The Receptor for Yolk Lipoprotein Deposition in the Chicken Oocyte*

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The final rapid growth phase of the chicken oocyte is characterized by massive uptake of hepatically synthesized yolk precursor proteins from the plasma. The two major yolk-forming components, very low density lipoprotein (VLDL) and vitellogenin (VTG), have been shown to interact with a 95-kDa protein present in detergent extracts of ovarian membranes; this protein is absent in hens of a mutant nonlaying chicken strain (Nimpf, J., Radosavljevic, M., and Schneider, W. J. (1989) J. Biol. Chem. 264, 1389–1398). Here, we have purified the 95-kDa protein by ligand and immunofinity chromatography and demonstrated its role in receptor-mediated endocytosis by ultrastructural immunolocalization, structural, and functional studies. The receptor was visualized exclusively in the oocyte proper and was absent from somatic cells, in agreement with the previously reported expression of two different lipoprotein receptors in somatic cells and oocytes, respectively, of laying hens (Hayashi, K., Nimpf, J., and Schneider, W. J. (1989) J. Biol. Chem. 264, 3131–3139). Amino acid sequences of tryptic fragments of the oocyte receptor were obtained, and its kinship to somatic low density lipoprotein receptors was confirmed through the demonstration of sequence conservation in three characteristic domains. In particular, the chicken receptor’s internalization sequence, Phe-Asp-Asn-Pro-Val-Tyr, is identical with that in low density lipoprotein receptors from mammals as well as Xenopus laevis. The ligand-binding properties, specificity, and kinetic parameters of the oocyte receptor were characterized in filtration assays employing pure ligands and receptor. In conjunction with ligand blotting experiments following limited protease digestion of the receptor, the binding assay data suggest that VTG recognizes a substructure of the VLDL-binding site. These studies establish that a cell-specific receptor mediates the endocytosis of VTG and VLDL into growing chicken oocytes and thus possibly plays a key role in control of oocyte growth.

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††† The abbreviations used are: VTG, vitellogenin; apo, apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; LRP, LDL receptor-related protein; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; R/O, restricted ovulator; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PBS, phosphate-buffered saline.

Yolk, the complex lipid-rich storage compartment of the female germ cells of oviparous species, serves as the major food source for the developing embryo. Most, if not all, yolk proteins are synthesized in and secreted from the liver, from where they are transported as plasma macromolecules to the oocytes within ovarian follicles. The major plasma proteins that are targeted to oocytes are vitellogenin (VTG), a lipoposphoglycoprotein (1, 2), and very low density lipoprotein (VLDL), a carrier of energy in the form of triacylglycerols (3–5). Previous studies (3, 6, 7) (reviewed in Ref. 8) on the mechanism for uptake of yolk precursor proteins from the plasma into rapidly growing chicken oocytes have provided indirect evidence for specific transport receptors in the membrane fraction of developing ovarian follicles. Using crude follicle membrane preparations and the technique of ligand blotting (9), we subsequently demonstrated that VLDL interacted with a 95-kDa membrane protein in saturable fashion and with high affinity (10). These studies also revealed an immunological relationship between the well known mammalian low density lipoprotein (LDL) receptor and the chicken protein. Interestingly, the 95-kDa protein is undetectable in cultured somatic cells such as chicken embryo fibroblasts or granulosa cells (11, 12). Instead, these cells express a 130-kDa receptor that mediates the regulation of cellular cholesterol homeostasis by analogy with the well known LDL receptor in mammalian cells (11, 13). Studies on a mutant nonlaying strain of chickens carrying a single gene defect (termed restricted ovulator (R/O) have established that the two proteins are the products of two different genes (11, 14). The findings in R/O hens also strongly suggested that the 95-kDa protein is a key component in mediating oocyte growth via yolk precursor protein uptake from the plasma. This functional role was further supported by our studies (14, 15), which revealed that the 95-kDa protein is capable of binding both VTG and VLDL; thus, the lack of deposition of these two yolk precursors into oocytes of R/O hens (14, 16–19) can be ascribed to the absence of the transport function normally performed by the 95-kDa membrane protein, hereafter designated the VTG/VLDL receptor. However, the exact localization of the receptor protein among the different cells in the ovarian follicle and, thus, its site of action, as well as its isolation and functional characterization, have not been reported.

In the present study, we have purified and characterized the VTG/VLDL receptor, analyzed its cellular distribution...
by immunocytochemistry, and studied its ligand-binding and biochemical properties in detail.

