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A comparative study on vitellogenin receptor of a lepidopteran insect (*Spodoptera litura)* and a decapod crustacean (*Scylla serrata*): Phylogenetic implication and co-evolution with vitellogenins

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Summary

Insects and crustaceans offer excellent model systems to study vitellogenesis in invertebrates. This paper gives a comprehensive account of the receptor-mediated yolk protein uptake in a lepidopteran insect *Spodoptera litura* and a decapod crustacean *Scylla serrata.* The vitellogenin (Vg) of both these arthropods has been characterized using electrophoretic and immuno-blotting techniques. The Vg's of both the animals are glycolipoproteins and exhibit immunological identity with their respective lipovitellins (Lv's). The physico-chemical characterization of vitellogenin receptors (VgR) of these arthropods revealed several similarities in their molecular weight and binding affinity with vitellins, which was increased considerably by divalent cation calcium. On the other hand, their binding affinity decreased significantly when treated with polyanionic suramin. These properties, along with the strong affinity of crab VgR for mammalian LDL and VLDL, qualify them as members of LDLR superfamily. The receptor-mediated endocytotic entry of Vg into the oocytes has been demonstrated by immunogold electron microscopic technique. The pathway starting from the formation of coated pits to the formation of mature endosomes has been traced using antibodies of Vg and VgR and gold-labeled secondary goat antibody. Use of green immunofluorescence techniques with FITC-tagged Vg antibody of *S. litura* produced additional evidence for endocytotic yolk protein uptake as well as uneven distribution of yolk within the oocytes.

Key words: Vitellogenin, vitellin, vitellogenin receptor, lepidoteran insect, decapod crustacean, oocyte

Introduction

There is considerable commonality between insects and crustaceans in respect of vitellogenin (Vg) synthesis and uptake into the oocyte during ovarian maturation. In both the cases Vg precursor molecule of the major egg yolk protein is synthesized outside the ovary and transported in the hemolymph to the oocytes. The higher dipteran flies, such as *Drosophila melanogaster,* are an exception to this rule in that synthesis of yolk peptides occurs in the ovary and the externally derived Vg is synthesized in the fat body (Bowens and Pathirana, 2002). Similarly, in the penaeid shrimps yolk proteins are synthesized both in the hepatopancreas and the ovary (Wilder et al., 2002). However, in the other large-bodied decapods such as crabs, lobsters, crayfish and freshwater prawns, almost all yolk precursor proteins are synthesized in the hepatopancreas, with only a limited synthesis within the ovary (Rani and Subramoniam, 1997; Yang et al., 2000; Chan et al., 2005). In addition to Vg, other major hemolymph proteins, such as the lipid transport lipoprotein lipophorin, are also deposited in significant quantities in the eggs of lepidopteran insects (Telfer, 2002). In the crustaceans too, hemolymph lipoproteins other than Vg are known to be sequestered into the oocytes during vitellogenesis (Subramoniam and Gunamalai, 2004). However, the sequestration of hemolymph Vg is invariably mediated by specific membrane receptors present on the oocytes. The Vg receptors (VgR) are also thought to mediate uptake of other lipid transport lipoproteins such as lipophorin in insects and low-density lipoproteins in birds into the eggs (Byrne et al., 1989).

VgR has been characterized from molecular perspectives mainly in birds, although insects and crustaceans have received some attention. Further, VgR of birds and other oviparous vertebrates are different from that of invertebrates in terms of molecular size and, probably, mode of ligand binding during yolk protein internalization (Li et al., 1988). In the present study an attempt has been made to compare VgR of a lepidopteran insect, *Spodoptera litura,* and a decapod crustacean, *Scylla*

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serrata, to throw light on their physico-chemical properties as well as binding affinities with Vg and other related ligands of phylogenetic interest. As a corollary, the nature of Vg in circulation in the hemolymph and its entry by endocytosis into the oocyte were also studied adopting immunoblotting and electron microscopy using immunogold-labeled vitellin molecules.

Materials and Methods

Purification and immunological detection of Vg

The hemolymph of the adult female S. *litura* was used for the purification of Vg following the method of Osir et al. (1986). After the removal of hemocytes, potassium bromide density gradient ultra-centrifugation was performed. The lipo-proteinaceous Vg was visualized as a yellow band in the potassium bromide gradient. The resultant layer was further purified by gel permeation chromatography using Sephadex G-200 column.

The Vg of mature female *Scylla serrata* was also separated from the hemolymph and purified thus. The purity of Vg was established in a native polyacrylamide gel electrophoresis followed by Western blotting using *S. serrata* anti-vitellin antibody. As for *S. litura*, *Manduca sexta* Vg-rabbit anti-serum, gifted by Prof. John H. Law, Centre for Insect Science, University of Arizona, USA, was used as the primary antibody.

Purification of LDL and VLDL

Rat LDL (g < 1.063g /ml) and VLDL (g < 1.006 g /ml) were prepared from plasma by potassium bromide density gradient centrifugation adopting the method of Chung et al. (1980), and the purity was checked adopting agarose gel electrophoresis (Heinecke et al., 1984).

Western blotting using antisera to Vg, LDL, VLDL or ApoB

The Vg purified from the crab was subjected to Western blotting to test the cross reactivity with its native antibody raised in rabbit as well as with antibodies of non-native lipoproteins, namely anti-human LDL,VLDL or ApoB antibodies, following Warrier and Subramoniam (2003).

