THE JOURNAL OF EXPERIMENTAL ZOOLOGY 274:163–170 (1996)

Vitellogenin Receptors During Vitellogenesis in the Rainbow Trout *Oncorhynchus mykiss*

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ABSTRACT Rainbow trout vitellogenin receptors have been characterized by ligand blotting and Scatchard analysis. Their evolution has been studied over a reproductive cycle in a broodstock of 2-year-old females. The receptors were prepared from ovarian membrane homogenates and were solubilized using n-octyl-β-D-glucopyranoside. The visualization of the receptor by ligand blotting using ^{125}iodine-vitellogenin after sodium dodecyl sulfate electrophoresis revealed the existence of one major binding component corresponding to a protein of 113 kDa. The Scatchard transformation of the binding data revealed a single class of binding sites with an apparent K_d of $1.8 \cdot 10^{-8}$ M/L. The variations of the binding characteristics (K_d and maximum binding) were investigated during vitellogenesis. This study revealed that the K_d was not affected by oocyte growth during vitellogenesis, but was highly decreased in ovulated eggs. The receptor number increased during the same period from 35 to 860 fM per oocyte, while the receptor number per mm^2 of oocyte membrane surface was doubled during the same period.

Oocyte vitellogenesis is one of the best examples of cell specialization for specific endocytosis of proteins. Vitellogenin (VTG) is a lipophosphoglycoprotein hepatically synthesized which constitutes the main yolk precursor of the egg. It reaches the ovary by the blood stream to be selectively taken up by the growing oocytes and enters the oocyte follicle by capillary vessels located in the theca

et al., '89), and fish (Stifani et al., '90a; Le Menn and Nuñez Rodriguez, '91; Chan et al., '91; Tyler and Lancaster, '93) has been shown to be a receptor-mediated mechanism, probably acting at the oolemma level just prior to endocytosis.

The aim of the present study is to characterize the VTG receptor system in rainbow trout oocytes and to analyze the evolution of the receptor char-

diphenylglycouril) were purchased from Sigma (St. Louis, MO). ^{125}Iodine (^{125}I; IMS 30) was purchased from Amersham (Arlington Heights, IL). Suramin was obtained from Mobay Chemical Corp. (NY).

Purification of rainbow trout VTG

VTG was purified from plasma of 17β-estradiol (E_2) treated fish receiving an E_2 cumulated dose of 5 mg/kg over a 15 day period. Blood was collected in heparinized syringes from the caudal artery, transferred to Ependorf tubes containing 1 mM PMSF to reduce protein degradation, and centrifuged for 5 min at 12,000*g*. Plasma aliquots were immediately frozen in liquid nitrogen.

VTG was obtained with one-step ion-exchange DEAE-Biogel A (Bio-Rad, Richmond, CA) chromatography using 100 mM Tris buffer (pH 7.8) with 2 mM calcium chloride and 1 mM PMSF. Elution was performed at 4°C with a linear gradient (0–250 mM) of sodium chloride (Fig. 1).

Fractions of the main peak were identified as VTG on sodium dodecyl sulfate-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE) compared to control males and vitellogenic females. The VTG migrated as a single band with an apparent molecular mass of 133 kDa. The fractions retained were pooled and concentrated on Amicon cell to the desired protein concentration determined by the Bradford ('76) method using ovalbumin as protein standard. It was not possible to determine the molecular mass of native VTG since the molecule is completely fractionated into 133 kDa subunits under non-reducing SDS electrophoresis. A determination by gel filtration on 6B Sepharose (Pharmacia, Piscataway, NJ) indicated for the native VTG a molecular mass around 450 kDa. We used this value in the calculations of specific activity and binding.

Iodination of VTG

Iodination of VTG with ^{125}I was performed by the iodogen method. Two milligrams of trout VTG and 10 µl (37×10^6 Bq) of ^{125}I Na (IMS 30) were added to 200 µg of iodogen coated in a glass scintillation vial. After 20 min the mixture was chromatographed on a 20 × 1 cm column Sephadex G 75 (Pharmacia) with 25 mM Tris buffer (pH 7.8) and 2 mM $CaCl_2$. The selected ^{125}I-VTG fractions were pooled filtered through 0.45 µm filters (MSI, Westboro, MA) and aliquots were stored (–30°C) in 50% glycerol. In these conditions the specific activities never exceeded 70,000 dpm/pM. The quality of the ^{125}I-VTG was checked by electrophoresis and immunoblotting (Fig. 2) using polyclonal rainbow trout VTG antibodies kindly provided by Dr. P.Y. Le Bail (INRA, Rennes, France).

