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Isolation of Plasma Membrane Complexes from *Xenopus* Oocytes

Doris A. Wall and Sushma Patel
Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York 10021

Summary. A method for the isolation of plasma membrane fractions from *Xenopus* oocytes has been developed, and the membranes have been characterized biochemically and morphologically. Plasma membrane complexes prepared by this procedure consisted of large sheets of the membrane, with associated vitelline envelope (a nonmembranous meshwork of fibers) and cortical (secretory) granules still attached. The morphology of cell surface microvilli and coated pits was well preserved. Cortical granules were removed by gentle homogenization in a low ionic strength medium, and integral and peripheral membrane proteins were then separated from vitelline envelopes by detergent extraction and phase separation in Triton-X-114. Biochemical characterization of the plasma membrane fractions indicated substantial levels of 5′-nucleotidase and alkaline phosphodiesterase activity associated with the oocyte cell surface, with 44–66% recovery of these markers in the final membrane preparations. Lectin blotting and lectin affinity chromatography with Concanavalin A and wheat germ agglutinin were used to characterize the major glycoprotein species associated with the plasma membrane complexes. Plasma membrane fractions prepared by this procedure should be very useful in both biochemical and morphological studies of membrane protein sorting in the *Xenopus* oocyte system.

Key Words plasma membrane · *Xenopus* · oocyte · glycoproteins

Introduction

The oocytes of nonplacental animals contain large stores of nutrient materials whose role is to support embryonic development until the organism can begin to feed. A large portion of the stored material consists of yolk proteins, which are synthesized as precursor molecules (vitellogenins) by the vertebrate liver, released into the circulation, and sequestered by oocytes via receptor-mediated endocytosis [42–44]. A well-studied example of this phenomenon exists in *Xenopus*, where approximately 80% of the total protein of a fully grown oocyte is yolk [9]. Fulfillment of their specialized function requires that these oocytes carry out endocytosis on a large scale, and that they create a membrane-bound intracellular yolk platelet compartment which occupies approximately 50% of the total volume of the fully grown cell. This compartment is a stable organelle until embryogenesis, when platelets disappear completely as the yolk within them is degraded [33, 46]. The formation of this large membrane-bound compartment provides an interesting opportunity to investigate the sorting of proteins on the endocytic pathway, since the target organelle is easily prepared in large quantity and the density of endocytic compartments increases rapidly with time as yolk proteins condense and eventually crystallize within them [40, 41]. In addition, the ability of *Xenopus* oocytes to translate exogenous mRNAs introduced by microinjection, and to target many exogenous proteins correctly, allows the introduction of membrane protein markers by this route [10, 12, 22, 25, 31, 32, 35–37]. The *Xenopus* oocyte system therefore possesses considerable advantages for the analysis of membrane protein sorting during endocytosis and of protein targeting during organelle biogenesis. Nevertheless, the potential of the oocyte system is currently limited by a lack of suitable cell fractionation schemes to allow isolation of purified membrane fractions.

We have begun studies of protein sorting by analyzing the membrane protein composition of the organelles at the beginning (plasma membrane) and end (yolk platelets) of the vitellogenin endocytic pathway. This paper presents a relatively simple scheme for the preparation of plasma membrane fractions from *Xenopus* oocytes and a biochemical and morphological characterization of these membrane preparations.

Materials and Methods

Animals and Reagents

Sexually mature *Xenopus* females were purchased from Nasco (Fort Atkinson, WI) and were maintained on a diet of liver and hamburger. Substrates for enzyme assays, Concanavalin A-Sepharose 4B (Con A-Sepharose: C-9017), methyl α-D-mannopyranoside (M-3752), Triton-X-114, and all lectins were pur-

chased from Sigma Chemical Company (St. Louis, MO). High purity sucrose for all homogenization buffers and density gradients was from Schwarz-Mann (Spring Valley, NY).

Preparation of Plasma Membrane Complexes (PMCs)

Oocytes were manually dissected from their surrounding follicles, and were brought to 2 ml volume per 100 oocytes with cold homogenization buffer (HB = 0.25 M sucrose, 10 mM HEPES, pH 7.4, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM PMSF, 1–5 μg/ml Pepstatin A, 1 μg/ml leupeptin). All subsequent steps were performed on ice. The cells were disrupted gently with 6–7 slow strokes in a Dounce homogenizer (Kontes Glass Company, Vineland, NJ), using the loose-fitting pestle. This procedure yielded large PMC sheets with adherent pigment granules as well as other contaminating organelles, which settled rapidly to the bottom of the homogenization vessel within 4–5 min. PMCs were clearly visible because of the pigment granules associated with them at this stage of their isolation. The complexes were collected from the bottom of the tube with a Pasteur pipette, resuspended in 4–6 ml of fresh HB, and agitated by pipetting up and down repeatedly with the same pipette, avoiding the formation of bubbles which can trap some of the complexes in a floating layer. The PMCs were allowed to settle again for 10–15 min, and the washing process was repeated several more times (usually five or six; *see below*) to remove residual contaminating organelles. The length of time needed for the PMCs to settle became longer as the membrane complexes become cleaner and took up to 30 min. Gentle rotation of the tube from side to side helped to dislodge complexes that adhered weakly to the sides of the tube during the settling process. All rinses of the PMCs were done in the same tube, rather than transferring them to a clean one for each rinse, because the complexes adhered tightly to the sides of a new tube, resulting in progressive loss of material in each subsequent wash. For the same reason, the same Pasteur pipette was used for all rinses.

The ease with which PMCs lost associated yolk and pigment granules was variable in preparations made from the oocytes of different animals, and the number of rinses used needed to be varied accordingly, to give a final preparation that appeared whitish in color by eye. The effectiveness of rinsing could be monitored during the preparation by phase contrast microscopy, where yolk platelets appeared as large refractile bodies, pigment granules as small black dots, and cortical granules as large, round, grayish structures. Purity was further assessed by the amount of yolk proteins present after analysis by SDS-PAGE, marker enzyme assay, and the extent to which contaminating organelles were seen in the EM (*see* Results). Oocytes from some animals gave membrane complexes that fragmented considerably during rinsing. This may be due to weaker vitelline envelope structure, since the integrity of the envelope appeared to be a major factor in preventing fragmentation of the complexes during their isolation. Collagenase-dissociated oocytes were usually not satisfactory for this procedure, because cortices from collagenase-treated cells tended to fragment into small pieces during homogenization and washing. Follicle cell contamination also varied from preparation to preparation, again depending on the animal used as a source of oocytes.

