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Cortical granule biogenesis is active throughout oogenesis in sea urchins

Michael Laidlaw and Gary M. Wessel*

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912, USA

*Author for correspondence

SUMMARY

Cortical granules are secretory vesicles formed in the eggs of most animals and are essential for the prevention of polyspermy in these organisms. We have studied the biogenesis of cortical granules in sea urchin oocytes by identifying cDNA clones that encode proteins targeted selectively to the cortical granules. These cDNA clones were identified by an immunoscreen of a cDNA library using antibodies to proteins of the fertilization envelope. Four different mRNAs were identified, ranging from 4 kb to 13 kb in length, that encoded proteins targeted specifically to cortical granules. Accumulation of these mRNAs began very early in oogenesis, in oocytes approximately 10-15 μm in diameter, and continued throughout oogenesis. The mRNAs reached peak abundance (on a per cell basis) in germinal vesicle stage oocytes, and the accumulation of each mRNA was linear with respect to oocyte growth. During breakdown of the germinal vesicle these mRNAs were degraded so that in eggs the mRNA signals were at background levels. Antibodies generated to recombinant proteins made from each of these cDNA clones showed that in the oocyte each cognate protein appeared early in oogenesis. These proteins accumulated only in cortical granules: no accumulation was seen in the cytoplasm, in Golgi, or in other vesicles, and no heterogeneity of the contents was seen within the population of cortical granules. Using these antibodies we show that cortical granules accumulated linearly throughout oogenesis. Prior to germinal vesicle breakdown, cortical granules accumulated throughout the cytoplasm of oocytes and increased in number as the oocytes enlarged; the rate of synthesis appeared equal at each stage. At oocyte maturation the cortical granules moved to the cell periphery coincident with the complete degradation of mRNAs encoding these proteins. These results show that the genes encoding cortical granule proteins are coordinately activated throughout oogenesis and that the pathway of protein biosynthesis and targeting to these regulated secretory granules is functional throughout oogenesis in this animal.

Key words: cortical granule, oogenesis, protein targeting, fertilization

INTRODUCTION

The major secretory vesicle of most eggs is the cortical granule. These vesicles line the egg cortex immediately underlying the plasma membrane and release their contents during the fertilization reaction. Exocytosis of the contents of cortical granules modifies the extracellular investments of the egg both mechanically and enzymatically, which leads to a slow block to polyspermy. In mammals, for example, cortical granule exocytosis results in the release of enzymes that alter the sperm receptors in the zona pellucida; e.g. conversion of ZP2 and ZP3 to ZP2f and ZP3f, respectively, that lead to the slow block to polyspermy (Moller and Wassarman, 1989; Ward and Kopf, 1993). In sea urchins, cortical granule exocytosis also results in proteolysis of the sperm receptor (reviewed by Foltz and Lennarz, 1993), as well as in the transformation of the vitelline layer into a thickened, fertilization envelope (reviewed by Shapiro et al., 1989). The later modification is an enzymatically catalyzed crosslinking of cortical granule proteins to the extracellular envelope, forming a mechanical barrier to further sperm penetration.

Cortical granules of sea urchins contain many different constituents to accomplish their varied tasks. Glycosaminoglycans (Schuel, 1985) and calcium ions (Cardasis et al., 1978) are stored within cortical granules at elevated concentrations and are released at cortical granule exocytosis during the fertilization reaction, as is a diverse protein population. The proteins include structural components destined for the fertilization envelope and an ovoperoxidase that catalyzes the crosslinking of these proteins within the envelope (Shapiro et al., 1989). Hyaline, a large glycoprotein that participates in the adhesion of early blastomeres, is released from cortical granules at fertilization (Hylander and Summers, 1982) as is a β-1,3-glucanase of unknown function (Epel et al., 1969; Wessel et al., 1987) and a protease that modifies the egg cell surface (Alliegro and Schuel, 1988; Schuel, 1985). Each of these proteins is targeted selectively to cortical granules and does not get packaged into other vesicles of the secretory pathway nor do they accumulate to detectable levels within the biosynthetic pathway (Hylander and Summers, 1982; Wessel, 1989). The targeting of these diverse proteins to the same vesicle suggests that some common feature, perhaps a physical property of the molecules or a shared amino acid sequence, is used in routing proteins to the cortical granule.

Although the mechanism of targeting molecules to secretory vesicles of the oocyte is unknown, results from electron microscopic examination of developing oocytes show that cortical granules form in proximity to Golgi organelles. Anderson examined cortical granule biosynthesis in the sea urchin *Arbacia punctulata* by electron microscopy and concluded that mature cortical granules form by a coalescence of coated, immature secretory vesicles that have budded from trans-Golgi saccules (Anderson, 1969). This and similar electron microscopic studies (Runnström, 1966; Verhey and Moyer, 1967; Schuel, 1985) provide the basis for our knowledge of cortical granule biosynthesis.

To address the mechanisms of cortical granule biosynthesis we sought to identify cDNA clones that encode proteins targeted specifically to cortical granules. Such cDNAs should be useful not only in learning more about the contents of these organelles, but also as tools to examine how cortical granules are made and how proteins are targeted selectively into this vesicle. Here we show the identification of four distinct mRNAs that encode proteins targeted specifically to the cortical granules of the sea urchin *Strongylocentrotus purpuratus*. Since cortical granules are found in the eggs of most animals, the mRNAs identified here may reveal features of cortical granule biosynthesis that are well conserved.

MATERIALS AND METHODS

Animals, gametes and embryos

Adult *Strongylocentrotus purpuratus* were obtained from Marinus (Long Beach, CA). Adults were induced to shed gametes by intracoelomic injection of 0.5 M KCl. Eggs were fertilized and embryos were cultured as described (McClay, 1986).

cDNA library immunoscreen and production of antibodies

Fertilization envelopes were isolated from eggs of *Strongylocentrotus purpuratus* which were activated with the calcium ionophore A23187 and treated with ATA (3-amino-1,2,4-triazole) as described (Weidman and Kay, 1986; Wessel, 1989). Antiserum generated to the resulting soft fertilization envelopes (SFE) was used to screen a λZAP cDNA library constructed from poly(A)+ RNA isolated from *Strongylocentrotus purpuratus* ovaries (Wessel et al., 1990). Phage plaques labeled by the antiserum were purified to homogeneity by repeated plating and immunolabeling and the cDNAs were recovered as a Bluescript plasmid (Short et al., 1988). XL1-Blue cells (Stratagene, Palo Alto, CA) bearing Bluescript plasmid-derived β-galactosidase/SFE peptides were induced with 10 mM IPTG for 2 hours, collected by centrifugation, and lysed by freezing and thawing. The lysate was then mixed with Freund's adjuvant and injected subcutaneously into female New Zealand white rabbits. Each rabbit received two boosts at 3-week intervals using the same immunogen in Ribi's adjuvant. Rabbits were bled from an ear artery 7-10 days following the last boost and the antisera were preabsorbed against XL-1 Blue cells.