Dot blotting of LDL, VLDL and HDL using anti-crab Lv antibodies

LDL, VLDL and HDL fractions were obtained from rat plasma by potassium bromide ultracentrifugation adopting the method of Chung et al. (1980), and those separated at different densities (LDL d<1.06 g/ml; VLDL d <1.006 g/ml, and HDL d <1.19 g/ml) were collected from a potassium bromide density gradient, dialyzed against PBS, and stored at -70ºC until use. Each of these lipoproteins, along with Vg from *S. serrata*, were dot blotted on a nitrocellulose paper and tested for their ability to bind anti-crab Lv antibodies (50 mg protein). The blot was developed using DAB to visualize the binding of the antibody.

Radioiodination of ligands

Vg, LDL and VLDL were radiolabeled with ^{125}I Na (Bhabha Atomic Research Center, Mumbai, India) by the lactoperoxidase method (Thorell and Johanssen, 1971). Protein-bound ^{125}I was separated from free ^{125}I in a Sephadex G -50 column (10 x 1 cm) (Sigma Chemical Company, MO, USA).

Preparation of oocyte membranes and solubilization

The crab ovary was used for preparation of oocyte membrane which was then solubilized to extract membrane proteins to obtain the VgR protein according to the method of Warrier and Subramoniam (2002). Briefly, crab ovary was homogenized in homogenization buffer, centrifuged at 100,000 x g, membrane pellets sonicated and subjected to Triton X 100 membrane protein extraction. The Triton extracts were centrifuged again under similar conditions and the supernatant containing the membrane proteins was stored at -70°C until use. Similar methods were used for the preparation of VgR in *Spodoptera*.

Electrophoretic separation, ligand blot and autoradiography of VgR of S. serrata

The Trition X-100 membrane extracts were subjected to SDS-electrophoresis on 7.5% polyacrylamide gel (0.1% SDS) under non-reducing conditions, according to Laemmli (1970). Proteins on the gel were transferred to nitrocellulose paper strips (pore size 0.4 µM) for 2 hr at 1mA/cm^2, keeping the system refrigerated at 4°C. The strips were incubated with buffer A containing 5% nonfat dry milk. After 2 hr, the blots were incubated in either ^{125}I-Vg, ^{125}I-LDL, or ^{125}I –VLDL (5 µg of protein in each case corresponding to 400 cpm/ng of protein) containing buffer A. Incubations were performed for 2 hr at room temperature under constant shaking. The blots were then rinsed with buffer A (4 x 10 min) and dried for 1 hr at 37°C. The dried blots were placed on film cassettes and exposed to photographic film (Kodak XAR-5) at -70°C for 3 days.

Ligand binding assay for Spodoptera *VgR*

Ligand binding assay for *Spodoptera* VgR was carried out by dot blot method using *Aedis aegyptii* VgR antibody and FITC-labeled secondary antibody, following the method of Persaud et al. (2003). The inhibitory effect

of suramin as well as the stimulatory role of Ca^{2+} were also investigated.

Purification of S. serrata *VgR proteins by gel filtration and HPLC*

The Triton X-100 extract was subjected to non-denaturing SDS-electrophoresis. A vertical slice of a single lane was excised and stained with Coomassie brilliant blue. The band comprising of VgR was identified corresponding to the band observed by autoradiography. The corresponding region (containing VgR) in the remainder of the lanes was cut and the proteins were eluted from gel fragments in 3 ml of TBS (pH 7.4) at 4°C for 24 hr. The elute was concentrated and 20 µg of protein was subjected to HPLC analysis using a Bio-sil SEC 250-5, 300 x 7.8 mm gel filtration column (Bio-Rad Laboratories, CA, USA). The fractions were collected every 60 sec and absorbance at 280 nm was monitored. Molecular weight standards (BioRad, USA), (thyroglobulin 670, bovine gamma globulin 158, chicken ovalbumin 44, equine myoglobin 17 kDa, and vitamin B_{12} 1.35), were subjected to the column and the elution time of the different proteins were estimated and compared to the elution time of the sample to determine the molecular weight. For confirming the Vg binding ability of the HPLC purified protein, the protein peak from the sample was dot blotted to nitrocellulose paper and subjected to ligand blotting using ^{125}I Vg described above.

Immunogold electron microscopy

Previtellogenic and vitellogenic ovaries of *S. serrata* were prepared for ultrastructural immunocyto - chemistry 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., Warrington, Pennsylvania, USA) gold embedding adopting the method of Barber et al. (1991).

Thin sections were cut and mounted on uncoated nickel grids. The filtered sections were then transferred without washing to a drop of primary antibody at a dilution of 1:1000, made in filtered phosphate-buffered saline for 2 hr at room temperature. Control grids were floated on non-immune serum. After washing with jets of filtered PBS, each grid was floated on a drop of protein - A gold [15 nm gold particles (Janssen Life Sciences Products, Belgium)] diluted 1:20 with filtered PBS for 1 hr at room temperature. The washing was performed using jets of filtered PBS followed by floating on drops of PBS. Final washing was carried out with jets of filtered distilled water and blotting with filter paper. The sections were dried and stained with uranyl acetate and lead citrate. The sections were examined in a Phillips CM 10 transmission electron microscope (Phillips, Alabama USA).

For *Spodoptera*, immunolocalization was followed using the anti-Vg and anti-VgR, respectively, of *M. sexta* and *A. aegyptii*. The secondary antibody used was protein-A gold antibody. The sections were stained with uranyl acetate and lead citrate and visualized in a FEI MORGAGNI 250 transmission electron microscope (FEI Company, Hillsboro, North America). In addition, fluorescent microscopic detection of Vg in the ovary in different maturity stages, using FITC tagged antibody, was also made following the method of Robinson et al. (2001).