Removal of Cortical Granules

Cortical granules were removed from washed PMCs by treatment with hypotonic medium (2 mM $MgCl_2$) containing the same protease inhibitors as the homogenization medium. The final settled PMC fraction (containing complexes from 200 oocytes in 0.3–0.4 ml of HB) was resuspended in 4 ml of 2 mM $MgCl_2$ and allowed to settle by gravity. Excess solution was then removed, and the PMCs were transferred to a 2-ml Dounce homogenizer and brought to 1 ml final volume. Homogenization with 20 strokes of the loose-fitting pestle was then used to dislodge the granules from their membrane association. A portion of the stripped PMCs were fixed and processed for light and electron microscopy, and the rest were centrifuged for 10 min in a microfuge to concentrate the membranes before they were analyzed by SDS-PAGE or subjected to detergent extraction as described below.

Manual Isolation of Vitelline Envelopes

Oocytes were manually dissected free of follicular tissue, using watchmaker's forceps. They were then placed in O-R2 solution [43] containing twice the normal amount of NaCl and were left for several minutes to allow a small degree of cell shrinkage to occur. The vitelline envelopes were then peeled from the surface of the oocytes with fine forceps and collected in a 1.5-ml conical microfuge tube. The envelopes were centrifuged for 10 min to form a compact pellet and then resuspended in 1 ml of 1% Triton-X-100 in HB by vigorous vortex mixing for several minutes. These detergent-extracted VEs were again centrifuged, and the pellet was solubilized for analysis by SDS-PAGE.

Enzyme and Protein Assays

Protein was assayed by the Bradford procedure [8], using the Bio-Rad dye reagent (Bio-Rad laboratories, Richmond, CA) and bovine gamma-globulin as standard, or by the Lowry procedure, as modified by Markwell et al. [21] with BSA as standard. β-N-acetyl-glucosaminidase [39], 5′-nucleotidase [1], NADPH-cytochrome *c* reductase [19], ATPase [24], succinate dehydrogenase [16], and alkaline phosphodiesterase [38] activities were determined as described in the cited references. Assay of 5′-nucleotidase activity by methods that entailed measurement of released inorganic phosphate was unsatisfactory for oocytes, because of extremely high backgrounds of phosphate in fractions derived from these cells. We presume this was due to the large quantities of highly phosphorylated yolk protein stored in these cells.

The homogenate produced by gentle procedures was nonhomogeneous and could not be divided easily into uniform aliquots containing equal amounts of the large PMCs. Determination of enzyme activities in homogenates was therefore performed on separately prepared homogenates of oocytes from the same animal, which were subjected to more vigorous homogenization (15–20 passes with the tight pestle of the Dounce homogenizer, followed by 5–6 passes through a 25-gauge syringe needle) than the samples of oocytes used for the PMC preparation itself.

Salt and Detergent Extraction

PMCs were extracted with 0.5–1 M NaCl, KCl, or KI in HB at pH values ranging from 7 to 11. In a typical experiment, PMCs from 125 oocytes were first incubated in the extraction medium for 60 min shaking on ice, with periodic sonication as described below for Triton-X-114 extraction. The extracted PMCs were centrifuged for 60 min at 100,000 × *g*, and the supernate was saved as the salt and/or high pH extract. High speed centrifuga-

tion was used because high salt treatment generally resulted in vesiculation of the PMCs, as is the case in the isolation of plasma membrane sheets from sea urchins [18]. The pellet was then subjected to detergent treatment by resuspension in a small volume (20–50 μl) of 1% Triton-X-100, 0.5% Na deoxycholate, 2 mM EDTA in HB, and sonication of this mixture for 10 min. The detergent-treated pellet was centrifuged for 5 min in a microfuge to separate extracted material from the insoluble residue. In some experiments, the membrane pellet was incubated for 30 min on ice, with vigorous vortex mixing at 10-min intervals.

Phase Separation in Triton-X-114

Separation of protein species by differences in their relative hydrophobicity was performed using the phase separation protocol of Bordier [7]. PMCs from 250 oocytes were suspended in 0.5 ml of HB and then centrifuged at 100,000 × *g* for 60 min to form a compact pellet. 200 μl of 1% Triton-X-114 in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4 was added to the membrane pellet, and the mixture was incubated for a total of 45 min of shaking on ice, with sonication for 60 sec in a bath sonicator containing ice water (Laboratory Supplies Company, Hicksville, NY) at the start and for every 15 min thereafter to help disrupt the pellet. After detergent solubilization, the membrane extract was centrifuged for 5 min in a microfuge, and the entire supernate used for phase separation. The final detergent pellet was approximately 20 μl in volume. SDS-PAGE analysis of the fractions obtained was performed on 8–15% gradient gels as previously described [17].

Lectin Blotting and Affinity Chromatography

Wheat germ and Concanavalin A (Con A) lectin blotting was performed according to the procedures of Bartles and Hubbard [4] and Bartles et al. [3], respectively. Con A-Sepharose columns of 1 ml bed volume were first washed with 10 ml of high salt buffer A (0.01 M Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$), and then with 10 ml of buffer A (same as high salt but with 0.15 M NaCl) containing 0.5 M methyl α-D-mannopyranoside [5]. The column was then rinsed with 20 ml of buffer A before detergent extracts of either PMCs or yolk platelet fractions were applied, and the sample was recirculated through the column for 1–1.5 hr at 4°C. The columns were then rinsed with buffer A, and unbound fractions were collected until the absorbance at 280 nm fell below 0.05. Bound proteins were then eluted by adding 0.5 ml of buffer A + 0.5 M methyl α-D-mannopyranoside and allowing the column to stand overnight in this buffer before eluting with the same buffer at 34°C the next day. 10 fractions of 1 ml each were collected, and the protein content of each was examined by SDS-PAGE followed by silver staining [48].

Results

PMCs Consist of Plasma Membrane, Cortical Granules, and Vitelline Envelope

PMCs consisted of three major components: (i) nonmembranous vitelline envelopes, (ii) the plasma membrane itself with microvilli and coated pits, and (iii) numerous cortical granules which remained attached to the membrane (Figs. 1 and 2). Inclusion of magnesium in the homogenization buffer was essential for maintenance of coated pit morphology, and for improved preservation of the normal microvillar structure. Lightly staining filamentous material was found in association with both the cytoplasmic and extracytoplasmic sides of the plasma membrane sheets, particularly with the microvillus cores (Fig. 2*C*). Fragments of rough endoplasmic reticulum were often present, as well as occasional mitochondria, pigment granules, and yolk platelets. In addition, some follicle cells often remained associated with the vitelline envelope. The frequency of follicle cell contamination was variable from preparation to preparation, and appeared to depend primarily on individual variation in the behavior of tissue obtained from different animals. The extent of contamination of PMCs with other organelles could be monitored quite readily by phase contrast microscopy, providing a relatively easy means of assessing purity during the course of the preparation.

