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Isolation of a Calcium-binding Phosphoprotein from the Oocytes and Hemolymph of the Blood-sucking Insect *Rhodnius prolixus**

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A novel calcium-binding phosphoprotein was isolated from the oocytes of the blood-sucking bug *Rhodnius prolixus*. This protein exhibits an apparent molecular mass of 18 kDa on gel filtration, but migrates as an 8-kDa band on *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine/SDS-polyacrylamide gels. It has a high content of serine (24% of the total number of residues), and phosphoserine is the sole amino acid phosphorylated *in vivo*. A similar protein was partially purified from the hemolymph. It resembles the oocyte form of the protein in its NH_2-terminal sequence and its ability to be taken up by growing ovaries. ^{45}Ca binding to the oocyte phosphoprotein was determined after SDS-polyacrylamide gel electrophoresis followed by blotting on nitrocellulose membranes. Titration of Ca^{2+}-binding sites shows a high capacity (≅50 mol/mol of protein), but a low affinity ($K_{0.5}$ ≅ 10^{-3} M). Based on these characteristics, we have named this protein *Rhodnius* calcium-binding phosphoprotein. It resembles phosvitin, a phosphoprotein present in the oocytes of nonmammalian vertebrates.

The storage of phosphoproteins as major components of egg yolk is a recurrent strategy in the animal kingdom. Vitellogenin, a phospholipoglycoprotein, is stored in oocytes during oogenesis (1–3). Biochemical aspects of its synthesis, uptake, and processing as well as its gene structure have been studied extensively in recent years in both vertebrates and invertebrates, and a number of differences in vitellogenins from these sources have been detected (4–6).

Inside the oocytes of nonmammalian vertebrates, vitellogenin (≅200 kDa) is the target of specific proteases whose activity gives rise to a set of smaller yolk polypeptides: lipovitellins (lipovitellin 1 ≅ 120 kDa; lipovitellin 2 ≅ 30 kDa) and phosvitins (28–35 kDa), which are ultimately associated with yolk platelets (4, 7–9). Phosvitin is one of the most phosphorylated proteins found in nature: more than half of its residues are serines, and most of them are phosphorylated (4, 10–12). There is a progressive increase in the length of this domain from that in fishes up to that found in the chicken (4, 13–15). The high degree of phosphorylation of phosvitins allows these proteins to bind cations such as iron, magnesium, and calcium (16–19). In *Xenopus*, after vitellogenin endocytosis, tightly bound calcium is associated exclusively with the phosvitin domain (20, 21). This binding is usually of low affinity but high capacity, and it has long been proposed to play a role in providing embryos with calcium required for bone and tooth formation (13, 22).

In invertebrates, vitellogenins contain less phosphate and fewer serines than in vertebrates (1, 4). Based on the amino acid sequence of different vitellogenins deduced from their cDNA, insect vitellogenins possess serine-rich stretches, but there is no evidence to show that these segments are removed inside the oocyte to produce an independent protein (5, 23–25). Thus, once inside the oocytes of insects, vitellogenin is called vitellin, but it does not suffer the proteolytic processing that generates a polyserine phosphoprotein like phosvitin in nonmammalian vertebrates.

During their development, insects do not build structures that require a large amount of calcium, such as bones and teeth. The reduction of the phosphorylated calcium-binding domain in insect vitellogenins suggests that the capacity of these proteins for the storage of this ion in the oocyte is smaller than with vertebrates and that it may also occur by other mechanisms (26). Although the requirement for calcium is also smaller, its homeostasis in growing oocytes and developing eggs is important for a number of events. Oocyte maturation, fertilization, and embryo development are general calcium-mediated processes common to both vertebrate and invertebrate species (27–29). In this study, we undertook a search for other maternally derived phosphoproteins besides vitellin with properties appropriate for the functions mentioned above, either in the oocytes or later during egg development. Here we describe the isolation of a low molecular mass calcium-binding phosphoprotein from the oocytes and hemolymph of the blood-sucking bug *Rhodnius prolixus* that resembles phosvitin from nonmammalian vertebrates in its high serine content and in its ability to bind calcium.

EXPERIMENTAL PROCEDURES

Insects—Insects were taken from a colony of *R. prolixus* maintained at 28 °C and 70% relative humidity. Normal mated females were fed on rabbit blood at 3-week intervals.

Oocytes and Hemolymph—During 3 months, ≅4000 oocytes were removed from vitellogenic females and stored at −20 °C. This was the rate-limiting step in RCBP[1] purification. Five days after a blood meal, chorionated oocytes were dissected free of ovarian tissues and washed with ice-cold 0.15 M NaCl. Oocytes were homogenized in a Potter-Elvehjem homogenizer in the presence of a mixture of protease inhibitors with final concentrations of 0.05 mg/ml each soybean trypsin in-

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[1] The abbreviations used are: RCBP, *Rhodnius* calcium-binding phosphoprotein; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine.

hibitor, leupeptin, lima bean trypsin inhibitor, and antipain; 1 mM benzamidine; 100 μM phenylmethylsulfonyl fluoride buffered with 20 mM Tris-HCl (pH 8.0); 10 mM NaF; 0.05 M NaCl; 1 mM EDTA; 1 mM EGTA; and 0.02% NaN_3. Oocyte homogenate was frozen overnight and then centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was collected and used as the starting material for the purification of phosphoprotein.