Results

Reactivity of Vg and Lv to rabbit anti-Lv antibodies

First, the reactivity of Vg and Lv to rabbit anti-Lv antibodies (50 mg) was tested by performing a Western blot. Both Vg and Lv appeared as dark bands (Fig. 1). In order to establish a relationship between crab Vg and Lv and the mammalian lipoprotein, LDL, Vg, and Lv fractionated on a 7.5% polyacrylamide gel was blotted to nitrocellulose and challenged with human anti-LDL antibodies (50 mg) followed by incubation with peroxidase labeled secondary antibodies. Bands corresponding to Vg and Lv appeared (Fig. 2) but with a lesser intensity than that observed in the Western blot using anti-Lv antibodies. However, cross reactivity with anti-VLDL antibodies was of meager intensity (not shown).

Reactivity of rat LDL and VLDL with anti-crab Lv antibodies

To confirm the relationship of these lipoproteins, LDL and VLDL from rat were dot blotted and challenged with anti-crab Lv antibodies. Rat HDL was also included in the blot. It was observed that LDL and VLDL reacted with anti-LV antibodies whereas HDL did not recognize these antibodies (Fig. 3). These results suggest that Lv of crab has epitopes that are also present in rat LDL and VLDL but not in HDL.

Binding characterization of crab VgR to Vg, LDL and VLDL

Initial experiments consisted of determination of the ability of ^{125}I Vg to bind the native oocyte membrane extract. Solid phase binding assay was employed using Triton X 100-solubilized oocyte membrane proteins and increasing concentrations of ^{125}I-labeled Vg as ligand. Binding of ^{125}I Vg was saturable at 20 µg (^{125}I Vg) protein and showed high affinity for binding. Scatchard analyses of the high affinity binding data gave a linear plot indicating

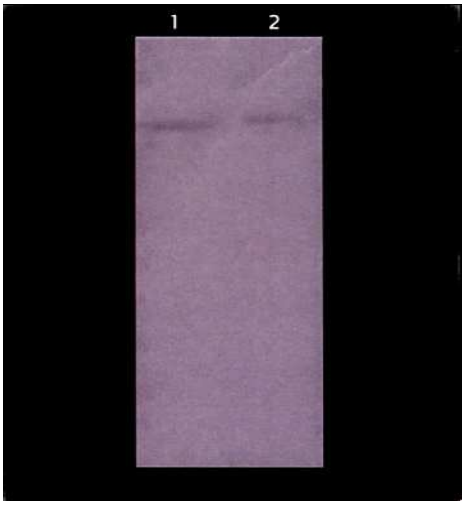

Fig. 1. Western blot of Vg (lane 1) and Lv (lane 2) using anti-Lv antibodies (50 mg). Vg and Lv reacted strongly with anti-Lv antibodies (From Warrier and Subramoniam, 2003).

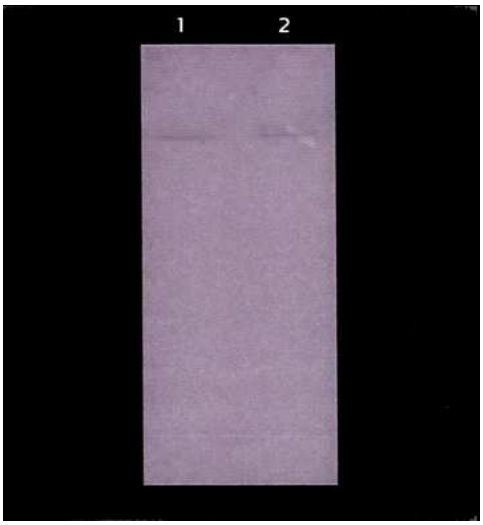

Fig. 2. Western blot analysis of crab Vg (lane 1) and Lv (lane 2) using human anti-LDL antibodies (50 mg). Vg and Lv of crab reacted moderately with anti-LDL antibodies (From Warrier and Subramoniam, 2003).

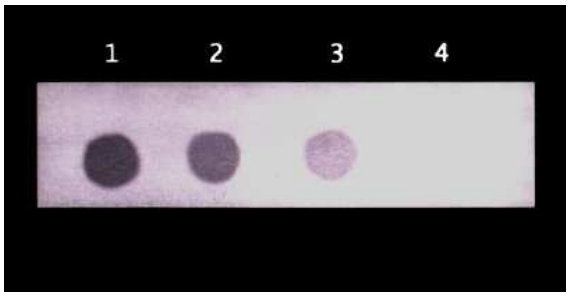

Fig. 3. Dot blot analysis of crab Vg (1), rat LDL (2), VLDL (3), and HDL (4) using anti-crab Vg antibodies (dilution 1:2,000). Anti-Lv antibodies react well with Vg, LDL, and VLDL but there is no reaction with HDL. (From Warrier and Subramoniam, 2003)

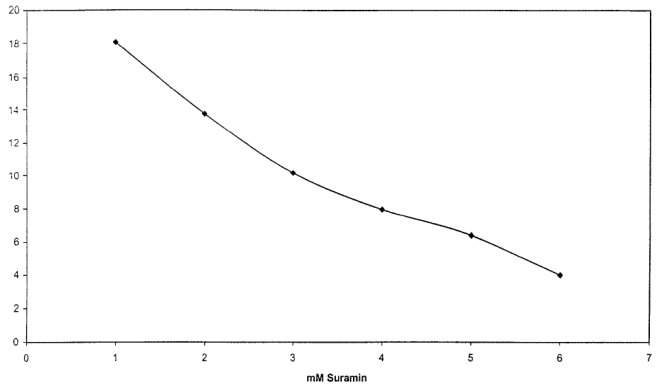

Fig. 4. Effect of suramin on the biniding of crab ^{125}I-Vg to oocyte VgR. Assay mixture (100 µl) contained oocyte membrane protein extract (20 µg) and ^{125} I-Vg (20 µg) in the presence of indicated concentration of inhibitor suramin (From Warrier and Subramoniam, 2002).