Electrophoretic Analysis of the PMCs

As expected from the morphological results, electrophoresis revealed vitelline envelope proteins to be major components of the final PMC preparations (Fig. 3). Comparison of PMCs and manually isolated VEs by SDS-PAGE and Coomassie Blue staining revealed seven major bands contributed by the VE, and numerous bands associated with the plasma membrane and associated filaments and cortical granules. The relative amount of yolk platelet contamination could be readily assessed by the amount of lipovitellins 1 and 2 seen after gel analysis (lipovitellin 1 is at 111–121 kDa, and lipovitellin 2 at 30–34 kDa; [45]). The results of this approach correlated well with the number of platelets seen morphologically.

Removal of Cortical Granules

The majority of cortical granules can be removed from the PMCs by gentle homogenization in 2 mM $MgCl_2$ (Figs. 1 and 2). Since some vesiculation of the PMCs occurs during this stripping procedure, the complex pattern of minor bands seen in the extract may result from the presence of plasma membrane vesicles that have remained in the supernate after the PMCs were spun in the microfuge (Fig. 4*B*, lane *Mg-Ext*). However, the polypeptides extracted by 2 mM $MgCl_2$ appear to be particularly enriched in a broad band centered at 35 to 45 kDa, which does not stain well by the silver procedure (Fig. 4*A* and *B*). In addition, a pair of high molecular weight bands at 150–200 kDa, and a low molecular weight band at 13 kDa also appear to be enriched in the

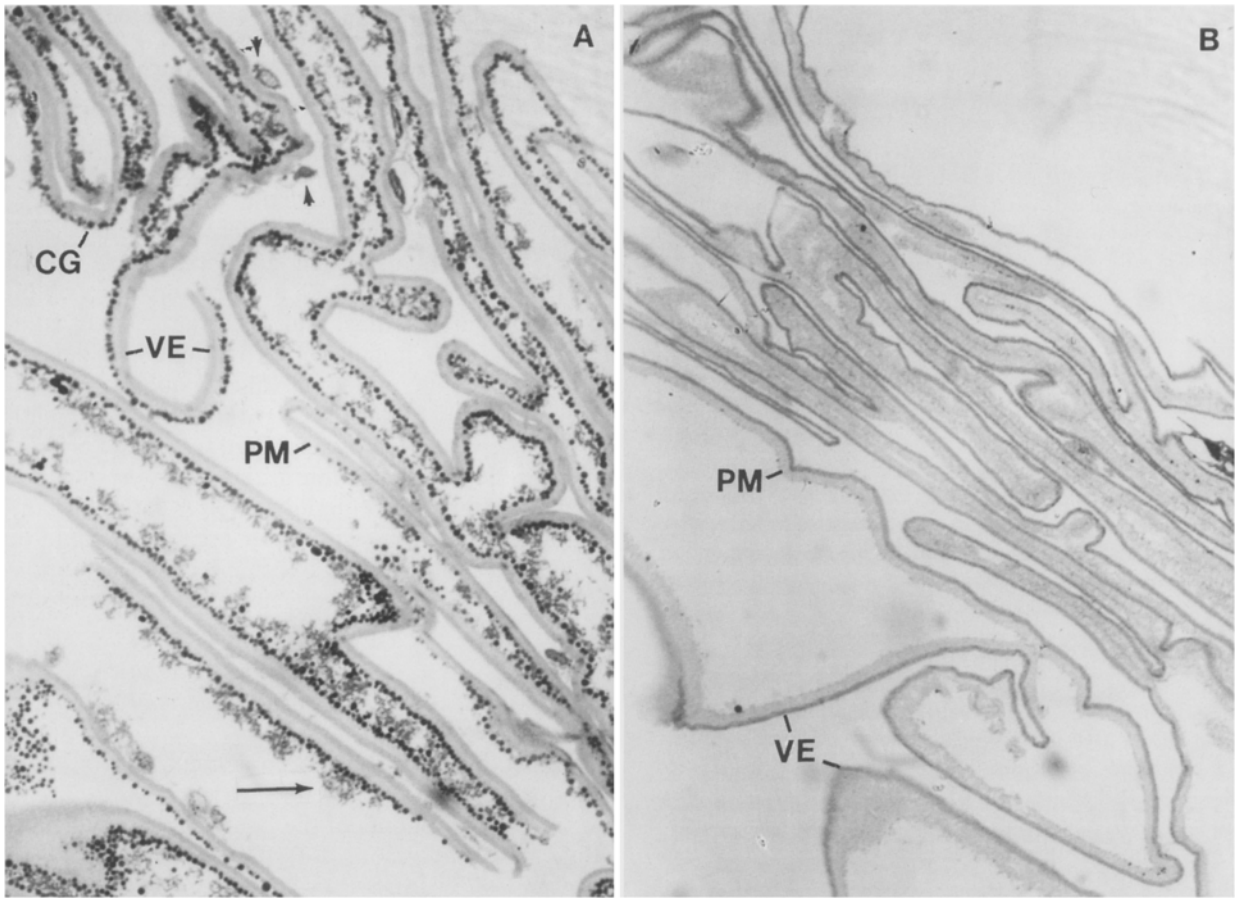

Fig. 1. Light micrograph of Toluidine blue-stained PMCs. (*A*) Typical PMC preparations consist predominantly of large sheets which include the vitelline envelope (*VE*), the plasma membrane (*PM*) itself, and darkly staining cortical granules (*CG*) attached to the cytoplasmic face of the membrane. Occasional follicle cells remain adherent to the vitelline envelope (e.g., at arrowheads). Yolk platelets are largely eliminated by the washing procedure, although other organelles remain associated with the cytoplasmic side of the PMCs in some regions (e.g., at arrow). (*B*) After treatment with 2 mM $MgCl_2$, the majority of cortical granules and other contaminating organelles are removed, while the vitelline envelope and plasma membrane itself remain quite intact. (1750×)

extract, suggesting that these may be components of the cortical granules. As expected, these polypeptides appear to be depleted from the PMCs remaining after cortical granule stripping (Fig. 4, *compare PMC* with *Mg-PMC* lanes). Furthermore, the extent of removal of these components correlates well with the extent to which the CGs are removed when they are examined morphologically.

