Solubilization and characterization of the chicken oocyte vitellogenin receptor

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This paper describes the biochemical characterization of the chicken oocyte plasma-membrane receptor for one of the major lipid-carrying yolk proteins, vitellogenin (VTG). The receptor was extracted from oocyte membranes with the non-ionic detergent octyl- β -D-glucoside and visualized by ligand blotting, with 125 I-VTG as a protein with an apparent M_r of 96 000, under non-reducing conditions. It exhibited high affinity for native chicken VTG (K_d 2×10^{-7} M) but was unable to bind VTG with reductively methylated lysine residues or phosvitin (the phosphoserine-rich intracellular cleavage product of VTG). Polyclonal antibodies to the 96 kDa protein inhibited VTG binding to the receptor and were able to precipitate functional VTG-receptor activity from oocyte-membrane detergent extracts with a concomitant removal of the 96 kDa protein. Antibodies directed against the mammalian receptor for low-density lipoprotein showed cross-reactivity with the chicken oocyte VTG receptor, raising the possibility that lipoprotein receptors in birds are structurally related to those in mammalian species.

INTRODUCTION

The major source of nutrients for the developing embryo of all oviparous (egg-laying) animals is the egg yolk which constitutes the non-organellar content of the oocyte. The oocyte's dramatic growth during the final stages of maturation is a result of massive deposition of yolk proteins [1], a process that has been termed vitellogenesis. In the domestic hen (*Gallus domesticus*), experimental evidence suggests that plasma vitellogenin (VTG), a lipophosphoglycoprotein, and triglyceride-rich lipoproteins of the very-low-density class, are the main precursors of yolk lipid components [2,3]. Both lipoproteins, as well as other yolk precursors, are synthesized in the liver of mature females under the control of oestrogen, secreted into the blood stream, transported to the ovary, and selectively taken up by the oocytes [4]. Specific cell surface receptors have been implicated in mediating their uptake by the mechanism of receptor-mediated endocytosis [5–11]. VTG, a collective term for a group of homodimeric proteins of 240 kDa subunits, is of particular interest in that after its selective uptake by the oocytes, it is proteolytically cleaved into the yolk proteins termed lipovitellins and phosvitins [12]. These cleavage products of VTG are stored during oogenesis and constitute a depot of amino acids, lipid, inorganic phosphate, and possibly calcium, during embryogenesis. Most of the lipid present on the VTG molecules is associated with the lipovitellin portion and is composed of approx. 60 % phospholipid and approx. 40 % triacylglycerols plus cholesterol [13]. Phosvitin is a glycosylated phosphoprotein containing approx. 60 % phosphoserine residues, and possibly minor satellite components [14]. In chicken, this complex yolk-protein pattern derives from the fact that there are three different VTG molecules which can be distinguished by a lack of immunological cross-reactivity, and by their different amino acid compositions, as well as peptide-cleavage patterns [15].

We have begun studies aimed at gaining a better understanding of the mechanisms involved in the control of oocyte growth. Here, we report the preliminary characterization of the chicken oocyte receptor for VTG. The receptor was solubilized by using detergent extraction from oocyte membranes and was identified by ligand blotting in conjunction with a solid-phase filtration assay.

MATERIALS AND METHODS

Materials

We obtained DEAE-cellulose DE-52 from Whatman, Protein A-Sepharose CL-4B and Sephadex G-25 columns PD 10 from Pharmacia, and Iodogen (1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycuril) from Pierce Chemical Co. Octyl- β -D-glucoside, phenylmethanesulphonyl fluoride (PMSF), leupeptin, aprotinin, phosvitin and bovine serum albumin were from Sigma; M_r standards from Bethesda Research Laboratories; nitrocellulose paper BA 85 from Schleicher and Schuell, Keene, NH, U.S.A.; Nuflow cellulose acetate membrane filters N25/45 UP from Oxoid; Swinnex W/O filtration units (Cat. No. SX0002500) from Millipore; [125 I]NaI (11–17 mCi/ μ g) from Edmonton Radiopharmaceutical Centre, Edmonton, Alberta, Canada, and suramin (sodium salt) from FBA Pharmaceuticals, NY, U.S.A. Other materials were obtained from previously reported sources [16].

Animals and diets

White Leghorn layers (8–18 months old) were obtained from a local poultry farm and maintained on layer mash, with a daylight period of 12 h. Adult female New Zealand rabbits were used for the production of polyclonal antibodies.

Abbreviations used: VTG, vitellogenin; LDL, low-density lipoprotein; PMSF, phenylmethanesulphonyl fluoride.