Preparation of ovarian membranes and VTG receptor solubilization

The ovarian follicles were crushed in 25 mM Tris (pH 8.0), 50 mM NaCl, 2 mM $CaCl_2$, and 1 mM PMSF (buffer A), with a loosely adjusted glass-teflon homogenizer. The mixture was filtered through gauze (100 µm mesh) and extensively

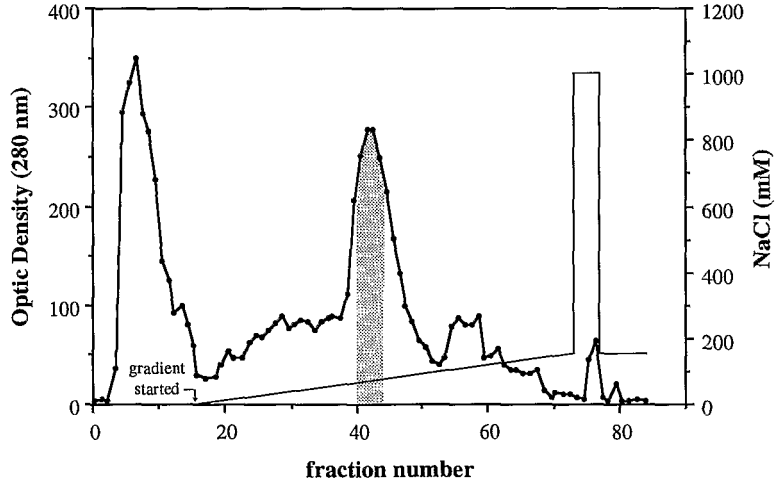
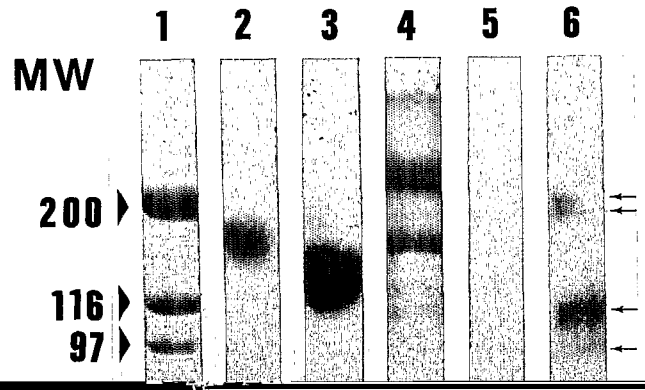


Fig. 1. Ion-exchange chromatography of rainbow trout VTG. One milliliter of 17β-estradiol treated fish plasma was applied to a 30 × 1 cm column of DEAE-Biogel A. Elution was performed with an NaCl linear gradient (0–250 mM) in 0.1 M Tris buffer (pH 7.8) containing 1 mM PMSF and 2 mM $CaCl_2$ at a flow rate of 36 ml/hr. Shaded area corresponds to the fractions retained from the VTG peak eluted at 75 mM NaCl.



proximately 0.1% of the initial ovary weight. This pellet was stored frozen in liquid nitrogen to be used in the binding experiments or for solubilization after resuspension in the appropriate buffer.

The solubilization of the 100,000*g* pellet was carried out in 150 mM Tris-maleate buffer (pH 6.0), 2 mM $CaCl_2$, 1 mM PMSF, 150 mM NaCl, and 36 mM n-octyl-β-D-glucopyranoside. The solution was stirred for 10 min at 5°C and centrifuged 1 hr at 5°C and 100,000*g*.

The supernatant containing the solubilized material was recovered and frozen in liquid nitro-

filtered through 0.45 μm cellulose acetate membrane filters (MSI) previously incubated for 60 min in buffer C. The membrane filters were then placed in appropriate tubes and counted for radioactivity in a Kontron γ counter. Scatchard analysis (Scatchard, '49) and other calculations are performed with Biosoft packages (Kinetic, EBDA, Ligand, Lowry) for Apple Macintosh computers. These programs have been adapted from those described previously by Munson and Rodbard ('80) and McPherson ('85).

