1, 4, and 7 were identical to lane B; 2, 5, and 8 to lane C; and 3, 6, and 9 to lane D. The nitrocellulose replicas were incubated with the indicated antibodies at a concentration of 1 μ g/ml. The second antibody, 125 I-labeled goat-anti-rabbit IgG, was used at a concentration of 5 μ g/ml (sp act, 320 cpm/ng) as described in Materials and Methods. Exposure of the dried nitrocellulose to Kodak XAR-5 film was for 12 hr at room temperature.

LH-VLDL, in that about twice the concentration of LH-VLDL was required to compete for 50% of 125 I-labeled LH-VLDL binding than that of CR-LDL (18.5 μ g/ml vs. 9.1 μ g/ml). In contrast to these ligands, apoII/PC had no inhibitory effect on the binding of 125 I-labeled LH-VLDL, even at a concentration at which CR-LDL and LH-VLDL completely abolished receptor binding (Fig. 6A). In another experiment, we tested whether an apoII-containing, apoB-free physiological ligand, namely LH-HDL, would behave similarly to reconstituted apoII. Fig. 6B demonstrates that both these particles did not compete for the binding of 125 I-labeled CR-LDL, whereas the unlabeled ligand was a very efficient competitor.

Ligand blotting

The chicken oocyte receptor for low and very low density lipoproteins is a 95-kDa protein as determined by SDS-PAGE under nonreducing conditions (21). The receptor protein can be visualized by ligand blotting with 125 I-labeled ligands after electrophoretic transfer to nitrocellulose of oocyte membrane proteins following separation on SDS-

polyacrylamide gels (21). We tested whether this method could be used to further demonstrate the lack of receptor binding of apoII/PC and of the plasma lipoprotein, LH-HDL. 125 I-labeled LH-VLDL bound to the receptor, which was identified as a discrete protein band of M_r 95,000 (Fig. 7, lane 1). The presence of increasing concentrations of unlabeled LH-VLDL or CR-LDL caused a significant reduction of 125 I-labeled VLDL binding; higher concentrations of these ligands than required in competitive binding assays (Fig. 6A) were necessary to abolish receptor binding. Nevertheless, the results in Fig. 7, lanes 1–6 demonstrate that ligand blotting under these conditions resulted in data that are qualitatively identical to those obtained in the filtration assay. ApoII/PC and LH-HDL were ineffective in displacing 125 I-labeled LH-VLDL from the 95-kDa oocyte receptor (Fig. 7, lanes 7–12); only at high concentrations of these ligands, a small decrease in 125 I-labeled VLDL binding was observed (Fig. 7, lanes 9 and 12). These data demonstrate that the reconstituted lipoprotein (apoII/PC) as well as the physiological particles (LH-HDL) displayed the same characteristics in both the filtration assay and in ligand blotting.

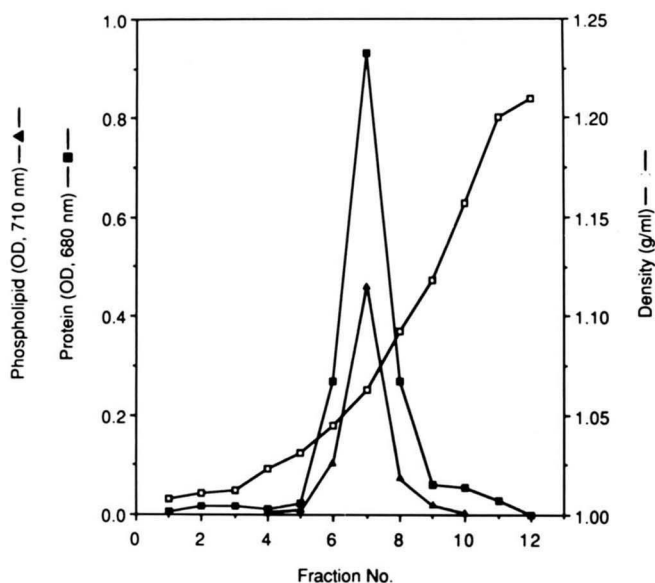


Fig. 4. Equilibrium density gradient centrifugation of apo-II reconstituted with egg phosphatidylcholine. ApoII was reconstituted with egg phosphatidylcholine in a ratio of protein/egg phosphatidylcholine 3:1 (wt/wt) and subjected to ultracentrifugation on a KBr density gradient as described in Materials and Methods. One-ml fractions were collected from the bottom, and their density (\square), phospholipid (\blacktriangle), and protein (\blacksquare) concentrations were measured as described in Materials and Methods.