Immunolabeling in situ

For immunofluorescent labeling, eggs, embryos, or pieces of ovaries were fixed in either Bouin's fixative for 2 hours at 22°C, or in modified Karnovsky's fixative containing 0.5% glutaraldehyde and 5% formaldehyde, as described (Wessel, 1989). Bouin's fixed material was embedded in paraffin and Karnovsky's fixed material was embedded in Spurr's resin (Spurr, 1969). Paraffin sections cut at 5 μm were rehydrated in phosphate-buffered saline containing 0.05% Tween-20 (PBST). Spurr's resin sections were cut at 1 μm, placed on aminopropyl-triethoxysilane-treated slides (Kingsley et al., 1993) and the resin removed by treatment with 5% KOH solution in ethanol for up to 1.5 minutes, and then rehydrated in PBST. Sections were incubated for 2 hours with their respective antiserum, diluted (100×) in PBST, washed in PBST, and then incubated for 2 hours in diluted (30×) affinity purified goat anti-rabbit antibody conjugated to fluorescein (GAR-FITC; Organon Technika, Research Triangle Park, NC). After washing in PBST, the slides were mounted with PBST-glycerol (1:9) containing 1 mg/ml para-phenylenediamine, and observed with a Zeiss fluorescence microscope equipped for epiluminescence and photographed on Kodak Tri-X film (ASA 400). Several controls were used to examine the specificity of the primary and secondary antibodies. These included using the preimmune antisera from each rabbit, using the secondary antibodies directly, using immune sera from rabbits injected with XL1-Blue lysates harboring plasmid-encoded fusion peptides to non-SFE sequences, and using affinity purified antibodies to non-relevant proteins. None of these antibody preparations resulted in non-specific labeling of the cortical granules. Antibodies against hyaline and β-1,3-glucanase were used as positive controls to identify signals specific to cortical granules (Wessel, 1989).

Whole-mount immunofluorescent labeling was performed on oocytes dissociated from ovarian tissues. Ovaries were chopped with a scalpel in calcium-magnesium-free seawater (CMF; McClay, 1986) and incubated for 30 minutes in CMF containing 1 mg/ml collagenase (Sigma). Cells were dispersed by passage through a Pasteur pipette and were then attached to poly-L-lysine coated coverslips. Cortical granule lawns were prepared on poly-L-lysine coated coverslips according to Vacquier (1975). Immature oocytes and cortical granule lawns were fixed with 3.7% formaldehyde in CMF for 20 minutes, then in 100% methanol at −20°C for 15 minutes, and washed with millipore filtered seawater (MFSW). The fixed cells were incubated with SFE 1 antiserum for 30 minutes at 23°C, washed with MFSW, and then incubated for 30 minutes at 23°C with GAR-FITC diluted 30×. The slides were washed with MFSW and observed under epi-fluorescence.

For electron microscopic immunolabeling, eggs and ovary explants were fixed with modified Karnovsky's solution, post-stained with 0.1% OsO_4, and embedded in Spurr's resin. Silver-gold sections (approximately 90 nm) were placed on nickel grids and incubated for at least 10 minutes in phosphate-buffered saline containing 10% fetal bovine serum (PBSF). Sections were then incubated for 1 hour in PBSF containing either immune or preimmune SFE antisera (diluted 100×). After washing in PBSF, sections were incubated for 1 hour in goat anti-rabbit antibodies conjugated to 15 nm colloidal gold particles (diluted 30× in PBSF; Jansenn). Sections were then gradually washed into PBS, postfixed with 2% glutaraldehyde, and stained with uranyl acetate and lead citrate. Sections were visualized at either 80 or 100 KeV with a Philips 410 electron microscope. Controls for these experiments were as described above for immunofluorescence labeling.

RNA isolation and analysis

Total RNA was isolated from ovaries of different stages, eggs and embryos and analyzed by RNA gel blots essentially as described (Bruskin et al., 1981). Recombinant cDNA clones encoding SFE proteins were excised from Bluescript plasmids with *Eco*RI and radiolabeled with [^{32}P]dCTP by random oligonucleotide primer labeling (Feinberg and Vogelstein, 1983) resulting in a specific activity of greater than 10^8 cts per minute/μg DNA.

RNA hybridization in situ

Ovaries of various developmental stages, eggs and embryos were fixed in 2% glutaraldehyde and prepared for in situ RNA hybridization as described previously (Angerer et al., 1987). ^{3}H-labeled antisense and sense transcripts were synthesized from the Bluescript plasmid-bearing cDNAs with T7 or T3 RNA polymerase to achieve a specific activity of 10^8 cts per minute/μg. Tissue sections were

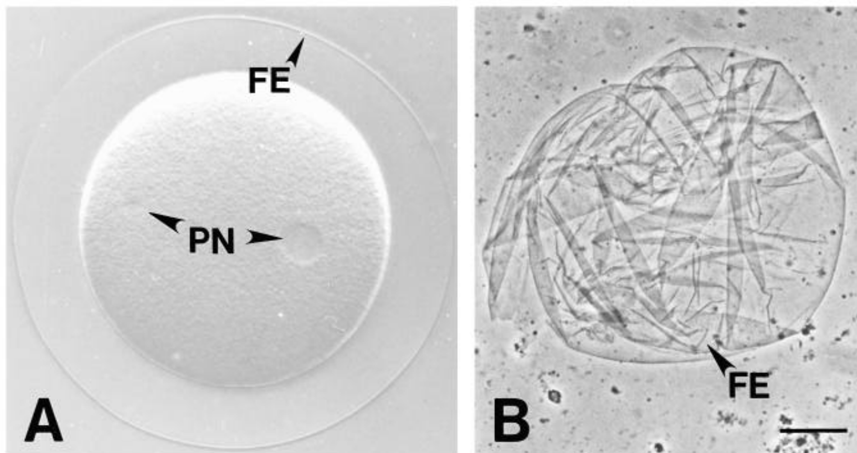

Fig. 1. (A) A fertilized egg showing the fertilization envelope that develops as a result of cortical granule exocytosis. Also evident are the male and female pronuclei on the left and right, respectively. (B) A fertilization envelope removed from a fertilized egg. A large population of envelopes can be isolated by batch procedures resulting in several milligrams of highly enriched envelope proteins. These envelopes were used to generate antibodies for an expression-screen of cDNAs that encode cortical granule proteins. FE, fertilization envelope; PN, pronucleus. Bar (in B), 20 μm.

hybridized with either sense or antisense probes, washed at Tm-5°C, and prepared for autoradiography as described (Angerer et al., 1987).