**EXPERIMENTAL PROCEDURES**

**Materials**—We obtained PMSF, aprotinin, leupeptin, Freund's complete and incomplete adjuvants, Triton X-100, Nonidet P-40, octyl $\beta$-d-glucoside, IODO-GEN, trypsin, and egg phosphatidylcholine from Sigma; DEAE-cellulose from Whatman; Na$^{14}$H from American Corp.; molecular weight standards from Bethesda Research Laboratories; CHAPS, endoproteinase Arg-C, endoproteinase Glu-C, and endoproteinase Lys-C from Boehringer Mannheim; CNBr-activated Sepharose 4B from Pharmacia LKB Biotechnology Inc.; Trans-Blot nitrocellulose paper from Bio-Rad; cellulose acetate membrane filters CA45 from Advantec, Japan; and suramin from FBA Pharmaceuticals, New York. All other materials were obtained from previously reported sources (10).

**Animals and Diets**—White Leghorn hens were purchased from the Department of Animal Science, University of Alberta and maintained on a layer mash. Roosters, obtained from the same source, were fed a grower mash. All chickens received a light period of 12 h. Oocytes were also collected during slaughtering by permission of Lilydale Poultry Sales, Edmonton, Alberta. Adult female New Zealand White rabbits were used for antibody production.

**Lipoproteins and Ligands**—VLDL was prepared from laying hens, whereas LDL and HDL were obtained from roosters. Lipoproteins were isolated by density ultracentrifugation, as described (10, 20). Yolk VLDL was prepared from freshly extruded yolks as described (20, 21). Reductively methylated ligands were prepared by incubation with sodium borohydride and formamide (22), except that sodium borohydride was added twice, and the addition of formaldehyde extended over 60 min. VTG was purified as described (23) by DEAE-cellulose chromatography.

**Antibody Preparation**—An eluate fraction from VLDL-Sepharose chromatography (see below and Fig. 1) was separated by SDS-polyacrylamide gel electrophoresis, stained briefly with Coomassie Blue, and destained in 5% (v/v) acetic acid, 10% (v/v) methanol. The band migrating at 95 kDa was excised, chopped into small fragments, and extracted with 0.1% SDS (w/v) octyl-$\beta$-D-glucoside. The gel pieces were incubated for 18 h at 23 °C in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS (24). Supernatant was removed and dialyzed exhaustively against a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl$_2$, 0.2% (v/v) Triton X-100. Rabbits were injected with 25 $\mu$g of eluted 95-kDa protein (1 ml) emulsified with 1 ml of Freund's complete adjuvant. Injections followed with 25 $\mu$g of protein in Freund's incomplete adjuvant on days 14, 21, and 28. Serum was obtained on day 35 and every 2 weeks thereafter for a period of 2 months. The IgG fraction was purified by Protein A-Sepharose chromatography (25). Nonimmune IgG was prepared before immunization.

**Protein Iodination**—VLDL was radiolabeled with $^{125}$I using the iodo monochloride method (10). Radioiodination of VTG, Protein A, and the VGT/VLDL receptor was performed using IODO-GEN (1,3,4,6-tetraaceto-3,6-di-iodophenylglycitol) (23).

**Purification of the VGT/VLDL Receptor**—Ovarian follicle membranes were prepared and membrane proteins were obtained by solubilization as described (10), except for increasing the volume of the solubilization mixtures. Briefly, membrane pellets (derived from 15–20 g of follicular tissue) were suspended in 4.5 ml of buffer containing 250 mM Tris maleate (pH 6.0), 2 mM CaCl$_2$, 1 mM PMSF, 2.5 mM EDTA, 5 $\mu$m leupeptin by aspiration through a 22-gauge needle. The volume was adjusted to 9 ml by the addition of H$_2$O and an appropriate detergent to yield 5–10 mg of protein/ml, 125 mM Tris maleate (pH 6.0), 2 mM CaCl$_2$, 0.5 mM PMSF, 1.325 g/m$^3$ ml aprotinin, and 2.5 $\mu$m leupeptin. As described under "Results," various detergents were utilized to solubilize membrane proteins, including (final concentrations in the solubilization mixture) 36 mM octyl $\beta$-d-glucoside, 30 mM CHAPS, 1% (v/v) Nonidet P-40, or 1% (v/v) Triton X-100. The suspension was incubated at 4°C for 10 min prior to centrifugation at 100,000 G for 1 h. The supernatant was either quick-frozen in liquid N$_2$ and stored at −70°C or used directly in purification experiments.

