Selective transport and packaging of the major yolk protein in the sea urchin

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Abstract

The major yolk protein of sea urchins is an iron-binding, transferrin-like molecule that is made in the adult gut. Its final destination though is the developing oocytes that are embedded in somatic accessory cells and encompassed by two epithelial layers of the ovary. In this study, we address the dynamics of yolk transport, endocytosis, and packaging during the vitellogenic phase of oogenesis in the sea urchin by use of fluorescently labeled major yolk protein (MYP). Incorporation of MYP into the accessory cells of the ovary and its packaging into yolk platelets of developing oocytes is visualized in isolated oocytes, ovary explants, and in whole animals. When MYP is introduced into the coelom of adult females, it is first accumulated by the somatic cells of the ovarian capsule and is then transported to the oocytes and packaged into yolk platelets. This phenomenon is specific for MYP and accurately reflects the endogenous MYP packaging. We find that oocytes cultured in isolation are endocytically active and capable of selectively packaging MYP into yolk platelets. Furthermore, oocytes that packaged exogenous MYP are capable of in vitro maturation, fertilization, and early development, enabling an in vivo documentation of MYP utilization and yolk platelet dynamics. These results demonstrate that the endocytic uptake of yolk proteins in sea urchins does not require a signal from their surrounding epithelial cells and can occur autonomous of the ovary. In addition, these results demonstrate that the entire population of yolk platelets is competent to receive new yolk protein input, suggesting that they are all made simultaneously during oogenesis.

Introduction

An important feature of oocyte development in many animal species is the accumulation of sufficient yolk to sustain embryogenesis. Yolk is a mixture of proteins, lipids, and carbohydrates, and in many species of egg-laying animals, it consists primarily of two predominant classes of storage proteins. One group is called vitellogenin (Vg) and is found in frog, nematode, fish, and some insects such as the mosquito (Wallace, 1978; Kimble and Sharrock, 1983; Sharrock, 1984; Byrne, 1989; Chen et al., 1997). The second group is referred to as yolk proteins (YPs) and is found in dipteran insects, including Drosophila melanogaster (reviewed in Bownes, 1992). Both classes of proteins are female-specific and are packaged into yolk platelets, yet they are different in terms of amino acid sequence, size, processing, and site of synthesis.

The major yolk protein (MYP) of sea urchins is distinct from both vitellogenins and yolk proteins. MYP is a 180-kDa glycoprotein selectively stored in the yolk platelets of oocytes, eggs, and embryos and accounts for 10–15% of the total cellular protein (Ichio et al., 1978; Kari and Rottman, 1980; Ozaki, 1980; Harrington and Easton, 1982). It has historically been classified as a vitellogenin based on its abundance and packaging into yolk platelets (Shyu et al., 1986, 1987). However, MYP is not a female-specific protein as are all other known yolk proteins since it is also synthesized and stored in the cells of the testis (Shyu et al., 1986; Unuma et al., 1998). The recent cloning and characterization of MYP (Brooks and Wessel, 2002) suggests that MYP is a transferrin-like...
iron binding protein rather than a vitellogenin. This classification better fits its physiological profile and suggests that it functions to transport iron in both sexes of the sea urchin to support the proliferative demands of gametogenesis. In females, its transport and packaging in the yolk platelets may provide an iron store to be used during embryogenesis.

MYP is predominately synthesized in the intestine of the sea urchin. This synthesis likely occurs in the adult animal and is transported to the ovaries where it is packaged into the yolk platelets. Throughout early development, the yolk platelets are evenly distributed throughout the cytoplasm of all cells, including the eggs, embryos, and larval stages. The concentration of yolk platelet signal is particularly evident in the gut of the gastrula and pluteus larvae, where it is likely involved in nutrient uptake.

Fig. 1. Oocytes package labeled MYP in vivo and develop normally. Whole animals were injected with Texas-Red MYP and Oregon-Green dextran and cultured for 69 h. Gametes were shed by KCl injection and the eggs were fertilized. The top panels (A–G; I–O) are DIC images, and the bottom panels (B–H; J–P) are the corresponding epifluorescence images of the live cells, with MYP in red and DNA in blue. Label above background was not detected in the green channel for dextran (results not shown, see also Fig. 2A). In ovaries (A, B), MYP is present in the somatic accessory cells and the yolk platelets of developing oocytes. Throughout early development, the yolk platelets are evenly distributed throughout the cytoplasm of all cells; shed egg (C, D) showing yolk packaging that took place prior to egg maturation in the ovary; 2-cell embryo (E, F); 32-cell embryo (I, J); mesenchyme blastula (K, L). During the later stages of development, yolk platelet signal is concentrated in the gut of the gastrula (M, N) and pluteus larvae (O, P). fe, fertilization envelope; mm, micromere; bar, 50 μm (A–N; O–P).
adult sea urchin as a 195-kDa protein precursor (Shyu et al., 1986). It is then secreted into the coelomic fluid, transverses the two epithelial layers of the ovarian capsule, and is absorbed by the somatic accessory cells where it is stored in large granules. These accessory cells are often referred to as nutritive phagocytes since they provide nutrition to developing gametes and they also recycle this nutrition by absorbing any unshed or degenerating germ cells at the end of the gametic cycle (Fuji, 1969; Walker, 1982; Harrington and Ozaki, 1986; Ozaki et al., 1986; Unuma et al., 1998, 2001).