Quantitative measurements

Ovaries from different times of the reproductive season, enriched for different stages of oocytes, were used to quantify both the number of cortical granules per oocyte and the relative SFE-mRNA by grain counts following in situ RNA hybridization. To quantify cortical granules, ovaries embedded in Spurr's resin were sectioned at 1 μm or eggs and oocytes were fixed for whole-mount analysis and were immunolabeled as described above with whole SFE polyclonal antisera and with monospecific antisera to each of the recombinant, cortical granule proteins. Oocytes labeled by immunofluorescence were photographed and the cortical granules counted from enlarged prints. The area of each cell was calculated following tracing of cell boundaries on polyacetate sheets. To compare mRNA levels in different stages of oocytes, 5 μm sections of ovaries that had been processed for in situ RNA hybridization were photographed under both dark-field and bright-field illumination and the silver grains were counted in oocytes sectioned through the nucleus. The area of each cell was calculated as above and in some cases serial sections were analyzed. Background grain values were calculated from areas over the slide that did not contain tissue sections and from signals resulting from hybridization with sense-strand probes. These data were collected from sections treated on the same slide in the immediate vicinity of each other from subsaturation exposures.

RESULTS

cDNA immunoscreen

Previous results showed that following the fertilization reaction several proteins of the cortical granules were incorporated in the fertilization envelope (Villacorta-Moeller and Carroll, 1982; Wessel, 1989). We took advantage of this result and used antisera generated against isolated fertilization envelopes (Fig. 1) to screen a cDNA expression library for cDNA clones that encode proteins targeted selectively to cortical granules. We did not know in advance when cortical granule constituents would be synthesized during oogenesis so we constructed a cDNA library made from RNA isolated from ovaries at different times of the breeding season. Although oocyte development in adults of *S. purpuratus* is not synchronous, we used ovaries enriched with oocytes of several different developmental stages, e.g. very young oocytes, mostly vitellogenic oocytes, germinal vesicle stage oocytes, and mature eggs. A cDNA library was constructed from poly(A)+ RNA using approximately equal amounts of RNA from each type of ovary.

An immunoscreen of over 250,000 plaques of this library resulted in 9 independent plaques that labeled with the antiserum. These cDNAs were cloned into the expression plasmid pBluescript and were then transformed into XL1-Blue

Table 1. Characteristics of cDNA clones isolated from an expression library screened with polyclonal antibody against fertilization envelope proteins

Clone name	Insert size (kb)	Apparent size of mRNA (kb)	Presence of antigen in cortical granule
SFE1	1.2	9.0	+
SFE6	3.2	4.0	−
SFE8	0.8	6.0	+
SFE9	2.2	4.5	+
SFE11	1.8	1.8	−
SFE13	1.4	13.0	+

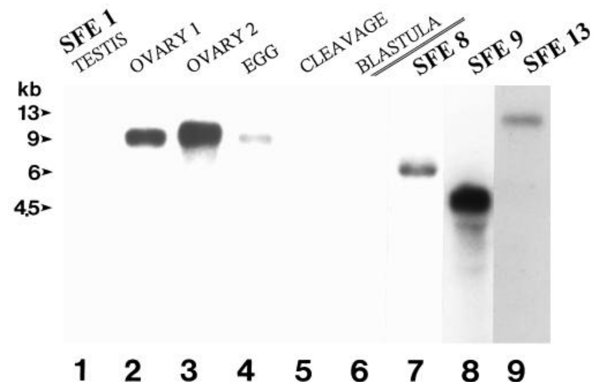

Fig. 2. RNA gel blots to identify mRNAs that encode cortical granule proteins. 10 μg of total RNA was loaded in each lane. Ovary 1, ovary from early in oogenesis; Ovary 2, ovary in peak of season; egg, unfertilized egg; cleavage, embryos during early cleavage. Lanes 7-9 contain RNA from the same stage ovary as that in lane 2. Each of the cDNAs described identify a single transcript. The relative abundance profile seen for SFE 1 in testis, ovaries, eggs, and in embryos represents the profiles seen also for transcripts of SFE 8, 9, and 13.

