Insect Biochem. Molec. Biol. Vol. 22, No. 8, pp. 803–816, 1992
Printed in Great Britain.

0965-1748/92 $5.00 + 0.00

CHARACTERIZATION OF THE SOLUBILIZED MOSQUITO VITELLOGENIN RECEPTOR

TARLOCHAN S. DHADIALLA, ALAN R. HAYS and ALEXANDER S. RAIKHEL*
Department of Entomology and Program in Cell and Molecular Biology, Michigan State University, East Lansing, MI 48824-1115, U.S.A.

(Received 10 April 1992; revised and accepted 27 July 1992)

Abstract—In this study we solubilized and characterized the receptor for the major egg yolk protein precursor, vitellogenin (Vg), from the yellow fever mosquito, *Aedes aegypti*. The receptor was solubilized from vitellogenic ovary membranes using octyl-β-D-glucoside (OG). Under equilibrium binding conditions, [^{35}S]Vg bound with high affinity ($K_d = 2.8 \times 10^{-8}$ M) to a single class of binding sites in solubilized ovary extracts. The solubilized receptor was present in ovarian extracts and bound selectively *A. aegypti* Vg and its storage form, vitellin (Vn). The receptor preparation was heat and trypsin sensitive. Binding of Vg to its receptor could be inhibited as well dissociated with suramin. The receptor was visualized by ligand-blotting as a 205 kDa protein under non-reducing conditions. It did not share immunological cross-reactivity with antibodies to chicken and locust Vg receptors. Vitellogenin, Vn and its purified subunits competed for binding to the receptor in the order, Vg ≈ Vn > Vn large subunit > Vn small subunit. Binding of dephosphorylated Vg was significantly reduced. Deglycosylated Vg, on the other hand, formed high molecular weight aggregates resulting in artifactually high binding which indicates importance of glycosylation for the stability of Vg molecule. During egg maturation, the number of receptor binding sites in ovaries correlated with the rate of Vg uptake and peaked between 24–30 h after which it reduced to no binding by 48 h post blood meal.

Key Word Index: receptor; vitellogenin; endocytosis; oocyte; mosquito

INTRODUCTION

Receptor-mediated internalization of nutritional and regulatory proteins from extracellular fluids by animal cells is an ubiquitous phenomenon. A protein molecule (ligand) binds to membrane-associated receptor and the receptor–ligand complex is internalized by clathrin-coated vesicles (Goldstein *et al.*, 1985; Keen, 1990; Pearse and Robertson, 1990).

In oviparous animals, an important physiological role of receptor-mediated endocytosis is the selective deposition of yolk protein precursors into developing oocytes. During this process, termed vitellogenesis, the major egg yolk precursor protein, vitellogenin (Vg), is synthesized by the faty body of insects or the liver of oviparous vertebrates for release into the circulating hemolymph or blood, respectively. The circulating Vg is then selectively internalized by developing oocytes where it is either stored as crystalline Vn as in insect eggs or cleaved into lipovitellin and phosvitin in eggs of oviparous vertebrates (Raikhel and Dhadialla, 1992).

Vitellogenins of insects, with the exception of yolk proteins of higher Diptera, are high molecular weight oligometric phosphoglycolipoproteins (Kunkel and Nordin, 1985; Raikhel and Dhadialla, 1992). *Drosophila* yolk proteins and mosquito Vg have also been shown to be sulfated (Baeuerle and Huttner, 1985; Dhadialla and Raikhel, 1990).

Considerable progress has been achieved in understanding the mechanisms of receptor-mediated yolk protein accumulation in oviparous vertebrates (Opresko and Wiley, 1987; Stifani *et al.*, 1988, 1990a–c). In contrast, similar research on insect oocytes was limited due to the amount of experimental material available (reviewed in Raikhel and Dhadialla, 1992). Since the efficient mode of egg production is one of the reasons that insects continue to be serious pests of medical, veterinary and agricultural importance an understanding of insect vitellogenesis is potentially important in devising novel methods of insect management.

The internalization of Vg in oviparous animals is a receptor-mediated process. This has been substantiated by demonstrating the specific uptake of Vg by growing oocytes of chicken (Roth *et al.*, 1976), the toad, *Xenopus* (Opresko and Wiley, 1987), and several insects: *Locusta migratoria* (Ferenz *et al.*, 1981; Röhrkasten and Ferenz, 1985), *Manduca sexta* (Osir and Law, 1986), *Rhodnius prolixus* (Oliveira *et al.*, 1986), *Hyalophora cecropia* (Kulakosky and Telfer, 1987), *Nauphoeta cinerea* (Kindle *et al.*, 1988), and *Aedes aegypti* (Koller *et al.*, 1989). Furthermore, binding of Vg or Vn to oocyte membranes isolated from both vertebrates and insects (Yusko and Roth, 1976; Yusko *et al.*, 1981; Osir and Law, 1986; König and Lanzrein, 1985; Röhrkasten and Ferenz, 1986; König *et al.*, 1988a, b; Dhadialla and Raikhel, 1991) as well as to solubilized Vg receptors (Röhrkasten and Ferenz, 1986; Stifani *et al.*, 1988) has been demonstrated. Finally, using ligand blotting, the solubilized Vg receptors of three oviparous vertebrates as well as two insect species has been visualized and their molecular weights determined

*Author for correspondence.

(Stifani *et al.*, 1988, 1990a–c; Röhrkasten *et al.*, 1989; Indrasith *et al.*, 1990).

Mosquitoes are by far the most important group of insect vectors, causing human losses through malaria, yellow fever, dengue and many arboviral and filarial diseases. Due to their medical importance we are interested in understanding hormonal, biochemical and molecular basis of egg maturation. Since Roth and Porter's (1964) observation of coated vesicles on mosquito oocytes, much of our knowledge on the accumulation and sorting of Vg and non-specific proteins in the mosquito oocytes has come through ultrastructural and high resolution cytochemical and immunochemical studies of the yellow fever mosquito. *A. aegypti* (reviewed in Raikhel, 1992; Raikhel and Dhadialla, 1992). At the biochemical level, the conditions for, and kinetics of, Vg uptake in *A. aegypti* oocytes have been determined (Koller *et al.*, 1989; Koller and Raikel, 1991) and the binding properties of mosquito Vg to membranes isolated from vitellogenic oocytes characterized (Dhadialla and Raikhel, 1991).

We report here the solubilization and characterization of binding properties of the *A. aegypti* Vg receptor. In addition we have done experiments to elucidate the receptor-binding domain on Vg and the role of glycosylation and phosphorylation of Vg in receptor–ligand interaction. Some of the preliminary data from this work has been reported in two reviews (Raikhel, 1992; Raikhel and Dhadialla, 1992).

EXPERIMENTAL PROCEDURES

Chemicals

All chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, Mo) unless stated otherwise. [^{35}S]methionine (1120 Ci/mmol) was purchased from ICN Radiochemicals (Irvine, Calif.) and DEAE–Sepharose CL-6B from Pharmacia. Endo-β-N-acetylglucosaminidase H (Endo H) and calf alkaline phosphatase (CAP) were from Boehringer Mannheim. Bio-Rad was the source for protein assay reagent, Econo-Pac desalting columns and molecular weight standards for electrophoresis. Safety Solve II scintillation cocktail was supplied by Research Products International. Antibodies to the chicken and locust (*L. migratoria*) Vg receptors produced in rabbits were gifts from Drs W. J. Schneider (University of Alberta, Canada) and Hans-Jorg Ferenz (University of Oldenburg, Germany), respectively. Purified *L. migratoria* Vg was kindly provided by Dr G. R. Wyatt (Queen's University, Kingston, Canada).

Insects

A colony of *A. aegypti* were maintained at 27°C as described by Lea (1964). Vitellogenesis was initiated in adult females 3–5 days after eclosion by feeding them on rats. The paired mosquito ovaries consist of about 75 ovarioles. At eclosion, each ovariole contains a germarium and a single follicle. Each follicle consists of seven nurse cells and an oocyte surrounded by a single layer of follicular epithelium. During vitellogenesis, the development of the follicles is synchronous.

Synthesis of radiolabeled vitellogenin

Vitellogenin, radiolabeled metabolically with [^{35}S]methionine was produced as described in detail by Koller *et al.* (1989) and Dhadialla and Raikhel (1990). To ensure the continued production of Vg, fat bodies were cultured *in vitro* in medium containing 10^{-5} M 20-hydroxyecdysone.