Separation of vitelline envelope and any residual yolk crystal and pigment granule melanin from the plasma membrane itself was achieved by taking advantage of differences in the relative detergent solubility of these different structures. PMCs were extracted with a mixture of 1% Triton-X-100, 0.5% Na deoxycholate, and 2 mM EDTA in HB as described in Materials and Methods. Numerous protein species were solubilized by treatment of PMCs with non-ionic detergents, while the vitelline enve-

Fig. 2. Isolated plasma membrane complexes from *Xenopus* oocytes. (*A*) Low power electron micrographs illustrate the three major components of the PMC fraction: the vitelline envelope (*VE*), plasma membrane (*PM*) itself with numerous microvilli, and attached cortical granules (*CG*). The major contaminating organelle is rough endoplasmic reticulum. (15,700×; bar indicates 1 μm). (*B*) Gentle homogenization in 2 mM $MgCl_2$ has little effect on the morphology of the plasma membrane, the filaments, or on the vitelline envelope, but eliminates most of the cortical granules from these preparations. 15,700×. (*C*) PMCs prepared without cortical granule removal. At higher magnification, coated pits are seen at the base of microvilli (arrows). A fine fuzzy (filamentous) meshwork appears to be associated with the core of microvilli, but similar material is also found to a lesser extent on the extracytoplasmic side of the plasma membrane sheets. Fragments of rough endoplasmic reticulum are also evident here. (27,000×; bar indicates 0.25 μm) (*D*) Coated pit morphology has not been altered significantly by treatment of PMCs with 2 mM $MgCl_2$. 27,000×

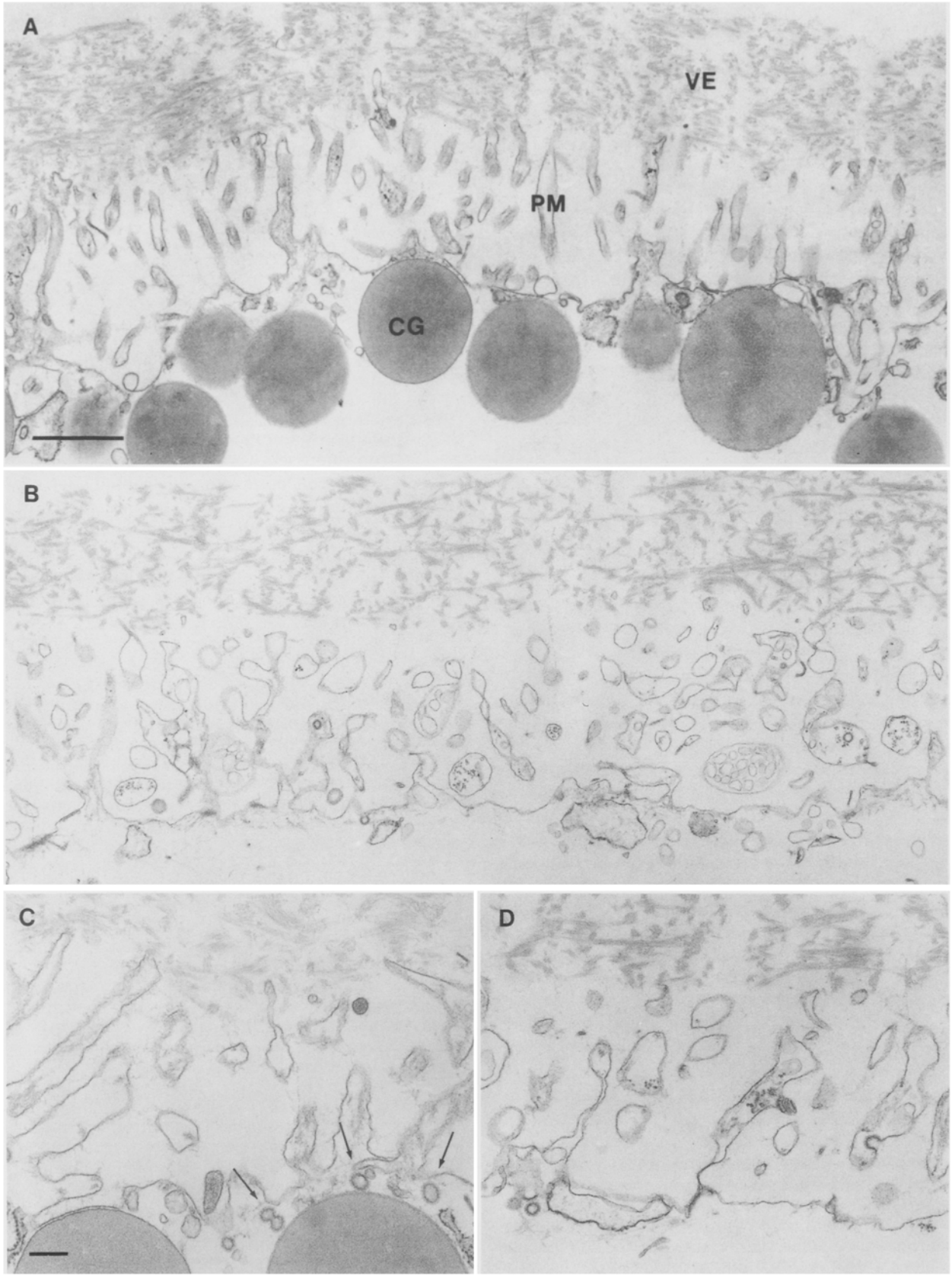
A
VE
PM
CG
B
C
D

Table. Activity of plasma membrane marker enzymes in isolated PMCs from *Xenopus* oocytes

	Protein[a]	5′-Nucleotidase[b]	Alkaline phosphodiesterase[c]
Whole oocytes (manual dissection)	367 ± 34	0.025 ± 0.013	0.005 ± 0.002
Plasma membrane complexes	1.25 ± 0.28	0.011 ± 0.003	0.0033 ± 0.0015
Relative enrichment[d]		125×	188×

Whole cell and isolated PMC enzyme activities were measured in the absence of detergent, in solution O-R2 containing the appropriate substrates. PMCs were assayed without cortical granule stripping. Values are given ± SD.

[a] μg protein/oocyte or PMC determined by the Lowry assay using a BSA standard.

[b] μmol AMP hydrolyzed/h/oocyte or PMC.

[c] μmol *p*-nitrophenol formed/h/oocyte or PMC.

[d] Calculated from the ratios of the specific activities.

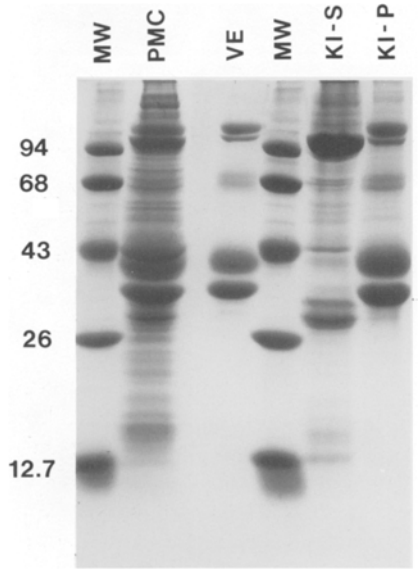

Fig. 3. SDS-PAGE analysis of PMC preparations. 100 μg each of total PMCs, manually isolated vitelline envelopes (*VE*), and the supernate (*KI-S*) and pellet (*KI-P*) of 0.5 M KI-treated PMCs were separated on an 8–15% gradient gel, and stained with Coomassie blue. The VE is composed of 6 major polypeptides, which are major bands in the PMC preparations and which remain insoluble after KI treatment. 0.5 M KI does solubilize yolk proteins, which are the major bands seen in the KI supernate at 100–120 kDa (lipovitellin 1) and as a doublet at 30–35 kDa (lipovitellin 2). Phosvitin does not stain with Coomassie blue. The amount of KI extracted material loaded on the *KI-P* lane is not sufficient to see the other relatively minor bands in this lane. *MW* = molecular weight markers

lope components and any contaminating yolk protein crystals and pigment granule melanin remained in the insoluble pellet (Fig. 4*A* and *B*). 78–99% of the 5′-nucleotidase activity recovered was associated with the detergent extracts, indicating that this treatment was effectively releasing integral membrane proteins. Examination of the residual pellet by electron microscopy confirmed that membrane bilayers had been completely disrupted, since no visible membrane vesicles or sheets were seen in a pellet consisting of yolk crystals, vitelline envelopes, and pigment granule contents (*data not shown*). Cortical granules could no longer be identified morphologically after the detergent extraction procedure.