DISCUSSION

The function of apoII, an apolipoprotein that is exclusively found in avian species, in the metabolism of triglyceride-rich lipoproteins in the laying hen is unknown. However, the dramatic induction of its hepatic synthesis by estrogens, concomitant with the onset of egg-laying, suggests that it might facilitate the uptake of triglyceride-rich lipoproteins via specific receptor(s) on the surface of oocytes. In the studies described here, we have addressed this possibility directly by determining whether this small apoprotein, when associated with lipid, would interact with the previously identified oocyte plasma membrane receptor for chicken lipoproteins of low and very low density (21). To this end, we first developed a rapid procedure for the purification and reconstitution of apoII. We then used the pure, lipid-associated apoII as well as HDL particles isolated from the plasma of laying hens (which contain apoII and apoA-I) in direct and competitive binding assays. Finally, ligand blotting was employed to further test the capacity of the oocyte receptor to interact with apoII.

The synthesis of apoII in the liver is under absolute control of estrogens (1, 2). In accordance with these reports, we did not detect any apoII in the plasma of untreated roosters but large amounts in the plasma of laying hens by Western blotting (Fig. 2). However, plasma apoII levels in LH were highly variable, and were reflected in a large

fluctuation in apoII/apoB ratios in the isolated VLDL ($d < 1.006$ g/ml) fraction. Consistently high apoII concentrations could be obtained in the VLDL of ER. After two rounds of ultracentrifugal flotation, preparations of ER-VLDL were free of albumin, and apoII could be extracted with a buffer containing supermicellar concentrations of octyl- β -D-glucoside following lyophilization and delipidation. Our attempts to reconstitute apoII with egg PC by detergent dialysis were based on previously reported findings by Jackson et al. (37). These authors showed association of apoII with dimyristoyl-PC (DMPC) by addition of DMPC vesicles to the apoprotein; the resulting complexes were isolated at densities between 1.09 and 1.11 g/ml and contained 5.3 mg of DMPC/mg of apoII. Here we used detergent dialysis to obtain maximal incorporation of apoII into egg PC-containing complexes. The discoidal apoII/PC particles became saturated with protein at about one molecule of apoII monomer per four

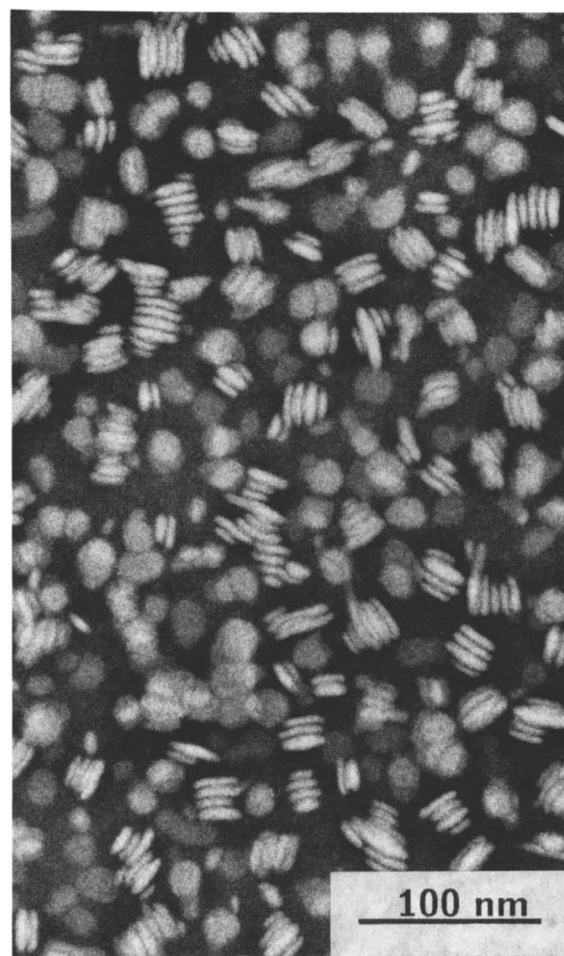


Fig. 5. Electron micrograph of reconstituted apoII. ApoII was reconstituted with egg phosphatidylcholine at a protein/lipid ratio of 3:1 (wt/wt) and isolated by KBr density gradient centrifugation as described in Materials and Methods. The particles were stained with 2% sodium phosphotungstate and processed for electron microscopy as described in Materials and Methods.