**VLDL-Sepharose Chromatography**—Yolk VLDL, prepared as described above, was coupled to CNBr-activated Sepharose 4B according to the manufacturer's instructions (26), at a ratio of 35 mg of VLDL-protein/g of dry gel. The VTG/VLDL receptor was coupled to CNBr-activated Sepharose 4B according to the method of Protein A-purified IgG/g of dry gel. The chromatography was performed similarly to that above, except that the gel was washed exhaustively with a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM NaCl, 2 mM CaCl$_2$, 1 mM PMSF, and 0.2% (v/v) Triton X-100, and then with 50 mM Tris-HCl (pH 8.0), 2 mM CaCl$_2$, 1 mM PMSF, and 30 mM octyl $\beta$-d-glucoside (buffer B). The bound protein was eluted in the presence of 0.5 M NH$_4$OH. Appropriate fractions were pooled and lyophilized.

**Binding Assays**—The solid phase filtration assay described previously (10, 23) was adapted as follows. Immunooaffinity-purified receptor was incubated in the precipitated unidimensional SDS-gel system or a regular gel apparatus. Electrophoresis was performed at 200 V at room temperature for 60 min for mini gels (8 × 6 × 0.15 cm) or at 35 mA/gel at 10 °C for 6 h for regular gels (16 × 16 × 0.15 cm). Samples containing 50 mM dithiothreitol were heated to 90 °C for 3 min, and samples devoid of reducing agent were not heated. Gels were calibrated with the following standards: myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), $\beta$-lactoglobulin (18 kDa), and lysozyme (14 kDa). Gels were stained in 0.1% Coomassie brilliant blue in 5% (v/v) acetic acid, 25% (v/v) methanol for 2 h and destained in 5% (v/v) acetic acid, 10% (v/v) methanol.

Ligand blotting with $^{125}$I-labeled VLDL (10) or $^{125}$I-labeled VTG (23) was performed in buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM CaCl$_2$, and 5% (w/v) bovine serum albumin. Western blotting experiments utilized buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl$_2$, and 5% (w/v) nonfat dry milk. The concentrations and specific radioactivities of the ligands and antibodies used in the incubation mixtures are indicated in the figure legends. Autoradiographs were obtained by exposing the dried nitrocellulose paper to autoradiography film at room temperature for the indicated times.

**Peptide Isolation and Sequence Analysis**—The oocyte receptor was purified as described above and subjected to electrophoresis on a 4.5–18% SDS-polyacrylamide gel and the protein was electrophoretically transferred onto a sheet of nitrocellulose with 20% methanol, 0.192 mM glycine, 25 mM Tris-HCl (pH 8.2) as the transfer buffer. The nitrocellulose filter was stained with Amido Black (30), the region where the 95-kDa protein was bound was sliced out, and the bound protein was digested on the membrane with trypsin (31). The resulting peptides were released into the supernatant and separated by reverse-phase high pressure liquid chromatography (32) using a Vydac C$_4$ column (150 × 2.1 mm) in a Waters peptide analyzer. Prominent peptides were sequenced on an Applied Biosystems model 477A sequenator using standard operating procedures.

**Protease Digestions**—Affinity-purified receptor (in buffer B) was incubated with endoproteinase Arg-C, endoproteinase Lys-C, endoproteinase Arg-C, endoproteinase Lys-C, or trypsin at various concentrations for the indicated periods at 37 °C.
Electron Microscopy—Oocytes and granulosa cell sheets (obtained as described in Ref. 33) were prepared for conventional electron microscopy by fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 4 h at room temperature. After rinsing in phosphate-buffered saline (PBS), the tissue was postfixed in 1% buffered osmium tetroxide for 1 h at room temperature. After a further buffer rinse, the tissue was dehydrated in a graded series of ethanol solutions and propylene oxide and embedded in Araldite. Thin sections were cut, mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.

Immunogold Cytochemistry—Tissue was prepared for ultrastructural immunocytochemistry by fixation in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature. Samples were then prepared for LR (Polysciences, Inc.) gold embedding as follows. The blocks of tissue were dehydrated in ethanol using the following series, with all steps being carried out at −20 °C: 50% ethanol, 45 min; 70% ethanol, 45 min; 1:1 90% ethanol and LR gold resin, 1 h; LR gold resin, overnight; LR gold resin plus initiator, 1 h; LR gold resin plus initiator, overnight. This was then polymerized in capsules under a UV lamp (360 nm) for 24 h. Thin sections were cut and mounted on uncoated nickel grids.

