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BINDING TO SPECIFIC RECEPTORS ON OOCYTE PLASMA MEMBRANES BY SERUM PHOSVITIN-LIPOVITELLIN

S. C. Yusko and Thomas F. Roth

University of Maryland Baltimore County, Catonsville, Maryland 21228

Specific binding sites for the serum complex of phosvitin and lipovitellin have been shown to exist on the outer surface of rapidly growing chicken oocytes. The existence and specificity of these sites were demonstrated by competition for binding to unfixed oocyte membrane fragments and by displacement of already bound and labeled phosvitin-lipovitellin from formaldehyde-fixed membranes. Only unlabeled phosvitin-lipovitellin competed with the ^{125}I-labeled complex for binding to the fragments or displacement of bound label; IgG isolated from egg yolks and bovine serum albumin were ineffective.

Protein transport into cells occurs in many cell types and is particularly manifest in oogenesis. For example, the yolk proteins phosvitin and lipovitellin in chickens (1, 2) and vitellogenin in the clawed-toed frog, Xenopus (3), are formed in the liver under the influence of estrogens and carried by the bloodstream to the ovary where they are incorporated into the yolk of oocytes. These proteins must cross the oocyte plasma membrane in order to accumulate in the yolk.

Immunoglobulins are another protein species important in protein transport since many young animals would die shortly after birth or hatching were it not for the presence of maternal immunoglobulins in their bodies. These antibodies are transported across the placenta in primates (4) or the yolk sac membranes in rabbits (5) to the fetus and afford immunological protection to the newborn until its own immune system is functional. This transport of proteins across the plasma membrane is believed to occur by a process involving micropinocytosis by the coated pit-coated vesicle mechanism (6, 7).

In the chicken egg yolk many proteins are sequestered, including gamma globulins (called γ-livetins for the egg) and serum albumin (α-livetins) as well as two yolk proteins, phosvitin and lipovitellin. Cutting and Roth (8) found that phosvitin and IgG were present in different ratios in the serum and egg yolk of chickens. Patterson et al. (9) obtained data indicating that chicken serum albumin and gamma globulins were accumulated at different rates. These data lend support to the idea that the process of accumulation may involve some selective mechanism, perhaps a recognition and binding step prior to incorporation by micropinocytosis. The involvement of a recognition and binding step implies the existence of receptor regions, possibly receptor proteins on the plasma membrane. Further support for this can be found in other systems. Sonada and Schlamowitz (10),

using formaldehyde-fixed yolk sac membranes from rabbits, found evidence that these membranes contain specific binding sites for gamma globulins.

The experiments in this paper were designed to determine whether specific binding sites exist on the oocyte cortex for the phosvitin-lipovitellin complex. With both fixed and unfixed chicken oocyte membranes, the data indicate that these membranes contain specific binding sites for the serum complex of phosvitin and lipovitellin. Gamma globulins and bovine serum albumin do not compete with the complex for binding to these sites.

MATERIALS

Agarose A-0.5 and A-1.5 were obtained from Bio-Rad. Ammonium sulfate from Schwarz/Mann, $Na^{125}I$ from New England Nuclear, Chloramine T from Eastman, DEAE cellulose from Whatman (DE 23). Live White Leghorn laying hens were purchased from a local slaughter house.

METHODS

Purification of Proteins

The phosvitin-lipovitellin complex (PvLv) was separated from the plasma of estrogenized White Leghorn roosters by the method of Beuving and Gruber (11) with DEAE cellulose ion exchange chromatography. After partial purification on DEAE, the preparation was further purified on a 40 × 2.5-cm column of Bio-Rad A-1.5 agarose. Protein-bound phosphate was determined by the method of Beuving and Gruber (12) except that the phosphomolybdic acid complex was not extracted into an organic phase (13).

IgG was purified from eggs by the method of Bernardi and Cook (14) and precipitated three times with 33% saturated ammonium sulfate at 0°C. The IgG was then separated from small quantities of a high molecular weight material on an agarose A-1.5 column. The IgG was stored at 4°C as the 33% ammonium sulfate precipitate.