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Purification of VTG

VTG was purified from laying-hen plasma as described by Deeley *et al.* [12] with some modifications. Briefly, about 10 ml of blood was drawn from the wing vein and collected in ice-cold tubes containing the following reagents (final concentrations): 16 mM-sodium citrate/1 mM-PMSF/2 μ M-leupeptin/aprotinin (2.5 μ g/ml). All buffers used in the purification of VTG by DEAE-cellulose column chromatography contained 2 mM- CaCl_2 and 1 mM-PMSF. After elution from the ion-exchange column [12], the fractions containing VTG were pooled and dialysed at 4 °C against buffer containing 150 mM-NaCl/0.2 mM-EDTA/1 mM-PMSF, pH 7.4. After dialysis, leupeptin (final concn. 2 μ M) and aprotinin (final concn. 2.5 μ g/ml) were added, and the protein was stored in portions at -70 °C. Protein concentrations were determined as described by Lowry *et al.* [17] with bovine serum albumin as standard; phosvitin concentrations were determined as described by Woods & Roth [18].

Protein radio-iodination and modification

VTG and phosvitin were radiolabelled with ^{125}I by the Iodogen method as described by Schneider *et al.* [19]. Reductively methylated VTG was prepared by treatment with formaldehyde plus NaBH_4 [20].

Preparation of oocyte membranes

All operations were performed at 0–4 °C. Follicles (3–15 mm diameter) were rapidly transferred to buffer containing 20 mM-Tris / HCl (pH 8) / 2 mM- CaCl_2 / 150 mM-NaCl/1 mM-PMSF/2 μ M-leupeptin (buffer A). Theca externa and theca interna were removed by dissection and the yolk extruded through an incision. The remaining material, consisting of basal lamina, granulosa cell layer, perivitelline layer and oocyte plasma membrane, was rinsed with buffer A until the wash fluid was free of yolk. The membranes were minced with scissors and subjected to homogenization in 10 ml of buffer A/g wet wt. with a Polytron homogenizer (30 s at setting 5 followed by two periods of 20 s, each at setting 8). Large debris was removed by centrifugation at 5000 *g* for 5 min and the resulting supernatant was subjected to centrifugation at 100 000 *g* for 1 h. The membrane pellets were suspended in buffer A by aspiration through a 22-gauge needle and re-sedimented by centrifugation at 100 000 *g* for 1 h; this was repeated once. The washed membrane pellets were quickly frozen in liquid N_2 and stored at -70 °C for up to 3 months before use.

Solubilization of chicken VTG receptors

All operations were carried out at 0–4 °C. In a typical solubilization experiment, membrane pellets obtained from the ovary of one chicken (weight of follicles 7–10 g) were suspended in 1.5 ml of buffer containing 250 mM-Tris/maleate (pH 6.0)/2 mM- CaCl_2 /1 mM-PMSF/2 μ M-leupeptin by aspiration through a 22-gauge needle. The suspension was sonicated twice for 20 s (Sonifier model W 185, Heat-System-Ultrasonics Inc., Plainview, NY, U.S.A.) with a microprobe at setting 6. Reagents were then added to adjust the suspension to a final volume of 3 ml containing the following components: 3–5 mg of protein/ml/125 mM-Tris/maleate (pH 6.0)/2 mM- CaCl_2 /160 mM-NaCl/0.5 mM-PMSF/1 μ M-leupeptin/36 mM-octyl- β -D-glucoside. The suspen-

sion was stirred at 4 °C for 10 min and undissolved material was removed by centrifugation at 100 000 *g* for 1 h. The clear supernatant, designated octylglucoside extract, was either quickly frozen in liquid N_2 and stored at -70 °C for up to 1 month or used immediately.

Electrophoresis, transfer to nitrocellulose and ligand blotting

One-dimensional electrophoresis according to Laemmli [21] was conducted on 4.5–18% polyacrylamide gradient slab gels. Electrophoresis was carried out at 10 °C and 35 mA/slab gel. When electrophoresis was conducted under reducing conditions, samples contained 10 mM-dithiothreitol and were heated at 90 °C for 5 min. Gels were calibrated with the following molecular-mass standards: myosin, 200 kDa; phosphorylase *b*, 97 kDa; bovine serum albumin, 68 kDa; ovalbumin, 43 kDa; α -chymotrypsinogen, 26 kDa; β -lactoglobulin, 18 kDa; lysozyme, 14 kDa. Gels were stained with Coomassie Blue. For ligand blotting experiments, samples were not reduced and not heated before application. Electrophoretic transfer of proteins to nitrocellulose was performed according to Daniel *et al.* [22]. Ligand blotting and Western blotting were carried out with 5% non-fat dry milk as blocking agent in buffer containing 25 mM-Tris/HCl (pH 8.0)/50 mM-NaCl/2 mM- CaCl_2 . The concentration and specific radioactivity of the ligand used in the incubation mixtures are indicated in the Figure legends. Autoradiograms were obtained by exposing the dried nitrocellulose paper to Kodak XAR-5 film at room temperature for the indicated times.