Purification of radiolabeled vitellogenin and unlabeled vitellin

Radiolabeled Vg from total proteins secreted by fat bodies cultured *in vitro* and unlabeled Vn from extracts of ovaries taken from females 20 to 24 h post blood-meal was purified as described by Dhadialla and Raikhel (1991). The purity of Vg and Vn was monitored by native or SDS–PAGE by using the Laemmli buffers (1970). Fractions containing purified Vg or Vn were concentrated by using Microsep centrifugal microconcentrators (Filtron Technology Corporation, Mass.) and the buffer in the concentrated fraction was exchanged for incubation buffer (IB). The composition of IB is the same as mosquito saline but buffered with 25 mM TES, pH 7.2 (Koller *et al.*, 1989).

Purification of vitellin subunits

Vitellin purified by ion-exchange chromatography was used to further fractionate its subunits by the method of Kawooya *et al.* (1989). Purified Vn was dialyzed against 20 mM Tris–HCl buffer, pH 8.4, containing 0.1 M NaCl and 1 mM EDTA, and then made to contain 8 M GdmCl and 2 mM DTT. The mixture was stirred for 30 min at 25°C, followed by 5 min in a boiling water bath. This solution (5 mg protein in 1 ml) was then applied to a Sepharose CL-6B column (2.5 × 90 cm) that was eluted with 20 mM Tris–HCl buffer, pH 8.4, containing 6 M GdmCl and 2 mM DTT. The fractions were monitored by SDS–PAGE and those which contained only the large (VnL: $M_r = 200{,}000$) or the small (VnS; $M_r = 66{,}000$) subunit were pooled, concentrated and dialyzed against IB. Unlike for *M. sexta* Vg, where the large subunit precipitates upon purification (Osir and Law, 1986), both the mosquito Vn subunits remained in solution.

Electrophoresis, transfer to nitrocellulose, ligand and immunoblotting

Electrophoresis of samples was carried out using the Bio-Rad Mini-PROTEAN II electrophoresis system and Laemmli buffers (1970). For electrophoresis under reducing conditions SDS-sample buffer contained 2 mM DTT and the samples were heated to 90°C for 5 min. The SDS-sample buffer for separating proteins under non-reducing conditions did not contain DTT and samples were not heated. Gels were stained with Coomassie Brilliant Blue to visualize the separated proteins. For ligand- or immunoblotting, electrophoretic transfer of proteins to nitrocellulose membrane (Bio-Rad's Trans-Blot Transfer membrane, 0.45 μm) was performed according to the method of Burnette (1981).

For ligand blots, nitrocellulose membranes were blocked with 5% non-fat dry milk/1% BSA in mosquito saline overnight. After incubating the blots for another 3 h in 1% BSA in mosquito saline, the blots were probed with radiolabeled Vg alone or radiolabeled Vg with unlabeled competitor for 6 h at 4°C. The concentration and specific activity of radiolabeled Vg used in the incubation mixtures is indicated in the figure legends. The probed blots were washed twice, 20 min each, in mosquito saline containing 5% milk. After a final wash with mosquito saline, the blots were dried and exposed to Kodak XAR-5 film at −70°C to obtain the autoradiograms.

For immunoblots, nitrocellulose membranes were blocked with 5% non-fat dry milk in 50 mM sodium phosphate, pH 7.2, containing 0.77 M NaCl. The rest of the procedure was as described by Hays and Raikhel (1990). Primary antibodies to the chicken and locust Vg receptor, produced in rabbits, were used at 1:50 dilution. Secondary antibodies (goat–anti-rabbit IgG conjugated to alkaline phosphatase) were used at 1:1000 dilution. The reaction was visualized with 5-bromo-4-chloro-3-indoyl phosphate, *p*-toluidine salt/nitroblue tetrazolium cocktail in 0.15 M bicarbonate/carbonate buffer, pH 9.6.

Isolation of ovary membranes and solubilization of membrane proteins

All operations were carried out at 4°C. Membranes from ovaries dissected 20 to 24 h after a blood meal were isolated after modification of the method by Dhadialla and Raikhel (1991). Usually 2000 ovary pairs in 5 ml mosquito saline containing a mixture of protease inhibitors (PI; 1 mM PMSF, 5 mM ε-aminocaproic acid, 1 mM benzamidine, 10 mg/ml aprotinin, 2 μg/ml each of antipain, chymostatin, leupeptin and pepstatin) were homogenized using a glass–Teflon homogenizer. The homogenate was centrifuged at 500 *g* for 10 min and the supernatant saved. The pellet was extracted thrice in 5 ml mosquito saline containing PI followed by centrifugation at 500 *g* for 10 min. Combined supernatants from above were centrifuged at 100,000 *g* for 60 min. The pellet was resuspended in B2 buffer (50 mM Tris–HCl, pH 8.4, containing 0.5 M NaCl) using glass–Teflon homogenizer and centrifuged at 100,000 *g* for 40 min. This step with the resulting pellet was repeated three more times. The pellet was resuspended in 125 mM Tris–Maleate buffer, pH 6.0, containing 2 mM $CaCl_2$, 160 mM NaCl, 1 mM PMSF, 5 mM ε-aminocaproic acid, 1 mM benzamidine and 2 μg/ml leupeptin (buffer C) and centrifuged at 100,000 *g* for 40 min.

The optimal amount of the detergent, octyl-β-D-glucoside (OG), needed for solubilization of membrane proteins was determined by solubilizing whole mosquito membrane proteins with various concentrations of OG (data not shown). 40 mM OG gave maximal amount of solubilized proteins. For solubilization of ovary membrane proteins, the membrane pellet obtained above was resuspended in 400 μl buffer C. The protein concentraton was measured using the BioRad protein assay. To the membrane suspension (3 mg/ml protein) was added 100 mM OG in buffer C to obtain a final concentration of 40 mM OG. This preparation was sonicated at the lowest setting for 5 s and incubated for 60 min before centrifugation at 100,000 *g* for 60 min. The supernatant was adjusted to 50% acetone and the precipitated protein centrifuged at 100,000 *g* for 30 min. The pellet thus obtained was resuspended in 5 ml mosquito saline using a glass–Teflon homogenizer. The suspension was centrifuged at 100,000 *g* for 40 min. The pellet was resuspended in 100–200 μl mosquito saline. After protein determination the solubilized extract (from here on referred to as OG extracts) was adjusted to a protein concentration of 3 μg/μl in 20 μl aliquots and stored at −70°C. The stored extracts retained Vg binding activity for 2–3 months at −70°C.

Solubilized OG extracts were also prepared of abdomens isolated from female mosquitoes 20–24 h post blood-meal. The abdomens containing adhering fat body, were dissected free of the gut and ovarian tissues and are referred to as fat body in the text. Solubilized fat body membrane OG extracts were stored as described above.

Assays for binding of radiolabeled Vg to membranes or OG extracts

The binding assays were done in polyethylene microcentrifuge tubes (500 μl) as described by Dhadialla and Raikhel (1991). Each reaction mixture consisted of IB containing 10 mg/ml BSA, unsolubilized membranes or OG extract of tissue membranes and [^{35}S]methionine labeled Vg (sp. act. 5–7 × 10^5 cpm/μg protein) as indicated in figure legends. Incubations were at 4°C for 90 min. Reaction mixtures containing unsolubilized membrane proteins were processed as detailed by Dhadialla and Raikhel (1991).

Binding to OG extracts was determined by filtering reaction mixtures through 0.2 μm poly(vinylidene fluoride) membrane filters (Millipore GVWP 02500). The filters had been pre-incubated overnight at 4°C with IB containing 1% BSA and 0.01% Vn to reduce nonspecific binding. Each filter with the reaction mixture was then washed with 15 ml IB containing 1% BSA and dried at 37°C overnight. The amount of radiolabeled Vg bound to its receptors was quantified by placing the dried filters in glass scintillation vials containing Bio-Safe scintillation cocktail and counting in a liquid scintillation counter (1209 RackBeta "PRIMO", LKB-Wallace). Appropriate controls to determine nonspecific binding of radiolabeled Vg were included as described by Dhadialla and Raikhel (1991). Specific binding was calculated as the difference between total binding and nonspecific binding. Nonspecific binding of radiolabeled Vg to filters in the presence or absence of Vn as the competitor was also subtracted from experimental values. Analysis of the data was as discussed by Dhadialla and Raikhel (1991).