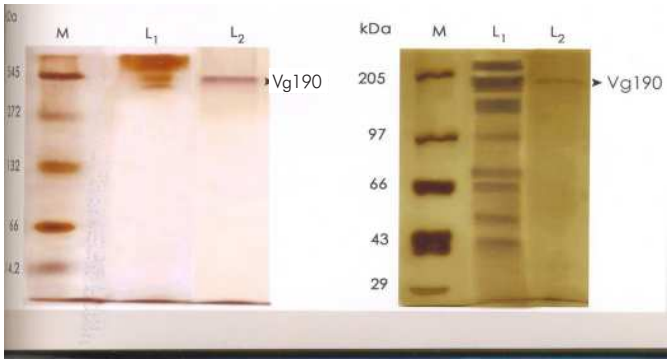

Fig.5. Electrophoretic analysis of the fractions containing *Spodoptera* Vg obtained during the purification procedure involving density gradient ultracentrifugation and gel filtration chromatography.

(A) 7% Native PAGE of the adult haemolymph protein of *Spodoptera litura* obtained by purification procedure. M – Marker; L1- Partially purified Vg in the fraction obtained by density gradient ultracentrifugation; L2 – Purified Vg in the fraction obtained from gel filtration chromatography.

(B) 7 % SDS – PAGE of the purified protein. M – Marker; L1- day '1' adult haemolymph; L2 – Purified Vg in the fraction obtained from gel filtration chromatography.

a single binding site on oocyte membrane for Vg. The apparent dissociation constant (Kd) was calculated to be 0.8 x 10^{-6} M and the maximum binding capacity (B_{max}) was found to be 0.212 µM.

Next, the question whether the receptor in this oocyte would recognize related ligands from mammals was addressed. For this, receptor binding studies were performed, as described previously, in which oocyte

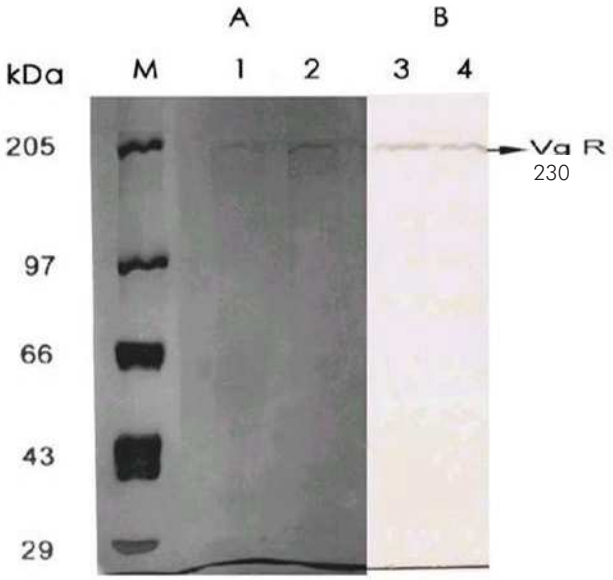

Fig. 6. 7% SDS – PAGE of the solublilized oocyte membrane extract of *Spodoptera litura* (A) and VgR showing positive signal for VgR antibody (B).

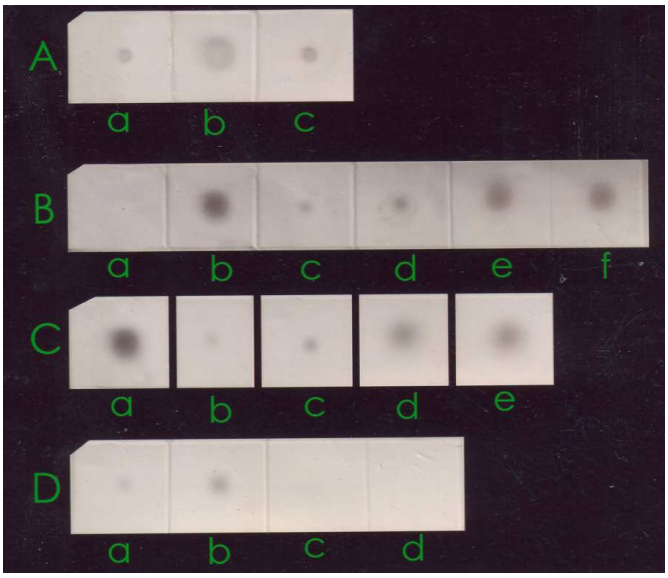

Fig. 7. *S. litura*. Receptor binding studies by modified dot blot analysis of the parameters effecting binding of Vg to its receptor.

A) Determination of the effect of Ca^{2+} concentration on binding of VgR (6µg/ml) to Vg (3µg/ml) using binding buffer containing (a) 1.5mM Ca^{2+} (b) 2mM Ca^{2+} (c) 2.5 mM Ca^{2+}

B) Analysis of binding activity at constant Vg concentration µg/ml but membrane concentration of (c) 4 µg/ml (d) 6 µg/ml (e) 8µg/ml (f) 10 µg/ml of binding buffer, with (a) male hemolymph, indicating control (b) 5µg/ml purified Vg as positive control .