For cytostaining, grids were floated on a drop of filtered 1% bovine serum albumin for 20 min and then transferred without washing to a drop of primary antibody, diluted if necessary with filtered PBS, for 2 h at room temperature. Control grids were floated on nonimmune serum or PBS. After washing with jets of filtered PBS, each grid was floated on three successive drops of PBS for 10 min each. Grids were then floated on drops of protein A-gold (15-nm gold particles; Janssen Life Sciences Products or E-Y Laboratories Inc.) diluted 1:10 with filtered PBS for 1 h at room temperature. Washing was performed using jets of filtered PBS followed by floating on drops of PBS as before. Final washing was carried out with jets of filtered distilled water and blotting with filter paper. Grids were stained with uranyl acetate and lead citrate.

Other Methods—Protein concentrations were determined by the Lowry method (34). The protein content of lipoproteins or samples containing detergent was determined by a modification (27) of the Lowry procedure.

RESULTS

In preliminary solubilization experiments, we determined that membrane preparations from follicles ranging in size from 3 to 15 mm in diameter yielded detergent extracts with the highest VTG/VLDL receptor content. There was no detectable difference in the efficiency of receptor protein solubilization by various detergents, including octyl glucoside, CHAPS, Nonidet P-40, and Triton X-100 (data not shown). Thus, unless the use of octyl glucoside was dictated by analytical procedures such as the solid phase binding assay (27), Triton X-100 was used in the purification scheme. Fig. 1 illustrates the rapid, two-step affinity chromatography procedure yielding pure VTG/VLDL receptor. The first separation step, chromatography on immobilized yolk VLDL, resulted in the isolation of two prominent proteins with apparent molecular weights on SDS-polyacrylamide gels in the absence of sulfhydryl-reducing agents of 95,000 and 380,000, respectively (lanes 2). The large protein did not bind to the affinity matrix used in the second purification step, consisting of immobilized IgG directed against the 95-kDa protein (lanes 3). In both affinity chromatography steps, extensive washing of the columns prior to elution was essential, and elution of bound proteins with dilute NH4OH was an efficient means of recovering active VTG/VLDL receptor. This is in agreement with previous observations during purification of the mammalian LDL receptor (35). The enrichment for receptor activity during successive stages of purification could be demonstrated by ligand blotting (Fig. 1, panels B and C) and immunoblotting (Fig. 1, panel D). With 125I-VLDL as ligand, the 95-kDa receptor, as well as the 380-kDa protein, a major component of the follicular membrane extract, was visualized. In studies to be reported elsewhere), this large protein has been shown to represent the chicken oocyte analogue of the chicken oocyte VTG/VLDL receptor at different stages of purification.

FIG. 1. SDS-polyacrylamide gel electrophoresis of the chicken oocyte VTG/VLDL receptor at different stages of purification. All samples were analyzed on a 4.5–18% SDS-polyacrylamide gradient gel. The molecular mass standards (Std.) (myosin, 200 kDa; phosphorylase b, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; and carbonic anhydrase, 29 kDa) were separated in the presence of, and all other samples in the absence of, 50 mM dithiothreitol. Panel A, lane 1, Triton X-100 membrane extract (20 μg of protein); lane 2, VLDL-Sepharose eluate (9.5 μg of protein); and lane 3, immunoaffinity-purified fraction (7 μg of protein). The positions of migration of the VTG/VLDL receptor (++) and of bands discussed in the text (○, ●) are indicated.

\(^2\) Stifani, S. Barber, D. L., Aebersold, R., Steyer, E., Shen, X., Nimpf, J., and Schneider, W. J. (1991) J. Biol. Chem. 266, in press.
FIG. 2. Ultrastructural immunolocalization of the VTG/VLDL receptor in granulosa cell sheets isolated from ovarian follicles. A, low power observation of an intact follicle shows the cells of the theca interna (th) separated by a basement membrane (bm) from the granulosa cell layer (g). The perivitelline layer (pv) separates the granulosa cells from the oocyte (oo). Yolk spheres (y) are visible in the oocyte. ×1,060. B, the isolated granulosa cell sheet shows an intact basement membrane (bm), granulosa cell layer (g), and perivitelline layer (pv). A piece of ruptured cytoplasm from the oocyte periphery (oo) is attached to the perivitelline layer. ×1,540. C, immunogold cytochemistry of granulosa cell sheets using affinity-purified anti-receptor IgG shows labeling primarily in the oocyte remnant (oo), with some gold particles in the perivitelline layer (pv) into which processes
FIG. 3. Ultrastructural immunolocalization of the VTG/VLDL receptor in intact ovarian follicles. A, in the yolk spheres, the receptor is concentrated in the electron lucent phase and markedly absent from the electron dense phase. $\times 7,140$. B and C, in the periphery of the intact normal oocyte, the anti-VTG/VLDL receptor antibody binds to the receptor on the luminal surface of coated vesicles. $\times 14,860$. D, in contrast to normal oocytes, coated vesicles in the periphery of intact oocytes from the R/O mutant are not immunoreactive with the antibody to the VTG/VLDL receptor. Note the electron dense contents of the vesicles from the mutant oocytes in comparison with those from the normal oocytes. $\times 16,980$.

pits on the surface and to intracellular vesicular structures (Fig. 2D). The 95-kDa protein was undetectable within and on the surface of granulosa cells (Fig. 2E), in agreement with previous biochemical findings demonstrating the absence of the VTG/VLDL receptor from these cells (12), as well as from fibroblasts (11).