Deglycosylation and dephosphorylation of radiolabeled Vg or unlabeled Vn

Vg or Vn were deglycosylated by a modification of the method of Raikhel and Bose (1988). Purified Vg or Vn was extensively dialyzed against 50 mM Na-citrate, pH 5.5, containing 100 mM NaCl and PI. The dialyzed samples were then incubated in the presence (Endo$^+$) or absence (Endo$^-$) or Endo H at 37°C for 16 h. These samples were then dialyzed against 20 mM Tris–HCl, pH 7.5, containing 250 mM NaCl before use for binding assays.

[^{35}S]Vg was dephosphorylated using CAP (CAP$^+$) in 20 mM Tris–HCl, pH 8, as described by Dhadialla and Raikhel (1990). Control [^{35}S]Vg was taken through all steps simultaneously but without CAP (CAP$^-$). The integrity of deglycosylated and dephosphorylated [^{35}S]Vg was verified by both native and SDS–PAGE. Analysis of the variously treated Vgs for deglycosylation by SDS–PAGE revealed a reduction in molecular weights of the large and small subunits of the deglycosylated Vg by about 10,000 and 13,000, respectively. Molecular weights of the untreated and sham deglycosylated Vg subunits were not effected. Similarly, the molecular weights of the dephosphorylated Vg subunits, analyzed by SDS–PAGE, were reduced by 7000 and 4000 for the large and small subunits, respectively. These results confirm our earlier observations (Dhadialla and Raikhel, 1990). When the same samples were separated by native-PAGE, the apparent molecular weight of untreated and sham treated Vg (for deglycosylation and dephosphorylation) was 380,000. Deglycosylated Vg produced a much focused band than intact Vg and had an apparent molecular weight of 325,000. There were additional high molecular weight bands, probably representing aggregates of deglycosylated Vg. There was no observable difference in molecular weights between dephosphorylated Vg and sham treated Vg when separated by native-PAGE.

Titration of suramin

To determine the optimal amount of suramin required for maximal inhibition of binding of Vg to its receptor, we used different concentrations of suramin in the reaction mixture.

The binding of radiolabeled Vg was increasingly inhibited with increasing concentrations of suramin in the reaction mixture. Suramin at a concentration of 0.3 mM inhibited binding of Vg to its receptor by 50%. With 1.5 mM suramin, the level of Vg binding was below that obtained with 100 molar excess unlabeled Vg as a competitor. For subsequent experiments suramin was used at a concentration of 5 mM.

RESULTS

Binding properties of the solubilized Vg receptor

In order to determine the time required for binding of Vg to its solubilized receptor to reach equilibrium, we incubated the reaction mixtures for 0–90 min at 4°C and analysed the data by a NLLSF program (Yamaoka *et al.*, 1981). As shown in Fig. 1, binding of Vg increased rapidly over the first 15 min and then increased very little between 60–90 min. The curve for specific binding is typical for first order kinetics,

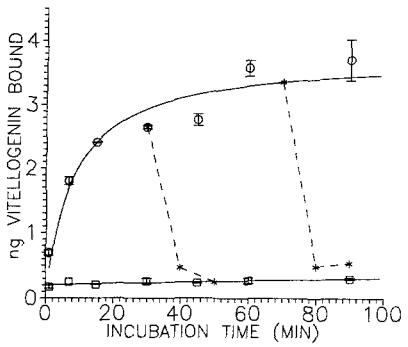

Fig. 1. Time dependence of Vg binding to OG solubilized ovary proteins and the effect of suramin on Vg binding. 6 μg of ovary OG extracted proteins were incubated with 0.3 μg [^{35}S]Vg (sp. act., 751,600 cpm/μg protein) in a total reaction volume of 120 μl at 4°C for the indicated times. Non-specific binding was determined by including 30 μg unlabeled Vn in parallel sets of reaction tubes. To another, sets of tubes containing ovary OG extracts and radiolabeled Vg, suramin (5 mM) were added after 30 or 70 min of reaction time. Binding and competition in these tubes was stopped 10 and 20 min after the addition of suramin. ○, specific binding of radiolabeled Vg; □, non-specific binding in the presence of excess unlabeled Vn; *, Vg binding in the presence of 5 mM suramin. The line for specific binding was drawn by NLLSF analysis of the data and the point at 70 min (*) is extrapolated from that. Data points represent the means ± SEM of three determinations.

indicating that the binding of Vg is to a single class of binding sites. Nonspecific binding, determined by including 100 molar excess of unlabeled Vn in parallel reaction mixtures as with [^{35}S]Vg alone, did not increase significantly over the 90 min period. Binding reactions for subsequent experiments were, therefore, for 90 min at 4°C.

Since suramin, a polysulfated polycyclic hydrocarbon, has been shown to be very effective in blocking the binding of various ligands such as vertebrate LDL (Schneider *et al.*, 1982), chicken Vg (Stifani *et al.*, 1988), *Manduca sexta* lipoprotein (Tsuchida and Wells, 1990) and locust and cockroach

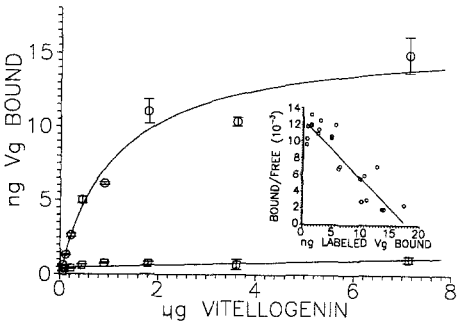

Fig. 2. Concentration-dependent binding of Vg to ovary OG extracts. 4 μg of OG solubilized proteins and increasing concentrations of [^{35}S]Vg (sp. act. 512,000 cpm/μg protein) were incubated in a reaction volume of 120 μl IB in the absence or presence of 100 molar excess of unlabeled Vn. ○, specific binding and; □, non-specific binding. The line for specific binding was drawn by NLLSF analysis of the data. Inset: Scatchard's plot of specific binding derived from the same data ($r^2 = -0.90$). Each point is a result of incubations done in triplicate.

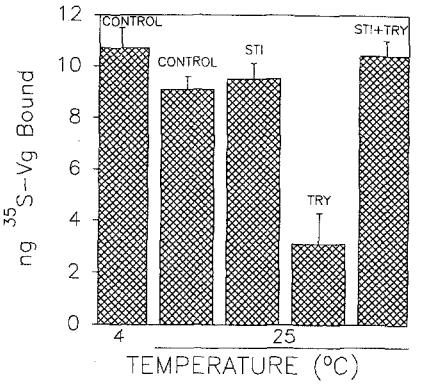

Fig. 3. Effect of trypsin on ovary OG extracts for Vg binding. Ovary OG extracts were incubated alone, with 30 μg soybean trypsin inhibitor (STI), 6 μg trypsin (TRY), STI and TRY together, or OG extracts alone at 25°C for 30 min after which 30 μg STI was added to tubes containing TRY. The contents in tubes were cooled to 4°C on ice. For each incubation 7.2 μg of the above treated or untreated OG extracts was incubated with 0.3 μg [^{35}S]Vg (sp. act. 553,600 cpm/μg protein) in the absence or presence of 30 μg unlabeled Vn. Each bar represents the mean specific binding ± SEM of three determinations.

Vgs (Röhrkasten and Ferenz, 1986; Röhrkasten *et al.*, 1989; Indrasith *et al.*, 1990) to their receptors and dissociation of lipoproteins from their receptors (Tsuchida and Wells, 1990) we tested its effect on binding of mosquito Vg to its receptor. Suramin (5 mM) was added to incubation mixtures 30 or 70 min after initiation of the binding reaction and the binding checked 10 and 20 min later. As shown in Fig. 1 (dotted lines), binding of Vg to its receptor was dissociated within 10 min after addition of suramin.

When fixed amounts of ovary OG extracts were incubated with increasing concentrations of radiolabeled Vg under equilibrium binding conditions, specific binding was saturable while non-specific binding increased only slightly in a linear manner (Fig. 2). The apparent K_d calculated by NLLSF analysis (Yamaoka *et al.*, 1981) of the data was 26 nM (using the native molecular weight of Vg and

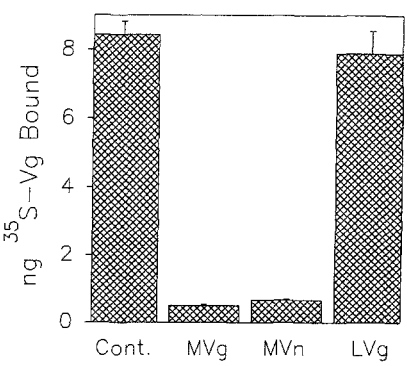

Fig. 4. Ligand binding specificity of Vg receptor in ovary OG extracts. 6 μg ovary OG extracts were incubated with 0.3 μg [^{35}S]Vg (sp. act. 590,990 cpm/μg protein) in a reaction volume of 120 μl IB either alone or in the presence of 30 μg unlabeled mosquito Vg, mosquito Vn or Vg of *L. migratoria*. Each bar represents the mean specific binding ± SEM of three determinations.