C) Concentration-dependent binding of Vg to its receptor at a constant membrane concentration (6µg/ml) and increasing Vg concentrations of (b) 2µg/ml (c) 2.5 µg/ml (d) 3µg/ml (e) 3.5µg/ml and (a) 5µg/ml purified Vg as positive control.

D) Detection of the binding activity of VgR (6 µg/ml) to Vg (3µg/ml) in the presence of varying concentrations of suramin (a) 4 mM (b) 4.5 mM (c) 5mM and (d) 5.5 mM in the binding buffer.

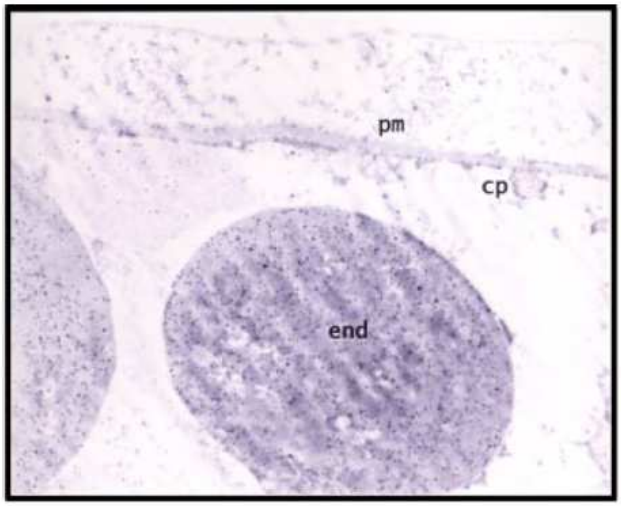

Fig. 8. Immunogold labeling of Vg in ultrathin sections of *S. serrata* oocyte and observed in TEM. Plasma membrane (pm) is visible and Vg labeling is seen in coated pits (cp) and is concentrated on the endosome (end). x 2900. (From Warrier and Subramoniam, 2002).

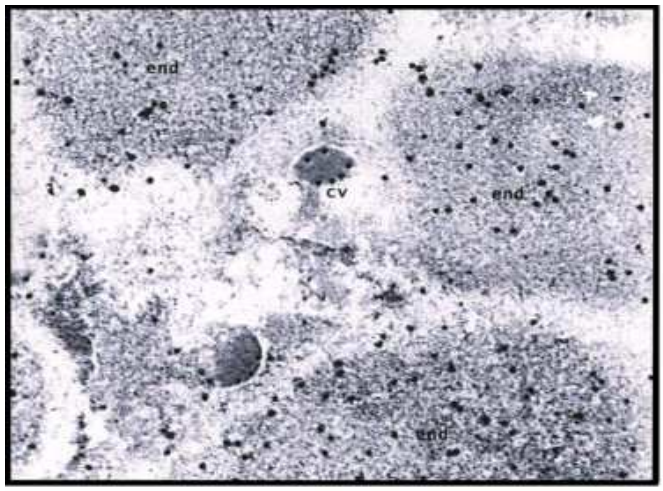

Fig 9. TEM. *S. serrata* oocyte. Vg labeling is seen along the luminal surface of the coated vesicle (cv). Vg particles are seen in endosomes (end) found close to the coated vesicles. Clustering of endosomes filled with Vg are seen. x 15,000. (From Warrier and Subramoniam, 2002).

membrane extract from *S. serrata* and ^{125} I-labeled LDL and VLDL from rat were used. Both these lipoproteins bound VgR in a saturable manner but with much lower affinity than binding of labeled Vg to VgR. LDL showed to have more affinity to VgR than VLDL. Analysis of the quantitative binding data of the binding curves resulted in Kd of 1.47 x 10^{-6} M and 4.2 x 10^{-6} M for LDL and VLDL, respectively (Table 1). Thus, the affinity of rat LDL to VgR was greater than the affinity of VLDL to VgR but lower than the binding affinity of crab Vg to VgR. Direct ligand binding of VgR with Vg and rat LDL and VLDL also supported this binding affinity. In order to establish a similarity between VgR from crab and VgR and LDL receptor of vertebrates, binding assays between VgR and ^{125} I Vg were performed in the presence of suramin, a known inhibitor of LDL receptor family. It was observed that ^{125} I Vg binding to VgR in the oocyte membrane extract was abolished in the presence of 6 mM suramin, leaving only a non-specific linear component (Fig. 4).

Table 1. Binding of different ligands to crab VgR

^{125} I labeled ligand	K_D (10^{-6} M)	B_{max} µM
Vitellogenin	0.8* ± 0.053	0.212 ± 0.077
VDL	1.47* ± 0.071	0.17 ± 0.091
VLDL	4.5* ± 0.081	0.037 ± 0.075

Dissociation constant (K_D) and maximum binding capacity (B_{max}) were estimated by Scatchard analysis. Values are given as the mean ± SEM of triplicate analysis.

- $P < 0.05$ (Students' t- test).

It has been shown earlier that calcium stimulates receptor binding in the case of apoE receptor (Hiu et al., 1986) and a lipophorin receptor of insects (Tsuchida and Wells, 1990). To further demonstrate that the crab VgR shares properties with other lipoprotein receptors, effect of Ca^{2+} on the binding of ^{125} I Vg to VgR was studied in a binding assay. Presence of Ca^{2+} markedly increased the binding of Vg to its receptor.