Filter assay for binding of ^{125}I -VTG to oocyte extracts

Octylglucoside extracts were diluted by addition of 7 vol. of buffer containing 50 mM-Tris/maleate (pH 6.0)/2 mM- CaCl_2 in order to adjust the detergent concentration to below its critical micellar concentration [23]. VTG binding sites were collected by centrifugation of the diluted extracts at 100 000 *g* for 1 h at 4 °C. The precipitate, designated precipitated octylglucoside extract, was resuspended by aspiration through a 22-gauge needle in buffer containing 25 mM-Tris/HCl (pH 8.0)/50 mM-NaCl/2 mM- CaCl_2 and used in the filter assay. The standard assay mixture (pH 8.0) of total vol. 100 μ l contained 12.5 mM-Tris/HCl/25 mM-NaCl/2 mM- CaCl_2 /16 mg of bovine serum albumin/ml, the indicated amount of protein of precipitated octylglucoside extract and the indicated concentrations of ^{125}I -VTG (see Figure legends). Incubation was for 90 min at room temperature and free ^{125}I -VTG was separated from receptor-bound ^{125}I -VTG by filtration as described [23]. It was absolutely necessary to filter the VTG solutions used in these experiments before assay since this protein tends to form aggregates that interfere with the assay by clotting the filters and giving high background levels. This pre-filtration was performed with Nuflow cellulose acetate filters (0.45 μ m) in Swinnex filtration units from Millipore, resulting in a protein loss of 5–10%. Assay filter background levels were less than 0.2% of the total radioactivity in the assay mixture. Filtered solutions were stored at 4 °C and not used for more than 2 days.

Immunological procedures

Antisera against the 96 kDa protein were raised in adult female New Zealand White rabbits by subcutaneous

Fig. 1. SDS/polyacrylamide-gel electrophoresis of chicken VTG

Chicken VTG was purified and radiolabelled with ^{125}I as described in the Materials and methods section. Electrophoresis was performed on a 4.5–18% SDS/polyacrylamide gradient gel under reducing conditions. Lane A, autoradiogram of ^{125}I -VTG; 1.5 μg of protein was loaded (specific activity 254 c.p.m./ng). This portion of the gel was dried and autoradiographed for 4 h. Lane B, M_r

injection of antigen prepared as follows. The protein, as visualized by ligand blotting in nitrocellulose replicas obtained from the same SDS/polyacrylamide gel, was cut out from the gel after electrophoresis under non-reducing conditions. Protein was eluted from the gel according to the procedure described by Hunkapiller *et al.* [24] and used for immunization. Rabbits received a first injection of antigen in Freund's complete adjuvant (day 0) and successive booster injections (days 14, 21, and 28) using incomplete Freund's adjuvant. On day 35, and once each week thereafter, the animals were bled from the ear vein and sera tested for the presence of antibody by Western blotting. IgG fractions were purified from sera on columns of Protein A–Sepharose CL-4B as previously described [25].

RESULTS

Purification and radiolabelling of chicken VTG

VTG was isolated from plasma of laying hens using the method described by Deeley *et al.* [12] with some minor modifications that improved the stability of the purified protein (described in the Materials and methods section). In particular, the presence of 2 mM- CaCl_2 and the protease inhibitors, leupeptin and aprotinin, in all isolation buffers resulted in preparations in which breakdown products of VTG were consistently absent upon storage at 4 °C for 5–7 days after isolation. When we attempted to radiolabel VTG with ^{125}I , we initially

standards, from top to bottom: myosin (200 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), α -chymotrypsinogen (26 kDa), β -lactoglobulin (18 kDa), lysozyme (14 kDa). Lane C, photograph of the gel used in lane A. Lane D, 3 μg of unlabelled VTG. Proteins were stained with Coomassie Blue. The side marks indicate the top of the separating gel.

Fig. 2. Binding of ^{125}I -VTG to precipitated octylglucoside extract of oocyte membranes

(a) Each assay tube contained the standard assay mixture (100 μl) with 33 μg of protein of precipitated octylglucoside extract and the indicated concentrations of ^{125}I -VTG (51 c.p.m./ng) in the absence (■) or presence (△) of 5 mM-suramin. High affinity binding (□) was determined by subtraction of non-specific binding (△) from total binding (■). (b) Scatchard plot of the high affinity binding data determined by the NIH program LIGAND [29]. The ratio bound/free is the amount of bound ^{125}I -VTG (μg of protein/ml) divided by the amount of unbound protein in the reaction mixture (μg of protein/ml).

