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Simple and Sensitive Detection of Vitellogenin Receptor(s) in Sakhalin Taimen (*Hucho perryi*)

Naoshi HIRAMATSU[1),2)], Haruhisa FUKADA[1)], Craig V. SULLIVAN[2)] and Akihiko HARA[1)]

Abstract

Oocyte membranes from vitellogenic ovarian follicles in Sakhalin taimen (*Hucho perryi*) were solubilized using a detergent, n-octyl-β-D-glucopylanoside. Taimen vitellogenin receptor(s) (VGR) in the extracts was visualized by enhanced chemical luminescence (ECL)-ligand blotting using biotinylated vitellogenin as a probe. The ovarian membranes revealed five receptor proteins in the ligand blotting under non-reducing condition. When the excess volume of non-biotinylated vitellogenin was added, or biotinylated bovine serum albumin was used as another probe, the receptor proteins could not be detected, indicating that the receptor proteins were specific for Vg. To obtain an information for the receptor binding site in Vg molecule, displacement of biotinylated Vg from ovarian membrane extracts by vitellogenin-derived competitive ligands was conducted. Lipovitellin could displace the labeled vitellogenin as well as vitellogenin, while β'-component and phosvitin showed less replacement, indicating that the major receptor-binding site is lipovitellin domain in Vg molecule.

Key words: Vitellogenin, Vitellogenin receptor, *Hucho perryi*, Receptor binding assay, Ligand blotting

Introduction

Oocyte vitellogenesis is one of the best examples of cell specialization for specific endocytosis of proteins. Vitellogenin (Vg), a precursor of egg yolk, reaches the ovary *via* the blood stream to be selectively taken up by growing oocytes, entering the ovarian follicle through capillary vessels located in the thecal layer. Vg reaches the germinal cell after passing through the basal lamina, intracellular spaces of granulosa cells, and then into the extracellular matrix of the granulosa epithelium and along the oocyte microvilli into the channels of the zona radiata until contacting the oolemma (Selman and Wallace, 1989). Internalization occurs on specialized areas of the plasma membrane of the oocyte leading to the formation of coated pits, pinching off vesicles of the oolemma and which entering the apical ooplasm. Vg internalization in amphibians (Opresko and Wiley, 1987; Stifani et al., 1990b), birds (George et al., 1987; Stifani et al., 1988), insects (Röehrkasten et al., 1989), and fish (Stifani et al., 1990a; Le Menn and Nuñez Rodriguez, 1991; Chan et al., 1991; Tyler and Lancaster, 1993; Nuñez Rodriguez et al., 1996; Tao et al., 1996; Tyler and Lubberink, 1996) has been shown to be a receptor-mediated mechanism, and a cell surface receptor for Vg (VGR) has been identified in these studies. A feature common to vertebrate VGRs is their high affinity for Vg. The molecular mass of VGR was first estimated by ligand blotting analysis to be 100 kDa in coho salmon, *Oncorhynchus kisutch* (Stifani et al., 1990a). In rainbow trout (*Oncorhynchus mykiss*), two current papers demonstrated the visualization of VGR by ligand blotting using radio-labeled Vg. Tyler and Lubberink (1996) detected 4 receptor proteins (220, 210, 110 and 100 kDa) with the main binding associated with the 210 kDa form, while Nuñez Rodriguez et al. (1996) also showed 4 bands (173, 168, 113 and 99 kDa) but the main binding was associated with the 113 kDa form. Thus, the molecular mass and number of VGRs remain to be definitively verified in salmonids. As to the binding site for VGR in Vg molecule, no study has been done in oviparous vertebrates. In this study, a simple detection system for the VGR was developed utilizing enhanced chemical luminescence ligand blotting (ECL-ligand blotting) in Sakhalin taimen (*Hucho perryi*). The receptor recognition moiety of Vg in the taimen was identified using a non-isotopic receptor assay for Vg using biotinylated ligand and 96-well microtiter plate.