The growth and differentiation of the nutritive phagocytes (NPs) are tightly linked to gametogenesis. Prior to gametogenesis, the follicular lumen of the gonads of both sexes of animals is filled with MYP-laden NPs. Then, as gametogenesis proceeds, the NPs regress as gametes develop. In males, the regressing NPs are depleted of MYP as spermatocytes develop, but this protein does not appear in the developing sperm cells and its fate in testis is not known (Unuma et al., 1998). In females, the depletion of MYP in the regressing NPs is coincident with the accumulation of MYP in the yolk platelets of ripening ova (Ozaki et al., 1986; Harrington and Ozaki, 1986). During this process, the MYP storage granules in the NPs break down and MYP is transported to the periphery of the oocyte where it is endocytosed (Takashima and Takashima, 1966; Tsukahara and Sugiyama, 1969; Tsukahara, 1971; Geary, 1978). During the vitellogenic phase of oogenesis (about a 1-month period depending on the species), the oocytes accumulate yolk proteins and grow to 10 times their original size to approximately 100 μm (depending on the species), the same size as a mature egg. Yolk formation in the sea urchin involves a combination of both heterosynthetic and autosynthetic mechanisms; MYP is packaged with another abundant yolk component, YP30, that is made by the oocyte (Wessel et al., 2000). The resultant spherical yolk organelles are membrane-bound and electron dense, 1–1.7 μm in diameter, distributed throughout the cytoplasm (Gross et al., 1960; Monroy and Maggio, 1963). They are present in large amounts and together account for 28–38% of the total egg weight (Harvey, 1932; Costello, 1939).

To elucidate the pathway of MYP entry to oocytes, we used fluorescently labeled MYP to follow its packaging dynamics both in vitro and in vivo. Sea urchins are amenable for investigating vitellogenic uptake of yolk protein precursors since oocyte growth is asynchronous and oocytes at all stages of development are found adjacent to one another within the inner epithelium of the same ovary lobe. These oocytes are easily dissected, cultured, and are optically clear.

The work here documents the transport and endocytosis of MYP in the ovary, and tests the hypothesis that yolk platelets are formed sequentially during oogenesis, as has been documented in frog oocytes (Danilchik and Gerhart, 1987). Instead, we find that the entire population of yolk platelets are made simultaneously during oogenesis, and that oocytes begin this process in mid-oogenesis, independent of the somatic accessory cells. These results will enable us to functionally evaluate the requisite mechanism for yolk targeting and deposition and may permit the identification of a discrete targeting signal if it exists.
Materials and methods

Handling of animals and gametes

Adult *Lytechinus variegatus* were obtained by Susan Decker (Miami, FL) or by ourselves (Beaufort, NC). They were maintained either in recirculating ASW tanks at 20°C or in running sea water tables with a diet of seaweed at the Duke University Marine Laboratory (Beaufort, NC). Females were shed by KCl (0.5 M) injection, and their ovaries were removed. For ovary explant studies, the ovary was left intact, rinsed several times, and incubated in sea water containing 100 μg/ml ampicillin (Sigma, St. Louis, MO). To obtain oocytes, ovaries were minced with a scalpel in sea water, larger ovarian fragments were removed by settling, and the supernatant containing dissociated somatic cells and free oocytes was transferred by pipette to fresh sea water. Individual oocytes were then isolated by mouth pipette to sea water containing ampicillin. To minimize damage to the cell surface, all glassware that came in contact with the oocytes was treated with SafetyCoat Nontoxic Coating (J.T. Baker, Phillipsburg, NJ). Overnight incubation of samples was at room temperature within a humidity chamber to avoid evaporation and to keep the salinity of the sea water optimal. Oocyte and egg stratification was conducted under high-centrifugation conditions in an isopycnic sucrose gradient as previously described (Wessel et al., 2002).

Membrane topology and endocytosis

FM1-43 (Molecular Probes, Eugene, OR), was resuspended in methanol at 1 mg/ml, then diluted in ASW to give a working concentration of 1 μg/ml. To evaluate endocytosis, eggs and oocytes were transferred to the FM1-43 in ASW. To quantitate FM1-43 endocytosis, confocal sections of live cells were acquired with a Zeiss LSM 410 laser scanning microscope and analyzed by using Metamorph software (version 4.6r5; Universal Imaging Corp, Downingtown, PA). The relative intensity of FM1-43 was calculated per area of each cell by using the region measurement function. The intensity of fluorescence was divided by the area of each cell to obtain a standardized value. Standardized value groups of different sized cells were averaged and compared by using Excel (Microsoft Corporation, WA). The values were subjected to ANOVA followed by a Student's *t* test.

Cytoskeletal inhibitors were used at the following concentrations: colchicine; 10 μM, and cytochalasin B: 1 μg/ml (Calbiochem, San Diego, CA: Schatten et al., 1986) both in ASW with 100 μg/ml ampicillin. DMSO (Sigma Chemicals, St. Louis, MO) was used for the solution vehicle and also in the control treatments at the same dilution and was found to have no effect in the assays here. For experiments testing cytoskeletal function, cells were preincubated with cytoskeletal inhibitors for 15 min and then incubated with Texas-Red-labeled MYP for 50 min. To evaluate endocytosis, oocytes were washed in ice-cold sea water and immediately visualized by using epifluorescence with a Zeiss Axioplan microscope.