Hemolymph was collected usually on the fourth day after a blood meal in the presence of phenylthiourea (30–130 μg/ml), 5 mM EDTA, and the mixture of protease inhibitors described above. This material was centrifuged at 11,000 × *g* for 15 min at room temperature and stored in liquid nitrogen until use.

Oocytes and Hemolymph Labeled Metabolically with $^{32}P_i$—Fifty adult females were fed with rabbit blood enriched with $^{32}P_i$ (10^9 cpm/ml of blood) (2), using a special feeder (30). Oocytes and hemolymph were collected and processed as described above.

$^{32}P_i$ Purification—Carrier-free $^{32}P_i$ was purchased from the Comissão Nacional de Energia Nuclear (São Paulo, Brazil) and purified by ion-exchange column chromatography on Dowex 1X-10 (31).

Purification of RCBP from Rhodnius Oocytes—Supernatants obtained from radioactive (≅300) and nonradioactive (≅4000) oocytes were combined and used as the starting material. To remove free $^{32}P_i$, supernatants were dialyzed overnight at 4 °C against buffer A (20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.02% NaN_3, 10 mM NaF, 1 mM benzamidine). The dialysate was then applied to a Sephadex G-75 gel filtration column (2.0 × 128 cm) equilibrated in buffer A. The column was eluted with a flow rate of 15 ml/h, and 2.5-ml fractions were collected. Aliquots of 0.1 ml were counted in a liquid scintillation counter, and protein content was measured by absorbance at 280 nm. [^{32}P]RCBP-containing fractions from the Sephadex G-75 column were pooled and applied to a DEAE Toyopearl 650 M column (1.4 × 24 cm) equilibrated in buffer A. The column was washed with buffer A, and a linear gradient of 100 ml containing 0.05–0.4 M NaCl in buffer A was developed. Protein content and radioactivity of fractions (0.1 ml) were measured as described above. Fractions containing [^{32}P]RCBP were pooled and dialyzed extensively against deionized water at 4 °C and then adjusted to 20 mM Tris-HCl (pH 7.5) using 0.2 M Tris-HCl. The dialyzed material was then applied to a Mono Q 5/5 Hr FPLC column (1-ml bed volume; Pharmacia Biotech Inc.) equilibrated in 20 mM Tris-HCl (pH 7.5). The column was washed and developed with a two-step gradient (0–0.5 and 0.5–1 M NaCl) at a flow rate of 1 ml/min. Protein content and radioactivity of fractions were measured as already described. Starting from 404 mg of oocyte homogenate, we obtained 0.26 mg of RCBP. Final protein yield was determined by the method of Lowry *et al.* (32). Taking into account the losses that occur during the purification procedures, RCBP composes 0.1–0.2% of total oocyte protein.

Purification of Phosphoproteins from Rhodnius Hemolymph—Hemolymph from insects fed with radioactive and nonradioactive blood was combined. Free $^{32}P_i$ was removed by dialysis overnight at 4 °C against buffer A. The dialysate was combined with 1 ml of 10-fold concentrated buffer A and purified essentially as described for RCBP from oocytes. Since ^{32}P-labeled hemolymph exhibits two low molecular mass ^{32}P-labeled peaks on Sephadex G-75 chromatography (HP 1 and HP 2; see "Results"), these peaks were collected separately and purified further using DEAE Toyopearl 650 M and Mono Q 5/5 Hr FPLC columns for HP 1 and DEAE Toyopearl 650 M for HP 2 as described above for RCBP from oocytes. Starting from 500 mg (≅9 ml) of hemolymph protein, we obtained 0.1 mg of HP 1 and 0.1 mg of HP 2.

Molecular Mass Determination—The apparent molecular mass of RCBP purified from oocytes was determined in a Smart system (Pharmacia Biotech Inc.) using a Superdex 75 PC 3.2/30 column equilibrated in 0.1 M phosphate buffer (pH 6.8) as described (33). The following proteins were used as standards: ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), ribonuclease (13.7 kDa), cytochrome *c* (12.3 kDa), and aprotinin (6.5 kDa).

SDS-Polyacrylamide Gel Electrophoresis (PAGE)—Polyacrylamide gels (15 × 15 × 0.1 cm) were run in the presence of SDS (34) at a constant current of 20 mA. Gels were stained (35), dried, and exposed to x-ray film at −70 °C. The following molecular mass standards were used: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (98 kDa), albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.3 kDa). The molecular mass of purified phosphoprotein was also determined under denaturing conditions (SDS-PAGE) using 16.5% acrylamide gels in the presence of Tricine (36). In this system, myoglobin-derived peptides were used as molecular mass standards (Sigma, MW-SDS-17S kit).