Materials and Methods

Fish

Mature female Sakhalin taimen was reared at Nanae Fish Culture Experimental Station, Faculty of Fisheries,

[1)] *Division of Marine Biosciences, Graduate School of Fisheries Sciences, Hokkaido University* (北海道大学大学院水産学研究科生命資源科学専攻生命機能学講座)
[2)] *Department of Zoology, North Carolina State University, Raleigh, NC, 27695-7617, USA*

Hokkaido University. Serum and ovaries were collected and stored at −30°C until use.

Purification of Vg and yolk proteins

Taimen Vg and yolk proteins were purified as described in our previous report (Hiramatsu and Hara, 1996).

Preparation of oocyte membrane extracts

Vitellogenic taimen ovarian follicles were stripped out of the ovary into 0.02 M Tris-HCl (pH 8.0) containing 2% NaCl and 0.01% NaN_3. The follicles were then crushed and washed with the Tris-HCl buffer until they were white in color. The follicle preparation was stirred in the Tris-HCl buffer containing 0.1, 0.5 or 1% n-octyl-β-D-glucopyranoside and aprotinin (3.75 TIU/ml) at 4°C for 2 hr, and then homogenized with glass homogenizer. The homogenized membrane was centrifuged at 10,000×g for 60 min. The supernatant was concentrated by an ultra filtration and centrifuged again to remove formed insoluble materials. The supernatant was collected as crude oocyte membrane extracts.

Biotinylation of taimen Vg

Taimen Vg was biotinylated as described in our previous study (Hiramatsu and Hara, 1997).

Electrophoresis and ligand blot analysis

One-dimensional electrophoresis was carried out according to Laemmli (1970) on 10% SDS-polyacrylamide gel. Electrophoretic transfer of proteins to PVDF membranes was done according to Kwon et al. (1990). After blocking with 5% non-fat skim milk for 30 min, the PVDF membranes with the transferred proteins were incubated for 2 hr in 0.02 M Tris-buffered saline-1% Tween (TBS-T) containing biotinylated Vg (50 μg in 25 ml of TBS-T) with or without a 250-fold excess of native (non-biotinylated) Vg. After twice washing with TBS-T for 15 min each, the membranes were incubated with streptavidin-HRPO at 1 : 10,000 dilution in TBS-T. After washing as described above, the membrane proteins that were coupled to biotinylated Vg and streptavidin coupled to peroxidase (avidin-HRPO, Boehringer Mannheim Biochem, Germany) were visualized by ECL Western blotting analysis system (Amersham International plc, England).

Receptor binding assays

Receptor binding assays were carried out in 96-well polystyrene ELISA microtiter plate (ICN Biomedicals, Horsham, PA, USA). Wells were coated with 100 μl of oocyte membrane extracts diluted at concentrations of 200 μg/ml with Tris-HCl buffer, pH 8.0, containing 2% NaCl and 0.02% NaN_3 for 4 hr at room temperature. The wells were washed three times with 200 μl of the Tris-HCl buffer containing 1% Triton X-100 (TBS-T). After blocking with 200 μl of 1% bovine serum albumin in the Tris-HCl buffer at 4°C overnight and washing with the TBS-T, displacement of biotinylated Vg (1 mg/ml) from oocyte membrane extracts by non-labeled Vg or Vg-derived yolk proteins, lipovitellin (Lv), phosvitin (Pv) and β'-component (β'), was conducted. Competitive ligands were added at serial dilutions of 100–0.3 μg/ml to the biotinylated Vg solution (final volume; 100 μl) and incubated for 20 hr at room temperature. After washing with TBS-T, each wells received 100 μl of avidin-HRPO diluted 1 : 10,000 with the Tris-HCl buffer, followed by incubation for 2 hr at room temperature. The plates were washed as described above and the color development was conducted at room temperature for 30 min in the dark by adding 150 μl of *o*-phenylenediamine (3 mg/ml 0.1 M citric acid-phosphate buffer pH 5.0 containing 0.02% H_2O_2) to each well. The reaction was stopped by adding 150 μl of 4 N HCl per well. The absorbance at 492 nm was measured using an ELISA plate reader (Bio-Rad 2550, Richmond, CA, USA).