Oocyte surface calculations

The total oocyte surface in mm^2 for an entire ovary is calculated as follows:

incubation of iodinated trout VTG with crude oocyte membrane preparations.

In order to determine the time required to reach equilibrium, appropriate tubes containing 85 μg of 100,000*g* membrane pellet (equivalent to 100 mm^2 of mid-vitellogenic oocytes, 2.5 mm of mean diameter) per tube were incubated for a maximum of 120 min at 22°C with 150,000 dpm of ^{125}I-VTG. The specific binding increased rapidly until 40 min and reached a plateau after 1 hr of incubation (Fig. 3), indicating that binding equilibrium was achieved. According to this result, the incubation time was fixed at 120 min in further experiments.

The saturability of the binding has been studied by adding increasing amounts of ^{125}I-VTG to

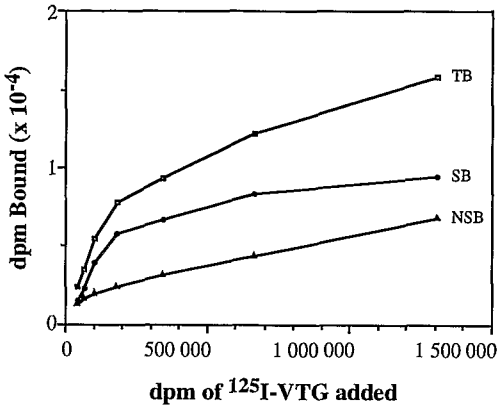


Fig. 4. Saturation of labeled VTG binding. Increasing amounts of ^{125}I-VTG (40,000–1,280,000 dpm) were added to the tubes containing the ovarian membranes (85 µg of protein per tube equivalent to 100 mm^2 of oocyte surface membranes). TB, total binding; SB, specific binding; NSB, non-specific binding. Each point corresponds to the mean of triplicate determinations. See Figure 3 for other details.

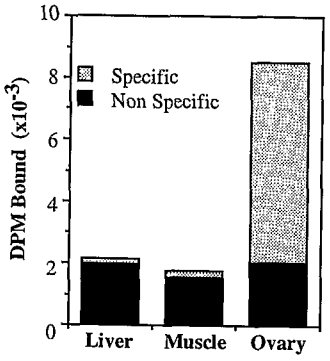


Fig. 6. Tissue specificity of trout VTG binding. See Figure 3 for other details.

Competitive displacement studies have been undertaken to confirm receptor specificity for VTG (Fig. 7). Various competitors (VTG, male plasma proteins, and suramin) were incubated with the labeled trout VTG and ovarian membranes. Trout VTG inhibited 90% of the binding at a concentration of 0.4 mg/ml; equivalent displacement was obtained with 1.4 mg/ml of suramin (1 mM). Total inhibition was

oocyte membrane preparations during vitellogenesis of a winter strain of females. The average oocyte diameter varied from 1.11 mm in July [corresponding to a gonadosomatic index (GSI) of 0.57] to 3.97 mm just prior to ovulation (corresponding to GSI of 9.84). The average diameter of the ovules obtained by stripping was 4.45 mm (Fig. 8).

During the same period the K_d values of the VTG receptors did not exhibit any significant variation (18–30 nM). A marked reduction in the K_d values (10 nM) was only observed in the stripped ovules (Fig. 9).

The oocyte receptor number during vitellogenesis (Fig. 10) increased very rapidly for the oo-

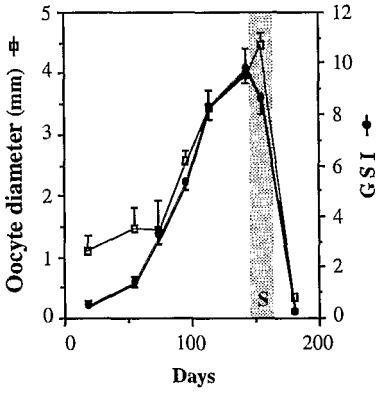


Fig. 8. Evolution of oocyte diameter and GSI during vitellogenesis in rainbow trout. S, spawning period. Values correspond to the mean of five females ± SEM.