Amino Acid Analysis—Purified RCBP from oocytes (1 nmol) was analyzed for its amino acid composition at the University of Arizona Biotechnology Protein Core Center using an ABI 420 A/H amino acid analyzer. The protein was hydrolyzed in vapor phase using 6 N HCl for 1 h at 155 °C and then derivatized with phenyl isothiocyanate to form phenylthiocarbamyl-derivatives, which were extracted and transferred to an on-line high pressure liquid chromatography apparatus for analysis at 254 nm. No corrections were made for losses during acid hydrolysis.

Phosphoamino Acid Analysis—[^{32}P]RCBP (0.1 mg) was digested in 6 N HCl at 100 °C for 2 h. The hydrolyzed sample was dried under vacuum in a Speed Vac concentrator and resuspended in 30 μl of deionized water. Paper electrophoresis was performed at pH 1.9 using formic acid, acetic acid, and water (25:78:89, v/v) for 8 h (450 V, 15 mA). Phospho amino acid standards (phosphoserine, phosphotyrosine, and phosphothreonine) were run in parallel with the sample. The total amount of phosphate associated with RCBP was not determined due to the small amount of RCBP available.

NH_2-terminal Sequencing—Samples were separated by SDS-PAGE and then electroblotted on polyvinylidene fluoride membranes as described by Matsudaira (37), followed by autoradiography at −70 °C. After localization, phosphoproteins were excised from membranes and microsequenced in the departmental protein microsequencing laboratory on a Porton 2095 microsequencer.

Uptake of ^{32}P-Phosphoproteins—Purified ^{32}P-phosphoproteins (2–5 μg of protein, 20,000–50,000 cpm in 5 μl of 0.15 M NaCl) were injected into the hemocoel of vitellogenic females on the third day after a blood meal using a 5-μl Hamilton syringe. Insects were kept at 28 °C until dissection; controls were kept in a flask maintained at 0 °C in an ice bath. At the desired times, ovaries and other organs were dissected, extensively washed in *Rhodnius* physiological saline (38), and homogenized in 0.15 M NaCl, and the radioactivity incorporated in the different organs was estimated by liquid scintillation counting. Other conditions were as described (39).

Calcium Binding Assay—Qualitative calcium binding was assayed as described (40). Briefly, after electrophoresis, proteins were electroblotted on nitrocellulose membranes. After transfer, membranes were washed for 1 h in three changes of a buffer containing 60 mM KCl, 10 mM imidazole (pH 6.8), and 5 mM $MgCl_2$, followed by a 5-min incubation at room temperature in the same buffer containing ^{45}Ca obtained from DuPont NEN and adjusted to 0.25 μM (1 mCi/liter). Finally, membranes were washed with distilled water for 5 min, dried, and exposed to Kodak X-Omat AR-5 film for 48 h at −70 °C. These experiments were carried out using purified [^{32}P]RCBP that had been stored at −20 °C for 6 months to allow ^{32}P decay. Under these conditions, no radioactivity from ^{32}P could be detected on the film. The phosphoprotein could be detected only on membranes incubated with ^{45}Ca.

Stoichiometry of ^{45}Ca Binding to RCBP—The stoichiometry of Ca^{2+} binding to purified RCBP was measured by the dot-blotting calcium binding assay (41). Final concentrations of ^{45}Ca ranging from 0.1 to 10 mM (specific activity = 200 cpm/nmol) in 10 mM imidazole (pH 6.8), 60 mM KCl, and 5 mM $MgCl_2$ were used. Nonspecific calcium binding was estimated using cytochrome *c* as described (41). ^{45}Ca bound specifically to RCBP was obtained by subtracting this nonspecific background from the total ^{45}Ca bound to RCBP.

RESULTS

Purification and Partial Characterization of RCBP from Oocytes—When oocyte supernatant is fractionated on a Sephadex G-75 column, the major ^{32}P label is associated predominantly with vitellin (fractions 60–80; Fig. 1*A*). A second peak of radioactivity in the low molecular mass range can also be detected (fractions 115–125). This ^{32}P-labeled protein was further purified by chromatography on ion-exchange columns (Fig. 1, *B* and *C*). Under these conditions, RCBP emerges as a single peak on a Mono Q column (Fig. 1*C*) and is homogeneous as judged by both SDS-PAGE and autoradiography (Fig. 2).

Analytical gel filtration using purified RCBP reveals an apparent molecular mass of 18 kDa (Fig. 3), although on Tricine/SDS-PAGE, it migrates as a band of ≅8 kDa. Anomalous mobility in SDS-PAGE is common to phosphoproteins and is related in part to the phosphorylation state of the molecule (33, 42). RCBP partially dephosphorylated with alkaline phosphatase migrates in SDS-PAGE with higher molecular mass (data not shown). Thus, for this study, the 18-kDa value was as-