Fig. 3. Ligand blotting of oocyte VTG receptors

Oocyte membrane octylglucoside extract (35 μ g of protein/lane) was subjected to electrophoresis on a 4.5–18% SDS/polyacrylamide gradient gel followed by transfer to nitrocellulose. Ligand blotting was performed as described in the Materials and methods section. All strips were incubated in 10 ml of buffer containing 2.6 μ g of 125 I-VTG/ml (254 c.p.m./ng) with the following additions: lane A, none; lane B, 130 μ g of unlabelled VTG/ml; lane C, 130 μ g of unlabelled methylated VTG (meVTG)/ml. The positions of migration of M_r standards are indicated. Autoradiography was for 12 h.

observed extensive fragmentation of the protein. However, with the Iodogen method, and keeping the specific radioactivity below 500 c.p.m./ng of protein, 125 I-VTG (free of breakdown products) was obtained. Fig. 1 shows the results of SDS/polyacrylamide-gel electrophoresis of a typical VTG preparation (lane D), and also demonstrates that after radiolabelling with 125 I, the VTG remained intact (lanes A and C). The preparations of unlabelled and 125 I-VTG presented in Fig. 1 were used in all experiments described herein.

Binding of 125 I-VTG to oocyte membrane octylglucoside extracts

Octylglucoside extracts of oocyte membranes were analysed for their ability to bind 125 I-VTG by a solid-phase filtration assay [23]. Binding reached identical maximum levels at 4, 23, and 37 $^{\circ}$ C after 40 min, showed a pH optimum of 8, and was linear with respect to oocyte membrane protein up to 0.6 mg/ml (results not shown). Under the standard conditions described in the Materials and methods section, binding of 125 I-VTG was saturable and showed high affinity (Fig. 2a). Furthermore, the saturable component of 125 I-VTG binding was abolished in the presence of 5 mM-suramin, leaving only a non-specific, linear component. Suramin is a polysulphated polycyclic hydrocarbon that has been shown to inhibit the binding of VTG to the locust oocyte receptor [26], and also inhibits the binding of human LDL [19], apoprotein E-containing lipoproteins [27], and platelet-derived growth factor [28], to their respective receptors. The same non-specific binding component found with suramin was observed when excess unlabelled VTG was present in incubation mixtures (results not shown).

Scatchard analysis by the NIH program LIGAND [29] of the high affinity binding data gave a linear plot indicating a single binding site on oocyte membranes for VTG (Fig. 2b). The apparent K_d was calculated to be 96 μ g/ml (approx. 2×10^{-7} M, assuming that dimeric VTG, M_r approx. 480 000, is the physiological ligand) and a maximum of 13 μ g of 125 I-VTG protein was bound per mg of protein of precipitated octylglucoside extract.

Identification and characterization of the VTG receptor

We applied the technique of ligand blotting [22] for visualization of the VTG-receptor protein (Fig. 3). Portions of oocyte membrane octylglucoside extracts were analysed by this method with 125 I-VTG as ligand, resulting in the identification of a protein with an apparent M_r of 96 000 under non-reducing conditions (Fig. 3, lane A). The presence of a 50-fold excess of unlabelled VTG in the incubation medium resulted in a dramatic reduction of 125 I-VTG binding to this protein (Fig. 3, lane B). In contrast, no effect on the binding of 125 I-VTG to the 96 kDa protein was observed in the presence of a 50-fold excess of VTG that had been subjected to reductive methylation (Fig. 3, lane C). The ligand blotting data were supported by parallel solid-phase filtration binding experiments; binding of 125 I-VTG to the precipitated octylglucoside extract was competitively inhibited by unlabelled VTG but was not significantly affected by methylated VTG (Fig. 4a). The lack of competition by methylated VTG was not due to breakdown of VTG during its chemical modification, since the derivatized VTG appeared intact upon inspection by SDS/polyacrylamide-gel electrophoresis (Fig. 4b).

The ligand specificity of the chicken oocyte VTG receptor was further analysed by ligand blotting (Fig. 5). We found that as much as a 780-fold molar excess of unlabelled phosvitin (M_r 36 000) failed to inhibit the binding of 125 I-VTG to the 96 kDa protein (Fig. 5, lane 3). In contrast, only a 50-fold molar excess of unlabelled VTG almost completely abolished receptor binding of 125 I-VTG (Fig. 5, lane 2). Suramin at a concentration of 5 mM totally inhibited binding of 125 I-VTG to the 96 kDa protein (Fig. 5, lane 4) in agreement with the solid-phase binding data presented in Fig. 2.