Molecular weight determination of* Spodoptera *Vg and VgR

For the determination of the molecular weight of Vg & VgR, the electrophoretic band corresponding to Vg and VgR run in a native PAGE was eluted, concentrated and applied to a HPLC gel filtration column. A major peak (A 280) was observed at 6.7 min. The elution times of the standard molecular weight proteins were determined by applying to the same column under similar conditions. A standard graph was plotted and the molecular weight of the major peak of the sample was determined to be 190 kDa for Vg (Fig. 5A) and 230 kDa for VgR (Fig. 6A), respectively. This was also confirmed based on the positive signals obtained in Western blot analysis (Figs. 5B, 6B).

Spodoptera *VgR ligand binding assay*

Using dot blot analysis, the binding activity of the Vg with the ovarian membrane extract protein was studied. Similar to *S. serrata*, the binding affinity was enhanced in the presence of Ca^{2+}, whereas suramin inhibited the binding significantly (Fig. 7).

Immuno-electron microscopic studies

To elucidate the cellular mechanism involved in the internalization of *S. serrata* Vg, immunogold electron microscopic study was carried out using anti-Vg as the primary antibody and gold labeled anti-rabbit secondary antibodies. Coated pits containing gold particles were visible at the plasma membrane in the oocyte, containing Vg-packed endosomes. Vg-labeled coated pits were also observed near the endosomes (Fig. 8). Some coated vesicles containing Vg at the luminal surface were observed to fuse into a maturing endosome (Fig. 9). Early endosomes were found to accumulate Vg but were electron-lucent; however, on granulation of the yolk, the early endosomes transformed into mature endosomes studded with electron-dense Vg particles. A characteristic feature of the vitellogenic oocyte was the presence of a large number of Vg-labeled endosomes. Fusion of endosomes was clearly evidenced (Fig. 10).

The electron microscopic studies of *Spodoptera* ovary, using gold-labeled anti-Vg and anti-VgR as primary antibodies, clearly evidenced receptor-mediated internalization of Vg into the oocytes. The invagination of oocyte plasma membrane to form the coated vesicles together with the formation of early and late endosomes as well as transitional yolk bodies were all revealed in their gold labeling with VgR antibody (Fig. 11). The presence of microvilli and the tubular vesicles without Vg, emanating from the transitional yolk body, were also discernible. The fusion of early endosomes to form the transitional yolk bodies, giving rise to the mature endosomes, was also seen (Fig. 12). The formation and maturation of the endosomes into the yolk bodies were similar to those in *S. serrata* described above. Another interesting observation from this immunogold labeling study was the unequal distribution of yolk within the oocyte.

Immunofluorescent detection of Vg in* Spodoptera *ovary

The uptake of vitellogenin into the ovary was also visualized adopting green immunofluorescent technique. The immunofluorescent signal for the presence of Vg was

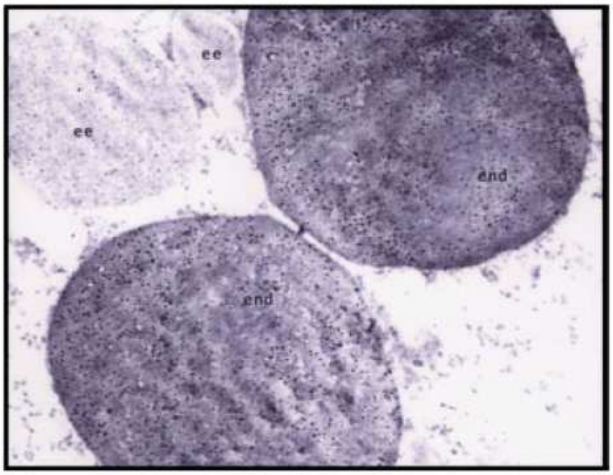

Fig. 10. TEM. *S. serrata* oocyte. Labeling of Vg. Electron-lucent early endosomes (ee) and in electron dense mature endosome (end). Extensive labeling of Vg is seen in the mature endosomes (end). x12000 (From Warrier and Subramoniam, 2002).

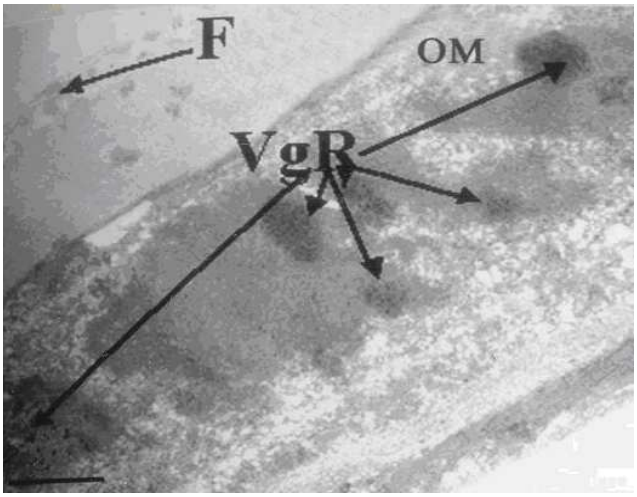

Fig. 11. TEM. *S. litura* oocyte. VgR is localized on the membrane (OM) of the oocyte, in the coated vesicle and coated pits. The follicular epithelium (F) is clearly visible. ×10,000.