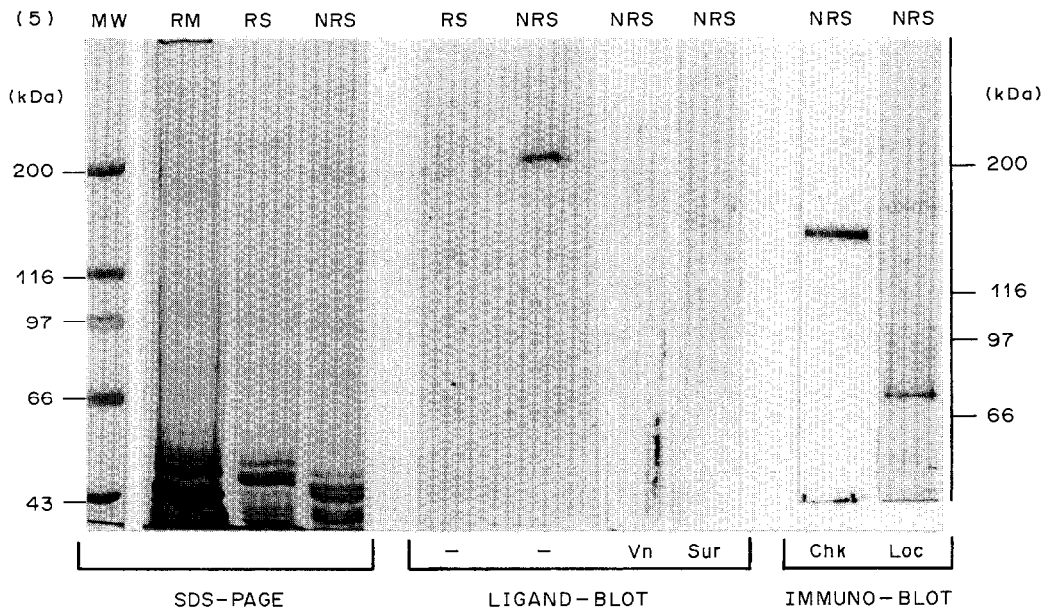

Fig. 5. Ligand blotting of solubilized mosquito Vg receptor. 30 μg ovary membrane protein (RM), or 12 μg ovary OG extracts (all other lanes) were separated on 6% SDS–PAGE under reducing (RS) or non-reducing (NRS) conditions followed by transfer to nitrocellulose. Ligand blotting was performed as described in the Materials and Methods section. All strips were incubated in 10 ml of buffer containing 36 μg/ml [^{35}S]Vg (sp. act. 436,100 cpm/μg protein) alone or 100 molar excess of unlabeled Vn (Vn) or 5 mM suramin (Sur). After washing, the blots were dried and exposed to X-ray film for 3–5 days at −70°C before developing the films to visualize the bound Vg receptor. The immunoblot of ovary OG extracts separated under NR conditions was probed with antibodies to chicken (Chk) or *L. migratoria* (Loc) Vg receptors, respectively. The molecular weight lane on the left shows the position and the molecular weights of the marker proteins used.

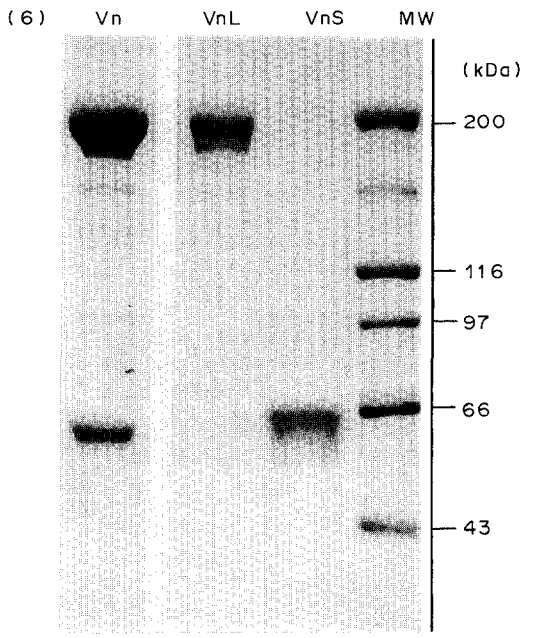

Fig. 6. Isolation of mosquito Vn subunits for determining receptor binding domain in Vg. Protein stained 5–10% SDS–PAGE to show the isolated Vn subunits from native Vn (Vn). VnL, 200 kDa large subunit; VnS, 66 kDa small subunit. The position of the Bio-Rad high molecular weight markers for SDS–PAGE are shown on the right.

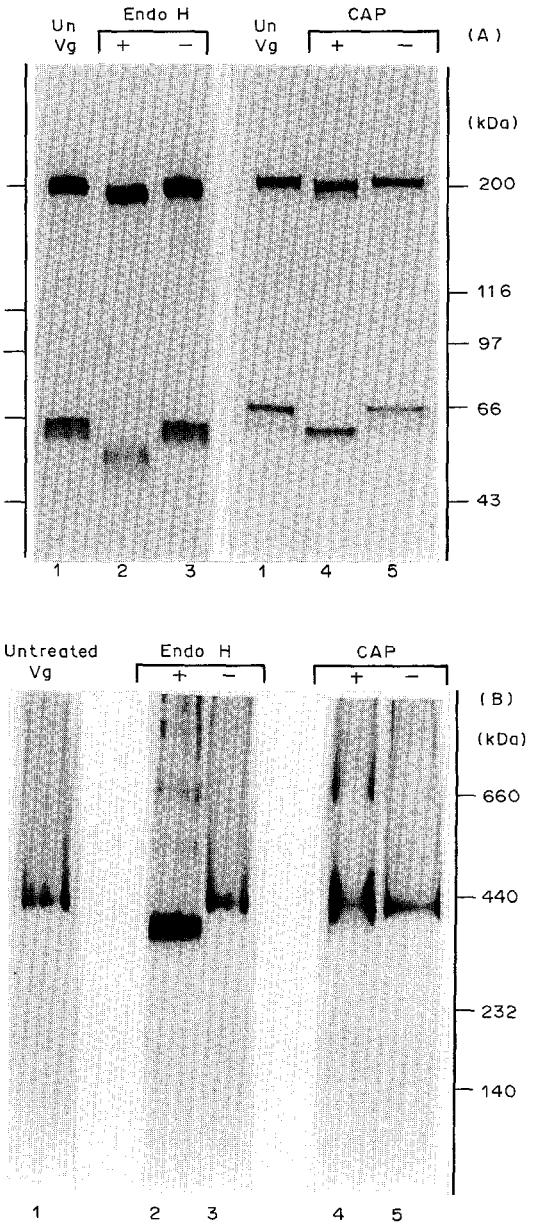

Fig. 8. SDS and native-PAGE of intact, deglycosylated and dephosphorylated [^{35}S]Vg. The proteins were separated on 5–15% polyacrylamide gels. After electrophoresis the gels were processed for fluorography as described in Experimental Procedures. (A) SDS–PAGE. Lanes 1, untreated Vg; 2 and 3, Vg treated with (+) and without (−) Endo H; 4 and 5, Vg treated with (+) and without (−) calf alkaline phosphatase. All lanes were loaded with 7000 cpm of the labeled Vg. The relative migration of Bio-Rad high molecular weight marker proteins for SDS–PAGE is shown on the right. (B) Native-PAGE. Samples in lanes 1–5 are the same as for SDS–PAGE and with similar loading. The position of high molecular weight marker proteins (Pharmacia) is shown on the right.

Vn to be 380,000). Analysis of the data by Scatchard's method (Scatchard, 1949) revealed an apparent K_d of 30 nM (Fig. 2, inset). Data from both the analysis indicates the presence of a single class of Vg binding sites in ovary OG extracts. The maximum amount of Vg bound, by extrapolation of this data, was 4.4 μg/mg OG extra protein.

Binding of Vg to its receptor was reduced when ovary OG extracts were exposed to heat or trypsin, indicating the proteinaceous nature of the Vg receptor. Preheating of the extracts at 80°C for 5 min almost completely destroyed the Vg binding activity (data not shown).