Fig. 4. Competition of unlabelled lipoproteins for binding of ^{125}I -VTG to oocyte VTG receptors

(a) Each assay tube contained the standard assay mixture (100 μl) with 36 μg of protein of precipitated octylglucoside extract, 15 μg of ^{125}I -VTG/ml (254 c.p.m./ng) and the indicated concentrations of unlabelled VTG ($\blacktriangle$) or unlabelled methylated VTG (meVTG) ($\square$). The values represent the average of duplicate incubations. (b) Chicken VTG was purified (lane 1) and its lysine residues methylated

Fig. 5. Specificity of binding of ^{125}I -VTG to oocyte VTG receptors

Oocyte membrane octylglucoside extract (35 μg of protein/lane) was subjected to electrophoresis on a 4.5–18% SDS/polyacrylamide gradient gel followed by transfer to nitrocellulose. Ligand blotting was performed as described in the Materials and methods section. All strips were incubated in 10 ml of buffer containing 1.7 μg of ^{125}I -VTG/ml (127 c.p.m./ng) with the following additions: lane 1, none; lane 2, 85 μg of unlabelled VTG/ml; lane 3, 100 μg of unlabelled phosvitin (PV)/ml; lane 4, 5 mM-suramin (SUR). The positions of migration of the M_r standards are indicated. Autoradiography was for 30 h.

Use of antibodies directed against the 96 kDa oocyte plasma-membrane protein

Polyclonal antibodies directed against the 96 kDa protein were prepared and IgG was isolated by chromatography on Protein A-Sepharose as described in the Materials and methods section. The IgG fraction, designated anti-OR-IgG, reacted exclusively with the 96 kDa VTG receptor as determined by Western blotting of oocyte octylglucoside extracts (results not shown). More importantly, anti-OR-IgG inhibited the binding of ^{125}I -VTG to its receptor. Fig. 6(a) shows that after incubation with increasing concentrations of anti-recep-

(lane 2) as described in the Materials and methods section. Electrophoresis was performed on a 4.5–18% SDS/polyacrylamide gradient gel under reducing conditions. Lanes 1 and 2 contain 2 μg of protein each and lane 3, M_r standards (STD; the same as in Fig. 1, lane B). Proteins were stained with Coomassie Blue.

Fig. 6. Inhibition of ¹²⁵I-VTG binding to oocyte VTG receptors by anti-receptor antibodies

(a) Oocyte membrane octylglucoside extract (35 μ g of protein/lane) was subjected to electrophoresis in a 4.5–18% SDS/polyacrylamide gradient gel followed by transfer to nitrocellulose. Ligand blotting was performed as described in the Materials and methods section. All strips were incubated in 10 ml of buffer with the following additions: lanes 2–5, anti-OR-IgG, lanes 7–10, control IgG, in the following concentrations: lanes 1 and 6, none; lanes 2 and 7, 40 μ g/ml; lanes 3 and 8, 80 μ g/ml; lanes 4

and 9, 160 μ g/ml; lanes 5 and 10, 320 μ g/ml. After incubation for 90 min at room temperature, the nitrocellulose strips were extensively washed with buffer and then incubated in 20 ml of buffer with 4.9 μ g of ¹²⁵I-VTG/ml (85 c.p.m./ng). The positions of migration of M_r standards are indicated. Autoradiography was for 8 h. (b) The regions of the nitrocellulose strips used in the experiment shown in (a) corresponding to receptor-bound ¹²⁵I-VTG (as indicated by autoradiography) were cut out and counted for ¹²⁵I radioactivity. Counts were plotted versus the concentration of IgG in the incubation buffer for anti-OR-IgG ($\square$; Imm.) and for control IgG ($\triangle$; Con.). The data are from one representative experiment; two other experiments gave identical results.

DISCUSSION

The present results demonstrate that chicken oocytes possess plasma membrane receptors for interaction with VTG. The receptor protein was solubilized from membranes by extraction with the non-ionic detergent octyl- β -D-glucoside. After solubilization, measurable VTG binding activity could be recovered in a precipitate obtained by decreasing the detergent concentration of the extract to below its critical micellar concentration. As previously shown [23], most of the octylglucoside is thereby removed, facilitating the quantification of receptor binding of lipoproteins. The precipitated octylglucoside extract was used in solid-phase filtration assays which showed that binding of ¹²⁵I-VTG to chicken oocyte membranes is specific, saturable, and displays high affinity. Scatchard analysis with the NIH program LIGAND [29] revealed a single binding site with a K_d of approx. 2×10^{-7} M (approx. 96 μ g of VTG/ml).