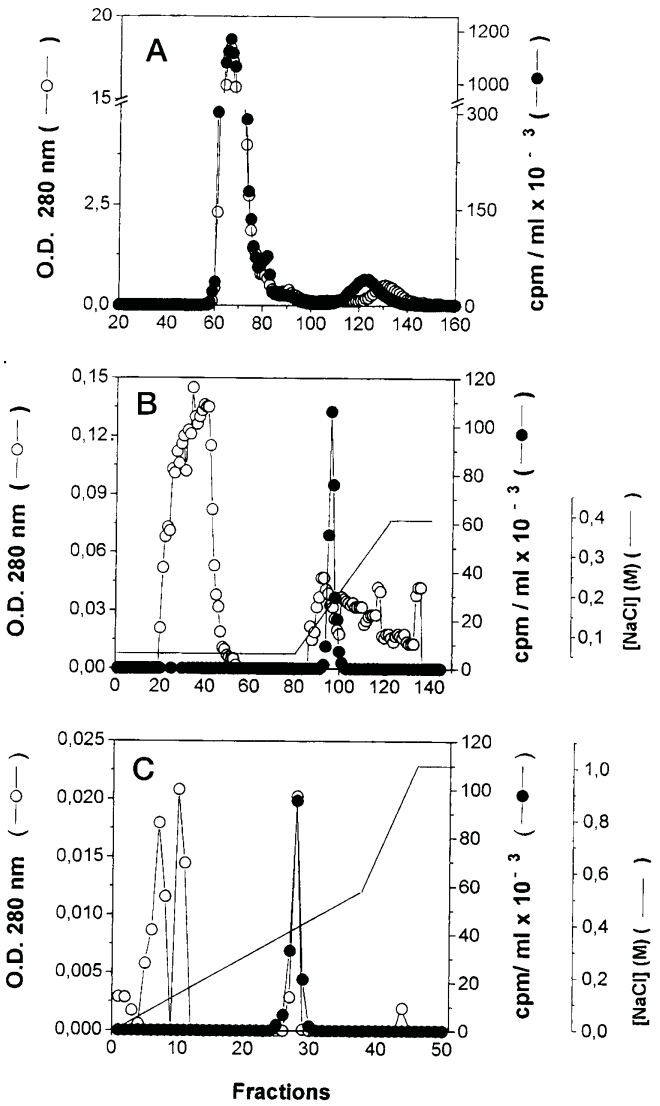

FIG. 1. **Purification of RCBP from oocytes.** Oocyte supernatant obtained from ^{32}P-labeled oocytes was combined with supernatant from nonradioactive oocytes and used as the starting material for the purification of RCBP. *A* shows the elution from a Sephadex G-75 gel filtration column (2.0 × 128 cm) packed in buffer A and eluted with the same buffer. Protein content was estimated from the absorbance at 280 nm (○). Radioactivity of samples was estimated by counting on a scintillation counter (●). In *B*, fractions 115–125 were pooled and applied to a DEAE Toyopearl 650 M column equilibrated in buffer A. The column was washed with buffer A, and proteins were eluted with a linear gradient (100 ml) from 0.05 to 0.4 M NaCl. Protein content and radioactivity of fractions were estimated as described for *A*. In *C*, fractions 90–105 containing [^{32}P]RCBP were pooled and dialyzed against deionized water and then adjusted to pH 7.5 using Tris-HCl and applied to a Mono Q 5/5 Hr FPLC column (1-ml bed volume) equilibrated in the same buffer. The column was washed and developed at 1 ml/min with two gradients of NaCl (0–0.5 and 0.5–1 M) in 20 mM Tris-HCl (pH 7.5).

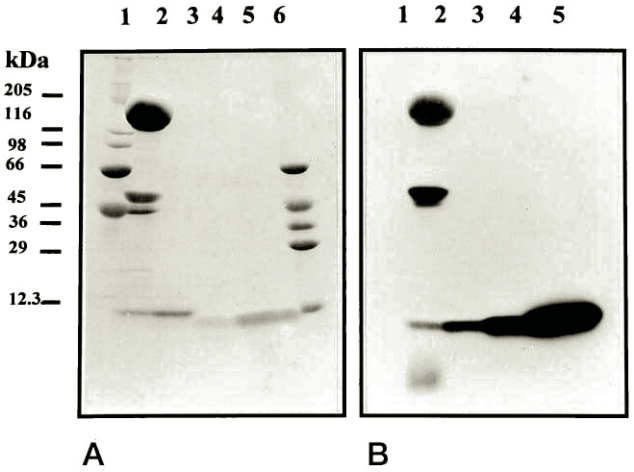

FIG. 2. **SDS-PAGE analysis of oocyte RCBP purification.** Samples from each step of the RCBP purification were subjected to SDS-PAGE on a 6–22.5% polyacrylamide separating gel. *A* shows the gel stained with Coomassie Blue; *B* shows the ^{32}P-labeled autoradiogram. *Lane 1*, high molecular mass standards; *lane 2*, dialyzed oocyte supernatant; *lane 3*, pooled fractions 115–125 from Sephadex G-75; *lane 4*, pooled fractions 90–105 from DEAE Toyopearl 650 M; *lane 5*, pooled fractions 25–30 from Mono Q; *lane 6*, low molecular mass standards. Prominent bands in *lane 2* are vitellin subunits (2).