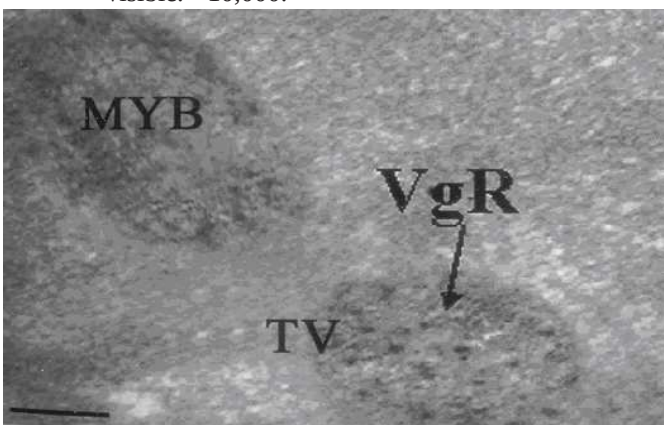

Fig. 12. TEM. *S. litura* oocyte. The tubular vesicles (Tv) containing VgR and the mature yolk body (MYB) without the signal for VgR are seen. × 9,000.

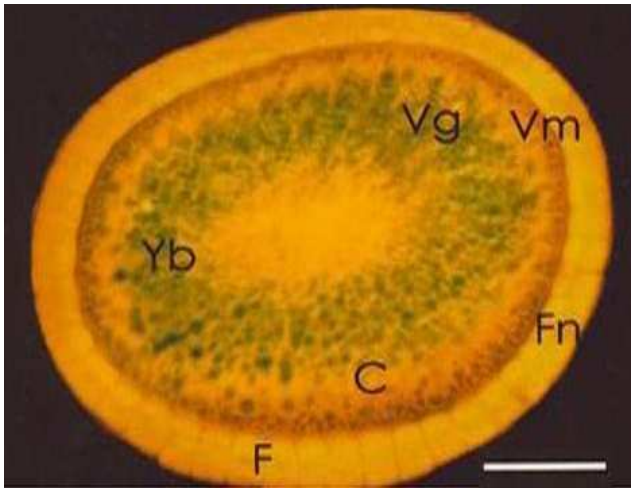

Fig. 13. Immunofluorescent localization of Vg in section of the ovary of *S. litura*. The FITC signals for the presence of Vg are localized exclusively in the oocyte while propidium iodide staining is totally limited to the follicle cells (F). The nuclei of the follicle cells (FN), the vitelline membrane (Vm) and yolk body (Yb) in the cytoplasm (C) are prominently seen. Scale bar – 10 µm.

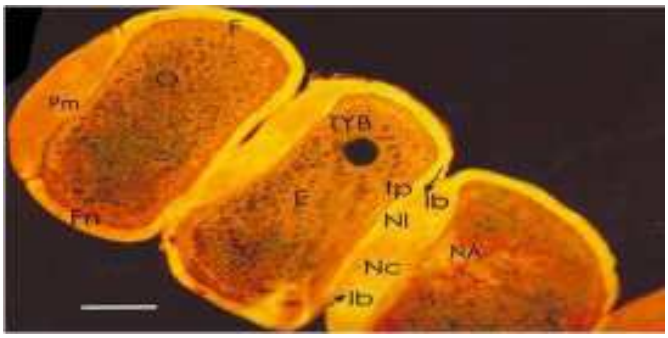

Fig. 14. Immunofluorescent image of the section of the ovary of *S. litura*. The FITC signal for the presence of Vg are seen only in the oocytes, while propidium iodide staining is limited to the follicle cells and nurse cells. The Vg are localized in the endocytotic vesicles (E) and the transitional yolk body (TYB) in the oocyte (O), surrounded by the follicle cells (F) containing the nucleus (Fn). The presence of outer peritoneal membrane (Pm) and the inner non–cellular tunica propria (Tp) is visible. The nurse cells (Nc), compound nurse cell nucleolus (Ni), intercellular bridge connecting a nurse cell to the oocyte (Ib), and transfer of nucleic acid (NA) are apparent. Scale bar – 5 µm.

localized exclusively in the oocyte, while the counterstain propidium iodide was limited to the follicle cells and nurse cells in the early and mid-stages of ovary development (Fig. 13, 14).

Discussion

Vitellogenesis is the most critical event of egg maturation in all arthropods. Recent studies have revealed that yolk proteins are produced as precursor molecules mainly in extraovarial sites such as fat body in insects (Engelmann,1970), myriapods (Prasath and Subramoniam, 1991) and arachnids (Taylor and Chinzei, 2002) and hepatopancreas in crustaceans (Wilder et al., 2002). Understandably, the vitellogenic system and, to a certain extent, its endocrine regulatory mechanisms are similar among insects, myriapods and arachnids, whereas crustaceans have a system with totally different endocrine regulation (Subramoniam, 1999). Despite differences in the hormonal control of vitellogenesis between insects and crustaceans, the mode of yolk precursor synthesis and its uptake into the ovary is quite similar, as revealed in the present study. In both insects and crustaceans, the yolk proteins are glycolipoproteins, lacking polyserine domains, although in *S. litura* the yolk protein is highly phosphorylated. The crustacean Lv, however, differs from that of insects in that the former is conjugated to carotenoid pigments. The authenticity of hemolymph Vg and the egg yolk proteins has been confirmed in the lepidopteran insect *S. litura* and the crab *S. serrata* by Western blotting. Similarities in primary structure and antigenicity between vitellins and Vg's in insects as well as crustaceans have been previously indicated (Sappington et al., 2002; Okuno et al., 2002). The antigenic similarity going beyond the family level is also evident in *S. litura*, as has also been demonstrated previously in the hymenopteran insect (Tufail et al., 2000). Recent homology studies made on the primary structure of crustacean vitellogenins have, however, revealed a closer similarity with large lipid transfer lipoproteins such as insect lipophorin and mammalian LDL than with Vg's of other animal groups (Subramoniam, 2002).