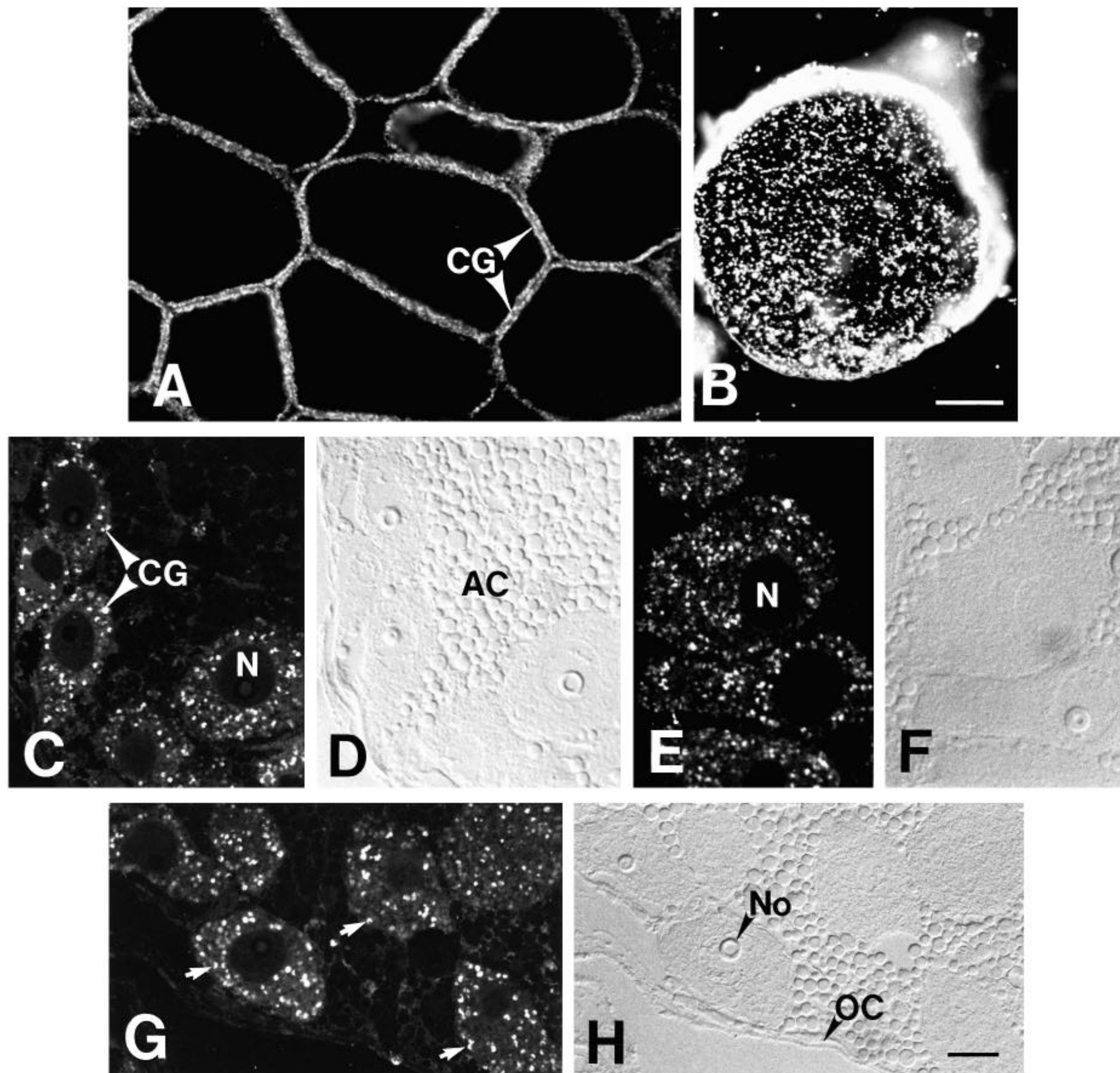

Fig. 3. Immunofluorescence evidence that SFE proteins are packaged specifically into cortical granules. Monospecific polyclonal antisera to each of the recombinant fusion peptides encoded by SFE cDNAs were used in an immunofluorescence assay on sections and whole mounts of eggs and ovaries. (A) Incubation of anti-SFE 1 on a section of ovary (*Strongylocentrotus purpuratus*) that is packed with eggs. SFE 1 labels particles of approximately 1µm within the cortex of each egg as predicted for cortical granules. No immunolabeling was detected in other areas of the cytoplasm of these cells nor in the lumen of the ovary. (B) A cortical granule lawn was made from eggs of *Lytechinus variegatus* (as described by Vacquier, 1975) and is shown from the cytoplasmic face of the egg. After incubation with anti-SFE 1 the lawn of cortical granules were selectively stained as particles approximately 1 µm in diameter. (C,D) Immunofluorescence and DIC optics, respectively. Incubation of SFE 8 with sections of ovary from early in the season. Adjacent sections were treated similarly with SFE 9, and SFE 13 (E,F and G,H respectively). In each case, label was seen throughout the cytoplasm in particles of approximately 1 µm as predicted for cortical granules synthesized during oogenesis. In some cases, the particles had a crescent shaped substructure (arrows in G for example). Note lack of immunosignal in prominent nuclei, in cells of the ovarian capsule, and in accessory cells. CG, cortical granules; N, nucleus; AC, accessory cells; No, nucleolus; OC, ovarian capsule. Bars in B and H each represent 20 µm, and the bar in H represents all photos except for B.

cells. Lysates of these transformants were analyzed by western blotting using the SFE antisera to authenticate the immunopositive cDNAs: eight of the nine cell lines showed production of a stable, SFE-positive fusion protein. DNA sequencing from the 5′ end of the cDNA from each of these cell lines verified that the cDNAs contained an open reading frame and none of these sequences showed identity to sequences in Genbank (sequence data not shown). These sequencing data and restriction maps of each cDNA showed that each recombinant was an independent isolate. RNA gel blots of ovarian RNA hybridized to inserts from each of the recombinants showed that six different sized transcripts were represented by these cDNAs, ranging from 1.8 kb to approximately 13 kb (Table 1, Fig. 2, and data not shown for SFE 6 and 11). The cDNAs SFE 1, 2, and 3 each hybridized to a transcript of the same size and to each other and were thus believed to represent the same transcript.

Proteins targeted to cortical granules

Antibodies generated to each of the recombinant proteins made from SFE 1, 6, 8, 9, 11, and 13 were used to determine the location of the encoded protein in eggs and developing oocytes (Table 1 and Fig. 3). Sections of mature eggs incubated with antisera SFE 1, 8, 9, and 13, showed strong immunolabeling within distinct granules (approximately 1 μm) either concentrated in the cortex of mature eggs, or dispersed throughout the cytoplasm in developing oocytes (Fig. 3). No other label was detected in the cytoplasm. Following fertilization, all immunolabel was lost from the zygote and no immunopostive signal was seen in the embryo at any stage of development (data not shown). From the size, location, and secretions of the immunolabeled structures, the proteins encoded by SFE 1,8,9, and 13 were believed to be associated with cortical granules. Antibodies made to cell lines SFE 6 and 11 show that these proteins are targeted not at the cortical granules, but at the vitelline layer (data not shown), an extracellular network of the mature egg that forms the framework upon which the fertilization envelope is constructed.

Thin sections of ovarian tissue processed for immunoelectron microscopy confirmed the association of SFE 1,8,9, and 13 proteins with the cortical granules (Fig. 4). Immunogold labeling showed that each of the four SFE proteins accumulated specifically within the species-characteristic spiral-lamellar substructure of the cortical granules. No label was detected in the homogeneous region of the cortical granule nor in any other organelle of the oocyte. Each cortical granule was labeled with each of the antibodies and although the signal intensity varied among the different sera, within each serum, all cortical granules contained comparable amounts of labeling. No heterogeneity in labeling or in intensity of labeling was seen among different cortical granules. These results show that the proteins encoded by SFE 1,8,9 and 13 are targeted selectively to cortical granules throughout oogenesis.