Enzymatic Activities of the PMC Fraction

Each PMC contained an average of 1.25 μg protein, and 5′-nucleotidase (0.006–0.014 μmol AMP/h/PMC), alkaline phosphodiesterase (0.0014–0.0044 μmol *p*-nitrophenol/h/PMC), and azide-insensitive ATPase (0.5–2.7 nmol ATP hydrolyzed/h/PMC) activity. Comparison with enzyme activities measured by incubation of whole oocytes in the reaction media indicated average yields of 44% (5′-nucleotidase) and 66% (alkaline phosphodiesterase) of the cell surface activity of these plasma membrane markers in PMC fractions, corresponding to 125- to 188-fold enrichments (Table). However, the complexes contained at least 80% of the total 5′-nucleotidase and alkaline phosphodiesterase activity recovered when activities were compared with total homogenate levels, suggesting some loss of activity during homogenate preparation.

Although azide-insensitive ATPase activity could be measured at the surface of oocytes by incubation of intact cells in the assay medium, ATPase activity did not rise linearly with increasing input of membrane fractions and was therefore considered an unreliable plasma membrane marker. Mitochondria contain the vast majority of the total oocyte ATPase activity, and contribute an azide-sensitive component of the total PMC ATPase lev-

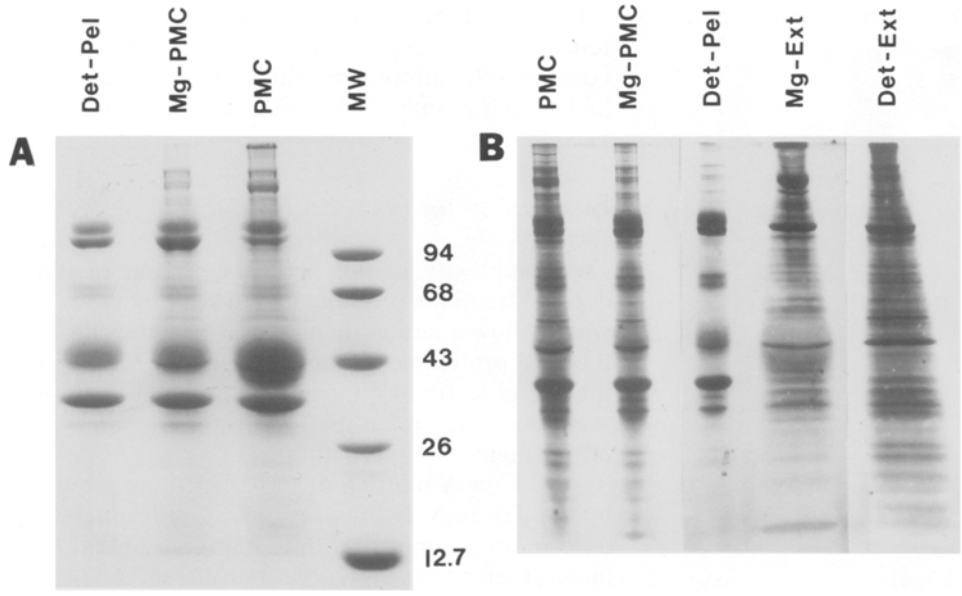

Fig. 4. SDS-PAGE analysis of PMCs after cortical granule stripping. Fractions produced by the Mg extraction protocol were analyzed by SDS-PAGE on 8–15% gradient gels and stained with either Coomassie blue (*A*) or silver (*B*). *PMC* = complexes before extraction; *Mg-PMC* = complexes after extraction; *Det-Pel* = detergent-insoluble residue; *Mg-Ext* = material released by $MgCl_2$ treatment; *Det-Ext* = detergent-soluble material; *MW* = molecular weight markers. Since the amount of protein obtained in the $MgCl_2$ and detergent extracts was not sufficient to be visualized with the Coomassie blue procedure, these fractions were only stained with silver. The pattern of proteins obtained was very similar in total PMCs before cortical granule removal and in $MgCl_2$-extracted PMCs, except for two high molecular weight bands at 150–200 kDa, a broad band at 35–45 kDa (poorly stained by silver), and a band at 13 kDa, which were enriched in the supernate after granule stripping. Detergent solubilization of the stripped PMCs gave a pellet consisting primarily of vitelline envelope components, while the supernate contained a complex mixture of polypeptide species

els (approximately 90% of the total homogenate ATPase activity is inhibited by the addition of 1 mM azide to the incubation medium). Mitochondrial contamination assessed by azide-sensitive ATPase activity and by electron microscopy was quite variable from preparation to preparation (addition of 1 mM NaN_3 to the reaction mixture in PMC assays inhibited 17–69% of the ATPase activity). Less than 1% of total cellular β-N-acetyl-glucosaminidase, a lysosomal marker enzyme, was recovered in the final PM complexes, despite the association of lysosomes with the peripheral cytoplasm of oocytes [39]. Galactosyl transferase activity was too low for reliable biochemical estimation in both homogenate and PMC fractions, while NADH/NADPH-cytochrome *c* reductase activity recovered in the membrane preparations was <0.5% of the homogenate levels. Although some fragments of rough endoplasmic reticulum were consistently found in the PMC fractions, we saw no morphological evidence for the presence of Golgi stacks.