Binding activity of ovary OG extracts trypsinized for 30 min at 25°C was reduced by 70% compared to untreated controls (Fig. 3). However, incubation of the ovary OG extracts with trypsin and soybean trypsin inhibitor together or with soybean trypsin inhibitor alone retained the binding activity of Vg to an extent similar to when the extracts were just incubated at 25°C for 30 min.

Previously we demonstrated that binding of mosquito Vg or Vn to the binding sites on isolated ovary membranes was specific for these proteins and that neither locust Vg nor rat IgG competed for binding to the mosquito Vg receptor (Dhadialla and Raikhel, 1991). In order to be sure that during solubilization of the Vg receptor on ovary membranes the specificity of Vg binding was not lost, we tested the ability of mosquito Vg and Vn as well as of locust Vg to compete for binding to the solubilized receptor. As shown in Fig. 4, while mosquito Vg and Vn competed almost equally with radiolabeled mosquito Vg for binding to the solubilized receptor, locust Vg did not. Mosquito Vn also competed with mosquito Vg for binding to its receptor on ligands blots (Fig. 5).

When tissue specificity for Vg binding was compared between ovary and fat body OG extracts, radiolabeled Vg bound predominantly to the former and very little to the latter (data not shown).

Identification of the Vg receptor by ligand-binding

When protein blots of ovary OG extracts, separated by electrophoresis in 6% polyacrylamide gels under reducing or non-reducing conditions, were probed with [^{35}S]Vg, a protein with an apparent molecular weight of 205,000 was identified only under non-reducing conditions (Fig. 5). The presence of 100-fold excess of unlabeled Vn or 5 mM suramin in the incubation mixture resulted in the loss of binding of radiolabeled Vg to the 205,000 mol. wt protein. The ligand blot analysis was also performed using solubilized ovarian membranes using 5–15 and 5–20% gradient gels. Under these conditions, in addition to a 205 kDa protein, radiolabeled Vg binds to several low molecular weight polypeptides with the most visible band of 15 kDa. The intensity of these bands, however, was significantly lower than that of the 205 kDa band. Suramin eliminates binding of Vg to the 205 kDa protein but not to the low molecular weight polypeptides (data not shown). From these data we conclude that the 205 kDa protein is the mosquito Vg receptor.

Recently, immunological cross-reactivity was reported for the chicken, *Xenopus* and fish Vg receptors (Stifani *et al.*, 1990a, b). In addition, investigators from the same group reported that in chicken internalization of low density lipoprotein and Vg was mediated via a single and the same peptide receptor as for Vg on the oocyte surface (Stifani *et al.*, 1990; Barber *et al.*, 1991). We have probed for immunological homology between the Vg receptors of *A. aegypti*, *L. migratoria* and chicken, using rabbit polyclonal antibodies against receptors from the latter two species. As shown in Fig. 5, antibodies to neither the chicken nor locust Vg receptor cross-reacted with the mosquito Vg receptor identified by ligand blotting. However, antibodies against both the chicken and locust Vg receptors recognized one or more antigens in the mosquito ovary OG extracts. While chicken Vg receptor antiserum recognized a mosquito ovary membrane peptide with $M_r = 147{,}000$, antibodies to locust Vg receptor cross-reacted with two prominent peptides ($M_r = 169{,}800$ and 74,000; Fig. 5). None of these cross-reacting proteins correspond to Vg receptor from chicken or locust (Stifani *et al.*, 1988; Röhrkasten *et al.*, 1989; Hafer and Ferenz, 1991).

Characterization of binding properties of Vg

Both Vg and Vn of *A. aegypti* consist of two subunits (Raikhel and Bose, 1988; Dhadialla and Raikhel, 1990). In order to determine which subunit has the domain for binding to the Vg receptor we separated the two subunits of unlabeled Vn by gel permeation chromatography (Fig. 6). We chose to use Vn subunits for competition against [^{35}S]Vg binding to its receptor, owing to the large amounts of Vn which can be obtained relative to Vg. Moreover, as shown in Figs 4 and 5, unlabeled Vg and Vn compete equally well for binding to the Vg receptor.

To determine the subunit binding domain, we measured the binding of radiolabeled Vg in the presence of 100 molar excess unlabeled intact Vn, VnL or VnS. The results in Fig. 7 show that while both subunits competed with Vg for binding to the receptor, neither was as effective in displacing radiolabeled Vg as intact native Vn. Nonetheless, the large subunit was more effective than the small subunit. In duplicate experiments, while VnL consistently competed with radiolabeled Vg for binding to its receptor, competition with VnS was variable. We noticed that competition with VnS was absent when purified VnS had been stored for even a few days in liquid N_2. Freshly prepared VnS or that stored at 4°C for a day always competed with intact Vg for binding to the receptor. On the other hand, even after storage for 1–2 weeks in liquid N_2, VnL was still an effective competitor, as shown in Fig. 7.

Next, we determined the role of phosphate and sugar moieties on Vg for its binding to the receptor. A batch of purified radiolabeled Vg was divided into three aliquots. One remained untreated and the other two were used for dephosphorylation or deglycosylation as described under Experimental Procedures. The integrity of the peptide chain in the dephosphorylated and deglycosylated samples was verified by native and SDS–PAGE (Fig. 8).

When receptor binding of dephosphorylated radiolabeled Vg (CAP^+Vg) was compared to that of CAP^-Vg, or untreated Vg, specific binding of CAP^+Vg to the Vg receptor was significantly reduced

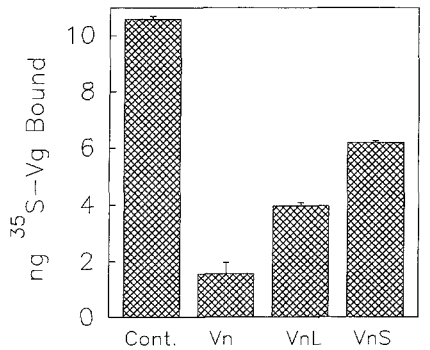

Fig. 7. Competition for Vg binding with native Vn and its subunits. 6 μg ovary OG extracts were incubated with 0.4 μg [^{35}S]Vg (sp. act. 367,700 cpm/μg protein) in total reaction volume of 120 μl IB containing; Cont., none; Vn, 40 μg intact Vn; VnL, 21 μg large Vn subunit, and VnS, 7 μg small Vn subunit. Each bar represents the mean ± SEM of three determinations.

(Fig. 9). On the other hand the specific binding of untreated Vg and CAP$^-$ Vg was similar. These results indicate that phosphorylation of mosquito Vg is necessary for recognition by its receptor.

Our experiments with deglycosylation of Vg have shown that the removal of sugars destabilizes the Vg molecule. The high molecular weight aggregates are evident in native-PAGE of Endo$^+$ Vg (Fig. 8). When variously treated Vgs for deglycosylation in incubation mixtures not containing ovary OG extracts were taken through the filtration step of the binding assay, the amount of deglycosylated Vg (Endo$^+$ Vg), trapped on the filter paper accounted for about 50% of that applied (data not shown). Some of the trapped Endo$^+$ Vg was displaced when 100-fold excess unlabeled Vn was included in the incubation mixture. Retention on the filter papers of sham-deglycosylated Vg (Endo$^-$ Vg) was twice as much as that obtained with untreated [^{35}S]Vg suggesting that exposure of Vg to buffers and pH conditions used in deglycosylation affects the stability of Vg. When the same experiments also included ovary OG extracts in the incubation mixtures, the resulting calculated "specific" binding of deglycosylated Vg was highly variable and difficult to interpret. We also tested radiolabeled Vg, Endo$^+$ Vg and Endo$^-$ Vg for uptake by ovaries cultured *in vitro*. There were no significant differences in the amount of the three types of radiolabeled Vgs associated with ovaries, possibly due to high binding of deglycosylated Vg to the ovarian sheath (data not shown). We conclude that binding and uptake after treatment of Vg with Endo H are artifactual by reason of deglycosylation conditions rendering Vg unstable.

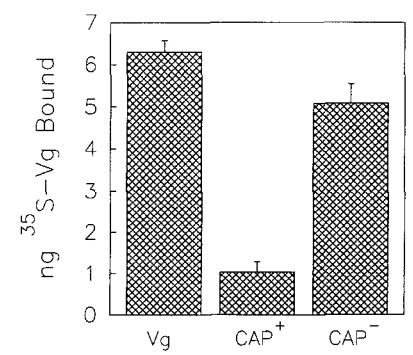

Fig. 9. Binding of native, dephosphorylated and sham dephosphorylated [^{35}S]Vg. 6 μg ovary OG extract was incubated with 0.39 μg ^{35}S-labeled native, dephosphorylated or sham dephosphorylated Vg (sp. act. 506,460 cpm/μg) in 120 μl IB in the absence or presence of 40 μg unlabeled native Vn. The data shows specific binding and represents the mean ± SEM of at least three determinations.