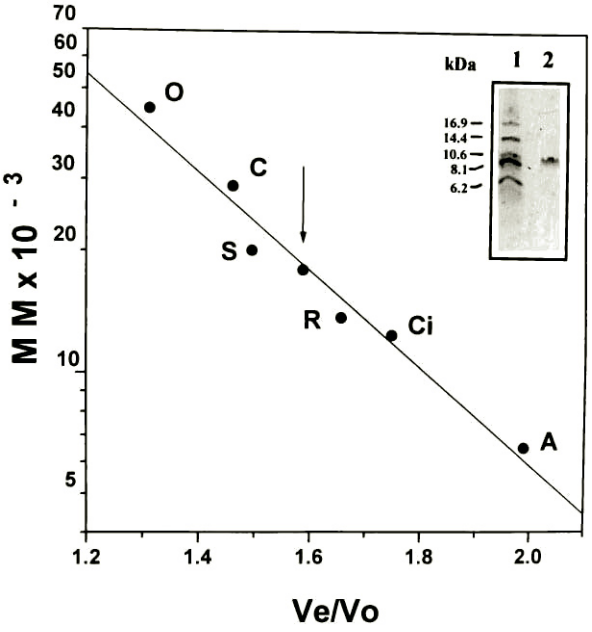

FIG. 3. **RCBP molecular mass determination.** The molecular mass of purified RCBP from oocytes was estimated both by gel filtration on a Smart system using a Superdex 75 PC 3.2/30 column and by SDS-PAGE on Tricine gels (*inset*). For gel filtration, the following proteins were used as standards: ovalbumin (*O*), carbonic anhydrase (*C*), soybean trypsin inhibitor (*S*), ribonuclease (*R*), cytochrome *c* (*Ci*), and aprotinin (*A*). *Ve*, elution volume; *Vo*, exclusion volume. The *arrow* indicates the position of RCBP. *Inset*, 20 μg of phosphorylated RCBP were applied onto a Tricine/SDS-16.5% acrylamide gel and stained with Coomassie Blue. *Lane 1*, molecular mass standards; *lane 2*, RCBP.

signed for RCBP.

Analysis of RCBP amino acid composition (Table I) shows that serine alone accounts for 24% of the total number of residues and that Asx (asparagine plus aspartate) and Glx (glutamine plus glutamate) together compose about 23%. RCBP is poor in aromatic amino acids. The phosphoamino acids were identified by high-voltage electrophoresis of an acid hydrolysate of [^{32}P]RCBP. Phosphoserine was the only phosphoamino acid detected (Fig. 4). Since RCBP phosphorylation occurred *in vivo*, the stoichiometry of serine phosphorylation could not be determined from these data.

Isolation of RCBP from Hemolymph—In analogy with other oocyte proteins from *Rhodnius* (39, 43), RCBP is also present in a hemolymphatic form. When hemolymph is chromatographed on Sephadex G-75 under the same conditions as those used for Fig. 1*A*, two ^{32}P-labeled peaks of low molecular mass are found (Fig. 5). These are designated HP 1 and HP 2 (hemolymphatic phosphoproteins 1 and 2). Both peaks were purified further on ion-exchange columns (see legend to Fig. 5). Samples from each step of the purification of HP 1 and HP 2 were analyzed by SDS-PAGE. The autoradiography of the gels summarizes the

TABLE I
Amino acid composition of RCBP from oocytes

RCBP from oocytes was analyzed for its amino acid content. The minimal chemical molecular weight calculated for 156 residues/mol is 17,018. No corrections were made for losses during acid hydrolysis.

Amino acid	Residues/mol
Asx	13.11
Glx	23.46
Ser	38.31
Gly	12.60
His	6.17
Arg	7.38
Thr	4.51
Ala	5.23
Pro	2.82
Tyr	0.52
Val	0.30
Met	10.67
Cys	ND[a]
Ile	7.36
Leu	8.38
Phe	3.91
Lys	11.13
Trp	ND
Total	155.86

[a] ND, not determined.

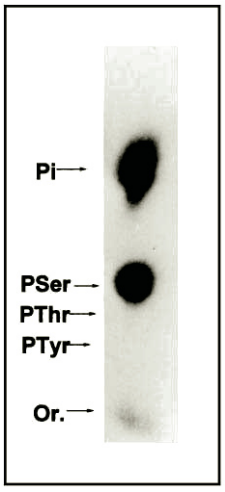

FIG. 4. **Phosphoamino acid analysis of oocyte RCBP.** Shown is an autoradiogram of phosphoamino acids separated by high-voltage paper electrophoresis following acid hydrolysis of purified [^{32}P]RCBP. *Pi*, free phosphate; *PSer*, phosphoserine; *PThr*, phosphothreonine; *PTyr*, phosphotyrosine; *Or.*, origin.