High Salt Extraction of PMC Preparations

Since our PMC preparations also included some residual cortical and pigment granules, yolk platelets, filaments, and peripheral membrane proteins, direct detergent treatment would release not only integral membrane proteins of the oocyte PM itself, but many of these other components as well. We therefore attempted to remove peripheral proteins first, through high salt or high pH extraction, before the detergent treatment. Exposure of PMCs to solutions of high ionic strength such as 0.5 M KI was quite effective in removing associated yolk protein contamination by solubilizing the crystals, as well as releasing many other unidentified protein species to the supernate (Fig. 3*A*, lane *KI-S*). Salt treatment was considerably more effective than alkaline treatment (up to pH 11) in this regard (*data not shown*). Neither treatment was effective in stripping cortical granules from the complexes. However, although high salt treatment left a residue which contained the expected VE components, few other proteins were visible by SDS-PAGE analysis of this material (Fig. 3*A*, lane *KI-P*). This apparent loss of integral membrane proteins was confirmed biochemically, since 33–71% of the recovered 5′-nucleotidase activity was found in the supernate after 0.5 M KI or 0.5 M KCl extraction. Lowering the salt concentration or shortening the exposure time were not effective in improving this extraction procedure, because significant decreases in 5′-nucleotidase release could only be achieved under conditions

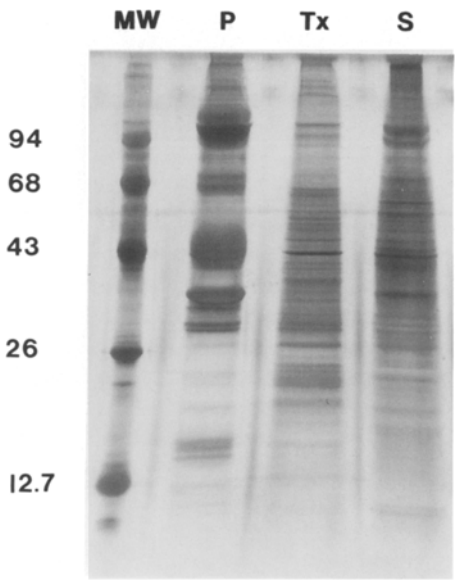

Fig. 5. Triton-X-114 extraction of PMCs. Complexes were prepared from 125 oocytes, and were extracted with 1% Triton-X-114 for 30 min on ice, with occasional sonication to assist in disruption of the pellet. After centrifugation in a microfuge to pellet insoluble material, the supernate was used for phase separation according to Bordier [7]. The proteins in the detergent and aqueous phases, and the insoluble pellet remaining after the original Triton-X-114 extraction were run on 8–15% SDS-PAGE gels and stained with silver. *P* = detergent insoluble pellet before phase separation; *Tx* = detergent phase after separation; *S* = aqueous phase after separation. Yolk proteins and vitelline envelope components are largely confined to the initial pellet, while a complex mixture of proteins partitions into each of the two phases after separation. The bands seen here in the detergent phase persist even after three rounds of Triton-X-114 extraction

that left much of the contaminating yolk proteins still associated with the PMC pellet (*data not shown*). We therefore decided to use Triton-X-114 phase separation for identification of integral and peripheral membrane proteins.

Phase Separation in Triton-X-114

Extraction of PMCs with 1% Triton-X-114 was as effective in solubilizing 5′-nucleotidase activity as the combination of Triton-X-100 and Na deoxycholate, releasing 77–87% of the recovered activity. Many of the detergent-solubilized proteins partition into the Triton-X-114 phase during separation according to the procedure of Bordier [7], suggesting that they are relatively hydrophobic integral membrane proteins (Fig. 5). The insoluble pellet remaining after Triton-X-114 extraction included yolk proteins at 111–121 and 30–34 kDa, the major components of the vitelline envelope, and a number of relatively minor species, e.g., at 15–17 kDa and >120 kDa (Fig. 5, lane *P*). Comparison of the proteins of the detergent and soluble phases revealed considerable differences, although many bands also had similar mobilities by this one-dimensional analysis.

Binding of Iodinated Lectins

The complexity of polypeptides present in PMC-derived fractions prompted us to seek methods that would allow characterization of a selected subset of the total protein population. We therefore turned to the use of lectin blotting and lectin affinity chromatography to focus on the glycoprotein composition of our membrane preparations.

^{125}I-Con A blotting of PMC fractions produced by the Triton-X-114 phase separation protocol was used in an attempt to differentiate peripheral from integral membrane glycoproteins (Fig. 6*A*). This procedure demonstrated a number of major Con A binding species, with three heavily stained bands associated with the detergent-insoluble residue (this pellet contains vitelline envelopes, as well as residual melanin granules and yolk crystals without membranes when examined by electron microscopy). We believe these to be components of the vitelline envelope, since they comigrate with major envelope proteins (Fig. 3), manually isolated vitelline envelopes give the same three bands on Con A blots (Fig. 6*A*, lane *VE*), and previous biochemical examination of envelopes has identified many components as glycoproteins [34, 47]. Of the remaining Con A binding glycoproteins, 3–4 high molecular weight species were associated with the high speed supernate, while the remaining glycoproteins partitioned mainly into the aqueous phase. The latter included a broad band at 35–45 kDa, very similar to the putative cortical granule protein depeleted after granule stripping in 2 mM $MgCl_2$. Relatively few minor species appeared to be associated with the detergent phase. These data suggest that the major glycoprotein species associated with the PMC preparations are either components of the vitelline envelope, peripheral membrane proteins, or possibly components of the cortical granules.

^{125}I-wheat germ lectin blotting also revealed a small number of major glycoproteins, three of which were vitelline envelope components found in both the detergent-insoluble pellet (Fig. 6*B*) as well as in isolated, detergent-washed envelopes (*not shown*). As was the case with Con A, the remaining major wheat germ agglutinin binding glycoproteins were predominantly associated with either the supernate of the first spin before detergent treatment, or partitioned into the aqueous phase. The considerable similarity in patterns obtained with these two