and 9, 160 μ g/ml; lanes 5 and 10, 320 μ g/ml. After incubation for 90 min at room temperature, the nitrocellulose strips were extensively washed with buffer and then incubated in 20 ml of buffer with 4.9 μ g of ¹²⁵I-VTG/ml (85 c.p.m./ng). The positions of migration of M_r standards are indicated. Autoradiography was for 8 h. (b) The regions of the nitrocellulose strips used in the experiment shown in (a) corresponding to receptor-bound ¹²⁵I-VTG (as indicated by autoradiography) were cut out and counted for ¹²⁵I radioactivity. Counts were plotted versus the concentration of IgG in the incubation buffer for anti-OR-IgG ($\square$; Imm.) and for control IgG ($\triangle$; Con.). The data are from one representative experiment; two other experiments gave identical results.

Fig. 7. Immunoprecipitation of oocyte VTG receptors

Octylglucoside extract (400 μ g of protein in 100 μ l) was mixed with 600 μ l of buffer containing 10 mM-sodium phosphate (pH 7.4), 150 mM-NaCl, 36 mM-octylglucoside (buffer C) and 1.75 mg of IgG/ml. Lanes A and D, anti-OR-IgG; lanes B and E, anti-(bovine LDL receptor) IgG; lanes C and F, control IgG. After incubation for 4 h at 23 °C, 100 μ l of a 50% (v/v) suspension of Protein A-Sepharose in buffer C was added. Following incubation for 2 h at 23 °C, the tubes were centrifuged for 2 min in an Eppendorf Microfuge. Both supernatants and precipitates were recovered. Of each supernatant, 60 μ l was loaded onto a 4.5–18% SDS/polyacrylamide gradient gel (lanes D–F). Each precipitate was washed three times with 1 ml of buffer C and then incubated with 100 μ l of buffer containing 62.5 mM-Tris/HCl (pH 6.8), 2.3% (w/v) SDS and 10% (v/v) glycerol for 15 min at 23 °C to liberate the Protein A-Sepharose-bound material. This was recovered in the supernatant following a 90 s centrifugation step in an Eppendorf Microfuge. Portions (50 μ l) of each supernatant were loaded onto lanes A to C of the gel. After electrophoresis and transfer to nitrocellulose, ligand blotting was performed with 6 μ g of VTG/ml (specific activity 254 c.p.m./ng) as described in the Materials and methods section. The positions of migration of the M_r standards are indicated. Autoradiography was for 16 h; after obtaining the photograph shown, the radioactive areas were cut out from the nitrocellulose strips and their radioactivity determined. The radioactivity in an equal size piece representing the background was determined for each lane and subtracted. The resulting radioactivities were: 8320, 1030, 30, 2700, 18320 and 23410 c.p.m. for lanes A, B, C, D, E and F, respectively.

Previously, Woods & Roth [8] reported a K_d value of 2.5×10^{-6} M for the binding of VTG to chicken oocyte membranes. In that study, suspensions of crude membrane fractions were used to determine 125 I-VTG binding in a centrifugation assay; it is possible that the observed

lower affinity of VTG-binding was due to contamination of membranes with adhering yolk proteins that would affect the binding of trace amounts of 125 I-labelled ligand. We have effectively removed yolk proteins by detergent solubilization and subsequent membrane protein recovery by detergent dilution. The precipitated extract had a maximum specific binding activity of approx. 13 μ g of VTG/mg of membrane protein, about 10-fold higher than the value reported by Woods & Roth [8], indicating that the detergent extraction significantly enriched the VTG receptors. High-affinity binding (K_d 4.2×10^{-8} M), similar to that reported here, was obtained by Röhrkasten & Ferenz [31] for the interaction of VTG with octylglucoside-solubilized oocyte membranes in the insect *Locusta migratoria*.

An interesting observation was that the binding of VTG to its receptor was inhibited by the drug suramin (Fig. 2a and Fig. 5, lane 4). Previous studies have shown that the binding of various ligands such as LDL [19], platelet-derived growth factor [28], and locust VTG [26] to their respective receptors can be blocked by suramin. Thus, the VTG receptor behaves similarly to other receptors involved in endocytosis utilizing the coated pit-coated vesicle mechanism.

When the technique of ligand blotting was applied to identify the oocyte membrane receptor, we visualized it as a protein with an apparent M_r of 96000 under non-reducing conditions. In contrast, when octylglucoside extracts were subjected to electrophoresis in the presence of disulphide reducing agents, the binding activity was lost, thus suggesting that intrachain disulphide bonds within the receptor molecule are necessary for retention of its biological activity (results not shown). This behaviour is also analogous to that of the receptors for certain other lipoproteins such as LDL [19] and apolipoprotein E-containing particles [27].