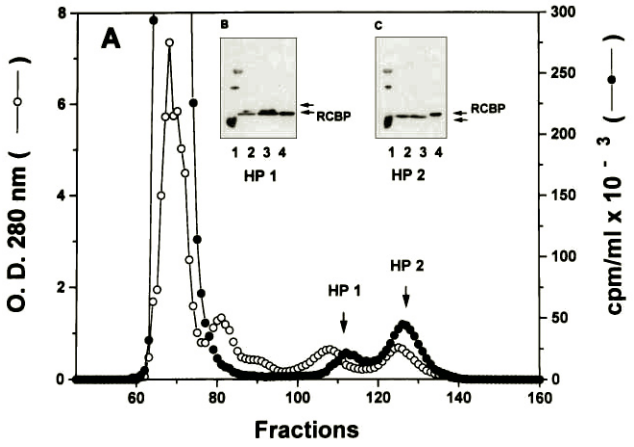

FIG. 5. **Purification of phosphoproteins from *Rhodnius* hemolymph.** ^{32}P-Labeled hemolymph was combined with nonradioactive hemolymph, dialyzed, and applied (in *A*) to a Sephadex G-75 gel filtration column (2.0 × 128 cm) equilibrated in buffer A and eluted with the same buffer. Protein content was estimated from absorption at 280 nm (○). Radioactivity of samples was estimated in a liquid scintillation counter (●). At the end of the run, fractions containing hemolymphatic phosphoproteins HP 1 and HP 2 were pooled and further purified as described for RCBP in the legend to Fig. 1*B*. *Inset B*, autoradiogram of SDS-PAGE (6–22.5% gradient) showing purification of [^{32}P]HP 1. *Lane 1*, dialyzed ^{32}P-labeled hemolymph; *lane 2*, pooled HP 1 fractions from DEAE Toyopearl; *lane 3*, pooled HP 1 fractions from Mono Q; *lane 4*, purified [^{32}P]RCBP from oocytes. *Arrows* show the two bands of HP 1; the *lower band* coincides with purified RCBP. *Inset C*, autoradiogram of SDS-PAGE (6–22.5% gradient) showing purification of [^{32}P]HP 2. *Lane 1*, dialyzed ^{32}P-labeled hemolymph; *lane 2*, pooled HP 2 fractions from Sephadex G-75; *lane 3*, pooled HP 2 fractions from DEAE Toyopearl; *lane 4*, purified [^{32}P]RCBP from oocytes. *Upper* and *lower arrows* show HP 2 and purified RCBP, respectively.

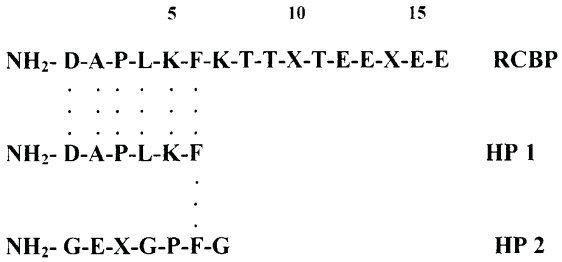

FIG. 6. **NH_2-terminal amino acid sequence.** Shown are the NH_2-terminal primary structures of RCBP isolated from oocytes, the faster migrating band of HP 1 from hemolymph, and HP 2.

purification steps (Fig. 5, *B* and *C*). The faster migrating band of the [^{32}P]HP 1 preparation (Fig. 5*B*) has the same mobility as the [^{32}P]RCBP isolated from oocyte and the same NH_2-terminal amino acid sequence (Fig. 6), thus suggesting that it may be a hemolymphatic form of RCBP. The NH_2-terminal amino acid sequence of HP 2 differs from that of RCBP in five out of six positions (Fig. 6), and it has a slightly higher mobility (Fig. 5*C*).

Uptake of [^{32}P]HP 1 and [^{32}P]HP 2 by the Ovary—The next experiments were carried out to determine whether [^{32}P]HP 1 or [^{32}P]HP 2 found in the hemolymph could be a precursor of [^{32}P]RCBP in the oocytes. Radiolabeled [^{32}P]HP 1 and [^{32}P]HP 2 were injected separately into the hemocoel of two different lots of vitellogenic females, and various organs were analyzed for ^{32}P-phosphoprotein uptake. Fig. 7*A* shows that [^{32}P]HP 1 is incorporated specifically by the ovaries, but not by the midgut or fat bodies. At 0 °C, as expected, the uptake of protein is inhibited. [^{32}P]HP 2 is not taken up by any of these organs.

In a second set of experiments, purified [^{32}P]RCBP from oocytes was injected into the hemocoel of vitellogenic females. Fig. 7*B* shows that [^{32}P]RCBP is taken up from the hemolymph into the ovaries over a period of hours. The ovaries of insects 16 h after injection were homogenized and analyzed by SDS-PAGE and autoradiography. The homogenate has a phosphoprotein of the same molecular mass as [^{32}P]RCBP (Fig. 7*B*, *inset*). This result indicates that the ovaries can take up [^{32}P]RCBP from the hemolymph.

Calcium Binding by RCBP—The amino acid composition and the evidence of phosphorylation indicate that RCBP may be negatively charged *in vivo*. In the next experiments, we examined its ability to bind Ca^{2+} specifically in the presence of Mg^{2+}. After SDS-PAGE (Fig. 8*A*), purified RCBP (^{32}P-labeled RCBP allowed to decay for 6 months) was transferred to nitrocellulose membranes and incubated with 0.25 μM ^{45}Ca for 5 min. Bovine brain calmodulin was included as a control. The autoradiogram shows ^{45}Ca bound to both RCBP and calmodulin (Fig. 8*B*). On a second gel, RCBP and calmodulin that were transferred to nitrocellulose membranes but not exposed to ^{45}Ca did not show any spots on the autoradiogram (Fig. 8*C*). This control shows that labeling observed in Fig. 8*B* is due to ^{45}Ca bound to RCBP and not to residual ^{32}P.