RNA accumulation through oogenesis

The accumulation of SFE mRNA during oogenesis was analyzed by in situ RNA hybridization. Sections of ovaries from different times during the breeding season were hybridized with ^{3}H-labeled sense and anti-sense RNA probes. These data show that transcripts for SFE 1,8,9 and 13 accumulated in all stages of oocytes beginning very early in oogenesis. No signal was seen in accessory cells, cells of the ovarian capsule, or in cells of the testis (data not shown). Oocytes in these animals develop from the germinal epithelium which lines the ovarian capsule. As oocytes develop, they increase in volume but remain in contact with the ovarian capsule until shortly before they mature when they move from the capsule and toward a centrally located lumen. Mature eggs are held within the lumen until spawning. The RNA signal of oocytes is dispersed throughout the cytoplasm though in larger oocytes, where the cytoplasmic volume is the

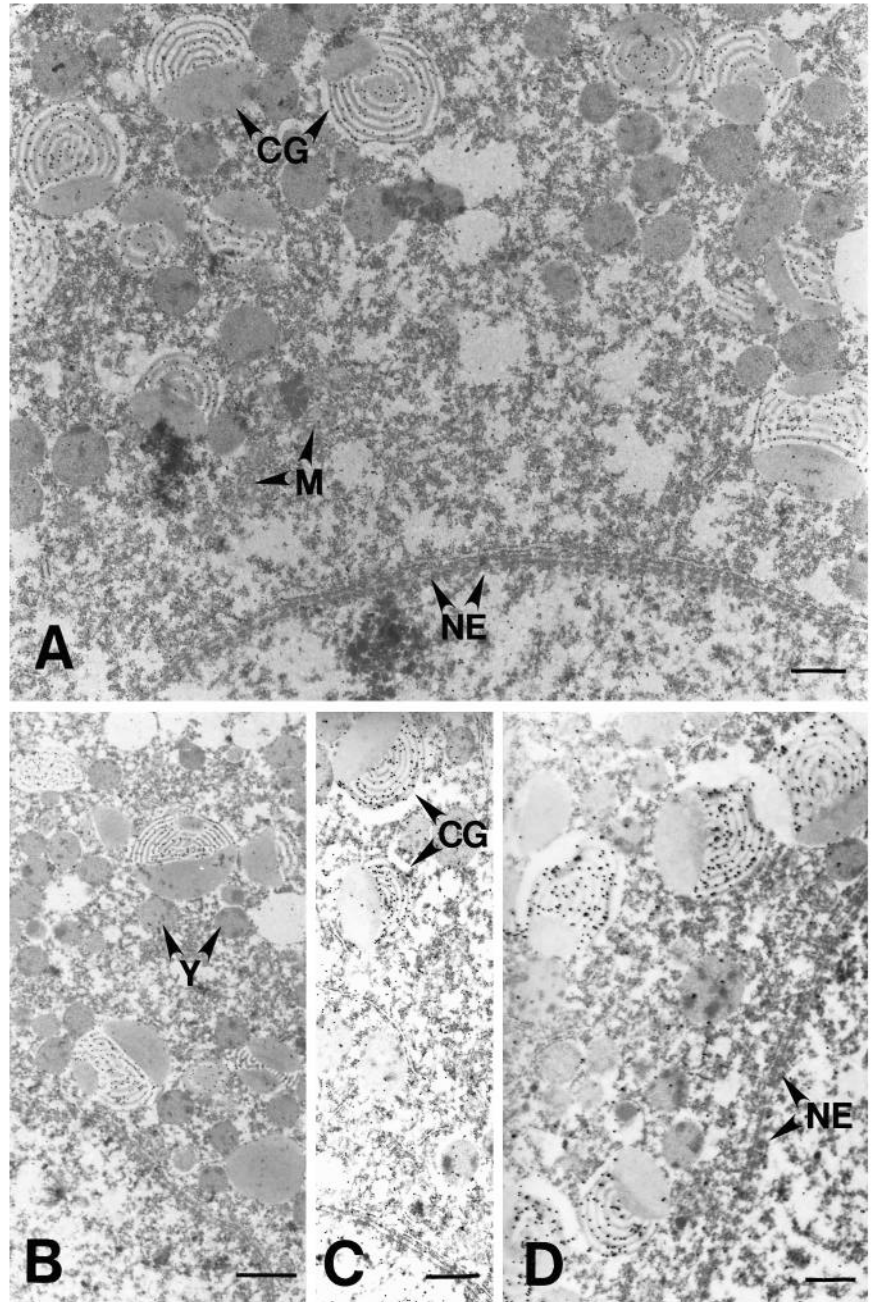

Fig. 4. Immunoelectron microscopic labeling of oocytes with SFE antisera. Thin plastic sections of immature oocytes were prepared for immunolabeling using colloidal gold conjugates. (A) SFE 1; (B) SFE 8; (C) SFE 9; and (D) SFE 13. Each experiment resulted in significant immunolabeling of cortical granules but not other organelles or cytoplasmic regions within the oocyte. Note the characteristic substructures of the cortical granules: a spiral lamella and an homogenous region. SFE immunolabeling is only detected in the spiral lamellar regions and is concentrated in the electron-dense lamellae. CG, cortical granules; M, mitochondria; Y, yolk platelet; NE, nuclear envelope. Bar, approx. 0.5 μm for all panels.

greatest, a slight but consistent concentration of RNA is seen surrounding the nucleus (Fig. 5). This concentration is likely due to the concentration of rough endoplasmic reticulum around the nucleus as seen by electron microscopy (Verhey and Moyer, 1967).

Prior to the breeding season, ovaries contained no RNA signal in any cells of the ovary (data not shown). Within one month however, ovaries contained developing oocytes, each of which contained significant cortical granule mRNA and proteins (Figs 2, 5). Thus the accumulation of SFE RNA and proteins is an early indication of oogenesis in this animal.