Second, immunocytochemical analysis was performed on thin sections prepared from intact ovarian follicles that had been surgically denuded of the external thecal layers and subsequently fixed. The receptor was present within the electron lucent phase of endocytic compartments termed yolk spheres, which are believed to represent storage organelles for yolk proteins (3, 8) (Fig. 3A; and cf. Fig. 2A). Importantly, the VTG/VLDL receptor was also clearly visualized in the periphery of the oocyte, primarily associated with coated pits and vesicles (Fig. 3, B and C). For control purposes, we performed experiments with thin sections of follicles from receptor-defective R/O hens (Fig. 3D). No significant immunoreactivity was observed in the mutant tissue, as expected from the previously demonstrated lack of expression of the 95-kDa protein in nonlaying chicken strain (14). A consistent observation was the electron-dense appearance of the contents of vesicles in the mutant as compared with normal oocytes, clearly visualized in the thin section shown in Fig. 3D. Incubations with nonimmune IgG resulted in the absence of gold particles in all tissue sections (not shown). Taken together, these results directly support our previous notion (11, 12, 15) that the 95-kDa protein is indeed specifically expressed by the oocyte and absent from somatic cells, and furthermore, mediates the uptake of lipoproteins via a coated pit/coated vesicle mechanism.

To obtain additional information on the structure of the oocyte receptor that might support the likely role of the 95-kDa protein in receptor-mediated endocytosis, we determined whether this protein showed sequence homology with the human LDL receptor. The purified protein was subjected to tryptic digestion, and amino acid sequence information of three fragments was obtained. As summarized in Fig. 4, all three sequences could be aligned with unique sequences in the LDL receptors from human, rat, rabbit, and Xenopus (40–43). Peptide I (Fig. 4) contains the tetrapeptide YWTD, a signature sequence of LDL receptors and related proteins that represents the consensus sequence of five repeated modules of approximately 45 residues each. Peptide II showed a 6/9 identity with a highly conserved sequence found in the epidermal growth factor-precursor homology domain of the LDL receptor in close proximity to the last of the five YWTD-containing modules (40). The most informative of the sequenced peptides showed a high degree of identity with a portion of the cytoplasmic domain of mammalian and am-
possibly due to the presence of inhibitory transport receptor. Components in impure preparations. Assuming an assay mixture with crude oocyte membrane extracts (10, 23, 46), purified receptor and the indicated concentration of radiolabeled ligand in the presence (■) or absence (○) of 3 mg/ml of unlabeled VTG. The specific activity of the laying hen "I-VTG was 165 cpn/ng. The amount of receptor-bound radiolabeled ligand was determined as described under "Experimental Procedures." High affinity binding (■) was calculated by subtracting nonspecific binding (□) from total binding (○). Each data point represents the average of duplicate determinations.

FIG. 5. Saturation curve for the binding of "I-VLDL to the purified 95-kDa receptor. Each assay tube contained the standard assay mixture (100 µl) with 7 µg of precipitated immunoaffinity-purified receptor and the indicated concentration of radiolabeled ligand in the presence (■) or absence (○) of 3 mg/ml of unlabeled laying hen VLDL. The specific activity of the laying hen "I-VLDL was 365 cpn/ng. The amount of receptor-bound radiolabeled ligand was determined as described under "Experimental Procedures." High affinity binding (■) was calculated by subtracting nonspecific binding (□) from total binding (○). Each data point represents the average of duplicate determinations.

phian LDL receptors. This region (III in Fig. 4) contains NFDNPVY, exactly the same as in the LDL receptors from man, rabbit, rat, cow, and Xenopus (43). Significantly, the sequence FXNPXY has been identified as the internalization signal of LDL receptors (44, 45), further substantiating our notion that the oocyte plasma membrane protein is a functional transport receptor.