The stoichiometry of Ca^{2+} binding to RCBP was determined

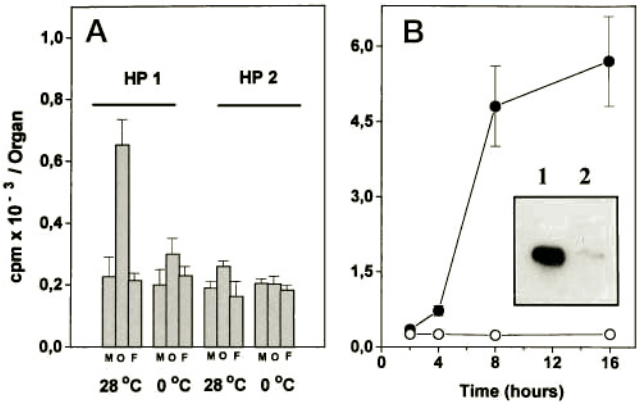

FIG. 7. **Uptake of phosphoproteins: HP 1, HP 2, and RCBP.** In *A*, vitellogenic females on the third day after feeding were injected with purified ^{32}P-labeled HP 1 or HP 2 (40,000 cpm/female) and kept either at 28 or 0 °C. After 4 h, the midgut (*M*), ovary (*O*), and fat body (*F*) were dissected and homogenized, and ^{32}P incorporation was measured by liquid scintillation counting. Each column shows mean ± S.D. of four insects. In *B*, the time course of [^{32}P]RCBP uptake from the hemocoel by *Rhodnius* ovaries is shown. Females injected with [^{32}P]RCBP were kept at either 28 °C (●) or 0 °C (○). At different times after the injection, ovaries were dissected and processed as described for *A*. Data show mean ± S.D. of four insects. *Inset*, an aliquot of the ovary homogenate taken 16 h after injection was run on SDS-polyacrylamide gel (20% acrylamide) and autoradiographed. *Lane 1*, purified [^{32}P]RCBP; *lane 2*, sample of the ovary 16 h after injection of [^{32}P]RCBP.

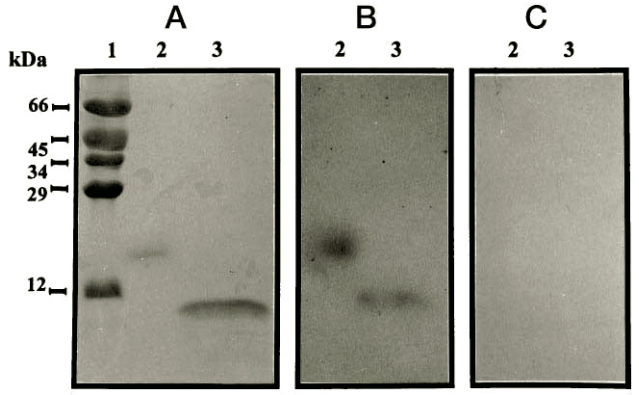

FIG. 8. **Binding of ^{45}Ca to RCBP and calmodulin.** Purified RCBP (50 μg) and calmodulin (2 μg) were subjected to SDS-PAGE (20% acrylamide) and transferred to nitrocellulose membranes. In *A*, the gel was stained with Coomassie Blue; *B* shows an autoradiogram of the nitrocellulose membrane incubated for 5 min with ^{45}Ca, as described under "Experimental Procedures." *C* is a control showing absence of radioactivity in decayed [^{32}P]RCBP samples that were transferred to nitrocellulose and autoradiographed without exposure to ^{45}Ca. *Lane 1*, low molecular mass standards; *lane 2*, bovine brain calmodulin; *lane 3*, decayed [^{32}P]RCBP.

by dot blotting, which uses less protein than other techniques (41). The titration was carried out with a constant amount of phosphoprotein while varying the concentration of $^{45}CaCl_2$ between 0.1 and 10 mM. Cytochrome *c* was used as a control for nonspecific ^{45}Ca binding (41). Immobilized on nitrocellulose discs, RCBP binds 80 mol of ^{45}Ca/mol of protein (Fig. 9) with a $K_{0.5}$ of 10^{-3} M. On subtracting the binding to cytochrome *c*, we find 50 mol of ^{45}Ca/mol of protein.

DISCUSSION

Vitellin is the major protein in the oocytes of *R. prolixus* (2, 39, 43), where it accounts for 86% of the total protein; RCBP corresponds to only 0.1–0.2%. As a result of metabolic labeling *in vivo*, the amount of ^{32}P covalently associated with each mole of RCBP is about five times higher than with vitellin (2, 39, 44). Invertebrate vitellogenins have less covalently bound phosphate than their vertebrate counterparts (4); the serine clusters are shorter (4–6), and the total number of phosphorylated residues is not known. There is no evidence for proteolytic processing of *Rhodnius* vitellogenin after endocytosis. This study is the first to describe a low molecular mass phosphorylated polypeptide associated with insect oocytes. Together, these data suggest that RCBP is a polyserine phosphoprotein that resembles phosvitin of the nonmammalian vertebrates, although it does not appear to be a product of postendocytotic processing since it is already present in the hemolymph. RCBP from oocytes and HP 1 from hemolymph are specifically taken up by the ovaries (Fig. 7), suggesting that an RCBP precursor may be acquired by the oocyte during growth. In both cases, the uptake is a temperature-dependent process, and the molecular mass of the accumulated protein is the same as that of the injected material (Fig. 7*B*, *inset*). These observations are consistent with the vectorial transfer of protein from hemolymph to oocytes, which has been demonstrated previously for other yolk proteins (1, 3). In addition to *Rhodnius* vitellogenin, a heme-binding protein present in the hemolymph was also found in the oocytes (43, 45).