Iodination of the Phosvitin-Lipovitellin Complex

Iodination of the PvLv was by the method of Brown and Reith (15) with the adaptation of Greenwood et al (16). The reaction mixture consisted of 50 μl of 0.2 M sodium borate buffer, pH 8.0, 100 μl containing 160–200 μg PvLv in saline, 30 μl of 0.33 mg/ml Chloramine T and 50 μl containing 1 mCi of $Na^{125}I$ in 0.05 M NaCl. After the reagents were mixed, an additional 100 μl of unlabeled PvLv was added and the non-protein-bound iodine removed by immediate sequential passage over two 2 ml Bio-Gel P-10 columns which had been pretreated with serum albumin to reduce protein absorption.

Isolation of Membrane Complex

White Leghorn laying hens were killed by decapitation and the ovaries removed and placed in Hanks' balanced salts solution (salts solution hereafter) (17). The bicarbonate and phenol red were omitted from the salts solution in all of the experiments in this paper. The large oocytes (1–3 cm diameter, orange color) were opened and drained of

yolk. The oocytes were then placed in salts solution and the cortex or membrane complex was dissected free of overlying connective tissue. Adhering yolk was removed by gentle shaking in the salts solution. The membrane complex used in these experiments consists of the oocyte plasma membrane with an adhering monolayer of follicular epithelial cells. A thick basement lamella overlays the follicular epithelium and a vitelline layer lies between the epithelium and the oocyte plasma membrane. The basement lamella and vitelline are acellular fibrous layers. The cortex was diced into pieces about 0.5–1 mm^2.

Fixation of Cortex

The fixation and washes were similar to those of Sonada and Schlamowitz (10). The pieces of cortex were fixed for 20 min in 6% freshly prepared formaldehyde at 0°C. Following fixation the membranes were washed four times for 10 min each in salts solution at 0°C. After this the membranes were washed twice in salts solution containing 3 mg/ml bovine serum albumin (BSA) for 30 min each at room temperature. To remove excess BSA the membranes were then rinsed twice in salts solution without BSA at room temperature for 15 min.

Labeling of Membranes

The fixed cortex pieces were incubated for 3 hr in 2 ml of salts solution, pH 7.4, containing 100 μg/ml of ^{125}I-labeled phosvitin-lipovitellin complex with a specific activity of 10^5 cpm/μg.

Washes

In order to reduce nonspecifically bound protein, the cortex pieces were washed eight times for 1 hr each in 3 ml salts solution with BSA at 25°C.

Specific Elution

After the last wash, the membranes were divided into four equal fractions. Each fraction was then eluted twice for 8 hr each time in the presence of either 2 mg/ml PvLv, 2 mg/ml BSA, 5 mg/ml IgG, or salts solution alone. After each specific elution, the membrane cortex pieces were separated from the supernatant by low speed centrifugation and the counts eluted from the membranes (supernatant) as well as those still bound to the membranes were determined.

The results were standardized by the same method as that used by Sonada and Schlamowitz (10) in that the counts eluted were expressed as the percent of those bound to the membranes just prior to the specific elution step.

Competition for Binding to Unfixed Membranes

The oocyte cortex was isolated as described earlier and sliced with small scissors into pieces about 1 mm^2 in area. The diced membranes were dispersed in salts solution containing 1 mg/ml BSA and, where necessary, the pH was adjusted to 7.4. The unlabeled proteins were dialyzed overnight vs salts solution. The pH of all solutions was ad-

justed to 7.4. The binding reaction took place in a total volume of 0.25 ml in 12 × 75 mm glass test tubes at 25°C. 1 mg/ml BSA was present in the reaction mixtures in order to stabilize membranes and to reduce nonspecific adsorption of labeled protein. The exact composition of the reaction mixtures is given in the Tables. In all cases the yolk cortex sections were the last ingredient added to the tubes. The binding of labeled proteins to membranes in the presence of competing proteins (unlabeled) was compared to tubes in which only the low concentration of the labeled protein was present.

Not all counts trapped on the filters were membrane associated. To determine that portion which was membrane associated, blank tubes were run which were identical to the experimental tubes except that membranes were omitted. The counts appearing in the blanks were subtracted from the total counts bound in the experimental tubes. The binding reaction was stopped by diluting the reaction mixtures 100-fold to 25 ml with salts solution containing 1 mg/ml serum albumin and then collecting the membranes on Whatman glass fiber (GF/C) filters by vacuum filtration. The filters were pretreated with serum albumin and unlabeled PvLv to reduce adherence of label to the filters. The dilution was accomplished by diluting the reaction mixtures to 5 ml in the reaction tube and pouring this into an additional 20 ml in the vacuum funnel before applying the vacuum. Membranes were subsequently washed by three sequential additions of 5 ml of salts solution without BSA. Less than 1 sec was required for each 5 ml wash to be completed. Filters were counted in a Nuclear Chicago gamma scintillation counter and the quantity of membrane protein on the filters was then determined.