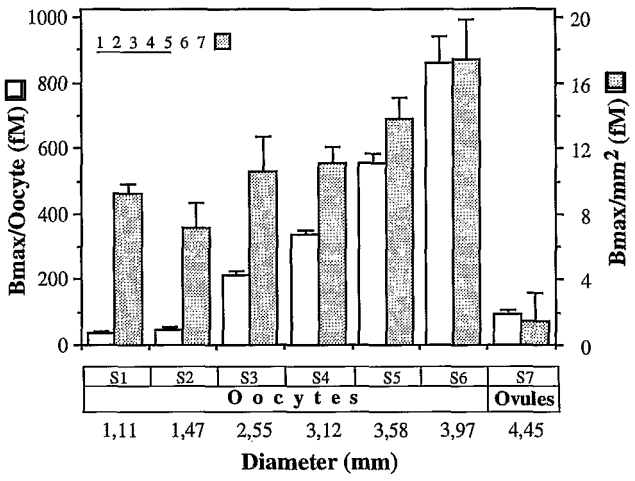


Fig. 10. Variations of the binding capacity of ^{125}I-VTG per oocyte and per mm^2 of oocyte membrane surface in vitellogenic oocytes and in stripped ovules. Values represent the mean of three determinations in triplicate ± SEM. Underlined numbers at the top of the figure indicate means that are not significantly different ($P \geq 0.05$) determined by analysis of variance.

25 times lower. These receptor numbers correspond to a VTG binding capacity of 20 ng per oocyte at the onset of vitellogenesis and 400 ng per oocyte at the end of vitellogenesis. The receptor

receptor number, which is equivalent to the number found in early vitellogenic oocytes and about 40 times less regarding the receptor number per mm^2. These findings demonstrate that the VTG receptor system in the oocytes is dramatically modified by the maturation-ovulation process.

Since the first demonstration of VTG receptors fish, carp, sea bass (Nuñez Rodriguez, unpublished results; Stifani et al., '90a), chicken (Woods and Roth, '80; Stifani et al., '88), and *Xenopus* (Stifani et al., '90b), the VTG receptor was found to be a single protein band with a molecular mass around 100 kDa. In the locust, cockroach, and mosquito, a single 200 kDa band has been

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., *72*:248–254.

Busson-Mabillot, S. (1984) Endosomes transfer yolk proteins to lysosomes in the vitellogenic oocyte of the trout. Biol. Cell, *51*:53–66.

Chan, S.L., C.H. Tan, M.K. Pang, and T.J. Lam (1991) Vitellogenin purification and development of assay for vitellogenin receptor in oocyte membranes of the tilapia (*Oreochromis niloticus*, Linnaeus 1766). J. Exp. Zool., *257*:96–109.

Dhadialla, T.S., A.R. Hays, and A.S. Raikhel (1992) Characterization of the solubilized mosquito vitellogenin receptor. Insect Biochem. Mol. Biol., *22(8)*:803–816.

George, R., D.L. Barber, and W.J. Schneider (1987) Characterization of the chicken oocyte receptor for low and very low density lipoproteins. J. Biochem. Chem., *262(35)*:16838–16847.

Goldstein, J.L., M.S. Brown, R.G.W. Anderson, and M.S. Brown (1979) Coated pits, coated vesicles and receptor mediated endocytosis. Nature, *279*:679–685.

Goldstein, J.L., M.S. Brown, R.G.W. Anderson, D.W. Russell, and W.J. Schneider (1985) Receptor-mediated endocytosis: Concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol., *1*:1–39.

Indrasith, L.S., H. Kindle, and B. Lanzrein (1990) Solubilization, identification and localization of vitellogenin binding sites in follicles of the cockroach, *Nauphoeta cinerea*. Arch. Insect Biochem. Physiol., *15*:213–228.

Kaplan, J. (1981) Polypeptide-binding membrane receptors: Analysis and classification. Science, *212*:14–20.

Lancaster, P., and C.R. Tyler (1991) Isolation of a membrane bound affinity protein from rainbow trout (*Oncorhynchus mykiss*) follicles. In: Reproductive Physiology of Fish. A.P. Scott, ed. University of East Anglia, Norwich, UK, p. 128.