Oocytes that have matured to the germinal vesicle stage appear to have maximal SFE RNA signal per cell whereas after germinal vesicle breakdown, the level of signal has decreased to approximate background levels. Although the timing of oocyte development is not well determined in these ovaries, germinal vesicle breakdown (GVBD) in related animals takes approximately 1 hour (Kishimoto, 1986; see also Grainger et al., 1986). Since a large mass of mRNA is stored in eggs following GVBD, the turnover of SFE mRNA is selective.

Cortical granule constituents accumulate synchronously

The production of SFE mRNA and cortical granules in developing oocytes was quantified. Relative RNA levels within a hybridization experiment were quantified by grain-counts and cortical granules were quantified following immunolabeling (Figs 6, 7). These data were then expressed on a per oocyte basis using size of the oocyte as an estimate of the oocyte developmental stage (Verhey and Moyer, 1967). The results show that the levels of each SFE mRNA increase throughout oogenesis similarly to the rate of increase in the number of cortical granules (Figs 6, 7) and that egg maturation is accompanied by the complete degradation of each mRNA (Figs 5, 7). We interpret these results as the genes encoding cortical granule proteins are transcribed continuously through oogenesis. The turnover of the family of SFE mRNAs at GVBD is likely the result of both the termination of SFE transcription and by degradation of the

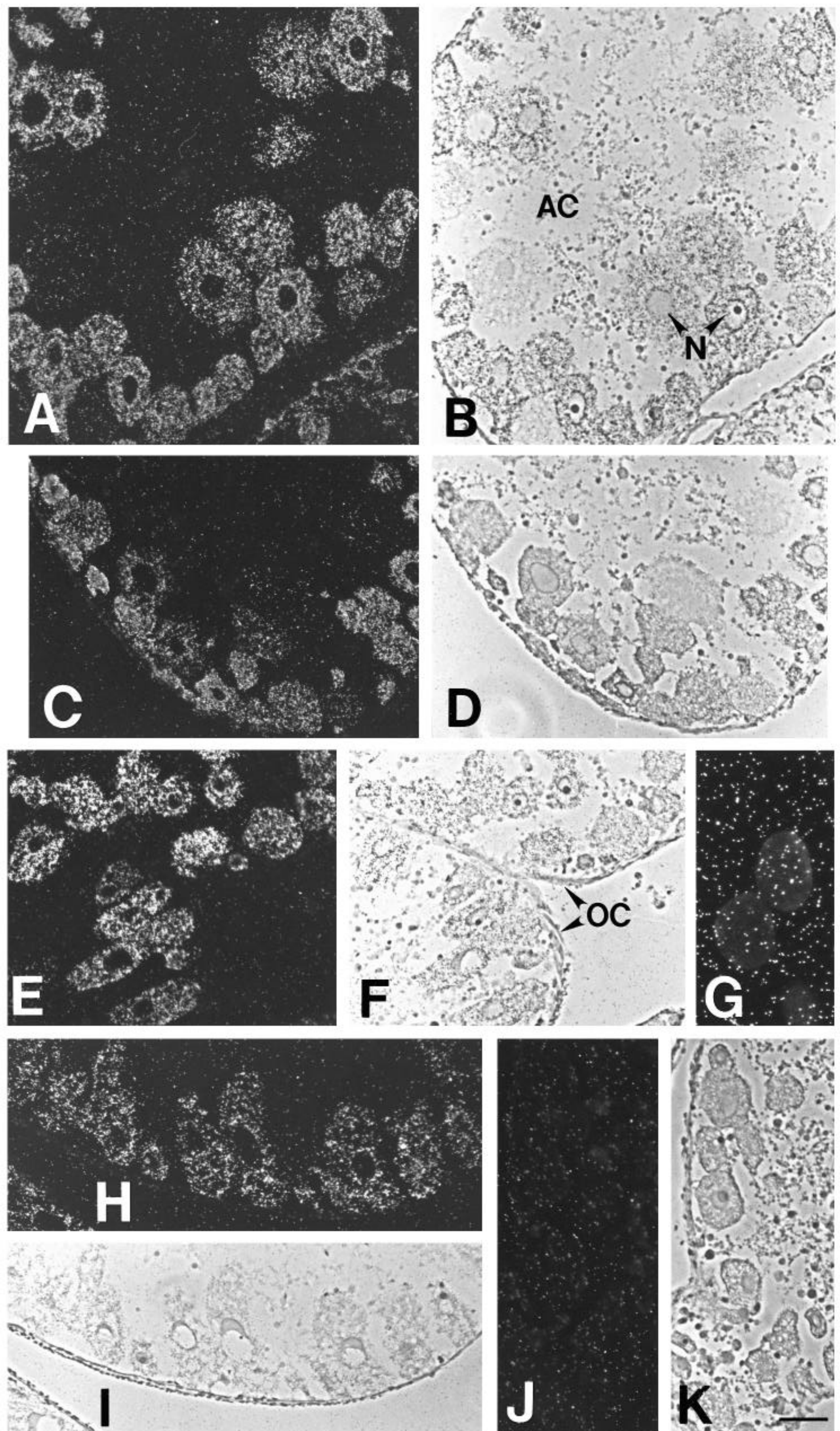

Fig. 5. Localization of SFE mRNAs in situ. Sections of ovaries and eggs were hybridized to either anti-sense (A-I) or sense (J,K) ^{3}H-labeled RNA probes. Hybridization results are shown both in dark-field and phase contrast optics respectively for SFE 1, (A,B); SFE 8 (C,D); SFE 9 (E,F); and SFE 13 (H,I). G shows eggs labeled with anti-sense SFE 9 (photographically overexposed), and J and K show hybridization results with the sense strand of SFE 1. N, nucleus; AC, accessory cells; OC, ovarian capsule. Bar (in K) approx. 50 μm.