Next, we were interested in the ligand-binding properties of the oocyte receptor, since previous studies employing ligand blotting (10, 15, 23) have shown that at least under those conditions the receptor binds both VLDL and VLDL. Figs. 5 and 6 demonstrate that the isolated protein had the capacity to bind "I-VLDL and "I-VTG with high affinity and in saturable fashion when analyzed by a solid phase filtration assay (27). To determine nonspecific binding of radiolabeled VLDL and VLDL and VLDL, we used excess unlabeled VLDL or suramin (23, 35), respectively. Scatchard analysis of the specific binding data revealed that each ligand interacted with a single class of binding sites, with Keq values of 3.4 µg/ml for VLDL and 2.8 µg/ml for VTG, respectively. These values are severalfold lower than those previously obtained in filtration binding assays with crude oocyte membrane extracts (10, 23, 46), possibly due to the presence of inhibitory or competing components in impure preparations. Assuming an M, of ~500,000 for apolipoprotein B, which is present as a single copy on VLDL and has been shown to mediate receptor binding (20), and of 420,000 for the circulating VTG-dimer (47, 48), half-maximal saturation of the receptor is reached at 6.8 nM apolipoprotein B and 6.7 nM VTG, respectively.

In order to determine the stoichiometry of the binding reaction, it was necessary to quantitate the amount of receptor protein retained by the filters used in the assay system, since it has previously been shown that in the filtration step required to separate receptor-bound from free ligand a considerable portion of LDL receptor protein applied to the filters was lost into the filtrate (49). To correct for these losses, we performed binding assays with "I-labeled pure receptor, which permitted direct measurements of the amount of receptor retained on the filter. Fig. 7 shows the results of an assay of "I-VLDL binding to the "I-labeled receptor. In the ab-
sence of ligand, approximately 3,000 cpm (35% of the radio-active receptor added) were retained by the filters, and the retained portion did not change when the $^{125}$I-labeled receptor had been incubated with unlabelled VLDL at concentrations up to 40 $\mu$g/ml. When $^{125}$I-VLDL instead of unlabelled VLDL was used in the incubations, a saturation curve was obtained; in the presence of excess unlabelled VLDL, only a linear component was observed (not shown). From these data and the specific radioactivities of both receptor and ligand, the amount of ligand bound to the receptor at saturation was calculated to be 7 ng of VLDL/mg of receptor. Assuming an $M_r$ of 90,000 for the protein moiety of the oocyte receptor and an $M_r$ of 700,000 for the protein moiety (apoB plus apoVLDL-II) of VLDL, maximum binding was approximately 0.93 mol of VLDL/mol of receptor. The calculated $K_a$ value for the interaction of the radiolabelled receptor with $^{125}$I-VLDL was 4.1 $\mu$g/ml, and thus the binding properties of the receptor did not appear significantly altered upon radioiodination (cf. Fig. 5, $K_a$ of 3.4 $\mu$g/ml for the unlabelled receptor). Unfortunately, several attempts to perform quantitative binding analysis with $^{125}$I-VTG were unsuccessful because a large non-specific component was elicted at the amounts of radiolabelled receptor required for reproducible binding reactions, compounded by the increased tendency of VTG to aggregate under these conditions.

To characterize further the dual binding capacity of the VTG/VLDL receptor, we performed ligand binding competition assays. Fig. 8 demonstrates that both unlabelled VLDL and VTG competed effectively with $^{125}$I-VLDL for binding sites on the receptor, with 50% reduction observed at $5 \mu$g/ml of either competitor. The discontinuity in the competition curve with unlabelled VTG is most likely due to the adverse properties of this lipoprotein mentioned above, since above 75 $\mu$g/ml of unlabelled VTG, $^{125}$I-VLDL retained by the filter rose sharply to 400% of control (not shown). In contrast to the inhibition by VLDL and VTG, no significant inhibition of $^{125}$I-VLDL binding was observed with either chicken high density lipoprotein (HDL, Fig. 8), which is devoid of apolipoprotein B, or reductively methylated VLDL. Modification of lysine residues of apolipoprotein B (10, 20) by reductive methylation has previously been found to abolish the binding of VLDL to detergent extracts of ovarian follicle membranes containing the 95-kDa oocyte receptor (10). In contrast to the competition by VTG and VLDL for $^{125}$I-VLDL binding, only VTG, but not VLDL, competed for $^{125}$I-VTG binding to the purified receptor (Fig. 9). With $^{125}$I-VTG as tracer ligand, the effects on filter retention encountered with $^{125}$I-VLDL (Fig. 8) were much less pronounced. Chicken HDL appeared to have a small inhibitory effect on $^{125}$I-VTG binding, but the displacement of $^{125}$I-VTG remained smaller than 25% even at high concentrations of unlabelled competitor. As observed with $^{125}$I-VLDL, reductively methylated of the autologous ligand abolished receptor binding of VTG. Furthermore, reductively methylated VLDL, as native VLDL, failed to compete with binding sites for $^{125}$I-VTG on the receptor (not shown). These data clearly demonstrate that the isolated oocyte receptor binds both major plasma lipoproteins that serve as yolk precursors in growing oocytes. Furthermore, the binding sites for the two ligands appear to have similar properties, but are likely not identical, with the binding pocket for the smaller VTG possibly being a substructure of the binding site for the larger and sterically less accessible VLDL particles.