Le Menn, F., and E. Burzawa-Gerard (1985) Effects of carp gonadotropin (cGTH), a fraction unadsorbed on concanavalin A-Sepharose obtained from cGTH on vitellogenesis in the hypophysectomized marine teleost *Gobius niger*. Gen. Comp. Endocrinol., *57*:23–36.

Le Menn, F., and J. Nuñez Rodriguez (1991) Receptor mediated endocytosis of VTG in fish follicle. In: Reproductive Physiology of Fish. A.P. Scott, ed. University of East Anglia, Norwich, UK, pp. 300–302.

McPherson, G.A. (1985) Analysis of radioligand binding experiments: A collection of computer programs for the IBM PC. J. Pharmacol. Methods, *14*:213–228.

Munson, P.J., and D. Rodbard (1980) LIGAND: A versatile computerized approach for the characterization of ligand binding systems. Anal. Biochem., *107*:220–239.

Opresko, L.K., and H.S. Wiley (1987) Receptor-mediated endocytosis in *Xenopus* oocytes. I. Characterization of the vitellogenin receptor system. J. Biol. Chem., *262(9)*: 4109–4115.

Opresko, L., H.S. Wiley, and R.A. Wallace (1980) Differential postendocytotic compartmentation in *Xenopus* oocytes is mediated by a specifically bound ligand. Cell, *22*:47–57.

Perry, M.M., and A.B. Gilbert (1979) Yolk transport in the ovarian follicle of the hen (*Gallus domesticus*): Lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. J. Cell Sci., *39*:257–272.

Röehrkasten, A., H.-J. Ferenz, B. Buschmann-Gebhardt, and J. Hafer (1989) Isolation of the vitellogenin-binding protein from locust ovaries. Arch. Insect Biochem. Physiol., *10*: 141–149.

Röhrkasten, A., and H.-J. Ferenz (1986) Solubilization of the locust vitellogenin receptor. Biochim. Biophys. Acta, *860*: 577–582.

Roth, T.F., J.A. Cutting, and A.S.B. (1976) Protein transport: A selective membrane mechanism. J. Supramol. Struct., *4*:527–548.

Scatchard, G. (1949) The attraction of proteins for small molecules and ions. Ann. N.Y. Acad. Sci., *51*:660–672.

Segaloff, D.L., and M. Ascoli (1988) Internalization of peptide hormones and hormone receptors. In: Hormones and Their Actions, Part I. B.A. Cooke, R.J.B. King, and H.J. van der Molen, eds. Elsevier Science Publishers BV (Biomedical Division), Amsterdam, pp. 133–149.

Selman, K., and R. Wallace (1989) Cellular aspects of oocyte growth in teleosts. Zool. Sci., *6*:211–231.

Silversand, C., S.J. Hyllner, and C. Haux (1993) Isolation, immunochemical detection, and observations of the instability of vitellogenin from four teleosts. J. Exp. Zool., *267*:587–597.

Stifani, S., R. George, and W.J. Schneider (1988) Solubilization and characterization of the chicken oocyte vitellogenin receptor. Biochem. J., *250*:467–475.

Stifani, S., F. Le Menn, J. Nuñez Rodriguez, and W.J. Schneider (1990a) Regulation oogenesis: The piscine receptor for vitellogenin. Biochim. Biophys. Acta, *1045*:271–279.

Stifani, S., J. Nimpf, and W.J. Schneider (1990b) Vitellogenesis in *Xenopus laevis* and chicken: Cognate ligands and oocyte receptors. J. Biol. Chem., *265*:882–888.

Tyler, C.R., and P. Lancaster (1993) Isolation and characterization of the receptor for vitellogenin from follicles of the rainbow trout, *Onchorhynchus mykiss*. J. Comp. Physiol. B, *163*:225–233.

Wallace, R.A. (1985) Vitellogenesis and oocyte growth in nonmammalian vertebrates. In: Developmental Biology. L.W. Browder, ed. Plenum Press, New York, pp. 127–177.

Woods, J.W., and F.R. Roth (1980) Selective protein transport: Identity of the solubilized phosvitin receptor from chicken oocytes. J. Supramol. Struct., *14*:473–481.