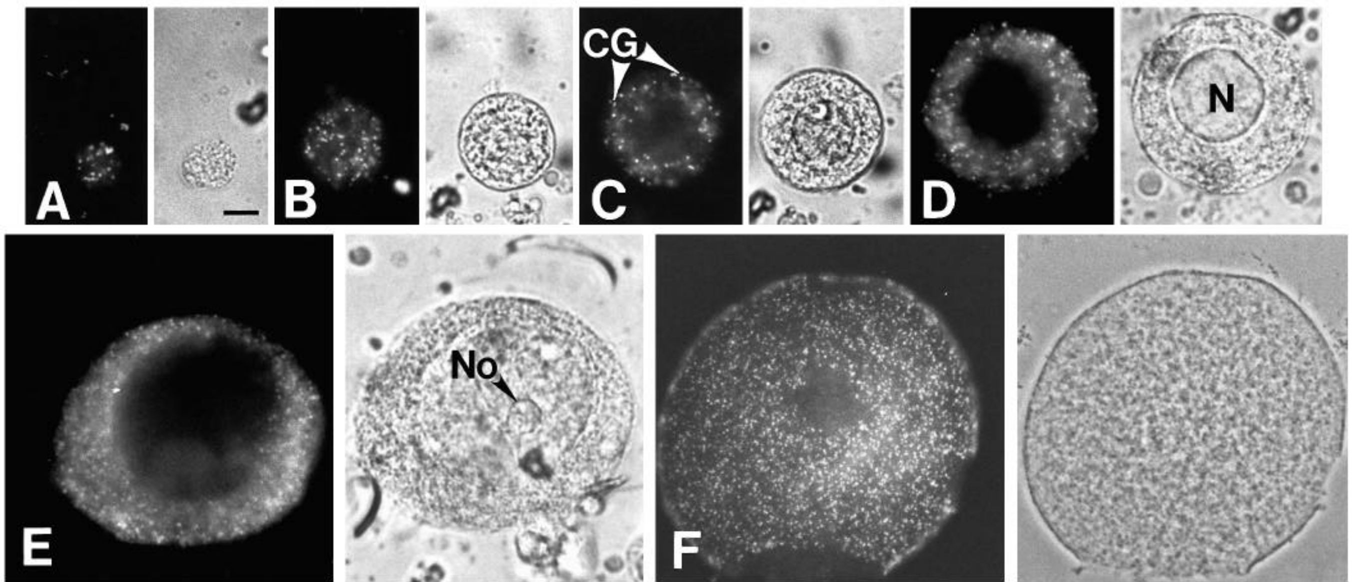

Fig. 6. Cortical granules accumulate throughout oogenesis. Whole-mount immunofluorescence assay using SFE 1 on different stages of oocytes isolated from a mid-season ovary. Each pair of panels shows the same cell using fluorescence and phase contrast microscopy respectively. Cortical granules are present in the smallest oocytes and accumulate throughout oogenesis. (F) The cell cortex of a mature egg that is slightly compressed. Other cells are shown in optical section of maximum cell diameter. CG, cortical granules; N, nucleus; No, nucleolus. Bar (in A), 20 µm.

RNA. During oogenesis each mRNA appears to be translated continuously and without storage. Each newly synthesized protein appears to be packaged directly into cortical granules and in concert with other cortical granule proteins. Thus the process of routing and packaging of the newly synthesized proteins (i.e. organelle biogenesis) is active until GVBD when the mRNA is effectively degraded.

DISCUSSION

A major function of developing oocytes is to synthesize and store proteins for use during both the fertilization reaction and early embryogenesis. For this task, oocytes have evolved an assortment of compartments that sequester proteins destined for the cell surface, the extracellular matrix, and the extra-embryonic layers (Alliegro et al., 1992). Here we examine the biogenesis of the major secretory vesicle of eggs, the cortical granules. Contents of the cortical granules function in the slow block to polyspermy in many animals throughout phylogeny and in sea urchins they also contribute to a protective environment for early embryonic development. In this report we have identified four mRNAs that encode proteins targeted selectively to cortical granules and show that the mRNAs, their cognate proteins, and discreet cortical granule structures are expressed coordinately, beginning very early in oogenesis. Each gene encoding cortical granule proteins appears to be activated at similar times in the oocyte and each protein is synchronously synthesized and packaged to generate a mature organelle. Since each cortical granule contains the same relative concentration of their various protein constituents throughout oogenesis, it is likely that cortical granules are constructed sequentially, i.e. the contents of a newly synthesized cortical granule are packaged before the contents for a second cortical granule accumulate. It does not appear that cortical granules are constructed partially and then modified by new protein addition over the course of oogenesis. It is also unlikely that mRNAs encoding each protein are stored for any appreciable time before translation, since cortical granules with a complete repertoire of contents are seen in oogenesis as soon as the encoding mRNA is detected. Although individual cortical granules may be resorbed or secreted during oogenesis, a net turn-over of cortical granules during oogenesis or at oocyte maturation was not apparent in our quantitation. In mouse oocytes however, significant turnover of cortical granules by precocious secretion is seen during formation of a cortical-free domain (Ducibella et al., 1990).

Several criteria were used to determine that the molecules identified here accumulated specifically in cortical granules. First, a variety of immunolocalization techniques were used that incorporated different protocols of fixation and embedding. These protocols included whole-mount preparations, paraffin sections and thick and thin plastic sections. Each protocol resulted in immunolabeling specifically within cortical granules for each of the four SFE proteins. Each of the SFE proteins were also secreted during the cortical reaction at fertilization as expected for proteins of the cortical granules (data not shown). Thus we believe the proteins identified in this screen are synthesized throughout the oocyte and then targeted to and concentrated specifically into cortical granules. We cannot exclude the possibility that some cortical granule proteins function or accumulate elsewhere in the oocyte, but the only detectable accumulation seen by each of our protocols is in vesicles with the morphology of mature cortical granules.

The morphology of secretory granules in most vertebrate somatic cells is a homogenous dense core, whereas cortical granules in sea urchins characteristically have a striking, and species characteristic morphological substructure. For example, *S. purpuratus* cortical granules contain a spiral lamellae, *Lytechinus variegatus* cortical granules have a

mottled, marble appearance, and *Arbacia punctulata*'s have a stellate morphology (Wessel, 1989). Notably, each cortical granule within a species is remarkably uniform in size and morphology. The eggs of *S. purpuratus*, for example, contain approximately 15,000 cortical granules, each with the same lamellar pattern with identical spacing, and each 1 µm in diameter. It is not clear how biogenesis leads to the distinct substructure particularly because each species' characteristic, vastly different substructure contains a similar protein composition (Wessel, 1989). Since most identified macromolecules are partitioned into distinct regions (Hylander and Summers, 1982; Alliegro and Schuel, 1988; Wessel, 1989; Somers et al., 1989), perhaps a substructure may be important in segregating molecules that might otherwise interact precociously.