Previous experimental work indicated that the locust VTG receptor apparently recognizes certain positive charges on the VTG molecule; modification of lysine residues reduced the binding of VTG to oocyte binding sites [26,31,32]. Hence, reductive methylation of lysine residues in chicken VTG was performed and the modified VTG was tested for its ability to inhibit the binding of 125 I-VTG to its receptor. Both solid-phase filtration assays and ligand blotting experiments demonstrated that methylated VTG failed to compete with 125 I-VTG for binding to the 96 kDa oocyte-membrane protein, while unmodified VTG abolished such binding effectively (Figs. 3 and 4). The possibility that this observation was due to extensive degradation of VTG by the use of NaBH_4 and formaldehyde in the methylation reaction, is highly unlikely, because a comparison by SDS/polyacrylamide-gel electrophoresis of native and methylated VTG showed only a very small change in the electrophoretic purity of the modified protein (Fig. 4b). Therefore, the results suggest that lysine residues may be essential for the interaction of VTG with its receptor. A similar finding was reported for the binding of apolipoprotein B in human LDL to its receptor [20].

Further analysis of ligand specificity of the VTG receptor revealed that excess unlabelled phosvitin, one of the intraoocytic processing products of endocytosed VTG, was unable to block the binding of 125 I-VTG to its receptor, even when present in a 780-fold molar excess (Fig. 5, lane 3). This finding, together with experiments that failed to show any binding of 125 I-phosvitin to

Table 1. Immunoprecipitation of oocyte VTG receptors

Portions of oocyte membrane octylglucoside extract (150 μ l containing 450 μ g of protein) were precipitated with the indicated IgG fractions at a concentration of 1.5 mg/ml in a total volume of 800 μ l as described in the legend to Fig. 7. The resulting supernatants (500 μ l) were diluted with 3.5 ml of buffer containing 50 mM-Tris/maleate (pH 6.0), 2 mM-CaCl₂, and the precipitated octylglucoside extract collected and resuspended in 500 μ l of buffer as described in the Materials and methods section. Binding of ¹²⁵I-VTG was measured at a concentration of 40 μ g/ml (specific radioactivity 127 c.p.m./ng), with each tube containing 30 μ l of resuspended precipitate, by the solid-phase filtration assay described in the Materials and methods section. Non-specific binding was determined from incubations containing 5 mM-suramin. Each value represents the average of duplicate incubations, with less than 5% variation.

IgG fraction	¹²⁵ I-VTG bound (ng/filter)			¹²⁵ I-VTG bound (% of control)
	Total	Non-specific	High affinity	
Control	628	14	614	100
Anti-OR	128	19	109	18
Anti-(bovine LDL receptor)	492	21	471	77
None	642	11	631	103

oocyte membrane proteins in ligand blotting experiments, or in the solid-phase binding assay at various pH values (results not shown), seems to rule out the possibility that the phosvitin portion of the VTG molecule is the one that is recognized by the VTG receptor. This finding is in contrast with previous reports by Woods & Roth [8] that claimed that phosvitin can bind to the same receptor as VTG. It should be noted, however, that these authors performed their binding experiments at pH 6.0. In the present studies, the pH optimum for VTG binding was shown to be 8.0, and no binding of either phosvitin or VTG was observed at pH 6.0. The possibility that the other portion of oocyte VTG processing, namely, lipovitellin, is recognized by the VTG receptor, can at present not be tested since lipovitellins are not sufficiently soluble to afford binding studies (they form the insoluble portion of egg yolk).

The ligand blotting experiments allowed us to identify the position where the VTG-binding protein migrated on SDS gels, to cut out the region of the gel containing the protein, and to elute it from the gel and use it for immunization of rabbits. The resulting polyclonal antibodies were used for further characterization of the 96 kDa protein. First, the antibody was capable of blocking the binding of ¹²⁵I-VTG to the 96 kDa oocyte membrane protein immobilized on nitrocellulose (Fig. 6). Second, the VTG-binding activity was precipitated from octylglucoside extracts with the anti-96 kDa antibody, but not with control IgG (Fig. 7 and Table 1). These findings demonstrate that the VTG-binding activity solubilized by octylglucoside from oocyte membranes represents the genuine VTG receptor.