Results

Detection of vitellogenin receptor by ligand blotting

SDS-PAGE and corresponding ligand blotting of oocyte membrane extracts from taimen are shown in Fig. 1. SDS-PAGE pattern revealed several bands with molecular mass from 175 to 16.5 kDa. Ligand blotting using biotinylated Vg as a probe showed some smear bands. These bands disappeared when the excess amount of non-biotinylated Vg was added. On the other hand, biotinylated bovine serum albumin showed no binding to the membrane extracts.

Effect of detergents on solubilization of oocyte membrane

Solubilization of oocyte membranes was conducted with three different concentrations (0.1, 0.5 and 1.0%) of n-octyl-β-D-glucopyranoside (Fig. 2). In spite of concentrations of detergent, same band pattern was observed in the ligand blotting. Five bands were visualized with molecular mass of $>$175 kDa, 90 kDa, 63 kDa, 46 kDa, and 35 kDa.

Subunit structure of vitellogenin receptor

Oocyte membrane extracts was applied to SDS-PAGE and ligand blots under reducing or non-reducing condi-

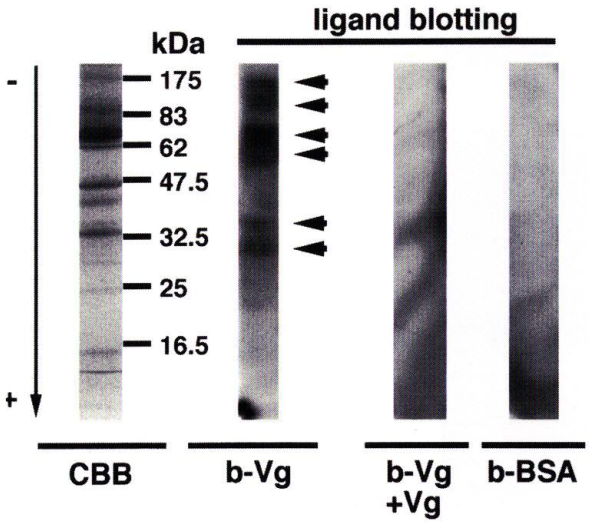

Fig. 1 Ligand blotting of oocyte membrane extracts from Sakhalin taimen ovary. The membrane extracts was applied to SDS-PAGE, followed by electroblotting, and then incubated with biotinylated vitellogenin (b-Vg) in the presence or absence of excess amount (×250) of non-labeled vitellogenin (b-Vg+cold Vg). Another probe, biotinylated bovine serum albumin (b-BSA), was used as well as b-Vg without competitive ligands to show a control binding for the membrane proteins. SDS-PAGE gel was stained with Coomassie Brilliant Blue (CBB).

tions (Fig. 3). Under reducing condition, the bindings corresponding to >175 kDa and 90 kDa found in non-reducing condition disappeared, while 35 kDa band was stained more strongly, suggesting the existence of subunit structure in VGR by disulfide bonds.

Displacement of biotinylated Vg from oocyte membrane extracts by Vg-derived yolk proteins

As shown in Fig. 4, native Vg (non-labeled Vg) and Lv reduced total binding of biotinylated Vg to the membrane extracts more than 90% at a concentration of 100 μg/ml, whereas higher affinity as a ligand was found in Vg than Lv when the concentration of the ligands were under 50 μg/ml. β' also showed that ~50% displacement at a concentration of 100 μg/ml, but Pv had almost no ability (~10%) to displace the binding.

Discussion

Major part of oocyte growth is due to uptake of Vg in fish (Selman and Wallace, 1989). Uptake rate of Vg has been considered to be regulated by changes of both numbers and turn over cycles of VGR. Thus, it is very important for understanding of regulation of oocyte growth to accumulate fundamental knowledge about VGR.