Preparation of coelomic fluid protein and fluorescent labeling

To fluorescently label MYP, coelomic fluid enriched with MYP was prepared as described (Brooks and Wessel, 2002). This protein was conjugated to fluorochromes by using both the FluoroReporter Oregon-Green 488 and Texas-Red-X protein labeling kits (Molecular Probes, Eugene, OR). The integrity of the protein was evaluated by SDS-PAGE, and the degree of fluorochrome labeling was determined according to the manufacturer’s instruction. In brief, we first calculated the molar extinction coefficient for MYP based on its full-length sequence (http://paris.chem.yale.edu/extinct.html). This value was then used along with the OD<sub>280</sub> and either OD<sub>495</sub> (Texas-Red) or OD<sub>496</sub> (Oregon-Green) of the MYP conjugation reaction to determine the extent of fluorochrome incorporation into MYP. With the known level of dye density in dextran (supplied by the manufacturer), we then determined that, when equal masses of dextran and MYP were used in an incubation, the dextran contained a 10-fold excess of fluorochrome abundance over MYP. As a control, partially iron-saturated human serum transferrin (Sigma, St. Louis, MO; 10 mg/ml) was also labeled.

In vivo MYP localization and quantitation

To identify internalized MYP in vivo, MYP-enriched fluorochrome-labeled coelomic fluid (see Protein labeling; Molecular Probes, Eugene, OR) was injected into the coelomic cavity of adult sea urchins by using a microfine 1-cc insulin syringe (Becton Dickinson, Franklin Lakes, NJ) to gently pierce the peristomal membrane surrounding Aristotle’s lantern. We calculated that the amount of MYP we injected represented 10% of the endogenous coelomic MYP. As a control, we coinjected Alexa-Green dextran (Molecular Probes, Eugene, OR) at an equal mass level of MYP that represents a 10-fold greater excess of fluorochrome than the MYP (see Fluorescent labeling calculation). Injected animals were incubated for 1–12 days, their eggs were collected by KCl injection, and then their ovaries were dissected. Eggs were fertilized and Tr-MYP label was visualized in live cells by epifluorescence with a Zeiss Axioplan microscope. To visualize DNA, Hoechst 33258 (Molecular Probes, Eugene, OR) was added to the cells to a final concentration of 10 μg/ml. Ovaries from these animals were dissected, fixed in 4% paraformaldehyde, and processed as previously described (Laidlaw and Wessel, 1994). Sections of ovary were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) to visualize DNA. Signals were visualized either by epifluorescence with a Zeiss Axioplan microscope or by laser-
scanning microscopy with a Zeiss LSM 410 confocal microscope.

To quantitate MYP endocytosis, confocal sections of the MYP-labeled ovary explant were analyzed by using Meta-morph Software (version 4.6r5; Universal Imaging Corp, Downingtown, PA). For each cell, the relative intensity of MYP was calculated per area of each cell by using the region measurement function. The intensity of fluorescence was divided by the area of each cell to obtain a standardized value. These values were statistically evaluated by using Excel (Microsoft Corporation, WA).

For electron microscopic immunolabeling, ovaries from animals incubated with Tr-MYP for 20 h were fixed in 4% formaldehyde in calcium-free artificial sea water to retain the chromophore and embedded in Spurr’s resin (Spurr, 1969). Silver–gold sections (approximately 90 nm) were placed on nickel grids and incubated for at least 20 min in phosphate-buffered saline containing 10% fetal bovine serum (PBSF). Sections were then incubated for at least 1 h in PBSF containing either immune or preimmune MYP antisera (Brooks and Wessel, 2002; diluted either 300× or 1000×) or antibodies to Tr (Molecular Probes, Eugene, OR; diluted 1:250). After washing in PBSF, sections were incubated for at least 1 h in goat anti-rabbit antibodies conjugated to 15-nm colloidal gold particles (diluted 30× in PBSF; Jackson Laboratories, West Grove, PA). Sections were then gradually washed into PBS, postfixed with 2% glutaraldehyde, and stained with uranyl acetate and lead citrate. Sections were visualized at 80 KeV with a Philips 410 electron microscope, and images were recorded by using a Hamamatsu ORCA slow scan CCD digital camera managed by Advanced Microscopy Technology Corporation software (Danvers, MA).

**In vitro MYP localization**

Ovary explants from individual animals were cultured overnight at room temperature in sea water that contained either Texas-Red MYP (Tr-MYP), Texas-Red-labeled human serum transferrin, unconjugated Texas-Red fluorochrome, or tetramethylrhodamine (TMR) dextran (10,000 MW; Molecular Probes, Eugene, OR), at 2.5 mg/ml. After a 24-h incubation, the ovaries were fixed in 4% paraformaldehyde and processed as previously described (Laidlaw and Wessel, 1994). To evaluate MYP endocytosis in individual cells, dissected eggs and free oocytes were transferred to ASW containing fluorescently labeled MYP (0.25 mg/ml). The oocytes that matured overnight were fertilized with sperm and visualized during early development. To evaluate selective MYP packaging, eggs and oocytes were incubated with both Oregon-Green MYP and TMR dextran, both at 0.25 mg/ml, for 30 min at room temperature to allow for a pulse of endocytosis. The cells were washed twice in ice-cold ASW, and images were recorded at different time points by using a scanning confocal microscope.