TABLE 1. Lipoprotein binding to chicken oocyte membrane octylglucoside extracts

¹²⁵ I-labeled Lipoprotein	K _d	B _{max} μg/mg protein
LH-VLDL	18.2 μg/ml	62.5
CR-LDL	5.2 μg/ml	39
ApoII/PC	>>1 mg/ml	
LH-HDL	>>1 mg/ml	

Saturation curves for the binding of radiolabeled lipoproteins and reconstituted apoII (apoII/PC) to precipitated octylglucoside extracts were obtained as described in Materials and Methods in a concentration range of 1–150 μg/ml for the ligands. K_d and B_{max} values were calculated from total binding data using the NIH program LIGAND (47). For all radiolabeled lipoproteins, nonspecific binding was determined in the presence of 500 μg/ml of respective unlabeled ligand. Each value represents the average of results obtained in two independent experiments, and is expressed in terms of protein content of the lipoproteins.

molecules of PC, a protein content about 18 times higher than that of the apoII/DMPC-complexes described by Jackson et al. (37). Nevertheless, the particles described here had a lower buoyant density (1.062 g/ml) than those described previously (d 1.09–1.11 g/ml), suggesting that apoII can associate with lipid bilayers in a variety of conformations depending on the procedure used for reconstitution. In studies to be reported elsewhere (R. George, J. Nimph, R. McElhaney, and W. J. Schneider, unpublished results), we have analyzed apoII/DMPC complexes obtained by detergent dialysis with differential scanning calorimetry, by measurement of circular dichroism, and

by fluorescence spectroscopy. The results suggest a significant influence of the single cysteine-residue at position 75 (20, 38) on the association of apoII with phospholipid which occurs presumably via interaction with a neighboring (residues 58–74) amphipathic helix (39).

In order to test whether lipid-bound apoII can interact with the chicken oocyte receptor, we did not rely on the use of apoII/PC alone; we also studied the binding characteristics of a relevant physiological particle. Namely, in the course of lipoprotein fractionations of laying hen plasma, we discovered that the HDL fraction (d < 1.21 g/ml) contained apoII in addition to apoA-I (Fig. 4). In experiments not shown, the buoyant density of these particles was determined as 1.096 g/ml, and a homogeneous peak was observed upon column chromatography in agarose (A-5m, Bio-Rad). Thus, by a variety of criteria, both apolipoproteins appear to be present on the same particles; future studies are planned to elucidate the synthetic and/or metabolic pathway(s) leading to their presence in the plasma of laying hens. The use of apoII/PC complexes rather than of free apoII in direct and competitive binding studies is suggested by the demonstration of Innerarity, Pitas, and Mahley (40) that apoE requires association with phospholipid in order to promote binding to the mammalian apoB,E-receptor.

Neither radioiodinated nor unlabeled apoII/PC bound to the oocyte receptor (Fig. 6 and Table 1); additional evidence for lack of recognition of apoII is the fact that laying hen-HDL, which contain both apoII and apoA-I, in the