Comparative data presented on insect and crustacean VgR's have brought out similarities in the physicochemical characteristics as well as their binding affinities with Vg molecules. Both of them are above 200 kDa in molecular weight and compare well with the recently characterized VgR of the shrimp *Penaeus monodon* (211 kDa) (Tiu et al., 2008) and other invertebrate Vg receptors such as that of the polychaete worm *Nereis virens* (190 kDa) (Baert and Slomianny, 1987). The molecular weight of these invertebrate VgR is however almost double that of vertebrate VgR's. Nevertheless, previous sequence studies have uncovered the fact that insect, nematode and vertebrate VgR's belong to the vertebrate low-density lipoprotein receptor family (Schonbaum et al., 1995). Similar to insects, Vg uptake into the ovary is also mediated by membrane receptors residing at the oocyte periphery (Laverdure and Soyez, 1988). In *S. serrata*, the VgR has a high molecular weight of 230 kDa, similar to that of insects.

In both *S. litura* and *S. serrata*, the binding of VgR with the labeled Vg has been augmented by the divalent cation, calcium. However, the polysulphated polycyclic hydrocarbon, suramin, inhibits the recognition of Vg by its receptor, both in the insect and the crustacean used in the present investigation. The polyanionic suramin, probably, forms complex with the positive residues of the receptor-binding sites of Vg, thereby preventing its interaction with VgR (Jung and Yun 2007). Suramin is also known to block VgR binding with Vg of vertebrates such as birds (Stifani et al., 1988), amphibians (Stifani et al., 1990) and fishes (Mananos et al., 1997). Suramin has an additional property of competing with lipoproteins such as human LDL and VLDL in their binding with the respective receptors. This inhibitory characteristic of suramin in receptor- ligand binding is congruent with the fact that VgR of insects, crustaceans and vertebrates belong to LDLR superfamily. Interestingly, VgR of birds also recognizes LDL and mediates its internalization into the oocytes (Schneider and Nimpf, 1993). Direct binding studies using ^{125} I –labeled crabVg, rat LDL and VLDL have, for the first time, revealed strong binding affinity of crustacean VgR with mammalian LDL and VLDL, suggesting that crustacean VgR also belongs to the LDLR super family (Warrier and Subramoniam, 2002). Recent elucidation of shrimp VgR primary structure and its homology with that of LDLR lends further support to this contention (Tiu et al., 2008).

In both insects and crustaceans, lipid-based energy metabolism is an important physiological feature. In this respect, inter-organ transport of lipid from the site of synthesis to the target organ is imperative. Insects have contrived efficient lipid transportation from fat body to flight muscle and ovary for flight and embryogenesis (Chino, 1985). Conversely, in crustaceans lipid transportation occurs in between hepatopancreas and ovary as well as epidermal cells to meet the energy requirement during egg maturation and molting (Lubzens et al., 1995). In insects, a distinct class of lipoprotein, lipophorin, is involved in lipid transport not only to flight muscles but also ovary to form an integral part of yolk bodies. Amino acid sequence studies of lipophorin have also shown that this lipoprotein belongs to the family of large lipid transfer lipoproteins (LLTP) (Atella et al., 2006). Interestingly, the

internalization of lipophorin into the oocyte is accomplished by a membrane receptor which is distinctly different from VgR (Sun et al., 2000). Nevertheless, VgR is also known to interact with lipophorin in its internalization into the oocyte in *Drosophila melangoster* (Schonbaum et al., 2000). Recent phylogenetic analyses in the German cockroach *Blatella germanica* have placed lipophorin receptor with the group formed by vertebrate LDLR, LPR8, VgR and VLDLR, which diverged from a common ancestor and diversified in parallel. Taken together, there appears to be a co-evolution of the LLTP and their respective receptors during the evolution of metazoans. Our present observations in *S. litura* and *S. serrata* lend further support in this regard.

The pathway of Vg within the developing oocytes of *S. serrata* was delineated employing immunogold electron microscopy using labeled antibodies directed against Vg. Vg was localized in the coated pits in the oocyte plasma membrane as clear invaginations into the cytoplasm. Within the cytoplasm Vg was also localized in the luminal surface of the coated vesicles. The coated vesicles lose their clathrin coat and fuse with one another to form early endosomes, which later mature into late endosomes. In the oocyte sections of *S. serrata*, early endosomes were present as identified by their shape and sparse labeling of Vg. In contrast, late endosomes were seen as spherical cytoplasmic organelles densely packed with Vg. The intense accumulation of Vg within the endosomes clearly indicates that the storage organelle of Vg in the crustacean is the endosome. The endocytotic pathway of Vg in the crustaceans is thus comparable to the receptor-mediated endocytosis of LDL in higher vertebrates and internalization of Vg in amphibian and mosquito oocytes, as evidenced by the sequential appearance of Vg labeling in the coated pits, coated vesicles and the early and mature endosomes (Raikhel and Dhadialla, 1992). Immunogold electron microscopic study using gold-labeled anti-Vg antibody as well as anti-VgR antibody in *S. litura* has not only revealed the receptor-mediated endocytosis but also clarified the participation of different cellular organelles in the formation of yolk bodies. Furthermore, the uneven distribution of yolk in the egg, where more yolk was observed in the animal hemisphere than in the vegetal hemisphere, is also evident from this immunogold labeling study.

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