**Immunolocalization assays in situ**

Immunofluorescence protein localization was performed on stratified Og-MYP in vivo labeled eggs and oocytes that were fixed in formaldehyde and processed as previously described (Laidlaw and Wessel, 1994). Colocalization to yolk was assessed by using a monoclonal antibody to the yolk-specific YP30 protein (Wessel et al., 2000; and data not shown). Preimmune sera and the primary antisera of the YP30 mAb were diluted 1:1, and the secondary antibody (CY3-conjugated affinity-purified goat anti-mouse IgG) was diluted 1:20 (Kirkgaard and Perry Labs, Gaithersburg, MD). Signals were visualized either by epifluorescence with a Zeiss Axiosplan microscope or by laser-scanning microscopy with a Zeiss LSM 410 confocal microscope.

For whole-mount surface immunofluorescence of live cells, eggs and dissected oocytes were first passed through cheesecloth two times to remove jelly, followed by an additional 5 min of incubation in ASW, pH 5.2 (with HCl), to remove residual jelly. Eggs and oocytes were washed two more times in ASW, pH 8.0. Aliquots of these cells were distributed to Eppendorf tubes, the volume was adjusted to 1.5 ml with cold ASW, and samples were placed on ice. Preimmune sera and the primary antisera of MYP were added (both 1:500), and the tubes were placed at 4°C in a Labquake (Berkeley, CA) for constant mixing. After a 30-min incubation, the samples were washed three times in ice-cold ASW, and the secondary antibody (Cy3-conjugated affinity-purified goat anti-rabbit IgG; Jackson Research Laboratories, Westgrove, PA) was diluted 1:200. Signals were visualized by laser-scanning microscopy with a Zeiss LSM 410 confocal microscope. Controls in this experiment included samples incubated with preimmune sera, a nonrelevant antibody, or secondary antibody alone. Each of these controls showed no detectable signal.

**Results**

**Ovaries incorporate MYP selectively**

Texas-Red MYP (Tr-MYP) and Alexa-Green dextran (at a 1:10 fluorochrome ratio) were injected into the coelomic cavity of whole animals and incubated for 1–12 days. Gametes from these animals were shed by KCl injection and the gonads were dissected. Epifluorescent images of live tissue of the ovary showed uniform Tr-MYP labeling in the ovarian capsule and within a population of nutritive phagocytes (NP)s of the somatic accessory cells, and intense punctate spheres, reminiscent of yolk platelets, scattered throughout the cytoplasm of the oocytes (Fig. 1A and B). The Tr-MYP label appears to be endocytosed specifically as Alexa-Green dextran label was absent from both somatic and gametic cells of the ovary. Testis from injected male animals showed similar Tr-MYP incorporation in the gonad capsule and accessory cells; however, label was absent from
spermatocytes (results not shown). Eggs shed from the Tr-MYP-injected females were observed by epifluorescence, and a subpopulation of eggs showed intense punctate fluorescent labeling (Fig. 1C and D). MYP-labeled eggs fertilized and proceeded normally through early development (Fig. 1E and F). The absence of fluorescence at the cell surface, in the nucleus, or fertilization envelope gives us confidence that the Tr-MYP had not been misdirected, for example, to vesicles that secrete the vitelline layer or to cortical granules. During early development the yolk spheres are present in the cytoplasm of all cells, including the micromeres at the 16-cell stage (Fig. 1I and J) similar to spermatocytes.

Fig. 3. The amount of MYP uptake is directly related to the oocyte’s developmental stage and not the availability of yolk precursor. Scanning confocal images of histological sections of ovary explant incubated with Texas-Red MYP for 24 h prior to fixation. The left panels are DIC images, and the right panels are the corresponding fluorescence images, with MYP in red and DNA staining in blue. AC, accessory cell; OC, ovarian capsule; Po, previtellogenic oocyte; Vo, vitellogenic oocyte; bar, 50 μm.

Fig. 4. The amount of endocytic uptake of yolk increases with oocyte size and recapitulates the in vivo endocytic activity. Ovary explants were incubated with Texas-Red MYP constructs for 24 h prior to fixation. Scanning confocal images of histological sections of ovary were taken and the relative fluorescence intensity of MYP was calculated per area of oocyte (y-axis) and compared with the diameter of each cell (x-axis) to calculate the relative endocytosis average. (A) The fluorescence intensity of MYP increases with oocyte size. (B) The small previtellogenic oocytes were pooled and averaged for comparison to the larger vitellogenic oocytes. The difference in MYP uptake in the larger oocytes (50–80 μm) compared with the smaller oocytes (20–50 μm) is statistically significant (P < 0.001) as determined by a two-sample t test.
the fate of yolk platelets previously reported for this animal (Wessel et al., 2000). The concentration of the yolk label to the developing gut is apparent during gastrulation and at the pluteus stage (Fig. 1M–P).