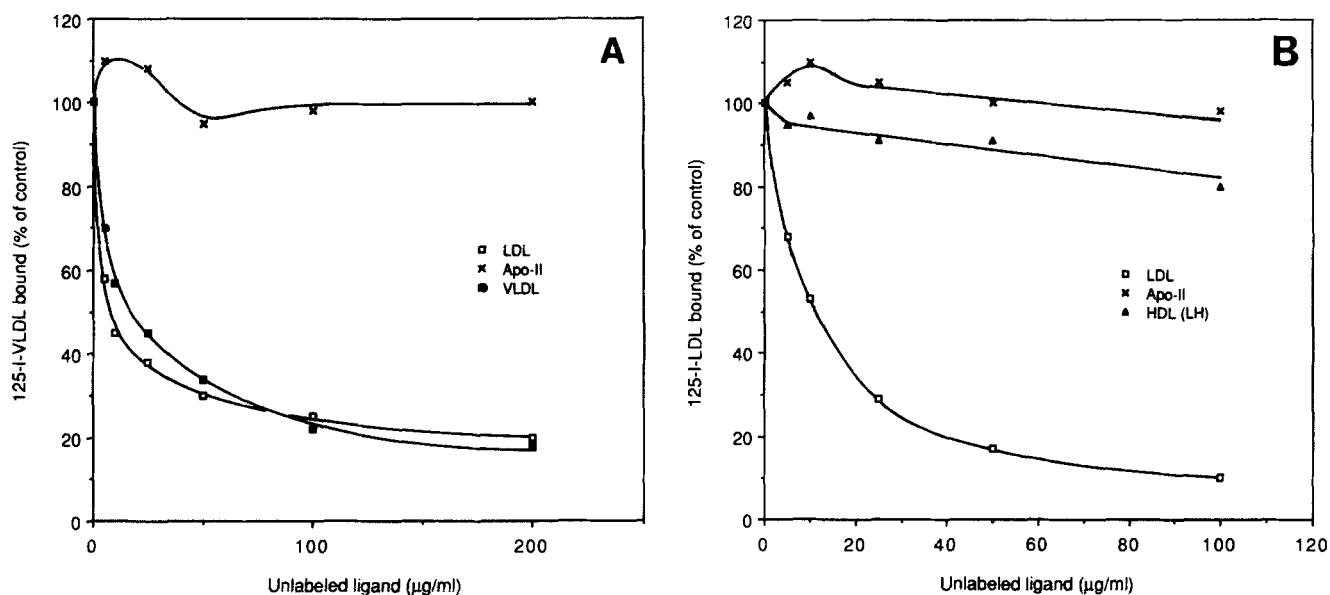


Fig. 6. Competition of unlabeled chicken lipoproteins and reconstituted apoII for binding of ¹²⁵I-labeled chicken lipoproteins to oocyte membrane extracts. Each assay tube contained the standard mixture (100 μl) with 12.8 μg of protein of precipitated octylglucoside extract and 18 μg/ml of ¹²⁵I-labeled LH-VLDL (panel A) with a specific activity of 230 cpm/ng, or 5 μg/ml of ¹²⁵I-labeled CR-LDL (panel B) with a specific activity of 190 cpm/ng. Binding experiments were performed in the presence or absence of the indicated concentrations of unlabeled ligands as described in Materials and Methods. The unlabeled ligands were: CR-LDL (□), LH-VLDL (■), LH-HDL (▲), and reconstituted apoII (x). Values represent the average of two determinations. The 100% control values were 31 μg/mg protein in A, and 20 μg/mg protein in B, respectively.