Finally, the finding that polyclonal IgG directed against the bovine LDL receptor cross-reacts to some extent with the VTG receptor raises the possibility that avian oocyte lipoprotein receptors are not only related to each other, but also may share structural features with mammalian receptors for cholesterol-carrying lipoproteins. In this context, we have recently shown that the anti-(bovine LDL receptor) antibody recognizes the chicken oocyte receptor for low- and very-low-density lipoproteins migrating on SDS/polyacrylamide gels under non-reducing conditions as a 95 kDa protein [33].

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REFERENCES

- Gilbert, A. B. (1971) in *Physiology and Biochemistry of the Domestic Fowl* (Bell, D. J. & Freeman, G. M., eds.), vol. 3, pp. 1163–1208, Academic Press, London and New York
- Paterson, R., Youngner, W. O. & Devon, E. J. (1962) *J. Gen. Physiol.* **45**, 501–513
- Perry, M. M. & Gilbert, A. B. (1979) *J. Cell Sci.* **39**, 257–279
- Wallace, R. A. (1985) in *Developmental Biology* (Browder, L. W., ed.), vol. 1, pp. 127–177, Plenum Press, New York
- Yusko, S. C. & Roth, T. F. (1976) *J. Supramol. Struct.* **4**, 89–97
- Roth, T. F., Cutting, J. A. & Atlas, S. D. (1976) *J. Supramol. Struct.* **4**, 527–548
- Yusko, S. C., Roth, T. F. & Smith, T. (1981) *Biochem. J.* **200**, 43–50
- Woods, J. W. & Roth, T. F. (1984) *Biochemistry* **23**, 5774–5780
- Krumins, S. A. & Roth, T. F. (1981) *Biochem. J.* **126**, 481–488
- Krumins, S. A. & Roth, T. F. (1985) *J. Cell. Biochem.* **28**, 281–288
- Opresko, L. K. & Wiley, H. S. (1987) *J. Biol. Chem.* **262**, 4109–4115
- Deeley, R. G., Mullinix, K. P., Wetekam, W., Kronenberg, H. M., Meyers, M., Eldridge, J. P. & Goldberger, R. E. (1975) *J. Biol. Chem.* **250**, 9060–9066
- Bernardi, G. & Cook, W. H. (1960) *Biochim. Biophys. Acta* **44**, 96–105
- Wallace, R. A. & Morgan, J. P. (1986) *Biochem. J.* **240**, 871–878
- Wang, S. Y., Smith, W. E. & Williams, D. L. (1983) *Biochemistry* **22**, 6206–6212
- Schneider, W. J., Goldstein, J. L. & Brown, M. S. (1985) *Methods Enzymol.* **109**, 405–417
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

18. Woods, J. W. & Roth, T. F. (1979) *J. Supramol. Struct.* **12**, 491–504
19. Schneider, W. J., Beisiegel, U., Goldstein, J. L. & Brown, M. S. (1982) *J. Biol. Chem.* **257**, 2664–2673
20. Weisgraber, K. H., Innerarity, T. L. & Mahley, R. W. (1978) *J. Biol. Chem.* **253**, 9053–9062
21. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
22. Daniel, T. O., Schneider, W. J., Goldstein, J. L. & Brown, M. S. (1983) *J. Biol. Chem.* **258**, 4606–4611
23. Schneider, W. J., Basu, S. K., McPhaul, M. J., Goldstein, J. L. & Brown, M. S. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 5577–5581
24. Hunkapiller, M. W., Lujan, E., Ostrander, F. & Hood, L. E. (1983) *Methods Enzymol.* **91**, 227–236
25. Beisiegel, U., Schneider, W. J., Goldstein, J. L., Anderson, R. G. & Brown, M. S. (1981) *J. Biol. Chem.* **256**, 11923–11931
26. Röhrkasten, A. & Ferenz, H.-J. (1987) *Wilhelm Roux's Arch. Dev. Biol.* **196**, 165–168
27. Hui, D. Y., Brecht, W. J., Hall, E. A., Friedman, G., Innerarity, T. L. & Mahley, R. W. (1986) *J. Biol. Chem.* **261**, 4256–4267
28. Williams, L. T., Tremble, P. M., Lavin, M. F. & Sunday, M. (1984) *J. Biol. Chem.* **259**, 5287–5294
29. Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–230
30. Tolleshaug, H., Hobgood, K. K., Brown, M. S. & Goldstein, J. L. (1983) *Cell* **32**, 941–951
31. Röhrkasten, A. & Ferenz, H. J. (1986) *Biochim. Biophys. Acta* **860**, 577–582
32. Röhrkasten, A. & Ferenz, H. J. (1985) *Wilhelm Roux's Arch. Dev. Biol.* **194**, 411–416
33. George, R., Barber, D. L. & Schneider, W. J. (1987) *J. Biol. Chem.*, in the press

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