To further examine the specificity of MYP incorporation into the gonads, ovary explants were cultured overnight in sea water containing different fluorescently labeled constructs. These included tetramethylrhodamine (TMR) dextran as a nonspecific fluorescent macromolecule, reactive Texas-Red (Tr) fluorochrome as a positive and unconjugated nonspecific fluorochrome marker, Tr-human serum transferrin that represents a transferrin family member related to the MYP sequence, and Tr-MYP. The targeting of Tr-MYP into the ovary and oocyte was selective as both

Fig. 5. Oocytes and eggs labeled in vivo with Texas-red MYP were subjected to isopycnic sucrose centrifugation to stratify organelles. The top panels are DIC images, and the bottom panels are the corresponding fluorescence images of the live cells, with MYP in red and DNA staining in blue. Texas-Red MYP-labeled vesicles stratify to the centrifugal end of the egg consistent with the position of yolk platelets (A, B), and during a 1-hour culture following stratification, the yolk platelets rapidly redisperse throughout the cytoplasm (C, D). pn, pronucleus; bar, 50 μm.
Fig. 6. Labeled MYP is correctly targeted and colocalizes with the yolk platelets of developing oocytes. Epifluorescence images of isolated oocytes that were incubated in vivo with Oregon-Green MYP. The top panel (A–B) is an image of a live oocyte and illustrates the yolk labeling distributed evenly throughout the cytoplasm of the oocyte prior to stratification. The bottom panels show an image of a single formaldehyde-fixed stratified oocyte that is labeled with a yolk-specific antibody to YP30. Overlay of the Og-MYP (D) and YP30 (E) signal indicates that exogenously added MYP colocalizes (F) with yolk platelets. gv, germinal vesicle; nc, nucleolus; bar, 50 μm.

Fig. 7. Ultrastructural immunolocalization of MYP and Texas-Red MYP in oocytes. Ultrathin sections of Tr-MYP-labeled ovaries were prepared for electron microscopic immunolabeling using antibodies to MYP (A–B), goat anti-rabbit antibodies alone (C), and Texas-Red chromophore antibodies (D–F). (A) The major yolk protein concentrated to yolk platelets is shown with homogenous granular-appearing organelles throughout the cytoplasm of the oocyte. The uniform distribution of endogenous MYP is (B) apparent in the larger platelets. The Texas-Red fluorochrome antibody (D–F) indicates Tr-MYP immunolabeling selectively in yolk platelets. This label is apparent in budding vesicles at the oocyte periphery (arrow, D) and is enriched at the periphery of the growing yolk platelets (E–F). yp, yolk platelet; bars, 0.5 mm.
TMR-dextran and Tr-human serum transferrin showed very limited incorporation that did not transverse the ovarian capsule (Fig. 2). On the other hand, the unconjugated Tr fluorochrome stained all of the cells within the ovarian capsule in an indiscriminate manner. Specific incorporation was only seen for Tr-MYP and it recapitulated the pattern seen in the in vivo incubated animals with intense labeling along the germinal epithelium of the ovarian capsule and within the somatic accessory cells. Punctate labeling reminiscent of yolk platelets was again seen in developing oocytes. However, not all oocytes incorporated label: smaller sized previtellogenic oocytes did not appear to incorporate label, while larger sized vitellogenic oocytes contained ample signal.

Fig. 8. MYP is endocytosed in vitro in a time-dependent manner and persists in vesicles. Isolated oocytes were incubated continuously with Texas-Red-labeled MYP. Images of live cells were recorded with a scanning confocal microscope over 90 min. (A) The first panel shows the DIC image, and the subsequent panels show the MYP fluorescent signal at different time points (40–90 min). (B) An oocyte from the above pool recorded after 20 h. The first panel shows an equatorial scan of the cell, and the second shows a subsurface scan of this same cell. gv, germinal vesicle; bar, 50 μm.
Fig. 10. MYP internalization is microfilament-dependent. MYP internalization was visualized in live cells following their culture in vitro in the presence or absence of cytoskeletal inhibitors. The left panels are DIC images, and the right panels are the corresponding fluorescence images of live cells with MYP in red. Treatment with the microtubule inhibitor colchicine (B) did not show a significant difference in MYP inception as compared with the control cells treated with DMSO alone (A), whereas treatment with the microfilament inhibitor cytochalasin D (C) has a dramatic inhibitory effect on MYP internalization. gv, germinal vesicle; nc, nucleolus; bar, 50 μm.

Fig. 11. Oocytes are more endocytically active than eggs. Isolated eggs and oocytes were continuously cultured with the lipophilic dye FM1-43 for 4 h at room temperature, and images of live cells were taken with a scanning confocal microscope. The DIC image is shown on the left with the corresponding fluorescence image on the right. gv, germinal vesicle; pn, pronucleus; scale bar, 50 μm.

Fig. 9. In vitro incubated oocytes package MYP, mature, are fertilizable, and develop normally. Oocytes and eggs were isolated from the ovary and cultured with Texas-Red MYP for 24 h prior to fertilization. The left panels are DIC images, and the right panels are the corresponding fluorescence images of live cells, with MYP in red and DNA staining in blue. (A) Oocyte. (B) Maturing oocyte extruding polar body (pb; MYP negative). (C) Fertilized egg with an elevated fertilization envelope (fe). (D) Two-cell-stage embryo. (E) Two-cell-stage embryo control. Bar, 50 μm.
MYP endocytosis is dependent on oocyte size

We further examined the finding that Tr-MYP is not incorporated into small oocytes. The Tr-MYP appeared to be evenly incorporated along the germinall epithelium of the ovarian capsule so there appears to be a uniformity of MYP entry into the capsule (Fig. 3A). Both small previtellogenic oocytes and large vitellogenic oocytes are attached to this germinall epithelium and are surrounded by MYP-positive accessory cells. However, only the larger sized oocytes endocytosed MYP (Fig. 3B). It thus appears that proximity of yolk precursor is not one of the limiting factors in this process. We were able to quantitate the differences in MYP endocytosis by measuring the relative fluorescent intensity of multiple oocytes within the ovary lobe, and we compared it with oocyte size. The relative fluorescent intensity was calculated per area of cell (y-axis) and compared with the diameter of each cell (x-axis; Fig. 4). These results indicate that the endocytic uptake of yolk is directly related to oocyte size, which correlates to its developmental state.