To shed further light on the ligand binding site(s) we subjected the pure receptor to limited digestion with proteases and analyzed the generated fragments for their ability to interact with VTG and/or VLDL by ligand blotting (Fig. 10). Digestion with endoproteinases Arg-C, Lys-C, or Glu-C produced the most consistent proteolytic fragmentation, with ligand binding peptides ranging from 50 to 85 kDa. Both $^{125}$I-VTG and $^{125}$I-VLDL bound to an identical set of fragments. Competition with unlabelled ligands demonstrated that each of the fragments exhibited the same binding properties as the intact receptor. Significantly, the ligand blotting experiments showed that labeled VLDL was displaced from the intact receptor and fragments by both VTG and VLDL, but binding of labeled VTG was only slightly affected by unlabelled VLDL. Thus, these findings are qualitatively in agreement with and provide an independent confirmation of, the filtration binding assay results of Figs. 8 and 9. Coomassie Blue staining revealed the presence of smaller fragments in digested receptor samples (not shown), but none of these appeared capable of binding either of the ligands, suggesting

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig8}
\caption{Effect of unlabelled ligands on the binding of $^{125}$I-VLDL to the purified VTG/VLDL receptor. Each assay tube contained the standard assay mixture (100 $\mu$l) with 15 $\mu$g of precipitated receptor and 4 $\mu$g/ml laying hen $^{125}$I-VLDL (58 cpm/ng) in the presence of unlabelled VLDL (●), reductively methylated VLDL (▲), VTG (■), or rooster HDL (△) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described under "Experimental Procedures." The 100% of-control value was 53 ng $^{125}$I-VLDL bound/filter.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig9}
\caption{Effect of unlabelled ligands on the binding of $^{125}$I-VTG to the purified VTG/VLDL receptor. Each assay tube contained the standard assay mixture (100 $\mu$l) with 13 $\mu$g of precipitated receptor and 3 $\mu$g/ml $^{125}$I-VTG (151 cpm/ng) in the presence of unlabelled VTG (●), reductively methylated VTG (■), VLDL (▲), or rooster HDL (△) at the indicated concentrations. The amount of receptor-bound radiolabelled ligand was determined by filtration as described under "Experimental Procedures." The 100% of-control value was 21 ng $^{125}$I-VTG bound/filter.}
\end{figure}
that both binding functions are closely linked and depend on the structural integrity of a substantial portion of the 95-kDa protein.

**DISCUSSION**

Interest in the 95-kDa protein arises from several observations. First, although it is immunologically related to and shares several biochemical properties with the mammalian LDL receptor, the key component in regulation of cellular cholesterol homeostasis, this receptor is undetectable in purified oocyte 95-kDa receptor. Immunoaffinity-purified receptor (150 μg) was incubated with 1.5 μg of endoproteinase Arg-C (lanes A), endoproteinase Lys-C (lanes B), endoproteinase Glu-C (lanes C), or without enzyme (lanes D) for 18 h at 37 °C in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM CaCl₂, 1 mM PMSF, and 30 mM octyl glucoside. Equal aliquots were separated by 4.5–18% SDS-polyacrylamide gel electrophoresis in the absence of reducing agents and transferred to nitrocellulose. Replicas were incubated in the presence of 125I-VTG (6 μg/ml; 264 cpm/ng) (upper panels) or 125I-VLDL (6 μg/ml; 36 cpm/ng) (lower panels). A 100-fold excess of unlabelled VLDL (center) or unlabelled VTG (right panels) was added to the incubations as indicated. Strips were exposed to Kodak AR film for 4 h (125I-VTG) or for 8 h (125I-VLDL), respectively.

The purification scheme reported herein enabled us to obtain first structural information on the 95-kDa oocyte receptor. Comparison with known primary structures of LDL receptors revealed extensive sequence identity in tryptic peptides of the chicken receptor with characteristic regions in the receptors of man, rabbit, rat, Xenopus laevis, and cow. One of the chicken receptor fragments contained the sequence YWTD repeats facilitates recycling of the chicken receptor likewise is capable of recycling between the cell surface and endocytic compartments.