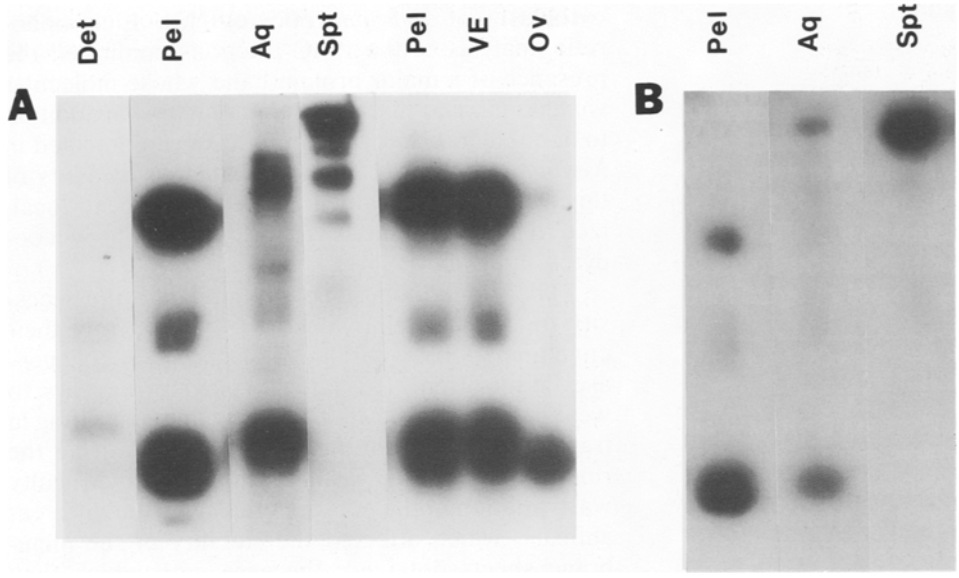

Fig. 6. Con A and wheat germ agglutinin binding proteins of PMC preparations. PMCs were prepared from 250 oocytes, solubilized in 1% Triton-X-114, and subjected to phase separation as described in Materials and Methods. Fractions were analyzed by transfer to nitrocellulose and blotting with ^{125}I-Con A (*A*) or with ^{125}I-wheat germ agglutinin (*B*) as described [3, 4]. Four fractions were examined: the detergent phase (*Det*), the aqueous phase (*Aq*), the residual pellet of detergent-insoluble material (*Pel*), and the supernate of the initial high speed spin used to concentrate the PMCs (*Spt*). The detergent phase of the WGA blot was omitted since no distinct bands were seen in this lane. For these blots, all of the high speed supernate and aqueous phase, half of the detergent-insoluble pellet, and 12 out of a total of 20 μl of the detergent phase were applied to the gel. In addition to the fractions derived from the phase separation protocol, isolated vitelline envelopes (*see* Materials and Methods) were run to determine their contribution to the overall pattern of lectin binding species (lane *VE*). The molecular weight marker is the glycoprotein ovalbumin (*Ov*) at 43 kDa. The major glycoprotein species detected by both lectins were associated with either the vitelline envelope (i.e., in the detergent-insoluble pellet), the aqueous phase, or the supernate of the initial spin before extraction (high molecular weight species near the origin). Proteins of the detergent phase made minor contributions to the total glycoprotein population detected by the blotting procedure

lectins suggested that the same glycoproteins often bound both lectins.

Lectin Affinity Chromatography

To isolate those glycoprotein species which bound to Con A, detergent extracts of PMC preparations were prepared and then applied to Con A columns without prior phase separation or cortical granule removal. Unbound and bound material was separated and analyzed by SDS-PAGE followed by silver staining. As can be seen in Fig. 7, a characteristic group of detergent-extracted PMC proteins bound to Con A-Sepharose, while the large majority of proteins remained unbound. The pattern was characterized by two prominent high molecular weight bands at >94 kDa, a series of minor bands between 94 and 43 kDa, and a diffuse band at 35–45 kDa. Warming the column and elution buffer was found to be essential for efficient elution of bound material with α-methyl mannoside (Fig. 7, lanes BD-4 and BD-34), as reported by a number of other workers [4]. The pattern of minor bands was not always reproducible, with some variation from preparation to preparation. The material applied to the Con A-Sepharose columns was essentially equivalent to the combined aqueous and detergent phases of the Triton-X-114 phase separation protocol. Comparison of the protein species seen here with the Con A blots of equivalent material (Fig. 6*A*) shows considerable similarity between the Con A binding patterns, although the resolution of the blotting method was poorer.

Comparison of the overall pattern of Con A binding proteins obtained from PMCs with that from detergent extracts of mitochondria, light and heavy yolk platelets obtained by sucrose gradient fractionation revealed substantial differences between the various organelles (*not shown*). Therefore, the Con A binding glycoproteins seen here could not be attributed to PMC contamination with these other organelles.

Discussion

Isolation of Surface Membrane Complexes

We have developed a relatively simple procedure for the preparation of large plasma membrane

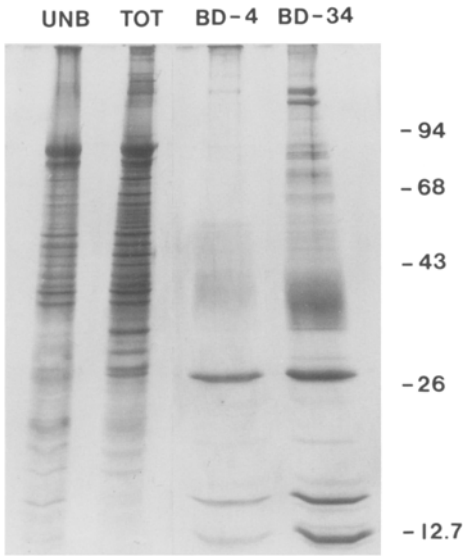

Fig. 7. Con A-Sepharose chromatography of PMC extracts. 250 PMCs were extracted at 4°C with 1% TX-114, and the extracts were centrifuged briefly to remove insoluble material. The detergent-soluble material was applied to a 2-ml Con A-Sepharose column, washed, and eluted with 1% α-methyl mannoside at either 4 or 34°C. *TOT* = total detergent extract before application to the column; *UNB* = unbound material eluted in wash without sugar; *BD*-4 = bound material eluted at 4°C by α-methyl mannoside; *BD*-34 = bound material eluted at 34°C with sugar. The low molecular weight bands at approximately 30 kDa or less which elute with the bound material are Con A, which leached slowly from the column. The elution conditions used did not appear to have removed all material from the column (e.g., in the 30–35 kDa range)

sheets from *Xenopus* oocytes, consisting of complexes of the vitelline envelope, plasma membrane, and cortical granules. The absence of any centrifugation steps, very gentle homogenization conditions, and inclusion of 2 mM $MgCl_2$ in the solutions used all contribute to excellent preservation of plasma membrane sheet size and morphology. The membrane sheets produced by this protocol retain microvilli and coated pits, and give good yields (44–66%) and enrichment (125- to 188-fold) of plasma membrane marker enzymes. Unlike oocyte plasma membrane isolation methods, which require individual dissection and rinsing of each membrane [30], the protocol described here is equally effective for any number of oocytes, making it suitable for use with small numbers of cells (e.g., after microinjection) as well as for preparative procedures using larger numbers.

Filaments associated with the microvilli are likely to include a substantial amount of actin, a protein normally associated with microvilli in other cells and which has been localized in the peripheral cytoplasm of *Xenopus* oocytes [13]. Electrophoretic analysis of the PMC fractions confirmed the presence of a major protein band whose molecular weight (45 kDa) resembled that of actin. In addition to actin, a 56-kDa cytokeratin has been detected in *Xenopus* oocytes [14], and substantial amounts of this intermediate filament protein have been localized to the subcortical cytoplasm of fully grown oocytes and eggs [15, 49].

The major limitation of our method is the necessity for manual removal of the oocytes from their enveloping follicle cell layers, rather than collagenase dissociation. The latter procedure appears to weaken the vitelline envelope structure, resulting in fragmentation of the membrane sheets during the rinsing process. A further unavoidable difficulty was variation in the oocytes obtained from different animals, which affected the stability of the membrane sheets obtained, the ease with which they could be rinsed free of contaminating organelles, and the number of follicle cells that remained adherent to the PMCs. The effectiveness of washing and extent of follicle cell contamination could be monitored by phase contrast microscopy during the course of the rinsing procedure. The oocytes from a few animals seemed particularly fragile and would fragment into pieces too small to recover by settling at 1 × *g*. Such cells were not suitable for PMC preparation by our protocol.