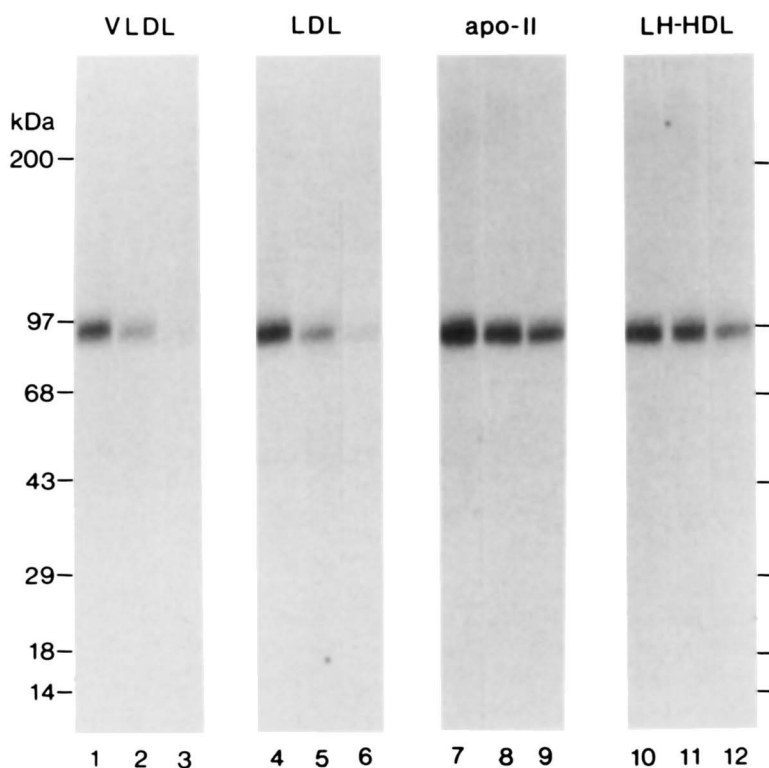


Fig. 7. Ligand blotting of oocyte membrane octylglucoside extracts. Oocyte membrane octylglucoside extract (10 μg of protein/lane) was subjected to electrophoresis on a 4.5–18% SDS polyacrylamide gradient gel under non-reducing conditions. Proteins were electrophoretically transferred to nitrocellulose, and ligand blotting was performed as described in Materials and Methods. All incubations contained 5 $\mu\text{g}/\text{ml}$ of ^{125}I -LH-VLDL (sp act: 230 cpm/ng) in the presence or absence of the indicated unlabeled ligands: lanes 1, 4, 7, and 10: none; lanes 2 and 3: 50 and 500 $\mu\text{g}/\text{ml}$ of LH-VLDL, respectively; lanes 5 and 6: 50 and 500 $\mu\text{g}/\text{ml}$ of CR-LDL, respectively; lanes 8 and 9: 50 and 500 $\mu\text{g}/\text{ml}$ reconstituted apoII, respectively; and lanes 11 and 12: 50 and 500 $\mu\text{g}/\text{ml}$ of LH-HDL, respectively. Exposure of the dried nitrocellulose sheets to Kodak XAR-5 film was for 24 hr at room temperature. M_r standards are indicated.

same experiments failed to recognize the receptor as well. This failure to bind cannot be due to an inhibitory effect of apoA-I on receptor interaction, since we have previously shown that HDL from untreated roosters which is devoid of apoII (Fig. 3 and ref. 21) does not inhibit oocyte receptor binding of chicken ^{125}I -labeled LDL or VLDL (21). Thus, we conclude that apoII is not a ligand for the chicken oocyte receptor for LDL and VLDL.

An interesting observation was the apparent higher affinity of the receptor for CR-LDL than for LH-VLDL (Table 1, Fig. 6A). Previously, we found very similar affinities for LH-VLDL and LH-LDL (21). Inasmuch as CR-LDL particles are smaller, contain more protein and less triglyceride, and have higher density than LH-LDL we believe that the different binding parameters for these particles reflect their differences in physical properties and relative apoB content rather than any contribution of the small amount of apoII in LH-LDL or of apoA-I in CR-LDL particles (data not shown, and refs. 41 and 42).

Finally, the value of ligand blotting (43) for the investigation of the questions addressed here could be demon-

strated by inhibition of ^{125}I -labeled VLDL binding to the 95-kDa receptor by VLDL and LDL, but not by apoII/PC and LH-HDL. The small amount of inhibition seen with high concentrations of apoII/PC and LH-HDL may have arisen from nonspecific interaction.

The findings reported here raise further questions along two lines. First, since we demonstrate here that apoB (the exclusive protein component of mammalian LDL) is the sole apolipoprotein through which VLDL and LDL bind to the oocyte receptor, it will be of interest to investigate whether apoE can interact with the avian oocyte counterpart of the mammalian somatic LDL receptor. Although chickens appear to lack apoE (44), the oocyte receptor's capacity to recognize it would indicate extensive structural similarities in the ligand binding regions of receptors in diverse animal kingdoms and offer insights into the phylogeny of these proteins. In this context, we know already that polyclonal antibodies to the bovine LDL receptor recognize the chicken oocyte receptor (21).

Second, since apoII does not function in VLDL delivery into oocytes, future investigations must address other

possible roles for this female-specific protein. We conceive the following alternatives. *i*) ApoII might protect the oocyte-destined VLDL particles from action of lipolytic enzymes such as lipoprotein lipase; ultrastructural studies (45) suggest that intact VLDL and not smaller remnants are transported into the oocyte. *ii*) While apoII does not interact with the oocyte receptor, this apolipoprotein might diminish receptor recognition of VLDL by tissues other than the oocyte, the liver in particular, in order to avoid pre-oocytic catabolism. *iii*) ApoII may serve a structural role in stabilizing the extremely triglyceride-rich nascent VLDL in laying hens; in this respect, an 18-kDa, adipokinetic hormone-induced protein in insect hemolymph termed apolipoprotein III has been shown to stabilize the lipid/water interface that increases during diglyceride transfer to lipophorin (46). Investigations along these lines are now underway. ■

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