A second peptide (peptide III in Fig. 4) harbors the sequence FDNPVY, the signal necessary for incorporation of LDL receptors into coated pits, a prime requirement for receptor internalization (55). The F, N, P, and Y residues are especially important, but substitutions with alanine at the D and V positions are tolerated (55). As discussed by Mehta et al. (43) for the X. laevis LDL receptor, the conservation of D and V residues in these positions might provide for a superior internalization signal. Interestingly, although FDNPVY is now known to be conserved in LDL receptors of six species, the corresponding sequences in LRPCs are different, but also conserved among the human (36) and chicken’ proteins. Namely, two presumptive internalization signals, IGNPTY and FTPNVPY, are present among the 100 cytoplasmic residues of the LDL receptors in chicken’ and man (36). Mammalian LRP is thought to function as endocytic receptor for chylomicron remnants and/or α₅-macroglobulin (56). The differences in internalization sequences between LRPCs and LDL receptors, including the chicken oocyte receptor, and the conservation of the pair of hexapeptides in avian and mammalian LRPCs suggest different evolutionary paths for the structural genes specifying these related proteins. In any case, the presence of a clearly defined internalization signal in the chicken oocyte protein further supports its in vivo function as transporter via a coated pit/coated vesicle mechanism.

The capacity of this receptor to import both VTG and VLDL into oocytes has been suggested previously (15), and the current ligand binding data obtained with the pure protein provide direct biochemical evidence. Both lipoproteins interact with the receptor with high affinity; we were able to determine, through a binding assay using radiolabeled pure

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*J. Nimpf et al., manuscript in preparation.*
receptor previously developed for the bovine LDL receptor (27), that each receptor molecule is capable of binding one VLDL particle. In these in vitro experiments, unlabelled VTG competed for the binding of radiolabeled VTG and VLDL, but unlabelled VLDL appeared unable to effectively block access of 125I-VTG to binding site(s) on the receptor. This property was shared by proteolytic fragments of the receptor, generated by digestion with various endoproteinases of defined specificity. It is conceivable that the VTG-binding site on the receptor is a substructure of the VLDL recognition site; the presence of VTG in the binding site(s) may decrease the affinity for VLDL, but the reverse appears not to be the case (Figs. 8-10). Presumably, the significantly different sizes of the two ligands would play an additional role in determining the relative amounts of VTG and VLDL being associated with the population of receptors on the oocyte surface. These considerations indicate that in the in vivo situation, simultaneous transport of VTG and VLDL via the 95-kDa receptor is entirely feasible. In this context, it would be desirable to determine the concentrations of these lipoproteins in the space between the perivitelline layer and the oocyte plasma membrane; however, no studies to address this question have been reported to date.

Following their release from capillaries in the theca interna, plasma components travel across the basement membrane, through gaps between the granulosa cells, and then through the acellular perivitelline matrix, the innermost aspect of which is lined by the oocyte plasma membrane harboring the receptor (Figs. 2 and 3). A possibly oversimplified view of the basement membrane is that of a filter (3) that precludes penetration of particles larger than 40 nm in diameter (55). We have previously shown that apo-VLDL-II, an apolipoprotein exclusively present in VLDL produced by laying hens, serves a dual role in that it protects the lipoprotein particles from lipolysis and is responsible for their smaller size (30-38-nm diameter) (5, 21, 57, and 58) as compared with apo-VLDL-II-free VLDL produced by roosters or immature hens (average diameter, 58 nm) (57). Nevertheless, previous observations suggest that, in the laying hen, VLDL particles accumulate in the basement membrane (53), possibly due to interaction of its sulfated proteoglycans with the apoB moiety of the lipoprotein (59). VTG has been shown to contain sequences similar to regions of apoB (50) and thus might behave in a similar fashion. Indeed, preliminary in vivo turnover experiments with gold-labeled VLDL and VTG indicate that proteoglycan binding sites for both ligands are saturated with the lipoproteins in the steady state, notwithstanding the passage of excess yolk precursor molecules through the basement membrane. Presumably, dynamic changes in the basement membrane, the intercellular spaces in the granulosa cell layer, and the perivitelline matrix provide one level of control for the relative concentrations of yolk precursor proteins encountering the oocyte surface for subsequent receptor-mediated transport into yolk. Fine-tuning of this control might be achieved by the receptor itself. Having identified and characterized the plasma membrane receptor for VTG and VLDL will greatly facilitate the delineation of the multistep pathway for yolk formation in the chicken oocyte and of its role in regulation of this giant cell's growth.

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Oocyte Lipoprotein Receptor

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