Preparation of plasma membrane fractions from unfertilized and fertilized eggs [26, 27] and from ovarian oocytes [30, 50] of *Xenopus* has been reported previously. In the work with eggs, nylon mesh filtration was used to remove vitelline envelopes from homogenates, followed by low speed centrifugation (150 × *g*, 5 min) to collect plasma membrane and cortex fragments as a fluffy layer on top of the yolk platelet pellet [26, 27]. The oocyte protocols utilized either manual rinsing of each individual membrane sheet [30] or a series of low speed centrifugations at 350 and 1500 × *g*, followed by density gradient centrifugation to isolate plasma membrane-cortical granule complexes [50]. During the initial development of our protocol, we encountered great difficulty in separating large plasma membrane sheets from the underlying yolk platelet pellet when we attempted to use a similar low speed centrifugation to collect PMCs, with considerable fragmentation of the PMCs during their resuspension after each spin. Allowing the PMCs to settle by gravity avoided this problem, since the large size of the complexes gave them a faster sedimentation rate then the platelets and other organelles and shearing damage during centrifugation could be avoided.

Electrophoretic Analysis of PMC Fractions

Biochemical analysis of *Xenopus* egg membrane fractions by SDS-PAGE on 4–30% gradient gels followed by Coomassie blue staining revealed a relatively small number (about 25) of major polypeptide bands, including the yolk proteins lipovitellin 1 and 2 and actin [26, 27]. Our Coomassie blue-stained PMC gels showed a similar pattern and number of bands when the shallower acrylamide gradient (8–15%) and contribution of vitelline envelope polypeptides were taken into account (Fig. 3). The major vitelline envelope polypeptides seen in our work were very similar to those previously reported by Wolf et al. [47] and by Richter [26]. However, the more sensitive silver staining protocol used here revealed many minor protein species associated with the PMCs, some of which are likely to be contributed by minor contaminating organelles, and some of which are probably *bona fide* plasma membrane constituents. Many of these relatively minor polypeptides were extracted by detergent (Fig. 4), unlike the vitelline envelope or yolk protein components. Phase separation using Triton-X-114 resulted in a roughly equal division of this complex group of polypeptides into the aqueous *vs.* detergent phases. By this criterion, many of these protein species are likely to be relatively hydrophobic integral membrane proteins. At present we do not know whether the similarity in electrophoretic mobility of many of the aqueous phase and detergent phase proteins means that the same molecules partition into both phases, or whether these are different molecules which could not be resolved by the one-dimensional electrophoretic analysis used. However, the striking differences in the distribution of lectin binding proteins between the aqueous and detergent phases suggests that the phase separation was quite effective, at least for these glycoproteins. In addition, 5′-nucleotidase activity was recovered predominantly in the detergent phase, as expected.

Cortical Granule Removal

A partial removal of cortical granules after treatment of *Xenopus* oocyte membrane fractions was originally reported by Richter and Tintschl [27], who used double distilled water as the stripping solution. Addition of 2 mM $MgCl_2$ and gentle shearing with the Dounce homogenizer improved the extent of granule removal that we obtained, while preserving membrane morphology. We did not achieve significant loss of cortical granules after PMC extraction with any of the high salt or high pH treatments used for removal of peripheral proteins, or with 1 M urea, 0.1% Na deoxycholate, or dilution into 1 M sucrose. The last protocol has been used for cortical granule stripping from sea urchin membrane complexes [18].

SDS-PAGE revealed several consistent changes in the polypeptide patterns seen before and after granule stripping. The major difference was loss of a broad band at 35–45 kDa, which stains poorly with silver (Fig. 4). A Coomassie blue staining band in the same size range was reported to be depleted after cortical granule removal from egg membranes by Richter and Tintschl [27], who also described a reduction in two high molecular weight polypeptides at 150–165 kDa. The 35–45 kDa glycoprotein is strongly reminiscent of the *Xenopus* oocyte lectin purified and characterized by Barondes et al. [28, 29]. Immunocytochemical localization detected this lectin in yolk platelets, cortical granules, and the vitelline envelope, with particular concentration in the granules when plastic sections were used for the localization experiments [28]. The lectin appears to be released upon cortical granule exocytosis [28].

Since hypotonic lysis would presumably rupture most of the cortical granules, their membranes may remain associated with stripped plasma membrane-vitelline sheets. No cortical granule membrane marker has been identified in *Xenopus*, preventing a direct test of this possibility. However, freeze-etching studies of *Xenopus* oocytes demonstrate a marked difference between plasma membrane and cortical granules in the size and density (number per μm^2 of membrane surface area) of intramembranous particles, with considerably larger and more numerous particles in the plasma membrane [6, and D. Wall, *unpublished observations*]. These results suggest that the relative amount of membrane protein contributed by the plasma membrane may be substantially greater than that derived from granule membranes.

Analysis of Lectin-Binding Glycoproteins

The lectin blotting experiments indicated that the major glycopeptides which bound to these two lectins were components of either the vitelline envelope, the cortical granules, or were peripheral proteins behaving like relatively hydrophilic species during phase separation in Triton-X-114. Only minor components appeared to be integral membrane proteins by this criterion, despite the presence of a large number of polypeptides in the detergent phase (Fig. 5). This result is in agreement with the Con A localization studies of Stenuit et al., who found that this lectin bound extensively to the vitelline enve-

lope, cortical granules, and periphery of yolk platelets, but that relatively little was found in association with the oocyte plasma membrane [34].

Those glycoproteins, which bound to Con A-Sepharose, should possess either high mannose, biantennary complex, or hybrid oligosaccharide side chains, any of which have been shown to bind to this lectin with high affinity [2, 11, 20, 23]. Since most of the glycoproteins which bound Con A also bound to wheat germ agglutinin after periodate-aniline-cyanoborohydride treatment, they most likely also contain residues terminating in either N-acetylglycosamine or sialic acid [4]. The most striking difference in affinity for the two lectins was demonstrated by the uppermost vitelline envelope component at approximately 120 kDa, which bound a much larger amount of iodinated Con A than wheat germ lectin, suggesting that this protein contained a relatively high number of either high mannose or hybrid type carbohydrate side chains that did not have terminal sialic acid or N-acetylglucosamine sugars.

The *Xenopus* oocyte is capable of expressing high levels of exogenous proteins by translation of injected mRNAs and appears to have the ability to target these polypeptides to their appropriate intracellular location, regardless of the species of their origin. This fact, together with recent advances in cloning of a variety of organelle content and membrane proteins, make the *Xenopus* oocyte a very useful system for investigation of the structural features of protein molecules that are responsible for their targeting within cells. The development of cell fractionation schemes applicable to this oocyte system will be important for such studies. Plasma membrane fractions prepared as described here should prove valuable for further biochemical and morphological studies of membrane protein sorting in these cells.

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