Determination of Membrane Protein

After counting, each filter was dispersed in 2 ml of 1 N NaOH and heated for 30 min at 100°C. The absorbance of the supernatant was read at 280 nm after compacting the filters and centrifugation. Using an extinction coefficient of $\epsilon = 60$ for a 1% solution, the results agreed well with those using a microbiuret method (18).

RESULTS

Beuving and Gruber (12) found that in the serum, in contrast to the yolk, phosvitin and lipovitellin existed in a tightly bound complex. When we placed this material on a molecular sieve (Bio-Rad A-1.5) three peaks were obtained (Fig. 1). The primary peak (the PvLv complex), having a molecular weight of $6–6.5 \times 10^{-5}$ daltons, was used for the experiments. Based on the absorbance at 280 nm, the major peak comprised 77% of the total with peaks 2 and 3 accounting for 7% and 15%, respectively. The protein-bound phosphate content of the complex (peak 1) was 3.5%.

To determine whether binding sites existed on the oocyte cortex for the PvLv complex, cortex fragments were incubated in the presence of ^{125}I–labeled PvLv and the amount bound to the fragments was measured. Binding to specific sites on the membranes should be subject to competition by large quantities of unlabeled protein of the same species, whereas counts due to entrapment would not be competible. The specificity of binding could be tested by having large quantities of other proteins present along with the small quantity of labeled PvLv. If the other proteins bound to the same site as PvLv, then competition would be shown by decreasing the counts bound to the membranes. In

TABLE I. Competition for Binding

Membranes plus	Bound ^{125}I-PvLv/tube cpm (avg)	Average after blanks subtracted and normalized for membrane protein cpm/mg protein	Difference from salts solution (competible counts) cpm/mg protein
Salts alone	9,429 ± 1,343	51,225 ± 7,491	
2 mg/ml IgG	8,397 ± 309	46,333 ± 2,646	4,892
2 mg/ml PvLv	5,906 ± 718	29,409 ± 4,964	21,816
Blanks (no membranes)			
Salts alone	1,045 ± 153		
2 mg/ml IgG	2,178 ± 98		
2 mg/ml PvLv	1,278 ± 160		

Incubations were for 1 hr at 25°C with gentle shaking. The reaction mixtures contained 1 mg/ml bovine serum albumin, diced unfixed yolk cortex, 33 μg ^{125}I-labeled PvLv (5×10^4 cpm), and 15 mM HEPES in a total volume of 250 μl of Hanks' balanced salts solution, pH 7.4. In addition, some tubes contained unlabeled protein in the quantities shown above. The values presented are the averages of three runs with the standard error of the mean.

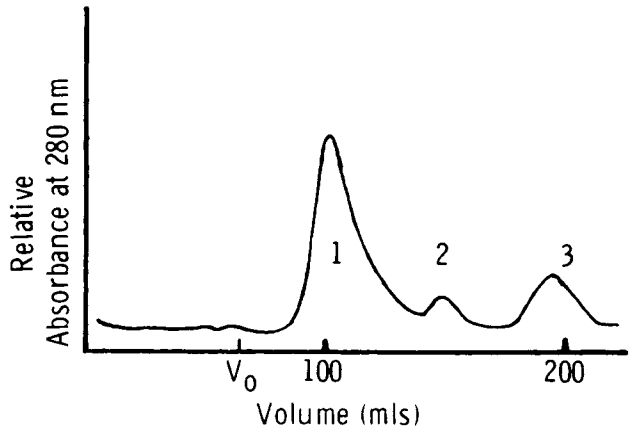

Fig. 1. Elution profile from an Agarose A-1.5 molecular sieve column (40 × 2.5 cm) of PvLv complex prepared by the method of Beuving and Gruber (11). The column volume was 200 ml and the void volume 63 ml, using the peak half height on the leading edge. The elution buffer was 0.15 M NaCl with 0.01 M sodium phosphate, pH 7.4, 4°C. The molecular weights determined by the elution volumes for peaks 1, 2, and 3, respectively, were $6-6.5 \times 10^5$, $1.8-2.2 \times 10^5$, and 10^4 daltons.