Exogenous MYP is correctly targeted to yolk platelets

To determine whether exogenous MYP is specifically targeted to yolk platelets, we first employed organelle stratification in eggs and oocytes. Individual oocytes or eggs that were labeled in vivo with Tr-MYP were centrifuged in an isopycnic sucrose gradient to stratify their organelles, and the position of the yolk platelets was assessed. Previous work has demonstrated that yolk platelets pellet to the dense centrifugal pole of the cell (Harvey, 1956) and the fluorescently labeled yolk platelets recapitulate this pattern (Fig. 5A). Surprisingly, the yolk platelets return to a uniform distribution in the cytoplasm relatively quickly following the stratification protocol (Fig. 5B). Since exogenously added MYP appeared to recapitulate endogenous MYP’s developmental program and appeared to localize to yolk-like granules during the vitellogenic phase of oogenesis, we further tested the success of this targeting by using an independent yolk-specific marker, YP30. The specificity of YP30 to yolk platelets has previously been addressed by electron microscopy (Wessel et al., 2000). YP30 is an oocyte-specific protein that accumulates to high levels during oogenesis and is selectively packaged in yolk platelets within the oocytes (Wessel et al., 2000). When stratified oocytes and eggs labeled in vivo with Og-MYP were colabeled with the YP30 antibody, the fluorescently labeled yolk platelets colocalized with the antibody (Fig. 6).

We also addressed the selective packaging of exogenous Tr-MYP at the ultrastructural level by making use of antibodies to the Texas-Red chromophore. Ovary explants were cultured with Tr-MYP for 20 h and assessed for incorporation of fluorescence into the oocytes. The tissues were then fixed, processed, and immunolabeled for electron microscopy by using anti-MYP as a marker of yolk platelets. The results show that MYP antibodies specifically labeled yolk platelets in oocytes as expected (Fig. 7) and that the label was distributed uniformly throughout the platelets, especially apparent in larger platelets (Fig. 7B). When antibodies to Tr were used, we also detected significant immunolabeling selectively in yolk platelets. In many planes of section, and especially in larger yolk platelets, this label was enriched at the periphery of the platelet (Fig. 7E and F). We conclude that exogenous Tr-MYP is internalized and packaged selectively in yolk platelets, reflecting the endogenous MYP dynamics, and that newly acquired MYP is added and remains at the growing yolk platelet periphery.

Labeled MYP is targeted to yolk platelets in isolated oocytes

To determine whether the ovarian accessory cells were necessary for yolk packaging in oocytes, we dissected oocytes and eggs from the ovary and incubated them continuously in sea water containing Tr-MYP. Similar to the in vivo and ovary explant experiments, isolated oocytes incorporated MYP selectively into yolk platelets. This endocytosis appears to function via active transport and not passive diffusion because, at 4°C, MYP labeling was not detected inside the cells (results not shown). After warming cells to room temperature (23°C), small punctate structure became apparent in the cytoplasm at the periphery of the cell within 30 min. Scanning confocal images of living oocytes endocytosing Tr-MYP over time is shown in Fig. 8. The endocytosis of Tr-MYP occurred in an even wave around the entire circumference of the oocyte. The label at the edge of the cell was more diffuse at earlier time points (40–60 min) and fewer distinct vesicles were apparent. At 90 min, diffuse labeling was still present at the edge of the cell, but internal areas contained distinct yolk vesicles. These vesicles persisted within the cytoplasm of the cell and were still visible during longer incubations (Fig. 8B, 20 h). Furthermore,
To examine the mechanism of MYP internalization, we examined oocytes cultured in the presence of cytoskeletal inhibitors. The cytoskeletal inhibitor concentrations we used effectively disrupt the specific cytoskeletal element, but do not damage oocytes as ascertained by recovery of the cells following treatment (Wessel et al., 2002). In these experiments, oocytes were isolated and incubated for 15 min either in the presence or absence of inhibitors. Oocytes were then cultured with labeled Tr-MYP for 50 min, washed, and directly visualized for MYP internalization. When the microtubule inhibitor colchicine was used, MYP was internalized generally as in the controls with punctate labeling of nascent yolk spheres present throughout the cytoplasm (Fig. 10A and B). However, in oocytes treated with the microfilament inhibitor cytochalasin D, MYP is seen at the cell cortex but internalization is not apparent (Fig. 10C).

Oocytes are more endocytically active than eggs

We had already determined that large oocytes were endocytically active for MYP, but not eggs or small oocytes (Fig. 4). We hypothesized that either they did not have the receptors for yolk or they were not endocytically active. To distinguish between these two possibilities, we looked at general endocytic activity and used the lipophilic dye, FM1-43, to quantitate this activity. FM1-43 is a nontoxic, watersoluble dye used to label plasma membranes and their derivatives, including in sea urchin eggs (Whalley et al., 1995). This dye is virtually nonfluorescent in aqueous medium, but upon insertion in the outer leaflet of the plasma membrane, it becomes intensely fluorescent. This dye is membrane-impermeable and is used to view plasma membrane dynamics, including the internalization of cell membranes during endocytosis. We incubated isolated eggs and oocytes of various sizes in sea water with FM1-43 and recorded endocytic activity using a confocal microscope. The results show that oocytes were far more endocytically active than eggs (Fig. 11). The amount of FM1-43 endocytosed for individual cells clustered into three distinct groups. Eggs were the least endocytically active, small oocytes were more active, and larger oocytes were most active. We conducted an ANOVA test on these data and found that the variance in FM 1-43 endocytosis among the three groups (small oocytes, large oocytes, and eggs) is significant (P < 0.001). We then proceeded to do a pairwise comparison of the three groups using the t test and found that each was statistically significant (large oocytes vs. eggs, P < 0.0005; small oocytes vs. eggs, P < 0.05; small oocytes vs. large oocytes, P < 0.005).