In previous studies (Nuñez Rodriguez et al., 1996; Tao et al., 1996; Tyler and Lubberink, 1996), radiolabeled Vg was used for ligand blotting as a probe to detect VGR. ECL-ligand blotting used in this study has several advantages over the autoradiography as follows : time of exposure to X-ray film is short (5 min) ;

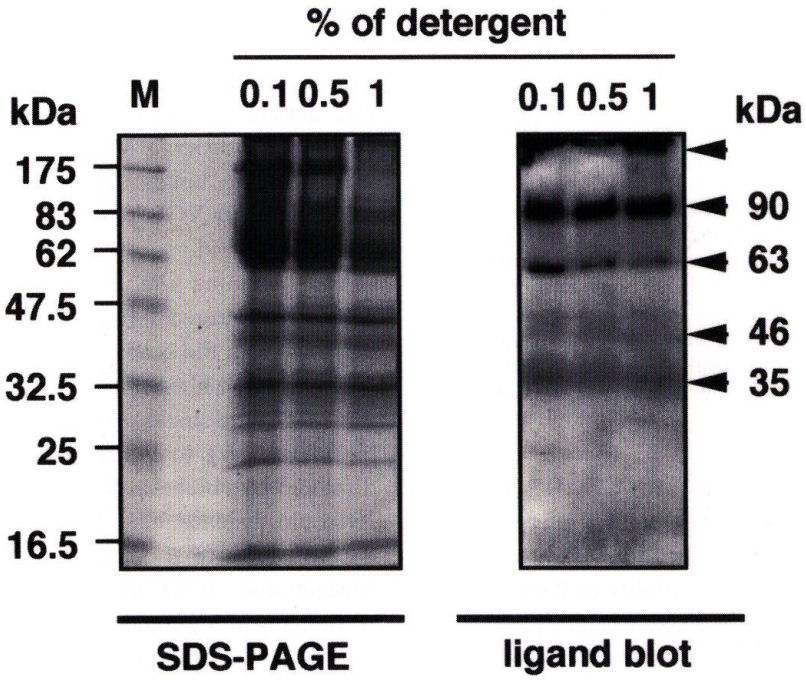

Fig. 2 SDS-PAGE and the corresponding ligand blotting of oocyte membrane extracts that solubilized by 0.1, 0.5, and 1.0% of a detergent, n-octyl-β-D-glucopyranoside. SDS-PAGE gel was stained with Coomassie Brilliant Blue. M : Prestained protein marker.

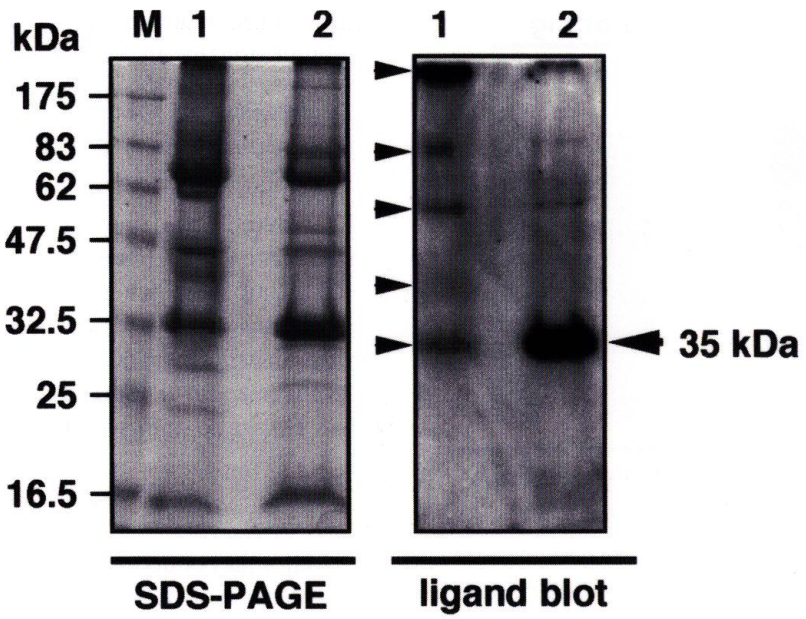

Fig. 3 SDS-PAGE and ligand blotting of oocyte membranes reduced (lane 1) or non-reduced (lane 2) with 2-mercaptoethanol. M : Prestained protein marker. SDS-PAGE gel was stained with Coomassie Brilliant Blue.