Vg receptor binding activity during oocyte development

In our previous study we demonstrated that the rate of Vg internalization by ovaries peaked between 24–30 h after a blood meal (Koller *et al.*, 1989). In this study we have investigated the developmental profile of Vg binding to ovary membranes during the first vitellogenic cycle to see whether the maximal rate of internalization corresponds to the highest amount of receptors per ovary. In the mosquito ovary, all oocytes develop synchronously throughout all the stages of egg maturation (Raikhel, 1992). It is, therefore, possible to use whole ovaries to study the changes in the number of Vg receptors during oocyte development.

Membranes prepared from ovaries taken from previtellogenic females as well as from vitellogenic females at various times after a blood meal were used for Vg binding. Figure 10 shows that the amount of Vg bound per ovary changes dramatically during egg maturation. Membranes from ovaries of

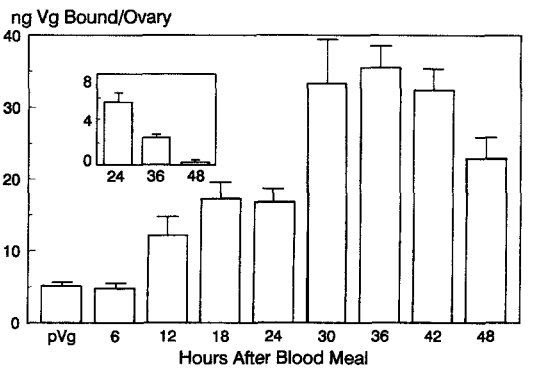

Fig. 10. Developmental profile of Vg binding to mosquito ovary membranes and its solubilized protein extracts during the vitellogenic cycle. Membranes from the indicated stages of ovaries were extracted as described in Experimental Procedures. 5 μg membrane protein was incubated with 0.4 μg [^{35}S]Vg (sp. act. 359,000 cpm/μg protein) in 80 μl IB. Non-specific binding was determined by incubating parallel sets of tubes as above but containing 40 μg unlabeled Vn as the competitor. The incubations were for 90 min at 4°C. The data show mean ± SEM specific binding and are from a typical experiment run in triplicate. Inset: membranes isolated from ovaries at 24, 36 and 48 h after a blood meal were solubilized with octyl-β-D-glucoside as described in Experimental Procedures. 8 μg of ovary OG protein was incubated in the presence of 0.4 μg [^{35}S]Vg (sp. act. 200,000 cpm/μg protein) in a reaction volume of 120 μl. Non-specific binding was determined by including 40 μg unlabeled Vn as indicated for the membrane binding assay, above. Each bar represents the mean specific binding ± SEM of three observations.

previtellogenic 3-day-old females, which are competent for vitellogenesis, exhibited relatively high binding activity. Binding first increased between 6–12 h and then again between 24–30 h after a blood meal. We were surprised to see a continued high level of Vg binding to membranes of ovaries taken between 36–48 h, a time when there is no uptake of Vg in ovaries (Koller *et al.*, 1989) and when chorion is deposited on the oocyte surface (Raikhel and Lea, 1991; Raikhel, 1992). This raised the question if Vg was not binding to the chorion fragments present in membrane preparations in a non-specific but displaceable manner. In order to confirm the nature of this high binding, we used OG extracts of membranes from ovaries taken at 24, 36 and 48 h after a blood meal for Vg binding (Fig. 10, inset). The results show that Vg binding does indeed decrease significantly at 36 h as compared to that observed at 24 h after a blood meal. At 48 h, there was very little or no binding at all. The difficulty in obtaining sufficient amounts of material prevented us from reproducing the entire experiment using OG extracts from ovary membranes.

DISCUSSION

In the present study we have solubilized Vg binding sites from ovary membranes isolated from vitellogenic mosquitoes and demonstrated that they have the properties expected of a receptor. Binding of [^{35}S]Vg to this solubilized receptor was saturable over time and equilibrium binding with first order kinetics was attained between 60–90 min at 4°C. The binding was selective for mosquito Vg and Vn, but not for Vg of another insect, *L. migratoria*. When tested for tissue specificity of Vg binding, radiolabeled Vg bound predominantly to solubilized ovary OG extracts and very little to solubilized OG extracts of fat bodies. Binding activity was sensitive to heat and protease treatment confirming that the Vg binding sites are of protein nature. Binding under equilibrium conditions demonstrated the existence of saturable single class of binding sites with a mean K_d of 28 nM (the mean value is derived after analysis of the data by NLLSF program and by Scatchard's method). This value is about 6-fold lower than that obtained in our earlier study for Vg binding to membranes isolated from the mosquito ovary (Dhadialla and Raikhel, 1991). It is possible that, solubilization of ovary membranes removes non-specific Vg binding components, which would result in a lower K_d value obtained in this report. Our present result is comparable with the 40 nM K_d for Vg binding to its receptor solubilized from *L. migratoria*, the only other insect for which such data have been obtained (Röhrkasten and Ferenz, 1986).

By ligand blotting of solubilized ovary OG extracts we found that [^{35}S]Vg bound to a single polypeptide with 205,000 mol. wt. The mosquito Vg receptor shared two properties in common with Vg receptors of *L. migratoria* and *N. cinerea* (Röhrkasten *et al.*, 1989; Indrasith *et al.*, 1990) and oviparous vertebrates (Stifani *et al.*, 1988, 1990a–c); (1) on ligand blots Vg bound to its receptor only in the absence of reducing agents, reinforcing the concept that intradisulfide bonds are necessary for receptor–ligand interaction, and (2) Vg receptor interaction could be inhibited as well as dissociated with suramin. Suramin, which is a negatively charged polysulfated cyclic compound, has been used as a competitor for the purification of the locust Vg receptor by affinity chromatography (Röhrkasten *et al.*, 1989). Indrasith *et al.* (1990) also used suramin to compete binding of *N. cinerea* Vg to its receptor on ligand blots. However, unlike results obtained by these authors, in our study suramin effectively displaced only Vg bound to its receptor (205 kDa) and not to low molecular weight proteins. Röhrkasten and Ferenz (1992) have recently shown importance of the lysine and arginine residues of Vg in high affinity binding to locust Vg receptor. These results also pointed out that the charge of the Vg and Vg receptor molecules is critial for their interaction.

The apparent molecular weight of the mosquito Vg receptor ($M_r = 205{,}000$), is similar to those for the cockroach, *N. cinerea* ($M_r = 200{,}000$; Indrasith *et al.*, 1990) and for *L. migratoria*, for which the weight was recently revised from 156,000 to 190,000 (Rohrkasten *et al.*, 1989; Hafer and Ferenz, 1991). The insect Vg receptors are twice larger than those of oviparous vertebrates; chicken ($M_r = 96{,}000$; Stifani *et al.*, 1988), *Xenopus* ($M_r = 115{,}000$; Stifani *et al.*, 1990c) and coho salmon, *Oncorhynchus kisutch* ($M_r = 100{,}000$; Stifani *et al.*, 1990a). The Vg receptors for the above three oviparous vertebrates are immunologically related and at least in chicken oocytes, the 96-kDa receptor mediates the internalization of both Vg and low density lipoprotein (Stifani *et al.*, 1990b). Polyclonal antibodies against *Locusta* Vg receptor recognized the Vg receptor from the polychaetous annelid, *Nereis virens*. The *Nereis* Vg receptor binds *Locusta* and *Schistocerca* Vg or Vn; the Vg receptors of both locust species bind the *Nereis* Vn (Hafer *et al.*, 1992). We tested for immunological relatedness between the Vg receptors of mosquito and those of *L. migratoria* and chicken. On immunoblots, rabbit polyclonal antibodies to neither the locust nor the chicken Vg receptors cross-reacted with the 205 kDa mosquito Vg receptor protein. The lack of cross-reactivity between antibodies to the locust Vg receptor and the mosquito Vg receptor supports our binding data in which *L. migratoria* Vg did not compete with mosquito Vg for binding to the receptor in either membrane preparation or in solubilized form (Fig. 4; Dhadialla and Raikhel, 1991).