The results for general endocytosis of FM1-43 compared with specific Tr-MYP endocytosis in ovary explants are shown in Fig. 12. The results show that, during the vitellogenic transition, at about 50 μm in diameter, an oocyte will increase general endocytosis by 2 fold, but will increase specific MYP uptake by approximately 10-fold. We conclude that transition to a vitellogenic oocyte includes both an increase in both general endocytosis, and especially selective MYP endocytosis.

MYP is present on the surface of both oocytes and eggs

Whole-mount immunolocalizations using a polyclonal antibody generated against MYP (Brooks and Wessel, 2002) indicated that this protein was present on the plasma membrane of both oocytes and eggs (Fig. 13B and C). MYP surface labeling of these cells was specific as labeling with MYP preimmune sera (Fig. 13A), a nonrelevant antibody, and secondary antibody alone did not result in any detectable signal (results not shown). The MYP label remained restricted to the cell surface at 4°C, but when these cells where allowed to warm to room temperature, MYP was endocytosed and packaged into vesicles by the oocytes but remained on the surface in eggs. This recapitulates our observation with exogenously added Tr-MYP and suggests that residual endogenous MYP is still bound to MYP receptors on the cell surface.

Oocytes specifically package MYP

We wanted to assess whether isolated oocytes, independent of their somatic accessory cells, would selectively endocytose MYP. This would determine if the level of selective MYP internalization in vivo is restricted to the somatic cells of the ovarian capsule or if each cellular constituent of the ovary was capable of this selection. In the future, this would help us determine MYP-receptor identity. We pulsed isolated eggs and oocytes with both Oregon-Green MYP (Og-MYP) and the fluid phase marker TMR-dextran for 30 min and then rinsed the cells in sea water. We then recorded confocal images of the cells at different time points. Thirty minutes following labeling (Fig. 14A), much of the label was colocalized in vesicles around the periphery of the larger sized oocytes. After an additional 4 h, however, almost all of the Og-MYP in large oocytes was selectively packaged in yolk platelets, while the red dextran signal was sorted to a different population of vesicles (Fig. 14B). The smaller sized oocytes did not appear to endocytose Og-MYP and neither did eggs, and this recapitulated our observation of the incorporation of MYP selectively in larger
vitellogenic oocytes. All of the cells had some dextran labeling, indicating that there was detectable fluid phase endocytosis as predicted by our FM1-43 labeling (Fig. 11). When these cells were incubated for even longer time points following the pulse labeling, the dextran label completely disappeared from all of the cells (results not shown), while the vitellogenic oocytes that had taken up Og-MYP retained this protein in yolk platelets, thus further demonstrating the

Fig. 13. MYP is present on the surface of both eggs and oocytes and is endocytosed by oocytes. Scanning confocal images of whole-mount immunolocalization of live cells. The left panels are DIC images, and the right panels are the corresponding fluorescence images. (A) Egg and an oocyte incubated with preimmune sera 1:1000. (B) Egg incubated with anti-MYP sera 1:1000. (C) Oocyte incubated with anti-MYP sera 1:1000; note the endocytic vesicles. gv, germinal vesicle; pn, pronucleus; bar, 50 μm.
selective uptake and packaging mechanisms of these cells independent of the somatic accessory cells.

Discussion

Oocytes excel in organelle biosynthesis and packaging. They produce exceedingly large numbers of mitochondria, cortical granules, and Golgi, and synthesize an elaborate extracellular matrix prior to fertilization (reviewed in Wessel et al., 2001). At the same time, they are capable of endocytosing and packaging massive amounts of protein, lipids, and carbohydrates and store them in specialized vesicles called yolk platelets. Morphological observations in eggs of insects and amphibians have provided most of our knowledge of vitellogenesis. Here, we made use of fluores-
ently labeled MYP to look at the dynamics of yolk platelet biogenesis in sea urchin eggs, an oligo- and isolecithal cell distinct from insects and amphibians.

Injection of labeled MYP into coeloms of whole animals or incubation of the fluorescent protein in ovary explants and isolated oocytes reported dynamics indistinguishable from endogenous MYP and enabled us to document this process. Thus, the use of labeled exogeneous MYP to follow the vitellogenic profile of endogenous yolk appears to be a viable approach to study MYP fate and dynamics (see also Danilchik and Gerhart, 1987). Early MYP endocytosis by oocytes is confined to a peripheral layer of the cytoplasm and the label is somewhat diffuse. This observation bears common themes to *Drosophila melanogaster*, other insects, fish, birds, and frogs, all of which have yolk receptor-mediated endocytosis via clathrin-coated pits (Busson et al., 1989; Snigirevskaya et al., 1997; Grant and Hirsh 1999; Richard et al., 2001). These pits invaginate at the oocyte surface and form tubulovesicular endosomal compartments that merge into multivesicular bodies that are precursors of yolk vesicles (Wall and Patel, 1987; reviewed in Sardet et al., 2002), and in sea urchin it appears that this internalization is dependent on microfilaments.