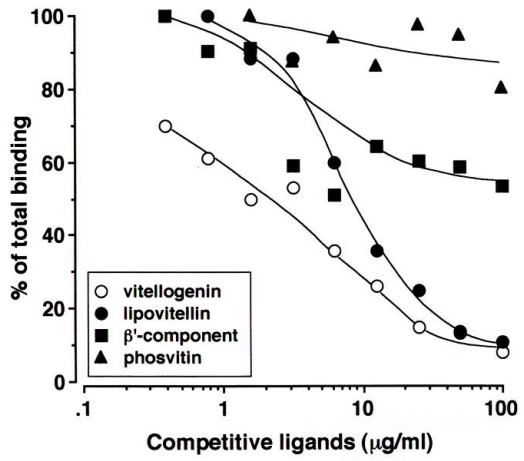

Fig. 4 Displacement of biotinylated Vg (b-Vg) from ovarian membranes by purified vitellogenin and yolk proteins. Total binding was determined by the incubation of b-Vg in wells immobilized with membrane extracts. From 0.3 to 100-fold excess amount of purified Vg and yolk proteins were added and incubated with b-Vg to see the displace ability. Symbols represent the average binding of b-Vg in triplicate incubations.

the sensitivity is extremely high as well as isotopic method ; biotinylation of Vg is simple and safety.

In the present study, five receptor proteins with high affinity to Vg, but without binding ability to BSA, were found in ECL-ligand blotting. These receptor proteins were identified as VGR because they could be displaced by native Vg (unlabeled Vg). Profiles of visualized taimen VGR with the main binding associated with the 90 kDa form was similar to those found in previous studies of rainbow trout (Nuñez Rodriguez et al., 1996). The binding with molecular mass over 175 kDa was also found in ECL-ligand blotting. This binding could be corresponded with the binding associated with the 220 and 210 kDa form in rainbow trout (Tyler and Lubberink, 1996). The bindings associated with lower molecular mass than 90 kDa (63, 46 and 35 kDa) have not detected in other studies. These bands might be degraded products of VGR proteins in taimen.

There has been no report on subunit structure of VGR. The main binding associated with the 35 kDa was detected in ECL-ligand blotting under reduced condition, indicating an existence of subunit structure with disulfide bonds. However, loss of the binding ability of other VGR proteins by the reduction remains to be considered.

VGR binding site in Vg molecule has not been definitively cleared. In chicken, Yusko et al. (1981) suggested that the binding site located in Pv domain in Vg molecule, while Stifani et al. (1990b) showed that the site was Lv I domain. In the present study, binding of biotinylated Vg to the membrane extracts was mainly displaced by unlabeled Vg and Lv. Unlabeled Vg and Lv could displace 90% of total binding of biotinylated Vg, while native β' showed 50% of displacement at a concentration of 100 μg/ml. These results suggest that β' domain in Vg molecule also has minor binding site to VGR, in addition to Lv domain which contains main binding site, in salmonids. VGR probably belongs to a family of receptors for lipoproteins including low density lipoprotein (LDL) and very low density lipoprotein

(VLDL). Chicken oocyte receptors for LDL, VLDL, and Vg is structurally related to each other and to mammalian receptors for cholesterol-carrying lipoproteins like apoB-100. Functional elements of receptor binding site in Vg molecule appear to have been conserved (reviewed by Specker and Sullivan, 1994). Fish oocytes are able to take up xenogenic Vgs from donors of a variety of vertebrate classes at rates similar to homologous Vg (Selman and Wallace, 1982). Peptides of the binding site will be useful for preparing the "universal antibody" to detect Vg over oviparous vertebrates. Interestingly, phosvitin did not inhibit binding of Vg to the receptor in this study and also in the study of Stifani et al. (1990b), suggesting that the phosvitin domain of Vg is not the site of receptor binding. This may also support the speculation that the phosvitin domain is a new component of the Vg gene.

From the results of the present study, it could be concluded that Vg is incorporated into oocytes *via* its specific receptor, VGR, in Sakhalin taimen. Simple detection and assay system for VGR developed in this study will be useful not only for understanding of the mechanism of Vg incorporation but also for evolutional studies on lipoprotein receptor family.

Acknowledgments

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