Another important aspect for receptor–ligand interaction concerns the domain of Vg involved in receptor binding. Our results suggest that both the subunits of *A. aegypti* are required for binding the native Vg molecular to its receptor. While both subunits competed with native Vg for binding to its receptor, neither one of them competed as well as the intact molecule. It is possible that in *A. aegypti* the single class of Vg receptors have bivalent binding sites for Vg subunits. In contrast, of the two cleavage products of chicken Vg, lipovitellin and phosvitin, Stifani *et al.* (1988, 1991) demonstrated that lipovitellin was responsible for binding to the Vg receptor. Similar experiments with Vg from the moth, *M. sexta*, indicated that the small subunit did not carry the receptor binding domain (Osir and Law, 1986). Since the purified large subunit of *M. sexta*

aggregates and, hence, could not be tested for binding, the authors construed from results with the small subunit that the receptor binding domain in this insect is in the large subunit. This is consistent with the fact, that in *M. sexta* Vg the small subunit is positioned inside the native molecule (Osir and Law, 1986). Based on immunological data (Raikhel and Bose, 1988) and data on Vg subunit-receptor binding in this report, we conclude that unlike *M. sexta* Vg, the two subunits of *A. aegypti* Vg are assembled such that both are exposed to the aqueous environment.

Results of binding experiments using untreated Vg and dephosphorylated Vg clearly indicate that phosphate moiety is required for binding of Vg to its receptor. The role of Vg phosphorylation for uptake or receptor binding has not been investigated for any other insect. Miller *et al.* (1982), however, demonstrated that dephosphorylation of riboflavin-binding protein and phosvitin decreased the uptake of these proteins by chicken oocytes.

Carbohydrate groups on proteins serve and important role for cell–cell interaction, protein targeting, recognition signals for ligand–cell surface receptors and host–pathogen interactions (Paulson, 1989). In vertebrates, the role of complex oligosaccharides of protein ligands in specific recognition by their receptors is well documented (Drickamer, 1991). Insects and vertebrate glycoproteins are fundamentally different with respect to asparagine-linked high mannose oligosaccharides. In insects the oligosaccharides are not modified during post-translational processing of proteins (Nordin *et al.*, 1984; Osir *et al.*, 1986). Although, insect Vgs are heavily glycosylated, their carbohydrate moieties remain sensitive to Endo H after Vg is secreted and even after its deposition into developing oocytes (Nordin *et al.*, 1984; Osir *et al.*, 1986; Raikhel and Bose, 1989; Dhadialla and Raikhel, 1990). It is, therefore, difficult to expect that unmodified high mannose oligosaccharide would serve as unique recognition entities during Vg receptor interaction.

The role of carbohydrate moiety on Vg for its uptake by oocytes and binding to its receptor has been investigated for only two insect species. Osir and Law (1986) and Osir *et al.* (1988) used Endo H to deglycosylate Vg and found that the carbohydrate moiety on *M. sexta* Vg was not necessary for Vg uptake or binding to follicle membranes. On the other hand, for *B. germanica* König *et al.* (1988b) found that Vn glycopeptides digested with α-mannosidase resulted in much higher binding of deglycosylated Vn to follicle membrane preparations than with untreated Vn. In another study, Gochoco *et al.* (1988) observed that endocytosis of radiolabeled 18S Vn by oocytes *in vivo* decreased 6-fold after treatment with α-mannosidase. König *et al.* (1988b) concluded that for *B. germanica*, high mannose oligosaccharides of Vn were necessary but not sufficient for binding to the Vg receptor. Our experimental approach, although similar to the one used by Osir and Law (1986) and Osir *et al.* (1988b), does not allow us to define the role of glycosylation of mosquito Vg for binding to its receptor. However, we can conclude that glycosylation is necessary for maintaining stability of Vg in its native state. Our finding is consistent with the long standing implication of glycosylation of proteins in maintenance of their stability (Olden *et al.*, 1982). Glycosylation of the contact site of A protein from the lower eukaryote, *Dictyostelium*, is important for stability but not its actual function in cell adhesion (Hohmann *et al.*, 1987).

In our study of the developmental profile of Vg receptor binding activity in oocytes during the first cycle of egg maturation, we found that binding activity was already present in previtellogenic ovaries. This is in agreement with morphological studies demonstrating that during previtellogenic development of oocytes there is an intensive formation of coated vesicles that renders the oocytes competent for Vg internalization (Raikhel, 1984; Raikhel and Lea, 1985). Thus, we can conclude that a substantial number of Vg receptors are synthesized in oocytes prior to the onset of vitellogenesis.

The only other insect for which binding of Vg to ovarian membranes at different developmental stages has been analyzed is the cockroach, *N. cinerea* (König and Lanzrein, 1985). This analysis, performed using isolated membranes, showed the continuous increase in Vg binding activity which peaked at the time of ovulation. However, by this time internalization of Vg decreases to background level (Kindle *et al.*, 1988). It is likely, that in these data there is also an inherent artifactual binding to the chorion fragments as demonstrated by our study. The approach we used included both isolated ovary membranes and their OG extracts. This allowed us to distinguish specific binding from artifactual binding of Vg to chorion fragments. When we tested the binding of radiolabeled Vg to solubilized OG extracts of membranes from ovaries taken at 24, 36 and 48 h, we noticed that the Vg binding activity from 48 h ovary OG extracts was largely lost and in 36 h ovary OG extracts it was dramatically reduced. We previously demonstrated that the uptake of Vg in ovaries ceases by 36 h after a blood meal (Koller *et al.*, 1989). Moreover, this time period overlaps with the time when the interfollicular spaces close and chorion is deposited on the oocyte surface (Raikhel and Lea, 1991; Raikhel, 1992), thus occluding the passage of Vg from the hemolymph to the receptors on the oocyte surface. The results of our Vg binding studies are, therefore, in agreement with the kinetics of Vg uptake in ovaries (Koller *et al.*, 1989). Finally, our data suggests that increase in the rate of internalization of Vg in *A. aegypti* ovaries during the first 30 h after a blood meal is, at least in part, due to an increase in the number of Vg receptors in developing oocytes.

In conclusion, we have solubilized the mosquito Vg receptor and characterized its properties. We have also characterized some properties of Vg with respect to its receptor binding domain. The role of carbohydrate and phosphate moieties on Mg for interaction with the Vg receptor was also investigated. Finally, we investigated the developmental profile for Vg receptor binding in mosquito ovaries during the first vitellogenic cycle. The knowledge gained through this study provides us with bases for further investigation of receptor-mediated accumulation of yolk protein precursors by the mosquito oocytes. Elucidation of this process, which is a cornerstone of egg maturation, could also help us understand how some

of the mosquito viruses gain entry into ovaries for transovarial transmission.

Acknowledgements—We thank Drs W. J. Schneider and Hans-Jorg Ferenz for antibodies against Vg receptors of chicken and *L. migratoria*, respectively, and Dr G. R. Wyatt for providing purified *L. migratoria* Vg. Finally, we wish to thank Dr F. Kezdy for his helpful suggestions and critical reading of the manuscript. This research was supported by the National Institutes of Health (AI-32154) and NATO grants to A. S. Raikhel.

REFERENCES

Baeuerle P. A. and Huttner W. B. (1985) Tyrosine sulfation of yolk proteins 1, 2, and 3 in *Drosophila melanogaster*. *J. biol. Chem.* **260,** 6434–6439.

Barber D. L., Sanders E. J., Aebersold R. and Schneider W. J. (1991) The receptor for yolk lipoprotein deposition in the chicken oocyte. *J. biol. Chem.* **266,** 18,761–18,770.

Burnette W. N. (1981) Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. *Analyt. Biochem.* **112,** 195–203.

Dhadialla T. S. and Raikhel A. S. (1990) Biosynthesis of mosquito vitellogenin. *J. biol. Chem.* **265,** 9924–9933.

Dhadialla T. S. and Raikhel A. S. (1991) Binding of vitellogenin to membranes isolated from mosquito ovaries. *Archs Insect Biochem. Physiol.* **18,** 55–70.

Drickamer K. (1991) Clearing up glycoprotein hormones. *Cell* **67,** 1029–1032.

Ferenz H.-J., Lubzens E. W. and Glass H. (1981) Vitellin and vitellogenin incorporation by isolated oocytes of *Locusta migratoria migratoriodes* (R.F.). *J. Insect Physiol.* **27,** 869–875.

Gochoco C. A., Kunkel J. G. and Nordin J. H. (1988) Experimental modifications of an insect vitellin affect its structure and its uptake by oocytes. *Archs Insect Biochem. Physiol.* **9,** 179–199.