We show here that the regulation of MYP uptake is autonomous of the follicular epithelium or the accessory cells of the ovary. This autonomy is not true of all organisms as in, for example, *Hyalophora cecropia* (silk moth), in vitro incubated oocytes can only incorporate vitellogenin if they are left intact with their follicle (Hausman et al., 1971). When *H. cecropia* oocytes are stripped of their epithelium and even left in the presence of this tissue, they will not endocytose vitellogenin (Anderson, 1971). It was found that the oocytes of this and other insects required a gap junctionally transmitted epithelial cell signal to facilitate endocytic yolk uptake (Adler and Woodruff, 2000). Sea urchin oocytes instead appear to be bathed in MYP from the germinal epithelium transport, or from nutritive phagocyte transport, and vitellogenic oocytes can selectively uptake this protein.

*Xenopus* oocytes, stripped of follicle cell interactions, are also capable of endocytosing exogenously labeled vitellogenin (Danilchik and Gerhart, 1987; Opresko and Wiley 1987; Busson et al., 1989). Similar to our results in sea urchin, the receptor-mediated endocytosis of amphibian vitellogenin is quantitatively the same over the surface of vitellogenic oocytes of all sizes. However, once formed, the *Xenopus* yolk platelets are displaced along a gradient with the concentration of yolk platelets becoming nearly 10 times higher in the vegetal hemisphere (Danilchik and Gerhart, 1987). This is part of the elaboration of the animal-vegetal axis of the *Xenopus* oocytes, coincident with partitioning of other molecules that participate in the regulation of development. We do not see any such gradient of yolk packaging in sea urchin oocytes, and instead find that essentially all yolk platelets actively store MYP throughout vitellogenesis.

It is not possible to know when the vitellogenic stage begins in this oocyte by strict morphological identification of yolk platelets (Verhey and Moyer, 1967) since the yolk platelet is comprised of two major populations of proteins of distinct origin. YP30 is made early within the previtellogenic oocyte and accounts for 50% of the mass of the mature yolk platelet (Wessel et al., 2000), whereas MYP is predominantly made outside the ovary and is imported into this same organelle beginning the onset of vitellogenesis. Thus, the timing of exogenous MYP endocytosis and packaging determined here by a dramatic increase in MYP endocytosis suggests that the vitellogenic stage begins when the oocyte reaches 40–50 μm in diameter. We have determined that access to yolk precursors is not a limiting factor for either the induction or cessation of yolk endocytosis. We see a rapid drop in this yolk inception once the large oocytes (equal in size to eggs) mature, indicating that some endocytic mechanisms change during meiosis. Endocytosis is reactivated at fertilization as demonstrated by the egg’s remarkable capacity for membrane retrieval following cortical granule exocytosis (Whalley et al., 1995; Ikebuchi et al., 2001), a process distinct from receptor-mediated endocytosis (Bement et al., 2000) as seen here for MYP.

The amount of exogenous MYP found in eggs of whole animals experiments was quite variable. We believe this represents a difference in exposure time of individual oocytes to the labeled MYP prior to oocyte maturation, and cessation of MYP endocytosis. An even greater population of eggs did not show detectable label, suggesting that these gametes were already stored within the ovarian lumen upon beginning the experiment. It is also clear from these studies that most, if not all, yolk platelets remain endocytically competent throughout oogenesis, regardless of their position within the oocyte. We do not, however, believe that yolk platelet positioning in the oocyte is static. Instead, the yolk platelets appear to rapidly diffuse throughout the cytoplasm, as seen by the cells that recover from strafication, and thus suggests that yolk platelets are regularly exposed to the surface endocytic uptake machinery, where they are freshly loaded on a regular basis.

Although we do not have the temporal resolution to determine the exact pathway of MYP transfer into oocytes, it appears that MYP can go directly from the ovarian capsule to the oocyte as well through the accessory cells. We therefore postulate that the signal(s) for MYP targeting may lie within the primary amino acid sequence or within post-translational modifications such as glycosylation. In *Drosophila*, it has been shown that *Escherichia coli*-produced yolk proteins, lacking normal posttranslational modifications, translocate to the *Drosophila* ovary upon microinjection into the hemolymph (Bowen et al., 2002). However, the exact final location of this foreign yolk protein in the ovary is pending immunocytochemistry.

The precision of MYP packaging into the yolk platelets of the sea urchin oocyte may reflect the important function of this protein. Recent results suggest that MYP is an iron transporter of the transferrin superfamily of proteins.
(Brooks and Wessel, 2002), characterized by their ability to bind iron tightly but reversibly (reviewed in Baker and Lindley, 1992). Iron is crucial for cell viability and its proper transport, and storage is critical as free iron can cause cellular damage and toxicity (reviewed in Boldt, 1999; reviewed in Nappi and Vaas, 2000). We have previously demonstrated that native MYP binds iron in vitro (Brooks and Wessel, 2002) and suggest that this protein may be a major regulator of iron homeostasis in the sea urchin. Proper targeting and sequestering of MYP within the yolk platelets would then be strongly selected not only to protect the developing oocytes but also to provide the iron that is necessary to sustain growth and proper packaging may involve the abundant yolk platelet protein YP30.

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