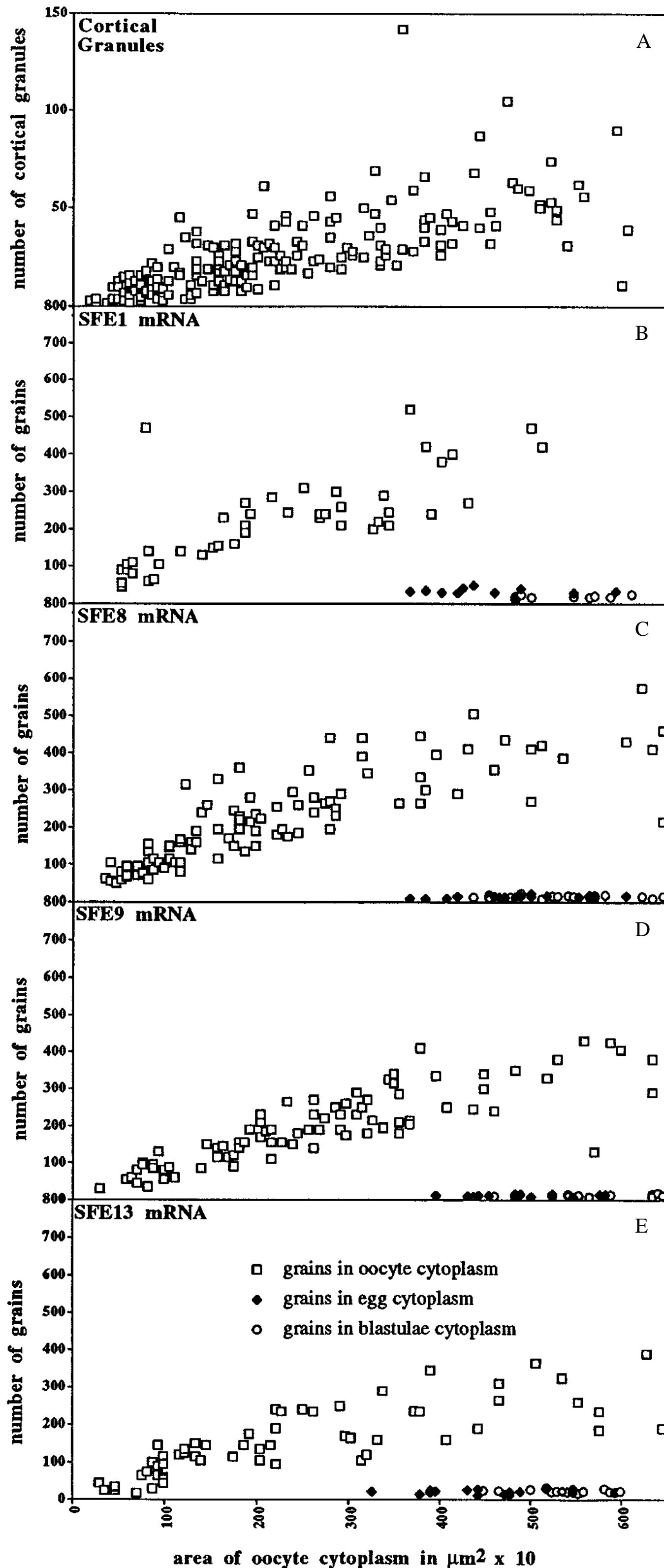

Fig. 7. Quantification of cortical granules and relative SFE mRNA during oogenesis. The results of immunofluorescence experiments and in situ RNA hybridizations were quantified by counting either immunolabeled cortical granules or grain number in sections of oocytes. These values are expressed on a per area basis from histological sections through maximum oocyte diameter. □, cortical granules (in A) or number of grains resulting from SFE mRNA hybridization (in B-E). ◆, grains resulting from SFE hybridization in mature egg; ○, grains resulting from SFE hybridization in blastula.

Each of the proteins identified in this study are packaged uniformly into cortical granules. No evidence was seen of content heterogeneity as reported by Anstrom et al. (1988) for the epitopes identified by monoclonal antibodies 1G8 (McClay et al., 1983) and B2C2 (Leaf et al., 1987). Thus, although variation in single epitopes may result from structural or processing variations, uniformity in protein content and abundance among cortical granules appears to be a general phenomena.

Each of the four mRNAs described here is degraded at oocyte maturation. The kinetics of this mRNA degradation was not precisely defined because we do not know when the mature eggs we examined underwent GVBD. However, this degradation is selective since many other mRNAs of the egg and early embryo do not show this loss (Davidson, 1986). Since each SFE mRNA is degraded at the same stage, they may share a 'rapid turnover determinant' that is activated at germinal vesicle breakdown and which may be independent of the adenylation state of the mRNA (Sachs, 1993).

The mRNA profile of SFE mRNA is similar to the profile of translation of a cortical granule protein in mouse oocytes. Pierce et al. (1992) used an antibody that cross-reacts with p75, a protein in cortical granules of mice, to examine the presence of p75 mRNA through oogenesis by in vitro translation. They found that p75 was synthesized in 20 µm oocytes, the smallest oocytes assayed, and increased many fold during oogenesis to a fully grown oocyte. Following germinal vesicle breakdown at maturation, the synthesis of p75 decreased over 90% following the profile shown here for SFE mRNA accumulation. Since cortical granules are near ubiquitous structures through phylogeny, these results suggest a conservation in the strategy of cortical granule biogenesis.

The cortical granule-specific proteins shown here likely share a common targeting mechanism that directs each protein to the developing cortical granule vesicle. Although the function of the proteins identified here is not known, it is clear that the contents of these regulated secretory vesicles are quite heterogeneous. Studies in neuronal and endocrine cells of vertebrates suggest that targeting of proteins to regulated secretory granules results both from the physical properties of the protein that enhance coaggregation, and from sorting receptors in the membrane of the trans-Golgi network that divert proteins into nascent secretory vesicles (Tooze and Stinchcombe, 1992). The cortical granules of sea urchins may employ additional

targeting signals since the granules are very heterogeneous in content and distinct from other types of regulated secretory vesicles. With the cDNA probes identified here we can begin to examine the inherent properties of cortical granule constituents that are important for segregation into cortical granules. The results presented here allow us to exclude the possibility that selective packaging of cortical granule constituents is the result of spatially or temporally restricted mRNA during oogenesis.

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