both Table I and Fig. 2 the pieces of unfixed oocyte cortex were incubated with labeled PvLv in the presence or absence of excess cold proteins. In both cases, unlabeled PvLv did compete with labeled material for binding. Indeed a significant portion (8–10%) of the added counts was bound in a competible manner after 1 hr. The time course of binding is shown in Fig. 2. Even after 2 hr the rate of the binding reaction is only slightly dimin-

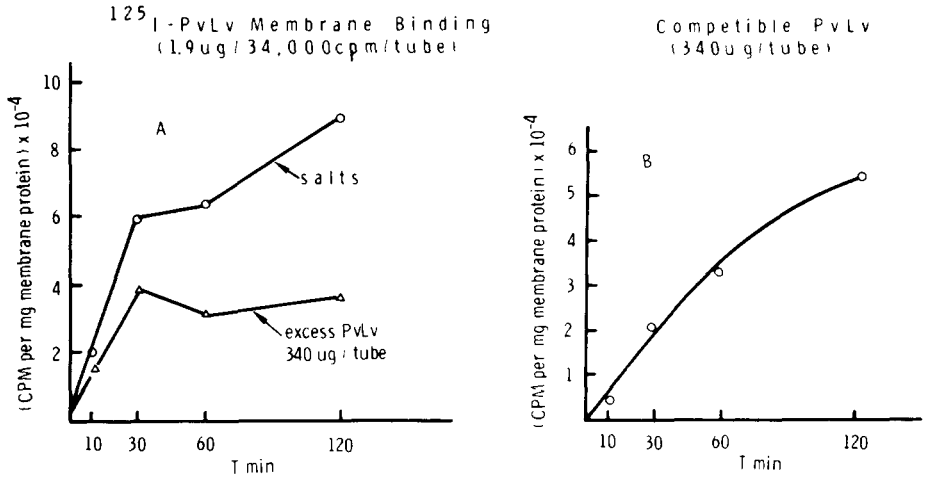

Fig. 2. Time course of binding to unfixed oocyte cortex. Diced, unfixed yolk cortex fragments were incubated for the designated times at 25°C with shaking. The reaction mixtures contained 1 mg/ml bovine serum albumin, 1.9 μg ^{125}I-labeled PvLv (3.4×10^4 cpm) and 15 mM HEPES in a total volume of 250 μl of Hanks' balanced salts solution, pH 7.4. The tubes with excess unlabeled protein contained an additional 340 μg per tube of unlabeled PvLv (△). Part A; the upper curve (○) represents the cpm bound to the membranes in the presence of labeled material only. The lower curve (△) presents data in the presence of excess unlabeled PvLv. Each point on the figure represents the average of two data points. Part B presents the competible binding which is the difference between binding in the presence and in the absence of excess unlabeled PvLv.

ished, indicating that neither equilibrium nor saturation of binding sites has occurred by this time.

IgG is a protein found in large quantities in egg yolk. Table I presents an experiment in which labeled PvLv is incubated with membranes in the presence of excess unlabeled PvLv or IgG. In contrast to nonlabeled PvLv, IgG did not compete with labeled PvLv for binding to the membranes. The slight decrease in binding in the presence of IgG is not statistically significant.

If the specific binding of PvLv to the membranes is reversible, then bound material should be specifically displaced in the presence of large quantities of unlabeled PvLv, but not by other proteins which do not bind to the same sites. To determine whether the bound label would be specifically eluted, the experiments shown in Table II were performed by using formaldehyde-fixed membranes. The fixation was necessary to preserve the membranes since the total time required for labeling, extensive washes, and specific elution was more than 24 hr. After the labeling and extensive washes with buffer, the membranes were divided into four portions and eluted with buffer, or buffer containing excess cold proteins. BSA was tested because it was present in the competition experiments of Table I to decrease nonspecific binding and possibly help stabilize membranes. Neither BSA nor IgG displaced PvLv from the membranes. The experiment was run on two other occasions with similar results.