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

Hafer J. and Ferenz H.-J. (1991) Locust vitellogenin receptor: an acidic glycoprotein with N- and O-linked oligosaccharides. *Comp. Biochem. Physiol.* **100B,** 579–586.

Hafer J., Fischer A. and Ferenz H.-J. (1992) Identification of the yolk receptor protein in oocytes of *Nereis virens* (Annelida, Polychaeta) and comparison with the locust vitellogenin receptor. *J. comp. Physiol.* **162,** 148–152.

Hays A. R. and Raikhel A. S. (1990) A novel protein produced by the vitellogenic fat body and accumulated in mosquito oocytes. *Wilh. Roux archs dev. Biol.* **119,** 114–121.

Hohmann H.-P., Bozzaro S., Merkl R., Wallraff E., Yoshida M., Weinhart U. and Gerisch G. (1987) Post-translational glycosylation of the contact site A protein of *Dictyostelium discoideum* is important for stability but not for its function in cell adhesion. *EMBO J.* **6,** 3663–3671.

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

Kawooya J. K., Wells M. A. and Law J. H. (1989) A strategy for solubilizing delipidated apolipoprotein with lysophosphatidylcholine and reconstitution with phosphatidylcholine. *Biochemistry* **28,** 6658–6667.

Keen J. H. (1990) Clathrin and associated assembly and disassembly proteins. *A. rev. Biochem.* **59,** 415–438.

Kindle H., König R. and Lanzrein B. (1988) *In vitro* uptake of vitellogenin by follicles of the cockroach *Nauphoeta cinerea*: comparison of artificial medial with hemolymph media and the role of vitellogenin concentration and juvenile hormone. *J. Insect Physiol.* **34,** 541–548.

Koller C. N. and Raikhel A. S. (1991) Initiation of vitellogenin uptake and protein synthesis in mosquito ovary. *J. Insect Physiol.* **37,** 703–711.

Koller C. N., Dhadialla T. S. and Raikhel A. S. (1989) Selective endocytosis of vitellogenin by oocytes of the mosquito, *Aedes aegypti*: an *in vitro* study. *Insect Biochem.* **19,** 693–702.

König R. and Lanzrein B. (1985) Binding of vitellogenin to specific receptors in oocyte membrane preparations of the ovoviviparous cockroach *Nauphoeta cinerea*. *Insect Biochem.* **15,** 735–747.

König R., Kindle H., Kunkel J. G. and Lanzrein B. (1988a) Vitellogenesis in the cockroach *Nauphoeta cinerea*: separation of two classes of ovarian binding sites and calcium effects on binding and uptake. *Archs Insect Biochem. Physiol.* **9,** 323–337.

König R., Nordin J. H., Gochoco C. H. and Kunkel J. G. (1988b) Studies on ligand recognition by vitellogenin receptors in follicle membrane preparations of German cockroach, *Blattella germanica*. *Insect Biochem.* **18,** 395–404.

Kulakosky P. C. and Telfer W. H. (1987) Selective endocytosis, *in vitro*, by ovarian follicles from *Hyalophora cecropia*. *Insect Biochem.* **17,** 845–858.

Kunkel J. G. and Nordin J. H. (1985) Yolk proteins. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology* (Edited by Kerkut C. A. and Gilbert L. I.), Vol. 1, pp. 83–111. Pergamon Press, Oxford.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Lea A. O. (1964) Studies on the dietary and endocrine regulation of autogenous *Aedes taeniorhynchus*. *J. med. Ent.* **1,** 40–44.

Miller M. S., Benore-Parsons M. and White H. B. (1982) Dephosphorylation of chicken riboflavin-binding protein and phosvitin decreases their uptake by oocytes. *J. biol. Chem.* **257,** 6818–6824.

Nordin J. H., Gochoco C. H., Wojchowski D. M. and Kunkel J. g. (1984) A comparison study of the sizeheterogeneous high mannose oigosaccharides of some insect vitellins. *Comp. Biochem. Physiol.* **79B,** 379–390.

Olden K., Bernard B. A., White S. L. and Parent J. B. (1982) Function of the carbohydrate moieties of glycoproteins. *J. cell. Biochem.* **18,** 313–335.

Oliveira P. L., Gondim K. C., Guedes D. M. and Masuda H. (1986) Uptake of yolk proteins in *Rhodnius prolixus*. *J. Insect Physiol.* **32,** 859–866.

Opresko L. K. and Wiley H. S. (1987) Receptor-mediated endocytosis in *Xenopus* oocytes. II. Evidence for two novel mechanisms of hormonal regulation. *J. biol. Chem.* **262,** 4116–4123.

Osir E. O. and Law J. H. (1986) Studies on binding and uptake of vitellogenin by follicles of the tobacco hornworm, *Manduca sexta*. *Archs Insect Biochem. Physiol.* **3,** 513–528.

Osir E. O., Anderson D. R., Grimes W. J. and Law J. H. (1986) Studies on the carbohydrate moiety of vitellogenin from the tobacco hornworm, *Manduca sexta*. *Insect Biochem.* **16,** 471–478.

Paulson J. C. (1989) Glycoproteins: what are the sugar chains for? *TIBS* **14,** 272–275.

Pearse B. M. F. and Robinson M. S. (1990) Clathrin, adaptors, and sorting. *A. rev. cell Biol.* **6,** 151–171.

Raikhel A. S. (1984) Accumulation of membrane-free clathrin-like lattices in the mosquito oocyte. *Eur. J. cell Biol.* **35,** 279–283.

Raikhel A. S. (1992) Vitellogenesis in mosquitoes. *Adv. dis. vect. Res.* **9,** 1–39.

Raikhel A. S. and Bose S. G. (1988) Properties of mosquito yolk protein: a study using monoclonal antibodies. *Insect Biochem.* **18,** 565–575.

Raikhel A. S. and Dhadialla T. S. (1992) Accumulation of yolk proteins in insect oocytes. *A. rev. Ent.* **37,** 217–251.

Raikhel A. S. and Lea A. O. (1985) Hormone-mediated formation of the endocytic complex in mosquito oocytes. *Gen. comp. Endocr.* **57,** 422–433.

Raikhel A. S. and Lea A. O. (1991) Control of follicular epithelium development and vitelline envelope formation in the mosquito; role of juvenile hormone and 20-hydroxyecdysone. *Tiss. Cell* **23,** 577–591.

Röhrkasten A. and Ferenz H.-J. (1985) *In vitro* study of selective endocytosis of vitellogenin by locust oocytes. *Wilh. Roux archs dev. Biol.* **194,** 411–416.

Röhrkasten A. and Ferenz H.-J. (1986) Properties of the vitellogenin receptor of isolated locust oocyte membranes. *Int. J. invert. reprod. Dev.* **10,** 133–142.

Röhrkasten A. and Ferenz H.-J. (1992) Role of the lysine and arginine residues of vitellogenin in high affinity binding to vitellogenin receptors in locust oocyte membranes. *Biochim. biophys. Acta* **1133,** 160–166.

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

Roth T. F. and Porter K. R. (1964) Yolk protein uptake in the oocyte of the mosquito *Aedes aegypti. J. cell Biol.* **20,** 313–332.

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

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

Schneider W. J., Beisiegel U., Goldstein J. L. and Brown M. S. (1982) Purification of the low density lipoprotein receptor, an acidic glycoprotein of 101,000 molecular weight. *J. biol. Chem.* **257,** 2664–2673.

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

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

Stifani S., Barber D. L., Nimpf J. and Schneider W. J. (1990b) A single chicken oocyte plasma membrane protein mediates uptake of very low density lipoprotein and vitellogenin. *Proc. natn Acad. Sci. U.S.A.* **87,** 1955–1959.

Stifani S., LeMenn F., Rodriguez J. N. and Schneider W. J. (1990c) Regulation of oogenesis: the piscine receptor for vitellogenin. *Biochem. biophys. Acta* **1045,** 271–279.

Tsuchida K. and Wells M. A. (1990) Isolation and characterization of a lipoprotein receptor from the fat body of an insect, *Manduca sexta. J. biol. Chem.* **265,** 5761–5767.

Yamaoka K., Tanigawara Y., Nakagawa T. and Uno T. (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharm. Dyn.* **4,** 879–885.

Yusko S. G. and Roth T. F. (1976) Binding to specific receptors on oocyte plasma membranes by serum phosvitin-lipovitellin. *J. supramolec. Struct.* **4,** 89–97.

Yusko S., Roth T. F. and Smith T. (1981) Receptor-mediated vitellogenin binding to chicken oocytes. *Biol. chem. J.* **200,** 43–50.