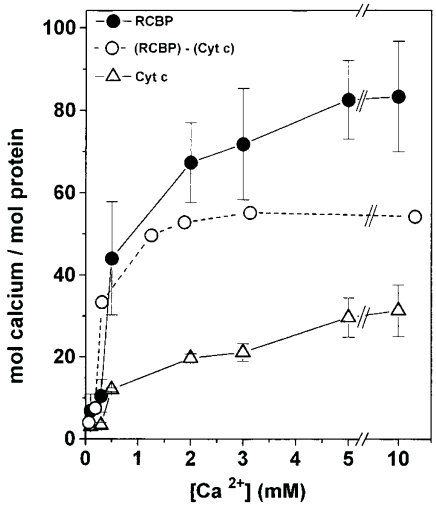

FIG. 9. **Oocyte RCBP calcium binding capacity.** Purified [^{32}P]RCBP was stored for 6 months in order to allow for the isotopic decay of [^{32}P]phosphate to occur. After this time, a negligible amount of radioactivity is associated with the phosphate moiety (see Fig. 8*C*). Then 100 pmol of protein were adsorbed on nitrocellulose discs, and ^{45}Ca binding was measured in the presence of 10 mM imidazole (pH 6.8), 60 mM KCl, 5 mM $MgCl_2$, and different concentrations of $^{45}CaCl_2$. Cytochrome *c* was used as a control. Each point is the mean ± S.D. of three independent determinations. ●, total calcium bound to RCBP; △, total calcium bound to cytochrome *c* (*Cyt c*); ○, specific binding to RCBP, where the binding to cytochrome *c* was subtracted from the total calcium bound to RCBP.

Zhang and Kunkel (46) demonstrated the presence of a high amount of calmodulin in the oocytes of *Blatella germanica*. Like RCBP, calmodulin is phosphorylated (47) and binds calcium. Distinction between the two proteins in this study is based on amino acid composition (Table I), mobility on Tricine/SDS-PAGE (Fig. 8*A*), and calcium binding affinity, which is much lower in RCBP (Fig. 9). The difference in calcium binding affinity of calmodulin and RCBP is due to the presence of the EF hand (48) motifs in the former, which confers the ability to bind calcium with high affinity. In RCBP, calcium probably binds to negatively charged amino acids and phosphorylated serines. There is no reason to suppose that the two proteins have similar roles. Calcium-binding phosphoproteins have been isolated and characterized from vertebrates (49–52), and some of them have been cloned. The yolk proteins vitellogenin and phosvitin have both been shown to bind calcium (19–21).

In titration experiments, phosvitin binds up to 100 mol of calcium/mol of protein (19). The calcium binding capacity of RCBP lies in the same range (Fig. 9).

Preliminary evidence suggests that RCBP is utilized by developing embryos during the initial 10 days post-oviposition (data not shown), while only 10–15% of vitellin is used up in this period (44), during which katatrepsis is completed and the yolk is enclosed by the embryo (53). The extensive degradation of vitellin then occurs inside the digestive system, providing the nutrients for growth as classically proposed for the function of yolk proteins (44). The difference in the timetable of vitellin and RCBP utilization suggests that these proteins may be localized in different compartments and may play different roles. During embryo development, the peripheral cell layer (blastoderm) gives rise to most cells of the body of the embryo (54) and encloses the central yolk part where vitellin is located. It is tempting to speculate that RCBP could be utilized for embryo formation together with vitellin, but that the latter is used primarily for embryo growth. Since *Rhodnius* vitellin also binds calcium, based on dot-blotting experiments (data not shown), both vitellin and RCBP have the potential to contribute to embryo formation and/or growth. The contribution of each of these proteins remains to be investigated. Both are dephosphorylated during embryogenesis (data not shown), and they may release the associated calcium for events such as cell adhesion, migration, and differentiation. Although RCBP composes a minor amount of total oocyte protein, it could be an important source of intracellular calcium, depending on its localization in the oocyte. This is an important point and is now under investigation. We are currently raising antibodies against RCBP to immunolocalize this molecule inside the oocyte. Ultrastructural observations showing the rearrangement of the endoplasmic reticulum around the yolk platelets during oocyte maturation (55) and the detection of calcium in yolk platelets (56) have led to the suggestion that this ion may also be required for an early step of yolk storage. Thus, knowledge of the distribution of calcium within the oocyte will indicate in the future how RCBP acts during *Rhodnius* oogenesis and development.

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