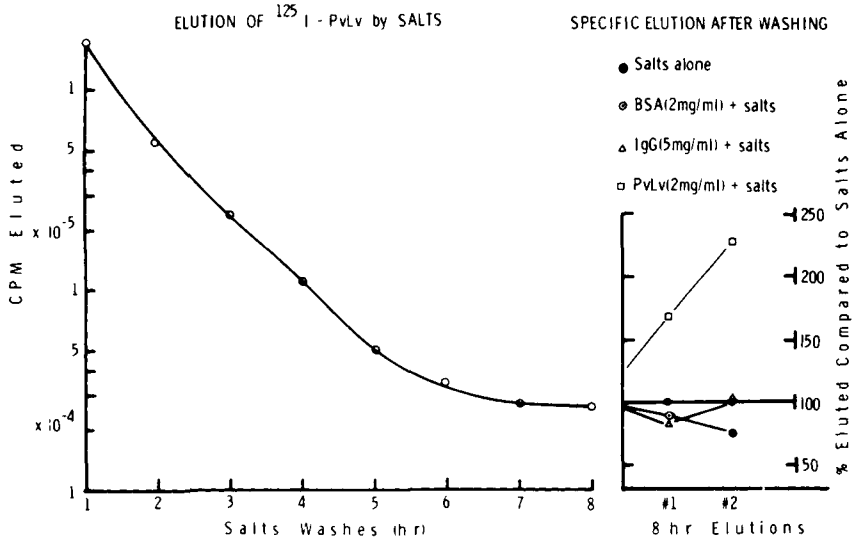

Fig. 3. Washes and elution by unlabeled proteins. Formaldehyde-fixed membranes were labeled for 3 hr as given in Methods. The membranes were then washed eight times for periods of 1 hr each with 3 ml of salts solution. The membranes were separated from the supernatant solutions by centrifugation and the supernatant solutions from each wash were counted for ^{125}I. Bound material was then eluted for two 8-hr periods in the presence of salts alone, or salts plus the unlabeled proteins indicated on the right (specific elution after washing). The specific elutions after washing are data also presented in Table II. For this figure the percent of bound counts eluted is presented relative to the elution by salts alone (100%).

TABLE II. Specific Elution of ^{125}I-Labeled Phosvitin-Lipovitellin Complex from Formaldehyde-Fixed Oocyte Cortex

	First elution			Second elution		
Membranes plus	cpm before elution	cpm eluted	% bound counts eluted	cpm before elution	cpm eluted	% bound counts eluted
Salts alone	206,210	28,396	13.8	172,371	11,625	6.7
2 mg/ml PvLv	200,978	46,734	23.3	148,674	22,649	15.2
2 mg/ml bovine serum albumin	208,200	26,114	12.6	178,525	9,443	5.3
5 mg/ml IgG	183,770	21,086	11.5	158,977	10,796	6.8

Cortex pieces, previously incubated with ^{125}I-labeled PvLv, were washed extensively and then incubated for two 8 hr periods in 2 ml of Hanks' balanced salts solution, pH 7.4, containing the unlabeled proteins shown. After each incubation, cortex sections were separated from the supernatant solutions and the ^{125}I-PvLv eluted from the sections was counted. Further details are presented in Methods.

For the experiment shown in Table II, the fixed, labeled cortex sections were washed eight times with the salts solution for periods of 1 hr each. The label eluted by these washes is shown in a semilog plot in Fig. 3. The wash data in the left portion of the figure appear to form a biphasic curve which initially follows first order kinetics. The biphasic nature may be due to two types of binding sites with different affinities. The right half of the figure shows the data from Table II presented relative to the elution with salts alone (100%). Only the PvLv complex displaced the already bound PvLv.

DISCUSSION

The competible binding of PvLv to the cortex sections and specific displacement of the bound material indicate the existence of binding sites for PvLv on these membranes. That these binding sites are specific is shown by the fact that IgG, a protein which is incorporated into egg yolks, did not compete with PvLv for binding to these sites, nor could it displace bound material. These data are more striking when it is realized that in the studies with unfixed and fixed membranes, respectively, the molar concentrations of IgG were more than three and seven-fold higher than those of PvLv. Therefore, IgG and the PvLv complex do not share the same binding sites. Cutting and Roth (8) as well as Patterson et al (9) reported data indicating selectivity in the process of protein accumulation in the chicken oocyte. If these binding sites are indeed involved in the accumulation process, it would explain the selectivity observed in the in vivo studies. Our results are similar to those of Sonada and Schlamowitz (10) who demonstrated the existence of selective binding sites for rabbit serum albumin and IgG on formaldehyde-fixed rabbit yolk sac membranes. These data are in agreement with the existence of two steps in the uptake process. The first step would involve the recognition and binding of the protein to special receptors on the plasma membrane prior to the incorporation step involving micropinocytosis via the coated pit and coated vesicle mechanism.

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